MEDICAL PARASITOLOGY

K.SHYAMASUNDARI
K. HANUMANTHA RAO

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Professor **Koka Shyamasundari** graduated from the Andhra University in 1954 with B.Sc degree and later with B.Sc (Hons) in 1956, and M. Sc by research in 1957. She worked as a demonstrator in the Department of Zoology, Andhra University till 1958 and was later elevated to the post of Lecturer in 1959. Her work leading to PhD, was on amphipods, both planktonic and fouling, with emphasis on neurosecretion and histochemistry. She was elevated to the post of Reader and Professor in succeeding years. Thirty one students have taken PhD, degree under her guidance. She has published 320 papers, most of them in International Journals (36 different foreign journals). She has presented 80 papers at both National and International Conferences. She has contributed significantly to histochemistry and histophysiology. She is the author of eleven books for intermediate, and B.Sc besides two monographs. She has published a book entitled "*Histochemistry in Focus*" in 2007, with professor K. Hanumantha Rao as the co-author. She is Fellow of Zoological society of India, Fellow of Zoological Society of Calcutta and Member of National Academy of Sciences. Allahabad.

Professor **Kona Hanumantha Rao** graduated from the Andhra University with B.Sc (Hons) degree in 1950 with Marine Biology as special subject. He started his early research with devotion to parasites of marine organisms. He took M.Sc (by research) degree in 1952 and D.Sc in 1958. He contributed extensively to lifecycle studies of trematode parasites of Visakhapatnam. He was trained in Biology of Helminthes while he was Colombo plan Scholar (1958–60) at University of Leeds, Tropical medicine, Dublin and Cambridge and London School of Tropical medicine and Hygiene. During this period, he became interested in Histochemistry of Mehlis glands, and established a strong school of histochemistry and Histophysiology at the Department of Zoology, Andhra University. Fifty students took Ph.D degree under his guidance in Marine Biology and Metazoan Parasitology (Histophysiology). He has published about 370 research papers and contributed significantly to histochemistry of scleroproteins in marine animals and parasites. He was fellow of National Academy of Sciences, Allahabad, Fellow of National Institute of communicable deseases, New Delhi, Fellow of Andhra Pradesh Academy of Sciences, Hyderabad.

As a Colombo plan scholar, he interacted with famous biologists like Prof. R. D. Keilin, Prof. H.W. Stunkard, Dr. J.G. Baer, Prof. G. Dubois., Prof. J.D. Smyth, Prof. J.C. Buckley, Prof. E.A. Paul Dr. T.Kerr., and Dr. J.B. Jennings.

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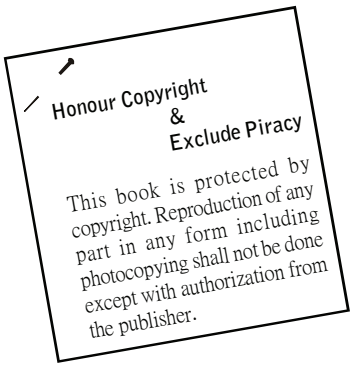

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VICE-CHANCELLOR

MESSAGE

It gives me great pleasure in writing this appreciation, in connection with the publication of the book "*Medical Parasitology*" by professors K. Shyamasundari and K. Hanumantha Rao. This book is a comprehensive treatise combining many aspects of parasitology including microbiology, protozoan and metazoan parasites and basic laboratory techniques (such as urine anlaysis, stool examination, haematology, immune system, preparation of whole mounts of invertebrates and their parts, methods for special organs, invertebrate staining methods and fixation and staining techniques in focus) for diagnosis. In short, as a book on medical parasitology, this book I am sure will be a welcome addition to the existing ones. Protozoan and metazoan parasites have been elaborately dealt with which is a special feature of the book.

The authors are teachers of vast experience in parasitology. Over the years Andhra University Zoology Department has established a study school of parasitology. Microbiology students and pupils of Medical college will find this book very informative and useful. I find that reputed professors Dr. G. Siva Rama Krishna (Professor of Medicine, A.M.C) and Dr. Jayakar (Professor of Microbiology, A.M.C.) have reviewed the book.

20.03.10
Beela Satyanarayana

FOREWORD

It gives me great pleasure to exercise a Foreword to this book on *Medical Parasitology*. Despite information explosions there is proverty of a concised book rich in essential details on Man and Microbes (Tiny living creatures).

Never before is so much in printed media and internet providing endless access to any subject under consideration. Nevertheless woefully time consuming to go through the information provided by these sources. This book is an attempt to meet the long felt need for quick reference on the subject.

The authors Dr. K. Shyamasundari and K. Hanumantha Rao who are qualified for decades of experience in the university atmosphere. The vision to see the need for such a book and conceptualized the same with photographs and illustrations is commendable.

The techniques detailed will find use in the laboratories for establishing the diagnosis of a disease state; under question reflecting the trend of investigating medicine overtaking clinical medicine.

Treatise, I am confident, shall be certainly useful to the scientific community at large, particularly those engaged in laboratory technology. My only hope is that this book will be made available to all libraries to be widely read. I deeply appreciate and whole heartedly congratulate the authors while lauding their effort for accomplishing the task of bringing out such a volume.

Prof. Dr. Siva Rama Krishna Rao
Ph.D, M.D, F.I.C.P, F. I AMS in India, F.C.C.P. USA
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PREFACE

Parasites and parasitic morbidity and/or disease seem to be a birth right of the tropics. This is a sequel to greater biodiversity fragmented in relation to biogeography and climatic shifts congenial to nocturnal enemies of humans, like mosquitoes. In fact in some cases parasite strategies have thwarted attempts of the scientific community to eradicate them. The best example running its course now is any kind of flu. Flu is no simple single strain strategy. At the base of parasitic diseases, man has recognized a dichotomy namely, animal diseases and diseases of man but there may be links between humans and animal (zoonoses).

We owe a great deal to the British Empire in the expansion of knowledge of tropical diseases especially of peninsular India with more or less warm climate plenty of room for growth of intermediate host like *Anopheles* (malaria), *Culex* (filaria) and *Aedes* (dengue). The School of Tropical Medicine and Hygiene at London is especially interested in this direction. We have at the present time many insitutions in India dealing with public health problems. Schools of Tropical Medicine, Calcutta, several laboratories of preventive medicine and more recently immunology laboratories. Likewise, there has been a growth of institutions dealing with veterinary medicine and animal health. We are just begining to understand what is known as zoonoses. That is parasites which normally infect and develop in animals can under certain circumstances infect humans and thrive with as much ease. India can now boast of being free of smallpox and plague but bad hygienic conditions are not allowing the control of malaria, dengue, filaria, typhoid, leprosy, etc. Dilution of efforts because of population explosion is the cause. A multiprolonged attack 1. Treatment, 2. Eradication of intermediate hosts (like mosquitoes) 3. Preventive measures (Prophylaxis) specific to each parasitic infection becomes a must, education is necessary.

The aim of the present books is to provide a brief but reasonably comprehensive information to the medical student as well as students of medical laboratory techniques. To be a comprehensive parasitologist of some worth and meaning the persent book has been designed. Equal emphasis is given to microbes and the protozoan and metazoan parasites. Two pertinent remarks can be quoted. Stoll 1947" had said that at any army center the medical orricer never heard of bancroftian filariasis (the disease) while soldiers saw it with dismay elephantiasis (the symptom) Philip 1987 had said that there is always some thing new under the parasitological sun.

The academic pursuits of British scientist, have laid the foundation of strong scientific Indian Society. The surgeon naturalist R.B.S. Sewell did monumental work in the field of marine biology of bay of Bengal. He was also the person who worked on life cycle stages of trematode parasites (Sewell, 1992). Ronald Ross discovered the life cycle of the malarial parasited in Secunderabad. Today there is a small "Ross Parasitology Lab" of Osmania University in Begumpet near Air-port in Secunderabad. Even today a "Ronald Ross day" is celebrated every year.

However, in the medical colleges parasitology is given a stepmontherly treatment. Many medical graduates are not fully informed except in some microbial areas like typhoid, cholera, etc.

In the preparation of this book and its completion we had the help of many colleagues and experts in several ways. Professor P.A. Jayakar, Professor of Microbiology, Andhra Medical College, had kindly reviewed the section on microbiology, Professor P. Siva Rama Krishna Rao, Professor of Medicine, Andhra Medical college and Dr. S. Meena, Professor of Pharmaceutical Chemistry, K.M. College of Pharmacy Madurai, have given valuable suggestions.

Our grateful thanks are due to Prof. B. Satyanarayana, Vice-Chancellor, Andhra University, for his message and encouragement. We are beholden to Prof. P.A. Jayakar and Prof. P. Siva Rama Krishna Rao (Andhra Medical College) for helpful suggestions. Thanks are due to Professors, D.E. Babu, Head of the Department, Department. of Zoology, Andhra University, B. Kishore, K. Sri Ramulu, Y. Prabhakar Rao, G. Gnanamani for help in many ways. We extend our appreciation to Major Dhinesh Sirpal (Medical Officer, NSTL, Visakhapatnam) and Dr. P. Sucharitha, Director, Institute of Preventive Medicine, Govt. of Andhra Pradesh, for their interest. We thank Sri K. Vijaya Kumar, Research Scholar, D. Ram Babu, Technical Assitant for their help.

Finally, we express our admiration to MJP Publishers, chennai, for their care and excellent get up.

K. Shyamasundari
K. Hanumantha Rao

CONTENTS

INTRODUCTION

The last one decade has witnessed a sea change in the graduate and post graduate courses in the life sciences departments. The mechanics of instruction and examination were subject to much experimentation. While change for the better and for more suitability for contemporary period is necessary, the important aspect is to not forget the foundation in basics required for any branch of science. Modernization of syllabus is one thing and bringing in interdisciplinary training is another. A compromise between these two and basics is mandatory. Can there be plum pudding without plums!

One such utilitarian course is that concerned with medical laboratory techniques, where normal physiology and altered physiology are targeted through clinical diagnostic techniques. What with altered life style of man in work or rest with a bewildering variety of food items most often adulterated not to speak of smoking or drinking and rapid industrialization the health and well being are jeopardized. Witness the health care centers, hospitals etc. whic h have increased beyond expectation and still not able to cope with the growing number of cases. The amount of anxiety and paucity of medical help are evident in 'swine flu' almost taking on epidemiological proportion.

There are some normal maladies which have been with us from time immemorial. Plague, smallpox, malaria, filaria, typhoid, cholera, to mention a few, are better known ones. A few of these have almost diappeared (smallpox and plague). Guinea worm infection also seems to be under check. These worms are visible and we see them with the patient with dismay. Now has come the invisible HIV. And the three musketeers—*Anopheles* (causing malaria), *Culex* (causing filaria) and *Aedes* (causing dengue fever)—have not left us altogether till today. This is tied up with sanitation and personal hygiene, and some parts of the globe have still to go a long way. There are other waterborne diseases with their emissaries. The study of diseases caused by viruses, bacteria, protozoa and helminthes is generally spoken of as medical parasitology, and this is a thoroughly neglected subject when it comes to metazoan parasites. In the medical colleges the emphasis is on microbiology (viruses and bacteria) and other aspects are virtually glossed over. It is for these reasons that a comprehensive book with equal emphasis on all aspects is considred a solution.

The axiom "Blood will tell" is well known. Blood is a sign of consanguinity (Heredity) and is a perfect benchmark of health and disease. Two major aspects alter blood chemistry in an artificial way—smoking and drinking. Fast foods and the poollutionof water are also major culprits.

Practically all parameters of blood are now amenable for quantification so that abnormal values are succinctly measured. This is the essence of clinical diagnosis. Much sophistication has occurred in the type of instruments and methodology—scanning, radioimmunoassay and what not.[1] What else is needed for the peace and happiness of a physician or surgeon. With all this, perhaps a physician or a surgeon may still require a sixth sense which experience enhances; it is only the so-called complications and late diagnosis that may kill a man now. Like many solutions in the field of book writing this may not be a perfect solution, yet appropriate proportion of devotion has been bestowed on every aspect within the limits of training period for a semester course in basics. Microbiology traditionally meant to designate the study of small organisms, the microbes, related to diseases like infuenza, cholera, plague, smallpox, tuberculosis, leprosy, etc. (viruses and bacteria). Although these are also parasites, the broader discipline parasitology has been set apart to the study of malaria and dengue, and of the filarial, nematode, cestode and trematode worms. Like physiology, microbiology, with emphasis on viruses and bacteria, was a monopoly of the medical colleges. Physiology and parasitology including the so-called microbes are now part of the pure science course. Perhaps the chief distinguishing feature between a microbes and multicellular parasite is the quickness and spontaneous production of strains of say influenza virus and so on. So the influenza virus can be as ancient as yesterday and as modern as tomorrow. In trypanosomes (sleeping sickness) the pellicle chemistry of the parasite is changed frequently so that attempts to deal with them become fruitless. Vaccines to combat such quick production of variations become infective. This is the burning question. Environmental degradation and improper hygienic conditions have only added to our misery specially in the tropics. It is thus necessary that every parasitologyist must lay emphasis on microbes as well as the multicellular parasites. A single textbook which encompasses the following is the need of today's parasitology student.

1. Microbes, protozoa, multicellular parasites
2. The diseases (pathology) they produce and epidemiology
3. A quantum of methods to detect them including what is known as clinical diagnosis.

The department of Zoology, Andhra University, has for several decades now excelled in the study of parasites of humans and animals, and established a standard laboratory to deal with microscopic diagnosis including histopathological techniques. Microbiology in its own right is assuming importance in universities.

In this book we wanted to give an opportuniy to a student to get basic information and training in clinical diagnosis (human), the lifestyle, structure and organization of parasites that infect and cause the several maladies which we see (smallpox, malaria, dengue, filaria, cholera, tuberculosis, plague and several known fevers) and modern ailments like HIV.

A small humorous anecdote may be pertinent here.

An Englishman who underwent surgery required blood transfusion. The blood of a Scotchman nearby matched the Englishman's blood. The Englishman paid him $ 50. A third transfusion was necessary and the Englishman merely thanked him. By now he had enough Scotch blood in him!

1 Magnetic Resonance Imaging Scan and CT scan

About toxicology the following story may be pertinent. At a certain place the adjoining river received all the effluents of the chemical plant. There was agitation and the daughter of the proprietor of the plant led the delegation. The father was surprised and said "Dorothy you are leading this agitation? The daughter replied "Daddy gone are those days when blood was thicker, now water is thicker". Waterborne and foodborne parasitic infections create havoc especially in rural India.

Really fresh water whether from rivers or lakes is becoming scarcer all over the globe. It has been said that DDT that has percolated into river Mississippi (USA) could be detected in the milk of Eskimo mothers in Iceland.

Medical colleges even today remain chiefly as microbe hunters. But it should be remembered that they may have to make a quantum jump in metazoan parasitology. Both water- and foodborne maladies must be given equal emphasis. Suppose a patient came with *Taenia saginata* (cestoda) infection, it is certain that he has eaten a barbeque or something related to beef. If a patient comes with Paragonimus (Trematode-lung fluke) infection the patient must have eaten undercooked crabs.

Zoonosis[1] is an aspect which must be emphasized. When parasites normally inhabiting the body of animals (birds or mammals) are found living and reproducing in humans, it is case of Zoonosis. A patient admitted in Guntur Medical College, Andhra Pradesh, died before diagnosis could be made. He was said to suffer from anemia. In the postmortem the intestinal contents (examined by us) showed enormous numbers of an echinostome trematode, *Artyfechinostomum*, as well as *Nector americanus*, the hookworm, which lacerates the intestinal wall. Simple examination of wall stools would have revealed the eggs of the trematode and the hookworm.

Fortunately in Andhra Pradesh most of the food items are well-cooked (boiled, fried with salt and spices). Thus, infective stages of parasitic worms do not withstand this treatment. However, some nematodes or schistosomes (trematodes) are different in that the infective larvae (hookworm larvae or schistosome larvae, cercariae) penetrate the patient's skin directly and enter the body. Amoebiasis or roundworm infections occur through contaminated water. Different levels of prophylaxis are necessary in such cases.

Some cestode parasites may be of biotechnological importance. The larvae of *Spirometra mansonoides* (human cestode) known as Sparganum occurs in the body cavity of various animals (Snake, mice, etc.). The larvae synthesize a growth hormone-like substance. Parasite-induced gigantism occurs in some intermediate hosts (especially snails).

In the case of *Diphyllobothrium latum* (fish cestode of man), there is selective absorption of Vitamin B_{12} by the proglottids. The host goes down with anemia. Thus it becomes necessary to find out the cause of anemia in each case. This shows how complicated the physiology of parasites may be.

Mal nutrition is an important factor especially in rural areas. Twenty percent of the world's undernourished are in India. While 7% of children are undernourished in China, in India it is 43%. Mal nutrition reduces disease resistance apart from engendering general debility.

1 Hanumantha Rao. K and K. Shyamasundari 1997 Zoonoses in Andhra Pradesh. Visakha. Sci-Journ 1 : 43–46 Acad. Sciences.

Adaptations of parasites are most diverse and interesting. There are two species which may exists as parasites or as free-living organisms. *Naegleria fowleri*, a protozoan, is common in soil and water. It can survive in warm water as well as in the human brain. Multiplication of *N.foweri* and degeneration brain can be rapid (meningocephalitis)[1]. Then there is the case of *Strongyloides stescoralis*, the human intestinal roundworms which can exist as a parasitic generation alternating with a free-living, soil-dwelling generation.

Humans provide a multitude of potential habitats within acidic or alkaline, and oxygen-rich or oxygen-poor. Stomach, intestine, lungs, brain, blood, cornea, skin, tissues and cells are the targets. Almost all parasties are prolific breeders because the hazards in the completion of their life cycles are many; consequently only a few may reachthe new hosts to become adults.

Resistance of pathogenic bacteria to antibiotic is fast becoming a grave medical and veterinary health problem all over the world. (Rawat *et. al.,* 2009, Proc. Nat. Acad. Sciences 79). Anthelmintics resistance involving particularly the nematodes is the most important concern of parasitologist round the world.

Biological control methods using predacious animals or even fungi are promising areas of the present day (Sanyal, 2009, *et. al.,* Proc. Nat. Acad. Sc. 79.).

All pesticides, apart from possessing the inherent danger of reaching tissues of human, are in effect biocides affecting useful organisms in the environment. Great care is necessary in this venture.

Sophistication in techniques is going fast. A very recent development is the perfection of a simple technique called Real Time Loop Amplification Methodology (RTLAM) to detect a HINI virus in one hour. It costs only Rs. 1500/- and is ideal for rural areas. The WHO kit takes a long time almost a day a find out a HINI-positive individual. Besides the kit costs up to Rs. 10,000/- requiring the need of a fluroscent detector. Research and development in this area are continuous processes which go on from one step to another in the road to perfection and cost-effectiveness and in order to meet the continuous evolution of parasites and their adaptions.

The best example of parasite adaption is that of the human cestode genus Taenia. Two species of Taenia are known. *Taenia solium* the pork tapeworm and *Taenia solium* the beef tapeworm. The cysticercus (bladder worm) of *T. solium* develops in the pig muscles (progtollids are swallowed by pig along with faeces).

The cysticercus of *T.saginata* develops in cattle. In this case since cattle are herbivorous, the proglottids move in a leech-like fashion and ascend up grass leaves awaiting foraging by cattle. The proglottids of *T.solium* are immobile and stay put in the faeces.

Hymenolepis nana the dwarf tapeworm of humans has been called 'a radically progressive cestode'. *H. nana* can develop into adult from hexacanth larva and then cysticercoid stage without intermediate host. The hexacanth penetrates the villus of intestine, become cysticercoid, and then breaks out into the lumen and gives rise tothe adult (Autoinfection). Worm burden may increase to such an extent that each worm is stunted. This is known as 'Crowding effect'. On balance, the parasite slogan is "Live and let live". La Rue (1938)[2] had said that a species by and large follows a general pattern of the family but may show deviation from it which we poor humans consider as normal.

1 Knutson, R.M. 1999 Fearsome Fauna pp 123 W.H.Freeman & Company.

2 La Rue, G.R. 1938. Life history studies and their relation to problems of taxonomy, J. Parasit 24 (1):1–11

Countries may differ in their nutritional status. For instance fish is cheaper with good protein and healthy omega fatty acids. It has been said that if all the fish that goes as chicken feed in America is diverted to Italy, the nutritional status of Italians can be improved by seven times than that existing. Even in a country like India, food items differ from season to season and from place to place. In Japan Gefulte fish is a dish of hot piping hot rice topped by raw fish. At least a dozen species of edible fish act as intermediate hosts of the human liverfluke *Clonorchis sinensis* common in Japan.

It is well worth digressing a little here. It has been said that over-nourishment in itself is a kind of mal nutrition; obesity and cardiac problems are the result. A balanced diet suitable to each country should be adopted. In India apart from under-nourishment a few other aspects extinguish life. Being a tropical country the usual problems are: 1. Heat exhaustion, 2. Tuberculosis, 3. Leprosy, 4. Helminthiasis (especially nematodes). It is well known that apart from Kwashiorkor, other causes of death may be venomous creatures. (cobra, King Cobra, Russell's viper, Krait and even scorpions). Tropical climate is conducive to these and the neutral enemies of humans (mosquitoes) although they are part of the ecosystem. Today with programmes of conservation of biodiversity, people require to be educated and every hospital must store multivalent antivenom. Although from the symptoms of a snake bite a clue is provided as to the identity of the snake, very often incidents in the nocturnal environment keep the doctor in the dark.

Professor David R. London (2001)[1], Registrar of the Royal College of Physicians of London said that as populations age the incidence of diseases increase and that there is ever-increasing consumer demand while Governments attempt to hold down health care costs. Professor Siva Rama Krishna Rao (2002)[2] said that importance must be given to fundamental skills of history-taking, and a sensible order of investigations should be adopted.

Dr. Peter Hotez, Research Professor, chairman, Department of Microbiology, Immunology and Tropical Medicine, Washington University, at a seminar on "Control of the Neglected tropical diseases: innovative approaches and role in U.S. Foreign Policy" (2010) said that there are approximately 70 million cases of hookworm and 30 million of lymphatic filariasis in India. Mahatma Gandhi suffered from hookworm infection. Gandhiji's efforts for peace in India may have been slowed down because of hookworms. This is one of the more dramatic examples of international peace and security. New drugs and vaccines of proven merit must be developed. Public expenditure on Nuclear projects to neglected tropical diseases is of the order of magnitude of 10,000 to one! Putting aside a small part of military expenditure toward tropical diseases would constitute a tremendous antipoverty step. In the field of tuberculosis there is a dismal picure. The irony is that with the availability of drugs for HIV, we are living with HIV but dying of tuberculosis. TB research has yet to see great progress. At a recent conference the biological and genetic information of *Mycobacterium tuberculosis* was discussed and presented (2010). About 1.7 million people die of TB annually: The Government's Open Source Drug Discovery (OSDD) is seized with the matter.

1, 2 P. Siva Ramakrishna Rao 2000. Common clinical challenges. A treatise on maladies and remedies.. Pp 892 Tat McGraw—Hill Publishing Company Limited, New Delhi.

1

BASIC EQUIPMENTS REQUIRED IN A LABORATORY

SPECTROPHOTOMETER/COLORIMETER

These instruments are used to measure the concentration of the substances in an unknown substance. The essential components of a spectrophotometer include (i) source of light, (ii) a monochromator, (iii) transparent vessels (cuvette) (iv) a photosensitive detector, (v) a meter a recorder.

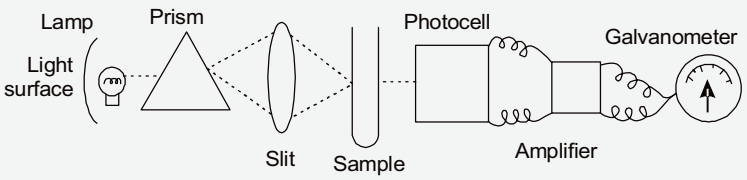


Figure 1.1 Essential components of spectrophotometer

Applications

1. Used to measure a number of biological compounds, e.g., proteins and nucleic acids.
2. Used for estimating enzymes, substrates, organic and inorganic gases and concentration of microorganisms.

CENTRIFUGE

Centrifugation is a widely used molecular separation method and is based on the principle that particles in an applied centrifugal field settle down due to gravity. The centrifuge consists of a metal rotor and a motor at a selected speed.

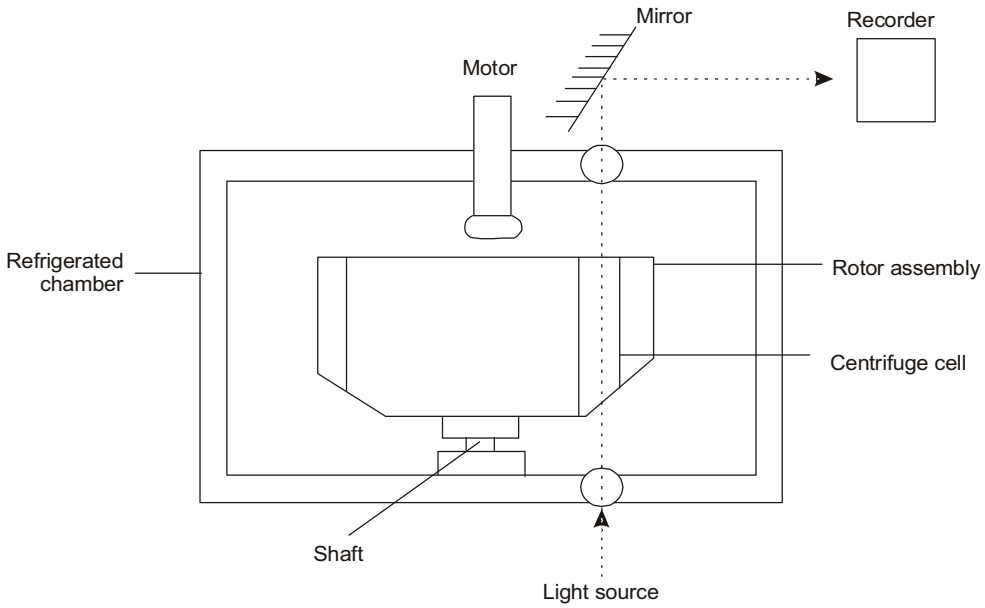


Figure 1.2 Essential components of centrifuge applications

Centrifugation techniques are concerned with separation, isolation and purification of whole cells, subcellular organelles (plasma membrane, polysomes, ribosomes, chromatin, nucleic acids, lipoproteins) and viruses.

ELECTROPHORESIS

It is a technique which involves the migration of charged particles or molecules in medium to oppositely charged electrodes under the influence of an applied electric field.

The electrophoresis instrument consists of two items, a) Power pack and b) Electrophoresis unit.

The power pack produces a stabilized direct current and has control for both voltage and current output.

The electrophoresis unit contains the electrodes, buffer reservoir, a support for the electrophoresis medium and a transparent insulation cover.

Applications

1. To determine the molecular weight of proteins, DNA sequence, etc.
2. To separate biological molecules such as amino acids, peptides, proteins, nucleic acids and nucleotides, etc.
3. To detect mutations in various genes in carcinogenesis.
4. In DNA foot printing.
5. In Southern and northern blotting technique.

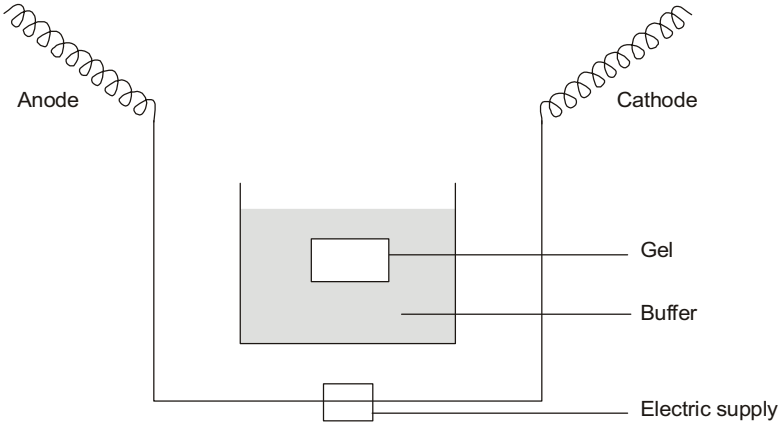


Figure 1.3 Horizontal electrophoresis

CHROMATOGRAPHY

Chromatography is one of the popular techniques for the separation of molecules. Every chromatographic technique should essentially possess two phases mainly stationary phase and mobile phase. Chromatography can be subdivided into different types namely paper chromatography, thin-layer chromatography, gas chromatography, ion-exchange chromatography, gel-filtration chromatography, affinity chromatography and HPLC (high-performance liquid chromatography), etc.

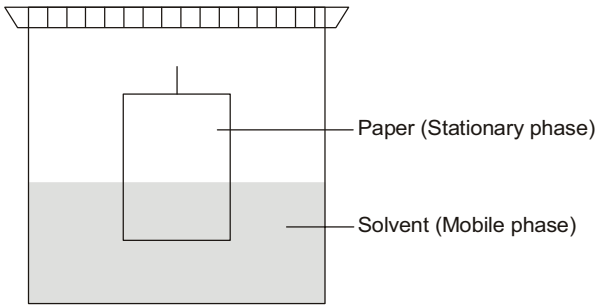


Figure 1.4 Separation by paper chromatography

Applications It is used in the separation of amino acids, proteins, nucleic acids, polysaccharides, complex lipids, plant pigments, drugs, pesticides, steroids and hormones, etc.

AUTOCLAVE

Autoclaving is the process of sterilization by saturated steam under high pressure above 100°C. It is a modified pressure cooker (or boiler, which may be horizontal or vertical). It consists of a chamber, thermometer, pressure gauge, door, filter, air inlet, jacket, air outlet, etc.

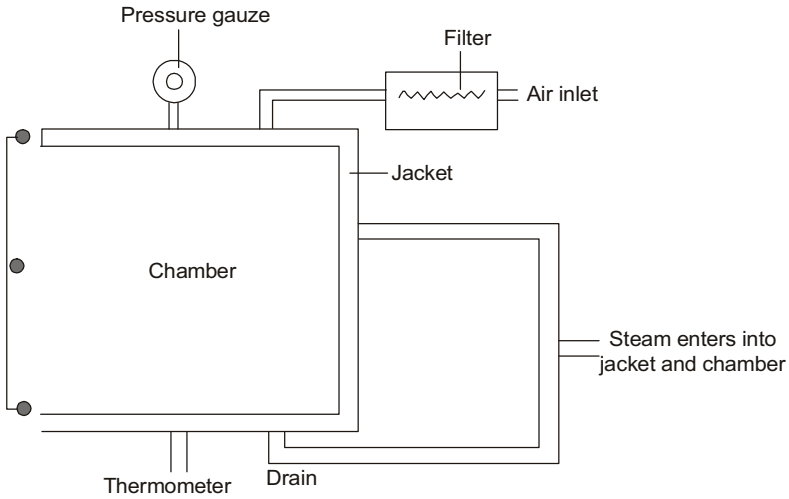


Figure 1.5 Autoclave

Uses It is used to sterilize culture media, rubber goods, syringes, dressings, etc. and is the surest method of destruction of bacterial spores.

pH METER (HYDROGEN ION CONCENTRATION)

One of the most common needs in biochemical equipment is the requirement to measure and control the pH of a reaction mixture. pH electrode is an example of an ion-selective electrode that responds to one specific ion in solution.

pH electrode consists of a thin glass porous membrane sealed a the end of a hard glass tube containing 0.1 M HCl.

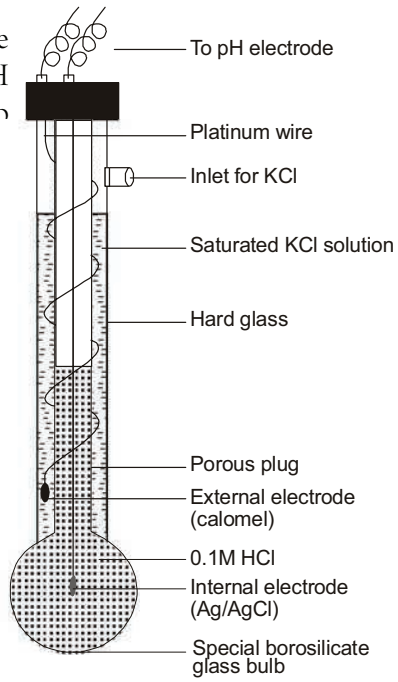


Figure 1.6 pH meter

Applications

1. To measure and control the pH of a reaction mixture
2. Analysis of sea water, serum
3. Analysis of drinking water, fertilizers
4. Analysis of air pollution

BALANCE

Single-pan balances are now more common than equal-arm balance because the weight can be measured faster and sensitivity remains constant. For most analytical purposes, a balance with maximum 100 to 200 g and $\pm$ 0.1 mg is required.

It consists of balance pan, balance weight and sensitivity adjustment. It is very useful for weighing chemicals in the laboratory.

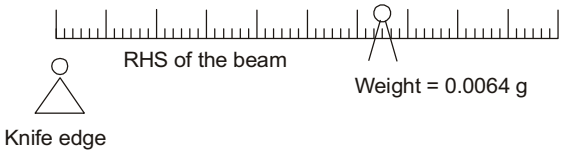


Figure 1.7 Scale

ANALYTICAL BALANCE

This balance is a lever of the first type. This rider balance is normally used in chemical laboratories. In this balance the beam is graduated in 10 equal divisions on either side of the fulcrum. Each of these big divisions is further subdivided into 5 or 10 equal divisions. These divisions can be moved into position with the help of a rider carrier operated from outside the balance box which serves to indicate accurately the weight of a substance to the third or fourth decimal places. The rider is usually made up of aluminium and itself weighs 10 mg. It is a general practice to use the rider on the right-hand side of the beam. The reading of a rider has been indicated in Figure 1.8.

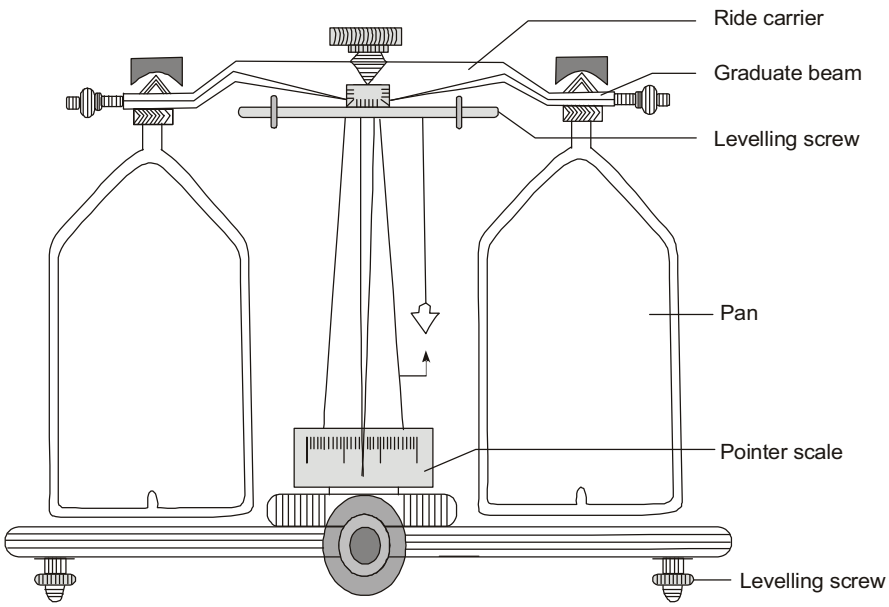


Figure 1.8 Analytical balance

Weight box It is a wooden box having the weights in the sequence as shown in Figure 1.. The weights are made of brass coated with chromium or nickel.

Fractional weights These are placed in small grooves in sequence in the weight box as 500 mg, 200 mg, 100 mg, 50 mg and 10 mg.

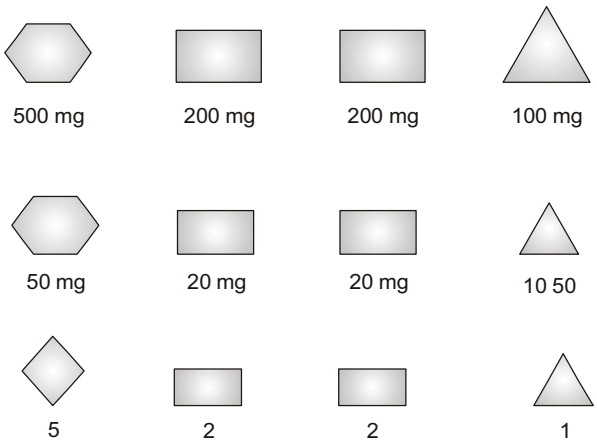


Figure 1.9 Fractional weights

FLAME PHOTOMETER

Principle Analysis is similar to that of spectrophotometry except that the place of the sample cell is taken by a flame. Thus it is the absorption or emission of specific wavelengths by excited atoms that is studied.

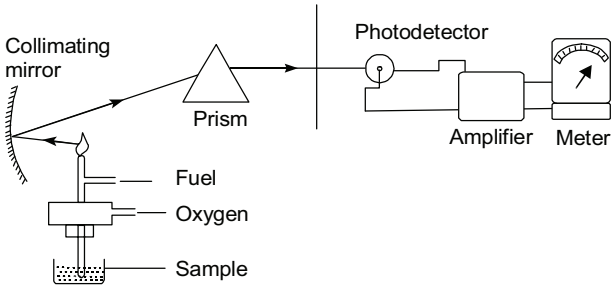


Figure 1.10 Flame photometer

The sample is evaporated into a flame. After evaporation of the water, two salt molecules are dissociated by the heat to an atomic vapour. A small percentage of the atoms is transformed into excited state by the absorption of decimal fraction of energy that displaces orbital 'S' to higher energy levels. The atoms immediately return to the ground state and in the process release the absorbed packets of energy in the form of light. The emitted light is of wavelength specific to each element and can be quantitated under carefully controlled conditions.

Components

1. Flame
2. Filter (monochromator)
3. Photomultiplier
4. Detector read-out system.

Applications

1. To assay about 20 elements in biological samples.
2. To determine trace metals in body fluids.
3. To directly measure sodium, potassium, calcium, magnesium, cadmium and zinc in physiological and pharmacological research.

MICROTOME

The microtome is an instrument designed to obtain sections of paraffin-embedded tissue. In an average histology laboratory, sections of tissues ranging from 5 μm to 12 μm thickness may be necessary to comprehend and interpret microscopic anatomy or cytology. The choice of thickness of sections depends upon the particular study required and the investigator must select judiciously.

The microtome (Figure 1.11) is a sturdy instrument whose main mechanism is to allow obtaining sections in a serial order at desired μm thickness. A carefully honed and appropriate knife is an important element of the set-up. In the main instrument there is a device, the block holder, to which a paraffin block with embedded tissue is affixed. A paraffin block has to be trimmed properly into a rectangle or cube before it is affixed to the block holder. The block holders come in several sizes. The front end of the microtome has the mechanism by which the block holder let into it is immovably held. At the rear end, there is a mechanism by which the thickness of the section is fixed (scale) and there is a rotating wheel together with a device allowing advancement of the block holder by the desired thickness upon each rotation of the wheel. The knife is inserted into a knife holder at the base of the anterior end. The knife is first fixed and then the knife holder is pushed towards the block holder. By appropriate manipulation, the knife edge is brought to the touching point of the paraffin block and the knife holder is now fixed. Now by synchronously rotating the wheel at the rear end with the right hand and holding a long needle in the left hand to salvage the sections which come as a ribbon, convenient lengths of the paraffin ribbon (15 to 20 cm) are obtained and placed as rows on a sheet of paper. Short lengths of the ribbon (4–5 cm) are cut with a blade and transferred to albuminized slides as 3 or 4 rows per slide. The sections are inundated with water and warmed on a slide heater for appropriate stretching. Water is now drained off and the slide is put on a slide rack for at least 24 hrs to dry.

Paraffin bath In the process of cutting thinner sections, the dehydrated and cleared tissue is subjected to a process of infiltration by immersing in molten paraffin (58°C) for shorter or longer time depending upon the specific tissue (mantle, liver, brain, etc.). The cabinet designed for holding molten paraffin in beakers, watch glasses, etc. and other containers is known as paraffin bath. It actually has an insulated glass

door and another door closing on that. A thermostatic arrangement keeps the temperature constant. The tissue is given two or three changes of molten paraffin. A beaker with molten paraffin is kept ready for embedding after infiltration. These are of various types depending upon the company manufacturing it.

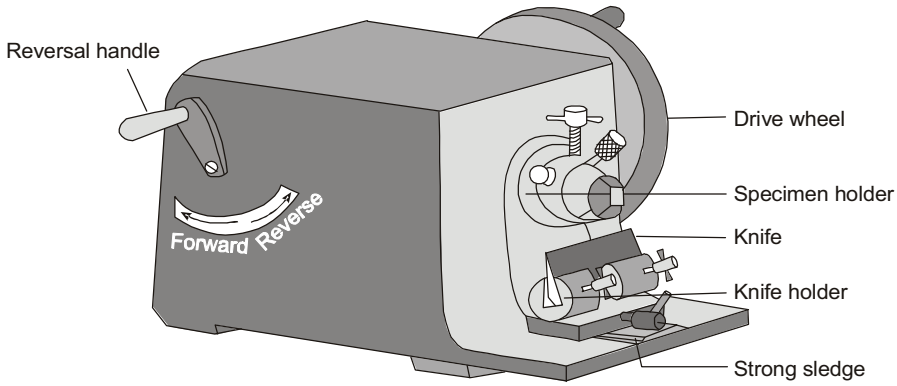


Figure 1.11 Rotary microtome

MICROSCOPE

Leeuwenhoek (1632–1723) of Holland seems to be the first person to observe microorganisms by constructing a simple microscope—a single tiny lens no more than a magnifying glass. For many organisms he had a separate microscope and his techniques of illumination are still a secret. He made about 419 microscopes.

The development of complex microscopes followed and Robert Hooke discovered cells for the first time through a primitive microscope he designed (Greek *micros* = a small skipsen to see). A microscope enables the size of retinal image of an object to be increased.

Magnification is the ratio of the enlarged image perceived to that formed on the retina of the unaided eye.

The light microscope has undergone evolution from the time of Leeuwenhoek. From a simple structure it has developed into what is called as compound microscope (Figure 1.12). It has more than one lens, the eyepiece lens and the objective lens. Light is allowed to enter from the face (though the objective) and enters a condenser which converges the light beam. An iris diaphragm is meant to control the amount of light passing through the specimen on the slide placed on the stage of the microscope. A mechanical stage allows manipulation in moving the slide. The objective magnifies the image before it is allowed to pass through the body tube to the eyepiece, the ocular lens.

The distance between the objective lens and slide with specimen can be changed as it allows the image to come into accurate focus by the adjustments.

1. The course adjustment knob
2. The fine adjustment knob.

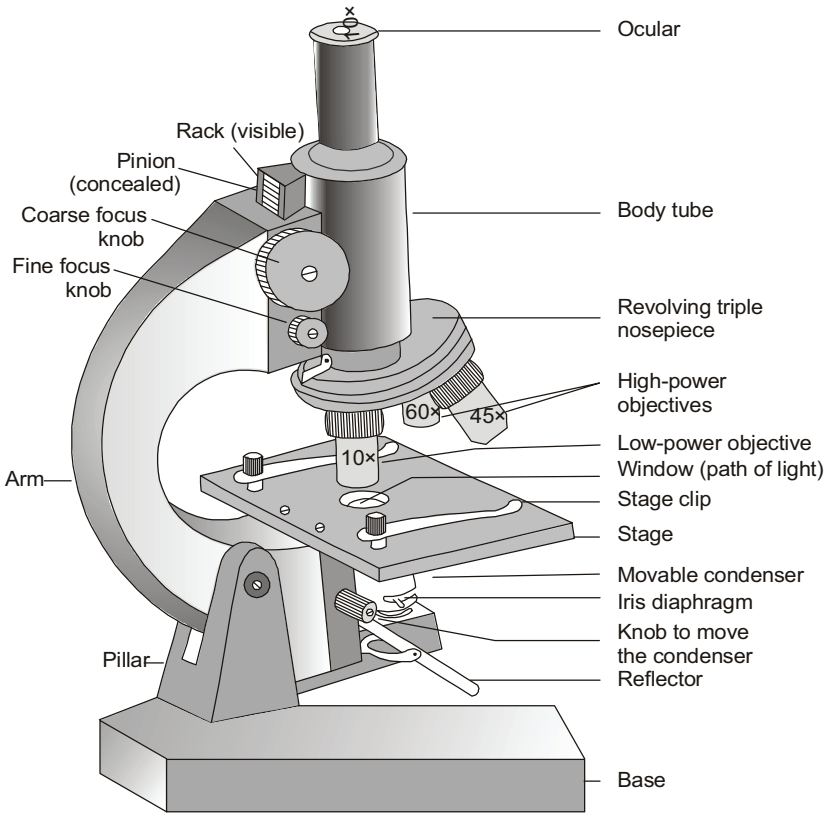


Figure 1.12 Compound microscope

In the coarse adjustment the specimen is brought into vision and the fine adjustment manipulation brings it into focus. Slowly a compound microscope is evolved which has interchangeable objectives 2×, 5×, 10×, 40× and 100× (different powers of magnification).

The eyepiece usually comes as 5× and 10× oculars and up to 30×. Changing of eyepiece or objective power may require refocusing. But most microscopes are now designed such that changing the objective will still keep the specimen in focus. This is called parafocal microscope magnification and resolving power.

Resolving power of the microscope or resolution is the ability to visually separate details of the specimen and it is not related to magnification.

$$\text{Magnification} = \frac{\text{Size of retinal image seen with the instrument}}{\text{Size of retinal image perceived with the unaided eye}}$$

The total magnification of the light microscope can be calculated by multiplying eyepiece (ocular) power with objective power.

Low power (3×) × (10×) = 30× magnification

(10×) × (3×) = 30 magnification

(10×) × (40×) = 400× magnification

Oil immersion – 10× × 100× = 100×

Measuring Micrometer (μm) previously called micron (μ) is equal to 0.00000 1 m = 10^{-6} m.

The second unit the nanometer (nm) was previously known as millimicron (mμ) is equal to 0.000000001 m – 10^{-9} m.

The third unit is Angstrom (Å). It is equal to 0.0000000001 m, 0.1 nm 10^{-10}.

A compound microscope with a single eyepiece (ocular) is called monocular. Most modern microscopes for an average laboratory are binocular microscopes with two eyepiece systems.

Dark field illumination, is necessary in some cases when specimens lack contrast with their background in a bright field.

In the case of some specimens like live spirochetes, spiral-shaped bacteria and larval stages of some organisms, the microscope has a condenser that prevents light from being transmitted through the specimen and causes the light to reflect off the specimen at an angle. When these rays are gathered together and focused, the object appears whitish opaque against a black background.

Condenser The correct usage of a condenser is important. The condenser and its iris diaphragm are not just for controlling the intensity of illumination. If the light is bright, the current should be reduced or a suitable filter must be used.

Phase-contrast Microscopy

Small organisms or cells may be difficult to observe in living condition. Stains like vital dyes which do not kill (neutral red or Janus green) may be useful sometimes. But to examine them unstained and alive, phase-contrast microscopy (Figure 1.13) is needed. In this microscope special condenser and objective lenses bring out differences in the refractive index of structures in the organism's cell, and the different degree of brightness of the parts enable us to perceive their nature.

In histochemical work only a small part of a cell may be stained, and a counterstain will be inconvenient. Phase-contrast is valuable in establishing the position of the stained structure in relation to the rest of the cell. One can determine the distribution of vital dyes or fix the cell and apply histochemical tests. Cell division and phagocytosis can be perceived.

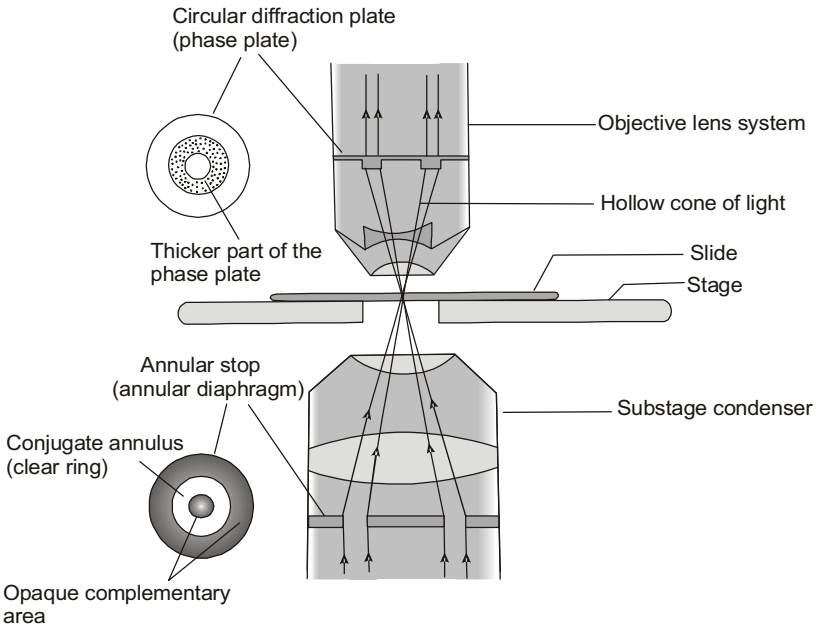


Figure 1.13 Phase contrast microscope

Interference Microscopy

With this microscope (Figure 1.14) a resolution higher than that of phase-contrast microscope is obtained. A short-depth field that is the thickness of the structure in focus at any given time can project a three-dimensional image.

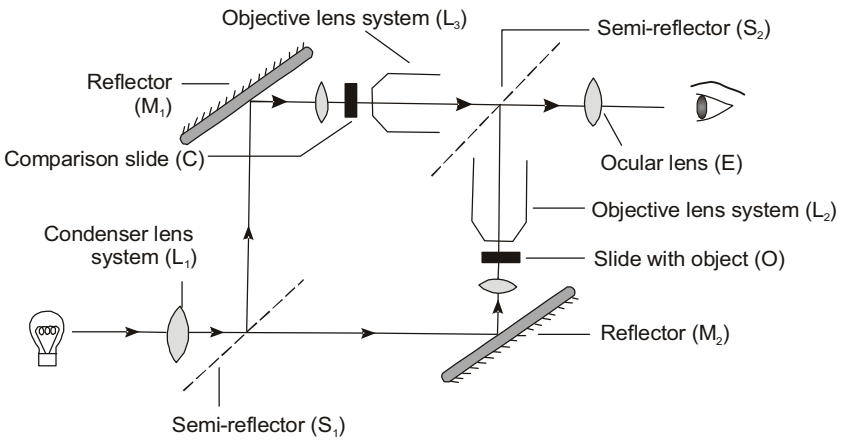


Figure 1.14 Interference microscopy

Whereas the phase contrast microscope has been a null method for the determination of refractive index. But it may be important sometimes to obtain knowledge of the optical path rather than refractive index. The development of interferometer microscope solved the problem.

The Smith Baker Microscopes

In this microscopy polarizing beam splitters are used. Birefringent plates are kept over the front of the condenser and objective. The condenser is illuminated with polarized light. A ray from the condenser is split into (1) an ordinary ray and (2) an extraordinary ray. The extraordinary rays are focused on the object and ordinary rays are laterally displaced and then brought to a focus.

The two sets of rays are brought to a common plane of polarization by inserting a rotatable polarizing sheet above the objective. Interference is brought about between a beam passing through the object and beam passing lateral to it.

Scanning Electron Microscope (SEM)

In scanning electron microscopy (Figure 1.15), electrons do not pass through the specimen but they are allowed to be emitted from the surface of the specimen. The specimen is fixed, dried and coated with a heavy metal (gold or platinum) and then scanned by a narrow beam of electrons. Only surface features can be examined. At first a primary beam of electrons scatters a quantity of electrons which is measured and from this the intensity of the second beam is manipulated moving in synchrony with the primary beam. An enlarged image of the surface is photographed and/or studied on screen.

Great depth of focus is achieved in SEM because the quantity of electrons scattered is related to the angle or surface of the beam. The result is a three-dimensional image. The SEM resolves objects close to 20 nm, the magnification going up to 50,000×.

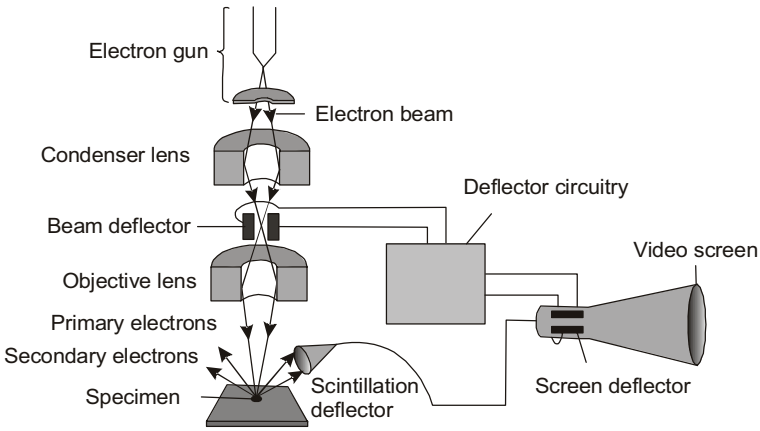


Figure 1.15 Scanning electron microscope (SEM)

Transmission Electron Microscope (TEM)

This is much larger than the light microscope and is upside down and is furnished with electromagnetic lenses. Electrons pass through the specimen to produce an image. The specimen is stained with electron-dense material. This enables some of the electrons to be scattered and some are focused to produce an image on a photographic plate or a phosphorescent screen. Because the scattered electrons are lost from the electron beam, the dense region of the specimen appears as areas of reduced electron flux and looks dark. The transmission electron microscope is shown in Figure 1.16.

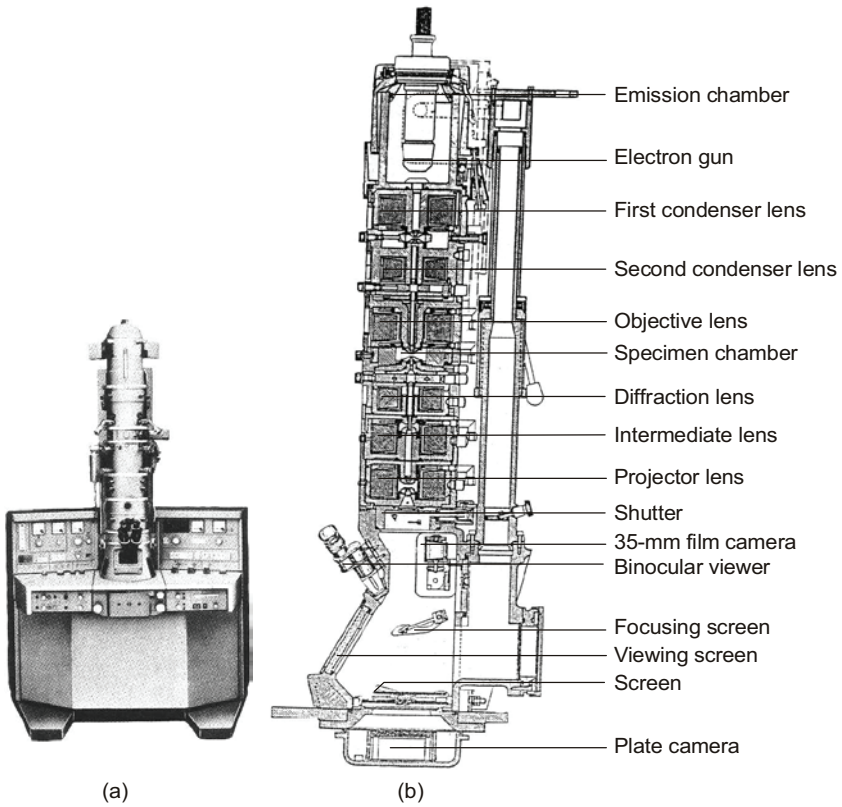


Figure 1.16 (a) Siemens Elmiscop 102 TEM (b) A high-resolution transmission electron microscope (a vertical sectional view)

Fluorescence Microscopy

A technique for localizing proteins within a cell is the fluorescent staining of cells and examining them with a fluorescence microscope (Figure 1.17). The dye absorbs light at one wavelength (excitation wavelength) and emits light fluoresce at a specific and longer wavelength. The dyes Rhodamine and Texas red emit red light. Cy 3 emits orange light. Usually a fluorescent dye–antibody complex can be designed for any specific macromolecule.

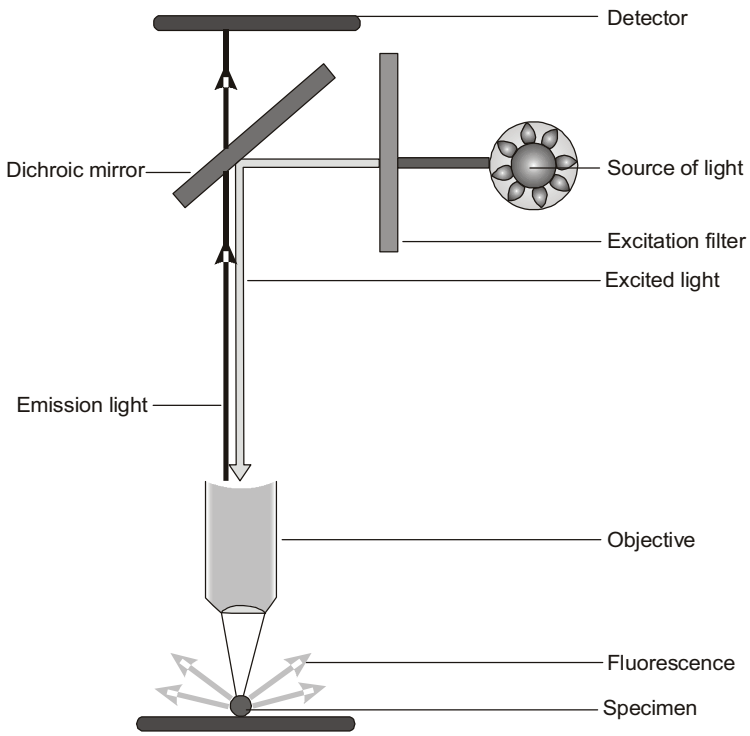


Figure 1.17 Schematic representation of the light path and the components in fluorescence microscopy

These complexes bind to the corresponding antibody which light up when illuminated by the exciting wavelength. This is the technique of immunofluorescence. Staining a specimen with two or three dyes that fluoresce at different wavelengths, multiple proteins in the cell can be localized.

Many substances such as drugs and carcinogens are strongly fluorescent. Their distribution in the body after administration can be traced. Fluorescent labelling of proteins and antibodies paved the way for detailed studies.

Confocal Microscopy

One can see fluorescent molecules in a single phase of focus. A small part of the sample is illuminated at any given point of time. The exciting light comes from a focused laser beam which rapidly moves to different spots. Images from different spots are recovered by a video camera and stored in a computer. The computer image can be displayed on a computer screen (Figure 1.18).

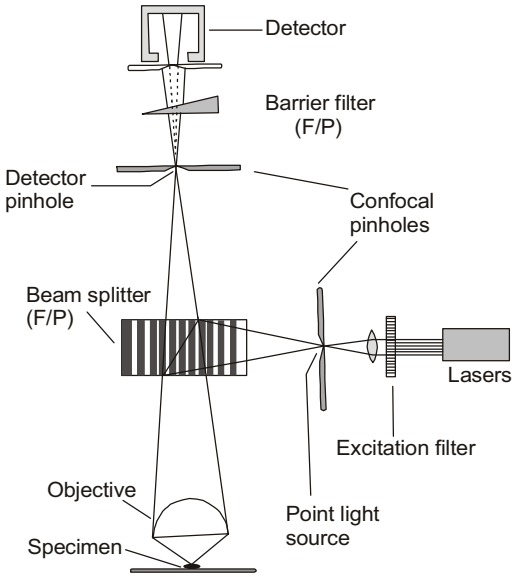


Figure 1.18 Optical path and working principle of confocal microscope

Deconvolution Microscopy

Confocal microscopes use a pinhole to exclude the out-of-focus light. The deconvolution microscopes collect all the light from several focal planes and mathematically reassign the out-of-focus light to its correct focal plane with a high-speed computer. This mathematical operation is known as deconvolution. Cross-sectional images rendered by a deconvolution microscope display greater details. The fluorescent labelling of the sample need not be intense. A three-dimensional image can be obtained through a computer which records individual images of planes at different depths of the sample and combines them all.

Ultraviolet Microscopy

Ultraviolet microscopy (Figure 1.19) as an imaging device has been generally ignored. Living cells may appear transparent in the visible range of the spectrum. However they absorb certain wavelengths in the infrared and ultraviolet ranges. Ultraviolet microscopy has received greater attention.

Proteins and nucleic acids were the targets of study. A series of photographs of the same cell at different wavelengths enable one to construct the absorption curve of a selected region of the cell. The absorption curve of cytoplasm shows only characteristics of a protein. This was the pioneering work of Casperson as early as 1936. Over the years the technique has been refined much. For example we have now the flying spot microscope. The basic principle is to use the scanned face of a cathode ray tube as light source, after passing through the specimen and condenser. It is used to reverse the direction of the light rays. The face of the cathode ray tube is placed above the eyepiece. The image of the moving spot is picked up by a photomultiplier. Now many modifications have been made.

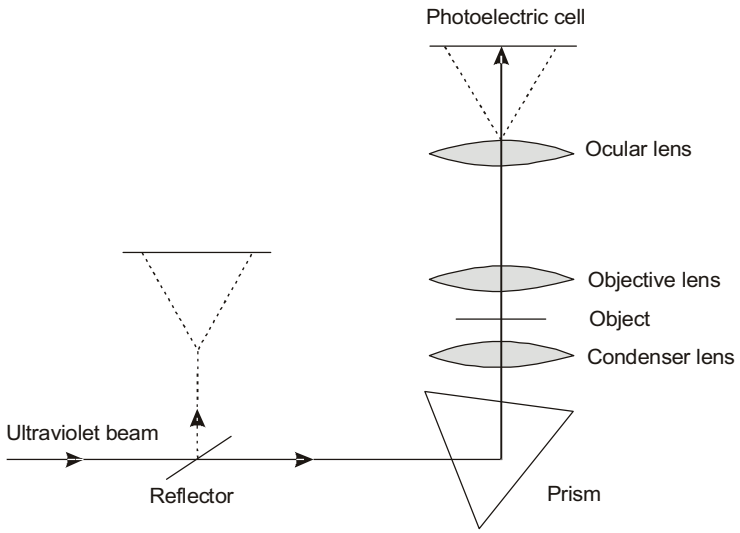


Figure 1.19 Schematic representatin of ultraviolet microscope

MICROPHOTOGRAPHY

Microphotography may broadly be defined as photography of live, fixed and stained biological material or any other type of material using the optics of a microscope. The camera suited for the purpose is fixed to the monocular tube of a microscope.

Even binocular microscope is always provided with a monocular which is ideal for microphotography.

An adapter can be utilized to fit on the camera to the monocular of the microscope. Any camera with its usual lens system removed can be used with appropriate adapters. It is best to use a camera with a shutter releaser of moderate length.

Films are of two kinds (a) black and white and (b) colour film (Kodak and Konica). For publications, usually black and white film is best unless the paper deals with histochemistry or a colourful small live animal. Usually black and white film of 200 ASA (moderate) is good. Colour film of 100 (Konica, etc.) is alright. Most often, the time of exposure with each type of film can be ascertained by practice or by guidance of an experienced colleague. For black and white film, it is customary to use filters placed at the place of emission of light. The choice of filters is determined by the contrast required which differs from sections to section and/or differently stained ones.

For instance, with iron haematoxylin/eosin sections, a red/orange filter combined with yellow will give sufficient contrast. To accentuate a region stained red, a blue filter may be necessary. This is only a concept. In practice sufficient experience is needed. This can be acquired by constant practice. An interplay of these filters may be necessary. For colour photography, filters are not necessary. At any rate there are special ones.

The aperture of the shutter of the microscope must be carefully adjusted. Cameras are provided with what is called the view finder from which one can know the extent/area of the section to be photographed. Accurate focusing has to be done and the light intensity has to be adjusted.

The time of exposure depends upon the extent of magnification. At lower magnification lesser time is needed. The shutter usually is adjusted to 'B' mark on the camera so that the shutter is open as long as one wants. This is the time of exposure.

The usual combinations used are eyepiece 5×, 10×, 15× and the objectives 2×, 10×, 40×, 100×. Appropriate combinations of eyepiece and objective must be chosen.

One important aspect of microphotography is measurement. Usually the eyepiece can be fitted with what is called ocular micrometer, which is a glass disc with a scale, say, divided into 100. The scale of the ocular has to be calibrated to know the exact measure of each division.

This is done for each combination of eyepiece × objective (5×10, 5×40, 10×10, 10×40, etc.) by calibrating with a stage micrometer where, say, 2 mm may be divided into 100 divisions. The eyepiece ocular scale impinges on the stage micrometer division, and measurements can be read off.

CAMERA LUCIDA

Camera lucida is a device (with prism and mirror arrangement, the prism part sits on the eyepiece and the mirror part is held at the end of horizontal device). The camera lucida is fitted to monocular so that the prism sits on the eyepiece. The mirror slanting is so adjusted that the entire view of the section is projected on the white sheet of paper placed adjacent to the microscope on the table. One can see the entire section projected as if on a screen on the white drawing paper. The ocular micrometer scale also can be screened thus. The ocular scale bar is drawn and measurement for each magnification can be indicated. All photographs must be accompanied by the appropriate scale bar (appropriate magnification) and the measurement indicated.

2

URINE ANALYSIS

Urine analysis is performed for the diagnosis of renal disorders, endocrine disorders (hormonal, diabetes), genetic abnormalities, pregnancy, parasite infection (*Schistosoma haematobium*), jaundice.

A normal person discharges 1500 ml of urine in a 24-hr period. Urine of a normal person is pale yellow in colour with a characteristic aromatic odour due to the presence of volatile organic acids. Freshly passed urine is slightly acidic (pH 6.0) which on standing becomes alkaline due to the release of ammonia from urea. Decomposition of urea occurs due to the release of urease by bacteria.

Urine contains 95% of water, the rest constituted by the soluble waste products—urea, uric acid, creatinine and excess of electrolytes (sodium, potassium, chlorides, calcium, phosphoric acid). The chemical composition of urine varies widely depending on the food and fluids. Normal urine after cooling becomes crystalline. Certain chemicals like urates, phosphates, leucocytes and epithelial cells are occasionally seen in normal urine but their increase in number may indicate a pathological state.

Normal urine contains a few erythrocytes (RBC), leucocytes (WBC), epithelial cells and crystals.

ELEMENTS FOUND IN URINE

Red blood cells (erythrocytes) These could be identified under high-power microscope. They are round, small, with crenated biconcave margins and without a nucleus.

By addition of 2 per cent acetic acid, they may be haemolysed. If RBC is present, the condition is called haematuria. If the RBC are analysed and haemoglobin is released into the urine then this condition is termed haemoglobinuria. This may be due to anaemia, incompatible blood transfusion, etc.

White blood cells or leucocytes They have a nucleus, margins are uneven and bigger in size than RBC and they do not lyse by the addition of 2 per cent acetic acid. An increase in WBC in urine may be attributed to renal infection either in the urinary bladder (crystals) or in the kidney (pyelonephritis).

Epithelial cells These are larger than both WBC and RBC. They have a nucleus and sides are angulated. If squamous cells are present, the condition is quite normal but if there is increased number, it indicates renal degeneration. The presence of transitional cells and renal tubular cells indicates the requirement for further medical examination.

Casts Usually their presence is attributed to renal disorders and is often associated with proteinuria. These casts are formed from gelled mucoproteins and are cylindrical with two parallel sides. Identification of the casts help in assessing severity of the renal disorder.

Hyaline casts These casts are as transparent as glass, cylindrical with parallel walls and ends are square-shaped. They dissolve rapidly in alkaline urine. Few hyaline casts are of no serious problem, but increased number indicates a mild damage in the kidney. When hyaline casts are covered by granules, they are known as granular casts. These are nothing but disintegrated products of cells trapped into protein matrix. Presence of granular casts indicates the pathological sign of kidney dysfunction. Protoneuria (2+ or more) when determined by chemical tests with granular cells in the sediment may indicate chronic nephritis. Wax and fatty casts have a transverse split, are yellowish in colour, and are always associated with renal disease. Waxy casts are easily recognizable and they have a high refractive index. This is the end product of cellular degeneration. Waxy casts in urine sediment indicate a serious pathological condition resulting in decreased urine flow.

Oval fat bodies Renal tubular epithelial cells which undergo fatty degeneration form oval fat bodies. The very presence of the fat globules and fatty casts suggests a serious pathological condition (nephritic syndrome) due to diabetes. These oval fat bodies cannot be recognized easily with crystal violet or methylene blue. A good method is to add a fat stain such as Sudan III or Sudan IV which colour the fat droplets red. Very often lipuria is accompanied by proteinuria.

Microorganisms Milky colour of the urine may be due to the presence of pus cells and bacteria. Possibilities of the presence of inorganic compounds of calcium and phosphate can be easily eliminated. If the presence of bacteria is suspected, Gram-staining can be carried out. If gram-negative bacilli are seen, it may be *Escherichia coli, Pseudomonas* or *Proteus*. The gram-positive bacterium which causes kidney infection is *Streptococcus pyogenes*. Laboratory culture of bacteria will further help in identification of pathogenic bacteria.

Yeast cells and *Candida* are seen in patients suffering from decreasing defence mechanisms mostly in old people under steroid treatment and diabetic patients. Yeast cells are similar to RBC, they are not lysed by 2 per cent acetic acid. If there are moving objects in the wet mount of urine sediments, presence of *Trichomonas vaginalis* is indicated. This is found in females and is the main cause of vaginitis. They are of rare occurrence in male patients. Ova of *Schistosoma haematobium* are rarely seen. This schistosome

(blood fluke) lives in the veins around the bladder and lays eggs that pass through the side of the bladder into urine. (*See* Chapter 5 for schistosome figures and description.)

URINOGENITAL ELEMENTS

Deposits of crystalline elements or unorganized elements of urinary sediments are constituted by inorganic substances. Generally crystals are not seen in fresh urine but appear after cooling. Some crystals are of chemical importance, e.g., cystine, tyrosine, leucine and sulphonamide.

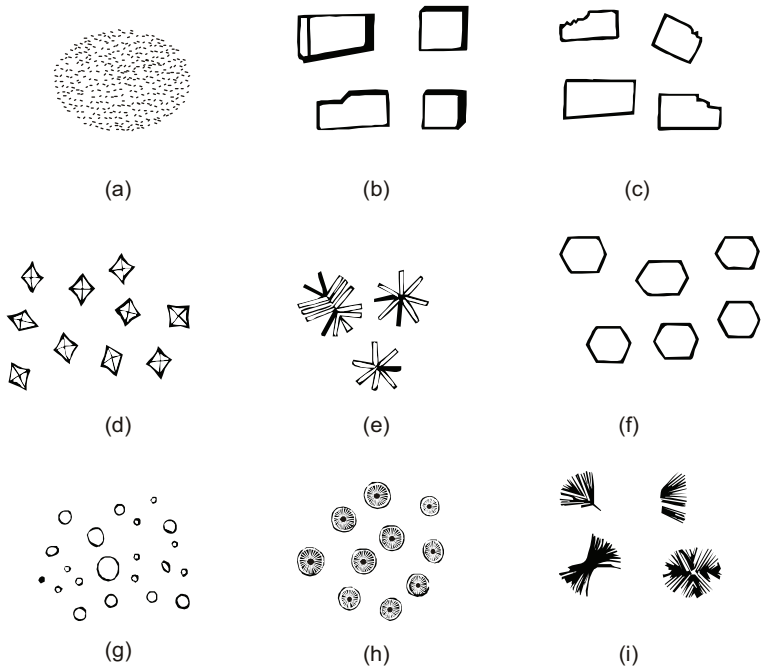


Figure 2.1 Crystals found in acid urine (a) Amorphous urates (b) Uric acid (c) Cholesterol (d) Calcium oxalate (e) Sodium urate (f) Cystine (g) Fat droplets (h) Leucine spheres (i) Tyrosine needles

In acid urine (Figure 2.1), calcium oxalate, uric acid and rate crystals are commonly expected. Calcium oxalate crystals clump together to form stones. Fresh urine does not contain uric acid and urate crystals. But they appear when urine is allowed to stand for some time. Excess deposits of uric acid and urate crystals may occur when there is slight change in uric acid metabolism. Calcium oxalate crystals appear as dumb-bells. They are insoluble in concentrated hydrochloric acid. Sodium urate and uric acid crystals are yellowish in colour and neither dissolve after heating with sodium hydroxide nor when treated with acetic acid or hydrochloric acid. The other crystals are those of sulphonamides, if patients are on sulphur drugs.

Common crystals found in alkaline urine (Figure 2.2) are phosphates, calcium carbonate and ammonium urate. Phosphate crystals are of different kinds—triple phosphate (amorphous), phosphate

(powdery), calcium phosphate crystals. Calcium phosphate crystals are large without a definite shape without any angular margins.

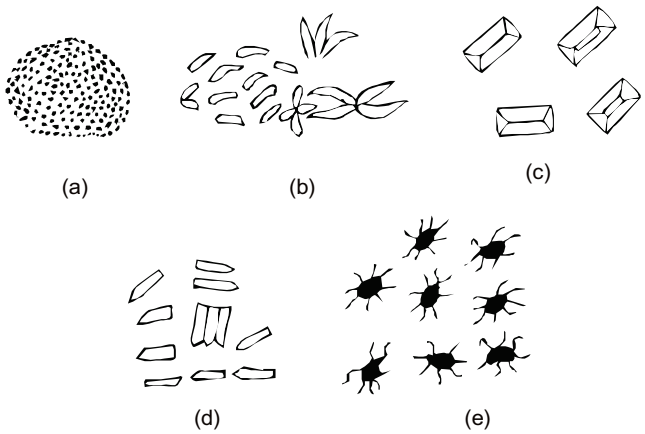


Figure 2.2 Crystals found in alkaline urine (a) Amorphous phosphate (b) Calcium carbonate (c)Triple phosphate (d) Calcium phosphate (e) Ammonium urate

Cystine These are identified by their hexagonal plates and are soluble in hydrochloric acid but not in acetic acid. Urine turns red in the presence of sodium nitroprusside.

Leucine and tyrosine These crystals are together found in patients with liver damage. Leucine crystals are oily spheres that are light yellow in colour, and sometimes cluster together. They are not soluble in hydrochloric acid. Tyrosine crystals have the shape of needles with a deep constriction in the middle. They may appear black. They are insoluble in acetic acid but dissolve in hydrochloric acid and ammonia.

CLINICAL TESTS FOR URINE

Glucose is not a normal constituent of urine. If it is present in overnight fasting urine, it indicates a pathological condition often associated with diabetes mellitus.

DETERMINATION OF GLUCOSE IN URINE

Benedicts Test

Reagents required

Solution 1

Crystalline copper sulphate	17.3 g
Distilled water	100 ml

Dissolve copper sulphate in distilled water.

Solution 2

Sodium carbonate	100.0 g
Sodium citrate	173.0 g
Distilled water	700 ml

Take these ingredients in a 1 litre flask and dissolve while stirring. If necessary, heat is applied until they are dissolved. Allow the solution to cool before mixing with solution 1.

Solution 3

Take a 1 litre flask, pour solution 1 containing solution 2. Mix well and make it to 1000 ml with distilled water. This solution lasts indefinitely. This solution is called alkaline copper reagent or Benedicts reagents.

Procedure

1. Arrange test tubes one for control, one for experimental.
2. With a pipette take 8 drops of urine into a test tube.
3. Add 5 ml of Benedicts reagent (solution 3) to this.
4. Mix well and place in a water bath for 5 min. Both time and temperature of the bath are critical.
5. Remove from the boiling water bath, cool to room temperature in a cold water bath for 5 min.
6. A positive reaction depends on the presence of a fine yellow, orange or brick red precipitate.

Results

The following indicate a negative result for glucose.

1. No change in the colour of the reagent (blue).
2. White or green precipitate formation from phosphates in urine.
3. Alteration of colour of the reagent without any precipitate formation.

The following indicate presence of glucose.

1. Greenish yellow precipitate of the mixed solution (approximately 0.5 g / dl of sugar)—1+
2. Large amount of yellowish green precipitate (0.75 g/dl of sugar)—2+
3. Orange precipitate. Some blue colour remains in the supernatant (1.0 g/dl of sugar)—3+
4. Large amounts of yellow, reddish yellow to red precipitate of the mixed solution. No blue remains in the supernatant (approximately 2.0 g/dl of sugar)—4+

DETERMINATION OF LACTOSE

Lactose is present in urine of lactating women. It is temporary and disappears when lactation ceases. In 3–5-day babies, there is deficiency in the enzyme· lactase, and their digestive system is not fully developed. Hence there is appearance of lactose in the urine.

Children and adults who are deficient in intestinal lactase contain lactose in the urine.

Osazone Method

Procedure

1. Take 5 ml of urine in a test tube.
2. To make it acidic, add few drops of glacial acetic acid.
3. To the test tube, add 1 g of a mixture of sodium acetate (2 parts) and phenyl hydrazine hydrochloride (1 part).
4. Place the tube for 30 min. in a boiling water bath.
5. Then cool it in a beaker containing tap water.
6. Take a small amount of deposit on a slide, put coverslip and study under a microscope.

Note: Sucrose does not react with phenyl hydrazine hydrochloride and also with the reducing reagents such as 'Benedicts' reagent (since it is not a reducing sugar).

DETERMINATION OF GALACTOSE

Galactose is present in the urine of children who are deficient in the enzyme which converts galactose into glucose. This deficiency is called galactosemia. By eliminating lactose and other sources of glucose in the diet this condition can be corrected. If it is not corrected in time the infant is likely to become mentally and physically deteriorated.

Orthotoluidine Test

Procedure

1. Take 5 ml of orthotoluidine reagent in a test tube.
2. To this add 5 ml of urine.
3. Place the tube in a boiling water bath for 10 min.
4. Observe the colour.

Results

No green colour, i.e., if original colour of the reagent (pale yellow) is still present, galactose is absent. If it turns green, galactose is present.

DETERMINATION OF FRUCTOSE

Fructose is present in urine of patients suffering from hepatic disorders.

Seliranoffs Test

Seliranoffs reagent is prepared by dissolving 50 mg of resorcinol in 33 ml of concentrated hydrochloric acid and diluted to 100 ml with distilled water.

Procedure

1. Take 5 ml of Seliranoffs reagent in a test tube.
2. To this add 0.5 ml of urine.
3. Place the tube in a boiling water bath for 5 min.
4. Observe the colour.

Results

No change in colour indicates that fructose is absent.

Colour change to red shows that fructose is present.

DETERMINATION OF KETONES

Where there is deficiency in carbohydrates or defect in carbohydrate metabolism, the body metabolizes increasing amounts of fatty acids. As a result ketone bodies increase in blood.

Rothera Test

The principle behind this test is that nitroprusside used in the test will react with both acetone and acetoacidic acid in the presence of alkali (ammonium hydroxide) to produce a purple-coloured compound.

Reagents required

Rothera's powder mixture

Sodium nitroprusside	0.75 g
Ammonium sulphate	20 g

Take the ingredients in a wide-mouthed flask and mix the ingredients thoroughly at room temperature (25ºC ± 5ºC).

Procedure

1. Take 5 ml of urine in a test tube with a pipette.
2. Add 1.00 g of Rothera's powder and mix well.
3. Add 1–2 ml of concentrated ammonium hydroxide from the sides of the tube.
4. Observe for pink–purple ring.

Results

No pink–purple ring	Ketone bodies absent.
Presence of a pink–purple ring	Ketone bodies present.

Note: Grade the results according to the intensity of the formation of ring as trace, + + + + + + and + + + +.

Legal's Test for Ketone Bodies

Take 10 ml of urine in a test tube and to it add few crystals of sodium nitroprusside. Acidify with glacial acetic acid. Overlay with strong liquor ammonia. Allow it to stand for 5 min. A violet ring indicates a positive test. The degree of positivity depends on the speed of the reaction.

Dunn and Shipley's Method

This is a simple method for qualitative determination of ketone bodies.

Reagents required

Sodium nitroprusside	1.0 g
Ammonium sulphate	20 mg
Anhydrous sodium carbonate	20 mg

Take the ingredients in a mortar and make it into a fine powder with the help of a pestle.

Procedure

1. Take two test tubes and label them as 'T' and 'C'.
2. Add a pinch of powdered mixture to both the tubes.
3. To the tube marked 'T' add a drop of urine.
4. To the tube marked 'C' add a drop of distilled water.
5. After 5 min., observe the colour of the reaction mixture.

Results

No violet colour in 'T'	Ketone bodies absent.
Violet colour in 'T'	Ketone bodies present.

No violet colour is produced in tube C. Colour produced in tube 'T' should be compared with that of tube 'C'.

TESTS FOR PROTEINS

Urine generally contains 2.8 mg of proteins. Their increase to 150 mg indicates that filtration of urine in the kidney is improper and the condition is termed as proteinuria.

Heat Test for Proteins

Reagents

3 per cent acetic acid (V / V)

Glacial acetic acid 3 ml

Distilled water 97 ml

Procedure

1. 10 ml of urine specimen is centrifuged. Supernatant is poured into a test tube. Sediment is used for microscopic examination.
2. Heat the supernatant. Care should be taken not to heat the bottom of the tube. Only upper part of the tube is heated.
3. If no cloudiness appears, it is a negative report.
4. If cloudiness appears remove the test tube from flame and add 2 or 3 drops of 3 per cent acetic acid and heat again. If the cloudiness disappears report as negative. If cloudiness still persists, it is positive for protein.

5. Degree of cloudiness can be graded as dissolution of precipitate indicates the presence of phosphates and carbonates. If it persists even after reaching the boiling point, it means protein-positive with albumin discharge.

Sulphosalicylic Acid Test

Sulphosalicylic acid precipitates any protein in the urine albumin or Bence–Jones protein. It is more sensitive and reliable than heat test.

Reagents

5% sulphosalicylic is obtained by mixing 5.0 g of sulphosalicylic acid in 100 ml distilled water.

Procedure

1. Centrifuge urine (1–2 ml) in a tube and to this add equal amount of sulphosalicylic acid.
2. Shake the tube and allow it to stand for 10 min.
3. Note degree of turbidity by looking at the illuminated tube.

Results

Grade and report the results as follows:

No turbidity or no increase in turbidity	Negative
Faint turbidity (0.010 g/dl)	Trace
Distinct turbidity, no granulation Co.050 g/dl	1 +
Turbidity with granulation but no flocculation (0.20 g/dl)	2 +
Turbidity with granulation and flocculation(0.50 g/dl)	3 +
Clumps of precipitate (1.0 g/dl)	4 +

Heat Test for Bence–Jones Protein

Presence of Bence–Jones proteins indicates the presence of multiple myeloma.

Bence–Jones proteins coagulate when heated to 45°–55°C and dissolve on boiling. Albumin coagulates above 60ºC and does not redissolve on boiling.

Reagents

33 per cent acetic acid (V / V)

Procedure

1. Centrifuge the urine specimen. Filter the supernatant. Check the pH. Add a few drops of dilute acetic acid if necessary.
2. Place 5 ml of urine specimen in a test tube.
3. Place the tube in a beaker of water and heat it, slowly raising the temperature. Keep a thermometer inside the tube and watch the temperature. If cloudiness appears at 40°C and 60°C it is Bence–Jones protein.
4. Boil the beaker to 100ºC. Bence–Jones protein will dissolve.
5. Filter the urine while hot. Albumin will coagulate above 60ºC.

6. Allow the water to cool. Urine specimen in the presence of Bence–Jones protein will cloud up again between 40°–60° C.

DETERMINATION OF ACETONE AND ACETIC ACID

Nitroprusside Test

Modification of Rothera's nitroprusside test is a qualitative test for acetone and acetic acid.

Reagents

Sodium nitroprusside	3.0 g
Ammonium sulphate	100.0 g
Sodium carbonate (anhydrous)	50.0 g

Crush the crystals of sodium nitroprusside into powder. To the powder add ammonium sulphate and sodium carbonate. Keep the powder in a screw-capped jar. It will last for one year.

Procedure

1. Take a small quantity of (1.0 g) above-mentioned powder in a test tube.
2. Add 3 drops of urine to moisten the powder.
3. Hold the tube against a white background.
4. If the colour changes to pink or violet, it indicates the presence of acetone and acetoacetic acid. A heavy quantity of acetone in the urine will result in the rapid formation of purple colour.

Results

No colour change	Negative
Slight purple colour	Traces
Deep purple colour	2+ to 3+
Rapid formation of purple colour	4+

DETERMINATION OF BILIRUBIN

Bilirubin is normally not found in urine. Presence of bilrubin in urine is detected before the clinical symptoms of jaundice.

Fauchet Test

Fauchet reagent

Trichloroacetic acid	25.0 g
Distilled water	50.0 ml
10 per cent ferric chloride	10.0 ml

Place them in a 100 ml volumetric flask. Let it dissolve, and transfer to polythene bottle.

Procedure

1. Check the pH of urine, it should be slightly acidic (lower than pH 7.0).
2. Take 10 ml of centrifuged urine. To that add 25 ml of 10 per cent barium chloride, mix well. It will become a milky white or yellow precipitate.
3. Filter the precipitate through a small filter paper.
4. When it is filtered, carefully unfold the paper on a dry filter paper and place both on a white tile.
5. Note the colour of the precipitate.
6. Add one drop of Fauchet's reagent on to the precipitate. If bile is present, a green or blue colour develops.

Ehrlisch's Test

Ehrlishch's reagent

Hydrochloric acid	20 ml
Distilled water	80 ml

Mix and add,

p-dimethyl aminobenzaldelyde 2 g

Stir the solution until aldehyde is dissolved. Add 500.0 g of sodium acetate to 500 ml of distilled water in a graduated beaker. Stir and heat the solution to 60ºC. After cooling transfer to a bottle.

Procedure

1. Add 1ml of Ehrlischs reagent to 10 ml of fresh urine in a test tube.
2. Warm the tube gently.
3. Watch colour change. If a rose colour appears, it indicates the presence of bilinogen. Proceed for the confirmatory test, i.e., mix 2 ml of urine with 2 ml of Ehrlishchs reagent. The immediate appearance of red colour indicates the presence of phosphobilinogen.
4. For confirmation add 4 ml of saturated aqueous solution of sodium acetate and mix well.
5. If the colour still persists presence of urobilinogen is suspected. If the colour fades, it is probably phosphobilinogen.

DETERMINATION OF BLOOD IN URINE

Blood is found in the form of RBC or when haemolysed, in the form of haemoglobin. Presence of RBC is detected by microscopic examination of the urinary sediment and this condition is called haematuria. Discharge of haemoglobin in urine is associated with renal lesions.

Benzidine Test

Reagents

Solution 1

Saturated solution of benzidine in glacial acetic acid (4 g to 100 ml). This solution lasts for weeks.

Solution 2

Hydrogen peroxide (3per cent V /V in distilled water). Prepare fresh.

Procedure

1. Mix equal parts of solution 1 and 2 in a test tube just before use.
2. To this add 2 ml of urine (previously boiled and cooled to avoid positive reactions).
3. The appearance of a green or blue colour within 5 min., indicates the presence of blood.

Results

Faint green	Traces
Green	1+
Greenish blue	2+
Blue	3+
Deep blue	4+

DETERMINATION OF BILE SALTS

Bile salts, when present, lower the surface tension of urine, and when sulphur powder is added on the surface of urine, sulphur particles sink to the bottom of the test tube. In the case of normal urine, sulphur particles float on the surface of the urine.

Procedure

1. Place about 10 ml of urine in a test tube.
2. Sprinkle dry sulphur powder on the surface of the urine.
3. Observe the sulphur particles.

Results

Sulphur particles sink to the bottom	Bile salts are present.
Sulphur particles float	Bile salts are absent.

DETERMINATION OF UROBILINOGEN

Procedure

1. Place 3 or 4 ml of urine in a centrifuge tube.
2. Add equal amount of 10 g/dl barium chloride. Mix well.
3. Centrifuge at 1500 rpm for 10 min. Then filter.
4. Take supernatant in another tube for urobilinogen test.
5. Add one or two drops of Fauchet's reagent to the sediment.
6. Add about 0.5 ml of Ehrlishch's reagent to the supernatant.

Result

In sediment

No change in the colour	Bile pigments absent.
Colour change to green	Bile pigments present.

In supernatant

Development of pale pink colour	Urobilinogen present.
Development of cherry red colour	Urobilinogen increased.

3

STOOL EXAMINATION

Faecal specimens should be collected in clean, wide-mouthed containers most often in a wax cardboard container with a tight lid. The specimen should not be contaminated with water or urine which may destruct the motile organisms. A minimum of three samples should be submitted. For accuracy especially in the case of amoebiasis, at least six samples should be provided to the laboratory. Collection of specimens on alternate days will give good results. Liquid specimens should be examined within 30 min. of passage or it should be preserved in polyvinyl alcohol (PVA). Of the PVA, SAF later gives excellent results. Stained smear gives an accurate examination of intestinal protozoa. In some laboratories copper sulphate is the component that is tried most frequently but does not yield expected results. Many laboratories try to avoid mercury compounds, but substitute compounds may not yield good results on preservation for protozoan morphology especially in a permanent stained smear.

PVA FIXATIVE

This is the most excellent and highly recommended for preserving protozoan cysts and trophozoites (Brooke and Goldman, 1949). PVA is a combination of modified Schaudins fixative and a water-soluble resin. The ratio of fixative to faecal matter should be 3 : 1, three parts of preservative to one part of faecal matter. This fixative keeps for several years. It can be either prepared in the laboratory or can be purchased. Protozoan, helminth eggs, and larvae could be used for helminth eggs which will prevent further development. The ratio of fixative to faecal matter for formalin fixative is the same as for PVA (3 : 1). This fixative is used for protozoan, helminth eggs and larvae. It is especially good for helminth eggs as this will prevent further development.

MIF SOLUTION

Merthiolate–iodine–formalin solution of Sapero and Lawless (1953) is good for helminth eggs, larval stages and also for some protozoans. However there are certain disadvantages with this fixative, since the iodine part in the fixative is not soluble.

SAF SOLUTION

This fixative is a combination of formalin and sodium acetate. It is a liquid fixative. The difficulty with this fixative is that the material may not adhere to the slide. So Mayers albumin is recommended which serves as an adhesive. The fixative furnishes best results in recombination with haematoxylin stain rather than trichrome stain.

Consistency of the stool may indicate the type of parasite or stage of the life cycle. Trophozoites are rarely found in liquid stools, cyst stages are found in formed or semi-formed specimens and rarely in liquid stools. *Ascaris lumbricoides, Enterobius vermicularis,* Tapeworm proglottids are rarely seen in the surface of the stool whereas *Hymenolepis nana, Trichuris trichiura* and hook worms are found. Blood in stool may indicate certain facts. High bleeding in stool indicates bleeding in lower bowel. Amoebic infection is certain in a mucous and bloody stool.

CLINICAL TESTS FOR STOOL

DIAGNOSTIC PROCEDURES

Direct Smear

Mix 2 mg of faecal material with a drop of physiological saline. This is in the form of uniform suspension under 22 × 22 mm coverslip. These smears are useful in detecting mobile trophozoite stages of Protozoa, helminth eggs and larvae. Helminth eggs, larval and protozoan cysts are also seen in the wet forms. A drop of iodine is placed at the edge of the coverslip. A weak iodine solution is recommended, or Lugols Dobell O'Conors and Autonis are also suggested. Gram's iodine often used for microorganisms is not used. Protozoan cysts stained with iodine reveal golden yellow cytoplasm, brown glycogen and pale nuclei. Chromatoidal bodies are not clear.

Buffered methylene blue is very effective at low pH (Nair,1953). It shows nuclear details. It stains cytoplasm in a pale blue shade and nuclei are dark blue.

1. *Concentration procedures* When faecal matter is directly mounted on a slide, it does not give a clear picture of the parasitic organisms present in the gastrointestinal tract. To overcome this, concentration procedures are adopted and a good number of procedures are available. Concentration procedures will enable us to identify cysts of *Giardia lamblia, Entamoeba histolytica, Entamoeba coli and Iodamoeba beutchlii,* but trophozoites could rarely be seen in concentration procedures. So to overcome this, a number of procedures are available such as floatation or sedimentation procedures. Floatation procedure permits the

separation of protozoan cysts and some helminth eggs, with the help of a liquid with high specific gravity. Parasites come to the surface and debris will remain at the bottom. This technique yields a clearer picture than the sedimentation procedure. However some dense eggs such as *Ascaris* egg (unfertilized), some helminth eggs, and some protozoa do not concentrate well with floatation method. Sedimentation procedure may give good result of protozoan eggs and larvae, but the sediment contains more faecal debris.

Zinc Sulphate Floating Procedure

In this method some helminth eggs and parasitic cysts float to the surface of a liquid with high specific gravity like zinc sulphate.

Procedure

1. Faecal suspension with half teaspoonful of faecal matter in 10–50 cc tap water.
2. Filter this suspension into a tube through two layers of gauze. Fill the tube with tap water up to 3 mm below the top and then centrifuge for one min. at 500 xg.
3. Decant, fill the tube with water and resuspend and centrifuge for 1 min. at 500 xmg.
4. Decant the water, add 2–3 ml of zinc sulphate solution resuspend the sediment, and fill the tube with zinc sulphate solution.
5. Centrifuge for two min. at 500 xg.
6. Without taking out the tubes from the centrifuge touch the surface of the suspension with a loop and then add the material on the loop onto a slide.

Result

Parasites should be detected.

Sedimentation procedure In this procedure, eggs, larvae and cysts are fixed in formalin. Morphological features are well-preserved.

FORMALIN–ETHER SEDIMENTATION TECHNIQUE

Procedure

1. Mix ½ teaspoonful of faeces with 70 ml of 10 per cent formalin, allow it to stand for 30 min. to fix properly.
2. Filter this suspension through two layers of gauze into a centrifuge tube.
3. To this add physiological saline up to ½ inch of top and then centrifuge for one min. at 500 xg.
4. Decant, resuspend the sediment in saline and centrifuge for one min. at 500 xg.
5. Decant and resuspend the sediment in 10 per cent formalin.
6. Add 3 ml of ether, stopper and shake vigorously.
7. Centrifuge for 3 min. at 500 xg. There are four layers in the tube. One layer is sediment at the bottom containing parasites, a layer of faecal matter on the top and a layer of the ether above this. Decant all fluid and prepare a wet mount.
8. Examine the slide.

Permanent Stained Smears

With permanent stained smears, intestinal protozoa can be easily detected and identified. Smaller protozoan organisms are often seen in the stained smear. Permanent stained smear is recommended for stool sample for routine examination. A good number of staining techniques are in vogue. The most commonly used method is iron–haemotoxylin method. Of late, trichrome stain is preferred in many laboratories.

Preparation of Fresh Material

As soon as the specimen arrives, take an applicator stick or brush to smear a small amount of stool on a clean slide and immerse it in Schaudins fixative and fix for 30 min. On the other hand if it is a liquid stool mix 3 or 4 drops of PVA with one or two drops of faecal matter on a slide and smear and allow it to dry at 35°C for several hours.

Preparation of PVA-Preserved Material

The material should remain in fixative at least for 30 min. After fixation the sample should be mixed and small amount of material should be poured on to a paper towel to absorb excess PVA. With an applicator stick or brush apply the stool material on to a slide and allow to dry at 37°C overnight. Then the smear is stained with trichrome stain (Gomori, 1950). This stain is good for both fresh and PVA-preserved material. This stain enables to distinguish cysts and trophozoites. When material is overstained, it can be destained in 90% alcohol which is acidified. With trichrome, the background debris is green, protozoans with blue-green to purple cytoplasm and red or purple-red nuclei and inclusions. Helminth eggs and larvae stain dark or purple.

With iron haemotoxylin stain, the background, the debris and organisms stain blue-grey to black. Iron haemotoxylin methods introduced by Spencer and Monroe (1976) and Tomkins and Miller (1947) are good.

The latter technique requires phosphotungstic acid as a destaining reagent.

Reagents required chromotrope 2R light green phosphotungstic acid glacial acetic acid.

Preparation of Trichrome Stain

Materials required

Chromotrope 2R	600 mg
Light green FCF	300 mg
Phosphotungstic acid	770 mg
Glacial acetic acid	1.0 mg
Distilled water	100 ml

Add the ingredients to glacial acetic acid in the following order—chromotrope 2R, light green and phosphotungstic acid. Allow it to ripen for 15 min. and then add 100 ml of distilled water.

Procedure

1. Prepare PVA-fixed faecal smear.
2. Keep in 70% ethanol for 5 min.

3. Again keep in two changes of 70% alcohol for 5 min. each.
4. Repeat the previous step.
5. Transfer to trichrome stain for 10 min.
6. Place in 90% ethanol acidified with acetic acid for 3 sec.
7. Differentiate in two changes of 100 per cent ethanol for 5 min.
8. Place in two changes of xylene or toluene for 5 min.
9. Mount in paramount.

Result

Debris	green
Protozoans	blue-green
Nuclei	red
Helminth eggs and larvae	dark red or purple

Modified Acid Stain for *Cryptosporidium* sp.

A number of stains are in vogue for identification of *Cryptosporidium* in humans, but the best is cold-modified acid-fast which gives best results. In patients with cysts in the stool, the highly specific and sensitive fluorescent method using a monoclonal antibody reagent reveals the presence of organisms.

Additional Technique for Gastrointestinal Specimens

Sometimes faecal examination fails to reveal the presence of *Entamoeba histolytica* despite repeating the procedure several times, then the material has to be obtained by sigmoidoscopy. Material from the mucosal surface has to be obtained either by aspiration or scraping with swabs and it has to be examined immediately so that moving trophozoites could be detected. There is a chance of microphages being mistaken for protozoans. For the preparation of permanent smear, the smear should be fixed in Schaudins fixative. Allow the smear to dry before staining, then the slide could be stained by any routine stain used for smear.

Faecal smears sometimes fail to reveal *Giardia lamblia* and *Strongyloides stercoralis,* the duodenal parasites. To detect this, stool specimen should be examined in an hour without using any preservative. Specimen should be centrifuged and the sediments should be examined as wet mounts to detect motile organisms.

Estimation of Worm Burdens

In certain helminth infections that have little clinical significance and as a result patient is not given treatment. If the number of the patient is small hence the parasites burden has to be estimated and this can be achieved by counting the number of eggs, passed in the stool.

The detection of egg count technique (Stall and Husheer 1926) has been widely used to estimate the number of adult worms especially *Ascaris* and *Trichuris.* Generally in hook worm and *Trichuris trichiura,* the egg count will decide whether the patient should be given therapy. 30,000 eggs of *Trichura* per gram indicates the presence of thousands of worms. Similarly 2000 to 5000 eggs per gram indicate severe infection.

Stool Dilution Egg Count Technique

Procedure

1. Keep the stool for 24 hrs and weigh it.
2. Take four grams of faeces.
3. Take this into tube and add 10 ml of 0.1 sodium hydroxide.
4. Add few glass beads to the tube and shake vigorously to obtain a uniform suspension.
5. Take 0.15 ml of the suspension and drain on to a slide. Without using the cover glass examine the slide and count the number of eggs.
6. Multiply egg count with 100 to obtain number of eggs per gram.
7. Egg count varies with the consistency of the faeces.

Recovery of Larval Stage Nematodes

Nematode infection gives rise to larval stages which hatch either in soil or in the tissues. They are useful in hook worm *Strongyloides* and *Trichostrongylus* infection. This technique is useful since the eggs of many species are identified and identification depends on larval morphology.

Harada–Mori Filter Paper Strip Culture

This method is useful for light infections. This method was originally described by Harada and Mori in 1955 and has been modified by several workers. Faecal matter should not be refrigerated since some of the nematodes are susceptible to cold and will undergo further development.

Procedure

1. Take 5 ml centrifuge tube and put 3–4 ml of distilled water.
2. Take filter paper strip (3/8 × 5 inches) and with this strip smear a thin film of 0.5 to 1g of faeces.
3. Cut one end of the filter paper strip so that the end is tapering. Now insert the tapering end of the filter paper into the tube.
4. Keep the tube at 24–28°C and to keep the original level add water. For two days there is evaporation and later culture becomes stabilized.
5. Faeces on the filter paper are kept moist due to capillary flow of water.
6. Leave the tube undisturbed for 10 days. Infective larvae start appearing after 5th day.
7. Draw out a small amount of fluid with a pipette and place it on a slide, active larvae are visible. They may be heat killed and can also be killed with iodine.
8. Hook worms like *Strongyloides, Trichostrongylus* could be detected.

Baermann Technique

In case the stool examination repeatedly gives a negative report, but the patient is suspected of *Strongyloides,* then Baermann technique is useful in recovering the larvae. In this technique the larvae migrate from faecal material through several layers of gauze into water. This water is centrifuged and the larvae concentrate at the bottom (Garcia and Bruckner 1988).

Procedure

1. Take a 6 inch funnel and attach rubber tubing with pinch clamp to bottom.
2. Place a large amount of faecal matter on the gauze so that it is covered with water.
3. Let the apparatus stand undisturbed for two hours.
4. Release the pinch clamp and draw 10 ml of fluid and spin down into a centrifuge.
5. Examine the sediment for larvae.

Hatching Procedure for Schistosome Eggs

Schistosome eggs recovered from either urine or stool are examined for viability. Movement of cilia on the flame cells indicate the presence of larvae.

Procedure

1. Take a stool specimen and thoroughly mix with saline and strain through two layers of gauze.
2. Let the material settle and pour off the supernatant. Repeat the process.
3. Decant the saline and add water. Transfer the fluid into a conical flask and fill with enough fluid up to the neck of the flask.
4. Cover the flask with a foil or black paper leaving 2 ml of fluid exposed to light.
5. Leave the flask in subdued light for 3 hrs at room temperature.
6. Now keep a bright light by the side of the flask.
7. The miracidia will migrate to the illuminated portion of the flask.

JSB Stain has Two Solutions

Solution 1

Eosin water soluble	0.2 g
Tap water	100.0 ml
Na_2 HPO_4 $2H_2O$	0.22 g
(Di sodium hydrogen phosphate)	
KH_2PO_4	0.74 g
Distilled water	100.0 ml

Solution 2

Tap water	400.0 ml
Dissolve and then add	
Sulphuric acid	1 per cent
Aqueous	3 ml
Potassium dichromate	
0.5 per cent aqueous	100.0 ml

Mix thoroughly the amorphous precipitate, methylene blue chromate is formed. This is heated over a low flame on a water bath. Though solution appears blue, on heating it turns green. 4–6 hrs of boiling is necessary. When solution turns blue cool it and allow the precipitate to dry in a dessicator. This

precipitate is ground with 1.75 g of $NA_2HPO_42H_2O$. Store it immerse sections in solution 1 for 10 sec. Wash and stain in solution 2 for 2 sec. Wash in acidulated water.

Cellophane Tape Preparation

The common pin worm or seat worm *Enterobius vermicularis* is common in children. Adult female migrates from the anus during the night and deposits eggs outside the gastrointestinal tract on the perianal area. Stool examination may not give positive results. Most infections are diagnosed by cellotape method. Usually eggs adhere to cellotape. Very rarely adult female is found on the surface of the formed stool. Scotch tape is taken and mounted tape is pressed firmly against the perianal region covering all sides. Then the tape is transferred to a glass slide with sticky side down. A drop of toluene is added for clearing and examined under microscope.

Urogenital Specimens

Vaginal discharges, urethral discharge and prostrate sections reveal the presence of *Trichomonas vaginalis*. These discharges are treated with a drop of saline and examined under low powers. Jerky movements of the organism can be noticed. Gradually the movement starts diminishing but undulating membrane can be observed.

Sputum Sputum should be collected from the lower respiratory passages and not from saliva. The specimen should be collected before brushing teeth. The formalin ether sedimentation technique can be used. Thick mucoid sputum can be centrifuged after the addition of an equal quantity of 3 per cent sodium hydroxide. The sputum contains brownish spots or "iron fillings" which may be *Paragonimus* eggs.

Peripheral blood may be useful in detecting microfilaria in stained and unstained smears. Blood should be collected between 10 pm and 2 pm.

Some additional procedures

Trichrome Stain

Cysts and trophozoites can be visualized with this stain that clearly shows the morphological features. It will provide a permanent record of the result.

Reagents required

Chromotrope 2 R

Light green SF

Phosphotungstic acid

Glacial acetic acid

Preparation of Stain

Take 600 mg of chromotrope 2R, 300 mg of light green, 700 mg of phosphotungstic acid in a dish and add 1ml of glacial acetic acid. Allow the mixture to ripen for 30 min. and then add 100 ml of distilled water. The stain is purple in colour.

Procedure

1. Prepare a fresh faecal smear with PVA.
2. Place in 70 per cent Ethanol and D Antonis iodine for 5 min.
3. Place in 2 changes of 70% ethanol for 2-5 min each.
4. Place in trichrome stain for 10 min.
5. Place in 90 percent ethanol acidified (1per cent acetic acid) for 3 sec.
6. Place in 100 percent ethanol (2 changes for 2–5 min).
7. Place in two changes of xylene or toluene for 2–5 min.
8. Mount the slide.

Result

Debris	green
Protozoans	blue-green to purple
Nuclei and inclusions	red or purple

Modified iron haemotoxylin stain:

As in previous case this stain will enhance the morphological features and will be a permanent preparation.

Reagents required

Albumin

Ethanol

Ferrous ammonium sulphate

Ferric ammonium sulphate

Hydrochloric acid

Picric acid

Ammonia

Basic fuchsin

Phenol crystals

Preparation of the reagents

Solution 1 (Stock solution)

Haematoxylin powder	10 g
95 % ethanol	100 g

Mix well until it dissolves. Ripen it for 2 weeks.

Solution 2 Mordant

Ferrous ammonium sulphate	10 g
Ferric ammonium sulphate	10 g
Hydrochloric acid	10 ml
Distilled water	1000 ml

Solution 3 Working solution of haematoxylin

Mix equal quantities of stock solution and mordant let the mixture cool thoroughly before use (at 2 hrs). This working solution should be made fresh every week.

Solution 4 Picric acid

Mix equal volumes of distilled water and saturated solution of picric acid to make 50 per cent saturated solution.

Solution 5 Decolorizer

Concentrated HCl	30 ml
Alcohol	1000 ml

Solution 6 70% alcohol ammonia

70% alcohol	50 ml
Ammonia	0.5 ml

pH should be 8. Bring it to 8 by adding ammonia.

Solution 7 Carbol fuschin (Kinyoun stain)

Solution A

Basic fuschin	
Ethanol (95%)	10 ml

Solution B

Phenol crystals	5 g
Distilled water	100 ml

If necessary heat it.

Mix solution A and B it will remain for one year at room temperature.

Procedure

Take an albuminized slide and add 1 drop of faecal matter from SAF concentration and spread it on the slide.

1. Allow the slide to dry at room temperature.
2. Wash in tap water (not running)—2 min.
3. Place slide in 70% alcohol—5 min.
4. Place slide in Kinyoun stain for 5 min.
5. Wash slide in running water for 1 min.
6. Place slide in solution 5 for 4 min.
7. Wash in running tap water.
8. Place slide in solution 3 for 8 min.
9. Wash slides in distilled water.
10. Place slide to solution 4 for 5 min.
11. Wash in running tap water for 10 min.
12. Transfer slide in solution 6 for 3 min.
13. Place the slide in 95% alcohol 5 min.

14. Place the slide in 100% alcohol 5 min.
15. Transfer slide to 2 changes of xylene 5 min.

Modified Acid Fast Stain for *Cryptosporidium parvum*

Reagents

Basic fuchsin

Phenol

95% alcohol

Concentrated Hcl

Methlene blue

Preparation of Reagents

Solution 1 Carbol fuchsin

Basic fuchsin	4 g
Phenol	8 ml
95% alcohol	20 ml
Distilled water	100 ml

Dissolve basic fuchsin in alcohol and then add water slowly while shaking. First melt phenol crystals at 56°C and then add 8 ml to the strain.

Solution 2 Decolorizer

95% alcohol	9.7 ml
Concentrated HCl	3 ml

Add Hcl to alcohol slowly.

Solution 3 Counter Stain

Methylene water	300 mg
Distilled water	100 ml

Cold Modified Acid Fast Stain (Kinyoun)

1. Centrifuge for 10 min. at 500 xg 19% formalinized stool.
2. Remove upper layer of sediment at 70% for 10 min.
3. Heat the smear at 70°C for 10 min.
4. Keep the slide in solution 1 for 3–5 min.
5. Wash in distilled water.
6. Flood the slide with solution 2 for 1 min.
7. Wash thoroughly.
8. Flood slide solution 3 for 1 min.
9. Washed with distilled water.

Result

Acid fast bacteria red

Hot Modified Acid Fast Stain

1. Centrifuge for 10 min. at 500 xg 10% formalin.
2. Remove deeper layer of sediment onto a slide.
3. Heat the smear at 70°C for 10 min.
4. Flood the slide with solution 1.
5. Heat the slide for 5 min.
6. Rinse the smear with water.
7. Decolorize with 5% aq. sulphuric acid for 30 sec.
8. Rinse the slide in distilled water and air-dry.
9. Flood the slide with solution 3 for 1 min.
10. Rinse with distilled water and air-dry.

Modified Trichrome Stain for the Microsporidia (Weber-green)

Reagents Required

Chromotrope 2R

Fast green

Phosphotungstic acid

Glacial acetic acid

90% ethyl alcohol

Preparation of Reagents

Solution 1 Modified Trichrome Stain

Add 3 ml of acetic acid to the stain ingredients allow the stain to ripen for 30 min. at room temperature. Then add 100 ml of distilled water. It will turn purple (dark). Store in a plastic bottle which will last for 1 year.

Solution 2 Acid alcohol

95% ethyl alcohol	995.5 ml
Glacial acetic acid	4.5 ml

Procedure

1. Make a smear of liquid (SAF).
2. Allow the smear to air-dry.
3. Place the slide in solution 1 for 90 min.
4. Rinse the slide in solution 2 for 10 sec.
5. Dip slides in 95 per cent alcohol several times.
6. Place in 100 per cent alcohol 10 min.

7. Place in xylene for 10 min.
8. Mount the slide.

Result

Spores are ovoid with spore wall pinkish red.

Modified Trichrome Stain for Microsporidia

Reagents Required

Chromotrope 2 R

Aniline blue

Phosphotungstic acid

Glacial acetic acid

Preparation of Reagents

Solution 1 Modified trichrome

Chromotrope 2 R	6 g
Aniline blue	5 g
Phosphotungstic acid	0.25 g
Glacial acetic acid	3 ml
Distilled water	100 ml

As in the previous case add 3 ml of acetic acid to the dry ingredients, allow the stain to ripen for 30 min. at room temperature. Now add 100 ml of distilled water and adjust pH (2.5) with 1 M HCl. Store in a plastic bottle. It lasts for one year.

Solution 2 Acid alcohol

(As described in previous case).

Procedure

Make a smear of liquid stool (SAF).

1. Allow the smear to air-dry.
2. Place the slide in absolute methanol 5 ml.
3. Place in solution 1 for 90 min.
4. Dip slide in solution 2 for 4–10 min.
5. Dip slide several times in 95% alcohol.
6. Place in 100 per cent alcohol for 10 min.
7. Place in xylene.
8. Mount the slide.
9. Examine under oil immersion microscope.

Result

Spores are ovoid, retractile, spore wall pinkish red, bacterial and other debris blue.

Staining of Thin Films—Giemsa Stain

By spreading the blood cells in a thin layer, the size of red cells, inclusions and extracellular forms can be more easily visualized.

Procedure

1. Fix blood film in absolute methanol 30 sec.
2. Allow slides to air-dry.
3. Dip slides in 1 part of Giemsa stock to 10–50 parts of Triton buffered water (pH 7.0 to 7.2) for 10 to 60 min.
4. Immerse slides briefly in triton X—100 buffered water.
5. Drain thoroughly and allow to air-dry.

Result

Erythrocytes pale grey	blue
Nuclei of white cells	purple or pale purple
Cytoplasm and eosinophilic granules	bright purple
Neutrophilic granules	deep pink purple
Parasitic forms	blue to purple
Nuclei	red

Staining of Thick Films—Giemsa Stain

A large amount of blood can be examined for parasitic forms by lysing the red blood cells and staining for parasites.

Procedure

The procedure to be followed for thick films is the same as for thin films except that the first two steps are omitted. If the slide has a thick film at one end and a thin film at the other end fix only the thin portion and then stain both parts of the film simultaneously.

4

HAEMATOLOGY

BLOOD—MORPHOLOGY

Blood is a form of fluid connective tissue type and is always in circulation in the body. In higher animals it is a viscous complex and in lower animals it is thin and watery. It is a major transporting medium and is always in movement in living bodies. It is heavier than water with a specific gravity ranging between 1.05 to 1.06. It is red in colour except in lower animals like *Amphioxus* and *Leptocephalus*. It is salty with a viscosity five times greater than water. The osmotic pressure is 7.6 and pH is 7.4 (alkaline). On centrifugation it separates into two main components, a light yellowish supernatant, plasma or the serum, and a reddish cellular portion, the corpuscles, at the bottom. Plasma forms about 55% and corpuscles about 45% of the blood volume.

Plasma is straw (light yellowish) coloured, homogeneous and sticky with a specific gravity of 1.025–1.034. It has some coagulants such as fibrinogen and anticoagulants like heparin. The liquid part of the plasma after removing the clot is known as serum. The organic components of blood are proteins, amino acids, nitrogenous waste products such as ammonia, urea, uric acid, enzymes, hormones, antibodies, fatty acids (cholesterol), vitamins, metabolic products (xanthene, hypoxanthine, creatinine, etc.) neutral fats, phospholipids and sugar (glucose).

Proteins which form 7% of the plasma are of three types. They are albumins, globulins and fibrinogen (3 : 1 ratio).

Albumins have a low molecular weight than globulin and they differ in solubility and saturation test. When saturated with ammonium sulphate, albumins get completely precipitated, whereas globulins are half-saturated. Albumins are formed in the liver and globulins in the reticuloendothelial cells, macrophages and lymphocytes.

Globulins are of three types, α–globulins, β–globulins and γ–globulins. There are two types of α-globulins— α_1-globulins and α_2-globulins. Globulin produces

i. an antibody called antitoxin which neutralizes the poisonous substances released by microorganisms,
ii. a substance called antilysin which helps in dissolving and destroying foreign proteins of the body,
iii. agglutinins which as the name indicates agglutinates foreign substances in the body,
iv. precipitants which help in precipitating foreign proteins and
v. opsonins which form substances which are responsible for making bacterial bodies suitable for phagocytosis by WBC. In a way globulins play a pivotal role in developing a defence mechanism against diseases produced by bacteria.

Fibrinogen, forming 4% of the plasma, is synthesized in the liver and is heat-resistant.

The functions of plasma proteins are closely associated with nutrition, transport and physicochemical aspects. Plasma proteins combine with lipids to form lipoproteins which are transported to different tissues of the body. Plasma proteins also play a major role in the maintenance of osmotic pressure, acid–base balance, water volume and viscosity.

Inorganic Components

0.9% of the plasma is constituted by inorganic components such as chlorides, bicarbonates, sulphates, phosphates of sodium, potassium, calcium and magnesium, besides iron, iodine and phosphorus.

The concentration of potassium and calcium differ in marine animals. The body concentrations of potassium and calcium are higher than that of surrounding sea water in marine animals. But in crustaceans it is little different. The concentration of magnesium and sulphates is lesser than the surrounding sea water.

Gaseous components of blood include oxygen, carbon dioxide, and traces of nitrogen. Oxygen and carbon dioxide are transported by the haemoglobin of the red blood cells. In plasma, oxygen is present in traces. Carbon dioxide in combination with alkaline radicals like carbonates and bicarbonates is present in both plasma and corpuscles.

Corpuscles

Corpuscles are of three types and float in plasma. They include red blood corpuscles or erythrocytes, white blood corpuscles or leucocytes and blood platelets or thrombocytes. Erythrocytes play a role in transporting oxygen and carbon dioxide, leucocytes defend the body against microorganisms and thrombocytes aid in blood clotting.

Red blood corpuscles or erythrocytes Erythrocytes are pale yellow in colour, when agitated become reddish in colour. Erythrocytes of venous blood are purple red and of arterial blood are scarlet red in colour. This colour variation reflects the oxygen concentration of haemoglobin. In lower vertebrates such as fishes, amphibians, reptiles and birds, erythrocytes are oval in shape and in higher vertebrates they are in the form of biconcave discs. In man they are either circular, biconcave or disc-shaped and are enucleated. The shape and enucleated condition reflects on progressive evolution.

The erythrocytes contain a spongy elastic substance called stroma (lipoproteins). The stroma envelops the erythrocytes externally in the form of a thin membrane made up of lecithin and cholesterol. This thin membrane has four layers of protein and two layers of lipid. This is permeable to ions of mineral salts. Within the loops of stroma there is a solution containing haemoglobin salts and some non-electrolytes. Haemoglobin concentration in blood is 12–15% in most mammals and birds, 6–10% in reptiles and amphibians and 6–11% in fishes.

White blood corpuscles or leucocytes WBCs lack the respiratory pigment and hence called leucocytes. The diameter is 10 μ, larger than the erythrocytes. These are broadly categorized into two groups—granulocytes and agranulocytes.

1. **Granulocyte** As the name suggests the cytoplasm of granulocytes is granular with an irregularly shaped large nucleus which is multilobed. These granulocytes grow in the red bone marrow from lymphocyte cells called myloblasts into three types called (i) basophils (ii) eosinophils and (iii) neutrophils.

   They play a major role in producing a factor, thrombokinase, which is very essential for the process of blood clotting. They measure 1.5–3 μ.

   i. **Basophils** They are also called as mast cells, and have multilobed nucleus. They measure about 10–15 μ in diameter. They take stains with basic dyes such as haematoxylin. Large number of basophils occur in bone marrow, less in connective tissue and a very few in circulation. They contain heparin, absorb toxins and play a role in the defence mechanism. Along with eosinophils, they are transformed into fixed connective tissue elements and thus help in the process of repair of chronic inflammations.

   ii. **Eosinophils** They stain with acid dyes such as eosin and measure 10–15 μ in diameter. The cytoplasm has granules and nucleus is bilobed. The eosinophilic content increases at the time of infection and allergic conditions. They are not phagocytic but bring out destruction and detoxification of toxins of protein origin. They occur in large numbers in the bone morrow.

   iii. **Neutrophils** Since they have multilobed nuclei, they are also called polymorphs. The nucleus stains with haematoxylin but the cytoplasm takes a pink shade with neutral dyes. They measure 10–15 μ in diameter exhibiting amoeboid movements. They constitute 65–70% of leucocytes. By phagocytosis they engulf bacteria and thus destroy them. Sometimes they are also termed as scavenger cells or phagocytes because they remove the diseased tissue of the body.

2. **Agranulocytes** As the name suggests there are no granules in the cytoplasm. They arise from lymphoblasts in the germ centres of lymph glands, spleen, other lymphoid tissue and also from reticuloendothelial cells of the red bone marrow.

   - Lymphocytes measure 10–16 μ in diameter and form 20–30% of the leucocytes. The nuclei are large and stain deep blue or purple. The cytoplasmic quantity is much more than the nucleus. They play an important role in the formation of fibroblastic cells which are good for tissue repair and regeneration. The most important role played by lymphocytes is the production of antibodies.

- Monocytes measure 12–20 μ in diameter and form 4–8% of the leucocyte content. The nucleus is bean-shaped or horseshoe-shaped or oval-shaped and takes a purple shade. The quality of cytoplasm is much more when compared to that of nucleus. They are motile and engulf bacteria by means of phagocytosis.

Blood platelets or thrombocytes They play a major role in producing a factor called thrombokinase which is very essential for the process of blood clotting. They measure 1.5–3 μ in diameter. They are colourless, flat, granular corpuscles and take stain with basic dyes. Their count is from 1.50000 to 400000 per ml. Any reduction in count is called thrombocytopenia.

Blood carries digested food substances like glucose, amino acids, polypeptides, fatty acids, glycerol, vitamins, minerals and water from the wall of the alimentary canal to the liver and other tissues where they are required. It transports O_2 from respiratory surfaces to the tissues and carbon dioxide in the reverse direction.

Blood also plays a major role in maintaining the body water volume at a constant level. The consistency is a very important factor in the regulation of osmotic pressure of the body fluids. Another important function is chemical coordination. It transports the hormones to the organs of action and this helps in coordinating the activities of various organs of the body.

It carries waste products like ammonia, urea, uric acid, creatin, carbon dioxide, etc. from the site of their origin to the concerned excretory organs for excretion.

By phagocytosis, the leucocytes engulf and devour the harmful and foreign substances such as bacteria and produce certain chemical substances called antibodies to attack or neutralize the toxins produced by the foreign substance.

It transports heat from deeper tissues to the surface of the body for evaporation. It transports water and salts to the sweat glands where the water is evaporated to reduce the body temperature.

It prevents excess loss of blood during injuries owing to its remarkable power of undergoing coagulation.

Blood Groups Identified in Human Beings

Based on the antigen-forming surface proteins of RBCs four groups—A, B, AB and O—are identified. Each group exhibits its own special features.

'A' group In this group antigens are 'A' type, antibodies are 'anti-B' type. 'A' type and 'O' type individuals can accept 'A' type only. Genotype 'A' group is expressed as IA, IH or IAi (I is a dormant gene)

'B' group It contains 'B' type antigens and 'anti-A' type antibodies. These individuals can accept blood from 'B' or 'O' group and donate blood to 'B', 'AB' types. Genotype is expressed IBIB or lbi.

'AB' group These individuals possess both 'A' and 'B' type antigens but antibodies are lacking. They can donate blood only to 'AB' group but accept blood from 'A', 'B', 'AB' or 'O'. They are called universal recipients.

'O' group No antigens present in this group but both 'anti'A1' and anti'B1' antibodies are present. These people can accept 'O' group only. This blood is known as universal 'donor' and its genotype is ii.

Identification of Blood Groups

Slide method is a common and simple method to identify blood groups. A prick is made on a finger with a sterilized needle. Then the finger is squeezed for flow of blood. First drop is wiped and then two drops of blood are collected over a clean sterilized slide one at either end.

One drop of 'anti-A' serum is added to the left drop of blood and a drop of 'anti-B' serum is added to the right drop. Then both the drops are mixed thoroughly with the help of a glass rod. The blood grouping may be studied after 10–15 min.

Blood	Leftside blood drop + anti-A serum	Rightside blood drop + anti-B serum
A	+	–
B	–	+
AB	+	+
O	–	–

\+ = Agglutinations results in clumping of blood cells.

– = Agglutinations never produce clumping.

Another blood drop is taken on a fresh slide and to it 'anti-B' serum is added and thoroughly mixed. If clumping is seen after 10–15 min., it is Rh-positive and absence of clumping shows that it is Rh-negative.

Usual haematological tests Usual tests are total count of blood cells (TC), differential count of WBC (DC), erythrocyte sedimentation rate (ESR) and packed cell volume (PCV).

Total blood count It is done by a specially designed slide called haemocytometer. Among haemocytometers, there are Neubauer chambers, improved Neubauer counter and Barker counting chamber.

For total blood count, 0.02 ml of blood is collected with a pipette. This is mixed with 4 ml of diluting fluid (31.3 g of trisodium citrate, 1ml formalin and is made up to 100 ml with distilled water).

The contents of the pipette are transferred to Neubauer counter and see that is equally distributed. Leave it undisturbed for two minutes and count the cells under microscope.

Number of RBC should be 5.5×10^{12} l (male) and 4.8×10^{12} l (female)

WBC or leucocytes Take 0.2 ml of blood into a pipette and add 0.4 ml of dilution fluid (dilution fluid is prepared by mixing 2% glacial acetic acid with a pinch of Gentian violet in 100 ml distilled water). Transfer the contents to Neubauer counter and observe it under microscope. WBC count should be 7.5×10^{9} l.

Differential Count (DC) of WBC

As already mentioned leucocytes are granular and agranular which are subdivided into lymphocytes, moncytes, eosinophils, basophils and neutrophils. Blood film is made as described on page 72–76,

Giemsa and Leishman's stains are used. Then the film is kept in phosphate buffer (pH 7.2) and then washed. The slide is dried and observed under oil immersion.

In normal person DC should be neutrophils 50–75%, eosinophils 1.8%, basophils 1% monocytes 2–10% and lymphocytes 20–35%.

Erythrocyte Sedimentation Rate (ESR)

Blood is collected and mixed with an anticoagulant and the contents are filled in ESR tube. Tube is placed vertically in a stand. RBC form into roaleaux and the sediment sinks and pack in ESR tube. The rate at which it occurs is the sedimentation rate, plasma fluid is left over the sedimentation in the ESR tube.

ESR in a normal person is 0.5 mm.

While in female it should be 0.7 mm.

If there is increase it indicates tissue degeneration, inflammation, TB, arthritis, etc.,

CHEMICAL ANALYSIS OF BLOOD

Blood is analysed for quantitative and qualitative estimation of various types of chemicals.

Serum Bilirubin

Preparation of Reagents

Solution 1 Catalyst

Anhydrous sodium acetate	123.0 g
Distilled water	200.0 ml

Add another 250 ml of distilled water.

Caffeine	50.0 g
Sodium benzoate	75.0 ml

Make up to 1 litre. This solution lasts for long time in refrigerator.

Solution 2 Sulphanilic acid

Take a volumetric flask and add

Distilled water	200.0 ml
1 M hydrochloric acid	69.0 ml
Sulphanilic acid	2.25 g

and make up to 500 ml.

Solution 3

Hydroxylamine hydrochloride	3.48 g
Distilled water	6.0 ml

and make up to 10.0 ml. This lasts for one week.

Solution 4 Fehling II

Distilled water	500.0 ml

Sodium hydroxide 100.0 g

Mix and cool under cold running water then add

Sodium potassium tartrate 350.0 g

and make up to 1 litre.

Solution 5

Take stock 1N HCl and make it to 1: 20 dilution.

Solution 6 Sodium nitrite

Sodium nitrite 2.6 g

Distilled water 100.0 ml

lasts for one week. Mix both, and this solution

Procedure

1. Make diazo reagent by adding 0.1 ml of sodium nitrite to 15.0 ml sulphanilic acid.

   Take 4 tubes-conjugated, reagent blank and standard.

Tubes	1	2	3	4
Total	Conjugated	reagent	blank	standard
Catalyst	4.6 ml		4.0 ml	4.0 ml
N/20 HCl		4.0 ml		
Serum	0.5 ml	0.5 ml		
Standard	–0.5 ml			

Mix all of tubes on vortex mixer. To each tube add 4.0 ml dilute reagent. Mix, let it stand for two min. To all tubes add 0.2 ml hydroxylamine stabilizer. Mix well. To all tubes add 2.0 ml and Fehling II. Mix by inversion. Allow it to stand for 5 min.

Now determine the absorbance of total and conjugated at 600 nm (or 590) setting sera absorbance with the reagent blast.

Bilirubin values are found by using the following formula:

$$\frac{\text{Total or conjugated absorbance standard value 1}}{\text{Standard absorbance}} = \text{Bilirubin in mg \%}$$

The final blue-green colour is stable for 20 min. The results are linear up to 6.0 mg %. Sera which give values above this should be reused after dilution with saline. Total bilirubin – Conjugated bilirubin gives Unconjugated bilirubin.

Serum Creatinine

Alkaline Picrate Method

Preparation of Reagents

Solution 1 Saturated picric acid

Dissolves excess of picric acid in distilled water.

Solution 2 Sodium hydroxide

Sodium hydroxide	10.0 g W/V
Distilled water	100 ml

Solution 3 Alkaline picrate solution

Saturated picric acid	10.0 ml
Sodium hydroxide	10 % (W/V) 1–2 ml

Solution 4 Stock standard creatinine solution

Creatinine zinc chloride	1.602 g

Make the volume up to 1000 ml with N/10 hydrochloric acid.

Solution 5 Standard creatinine working solution

Dilute 1.0 ml stock solution to 100 ml with distilled water.

Procedure

1. Mix thoroughly

Plasma serum	2.0 ml
Distilled water	2.0 ml

10% W/V sodium tungstate solution 1.0 ml.

2/3 N Sulphuric acid	1.0 ml

Then centrifuge at 2500 rpm for 5 min

2. Take two test tubes and mark them as Test and Blank.

	Test standard		Standard blank	
	1		2	
Supernatant				
Fluid from	3.0 ml			
Standard creatinine				
Solution	-	1.0 ml		3.0 ml -
Distilled water	-	2.0 ml	-	3.0 ml
Alkaline picrate				
solution	1.5 ml	1.5 ml	1.5 ml	1.5 ml

Mix thoroughly and leave at room temperature for 10 min.

3. Read the absorbance of the test and standard solutions in a photo-colorimeter using a green filter (520–540 nm) setting the zero optical density with the blank solution.

Using standard I

$$\frac{\text{OD test}}{\text{OD standard}} \times 0.01 \text{ mg} \times \frac{100 \text{ ml}}{1.0 \text{ mg\%}}$$

$$\frac{\text{OD test}}{\text{OD standard}} \times 1.0 \text{ mg} = \text{Creatinine mg\%}$$

Using standard II

$$\frac{\text{Test (reading) OD}}{\text{OD standard}} \times 0.03 \text{ mg} \frac{100 \text{ ml}}{1.0 \text{ ml}}$$

$$\frac{\text{OD test}}{\text{OD standard}} \times 3.0 \text{ mg} = \text{Creatinine mg\%}$$

Haemoglobin

It has two components called "Haeme" (non-protein part) and globin (Protein part). Haemoglobin is estimated in several ways, its intensity of colour, its iron content and its capacity of combining with oxygen and carbon dioxide. Haemoglobin can be estimated in the following ways.

Acid–Base Method

0.05 ml of blood is taken from a vein and later 4 ml of 1N HCl is added to it. It is thoroughly shaked and left undisturbed for half an hour. After 5 min. 0.95 ml of 0.1N sodium hydroxide is added. Now 1% haemoglobin can be directly read from photoelectric colorimeter using yellow filter.

Sahl's Haemoglobinometric Method

This is in practice quite regularly. 0.02 ml of blood is collected into haemoglobin pipette and 0.1N HCl is added to this. After shaking the pipette it is transferred to Sahl's haemoglobinometer. Till the colour matches with the standard colour, distilled water is added. Now both the colours are compared and percentage is known from the standard value. If there is no colour 0.04 ml is added to 0.1 N HCl (double) to decrease the quantity of haemoglobin.

Cyanomethanoglobin Method

Reagents Required

Sodium bicarbonate	1 g
Potassium cyanide	0.05 g
Potassium ferricyanide	200 mg
Distilled water	1000 cc

This is called Drabkin's solution.

To 20 ml of blood, 5 ml of Drabkin's solution is added. After mixing well, read in a photocolorimeter at 540 mm (green filter). For this method certified haemoglobin solution may be procured from a reputed laboratory. A linear graph may be obtained by putting the known Hb concentration against the colorimetric optical density reading. For future the corresponding haemoglobin value can be directly read from the calibration curve after knowing the optical density of particular unknown blood sample.

Sheard–Sanford Oxyhaemoglobin Method

To 0.1 ml of blood 20 ml of 0.1% sodium carbonate is added and read the optical density in photometer at 540 nm in 30 min. Photometer calibration should be based on blood iron determination or oxygen carrying capacity determination.

Alkali haematin method, gasometric method are there but not frequently applied. Specific gravity method is quite good. Normal specific gravity of blood sugar is from 1.048 to1.066. From specific gravity of unknown sample its haemoglobin is calculated.

Estimation of Blood Urea Nitrogen

The most important excretory product of metabolic activity is ammonia. In ornithine cycle, liver ammonia is converted into urea.

Urea content in normal man should be 10–18 mg/dl.

The urea of serum is hydrolysed by specific enzyme, urease and is converted to ammonia and carbon dioxide. The reaction is buffered with EDTA.

Berthelot Method

Urease	150 mg
EDTA	1 g
Distilled water	80 ml

Adjust pH to 6.5. Make up to 100 ml. Store in refrigerator

Phenol Colour Reagent

Solution 1 Phenol colour reagent

Phenol crystals	25.0 g
Distilled water	400 ml

Mix this in 500 ml volumetric flask.

In another flask take

Sodium nitroprusside	125 mg
Distilled water	50 ml

Add this to solution 1 and make it 500 ml with distilled water.

Solution 2 Alkaline hypochlorite reagent

Sodium hydroxide	12.5 g
Distilled water	400.0 ml

Add about 25 ml of commercial bleach and make up to 500 ml with distilled water.

Working standard solution

Pure urea	215 mg
Distilled water	500 ml

Mix well, shake and store in refrigerator.

Add solution 3 and 4.

Procedure

1. Take three test tubes and mark them as test 'T', blank 'B' and standard 'S' and into the tubes marked 'T', 'B', 'S' take 0.2 ml of buffered urease solution and add 20 ml of serum to the tube marked 'T' and 20 ml working standard solution to tube 'S'.
2. Incubate all three tubes for 15 min. at 37°C.
3. Remove them from bath and add 1ml of phenol colour reagent to the three tubes. Shake, mix and add 1ml of alkaline hypochlorite reagent and mix again.
4. Again place them in a water bath (37°C) for 15 minutes.
5. Remove the tubes from water bath and add 10 ml of distilled water to all the tubes and mix well.
6. Read the optical density of the test 'T' and standard 'S' at 630 nm in spectrophotometer using the blank solution 'B' at '0' absorbance.
7. If the absorbance reading of test solution is above 0.8, then dilute both test and blank solutions with distilled water until the absorbance of test solution falls with in the range of 0.2 to 0.8.

Calculation

Optical density = Absorbance = OD

$$\frac{\text{OD of test}}{\text{OD of standard}} \times 20 = \text{BUN mg per cent}$$

Calculation of standard is 20 mg%

Blood urea mg% =BUN mg percent × 2.14

Oxime Method

When diacetyl monoxime reacts with urea a pink colour is developed. Ferric ions and other activations intensify the colour. The intensity is measured calorimetrically at 520 nm.

Preparation of Reagents

Solution A Oxime solution

Take a 1 litre flask and add the following.

Diacetyl monoxime	1 g
(2, 3-bitanedione monoxime)	
Thiosemicarbozide	0.2 g
Sodium chloride	9 g

Dissolve the ingredients in distilled water and make up to 1000 ml. It lasts for three weeks.

Solution B Acid solution

Take a 1 litre volumetric flask and add the following cautiously in 800 ml distilled water.

Concentrated sulphuric acid	60 ml
Phosphoric acid (85%)	10 ml
Ferric chloride	0.1 g

Dissolve, shake, make the volume up to 100 ml with distilled water. It lasts for 2 months.

Solution C Standard solution

i) Benzoic acid

Benzoic acid (0.2 % w/v in water)	2 g
Distilled water	1000 ml

Mix it in a beaker. This solution can be used for preparing and diluting the standard solution.

ii) Stock urea standard solution (1 g/dl)

Dry urea	1 g
Saturated benzoic acid solution	50 ml

Take 100 ml volumetric flask and add the above-mentioned ingredients. Keep it in a refrigerator. It lasts for six months.

iii) Urea working solution (40 mg/dl)

Stock solution	4.0 ml
Benzoic acid solution	100 ml

Mix them in 100 ml volumetric flask. If necessary dilute the working standard to a further 1 : 10.

Procedure

A. 1 : 20 dilution of serum and standard.

1. Take two test tubes (15 ml) and mark them as 'T' and 'S'.
2. Add 1.9 ml of distilled water in each tube.
3. Add 0.1 ml of serum and standard respectively in tubes 'T' and 'S'.

B. Colour reaction

1. Take 3 test tubes, mark them as 'T', 'S', and 'B' for test, standard and blank.
2. Take 3 ml of colour reagent in each tube.
3. To this add 3 ml of acid reagent in each tube.
4. Add 0.25 ml of diluted specimen and the standard with the help of 1ml pipette. Add an equal volume of distilled water in the tube marked 'B'.
5. Mix the contents of each tube thoroughly.
6. Place the tubes in a water bath (boiling) for 10 min.
7. Cool under running tap water.

Measurement of colour intensity

Take three cuvettes and mark them as 'T', 'S' and 'B' and transfer the contents.

Set the instrument 0% T without allowing the light to pass through cuvettes use the blank for setting 100% T or 0 absorbance.

Calculation

$$\text{Urea concentration (mg / dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 40$$

Extraction of Urea by Titration Method

Solution A

Potassium oxalate	30 g
Distilled water	100 ml

Solution B

Trichloroacetic acid	10 g
Distilled water	100 ml

Solution C

Mercuric chloride	5 g
Distilled water	100 ml

Procedure

1. Take a test tube and take 5 ml of venous blood and add 0.1 ml of solution A and 0.1 ml of solution B. Shake well and allow it to stand for 15 min.
2. 5 ml of supernatant liquid is transferred to a conical flask and titrated against solution C.
3. Titration is stopped when the contents turn reddish brown. Burette reading is taken and calculation is made as follows.

Quantity of mercuric chloride solution (solution C) × concentrated mercuric chloride × molecular weight of urea + molecular weight of mercuric chloride × 10.

Harrison's Method

Solution 1

Potassium chloride	150 g
Mercuric chloride	200 g
Distilled water	1000 ml

Solution 2

Sodium hydroxide	10 g
Distilled water	100 ml
Nessler's reagent	
Solution 1	150 ml
Solution 2	700 ml

Make up to 1000 ml.

Procedure

Take 0.2 ml of blood and add 3.2 ml of water, 200 mg of soya bean powder. Shake well and incubate at 40–50°C for 15 min. Then add 0.3 ml of 10% sodium tungstate and 0.3 ml of 2/3 $NH_2 SO_4$. Shake well and centrifuge after 3 min. Take

2 ml of supernatant fluid in a test tube and add 5 ml of ammonia, 1 ml of Nessler's reagent. Colour developed is read in photocolorimeter at 480 nm after comparing it with known standard.

2 ml of supernatant fluid = 0.1 ml blood

2 ml of standard urea = 0.1 mg urea.

$$\text{Blood urea} = \frac{\text{Colorimetric value of the sample at 400 mm}}{\text{Colorimetric reading of standard at 400 mm}} \times 100$$

Estimation of Serum Proteins

The concept involves the estimation of proteins reacted with alkaline solution of cupric ions and the intensity of the purple colour obtained with peptide ions is compared with known concentration of protein.

Solution 1 Sulphate–Sulphite solution

Sodium sulphate	208 g
Sodium sulphite	70 g
Distilled water	900 ml
Concentrated H_2SO_4	2 ml

Take a 2 litre flask, add the above-mentioned ingredients slowly, stir and make to the mark with water. The pH 7.0 should be maintained. Always keep this at 37°C incubator in a stoppered bottle.

Solution 2 Stock Biuret reagent of Weichselfbaum

Rochelle salt	45 g
0.2N Sodium hydroxide	400 ml
Copper sulphate	15 g

Take this in a 1 litre flask and make up to 1000 ml with 0.2N sodium hydroxide.

Solution 3 Biuret working solution

Stock Biuret (solution 2)	200 ml
0.2 Sodium hydroxide	800 ml
Potassium iodide	5 g

Take this in a 1 litre flask.

Solution 4 Tartrate iodide solution

Rochelle salt	9 g
0.2N sodium hydroxide	
with 5 g potassium iodide	1000 ml

Solution 5 Ether

Solution 6 Standard serum

For this, pooled sera is collected and protein content by Kjeldahl's method is estimated and it should be kept in a deep freeze or else commercially available protein serum can be obtained.

Procedure

Take 10 ml of solution in a centrifuge (90 × 15 m) and pour on to it a layer of 0.4 m serum, invert to mix and from this take 2 ml of the mixture and add 10 ml of solution 3 into a tube.

Albumin

Add about 3 ml of ether to the rest of the serum sulphate sulphite mixture, stopper and shake. Then centrifuge for 5 min. After that tilt the tube and insert a pipette into the clear solution below the globulin layer. Do not disturb the precipitate. Pipette 2.0 ml of it and add on of Biuret reagent.

Add 2 ml of solution 1 to 5.0 ml of tartrate iodide solution (solution 4) Beret black; add 2 ml of standard serum (solution 6) standard; pipette 0.4 ml of the standard serum into 6.0 ml of solution 1 and transfer 2.0 ml of the mixture into 5.0 ml of the Biuret reagent (solution 3) in a test tube standard serum black; pipette this as described for the above test. Serene shake all the tubes and place them in a water bath at 37°C for 10 min. Cool for 5 min. at room temperature then read in absorptiometer at 55.5 nm read the calculation

$$\frac{\text{OD of unknown serum black}}{\text{OD of standard serum black}} \times \text{Concentration of standard} = \text{Grams protein}$$

Estimation of protein by Biuret method

Reagent Preparation

Solution 1

Sodium potassium tartarate	45 g
Copper sulphate	15 g
0.2N Sodium hydroxide	400 ml

Take a 1 litre volumetric flask and add the above-mentioned ingredients, allow them to dissolve. Then add 5 g of potassium iodide and make up to 1000 ml with 0.2N sodium hydroxide. This is the stock solution.

Working solution

Stock solution	100 ml
Na OH solution	500 ml

Solution 2

Calcium chloride	25 g
Distilled water	100 ml

Solution 3 Ethers

Solution 4 Alkaline tartarate solution

Potassium tartarate	45 g
0.2 N Sodium hydroxide	400 ml

100 ml of this is made up to 500 ml with NaOH to get the working solution.

Solution 5

Sodium chloride	9 g
Distilled water	100 ml

Solution 6 Standard serum solution

Procedure

To 4 ml of venous blood, EDTA salt or heparin is added and centrifuged at 3000 rpm. Precipitate constitutes the blood cells and plasma forms the supernatant.

To 2 ml of supernatant plasma add 50 ml of 0.9% sodium chloride and 2 ml of solution 2. It is left undisturbed at 37ºC for atl east 2 hrs when plasma gets solidified to form a clot. This can be separated and fibrinogen can be estimated as 0.9 % from this. This clot is washed in 0.9% sodium chloride solution and dissolved in 5 ml solution 1 (working solution). Afterwards it is slightly warmed and to it 3 ml of distilled water is added. Then its optical density is read as 540 nm in photoelectric colorimeter under a green filter.

Take a test tube and add 0.5 ml of serum after removing the clot and to it add 5.5 ml of sodium suphate solution. Shake well for 30 sec. and then centrifuge for 5 min. to get the precipitate containing globulins.

To 2 ml of supernatant fluid from the above centrifuge, 5 ml of Biuret reagent is added. Its optical density is read in photoelectric colorimeter using green filter at 540 nm for estimating albumins.

5 ml of pure serum from the first centrifuge is taken in a tube and to it 5.5 ml of solution 1 (working solution) is added and OD is read at 540 nm using green filter for estimating total proteins.

From this the OD value for albumins is subtracted to get the total globulins.

OD values could be compared with OD value of standard serum to get protein quantity.

Plasma or Serum Creatinine

Alkaline picrate method

The principle is when plasma or serum is diluted with distilled water proteins get precipitated by addition of tungstic acid. To the free filtrate alkaline picrate is added. A red colour is developed which is proportional to the amount of creatinine in the filtrate.

Preparation of Reagents

Solution 1

Saturated picric acid put excess of picric acid in a bottle containing distilled water.

Solution 2

Sodium hydroxide w/v	10 g
Distilled water	100 ml

Solution 3 Alkaline picrate solution

This solution should be fresh at each experiment.

Saturated picric acid	10 ml
Solution 2	2.0 ml.

Mix well. The colour of the alkaline picrate should not be more than twice as deep as the colour of the picric acid.

Solution 4 Stock standard creatinine solution

Pure creatinine zinc chloride	1.602 g
N/10 Hydrochloric acid	1000 ml

Solution 5

Stock standard ceratinine	1.0 ml
Distilled water	100 ml

This solution lasts for a week.

Procedure

In a clean centrifuge tube take

Serum	2.0 ml
Distilled water	2.0 ml
10% (w/v) sodium	1.0 ml
Tungstate solution	1.0 ml
2/3 N sulphuric acid	1.0 ml

Mix thoroughly and centrifuge at 2500 rpm for 5 min. Take four tubes and mark them as 'T', 'S_1', 'S_2', 'B'.

	T	S_1	S_2	B
Supernatant fluid from 1 (above fluid)	3.0	–	–	–
Standard creatinine (Solution 5)	–	1.0 ml	3.0 ml	–
Distilled water	–	2.0 ml	–	3.0 ml
Alkaline picrate solution (Solution 3)	1.5 ml	1.5 ml	1.5 ml	1.5 ml

Mix the contents in each tube and leave them at room temperature for 10 min. Read the absorbance of the test and standard solution in photocolorimeter using a green filter 520–540 nm setting the zero optical density with the blank calculation:

Standard I

$$\frac{\text{OD test}}{\text{OD standard}} \times 0.01\,\text{mg} \times \frac{100\,\text{ml}}{1.0\,\text{ml}} = \text{Creatinine mg\%}$$

$$\frac{\text{OD test}}{\text{OD standard}} \times 0.01\,\text{mg} = \text{Creatinine mg\%}$$

Using standard II

$$\frac{\text{Test (reading) OD}}{\text{OD standard}} \times 0.03\,\text{mg} \times \frac{100\,\text{ml}}{1.0\,\text{ml}} = \text{Creatinine mg\%}$$

$$\frac{\text{OD test}}{\text{OD standard}} \times 3.0\ \text{mg} = \text{Creatinine mg\%}$$

Normal values 0.5 to 1.6 mg % of plasma or serum.

Estimation of Cholesterol

Cholesterol occurs in free form, it is an organic substance. It is synthesized from acetyl co-enzyme A. It plays a major role in liver metabolism.

Regents

Solution 1 Ethanol–ether mixture

Ethanol	3 parts
Ether	1 part

Solution 2 Acetic anhydride–sulphuric acid mixture

Acetic anhydride	20 ml
Concentrated sulphuric acid	1 ml

This should be prepared afresh.

Solution 3 Standard solution of cholesterol

Cholesterol	250 ml
Chloroform	100 ml

Procedure

Take 0.2 ml of venous blood in a test tube. To this add 1 ml of solution 1 and shake it for half an hour and allow it to stand. Then centrifuge it. Take a clean beaker and take supernatant fluid and dry it in a water bath. A powder is formed.

To this add 5 ml of chloroform and 2 ml of solution 2 shake well and leave it undisturbed for 15 min. The colour developed is read on photoelectric colorimeter at 680 nm using a red filter.

$$\text{Cholestrol} = \frac{\text{Colorimetric value of the blood sample}}{\text{Colorimetric value of standard}} \times \text{Dilution factor}$$

Plasma or serum creatinine

Estimation of Glucose

Glucose values have to be estimated at least half an hour after blood collection or sodium fluoride is added to blood to stop glycolysis.

Alkaline reduction method is adopted for glucose estimation.

Solution 1 2N sulphuric acid

Concentrated sulphuric acid	2 ml
Distilled water	100 ml

Solution 2 Phosphomolybdic acid reagent

Molybdic acid	17.5 g
Sodium tungstate	2.5 g
1N Sodium hydroxide	100 ml
Distilled water	100 ml

In a volumetric flask take the above-mentioned ingredients are added and boil for 40 min. to allow ammonia to evaporate. Then it is cooled and to it is added 62.5 ml of phosphoric acid. This is made up to 250 ml with distilled water.

Solution 3 Sodium tungstate

Sodium tungstate	100 g
Distilled water	200 ml

Solution 4 Alkaline copper tartarate

Sodium carbonate (anhydrous)	200 g
Tartaric acid	3.75 g
Copper sulphate	2.25 g
Distilled water	200 ml

Make it up to 500 ml.

Solution 5 Standard glucose solution

Pure glucose	0.25 g
Distilled water	100 ml
Benzoic acid	1 g

This is the stock solution. By adding distilled water different concentrations could be obtained.

Take 0.1ml of venous blood and add 0.2 ml of solution 3 and 35 ml of distilled water. Now add 0.2 ml of solution 1 and shake. Allow it to stand for 10 min., filter. Filtrate contains glucose. Take 2 ml of filtrate and to it add solution 4. Warm it for 10 min., cool it and then add 2 ml of solution 2. Allow it to stand for 10 min. Add distilled water to make it up to 12.5 ml shake well.

This solution is kept in photoelectric colorimeter for reading the optical density using dark red filter at 440 nm.

Glucose in Blood

After drawing the standard graph, amount of glucose is estimated from the graph.

Normal blood values 60–100 mg% (Fasting)

120 mg (post-prandial)

Toluidine Blue Method

Solution 1 3% trichloroacetic acid (TCA)

Pure TCA	30.0 g
Distilled water	1000 ml

Solution 2 *o* -Toluidine blue reagent

Pure thiourea	1.5 g
Glacial acetic acid	200 ml

Take solution 1 in a volumetric flask and gently heat it, add 60 ml *o* -toluidine blue, mix and make it 1 litre with glacial acetic acid. Keep in an ambour-coloured bottle.

Solution 3 Stock standard glucose (stock)

Pure (anhydrous) glucose	1.0 g
Saturated solution of benzoic acid	100 ml

Solution 4 Working solution

Stock solution	1 ml
Saturated solution of benzoic acid	100 ml

Procedure

1. Take 0.1 ml of blood serum and add 3.0%, w/v of solution 1 in a large test tube. Mix it and allow it to stand for 5 min. and centrifuge for 10 min. at 2500 rpm.
2. Take three tubes and mark them as 'T', 'S' and 'B'.
3. Take 1 ml of clear supernatant from step 1 marked 'T' (test) into the 'B' (blank) and 1.0 ml of distilled water. In the tube 'S' (standard) pipette 1.0 ml of working standard solution (solution 4).
4. Add 5.0 ml of toluidine blue to all these tubes.
5. Heat in boiling water bath after closing the tubes with aluminium caps.
6. Cool in cold tap water for 4 min.
7. Read optical density of test and standard at 630 nm in spectrophotometer setting zero optical density with blank.

$$\text{Calculation} = \frac{\text{OD test}}{\text{OD standard}} \times \text{mg glucose per cent}$$

Serum Uric acid

Phosphostungstate method

Preparation of reagents

Solution 1 Phosphotungstic acid reagent

Sodium tungstate	50 g
82% Orthophosphoric and	32.0 ml

Take a round bottomed flask, reflex the mixture for 2 hrs. Take out the flask from condenser attached and add a drop of bromine. Boil for few minutes on a flame and then dilute to 1000 ml in a graduated cylinder. It is customary to use 32 g of lithium sulphate which is added to the reagent mixture after making the volume to 1000 ml. Lithum sulphate is dissolved by thorough mixing. It increases the sensitivity and stability of the reagent.

Solution 2

Trisodium phosphate	1.0 g
Water	1000.0 ml

Solution 3 (Alkalizing reagent)

Anhydrous sodium carbonate	100.0 g
Urea	200.0 g
Triethanolamine	800.0 ml
Make up to	1000 ml

Alternatively prepare 14% sodium carbonate solution by dissolving 70.0 g of anhydrous sodium carbonate in water and dilute to 500 ml. The former reagent is powerful over the latter. Store in a polythene bottle at room temperature.

Solution 4 Uric acid standard

a) Stock standard

In a 1 litre beaker take 500 ml of distilled water.

i. Add 1.0 g of lithium carbonate and dissolve by shaking.

ii. Add 500 mg of pure picric acid, stirr until dissolved. Warm up to 60°C.

iii. Add 5.0 ml of 40% formaldehyde.

iv. Add 400 ml of water.

v. Mix and adjust the pH with addition of drops of dilute acetic acid (1 vol of glacial acetic acid and 3 vol of water).

vi. Transfer the above solution to 1 litre flask and dilute the standard stock to 1000 ml, put the stopper and mix by inversion.

Keep the stock standard solution in a refrigerator. It is stable for several months.

Take out as needed only (10–20 ml). Let the stock solution be adjusted to room temperature before taking the liquid for dilution.

b) Working standard (5 mg/dl)

Dilute the stock standard (1–10) by placing 1.0 ml of the stock in a 100 ml volumetric flask and dilute it up to the mark with water and keep at room temperature for ready use. Do not store for prolonged period at room temperature.

Procedure

1. Take three centrifuge tubes with tapering ends (15 ml capacity) and place them in a rack, label them as 'T', 'S' and 'B' which correspond to test, standard and blank respectively.
2. Add 0.5 ml of the serum specimen in (T), working standard in (S) and water in (B).
3. Add 1.5 ml of phosphotungstic acid solution to all the tubes and shake well.
4. After 3 or 4 min. centrifuge the specimen tube.
5. Transfer 1.0 ml of clear supernatant to approximately labelled tubes T, S, B. Transfer 3.0 ml of the alkalizing reagent and mix well.
6. After 20 min. read the absorbance of the sample and standard against the blank (1680–700 nm).

Calculation

Concentration of uric acid (mg/dl) = (A/A5) x 5

A5 = Absorbance of standard solution

5 = Concentration of the standard.

Serum Calcium

Titrimetric method

Preparation of Reagents

Solution 1

Indicator calcium (1-2 hydroxy -1 naphthylazo) -2-naphthol -4 sulphonic acid)

50.0 ml of calcium is dissolved in 10.0 ml of ethyl alcohol and this solution lasts for several weeks at room temperature. Other suggested suitable dyes—Eriochrome R 2002, Eriochrome, S.E., methyl thymol blue and calcofast blue 2 G.

Calcein, Murexide, Patton-Reeder are not suitable dyes.

Solution 2

Sodium hydroxide (2N)

30.0 g of sodium hydroxide is dissolved in 500 ml of water in a volumetric flask. After cooling the solution is made up to 1000 ml.

Solution 3

EDTA

a) Stock solution (0.05 M). Take a one litre flask and add 14.0 g of disodium EDTA dehydrate. By adding 1000 ml water mix well and allow the salt to dissolve.

b) Working solution

Dilute this stock EDTA solution to 1 : 50.

c) Standardize the EDTA solution by titrating with calcium standard.

Solution 4

Stock standard (0.40 mg/ml) weight 0.1001 anhydrous calcium carbonate.

Keep the salt at 110°C overnight in and then cool in dessication. Take a 100 ml volumetric flask and transfer the calcium carbonate. Add 15 ml of water with 2.0 ml of 1NHCl. Heat at 60°C to facilitate solubility.

Solution 5

Working solution of calcium (10 mg/dl). Dilute 25.0 ml of stock standard with 100.0 ml water in a volumetric flask.

Procedure

1. Fill a burette with diluted solution of EDTA.
2. Take two clean 100 ml Erlenmeyer flasks and mark them as 'T' (test) and 'S' (standard).
3. Add 1.0 ml of serum to be tested in the flask marked 'T' and 1.0 ml working standard solution of calcium (0.1 mg) in the flask marked 'S'.
4. Add 4 ml of sodium hydroxide solution and 2.5 ml of distilled water to both flasks. Mix and add 2 drops of indicator solution.
5. Titrate the contents of the flask (Test and standard) with the diluted EDTA solution held on the burette. Continue titration until colour changes from purple to light blue. Note the volume of the titrant (EDTA) needed for the test solution (T_1) and for the standard solution (T_2).
6. Calculate the calcium concentration of serum … calcium concentration in serum (mg/dl) = $T_1/T_2 \times 10$.

   T_1 = Volume of titrant (EDTA) needed for the test reason

   T_2 = Volume of titrant (EDTA) needed for the standard.

   10= Concentration of standard (10 mg/dl)

   Conversions (a) mg/dl × 1.25 = mmol/L

   (b) mg/dl × 0.5 = mEq/L

Fehling Test for Sugar

Solution 1

Copper sulphate	34.64 g
Distilled water	500.0 ml

Solution 2

Sodium potassium tartarate	173.0 g
Potassium hydroxide	100.0 g
Distilled water	500.0 ml

Mix 1 ml each of solution 1 and solution 2 in a test tube and to it add 6 ml of distilled water. Mix and boil the solution. Add few drops of urine at a time to the solution and avoid boiling urine quantity should be less than the quantity of the reagent. A red or yellow precipitate shows the presence of sugar.

Rubners Test for Lactose

In a test tube take 10 ml of urine and add 2.5 g of lead acetate. Shake, filter and boil the filtrate and then add 1.5 ml of ammonium hydroxide (sp. g 0.880). On cooling a red precipitate develops which indicates the presence of lactose.

Selincinoff's Test for Fructose

Solution 1 Seliwanoff's reagent

Resorcinol	50 mg
Hydrochloric acid	33.0 ml
Distilled water	100.0 ml

This solution lasts for 3 weeks.

Take 2 ml of solution in a test tube and add 0.3 ml of urine and heat in a water bath for five min. If fructose is present a deep reddish colour develops.

Tauber's Test for Pentose

Solution 1 Tauber's reagent

Benzedine	4.0 g
Glacial acetic acid	100.0 ml

Dissolve benzedine in acetic acid. This solution lasts for four days. Take 1.0 ml of urine in a test tube add 0.5 ml of solution 1. Boil for 30 sec. Add 1.0 ml of water. A pink or red colour denotes the presence of pentose. If there is no pentose the mixture is yellowish in colour.

Smith's Test

Take a test tube and add 3.0 ml of urine and then add equal volume of tincture iodine and water in equal parts. If bile pigment is present a bright green ring develops at the junction of the fluids.

BLOOD FILM EXAMINATION

A large number of parasites are found in blood such as malarial parasites, microfilaria and trypanosomes. To study these two types of blood films (a) thin blood film (b) thick blood film have to be prepared.

(a) Thin film can be made by a finger prick. A small drop is spread on a clean non-greasy slide and with a spreader uniform smear is made. It is allowed to dry and later stained.

(b) Thick film is made with a big drop of blood which is put in the centre of the slide and spread with the help of a needle or slide at least ½ an inch area and dried.

Blood films must be prepared within one hour after the blood is drawn. Otherwise organism morphology may not be clear. Blood films should be stained as soon as possible and delay of more than three days may result in failure to demonstrate staining characteristics of individual species.

The most common stains are of two types, the Wright's stain and Giemsa stain. In Wright's stain the fixative is combined with the staining solution. As a result of this both fixation and staining occur at the same time. Thick films are lysed while processing.

Giemsa stain is the other one. Here the fixative and stain are separate. So the thick film is fixed with absolute methanol (acetone free) before staining thick films as it will be lysed during the staining process.

When slides are removed from either staining, they are air-dried in a vertical position. Then they can be examined under oil immersion by placing oil directly on the uncovered blood film. If permanent slides are required for record, permanent mount should be made.

Staining of Blood Films

Blood cells have both acidophilic and basophilic structures. The nuclei are basophilic and take a blue shade the basophilic granules also take a blue shade. Haemoglobin takes a red shade. Various stains such as Wright's, Leishman's, Geimsa and Jenner stain are in vogue. In most laboratories methylene blue is used as a basic stain and toluidine blue in some. Most laboratories use eosin as the acid stain though azure I and II are also used.

Buffer solutions commonly used are

Solution 1

Sodium hydroxide	8 g
Distilled water	1000 ml

Solution 2

Potassium dihydrogenphosphate	27.2 g
Distilled water	1000 cc

Take 23.7 cc of solution 1 and 50 cc of solution 2 and mix it. 20 cc of the mixture is added to 100 cc of distilled water (pH 6.8).

Thick blood smear examination enable you to test larger quantity of blood but it has its own disadvantages in the sense that morphology is distorted resulting in non-identification. In this smear

blood cells concentrate in the centre of the film. Initially the slide should be examined under low magnification (10 × objective) to detect microfilariae, *Babesia* spp., *Trypanosoma* spp and malarial parasites are best detected under oil immersion (100 × objective). Presence of brown pigment granules may indicate the presence of malarial parasites. RBC will concentrate at the peripheri of the thick film and this gives a clue for malarial diagnosis.

Microfilaria occur in small numbers in thin films. They are commonly found at the edge of the film or at the feathered end of the film because they are carriers of these sites while spreading whereas RBC are drawn out into one single distinct layer of cells. These can be examined for malarial parasite, etc., using stains like Wright's stain.

Stains used

1. Wright's Stain

Wright's stain powder (BDA)	200 mg
Methylene blue (acetone free)	100 ml

Allow it to stand for a few days.

Procedure for Wright's staining

1. Flood the slide with stain 1–2 min.
2. Dilute it with equal amount of buffer.
3. Allow it to stand for 5 min.
4. Flood off with tap water.

2. Leishman's Stain

Leishman's powder	150 mg
Methyl alcohol	133 ml

If it does not dissolve, powder the stain and dissolve and store it.

Procedure for Leishman's staining

1. Flood the slide with stain for 20 min.
2. Dilute it with double amount of buffer.
3. Excess stain is flooded with distilled water.
4. Air-dry the slide.

3. Giemsa stain

Giemsa powder	300 mg
Glycerine	25.0 ml
Methyl alcohol	25.0 ml

This is the stock solution. Just before use dilute 1 ml of stain with 9 ml of buffered distilled water.

Procedure for Giemsa staining

1. Fix the blood film with methyl alcohol for 5 min.
2. Air-dry.
3. Flood the slide with stain for 15 min.

4. Wash with tap water.

Giemsa stains give the following results.

Erythrocytes	Pale red
Nuclei of leucocytes	Purple
Cytoplasm and Oesinophil granules	Dark pink or purple

Staining of Thick Films

Thick films have to be dehaemoglobinized before staining. The slide is kept in distilled water for 10 min. dried and stained with any of the stains mentioned above. They must not be fixed before staining. Common stains are field's stain and Simeon's stain.

Field stain A

Methylene blue	800 mg
Azure I	500 mg
Disodium hydrogen phosphate	6.25 g
Distilled water	500 ml

Field stain B

Eosin (water soluble)	1.0 g
Na_2HPO_4	5.0 g
KH_2PO_4	6.25 g
Distilled water	500 ml

Grind the ingredients and dissolve. Allow the stain to ripen for four hours and filter.

Procedure

1. Immerse the film in solution A for a sec.
2. Rinse in distilled water by waving.
3. Immerse in solution B for a sec.
4. Rinse in distilled water by waving for 2–3 sec.
5. Place vertically in a rack.

Simeon's Modification of Boyes and Sterevel's Method

This stain can easily be replaced for Leishman's or Wright's stain.

Solution 1

Eosin	1 g
Distilled water	1000 ml

Solution 2

(A)	Methylene blue	1 g
	Distilled water	75 ml
(B)	Potassium permanganate	1.5 g
	Distilled water	75 ml

Mix solutions A and B in a flask. A precipitate is formed. Flask is kept in a water bath and boiled for 30 min. during which time the precipitate dissolves.

Procedure for Thin Films

1. Fix the smear by immersing it in rectified spirit for 1 min.
2. Rinse in tap water.
3. Immerse in solution for 1–10 sec.
4. Rinse in tap water.
5. Immerse in solution for 2–15 sec.
6. Rinse in tap water for 4 sec.
7. Allow it to dry.

Procedure for Thick Films

1. Dehaemoglobinize by immersing in tap water.
2. Immerse in solution for 6 sec.
3. Wash in tap water.
4. Immerse in eosin solution for 1–2 sec.
5. Wash in tap water and allow it to dry.

5

PARASITOLOGY

This chapter is devoted to parasites infecting humans with a brief note on the diseases and the pathogenicity they cause. Protozoan and some metazoan endoparasites and the structural characteristics of their eggs and cysts are described here.

Many parasitic organisms which infect humans are distributed worldwide. Humans act mostly as the definitive host especially for roundworms, hookworms, filarial worms, etc., and intermediate host for malarial parasites and hydatid cysts. Tropical countries stand unique by providing characteristic types of parasitic infections.

Most often humans get infection through contaminated food, water, soil and transmission from domestic animals, insects which act as vectors and other infected persons and rarely by auto infection. Of these, the major and common mode of infection is through contaminated food, water and soiled fingers. Most of the intestinal parasites which enter the body this way, are infective stages such as cysts or embryonated eggs or even larval forms. Swallowed cysts through food or water commonly cause amoebic dysentery and other intestinal infections. In the case of roundworm, whipworm and pinworm (nematodes) infective stages (embryonated eggs) are swallowed. Larval infection takes place when improperly cooked beef or pork or fish are eaten. Guinea worm infection occurs through consumption of water containing the intermediate host *Cyclops* which carries the infective larval stages.

Hookworm infection is common in people who walk bare-footed on contaminated soil. The larva penetrates the skin. Schistosomiasis is acquired through the cercareal larvae which penetrate the skin. Gingivitis and trichomonasis are transmitted from person to person by kissing in the former case and intercourse in the latter.

When humans are prone to so many infections, clinicians and laboratory personnel should be aware of the appropriate procedures for their identification. They must be able to identify and provide most accurate diagnostic test results.

Laboratory plays a major role in morphological identification of the parasites. Parasites, when compared to bacteria, are large and possess a distinct shape and structure which enable their specific diagnosis on morphological grounds. Morphological diagnosis of parasite consists of two steps–detection of parasite or its parts and identification—and depends upon the skill and expertise of the technician in the identification of the parasite.

The various human parasites could be classified into five major sub-divisions. They include protozoans (amoeba, flagellate, ciliates, sporozoans, coccidians and microsporidians), the platyhelminthes (trematodes and cestodes), acanthocephalans, nematodes or roundworms and the arthropods (insects, spiders, and ticks). The main groups included here are protozoans, platyhelminthes and nematodes.

The list given in the next section includes those which are clinical, relevant in the context of human parasitology. This list provides some insight into parasite grouping thus leading to better understanding of organisms' morphology, parasite infection and appropriate clinical diagnostic approach. Identification of these parasites depends on correct specimen collection and adequate fixation. Improperly submitted specimens may result in failure to identify the organism.

LABORATORY METHOD FOR IDENTIFICATION OF PARASITES AND THEIR INFECTIONS

Parasitic organisms that infect humans are worldwide in distribution. Parasites of human beings come under five major groups:

1. Protozoa—amoebae, flagellates, ciliates, sporozoans, coccidians and microsporidians
2. Platyhelminthes (cestodes and trematodes)
3. Nematodes—roundworms, pinworms , etc.
4. Acanthocephalans
5. Arthropods—insects, spiders and mites

In this chapter only the first three groups are given importance and an elaborate account is given to those which are clinically relevant human parasites.

Morphological details are essential for the identification of parasites which in turn depend mostly on specimen collection and type of fixation. Care should be taken for these two steps, otherwise it will result in misidentification of the organism.

The following list furnishes information on the body sites infected with parasites.

This chapter provides information on laboratory techniques and morphological details for correct identification of the most common human parasites.

PROTOZOA

Intestinal Protozoans

Amoeba

Entamoeba histolytica
Entamoeba hartmanni
Entamoeba coli
Entamoeba polecki
Entamoeba gingivalis
Endolimax nana
Iodamoeba beutchlii

Flagellates

Giardia lamblia
Trichmonas hominis
Trichomonas tenax
Dientamoeba fragilis
Chilomastix mesnili
Enteromonas hominis
Retortamonas intestinalis

Ciliates

Balantidium coli

Microsporidia

Blastocystis hominis

Coccidia

Cryptosporidium parvum
Isospora belli
Sarcocystis hominis
Sarcocystis suihominis
Sarcocystis lindemanni

Protozoa in Blood and other Tissues

Sporozoa

Plasmodium vivax
Plasmodium ovale
Plasmodium malariae
Plasmodium falciparum

Flagellates

Leishmania tropica
Leishmania braziliensis
Leishmania donovani
Leishmania mexicana
Trypanosoma gambiense
Trypanosoma rhodesiense
Trypanosoma cruzi
Trypanosoma rangeli

Protozoa in other Body Sites

Flagellates

Trichomonas vaginalis

Coccidia

Toxoplasma gondi

TREMATODES

Intestinal

Fasciolopsis buski
Echinostoma ilocanum
Heterophyes heterophyes
Metagonimus yokogawi
Artyfechinostomum mehrai

Liver and Lungs

Dicrocoelium dendriticum
Opisthorchis sinensis
Fasciola hepatica
Paragonimus westermani

Bile Passage

Clonorchis sinensis

Bile Tract

Opisthorchis felineus

Caecum and Colon

Gastrodiscoides hominis

CESTODES

Intestinal

Diphyllobothrium latum
Diphylidium caninum
Hymenolepis nana
Hymenolepis diminuta
Taenia solium
Taenia saginata

Tissues

Echinococcus granulosus
Echinococcus multilocularis
Multiceps multiceps

NEMATODES

Intestinal

Ascaris lumbricoides
Enterobius vermicularis
Ancylostoma duodenale
Necator americanus
Strongyloides stercoralis
Trichuris trichiura

Tissues

Trichinella spiralis

Cutaneous

Ancylostoma braziliense
Angiostrongylus contonensis
Gnathostoma spinigerum

Connective Tissues

Dracunculus medinensis

Blood

Wuchereria brancrofti
Brugia malayi

Loa loa
Onchocerca volvulus
Mansonella ozzardi
Dirofilaria immitis
Dipetalonema perstans
Dipetalonema streptocercum

Protozoa

Protozoans are unicellular or single-celled microorganisms having a single nucleus or nuclei and cytoplasm. Structurally the protozoa can be compared to a single animal cell and functionally they are comparable to a whole animal in performing various functions such as protection, locomotion, nutrition, excretion and reproduction. Each protozoan cell possesses almost all metazoan organelles such as nucleus, Golgi, mitochondria and ribosomes; some protozoans may possess special organelles not recognized in metazoans.

Parasitic protozoa, unlike their free-living counterparts, exhibit a more restricted life being confined to a particular host, whereas free-living protozoa occur everywhere ranging from snowy mountain heights to deep seas. Protozoans exhibit a variety of shapes. Usually a protozoan cell is bound by a membrane supported by contractile fibres. Cytoplasm could be differentiated into outer cytoplasm and inner endoplasm. Ectoplasm is mostly concerned with locomotion (extending pseudopodia or flagella), nutrition (engulfment of food by extension of pseudopodia), and excretion by contractile vacuoles, and serves as a protective device. Endoplasm has a nucleus surrounded by nuclear membrane. In some protozoans nucleus is a mass of chromatin and in others it has a nuclear membrane, containing nuclear sap where karyosome is present. Nucleus contains one or more nucleoli. Endoplasm accommodates Golgi apparatus, mitochondria, endoplasmic reticulum, ribosomes, food vacuoles, contractile vacuoles and so on.

Most of the parasitic protozoans have an active stage and a resting stage, the active stage being trophozoite stage. Trophozoite in other words is an active feeding stage where food is made available by diffusion from the surrounding environment or by active transport through plasma membrane. Sometimes photocytosis may occur. In some protozoans an organelle called cytosome akin to mouth is present. Resting stage is the cyst stage, which lies dormant for long periods. This is also an infective stage for the vertebrate host.

Protozoans reproduce usually by binary fission or multiple fission and by budding. Some reproduce asexually by genetic change. In binary and multiple fission, the nuclear division is followed by the cytoplasmic division. This division may be longitudinal as in flagellates, or may be transverse as in ciliates. Malarial parasites have two cycles in their life history, involving schizogony (asexual) and sporogony (sexual). In schizogony, nuclear division results in the formation of merozoites. In ciliates, sexual division is by conjugation involving nuclear exchange between two organisms. In sporogony, male and female gametocytes are produced which after fertilization give rise to a zygote which in turn produces sporozoites.

Some parasitic protozoa involve only one host in their life cycle. Others use two hosts with sexual phase in one host and asexual phase in another host. The former is the definitive host and the latter is the intermediate host. According to Heyneman (1998), human protozoan parasites are classified under three phyla as follows:

1. Sarcomastigophora which includes flagellates and amoeba.
2. Apicomplexa which includes sporozoans
3. Ciliophora which includes ciliates

These three groups include a number of human protozoans.

Mastigophora

1. Flagella are the locomotor organs.
2. There may be one or more flagella.
3. Undulating membrane is present.
4. Sometimes flagellum runs along the undulating membrane.
5. A kinetoplast is invariably present from which arises the flagellum (*Trypanosoma* and *Leishmania*)
6. Sometimes kinetoplast is absent but flagella may be present (two or many). There are intestinal flagellates (*Giardia, Trichomonas*), *Chilomastix* and *Dientamoeba*.

Sarcodina

1. Organs of locomotion are pseudopodia.
2. Contractile vacuoles and food vacuoles are present.
3. Life cycle has two stages—a trophozoite and a cyst stage.
4. Cyst stage is the infective stage to vertebrate host.

Examples are *Entamoeba, Endolimax, Iodamoeba, Naegleria* and *Acanthamoeba*.

Sporozoa

Members of this group infect blood and other tissue, exhibit complex life cycle that introduce asexual and sexual generations. The four species of *Plasmodium* are included in this group and the disease caused is malaria. Isospora and Microsporidia are found in intestinal mucosa. These species are frequently reported from patients suffering from AIDS (Cohen *et al.,* 1984; Navin and Jurane, 1984; Ng *et al.,* 1984).

1. They have a complete life cycle with intermitting and asexual reproductive stages.
2. They involve two hosts, a definitive and an intermediate host.
3. They produce spore-like cysts, e.g., *Plasmodium* spp.

Coccidia

Toxoplasma gondii, the coccidian enters humans via ingestion although several stages in its life cycle are reported from other hosts particularly cats. Trophozoites can be identified in squash preparation from brain tissue and lymph nodes. Examples are *Cryptosporidium, Sarcocystis, Toxoplasma* and *Babesia*.

Ciliates

1. Chief locomotor organs are cilia, which are extensions of the cytoplasm.
2. There are two nuclei—the macro and micronucleus.

3. Sexual reproduction by conjugation.
4. Excretion by cytophase.

Balantidium coli is a giant intestinal ciliate. This is the only human ciliate parasite.

Human protozoan parasites

Intestinal Protozoa

Amoeba

Entamoeba histolytica
Entamoeba hartmanni
Entamoeba coli
Entamoeba gingivalis
Entamoeba polecki
Endolimax nana
Iodamoeba beütchlii

Flagellates

Giardia lamblia
Trichomonas hominis
Trichomonas tenax
Dientamoeba fragilis
Chilomastix mesnili
Enteromonas hominis
Retortamonas intestinalis

Ciliates

Balantidium coli

Coccidians

Cryptosporidium parvum
Isosporalelli
Sarcocystis hominis
Sarcocystis suihominis
Sarcocystis lindemanni
Toxoplasma gondii

Microsporidians

Blastocystis hominis
Nosema composi
Plestophora sp.

Haemoflagellates

Trypanosoma gambiense
Trypanosoma rhodesiense
Trypanosoma bruci
Trypanosoma cruzi
Trypanosoma rangeli
Leishmania donovani
Leishmania mexicana
Leishmania tropica
Leishmania braziliensis

INTESTINAL AMOEBAE

Entamoeba histolytica

This parasite (Figure 5.1) was first discovered by Losch (1875). It has worldwide distribution and is common in the tropics than in temperate countries. It inhabits the lumen of the large intestine of human beings, dogs, cats, pigs, rodents and primates. It actually invades intestinal mucus.

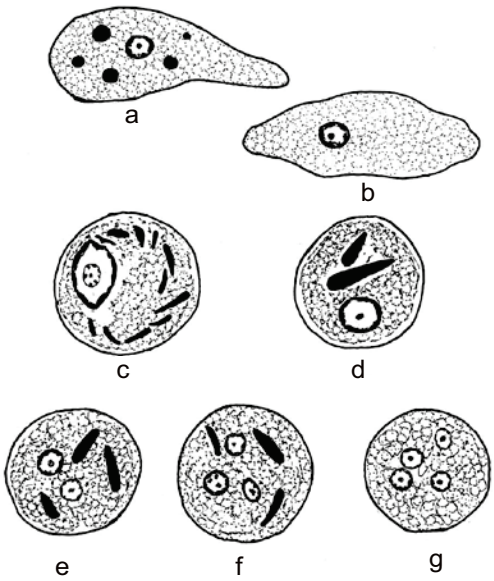


Figure 5.1 *Extamoeba histolytica;* a, b—Trophozoites, c,d—Early cysts, e–g—Cysts

Trophozoite

The trophozoite is motile and irregular in shape, and measures about 15–30 μm in diameter. It may contain red cells, but no bacterial inclusions. Fingerlike pseudopodia help in motility. It has an irregular

shape inhibiting amoeboid movement. There is a single nucleus, which can be noticed in unstained preparation. Peripheral chromatin has fine granules, which are evenly distributed. Karyosome is compact, centrally located and sometimes eccentric. Cytoplasm is finely granular and could be differentiated into ectoplasm and endoplasm. Ectoplasm is clear and distinctly separated from endoplasm which contains a number of vesicles embedded in a cytoplasmic matrix. These vesicles have ingested red blood cells. There is no rough endoplasmic reticulum or Golgi system. Ribosomes are arranged helically. There are microfilaments at the site of attachment below the plasma membrane. They form phagocytic channels. Nuclear structure is clear with haemotoxylin, eosine stain or trichrome stain, and is 4–7 μm diameter. The nuclear membrane is clear and lined by closely packed granules of chromatin. Karyosome is present in the centre of the nucleus as a small mass of chromatin.

Precyst

These stages are colourless, round or oval cells, smaller than the trophozoites and larger than the cyst. They have a round nucleus but no cyst wall and no ingested material. Rarely chromatoid bodies are seen in the cytoplasm.

Cyst

Cysts are spherical or oval in shape, 10–16 μm thick. Immature cysts have a single nucleus and mature cysts contain four nuclei. Mostly the nuclei are not visible but rarely appear as refractive bodies. In the young cyst, cytoplasm contains vacuoles with chromatoid bodies, which take bluish violet stain with haemotoxylin like the chromatin of the nucleus. The mature cyst also contains paracrystalline aggregation of ribosomes.

Epidemiology

E. histolytica has worldwide distribution. It can be ranked third among leading parasites causing fatal diseases, first two being malaria and schistosomiasis. At least 50–80% of the population in the tropics are affected.

Pathogenesis

E. histolytica penetrates the intestinal mucosa, forms ulcers, multiplies and finally lead to mucosal damage and haemorrhage. The amoebae feed on red blood cells. A bloody dysentery (amoebic dysentery) is the result of the invasion. Amoebic ulcers erode blood vessels and as a result amoebae get into blood circulation and are thus carried to other organs. They get established in blood vessels and form amoebic ulcers or abscesses. Liver is more prone for these abscesses but lungs and brain are also invaded. Amoebic liver abscesses, peritoneal amoebiasis, pericardial amoebiasis are some of the diseases for which *Entamoeba histolytica* is the causative agent.

Entamoeba hartmanni

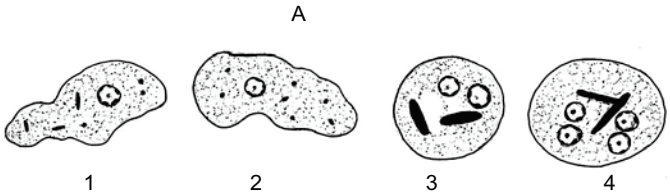


Figure 5.2 *Entamoeba hartmanni*, 1 and 2—Trophozoites, 3 and 4—Cysts

Trophozoite

Diameter ranges from 5–10 μm. No motility is noticed. There is a single nucleus which cannot be detected in unstained preparations. Karyosome is compact and located either centrally or eccentrically. Cytoplasm is finely granular. Bacteria and red blood cells are noticed as inclusions.

Cyst

Diameter is 5–10 μm. Immature cyst has one or two nuclei and mature cyst has four nuclei. Chromatin is in the form of granules and is evenly distributed. Karyosome is small, compact, centrally located and chromatid bodies are elongate with blunt round ends.

Entamoeba coli

This was first discovered by Lewis (1870) and Cunningham (1871) in Calcutta, and is worldwide in distribution.

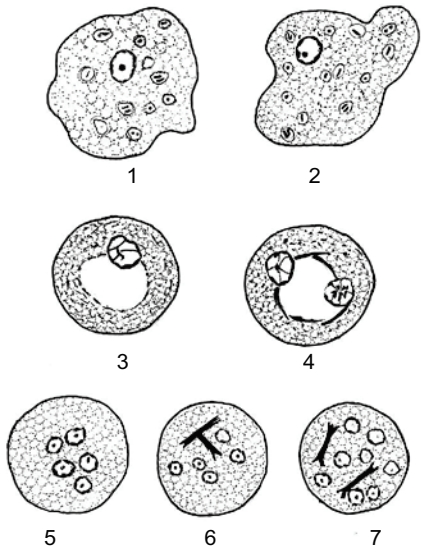


Figure 5.3 *Entamoeba histolytica*, 1 and 2—Trophozoites, 3 and 4—Early cysts, 5–7—Cysts

Trophozoite

It ranges in size between 20 and 25 μm. Pseudopodia are blunt and motility is sluggish. There is a single nucleus, which is visible in unstained preparations. Cytoplasm is dense with many food vacuoles. Chromatin is clumped and unevenly arranged on the membrane giving a dark ring appearance. Karyosome is not compact but is darkly stained. It may or may not be eccentric. The granular cytoplasm is vaguely differentiated into cytoplasm and endoplasm. Yeast and other debris form inclusions. Tropozoite inhabits large intestine but it is non-pathogenic.

Cyst

Size ranges from 10 to 35 μm. It has a spherical shape, but is rarely oval or triangular. Mature cysts have 8 nuclei or even more, whereas immature cysts have two nuclei. Chromatoid bodies are sphincter-shaped with round pointed ends.

Epidemiology

Common mode of transmission is through faecal contamination and the pathogen reaches the mouth. Its prevalence is reported in the tropics, USA, Europe and in countries with poor hygienic conditions. Though monkeys and dogs are infected, human infection is acquired from human sources.

Pathogenesis

E. coli is a lumen parasite that is non-pathogenic, there are no symptoms of infection.

Entamoeba polecki

It is usually a parasite of monkey and dog but there are occasional reports in man. The trophozoite of *E. polecki* resembles that of *E. coli* with a nucleate cyst. It measures 10–20 μm and cyst is 5–11 μm.Trophozoite exhibits sluggish movements. Inclusion bodies are oval. This is a parasite in the intestine of dogs, goats and monkeys. According to some it is intermediate between *E. histolytica* and *E. coli* but more investigations revealed its identity as a distinct species. It is non-pathogenic unlike *E. coli*.

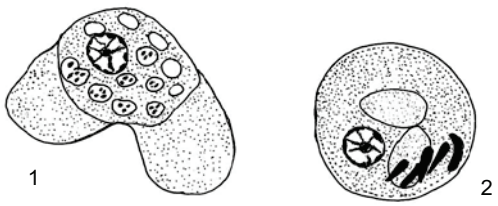


Figure 5.4 *Entamoeba polecki*, 2—Trophozoite

Epidemiology

This parasite is cosmopolitan and usually attacks the large intestine of monkeys and dogs but its prevalence in humans has been rarely reported. The trophozoites of *E. polecki* have resemblance to those of *E. coli* in exhibiting sluggish movement. Cysts are uninucleated with ovoid inclusion bodies.

Pathogenesis

Pathogenicity in human and reservoir hosts is rarely reported.

Entamoeba gingivalis

This was first described by Gross in 1849. This parasite has worldwide distribution. Peculiarly in this form only the trophozoite is present, the cyst stage being absent. It inhabits the gingival areas around mouth of humans and other mammals. The diameter is 5–30 μm. There are a number of food vacuoles. Nucleus is lined by a beaded peripheral chromatin. Consumed bacteria form inclusions. The nucleus has a central karyosome.

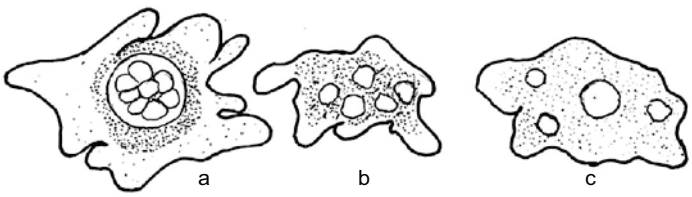


Figure 5.5 *Entamoeba gingivalis*, a & b—Unstained, c—Stained with haemotoxylin

Epidemiology

Transmission is via droplet spray from the mouth of an infected individual to another during close contact or from contaminated drinking glasses or other dishes. Incidence of infection is 70–95 per cent.

Pathogenesis

It is actually a commensal found primarily on exuva from the margins of the gums from between the teeth or from dentures. This is associated with *Trichomonas tenax* or with oral bacteria or spirochetes.

Endolimax nana

Endolimax nana is a cosmopolitan intestinal amoeba of humans. It is also found to occur in pigs, and primates. It is most often confused with *E. histolytica*.

Trophozoites

These are small usually 8–10 μm in diameter. Movement is by pseudopodia but they fail to produce directional locomotion. There is generally a single nucleus rarely noticeable in unstained preparations. Peripheral chromatin is absent. There is a large irregular karyosome. Cytoplasm is granular, and vacuolated. Bacteria are seen as inclusions.

Cyst

They are 6–10 μm in diameter with a retractile cyst wall. Nuclear structure and the appearance of the cytoplasm closely resemble those of *Iodamoeba beutchlii*. The shape of the cyst varies from spherical, ellipsoidal to ovoidal. There are four nuclei in mature cysts and two in immature cysts. There is no peripheral chromatin. Karyosome is small. In *E. nana*, no chromatoidal body could be seen in stained preparations.

Epidemiology

The source of infection is similar to that of other amoebae, from cysts. Infection is by ingestion of viable cysts in polluted water, food or from contaminated objects. Cysts of *E. nana* cannot tolerate desiccation and other unfavourable conditions. Infection is at its peak in warm moist climate under poor hygienic conditions.

Pathogenesis

E. nana is non-pathogenic and produces no significant symptoms, although at times these may be associated with symptoms which are erroneously attributed to this amoeba.

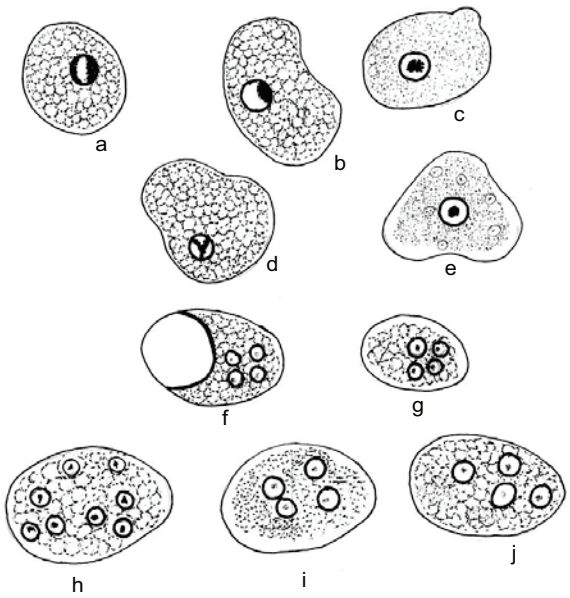


Figure 5.6 *Endolimax nana*, a–e—Trophozoites, f–j—Cysts

Iodamoeba beutchlii

This is the most common amoeba of swine. Pigs were the original hosts and this parasite has been recorded in human beings frequently.

Trophozoite

It greatly varies in size, the range being 12–15 μm in diameter. It is very sluggish. There is a single nucleus not detectable in unstained preparations. Peripheral chromatin is absent. Karyosome is large and surrounded by retractile granules. Cytoplasm is highly vacuolated and coarsely granular. It contains one or more glycogen masses, which are detectable with iodine staining. Bacteria, yeast, blood cells and debris are not found.

Cyst

Ranges from 6–12 μm in diameter. The shape of the cyst is either oval, ellipsoidal, pyriform or irregular, eccentric with retractile granules on one side. Chromatoidal bodies are absent. The cysts are prominent in iodine preparations because of the presence of the large dense glycogen-containing vacuole.

Epidemiology

Transmission of *Iodamoeba beutchlii* from one person to another takes place by injection of viable cysts through contaminated food, water and, sometimes, objects. This amoeba is not as common as *Entamoeba coli* or *Endolimax nana*. Percentage of infection is in the range of 2.5–5 per cent in temperate countries, whereas in the tropics, it is much higher. Rarely hog species may be responsible for infections.

Pathogenesis

Iodamoeba beutchlii is considered to be a non-pathogenic parasite and occupies the lumen of the large intestine. Trophozoite of this species occasionally invades the intestinal wall and migrates to the extra-intestinal viscera such as lungs and brain. In extra-intestinal regions, the parasites are found in multiple lesions (Derrick, 1948).

1. *Giardia lamblia*
2. *Trichomonas intestinalis*
3. *Trichomonas hominis*
4. *Trichomonas tenax*
5. *Chilomastix mesnili*
6. *Enteromonas hominis*
7. *Retortamonas intestinalis*

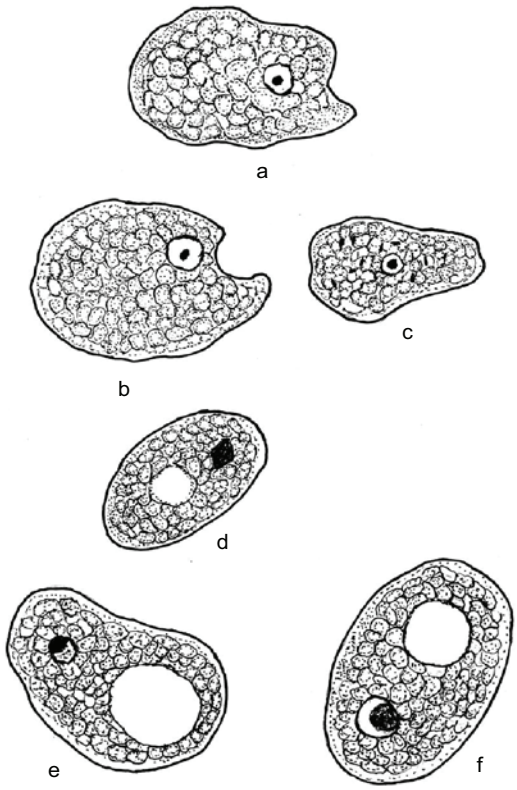


Figure 5.7 *Iodamoeba beutchlii*, a–c—Trophozoites, d–f—Cysts

INTESTINAL FLAGELLATES

Giardia lamblia

This was first discovered by Leeuwenhoek(1681). This genus name is after the Giard of Paris and the species name is after the Lamble of Prague. This organism inhabits the duodenum and jejunum of human beings. This is the causative agent of giardiasis. The trophozoite is motile, pear-shaped, measuring 10–12 μm in length and 5–10 μm in breadth. Generally, the organism is trapped in the mucosa of duodenum. There are eight pairs of flagellae, of which four are lateral, ventral and two caudal. Two nuclei are present. Karyosome is centrally located, prominent and in cystic phases. The two axostyles extend through the whole length of the body in the middle line. On the ventral surface there is an adhesive disc, which is attached to the intestinal wall. There are two parabasal bodies (according to Baker these are incorrectly called parabasal bodies whose function is not clearly known) lying posterior to the sucker. The ventral disc occupies a major portion of the ventral surface.

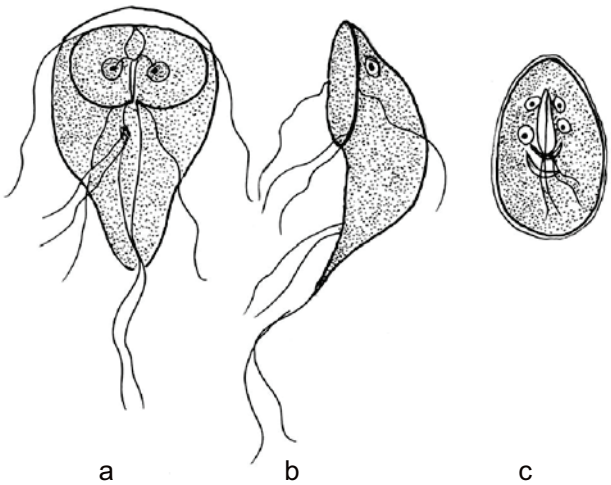


Figure 5.8 *Giardia lamblia*, a—Trophozoite ventral view, b—Profile view, c—Cysts

As the parasites pass into the colon, they encyst. Cysts are found in enormous numbers in stool. Cysts are infective stages and are oval in shape measuring 8 × 11 μm in size. The cysts contain two nuclei in immature stage and four nuclei in infective stage, placed at the anterior end; with a confused jumble of flagellae. There is a comma-shaped parabasal body. These cysts are ingested along with contaminated food and water. Children develop chronic diarrhoea.

Epidemiology

G. lamblia has worldwide distribution. Human beings are infected by contaminated water or blood containing *Giardia* cysts or by direct faecal contamination as seen in the cases of children who spend more time in day-care centres. Epidemics have been reported from USA when there is a failure in sewage disposal facilities or contaminated water supply. Cysts survive for two to three months in water. Sometimes people in wilderness also get infection from horse, cattle, sheep, deer or pet dogs and cats which harbour the parasite. This is an evidence to show that human infection can also be through zoonosis.

Pathogenesis

Trophozoite gets attached to the mucosal surface of the duodenal wall with the aid of the ventral disc of the sucker and damage the wall, leading to atrophy of the cells. They cause infection, slight inflammation of the duodenal and jejunal mucosa with diarrhoea and atrophy of the villi. This leads to loss of weight, distention, abdominal cramps and frequent flatulence. Children are more prone to this than adults. Diagnosis is by finding cysts in stool or trophozoite and cysts in liquid stools.

Trichomonas vaginalis

Three trichomonads are flagellated with 3–5 anterior flagellae and an undulating membrane. This is the largest of the three trichomonads infecting humans. *T. vaginalis* is oval or pear-shaped with a short undulating membrane almost lined by a flagellum, four anterior flagella which measure about 13 μm in length and width from 2 to 4 μm. The lateral flagellum ends at the middle of the cell. This also contains a prominent axostyle. The parabasal body with parabasal filament is large. The parabasal covers around the nucleus and the cytosome is not conspicuous. Nuclear chromatin is uniformly distributed. No cysts are formed. The trophozoite is transmitted through sexual intercourse.

Epidemiology

Cyst stage is lacking in *T. vaginalis*. During sexual intercourse, the parasite in the trophozoite stage is transmitted. Population survey reveals that females between 30–50 years are more prone to this than the young adults. Adult males are also susceptible. There are reports of its presence in newborn babies (Little wood and Kholer, 1966). Males act as vectors and infected female is the reservoir host of *T.vaginalis* which is distinct from *T. hominis,* a parasite of the human intestinal tract. Vaginal trichomonasis is not due to faecal contamination of female genitalia.

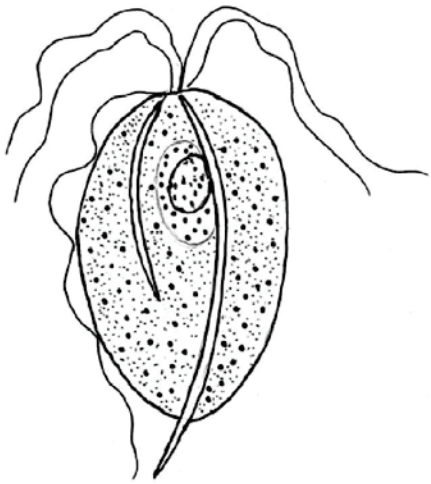


Figure 5.9 *Trichomonas vaginalis*

Pathogenesis

T. vaginalis lives in the vaginal, urethral and prostate tissues. Once it gains entry into the vagina, it causes degeneration, desquamation of the vaginal epithelium resulting in the inflammation of tissue layers.

Clinical diagnosis is by the demonstration of trophozoite in Giemsa-stained smears obtained from smears of vaginal discharge. Wet smears can be examined microscopically with a drop of saline and motile trophozoites can be spotted.

Trichomonas hominis

It is pear shaped and its size ranges from 11–12 μm with rapid jerky movements. There is a single nucleus, not noticeable in unstained preparations. There are four flagella of which three are anterior and one posterior. Axostyle protrudes beyond posterior end, may be visible. Undulating membrane extends through the length of the body. There is no cyst.

Epidemiology

There is no cyst stage in the life cycle. So transmission takes place in the trophozoite condition but not in the quiescent form. According to Weyman and O'Conner (1917), filth flies most probably serve as mechanical vectors. Once they gain entry through the mouth when taken along with contaminated food or water, they pass on to the stomach and small intestine. This parasite cannot be transplanted into the vagina.

Pathogenesis

There are reports of *T. hominis* being pathogenic causing intestinal disorder. Medical attention is required, though the symptoms are not of serious nature.

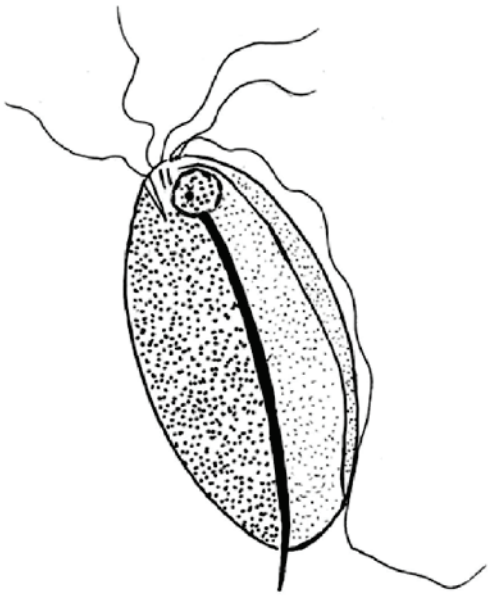


Figure 5.10 *Trichomonas hominis*

Trichomonas intestinalis

Size range is 10–15 μm. It is pear-shaped. There are commonly four but at times more flagellae. Three of them extend anteriorly. An axostyle extends the whole length of the body projecting beyond caudal end process. Undulating membrane extends along one side. Motility is by undulating flapping movements. There is no cyst.

Trichomonas tenax

T. tenax (Figure 5.11) is a parasite found in the gums measuring 5–12 μm in length and 7–10 μm in width. It is a pyriform flagellate known to occur in the trophozoite stage, appearing slender than *T. vaginalis*. There are four flagella, which are anteriorly directed, and one flagellum runs along the undulating membrane. There is a chromatin basal rod of the same length as the undulating membrane and a single blepharoplast. A parabasal apparatus, a thick axostyle is present originating near the blepharoplast. Nucleus is ovoidal and a cytostome is situated at the anterior end opposite to the undulating membrane.

Epidemiology

The incidence of infection is 0.25 per cent and this mainly depends on the exposure and oral hygienic conditions. The exact method of transmission of *Trichomonas tenax* is not clearly known but the positivity of exposure which is the result of droplet spray from the mouth, kissing or contaminated dishes and drinking glasses.

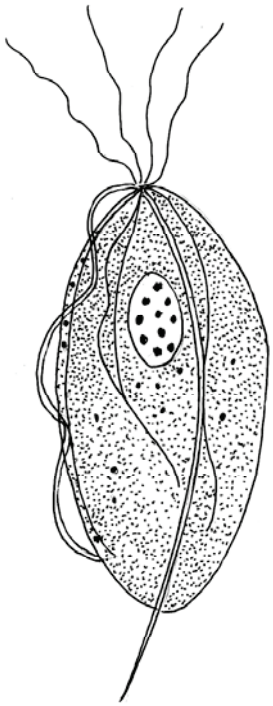


Figure 5.11 *Trichomonas tenax*

Pathogenesis

Trichomonas tenax is a non-pathogenic commensal found in the human oral cavity lodged in the tartar around the teeth and in the margins of the gums. Occasionally these parasite have been reported from the respiratory tract of humans (Watson and Buchanan, 1963).

Dientamoeba fragilis

For long this was included with the amoeba but in recent years it got the recognition as a flagellate and is classified along with *Trichomonas*. Trophozoite stage is like that of amoeba stage, size 9–12 μm with angular pseudopodia, broad, lobed and serrated. It is often bilobate or bean-shaped. Mostly the trophozoite has a single nucleus and has rarely, many nuclei, though not detectable in unstained preparation (Figure 5.12). Peripheral chromatin is absent. Karyosome has a cluster of 4–8 granules. No visible flagellae. Cytoplasm is finely granular and vacuolated inclusions containing bacteria and yeast. There is no cyst as in *Trichomonas*. It is actually a parasite of human beings but is also found in sheep, monkeys and apes. Infection is mild with slight abdominal pain, flatulence, diarrhoea and vomiting with loss of weight as in the case of giardiasis.

Epidemiology

Infection is highly prevalent in asylums. Transmission is usually by infection of trophozoite present in contaminated food, water and soiled fingertips. Experiments to infect humans and monkeys *Maccaca mullata* with cultured organisms orally did not give positive results. Burrows and Swardlow (1956) however found, that the common pinworm, *Enterobius vermicularis* acts as a vector because the pinworm eggs contained small amoeboid organisms most probably the *Dientamoeba fragilis*.

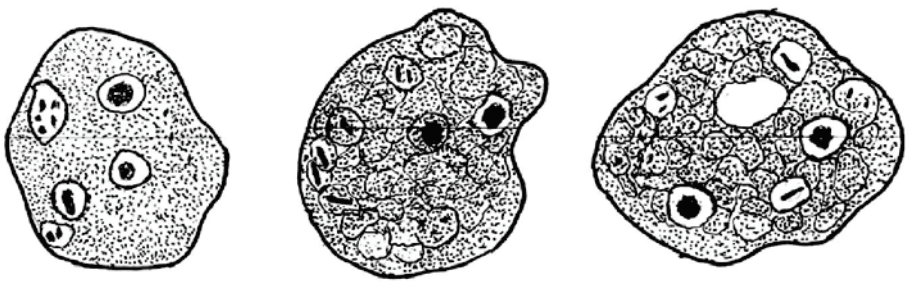


Figure 5.12 *Dientamoeba fragilis*, a—Uninucleate, b and c—Binucleate

Pathogenesis

Generally *Dientamoeba fragilis* do not invade tissues but when it settles down in the granular crypts of large intestine it causes superficial irritation, secretion of excess mucous, lack of dehydration of faeces, vomiting, nausea, low fever, diarrhoea with severe pain in the abdomen and hyper mortality of the bowel. Treatment is same as for *Entameoba histolytica* infection.

Chilomastix mesnili

Trophozoite

It is pear-shaped, 6–24 μm in diameter and resembles *Trichomonas*. It exhibits rotary or spiral movement, unlike trichomonads. There is a single nucleus not visible in unstained preparation. There are four flagella, three anterior and one in cytostome which is very prominent and extends almost half the length of the body (Figure 5.13). There is a spiral groove across the ventral surface of the body.

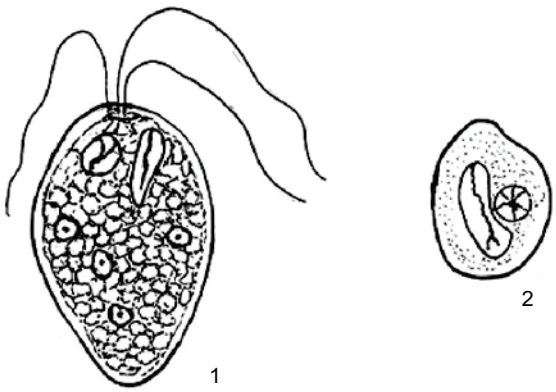


Figure 5.13 *Chilomastix mesnili*, 1—Trophozite, 2—Cyst

Cyst

It is lemon-shaped with a knob at the anterior end and has a diameter of 8 μm. Nucleus is single but not visible in unstained preparation. It is supported by fibrils; a curved fibril alongside of cytostome is referred to as "shepherd crook".

Epidemiology

The incidence of *C. mesnili* is 1–10 per cent or sometimes more. When cysts of *C. mesnili* from the stool of infected person gain entry into the mouth of healthy individual, transmission take place. Monkeys are known to harbour chilomastix but this does not resemble *C. mesnili* and there is no chance of any type of transmission from monkeys to man.

Pathogenesis

C. mesnili does not produce any symptoms and it is more or less a harmless commensal.

Enteromonas hominis

Trophozoite

It is oval in shape and is 8–9 μm exhibiting jerky movements. Single nucleus, as usual not visible in unstained preparations. There are four flagella, of these three are anteriorly situated, one extending freely either posteriorly or laterally (Figure 5.14).

Cyst

It is oval or elongate, and is 6–8 μm in diameter. Nuclear number varies from 1–4. Two nuclei lie at opposite ends of cyst, and are not visible in unstained preparation. It resembles cysts of *Endolimax nana*. Fibrils are not seen.

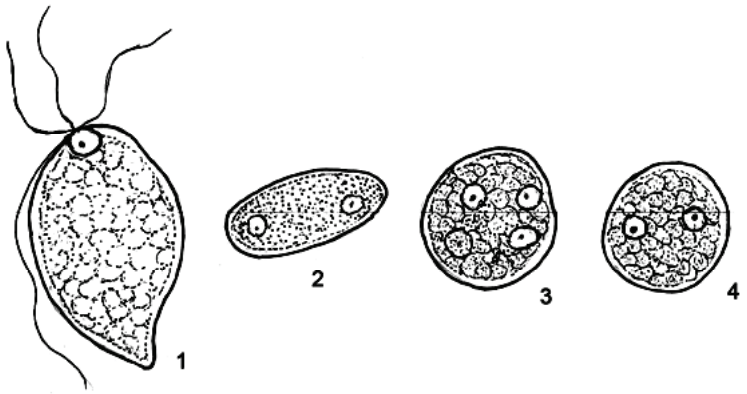


Figure 5.14 *Enteromonas hominis,* 1—Trophozoite, 2–4—Cysts

Epidemiology

It may be presumed that infection with *E. hominis* is the result of ingestion of food and water contaminated with cysts. There is no evidence for this.

Pathogenesis

Most often it has been detected from diarrhoea stools. But there are no symptoms, hence it is considered as a harmless commensal.

Retortamonas intestinalis

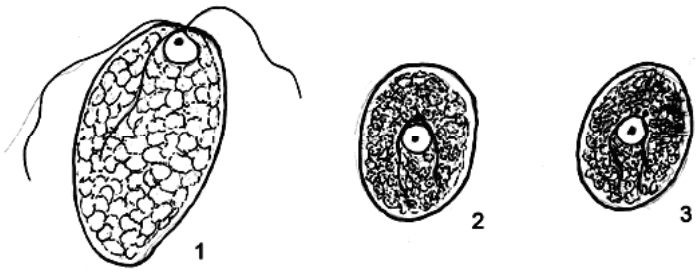


Figure 5.15 *Retortamonas intestinalis*, 1—Trophozoite, 2–3—Cysts

Trophozoite

Pear-shaped or oval, 6–7 μm, exhibits jerky movements. There is a single nucleus, not visible in unstained preparations. There are two flagella—one anterior and one posterior. Cytostome is present extending to half the length of the body (Figure 5.15).

Cyst

It is pear-shaped or lemon-shaped with a diameter range of 4–7 μm. Single nucleus, not clear in unstained preparations. Cyst almost resembles *Chilomastix* cyst with supporting fibrils extending above the nucleus.

Epidemiology

Food, water and unclean objects are the source of infection when they are contaminated with cysts in the stools of infected individuals. Unknowingly these cysts are engulfed along with food and water. The incidence rate is so low that thousands of stool samples have to be examined to locate the organism. Its related species are reported from guinea pigs, monkeys, rabbits and so on but this particular protozoan has been reported from other hosts.

Pathogenesis

Though *R. intestinalis* has been commonly reported from diarrhoea stool, its pathogenicity has not been proved.

Intestinal Coccidia

Cryptosporidium parvum

Isospora belli

Sarcocystis bovohominis

Sarcocystis suihominis

Sarcocystis lindemanni

Toxoplasma gondi

Intestinal coccidians

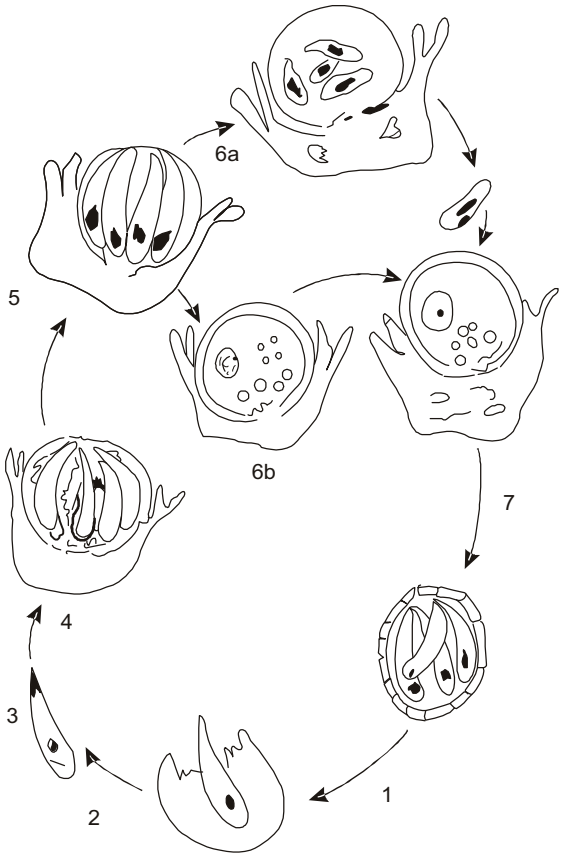
Cryptosporidium parvum

This was first described by Tyzzer (1907) and was described as *Cryptosporidium muris* in the gastric mucosa of laboratory mice. This was included in coccidia in 1912, the same author demonstrated a similar parasite in the small intestine and recognized it as a different species and named it as *Cryptosporidium parvum*. Till 1955 it was considered to be non-pathogenic, but in 1974 a similar disorder was noticed in calves. In 1976, Nine *et al.* first reported this in humans. It is a threat to HIV-infected individuals.

Cryptosporidium parvum is responsible for intestinal disorders and its presence has also been detected in immuno-suppressed patients particularly with AIDS (Garcia *et al.,* 1983, Navin and Juraneck, 1984) and it is a threat to HIV-infected individuals. (First report in humans was in 1976 by Nine *et al.,* 1976).

Cryptosporidium parvum infects humans, cattle, sheep, goat, deer, horse, buffaloes. Cats and non-mammalian hosts (Current and Bick, 1989). The life cycle (Figure 5.16)is completed in a single host. It is ingested in the oocyst stage, which is already sporulated before shedding. The oocyst is 4–6 μm in diameter. The ingested oocyst reaches the small intestine and later develops into micro- and macrogametocytes. Sexual multiplication takes place resembling the formation of the zygote that develops

into thin-walled oocysts. Thick-walled oocysts pass out along with faeces and thin-walled oocyst may lead into auto-infection (Angus, 1990).



1—Sporulated oocysts in faeces, 2—Encystment in intestine, 3—Free sporozoites in intestine, 4—Type 1 meront with 6–8 sporozoites, 5—Type 2 meront with 4 merozoites, 6—a. Microgametocyte with 16 microgametes, b. Microgamete fertilizes macrogamete, 7—Thick-walled oocyst

Figure 5.16 *Cryptosporidium parvum*

Isospora belli

This coccidian was first described in 1915 but it did not receive much attention because of its rareness in developed countries. But in recent years it has gained importance because of its association with AIDS patients. The infective stage is the oocyst.

Man is infected by ingestion of oocyst present in food or drink. The parasite stage inhabits the epithelial cells of the small intestine. Schizogony and sporogony take place in the human host. During schizogony, sickle-shaped merozoites are produced in the epithelial cells. Male and female gametocytes develop in the same infected cells. After fertilization, zygote develops into oocyst that comes out along with faeces.

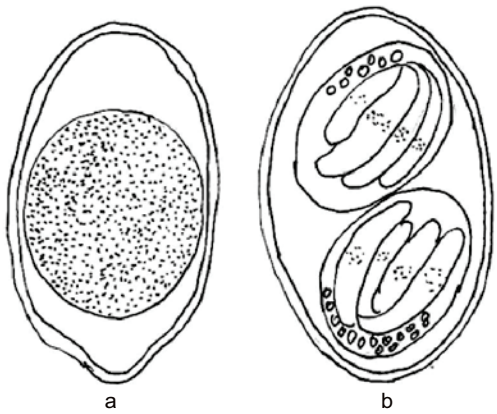


Figure 5.17 *Isospora belli* a—Immature cyst, b—Mature cyst

The oocyst is flask-shaped with transparent wall and measures 25 × 25 μm. It contains a single sporoblast. The oocyst matures inside the body and develops into two sporocysts each of which contain four sporozoites. This is the infective stage. At this stage when swallowed by the host the sporzoites reach and attack the intestinal epithelial cells and thus schizogony commences. Oocysts are the diagnostic stages in the stool. Sometimes, other stages in the life cycle could also be seen in the fluid taken from the intestinal tract.

Epidemiology

Dogs are supposed to be reservoir hosts. It is quite evident that food and water contaminated with faeces contain the ripe cysts. However how animals acquire infections from human *Isospora* is not clearly known.

Pathogenesis

Humans get infection by accidental ingestion of sporozoites, which burst out from ripened oocytes of *Isospora belli*. The mucosa of ileium or caecum gets eroded. The sporozoites transform into the trophozoites that are released and that start attacking other mucous cells. This results in extensive damage to the cells. Ultimately this leads to mucous diarrhoea and is a characteristic symptom of the disease.

Sarcocystis spp.

Sarcocystis is a common parasite of rodents, cow, pig and other herbivores but as human parasites they are not significant. Life cycle of *Sarcocystis* is similar to that of *Toxoplasma* but extra-intestinal development does not occur in the definitive host. Another difference is that the zooites of *Sarcocystis* are larger than those of *T. gondi*. Man is the definitive host of *S. bovihominis* and *S. buihominis*, the intermediate hosts of which are ox and pigs respectively.

The life cycle has both sexual and asexual phases, sexual phase in the intestinal gut mucosal cells of carnivores, and asexual phase in muscle and other cells of herbivores. Depending on the species, man serves as definitive as well as intermediate host for *Sarcocystis bovihominis* and *S. suihominis* and *S. lindemanni* respectively.

Sarcocystis produces cysts in the muscles of intermediate host measuring about 0.1 mm to several centimetres long. The cysts have a number of merozoites, which when released from a *Sarcocystis* in the

gut of a definitive host, the intestinal mucosal cells are invaded. They enter into a sexual stage to produce male and female gametes. Fertilization follows. The zygote develops into oocyst each having two sporocysts infective to the intermediate host. These oocysts pass out with the faeces and are ingested by the intermediate host. The sporocysts in the gut each release four sporozoites. The sporocyst penetrates the gut wall and reaches the vascular endothelial cells. They undergo schizogony and produce merozoites, which attack muscle fibres and develop into sporocysts.

Epidemiology

Infections in human beings are by ingestion of raw or poorly cooked lamb, pork, beef and other meat products.

Pathogenesis

Heavy sarcocyst infections sometimes result in the death of animals, e.g., mice, sheep and swine. It is not yet clear that the parasite is pathogenic to humans. However, subcutaneous swelling, eosinophilia and heart failure are attributed to *Sarcocystis* infection. There are reports of *Sarcocystis* being found in larynx, heart and tongue as well as skeletal muscle.

Sarcocystis lindemanni

It has an elongated fusiform shape, both ends being more or less pointed. Parasite is enclosed in a membrane and contains round and crescent-shaped spores. They appear as small streaks embedded in the muscle fibres and each parasite consists of a cylindrical white tube with lobulated surface. Both ends are pointed. The outer membrane exhibits radial striations, which extend as prolonged thus dividing the tube into boxes or compartments. Outer compartment has round cells and inner one has spores or trophozoites. Trophozoite has one end rounded and the other pointed. Nucleus lies nearer the round end. There are some types of muscle striations extending from one end to the other. A small polar ring is present at the pointed end. A number of trophozoites are present in a parasite. Parasites are usually found in the muscle of larynx, oesophagus, diaphragm, chest and abdomen, and rarely in heart muscle.

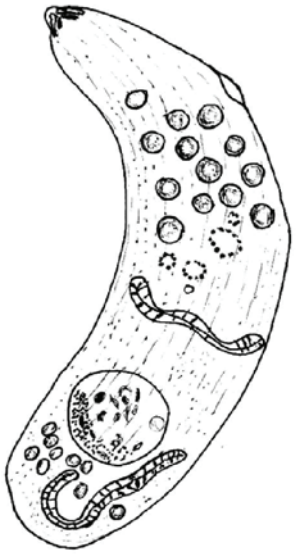


Figure 5.18 *Sarcocystis lindemanni*

Epidemiology

Laboratory animals can be infected artificially by feeding them with infected faeces or muscle strips. So it is easy for man to get infected with contaminated food and water.

Pathogenesis

No symptoms were noticed in persons infected with *S. lindemanni.* This parasite is usually located in myocardium, muscles of larynx, tongue and limbs. For sheep and other animals infection is fatal. The pathogenical symptoms are not very serious in humans.

Toxoplasma gondi

It is a coccidian protozoan (Figure 5.19) of world wide distribution. It infects a wide variety of animals and birds but does not cause any serious disease. Cats and similar genera of the family Felidae are the final hosts where the oocysts producing sexual stage develop.

Figure 5.19 *Toxoplasma gondi*

The trophozoites are pyriform ovoidal in shape measuring 4–6 μm long and 2–3 μm wide. Nucleus has a membrane and a central karyosome. In general morphology, it resembles *Leishmania* species but differ in the absence of kinetoplast. Innumerable numbers of parasites are observed in mononuclear and endothelial cells. In haemotoxylin preparations, nucleus shows a membrane surrounding it and a karyosome. In human beings these parasites are found in smears of exudates singly, free or intracellular, or in cystlike masses.

These organisms attack the mucous cells of the cat's small intestine and develop into schizonts and gametocyte. Sexual fusion between gametes results in the formation of oocysts. The oocysts migrate to the lumen of the gut from where they pass out with faeces. These oocysts resemble those of *Isospora*. Each oocyst has two sporocysts, which after 48 hrs develop into four sporozoites. Each oocyst has eight sporozoites, which after ingestion by some birds or any suitable mammal or human infect the duodenum and thus eight sporozoites are released. They penetrate through the wall, circulate inside the body and start invading macrophages, in which they form trophozoites. These trophozoites multiply into crescent-shaped cells, which invade the nervous system, especially the brain and the eye, and form cysts. This is the chronic stage of the disease. These cysts are infective. In human beings *T. gondi* produces what is called toxoplasmosis. Most of the human infections are non-significant. Sometimes it is fatal in patients with AIDS.

Epidemiology

Toxoplasmosis is acquired congenitally or from external sources. Newborns are the worst affected. The method of transmission is not clearly known. One method is by feeding the animal material with parasites via intranasal, extranervous, interperitoneal, sub-cutaneous or intracerebral inoculation (Sobin and Olisky, 1987). Inadequately cooked or undercooked pork when eaten, may positively become a reason for infection in rodents. The major source of infection is by ingestion of oocyst present in cat

faeces. This is supposed to be the primary source of infection. Infections are noticed in all types of climatic conditions in humans and other warm-blooded animals.

Pathogenesis

When the parasite is released into circulation, lesions are observed in the circulation. Tissue necrosis is observed in association with thrombosis of small vessels. Mononuclear cells are observed at the periphery of the lymph nodes. It is possible to identify the parasites with the help of immunocytochemical stains. *Toxoplasma* is noticed in the tissues of the patient who were immunosuppressed. In toxoplasmic encephalitis, central necrosis is common in AIDS patients, where the heart, liver, lungs and brain show necrosis and thrombosis.

Congenital toxoplasmosis affects the central nervous system. Encephalitis is common in brain tissues. Glial nodules are formed, central tissue destruction results from hydrocephalus. In the eye, the central region shows destruction. Human infections are non-significant. Sometimes it is fatal in patients with AIDS.

MICROSPORIDIA

Enterocytozoon bieneusi

This is a microsporidial infection prominent in AIDS patients. The microsporidia are an assemblage of intracellular parasites. This parasite has unicellular spores with a coiled tubular polar filament through which the sporoplasm is discharged into the host cell. This later develops into a schizont, which is oblong with two, eight or more nuclei, which become merozoites. These merozoites undergo series of sexual and asexual divisions resulting in spore production.

All vertebrates especially fish, and many invertebrate groups, especially insects, are infected. Transmission is through food and water contaminated with spores. Few reports were obtained from infected AIDS patients. The species that known to infect human beings are *Encephalitozoon cuniculi, Enterocytozoon bieneusi*, *Pleistophora* sp. and *Nosema connori*.

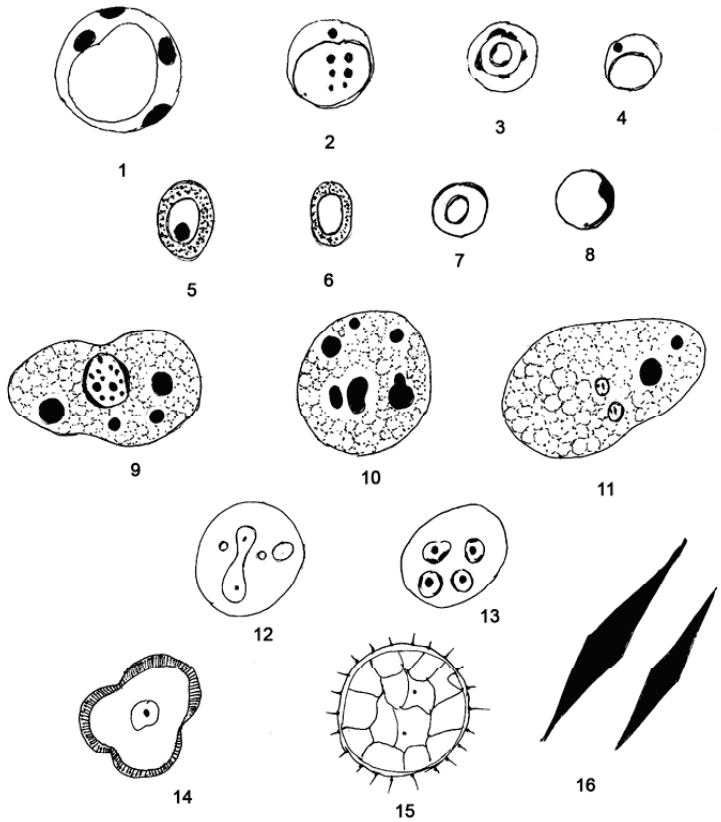
Microsporidian stages, which are infective, have small spores about 5 × 2 μm in size. These are ingested orally. The ingested spore ejects a hollow tubular polar filament through infective sporoplasm into the cytoplasm of the host cell. Within this host cell, successive cycles of merogony followed by sporogony occur leading to the production of many spores.

Encephalitozoon cuniculi is a common parasite of rodents, rabbits and carnivores. Monkeys are rarely infected. Two cases, one from Japan and other from Sweden were reported.

Blastocystis hominis

This is a fungal genus with a number of species. *Blastoscytsis hominis* occurs frequently in many samples of stools. It is closely attached to *Schizosaccharomycetes*. Young specimens are small 2 μm – 3 μm in diameter. There is a central spherical mass, the so-called paraglycogen body in the cytoplasm. With the growth of the organism, vacuolation of cytoplasm becomes evident. The organism grows to attain a size

varying from 8 to 35 μm. When mature the organism displays two areas 1) a central refringent paraglycogen mass. 2) an investing capsule with brightly refringent nuclei.



1,2,4—*Blastocystis hominis*, 3,5–8—Yeast cells, 9—Marcrophage with nucleus, 10 and 11—Deteriorated macrophage without nucleus, 12 and 13—Polymorphonuclear leucocytes, 14-15 Pollen grains, 16—Charcot–Leydon crystals

Figure 5.20 *Blastocystis hominis*

When making an emulsion of the stool, the outer capsule is pushed aside giving an "over rimmed type" resembling an engagement ring. Sometimes the outer capsule occurs on both sides of the paraglycogen mass giving "double rimmed type". Thus frequently it is misidentified as the cyst of *Entamoeba histolytica*. Division is mainly by binary fission. The outer capsule becomes elongated and the organism divides across into two. Occasionally a second division occurs before the completion of the first so that multiple fission stages appear.

In culture, two other methods of reproduction have been noticed. Hernia-like knobs are formed in the outer capsule and into each knob a nucleus enters. These buds are pinched off ultimately giving rise to several young forms. In old culture a third method of reproduction may be seen. This is called endogenous bud formation. Thus nuclei break down into smaller bits which later fuse to give rise to

small daughter nuclei. Each daughter nucleus acquires a small amount of cytoplasm, with the formation of a big acrospine containing small buds. This ruptures and the buds are liberated.

In most stools all these forms can be seen. Finally it should be staged that the impotence of *Blastocystis hominis* is that the student must not compare the sum of these stages for protozoa cysts.

INTESTINAL CILIATES

Balantidium coli

This is the largest protozoan parasite occurring in the intestine of man. There are two stages in the life cycle, the trophozoite and the cyst. Trophozoite is ovoidal in shape and covered with cilia. The cilia originate from basal granules situated under the cell membrane. The anterior end is little pointed with a slightly curved depression on one side of the long axis. The depression almost appears inverted. This is called the cytostome. Posterior end is somewhat broad and rounded. The size of trophozoite varies from 50–200 microns in length and 40–70 microns in width. The cytoplasm contains numerous food vacuoles and a couple of contractile vacuoles. The characteristic feature is the presence of two nuclei, the large macronucleus and the small micronucleus. Macronucleus is bean-shaped and densely packed with chromatin granules. The micronucleus is situated in the centre of the inner curvature of the macronucleus.

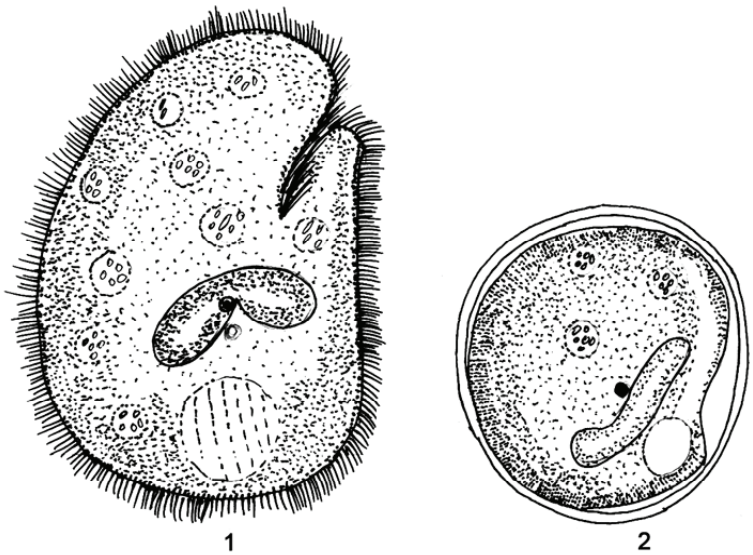


Figure 5.21 1— *Balantidium coli*, 2—Cyst

B. coli normally lives in the large intestine of man, monkeys and pigs. Trophozoites feed on the cells of the intestinal wall. They undergo transverse binary fission. This is followed by the division of the nuclei followed by division of cytoplasm resulting in the formation of two separate daughter organisms. Encystation of the trophozoite occurs in the faecal material which, down the bowl, becomes dehydrated. In the process, the organisms may partially round up. A cyst wall is formed around.

TREMATODA

Intestinal helminthes infecting humans fall under two phyla the platyhelminthes and nematodes. The flatworms, as the name suggests are flat, leaf-shaped, soft-bodied, syncitial worms. They have a pair of suckers. In this group of platyhelminthes there are two classes, the Trematoda and the Cestoda.

Tremotodes are leaf-shaped, elongated worms with two suckers and hooks for attachment. The digestive system is bipartite, except in snake trematode. *Atrophecaecum* anus does not open. They lack a cuticle but a cellular epithelium is present. The following are some of the characteristic features.

1. Unsegmented worms of different sizes.
2. All are hermaphrodites except schistosomes.
3. Alimentary canal is incomplete with bifurcated oesophagus in front of a ventral sucker and two or more intestinal caecae.
4. Acetabulum is the attachment organ.
5. Both nervous and excretory systems are present.
6. Highly developed reproductive system.
7. All eggs may be operculated or not, except schistosome egg.

Human Trematodes

1. *Fasciola hepatica*
2. *Fasciolopsis buski*
3. *Clonorchis sinensis*
4. *Opisthorchis felineus*
5. *Heterophyes heterophyes*
6. *Metagonimus yokogawai*
7. *Opisthorchis viverium*
8. *Paragonimus westermani*
9. *Dicrocoelium dendriticum*
10. *Gastrodiscoides hominis*
11. *Echinostoma ilocanum*

Species differ in their morphology, life cycle as well as infection sites. Schistosomes are dioecious, exhibiting sexual dimorphism. Another interesting feature is that they lack an encysted stage or a second intermediate host. They penetrate the skin and get lodged in the vascular system, whereas all other flukes are monoecious and encyst in a second intermediate host. Man gets infection by ingesting encysted metacercariae. Most of the trematodes possess operculated eggs which could be recovered from the stool by the sedimentation concentration technique. Floatation method does not give satisfactory results.

Most of the eggs of trematodes are very similar in size (Figure 5.24) and morphology. The eggs of *Clonorchis, Heterophyes, Metagonimus* are small and almost similar but larger than the above-mentioned forms.

Eggs of *Paragonimus westermani* are found in stools as well as in sputum. These eggs resemble those of *Diphyllobothrium latum.* Eggs of *Schistosoma* spp. are characterized by having a prominent lateral spine, and eggs are non-operculated.

Life cycles of trematodes are complicated. A sexual reproductive phase has several generations of larval stages in a snail which forms the first intermediate host. The life cycle of human trematode is initiated by eggs which are passed to freshwater through faeces.

The eggs hatch into ciliated larvae called the miracidium. Eggs of *Clonorchis sinensis, Opisthorchis felineus, Opisthorchis viverrini* and *Heterophyes heterophyes* are too small to be eaten by the snail host. In the body of the snail, development of different stages follows. Snail hosts are specific for each species of the fluke. In the snail's viscera, a series of larval stages are developed—sporocyst, redia, cercaria and metacercaria.

The rediae migrate to the digestive system of the snail or gonad and develop into cercariae. These cercariae are liberated each day. They swim about with lashing movements whereas schistosomes pass through two generations of sporocysts without rediae. The sporocyst (last generation) develops into cercariae with forked tail with which it hangs from the water surface. This posture enables them to invade the skin of the human host or any other vertebrate host. The encysted or metacercarial stage found in all other fluke's life cycles of humans is absent. This is the general pattern of development. Variations occur.

Fasciolopsis buski encysts on vegetation. Metacercariae gain entry into the host, when host eat raw vegetation. Maturity occurs in intestine.

Fasciola hepatica also encysts on aquatic vegetation. They gain entry through drinking water when they are washed off into the water and eaten by sheep and other cattle. Adult flukes are occasionally found in man. They penetrate the gut wall, then liver and finally the bile duct of the definitive host, the sheep.

Paragonimus westermani produces a number of cercariae in the infected host (snail), leaves the snail, crawls below the aquatic substrate with the help of adhesive tail in search of a crustacean (the second intermediate host) a crayfish or a crab. When these crustaceans are eaten raw or undercooked, metacercariae get encysted in human gut and young are released into the gut.

Other flukes of human beings are encysted in various fresh water fish. Cysts that gain entry into human beings are digested in the duodenum.

Heterophyes heterophyes lives in the intestine eroding the mucous membrane, *Clonorchis sinensis, Opisthorchis felineus, Opisthorchis viverrini* encyst in a variety of fresh water fishes and gain entry into the intestine of human beings by eating smoked, raw or pickled fish. Young encysted from metacercariae in digested fish flesh pass into the liver through the bile duct.

Echinostoma ilocanum

This is a parasite living attached to the wall of the small intestine and measures 2.5–6.5 mm in length and 1–1.35 mm in breadth (Figure 5.23). Anteriorly there is a circumoral disc surrounded by a row of 40–50 spines. The body is covered over by plaquelike scales. The oral sucker lies in the centre of the oral disc and the ventral sucker is also anterior in position. Testes lie one below the other. Ovary lies anterior to the vitellaria and occupies the lateral border at the posterior end.

Eggs are straw-coloured, operculate (Figure 5.24 (a)), 83–116 μm in diameter. They are not mature when passed in the stool but once they are dropped in water, they reach maturity in 6–15 days, and hatch.

The miracidium penetrates the snail tissues, the snail host being *Gyraulus convexiusculus* and passes through the mantlefold and gills, develops into redia, daughter rediae and finally cercariae in 42–50 days.

The cercariae have a simple tail and resemble a miniature adult, having the same number of circumoral spines and mature sex organs. Cercariae escape from the host tissue, lead a brief free-swimming life, seek second intermediate host, and encyst in the fresh water snail of the genus *Pila conica*. These snails are eaten raw by natives. So human infection is by consuming raw snails which are infected with encysted cercariae.

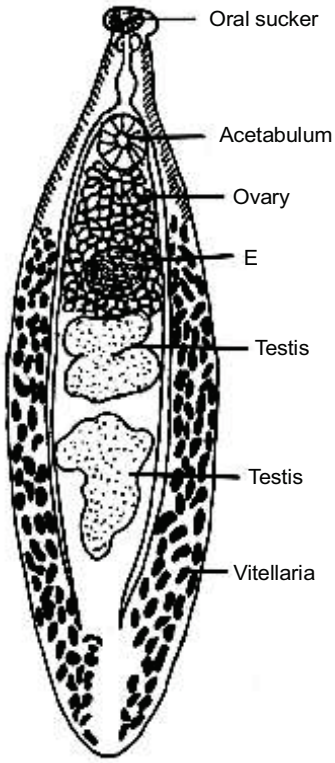


Figure 5.22 *Echinostoma ilocanum*

Epidemiology

Common in Korea, Indonesia, Philippines, Malaysia, Thailand, Taiwan where people have the habit of eating raw sea food.

Pathogenesis

The organism causes inflammation and ulceration of the jejunal mucosa. Echinostomes are not highly pathogenic but heavy infections may cause diarrhoea, abdominal discomfort and anaemia, though not of life-threatening nature.

Fasciola hepatica (Linnaeus, 1758) is a parasite of sheep and cattle. This was first discovered 500 years ago before most others (deBrie, 1379). Its complete life cyle was done by Luckart (1882) and Thomas (1883). This paved way for the elucidation of life cycles of other trematodes.

This is a parasite of sheep and cattle causing the disease "liver rot". It inhabits many domesticated and wild herbivores and has worldwide distribution.

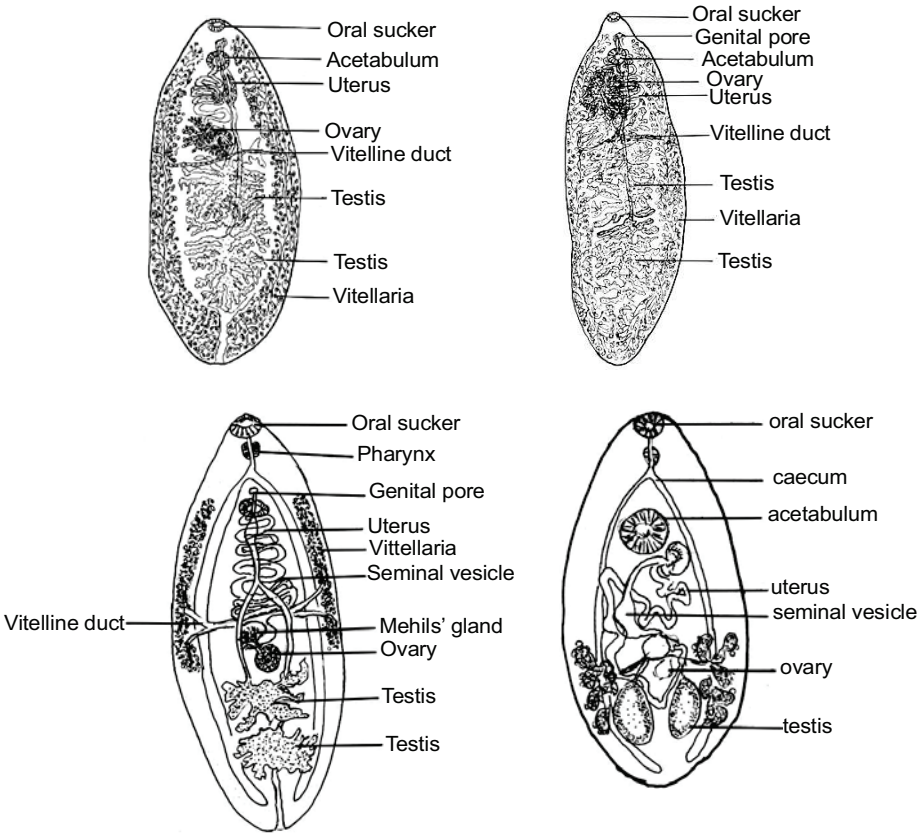
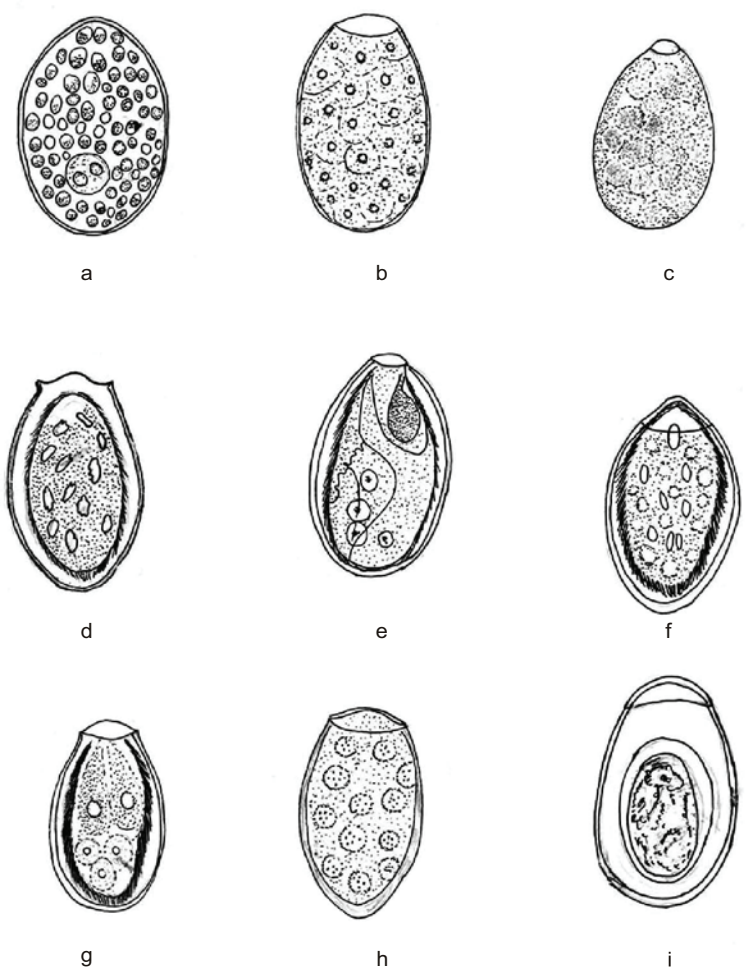


Figure 5.23 *Echinostoma ilocanum*

The adult worm lives in the bile duct of the final host and measures 2.5 cm × 8.13 cm, with a narrow anterior extremity containing the oral sucker. It is grey-coloured with dark edges. A little distance (3 mm) from the anterior extremity is situated the ventral sucker larger than the oral. Intestinal caecae branch out into diverticula. Ovary is situated in the posterior end, anterior to the testis and it is racemose. A short uterus is situated anterior to the ovary. Genital pore is median and cirrus is extensile.

Eggs are excreted in the faeces. Egg is oval (Figure 5.24b) (130–140 μm × 63–90 μm) brown, and has tanned appearance with ovum and yolk cells. A miracidium larva emerges out from the egg within 3 weeks and searches for a lymnaeid host. Several species of *Lymnaea* serve as hosts.



a—*Echinostoma ilocanum*, b—*Fasciola hepatica*, c—*FasciolIppsis buski*, d—*Clonorchis sinensis*, e—*Optisthorchis felineus*, f—*Heterophyes heterophyes*, g—*Metagonimus yokogawai*, h—*Paragonimus westermani*, i—*Dicrocoelium dendriticum*

Figure 5.24 Trematoda eggs

L. cubensis serves as intermediate host depending on its distribution in different parts of the world. In the snail host, miracidium develops into sporocyst which later develops into rediae giving rise to free-swimming metacercariae. It takes at least 2 months for the miracidium to develop into these successive stages. Cercariae are released from the body of the snail and settle on grass and start secreting mucus to form cyst containing metacercariae. These cysts are infective upon ingestion by sheep and other hoots. They enter the duodenum, penetrate the intestinal wall and then migrate to the body cavity and then to the bile duct where they mature into adults.

Epidemiology

Infection in humans is rare, though infection in other animals is severe and of high prevalence, especially in cattle. Human infection depends upon temperature (10–30°C) and their habit of eating water plants from water contaminated with animal faeces. Humidity and rainfall of high range may favour infection.

Pathogenesis

F.hepatica causes considerable damage to sheep and cattle. Acute cases result in severe pathology such as destruction of parenchymal tissues, haemorrhage. Repair mechanisms sometimes lead to fibrosis, increased pressure and atrophy of the liver.

Fasciolopsis buski (Lankester (1857), (1902))

This is the giant intestinal fluke. Its infection is mostly confined to Asian countries like China, India, Bangladesh, Thailand, Malaysia, Myanmar, and Sumatra. It is a parasite found in pig and dog, and inhabits the small intestine. This is the largest trematode of man measuring 3 cm × 12 cm and is 3 cm thick. It is oval, elongated and flesh-coloured (Figure 5.25). Body is covered by transverse rows of spines with numerous spines near the ventral sucker. Acetabulum is close to the ventral sucker and it is prolonged into a cavity dorsally and backwards. Intestinal caecae are without lateral branches. Genital pore is medially placed anterior to the ventral sucker. Both testis and ovary are branched.

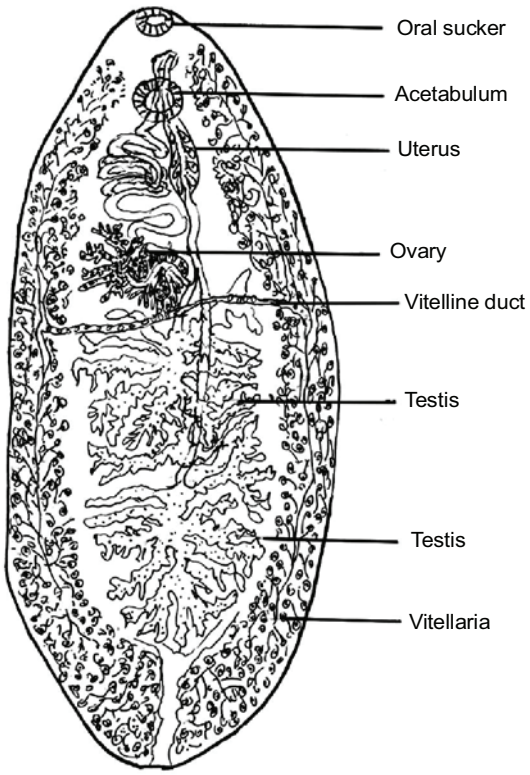


Figure 5.25 *Fasciolopsis buski*

Egg measures on an average 140 × 80 μm and is operculated. Presence of eggs in the stool confirms the infection. After 3–7 weeks, the egg hatches into a miracidium in the snail host *Segmentina hemisphaerula.* Miracidium later develops into sporocyst within 3 days, which later develops into redia, daughter redia and finally cercaria. The whole cycle is completed in 2 months. Cercaria is oval in shape, lophocercous, with a well-developed digestive tract, a muscular bladder and collecting lobules. These cercariae as usual encyst on water plants and transform into metacercariae. Man and pig become infected through the consumption of viable metacercariae attached to the water plants like water hyacinth, water caltrops, water chestnut, water bamboo, lotus roots and wild rice shoots.

F. buski encysts in the duodenum and gets attached to the duodenal and jejunal walls. The larvae mature in 3 months and start producing as many as 25,000 eggs per day. If these eggs reach water sources, the cycle repeats.

Epidemiology

In Thailand, certain places where there is heavy rainfall and flooding resulting in faecal contamination of water are sources of infection. In addition to this, there is large-scale usage of pig and human faeces as manure in certain areas and these regions are prone to infection. In China, there are reports of sources of infection through contaminated drinking water. Children over 5 years of age are more prone to infection since they eat and enjoy water plants while in play.

Pathogenesis

When the parasites occur in small numbers, they do not cause any harm, but when they occur in large numbers, they cause intestinal obstruction, followed by intestinal ulceration which will indirectly interfere with digestion. Ulcers sometimes bleed due to capillary damage.

Symptoms

Diarrhoea, flatulence, loss of appetite, vomiting, mild colic pain and ultimately fever and eosinophilia. Since eggs are laid in large numbers, faecal infection is quite easy.

Clonorchis sinensis (Cobbold (1875), Looss (1907))

Clonorchis sinensis (Figure 5.26) parasitizes man and also biliary passages of cats, dogs, rats, pigs, camel, etc. It is mostly found in bile ducts rather than in gall bladder or duodenum, pancreas and pancreatic duct. It is like a spatula measuring 10.25 × 2.5 mm. Anterior end is tapering with a small tegument. Oral sucker is larger than the ventral sucker and with simple intestinal caecae. Genital pore is situated anterior to the ventral sucker. Testes are located in the posterior extremity lying one behind the other. Ovary is trilobed. First the spermatozoa mature and pass on to the uterus, from there to the spermatheca where they are stored to receive ova which get fertilized there.

Eggs are small, measure 20–30 × 15–17 μm, (Figure 5.22d), are yellowish brown in colour and operculated. These are the smallest eggs of all trematodes found in man. These eggs lie viable for 5 weeks. Before that they have to find their way into the body of the snail before the miracidium hatches out. Miracidium first penetrates the wall of the Oesophagus. The cilia are cast off and develop into sporocyst.

Within the sporocyst, rediae are developed which grow and then the sporocyst bursts letting out rediae to attack the liver through the oesophagus. This lasts for 3–4 weeks.

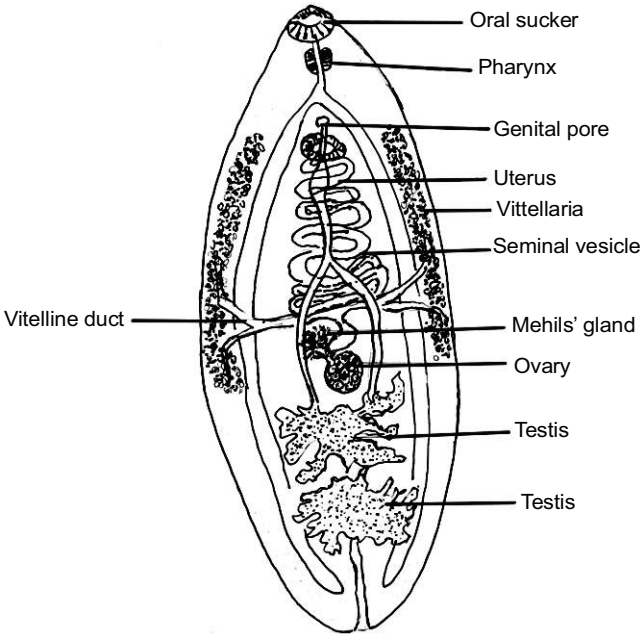


Figure 5.26 *Clonorchis sinensis*

Cercariae are lophocercous with a blunt tail, measure 450–550 × 100–200 μm, and escape through the birth pore of the rediae. There are two eyespots. The cercariae that come out of the snail must find another host which is a fish belonging to the family Cyprinidae and Anabantidae within 48 hrs. In fish they encyst in the muscles or beneath the scales as metacercariae. These start secreting a viscous fluid around the cyst which in turn is capsulated by a fibrous layer from the fish tissues. Man gets infection when the fish muscle is eaten raw or pickled. The cyst is resistant to the digestive juice of human.

The cyst wall slowly gets digested by secretions of duodenum and they escape and attack the mucosa. Maturity requires 26 days and it slowly reaches the bile duct. Lifespan is 12 years.

Epidemiology Dogs and cats are also infected besides man. In North China, human infection is rare. The definitive hosts acquire infection by consuming raw or pickled or salted fresh water fish which are infected with metacercariae. Even improperly cooked food brings out infection. People of Hawaii get these infections by consuming frozen, dried or pickled flesh of fish imported from Japan or China. Water polluted with faeces of reservoir hosts is the source of infection of the molluscan and subsequently fish hosts. *Clonorchis* infections are found in orientals in all parts of the world.

Pathology The larvae reach the bile ducts, and this results in inflammation in biliary epithelium. When larvae are in contact with epithelium, encapsulating fibrosis of the ducts results. When larvae are

numerous, they clog the duct. Walls become thickened and the surrounding liver portion gets affected and finally in severe cases leads to cirrhosis, liver enlargement, diarrhoea with blood oedema and abdominal discomfort. Diagnosis is based on the spotting of eggs in faeces.

Opisthorchis felineus (Revolta, 1884)

This parasite measures 8–11 × 1.5–2 mm, inhabits liver, bile ducts, pancreas and the lungs (Figure 5.27).

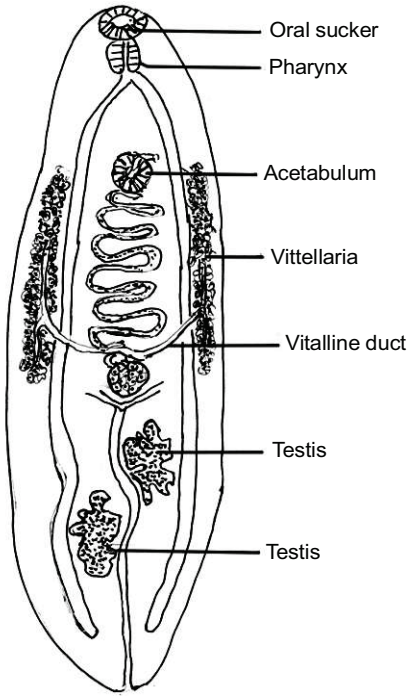


Figure 5.27 *Opisthorchis felineus*

Life cycle

The intermediate host is a snail belonging to the family Bithynidae *(Bithynia leachi, Bithyknia tentaculata)*. The eggs (Figure 5.22e) consumed by snail have a fully developed miracidium which develops into sporocyst in the intestine (1.2 – 1.85 mm). In a month's time rediae develop which later give rise to cercariae. Cercariae measure 430 – 670 × 40 – 50 μm (Figure 5.28). These come out of the snail and are phototactic.

Second intermediate host is the fish belonging to the family Cyprinidae *(Cyprinus carpio* and many others). The cercariae penetrate and develop into metacercariae (220 × 160 μm). When such infected fishes are consumed, they pass through stomach and enter the bile duct. The entire life cycle is completed in 4 months.

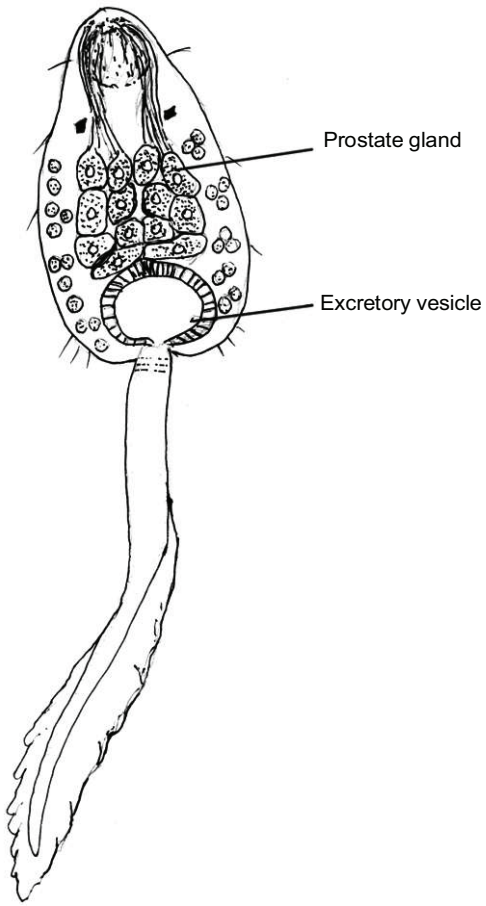


Figure 5.28 Cercaria of *Opisthorchis felineus*

Opisthorchis viverini (Poirier, 1886)

The definitive hosts are the dogs and civet cats. *O. viverini* differs from *O. felineus* in the following characters. VitellarIa are segregated into few clusters. Ovary and testes are closer. Egg size is 24 × 15 μm. It is an important fluke in Thailand. The snail hosts are *Bithynia funiculata B. goniophalus* and *B. laevis*. Second intermediate host *Puntius orphoides, Hampala dispar, Cyclocheilichthys siaja.* Important clinical findings are flatulence after meal and jaundice. Fish-eating mammals like dogs and cats serve as hosts.

Epidemiology

O. felineus is prevalent in animals throughout Europe, especially Siberia where people enjoy eating thin slices offrozen or highly salted fish.

O.viverini

In Thailand 35% of the people are prone to infection where raw fish dishes are a delicacy. Uncooked fermented fish is eaten daily, which contain metacercariae.

Pathogenesis

Liver enlargement, chronic inflammation of lymphocytes, monocytes, eosinophilia and epithelial hyperplasia.

Heterophyes heterophyes (Siebold 1852)

It is a parasite of the small intestine of humans, worldwide in distribution and occurring in Egypt, China, Japan, Brazil, Korea, Spain, France and Greece. Rats, fox, dog, wolf, jackal and cat also serve as hosts. It is a small parasite, pyriform in shape and measures 1–1.7 × 0.3–0.7 μm (Figure 5.29). It imparts a coffee colour to the intestinal wall. Ventral sucker is three times larger than the oral sucker. Tegument is thick with closely set narrow scales. The genital sucker is situated on the posterior border of the ventral sucker with a diameter of 150 μm and set with 60–90 spines which are multidigitate. Uterus is in the form of a brown patch in the centre. In the digestive system, the prepharynx is short, oesophagus is long and the intestinal caecae extend to the posterior extremity. Vitelline glands are situated posteriorly, arranged in two clumps. Near the ventral sucker lies the gential pore postero-laterally. The pore is encircled by a ring armed with 70 chitinous teeth. Ovary is spherical and median. Testes are oval and posterior. Receptaculum seminis is large and uterine coils are few. Laurer's canal and seminal vesicle are present.

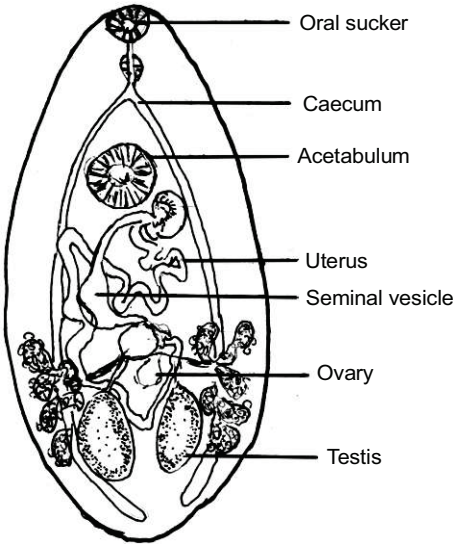


Figure 5.29 *Heterophyes heterophyes*

Eggs are minute, operculate and ovoidal (Figure 5.22f) with a light brown shade. Egg measures 20–30 × 15–17 μm. When eggs are deposited, each egg contains a ciliated miracidium with a bilateral symmetry. Hatching depends upon its coming in contact with a suitable host, the snail. When they are eaten by the snail host (*Cerithidia cingulata, Tymphotomus micropteres, Melanoides tuberculata,* etc.), these miracidia develop into sporocyst and later into cercariae with a well-developed eye and a membranous tail.

The cercariae, on finding a second intermediate host (*Mugil cephalus* (Mullet)), develop into metacercariae which encyst under the scales of the fish.

Epidemiology

Heterophyes is mainly confined to Asia (Japan, Korea, Thailand, Taiwan, Philippines and China). Human infection is by consuming infected raw or pickled or salted fish. These parasites are common in cats, dogs, foxes and other fish-eating mammals in addition to humans. When infected persons release faeces into water, it gets polluted and forms the source of infection.

Pathogenesis

Intestinal mucosa inflames at the sites of attachment of parasites. Because of parasites, diarrhoea, colic pain, irritation, etc., are some of the pathogenic symptoms. Sometimes the parasite reaches the cardiac valves, ultimately leading to cardiac failure. Sometimes they are carried to the brain where they cause cerebral haemorrhage.

Metagonimus yokogawai (Katsurada, 1901)

It has worldwide distribution. It inhabits the small intestine of man, cats, dogs, pigs, fish-eating birds also serve as hosts. It is a very small parasite (Figure 5.30), measuring 1 – 2.5 μm by 0.4 – 0.75 μm and its shape is almost similar to *Heterophyes heterophyes.* Ventral sucker is slightly deflected to the right. Closely associated with the acetabulum is the genital opening, the outer rim of which is fused with the acetabulum. Tegument is provided with spines. Testes are ovoid and posteriorly situated. Ovary and receptaculum are situated in front of the testis and are median in position. Yolk glands are scattered in the posterior third of the body. Uterus is situated between the testes and ventral sucker. Seminal vesicle lies in front of the ovary.

Egg measures 27–28 μm × 16–18 μm and has a close resemblance to that of *Clonorchis sinensis*. Egg has an ovoid shape.

Life Cycle

First intermediate host ingests fully developed unhatched eggs. First sporocyst generation gives rise to two redial generations and finally lophocercous cercariae are formed in the first intermediate hosts, the snail *Semisulcospira libertina* and *S. eoreana.* The cercariae have pigmented eyespots and numerous penetration glands. The tail is long and membranous. After swimming vigorously in the water, the cercariae enter the fish host. *Plecoglossus altivelis, Cyprinus carassius* are some. These cecariae encyst under the scales in the skin or in the flesh of the fish. The metacercariae measure 150 × 100 μm. Ingestion of raw or pickled fish is the source of infection. Fish-eating mammals and pelicans serve as reservoir host.

Epidemiology

Man gets infection by eating uncooked fresh water fishes of the genus *Plecoglossus* and *Odontobatis.* Birds like pelicans and fish-eating mammals form the reservoir hosts. Original source of infection is the polluted water where intermediate hosts live.

Pathogenesis

Slight inflammation develops at the sites of attachment. This is followed by sloughways of the mucosa or necrosis of the mucosal cells. At the sites of attachment, suckers irritate the mucosa. Thus excess mucosa

is secreted which results in possible erosion of the wall. Sometimes they get infiltrated into the capillaries and reach the brain and spinal cord or myocardium. Symptoms are mild diarrhoea especially when the parasite occurs in great numbers. Host tissues fail to encapsulate the parasites and this may pave the way for their entry into the capillaries.

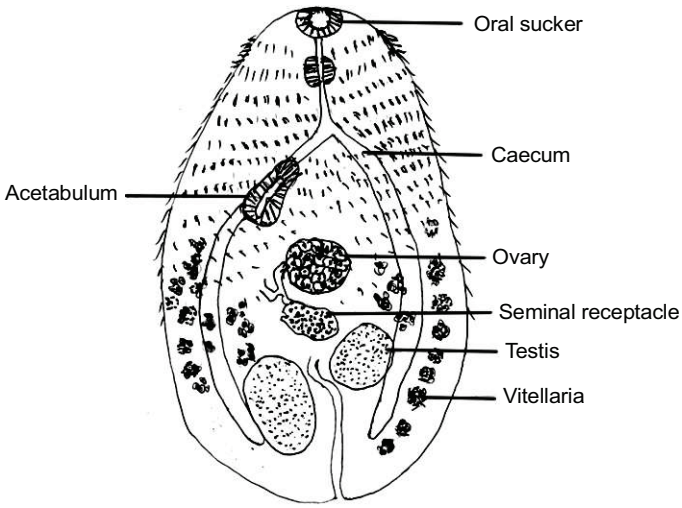


Figure 5.30 *Metagonimus yokogawai*

Paragonimus westermani (Kerbert, 1878)

This parasite measures 8–20 × 6–9 μm and is somewhat oval in shape (Figure 5.31). The oral sucker is subterminal. Ventral sucker is larger than the oral sucker, almost and somewhere in the central part of the body. Body is transluscent and reddish brown in colour. It has a short oesophagus, pharynx and a bifurcated intestine. Genital pore is situated near the ventral sucker. Testis is tubular, ovary is branched on either side of the median line posterior to the ventral sucker. Saclike uterus lies opposite to the ovary. Well-developed vitellaria extend the whole length of the body. There is a shell gland, Laurer's canal. There is no cirrus pouch and cirrus organ.

Life cycle The eggs are in the form of pockets in the lungs and find their way into the water by sputum or faeces. Within 15 days to seven weeks, a ciliated miracidium emerges out. It has a ciliated covering arranged in four rows at the anterior end. The excretory pore is in the form of a rosette. It makes entry into the snail host. In about two months it develops into sporocyst and later rediae. Each redia contains cercariae which are microcercous and which meaure 200 × 70–80 μm and are ellipsoidal in shape. Tail is in the form of a knob. It has an anterior stylet and the whole body is covered with spines. The second intermediate hosts are crabs or crayfish (fresh water). The cercariae bore into these hosts. In crabs, the metacercariae encyst in the liver, muscles and the gills. When humans consume such infected crabs, the metacercariae enter the stomach where the cyst wall is digested. From the abdominal cavity, they penetrate the diaphragm, lungs and finally the bronchiole.

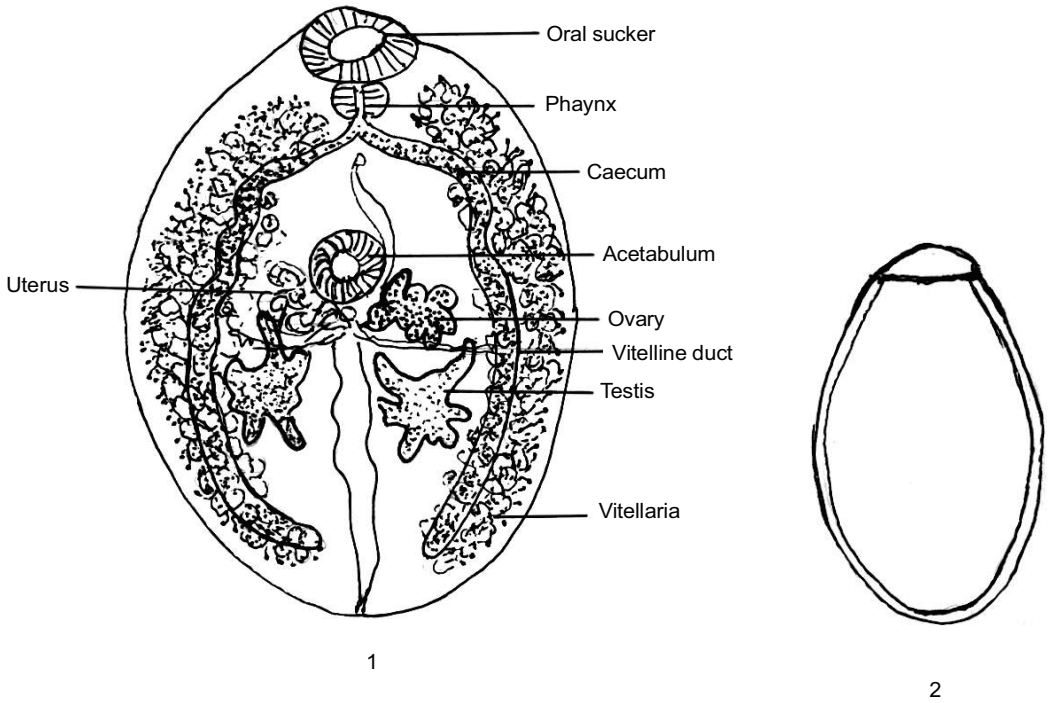


Figure 5.31 *Paragonimus westermani*

Dicrocoelium dendriticum (Rudolphi (1818), Looss (1897))

It is a parasite of ruminants but humans seem to harbour them in almost all European countries. This parasite is flat, transparent and lancet-shaped, and 5–15 μm long and 1.5–2.5 μm broad (Figure 5.32). Integument is devoid of spine. The acetabulum is situated in the anterior end. Testes are lobed, obliquely arranged anterior to the ovary just a little behind the acetabulum. Ovary lies on the right side. Cirrus pouch is bottle-shaped. Small receptaculum seminis and Laurer's canal are present. Vitelline glands occupy the middle third of the body and the ootype is situated anterior to the mid-ventral plane of the body. Uterus is coiled, extending to the posterior part of the body and ascending to the genital pore.

Eggs are thick-shelled, operculate, golden brown in colour and measure 38–45 μm × 22–30 μm. Fully embryonated eggs pass along with faeces and are resistant to desiccation. Now it is time for them to gain entry into the body of the land snail of the species *Zefrina detrita* and *Helicella candidula*.

The miracidium is released into the digestive tract of the snail, which penetrates the intestinal epithelium and ultimately reaches the hepatopancreas. The sporocyst gives rise to the daughter sporocysts which give rise to cercariae. The whole procedure is completed in 3–5 months. The cercariae leave the sporocyst, migrate to the respiratory chamber in the form of stone balls which are cemented by mucus. This mucus is secreted by mucous glands situated in the posterior region of the cercariae. The mucous balls are released individually or in cluster. These stone balls have to be swallowed by the second intermediate host, the ant of the genus *Formica.* The cercariae penetrate the intestinal wall. Then the cercariae encyst,

and metacercariae are formed in the abdominal cavity. If humans happen to consume these infected ants, the cyst wall is digested and young flukes migrate to the bile system, and maturation of flukes takes place in about 50 days.

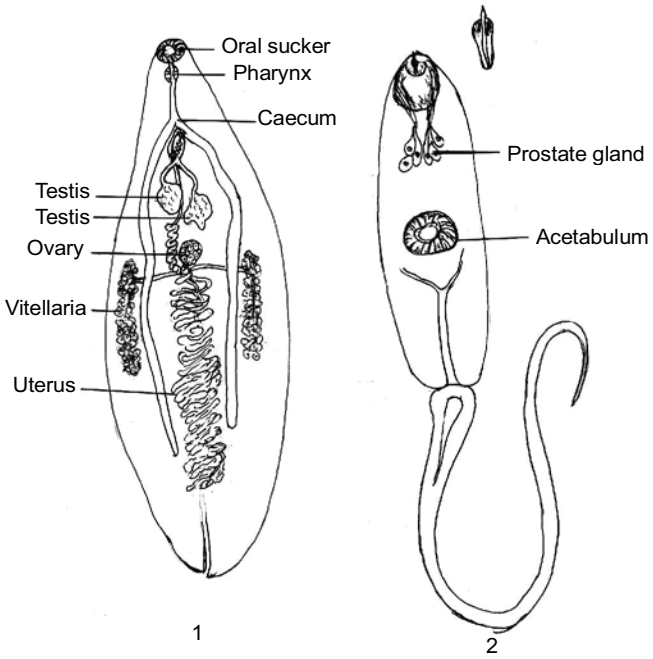


Figure 5.32 1— *Dicrocoelium dendriticum,* 2—Cercaria of *D. dendriticum*

Epidemiology

When there is bright sunshine, the cercaria leaves the snail host. After getting released from the sporocyst, they migrate to the respiratory chamber of the snail and several hundreds of them agglutinate into stone balls. The snail crawls on the grass leaving behind the stone balls. The suitable intermediate host is the ant *Formica fusea*. These infected ants gain entry into the mammalian definitive host along with the grass on which it preys.

Pathogenesis

No remarkable damage is noticed in infected hosts. However, in human beings, flatulence, dyspepsia, constipation, enlarged liver, diarrhoea and vomiting are some of the symptoms.

Gastrodiscoides hominis (Lewis & McConnell (1876), Leiper (1913))

This parasite is widely distributed in India, Malaysia, Pakistan, Philippines, Guyana, Vietnam, Assam, Myannmar and so on. Pig is the common reservoir host. Rhesus monkey is also found to be infected in India. The parasite is reddish and can expand its body to 1 cm. But preserved specimen measures 5.7 × 3.4 mm with anterior conical and posterior discoidal end (Figure 5.33). Genital papillae are prominent

and the genital pore is situated near the oral sucker. Acetabulum is situated posteriorly and measures 2 mm diameter. Cuticle is aspinosus. Alimentary canal has a pharynx which has two pear-shaped pharyngial pouches. Oesophagus is bulb like from where the intestinal caecae branches start, and caecae extend up to the acetabulum. Testes are lobulated and situated between the intestinal caecae. A seminal vesicle is present but there is no cirrus. Ovary is median and situated between the two testicular lobes. Shell gland is ovoidal and placed near the ovary. Receptaculum seminis lies anterior to it. Uterus is short. Laurer's canal is present. Vitellaria extend up to the mid-third of the body.

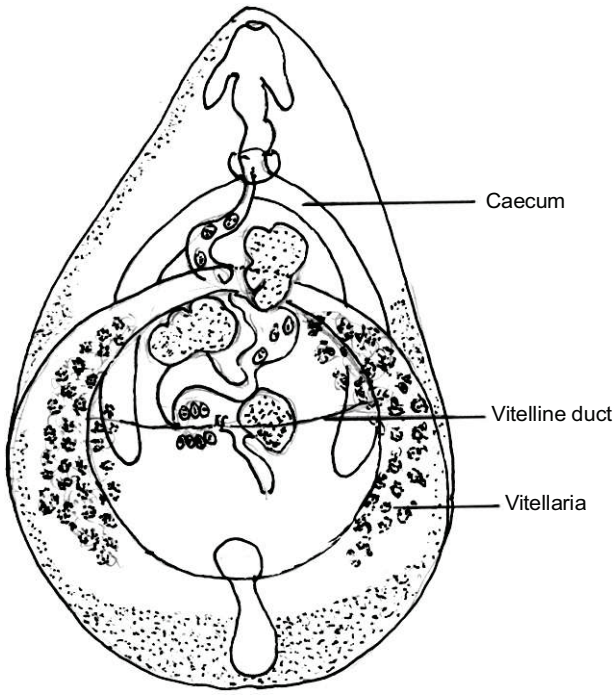


Figure 5.33 *Gastrodiscoides hominis*

Eggs are oval measuring 150 × 60 μm with an operculum. Adults live in the digestive tract (caecum and colon) of man, pig and deer. Eggs which pass in stool develop into miracidia. These enter a fresh water snail, *Helicorhus coenosus,* where they develop into sporocysts and rediae which develop into cercariae. At this stage, they leave the snail and encyst on grass as metacercariae which are the infective stages. When swallowed by the definitive host, life cycle repeats.

Artifechinostomum mehrai

Body is elongate, spinose, and has a head collar with a crown of about 43 spines (Figure 5.34). Corner spines are large. Marginal spines are in a single row laterally and a double row dorsally. Small oral sucker is acetabalum is large. Two testes tandom deeply lobed in posterior half of body. Cirrus pouch is long and genital pore is immediately preacetabular. Ovary is oval, submedian in the equatorial third of the body. Seminal receptacle

is present. Laurer's canal is present. (Hanumantha Rao, 1963). Uterus is long with a number of eggs. Vitellaria are present in lateral folds from behind the acetabular region to the posterior extremity.

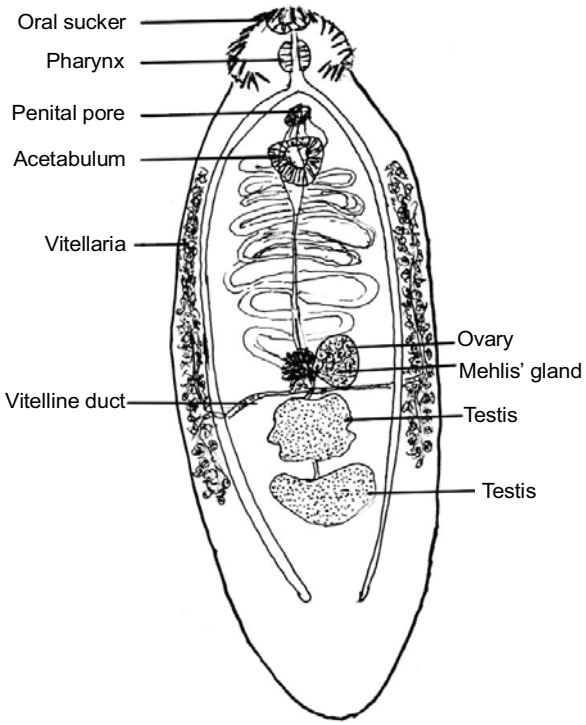


Figure 5.34 *Artifechinostomum mehrai*

Specimens of *A. mehrai* were recorded from the intestine of a girl in Andhra Pradesh (Hanumantha Rao, 1963). Most probably the girl acquired infection by eating raw snails *(Pila virens).*

Cercaria andhraensis is reported from *Pila virens*. Metacercariae occur in the same snail host.

Pathogenesis

The crown of spines destroy the mucosa of the intestine and to some extent they are haematophagus and may precipitate anaemia in humans. Although they are parasites of birds, they are of zoonotic significance.

CESTODES

Cestodes or tapeworms are ribbonlike worms with chains of segments. These are highly specialized flatworm parasites. Since Cambrian era, their ancestors diverged from free-living flatworms to parasitize the earliest vertebrates. Most of the tapeworms require a host to adjust the different stages of their life cycle. Mostly adult tapeworms are harboured in the gut of the definitive host (vertebrate) with four species adapted specifically to human beings. As already mentioned each segment has a complete set of male and female reproductive systems. They are known for their extreme adaptations for parasitic life. Mouth and digestive system are totally absent. For their nutrition they absorb pre-digested food through millions of sub-microscopic hair-like extensions, the microtriches, which interdigitate the host villi.

The tapeworm has a well-developed scolex with suckers, grooves (bothria) or hooks. These suckers are controlled by muscles and these provide a firm attachment ground to the parasite, i.e., it gets a firm hold to the wall of the intestine. The scolex is followed by a growing neck region (the strobila) to a series or a chain of a large number of segments or proglottids. These proglottids mature towards the distal end of the worm. These gravid proglottids with mature reproductive organs produce eggs as many as 50,000 eggs per day.

All larval stages are parasitic. Adults usually live in the intestine, and larvae live in the tissues of the intermediate host. Intermediate host may be either a vertebrate or an invertebrate. Sometimes both larvae and adults are found in humans depending upon the species. Based on morphological variations, the human cestodes could be categorized into (a) cyclophyllidean cestodes and (b) pseudophyllidean cestodes.

In cyclophyllidean cestodes, scolex has four cuplike suckers. Uterus does not have a separate opening. There is a common genital pore on the lateral side of each segment. Eggs are non-operculated. There is no ciliated embryo. These cestodes have an exclusively terrestrial life cycle involving a single intermediate host, which is either an invertebrate or a vertebrate. When the intermediate host ingests the eggs, it gets infected and the eggs develop into larvae and migrate to the tissues. If the intermediate host is eaten by a suitable definitive host, the life cycle is completed where it develops into the adult.

In pseudophyllidean cestodes, scolex has two slitlike grooves. Uterus opens out independently. There is a common genital pore in the mid-ventral line. Eggs are operculated and produce ciliated embryos. The life cycle is complicated, the first intermediate host being an aquatic invertebrate infected with a procercoid larval stage of the parasite. When this is ingested by a second intermediate host which may be either a fish or a reptile, the parasite develops into plerocercoid larva. After completing the cycle in some intermediate hosts, the larva must seek a definitive host where it develops into an adult.

Some important intestinal cestodes of humans are the following.

1. *Diphyllobothrium latum*
2. *Dipylidium caninum*
3. *Taenia solium*
4. *Taenia saginata*
5. *Echinococcus granulosus*
6. *Echinococcus multilocularis*
7. *Hymenolepis nana*
8. *Hymenolepis diminuta*
9. *Multiceps multiceps*

Taenia saginata (Goeze, 1782 (Beef tapeworm)

Taenia saginata is a whitish, semi-transparent worm reaching a length of 4–10 m and contains as many as 2,000 segments. The scolex is pear-shaped and without a rostellum or rostellar hooks (Figure 5.35a). Adult worms live with their heads firmly attached to the mucous membrane of the small intestine. The scolex has four hemispherical suckers which are frequently pigmented. The suckers are the sole

organs of attachment. Neck is slender and long, not more than one half as broad as the head. This is followed by a chain of segments—immature, mature (Figure 5.35b) and gravid proglottids—which is three times longer. As many as testes (300 – 400) and in the absence of accessory ovarian lobe. There are as many as 97,000 eggs in each gravid segment (Figure 5.35c). The uterus has 15–20 main lateral branches which is a diagnostic character. When the gravid proglottid detaches itself from the strobila, the ova are expelled. The eggs are globular, 30–40 × 20–30 μm, and cannot be distinguished from *T. solium*. These eggs have a double-shelled embryophore with an oncosphere inside. The egg has an outer shell, a chorionic membrane and two oncospharal membranes.

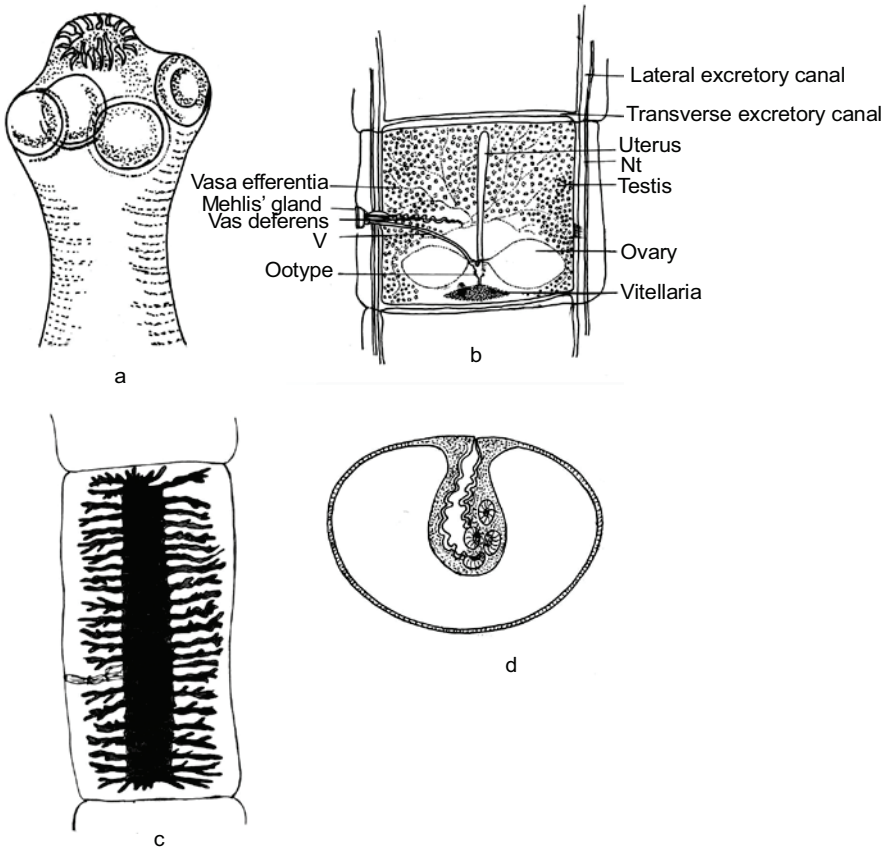


Figure 5.35 a—Scolex of *Taenia saginata*, b—Mature proglottid, c—Gravid proglottid showing uterine pattern, d—Cysticercus

Life cycle Gravid proglottids pass out in stool to the exterior and get settled on grass. There they disintegrate. When the intermediate host ox happens to eat the grass the eggs reach the duodenum, where the oncospheres are set free. They migrate to the small intestine where they penetrate the wall and from there they get into circulation and are carried on to the pterygoid muscle, diaphragm and the tongue. In the muscle in 60–75 days they metamorphose into the bladderworm (cysticercus).

This cysticercus measures 7.5–9 × 5.5 μm. The scolex is unarmed, ovoid in shape and milky white. The cysticercus is devoid of hooks on the scolices and this is a distinguishing character in which they differ from other cysticerci. They live for eight months in the ox and their further development depends upon their entry into humans who are the definitive host and who get infected by eating flesh with cysticercus. The bladder is digested and the liberated scolex gets a firm hold on the wall of the intestine.

Cysticercus It has a small invaginated scolex (Figure 5.35d). It has hairlike processes, a peripherous collaginous fibrous layer, two muscle layers, calcareous corpuscles, peripheral cells, flame cells, a duct system embedded in a loose fibrous net and a central band of muscles. The different cestode larvae could be distinguished in human tissues by variations in their structures.

Epidemiology Humans are infected by eating raw beef containing cysticercus larvae. Cattle get infected by grazing on ground polluted by human faeces containing the eggs.

Pathogenesis Because of the size of the worm, they produce acute intestinal stoppage. Sometimes proglottids get lodged in the lumen of the appendix and cause appendicitis. When the incubation period is over, diarrhoea starts and there is loss of weight.

Taenia solium (Linnaeus, 1750) (Pork tapeworm)

This is a parasite of the small intestine with its body wound back and forth in the lumen of the small intestine with 1 mm diameter, globular, quadrangular, four large cup-shaped suckers. Rostellum is short and pigmented and studded with a double row of 20–50 hooklets (Figure 5.36a). The four circular suckers measure 0.5 mm in diameter. The anteriormost immature proglottids are small, broader than long, whereas the more posterior mature ones (Figure 5.36b) measure 12 × 6 mm and are almost square-shaped, and gravid proglottids (Figure 5.36c) are longer than broad. Proglottid number is 1000. The genital pore is marginal surrounded with thick lips and these pores alternate irregularly between right and left margins. On the lower side of vas deferens is the vaginal tube which ends in the ootype. The ovarian lobes are symmetrical and there is an accessory lobe on the side of the genital pore. Vitelline follicles are elliptical, the oviduct receives the common vitelline duct and vagina before it opens into the ootype. Uterus is situated medially with 7–13 cercae or diverticulae. The follicles of the testes (150–200) eggs and they pass out along with faeces. The egg measures 31–56 μm in diameter and is spherical, with no operculum (Figure 5.37f). It has 2 shells, one is formed by the embryo and the other by vitelline membrane which gets disintegrated when it passes out along with the faeces. The egg contains 6 hooked oncospheres.

Life cycle The terminal gravid proglottids frequently get separated from the strobila and they pass out in faeces. The segments disintegrate while the eggs remain viable for many weeks. These eggs are eaten by the pig, the intermediate host. The oncosphere penetrates the gut wall and gets entry into the bloodstream and finally gets lodged in the muscles. At this stage it is called a cysticercus. Pig muscle containing cysticercus is called measly pork. Man gets infected when measly pork is consumed. In the alimentary canal, the bladder of the cysticercus is absorbed by the digestive juices, the scolex and head are evaginated and then they migrate to the small intestine where the scolex holds on to the wall of the gut slowly forming proglottids. Man is occasionally infected by cysticerca, so also pig and monkey.

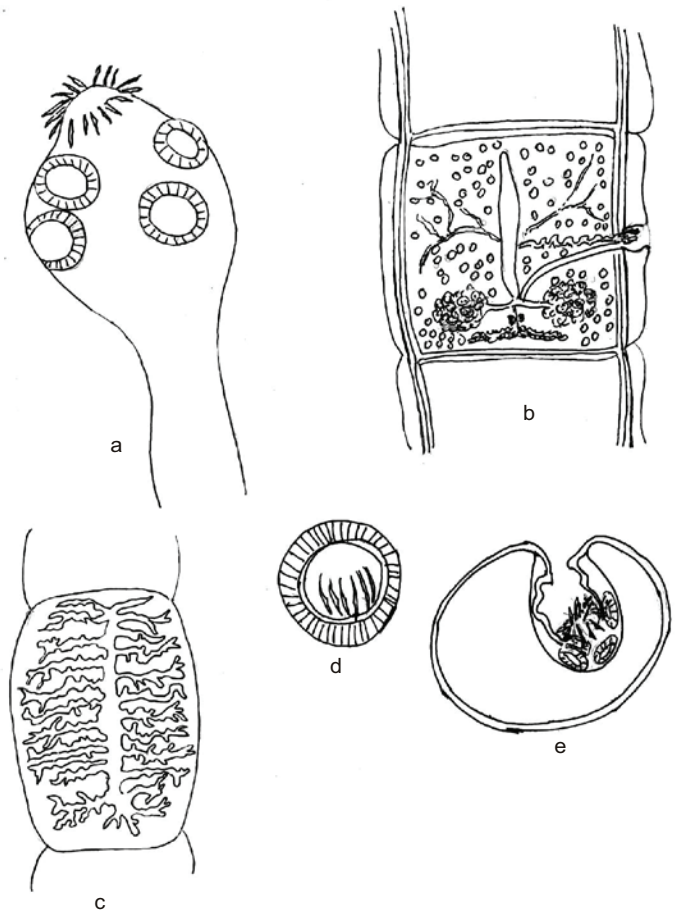


Figure 5.36 a—Scolex of *Taenia solium*, b—Mature proglottid, c—Gravid proglottid showing uterine pattern, d—Egg, e. *Cysticercus*

Epidemiology

Humans are the definitive hosts. Cysticercal stages were also reported from Thailand gibbons. Human infection is due to consumption of frozen pork or measly pork. Sometimes cats, dogs and sheep harbour cysticercus stage.

Pathogenesis *Taenia solium* at the site of attachment may cause irritation to the mucosa. The adult worm does not cause serious damage but sometimes is the cause of abdominal discomfort, indigestion, diarrhoea, alternated with constipation.

Diagnosis is by recovery of gravid proglottids and a number of uterine branches.

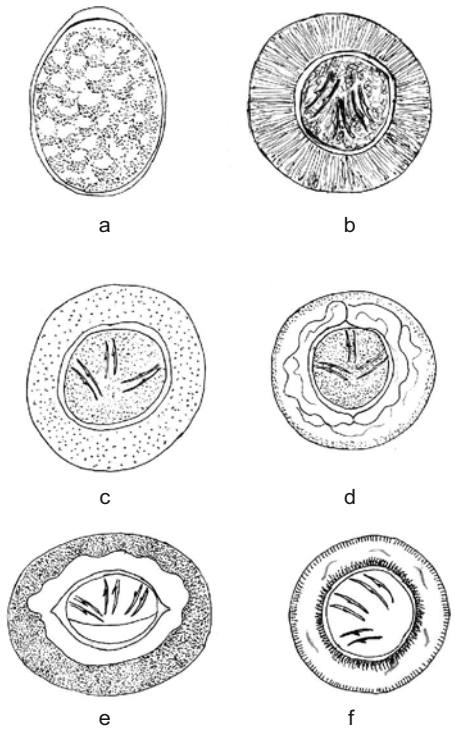


Figure 5.37 Egg of cestodes, a—*Diphyllobothrium latum*, b—*Echinococcus granulosus*, c—*Dipylidium caninum*, d—*Hymenolepis diminuta*, e—*Taenia solium*

Diphyllobothrium latum (Linnaeus, 1785)

It is a pseudophyllidean cestode commonly called fish tapeworm. It is greyish translucent growing to a length of 3–10 m with more than 3,000 proglottids. It lies coiled in the small intestine. It commonly occurrs in countries like Far East, Japan, China, Europe and America. Its longevity is 13 years. The scolex is small, spoon-shaped about 23 mm long and 1 mm broad, without rostellum and hooks but there are 2 longitudinal ridges or grooves, one dorsal and one ventral. Behind the scolex is the unsegmented neck region several times longer than head. This is followed by a chain of proglottids with immature and mature proglottids. The proglottid number varies from 3,000 to 4,000. Mature segments are broader than they are long and filled with male and female genitalia. The various parts/stages of diphyllobothrium are shown in Figure 5.38.

Testes are in the form of follicles, laterally situated in the dorsal plane. The vasa efferentia unite into vas deferens just in front of the ootype and proceeds in the form of a convoluted tube travelling towards the common genital atrium. A seminal vesicle and cirrus region are situated near the genital atrium. Ovary is bilobed lying at the posterior region of the proglottid with ootype in between the lobes, and is surrounded by Mehlis' gland. From the ootype arises the vagina which opens out near the male genital pore. The oviduct and the common vitelline duct join the ootype from where the uterus arises and

travels in a convoluted manner up to the uterine pore. In the ootype, fertilization takes place and simultaneously the yolk material and the shell are added, and the egg passes to the uterus.

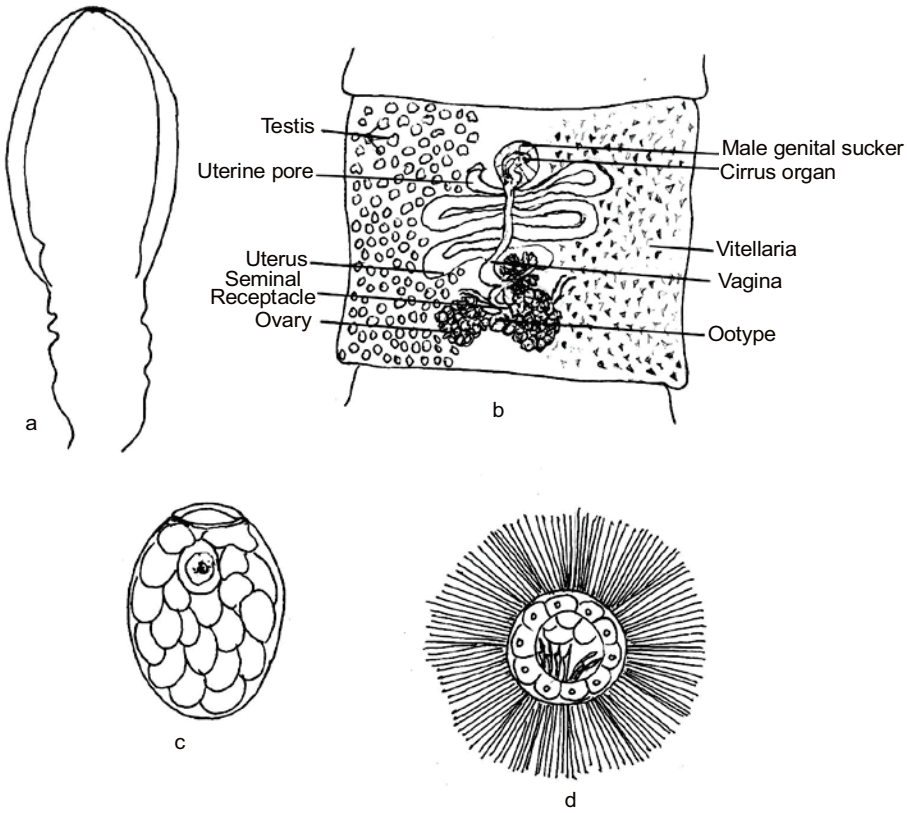


Figure 5.38 *Diphyllobothrium latum;* a—Head of *D. latum*, b—Mature proglottid, c—Egg, d—Free swimming coracidium

Eggs as many as 35,000–100,000 are discharged every day. Eggs are ovoid, operculate with a brown shell and measure 70 × 45 μm (Figure 5.37a) There is a knob at the other end. Eggs are resistant to chemicals but become non-viable when subjected to desiccation and putrefaction. Eggs pass in faeces in large numbers and they are infective to man when the egg is passed in water. Upon entering water, the operculum is lifted. They require 11–15 days for development. The egg contains an immature embryo. A ciliated six-hooked coracidium emerges (20–30 μm) which swims about with the help of the cilia. Its viability depends on the temperature. Upon maturing, the embryo escapes through the operculate slit of the shell, the embryonal envelopes are cast off and the ciliated embryo swims in water. Within 12 days it must find an appropriate intermediate host. Normally it gains entry into a fresh water crustacean like *Cyclops strenius, Diapotamvs vulgaris,* etc. In the digestive tract the outer layer is digested. The hooks penetrate into the wall of the gut and the larva migrates to the body cavity of the *Cyclops*. At this stage it perishes. This is the precercoid larva. It has an oval shape and is 50–60 μm long with 6 terminal hooklets.

If such *Cyclops* happens to be swallowed by fish, it forms the second intermediate host. The fishes like perches, salmon, and trout act as second intermediate host. After reaching the stomach of the fish, the precercoid penetrates the body cavity and after 3 or 4 days it encysts and becomes the pleurocercoid which has a length of 6 mm and settles in the muscles and tissues. The pleurocercoid develops bothrea, nervous system and excretory system. It is now the infective stage. When improperly cooked or raw fish are eaten by humans pleurocercoid develops into adult in 5–6 weeks as *Diphyllobothrenum.*

Epidemiology Domestic dogs, cats, mongoose, sea lions, foxes, bears, pigs, seals and walruses are tdefinitive hosts apart from humans. Eggs released along with human along with human faeces mature and hatch and produce infection. The first intermediate host are the copepods. Fish get infection when they consume the copepods. When such infected fish are eaten as pickles or as improperly cooked food, humans get infected.

Pathogenesis There is no evidence of damage but in some patients, mechanical obstruction of the bones is noticed when these parasites occur in large numbers in a patient who may suffer from pernicious anaemia. Diagnosis is by detection of eggs in the faeces.

Hymenolepis nana

This is commonly called dwarf tapeworm. It is unique amongst cestodes in that the life cycle is completed in humans alone without other host species. Interestingly the same individual acts as intermediate and definitive host. The parasite is small growing to a length of 40 mm with 1 mm diameter. Scolex is minute, rhomboidal in shape (0.2 m in diameter) with 4 suckers which are hemispherical. Rostellum is very small, studded with 20 – 30 spines in a ring. This is followed by a long and slender cervical region. Posteriormost proglottids are wider and broader than those of the anterior end and the posterior strobila are almost rounded. When distal proglottids disintegrate, the eggs are released. Egg measures 35–45 μm in diameter, and is almost spherical and hyaline bearing an oncosphere enclosed in an inner envelope. This envelope has a polar thickening from which arise 4–8 polar filaments. Oncosphere bears 3 pairs of lancet-shaped hooklets.

Embryonated eggs (Figure 5.37d) passed in faeces by chance are ingested by the host, and reach the stomach, where they hatch, and then reach the intestine. The oncosphere with the help of hooks, gets firm hold on the wall of the intestine and almost penetrate into the villi of the small intestine and metamorphose into cysticercus which migrates into the intestinal lumen and attacks other villi of the small intestine. In about a fortnight, they develop into mature worms.

Epidemiology

A typical example of human dwarf worm without the involvement of intermediate host in the life cycle. Children are more prone to infection than adults.

Pathogenesis

This parasite produces irritation in the intestinal mucosa. Sometimes headache, dizziness, inanition, pruritus of the nose and anus, abdominal discomfort, diarrhoea, etc., are some of the symptoms.

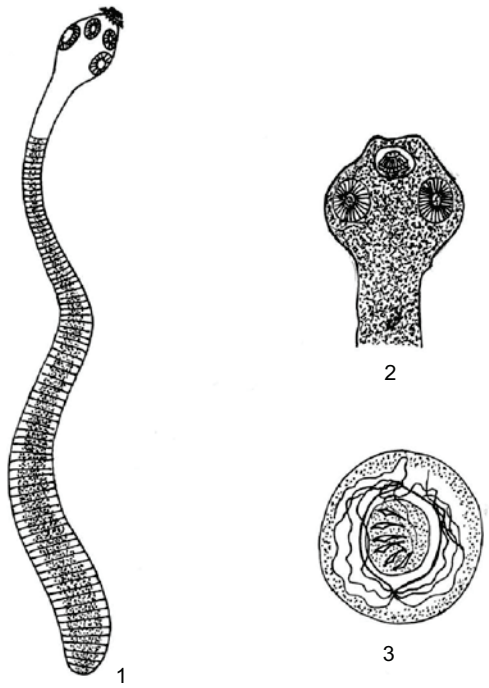


Figure 5.39 *Hymenolepis nana*, 1—Complete worm, 2—Scolex enlarged, 3—Egg

Hymenolepis diminuta (Ruldolphi (1819), Blanchard (1891))

Worms grow to a length of 20–60 cm. The cervical region is very narrow (0.5 mm) but slowly the width gradually increases to 3–4 mm. Scolex is small, spherical with 4 cup-shaped suckers. At the apex there is a cavity into which the pyriform rostellum can be invaginated. The posterior mature proglottids (Figure 5.40a) measure 0.75 mm in length and 2.5 mm in breadth. Each proglottid bears 3 ovoidal testes. Gravid proglottids first detach from the strobila and release the eggs (Figure 5.40b) after disintegration. These eggs are passed out along with the faeces.

Eggs have a transparent outer membrane, and are spherical in shape and slightly yellowish in colour (Figure 5.37 d) with a diameter of 60–80 μm × 72–86 μm. The oncosphere is surrounded by an inner membrane which has two polar thickenings but lacks polar filaments. There is gelatinous fluid between two membranes. There are 6 lanceolate hooklets exhibiting a fan pattern. Eggs are highly sensitive to temperature above 60°C but highly resistant to chemicals, putrefaction and also desiccation.

A number of arthropods which are scavengers in their larval and adult stage serve as intermediate hosts and once they swallow the egg, it enters the intestine where it hatches and then penetrates into the haemal cavity with the help of oncospheres. In the haemal cavity the egg metamorphoses into a cysticercoid larva (Figure 5.40 c and Figure 5.40d).

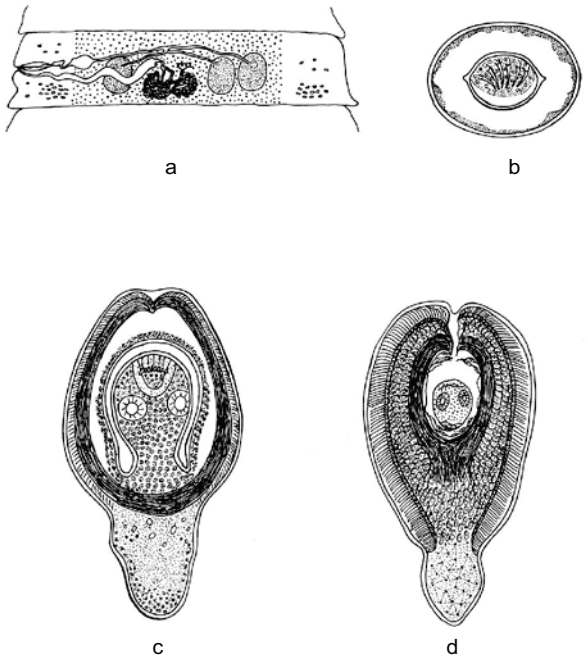


Figure 5.40 *Hymenolepis diminuta*, a—Mature proglottid, b—Egg, c—Cysticercoid of *H.nana*, d—Cysticercoid of *H. diminuta*

Epidemiology

All age groups are susceptible. Accidental ingestion of intermediate hosts results in infection.

Pathogenesis

Less common in humans. However usual pathology associated with tapeworms is characteristic. In addition Cashema may occur.

Dipylidium caninum (Linnaeus (1750), Ralliet (1863))

The strobila of *Dipylidium caninum* grows to a length of 100–700 mm with a chain of elliptical proglottids. Rostellum is small, rhomboidal in shape, with a diameter of 250–500 μm. Rostellum bears 4 cropped suckers capable of protrusion. The rostellum has 1–7 circlets of spines each of which has a large round base and a curved arm. Anterior spines are larger and posterior spines are the smallest. The rostellum is followed by a short slender neck. Immature proglottids are broader than longer. Each mature proglottid (Figure 5.41b) is provided with a double set of reproductive organs. The genital atrium is situated on the lateral side of the proglottid. Receptaculum seminis is absent. Gravid proglottids are filled with uterine block, each with 8–15 eggs covered by an embryonic membrane.

Eggs measure 25–40 μm in diameter. They are spherical in shape and provided with delicate hooklets (Figure 5.37c) measuring 12–15 μm in length. These eggs are released in the form of capsules which get deposited on the ground. These have to be ingested either by dog or cat fleas which are the ectoparasites.

Once they are picked up by the fleas they reach the intestine, then the haemal cavities where they develop into procercoid and later into cysticercoid larvae. When the fleas die by chance, procercoids also die. Some are resistant to insects, metamorphosis and reach the adult stage. Man gets infection by accidentally consuming infected insect hosts.

Epidemiology Most of the cases have been in children because of very common occurrence in dogs and cats. Human cases do occur.

Pathogenesis Children are seldom infected with more than one worm. Slight intestinal disturbances, indigestion, loss of appetite. Toxic nervous manifestation are the diagnostic symptoms.

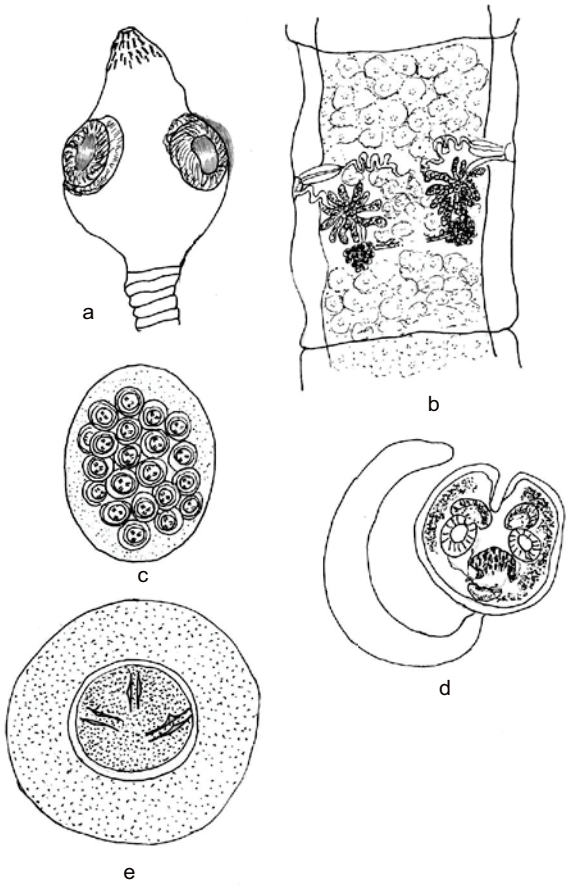


Figure 5.41 *Dipylidium caninum*, a—Scolex enlarged, b—Mature proglottid enlarged, c—Cluster of eggs, d—Cysticercoid larva enlarged, e—Single egg enlarged

Echinococcus granulosus (Batsch (1786), Rudolphi (1805))

This parasite (Figure 5.42) is minute and inhabits the villi of small intestine of the dog and other members of Canidae. It measures 3–6 mm in length. Scolex is 300 μm in diameter, pyriform, equipped with

4 suckers and nearly 50 hooklets. The body has interestingly one immature, one mature and one gravid proglottid. Immature proglottids are small and narrow and the gravid is the longest. The uterus in the gravid has lateral branches, which appear like twisted coils. When the uterus bursts, it releases few eggs.

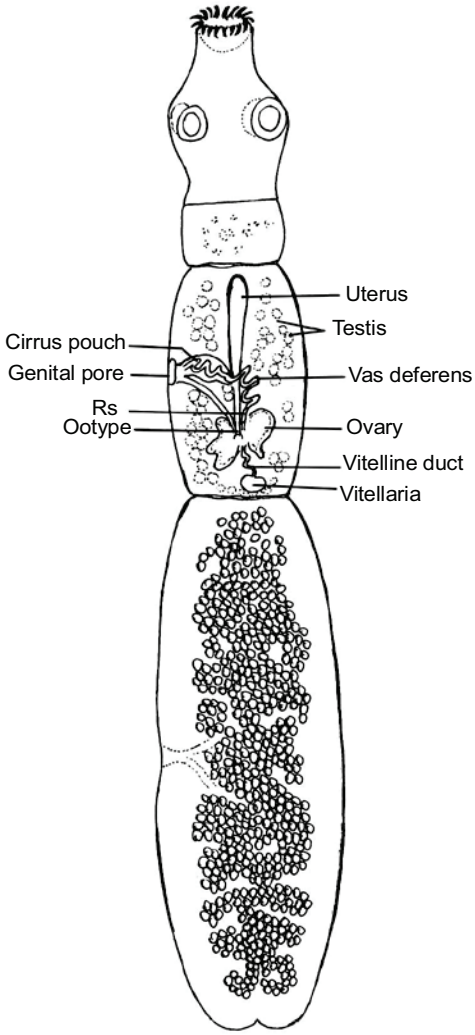


Figure 5.42 *Echinococcus granulosus*

These eggs strikingly resemble the eggs of *Taenia solium* and *Multiceps multiceps.* (Figure 5.37b). When these eggs happen to be swallowed by sheep, the intermediate host or even humans, the egg reaches the duodenum where it hatches into onchosphere which through the intestinal wall migrates to the mesenteric venules and finally gets lodged in capillaries of various organs and tissues. Some important sites of infection are the liver, lungs and other focci. Most of these lie attached to mononucleate leucocytes and perish at the sites where they are lodged; however some survive. Those that survive reach a diameter of 40 μm by the fourth day. During this time, each one develops a cystic cavity. After 3 weeks, the cyst

measures a diameter of 250 μm. At this stage, the host tissue reacts conspicuously. The larva is surrounded by an envelope of fibrous cyst wall secreted by the host tissue. After 30 days, these cysts measure 1 mm and in 5 months, they measure about 10 mm. By this time, the inner surface starts to produce hollow brood capsules. These capsules get attached by slender stalks. With age, more brood capsules form and the older brood capsules differentiate into a number of scolices (3–30). Due to pressure, the mother cyst develops buds which get detached and the detached buds in turn develop buds independently which get attached and develop independently as daughter cysts. The fluid of the cyst is colourless. After 20 years, they reach independent life.

Epidemiology

Humans are infected by the larval or hydatid stage. Dog is the definitive host. Fox, jackal and hyaena are also prone to this. Sheep, cattle and pigs are commonly infected. Horses, monkeys and camels are reservoir hosts. When the definitive host consumes the viscera of infected larval host, they become infected. Infection in humans is acquired in childhood through association with infective dogs.

Pathogenesis

Hydatid disease is serious.

Echinococcus multilocularis (Leuckart (1863), Vogel (1757))

This is a canine parasite (Figure 5.43) living attached to the small intestine. Canines are the definitive host. This is smaller than *E. granulosus* (1.2 to 3.7 mm vs 30 to 60 mm). In the mature proglottid, the genital pore is situated at the anterior end. Their number is 16 – 26 lying posterior to the cirrus pouch. As in *E. granulosus,* uterus is devoid of lateral outpockets. The eggs resemble those of *Taenia* and are extremely resistant to extreme cold temperatures.

Fox is the principal host and mice are the intermediate hosts. The parasite affects the liver. Cysts are numerous and found in clusters hence the name *multilocularis.*

Epidemiology Man gets infection sporadically. It is due to direct contaminative contact with fox's excreta especially when ground gets polluted.

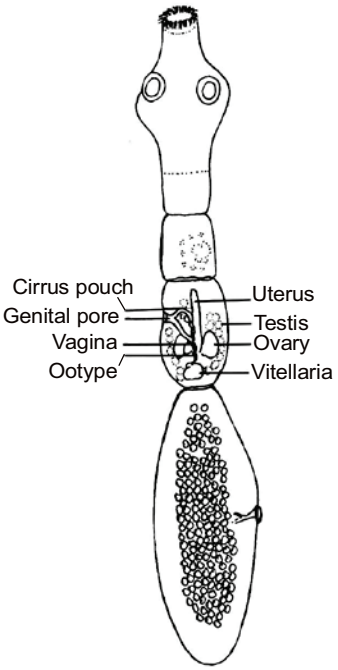


Figure 5.43 *Echinococcus ultilocularis*

Multiceps multiceps (Lake (1780), Hall (1910))

Adults live attached to the small intestine of members of Canidae (fox, dog and wolf). Adult measures 40–60 cm with an armed rostellum. Scolex is pyriform (0.8 mm in diameter) and hooklets (22 – 32) are

arranged in a double ring. These hooklets are of two sizes, the larger ones measuring 150–170 μm and smaller ones 90–130 μm. The uterus has 18–26 lateral branches on each side of the main stem.

The eggs are 30–31 μm in diameter. Dog, wolf and fox are the definitive hosts. Herbivorous mammals like. sheep, goat, cattle, horse, antelopes and gazelles harbour the larval stages. In addition *Macaca radiata* and *M. mullata* also form the hosts. They get infected by accidentally consuming contaminated dog faeces. On reaching the intestine of the intermediate host, the egg hatches into onchosphere and finally gets lodged in the various tissues of the body, but favourite sites are the brain and the spinal cord. The larva metamorphoses into crenurus a bladder worm. The larva has multiple scolices. Each scolex is a replica of the adult worm and develops into an adult worm. Dog gets infection by accidentally consuming the infected brain of sheep.

Nematoda

The group Nematoda is a widespread and successful group consisting of a number of small worms, which occupy almost every habitat. i.e., terrestrial, marine and fresh water. Added to this they gain fame as the best known parasites of every class of vertebrates and invertebrates including insects as well as plants. In vertebrate hosts they infect almost all tissues and organs. Some of them are much larger in size than their free-living counterparts.

The larval or the juvenile stages are spent in the intermediate host and the adult stage in definitive hosts. In some instances, all the stages are passed in the same host as in the case of *Trichinella spiralis.*

The following are some of the important characters of parasitic nematodes.

- Cuticle is non-cellular which is shed periodically.
- Provided with musculature.
- Fully developed digestive system is present.
- Reproductive system is well-developed.
- Eggs and the larval stages are well suited to survive in the external environment or in the intermediate host.

More than a dozen species of nematodes are human parasites and more than a dozen species are zoonotic human parasites. More than 1 billion people are hosts of *Ascaris lumbricoides* (roundworm), more than 800 million people have hookworm (*Ancylostoma duodenale*) infection and several hundreds of millions of people suffer from pinworm (*Enterobius vermicularis* and *Wuchereria bancrofti*) infection.

Intestinal nematodes gain entry into humans via contaminated food, water and soil. The infective eggs of *Ascaris* and *Trichuris trichiura* are highly resistant to dessication and other environmental factors *Strongyloides stercoralis* infects the skin (third stage larvae). Spiralis infection is by consuming undercooked meat and pork which is infected with the encysted larvae. Pinworm infection is by accidentally encysting through soiled fingers and clothing. So they are considered as urban parasites. *Trichinella* infection is by encysted larva present in the meat.

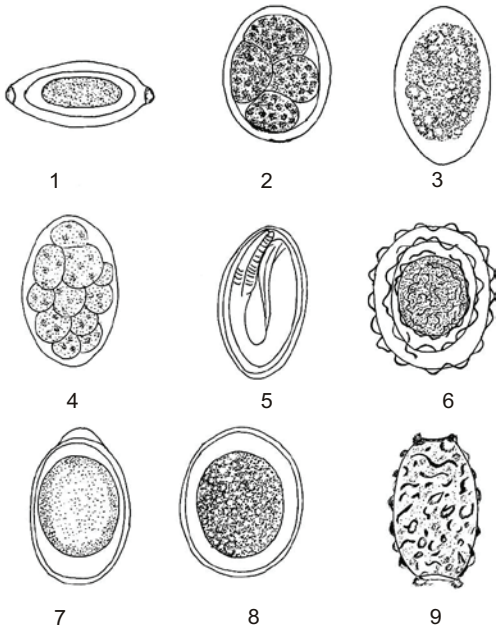
Wuchereria bancrofti, Brugia malayi are transmitted by mosquito bite. *Loa loa,* the eyeworm, is transmitted by the deer fly of the genus *Chrysops*. *Onchocercus volvulus* is transmitted through blackflies of the genus *Simulum, Masonella* by midges of the genus *Culicoides*. Infection of *Dracunculus medinensis,* the

guinea worm, is via aquatic crustacean, the copepod. These encysted larvae are released from blisters in the skin. The copepods consuming the larvae and when these infected copepods are consumed through drinking water inadvertently the larva enters the human. In one year the worms mature and mate. The females travel to the skin and cause blisters which are ready to infect a copepod.

Intestinal parasites can be tolerated when they are in small number, but when they occur in large numbers they cause malnutrition and retarded growth. The same problem is faced with hookworms, which are the causative agents of anaemia.

Wuchereria and *Brugia* are responsible for elephantiasis (enlargement of limbs, breasts and genitalia) and this is an immunopathological response. *Onchocerca* causes severe damage, embryos getting into the intestinal fluids of the skin and subdermal tissues causing a lot of changes in the skin.

NEMATODES



1. *Ascaris lumbricoides,* 2. *Gnathostoma spinigerum,* 3. *Ancylostoma duodenale*
4. *Ancylostoma braziliense,* 5. *Necator americanus,* 6. *Strongyloides stercoralis*
7. *Enterobius vermicularis,* 8. *Trichuris trichiura,* 9. *Trichinella spiralis*
10. *Angiostrongylus cantonensis,* 11. *Dracunculus medinensis*

Figure 5.44 *Ascaris lumbricoides*

Ascaris lumbricoides (Linnaeus, 1758)

It is the largest human intestinal parasite (Figure 5.45). Adult female is larger than the male with an elongated cylindrical body, with a blunt anterior end and tapering posterior end. It measures 20–35 cm

× 3–8 mm and the male 15–30 cm × 2–4 mm. The worm is pale, brown or whitish in colour with prominent lateral lines longitudinally running the entire length of the body. The mouth is situated at the anterior end and is guarded by 3 lips with fine denticulated ridges. Of the three lips, one is dorso-median and the other two are ventro-lateral. Buccal cavity is triangular. The posterior end of male is curved. Reproductive system consists of testes and vas deferens, ejaculatory duct which is coiled towards its posterior end and opens into the cloaca. Associated with male reproductive system is a pair of spicules situated in a pocket. Gubernaculum is absent. The male tail is conical with two rows of tactile papillae mostly pre-anal and few post-anal. In the female, the vagina is directed backward, paired genital tubes are present, each having uterus, receptaculum seminis, oviduct and an ovary. At a time these worms can lay as many as 27 million eggs and per day as many as 200,000 eggs are laid.

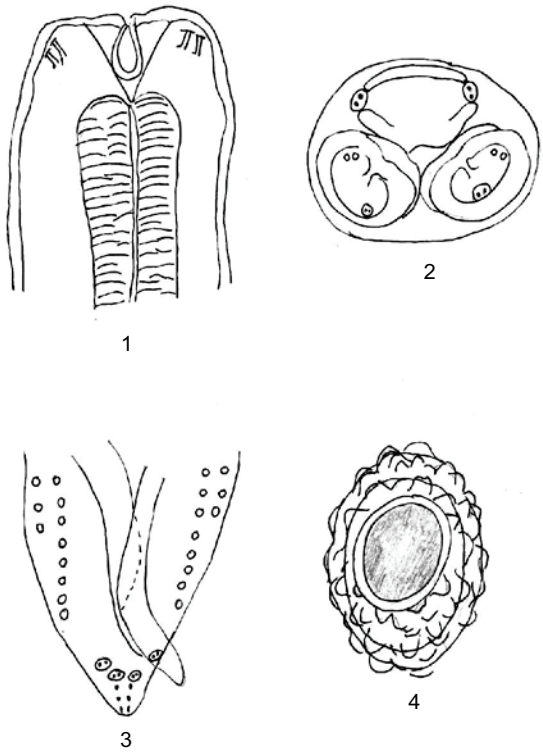


Figure 5.45 *Ascaris lumbricoides*, 1—Anterior extremity of male worm, 2—Head of worm showing lips and papillae, 3—Posterior end of male, 4—Fertilized egg

Egg measures 50–70 × 40–50 μm with an elliptical shape or ovoidal broad (Figure 5.44 f) with a transparent shell having a rough albuminous coat and with lipoidal vitelline membrane inside (not found in unfertilized eggs) and a thick transparent middle layer. These eggs when they pass in the faeces do not have a differentiated embryo, and no segmentation. These eggs are resistant to desiccation, low temperatures, putrefaction of the medium and strong chemicals, and they lie dormant. With advent of

favourable conditions, the eggs are stimulated. Within 2–4 months, the coiled up embryo inside the egg is seen moving. The embryo will come out only when the egg is swallowed. Once it is swallowed, it passes down to the duodenum, and is softened by the digestive juices. It releases the larva which becomes activated and is called the rhabditiform larva. It penetrates the mucous membrane, enters the blood via the heart and the lungs and then to the alveolus capillaries. Here they burrow in the wall of the alveolus, enter the respiratory tree and finally move to the trachea. Most of the larvae are again swallowed for the second time from the trachea and they reach the small intestine. This second invasion brings about severe allergy and low blood pressure. The whole process takes place in 10–14 days during which period the larva moults twice. The larva measures about 1.75–2.37 mm. Then the larva migrates to the intestine by the fifth day. The larva moults again between the 25th and 29th day. Incubation period in humans is about 60–70 days. Humans gets infection by ingesting fully embryonated eggs from the soil, or food and consuming drinks, contaminated with viable eggs.

Epidemiology

Human infection occurs by ingestion of fully embryonated eggs present in polluted soil, food and drink. All age groups may be heavily infected. In areas where human faeces are used as fertilizers, infections occur through vegetables contaminated with eggs. *Ascariasis* is known as household infection propagated within the house.

Pathogenesis

Complications due to *A. lumbricoides* arise out of the need for extra-intestinal migration. The migration of the larva through the lungs may precipitate in pneumonia. In most nematode infections eosinophilia is common. Adults in the intestine cause abdominal discomfort, colic pains, vomiting and diarrhoea. In heavy infections, worms become tangled masses blocking the host intestine. In severe infections worms up to 1500 have been recovered from the host.

Irritation of mucous membrane and appendicitis occur. Release of toxic substances from the worm cause convulsions, delirium, etc. *Ascaris*-infected school children have stunted growth.

Gnathostoma spinigerum (Owen, 1838)

Adult worms parasitize wild and domestic cats and dogs which form important reservoirs. The males measure 11–25 mm and females, 25–54 mm (Figure 5.46). These are transparent, reddish tinged nematodes with sub-globose cephalic swelling separated from the remainder of the body by a cervical constriction. Anteriorly, half the body is studded with spines which are leaflike. Behind the cervix these spines are tridented and broad and below the cervix they are pointed. These spines are species-specific. The posterior part of the body is aspinosus. Anteriorly the cephalic region of the body is covered with 4–8 transverse rows of sharp recurved spines. Mouth is guarded by fleshy lips. Oesophagus is surrounded by 4 cervical glands which fuse in pairs and open through ducts.

The male has a pseudobursa with four pairs of preanal papillae. Copulatory spicules are chitinous and rodlike, measuring 1.1 mm and 0.4 mm respectively. Vagina is long. The other genital tubes are present.

Eggs are 65–70 × 38–40 μm size, ovoid, transparent, superficially pitted, (Figure 5.44 g, h and i) have a plug at the anterior end and which is mucoid, when they are laid they are embryonated. Adults live in

lesions in the stomach wall, of cats and dogs. Eggs come out of the lesion and they are discharged in faeces into water where they embryonate and hatch. From the egg emerges out 1st stage larva measuring 223–275 × 13.4–17.4 μm provided with an anterior round end studded with spines. It gains entry to a crustacean, the *Cyclops* and penetrates into the haemocoel and undergoes metamorphosis in 10–14 days into the 2nd stage larva (350–450 × 60–65 μm). This larva has a head bulb armed with four rings of spines and two pairs of cervical glands.

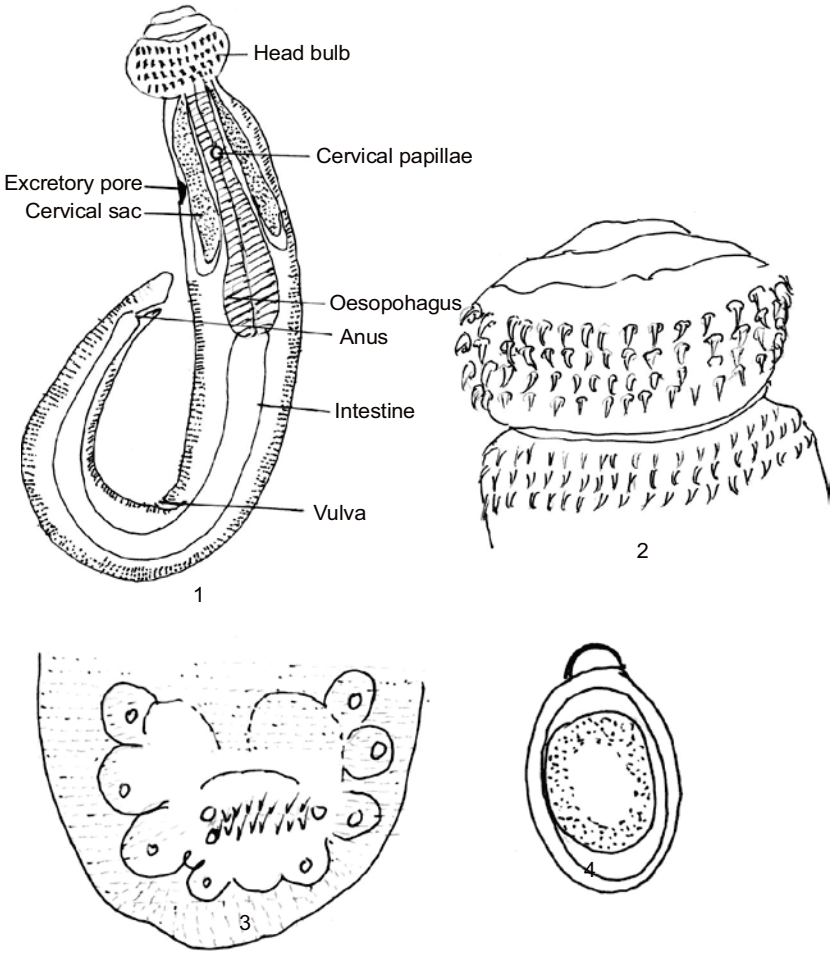


Figure 5.46 *Gnathostoma spinigerum*, 1—Third stage larva—lateral view, 2—Head of third stage larva, 3—Posterior end of male, 4—Fertilized egg

The third stage larva develops in a second intermediate host which may be a snake like python or cobra in India, a fresh water fish in Philippines, a frog in Thailand or crabs, amphibians, reptiles, mammals and chicks in Thailand, as well as humans where it does not undergo complete maturation. Complete maturation occurs in cats and dogs.

So the cycle is as follows:

Adults live in the lesions of the stomach wall of cats and dogs and they are extruded from lesions and discharged in faeces into water where they embryonate and hatch.

The larvae are ingested by the crustacean *Cyclops* where they develop into 2nd stage larva provided with head bulb bearing 4 rows of spines.

When such infected *Cyclop*s is eaten by the fish, snake and the frogs (depending upon the country) the larva develops into 3rd stage larva.

When a suitable definitive host eats the infected flesh of the fish, the larva matures in the stomach in about six and half months, completing the cycle. Humans are infected by consuming improperly cooked infected flesh of fish.

Epidemiology

Not much is known. However, tendency for the infection to be common in females is significant. Inadequately cooked and processed meat is responsible for infection (domestic duck and chick).

Pathogenesis

Gnathostomiasis interna refers to the coiled worm which lies inside tumours of the digestive tract of reservoir host. However in humans the immature worms occur in the peripheral tissue (subcutaneous layers). This is known as gnathostomiasis externa.

Abscess pockets Formation of deep cutaneous or subcutaneous tunnels in which the larva migrates. 'Larva migrations' cause creeping eruption. High eosinophilia and lymphocytosis may occur. Ocular involvement may also be seen.

Ancylostoma duodenale (Dubini, 1843)

This is commonly called the hookworm. Both male and female are stout, cylindrical and anteriorly constricted. Females measure 1–1.3 cm × 0.6 m, cylindrical in shape, posteriorly expanded, creamy grey in colour and covered with a tough cuticle and provided with a pair of lateral cervical papillae below the circumesophageal ring (Figure 5.47). Vagina is at the posterior one-third of the body.

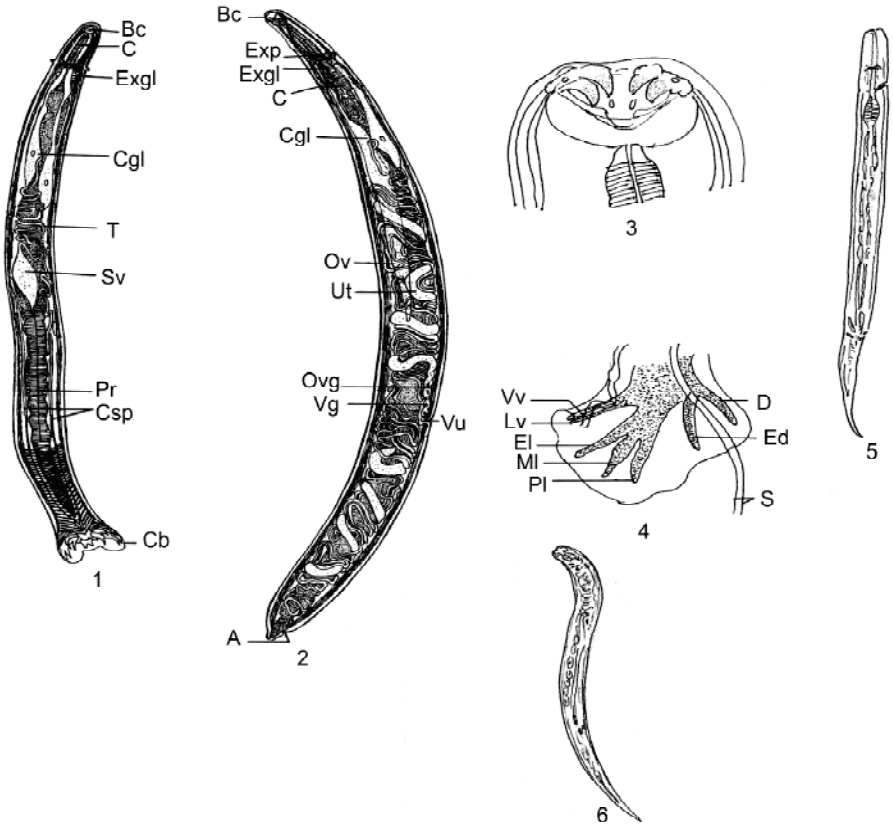
Adult worms live in the duodenum, jejunum and ileum. The body is so curved that the anterior aspect is concave and the ventral aspect convex. Mouth is not at the tip but directed dorsally. Buccal capsule is prominent with two pairs of horselike teeth.

Male worm is 8–11 mm in length and 0.4 m thick. The posterior end is expanded with copulatory bursa supported by rays. The ray pattern is species-specific. Cloaca is situated in the bursa. The rectum and genital canal open into the bursa. There is a pair of long retractile bristlelike copulatory spicules the tips of which project from the bursa.

The female is longer than the male and measures 10–13 mm long and 0.6 mm thick. The posterior end is conical with sub-terminal anus. Vulva opens on the ventral side at the junction of the middle and posterior third of the body. The vagina leads into coiled tubes, the ovarian tubes. During copulation the

male attaches its copulatory bursa to the vulva. The copulatory pairs assume a Y-shaped figure. In both sexes, well-differentiated cephalic glands are present. They secrete an anti-coagulating ferment. The excretory pore is ventrally placed at the level of the oesophagus. The buccal capsule has a chitinous living with 2 pairs of sharp teeth on the ventral side.

Eggs are ovoid and measure 60 × 60 μm (Figure 5.44 b). It has an acting hyaline shell membrane. When eggs are released, the egg contains an unsegmented ovum. When passed in the faeces, the egg contains a segmented ovum with 4 or 8 blastomeres and with a space between the ovum and the egg shell. A female can lay as many as 25,000 to 30,000 eggs a day and 55 million during its lifetime.



Hb—head lobe, Ep—excretory pore, Cs—cervical sac, A—anus, Vu—vulva, D—dorsal ray, Ml—mediolateral ray, Pl—postero lateral ray, S—spicules, Vv—ventro ventral ray, Exgl—excretory gland, Cg—paired cephalic glands, Bc—Buccal capsule, Pr—prostate gland, Csp—copulatory spicules, Ovg—oo ejector

Figure 5.47 *Ancylostoma duodenale* 1—Male, 2—Female, 3—Anterior and showing buccal capsule, 4—Posterior end of male with bursal rays and spicules, 5—Filariform larva of *A. duodenale*, 6—Rhabditifrom larva of *A. duodenale*

Eggs freshly passed in faeces are not infective for man. When eggs are deposited in soil under favourable conditions like shady environment, or moist and decaying vegetation, a rhabditiform larva emerges out.

It measures 250 μm long and feeds on bacteria and organic matter in the soil, moults first time on 3rd day and second time on 5th day to become the infective filariform larva. It is 500 μm long with a pointed tail. This is the non-feeding stage. They live in the soil for about 5 weeks. They are attached to grass blades.

When a person walks bare-footed in soil containing filariform larva, the latter penetrate the skin (sub-cutaneous tissue). The soft skin between the toes is the favourite site for penetration. For people working in farms, the larvae may penetrate the skin of the hands. The larvae enter the venules of the subcutaneous tissue, and is carried in the circulation to the right heart and to the lungs. In the lungs they break the capillaries and reach the alveoli, from where they migrate to the jejunum, where they moult and develop a temporary capsule with which they get attached to the mucosa. Here they feed and grow and undergo the fourth and final moult during which they develop a regular buccal capsule growing into adults. In about six weeks they become sexually mature and start laying eggs.

Oral infection is rare. The larvae penetrate the buccal mucosa, reach the venous circulation and complete the migration via lungs. Alternatively the larvae may be swallowed and develop directly into adults in the small intestine without a tissue phase.

Epidemiology

Epidemiology of hookworms is linked to the following closely integrated factors:

Adequate source of human infection Defaecation habits—eggs deposited in areas of favourable conditions, appropriate environment for development of the eggs (moisture, warmth).

Soil-shade and sandy.

Opportunity for filariform larva to come into contact with human skin.

In tropical and subtropical countries these conditions remain optimal throughout a greater part of the year. Prolonged rainfall or dry and cold seasons may be detrimental for the survival of larvae.

Pathogenesis

The hookworm larvae, itching and inflammation of the skin and development of pustular sores may occur. *A. braziliense* may fail to find these ways beneath the germinative layer and become unable to reach blood or lymph vessels. Thus they may enter airlessly under the skin-cutaneous larva migrans (creeping eruption). Next they cause pulmonary infection and even pneumonia symptoms. The larva may be encapsulated. Eosinophilia and leucocytoses are common. When the worms are migrating in the intestine, nausea, abdominal discomfort are common. The important effect's because of their habit of blood drinking anaemia is the frequent condition. In severe cases haemoglobin may be reduced to 30%. Appetite is capricious.

In children, physical and mental retardation may appear. In pregnant mothers still births are common.

Ancylostoma braziliense (DeFaria, 1910)

It is found in dogs and cats. It is a rare parasite in the small intestine. It is part of mixed hookworm infection in humans in India, Malaysia and Thailand. Males measure 7.75–8.5 mm in length × 0.35 mm in diameter and females measure 9–10 m × 0.375 m (Figure 5.48). Unlike *A. duodenale, A. braziliense* has a buccal capsule within conspicuous median teeth and a pair of outer teeth. The bursa is also conspicuous,

and is supported by short stubby rays. The eggs are almost similar to those of *A. duodenale* and are indistinguishable from the latter. The life cycle pattern is same as that of *A. duodenale*. Humans are not suitable hosts. The larva does not enter the bloodstream but wanders under the skin.

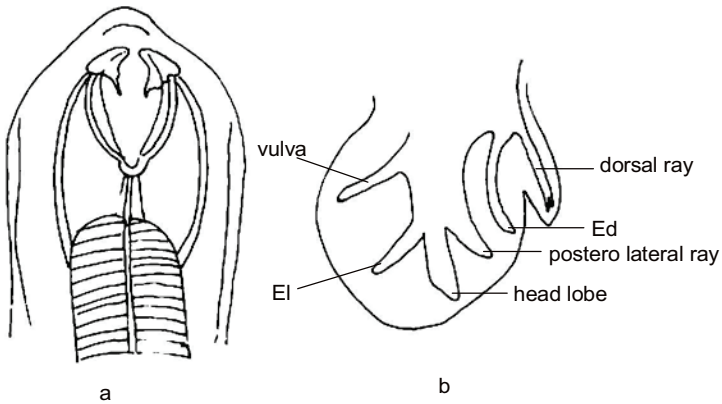


Figure 5.48 *Anclyostoma braziliense;* a. anterior end, b. posterior end

Necator americanus (Stiles, 1902)

It is a parasite in the small intestine of humans, gorilla, rhinoceros, pangolin and some rodents. *Necator americanus,* unlike *A. duodenale,* is a short slender worm (Figure 5.49). The female grows to a length of 0.9–1.1 cm × 0.4 mm. Vulva is placed slightly in the middle of the body and when it copulates, a Y-shaped figure is attained as in *A. duodenale*. Male grows to a length of 7.9 × 0.3 mm, has the copulatory bursa closed and blunt and there is a short median lobe which appears as if divided. The dorsal ray branches at the base into divergent arms with bipartite lips. The base of the dorsal and dorso-lateral rays is short. Two separate spicules unite to form a single terminal 'fish book' barb. The living worms are greyish yellow and at times reddish.

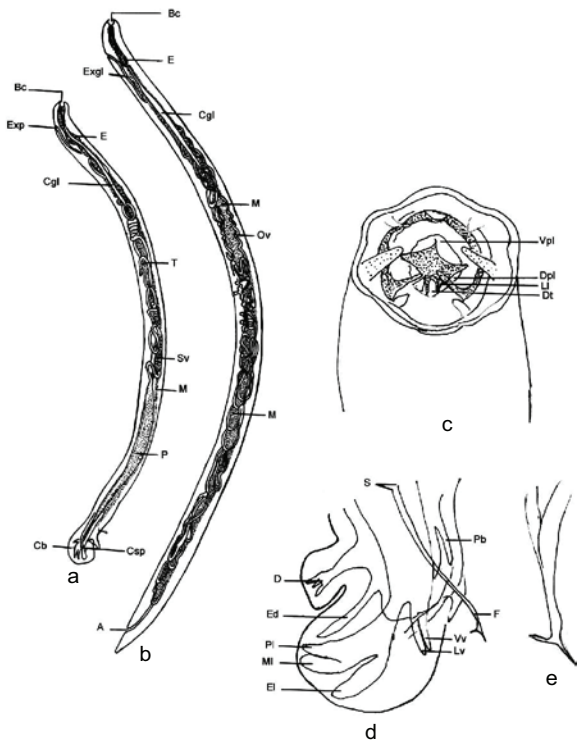
In the female, there is a sudden bend of the head and it is quite distinctive. Buccal capsule has an irregular border and is small. Unlike *A. duodenale,* it has a ventral pair of cutting plates (four hooklike teeth in *A. duodenale*). Chitinous plate replaces the first pair of dorsal teeth. The outlet of the dorsal gland constitutes a "dorsal rib" or tooth which projects into the oral cavity. Placed deep under the capsule are 2 pairs of lancets, one pair dorsal and the other, submedian.

Egg measures 64–75 × 36–40 μm. The third stage larva has a larger buccal vesicle and there is an intervening space between oesophagus and midgut. In this respect, it differs from *Strongyloides stercoralis*. The female lays as many as 6000 – 20,000 eggs a day. The lifespan of the worm is five years.

Life cycle Eggs when laid in the lumen of the intestine have two, four or eight blastomeres. When they are extracted along with faeces on damp soil, they start developing. Under favourable conditions, the rhabditiform larva escapes out of the egg and feeds on bacteria in the faeces. The rhabditiform larva has a bulbous (double) oesophagus.

It feeds voraciously and on the third day it moults (length 400 μm) and the oesophageal bulb disappears on the fifth day (length 500–700 μm) and the larva elongates and becomes fully developed.

Now it leaves the faeces and enters the soil, moults again to become the filariform larva or the third stage larva. This larva has well-developed mouth capsule, a muscular oesophagus and a protective sheath. The larva is incapable of swimming in water. The protective sheath is useful in withstanding desiccation. It can live for 2 years provided the conditions are optimum. Direct sunlight, salt, water or flooding is fatal for the survival.



Cb—copulatory bursa, Cgl—Paired cephalic glands, Csp—Copulatory spicules, E—Oesophagus, Exgl—Excretory gland, Exp—Excretory pore, M—Midgut, Pg—Prostate gland, Sv—Seminal vesicle, T—Testes, D—dorsal ray, E—Externo dorsal ray, F—Bursel terminus of spicules, Lv—Latero ventral ray, Md—Medio lateral ray, Pb—Pre bursal ray, Pl—Postero lateral ray, Vv—Ventro lateral ray, BC—Buccal capsule, Ov—Ovary, Vpl—Ventral cutting plate, Dpl—Dorsal cutting plate, Li—Lateral land set, Dt—Dorsal tooth

Figure 5.49 *Necator americanus*, a—Adult male, b—Adult female, c—Anterior end showing buccal capsule, d—Posterior end of male showing bursal rays and spicules, e—Spicules

Now it is time to enter the host and when it comes in contact with the host, it penetrates the skin and the protective sheath is discarded. It later enters the lymphatics, then to the bloodstream and migrates to the lungs by the third day. *A. duodenale* can infect humans via skin as well as mucous membrane of the mouth, whereas *N. americanus* gains entry through the skin. From the lungs, it reaches the alveoli,

bronchioles, travels via trachea and oesophagus to the stomach. It moults for the third time during this migration. This larva develops a buccal capsule when it reaches the intestine on the seventh day, where it moults again (4th moult), the terminal buccal capsule is converted into provisional buccal capsule with the mouth without teeth opening into it. On the fifteenth day the, "provisional buccal capsule" is replaced by adult buccal capsule and bursa in the male. It becomes sexually mature in about 3–5 weeks, copulates and produces eggs (fertile). *N. americanus* lays as many as 9000 eggs per day.

Epidemiology and Pathogenesis

The epidemiology and pathogenesis are the same as for other hookworms.

Strongyloides stercoralis (Bavay, 1876)

The various stages in the life cycle of *Strongyloides stercoralis* are shown in Figure 5.50 a–g.

Free-living stage

This phase of life cycle takes place in warm climate where moisture favours development, abundant faecal matter is available in the soil. There are 100% chances of free-living existence and multiplication. The rhabdoid and rhabditoid male is fusiform and measures 0.7 mm length by 40–50 μm diameter. A gubernaculum and 2 spicules are present. Caudal aloe is lacking. Tail is curved.

Rhabditoid female is stout and measures 1 mm by 50–75 μm. Uterus is 2-horned with a vulva opening near the middle region on the ventral side. Eggs discharged from fertilized female are embryonated, which hatch in a few hours. The first stage or rhabditoid larva has a characteristic shape and the muscular oesophageal structure is of great importance. The buccal cavity is small. The larvae feed on organic matter in the soil, moult, grow rapidly and after 3 months develop into an adult.

They lead a free-living life indefinitely. When conditions are not favourable, the rhabditoid larva metamorphoses into the filariform stage, where the worms are long and delicate, with a long oesophagus.

Parasitic stage

It was thought that embryos were produced by parasitic parthenogenic female. But it is clear now that parasitic males also exists. It has an oesophagus with club-shaped anterior part with a posterior constriction and a posterior bulb. A gubernaculum, a pair of copulatory spicules are present. This adult male resembles the adult male of free-living generation.

Parasitic males are not found in human infection because they cannot invade the intestinal wall; so they are eliminated as soon as the females begin to oviposit.

Female measures 2.5 × 9.34 mm, with a tapering anterior end and posteriorly a conical tail is present. Mouth is guarded by 3 lips and leads into an oesophagus occupying a quarter of a length of the body. Vulva lies in the posterior third of the body. Uterus is prominent and is filled with eggs. These eggs are released into the lumen of the bowel when they are in the advanced stage of development. Rarely they are found in 50–58 × 34 μm. These eggs hatch into embryos 0.2–0.3 × 0.013 mm. They have a double-bulb oesophagus which resembles the rhabditiform larvae of *Ancylostoma* and *Necator.* Now they come

out along with the faeces and in 3–5 days they are converted into male and female free-living forms. These have a rhabditiform and double-bulb oesophagus which is muscular.

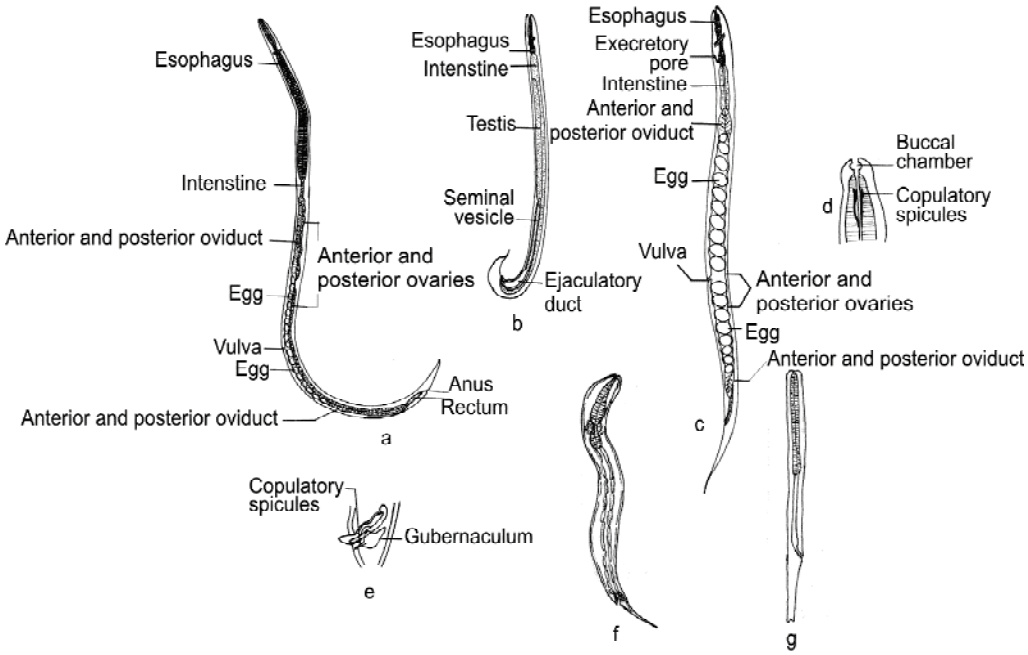


Figure 5.50 *Strongyloides stercoralis*, a—Parasitic female, b—Free-living female, c—Free-living male, d—Anterior end of parasitic male, e—Copulatory spicules and gubernaculum of male, f—Rhabditiform larva, g— Filariform larva

Male is a free-living form and measures 0.7 × 0.035 mm. Tail is curved ventrally. Two spicules are present. The free-living female measures 1 × 0.05 mm. Vulva is situated in the middle of the body. Uterus measures 70 × 40 μm and contains thin-shelled eggs.

Copulation takes place in faeces, and rhabditiform larvae are produced. They cannot be distinguished from those derived from a parasitic female. After 3–5 days, these larvae develop into filariform larvae, the infective stage which re-enters the humans via the skin or the buccal mucosa as in *Ancylostoma* or *Necator*, but they lie alive for several weeks, in the soil. In filariform larvae the eosophagus is very long, occupying half the length of the body. These filariform larvae make their entry into the small intestine and develop into parasitic females.

When the climatic conditions are favourable, the sexual phase in the faeces is omitted and rhabdtiform larvae produced by the female may develop directly into filariform larvae, which infect the definitive host.

Epidemiology

Infection is through soil contaminated with dog and cat faeces, when people take bath with bare feet, or crawl underneath for plumbing work. In sub-tropical countries, exposure is a common factor especially during summer months.

Pathology

Filariform larvae cause haemorrhages at the site of invasion, accompanied by congestion and oedema. The larvae migrate to cutaneous blood.

Enterobius vermicularis (Linnaeus, 1758)

The adult worms live in the caecum, appendix, the adjacent regions of the ascending colon and the ileum. They live with their heads attached to the mucosa of the intestinal wall. The parasite is small, white and spindle-shaped. A true buccal capsule is absent but the worm has 3 lips and a dorso-ventral bladder transversely striated. Male is rarely seen. It measures 2.5 mm in length and 0.1 to 0.2 in greatest diameter (Figure 5.51b). Posterior end is strongly curved spirally. The curved end bears 6 sensory papillae and a single spicule. A gubernaculum is lacking. Copulatory bursa is reduced and termed as "caudal aloe" supported by a pair of anterior pedunculate papillae.

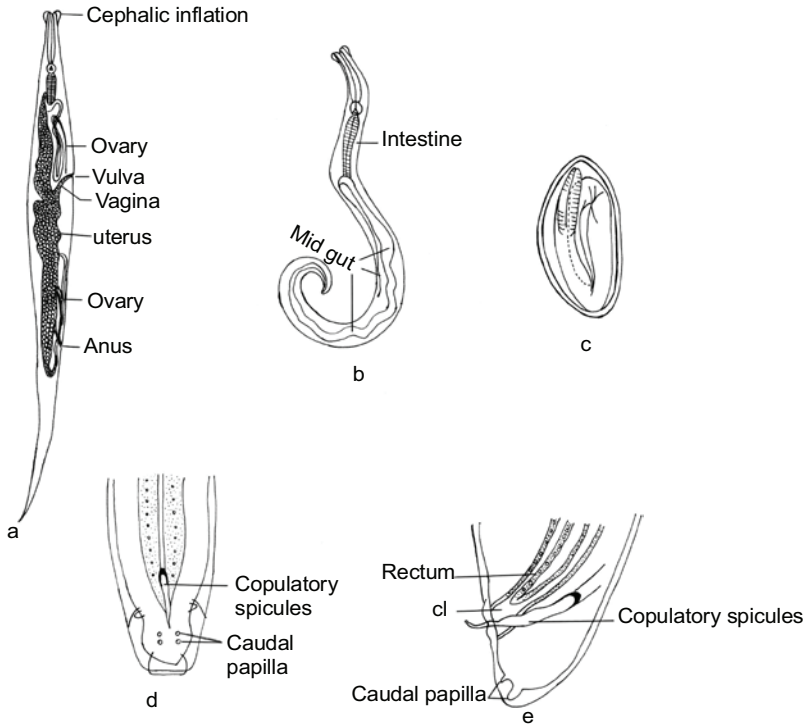


Figure 5.51 a—*Enterobius vermicularis*, b—Male, c—Embryonated eggs, d—Posterior end of male ventral view, e—Posterior end of male lateral view

Female (Figure 5.51a) measures 8–13 mm in length and 0.3–0.5 mm in breadth and has a long pointed tail; the anus is 2 mm from the posterior extremity. Vulva opens on the ventral side in the middle of the body. Vagina is long and travels posteriorly and joins the paired genital organs, i.e., uteri, oviducts and the ovarian tubules which are coiled several times.

In the gravid female, the uterus is transversely distended. A gravid female lays as many as 10–15,000 eggs within few minutes, and dies when egg-laying is completed. The female leaves the intestinal wall and falls in the lumen of the intestine, passing down the colon and out of the anus to crawl on the perianal and supra anal skins and in female, the parasites at times enter the vagina. The migration occurs from 15–43 days after the ingestion of infective pinworm egg.

The egg measures 50–54 × 20–24 μm (Figure 5.51c). It has a characteristic shape, flattened on one side. It is colourless with a double shell, and contains a more or less fully formed embryo. Egg has an outer thick albuminous layer, a blue hyaline inner layer and shell proper.

Life cycle No intermediate host is involved in the life cycle for subsequent development and there is no multiplication of worms inside the body. The shell of egg gets weakened by the digestive juices and the larva breaks out of the shell and starts invading the glandular crypts and penetrates into the glands and stroma where it coils up.

The lifespan of *E. vermicularis* is 37 to 93 days. The female worm comes down the intestine and lies in the faeces. The fertilized worm migrates to the anal region to lay eggs in the perianal skin and perineum.

The crawling of the gravid females produces an itching sensation. After a few hours the embryo develops rapidly and grows to a length of 140–150 μm. The eggs enter the mouth due to nails soiled with faecal matter under finger nails, reaches the digestive system where it hatches. Liberated larvae after 2 months reach the large intestine where they mature. The whole cycle is completed in 2–4 weeks.

Epidemiology It is worldwide in distribution and children are more prone to this disease. It is common in groups or institutions like school and asylums. Though it is a human infection, chimpanzees and gibbons could also be infected.

Pathology Due to its habitation in upper part of colon, caecum and lower ileum, minute ulcerations are seen. Sometimes haemorrhage could be noticed. Symptoms are more obvious when gravid females migrate out by the anus on to perianal skin to deposit eggs where they cause pruritus.

Trichuris trichiura (Linnaeus, 1771)

This parasite lives attached to the wall of the caecum. Humans are the typical hosts, although there are reports of the parasites infecting pigs and some monkeys. Worms are creamy with a fleshy posterior end. The oesophagus is delicate with anterior muscle with stylet and posterior muscles dilating at its more distal end.

Male grows to a length of 30–40 mm, and has an anterior attenuated portion with cellular oesophagus which is half as long again as thicker posterior portion. The caudal portion is curved with a single spicule armed with spines (Figure 5.52a).

The female grows to a length of 30–34 mm with an anterior attenuated portion, twice as long as the posterior portion. Uterus occupies the posterior portion fully packed with eggs. Ovary is saccular and runs forward from the posterior end. Females preponderate over males in a proportion of over 400 to 1.

Eggs measure 50 × 22 μm, and are brown in colour and have a peculiar barrel shape. It has a thick shell with a plug at both ends. Eggs contain an unsegmented embryo. They are discharged and passed in the stool.

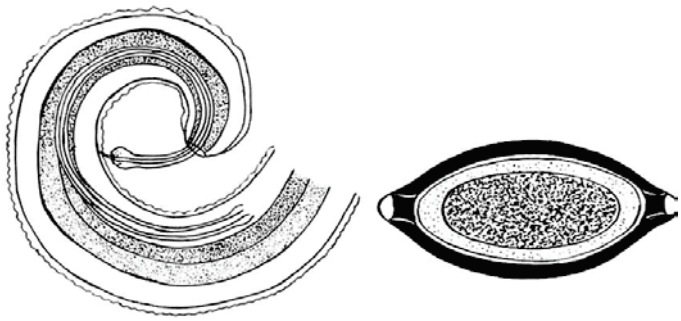


Figure 5.52 *Trichuris trichiura,* a—Posterior end of male showing copulatory sheath, b—Egg of *Trichuris trichiura*

Epidemiology

It is common in places where rainfall and, humidity are high, and which are shady and have poor sanitation and in contaminated soil. Mostly it is transmitted by soil contaminated with eggs. In Malaysia, children are more prone to this infection. This infection is associated with *Ascaris.*

Pathology

When they occur in large numbers they spread to the colon and then to rectum. Haemorrhage, stools with lot of mucus and dysentery are some of the symptoms.

Trichinella spiralis (Onem, 1835)

Trichinella spiralis is a small white worm visible to the naked eye and lives in the small intestine (Figure 5.53). When humans consume raw flesh or improperly cooked flesh they are infected with *T. spiralis.* The cysts are digested in the stomach by digestive juices. After few hours they encyst in the duodenum where they attack the jejunal and duodenal mucosa and later moult four times and develop into a minute threadlike adult males and females.

The male measures 1.6 × 0.04 mm in length and breadth, is 40–60 μm in diameter. At the caudal end the cloaca is situated and is guarded by 2 pairs of papillae situated at the anterior end. Uterine tube is coiled and runs anteriorly. The anus is at the posterior terminus. Female lives for a month and produces 1500 larvae per day. The larva measures 100 × 6 μm.

Life cycle Uterus is filled with eggs but the developing embryo breaks the shell, comes out and falls into the uterine tube. The larvae migrate to the lymphatics and enter the bloodstream and reach all parts of the body. Now a cyst is formed, and is surrounded by the cyst wall encapsulated by the host tissues. The capsule has blunt ends and is an adventitious ellipsoidal sheath.

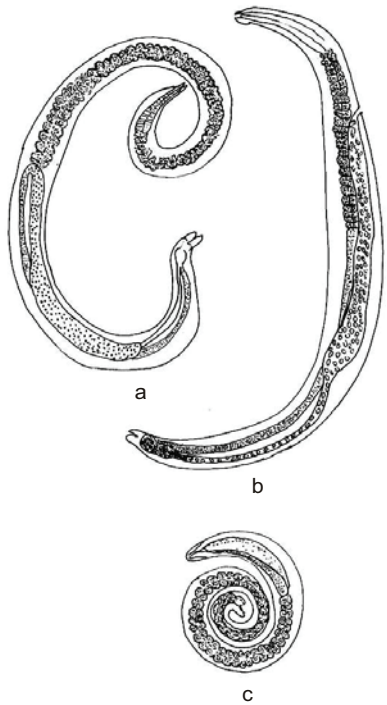


Figure 5.53 *Trichinella spiralis*, a—Adult male, b—Adult female, c—Larva

The long axis of the larva is parallel to the long axis of the muscle fibres. The encysted larva remains viable for many years with the help of proteins converted from amino acids of the host. These amino acids are first transferred to the cyst. These cysts are consumed by a suitable vertebrate host where the cysts are digested in the stomach and from there the larvae migrate to the jejunum and duodenum, attacking the mucosa. The larvae undergo four moults and develop into adult male or female. These enter the bowel and re-enter the mucosa, penetrate the villi and reach the muscularis glands. All these changes take place in about 4–16 weeks. Then the larvae are carried to the right heart and then to the lung, get into arterial circulation and finally get lodged in the striated muscle where they encyst.

Epidemiology

Rats are common reservoir hosts. Marked cannibalistic tendencies exist in them. Rodent reservoirs transmit infection to dogs, cats, and wild animals. There are two epidemiological phases.

- Sylvatic wild mammals with no connection to humans or domestic animals
- Synantropic connection with animals in close association with humans

Trichinellosis is mainly linked with man and pigs. The name "garbage worm" is given because of the habit of feeding pigs with unsterilized pork pickles.

Pathogenesis

Adult intestinal worms have short lifespan (3 months). The larvae are made pathogenic. Numerous larvae enter the blood circulation (the adult is viviparous) and reach various tissues but undergo further development in active voluntary muscles (ribs, lungs, eye and limbs). In muscles they roll themselves spirally and a cyst is formed. The cyst wall may start calcifying. and the worm may die.

Angiostrongylus cantonensis

Male is a delicate filiform worm growing to a length of 15.5–22 mm in length × 0.35+ mm in breadth. Cuticle is smooth but at the anterior and posterior extremities it is thickened. Mouth is guarded by 3 lips of which the dorso-median has two papillae and the lateral ones each have one papilla. Body appears transparent with feeble transverse striations. In addition to these papillae, there are 4 pairs of papillae on the external border of the head. Mouth leads directly into oesophagus as there is no buccal capsule. Oesophagus is 0.29 – 0.33 mm long by 0.05 mm at maximum breadth. Excretory pore is situated near about the junction of the esophagus and intestine. Spicules are in the form of striated rods 1.2 mm in length. There is no gubernaculum. Bursa is well-developed and a pair of adanal papillae is present.

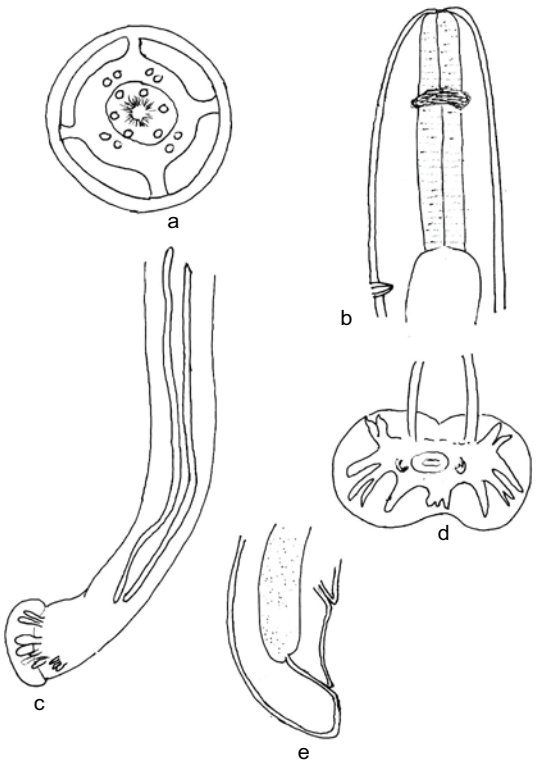


Figure 5.54 *Angiostrongylus contonensis*, a—Head on view of buccal extremity, b—Lateral view of anterior end, c—Lateral view of posterior end of male showing two copulatory spicules and bursa copulatrix, d—Posterior view of bursa, e—Posterior extremity of female, lateral view

The female is 18.5–33 mm long and 0.28–0.5 mm in maximum breadth. All structures are almost similar to those of male. Uterine tubes are spirally wound. The cuticle is so transparent that spiral uterine tubes and blood-filled intestine could be seen. Vulva is in the form of a slit. Anus is situated at the posterior extremity. The various parts of the male and female worm are depicted in Figure 5.54.

The eggs are oval in shape, (Figure 5.44d) measure 46–48 × 68–74 μm in diameter and possess a hyaline shell. They are embryonated at the time of deposition. A female can lay as many as 15,000 eggs a day.

Eggs hatch in the lungs of the rats and the first-stage larvae then migrate to the trachea by penetrating the respiratory tract. Finally they are discharged along with faeces. They have to find a molluscan intermediate host and *Agriolimax* is the intermediate host. They moult twice in the intermediate host in about 7 days. The slugs are eaten by rats, and the larvae with their casts are freed in the stomach of the rat by digestive juices. Then they travel along the ileum and slowly reach the bloodstream and get into the central nervous system for 15 hrs. In the cerebrum they moult for the third time in six or 7 days and the final moult on the 11th to 13th day. Young ones come out and crawl in the brain. They remain here for two weeks. Then they migrate to the lungs by 28–31st day via the venous system, reach the right heart and finally the pulmonary artery and start laying eggs. Life cycle is similar to that of *W. bancrofti*. This microfilaria is also nocturnal and development in mosquito is of shorter duration 6–8½ day. The larva undergoes 2 moults in Mansonia. A buccal cavity is formed from cephalic space, oesophagus from the nuclei of the anterior part of the nuclear column, rectum and anus from four G cells. After the first moult, the tail with two nuclei is shed.

Epidemiology

Not clearly known.

Pathogenesis

Eosinophilia, meningencephalitis have been reported in a patient. Tissue necrosis was seen. In one case headache and fever persisted. This patient died and autopsy revealed necrotic brain tissue.

Crystals occur in meninges. Vascular congestion was prominent.

Dracunculus medinensis (Linnaeus, 1758)

Dracunculus medinensis is commonly called the guinea worm. In olden days, a peculiar device was used to extract the worm from the body by twisting it on a stick slit. This system persists in endemic areas. This worm is widely distributed in Africa, Middle East, Iraq, Iran, Arabia, Pakistan and India.

The female is long and measures 60 cm × 1.5–1.7 cm. Body is cylindrical and smooth. Tail is pointed forming a sort of a blunt hook. The head is round with a cephalic shield. Mouth is triangular and surrounded by six papillae with an outer circle of four double papillae. Behind the nerve ring, a pair of cervical papillae is present which are laterally disposed. Oesophagus has a single bulb. The alimentary canal is small. Uterus is branched as a result of which alimentary canal is pushed to a side. Vulva is very small situated in the middle of the body. Major portion of the body is filled with uterus which is loaded with embryos. A pair of ovaries and oviducts are present at the posterior extremity.

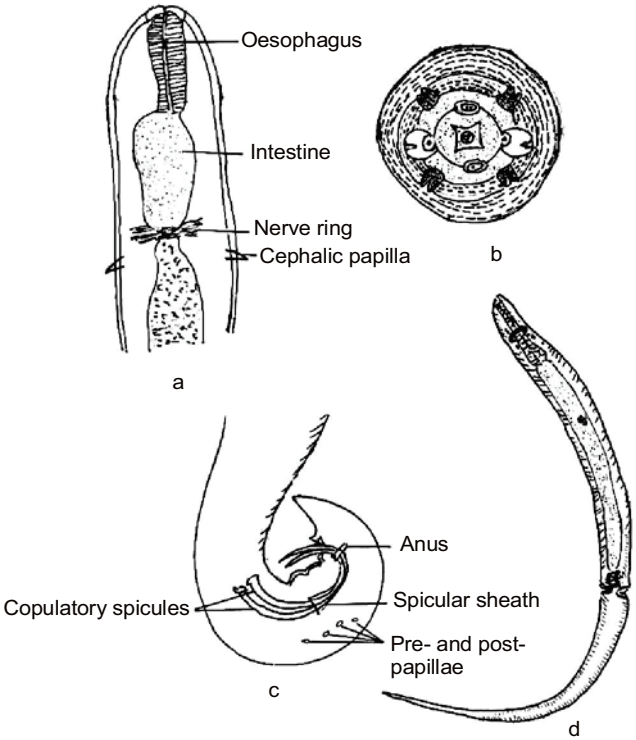


Figure 5.55 *Dracunculus medinensis*, a—Anterior end of female—ventral view, b—Head of view of worm, c—Posterior end of male—ventro lateral view, d—Larva of *Dracunculus medinensis*

The male measures 40 mm in length 1.2–2.9 × 0.4 mm in breadth. The posterior end is coiled with 10 pairs of caudal papillae of which 4 are pre-anal, and six post-anal. The copulatory spicules are subequal. After copulation, the male dies. Figure 5.55 shows the various parts of male and female of this organism.

The embryo measures 500–750 × 17 μm and is flattened. Body shows transverse striation. Tail is long, slender with a rounded head. The alimentary canal consists of a bulblike oesophagus and rudimentary anus.

Life cycle Man is the definitive host. The adult worm discharges larvae when they are ingested by a crustacean (copepod), the intermediate host. When humans drink accidentally the contaminated water infected with *Cyclops*, they get the infection. This is the third-stage larvae present in the haemocoel. In the body of humans, the *Cyclops* is killed by the gastric juices and the worms are released and penetrate the duodenal wall and attack the sub-cutaneous tissues. In the tissues the larvae develop into male or female within a span of 4 months. After copulation, male dies. The fertilized female grows, migrates within the connective tissues and after six months they come to lie in the sites where it is likely to come in contact with water. The common site is the leg. Arms, shoulders, feet, genitalia are also affected.

The female worm lies below the skin and secretes some kind of a toxin which irritates the skin and forms blister. This blister bursts and forms an ulcer with a small hole at its base through which the female

protrudes. When the ulcer comes in contact with water, the female discharges a milky fluid loaded with a number of larvae. This process lasts for 3 weeks.

The larvae swim about for a week and gain entry into the fresh water crustacean *Cyclops*. The larvae penetrate the gut wall of the crustacean, enter the body cavity where 2 ecdyses take place in 2–4 weeks and enter into third-stage larva. The entire cycle takes about a year.

Epidemiology

Period of infection coincides with rainy season. Infection is common in children and adults up to 35 years. Later the incidence falls. Infections are common in areas of step wells. Jodhpur in Rajasthan is a highly endemic area.

Pathogenesis

The guinea worm pierces the layers of the skin through the anterior end of the body and irritates the tissue to cause a blister. The blister breaks and exposes a shallow ulcer with a hole in the middle. When the ulcer comes into contact with water the worm's uterus projects out and a milky fluid containing larvae is issued. The larvae are released in puffs and ultimately the uterus dries up.

BLOOD PARASITES

Human haemoflagellates include the genera *Trypanosoma* and *Leishmania.* Human trypanosomes are of two types: 1) Those which cause African sleeping sickness, a disease transmitted by tsetse fly of the genus *Glossina.* These parasites include *Trypanosoma gambiense, T. bruci, T. rhodesiense* 2) The second variety is the American 'chagas' disease which is transmitted by cone-nosed bugs of the genus *Triatoma.* This includes *Trypanosoma cruzi.*

Leishmania has a number of species infecting humans, and it is the causative agent of a number of human infections such as cutaneous (oriental sores), mucocutaneous (espundia) and visceral (kala-azar) leishmaniasis. The transmission of this disease is by sand flies of the genus *Phlebotomus, Lutzomyia* and *Psuchodophygus.*

Trypanosomes are pleomorphic forms. In human blood they occur as trypomastigotes which have an elongated body with a longitudinal lateral undulating membrane and a flagellum lining the edge of the membrane and at the anterior end it extends as a whiplike extension. Kinetoplast, a darkly staining organ, is situated adjacent to the blepharoplast from which the flagellum arises.

Other polymorphic or developmental forms are amastigote (leishmanial intracellular stage), promastigote (flagellated extracellular stage), a lanceolate form (where an undulating membrane is lacking with a kinetoplast at the anterior end), an epimastigote form (elongated extracellular stage with a more posteriorly placed kinetoplast almost near the nucleus and with an undulating membrane).

In *Trypanosoma cruzi,* the amastigote, promastigote and epimastigote stages occur in humans, and in vectors trypomastigote stage occurs. In *Trypanosoma gambiense, T. brucei* and *T. rhodesiense*, the trypomastigote stage occurs in tsetse fly and only trypomastigote stage occurs in humans.

In *Leishmania,* life cycle has only two stages, promastigote and amastigote. The promastigote is found in vectors.

Trypanosomes

Like species of *Plasmodium*, trypanosomes introduce two hosts in their life cycle, a vertebrate and invertebrate host. Three species of trypanosomes can infect humans.

In mammals *Trypanosoma* occurs in blood as an elongated mature trypomastigote. In vectors, a multiplying epimastigote is formed first and later develops into infective trypomastigote stage.

Trypanosoma gambiense

Morphology

Trypanosoma gambiense (Figure 5.56) and *T. rhodesiense* are indistinguishable except in size, shape of the body and length of the flagellum. A large round kinetostome is characteristic. Tissue forms are commonly found in musles, liver and brain. They develop from amastigote forms and later multiply to form an intracellular colony often invading the host cells. *Trypanosoma rangeli* common to South and Central America infects humans without causing a specified disease so it has to be carefully distinguished from other pathogenic species.

The general morphology is, it is an elongated form measuring 12–30 μm long. Reservoir is situated posterior to the nucleus. Still posterior is the kinetoplast. The flagellum is attached to the body by an undulating membrane. With Giemsa, or Ramanovsky stains, kinetoplast takes a red shade, cytoplasm a blue shade and nucleus a red shade.

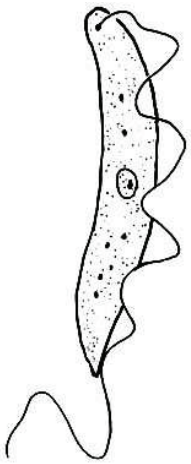


Figure 5.56 *Trypanosoma gambiense*

Figure 5.57 *Tryphosoma hodesiense*

Epidemiology

The main vector in the transmission of *T. gambiense* from man to man is the tsetse fly *Glossina*. Both male and female flies serve as vectors after a cycle of development. *Glossina palpalis* and in some regions *Glossina trachinoides* are the usual species. In the flea the cycle is 12–30 days. Transmission is through the bite of the fly.

When the parasites have gone through the metacyclic phase, not only is Gambian trypanosomiasis transmitted to man, but also other trypanosome infections, but they are fatal to domestic animals.

Pathogenesis

T. gambiense produces a chronic disease which may be categorized into 3 phases following an incubation period of 2–23 days.

1. Trypanosomes in bloodstream.
2. Trypanosomes in lymph nodes.
3. Trypanosomes invading central nervous system.

When the proboscis of the infected fly (metacyclic phase) pierces the skin of a healthy person, the parasite gains entry into the tissues of the site of infection. First an inflammation is noticed which slowly subsides within a week or two. Then they enter the bloodstream, multiply and produce parasitemia. They start producing injurious effects on every tissue and organ. The condition is quite clear in the lymph nodes. Here endothelial cells proliferate and thus results in the infiltration of leucocytes. When they gain entry into the central nervous system, there is infiltration of lymphocytes, plasma cells and endothelial cells in the brain substance. Sleeping sickness is due to chronic inflammation of the lymphatic system around blood vessels.

T. brucei

In vertebrate hosts, that is humans and mammals, only trypomastigotes occur as long slender forms with anterior flagellum which undergoes longitudinal binary fission in the blood and tissue fluids, whereas shorter forms with or without flagellum do not undergo division in the vertebrate host but continue the life cycle in the vector, the bloodsucking dipterans of the genus *Glossina*. Both sexes feed on blood. There is a chance of slender forms getting transformed into broader forms but not vice versa. The trypomastigote undergoes binary fission and starts developing in the vector. Unlike the forms in mammals, they have a large single mitochondrium and a functional Kreb's cycle whereas the stage occurring in mammals have only an inactive mitochondrium. Respiration is anaerobic. The pro-cyclic trypomastigotes, to start with, are confined to a chitinous tube which forms a lining to the midgut of the tsetse fly but slowly come out of the peritrophic membrane so that they are not expelled along with the faeces . Slowly they migrate to the lumen of the salivary glands.

In the salivary glands the epimastigotes appear and get attached to the wall of the salivary glands by means of flagella. These epimastigotes slowly transform again into short trypomastigotes. These have a short flagellum. These are the infective metacyclic trypomastigotes and are ingested to the appearance of metacyclic forms in the salivary glands. For reasons not known, trypanosomes ingested by the flies fail to complete the life cycle, even 1% fail to get established or develop beyond the mid gut. But once the fly becomes infective it retains it to rest of the life.

Once the infective trypomastigotes are injected into the susceptible mammal, they transform into slender forms and commence division. To begin with, they are restricted to tissue fluid at the site of the bite, but a few days later they spread through the body via the bloodstream and finally get established in the central nervous system and appear in the cerebrospinal fluid.

Geographical distribution *T. brucei* is found in tropical Africa because the *Glossina* species also has restricted distribution.

Trypanosoma cruzi

In infected mammals, trypomastigotes develop in the bloodstream and the intracellular amastigotes in the muscle cells. The trypomastigotes do not multiply but disseminate the infection around the body of the mammalian host and seek to infect the vectors (large bloodsucking bugs). When the bug sucks the blood of the infected person, trypomastigotes get transformed into empimastigotes in the midgut. There they undergo division and slowly pass on to the hindgut and get transformed into small metacyclic trypomastigotes which are expelled along with faecal matter. Generally insects feed on sleeping persons. By chance if faecal matter is transferred by scratching or rubbing fingers on the eyes, the trypomastigotes penetrate the conjuctiva. Besides human beings, pet animals like cats, dogs, rodents, raccoons and opossums serve as reservoirs of human infection.

Figure 5.58 *Trypanosoma cruzi*

The metacyclic trypomastigotes enter the host cells, transfer into amastigotes and undergo division by binary fission until the cell ruptures. Before the rupture of the cell the trypomastigotes elongate, measure 20 μm with a sharply pointed posterior end and an anterior extension of the flagellum beyond the end of the cells. The pleomorphism noticed in *T. gambiense* is not so prominent as in *T. cruzi*. According to some investigators, two forms could be distinguished. Slender forms ready to reinvade host cells and broader forms which are infectious.

T. cruzi is widely distributed throughout South America and Central America and Southern USA.

Epidemiology

Transmission from man is by bug *Panstrongylus megistus.* When the bug bites a man its faeces containing the metacyclic trypanosomes are rubbed onto the wound. While biting, the arthropod defaecates and the punctured wound becomes contaminated with the faecal matter. In the bug, *T. cruzi* remains for about a week in the midgut after its bite. The bug becomes infective within 8–10 days after biting an infected host and remains infective for as long as 2 years (growing from larva to adult). Adults are the transmitting agents.

Pathogenesis

T. cruzi deposits semi-liquid faeces on the human skin especially near the eye or around the nares or lips. When the bug is taking the blood meal the metacyclic stages are engulfed by histocytes and start invading adipose cells of the simultaneous tissue and also muscle cells. They develop into leishmania forms in 3 days. The parasitized cells rupture on the fourth day and infiltration of leucocytes, monocytes and lymphocytes takes place resulting in the oedema of the area.

The leishmania forms get distributed through blood or lymph channels and enter the lymph nodes, lungs, spleen, liver and bone marrow where they multiply, muscle fibres, neuroglia, microglia of the brain cortex and adrenal cortex, thyroid gland, sex organs and intestinal mucosa. In these tissues they multiply and when large number are formed, they escape from the respiratory tissue cells into the bloodstream. They are found as trypanosome forms and this is followed by fever.

Trypanosoma rangeli

This parasite has been recorded in Columbia, Chile and Brazil, and measures 31 μm in length. These are longer than *T. cruzi*. Undulating membrane is broad, flagellum is anterior in position, at least half the length of the body. Nucleus is ovoid, blepharoplast is minute lying at the posterior terminus of the undulating membrane. Reproduction is by longitudinal fission. Peculiarly the parasite is non-pathogenic in vertebrate hosts and pathogenic in invertebrate hosts. In the bloodstream of humans it exhibits typical trypanosome form.

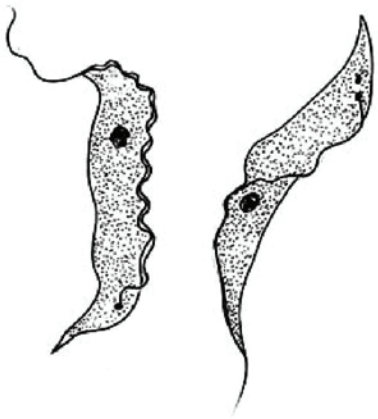


Figure 5.59 *Trypanosoma rangeli*

The forms seen in human beings are trypomastigotes which are longer than those of *T. cruzi*. It completes its development in the haemolymph and salivary glands. From now onwards it is transmitted to mammals by inoculation in the salivary glands of the bug. These are larger than those of *T. cruzi,* and *T. rangeli* is pathogenic in invertebrate host.

Pathogenesis

Infection leads to hyperplasia of the system. Liver enlarges, so also the spleen.

Leishmanial Parasites

Like trypanosomes these also have introduced two hosts, a vertebrate and an invertebrate, in their life cycle. In the vertebrate hosts they are intracellular amastigotes and in the invertebrate hosts they are promastigotes which are elongate motile flagellates, with a kinetoplast at the anterior end of the cell and nucleus in the middle. Amastigote as well as promastigotes divide by binary fission and sexual reproduction is absent.

Amastigote is intracellular parasite inhabiting the reticuloendothelial system. It is oval in shape measuring 1.5–5 μm, a nucleus and kinetoplast are present. As the vector sucks blood from humans it introduces the promastigote form, and thus infection starts. Then the parasite from the sites of bite enter the reticulo-endothelial system (Bone marrow, liver, and spleen).

Leishmania donovani

Leishmania donovani was first described by Leishman. It is ovoidal and 2–5 μm in diameter. Cytoplasm is vacuolated. When smears are stained with Leishman's, Giemsa or Wright's stains cytoplasm takes a blue shade, and the large oval nucleus a red shade. Cytoplasm is enclosed in a limiting membrane. At right angles to the nucleus is the kinetoplast which takes a purple shade. Kinetoplast has a parabasal body. From a dotlike blepharoplast arises the axonema and it extends up to the tip of the cell. There is an unstained vacuole lying at the side of the kinetoplast. Promastigote phase is spindle-shaped, 15–20 μm long with flagellum measuring 20–30 μm in length. Kinetoplast is at the anterior end, and nucleus is centrally placed at the base of the flagellum.

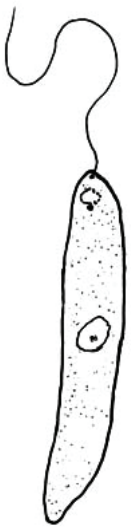


Figure 5.60 *Leishmania donovani*

The vector species of *Leishmania* which infect humans are sandflies of the genera *Phlebotomus* (in Africa, Asia and Europe), and *Lutzomyia* and *Psychodopygus* (in South and Central America). The leishmania species infecting humans and transmitted by mosquito can be divided into 3 groups.

1. *L. donovani* causing visceral leishmaniasis (kala-azar).
2. *L. tropica* causing cutaneous leishmaniasis (oriental sores, Baghdad boils wet and dry cutaneous sores)
3. *L. braziliensis* causing mucocutaneous leishmaniasis (espundia).

Life cycles After feeding, the sandflies ingest amastigotes which escape from the cell and transform into promastigotes which inhabit the insect's midgut. There they colonize. Ultimately promastigotes develop in the fly's hindgut and later spread forwards into the foregut of the vector and finally into the proboscis where the metacyclic promastigotes are formed.

When the sandfly feeds on blood, it transmits the infective promastigotes by bite. They enter the phagocytes and transform into amastigotes after phagocytosis by macrophages. There they multiply filling the cytoplasm of the macrophages. The infected cells burst, the parasites are released and again phagocytosed and the cycle repeats, producing lesions on the skin or vascular infection depending on the species of parasite and the host response.

Geographical distribution Visceral leishmaniasis also known as kala-azar is found in S. America, in tropical East Africa, Southern Europe and in parts of India and Central Asia. Cutaneous leishmaniasis is prevalent in Asia and tropical Africa.

North Africa vectors are species of *Phlebotomus* and reservoir hosts are rodents. In central and South America, cutaneous leishmaniasis is caused by a number of species which are yet to be well-defined. The African and Asian species and in South Africa the causative agent is *Leishmania braziliensis*.

Leishmania donovani the sole monarch of kala-azar, spreads from the site of bite and multiply in reticuloendothelial cells, especially the macrophages in spleen, lymph nodes, liver and the bone marrow. Symptoms are hyperplasia of the spleen, emaciation followed by weakness and irregular fever. Unnoticed and undiagnosed cases end fatally.

Epidemiology

Kala-azar is a dramatic disease. Transmission cycle is probably from humans to sandfly and to humans. A possible method of transmission is through the ingestion of food or drink contaminated with *L. donovani*. This parasite may be found in the faeces and urine of kala-azar patient and susceptible animals such as hampsters which eat the material containing the organism obtained from liver or spleen of such patients. While hampsters feeding upon such hampsters die of such infection.

Pathogenesis

When the leptomonas stage of the parasite is inoculated into the victim's skin by an infected sandfly, the nearby macrophages engulf the organisms which metamorphose into leishmania stage within the cytoplasm of the host cell. Here the leishmania multiply and remain quiescent for many weeks or months. Some of the parasitized macrophages are carried from the skin to the viscera where they lodge and parasites rapidly develop in the spleen, liver, bone marrow and other centres of the reticulo-endothelial activity, the leishmanias are taken up by fixed macrophages such as kupffer cells in the liver, multiply in these cells and destroy them.

Kala-azar bodies increase in number in the viscera, there is an increased but usually unsuccessful attempt at phagocytosis on the part of the macrophages. This results in excess production of macrophages with a corresponding depletion in the number of polymorphs, nuclear leucocytes causing at first monocytosis and neutropenia with granular cytopenia. The involvement of red bone marrow is responsible for diminished production of erythrocytes with ensuing anaemia. The neutropenia allows bacteria and other secondary invaders to attack the tissue without a defence on the part of the host.

L. tropica and *L. mexicana* create dermal lesions called oriental sores or Delhi boils at the site of bite by the sandfly. Due to proliferation of amastigote both intracellularly and spreading extracellularly causes penetration into the epidermis causing ulceration.

Epidemiology

Sandflies are the vectors of the disease. These sandflies are *Phlebotomus papatasii* and *P. sergenti.* Oriental sores are universal and at times several sores are produced by scratching the serum exuding from the primary sore. This may be spread to normal skin.

Pathogenesis When an infected sandfly bites and sucks the blood, the leptostoma stages of *Leishmania tropica* are introduced into the skin. The parasites are engulfed by local white cells. In macrophages the parasites round to become leishmania form, multiply and when macrophages rupture, leishmania forms are liberated and taken up by others in the vicinity.

L. braziliensis

This species causes monocutaneous lesions. The lesions grow slowly. From the site of inoculation, migration occurs rapidly to the non-pharyngeal region. Nasal sputum gets destroyed slowly. This parasite is almost similar morphologically to *L. donovani,* and *L. tropica*. It exhibits tissue cells and large mono membrane of the nose, mouth and pharynx. Life cycle is identical with *L. donovani* and *L. tropica*. It causes a serious diseases called espundia. The parasite is widespread in the jungles of Brazil, Peru and in South American States causing severe destruction of mucous membrane of mouth, nose and pharynx.

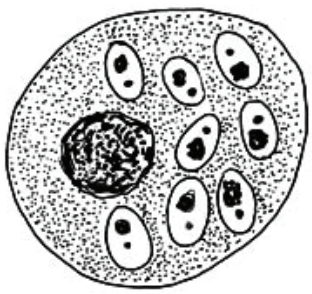


Figure 5.61 *Leishmania braziliensis*

Epidemiology

This disease assumes epidemic proportions when human beings in endemic foci go into the forests to cut bamboo or obtain forest products. Infections are common all through the year. Human inoculation occurs most commonly following high incidence of the appropriate species of *Phlebotomus.*

Pathogenesis

Following inoculation of the leptomonas stage of *L. braziliensis* by an infected sandfly, a small papilla first appears. This soon transforms into a red itching vesicle. Within 1–4 weeks the lesions begin to show ulceration often with round or oval contour but at times with ragged irregular edge, in either type with raised undulated margin. Histologically the elevated region shows epithelial hyperplasia and intense dermal inflammation with oedema.

Leishmania tropica

This parasite is almost similar to *L. donovani* and *L. braziliensis*. In humans it occurs in leishmania form and in sandfly in the leptomonas form. It is actually a parasite of the skin found in the endothelial cells of capillaries in lymph nodes with large mononuclear cells. It just starts as a pimple which slowly erupts and spreads. These eruptions are detectable on face, hands, feet, legs and so on. Life cycle is similar to that of *L. donovani*.

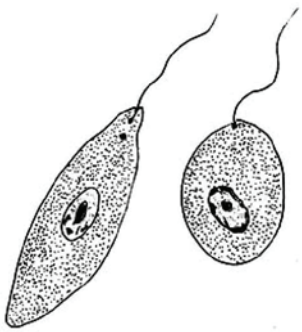


Figure 5.62 *Leishmania tropica*

Leishmania mexicana

Widely,distributed in central and South Africa especially in Mexico. It causes ear ulcers called chiclero ulcers. This infection may spread to viscera involving liver and spleen.

BLOOD SPOROZOANS

Malarial Parasites

Malaria is a very important parasitic disease which claimed importance in medical history. It is of serious public health importance. Though it has been eradicated to some extent in certain parts of the world, it still prevails in some remote places and interior villages. It is a widespread disease. At least 2.5 million people all over the world are affected. Africa is the worst affected country and at least 20 million people get affected every year.

This disease claims importance from time immemorial. Chills, intermittent fever and shivering was reported in olden days even in the 5th century BC. These gave a clear clinical picture and prevalence of this

disease. Even in olden days, Romans and Greeks could connect the prevalence of this disease to stagnant waters, and measures were taken to control the disease by going in for effective drainage. Actually the name malaria was given to this disease in the 18th century in Italy.

Later in late the 19th century, the causative agent for malaria was discovered by Lavern (1886), and Golgi described the asexual reproduction of the parasite in red blood cells (erythrocytic schizogony) and the life cycle was called 'Cycle of Golgi'. It was Ramanovsky who developed a method of staining of malarial parasite in 1891. Three species—*Plasmodium vivax, Plasmodium malariae* and *Plasmodium falciparum*—were reported to infect man and were described between 1889 and 1890 in Italy. In 1922 *Plasmodium ovale* was identified.

Transmission of disease was established in 1897 by Ronald Ross from Secunderabad, India. He studied the life cycle in detail in mosquito. This cycle sporogony was named after Ronald Ross as 'Cycle of Ross'. This has enabled to control and eradicate the disease.

Female *Anopheles* mosquito is responsible for transmission of this disease to human beings. Though male feeds on fruit juice, female requires a blood meal and two blood meals are required to lay the eggs. Bird malaria is transmitted by *Culex, Aedes* and other mosquitoes. For other mammals like monkeys, apes and rodents again *Anopheles* serves as vector.

Natural infection in birds is caused by several species of *Plasmodium*. Monkey malaria species are used for experimental studies like *P. cyanomolgi, P. inui, P. shortti* and *P. kowlesi. P. kowlesi* is a parasite of Rhesus monkey and this is supposed to infect aborigines in Malaysian jungles.

Life cycle Life cycle involves two stages, asexual life cycle (schizogony) in humans and sexual life cycle (sporogony) in female *Anopheles* mosquito. Schizogony takes place in two locations, in the red blood cells (erythrocytic cycle) and in the liver cells (exoerythrocytic cycle) which is a very important step before the parasite can invade erythrocytes. It is called pre-erythrocytic schizogony. The products of schizogony are merozoites.

The sexual phase takes place in the female *Anopheles* mosquito even though the sexual forms of the parasites originate in human red blood cells. Maturation and fertilization take place in the mosquito giving rise to a large number of sporozoites. So this multiplication stage is called sporogony.

So the life cycle of malarial parasite involves alternation of generations, i.e., asexual and sexual cycles occur alternately. This involves alternation of two hosts—asexual phase being completed in humans and sexual phase in the mosquito. Mosquito forms the definitive host and man is the intermediate host.

When female infected mosquito bites a person, he/she gets infected. Sporozoites which are the infective stages of the parasites are stored in the salivary glands of the mosquito. When mosquito sucks the blood these sporozoites are injected into the blood capillaries with its syringelike proboscis. The sporozoites get into the bloodstream where some of them are destroyed by phagocytes but some of them reach the liver and enter the hepatocytes.

Exoerythrocytic Stage

In an hour after their injection by the mosquito, the sporozoites reach the liver, enter the hepatocytes and initiate pre-erythrocytic schizogony. To start with, sporozoites are spindle-shaped, elongated bodies which

very soon assume a round shape inside the liver cell. They grow in size and undergo repeated nuclear divisions. Each daughter nucleus is surrounded by a bit of cytoplasm. This stage is called pre-erythrocytic or primary exo-erythrocytic schizont. With the growth of the schizont, the hepatocyte becomes distended and the nucleus is pushed to a side towards the periphery. There is no pigment in the liver schizont. In a fortnight, the schizont matures and bursts releasing thousands of merozoites which enter the bloodstream and infect the erythrocytes by a process of invagination. The duration of pre-erythrocytic phase in the liver, the size of mature schizont and the number of merozoites produced varies with the species of the parasite.

In *P.vivax* and *P.ovale,* some sporozoites after entering the hepatocytes remain inert in a stage called hypnozoites which are uninucleate forms with a size of 4–5 μm diameter. They remain for long during which period some hypnozoites are transformed into schizonts and release merozoites. These merozoites attack erythrocytes producing clinical relapse. This is the present concept as far as *P. ovale* and *P. vivax* are concerned, whereas in *P. falciparum* and *P. malariae* no hypnozoites are formed. But few erythrocytic parasites present in the bloodstream, multiply in sufficient numbers resulting in clinical disease. *P. malariae* infection becomes chronic and lasts for periods sometimes up to 40–50 years.

Erythrocytic Stage

The merozoites after release by the pre-erythrocytic schizont attack the blood cells. Merozoites are bean-shaped measuring 1.5 μm in length. They attack the erythrocytes by their apex. Merozoites enter the erythrocytes by endocytosis and the red cell membrane seals itself to form a vacuole enclosing the merozoites. It takes about 30 seconds for the entry into the red blood cells. Then the merozoite rounds up and loses its organelles. Now it has round shape with a vacuole in the centre, the cytoplasm being pushed to the periphery with nucleus at one pole. With Giemsa or Ramonovsky's stain, cytoplasm takes a blue shade, nucleus takes a red shade and the vacuole remains unstained. This differential staining gives the appearance of a ring shape to the parasite. At this stage it is called the signet ring stage or the ring form.

The parasite starts feeding on haemoglobin of the erythrocyte and instead of metabolizing the haemoglobin completely, it leaves a residue called malarial pigment. This pigment has iron, which accumulates in the body of the parasite as dark granules. As the parasite grows, these granules become prominent. When the parasitized cells rupture, this pigment is released and is taken up by reticulo-endothelial cells. Such pigment laden cells in the internal organs are an indication of previous malarial infection.

Ring form grows in size, assumes irregular shape and shows amoeboid movements. This is the amoeboid form. When this amoeboid form has reached a certain size, its nucleus starts dividing. The parasite within the erythrocyte is now called a trophozoite. The ring form is the early trophozoite and the amoeboid form the later trophozoite.

From the time the nucleus starts dividing, the parasite within the erythrocyte is called the schizont. To start with, the nucleus undergoes repeated division while the cytoplasm remains undivided. This is the early schizont. Then this is followed by cytoplasm bits enclosing each nucleus. This is the late schizont stage. In this mature schizont can be seen a number of merozoites each with a nucleus and surrounding cytoplasm. Now the schizont ruptures releasing merozoites into circulation. These

merozoites invade fresh erythrocytes where they undergo same type of development. This cycle is repeated a number of times till the host develops immune response.

The duration of the erythrocytic schizogony varies according to the species of the parasite. It is about 48 hrs in *P. vivax, P. falciparum* and *P. ovale* and 72 hrs in *P. malariae*. The periodicity is termed as tertian quartant.

P. vivax Benign, tertian (occurs after every 48 hrs) or every third day. It is more dangerous than *P. falciparum* malaria which is called malignant tertian.

P. falciparum Malignant tertian - cycles are not synchronized in a regular way. It is also pernicious malaria because of its lethal nature.

P. malariae Quartant malaria—occurs every fourth day as it has a cycle of 72 hrs.

P. ovale Tertian-tertian periodicity.

Gametogony

After a series of cycles of erythrocytic schizogony, some merozoites instead of becoming schizont, develop into gametocytes within the red blood cells. These gametocytes grow in size and fill the red blood cell but the nucleus remains undivided. Development of gametocytes usually takes place within the internal organ, spleen or bone marrow. Mature forms appear in peripheral blood. Mature gametocytes have a round shape except in *P. falciparum* where they are crescent-shaped. In the female gametocyte or the macro-gametocyte, the cytoplasm takes a pale blue shade and nucleus takes a pale shade. Nucleus is large. There are large pigment granules. Macro-gametocytes are more in number than micro-gametocytes.

Though these gametocytes are incapable of causing any illness they are very important for transmission of the infection. These gametocytes do not undergo further development in the vertebrate host. They must be again introduced into the mosquito host or else they die.

Life Cycle in Mosquito

When a female *Anopheles* mosquito sucks the blood along with parasitized erythrocytes, asexual forms of malarial parasite are digested but the gametocytes are set free in the stomach of the mosquito. The macro-gametocyte divides into 5–8 nuclei from each of which protrudes a long actively motile whiplike filament. These filaments are the male gametes or macro-gametes. They lash about for sometime and then break. This is called exflagellation.

The macro-gametocytes do not divide but undergo a process of maturation to become the macrogamete. It is fertilized by one of the microgamete and this results in the formation of a zygote. This process takes place within ½ to 2 hrs after the blood meal. To start with, the zygote is non-motile, round in shape, after which it elongates and within 24 hrs starts moving, assumes fusiform body fin with an apical complex anteriorly. This is called the ookiniete stage. This ookiniete penetrates the wall of the stomach and settles below the basement membrane. It assumes a round shape and measure 6–12 μm in diameter. This stage is called oocyst. So the stomach of mosquito has hundreds of oocysts.

The oocyst grows in size, and assumes a spherical shape measuring 6–12 μm in diameter. The oocyst matures and increases in size and the nucleus undergoes multiple divisions. This process is

sporogony where within the oocyst, thousands of sporozoites are produced. Each sporozoite measures 10–15 μm in length with centrally located nucleus and an internal apical complex. The oocyst ruptures and the sporozoites are released into the haemocoel. The sporozoites find their way into the salivary gland and finally into the salivary ducts. Now the mosquito is infective and when it sucks blood, the sporozoites are injected into the capillaries thereby initiating human infection.

Time taken for sporogony in the mosquito varies with species and also temperature conditions.

Morphological Features of Various Malarial Parasites

Plasmodium vivax

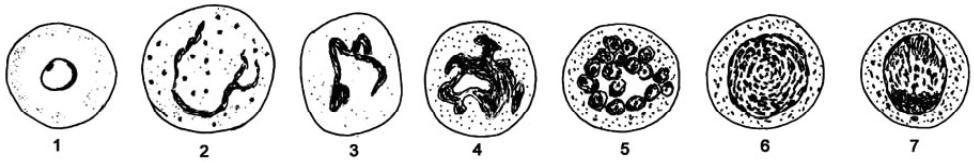


Figure 5.63 *Plasmodium vivax*, 1. Early trophozoite, 2. Late trophozoite with Schuffner's dots, 3. Late trophozoite with amoeboid cytoplasm, 4. Late trophozoite, 5. Mature schizont with 18 merozoites and compact pigment, 6. Micro-gametocyte with diffused chromatin, 7. Macro-gametocyte with compact chromatin

1. Parasitized blood cells are larger (1½ to 2) than the normal cell. They are oval to round in shape, and all infected cells have Schuffner's dots. Cytoplasm is pale, all developmental stages are present in peripheral blood cells.
2. In young trophozoite, the cell is almost occupied by the ring. Chromatin is healthy.
3. The growing trophozoite is an irregular amoeboid shape. Vacuole is still present. There is some increase in brown pigment.
4. Mature trophozoite is also irregular, amoeboid, occupies almost entire cell, fine brown pigment is present.
5. In schizont stage cytoplasm is in the form of bands, chromatin division occurs, pigment in clumps.
6. Macro-gametocyte is round or oval, with homogeneous cytoplasm, chromatin is compact, light brown pigment is present.
7. Micro-gametocyte has pink to purple chromatin which is surrounded by pale colourless pigment which is evenly distributed.

Plasmodium malariae

1. Size is normal. Schuffner's dots are absent. Cytoplasm is normal, ring stage is brief, growing and mature trophozoites and schizonts are present.
2. In young trophozoite ring is small and occupies 1/6th of the cell, chromatin is heavy like a dot. Pigmentation starts appearing.

3. Growing trophozoite is not amoeboid but band-shaped, chromatin is hidden by coarse dark brown pigment.
4. Mature trophozoite without vacuoles which disappear early, cytoplasm compact, oval or band-shaped almost filling the cell. Chromatin is marked by peripheral dark brown pigment.
5. Schizont is almost similar to that of *P. vivax*, dark large pigment granules distributed either peripherally or centrally.
6. Mature schizont with 8 merozoites in rosettes or irregular, clusters almost occupying the cell with the brown-green pigment centrally localized.
7. Macro-gametocytes almost similar to *P. vivax* with much darker and coarse pigment. Macro-gametocytes are fewer in number.
8. Micro-gametocyte is also similar to that of *P. vivax*, fewer in number, pigment is dark and more coarse.

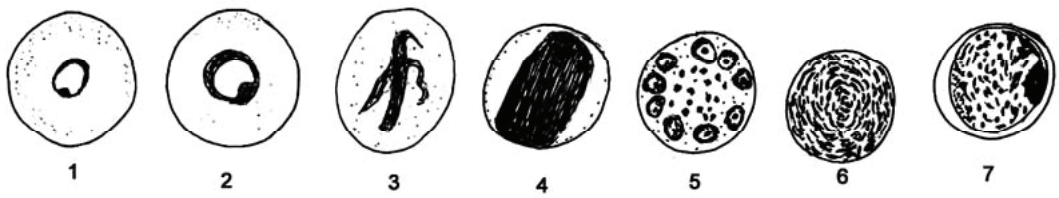


Figure 5.64 *Plasmodium malariae*, 1. Early trophozoite, 2. Early trophozoite with thick cytoplasm, 3. Early trophozoite with band form, 4. Late trophozoite with heavy pigment, 5. Mature schizont with merozoites (9) arranged in a rosette, 6. Micro-gametocyte with dispersed chromatin, 7. Macro-gametocyte with compact chromatin

Plasmodium falciparum

1. Parasitized blood cells are normal, no Schuffner's dots, rarely comma-shaped, red dots, the Mauer's dots, are present. Cytoplasm with bluish tinge. All developmental stages are not present. Young ring forms and few gametocytes are present.
2. Young trophozoite with a small ring with small chromatin dot. Small vacuoles are surrounded by scanty cytoplasm. Cell is with multiple rings.
3. Growing trophozoite with heavy ring with fine pigment granules.
4. Mature trophozoite is not seen in peripheral blood. All developmental stages after ring forms occur in capillaries of viscera.
5. Schizonts are not seen in peripheral blood.
6. Mature schizonts are not seen in peripheral blood.
7. Macro-gametocyte is crescent- or sausage-shaped, near the chromatin dot. Black pigment is centrally located.

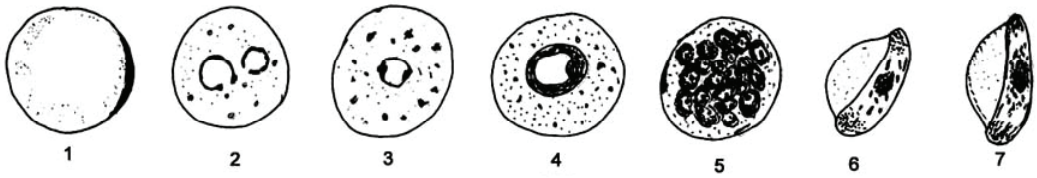


Figure 5.65 *Plasmodium falciparum*, 1. Early trophozoite, 2. Early trophozoite with one ring in the form of head phone, 3. Early trophozoite with Mauer's dots, 4. Late trophozoite with larger ring and Mauer's dots, 5. Mature schizont with merozoites, 6. Micro-gametocytes with dispersed chromatin, 7. Macro-gametocytes with compact chromatin

Plasmodium ovale

1. 60% of the parasitized cells are larger than normal, oval, 20% of them with irregular frayed edges. Schuffner's dots are present in all stages including ring forms. These dots are larger and darker. Cytoplasm is pale, all developmental stages are present in cells of peripheral blood.
2. In young trophozoite, the ring is large, amoeboid as in *P. vivax*.
3. The ring shape is maintained in growing trophozoite stage.
4. The ring shape is more compact than in *P. vivax*.
5. Mature schizont with 8 merozoites in rosettes or irregular clusters.
6. Schizont is smaller, more compact than in *P. vivax*.
7. Macro-gametocyte smaller than in *P. vivax*.
8. Micro-gametocyte smaller than in *P. vivax*.

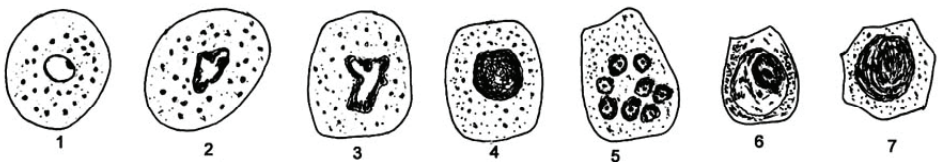


Figure 5.66 *Plasmodium ovale*, 1. Early trophozoite with Schuffner's dots, 2. Early trophozoite (note enlarged RBC), 3. Late trophozoite in RBC with fimbricated ridges, 4. Developing schizont with irregular shape, 5. Mature schizont with merozoites (8) arranged irregularly, 6. Micro-gametocytes with dispersed chromatin, 7. Macro-gametocytes with compact chromatin.

Blood Flukes—schistosomes

These parasites are also provided with oral sucker and acetabulum with the help of which the worm attaches itself to the walls of the blood vessels. The alimentary system has a mouth situated at the anterior

extremity, leading to the oral cavity which in turn leads to the oesophagus which then bifurcates. The two caecae unite posteriorly ending blindly. Anus is absent. They feed on the blood in which the worms live.

The excretory system consists of two longitudinal canals which open posteriorly and are led by connecting tubules. The function of flame cells is to filter the fluid wastes into the tubules with the help of the cilia they are provided with.

The nervous system is simple with an oesophageal ganglion and commissure encircling the oesophagus and two longitudinal nerve cords travelling to the posterior end.

The male reproductive system consists of testis lying in the dorsal median line posterior to the ventral sucker. A vas deferens arises from each testis which unite into a seminal vesicle near about the genital pore situated in the median line posterior to the ventral sucker.

Male worms are flat, leaf-like and folded to form the gynaecophoral canal into which the female worm gets enclosed almost its entire length. The female reproductive organ consists of an elongated ovary at the posterior end. The ovary leads into an oviduct which travels forward and joins the vitelline duct from the vitellaria and forms a common reproductive duct which travels forward and enters the ootype, a large egg-shaped chamber which receives the ducts of the Mehlis' gland. The ootype opens into the uterus anteriorly which passes forwards to open into the genital pore.

Life cycle Eggs are passed in urine as in *S. haematobium* or faeces as in *S.mansoni, S. japonicum, S. meckongi, S. intercalatum*. This is not a hard-and-fast rule, sometimes eggs of *S.haematobium* are passed in faeces and *S.mansoni* in urine.

When the egg is dropped into water, it contains fully embryonated miracidium. The miracidium is expelled from the egg and swims vigorously with its ciliated epidermis for 12 hrs and searches for a snail host. The miracidia are provided with sensory receptors to locate the snail host. The miracidia are also provided with an apical papilla, apical gland and pair of lateral glands, which help in penetrating into the snail tissues. When it comes across a suitable (most often) snail host, it develops into the next stage the mother sporocyst. The ciliated epidermis is cast off and a syncitial tegument develops with numerous microvilli.

Within the mother sporocyst, the germ cells give rise to daughter sporocysts which soon leave the mother sporocyst in about a week and migrate to the digestive gland of the snail and slowly develop into the next stage, the cercariae. Several thousands of cercariae develop. From the time the miracidium penetrates into the snail to the development of cercariae, the time lapse is 3–4 weeks for *S. intercalatum*, 4–5 weeks for *S.mansoni*, 5–6 weeks for *S.haematobium* and *S.meckongi* and 7 weeks for *S.japonicum*.

The mature cercariae come out of the sporocyst, escape from the host and swim about freely in water. It has a bifurcate tail, it measures less than 0.5 mm, it has an oral organ, a small acetabulum, a mouth, oesophagus, a pair of short caecae and an excretory system of flame cells with tubules and ducts leading to the excretory bladder situated at the posterior end of the body. It has 3 sets of glands, the head glands, the preacetabular (4) and post-acetabular (6) glands. The secretions of the post-acetabular glands help in attaching to the skin of the vertebrate host.

At a time, 2000–3000 cercariae are shed out from the snail per day. These cercariae are influenced by light gravity. They do not feed while swimming, depending on glycogen reserves. They swim to the

surface and sink from time to time. Their lifespan is short (48 hrs) and depends on external factors like temperature and turbulence. Within this period, they must come across a vertebrate host.

When it comes across a suitable definitive host, it penetrates, sheds off its trail and transforms into schistosomulum. It attaches to the skin for 2–4 days and migrates to the venous system and then migrates to the lungs. It transforms into longer and thinner wormlike structure and gets into the pulmonary vein, leaves the lungs and passes to the heart circulation they make several circuits and get into hepatic portal system. Within 25 days paired worms are found in liver and then they leave the liver. They must have mated by now and migrate to the veins of visceral plexus (*S. haematobium*), mesenteries veins in the case of *S.mansoni, S.japonicum, S.mekongi and S.intercalatum,* where they start laying eggs.

The time taken for the young ones to lay eggs from the time the cercariae penetrate is 30–50 days.

The female leaves the male and enters the finest vessel to deposit eggs which make their way from the venules into the tissues. Most of the eggs pass through the mucosa to be excreted in urine or faeces.

In the case of *S. haematobium,* the eggs reach the wall of the bladder but rarely into the rectum, those of *S. mansoni, S. meckongi, S. japonicum, S. intercalatum* reach the wall of the rectum. Some eggs of all species remain in the liver, genital tract, lungs, central nervous system and other organs.

The life cycle of all species of schistosomes infecting humans has a common route starting from sexual generation in the vascular system of the definitive host, asexual phase in the fresh water intermediate snail host till the cercariae return to the mucosa when humans are exposed to infected water and after passage through the tissues as schistosomes—developing into males and females. Their future growth takes place in the intra-hepatic vessels.

Intermediate hosts of *S. haematobium* is species of *Bulinus-Bulinus africanus,* of *S. intercalation* is *Bulinus forskah,* of *S.mansoni* is the snail of the genus *Biomphalaria,* and of *S. japonicum* is *Oncomlama hupensis.*

Epidemiology

Of all the parasitic infections in humans schistosomiasis is worldwide and widespread. It can be ranked second, malaria being first. It is very common in rural areas of developing countries.

Infections are mainly through human source. In some areas like Tanzania, wild baboons carry the infection and occasionally the rodent *Gerbil* can be infected (Egypt). Defaecation habits of humans contribute to epidemic. Migration of infected humans and extension of irrigation systems are routes of import of pathogenesis. This condition is similar to *S. japonicum*. Lesions produced by *S. mansoni* and *S. japonicum* are almost similar although in *S.mansoni,* the much smaller number of eggs delay the process of granuloma formation. In both the cases the organs and lesions most directly involved are the rectum and colon. The severity of the infection depends on worm burden. Three stages (1) incubation (2) egg deposition and expulsion (3) tissue proliferation and repair.

Dermatitis associated with cercarial penetration involves cellular infiltration of the area. Infiltrative changes in the liver leads to acute hepatitis. During the growth of the larva, hyperemia in the wall of the small intestine is noticed. Maturing of the worms occurs in the superior mesenteric venules. The eggs are sausage-shaped, chains accumulate in the smaller mesenteric veins. Anaemia, thrombosis of the mesenteries and the portal vessels, development of papillomalta from mucosa are the final pathological condition.

Schistosoma haematobium

Adults live in venous circulation mostly but sometimes in portal bloodstream. The male grows to a length of 10–15 mm in length and 0.8 to 1 mm in breadth. Entire body is covered over by tubercles and has as usual the oral sucker and acetabulum which is larger than the oral sucker. Body is unfolded to the posterior extremity to form the gynaecophoral canal which accommodates female during copulation and oviposition. Oesophagus is surrounded by a cluster of glands. The intestinal caecae run posteriorly to form a single trunk which ends bluntly. Testes lie behind the ventral sucker. From each testes arises a vas eferens, which enters into the common vas deferens which dilates to form vesicula seminalis which in turn opens through the genital pore behind the acetabulum. Prostate glands are lacking.

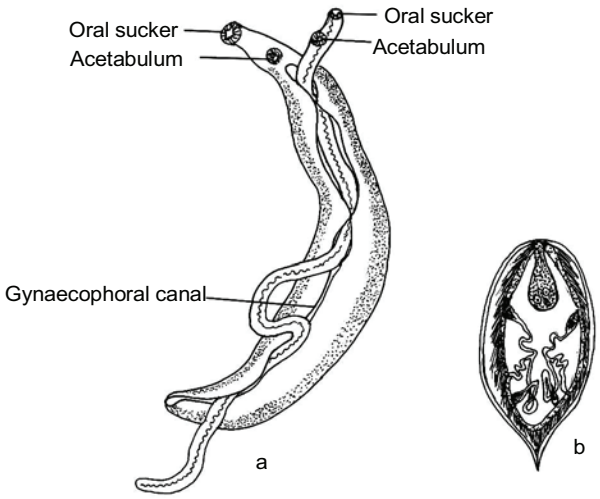


Figure 5.67 a—*Schistosoma haematobium*, b—Egg of *S. haematobium*

The female unlike male is long and slender and grows to a length of 20 0.25 mm. The ovary leads into oviduct which opens into ootype. The ova are fertilized in the ootype, the vitelline material is received from vitelline glands, the shell and then they are passed into the uterus.

Eggs already contain a miracidium inside by the time they are evacuated into the urine and stool. The egg breaks and passes through the mucosa into the lumen with adhering blood. The eggs pass out through urine. The eggs have a brown, transparent shell with a distinct terminal spine and measure 112 – 170 μm by 40–70 μm.

Miracidia comes out of the egg and leads a free swimming life and it must come in contact with an appropriate snail host within 4–8 weeks. Within this time it gives rise to first and second generations of sporocysts and finally into a forked cercariae. The molluscan host is *Bullinus truncatus*.

The cercaria has an ovoid body (140–240 μm × 57 100 μm) with a forked tail. It has an anterior sucker and small ventral sucker surrounded by spines. It has 5 pairs of penetration glands of which 2 are anterior loaded with granular material and 3 are posterior with homogeneous basophilic material. Secretion of the glands is released near the anterior dorsal margin of the oral sucker.

After escaping from the snail host the cercaria swim vigorously exhibiting sinking movements. When man accidentally takes a bath in infected (cercaria) water the cercaria come in contact with the skin. The tail is cast off within 24 hrs the cercaria penetrates the peripheral venules, get transported to the heart and then to the pulmonary capillaries, and finally to the systemic circulation and start the cycle.

Epidemiology

This can be considered as most significant not human host in some endemic foci, the entire population in some localities may show the infection. The expansion of irrigation facilities are ever increasing in the areas of infection. The Aswan Dam for instance has created facilities for the transport of the snails far and wide. Children are more frequently infected in certain endemic areas. Some snails are sewage feeders. All these ecological conditions fasten the infection in to epidemic proportions.

Pathogenesis

When cercaria penetrate the skin, little exuviate of the tissues may be caused. No extensive local damage occurs. The tail less larvae called schistosomulae reach the right side of the heart and then into pulmonary vessels. In the lungs little haemorrhage and leucocyte infiltration develops around the worm. Approximately three weeks after entry into the skin the young worms reach the interior mesenteric vein. The mature worms, (12 weeks old) begin to lay terminal spined egg. The spined egg escapes through perivascular testis into the lumen. Blood and necrotic cells accumulate. The wall of the urinary bladder is damaged primarily. Eggs which are not able to escape provoke the formation of abscess. Fibrosis may occur. Bacterial invasions may complicate the infection in the tissues. This type of urinary bilharziaris, chronic cystitis, generalized hyperplasma with purulent delire are common. Lesions may enter into form on the penis and even elephantiasis may result.

Schistosoma mansoni

These worms are almost similar to *S. haematobium.* Male is 6.4 to 12 mm in length and females 7.2–17 mm. Body is covered over by tentacles which are prominent. In male, the testis is 6.9 μm with minute sensory papillae and with tufts of hairs.

In female, the ovary is at the anterior end, the receptaculum seminis lies posterior to the ovary. Uterus is short and already contains eggs with characteristic lateral spines. The eggs break the venules, get into the mucosal layers of the intestine and escape into the lumen of the bowel and finally pass into faeces. The egg has a yellowish brown shell which is transparent with characteristic lateral spines. It measures 114–175 μm in length and 45–68 μm in breadth. The eggs are in mature stage when they are discharged. Miracidia escape from the egg and they possess penetration glands. A small primitive gut is present. Miracidia have the ability to locate the appropriate snail host which in this case is *B. glabarata*. In 4 weeks time the miracidia develops into one or two generations of sporocysts and finally cercaria. The cercaria resembles that of *S.japonicum*. It possesses 2 pairs of preacetabular and 3–4 pairs of post-acetabular penetration glands.

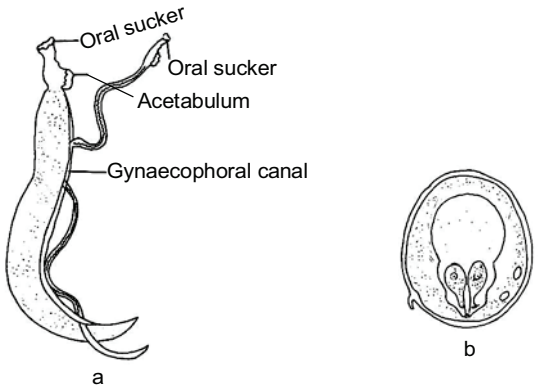


Figure 5.68 a—*Schistosoma mansoni*, b—Egg of *S. mansoni*

Schistosoma japonicum

It almost resembles *S. mansoni* and *S. haematobium*. Body is devoid of tubercles. Male measures 12–20 mm × 0.5–0.55 mm. Integument is covered over by minute spines which are more prominent at the gynaecophoral canal and suckers. Testes is 7 in number. Female is 26 mm × 0.3 mm and the body is covered by spines. Ovary is median and vitellaria are situated postero-laterally. Uterus is a long straight tube. Ootype is anterior in the mid-line. Eggs measure 67 × 50 μm. The eggs which are inside the superior mesenteric vein enter the small intestine. The ovi-posited eggs break the mucosa and sub-mucosa and get dropped into the lumen of the bowel.

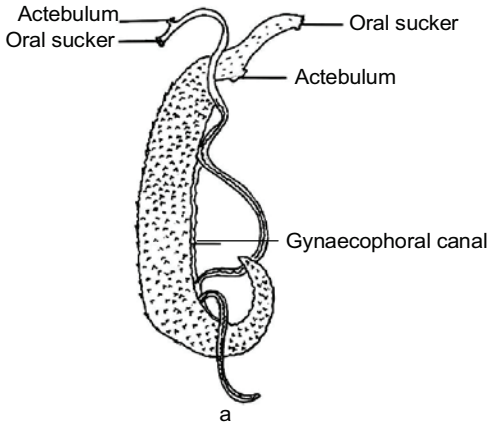


Figure 5.69 *a—Schistosoma japonicum*

The eggs pass along with faeces and under favourable conditions develop into miracidium which escapes out of the egg and swims about, and sheds its embryonic membrane. It should come in contact with an appropriate snail host, as in other species within 5–7 weeks it develops into first and second generation of sporocyst and then into forked cercaria. The cercaria come out of the snail. It measures 100–160 μm × 66 μm. This almost resembles that of *S. mansoni* and *S. japonicum*. It has 8 flame cells. When the cercaria comes in contact with the skin of mammals, the tail is cast off and within 24 hrs

migrates to the cutaneous capillaries, then to the venous circulation, from where it is carried to the right heart, lungs, then to the left heart and finally to the systemic circulation. Within 6 weeks the worms mate and females start laying eggs.

Blood Nematodes

Wuchereria bancrofti (Cobbold 1877, Seerat 1921)

It is a threadlike worm, white in colour and inhabits lymph glands and vessels. Sexes live coiled together and it is a bit difficult to separate them. Cuticle is smooth. Male measures 4 mm × 0.1 mm, coiled tail with two spicules. Spicule is grooved on the ventral side. Distal portion is whiplike ending in a hood and there are 15 pairs of minute sensory caudal papillae. In the posterior wall of the cloaca, there is a saddle-shaped thickening forming a shield. In *W. bancrofti* there is a characteristic accessory process. There are 12 pairs of circumoral papillae of which eight pairs are pre-anal and 4 pairs are post-anal. There are two pairs of large papillae which are sessile and at the tail a solitary pair of minute size.

The female measures 6.5 cm × 0.2–2.8 m. The anterior end is tapering with a round swelling. There are some sessile papillae on the head. The oral aperture leads to oesophagus which is cylindrical in shape. The tubular intestine is 1/5th of the total diameter and posteriorly opens into the rectum. The caudal end is narrow ending abruptly in a rounded fashion. Vulva is behind the anterior extremity. Vagina is swollen and leads into the uterus which divides into 2 coiled tubes occupying a major portion of the body and wider than the intestine. There is a pair of ovaries and oviducts.

Eggs are enclosed in a chorionic membrane, which forms a protective sheath to microfilariae. The female liberates the eggs which migrate to the bloodstream via the lymphatics. The microfilariae measure 216 μm. Within the egg the embryo lies curled up.

The microfilaria which measure 280 × 7 μm has no definite shape. Under high magnification, the microfilaria is enclosed in a sheath. Sheath is longer than the microfilaria. It takes a mauve shade with Giemsa stain. Within the sheath the microfilaria moves up and backwards and upwards. The nature of the sheath is the subject of controversy. The general opinion is that the sheath is nothing but an outstretched vitelline membrane but in certain other microfilaria, it is not the vitelline membrane but the sheath is developed during its sojourn in the blood. Somewhere in the middle third of the body, some kind of granular material is present which may be considered as the primitive gut. The whole body has transverse striations which could be considered as the muscular layer. At one-seventh of the length from the head there is a break, which could be considered as the nerve ring and at the fifth of the length there is a triangular 'V' shaped path or 'V' spot which is supposed to be the excretory cell and excretory pore. Anus is at the posterior end, a short distance from the tail, or cloaca and is called the posterior 'V' spot. When stained, some cells get deeply stained and they form the G_1–G_4 cells, the genital cells and the body appears to be fully packed with cells. A short fang shoots out at regular intervals from the uncovered cephalic end.

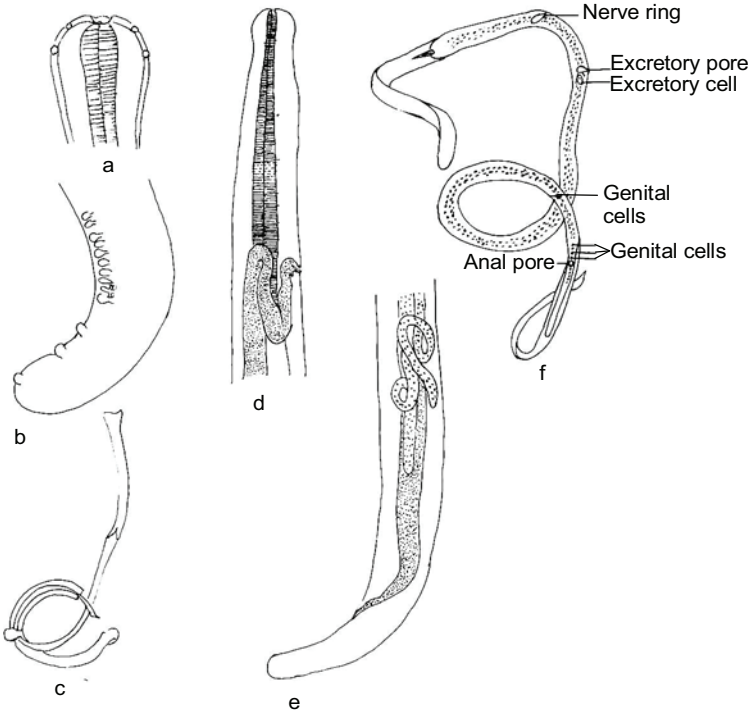


Figure 5.70 *Wuchereria brancrofti*, a—Anterior end, b—Posterior end, c—Spicules and gubernaculam, d—Anterior end of female, e—Posterior end of female, f—Microfilaria of *Wuchereria bancrofti*

Microfilaria pass through the peripheral capillaries. They are more active at night than during day.

Life cycle Microfilaria circulate in the bloodstream. In many Asian countries they exhibit nocturnal periodicity. They occur in large numbers in peripheral blood at night between 10 p.m. and 4 a.m. During daytime they are found in capillaries of the lungs, kidneys or in other great vessels. In certain countries like Malayan archipelago and Pacific Islands, they occur in peripheral circulation both at day and night with a peak period in the afternoon. The life cycle was first worked out by Mansoon in 1878 in China.

Life cycle in the mosquito The mosquito *Culex pipiens quinquefasciatus,* is the vector which transmits the organism from one human to another. Within an hour, after entering the mosquito, in the stomach the sheaths are cast off and they penetrate the stomach wall. While penetrating, there is a chance of the microfilaria being damaged by the buccopharyngeal armature of the mosquito. The embryos collect at the anterior end of the stomach, later migrate to the midgut.

Man is the definitive host. There is no reservoir host for *W. bancrofti*. The intermediate host is the female mosquito of the genus *Culex*. In India, the vector is *Culex pipiens quinquefasciatus*.

Further development of microfilariae depends upon their entry into the male *Culex* mosquito. If this does not happen the microfilaria die. The lifespan is about 2–3 months when *Culex* feeds on the

blood of infected humans, the sheaths are cast off, then penetrate the stomach wall and finally reach the thoracic muscles where further development takes place. After 12 days, they metamorphose into the 1st stage larva. It has a sausage shape with a tail and measure 225–325 × 15–30 μm. It moults twice a week grows considerably and develops into the second stage larva, which measures 225–325 × 15–30 μm. It develops further in a week, becomes elongated and all external structures are developed. Now it is the third stage larva. This is the filariform larva and measures 1500 –2000 × 5–28 μm. This is infective, active, motile larva. They migrate to the proboscis of the mosquito. Now it is ready to infect the human host when mosquito takes a bite.

Microfilaria does not develop further in the mosquito. From the time the microfilaria enters the mosquito, to the development of the infective stage in the proboscis, 10–20 days are required for the whole period. This period varies with environmental factors like humidity, temperature and also vector species.

When a mosquito with infective stages bites a person and takes a blood meal, it introduces microfilaria. The larva accumulates at the site of bite, penetrates the skin, enters the lymphatic vessels and is carried to lymph nodes where it develops into adult form either male or female. In about 6 months the larvae become sexually mature and mate.

A female releases as many as 2,50,000 microfilaria per day. They pass through the thoracic duct and pulmonary capillaries to the peripheral circulation. From the time microfilaria enter the human host till the appearance of microfilaria in circulation, it takes 8–12 months. This is the incubation period.

Pathogenesis

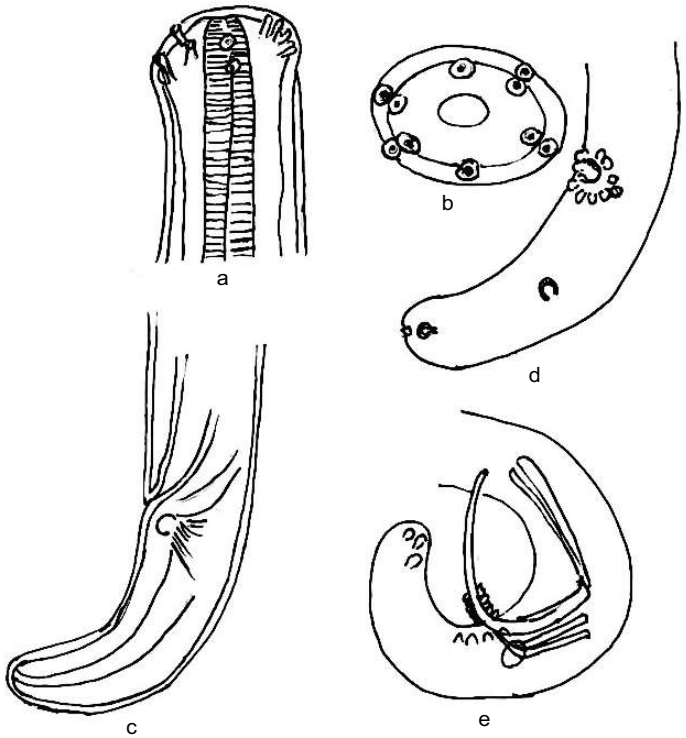
The typical manifestation of filariasis is due to blockage of lymph nodes and vessels. The affected lymph nodes and vessels show hyperplasia. The wall of the vessels get thickened and the lumen narrows. As a result the lymph vessels dilate and finally result in oedema or filariasis.

Brugia malayi (Brug, 1927)

This was named after Brug who in 1927 described a new type of microfilaria in Sumatrans and in 1940 Rao and Maplestone described it in India. It is worldwide in distribution occurring in India, Far East Indonesia, Philippines, Thailand, Malaysia, China, South Korea, Vietnam and Japan.

Adults are similar to *W. bancrofti* but smaller with a long cephalic space with double stylets, the tail carries two distinct nuclei. The female measures 52 mm in length × 160 μm. Vulva is at the anterior extremity with a caudal rounded end. Male measures 22–23 m in length and 88 μm in diameter. Anus is situated near about the posterior tip. A pair of papillae are present, one in front of the cloaca and another behind. A small gubernaculum is present with two spicules, one shorter and the other longer. The mosquito *Mansonia longipalpis* serves as the intermediate host.

Life cycle is similar to that of *W. bancrofti*. This microfilaria is also nocturnal, and development in mosquito is of shorter duration, 6–8 ½ days. The larva undergoes 2 moults in *Mansonia*. A buccal cavity is formed from cephalic space, oesophagus from the nuclei of the anterior part of the nuclear column and anus from four G cells. After the first moult, the tail with two nuclei is shed.



1,2,3,4—Gentail cells, Nr—Nerve ring, Ep—Excretory pore, Ec—Excretory cell

Figure 5.71 *Wuchereria bancrofti*, a—*Brugia malayi*—Anterior end of female, b—View of Head, c—Posterior end of female, d—Posterior end of male, e—Posterior end of male-lateral view

Loa loa

Loa loa commonly called eyeworm causes loiasis. It was first described in the eye of a patient in West Indies in 1770. It is limited to areas in forests of West and Central Africa.

Adult worms grow to a length of 30–7 mm and 0.3 to 0.5 mm in thickness. The worm is filiform, cylindrical, semitransparent and white in colour. Body is with protuberances which are translucent more in females. They do not exhibit any pattern in their distribution. In female they are distributed all through the length of the body whereas in male in the extremes they are lacking. Mouth is not guarded by lips or papillae one dorsally and other ventrally. The alimentary canal consists of a funnel-shaped mouth leading into a slender oesophagus, following into the intestine and into finally a short attenuated rectum.

The male measures 3–34 cm × 0.35 – 043 m tapering posteriorly to a tail which is curved ventrally with two lateral expansions of the cuticle. Somewhere in the middle, 0.08 mm from the tail, there is an orifice, the anogenital orifice with two unequal spicules surrounded by thick labia. There are 4 globular pedunculated arranged antero-posteriorly in order of their size, and a single post-anal papilla.

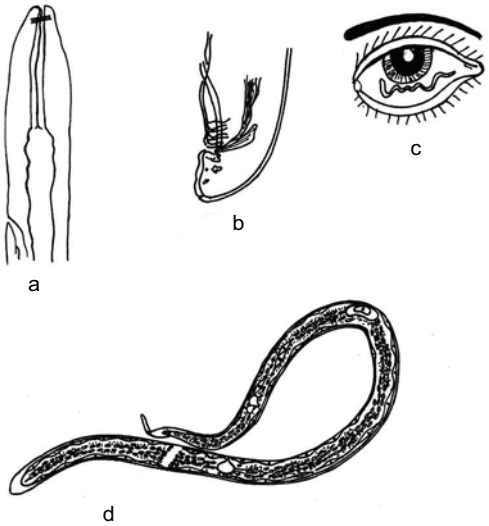


Figure 5.72 *Loa loa*, a—Anterior end, b—Tail of male showing spicules and papillae, c—*Loa loa* in eye, d—Microfilaria of *Loa loa*

Female grows to a length of 5–7 cm × 0.55 mm, is straight with rounded posterior extremity. Vulva is situated 2.5 mm below the anterior tip. It is situated on a small elevator. Vagina which is 9 mm long divides into two uterine tubes extending the length of the body. Ovaries are at the narrowed end full of eggs at all stages of development. Reproduction is ovoviviparous.

Life cycle Microfilariae resemble those of *W.bancrofti* in size and structure. Microfilariae are taken up by the fly *Chrysops* with the blood meal. It enters the stomach where the sheaths are cast off within 3 hrs and later penetrates the stomach wall, and enters the thoracic muscles. It takes 10 days to complete development in the body of *Chrysops*. During the first 3 days a torpedo shape, on fourth and fifth days, the worm lengthens and by sixth day it assumes the appearance of cork screw. Now it moults. Till now the tail was pointed. Now the shape changes to one with rounded trilobed posterior extremity. By tenth day it grows to a length of 2 × 0.025 mm and by this time two moults are over. The third stage is the infective stage moves forwards to the head region and get lodged in large numbers to be transported to proboscis. When *Chrysops* takes a blood meal, it is introducing these infective stages on the skin which they penetrate.

Epidemiology

Infections among humans is primarily among villagers and involves *Chrysops sihacea,* the dung fly.

Pathogenesis

Itchy oedematous swellings may appear and disappear periodically (calabar swellings).

Loa microfilaria encephalitis, penetration into brain, spinal cord may occur.

Onchocerca volvulus (Leuckard, 1893)

Onchocerca volvulus is found mainly in tropical Africa and to some extent in central and South America and to a little extent in Yemen and South Africa.

Adult worms are found in tumours in subcutaneous connective tissue of persons infected. Worms are white, filiform and opalescent. The head is rounded. The cuticle exhibits transverse ridges. The posterior end is curved. The male measures 2–4 cm × 0.2 mm, alimentary canal is a straight tube ending in anus. The tail ends up in a slight spiral with a bulbous tip. 2 pairs of pre-anal and 2 pairs of post anal papillae are present with an intermediate large papilla. Two unequal spicules protrude from the cloaca.

Females measure 60–70 cm × 0.4 mm. Head is round and tail is curved. The vulva is situated at the anterior end, striations found in male are not so prominent in female. In female the presence of two striations in the inner layer of the cuticle is a characteristic feature. It is ovoviviparous and the egg is provided with a striated shell with pointed processes at each pole. Usually the males outnumber the females—4 males and 2 females in each nodule.

The microfilariae are without sheaths and are seen in the fluid in the nodule cavity. The 'V' spot is present at the anterior end. The microfilariae are non-periodic. They are found in the skin in cervical lymph glands in humans and sometimes in blood and rarely in urine. They are also present in conjunctiva. These worms are associated with eye symptoms. Humans are the definitive hosts and black fly of the genus *Simulum* is the intermediate host. These flies are poor feeders and suck the blood and tissue fluids. The fly when it bites, extracts microfilaria from the upper layers of the skin. The microfilariae enter the stomach, penetrate its walls and then enter the thoracic muscles, where they undergo further development. Ecdysis takes place twice. On the seventh day the larva measures 0.65 mm. These larvae migrate to the proboscis and this is the infective third stage. When an infective *Simulum* bites a healthy person, infection is transmitted. The adult worm's lifespan in humans is 15 years. At the site of the bite, the microfilariae develop into adults.

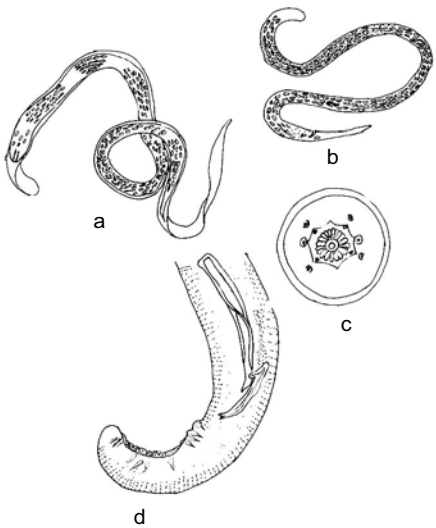


Figure 5.73 a—Microfilaria of *Brugia malayi*, b—Microfilaria of *Onchocerca volvulus*, c—View of Head, d—Posterior end of male lateral view

Pathogenesis

Microfilariae cause lesions in the skin and eyes. Ocular diseases are photophobia, blurring vision and finally total blindness.

Mansonella ozzardi (Manson, 1897)

It is mostly confined to South America and the West Indies. Adults are found in the peritoneal and pleural cavities of humans. The male measures 24–28.4 mm long 0.07–0.08 mm in diameter. One end is coiled and has 2 spicules and a caudal alae. The female measures 32.2–61.5 mm × 0.13–0.16 mm in diameter and has a vulva at the caudal end. Ovaries are highly coiled at the posterior end. Vagina leads into the uterus which is paired and occupies the major portion of the body. Adult worms live embedded in adipose tissues and in the mesentery. A sheath is lacking for microfilariae which measures 207–232 μm in diameter. The anterior end is round and tail is attenuated.

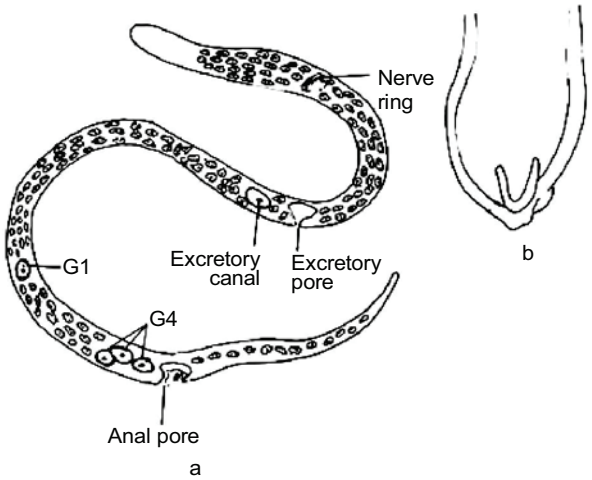


Figure 5.74 *a—Microfilaria of Mansonella ozzardi*, b—Posterior end of female, G1–G4—Genital cells

Life cycle The insect vectors are midges and *Simulum*. When vectors take a blood meal from infected host the microfilariae are ingested and they migrate to thoracic muscles in 24 hrs, then they moult twice and the third stage larva which is infective 90.7 mm in length. This larva migrates to the head region to reach the proboscis. All these changes occur in 8 days time, from the time the infective blood is ingested.

Epidemiology

This has been inadequately studied.

Pathogenesis

Adult worms produce little tissue reaction. Sometimes hydrocoele, enlarged lymph glands are the symptoms.

Dipetalonema perstans (Manson, 1891)

This worm is extremely distributed in tropical Africa and coastal South America. The adult worms live in body cavities especially in peritoneum, pleura and rarely in pericardium. Body is long and cylindrical, and a smooth mouth is lacking. In both the sexes the tail is peculiar in having a chitinous covering of the

extreme tip and it is split into two minute appendages. The female possesses four circular appendages at the posterior extremity.

The male measures 4.5 cm × 0.06 mm, and is as usual, smaller than female. The cloaca has four pairs of pre-anal and one pair of post-anal papillae and two spicules which are unequal in size.

The female measures 7–8 cm × 0.12 mm. Head is club-shaped and vulva is situated at the head end 1.2 cm from head. Anus is situated in the concavity formed by the tail.

Microfilaria is lacking a sheath, measures 200 × 4.5 μm. It can elongate and contract to any length. As a result measurement varies to a great extent. It is smaller than *W. bancrofti* and *Loa loa.* Caudal end is rounded 'V' spot is anterior, there is no tail spot, or granular mass, and it moves freely in blood. The worm does not show any periodicity.

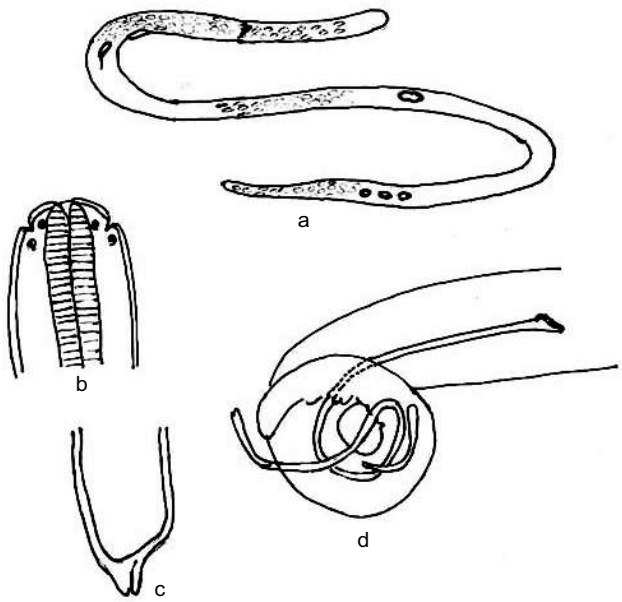


Figure 5.75 a—Microfilaria of *Dipetalonema perstans*, b—Anterior end of worm showing distinct papilla, c—Caudal end of female, d—Caudal end showing distinct papillae

Life cycle Vectors are insects of species of *Cuculoides.* Microfilaria are ingested along with blood meal. They penetrate the stomach and develop in thoracic muscles. Third stage larva comes out within 6–9 days. They are 0.7 mm long and are infective.

Dirofilaria immitis

These worms are creamy white in colour and slender. They measure 25–30 cm and the male is 12–15 cm in length. Oesophagus is divided into an anterior muscular and posterior granular parts. In

male the tail is spirally coiled and bursa is absent. Cloacal papillae are 4–6 pairs and there are spicules of unequal size. The ulva of the female opens in the anterior part of the body. Microfilariae measure 307–322 μm × 6.7–7.1 μm. In dogs these worms are found in connecting large vessels of the right heart.

Epidemiology

Human infection is due to the bite of *Cuculoides*.

Dipetalonema streptocercum

The microfilaria are without a sheath. Body is straight except at the posterior end which is strongly bent to form a curve. Length ranges from 180–240 μm and 3 μm diameter. The anterior extremity is bluntly rounded. Adult worms have not been detected in human hosts but believed to bear close resemblance to those described in the subcutaneous tissues of the chimpanzee.

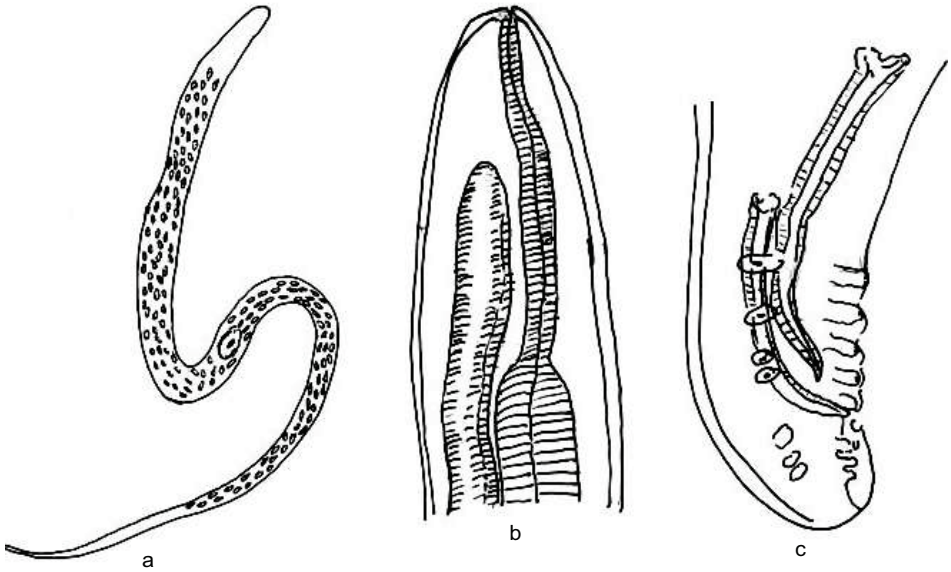


Figure 5.76 a—Microfilaria of *Dirofilaria immitis*, b—Anterior end of female, c—Posterior end of male

6

IMMUNITY

The principal constituent of the blood vascular system is the blood in vertebrates. Red blood corpuscles (RBC) or erythrocytes, white blood corpuscles (WBC) or leucocytes and platelets are suspended in the blood. RBCs play a role in conducting gases, WBCs establish a defence system and this prevents the invasion of the parasites and other harmful microbes.

Immunity can be defined as the activity which is related to the identification of the foreign proteins, i.e., antigens, and regulate or initiate the production of antibodies to fight against the invaded proteins, kill them and thus protect the organism from diseases.

Once a foreign body or organism enters the body, it acts as a foreign protein or antigen. In response to this, specific antibodies are produced in the body. Different types of antibodies are produced in response to different antigens. Three plasma proteins, albumin, fibrinogens and globulins are recognized. Globulins play a very pivotal role in the formation of antibodies. When a correct antibody is required, a number of them are produced. Organisms can eliminate the antigens and make them inert. Another important role played by antibodies is to help in gaining resistance against such organisms. This activity of establishing resistance against the disease-causing organisms is termed as immunity.

ACTIVE NATURAL IMMUNITY

Any pathogenic foreign organism entering the body is eliminated without any internal intervention. Temporary uneasiness and suffering will be developed but true pathogenicity is not established. Such protective system naturally occurs in human beings and as such this type of built-in resistance of the body in other words is known as the natural immunity. This is acquired by the influence of the environment in a natural way without any artificial means. Sometimes, natural immunity may be acquired by the non-supply of certain nutrients.

Natural susceptibility may be developed by artificial means. Bacon *et al.* (1951) have demonstrated that the purines are necessary for the growth of *Salmonella typhimurium*. They are unable to synthesize their own purines. When *S. typhimurium* was injected into mice, the mice were unable to show any susceptibility. *Para*-aminobenzoic acid (PABA) is important for the growth of *Plasmodium barghei,* a causative agent of malaria. Milk is deficient in PABA. When rats were fed with milk they became resistant to *P. berghei* and when PABA was supplemented in the milk they developed susceptibility.

All persons of a population having same genotype may not exhibit similar type of immune response. Similarly immunity exhibited in a particular environment at a particular time shows change in a changed place and environment. A person who is immune to a particular type of disease at a particular season may be immune to the same disease in the same environment.

Body secretions such as tears, enzymes, saliva, sweat, etc. also assist in preventing the entry of pathogenic organisms.

ACTIVE ARTIFICIAL IMMUNITY

When living organisms are exposed to X-rays and UV-rays, etc., they can be attenuated. Such attenuated organisms, toxoids, or dead organisms which have antigenic nature cannot act as antigens when they find entry into the blood of an individual. Antibodies are produced in response to antigenic stimulus and this helps in establishing immunity. Antibodies are produced at a lower rate. When the real pathogens enter at a lower rate in the initial stages, already existing antibodies kill them and at the same time stimulate their quick production. Such antibodies protect the organisms against that specific disease for a very long time.

ACQUIRED NATURAL IMMUNITY

When the foetus is developing in the uterus, antibodies which are already present in mother's blood enter the child either through placenta or through milk. So naturally infants acquire natural immunity through antibodies and fight against certain diseases. Such immunity is called acquired natural immunity.

ACQUIRED ARTIFICIAL IMMUNITY

This is the immunity established temporarily by way of drops or infecting antitoxins or immunoglobulins into the body of recipients, e.g., anti-tetanus serum, antidiphtheria serum, and immunoglobulins. As soon as they enter the body, immunity is developed quickly but lasts for a very short duration providing protection temporarily but body becomes non-immune. When antibodies disapppear, the body may need a booster dose.

PLASMA PROTEINS AND IMMUNOGLOBULIN

Three types of proteins namely albumins, globulins and fibrinogens are present in blood plasma. Plasma without fibrinogen is termed as "serum".

The following is the concentration of blood proteins:

Albumins	3.87–5.3 g / 100 ml
Globulins	1.8–3.6 g / 100 ml
Fibrinogens	0.2–0.4 g / 100 ml

Albumins These are synthesized in the liver and their lifespan is for 17–20 days. The major role played by albumins is the contribution of oncotic pressure of colloidal proteins. Their molecular diameter is much less than that of globulins and as a result more oncotic pressure is exerted. When albumin concentration falls below 2%, it will result in nephritic syndrome. Decrease of albumins in blood leads to such chronic condition like liver cirrhosis. Albumins carry calcium, bilirubin, free fatty acids, etc.

Fibrinogen is the least soluble of the plasma proteins. It is synthesized in the liver and has six polypeptide chains. Molecular weight is about 3,40,000. Each molecule of fibrinogen is fibre-shaped with negatively charged ends which contribute to water solubility as well as to rupture of the termini of other fibrinogen molecules and thus prevent aggregation.

Globulins They have a complicated structure, the molecular weight being 90,000 or above and as such they contribute to lesser oncotic pressure than albumins. In diseases related to kidneys, albumins are lost to a great extent than globulins. This is mostly because of filtration which is dependent upon molecular size. Globulins are categorized (at a pH level of 4.6) into three groups α-globulin β-globulin γ-globulin and at pH 8.6 the α-globulin are further classified into α_1-globulins and α_2-globulins. These two globulins are also called as glycoproteins and are produced in the liver. They contain carbohydrates. About 3% of β-globulins and 5% α-globulin carry lipids and are called lipoproteins. An increase in the level of β-globulin is noticed in persons suffering from pneumonia, typhoid, cholera and meningitis and increase in α-globulins is noticed in people with tuberculosis (pulmonary) and leprosy.

γ-globulins with a molecular weight of 1,56,000 were thought to be proteins and function as antibodies. But recent studies have shown that the term immunoglobulins has been applied to those proteins which show antibody activity. γ-globulins which do not show the antibody activity are associated with β-globulins and α-globulins. Unlike other proteins γ-globulins are not produced in the liver but are synthesized in plasma cells, lymploid tissues of thymus, reticular cells, spleen, lungs and bone marrow. If the level of γ-globulins increases, it is noticed in people with infective hepatitis, cirrhosis of liver, tuberculosis, Kala azar, malaria, etc.

Immunoglobulins are proteins. These function either as antibodies or chemically related antibodies. According to the World Health Organization (WHO), immunoglobulins must be proteins of animal origin and exhibit a common structure function as antibodies.

Immunoglobulins are glycoproteins composed of polypeptides (82.96%) and carbohydrates (4.18%). The phospholipid component has two light and two heavy chains connected by three covalent linkages and some non-covalent forces. The heavy chains have carbohydrate moiety. All four chains are governed by specific amino acid sequence. The light chains are of two distinct antigen types called the Kappa type and lambda type. These two types are seen for each class of immunoglobulin. The heavy chains occur as α, β, γ and ε types. The type of heavy chains make the immunoglobulin to be categorized into five classes, i.e., IgG, IgA, IgM, IgD and IgE. Each class is composed of thousands of antibodies.

The two heavy chains make these five classes of immunoglobulins to fall under two categories, i.e., IgG into IgG Kappa, IgG lambda type and IgA as IgA Kappa and IgA lambda and so on.

IgG It forms the major constituent, i.e., 75% of the total immunoglobulin. Another important character is that it is the only immunoglobulin that crosses the placenta and is capable of fixing the serum component. It is again categorized into four classes IgG_1, IgG_2, IgG_3 and IgG_4. IgG has four chains, two light and two heavy chains, linked by covalent disulphide bonds assuming the total shape of the molecule '**γ**'. Carbohydrate moiety is attached to one of the heavy chains. The half-life of IgG is about 23 days. Before destruction, it forms a complex with antigen and then undergoes phagocytosis in the reticuloendothelial cells. The structure of immunoglobulin can be studied by the cleavage (selective) of the molecule using pepsin or papain.

IgA These immunoglobulins are found prominently in the body secretions exposed to environment for example, saliva, tears, respiratory mucus, breast milk, gastro-intestinal secretions, urinogenital secretions, etc. IgA has four chained molecules (molecular weight is 1,70,000). Secretory IgA has two such units (chain molecule) and in addition a J chain (molecular weight is 25,000) and a secretory component.

IgM This immunoglobulin is found on the surface of the lymphoid cells. It makes its first appearance as an antibody phylogenetically. It readily responds to antigen injections. It plays a very important role in providing immunity against bacteria and viruses.

IgD Its functional aspect is not clearly known. But there are reports that IgD activity is the highest against certain antigens like insulin, milk proteins, diphtheria, thyroid, etc.

IgE The primary role is to develop allergy or hypersensitivity to certain antigens or foreign substances.

IMMUNE RESPONSE

Immune response can be termed as a response of living organisms to reject or attack the foreign unwanted or undesirable substances in the body. This is brought about by the effector cells, the important of which are leucocytes, plasma cells, macrophages found in the lymph nodes, spleen, thymus, tonsils and lymphoid follicles of gastro-intestinal tract. The types of leucocytes or WBC such as neutrophils, eosinophils, basophils, lymphocytes and monocytes cause the immune response by engulfing the foreign substance by phogocytosis or through the production of antibodies, where the plasma cells are derivatives of monocytes (B cells) which secrete the antibodies. The macrophages are derived from monocytes (T cells) to perform phagocytosis. Though this structure varies from place to place, they have a common distinctive feature, that is, the presence of numerous cytoplasmic granules to ingest the foreign substance.

When an antigen enters the body, the response against it is called the primary response. When there is subsequent entry of the same antigen, the response is called the secondary response. The time gap between the primary and secondary response is 3–10 days, i.e., entry of the antigen and production of the antibody. This gap period is the latent period when the antibodies are in large numbers but perish in two or three days. These antibodies are immunoglobulin M type (IgM). During the second dose of antigens which is called the booster dose, the antibodies are produced in great numbers to protect the

organism from foreign invading organisms. This belongs to the IgG group. This group of antibodies function for long periods hence a strong immunity is developed.

The harmful effects produced by antigens are readily responded by antibodies and this protects the individual. Once the antigens find entry, they stimulate the formation of antibodies to eliminate themselves from the body and thus a temporary immunity is developed.

Lymphocytes (one of the variations of WBC) play a vital role for the establishment of such immune response. These cells are formed from the primordial stem cells of the bone marrow. These cells make entry into thymus and smaller lymphocytes are transported to spleen lymph nodes and body cells.

In humans, thymus gland is a bilobed structure lying between the heart and the thyroid gland, and the lymphocytes passing through thymus gland come out as T cells. Other cells passing through other glands are called β cells. T cells by themselves cannot produce antibodies but influence β cells to regulate the production of antibodies.

This phenomenon is called CMI or cell-mediated immunity. All antigens that enter the body are recognized by T and B cells but the latter cannot identify the antigens entering directly into the circulatory system. B cells perish in 5–7 days whereas T cells last for one year.

To a large extent the antigens are distracted by macrophages, monocytes and any remaining are disintegrated and removed by T and B cell system. Based on the specificity of antigens, two types of immune system has been recognized in man.

1. Cell-mediated immunity (CMI)
2. Antibody-mediated immunity (AMI)

Cell-mediated Immunity

The mediator is T cell. Fungal cells, viruses, protozoa and bacteria stimulate T lymphocytes, find entry, into the body and reach the located areas of the parasite and drive them out through the circulatory system. At the sight of antigens, the T cells change the immature lymphoblasts and produce several low molecular proteins, the lymphokines, which can be further classified into three types (a) Effectors cells, (b) regulatory cells, and (c) memory cells.

Effector cells They are actually involved in providing immunity, these in turn are divided into (i) killer cells (ii) delayed hypersensitivity lymphocytes.

i. **Killer cells** They get attached with the antigen cell damage, the plasma membrane, thus leading to the lysis of the cell.

ii. **Delayed hypersensitivity lymphocytes** These lymphocytes cannot directly attack the antigens but they release lymphokines which hasten the phagocyte activity and thus establish immunity. Six types of lymphocytes are released from these cells.

   a. **Migratory inhibition factor (MIF)** helps in macrophage movements to be reduced and thus the macrophages cluster around T cell and activate them.

   b. **Chemotactic factor (CF)** Through this, chemicals are released which attract the macrophage cells to the place of antigens.

c. **Macrophage-activating factor (MAF)** Their main role is to activate the macrophage cells containing large number of lysosomes and quicken phogocytosis.
d. **Specific macrophage arming factor (SMAF)** The phagocyte capacity of macrophage is enhanced to destroy the identified and specific antigens.
e. **Lymphotoxins** Unspecified pathogens are destroyed by lymphotoxins.
f. **Immune interferon** This hastens the lytic activity of F cells and thus inhibits the synthesis of antiviral proteins in neighbouring cells.

Regulatory cells As the name indicates, they regulate the T lymphocytes which in turn coordinate the AMI and CMI by regressing the intensity of resistance.

These regulatory cells are of two types:

a. Hyper T cells which assist the B cells to initiate the activity of antibody.
b. Superior T cells which suppress the immune reactions in the host. They directly intervene with the activity of B cells.

Memory cells Once the antigens are eliminated from the body they remain passive. Subsequent entry of antigens is immediately recognized and antibody production is promoted. The antibodies are produced in large numbers so that the antigens are eliminated at the earliest. The memory cells establish long-term immunity.

Antibody-Mediated immunity (AMI) or Humoral immunity (HI)

B lymphocytes play an important role in this type of immunity. These cells release antibodies into the body fluids and thus antigenic activity is inhibited. When B cells are released from the bone marrow, they come in contact with antigens and grow into large blast cells. They divide and form small cells (micro cells) which attack the antigens through the production of antibodies. Hence they are called immuno-competent cells. The micro cells are called plasma cells. Immune-response genes regulate the production and function of these cells. Antibody production is at a very fast rate, thousands being produced per second under the influence of genes till the antigenic activity is completely damaged.

After few days, plasma cells perish and a second generation is produced to replace them. These are B cells with memory function, and their lifespan is quite long in lymph nodes and they regulate the production of antibodies again through the production of plasma cells.

Natural Killer Cells—Interferons

T and B cells play a major role in establishing immunity. Release of interferons from viruses produces killer cells and regulate the action of immune interferons. These interferons kill viruses and virus-infected cells.

Antigen–Antibody Interactions

Antigens are solely responsible for the stimulation in the formation of antibodies. Former opinion was that γ-globulins play a great role in establishing immunity. But the recent opinion is that IgG, IgA, IgM, IgD and IgE are solely responsible for providing resistance against diseases in humans. As already stated,

every globulin occurs in two states K and L types. Each type possesses two heavy chains of γ and β type with a molecular weight of 12,000 and heavy chain of H or A type with a molecular weight of 55,000. Specific antibodies are formed by the stimulation of antigens. This antigen–antibody interaction resemble lock and key arrangement. Antigen–antibody interactions fall under five types.

1. ***Agglutinations*** Antibodies which are bivalent and trivalent types bind with insoluble microscopic antibodies and form insoluble clumps of cells during agglutination. This activity occurs in a very short time. Phagocytes can easily identify and engulf them, e.g., clumping of cells in blood transfusion.
2. ***Precipitation*** Antigens which are soluble interact with antibodies and thus an insoluble precipitate is formed. This procedure is slow and hence it is not visible. T lymphocytes help in establishing immunity by bringing out an equilibrium between antigens and antibodies.
3. ***Lysis*** When bacteria and parasitic organisms (pathogenic) gain entry into the body, antibodies are immediately formed at specific sites and plasma membranes disintegrate by the formation of complement system. This is the first step in the lysis of the pathogen. This is one method by which the distribution and differentiation of gram-negative bacteria is stopped by antibodies. There are eleven factors proposed by Jowles Bordo (1895) which play a role in lysis. These factors are C_1, C_2 – e.g., these C factors are contributed by IgG and IgM. The proteins of C factors damage the cell membrane of the pathogen much before antigen–antibody interaction. This is the lysed membrane and cytoplasm flows out and leads to the degeneration of the pathogen. The lysed cells are sent out through the capillaries whose permeability increases.
4. ***Opsonization*** Opsonins are formed by proteins of C_3, C_5 and some antibodies. These facilitate to identify antigens immediately by way of F phagocytes and phagocytosis is accelerated. Opsonization is nothing but phagocytosis which takes place under the activity of opsonization. A stimulus from opsonins is released by the histamines from WBC and as a result the permeability of blood capillaries and contraction of smooth muscles are increased. An acute antimicrobial inflammatory reference is established from the complementary system.
5. ***Neutralization*** Antitoxins released from antibodies neutralize toxins released from antigens. The harmful effect is controlled and antigens are made inactive.

IMMUNOLOGICAL TECHNIQUES

Serology is the diagnosis of a disease through antigen–antibody interaction. When people are attacked by a pathogen, the antibody quantity will be considerably great. By conducting certain tests the disease may be confirmed. There are quite a good number of immunological tests for the diagnosis and to take preventive measures against the parasites in latent state inside the host.

7

MICROORGANISMS

BACTERIA

Bacteria are very small organisms ranging between 0.5 and 1.0 μm and could be distinguished and diagnosed by differences in shape, certain morphological features and their staining affinities to different stains. They react to single dye, mixed dyes and polychromed dyes.

Most bacteria have one of three shapes. They may be spherical (cocci), rod-shaped (bacilli) or spiral (spirilla). Some may occur as short rods referred to as coccobacillus. A few others possess different shapes. Square cells were also reported from the salty pool of Senai peninsula in Egypt in 1980.

Thus bacteria could be classified into (Figure 7.1)

1. Cocci - Which are spherical or oval
2. Bacilli - Rod shaped
3. Vibrios - Comma-shaped, curved rods, exhibiting vibrating motility
4. Spirilla - Rigid spiral forms
5. Spirochetes - Spiral-shaped
6. Mycoplasmas - Without cell wall hence lack stable morphology. They occur as round or oval with interlocking filaments.

Pathogenic bacteria may be classified on the basis of

1. Staining reactions (gram-positive, gram-negative and acid-fast staining)
2. Morphological features (cocci, bacilli, coccobacilli)
3. Growth conditions (aerobic and anaerobic)
4. Biochemical reactions

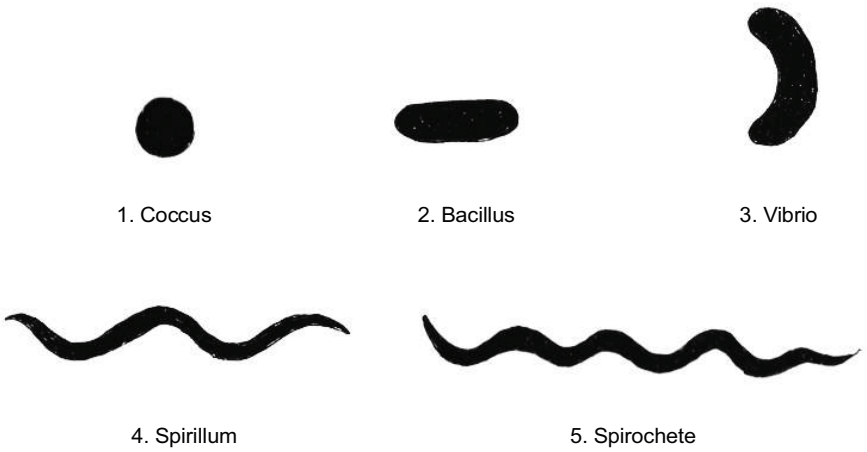


Figure 7.1 Shapes of bacteria

Gross Classification of Pathogenic Bacteria

Gram-positive

Cocci (aerobic)	*Streptococcus pyogenes*
	Streptococcus pneumoniae
	Staphylococcus aureus
	Staphylococcus epidermitidis
Bacilli (aerobic)	
i. Spore formers	*Bacillus anthracis*
ii. Non-spore formers (anaerobic)	*Corynebacterium diphtheriae*
i. Spore formers	*Clostridium tetani*
	Clostridium botulinum
	Clostridium perfringens
ii. Non-spore formers	*Lactobacillus*

Gram-negative

Cocci	*Neisseria gonorrhoeae*
	Neisseria meningitidis
	Neisseria catarrhalis

Bacilli

i.	Aerobic	
	a) Fermenters	Enterobacteriaceae
	b) Non-fermenters	Pseudomonas
ii.	Anaerobic	Bacteroidis
Coccobacilli (aerobic)		*Bordetella pertussis*
		Haemophilus influenzae
		Brucella abortus
		Pasteurella haemolytica
Acid-fast bacteria		*Mycobacterium tuberculosis*
		Mycobacterium leprae

Others

i.	Spirochetes (stain with aniline dyes)	*Treponema*
		Borrelia and *Leptospira*
ii.	Filamentous bacteria	
	a) filamentous weak acid fast	*Nocardia asteroides*
	b) aerobic and gram-positive	*Actinomycetes irraelli*

Bacteria occur as independent cells attached to one another. Division is by binary fission. The resulting cells each lead an independent life of all other cells. The place of division of bacteria plays a major role in their arrangement. If bacteria divide in one plane, it results in chains, while those dividing in several planes form clusters.

Cocci are arranged in several characteristic arrangements(Figure 7.2) depending on the plane of division and cocci are arranged in patterns whereas bacilli (Figure 7.3) occur singly or in pairs and some form chains and long branched multinucleate filaments called hyphae. The bacteria that form long chains are termed as streptococci, those which occur as two cells and bridged together are referred to as diplococci and those which occur in clusters are called staphylococci. Still others form cuboidal pockets with eight or more cells. Long branched multinucleated filaments are seen in *Streptomyces*. Curved bacteria have a twist (Figure 7.4). The single twist gives them vibroid shape. For example, spirilla are rigid helical bacteria. Some bacteria are pear-shaped (*Pasteurella*), lobed spheres *Sulfolobus* rods with square ends *Bacillus anthracis,* some discs arranged like stacks of coins *Caryophanon* and rods with sculptured (helically) surfaces *Seliferia*.

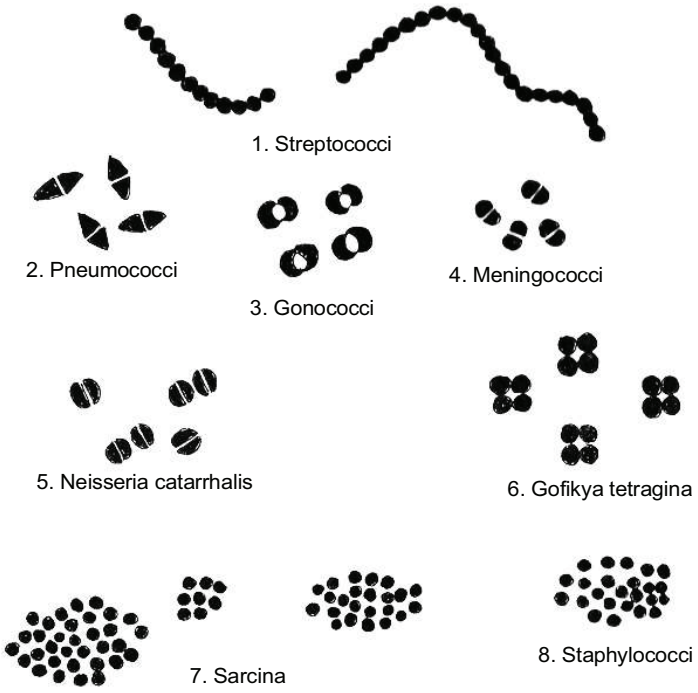


Figure 7.2 Arrangement of cocci

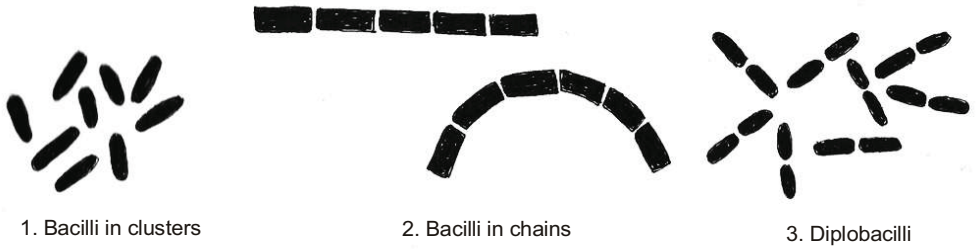


Figure 7.3 Arrangement of bacilli

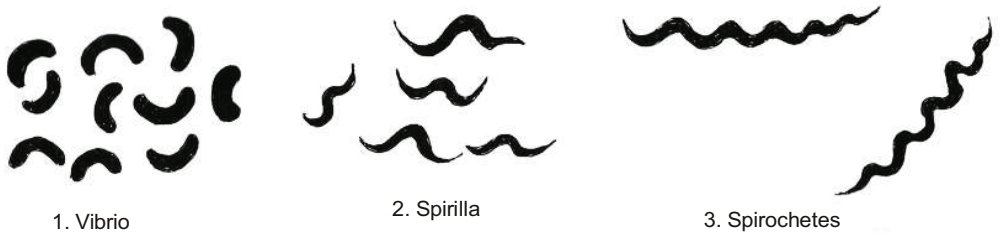


Figure 7.4 Arrangement of curved bacteria

Cell Wall

The bacterial cell wall has an outer cell envelope which has two components, a rigid cell wall and below it plasma memberane and cytoplasm. Protoplasm comprises the cytoplasm, cytoplasmic inclusions like ribosomes, mesosomes, granules, vacuoles and the nuclear body. Some bacteria have a third layer, the capsule or the slime layer. All these layers are collectively called as cell envelopes.

The bacterial cell wall is composed of sub-units found no where else in nature. The cell wall and component parts may produce symptoms of disease. The cell wall is the site of action of some of the effective antibodies. Some bacteria possess some additional structures, they have filamentous appendages which protrude from cell surface. The flagellae are for locomotion.

Staining procedures for bacteria The chemical composition of the wall determines the gram-staining properties of the cell. The shape of the organism is also determined by the cell wall, for example cylindrical shape is due to cylindrical wall and spherical wall gives the organism a spherical shape.

As already mentioned different bacteria react differently when treated with the same dye. Based on this feature most bacteria are separated into two groups, the gram-positive and the gram-negative.

Gram-positive bacteria when treated with crystal violet retain the stain after they are treated with iodine and washed with alcohol, whereas in gram-negative bacteria, alcohol washes the crystal violet–iodine complex. Apparently the dye–iodine complex becomes trapped in alcohol in gram-positive bacteria and on the other hand the cell wall is permeable in gram-negative bacteria. Flagellum is the major organ for movement. Flagella are frequently arranged. In some bacteria, there is single flagellum. Others have tufts. In still others along the sides of the body, flagellae are inserted at many points. This flagellar arrangement has a great bearing in the identification of bacteria.

As it is difficult to observe the transparent rapidly moving organism under the light microscope, cells are first killed and later treated with dyes. The entire organism or some parts stain prominently in contrast to the unstained parts.

First a drop of liquid containing the organism is placed on a slide and fixed to the slide by passing slide over a flame. The organism gets fixed and is later treated with the dye.

A number of staining procedures are in vogue. Some dyes will stain a particular component and some will stain only a particular group of organisms, thus making it easy to classify the organisms based on their staining characteristics.

There are two varieties of stains which colour the components and have a strong affinity for the dyes or vice-versa. The negative stains never penetrate the cell and as such it is highly visible with a contrasting dark background. Negative stains are used to demonstrate surface structures which are not stained by positive stains.

The dye methylene blue is usually used on fixed bacteria. The dye stains the entire cell blue without staining the background material. Methylene blue is a basic dye (with a positive charge) that strongly binds the nucleic acids DNA and RNA of the cells (which have negative charge). Internal structures are not visible.

On the other hand, acidic dyes (having negative charges) such as eosin, acid fuchsin and Congo red, stain basic compounds in the cell especially proteins carrying positive charges (composed of amino acids). Sudan black B is used to localize fat droplets in bacteria.

Gram's iodine

Reagents required

Crystal violet
Ethanol
Ammonium oxalate
Iodine
Potassium iodide
Absolute alcohol
Acetone
Safranin

Preparation of reagents

Solution 1 Crystal violet stain

Solution A

Crystal violet	2.0 g
95 per cent alcohol	10 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix solution A and B and store for 24 hrs. Filter it.

Solution 2

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Dissolve potassium iodide in water and then add iodine.

Solution 3 Decolorizer

Absolute alcohol	250 ml
Acetone	250 ml

Mix them and store.

Solution 4

Safranin O	0.34 g
Absolute alcohol	10.0 ml
Distilled water	90.0 ml

Grind the dye in alcohol and then add water.

Procedure

1. Fix bacteria or smear by passing the slide over a flame.
2. Cover the slide with solution 1 (crystal violet).
3. Pour off the stain; flood the slide with solution 2.
4. Drain off iodine and rinse in water.
5. Decolorize in solution 3 for 3–5 sec.
6. Flood the slide with solution 4 for 4–10 sec.
7. Drain off and allow it to dry.

This differential behaviour of bacteria in taking the stain is attributed to the chemical structure of the cell wall. The cell wall of gram-positive bacteria is different from that of gram-negative bacteria.

Gram-positive bacteria

Staphylococcus spp.

Streptococcus spp.

Pneumococcus spp.

Corynebacterium spp.

Anthrax spp.

Clostridium spp.

Gram-negative bacteria

Neisseria spp.

Enterobacteriaceae

Pseudomonas spp.

Vibrio spp.

Haemophilus spp.

Bordetella spp.

Brucella spp.

Pasteurella spp.

Acid-fast stain is also very popular especially in diagnostic laboratories engaged in detecting *Mycobacterium.*

Zeihl Neelsen acid-fast stain

Reagents required

Basic fuchsin

95 per cent alcohol

Phenol

Hydrochloric acid

Methylene blue

Preparation

Solution 1 Carbol fuchsin solution

Solution A

Basic fuchsin	3 g
95 per cent alcohol	100 ml

Dissolve the dye in alcohol and gently heat it.

Solution B

Phenol	10 g
Distilled water	200 ml

Mix phenol in water and heat up to 56°C.

Solution C

Mix 10 ml of solution A with 90 ml of solution B. Filter it.

Solution 2 Acid alcohol solution

Conc. HCl	3 ml
Absolute alcohol	97.0 ml

Solution 3 Methylene blue

Methylene blue	0.3 g
Distilled water	100 ml

Dissolve and filter.

Procedure

1. Prepare a smear and heat it over a flame.
2. Flood the slide with solution 1 (solution C) for five min.
3. Rinse in water.
4. Decolorize it with solution 2 for 3 min.
5. Wash in running water.
6. Counterstain with solution 3 for 10 min.
7. Wash, blot-dry. This is followed by gentle heating of the slide.

The acid-fast nature is mainly due to the presence of mycolic acid (lipids) in their cell walls. This type of bacteria are species under the genus *Mycobacterium,* which are causative agents of tuberculosis and leprosy. The lipid does not permit Gram stain to penetrate the cell wall.

SOME IMPORTANT BACTERIA

Staphylococcus aureus

These are spherical in shape measuring 1 μm in diameter and the characteristic feature is their arrangement in clusters resembling grape bunches. Sometimes they may be found singly or in pairs or in the form of chains having 3 or 4 cells. No motility, or capsule formation is seen and they are also non-sporing.

Almost 30% of the people have staphylococci in the nose. Staphylococci may also come from infected domestic animals such as cows. Mode of transmission may be by direct contact.

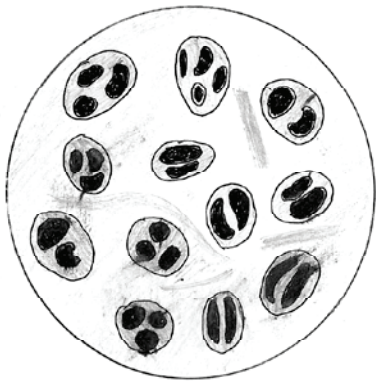


Figure 7.5 *Staphylococcus aureus*

Streptococcus pyogenes

These are spherical to oval, measuring 0.5 to 1.0 μm in diameter. These are arranged in chains due to cocci dividing in one plane only and daughter cells fail to separate. Length of the chains forms an important character in classification. These are also non-motile and non-sporing but capsulated. *Streptococcus* is responsible for respiratory infections, sore throat in tonsilitis, scarlet fever which was once very prevalent in colder countries, skin infections such as erysipelas and impetigo.

Figure 7.6 *Streptococcus pyogenes*

Pneumococcus

Small elongated cocci with one broad end, one narrow end giving lanceolate appearance. They occur in pairs with broad ends in opposition. They are capsulated, each capsule enclosing each pair. They stain with aniline dyes and are gram-positive.

In humans *Pneumococcus* causes bronchio-pneumonia meningitis which is the most serious pneumococcal infection. This disease is common in children.

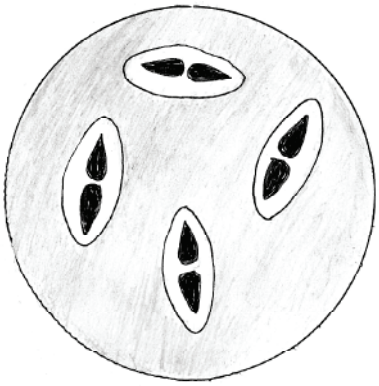


Figure 7.7 *Pneumococcus*

Corynebacterium diphtheriae

It is slender rodlike measuring 3.6 × 0.68 μm. The bacilli are pleomorphic, non-motile, non-capsulated cells exhibiting septa. They are gram-positive when observed in smears. The bacilli are arranged in a characteristic fashion, and are seen in pairs. This is a causative agent of diphtheria.

Figure 7.8 *Corynebacterium diphtheriae*

Bacillus anthracis

This is one of the largest pathogenic bacteria measuring 3.10 × 1.16 μm. It may occur singly or in pairs or in chains. The whole chain is surrounded by a capsule. Spores are formed in cultures but never in animal

body. Spores are oval or elliptical in shape. They are gram-positive and non-acid-fast and non-motile. Anthrax is a disease of cattle, sheep and horses. Human anthrax is contracted through the skin, and the face, neck, hands, arms and back are the usual sites of infection.

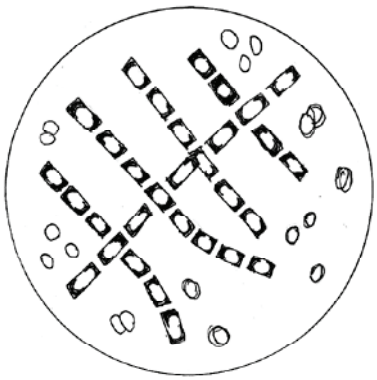


Figure 7.9 *Anthrax bacilli*

Clostridium welchi

It is a gram-positive bacillus with rounded or truncated ends measuring 4.6 × 1 μm. They occur either singly or in chains or in bundles. They are pleomorphic and non-motile and form capsules. Spore formation is seen, spores being central or sub-terminal.

This is the causative agent of gas gangrene, food poisoning, narcotizing colitis and urinary tract infections.

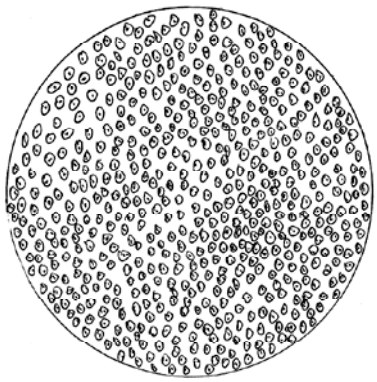


Figure 7.10 *Clostridium welchi*

Clostridium tetani

It is a short gram-positive slender bacillus, measuring 4.8 × 0.5 μm. It has rounded ends occurring either singly or in chains. They are spore-forming, spores are spherical, terminal and bulging and as such the bacillus has a drumstick appearance. It is the causative agent of tetanus.

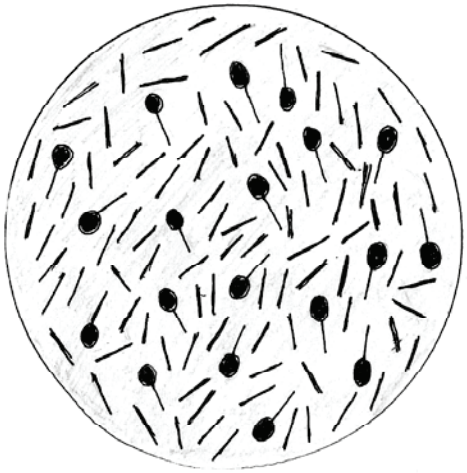


Figure 7.11 *Clostridium tetani*

Escherichia coli

It is gram-positive appearing as straight rods, measuring 1.3 μm arranged singly or in pairs. There is no spore formation, and is motile and capsulated. It is the causative agent of urinary tract infection, diarrhoea, gastroenteritis and pyogenic infections.

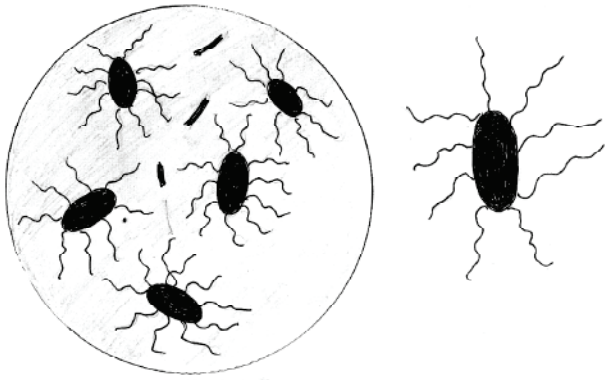


Figure 7.12 *Escherichia coli*

Shigella

These are rodlike, gram-positive bacteria measuring 0.5 × 1.3 μ in size, and are non-motile, non-spore-forming and non-capsulated. This is the causative agent of bacillary dysentery.

Salmonella typhi

These are rodlike, gram-negative organism, measuring 1.3 × 0.5 – 0.8 μm in diameter. They are motile, with no capsule formation or spore formation. The bacilli are causative agents of enteric fever, septicaemia, gastroenteritis or enteric fever including typhoid fever.

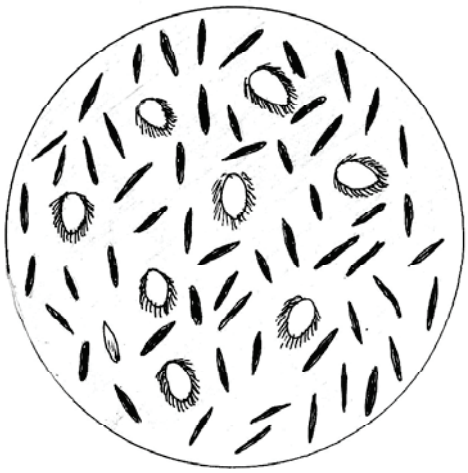


Figure 7.13 *Salmonella typhi*

Vibrio cholerae

These are curved, cylindrical rods measuring 1.5 × 0.2 – 0.4 μm in diameter with rounded ends or slightly pointed ends. The cell is typically comma-shaped. They are motile with a single polar flagellum, and are gram-negative but stain with aniline dyes and are non-acid-fast. This is the causative agent of cholera.

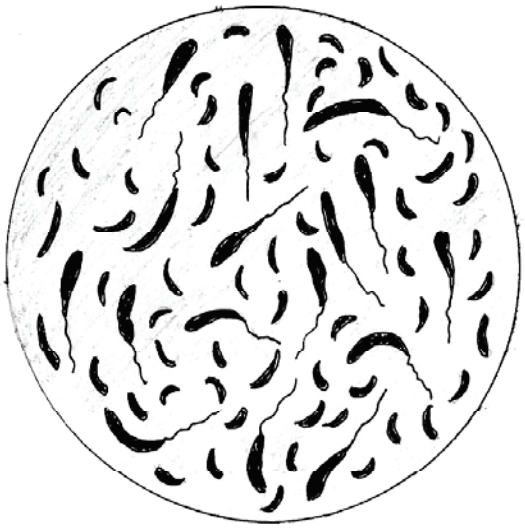


Figure 7.14 *Vibrio cholerae*

Pseudomonas aeruginosa

This is a single bacillus measuring 1.5 – 30 × 0.5 μm. It is motile with a polar filament, non-capsulated and gram-negative. This is the causative agent of infantile diarrhoea.

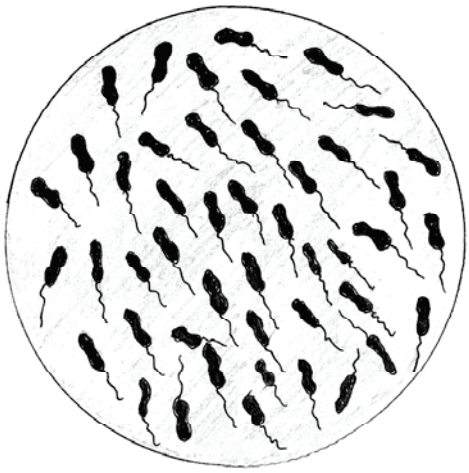


Figure 7.15 *Pseudomonas aeruginosa*

Yersinia pestis

This is the plague bacillus. It is a short hump bacillus, ovoid in shape, measures 1.5 × 0.7 μm in size. Ends are rounded and with convex sides, and the bacteria occur in short chains or in small groups. Polymorphism is common. It is gram-negative and the causative agent of plague.

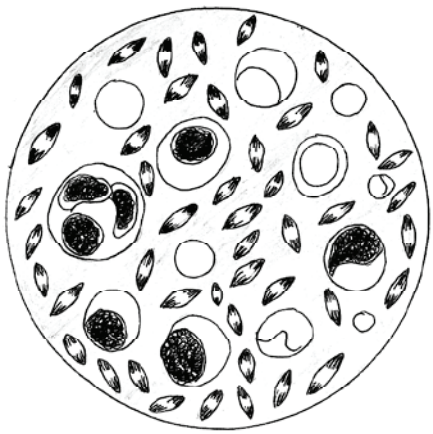


Figure 7.16 *Yersinia pestis*

Neisseria meningitidis

They are gram-negative cocci, either oval or spherical in shape 0.6 – 0.8 μm in diameter, arranged in pairs with one side flattened, the long axis being at right angles to the line joining the two cocci in pair. Size variation, shape variation and also variations in staining properties are noticed. These are non-motile. They are causative agents of cerebrospinal meningitis and meningococcal septicaemia.

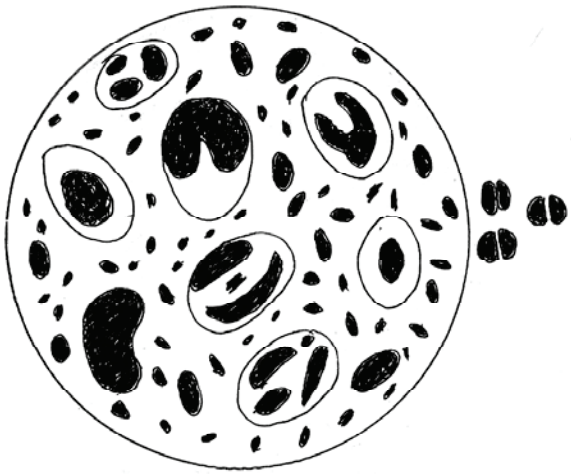


Figure 7.17 *Neisseria meningitidis* (Enlarged view to show flat adjacent sides of cocci)

Neisseria gonorrhoeae (*Gonococcus*)

These bacteria resemble meningitidis in many aspects. This organism appears as diplococcus with adjacent sides concave, appearing kidney-shaped or pear-shaped. *Gonococcus* possesses pits on the surface which aid in adhesion of the cocci to the mucosal membrane. Pitiated gonococci agglutinate human blood cells. This is the causative agent of gonorrhoeae which is acquired by sexual contact.

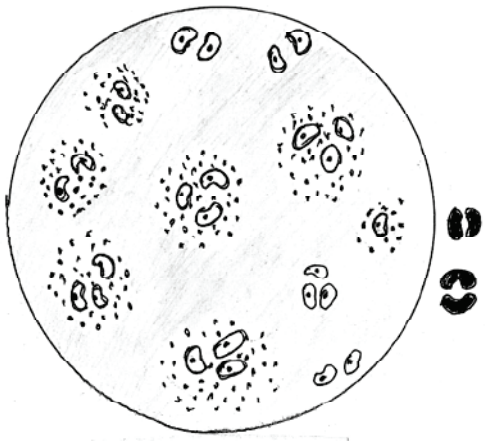


Figure 7.18 *Neisseria gonorrhoeae* (Enlarged view of kidney-shaped cells)

Haemophilus influenzae

It is small and measures 1.5 × 0.3 μm. It is non-motile, non-sporing and pleomorphic. It is gram-negative but stains with Leoffler's methylene blue or dilute carbol fuchsin. The most serious disease caused by *H.influenzae* is meningitis. Ninety per cent of the people affected succumb to this disease.

Figure 7.19 *Haemophilus influenzae*

Bordetella pertussis

This gram-negative bacillus is small and ovoid and measures 0.5–0.7 × 0.6–1.5 μm in size. It is arranged either singly or in chains, and is non-motile, non-capsulated, and non-sporing. It is the causative agent of whooping cough in children, characterized by paraxyons, cough and respiratory distress.

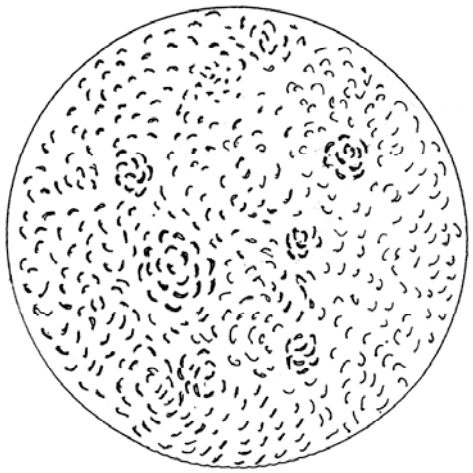


Figure 7.20 *Bordetella pertussis*

Mycobacterium tuberculosis

It is a straight or curved rod measuring 1.4 × 0.1 – 0.8 μm. It occurs either singly or in pairs or in clumps. Occasionally branching forms may be seen. It is non-motile, non-sporing and non-capsulated, gram-positive and with Ziehl-Neelsen's carbol fuchsin methods or fluorescent dyes, the bacteria resist decolorization. This is the causative agent of tuberculosis.

Figure 7.21 *Mycobacterium tuberculosis*

Mycobacterium leprae

It is straight or slightly curved rod measuring 1.8 × .2–0.5 μm in diameter, polar bodies may be present. Branching is observed. It is gram-positive and acid-fast. They occur either singly or in groups. This is the causative agent of leprosy.

Figure 7.22 *Mycobacterium leprae*

Spirochetes

Spirochetes have usually a helical shape but they are capable of twisting or controlling their shape. A special kind of flagellum is located between the outer membrane and the protoplasmic cylinder, i.e., they are located in the periplasmic space of the cell. Ultrastructural details of the protoplasmic flagella are similar to that of ordinary flagella with a basal body with discs. Flagellae help during swimming of the spirochetes. Motility depends purely on ordinary flagella since periplasmic flagella cannot be extended outside the cell wall. The exact mechanism of motility is not clearly understood. Unlike bacteria which swim best in media of low viscosity, spirochetes do it in media of high viscosity. In addition, spirochetes are also capable of creeping and crawling when in contact with solid surface.

Spirochetes are responsible for the relapsing fever syphilis. They divide by transverse fission.

Borrelia recurrentis

These are irregularly shaped with one or both ends pointed. It consists of five to ten loose spiral cells at intervals of about 2 μm. Exhibits mobility with lashing movements. Stains with Giemsa stain and gram-negative. It is the causative agent of relapsing fever.

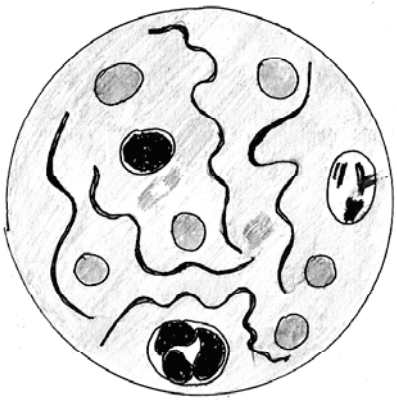


Figure 7.23 *Borrelia recurrentis*

Borrelia vincenti

It is a motile spirochete measuring 5–20 μm long and 0.2–0.6 μm wide. It takes carbol fuchsin stain and it has 3–8 coils of varying sizes. It is gram-negative. It is actually a commensal but under pre-disposing conditions like nutrition or viral infection, it may cause gastrostomatis or uropharyngitis.

Figure 7.24 *Borrelia vincenti*

Leptospira

These are very delicate measuring 6–20 μm long and 0.1 μm thick. They have a number of coils all set together. With great difficulty they could be observed under dark ground illumination or under electron microscope. They give the appearance of an umbrella handle because their ends are hooked. They stain with Giemsa but are gram-negative and actively motile.

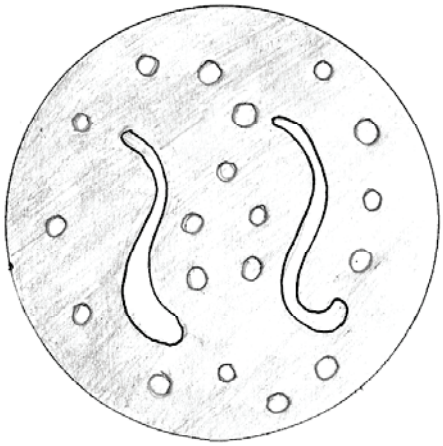


Figure 7.25 *Leptospira*

Treponema pallidum

It is a thin delicate spirochete with both ends pointed, about 10 μm in length, 0.1–0.2 μm in width. There are about 10 angular spirals. They are motile rotating around the long axis. Interestingly they exhibit backward and forward movements, simultaneously flexing the body. These movements could be observed under dark ground microscope. They are gram-negative. *T. pallidum* is the causative agent of syphilis.

Figure 7.26 *Treponema pallidum*

Mycoplasma

These are the minutest free-living microorganisms and are polymorphic. They are in the form of granules and filaments differing in size. Granules vary in size from 125–250 μm and larger granules about 500–1000 μm in size. These larger bodies are disc-balloon- or star-shaped. They are gram-negative but stain with Giemsa stain and have no spore formation, no flagellae, and no cell wall, and are non-motile.

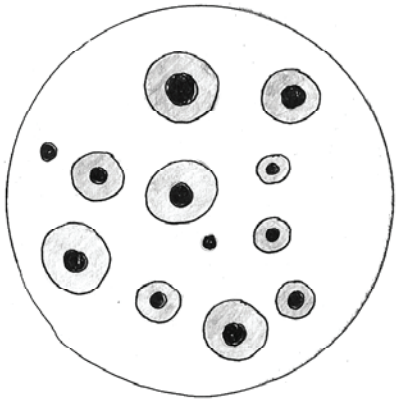


Figure 7.27 *Mycoplasma*

CULTURE MEDIA FOR BACTERIA

The best way of studying bacterial populations is to grow them separately on culture media and obtain as pure culture for study.

The first person who used liquid media was Louis Pasteur. This was not a successful method since bacteria grown in culture media did not exhibit specific characteristics. Added to this, it is difficult to isolate different types of bacteria from mixed populations. But still they have their own uses. Liquid media helps when large volumes of bacteria have to be tested and also helps in preparing bulk cultures of antigens.

On the other hand solid media enable bacteria to produce discrete visible growth. When proper doses are inoculated, bacteria are capable of producing colonies. Identification is easy because colony morphology is distinct and pigmentation is perfectly visible.

The oldest method of preparing solid medium was with potato (Robert Koch). A little later gelatin was used for solidification. Even this did not give satisfactory results. Later Fran Hesse (wife of one of the investigators) suggested agar to solidify culture media. Now use of agar (agar-agar) is in vogue. Agar is constituted by long polysaccharides (long chains), in organic salts and protein substances to a little extent. It melts at 98°C and sets at 42°C. It is available from a few types of sea weeds. 2% agar is sufficient for solid media.

Another medium is peptone which gained popularity. It is a mixture of proteins which constitute proteases, polypeptides and amino acids and a variety of inorganic salts such as phosphates, potassium and magnesium.

The following are some media.

Simple media This consists of meat extract, peptone, sodium chloride and water. 2% agar is added to nutrient broth. This is the simplest media and most of the laboratories are in favour of this medium.

Complex media These are for special purposes to study certain characteristics. This includes special nutrients which are required for a particular bacterium under consideration.

Enriched medium Blood, serum and egg are added to a basal medium. This is for the growth of bacteria which are more exact in their nutritional needs and which need additional source of nutrition. This is achieved by adding blood and serum to the nutrient agar.

Enrichment media In mixed cultures, a particular bacteria which has to be isolated is overgrown by the unwanted bacteria, in other words, the pathogenic bacteria are overgrown by non-pathogenic and commensal bacteria. *Salmonella typhi* is overgrown by *Escherichia coli* in cultures from faeces. Under these circumstances, substances which stimulate the growth of the bacteria to be studied, suppress the unwanted bacteria. If such substances are added to the medium, the culture will increase the growth of the wanted bacterium. Such media are called enrichment media.

Selective media As mentioned in the previous case, if inhibitory substance is added, it will allow the growth of the required bacterium to form colonies.

Differential media Substances incorporated into the media enables to differentiate the characteristics of bacteria and distinguish them. An example is Mc Conkey's medium consisting of lactose, agar, lactose, natural red peptone and taurocholate brings out clearly lactose fermenters as pink colonies whereas non-lactose fermenters are colourless.

Peptone water media

Peptone	10 g
Sodium chloride	5 g
Distilled water	1000 ml

Dissolve the ingredients by steaming (pH 7.5). Filter and distribute them in separate bottles, sterlize at 15 lb pressure.

Nutrient broth

Peptone	10 g
Sodium chloride	5 g
Meat extract	10 g
Distilled water	1000 ml

Dissolve the ingredients by steaming (pH 7.6). Phosphates get precipitated which should be extracted by filtration. Distribute them in separate bottles and sterilize at 15 lb pressure for 20 min.

If glucose broth is required add 1% glucose to this nutrient broth.

Nutrient agar As mentioned above agar-agar is a long chain of polysaccharides obtained from some seaweeds. To the nutrient broth add 2% agar to become nutrient agar and then autoclave at 15 lb for 20 min. Distribute in different flasks and sterilize at 15 lb for 20 min.

Blood agar First melt the nutrient agar and then cool at 50°C and then add 5–10% sterilized defibrinated blood. After mixing, it is distributed in different Petri dishes which are sloped (rabbit blood or blood from bank may also be used).

Chocolate agar As in the case of blood agar, add blood to nutrient agar. Mix well and heat up to 80°C for 10 min. Then it is distributed in Petri dishes as desired.

Sugar media This is useful for studying biochemical reactions of bacteria. Add 1% of the required sugar to peptone which is sterilized and add 1% Andrade's indicator (Dissolve 500 mg of acid fuchsin to 100 ml of distilled water and 16 ml of normal sodium hydroxide. Leave it overnight undisturbed. The colour should change from pink to brownish red and then to yellow.) Distribute into sterile tubes containing inverted Durhams fermentation tubes. Indicator will help in the study of acid formation by bacteria. Reddish pink colour indicates that acid is produced.

Hiss's serum water sugars

This medium is useful in studying the biochemical reaction of *Neisseria, Corynebacterium* and other bacteria which grow in serum.

Ox serum	1 part
Distilled water	3 parts

pH should be adjusted to 7.5. Add 1% Andrade's indicator and 1% sugar and sterilize.

Loeffler's serum slopes (Useful for culturing diphtheria bacilli)

Ox serum	3 parts
1% glucose broth	1 part

Ox's blood is collected under sterile conditions and the serum is separated asceptically. Insipissate the medium at 75°C for 1 hour.

Media for growth of anaerobes

1. *Thioglycollate medium* To nutrient broth, add 0.1% sodium thioglycollate, 0.05% powdered sugar and 1% glucose and also add 1/500000 methylene blue. Sterilize the solution for 15 minutes at 10 lb. Anaerobic condition is maintained by thioglycollate. Methylene blue acts as an indicator.

Robertson's cooked meat medium Take 500 g of ox heart (free of fat) and put in 500 ml of distilled water. Boil it for some time. Meat should be added to boiling distilled water. Drain of the water through muslin cloth, and then allow it to dry on the same cloth. Take 2.9 g of this dried meat in a bottle and to this add 10 ml of nutrient broth or the infusion broth filtered from the meat. Add 0.25% of sodium chloride and 0.5% peptone (pH 7.7). Close the bottle and autoclave at 15 lb for 20 min.

Mac Conkey's medium

Peptone	20 g
Sodium taurocholate	5 g
Distilled water	1000 ml
Sodium chloride	5 g

Dissolve the ingredients and steam them and adjust the pH to 7.5. To this add 2% agar and melt it in an autoclave at 15 lb for 20 minutes, then clear it with white of egg. Then add 10 g of lactose, 7–10 ml of 1% neutral red to the medium. Now distribute the ingredients in 200 ml flasks and sterilize by steaming for 3 days in Arnold's steam sterilizer. If *Escherichia coli* are present (which produce acid from lactose) pink-coloured colonies are formed. These are called lactose fermenters. *Salmonella typhi* produce colourless colonies. They are non-lactose fermenters. Sodium taurocholate in the ingredients inhibits the growth of gram-positive bacteria and promotes the gram-negative enteric bacteria.

Dien-Donnes medium

This is a selective medium for isolation of *Vibrio cholerae* has equal parts of defibrinated blood and normal caustic soda solution taken and heated in a steam, sterilize for 30 min. This mixture is added in the ratio 1 : 7 to 3 per cent nutrient agar and mixed thoroughly.

McLeods medium This is a good medium for isolation of *Corynebacterium diphtheriae*.

Take 10 ml of nutrient agar buds melted and cooked to 75°C. To this, add 15 to 20 drops of defibrinated blood and 5 drops of 2 per cent potassium tellurite solution (2 g of potassium tellurite in 100.0 ml of distilled water).

Lowenstein and Jensen medium This is an excellent medium for isolation and cultivation of *Mycobacterium tuberculosis*.

Solution

Potassium dihydrogen phosphate	0.4 g
Magnesium sulphate	0.4 g
Magnesium citrate	0.1 g
Asparagines	0.6 g
Glycerol	2.0 ml
Distilled water	98.0 ml

Take the ingredients into a beaker and heat in a steam bath for two hours. Take 30.0 g of potato starch to every 600.0 ml of salt solution. Then heat it in a water bath for 20 min. while stirring. Now add 500 ml of egg white to 100 ml of yellow of egg. Add distilled water to make up to 1000 ml and beat it well. Now take 600 ml of starch–salt mixture and add 1000 ml of egg beat. To this add 2.0 ml of 2 per cent malachite green and mix well. Take 6 screw-capped bottles and put 5 to 6 ml of the above mixture and insipissate in slanting position for half an hour at 80°C. Then sterilize it for half an hour at 75°C.

Aronson's medium

This medium is useful in isolation and identification of *Vibrio cholerae*.

Agar	35.0 g
Meat extract	10.0 g
Sodium chloride	5.0 g
10 per cent sodium carbonate	60.0 ml
20 per cent saccharose solution	50.0 ml
20 per cent glucose	50.0 ml
Basic fuchsin (saturated solution)	4.0 ml
10 per cent sodium sulphite	20.0 ml
Distilled water	1000 ml

Take agar and soak it in distilled water overnight and to this add peptone, sodium chloride and lab lemco and heat in the steam sterilizer for 5 hrs. Allow it to stand for some time. Take flasks and fill with 100 ml of the supernatant. Mix all ingredients except sodium sulphite solution. Sodium sulphite solution is sterilized by boiling repeatedly. To 100 ml of agar mixture, 6 ml of 10 per cent sodium carbonate solution is added and sterilized at 100°C for 15 min. When it is hot add 5 ml of saccharose solution, 5 ml of glucose solution, 0.4 ml of basic fuchsin and 2 ml of sodium sulphite.

Tetrathionate broth It is an enrichment medium in the isolation of *Salmonella typhi* and other *Salmonella*.

A)	Protease peptone	5.0 g
	Bile salts	1.0 g
	Distilled water	500.0 ml
B)	3% Sodium thiosulphate	500.0 ml
C)	0.1% Brilliant green	11 ml per litre
D)	25% iodine	25 ml per litre

Solution A Dissolve the peptone and bile salts in distilled water. Then add calcium carbonate and autoclave the solution 121°C for 20 min.

Solution B Dissolve sodium thiosulphate in distilled water and distribute in 500 ml amounts into screw-capped bottles. Sterilize by heating in steam at 100°C for half an hour. Label and store.

Solution C Dissolve 0.1 g of brilliant green in 100 ml of distilled water. Label and store.

Solution D Dissolve 20.0 g of potassium iodide in 50 ml of distilled water. To this add 25.0 g of iodine, mix well and make up the volume to 100 ml. Label and store.

Take one bottle of solution A and add 500 ml of solution B and 11 ml of solution C and mix thoroughly. Take 30-ml screw-capped bottles and in each bottle put 12 ml of the mixture. Store at 4°C. Before inoculation add 0.3 ml of solution D to each bottles.

Inoculate one tube with two or three large loopfuls (about 1g) from a known *Salmonella* species faeces. Incubate at 37°C for 18 hrs. Plate on a suitable selective medium and examine growth after 18 hrs incubation at 37°C.

BACTERIAL CULTURE

All samples for culture must be collected in a sterile container taking into account all aseptic precautions from the time of collection till the final stage of reading the test.

Urine Urine is collected by a sterilized catheter by catheterization, or a sample of mid-stream specimen is to be collected in a sterile container.

Fluid In the case of CSF and exudates, collect about 5 to 10 ml of fluid in a sterile bottle after putting a little amount of citrate solution to prevent coagulation.

Blood Take about 10 ml of blood and immediately place in bottles containing the appropriate broth, e.g., 50 ml of bile broth, 25 ml of Harley's broth or liver broth.

Swabs Take a cotton-tipped wire or wooden swab after sterilizing well and observing all aseptic precautions. Collect the material which is to be investigated and replace in a sterile test tube.

Sputum Collect sputum in a sterile container.

Faeces Clean the bed pan and put a little spirit in it and flame it. Allow the bedpan to cool collect the motion in the bedpan. Make sure that it is not contaminated with urine. Take a little stool in a sterile test tube or a bottle.

INOCULATION OF CULTURE TUBES

The culture tubes containing specimens are held almost parallel to the table-top to avoid air contamination in between the thumb and fingers of left hand. A sterile loop is taken in the right hand (the loop is heated red hot). The cotton plug of the tube is removed by grasping it between the little finger and palm of the right hand. The sterile loop is immediately introduced into the culture tube and held for some time to cool the loop. Then the loop is dipped into the material or the specimen and the loop is removed without touching the sides of the tube. The charged loop is then inoculated into the fresh culture observing all the above procedure. If it is stout or slope, the material is gently rubbed or spread over the surface by gentle side-to-side stroke. If it is fluid medium, the loop is just immersed in it and gently shaken or rubbed against the wall of the tube. After inoculation the loop is immediately sterilized. The mouth of the tube is flamed before and after inoculation and plugged with cotton. Label the culture with name and date and immediately put it in the incubator.

Streak plate metod The culture material is spread on the surface of the medium in the Petri dish with the help of the loop.

Anaerobes Bacteria grow well in the presence of carbon dioxide. To grow these organisms inoculated medium is placed in the desiccator. A lighted candle is placed in the desiccator and the lid is closed. After some time the handle is removed from the desiccator, the lid closed and the desiccator is placed in the incubator.

FUNGI

These are without chlorophyll and are eukaryotic chemo organotropic organisms. The thallus or body of fungus is of a single cell as in yeasts and with filaments 5–10 μm across which are commonly frenched. Some fungi are dimorphic, i.e., they exist in two forms. Some of the pathogenic fungi of humans and other animals have a unicellular form in their hosts but when they grow saprophytically either in laboratory culture medium or in soil, they have a filamentous mould form. So a fungal colony is either a mass of yeast cells or it may be a filamentous mat of mould.

Actinomycetes fungi also anastomose to form filaments and mycelia and produce sexual spores which are the result of the fusion of two cells. Sexual spores are formed by the differentiation of spore-bearing cells of the spore-bearing hyphae without fusion. In some cases tips of the hyphae fragment and produce the spores called conidia (Penicillium). Fungi can be stained easily.

A few fungi are pathogenic, causing serious diseases like meningitis, European blastomycosis and some infections of skin and mucous membrane.

Yeast cells in general are larger than bacteria with their size varying from 1.5 μm in width and 5–30 μm in length. They are commonly egg-shaped, some are elongated and others are spherical. Yeast cell is surrounded by a true cell wall. They are non-cellular, unnucleated and are capable of budding and fusion.

ACTINOMYCETES

They have a system of anastomosing filaments which are known as hyphae. They intertwine and anastamose greatly to form a colony called mycelium. Sometimes these anastamosing filaments break up to form bacillarylike bodies which cannot be distinguished from bacteria. Sometimes the protoplasm of hyphae aggregate to form sporelike bodies which are gram-positive. According to some they are transitory between the bacteria and fungi.

RICKETTSIA

These are very small, gram-negative microorganisms and are obligate parasites able to grow only in host cells. They cause 'typhus', spotted fever and associated diseases. Appearing as bacilli or cocci, they may occur either singly or in pairs or in dense masses. Some of them are specific to cytoplasm and others are found in nucleoli. Rickettsia could easily be stained with Giemsa stain or with Machiavello's method. Some of the diseases caused by them are classical typhus fever, murine typhus fever, rickettsial pox and scrub typhus.

VIRUSES

Viruses are too small and cannot be seen with naked eye. They are so small that they easily pass through filters. Viruses are responsible for causing serious diseases like yellow fever, poxes, influenza, measles, mumps, rabies, colds, infective hepatitis, encephalitis and poliomyelitis. Viruses have infectious particles called inclusion bodies of different sizes. They are found in the cytoplasm or nucleus of infected host cells. These inclusion bodies can be demonstrated by special staining methods. Many viruses like rabies, poliomyelitis and measles form inclusion bodies which are also seen in other infections like psittacosis and trachoma.

8

FIXATION AND STAINING METHODS

One cannot conduct histological and histochemical investigations except for certain activities like enzymes on living cells as the integrity of the tissue or cells may be damaged. It is therefore necessary to 'fix' the tissue and subsequently localize the chemical constituents. Tissues should be fixed immediately after death and the fixative should be 20 times the bulk of the tissue. After death the tissues are prone to degenerative changes. Autolysis sets in and there is distortion of cell structure. To avoid all this it is necessary to fix the tissue immediately after death. If the fixative is not ready on hand, the tissue can be kept in freezing chamber.

There are a number of fixatives of which formaldehyde (40 per cent) takes the pride of being the most popular and routinely available fixative. Formaldeyde reacts with proteins and amino acids like lysine, arginine, histidine, glutamine, aspargin, cystein, tyrosine and tryptophan.

SOME COMMON FIXATIVES

Formol calcium (Baker, 1944)

Formalin	10.0 ml
Calcium chloride	2.0 g
Distilled water	100.0 ml

This is good to preserve lipids and phospholipids. Post-treatment is necessary after fixation (24 hrs) in 2 per cent potassium dichromate at room temperature for 24 hrs and at 60°C for another 24 hrs.

Acetic alcohol formalin

Formalin	5.0 ml
Glacial acetic acid	5.0 ml
70 per cent alcohol	90.0 ml

This is a fixative for glycogen. It penetrates rapidly. Fixation time is 4 hrs.

Zenker's stock solution

Potassium dichromate	2.5 g
Mercuric chloride	5.0 g
Distilled water	100.0 ml

Dissolve potassium dichromate and mercuric chloride in water by heating. This is the stock solution.

Zenker's stock	100.0 ml
Zenker's working solution	
5 per cent glacial acetic acid	5.0 ml

Helly's fluid can also be prepared from Zenker's stock solution. Instead of acetic acid, formaldelyde is added.

Zenker's stock	100.0 ml
5 per cent formaldehyde	5.0 ml

Bouin's fluid

This is well known as a routine fixative recommended especially for Masson's staining technique.

Picric acid (4 per cent aqueous)	150.0 ml
40 per cent formaldehyde	50.0 ml
Glacial acetic acid	10.0 ml

Post-treatment after washing in Scott's tap water is necessary.

Scott's tap water

Sodium bicarbonate	2.0 g
Magnesium sulphate	20.0 g
Distilled water	1000.0 ml

Add a pinch of thymol to prevent moulds.

Carnoy's fixative

Absolute alcohol	60.0 ml
Chloroform	30.0 ml
Glacial acetic acid	10.0 ml

Carnoy's fixative penetrates rapidly. Nuclear preservation is excellent.

Susa fixative

Saturated mercuric chloride	50.0 ml
Trichloroacetic acid (TCA)	2.0 g
Glacial acetic acid	4.0 ml

40 per cent formaldehyde	20.0 ml
Distilled water	20.0 ml

It is a choice fixative, substitute for Zenker's fluid. Post-treatment in iodine alcohol (few crystals of iodine in 70 per cent alcohol) for 24 hrs is required.

DECALCIFICATION

In some tissues especially bone in vertebrates and among invertebrates, molluscan shells, crustacean cuticle or exoskeleton and tissues with spicules and small setae, excess calcium and such tissues require decalcification. The size of the tissue should be kept to a minimum of 4–5 mm to ensure adequate fixation. For such tissues, overnight soaking in water usually softens the tissue.

Before decalcifying the tissue, it should undergo routine fixation. There are a number of decalcifying agents with or without acid interference.

There are several ways of decalcifying the tissue. It may be affected by dilute mineral acids or chelating agents or by electropohoretic removal of calcium. To assure the complete removal of calcium, there is one method by which it can be tested. For a long time radiography of the tissue was in use but this method cannot be followed with the tissues fixed in fluids containing mercuric chloride. The following is the best way to test whether calcium is completely removed. 5–6 ml of decalcifying fluid is neutralized with N_2NaOH and then 1ml of 2 per cent sodium or potassium oxalate is added. If the fluid is still turbid, that means calcium is not completely removed from the tissue and decalcification is not complete. Absence of turbidity after five min. indicates that the tissue fluid is free from calcium.

Most often hydrochloric acid, acetic acid and nitric acid are used as decalcifying agents resulting in unsatisfactory histochemical methods.

Nitric Acid Method

Sections are decalcified in large quantities of 5 per cent aqueous nitric acid for 4 days. Solution should be changed every day and washed in running water for 24 hrs. Neutralize then in 10 per cent formalin to which excess quantity of magnesium carbonate is added. Then wash in running water for 48 hrs. Then proceed with usual dehydration, clearing and embedding. The disadvantage with this method is that exposure to nitric acid for long periods may cause impairment of nuclear staining.

Formic Acid–Sodium Citrate Method

Solution 1

Sodium nitrate	50.0 g
Distilled water	200.0 ml

Solution 2

Formic acid 90 per cent	125.0 ml
Distilled water	125.0 ml

Mix solutions 1 and 2. Decalcify for 5–14 days in the above solution. Solution should be changed daily.

Decalcifying fuids

Jenkin's fluid

Absolute alcohol	73.0 ml
Distilled water	10.0 ml
Chloroform	10.0 ml
Glacial acetic acid	3.0 ml
Hydrochloric acid	4.0 ml

Volume of the fluid should be 40–50 times the bulk of the tissue. After decalcification, transfer to absolute alcohol, fixation is for 16–48 hrs. Then post-fix in neutral formalin for 48 hrs. Avoid prolonged treatment with decalcifiers.

Gooding and Stewart's fluid

Formic acid	5–25.0 ml
Formalin	5.0 ml
Distilled water	100.0 ml

It is a good decalcifying fluid causing minimum damage to the tissues. Decalcification is complete in 2–4 days depending on the thickness of the tissue.

Lillies fluid

2 per cent picric acid	85.0 ml
(2 g in 100 ml water)	
Concentrated formaldehyde	10.0 ml
Formic acid (90–95 per cent) (95 ml / 5 ml water)	5.0 ml

The tissue is kept for one or two days in fluid and 2–3 days in 70 per cent alcohol till yellow colour disappears.

Decalcification of molluscan shell

Shell is first cleared of foreign material adhering to it and thoroughly washed with distilled water. Then shell is fixed in 5 per cent formaldehyde overnight and decalcified with 2 per cent acetic acid . If the shell is fragile, only 1 per cent acetic acid is used and for heavy shells, higher concentrations are used. After the decalcification process, the material is again treated with 5 per cent formaldehyde for five hours and washed in running water overnight. Then it is transferred to 70 per cent alcohol and dehydrate.

From practice it has been found that gastropod shells, when put in Susa for longer periods (more than 20 days), are softened and no further decalcification was necessary for sectioning. No cellular or structural or functional damage was noticed though animals were in susa for one month.

TISSUE PROCESSING

For microscopical investigations, tissues have to be sectioned. For this it is necessary to impregnate the tissue with a medium which after solidification facilitates the sections of desired thickness to be cut. Sections can also be cut with frozen tissue on cryostat or on freezing microtome.

For histology, the most routinely used medium with satisfactory results is paraffin wax (m.p 56–58°C). Before cutting the section or even embedding in paraffin wax, the tissue must pass through

a) Complete fixation
b) Removal of water by dehydration
c) Clearing with clearing agent which is completely miscible with both dehydrating agent and paraffin embedding wax
d) Embedding

a) Complete fixation As already mentioned above different fixatives are used to fix the tissue. Some fixatives like Zenker, Susa, Bouin's and formol calcium require post-treatment as stated already. Once the tissue is completely fixed, it is washed.

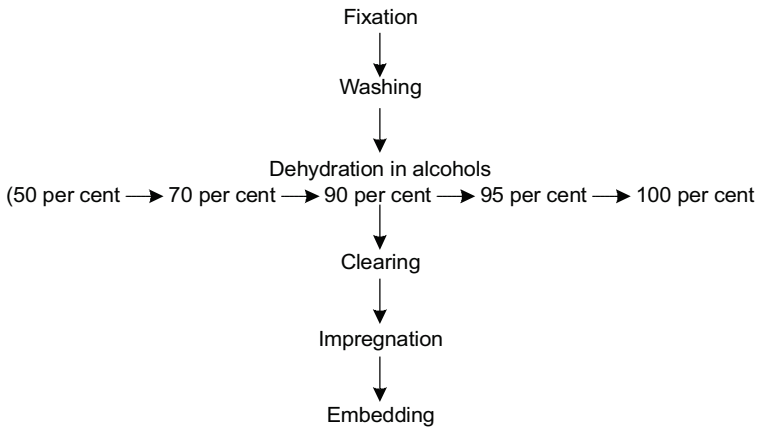
b) Dehydration It is achieved by passing the tissue in graded series of alcohol, i.e., 50 per cent, 70 per cent, 90 per cent, 95 per cent, 100 per cent. The time required for dehydration depends upon the type and size of the tissue.

c) Clearing A medium is chosen to remove the dehydrating agent and it should mix with alcohols as well as embedding media. There are several reagents suitable for this. The most common and frequently used is xylene (xylol). Toluene, chloroform, benzene, carbon tetrachloride, carbon disulphide, petrol, amyl acetate, methyl benzoate, cedar wood oil and clove oil are some of the clearing agents.

d) Embedding Researchers adopt different methods of embedding. A number of receptacles are suggested to be used as moulds. The most commonly used is 'L' piece. Embryo cups, Petri dishes, watch glasses, shallow dishes, shallow dishes having sloping slide are also suitable.

Paraffin finds a popular place in the laboratory due to the ease with which large number of tissue blocks are processed in a short time.

Schedule for processing the tissue



Embedded blocks are now ready for sectioning. The blocks can be trimmed into squares. Use metal or wooden block holders. Adjust the microtome for section thickness (5–6 μm). Raise the tissue carrier and place the block holder with its mounted tissue block, tighten the clamp of the tissue carrier on to the stem of the block holder.

Insert the microtome knife and tighten its clamps. The knife should beat a proper angle so that sections adhere to each other serially. Sectioning can be done in such a way that they move down in the form of a ribbon, each section adhering to the preceding as well as following sections. When the sections are ready, they have to be placed in a serial order on slides. Usually egg albumin is smeared over the surface of the slide with the finger.

Mayer's egg albumin

Egg white	50.0 ml
Glycerine	50.0 ml

Mix thoroughly, filter and add a piece of thymol crystal.

Sections are placed on albuminized slides with 10 drops of water. Then the sections are stretched on a hot plate in an oven adjusted to 50°C. Care is taken to see that the wax does not melt completely. This is followed by air-drying the slides for one or two days and later staining according to the requirement. First the slides are immersed in xylol to remove the wax and later hydrated to water through graded series of alcohol (100 per cent → 95 per cent → 90 per cent → 70 per cent → 50 per cent and down to water. Now they are ready to be stained as per the requirement.

ROUTINE STAINING PROCEDURES

HAEMATOXYLINS

Haematoxylin is a powerful dye staining through purple-blue or blue-black. If mordanted with iron, it is exceedingly good for mitotic study. Chromatin takes black or blue-black colour. Usually the type of mordant used influences the type of tissue that takes the stain. Some haematoxylin staining requires the tissues to be pre-treated with a mordant. An example is Heidenhains's iron haematoxylin.

Iron Haematoxylin

In this iron solutions are used both as oxidizing agents and mordants. Ferric chloride and ferric ammonium sulphate are the iron salts.

Reagents required

Iron alum

Haematoxylin

Absolute alcohol

Eosin

Preparation of reagents

Solution 1

Iron alum	4.0 g
Distilled water	100.0 ml

Solution 2 Stock solution

Haemaloxytin	10.0 g
Absolute alchohol	100.0 ml

Allow it to ripen for six months.

Solution 3 Working solution

Solution 2	5.0 ml
Absolute alcohol	95.0 ml

Fixation

Avoid mercuric-chloride-containing fixatives.

Procedure

1. Deparaffinize and hydrate the slides to water.
2. Mordant in solution 1 for 30 min.
3. Wash in running water.
4. Stain in solution for 3 for 30 min.
5. Wash in running water.
6. Destain in 2 per cent iron alum until the nuclei are sharply coloured.
7. Wash in running water.
8. Counterstain in 1 per cent eosin for 20 seconds.
9. Rapidly dehydrate, clear and mount in Canada balsam.

Result

Nuclei	Deep black
Cyloplasm	Eosin colour

Delafield's Haematoxylin (Carleton & Leach, 1947)

The longevity of this haematoxyltin is same as Ehrlisch's haematoxylin.

Reagents required

Haematoxylin
Ethyl alcohol
Ammonia alum
Glycerol

Preparation of reagent

Solution 1

Haematoxylin	6.0 g
Ethyl alcohol	50.0 ml

Solution 2

Ammonia alum	55.0 g
Distilled water	100.0 ml

Solution 3

Glycerol	150.0 ml
Ethyl alcohol	150.0 ml

First mix haematoxylin in ethyl alcohol and ammonia alum in water separately. Now mix both solution 1 and 2, filter and then add solution 3 after 3 days.

Fixation

Any general fixative

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to Delafield's haematoxytlin.
3. Wash in running water.
4. Transfer to 70 per cent alcohol.
5. Differentiate in acid alcohol (2 to 3 drops of HCl in 60 ml of 70 per cent alcohol).
6. If nuclei are still deep blue, repeat the process.
7. Transfer to 70 per cent alcohol.
8. Counterstain if desired.
9. Wash, dehydrate, clear and mount.

Mayer's Haematoxylin

Add 1.0 g of haematoxylin to 1 litre of distilled water. Heat until dissolved. Add 1.0 g of citric acid and 50.0 g of chloral hydrate. Allow to ripen for 6–8 weeks.

Fixation

Any general fixative

Procedure

1. Deparaffinize and hydrate the slides to water.
2. Stain in Mayer's haematoxylin for 10 min.
3. Wash in running water for 3 min.
4. Counterstain with eosin for 1 min.
5. Wash quickly, dehydrate, clear and mount.

Result

Nuclei	Deep blue
Cytoplasm	Deep pink

Weigert's Haematoxylin (Lillie & Henderson, 1960)

Reagents required

Haematoxylin

Absolute alcohol

Ferric chloride

Hydrochloric acid

Preparation of reagents

Solution 1

Hematoxylin	1.0 g
Absolute alcohol	100.0 ml

Solution 2

30 per cent ferric chloride	4.0 ml
(30 g in 100 ml water)	
Distilled water	100.00 ml
Hydrochloric acid	1.0 ml

Mix equal parts solutions of 1 and 2 just before use. The staining solution should be violet-black in colour.

Solution 1 must be allowed to ripen at least one week before it is used.

Fixation

Any general fixative.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in haematoxylin for 3–5 min.
3. Wash in running water.
4. Counterstain with eosin for 30 sec.
5. Wash, dehydrate, clear and mount.

Result

Nuclei	Black
Other elements	eosin colour

Ehrlisch Haematoxylin

Reagents

Haematoxylin

Ammonia alum

Ethyl alcohol

Glycerin

Preparation of reagents

Haematoxylin	2.0 g
Ammonia alum	3.0 g
Ethyl alcohol	100.0 ml
Glycerine	100.0 ml
Distilled water	100.0 ml

Ripen for 6–8 weeks. For immediate use it can be ripened with 0.24 g of sodium iodate. If 100.0 ml of glacial acetic acid is added it keeps for years.

Some CounterStains for Haematoxylin

Eosin (Putt 1948)

Eosin Y	1.0 g
Potassium dichromate	0.500 mg
Saturated aqueous picric acid	10.0 ml
Absolute alcohol	10.0 ml
Distilled water	80.0 ml
Acetic acid (if necessary)	1 drop

Eosin–Orange G

1 per cent eosin Y in 95 per cent alcohol	10.0 ml
Orange saturated solution	5.0 ml
95 per cent alcohol	45.0 ml

Orange G

Orange G	1.0 g
Phosphotungstic acid	5.0 g
95 per cent alcohol	100.0 ml

SPECIAL STAINING PROCEDURES

TECHNIQUES FOR CARBOHYDRATES

Best Carmine Method for Glycogen (Best 1906)

Fixative

Paraffin, freeze-dried and frozen sections.

Reagents required

Carmine
Potassium chloride
Ammonia (–880)
Methyl alcohol
Absolute alcohol
Distilled water

Preparation of reagents

Solution 1 Best's carmine

Carmine	2.0 g
Potassium carbonate	1.0 g
Potassium chloride	5.0 g
Distilled water	60.0 ml

Boil the solution for 5 min. (gently), and then cool and filter. Add 20 ml of ammonia to the filtrate.

Solution 2 Best's Carmine working solution

Stock solution	12.0 ml
Ammonia	18.0 ml
Methyl alcohol	18.0 ml

Solution 3 Best's differentiator

Absolute alcohol	8.0 ml
Methyl alcohol	4.0 ml
Distilled water	10.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Keep the sections in 1 per cent celloidin for 5 min.
3. Keep the sections in tap water.
4. Transfer to alum haematoxylin.
5. Wash in tap water.
6. Place sections in solution 2.
7. Rinse briefly in solution 3 giving 2 changes for 20 sec. each.
8. Wash in 90 per cent alcohol.
9. Place in absolute alcohol.
10. Clear in xylene and mount in DPX.

Result

Glycogen	Red
Nuclei	Blue

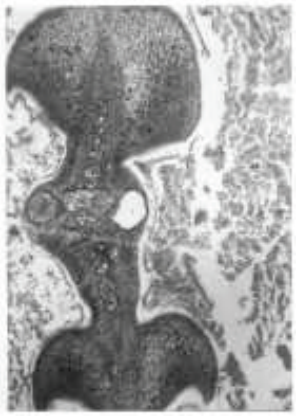


Figure 8.1 Section of the cestode *Penetrocephalus ganapathii* stained with best carmine for glycogen (*See* Plate 1.1)

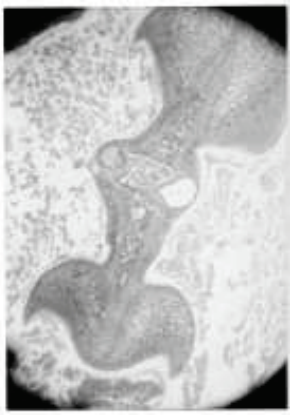


Figure 8.2 Same after treatment with α amylase—negative to best carmine (*See* Plate 1.2)

Remarks

This method was first introduced by Best (1906) and later modified by several investigators. Mucin also stains but not as intensely as glycogen. Any tissue section known to contain glycogen should be used as control. It is better to use diastase in the incubating medium.

Periodic Acid/Schiff (PAS) Reaction

Fixation

All types

Reagents required

Periodic acid

Basic fuchsin

Hydrochloric acid

Potassium metabisulphite

Activated charcoal

Preparation of reagents

Solution 1 Periodic acid

Periodic acid	1.0 g
Distilled water	100.0 ml

Solution 2 Schiff's reagent

Boil 1.0 g of basic fuchsin in 100.0 ml of distilled water. Shake the flask, allow it to cool and filter. To the filtrate add 20.0 ml of 1N hydrochloric acid and 1.0 g of potassium metabisulphite. Store the solution in dark for 24 hrs. After that add 200–300 mg of activated charcoal. Filter and store in amber-coloured bottles at 4°C.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place in solution 1 for 10 min.
3. Wash in tap water.
4. Treat with solution 2 for 30 min.
5. Wash in tap water.
6. Counterstain in 0.5 per cent light green for 5 min.
7. Wash in distilled water.
8. Dehydrate, clear and mount.

Result

PAS-positive material	Magenta
Nuclei and proteins	Green

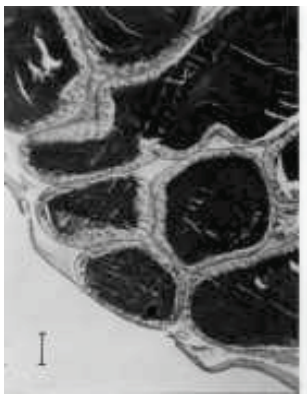


Figure 8.3 Section of accessory gland of the crab *Ocypoda platytarsis*. Secretions showing strong positivity to PAS (Carbohydrates) (*See* Plate 1.3)

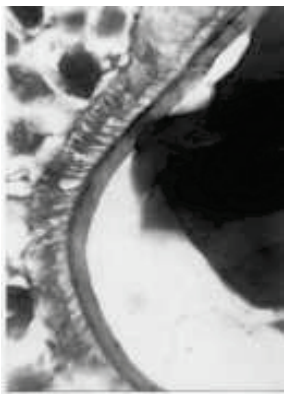


Figure 8.4 Section of style sac of the gastropod *Gabbia orcula* stained with PAS /light green. Style sac cells show positivity to light green (Proteins) 21μm (*See* Plate 1.4)

Mayer's Muci Carmine (Mayer 1896, modified by Southgate, 1927)

Fixation

All types

Reagents required

Carmine

Aluminium hydroxide

Aluminium chloride (anhydrous)

Absolute alcohol

Distilled water

Preparation of reagents

Solution 1

Carmine	1.0 g
Aluminium hydroxide	1.0 g
Absolute alcohol	50.0 ml
Distilled water	50 ml

Shake well and then add 500 mg of aluminium chloride. Boil the solution for 3 min. Allow it to cool and make up to the original volume with 50 per cent alcohol and filter. This solution lasts for one year. For use dilute the stock at 1 : 4 ratio with distilled water. Store at 4°C.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain nuclei with haematoxylin for 10 min.
3. Wash in tap water.
4. Differentiate in 1 per cent acid alcohol.
5. Wash in water.
6. Stain in staining solution for 30 min.
7. Wash, dehydrate, clear and mount in DPX.

Result

Mucosubstances	Red
Nuclei	Blue
Connective tissue	Pale gray

TECHNIQUES FOR MUCOPOLYSACCHARIDES

Alcian Blue (pH 2.5)

Reagents required

Alcian blue 8GX

Acetic acid

Preparation of reagents

Alcian blue 8GX	1.0 g
3 per cent acetic acid	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in solution 1 (alcian blue) for 30 min.
3. Wash in running tap water.
4. Dehydrate, clear and mount in Canada balsam.

Result

Weakly acidic sulphated mucopolysaccharides	
Hyaluronic acid and sialomucins	Blue
Strongly acidic chromotropes	Light blue

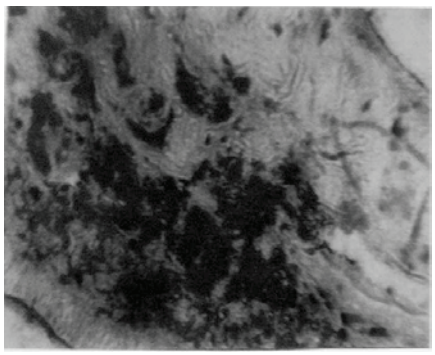


Figure 8.5 Section of foot of *G. orcula* stained with alcian blue at pH 2.5. Foot sole glands showing weakly acidic sulphated mucopolysaccharides. 21 μm (*See* Plate 1.5)

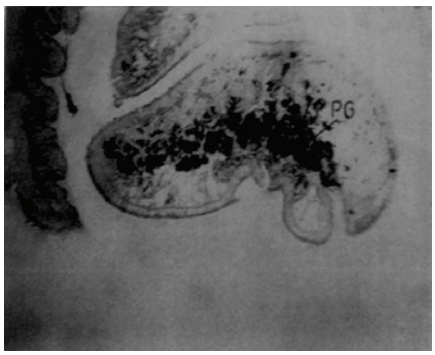


Figure 8.6 Pedal glands of *G. orcula* showing strong alcianophilia with alcian blue at pH 2.5. PG: pedal gland 90 μm (*See* Plate 1.6)

Alcian Blue (pH 1.0)

Reagents required

Alcian blue 8GX

Hydrochloric acid

Preparation of reagents

Alcian blue 8GX	1.0 g
0.1N Hydrochloric acid	100.0 ml
(1ml HCl in 100 ml distilled water)	

Procedure

1. Hydrate slides to water.
2. Stain in alcian blue solution for 30 min.

3. Blot and dry with filter paper.
4. Dehydrate, clear and mount.

Result

Sulphated mucosubstances	Greenish blue
Non-sulphated mucosubstances	Unstained

Rationale

Steedman (1950) first introduced alcian blue for acid mucosubstances. In this technique alcian blue stains with salt linkage to the acidic group in acid mucosubstances. By altering the pH of the alcian blue solution, different types of acid mucosubstances can be distinguished. At pH 0.2, only the strongly sulphated mucosubstances stain. By slightly increasing the pH to 1.0, both weakly and strongly sulphated mucosubstances stain. At pH 2.5 only acid mucosubstances take the colour. According to Cook (1974) even these are not satisfactory techniques. It is better to use critical electrolyte concentration (CEC) method to obtain precise results. This method was first used by Scott & Dorling (1965).

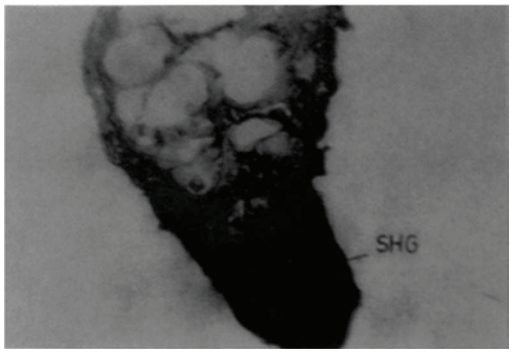


Figure 8.7 Section of mantle of *G. orcula* stained with alcian blue at pH 1.0. Shell gland has taken a deep blue shade (sulphated mucosubstances) (*See* Plate 1.7)

Alcian Blue at pH 2.5 and pH 1.0/PAS

Reagents required

Alcian blue 8GX

Periodic acid

Schiffs reagent

Preparation of reagents

Solution 1

Alcian blue pH 2.5—As described on page

Alcian blue pH 1.0—As described on page

Solution 2

Periodic acid	1.0 g
Distilled water	100.0 ml

Solution 3

Schiff's reagent as described in this chapter.

Procedure

Take 2 slides for AB pH 1.0 and pH 2.5

1. Deparaffinize and hydrate slides to water.
2. Place in solution 1 (alcian blue) for 30 min.
3. Wash in distilled water.
4. Treat with solution 2 for 10 min.
5. Wash in water.
6. Stain in solution 3 for10 min.
7. Rinse in sodium metabisulphite.
8. Wash in distilled water.
9. Dehydrate, clear and mount in Canada balsam.

Result

Hyaluronic acid and sialomucins (both weakly and strongly acidic)	Blue
Sulphated mucosubstances at pH 1.0	Blue
Neutral mucosubstances stain at pH 2.5	Red
Sialomucins at pH 1.0	Red

Remarks

A very good technique to distinguish acid from neutral mucins.

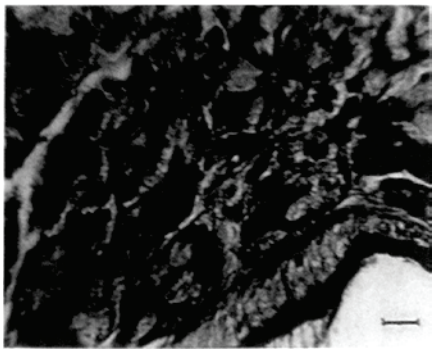


Figure 8.8 Section of oesophagus of the mole crab *Emerita asiatica*. Oesophageal glands have stained red with alcian blue pH 1.0/PAS—Sialomucins are indicated. 100 μm (*See* Plate 1.8)

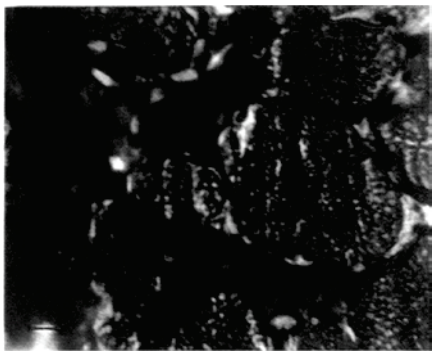


Figure 8.9 Section of vas deferens of *O.platytarsis* stained with alcian blue pH 2.5/PAS. A part of secretions stained red are neutral mucopolysaccharides. Hyalo and sialomucius have taken a blue shade 24 μm (*See* Plate 2.1)

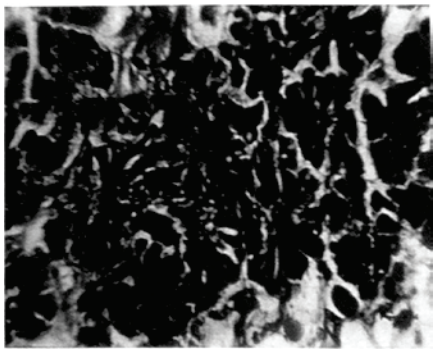


Figure 8.10 Oesophageal glands of E. asiatica stained with alcian blue pH 2.5/PAS. Glands stained red neutral mucopolysaccharedes. 21 μm (*See* Plate 2.2)

Toluidine Blue Method (Kramer and Windrum, 1955)

Reagents required

Toluidine blue

Ethyl alcohol

Preparation of reagents

Toluidine blue	100 mg
Absolute alcohol	30.0 ml
Distilled water	70.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to staining solution for 15 min.
3. Wash in distilled water.
4. Mount in glycerine jelly.

Result

Acid mucopolysaccharides	Pink (metachromatic)
Nuclei	Blue

TECHNIQUES FOR PROTEINS

Mercury Bromophenol Blue Method for General Proteins

Fixation

Any general fixative—Susa is preferable.

Formalin is recommended.

Reagents required

Mercuric chloride

Bromophenol blue

Preparation of reagents

Solution 1 Staining solution

Mercuric chloride	Saturated
Bromophenol blue	1.0 g
Distilled water	100.0 ml

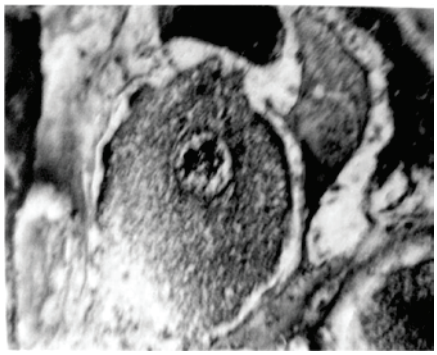


Figure 8.11 Section of ovary of the terrestrial crab *Cardisoma cornifex*. Stage 4 oocytes showing bromophenol blue positive protein bodies. 90 μm (*See* Plate 2.3)

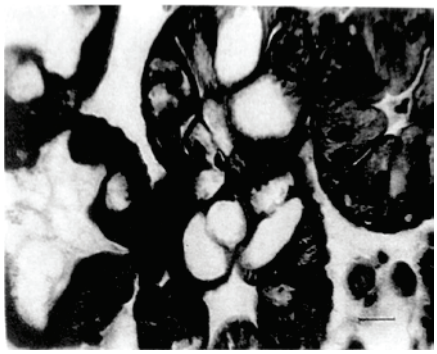


Figure 8.12 Hepatopancreatic cells of *E.asiatica* stained with bromophenol blue. 12 μm (*See* Plate 2.4)

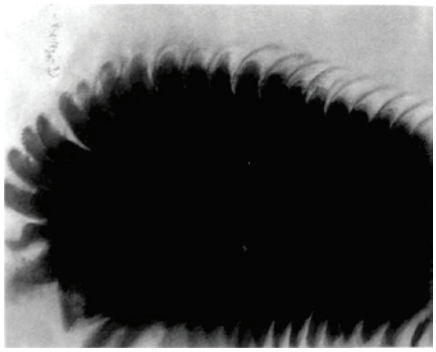


Figure 8.13 Radule of *G. orcula* stained with light green (proteins) 21 mm (*See* Plate 2.5)

Procedure

1. Hydrate slides to water.
2. Transfer slides to solution 1.

3. Wash in tap water.
4. Dehydrate, clear and mount.

Result

Basic proteins	Deep blue

Perfeormic Acid/Alcian Blue Method for Disulphides (Adams and Sloper, 1956)

Fixatives

Formalin, Carnoy, Susa

Reagents required

Formic acid

Hydrogen peroxide

Sulphuric acid

Alcian blue 8GS

Preparation of reagents

Solution 1 Performic acid (oxidizing solution) (Pearse, 1968)

98 per cent formic acid	40.0 ml
100 V of H_2O_2	4.0 ml
Sulphuric acid	0.5 ml

Solution 2 Staining solution

Alcian blue 8 GS	1.0 g
98 per cent sulphuric acid	2.7 ml
Distilled water	47.2 ml

Procedure

1. Deparaffinize and hydrate the slides in water.
2. Keep sections in solution 1 (oxidizing solution) for 5 min.
3. Wash in tap water.
4. Dry sections.
5. Rinse in tap water.
6. Transfer to solution 2 (alcian blue) for 1 hr.
7. Wash in running tap water.
8. Dehydrate, clear and mount.

Result

Disulphides	Dark blue

Rationale

Adams and Sloper (1955) were the first to introduce to demonstrate cysteins in paraffin sections. With cryostat and freeze-dried sections, this is an excellent technique which gives best results. Performic acid oxidizes the cysteins to cysteine sulphuric acid which stains with the basic dye alcian blue.

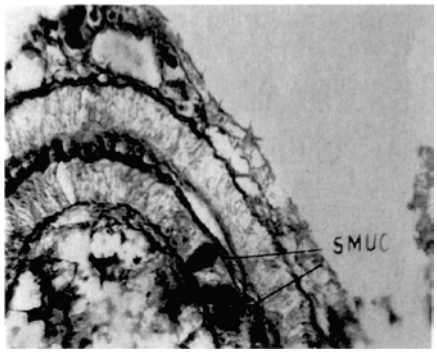


Figure 8.14 Mocous cells of stomach region of *G. orcula* stained with performic acid/alcian blue. Blue shade indicates the presence of disulphides (S–S); Stomach mucous cells. 21 μm (*See* Plate 2.6)

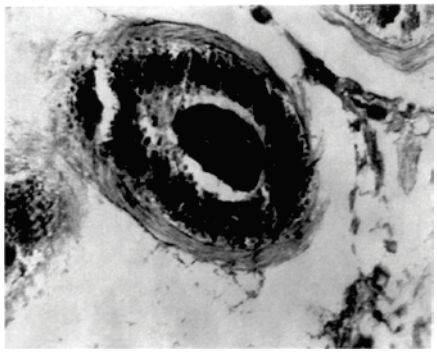


Figure 8.15 Vas deferens of *G. orcula* is rich with disulphides (performic acid/ alcian blue). 21 μm (*See* Plate 2.7)

Ferric Ferricyanide Technique for Sulphydryls

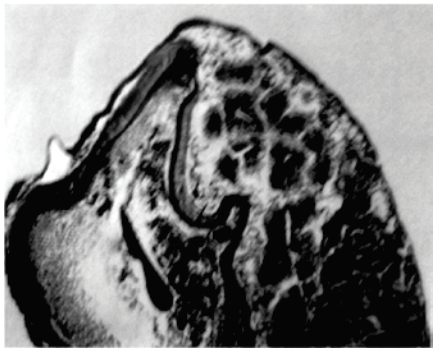


Figure 8.16 Gastric shield of *G. orcula* stained with ferric ferricyanide showing the presence of sulfhydryl groups (SH); GS: Gastric shield 21 μm (*See* Plate 2.8)

Fixation

Any general fixative

Reagents required

Potassium ferricyanide

Ferric chloride

Preparation of reagents

Solution 1 Potassium ferricyanide

Potassiuim ferricyanide	1.0 g
Distilled water	100.0 ml

Solution 2 Ferric chloride solution

Ferric chloride	1.0 g
Distilled water	100.0 ml

Solution 3 Working solution

4.0 ml of solution 1 and 3.0 ml of solution 2 and 6.0 ml of distilled water.

All these reagents should be prepared just before use.

Procedure

1. Deparaffinize and bring slides to water.
2. Immerse slides in solution 3 for 10 min.
3. Wash in tap water.
4. Dehydrate, clear and mount.

Result

Sulphydryls	Blue
Nuclei	Red

TECHIQUES FOR AMYLOIDS

Crystal Violet Method (Lief, 1947)

Fixation

10 per cent formalin or alcohol

Reagents required

Crystal violet

Hydrochloric acid

Preparation of reagents

Solution 1 Crystal violet stock solution

Crystal violet	15.0 g
95 per cent alcohol	100.0 ml

Solution 2 Working solution

Solution 1	10.0 ml
Distilled water	300.0 ml
Concentrated HCl	1.0 ml

Procedure

1. Dewax and bring sections down to water.
2. Transfer to solution 2 for 5 min.
3. Wash in distilled water.
4. Mount in glycerine jelly.

Result

Amyloid	Purple
Background	Blue

Congo red method modified by Highman (1946)

Fixation

All types—Frozen sections are recommended

Reagents required

Congo red
Potassium hydroxide
Absolute alcohol

Preparation of reagents

Solution 1

Congo red	0.500 mg
Absolute alcohol	50.0 ml
Distilled water	50.0 ml

Solution 2

Potassium hydroxide	0.200 mg
Absolute alcohol	30.0 ml
Distilled water	20.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place in solution 1.
3. Wash in distilled water.
4. Transfer to solution 2.

5. Wash in tap water.
6. Dehydrate, clear and mount.

Result

Amyloid	Orange-red
Elastin	Orange

TECHNIQUES FOR NUCLEIC ACIDS

DNA Feulgen Nuclear Reaction (Feulgen and Rosenbeck, 1924)

Fixation

Any fixative

Reagent required

Concentrated hydrochloric acid

Schiff's reagents

Potassium metabisulphite

Preparation of reagents

Solution 1 (HCl)

Concentrated HCl	8.5 ml
Distilled water	91.5 ml

Solution 2

Schiff's reagents

Described in periodic acid/Schiff's reaction.

Solution 3 (Potassium metabisulphite solution)

10 per cent potassium metabisulphite (10 g/100ml distilled water)	5.0 ml
N-hydrochloric acid	5.0 ml
Distilled water	9.0 ml

Procedure

1. Hydrate slides to water.
2. Place sections in solution 1 at room temperature for 1 min.
3. Transfer sections to solution 2 (Schiff's) for 45 min.
4. Rinse sections in solution 3 (3 changes), 2 min. each.
5. Rinse in distilled water.
6. Couterstain with light green if necessary for 2 min.

7. Wash in water.
8. Dehydrate, clear and mount.

Result

DNA	Purple
Cytoplasm	Green

Remarks

Caution to be taken for hydrolysis time. It depends upon the fixative. N-HCl should be preheated.

Retionale

It was first introduced by Feulgen and Rosenbeck (1924). It is a specific technique for DNA. During hydrolysis, only deoxyribose sugar reacts with N-HCl and ribose sugar does not interfere and is not hydrolysed. During hydrolysis free aldehydes result when treated with Schiff's reagent produces a coloured compound with DNA. Results depend upon the hydrolysis time which varies with fixative. If hydrolysis is prolonged and exceeds the time limit, intensity of stain becomes weaker and weaker and ultimately disappears. It is better to avoid Bouin's fixative because over-hydrolysis takes place during fixation. Formol saline and Carnoy are the best. Immersion in cold N/1 hydrochloric acid before and after treatment at 60°C is desired.

Hydrolysis in N-HCl at 60°C (Bauer,1932)

Fixative	Time in min.
Bouin's	Not recommended
Helly	6
Carnoy	8
Flemings	8
Formalin	8–10
Zenker-formol	5
Newcomers	20
Regaud	14
Susa	18
Zenker	5
Champy	25

Pyronin-methyl green for nucleic acid (Elias 1969)

Fixation

Carnoy

Reagents required

Methyl green

Acetate buffer (Walpole)

Pyronin

Preparation of reagents

Solution 1

Methyl green	500 mg
Acetate buffer	100.0 ml
Pyronin G or Y	200 mg

Procedure

1. Deparaffinize and hydrate slides to water.
2. Treat with solution 1 for 1 hr at 37°C.
3. Rinse in cold distilled water.
4. Rinse in butanol.
5. Dehydrate in butanol giving 2 changes of 5 min. each.
6. Clear and mount.

Result

Nuclear and cytoplasmic basophilic substance	Red

Thionine methyl green for nucleic acids (Roque *et al.*, 1965)

Fixation

4 per cent formaldehyde in 1 per cent sodium acetate for 3 hrs.

Reagents required

Methyl green

Thionine

Citrate buffer

Preparation of reagents

Solution 1

Methyl green	100 mg
Thionine	16 mg
Citrate buffer pH 5.8	100.0 ml

First dissolve thionine in little water and then add buffer and methyl green, Shake well, filter. Methyl green is purified with chloroform extraction.

Procedure

1. Dewax and hydrate slides to water.
2. Treat with solution 1 for 30 min. at 40°C.
3. Rinse in distilled water.

4. Dehydrate in a mixture containing 80.0 ml of 3 butyl alcohol and 20 ml of absolute alcohol giving 3 changes.
5. Rinse in absolute alcohol, clear and mount.

Result

Chromatin	Green or blue-green
Nuclear and cytoplasmic basophilic substance	Red

RNA, DNA– Methyl green pyronin method (Pappenheim, 1899, Unna, 1902; Bancroft and Cook, 1994)

Fixation

All types; preferably freeze-dried

Reagents required

0.1 M acetate buffer

Pyronin Y

Methyl green

Chloroform

Preparation of reagents

Solution 1 Methyl green

Dissolve 2 g of methyl green in 100 ml of distilled water. Place this solution while stirring in a separating funnel. Add 100.0 ml chloroform and shake well. Discard contaminated chloroform. Repeated extraction with chloroform is necessary.

Solution 2 Pyronin Y

Pyronin Y	2.0 g
Distilled water	100.0 ml

Solution 3 Staining solution

Methyl green (Solution 1)	7.5 ml
Pyronin Y (Solution 2)	12.5 ml
Acetic buffer (pH 4.8)	30.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer slides to solution 3 for 4–10 min.
3. Blot and dry.
4. Dip rapidly in absolute acetone.
5. Dip rapidly in 10 per cent acetone in xylene.
6. Dip rapidly in 50 per cent acetone in xylene.

7. Transfer to xylene.
8. Transfer sections to fresh xylene and mount in DPX.

Result

DNA	Green
RNA	Red

Rationale

First introduced by Pappenheim (1899), this was later modified by Unna (1902); Taft (1951), Trevan and Sharrock (1951), Brachet (1953) and Kurnic (1955). Kurnic (1955) has suggested that while methyl green blends with DNA, two sites are involved and two phosphoric groups of the DNA combine with the amino group.

Methyl green-pyronin Y method for RNA–DNA (Treven and Sharrock, 1951 modified)

Fixation

Neutral fixatives, neutral buffered formalin

Reagents required

Acetate buffer pH 4.8

5% per cent pyronin Y

2% per cent Methyl green

Preparation of reagents

Solution 1 Methyl green–Pyronin Y solution

2 per cent methyl green (chloroform washed)	10.0 ml
5 per cent pyronin Y	17.5 ml
Distilled water	250.0 ml

Solution 2 Acetic buffer

Acetic buffer (pH 4.8)

Solution 3 Working solution

Solution 1	25.0 ml
Solution 2	200 ml

Procedure

1. Deparaffinize and bring down slides to water.
2. Dip in distilled water and then blot.
3. Transfer to solution 3 (working solution) for 20–30 min.
4. Rinse rapidly in distilled water.
5. Dehydrate, clear and mount.

Result

DNA	Green to bluish green
RNA	Red

Gallocyanin–chrome alum method for RNA and DNA (Einarson, 1932, 1951)

Fixation

All types

Reagents required

Gallocyanin

Chrome alum

Preparation of reagents

Solution 1 Chrome alum–gallocyanin

Dissolve 5.0 g of chrome alum in 100.0 ml of distilled water and then add 150 mg of gallocyanin. Heat the solution to boiling for 5 min. Cool it and adjust it to 100.0 ml.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer slides to solution 1 for 18–48 hrs.
3. Wash thoroughly in tap water.
4. Dehydrate, clear and mount.

Result

DNA and RNA	Blue

Rationale

This method was first introduced by Einarson (1932) for Nissl granules. Later in 1951 this technique was applied to demonstrate nucleic acids. In this technique phosphoric acid residue, molecules of nucleic acids combine with gallocyanin at pH 1.0. At pH 2.0 and above, other tissues stain. Thus it is important to maintain pH 1.0.

DNA–Napthoic acid hydrazine–Feulgen method (Pearse, 1951)

Fixation

All types

Reagents required

Veronol acetate buffer

2-hydroxy-3-naphthoic acid hydrazine

Acetic acid

Fast blue B

N-hydrochloric acid

Preparation of reagents

Solution 1 Acid water

Concentrated HCl	8.5 ml
Distilled water	91.5 ml

Solution 2 Fast blue B solution

Fast blue B	50 mg
Veronol acetate buffer (pH 7.4)	50.0 ml

This buffer should be prepared before use.

Solution 3 NAH solution

2-Hydroxy-3-naphthoic acid hydrazide	50 mg
Absolute alcohol	30.0 ml
Conc. acetic acid	3.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Immerse briefly in solution 1 (room temperature).
3. Transfer sections to solution 1 at 60°C.
4. Again transfer to solution 1 at room temperature for 1 min.
5. Transfer to distilled water for 1 min.
6. Transfer to 50 per cent alcohol for 10 min.
7. Transfer sections to solution 3 (NAH) at room temperature.
8. Transfer to 50 per cent alcohol for 10 min.
9. Transfer to distilled water for 1 min.
10. Transfer sections to solution 2 (fast blue B) for 3 min.
11. Dehydrate, clear and mount.

Result

DNA	Bluish purple
RNA	Purple red

Rationale

This method can also be used as a control for Feulgen reaction. When sections are hydrolysed at 60°C, free aldehydes are released and they combine with 2-hydroxy-3-naphthoic acid hydrazide. This is then coupled to Fast blue B producing a purplish blue colour at the site of coupling. Though dinitrophenyl hydrazine was used in the ratio 2 : 4 by Danielli (1947), best results are obtained with 2-hydroxy-3-naphthoic acid.

RNA-DNA– Acridine orange (Bertalanffy and Nagy, 1962)

Fixation

Freeze-dried, frozen cryostat

Paraffin—Best fixatives are acetic ethanol and 70 per cent alcohol

Reagents required

0.2 M phosphate buffer (see page)

Calcium chloride

Acridine orange

Acetic acid

Preparation of reagents

Solution 1 (Acridine orange solution)

Acridine orange	50 mg
Distilled water	100.0 ml

The pH of the solution should be 6.0 with addition of phosphate buffer. The volume is made to 50 ml.

Solution 2 Phosphate buffer

Solution 3 (Calcium chloride solution)

Calcium chloride	11.0 g
Distilled water	50.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Rapid dip in 1 per cent acetic acid for 15 sec.
3. Rinse in distilled water.
4. Transfer slides to solution 1 (acridine orange) for 2 min.
5. Place sections in solution 2 (phosphate buffer) for 1 min.
6. Differentiate in solution 3 for 20 sec.
7. Bring sections again to solution 2 (phosphate buffer).
8. Mount sections wet and examine under fluorescence microscope.

Result

DNA	Red
RNA	Light green

Rationale

Clarity and perfection of the technique depend on the fixative, concentration of acridine orange and pH of the working solution (6.0). If the concentration of the stain is more than what is required, red colour will overwhelm green colour.

Menzies Method (Menzies, 1963)

Fixation

10 per cent neutral buffered formalin

Reagents required

Tetrahydrofuran
Hydrochloric acid
Azure B
Azure II
Basic fuchsin
Glacial acetic acid

Preparation of reagents

Solution 1 Hydrochloric acid tetrahydrofuran

Hydrochloric acid	1.0 g
Tetrahydrofuran	90.0 ml
Distilled water	250.0 ml

solution 2 1 per cent basic fuchsin solution stock

Azure B	1.0 g
Distilled water	100.0 ml

Solution 3 0.3 per cent basic fuchsin solution

Basic fuchsin	100 mg
Distilled water	100.0 ml

Solution 4 (Azure B basic fuchsin solution)

Solution 2	30.0 ml
Solution 3	8.0
Glacial acetic acid	2.0 ml

Procedure

1. Deparaffinize and bring down slides to water.
2. Place in solution 1 at 37°C for 5 min.
3. Directly transfer to solution 4 for 30 min.
4. Rinse in acetone.
5. Clear in xylene and mount.

Result

DNA	Red
RNA	Blue

Spicer's method for nucleic acids (Spicer, 1916 b)

Fixation

Bouin's solution

Reagents required

Basic fuchsin
Sodium metabisulphite
Citric acid
Disodium phosphate
Methylene blue

Preparation of reagents

Solution 1 Schiff's reagent

Solution 2 (0.5 per cent sodium metabisulphite solution)

sodium metabisulphite	500 mg
Distilled water	100.0 ml

Solution 3 0.1 M citiric acid solution

Citiric acid	19.21 g
Distilled water	1000.0 ml

Solution 4 0.2 M disodium phosphate solution

Disodium phosphate	28.40 g
Distilled water	1000.0 ml

Solution 5 Methylene blue solution

Methylene blue	10 mg
Solution 3 citric acid 0.2 M	28.6 ml
Solution 4 Disodium phosphate	11.4 ml

Procedure

1. Dewax and hydrate slides to water.
2. Transfer to solution 1 for 10 min.
3. Rinse in three changes in solution 2.
4. Wash in running water.
5. Transfer to solution 5 for 80 min.
6. Dehydrate, clear and mount.

Result

DNA	Red
Chromosomes	Red

Chromatin	Red
Cytoplasmic RNA	Blue

TECHNIQUES FOR LIPIDS

Oil Red 0 Method (Lillie and Ashburn, 1943)

Fixation

Formalin calcium, frozen cryostat post-fixed

Reagents required

Oil red O

Triethyl phosphate

Haematoxylin

Preparation of reagents (Oil red O solution)

Oil red O	1.0 g
Triethyl phosphate	60.0 ml
Distilled water	40.0 ml

Add distilled water to triethyl phosphate and then add the dye. Heat the solution, stirring constantly, cool and filter. This forms the stock solution which should be filtered before use.

Procedure

1. Wash sections in distilled water.
2. Transfer sections to 60 per cent triethyl phosphate.
3. Stain in oil red O solution at 20°C for 15 min.
4. Wash section in 60 per cent tri ethylphosphate 30 sec.
5. Wash in distilled water.
6. Stain sections in haemataxylin for 1 min.
7. Wash and mount in glycerine jelly.

Result

Lipids	Red
Nuclei	Blue

Osmium tetroxide method (Mallory, 1944)

Fixation

10 per cent neutral filtered formalin

Reagents required

Osmium tetroxide

Preparation of reagent

Solution 1

Osmium tetroxide	1.0 g (ampoule)
Distilled water	100.0 ml

With a file make a deep constriction on the ampoule and drop into distilled water and shake vigorously so that ampoule breaks. Osmium tetroxide dissolves in water. This method is to prevent from breathing fumes.

Procedure

1. Cut 10–15 μ on frozen sections.
2. Treat with osmium tetroxide for 24 hrs.
3. Wash in distilled water giving several changes for 12 hrs.
4. Treat with absolute alcohol for 5 hrs.
5. Wash well in distilled water.
6. Mount in glycerine jelly.

Result

Lipids	Black
Background	Brown

Sudan Black B for Bound Lipids in Paraffin Sections

Preparation of reagent

Sudan black B saturated in 70 per cent alcohol.

Procedure

1. Bring sections to 70 per cent alcohol.
2. Stain for 30 min. in Sudan black solution.
3. Rinse quickly in 70 per cent alcohol.
4. Wash in running water.
5. Countertstain in Mayer's haemalum.
6. Wash in water.
7. Mount in glycerine jelly.

Result

Lipids stain black.

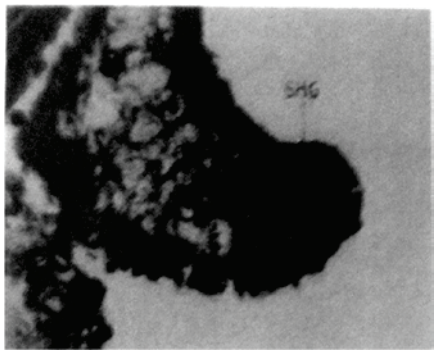


Figure 8.17 Shell gland of G. orcula showing positivity to Sudan black B (Lipids). the 21 μm; SHG: Shell gland. (*See* Plate 3.1)

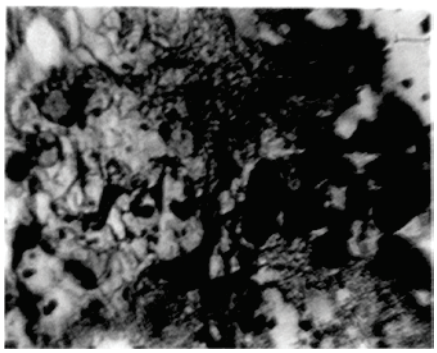


Figure 8.18 Reserve cells in intermoult cuticle of *E.asiatica* stained with Sudan black B. Reserve cells are loaded with lipids 21 μm. (*See* Plate 3.2)

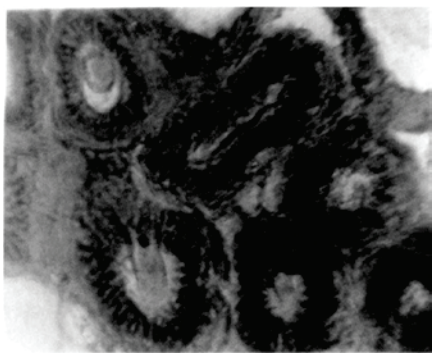


Figure 8.19 Vas deferens of *G. orcula* stained with Sudan black B showing the presence of acid lipids 104 mm. (*See* Plate 3.3)

Copper Phthalocyanin for Phospholipids (Klüver & Barrera 1953)

Fixation

Formol calcium for frozen section

Reagents required

Luxol fast blue G

Lithium carbonate

Neutral red

Preparation of reagents

Solution 1 Luxol fast blue solution

Luxol fast blue	10 mg
95 per cent alcohol	100.0 ml

Solution 2 Lithium carbonate solution

Lithium carbonate	50 mg
Distilled water	100.0 ml

Solution 3

Neutral red	1.0 g
Distilled water	100.0 ml

Procedure

1. Bring sections to absolute alcohol.
2. Place sections in solution 1 for 6–18 hrs at 60°C.
3. Rinse in 70 per cent alcohol and wash in water.
4. Differentiate insolution 2 for 30 min.
5. Rinse in water.
6. If desired counterstain in solution 3 for 5 min.
7. Dehydrate, clear and mount in Canada balsam.

Result

Phospholipids except sphingomyelins stain blue.

A dark blue-reddish shade denotes the presence of phospholipids.

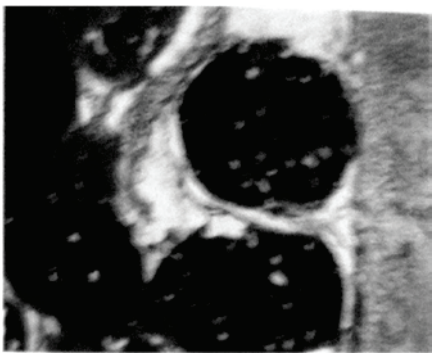


Figure 8.20 Section of ovary of *Cardisoma* the

Figure 8.21 Neurosecretory cell in

cornifex. Stage 4 oocytes loaded with phospholipid bodies (copper phthalocyanin) 21 μm. (*See* Plate 3.4)

brain *E. asiatica* stained with copper phthalocyanin 90 μm. (*See* Plate 3.5)

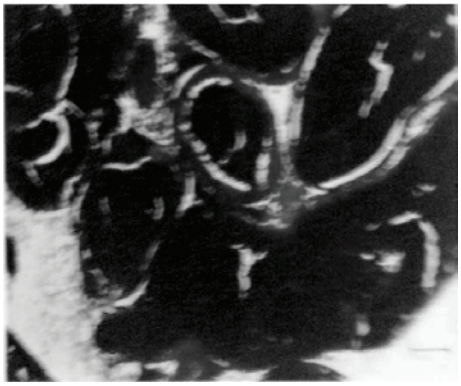


Figure 8.22 Secretions of accessory glands of *O.platytarsis* stained with copper phthalocyanin 24 mm. (*See* Plate 3.6)

Acid haematein for phospholipids (Hori, 1963)

Fixation

Either in formol calcium (10 per cent formalin in 1 per cent calcium chloride Formalin calcium / cadmium (1 per cent cadmium chloride is added to formalin calcium)

Glutaraldelyde calcium chloride (2:5:25)

Reagent required

Haematoxylin

Sodium iodate

Glacial acetic acid

Borax

Potassium ferricyanide

Preparation of reagents

Solution 1 Acid haematein

Haematoxylin	50 mg
0.01 per cent sodium iodate	50.0 ml

Heat to boil, cool and then add 1.0 ml of glacial acetic acid.

Solution 2

Borax	0.250 mg

Potassium ferricyanide	0.250 mg
Distilled water	100.0 ml

Store in refrigerator.

Procedure

1. Cut frozen section of 1μm thick.
2. Post-chromate sections in 5 per cent aqueous potassium dichromate at 60°C for 4 hours.
3. Wash in distilled water.
4. Treat with solution 1 for 30 min. at 37°C.
5. Wash in distilled water.
6. Differentiate in solution 2.
7. Dehydrate, clear and mount.

Result

Phospholipids	Blue to blue-grey

TECHNIQUES FOR PIGMENTS

Dunn–thomson Method for Haemoglobin (Dunn–Thomson,1945)

Fixation

All types

Reagents required

Acid fuchsin
Haematoxylin
Iron alum
Picric acid

Preparation of reagents

Solution1 Haematoxylin

Haematoxylin	250 mg
Distilled water	100.0 ml

Solution2 Van Gieson stain

1per cent acid fuchsin	13.0 ml
Saturated picric acid	100.0 ml

Solution 3 Alum

Iron alum	4.0 g
Distilled water	100.0 ml

Procedure

1. Hydrate slides to water.
2. Place in solution 1(aqueous haematoxylin) for 15 min.
3. Wash in water.
4. Mordant in solution 3(iron) for 1 min.
5. Transfer to 0.25 per cent aqueous haematoxylin.
6. Rinse in water.
7. Transfer to solution 2(Van Gieson) for 15 min.
8. Rinse in 95 per cent alcohol for 3 min.
9. Rinse in 10 per cent alcohol, clear in xylene and mount in DPX.

Result

Haemoglobin	Greenish black

Remarks

Any fixative with an acid ingredient should be avoided.

Leucopatent Method for Haemoglobin: (Dunn–Thomson)

Fixation

Formalin-paraffin sections

Reagents required

Patent blue V
Powdered zinc
Glacial acetic acid
Hydrogen peroxide
Mayer's haemalum

Preparation of reagents

Solution 1 Stock solution

Patent blue V	1.0 g
Distilled water	100.0 ml
Powdered zinc	2.0 g
Glacial acetic acid	2.0 ml

In a round-bottomed flask, mix all the ingredients. Then boil the solution gently for about 15 min. till the solution becomes straw-coloured. Allow it to cool and then filter and store it.

Solution 2 Working solution

Solution 1	30.0 ml
Glacial acetic acid	6.0 ml
3 per cent (10 V) H_2O_2	3.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in solution 2 (working solution) for five min.
3. Rinse in water.
4. Counterstain in Mayer's haemalum for one min.
5. Rinse in water.
6. Dehydrate, clear and mount.

Result

Haemoglobin	Dark blue-green
Eosinophil and neutrophil granules	Dark blue
Nuclei	Red

Rationale

It is based upon the demonstration of haemoglobin peroxidase and has replaced similar peroxidase methods such as carcinogenic benzidine method. Since haematoxylin is a peroxidase method otherwise oxidase containing elements will also be determined.

Puchtler and Sweat Method for Haemoglobin and Haemosiderin (Puchtler & Sweat, 1963)

Fixation

Zenker formol

Reagents required

Potassium ferrocyanide
Hydrochloric acid
Tannic acid
Phosphomolybdic acid
Phloxine B
Methanol
Glacial acetic acid

Preparation of reagents

Solution 1 2 per cent potassium ferrocyanide

Potassium ferrocyanide	2.0 g

Distilled water	100.0 ml

Solution 2 2 per cent Hydrochloric acid – stock

Concentrated hydrochloric acid	2.0 ml
Distilled water	100.0 ml

Solution 3 Potassium ferrocyanide–hydrochloric acid working solution

Solution 1	50.0 ml
Solution 2	50.0 ml

Solution 4 Tannic acid solution – 5 g + 100 ml water

Solution 5 1 per cent phosphomolybdic acid (1 g/100 ml water)

Solution 6 Phloxine B solution

Phloxine B	5.0 g
Glacial acetic acid	50.0 ml
Methyl alcohol	90.0 ml

Solution 7 Methanol glacial acetic acid solution

Methanol	95 ml
Glacial acetic acid	5 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in solution 3 for 30 min.
3. Rinse in distilled water.
4. Transfer to solution 4 for 10 min.
5. Rinse in distilled water.
6. Place in solution 5 for 10 min.
7. Rinse in distilled water.
8. Transfer to solution 6 for 5 min.
9. Differentiate in solution 7.
10. Dehydrate, clear and mount.

Result

Haemoglobin	Red
Haemosiderin	Blue-green or dark blue

Gmelin Method for Bile Pigments

Fixation

All types of fixatives especially formalin

Reagents required

Concentrated nitric acid

Absolute alcohol

Preparation of reagents

Solution 1 Nitric acid alcohol

Concentrated nitric acid	5.0 ml
Absolute alcohol	5.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Flood the slide with solution 1.
3. Excess stain is drained off with filter paper.
4. Place the coverslip and ring the coverslip with paraffin.

Results

Bile pigment	Reddish green

Rationale

Tissues fixed in formalin can be used in this method. Concentrated nitric acid damages the tissues. Oxidation is completed in 3 stages producing 3 different colours, viz., red, purple and green. Nitric acid is replaced by nitrous acid which on oxidation also exhibits 3 colours, viz., green, purple and red. Both preparations are temporary.

Bile Pigment Staining (Bilirubin) (Glenner, 1957)

Fixation

Fresh frozen sections (Adamstone and Taylor, 1948) or formalin fixed—fixation time 6 hrs—carbowax sections.

Reagents required

Potassium dichromate

Potassium dihydrogen phosphate

Haematoxylin

Preparation of reagents

Solution 1 Potassium dichromate solution

3 per cent potassium dichromate (3 g/100 ml water)	25.0 ml
Buffer pH 2.2 (0.1 NHCl)	8.0 ml
0.1N Potassium dihydrogen phosphate	170 ml

Procedure

1. Place sections in solution 1 for 15 min. at room temperature.
2. Wash in running water for 5 min.
3. Counterstain in haematoxylin.
4. Dehydrate rapidly.
5. Clear and mount.

Result

Bilirubin	Emerald green

Rationale

Bile is oxidized to green biliverdin by the iodine solution.

Glenner's Method for Bilirubin (Haemosiderin and Lipofuchsin, 1957)

Fixation

Frozen sections or cryostat sections

Reagents required

Potassium dichromate

Acetic acid

Potassium ferrocyanide

Oil red O

Preparation of reagents

Solution 1 Potassium dichromate

Potassium dichromate	2.0 g
Distilled water	100.0 ml

Solution 2 Potassium ferrocyanide

Potassium ferrocyanide	2.0 g
Distilled water	100.0 ml

Procedure

1. Cut frozen sections.
2. Immerse sections in solution 1 for 5 min.
3. Transfer sections to a solution containing equal parts of 5 per cent acetic acid and freshly prepared solution 2 for 20 min.
4. Rinse in running water and treat with buffered dichromate solution for 15 min.
5. Rinse in water.

6. Transfer sections to Oil red O solution (saturated solution, i.e., 0.25–0.5 per cent in isopropyl alcohol) for 20 min.
7. Rinse in 70 per cent alcohol and remove excess stain.
8. Wash in running water.
9. Mount in Apathy's medium.

Result

Bilirubin	Green
Haemosiderin	Blue
Lipofuchsin	Red

Stein's Technique for Bilirubin

Procedure

1. Hydrate slides to water.
2. Transfer sections to a mixture of 3 parts of Lugol's iodine and 1 part of tincture of iodine for 6–12 hrs.
3. Decolorize with 5 per cent aqueous solution of sodium sulphate for 15–20 sec.
4. Counterstain if desired nuclei in alum carmine 1–3 hrs.
5. Wash well.
6. Dehydrate in acetone, clear in xylene and mount in Canada balsam.

Result

Bilirubin	Green
Nuclei	Red

Hall's Method for Bilirubin (Hall, 1960)

Fixation

10 per cent buffered neutral formalin

Reagents required

Trichloroacetic acid

Ferric chloride

Picric acid

Acid fuchsin

Preparation of reagents

Solution 1 Fouchet's solution

Trichloroacetic acid	25.0 g
Distilled water	100.0 ml

To this solution add 10 per cent ferric chloride—10.0 ml solution 1

Ferric chloride solution

Ferric chloride	10.0 g
Distilled water	100.0 ml

Solution 2 Van Gieson's solution

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1.
3. Wash in running water.
4. Place in solution for 2–5 min.
5. Dehydrate, clear and mount.

Result

Biliverdin	Green
Collagen	Red
Muscle	Yellow

Long Ziehl-neelsen Method for Lipofuchsin

Fixation

Any general fixative

Reagents required

Basic fuchsin
Phenol
Absolute alcohol
Haematoxylin

Preparation of reagents

Solution 1 Carbol fuchsin

Basic fuchsin	1.0 g
Phenol	500 mg
Absolute alcohol	10.0 ml
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Immerse sections in solution 1 for 3 hrs at 60°C.
3. Wash in tap water.

4. Differentiate in acid alcohol.
5. Wash in tap water.
6. Counterstain in haematoxylin.
7. Dehydrate, clear and mount.

Result

Lipofuchsin	Red
Nuclei	Blue

Rationale

Since lipofuchsin is lipid in nature, it exhibits the property of acid fastness due to tubercle bacilli. With increasing staining period, the colour becomes brighter.

Sudan black B and aldehyde fuchsin techniques have also been considered to be revealing lipofuchsin.

Melanin, Lipofuchsin and Argentaffin—Schmorl's Method

Fixation

Any fixative

Reagents required

Ferric chloride

Potassium ferricyanide

Safranin

Acetic acid

Preparation of reagents

Solution 1 Ferric chloride solution

Ferric chloride	50 mg
Distilled water	50.0 ml

Solution 2 Potassium ferricyanide solution

Potassium ferricyanide	500 mg
Distilled water	50.0 ml

Solution 3 Staining solution

Solution 1	37.5 ml
Solution 2	5.0 ml
Distilled water	7.5 ml

Procedure

1. Hydrate slides to water.

2. Immerse in solution 3 for 30 sec; watch staining intensity. If the colour is intense take out the slide.
3. Differentiate in 1 per cent acetic acid.
4. Wash in tap water.
5. Dehydrate, clear and mount.

Result

Melanin	Deep blue
Argentaffin granules	Deep blue
Lipofuchsin	Deep blue

Rationale

Ferric chloride and potassium ferricyanide reduce ferric ferricyanide to ferrocyanide when a blue precipitate develops. Staining time should be controlled. Melanin and lipofuchsin take the stain earlier than argentaffin.

Modified Giemsa stain for chromaffin cell granules

Reagents required

Giemsa stain

Acetic acid

Preparation of reagents

Solution 1 Giemsa stain

Giemsa's stain	2 ml
Distilled water	48.0 ml

Adjust pH to 6.8 with 0.5 acetic acid

Procedure

1. Deparaffinize and hydrate slides to water.
2. Rinse in distilled water.
3. Place in dilute solution 1 overnight.
4. Rinse in distilled water.
5. Wash in 0.5 per cent acetic acid.
6. Wash, dehydrate, clear and mount.

Result

Chromaffin granules	Greenish yellow
Nuclei	Blue

Argentaffin Cell Granules—Alkaline Diazo Method

Fixation

Formalin

Reagents required

Diazonium salt Fast Red B

0.1 M veronol acetate buffer pH 9.2

Fast red salt B

Haematoxylin

Preparation of reagents

Solution 1 Staining solution

0.5 M veronol acetate buffer (pH 9.2) containing 1 mg or 1 ml diazotate of 5-nitroanisidine (Fast red salt B)

Solution 2 Mayer's haemalum (*See* same chapter)

Procedure

1. Deparaffinize and hydrate slides to water.
2. Immerse slides in staining solution 1 for 30 sec. at 4°C.
3. Wash.
4. Stain nuclei in solution 2 for 5 min.
5. Wash.
6. Dehydrate, clear and mount.

Result

Argentaffin Orange-red

Nuclei Blue

Rationale

At pH 9.2, Fast red salt B added to veronol acetate engenders coupling between phenolic components of argentaffin granules and Fast Red Salt B. The resulting compound is orange-red.

Nor-adrenaline Fluorescence Technique

Fixation

Freeze-dried or formalin-frozen

Procedure

1. Fix in formol saline for 24 hrs.
2. Cut frozen sections of 10 m thickness.
3. Mount in glycerine jelly.
4. Examine under fluorescence microscope.

Freeze-dried

1. Freeze-dry.
2. Fix in formalin vapour.
3. Embed in paraffin wax.
4. Cut 8 μ thick sections.
5. Mount in light petroleum.
6. Examine under fluorescence microscope.

Result

Strong fluorescence (Yellowish – Green) Presence of nor-adrenaline

Chromaffin Reaction for Nor-adrenaline and Adrenaline (Hillarp and Hokfelt, 1955)

Fixation

Fresh sections

Reagents required

Potassium dichromate

Potassium chromate

Preparation of reagents

Solution 1 Potassium dichromate solution

Potassium chromate	5.0 g
Distilled water	100.0 ml

Solution 2 Potassium chromate solution

Potassium dichromate	5.0 g
Distilled water	100.0 ml

Solution 3 Working solution

Solution 1	10 vol. or 100.0 ml
Solution 2	1 vol. or 10.0 ml

Procedure

1. Place fresh slices in solution 3 for 16 hrs and at room temperature.
2. Wash in 3 changes of distilled water.
3. Mount in glycerine jelly.
4. Dehydrate, clear and mount.

Result

Adrenaline	Dark brown
Nor-adrenaline	Yellow-brown

Iodate Method for Nor-adrenaline: (Hillarp And Hokfelt, 1955)

Fixation

Fresh

Reagents required

Potassium iodate

Formalin

Preparation of reagents

Solution 1 Potassium iodate solution

Potassium iodate	10.0 g
Distilled water	100.0 ml

Procedure

1. Keep fresh slices of tissue in solution 1 for 16 hrs at room temperature.
2. Place them in 10 per cent formalin for two hours.
3. Cut sections of 20 μ thickness.
4. Wash and counterstain nuclei if desired.
5. Mount sections on slides, dehydrate, clear and mount.

Result

Nor-ardrenaline—Brown

REMOVAL OF PIGMENTS

1. Permanganate Method (Lillie, 1965)

1. Hydrate slides to water.
2. Transfer slides to 0.1 per cent potassium permanganate for 12–24 hrs.
3. Wash in running water.
4. Transfer slides to 1 per cent oxalic acid for 1 min.
5. Wash and proceed to stain.

2. Performic or Peracetic Acid Methods

Bleaching is done by immersing slides in either performic acid or peracetic acid for 1–2 hrs.

Performic acid Add 8 ml of 90 per cent formic and to 31 ml of 30 per cent H_2O_2 and 0.22 ml of concentrated sulphuric acid, store it below temperature of 25°C. 4.7 per cent of performic acid is formed which will last only for few hours.

Peracetic acid Add 95.6 ml of acetic acid to 259 ml of 30 per cent H_2O_2 and 0.22 ml of concentrated H_2SO_4.

Allow it to stand for 3 days. Add 40 mg of disodium phosphate. Store at 1°C. This stands for several months.

3. Chlorate Method

Immerse sections in a mixture containing 50 per cent alcohol to which little of potassium chlorate and a few drops of HCl are added. Before staining, wash.

4. Bromine Method

Wash in 1 per cent bromine water. Immerse slides for 24 hrs .

5. Chromic Acid Method

Immerse slides in a mixture containing 1 per cent chromic acid and 5 per cent calcium chloride for 8–12 hrs.

6. Peroxide Method

Immerse slides for 24–48 hrs in 10 per cent H_2O_2; wash well before staining. This is a specific method for melanin.

Formalin Pigment

Brown or black pigment in the form of crystalline granules is formed by formalin, which are considered as haematein derivatives.

Baker Method

One per cent potassium hydroxide is added to 80 per cent alcohol or picric acid until precipitate is removed.

Murdock Method (1945)

Place sections in a mixture of

3 per cent H_2O_2	25 ml
Acetone	25 ml
Ammonia	1 drop

Add ammonia dropwise till precipitate dissolves.

Barrett Method (1944)

Place section in a saturated solution of picric acid in alcohol for 10 min. to 2 hrs.

Pearse Method (1960)

Immerse slides in 90 per cent formic acid.

Malarial Pigment

This is also in the form of brownish black pigments.

Gridley Method (1975)

1. Hydrate slides to water.

2. Bleach for 5 min. in a mixture of

Acetone	50 ml
3 per cent H_2O_2	50 ml
28–29 per cent ammonia	1.0 ml

or bleach overnight in 5 per cent ammonium sulphide. Wash well before staining.

Haemosiderin

This is in the form of yellowish brown or greenish brown pigment. It does not dissolve in acids and alkalis. It can be identified by Perl's test.

Bile pigments

These are yellowish green pigments. Bleaching is not effective, and they are argentaffin-positive. It can be converted to biliverdin with H_2O_2, Lugol solution and nitrous acid.

TECHNIQUES FOR MINERALS

Perl's Prussian Blue Reaction for Iron (perl, 1867)(Plate 50, Fig. 1)

Fixation

Any general or frozen section

Reagents required

1. Potassium ferricyanide
2. Hydrochloric acid
3. Neutral red

Preparation of reagents

Solution 1 Potassium ferricyanide solution

Fresh potassium ferricyanide	2.0 g
Distilled water	100.0 ml

Solution 2 Hydrochloric acid

2 per cent hydrochloric acid	2.0 ml
Distilled water	249.0 ml

Solution 3 Staining solution

2 per cent hydrochloric acid	2.0 ml
2 per cent potassium ferricyanide	25.0 ml

(Solution 1)

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to freshly prepared solution 3 (Staining solution) for 30 min.
3. Wash in water.
4. Counterstain if desired in neutral red.
5. Wash rapidly.
6. Dehydrate, clear and mount.

Results

Ferric alum	Blue
Nuclei	Red

Rationale

First introduced by Perl (1867). Both paraffin and frozen sections could be treated in this method. 2 per cent hydrochloric acid liberates iron and ferric ions. Later it reacts with potassium ferricyanide to form potassium ferric ferricyanide. The end product is an insoluble blue compound.

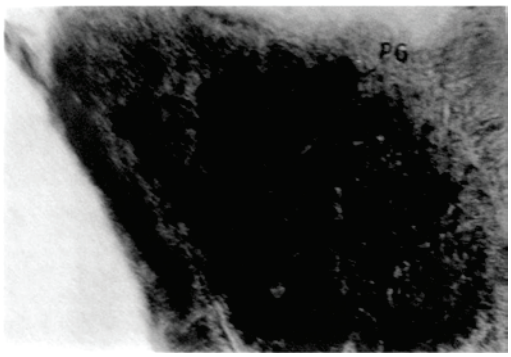


Figure 8.23 Pedal gland of *G.orcula* stained with Prussian blue positivity to Prussian blue indicates the presence of ferritn PG: Pedal gland 21 mm. (*See* Plate 3.7)

Iron Reaction: Dinitro-resorcinol (Humphrey, 1935)

Fixation

Formalin

Any general fixative with acid ingredients. 10 per cent buffered formalin is also preferable.

Reagents required

1. Dinitroresorcinol
2. Ammonium sulphate

Preparation of reagents

Solution 1

30 per cent ammonium sulphide—analytical.

Solution 2

Saturated aqueous dinitroresorcinol or 3 per cent in 50 per cent alcohol.

Procedure

3. Rinse in water.
4. Transfer to solution 2 (dinitroresorcinol) for 24 hrs.
5. Wash in water.
6. Dehydrate, clear and mount.

Results

Iron	Dark green
Background	Brown

Remarks

An old solution seems to work well. Saturated solution of dinitroresorcinol should have excess salt.

TurnBull Blue Method for Ferrous Iron (Pearse, 1953)

Fixation

10 per cent buffered formalin

Any other fixative without acid ingredient

Reagents required

1. Ammonium sulphide
2. Potassium ferricyanide
3. Hydrochloric acid
4. Safranin O

Preparation of reagents

Solution 1 Saturated ammonium sulphide

Saturated ammonium sulphide—Analytical

Solution 2 Potassium ferricyanide

Potassium ferricyanide	20.0 g
Distilled water	100.0 ml

Solution 3 Hydrochloric acid

Hydrochloric acid	1.0 ml
Distilled water	100.0 ml

Solution 4 Safranin O solution

Safranin O solution	200 mg
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Wash in distilled water.
3. Transfer to solution 1 (yellow ammonium sulphide) for 1–3 hrs.
4. Rinse in distilled water.
5. Transfer to a freshly prepared mixture containing equal parts of solution 2 and solution 3 for 20 min.
6. Rinse in distilled water.
7. Counterstain with solution 4 (Safranin O) for 2–5 min.
8. Rinse in 70 per cent alcohol.
9. Dehydrate, clear and mount.

Result

Ferrous and ferric iron	Deep blue

Alizarin Reds: (Puchtler *et al.*, 1969)

Fixation

Carnoy, avoid using formalin or Zenker formol

Reagents required

Alizarin red S

Phosphate or barbital buffer, pH 9

Preparation of reagents

Solution 1

Alizarin red S	500 mg
Phosphate buffer, pH 9	100.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in solution 1 for 30–60 sec.
3. Differentiate in buffer (pH 9) for 5 sec.
4. Dehydrate, clear and mount.

Result

Calcium	Orange-red

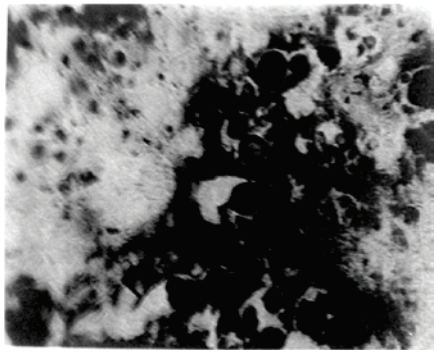


Figure 8.24 Section of foot of *G. orcula* stained with alizarin red S. Spherules show calcium; CS: Calcium spherules 90 μm. (*See* Plate 3.8)

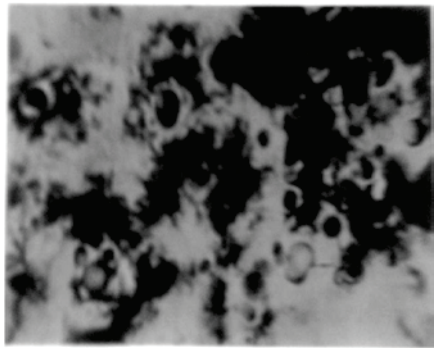


Figure 8.25 Section of mantle of *G. orcula* showing calcareous spherules full of calcium (alizarin red S) 21 μm. CS: Calcium spherules (*See* Plate 4.1)

Vonkosa Method for Calcium: (Vonkosa, 1901)

Fixation

Neutral fixatives

Reagents required

Silver nitrate

Sodium thiosulphate

Neutral red

Preparation of reagents

Solution 1 Silver nitrate solution

Silver nitrate	750 mg
Distilled water	50.0 ml

Solution 2 Sodium thiosulphate

Sodium thiosulphate	2.5 g
Distilled water	50.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer slides to solution 1 (1.5 per cent silver nitrate) for 15–30 min.
3. Wash in distilled water.
4. Transfer to solution 2 (hypo solution) for 1 min.
5. Wash in distilled water.

6. Counterstain in 2 per cent neutral red for 30 sec.
7. Wash in distilled water.
8. Dehydrate, clear and mount in Canada balsam.

Results

Calcium deposits	Brown to black
Nuclei	Red

Rationale

Calcium carbonate or calcium phosphate subjected to silver nitrate engenders calcium to be replaced by silver. With sunlight or ultraviolet light the silver salt undergoes reduction to metallic silver. When such tissue sections are subjected to sodium thiosulphate solution, calcium deposits turn black.

Carr's Method for Calcium (Carr *et al.*, 1961)

Fixation

10 per cent neutral buffered formalin

Reagents required

Sodium hydroxide

Chloranilic acid

Light green

Preparation of solutions

Solution 1 Staining solution

Distilled water	100.0 ml
Sodium hydroxide	400 mg
Chloranilic acid	1.0 g

After all acid has dissolved. Filter

Solution 2 Counterstain

Light green	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize and bring down slides to water.
2. Place in solution 1 for 30 min.
3. Wash in running water for 15 min.
4. Transfer to solution 2 for 5 min.
5. Rinse in distilled water.
6. Dehydate, clear and mount.

Result

Calcium	Red-brown
Background	Green

Benzidine Method for Copper

Fixation

Formalin

Reagents required

Benzidine hydrochloride

Ammonium thiocyanate

Neutral red

Preparation of reagents

Solution1 Benzidine solution

Benzidine hydrochloride	10 mg
Ammonium thiocyanate	30 mg
Distilled water	5.0 ml

Solution 2 Counterstain

Neutral red	1.0 g
Distilled	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Flood slides with solution 1 for 10 min.
3. Rinse in distilled water.
4. Counterstain with solution 2 for 15 sec.
5. Rinse in water.
6. Dehydrate, clear and mount.

Result

Copper deposits	Blue

Mallory and Parker's Haematoxylin Method for Lead and Copper

Fixation

Formalin or alcohol

Reagents required

Haematoxylin

Potassium hydrogen phosphate

Preparation of reagents

Solution 1

Dissolve 10 mg of haematoxylin in few drops of 95 per cent alcohol. To this, add 10 ml of 2 per cent potassium dihydrogen phosphate (filtered) solution. It should be made fresh before use.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in freshly prepared solution 1(haematoxylin) for 2–3 hrs at 56–60°C.
3. Wash in running water.
4. Dehydrate.
5. Clear in terpineol and mount in terpineol balsam.

Result

Lead	Dark grey-blue
Copper	Blue
Haemosiderin	Black

Rhodizonate Method for Lead Salts

Fixation

Neutral fixative

Reagents required

Sodium rhodizonate

Acetic acid

Preparation of reagent

Solution1 Staining solution

Sodium rhodizonate	200 mg
Distilled water	99.0 ml
Acetic acid	1.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1(rhodizonate solution).
3. Rinse in water.

4. Stain in 0.1 per cent light green in 1 per cent acetic acid.
5. Rinse in water.
6. Mount in glycerine jelly.

Result

Lead salts	Red
Background	Green

Remarks

In this technique sodium rhodizonate reacts with lead in the tissue.

Chromate Method for Lead Salts

Fixation

Cold microtome	Frozen
Formalin	Paraffin

Reagents required

Potassium dichromate

Acetic acid

Toluidine blue

Preparation of reagent

Solution 1 Potassium chromate solution

Potassium dichromate	2.0 g
1 per cent aqueous acetic acid	100.0ml

Procedure

1. Fix fresh pieces in Regaud's solution for 2 days.
2. Deparaffinize paraffin sections and bring them to water.
3. Stain in solution 1 for a few days.
4. Wash tissue in running water for 6 hrs.
5. Dehydrate, clear and embed in paraffin wax.
6. Cut 10 μ thick sections.
7. Deparaffinize and bring down slides to water.
8. Stain with 0.5 per cent toluidine blue.
9. Dehydrate, clear and mount.

Result

Sites of lead	Yellow

Solochrome Azurine Method for Aluminium and Beryllium

Fixation

Any general fixative

Reagents required

Solochrome azurine

Preparation of reagent

Solution 1 Working solution

Solochrome azurine	200 mg
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer slides to solution1 (Solochrome azurine solution).
3. Rinse in distilled water.
4. Dehydrate, clear and mount in DPX.

Result

Aluminium	Deep blue
Beryllium	Deep blue

Remarks

Both aluminium and beryllium take blue colour. The two can be differentiated by preheating the slide with an alkali when aluminium is removed. Beryllium remains unaffected.

Naphthochrome Green B Method for Aluminium and Calcium: (Denz, 1949; Pearse 1960)

Fixation

Any general fixative

Reagents required

Naphthochrome green B

Preparation of reagent

Solution 1 Staining solution

Naphthochrome green B	150 mg
Distilled water	100 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer slides to solution 1(naphthochrome green B) for 5–10 min.

3. Wash in distilled water.
4. Dehydrate, clear and mount in Canada balsam.

Result

Aluminium	Deep, brownish red

TECHNIQUES FOR CONNECTIVE TISSUES

Elastin Staining

Verhoeff's Elastin stain

Fixation

Any general fixative

Reagents required

Potassium
Iodine
Ferric chloride
Ponceau S
Acetic acid

Preparation of reagents

Verhoeff's stain

Dissolve 3 g of haematoxylin in 66 ml absolute ethyl alcohol. Cool, filter and add 24 ml of 10 per cent aqueous ferric chloride and 24 ml of Verhoeff's iodine solution. This lasts for 2 weeks.

Solution 1 Verhoeff's iodine

Potassium	4.0 g
Distilled	100.0 ml
Dissolve and add iodine	2.0 g

Solution 2 Ferric chloride solution 10 per cent

Ferric chloride	10.0 g
Distilled water	100.0 ml

Solution 3 Ferric chloride solution 2 per cent

10 per cent ferric chloride	20.0 ml
Distilled water	100.0 ml

Solution 4 Picro-ponceau solution

Ponceau S, C.127195-1 per cent aq.	10.0 ml
Picric acid saturated	86.6 ml
Acetic acid 1 per cent aq.	4.0 ml

Procedure

1. Deparaffinize and bring slides to 70 per cent alcohol.
2. Stain in solution 1 (Verhoff's stain) for few minutes.
3. Rinse in distilled water.
4. Differentiate in solution 3 (2 per cent ferric chloride) for few min. If the section is destained bring them back to solution 1.
5. Transfer to 5 per cent sodium thiosulphate for 1 minute.
6. Wash in running water.
7. Counterstain in solution 4 (Picro-ponceau).
8. Differentiate in 95 per cent alcohol.
9. Dehydrate, clear and mount.

Result

Elastic fibres	Brilliant black
Nuclei	Blue to brownish black
Collagen	Red

Remarks

Verhoeff's stain can be combined with Perl's staining to show both iron and elastin (Pickett and Klavins,1961).

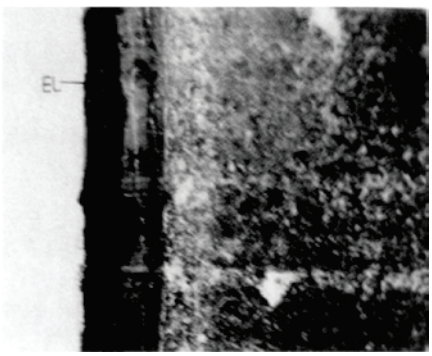


Figure 8.26 Sagittal section of spermathecum of *Cardisoma cornifex* stained with verhoeff. Outer wall is positive (Elastin); El: Elastin 90 μm. (*See* Plate 4.2)

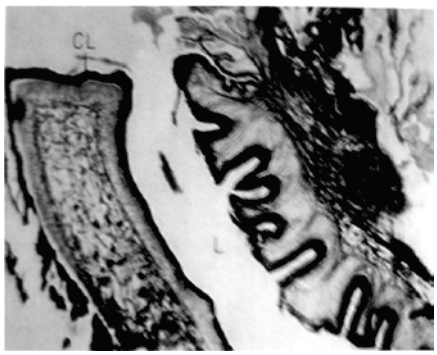


Figure 8.27 Section of oviduct of *C. cornifex* stained with verhoeff. Inner most wall is showing elastin nature 21 μm. (*See* Plate 4.3)

Iron gallein Elastin Stain (Churukian and Schenk,1976)

Fixation

Any general fixatives

Reagents required

Gallein

Ethyline

Ferric chloride

Concentrated HCl

Preparation of reagents

Solution 1

Gallein	1.0 g
Ethylene glycol	20.0 ml

Add 80 ml absolute alcohol and mix.

Solution 2

Ferric chloride	4.0 ml
Distilled water	95.0 ml
Conc. hydrochloric acid	1.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in solution 1 for 30 min.
3. Wash in running water.
4. Immerse in solution 2 for 2 min.
5. Wash in water.
6. Counterstain if necessary.

Result

Elastin	Black

Basic Fuchsin Stain (Horobin *et al.*, 1974)

Reagents required

Basic fuchsin

Ferric chloride

Hydrochloric acid

Preparation of reagent

Solution 1

Add 2.0 g of basic fuchsin to 200 ml of boiling water. Then add 25 ml of 30 per cent ferric chloride and again boil for 5 min. Filter and dry the precipitate. Dissolve the precipitate in 200 ml of 95 per cent alcohol. Store this solution in refrigerator. This solution lasts for several months.

Procedure

1. Dewax and hydrate slides to water.
2. Keep in solution 1 for 30 min.
3. Differentiate in 70 per cent alcohol.
4. Differentiate in 1per cent HCl in 70 per cent alcohol.
5. Dehydrate, clear and mount.

Result

Elastin	Deep purple
Basophilis	Purple

Orcinol-new Fuchsin (Fullmer and Lillie,1956)

Fixation

Any general fixative

Reagent required

New fuchsin

Orcinol

Ferric chloride

Preparation of reagent

Solution1

New fuchsin C. 142520	2.0 g
Orcinol	4.0 g
Distilled water	200.0 ml

Boil for 5 min. and then add 25 ml of ferric chloride ($FeCl_2$)29.1per cent 25 ml. Boil for 5 min., collect, precipitate on a filter paper and dissolve in 100 ml of 95 per cent ethyl alcohol.

Procedure

1. Deparaffinize and transfer slides to absolute alcohol.
2. Stain in solution 1 at 37°C for 15 min.
3. Differentiate in 70 per cent alcohol giving 3 changes of 5 min. each.
4. Dehydrate in 95 per cent absolute alcohol, clear and mount.
5. If desired counterstain with safranin.

Result

Elastin fibres	Deep violet
Collagen	Unstained

Aldehyde Fuchsin (Gomori, 1950a)

Fixative

Any fixative without dichromate

Reagents required

Potassium permanganate

Sulphuric acid

Sodium bisulphite

Basic fuchsin

Paraldehyde

Hydrochloric acid

Preparation of reagents

Solution 1 Potassium permanganate

Potassium permanganate	300 mg
Distilled water	100.0ml
H_2SO_4	0.3 ml

Solution 2 Sodium bisulphite (2.5 per cent)

Sodium bisulphite	2.5 g
Distilled water	100.0 ml

Solution 3 Aldehyde fuchsin

Add 1 g of basic fuchsin to 200 ml boiling water. Boil, cool and filter. Add 2 ml of conc. HCl and 2 ml paraldehyde. Leave stoppered at room temperature. When mixture has lost reddish colour and is deep purple (3–4 days) filter it and discard filtrate. Dry precipitate on filter paper in an oven. Remove and store in a bottle. To make staining solution dissolve 0.25 g in 50 ml of 70 per cent alcohol. It keeps for 6 months.

Procedure

1. Hydrate slides to water and remove $HgCl_2$.
2. Oxidize in solution 1 for 1 min.
3. Rinse in distilled water.
4. Bleach in solution 2 until permanganate colour is removed.
5. Wash.
6. Transfer to 70 per cent alcohol.
7. Stain in solution 3 (aldehyde fuchsin).
8. Wipe off the back of slide and rinse in 95 per cent alcohol.
9. Dehydrate, clear and mount.

Result

Elastin	Deep purple
Mast cells, etc.	Purple

Comment

Can be combined with pearl's reaction to show both iron and

Orcein Method (Bickett and Klavins, 1965)

Fixation

Any general fixative

Reagents required

Orcein

Hydrochloric acid

Preparation of reagents

Solution 1

Orcein	1.0 g
70 per cent ethyl alcohol	100.0 ml
Hydrochloric acid	1.0 ml
(Assay 37.38 per cent)	

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in solution 1 for 30–60 min.
3. Wash briefly in distilled water.
4. Dehydrate in 95 per cent alcohol for 2 min.
5. Differentiate in absolute alcohol.
6. Rinse in fresh absolute alcohol.
7. Clear and mount.

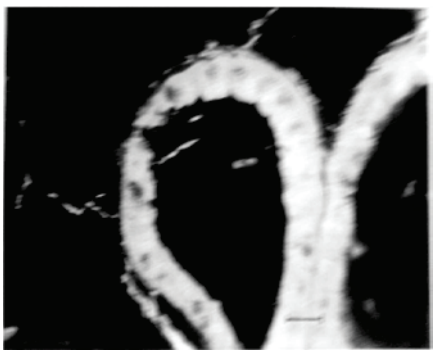


Figure 8.28 Section of accessory gland of *O. platytarsis*. Secretions showing the presence of elastin. Stained with orcein 30 μm. (*See* Plate 4.4)

Result

Elastin	Red

Van Gieson's Picrofuchsin Method

Fixation

Any alcohol

Reagents required

Harris haematoxylin

Acid fuchsin

Picric acid

Preparation of reagents

Solution 1 Harris haematoxylin

Dissolve 1.0 g of haematoxylin in 10.0 ml ethyl alcohol. Dissolve 20.0 g of potassium or alum inium alum in 200 ml water and boil. Add hematoxylin and boil for ½ minute. Add 0.5 g of mercuric oxide and cool rapidly. Add a few drops of glacial acetic acid. It does not keep longer than a month or two.

Solution 2 Van Gieson No. 1

1 per cent aq. acid fuchsin	5.0 ml
Saturated aq. picric acid	100.0 ml

Solution 3 Saturated picric acid

Picric acid saturated in 95 per cent alcohol	86.8 ml
Terpineol or organum for celliodin sections	

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain nuclei deeply (about 10 min.) in solution 1 (Harris haematoxylin).
3. Stain in solution 2 (Van Gieson's solution) for 1 min.
4. Wash quickly in water.
5. Decolorize and differentiate in 95 per cent alcohol satuarated with picric acid (solution 3) until sections are pale yellow.
6. Dehydrate in alcohol, clear and mount.

Result

Collagen	Bright red
Nuclei	Blue or black
Other tissue elements	Yellow

Biebrich Scarlet—Picro Aniline Blue

Fixation

Any general

Reagents required

Biebrich scarlet
Glacial acetic acid
Aniline blue
Picric acid

Preparation of reagents

Solution 1 Weigert's haematoxylin (Same chapter)

Solution 2 Biebrich scarlet solution

Biebrich scarlet	200 mg
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Solution 3 Aniline blue solution

Aniline blue	100 mg
Saturated aq. picric acid	100.0 ml

Solution 4 1 per cent aq. acetic acid

Procedure

1. Deparaffinize and bring sections to water.
2. Stain in solution 1 (Weigert's haematoxylin) for 5 min.
3. Wash in water.
4. Stain in solution 2 (Biebrich scarlet) for 4 min.
5. Rinse in water.
6. Stain in solution 3 (aniline blue) for 5 min.
7. Wash in solution 4.
8. Dehydrate, clear and mount.

Result

Erythrocytes	Orange
Muscles	Pink
Cytoplasm	Pink
Nuclei	Black
Reticulum	Blue
Connective tissue	Blue

Mallory's Aniline Blue for Collagen

Fixation

Zenker, mercury-containing fixative

Reagents required

Acid fuchsin

Aniline blue

Orange G

Phosphotungstic acid

Preparation of reagents

Solution 1 Acid fuchsin solution

Acid fuchsin	500 mg
Distilled water	100.0 ml

Solution 2 Aniline blue–orange G solution

Aniline blue (water-soluble)	500 mg
Orange G	2.0 g
1 per cent phosphotungstic acid	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place in solution 1 (acid fuchsin solution) for 1 min.
3. Transfer directly to solution 2 (Aniline blue - orange G solution) for 20–60 min.
4. Rinse in 95 per cent alcohol giving several changes.
5. Dehydrate, clear and mount.

Result

Collagen	Intense blue
Hyaline tissue	Blue
Erythrocytes and myelin	Yellow to orange

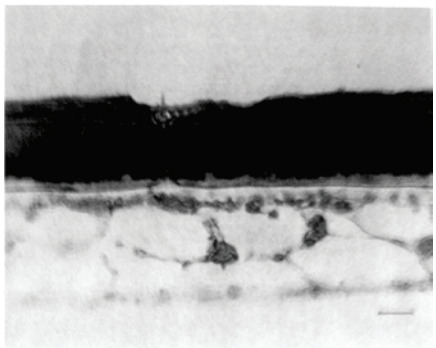


Figure 8.29 Intermoult cuticle of *E. asiatica* showing collagenous nature with aniline blue 20 μm. (*See* Plate 4.5)

Silver impregnation for reticulin Subbed slides are good. No loss of sections

Del Rio-Hortega Method

Fixation

Any good general fixative

Reagents required

Silver nitrate

Lithium carbonate

Preparation of reagents

Solution 1 Ammoniacal silver carbonate

10 per cent silver nitrate	10.0 ml
Saturated lithium carbonate	10.0 ml

A precipitate occurs. Decant and wash precipate 5 times. Add 25 ml of distilled waer. Add ammonia (28 per cent) drop by drop till precipitate dissolves. Add 95 per cent alcohol 100 ml and filter. Warm at 50°C for 30 min. Filtering should be repeated every time before use.

Solution 2 Formalin

Formalin	10.0 ml
Distilled water	50.0 ml

Solution 3 Gold chloride solution

Gold chloride 1 per cent	12.5 ml
(1 g + 100 ml water)	
Distilled water	50.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Treat with 0.2 per cent potassium permanganate (200 mg + 100 ml water) for 2 min.
3. Wash in distilled water.
4. Bleach in 5 per cent oxalic acid (5 g + 100 ml water) for 3 min.
5. Wash in distilled water for 10 min.
6. Impregnate with solution 1 (silver solution) at 37° C for 30 min. (do not expose to light).
7. Rinse quickly in distilled water.
8. Reduce in solution 2 (formalin solution) for 3 min.
9. Wash in distilled water for 3 min.
10. Tone with solution 3 until yellow colour turns purple-grey.
11. Rinse in distilled water.
12. Fix in sodium thiosulphate (5 g + 100 ml water) for 3 min.

13. Wash in running water.
14. Counterstain (if desired) in haematoxylin.
15. Dehydrate, clear and mount.

Result

Reticulin	Black
Collagen	Red to rose
Nuclei	Black or blue
Cytoplasm	Greenish yellow
Muscle fibres, elastin	Light yellow

Gridley's Method (1951)

Fixation

Any good general fixative

Reagents required

Silver nitrate

Sodium hydroxide

Periodic acid

Preparation of reagents

Solution 1 Ammoniacal silver oxide

5 per cent silver nitrate	20.0 ml
(5 g + 100 ml water)	
10 per cent sodium hydroxide	20 drops
(10 g + 100 ml water)	

Add 28 per cent ammonia dropwise to the above mixture until precipitate dissolves. Add distilled water up to 50 ml.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Treat with 0.5 per cent periodic acid (500 mg + 1000 ml water) for 15 min.
3. Rinse in distilled water.
4. Treat with 2 per cent silver nitrate (2 g + 100 ml water) for 30 min. at room temperature.
5. Rinse in 2 changes of distilled water.
6. Impregnate in solution 1 (ammoniacal silver solution) for 15 min. at room temperature.
7. Rinse in distilled water.
8. Reduce in 30 per cent formalin(30 ml +70 ml water) for 3 min.
9. Rinse in distilled water giving 4 changes.

10. Tone in gold chloride (10 ml 1 per cent stock solution/40 ml water) until yellow-brown colour has changed to lavender-grey.
11. Rinse in distilled water.
12. Fix in 5 per cent sodium thiosulphate (5 g+100 ml water) for 3 min.
13. Wash in running water.
14. Counterstain if desired.
15. Dehydrate, clear and mount.

Result

Reticulin fibres	Black
Other tissue elements	Depends on Counterstain used

Wilder's Method (1935)

Fixation

Any general fixative

Reagents required

Phosphomolybdic acid

Uranium nitrate

Silver nitrate

Ammonia

Preparation of reagents

Solution 1 Phosphomolybdic acid

Phosphomolybdic acid	10.0 g
Distilled water	100.0 ml

Solution 2 Uranium nitrate solution

Uranium nitrate	1.0 g
Distilled water	100.0 ml

Solution 3 Ammoniacal silver nitrate

Add ammonia (28 per cent) to 5 ml of 10 per cent silver nitrate drop by drop. Until the precipitate formed dissolves, add 5 ml of 3.1 per cent sodium hydroxide. Dissolve the precipitate with addition of ammonia and make the solution up to 500 ml with distilled water.

Solution 4

Distilled water	50.0 ml
Formalin	0.5 ml
Uranium nitrate (1 per cent)	1.5 ml

Make fresh solution each time.

Solution 5 Gold chloride solution

Gold chloride stock solution (1 g/100 ml)	10.0 ml
Distilled water	40–80 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Wash.
3. Treat with solution 1 (phosphomolybdic acid) for 1 min.
4. Wash in running water for 5 min.
5. Treat with solution 2 (uranium nitrate) for 5 sec.
6. Rinse in distilled water.
7. Impregnate with solution 3 (ammoniacal silver nitrate) for 1 min.
8. Dip quickly in 95 per cent alcohol, then into solution 4 (reducing solution) for 1 min.
9. Wash in distilled water.
10. Tone in solution 5 (gold chloride) until yellow colour turns purplish grey.
11. Rinse in distilled water.
12. Fix in sodium thiosulphate 5 per cent (5 g/100 ml water) for 3–5 min.
13. Wash in running water.
14. Counterstain if desired.
15. Dehydrate, clear and mount.

Result

Reticulin fibres Black

Comments

Phosphomolybdic acid can be used as an alternative to potassium permanganate. Sensitization with uranium nitrate reduces the time and eliminates the heat required by reticulin methods. Staple and Grizzle (1986a, 1986b) recommend methods based on the argyrophil reaction for reticulum and argentaffin granules.

TECHNIQUES FOR FIBRIN

Mallory PTAH Method for Fibrin

Fixation

Any general fixative

Reagents required

Potassium dichromate

Potassium permanganate

Oxalic acid

Haematoxylin

Phosphotungstic acid

Preparation of reagents

Solution 1 Potassium dichromate solution

3 per cent potassium dichromate	3 parts
10 per cent hydrochloric acid	1 part

Solution 2 Potassium permanganate solution

0.5 per cent permanganate	47.5 ml
3 per cent sulphuric acid	2.5 ml

Solution 3 Oxalic acid solution

Oxalic acid	1.0 g
Distilled water	100.0 ml

Solution 4 PTAH

Haematoxylin	100 mg
Phosphotungstic acid	2.0 g
Distilled water	100.0 ml

Allow to ripen for 6 months.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Postchrome in solution 1 for 30 min.
3. Wash in water.
4. Oxidize in solution 2 for 1 min.
5. Wash in water.
6. Bleach in solution 3.
7. Rinse in distilled water.
8. Transfer to solution 4 for 12–24 hrs.
9. Dehydrate, clear and mount.

Result

Fibrin	Dark blue
Nuclei	Light blue
Collagen	Rose-red

Picro-mallory V for Fibrin (Lendrum *et al.*, 1962)

Fixation

Any general fixative

Reagents required

Picric acid
Orange G
Lissamine fast yellow
Acid fuchsin
Acetic acid
Phosphotungstic acid
Acid blue
Light green

Preparation of reagents

Solution 1

~~Saturated picric acid in 200 ml ethanol. Then add 400 mg orange G, 400 mg Lissamine yellow 26 (yellow mordant).~~

Solution 2 Acetic acid fuchsin

Acid fuchsin	1.0 g
Acetic acid 1 per cent	100.0 ml

Solution 3 PTA solution

Phosphotungstic acid	1.0 g
Distilled water	100.0 ml

Solution 4 Light green solution

Light green	2.0 g
Distilled water	100.0 ml

Solution 5 Mordant

Yellow mordant (solution 1)	30.0 ml
Ethanol (80 per cent)	70.0 ml

Procedure

1. Hydrate slides to water.
2. Immerse in solution 5 for 5 min.
3. Wash.
4. Stain in solution 2.
5. Rinse in tap water.
6. Transfer to solution 3.
7. Rinse in tap water.
8. Stain in solution 4 for 2 min.
9. Rinse in tap water.
10. Dehydrate, clear and mount.

Result

Nuclei	Blue-black
Collagen	Bluish grey
Fibrin	Red

Maritius–scarlet–Blue Method for Fibrin (Lendrum *et al.*, 1962)

Fixation

Any general fixative

Reagents required

Maritius yellow
Phospohotungstic acid
Brilliant crystal scarlet 6R
Acetic acid

Preparation of reagents

Solution 1 Maritius yellow solution

Maritius yellow	500 mg
Ethanol (95%)	100.0 ml
Phosphotungstic acid	2.0 g

Solution 2 Crystal scarlet solution

Brilliant crystal scarlet	1.0 g
2.5 per cent acetic acid	100.0 ml

Solution 3 PTA solution

Phosphotungstic acid	1.0 g
Distilled water	100.0 ml

Solution 4 Soluble blue

Soluble blue	1.0 g
1 per cent acetic acid	100.0 ml

Procedure

1. Hydrate slides to water.
2. Stain with solution 1 for 2 min.
3. Wash in water.
4. Stain in solution 2 for 10 min.
5. Wash.
6. Treat with solution 3 for 5 min.

7. Wash.
8. Treat with solution 4 for 10 min.
9. Wash.
10. Dehydrate, clear and mount.

Result

Fibrin	Red
Nuclei	Blue-black
Red cells	Yellow

Masson 44/41 Method for Firbin (Lendrum *et al.*, 1962)

Fixation

Any general fixative

Reagents required

Phosphotungstic acid

Picric acid

Acid red 44 LB

Mercuric chloride

Celestian blue

Brilliant crystal scarlet 6R

Naphthalene blue black CS

Procedure

1. Remove wax with xylene and rinse with trichloroethylene and immerse in a closed jar of trichloroethylene for 48 hrs.
2. Rinse in absolute alcohol.
3. Place in a closed jar containing absolute ethanol saturated with picric acid containing 3 per cent mercuric chloride.
4. Wash and remove mercury by treating with Lugol's iodine followed by thiosulphate.
5. Wash till yellow colour disappears.
6. Stain nuclei with celestian blue haemalum sequence. Rinse in tap water.
7. Differentiate with 0.25 per cent HCl in 70 per cent ethanol.
8. Wash in running water.
9. Stain with brilliant crystal scarlet 6R in 1 per cent acetic acid for 5 min.
10. Rinse in water and treat with 1 per cent PTA for 5 min.
11. Rinse in water and treat with 1 per cent naphthalene blue black CS in 1 per cent aqueous acetic acid for 30 min.
12. Wash, dehydrate, clear and mount in DPX.

Result

Nuclei	Black
Red cells	Red
Fibrin	Deep black

Fraser–Lendrum Method for Fibrin

Fixation

Zenker's

Reagents required

Celestian blue
Ferric ammonium sulphate
Haematoxylin
Orange G
Picric acid
Acid fuchsin
Acetic acid
Phosphotungstic acid
Light green

Preparation of reagents

Solution 1 Celestian blue

Ferric ammonium sulphate	2.5 g
Distilled water	50.0 ml

Dissolve for 12 hrs at room temperature and add

Celestian blue	250 mg

Boil for 3 min. and filter and add 7 ml glycerine.

Solution 2 Mayer's haematoxylin

Solution 3 Orange G – picric acid solution

Saturated picric acid in 80 per cent	200.0 ml
Orange G	400 mg

Solution 4 Acid fuchsin solution

Acid fuchsin	1.0 g
Distilled water	99.0 ml
Glacial acetic acid	1.0 ml

Solution 5 MacFarlanes stock solution

Phosphotungstic acid	25.0 g
Picric acid	2.5 g
95 per cent alcohol	100.0 ml

Solution 6 Working solution

Solution 5	40.0 ml
95 per cent alcohol	40.0 ml
Distilled water	20.0 ml

Solution 7

Light green	2.0 g
Distilled water	98.0 ml
Glacial acetic acid	1.0 ml

Solution 8

Solution 3	30.0 ml
80 per cent alcohol	70.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1 for 5 min.
3. Wash in tap water.
4. Transfer to solution 2 for 5 min.
5. Wash in tap water.
6. Transfer to solution 3 for 5 min.
7. Wash in tap water.
8. Keep slides in solution 4 for 5 min.
9. Wash in tap water.
10. Transfer to solution 8 for 15 sec.
11. Wash in tap water.
12. Transfer to solution 6 for 5 min.
13. Wash in tap water.
14. Counterstain in solution 7 for 1 min.
15. Dehydrate, clear and mount.

Result

Fibrin keratin	Red
Collagen	Green
Erythrocytes	Orange

TECHNIQUES FOR KERATIN

Ayoub Shiklar Method for Keratin and Prekeratin (Ayoub and Shiklar, 1963)

Fixation

10 per cent buffered neutral formalin

Reagents required

Fuchsin

Aniline blue

Orange G

Preparation of reagents

Solution 1 Acid fuchsin solution

Acid fuchsin	5.0 g
Distilled water	100.0 ml

Solution 2 Aniline blue–Orange G solution (*See* same chapter)

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1 for 5 min.
3. Transfer directly to solution 1 for 5 min.
4. Transfer direct to 95 per cent alcohol.
5. Dehydrate, clear and mount.

Result

Keratin	Brilliant red
Connective tissue	Deep blue

Rhodamine B Method for Keratin

Fixation

Any fixative or 10 per cent formalin

Reagents required

Rhodamine B

McIvaine buffer

Toluidine blue

Preparation of reagents

Solution 1

Toluidine blue	100 mg
Distilled water	100 ml

Solution 2

Rhodamine B	100 mg
McIvaine's buffer (pH 3.6)	100 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1 for 10 min.
3. Wash in distilled water.
4. Transfer to solution 2 for 10 min.
5. Wash in distilled water.
6. Dehydrate, clear and mount.

Result

Keratin stained red.

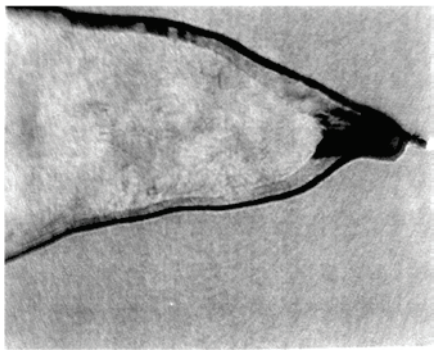


Figure 8.30 First day postmoult cuticle of *E. asiatica* showing the presence of keratin as evidenced with Rhodamine B; 30 μm. (*See* Plate 4.6)

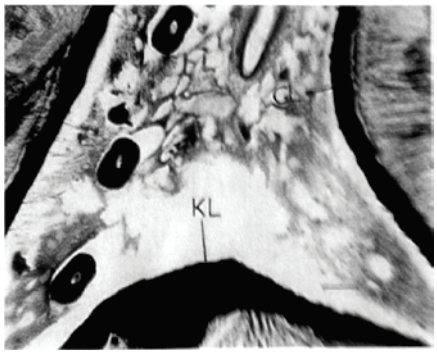


Figure 8.31 Oesophageal lining of *E. asiatica* showing the presence of both collagen as well as keratin layers when Stained with orcein–aniline blue–orangeG.; KL: Keratin layer; CL: Collagen layer 21 μm. (*See* Plate 4.7)

TECHNIQUES FOR ENZYMES

Azo-dye Coupling Method for Alkaline Phosphatase

Reagents required

0.1 M tris buffer stock solution

Sodium α - naphthyl phosphate

Diazonium salt (Fast red TR)

Preparation of reagents

Solution 1 Incubating medium

Sodium naphthyl phosphate	10 mg
0.1 M tris buffer (stock solution) pH 8.0	10.0 ml
Diazonium salt	10 mg

pH of incubating medium should be adjusted to 9.0 –9.4. Initially sodium naphthyl phosphate is dissolved in the buffer. Diazonium salt is now added and the solution is mixed well. This has to be used immediately.

Sections

Cryostat post-fixed, pre-fixed and freeze-dried sections embedded in paraffin.

Procedure

1. Bring sections to water and incubate in solution 1 at room temperature for 10–60 min.
2. Wash in distilled water.
3. Counterstain in 2 per cent methyl green.
4. Wash in distilled water.
5. Mount in glycerine jelly.

Result

Alkaline phosphatase activity	Reddish brown
Nuclei	Green

Rationale

First described by Menton *et al.* (1944), it was later modified by Gomori (1951). The incubating medium should be at pH 9.2. From the substrate, the enzyme liberates naphthyl which couples with diazonium salt to form an insoluble azo dye at the sites of enzyme activity. The choice of the diazonium salt and pH of the incubating medium determine the quality of staining.

Remarks

pH of the incubating medium must be 9.2. Paraffin section requires more time for incubation.

Calcium Cobalt Method for Alkaline Phosphatase (Gomori,1941)

Fixation

Cold acetone	Cold formalin
Paraffin	Frozen

Reagents required

Sodium β-glycerophosphate
Sodium diethylbarbiturate
Calcium chloride
Magnesium sulphate
Cobalt nitrate
Yellow ammonium sulphide

Preparation of the reagents

Solution 1 Incubating medium

3 per cent sodium β-glycerophosphate	10.0 ml
2 per cent sodium diethyl barbiturate	10.0 ml
Distilled water	5.0 ml
2 per cent calcium chloride	20.0 ml
5 per cent magnesium sulphate	1.0 ml

Solution 2 Cobalt solution

Cobalt nitrate or acetate	2.0 g
Distilled water	100.0 ml

Procedure

Fixation procedure as mentioned above.

1. For paraffin sections, dewax with light petroleum.
2. Bring them down to water via acetone.
3. Place the section in solution 1 for 16 hrs at 37° C.
4. Rinse in running water.
5. Transfer to solution 2 for 3–5 min.
6. Rinse in distilled water.
7. Flood slides with dilute yellow ammonium sulphide.
8. Wash in water.
9. Dehydrate, clear and mount.

Result

Sites of alkaline phosphatase activity	Black

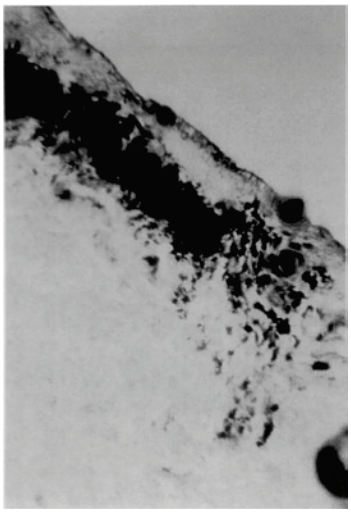


Figure 8.32 Section of foot of the bivalve Mytilopsis sallei showing alkaline phosphatase activity in the enzyme gland- Calcium cobalt method. (*See* plate 4.8)

Azo Dye Method for Leucocyte Phosphatase, (Kaplow, Monis, Hayhoe)

Procedure

1. Take smear from venous blood and dry in air.
2. Fix the smear in ice-cold 10 per cent formalin-methanol (40 per cent formaldehyde 10 ml + methanol 90 ml) for 30 sec.
3. Incubate the slides in the following medium at 22°C for 10 min.

0.1 M Tris buffer (pH 9.2)	25.0 ml
Sodium naphthol phosphate	20 mg
Fast garnet GBC salt	30 mg

4. Wash in running water.
5. Counterstain with Mayer's haemalum for 3 min.
6. Wash in water.
7. Mount in glycerine jelly.

Result

Sites of alkaline phosphatase activity	Brown

Naphthol AS-phosphate Azo Dye Method (Burstone, 19577a)

Fixation

Freeze-dried or cold acetone–paraffin

1. Bring down sections to water via xylene and acetone.
2. Transfer to incubating medium at 22°C for 30 min.
3. ASD salt—40 mg + 1,000 ml distilled water.

   Or any diazonium salt

   Fast red

   Violet LB salt

   Fast blue RR

   Red RC

   Red TR are recommended.
4. Wash rapidly in water.
5. Mount in PVP medium.

Result

Sites of alkaline phosphatase activity	Red or blue

Acid Phosphatase The Naphthol AS-BI Phosphate Method: (Burstone, 1958 modified by Barka, 1960) (Simultaneous coupling with substituted naphthols)

Fixation

Cryostat post-fixed, prefixed, freeze-dried and frozen

Reagents required

Veronol acetate buffer

Naphthol AS –BI phosphate

N˝N dimethyl formamide

Pararosaniline hydrochloride

Hydrochloric acid

Sodium nitrite

0.1N sodium hydroxide

Preparation of reagents

Solution 1 Substrate solution

Naphthol AS – BI phosphate	50 mg
Dimethyl formamide	5.0 ml

Solution 2 Buffer solution

Veronol acetate buffer stock A

Veronol acetate buffer

Sodium acetate	1.1942 g

Veronol sodium barbiturate	2.94 g
Distilled water	100 ml

Add 1ml of veronol acetate solution to M/10 hydrochloric acid and distilled water.

Solution 3 Sodium nitrite solution

Sodium nitrite	400 mg
Distilled water	10.0 ml

Solution 4 Pararosaniline stock

Pararosaniline hydrochloride	1g
Distilled water	20 ml
Conc. HCl	5 ml

Pararosaniline is dissolved in distilled water and then HCl is added. Heat the solution gently, cool, filter and store in a refrigerator.

Solution 5

Distilled water

Solution 6 Incubating medium

Solution 1	0.5 ml
Solution 2	2.5 ml
Solution 3 and 4	0.8 ml and 0.4 ml of solution 3 and 4 mixed before adding to the incubating solution
Solution 5	6.5 ml

The pH of the solution should be 4.7–5.0, it is adjusted with 0.1N NaOH

Procedure

1. Sections are placed in solution 6 (incubating medium) and incubated at 37°C for 15–60 min.
2. Wash in distilled water.
3. Counterstain with 2 per cent methyl green.
4. Wash in distilled water.
5. Mount in glycerine jelly or dehydrate quickly in alcohol and mount in DPX.

Result

Sites of acid phosphatase activity	Red
Nuclei	Green

Remarks

Solution 3 (sodium nitrate) should be fresh. pH should be 4.7–5.0. Solution should be filtered.

Rationale

Burstone (1958) recommended AS-BI phosphate instead of naphthol AS the best. Barka (1960) suggested the use of hexazonium pararosaniline as the diazonium salt in the simultaneous coupling method. This diazonium along with AS-BI phosphate gives accurate localization and he recommended this.

Glucose 6-phosphatase Lead Method (Wachstein and Miesel, 1956)

Reagents required

Glucose 6-phosphate (potassium salt)

Tris maleate (pH 6.7)

2 per cent lead nitrate

Ammonium sulphide

Preparation of solution 1 Incubation medium

0.12 per cent glucose 6-phosphate (Aq.)	4.0 ml
0.2 M tris maleate (pH 6.7)	4.0 ml
2 per cent lead nitrate	0.6 ml
Distilled water	1.4 ml

Sections

Cryostat unfixed. Frozen unfixed.

Procedure

1. Incubate unfixed sections in solution 1 for 5–20 min. at 37°C.
2. Wash in distilled water giving 3 changes.
3. Transfer sections to 1 per cent ammonium sulphide for 2 min.
4. Wash in distilled water.
5. Again fix sections in 10 per cent formaldehyde for 15–30 min.
6. Wash in distilled water.
7. Mount in glycerine jelly.

Result

Sites of glucose 6-phosphate activity	Brownish black

Rationale

Chiquonine (1954) described the method and modified it in 1955. The present technique was first described by Wachstein and Meisel (1956). In glucose 6-phosphate the potassium salt is the substrate, lead nitrate in the incubating medium produces a precipitate with the released phosphate. The lead precipitate on treatment with ammonium sulphide becomes the discernible lead sulphide.

Glucose 6-phosphate being a sensitive enzyme, the reaction is advocated to be done with unfixed sections. Formol saline destroys it. Confusing as it may appear, the same method is applicable to both acid and alkaline phosphatases. By stringent control sections can be avoided.

Adenosine Triphosphatase: Lead Method (Wachstein and Meisel, 1960)

Reagents required

Adenosine triphosphate

Tris buffer (pH 7.2)

2 per cent lead nitrate

2.5 per cent magnesium nitrate

Ammonium sulphide

Preparation of reagents

Solution 1 Incubating medium

0.125 per cent adenosine triphosphate	4.0 ml
Tris buffer	4.0 ml
2 per cent lead nitrate	0.6 ml
2 per cent magnesium nitrate	1.0 ml
Distilled water	0.4 ml

Sections

Frozen fixed

Cryostat unfixed

Cryostat prefixed

Procedure

1. Carefully incubate free-floating sections in solution 1 for 10–60 min. at 37°C.
2. Wash in distilled water.
3. Transfer to 1 per cent ammonium sulphide for 2 min.
4. Wash in distilled water.
5. Again fix sections in 10 per cent formaldehyde for 15–30 min.
6. Wash in distilled water.
7. Mount in glycerine jelly.

Result

Glucose 6-phosphate activity	Brownish black

ESTERASE

Non-specific Esterase: α-naphthol Acetate Method Using Fast Blue B (Gomori, 1950a)

Reagent required

α-naphthyl acetate

Acetone

0.2 M phosphate buffer (pH 7.4)

Fast blue B

Preparation (Incubating medium) of solution 1

α- naphthyl acetate	5 mg
Acetone	0.1 ml
0.2 M phosphate buffer (pH 7.4)	10.0 ml
Fast blue B	30 mg

α-naphthyl acetate is first dissolved in acetone and to this is added phosphate buffer and mixed thoroughly. Fast blue B is then added and the solution is filtered.

Section

Cryostat prefixed

Cryostat post-fixed

Freeze-dried

Paraffin sections

Procedure

1. Bring section down to water.
2. Transfer sections to solution 1(incubating medium) for 30 sec. to 15 min. at room temperature.
3. Wash in running water for 3 min.
4. Counterstain in Mayer's carmalum 3 min.
5. Wash in running tap water for 3 min.
6. Mount in glycerine jelly.

Result

Sites of esterase activity	Reddish brown
Nuclei	Red

Rationale

All types of esterase activity may be revealed. The pH should be 7.4. The simultaneous coupling was first described by Nachlas and Seligman (1949a). β-naphthyl acetate was first used with diazo fast blue B as a coupling agent. Gomori (1952a) used α-naphthyl instead of β-naphthyl acetate because the resulting azo dye was not soluble in water.

Esterase in the tissue attacks α-naphthyl acetate and releases α-naphthyl which combines with Fast Blue B to produce an insoluble azo dye at the site of enzyme activity. Cholinesterase may be involved here, but can be inhibited by using eserine (10^{-5}M).

Non-specific Esterase: α-Naphthyl Actate Method Using Hexazotized Pararosaniline (Gomori, 1950a; Davis and Ornstein,1959)

Reagent required

α-naphthyl acetate

0.2 M phosphate buffer (stock solution)

Acetone

Pararosaniline hydrochloride

HCl

Sodium nitrate

Preparation of reagents

Solution 1 Substrate solution

α-naphthyl acetate	50 mg
Acetone	5 ml

Solution 2 Buffer solution

0.2 M phosphate buffer, stock solution A

Solution 3 sodium nitrate solution

Sodium nitrate	400 mg
Distilled water	10 ml

Solution 4 Pararosaniline–HCl stock

Pararosaniline hydrochloride	2 mg
2N Hydrochloric acid	50 ml

Heat, cool to room temperature.

Soution 5 Preparation of incubating medium

Solution1	0.25 ml
Solution 2	7.25 ml
Solution 3	0.8 ml (0.4 ml of solution 3 and 4 are mixed before adding to the mixture)
Solution 4	2.5 ml

Final pH should be 7.4.

Procedure

1 Bring sections to water.

2 Keep slides in incubating solution 5 for 2–20 min. at 37°C.

3 Wash in running water.

4 Counterstain with 2 per cent methyl green.

5 Wash.

6 Dehydrate rapidly through alcohols and clear in xylene and mount.

Result

Esterase	Reddish brown
Nuclei	Green

Rationale

This is similar to the previous method except that hexazonium pararosaniline is used instead of Fast Blue B. This coupling agent for esterase was used by Davis and Ornstein (1959). It is better than the previous method because sections can be dehydrated through alcohol series to xylene and mounted in a synthetic mounting medium.

Non-Specific Esterase: Indoxyl Acetate Method (Holt & Withers, 1952)

Reagents required

5-Bromo-indoxyl acetate

Ethanol – 0.1 M tris buffer (pH 6.8)

Potassium ferricyanide

Potassium ferrocyanide

Calcium chloride

Preparation of solution 1 Incubation medium

5-Bromo-indoxyl acetate	1 mg
Ethanol	0.1 ml
Tris buffer(0.1 M) (pH 6–8)	2.0 ml
Potassium ferricyanide (0.05 M) (1.6 per cent)	1 ml
Potassium ferrocyanide (0.05 M) (2.1 per cent)	1 ml
Calcium chloride (0.1 M) (2.1 per cent)	1 ml
Distilled water	5 ml

The 5-bromo-indoxyl acetate is dissolved in ethanol and the buffer is then added. The remaining chemicals are dissolved in distilled water and the solution is mixed. Solution should be freshly prepared.

Sections

Cryostat pre- and post-fixed

Freeze-dried

Frozen

Paraffin

Procedure

1. Bring sections to water.
2. Keep slides in solution 1 and incubate at 37°C for 15 min. to 1 hr.
3. Rinse in tap water.
4. Counterstain in Mayer's carmalum for 5 min.
5. Rinse in tap water.
6. Mount in glycerine jelly or dehydrate quickly and mount in DPX.

Result

Esterase activity	Black
Nuclei	Red

Lipases

These are a group of enzymes which are capable of hydrolysing long-chain esters with more than seven carbon bonds which contain saturated fatty acids. Liver, pancreas and adrenals are the sources of lipases. Here again there is overlap between lipases and non-specific esterases and between both can hydrolyse simple esters. Lipase method is purely based on the enzyme hydrolysing the tween 60 to produce calcium ions which combine with fatty acids to form insoluble, calcium soaps which when treated with lead ions and finally ammonium sulphide form dark brown or black deposits of lead sulphide at the sites of enzyme activity.

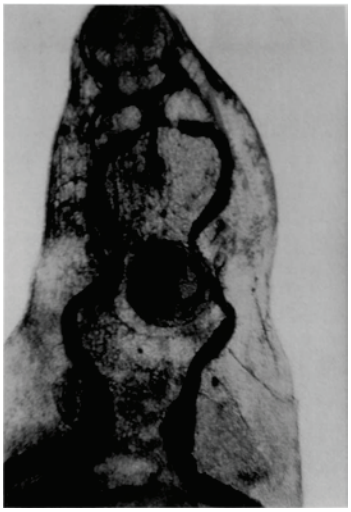


Figure 8.33 *Elongoparorchis pneumatis* a trematode parasite in the air bladder of the marine cat fish.

Note: Nervous system is showing esterase activity–Stained with 5-bromoindoxyl acetate (*See* Plate 4.9)

Lipase: Tween Method (Gomori, 1952a)

Reagents required

Tween 40, 60 or 80 (tween 80 preferred)

Tris buffer (pH 7.2)

Calcium chloride

Thymol

Lead nitrate

Ammonium sulphide

Preparation of reagents

Solution 1

Tris buffer(pH 7.2)

Solution 2 Tween

Tween 40, 60 or 80	5.0 g
Tris buffer (pH 7.2)	100.0 ml
Thymol	a few small crystals

Solution 3 Calcium chloride solution

Calcium chloride	200 mg
Distilled water	10.0 ml

Solution 4 Lead nitrate solution

Lead nitrate	1.0 g
Distilled water	50.0 ml

Solution 5 Preparation of incubation medium

Solution 1	9.0 ml
Solution 2	0.06 ml
Solution 3	0.3 ml

Sections

Formalin-fixed, free-floating

Cryostat prefixed

Paraffin sections

Procedure

1. Bring sections to water.
2. Incubate sections at 37°C for 2–8 hrs in solution 5. For paraffin sections required incubation is 24 hrs.
3. Rinse sections in 3 changes of distilled water.
4. Place sections in preheated solution 4 (lead nitrate solution) at 55°C for 10 min.
5. Rinse sections in distilled water.
6. Transfer sections to 1% ammonium sulphide for 3 min.
7. Rinse in distilled water.
8. Counterstain in Mayer's carmalum for 5 min.
9. Wash in tap water.
10. Mount in glycerine jelly.

Result

Sites of lipase activity	Yellow brown black
Nuclei	Red

Rationale

First described by Gomori (1945), it is not a specific test because tweens are hydrolysed also by some non-specific esterases. Gomori (1952a) modified it. Tween 60 and 80 are used besides lead nitrate and calcium chloride. The lipase in the tissue hydrolyses tween to release fatty acids. Fatty acids combine with lead nitrate to release lead which when treated with dilute ammonium sulphide precipitates as black lead sulphide at the site of the enzyme activity.

Cholinesterase: Myristoyl Choline Method (Gomori, 1948)

Reagents required

Myristoyl choline

0.1 M veronol acetate buffer

Cobalt acetate

Calcium chloride

Magnesium chloride

Ammonium sulphide

Thymol

Preparation of reagents

Solution 1

Cobalt acetate	400 mg
Distilled water	80.0 ml
0.1 M veronol acetate (pH 7.6)	20.0 ml
Magnesium chloride	1 mg
Calcium chloride	1 mg
Manganese chloride	1 mg
Thymol	A few small crystals

Solution 2 Myristoyl solution

Myristoyl choline	70 mg
Distilled water	10.0 ml
Thymol	A few small crystals

This solution should be in refrigerator (4°C).

Solution 3 Incubating medium

Solution 1	10.0 ml
Solution 2	0.2 ml

Sections

Cryostat prefixed, formalin fixed, cryostat post-fixed, paraffin

Procedure

1. Bring down sections to water.
2. Incubate sections for 1–4 hrs for 37°C. Paraffin sections require 2–16 hrs.
3. Wash in distilled water for 2 min.
4. Transfer sections to 2 per cent ammonium sulphide.
5. Wash in distilled water.
6. Counterstain in 0.05 per cent of incubating medium for 30 sec.
7. Wash in tap water.
8. Mount in glycerine jelly.

Result

Sites of cholinesterase activity	Dark brown to black

Rationale

Gomori (1948) used myristoyl as a substrate. The incubating medium includes cobalt acetate in veronol acetate medium. Calcium, manganese and magnesium are activators. Cholinesterase in the tissue splits myristoyl choline and releases fatty acids, which combine with cobalt acetate to form cobalt salt. On adding dilute ammonium sulphide, a precipitate of cobalt sulphide develops at the sites of enzyme activity.

Cholinesterase: Thiocholine Method (Gerebtzoff, 1959) (Modified by Filipe and Lake, 1983)

Reagents required

Acetyl thiocholine iodide

0.1 M acetate buffer (pH 5.0–6.2)

Cupric sulphate

Glycerine

Ammonium sulphide

Preparation of solutions

Solution 1 Buffer

0.1 M acetate buffer (pH 5.0–6.2)

Solution 2 Staining solution

Acetyl thiocholine iodide	15 mg
Cupric sulphate	7 mg
Distilled water	1.4 ml

The solution is centrifuged at 4,000 r.p.m. for 15 min. and the supernatant is used.

Solution 3 Glycerine

Glycerine	375 mg
Distilled water	10.0 ml

Solution 4 Cupric sulphate solution

Cupric sulphate	375 mg
Distilled water	10.0 ml

Solution 5 Incubating medium

Solution 1	5.0 ml
Solution 2	0.8 ml
Solution 3	0.2 ml
Solution 4	0.2 ml
Distilled water	3.8 ml

The pH of the incubating medium varies with the type of the tissue. Tissues with high cholinesterase activity are incubated at pH 5.0 and others at 6.2.

Sections

Cryostat-prefixed, formalin-fixed—free floating, cryostat-post-fixed, paraffin sections

Procedure

1. Place sections in incubating medium for 10–90 min. at 37°C.
2. Rinse in 2 changes of distilled water.
3. Transfer sections to 2 per cent ammonium sulphide.
4. Wash well in distilled water.
5. Counterstain if necessary.
6. Wash in tap water.
7. Mount in glycerine jelly.

Result

Cholinesterase activity	Brown

Rationale

Koelle and Friedenwald (1949) described the method, it is extensively used for both types of cholinesterase.

This incubating medium combines acetyl thiocholine iodide, copper sulphate and acetate buffer. Cholinesterases in the tissues hydrolyse the acteyl thiocholine to produce thiocholine which combines with copper ions to form copper thiocholine. The precipitate on treatment with 2 per cent ammonium sulphide is converted to copper sulphide visible as a precipitate.

Thiolactic Acid Method for Cholinesterase (Crevier and Belanger, 1955)

Fixation

Formalin-fixed, frozen

Fresh

Preparation of reagents

Solution 1

0.12 M thiolactic acid	0.75 mg
0.001 M lead nitrate	27 mg
0.1 M Na_2SO_4	83 ml
McIvaine's buffer (pH 6.2)	17.0 ml

Procedure

1. Incubate in solution 1 at 22°C for half to 1 hr.
2. Wash in ice-cold water.
3. If desired counterstain in 0.02 per cent basic fuchsin.
4. Wash in ice-cold water.
5. Dehydrate and clear in xylene.
6. Mount in paramount.

Result

Cholinesterase activity sites revealed as black deposits.

Succinate Dehydrogenases (Pearse, 1972)

Reagents required

34:5-dimethyl thiazolyl –2)5-diphenyl tetrazolium bromide (MTT) or nitro blue tetrazolium (NBT)

Tris buffer (pH 7.4)

Cobalt chloride (if MTT is used)

Sodium succinate

Formol saline

Methyl green

0.05 M magnesium chloride

Preparation of reagents

Solution 1 Sodium succinate substrate solution

Sodium succinate	6.75 g
Distilled water	8.0 ml
N-HCL	0.05 ml

Add sodium succinate to water and stir and then add NHCl. Final pH should be 7.1 and make up the volume to 10 ml. Keep it at very low temperature (freezing).

Solution 2 Stock tetrazolium solution

MTT (1 mg per 1 ml distilled water)	2.5 ml
Tris buffer	2.5 ml

0.5 Cobalt chloride	0.5 ml
0.05 Magnesium chloride	1.0 ml
Distilled water	2.5 ml

Final pH is 7.0. Adjust with tris buffer N-HCl. Keep at freezing temperature.

Solution 3 Formol saline

10 per cent formol saline

Solution 4 Methyl green solution

2 per cent methyl green

Solution 5 Incubating medium

Solution 2 stock tetrazolium solution	0.9 ml
Solution 1 succinate substrate solution	0.1 ml

Fixation

Cryostat-unfixed

Frozen-unfixed

Procedure

1. Flood sections with solution 5 (incubating solution) for 30 min. at 37°C.
2. Pass them on to solution 3 (formol saline) for 10–15 min.
3. Wash in running water for 2 min.
4. Counterstain with solution 4 (methyl green) for 5 min.
5. Rinse in distilled water.
6. Mount in glycerine jelly.

Result

Succinate dehydrogenases	Black
Nuclei	Green

Rationale

From the substrate (sodium succinate) dehydrogenases, release hydrogen, which reduces the tetrazolium salt to yield formazan. Formazan is then chelated to cobalt ions resulting in a brightly coloured insoluble deposit.

In NBT technique hydrogen is released by succinate dehydrogenase from substrate. This in turn reduces the nutro-BT to produce a coloured, water-insoluble formazan.

Glucose 6-phosphate Dehydrogenase (Pearse, 1972)

Reagents required

3(4:5)-dimethyl thiazolyl-2-5-diphenyl tetrazolium bromide

MTT or Nitro-blue tetrazolium (NBT)

Tris buffer (pH 7.4)

Cobalt chloride (MTT is used)

Glucose 6-phosphate

Formol saline

2 per cent methyl green

Nicotinamide adenine dinucleotide phosphate (NADP)

0.8 M magnesium chloride

Preparation of solution

Solution 1 Glucose 6-phosphate substrate solution

Glucose 6-phosphate	300 mg
Distilled water	0.8 ml
N-HCl	0.06 ml

Glucose 6-phosphate is dissoloved in distilled water. pH should be 7.1, neutralize it with N-HCl. The total volume should be 1 ml. Keep the solution in froze condition.

Solution 2 Stock MTT or NBT tetrazolium

Solution 3 10 per cent formol saline

Solution 4 2 per cent methyl green

Solution 5 Solution 2 (stock terazolium solution)	0.9 ml
Solution 1 Glucose 6-phosphate substrate solution	0.1 ml
Coenzymes NADP	2 mg

Sections

Frozen-unfixed

Cryostat-unfixed

Procedure

1. Flood sections with solution 5 incubating medium for 30 min. to 1 hr at 30°C.
2. Transfer sections to solution 3 (10 per cent formol saline) for 10–15 min.
3. Wash well in tap water.
4. Counterstain in solution 4 (2 per cent, methyl green).
5. Wash in tap water.
6. Mount in glycerine jelly.

Result

Glucose 6-phosphate dehydrogenase	Black formazan deposit with MTT
Nuclei	Green

Methods for Glucose 6-Phosphatase

Fixation

Cold microtome

Reagents required

Potassium glucose 6-phosphate

Tris buffer

Lead nitrate

Preparation of reagents

Solution 1(Incubating medium)

0.125 per cent potassium glucose 6-phosphate	20.0 ml
0.2 M tris buffer ( )	20.0 ml
2 per cent lead nitrate	3.0 ml
Distilled water	7.0 ml

Procedure

1. Cut 10–15 μm frozen sections.
2. Treat them with incubating medium for 5–15 min. at 32°C.
3. Wash in distilled water.
4. Treat with yellow ammonium sulphide.
5. Wash in water.
6. Post-fix in 6 per cent neutral formaldehyde.
7. Mount in glycerine and ring the coverslip with nail polish.

Result

Sites of glucose 6-phosphatase activity	Brownish black

TECHNIQUES FOR MICROORGANISMS

Gram Staining

With Gram-staining technique, the gram-positive organisms take the colour of the dye (crystal violet). Iodine is the mordant and after mordanting, a precipitate is formed. This precipitate is soluble in water.

Gram Weigert Method (Krajian and Grad wohl, 1952)

Fixation

Zenker's formal in or 10 per cent formalin

Reagents required

Eosin

Crystal violet

Aniline oil

Potassium iodide

Iodine

Preparation of reagents

Solution 1 Eosin solution

Eosin	1.0 g
Distilled water	100.0 ml

Solution 2 Staining solution

Crystal violet	5.0 g
95 per cent ethyl alcohol	10.0 ml
Aniline oil	2.0 ml
Distilled water	88.0 ml

First mix aniline oil in water. Then add crystal violet solution (dissolved in alcohol). This solution lasts for several months.

Solution 3

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml

Dissolve potassium iodide in water and then add iodine.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Transfer to solution 1 for 5 min.
3. Rinse in water.
4. Place in solution 2 (crystal violet) for 10 min.
5. Wash and flood the slides with solution 3 (Gram's iodine).
6. Blot with filter paper.
7. Flood the slides with equal parts of aniline oil and xylene.
8. Clear in xylene and mount.

Result

Gram-positive bacteria and fungi	Violet
Gram-negative bacteria and fungi	Do not stain
Fibrin	Blue-black

Rationale

Differential staining depends on the permeability of the cell membrane (Bartholomew and Mittwer, 1950, 51). In gram-positive bacteria, the linkage is between the acid groups of bacteria and alkaline group of the dye. Iodine forms a dye complex which is dissociated with dye alcohols. Cell membrane is permeable to alcohol. Thus decolorization occurs and the reaction is gram-negative. On the other hand if the membrane is not permeable, decolorization does not occur and the reaction is gram-positive. Others who dealt with Gram-staining are Bartholomew and Finklestein (1965) and Mittwer *et al.* (1950).

Acid Fast Bacteria Staining: Ziehl-Neelsen (Putt's modification, 1951)

Fixation

Any general fixative. 10 per cent formalin preferred.

Reagents required

New fuchsin

Phenol

Methyl alcohol

Glacial acetic acid

Methylene blue chloride

Lithium carbonate

Preparation of reagents (New fuchsin)

Solution 1

New fuchsin	1.0 g
Phenol	5.0 g
Ethyl or methyl alcohol	10.0 ml
Distilled water	100.0 ml

Solution 2 Acetic alcohol

Glacial acetic acid	5.0 ml
Absolute ethyl alcohol	95.0 ml

Solution 3 Counterstain

Methylene blue chloride	0.5 g
Absolute ethyl alcohol	100 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Immerse in solution 1 for 3 min.
3. Treat with lithium carbonate (saturated) for 1 min.
4. Differentiate in solution 2 acetic alcohol—pale pink.
5. Rinse in absolute alcohol for 2 min.

6. Counterstain in solution 3.
7. Wash, dehydrate, clear and mount.

Result

Acid-fast bacteria	Red
Mast cell	Deep blue
Other bacteria	Blue

Harada Method (1973)

Fixation

10 per cent neutral buffered formalin

Reagents required

Basic fuchsin
Phenol
Methylene blue
Potassium hydroxide

Preparation of reagents

Solution 1 Carbol fuchsin

Basic fuchsin alcohol (saturated)	10.0 ml
5 per cent aqueous phenol	90.0 ml

Solution 2 Loffer alkaline methylene blue

Methylene blue	3.0 g
Absolute alcohol	30.0 ml
Potassium hydroxide (0.01 per cent aqueous)	100.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in 1 per cent potassium permanganate for oxidation for an hour.
3. Rinse in water.
4. Transfer to solution 1 for 5 min.
5. Bleach in 1 per cent oxalic acid for 3 min.
6. Rinse in distilled water.
7. Decolorize in 1 per cent HCl in 70 per cent alcohol for 20 sec.
8. Wash in water.
9. Counterstain in solution 2 (dilute in the ratio 1 : 9 with distilled water) for 15 sec.
10. Rinse in tap water.
11. Dehydrate clear and mount.

Result

Acid-fast bacteria	Red
Mast granules	Deep blue
Other bacteria	Blue
Nuclei	Blue

Kin Youn's Carbol Fuchsin Method (Marti and Johnson's modification 1951)

Fixation

Any general fixative. 10 per cent formalin

Reagents

Basic fuchsin
Phenol
Nitric acid
Malachite green
Tergitol

Preparation of reagents

Solution 1 (Carbol fuchsin)

Basic fuchsin	4.0 g
Phenol	8.0 g
95 per cent ethyl alcohol	20.0 ml
Distilled water	100.0 ml

Add 1 drop tergitol up to 7 to every 30 ml or above.

Solution 2 Acid alcohol

Conc. nitric acid	0.5 ml
95 per cent ethyl alcohol	95.0 ml

Solution 3 Malachite green

Malachite green oxalate	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place in solution 1 (Carbol fuchsin) for 30 min.
3. Wash in running water for 5 min.
4. Decolorize in solution 2 (acid alcohol) for 3 min.
5. Wash in running water.

6. Rinse in 95 per cent alcohol.
7. Wash in running water.
8. Transfer to solution 3 (malachite green) for 30 sec.
9. Wash in water.
10. Dehydrate, clear and mount.

Result

Acid-fast bacteria	Red
Tissue element	Green

Fite-formaldehyde Method (Wade's modification, 1957)

Fixation

Zenker preferably

Reagents required

New fuchsin
Phenol
Methyl alcohol
Acid fuchsin
Picric acid
Potassium permanganate
Oxalic acid

Preparation of reagents

Solution 1 Phenol new fuchsin

New fuchsin	500 mg
Phenol	5.0 g
Ethyl or methyl alcohol	10.0 ml
Distilled water	100.0 ml

Solution 2 Van Gieson modified

Acid fuchsin	10 mg
Picric acid	100 mg
Distilled water	100.0 ml

Procedure

1. Deparaffinize in turpentine–paraffin oil (2 : 1) giving 2 changes.
2. Drain and blot excess fluid.

3. Stain in solution 1 (Phenol fuchsin) overnight.
4. Wash in tap water.
5. Flood with formalin for 5 min.
6. Wash in water.
7. Treat with 5 per cent sulphuric acid (5 ml/95 ml water) for 5 min.
8. Wash.
9. Treat with1 per cent $KMnO_4$ (1 g/100 ml water) for 3 min.
10. Wash.
11. Bleach in 2 : 5 % oxalic acid (2.5 g/100 ml water) for 30 sec.
12. Wash.
13. Place in solution 2 (modified Van Gieson) for 3 min.
14. Rinse in 95 per cent alcohol.
15. Dehydrate, clear and mount.

Results

Acid-fast bacteria	Deep blue
Connective tissue	Red
Other tissue elements	Yellow

(Also refer Beamer and Firminger, 1955), Tilden & Tanaka, 1945.

Wade's Method for Acid-Fast Bacteria (Wade, 1952)

Fixation

Zenker

Reagents required

Terpentine
Potassium permanganate
Paraffin oil
Phenol crystals
New fuchsin
Oxalic acid
Sulphuric acid

Preparation of reagents

Soluiton 1 Deparaffinizing solution

Turpentine	2 parts
Paraffin	1 part

Solution 2 Carbol new fuchsin

New fuchsin magenta III	500 mg
Pheno crystals liquid	5.0 ml
100 per cent alcohol	10.0 ml
Distilled water	100.0 ml

Solution 3 5 per cent sulphuric acid

Conc. Sulphuric acid	5.0 ml
Distilled water	95.0 ml

Solution 4 1 per cent potassium permanganate

1 g/100 ml water

Solution 5 2 per cent oxalic acid

2 g/100 ml water

Solution 6 Modified Van Gieson

Acid fuchsin	10 mg
Picric acid	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize in solution 1 and hydrate slides to water.
2. Place in solution 2 overnight.
3. Wash in distilled water.
4. Place in concentrated formalin until sections are blue.
5. Wash in running water.
6. Place in solution 3 for 1 min.
7. Wash in running water.
8. Transfer to solution 4 for 3 min.
9. Rinse in tap water.
10. Transfer to solution 5 for 2 min.
11. Rinse in tap water.
12. Place in solution 6 for 5 min.
13. Wash.
14. Dehydrate and mount.

Result

Acid-fast bacilli	Deep blue
Connective tissue	Red
Background	Yellow

Warthin-Starry Silver Method (Kerr, 1938; Faulkner and Lillie, 1945; Bridges and Luna, 1957)

Fixation

10 per cent formalin

Reagents required

Citric acid
Silver nitrate
Hydroquinone
Gelatine
Triple-distilled water

Preparation of reagents

Solution 1 Acid water

Triple-distilled water	100.0 ml
Citric acid	10.0 g

Solution 2 2 per cent silver nitrate

Silver nitrate	2.0 g
Acidulated water	100.0 ml

Solution 3 1 per cent silver nitrate

Silver nitrate	1.0 g
Acid water	100.0 ml

Solution 4 0.15 per cent hydroquinone

Hydroquinone	150 mg
Acidified water	100.0 ml

Solution 5 5 per cent gelatin

Gelatin (extra pure)	5.0 g
Acidified water	100.0 ml

Solution 6 Developer

Solution 2	2 per cent silver nitrate	1.5 ml
Solution 5	5 per cent gelatin	3.75 ml
Solution 4	0.15 per cent hydroquinone	2.0 ml

Preheat solution to 55–60°C, mix in order while staining.

Procedure

1. Deparaffinize and hydrate slides to solution 1.
2. Impregnate in solution 3 (1 per cent silver nitrate) at 55–60°C for 30 min.

3. Place slides in a coplin jar and pour warm solution 6 (developer) till the sections become golden brown or yellow.
4. Rinse with warm tap water.
5. Dehydrate, clear and mount

Result

Spirochetes	Black
Background	Yellow

Levaditi Method for Block Staining (Mallory, 1944)

Fixation

10 per cent formalin

Reagents required

Silver nitrate

Pyrogallic acid

Formalin

Preparation of reagents

Solution 1 Silver nitrate

Silver nitrate	1.5–3.0 g
Distilled water	100.0 ml

Solution 2 Reducing solution

Pyrogallic acid	4.0 g
Formalin	5.0 ml
Distilled water	100.0 ml

Procedure

1. Rinse blocks of tissue in tap water.
2. Place in 95 per cent ethyl alcohol.
3. Transfer to distilled water.
4. Impregnate with solution 1 (silver nitrate) at 37°C for 3–5 days.
5. Wash in distilled water.
6. Keep in solution 2 and reduce at room temperature in dark for 24–72 hrs.
7. Wash in distilled water.
8. Dehydrate, clear in cedar wood oil, infiltrate with paraffin.
9. Embed, take 5μ sections and mount on slide.
10. Dewax with xylene and mount.

Result

Spirochetes	Black
Background	Brownish yellow

Remarks

All glassware should be cleaned with potassium dichromate + sulphuric acid. All solutions must be fresh, coat forceps with paraffin. Use triple-distilled water.

Krajian Silver Stain for Spirochetes (Krajian, 1939 Modified by Waltar Smith *et.al.*, 1969)

Fixation

10 per cent formalin for 24 hrs

Reagents required

Acetone
Glycerine
Uranium nitrate
Gum mastic
Silver nitrate hydroquinone
Sodium sulphite
Formaldehyde
Sodium thiosulphate

Preparation of reagents

Solution 1 Mordant solution

Acetone	30.0 ml
Ethanol (90 per cent)	30.0 ml
Uranium nitrate	3.0 g

Preparation should be done in a clean Erlenmeyer flask. After dissolving the ingredients, keep the solution in an amber-coloured bottle in a refrigerator.

Solution 2 Gum mastic

Gum mastic	25.0 g
Absolute ethanol	35.0 ml

Put this in a clean flask and shake frequently at least for 5 days till the solution is clear. Now the solution is stable and can be stored for a long time. Only the clear portion of the solution is used.

Solution 3 Dilute gum mastic solutions

Everyday, add 70 drops of saturated alcoholic gum mastic, i.e., solution 2, to 100 ml of 95 per cent ethanol.

Solution 4 Silver nitrate stock solution

Silver nitrate	10.0 g
Triple-distilled water	100.0 ml

Store in an amber-coloured bottle in a refrigerator.

Solution 5 Developer

Hydroquinone	620 mg
Sodium sulphate	200 mg
Formaldehyde	5.0 ml
Acetone	5.0 ml
Pyridine	5.0 ml
Saturated gum mastic (Solution 2)	5.0 ml
Triple-distilled water	30.0 ml

Mix this in order and it should be prepared very fresh, i.e., just before immersing the slides in the solution. Heat it in a water bath at 60°C.

Solution 6

Sodium thiosulphate	5.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize and bring down to triple-distilled water (2 sets of slides—one as control).
2. Place the slides in solution 1 at 60°C in a water bath for 10 min.
3. Rinse in distilled water.
4. Transfer slides to water.
5. Rinse in distilled water
6. Take solution 4 in a coplin jar and place the slides and heat in a water bath at 75°C for 7 min.
7. Now prepare the final mixing of solution 5 (developer) and heat at 60°C in a water bath.
8. Then expose the slides to light, again dip and expose 5–7 times.
9. Wash in distilled water.
10. Transfer the slides to solution 6 for 5 min.
11. Rinse in distilled water.
12. Dehydrate, clear and mount.

Result

Spirochetes	Black
Background	Brown

Gomori's Methanamine Silver Nitrate Method (Grocott's adaptation, 1955, Mowry's modification, 1959)

Fixation

10 per cent formalin

Reagents required

Silver nitrate
Methanamine
Borax
Light green
Glacial acetic acid
Periodic acid
Chromic acid
Sodium bisulphite

Preparation of reagents

Solution 1 Methanamine silver nitrate stock solution

5 per cent silver nitrate (5 g/100 ml water)	5.0 ml
3 per cent methanamine (3 g/100 ml water)	100.0 ml

Solution 2 Working solution

5 per cent borax (5 g/100 ml water)	2.0 ml
Distilled water	25.0 ml
Methanamine stock (Solution 1)	25.0 ml

Solution 3 Light green stock solution

Light green yellowish	200 mg
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

Solution 4 Working solution

(Solution 3) Light green stock solution	10.0 ml
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Oxidize in 0.5 per cent periodic acid for 45 min.

3. Wash in water.
4. Oxidize in 5 per cent chromic acid (5 g/100 ml water) for 45 min.
5. Wash.
6. Transfer to sodium bisulphite (2 per cent) (2 g/100 ml of water) for 5 min.
7. Wash in tap water.
8. Wash in distilled water.
9. Transfer to solution 2 (methanamine silver nitrate) 58°C for 30 min.
10. Wash for several changes.
11. Tone in gold chloride.
12. Rinse in distilled water.
13. Place in 5 per cent sodium thiosulphate (5 g/100 ml water) for 3 min.
14. Wash in running water.
15. Counterstain in solution 4 (light green) for 30 sec.
16. Dehydrate, clear and mount.

Results

Fungi	Black
Background	Light green

Metachromatic Method (Kelly *et al.*, 1962)

Fixation

10 per cent formalin

Reagents required

Sulphuric acid

Toluidine blue

Acetic acid

Preparation of reagents

Solution 1 Sulphation reagent

Concentrated sulphuric acid is added dropwise to equal volume of acid diethyl ether.

Solution 2 Toluidine blue

Toluidine blue	10 mg
3 per cent aqueous acetic acid	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Air-dry for 10 min.

3. Keep slides in solution 1.
4. Wash.
5. Transfer to solution 2.
6. Wash in 3 per cent acetic acid.
7. Dehydrate, clear and mount.

Result

Fungi	Red
Background	Pale blue or colourless

(*See* also Schneider, (1963))

Gridley's Method For Fungi: (Gridley, 1953)

Fixation

10 per cent neutral buffered formalin

Reagents required

Chromic acid
Metanil yellow
Basic fuchsin
Paraldehyde
Hydrochloric acid
Potassium metabisulphate
Charcoal

Preparation of reagents

Solution 1 4 per cent chromic acid solution

Chromic acid	4.0 g
Distilled water	100.0 ml

Solution 2 Feulgen's reagent,

Solution 3 Aldehyde fuchsin,

Solution 4 2.5 per cent metanil yellow

Metanil yellow	250 mg
Distilled water	100.0 ml
Glacial acetic acid	0.25 ml

Procedure

1. Dewax and bring down slides to water.
2. Oxidize in solution 1 for 1 hr.
3. Wash in running water till colourless.

4. Transfer to solution 2 for 15 min.
5. Wash in running water for 10 min.
6. Rinse in 70 per cent alcohol.
7. Transfer to solution 3 for 30 min.
8. Differentiate in 70 per cent alcohol.
9. Counterstain with solution 4 for 1 min.
10. Rinse in distilled water.
11. Dehydrate, clear and mount.

Results

Mycelia	Deep purple
Conidia	Deep rose
Background	Yellow
Elastic fibres	Purple

Grocott's Method For Fungi (Grocott, 1955)

Fixation

10 per cent neutral buffered formalin

Reagents required

Chromic acid
Silver nitrate
Methanamine
Borax
Sodium bisulphite
Gold chloride

Preparation of reagents

Solution 1 4 per cent chromic acid solution
(4 g/100 ml water)

Solution 2 5 per cent silver nitrate solution
(5 g/100 ml water)

Solution 3 3 per cent methanamine solution

Hexamethylenetetramine	3.0 g
Distilled water	100.0 ml

Solution 4 5 per cent borax solution

Borax	5.0 g
Distilled water	100.0 ml

Solution 5 Methanamine–silver nitrate stock

Solution 2	5.0 ml
Solution 3	100.0 ml

Solution 6 Methanamine–silver nitrate working solution

Solution 5	25.0 ml
Distilled water	25.0 ml
Solution 4	2.0 ml

Solution 7 1 per cent sodium bisulphite solution

Sodium bisulphite	1.0 g
Distilled water	100.0 ml

Solution 8 0.1 per cent gold chloride

Gold chloride water	100 mg
Distilled water	100.0 ml

Solution 9 2 per cent sodium thiosulphate solution

Solution 10 0.2 per cent light green solution

Light green	200 mg
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

Procedure

1. Dewax and bring down slides to water.
2. Place in solution 1 for oxidizing for 1 hr.
3. Wash in running water.
4. Place in solution 7 for 1 min.
5. Wash in running water.
6. Transfer to solution 6 at 58°C for 1 hr.
7. Rinse in distilled water.
8. Differentiate in solution 9 for 1 min.
9. Tone in solution 8 for 5 min.
10. Rinse in distilled water.
11. Counterstain with solution10.
12. Rinse in distilled water.
13. Dehydrate, clear and mount.

Result

Fungi	Black
Mucin	grey

Mycelia	Rose
Background	Green

Castaneda's Method (Gradwohl, 1963)

Fixation

Regaud's or any general fixative

Reagents required

Dibasic sodium phosphate
Monobasic sodium phosphate
Methylene blue
Potassium hydroxide
Formalin
Safranin

Preparation of reagents

Solution 1 Buffer solutions

Solution 1a

Dibasic sodium phosphate (Na_2HPO_4)	23.86 g
Distilled water	1000.0 ml

Solution 1b

Monobasic sodium phosphate anhydrous (Na_2HPO_4)	11.34 g
Distilled water	1000.0 ml

Solution 2 Working solution

Solution 1a	88.0 ml
Solution 1b	12.0 ml
Formalin	0.2 ml

Solution 3 Methylene blue solution

Dissolve methylene blue	21.0 g
95 per cent alcohol	300.0 ml
Dissolve potassium hydroxide	0.1 g
Distilled water	1000.0 ml

Mix methylene blue solution and potassium hydroxide solution and allow to stand for 24 hrs.

Staining solution

Solution 4

Mix solution 2 with solution 3.

Procedure

1. Hydrate slides to 50 per cent alcohol.
2. Place in solution 4 (methylene blue solution) for 2–3 min.
3. Wash in running water for 30 seconds.
4. Counterstain in 1 per cent safranin (1 g/100 ml water) for 2 min.
5. Rinse in 95 per cent alcohol.
6. Dehydrate, clear and mount.

Result

Rickettsia	Light blue

Ordway-macchiavellow Method: (Gradwohl, 1963)

Fixation

Regaud's fixative

Reagents required

Porrier's blue

Eosin bluish

Preparation of reagents

Solution 1

Porrier's blue 1 per cent (1 g/100 ml water)	10.0 ml
Eosin bluish 0.45 per cent (0.450 mg/100 ml water)	15.0 ml

Solution 2

Mix the two before use and then add 25.0 ml distilled water while stirring.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place in staining solution 2 for 8 min.
3. Decolorize in 95 per cent ethyl alcohol till slides are pale bluish pink.
4. Dehydrate, clear and mount.

Result

Rickettsia	Bright red
Inclusion bodies	Bright red

Wolback's Variation of the Giemsa Stain for Rickettsiae (Wolback *et al.*, 1922)

Fixation

Zenker's fixative

Reagents required

Giemsa

Methanol

Rosin

Sodium carbonate

Preparation of reagents

Solution 1 Giemsa stain solution

(Prepare stock solution of Giemsa as Good Burn and Marmion stain)

Giemsa stock	2.5 ml
Distilled water	100.0 ml
Absolute methanol	2.5 ml
0.5 per cent sodium carbonate	0.25 ml

Solution 2 Colophonium alcohol

Rosin (Colophonium)	10.0 g
95 per cent ethanol	100.0 ml

Solution 3 For working solution

Solution 2—10 per cent rosin	0.5 ml
85 per cent ethanol	100.0 ml

Procedure

1. Deparaffinize and bring down sections to water.
2. Immerse in solution 1 for 1 hr. Repeat it at 1 hr interval overnight.
3. Rinse in distilled water.
4. Differentiate in 95 per cent alcohol and later place in solution 3 and expose to sunlight.
5. Dehydrate, clear and mount.

Results

Rickettsiae and chlamydiae	Reddish purple
Nuclei	Blue-purple
Cytoplasm	Blue

Pinkerton's Method For Rickettsiae: (Simmons and Gentzkow, 1944)

Fixation

Zenker's Regaud's

Reagents required

Methylene blue

Basic fuchsin

Citric acid

Preparation of reagents

Solution 1 1 per cent methylene blue solution

Methylene blue	1.0 g
Distilled water	100.0 ml

Solution 2 0.25 per cent basic fuchsin

Basic fuchsin	250 mg
Distilled water	100.0 ml

Solution 3 0.5 per cent citric acid solution

Citric acid	500 mg
Distilled water	100.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in solution 1 overnight.
3. Rinse in 95 per cent alcohol.
4. Rinse in distilled water.
5. Transfer to solution 2 for 30 min.
6. Decolorize rapidly in solution 3.
7. Dehydrate, clear and mount.

Result

Rickettsia	Bright red
Nuclei	Blue

Lendrum Method for Inclusion Bodies (Lendrum, 1947)

Fixation

Zenker's fixative

Reagents required

Mayer's haematoxylin

Phloxine

Tartrazine

Preparation of reagents

Solution 1 Mayer's haematoxylin

Solution 2 0.5 per cent phloxine solution

Phloxine	500 mg
70 per cent alcohol	200.0 ml
Calcium chloride	1.0 g

Solution 3 2.5 per cent tartrazine solution

Tartrazine	2.5 g
Ethylene glycolmonoethyl ether	100.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Place in solution 1 for 10 min.
3. Wash in running water till sections are blue.
4. Place in solution 2 for 30 min.
5. Rinse in distilled water.
6. Place in solution 3 until inclusion bodies are red.
7. Dehydrate, clear and mount.

Result

Inclusion bodies	Red
Nuclei	Blue
Background	Yellow

Page-green Method For Inclusion Bodies (Page and Green, 1942)

Fixation

Any general Fixative

Reagents required

Hydrochloric acid

Beibrich scarlet

Orange G

Fast green

Phosphotungstic acid

Phosphomolybdic acid

Acetic acid

Haematoxylin

Ammonia

Preparation of reagents

Solution 1 1 per cent acid alcohol

Concentrated HCl	100.0 ml
Distilled water	100.0 ml

Solution 2 S. Lott's staining solution

50 per cent alcohol	100.0 ml
Biebrich scarlet	1.0 g
Orange G	250 mg
Fast green FCF	75 mg
Phosphotungstic acid	500 mg
Phosphomolybdic acid	0.5 g
Glacial acetic acid	2.0 ml

Solution 3 Harris haematoxylin

Solution 4 Ammonia water solution

Tap water	1000.0 ml
Ammonium hydroxide (20 per cent)	2.3 ml

Procedure

1. Dewax and bring down slides to water.
2. Place in solution 3 for 5 min.
3. Rinse in water.
4. Differentiate in solution 1 until no haematoxylin comes out.
5. Wash in water.
6. Blue sections in solution 4.
7. Wash in running water for 10 min.
8. Transfer to solution 2 for 1 min.
9. Rinse in 95 per cent alcohol.
10. Dehydrate, clear and mount.

Result

Inclusion bodies	Brilliant red
Connective tissue	Light green
Elastic tissue	Purplish red
Keratin	Orange
Erythrocytes	Orange-red
Nuclei	Blue

9

PREPARATION OF PERMANENT WHOLE MOUNTS OF INVERTEBRATES OR THEIR PARTS

All invertebrates cannot be treated alike as far as fixatives and their applications are concerned. However, certain generalized applications to the most common forms could be made. Fixatives cannot be applied directly to all forms since some groups like coelenterates withdraw the tentacles, the appendiculated forms contract their appendages, and the soft-bodied forms roll up into balls. To avoid this, certain anaesthetizing and narcotizing agents have to be administered before killing and fixing the specimens. As soon as narcotization is complete, one can proceed with fixation before the death of the organism.

SOME NARCOTIZING AND ANAESTHETIZING AGENTS

Magnesium chloride or Magnesium sulphate

These two are popular and extensively used narcotizing agents, in anaesthetizing sea anemones, corals, tunicates, annelids, nudibranchs. Magnesium sulphate is tied in a bag and suspended in water to touch its surface. Another way is to siphon out 30% of solution directly on the organism. To ensure complete narcotization, light pricking with needle (without damaging the tissues) is necessary. If there is no response then the organism is considered sufficiently narcotized.

Menthol

In a bowl of water, sprinkle menthol on the surface and narcotize big animals by leaving overnight. Menthol mixed with chloral hydrates in the ratio 45.0 g and 55.0 g respectively works well. The powders are ground and sprinkled on the surface of water. It is good for sessile animals such as bryozoans, coelenterates like hydra and even some flukes. Larger animals require longer exposure. When contractions and movements cease, the organism can be fixed. For some molluscs, tunicates, hydrozoans, turbellarians, annelids, chloral hydrate alone is sufficient.

Cocaine

One per cent solution of cocaine (1 g/100 ml water) is added to the water so as to touch the surface of nudibranchs, rotifers and bryozoans. Eucaine hydrochloride also serves the purpose.

Chloretone

It is a slow narcotizer and 0.33 to 1.0 per cent could be used for narcotizing. This is especially useful for monogeneans.

Chloroform

For aquatic animals both, marine and freshwater, chloroform can be sprinkled on the surface of the water. Insects and arachnids are narcotized in this way.

Ether and Alcohol

Ether can be used as chloroform for insects whereas alcohol is added dropwise to water and the proportion should not exceed 10 per cent. Among terrestrial animals, it is good for earthworms and is also recommended for freshwater forms.

Asphyxiation

Water is boiled to remove all oxygen. Cool the water, and immerse animals in the non-oxygenated water. This is especially good for gastropods.

Cold Treatment

The organisms are kept in a freezing chamber or in a mixture of ice and salt or in ice-cool water until relaxation occurs. Later specimens are transferred to lukeworm water before fixing.

Hanley's solution (Gray, 1954)

Water	90.0 ml
Ethyl cellosolve—1	10.0 ml
Eucaine hydrochloride	300 mg

The solution is added to water containing animals. It is recommended for rotifers and hydrozoans.

Propylene phenoxetol (Owen, 1965, Owen and Steedman, 1956, 1958)

Take 5 ml of this solution and add 15–20 ml of seawater where animals are kept. This solution is recommended for molluscs.

SOME METHODS OF HANDLING

Porifera

For small poriferans the following mixture can be used.

Osmium tetroxide	2.5 g
Mercuric chloride	9.0 g
Water	250.0 ml

Large sponges can either be fixed in Gilson's or Carnoy fluid.

Decalcifying fluid for calcareous sponges 70–80 per cent alcohol + 3 per cent hydrochloric acid is used for silicious sponges. 80 per cent alcohol + 5 per cent hydrochloric acid is used for calcareous sponges. After decalcification and desilicification, transfer them to 80 per cent alcohol. A paraffin-coated container is best suited for the procedure.

Desilicification in a solution containing

80 per cent alcohol	100.0 ml
5 per cent hydrochloric acid	

This acid should be added to alcohol slowly in a wide-mouthed container which should be coated internally with paraffin wax. Animals can be later transferred to 80 per cent alcohol.

Coelenterates

Techniques differ from group to group.

Hydra Place hydra in a Petri dish. When the animals are properly extended pour on to them a solution containing

Saturated mercuric chloride	95.0 ml
Glacial acetic acid	5.0 ml

This solution should be poured starting from basal disc towards oral end. This will prevent the withdrawal of tentacles.

Sea anemones First, anaesthetize animals with either menthol or 30 per cent magnesium chloride. These solutions should be added slowly for a period of one or two hours. When no contraction is evident, pipette out excess solution, leaving sufficient solution to cover and then add Susa or Bouin's or any other fixative having mercuric chloride or 10 per cent formalin. After they are properly fixed, transfer them to fresh fixative. Animals harden later.

Scyphozoans

To a jelly fish in seawater add slowly 10 per cent formalin, with constant stirring. The concentration should be 10 ml to 100 ml in water. After 3–4 hrs, transfer specimens to fresh formalin.

Medusae Medusae should be anaesthetized with either cocaine or magnesium chloride. Continue adding the anaesthetizer until contractions cease. Transfer them to 10 per cent neutral buffered formalin.

Corals

Corals have to be narcotized with magnesium sulphate and later fixed in a solution containing saturated mercuric chloride and 5 per cent acetic acid. This solution should be heated before fixing the animal.

Apart from all these methods, simple freezing may help proper fixation.

Planarians

Animals have to be starved at least two days before fixation. Put planarian in a small quantity of water in a Petri dish. When properly extended ,add a drop or two of 2 per cent nitric acid directly. Then add Gilson's or saturated mercuric chloride in saline. Transfer it to a fresh fixative. Dawar (1973) recommended the following relaxing and fixing agent.

Magnesium sulphate	2.50 g
Formaldehyde	4.5 ml
Conc. nitric acid	2.0 ml
Distilled water	200.0 ml

This has to be conducted at room temperature for 24 hrs. From our experience it has been observed that hot Susa is good for specimens which are to be sectioned.

Platyhelminthes

Trematodes First they can be anaesthetized with menthol. First soak a filter paper in Gilson's fluid and place it on a glass plate. Take another soaked paper and place it on the worm. Apply proper pressure (taking caution not to destroy the tissue). The two glass plates could be tied with rubber bands at either end. Leave it in this position at least for 12 hrs in fixative. Remove the glass plates and filter paper and transfer the worm to fresh fixative and leave overnight.

For small trematodes put them in a small dish, and shake with small quantity of 0.5–1.0% salt solution. After 5 min. add saturated mercuric chloride containing 2 per cent acetic acid and again shake for several minutes. Change to fresh fixative for 6–12 hrs. Remove the worm and place it on a slide and tie it with two rubber bands or if it is too small with a cover slip. Pipette fixative between two slides and then leave the slides in a Petri dish containing fresh fixative (Gower, 1939). For sectioning, stretch specimen on a dry filter paper and stretch it stroking gently with camel hair brush. The specimen now remains stuck to the filter paper. Now put appropriate fixative (Susa, Bouin's, FCa, etc.) dropwise. Let the specimen remain for one hour in the fixative.

Cestodes Put the worm first in a freezing chamber until relaxed (overnight). Then flatten the worm in between two slides. Tie thread around the slides to keep them together (Demke 1952). If a museum specimen mount is required, bind the worm on a long glass plate and pour fixative on it. Sometime after chilling, they can be relaxed in 70 per cent alcohol or hot 5 per cent formalin. Small cestodes can be placed on a tall container with an applicator stick and fixative poured on it. When they hang straight, immerse them in fixative.

Nemertines Nemertines are best fixed in a solution containing saturated mercuric chloride and acetic acid in the ratio 95 : 5.

Rotifers These are narcotized with the following fluid containing

2 per cent benzamine hydrochloride	3 parts
Distilled water	6 parts
Cellosolve	1 part

After narcotization is complete, add 10 per cent neutral buffered formalin.

Nemathelminthes / Nematodes

Worms are initially placed in physiological saline. Solution is shaken well. To straighten nematodes, pour hot glycerine alcohol copiously on the specimen (80 per cent alcohol and 10 per cent glycerine). They are then transferred to glycerol (80 per cent alcohol and 10 per cent glycerol).

If small nematodes are to be treated, place them in a watch glass and apply gentle heat taking care not to destroy the tissue and then transfer them to a solution containing

95 per cent alcohol	12.20 ml
Glacial acetic acid	10.0 ml
Formalin	6.0 ml
Distilled water	40.0 ml

If a few drops of saturated aqueous picric acid are added, the worm takes up colour. If a whole mount has to be prepared, place the animal in glycerol–alcohol mixture. Allow the alcohol to evaporate. When alcohol is completely evaporated, only pure glycerine is left. Now the worm can be mounted in glycerine jelly. The same procedure can be followed for helminth ova.

When blood has to be tested for microfilaria, take blood smears at midnight as it is done for any other smear taken. Dry the slide and fix it in mercuric chloride containing fixative and stain with Delafield's haematoxylin. An alternate method to smear is to dry the blood, dehaemoglobinize in 5 per cent acetic acid, air-dry, fix in methyl alcohol and stain with Giemsa.

Bryozoa

Bryozoans are to be anaesthetized first with menthol. Marine bryozoans can be fixed in a solution containing 10 per cent chromic acid (10 g/100 ml water) 10.0 ml + 10 per cent acetic acid, 10.0 ml + 8.0 ml distilled water. Freshwater bryozoans can be fixed in 10 per cent formalin.

Annelida

When earthworms are to be sectioned, starve them for two days on moist filter paper to keep the intestine free of grit or any other hard material. Instead of starving them, they can be fed with cornmeal and agar (1:1) or chopped lettuce for a couple of days. (Cocke, 1938), or with agar only (Becker and Roudabush, 1935). When no more grit is evident in faeces, place the worms in a Petri dish, add water, straighten the worm and then siphon out 50 per cent alcohol on it, so that the solution is 10 per cent. After narcotizing, fix it either in Bouin's or Susa. Wash the worms thoroughly and transfer to tertiary butyl alcohol (24 hrs) and then transfer to butyl alcohol saturated with paraffin (56°–60°) for 24 hrs and pure paraffin for 24 hrs and then embed.

Marine animals should be kept in clear seawater for 2 days and then anaesthetized with chloroform and fixed in either Bouin's or mercuric chloride fixative.

Arthropoda

Put cotton soaked either in ether or chloroform or potassium cyanide in a screw-capped bottle or a wide-mouthed bottle. Place the insect inside the bottle. As soon as the insect is anaesthetized, place it on a glass plate and spread the appendages with another plate and drain fixative in between the slides.

For whole mounts, rapidly penetrating fixative should be used. Picro-sulphuric, sublimate fixatives, mixtures containing nitric acid, alcohol or Bouin's could be used.

For whole mounts, clearing of exoskeleton is difficult but is an essential process. Body contents have to be transparent or must be removed. Lactophenol mounting will serve the purpose. As the arthropod cuticle is tough and heavily pigmented, the pigment has to be bleached by hydrogen peroxide for 12 hrs. Fleas and ticks have to be bleached with 10 per cent potassium hydroxide for 8–12 hrs. This clears the body contents. Sometimes acid corrosives are preferred by some because it does not soften the integuments as much as alkaline which is corrosive.

Acid corrosives

Glacial acetic acid	1.0 ml
Chlorol hydrate	1.0 g
Water	1.0 ml

Vyas (1972) recommends a fluid containing

Glycerine	12.0 ml
Formaldehyde	2.0 ml
Distilled water	100.0 ml

Add few crystals of thymol.

This serves to preserve both exoskeleton and soft parts. When the specimens are large, this fluid can be injected directly. Potassium hydroxide is not advisable for small delicate insects. Equal parts of chlorol hydrate and phenol are suggested to clear them. Leave them in this fluid for two weeks. If clearing is not satisfactory, transfer the solution to 40°C for two days. Later transfer them to absolute alcohol and mount in lactophenol.

Because of the chitinous cuticle, sectioning becomes difficult when dioxane method or double embedding is preferred. Sometimes tissue blocks may be soaked overnight or for several days.

Modified Carnoy

Absolute isopropyl alcohol	6 parts
Chloroform	3 parts
Formic acid	1 part

Fixation, dehydration and infiltration are processed under reduced pressure.

Refer Beckel (1959) for sectioning methods. For better sections, see Barros-Pita (1971), Roden (1975), Nelson (1974) and Kimmel and Jee (1975).

Molluscs

Snails are put in boiled water and propylene phenoxetol is added. For bivalves, the shells should be decalcified with 3–4 per cent nitric acid. If the soft parts are to be fixed, keep the valves apart and put the entire animal in the fixative. After proper fixation, dissect out the soft parts and fix again in a fresh fixative.

To decalcify bivalve shells, fix in 5 per cent formaldehyde overnight and decalcify with 2 per cent acetic acid. After decalcification, shell is put in 5 per cent formaldehyde for 5 hrs and then washed in water

overnight. The material is transferred to 70 per cent ethanol, dehydrated and cleared in creosote. Anderson (1971) recommends a decalcifier fixative (2 : 1) for good cellular details. All molluscs with calcareous shells can be treated in a similar manner.

Nudibranchs On to the nudibranchs kept in sea water add 1 per cent neutral buffered formalin slowly dropwise every 15 min.

Fresh water molluscs Warm the water slowly in which they are held. This will make them extend the foot. When there is no response for needle prick, fix them in Gilson's, Bouin's or Zenker.

Echinoderms

Narcotize echinoderms by sprinkling menthol on the surface of water. Magnesium chloride or magnesium sulphate can be added to water. Initially inject the fixative (mercuric chloride acetic acid) into the top of the ray. This will spread even into the tube feet. Then drop the animal in the fixative (Moore, 1962).

STAINING INVERTEBRATES

Staining of sections depends on the choice of the stain and relative fixation. Delicate transparent forms like hydra, hydroids, daphnids, bryozoans, medusae, flukes, tapeworms small annelids, tunicates and ammocetes larvae can be stained with alum carmine or Kornhauser haematein as an alternative.

Some trematodes and tapeworms have thick muscle layer apart from tegument and the body appears opaque and the internal anatomy remains obscure. In such cases, stain the worms, dehydrate, clear in a good oil like cedar wood oil or tarpenol on a glass plate and put it in a dish and keep the dish under a binocular microscope. Alternate method is to stain and then wash in water. Then transfer to 0.5 per cent potassium permanganate. Then the worm turns to greenish brown colour. Remove it immediately to distilled water for 5 min., then transfer the worms to 2–3 per cent oxalic acid. It is bleached and the greenish brown sheen is lost. Wash in running water, dehydrate, clear and mount.

After destaining, the specimen can be turned blue with sodium carbonate solution. Other references concerning invertebrate staining are:

- Beckel and Roudabush, 1945
- Galigher and Kozloff, 1964
- Gatenby and Beams, 1950
- Gray, 1954
- Mahoney, 1968
- Pantin, 1946

Preparation of Chick Embryos

Break the shell of the egg with the handle of a scissors at the air space. Cut the shell along the long axis avoiding damage by scissors. Keep the egg on the embryo cup and immerse in physiological saline at 37°C. Carefully remove half of the shell. The embryo can be seen floating on the yolk. With small scissors

cut quickly around. Grip one edge with forceps and gently slip a watch glass under the chick embryo. Lift the watch glass with the embryo with little saline. Any yolk particles coming along with the embryo can be pipetted out. Straighten out the embryo to ensure that there are no folds. Gently pipette saline on the embryo; then draw out all saline, traces of yolk and vitelline membrane with a pipette.

Cut filter paper into small triangles and place them along circular margin of the area all around to stretch. With a pipette apply Bouin's or Gilson's fixative. Leave overnight to harden the embryo. Remove embryo from the dish and transfer to fresh fixative and wash it. Alum carmine or Ehrlich haematoxylin are suitable stains. After Ehrlich haematoxylin staining, destain appropriately and blue with sodium bicarbonate or ammonia. Wash, dehydrate, clear and mount. While placing the coverslip, avoid undue pressure. This method could be applied to reptilian and mammalian embryos and amphibian larvae.

Whole Mounts

Whole mounts of small specimens, are usually mounted in resins in toluene, glycerine jelly or gum arabic.

Fixation

1. Place specimen in 70 per cent alcohol.
2. Transfer to 95 per cent alcohol.
3. Transfer to bleaching fluid

70 per cent alcohol	10.0 ml
Chlorox	4 drops

or

Water	500 ml
H_2O_2	50.0 ml
With traces of ammonia	24 hrs

Procedure

1. Wash with 70 per cent alcohol giving 3 changes.
2. Dehydrate in 95 per cent alcohol.
3. Clear in creosote and xylene mixture.
4. If suddenly put in a mountant, the tissue may become brittle. To avoid this, impregnate gradually by adding a drop of mountant each day and stir carefully.
5. Now mount the specimen. If the specimen is large ,it should have support under the cover glass. Support may be in the form of cover glass bits or cutting glass rods, circle or squares. Place a drop of mountant on the slide and place specimen in the centre of the mount and mounted cover glass. If many specimens are to be put on the same slide, there is a chance of drifting. To avoid this air-dry the mountant, then place the coverslip.

If celloid is required in mounting, dehydrate through absolute alcohol, alcohol ether and then into thin colloidin. Put specimens in Petri dish with celloidin. After the solvent evaporates squares of colloidin are cut, dehydrated, cleared and mounted. Advantage of this method is that no supports are required while mounting with a coverslip. PVA is also used as a mounting medium (Rubin, 1951). Courtright (1966) used polyester resins.

Glycerol Jelly Mounts

Most materials fixed in various fixatives or even frozen sections may be mounted directly from water to glycerol jelly. If there is danger of specimens collapsing, transfer the material from 70 per cent alcohol or water into a mixture containing 10–15 per cent glycerol in alcohol or water. Keep the dish containing glycerol alcohol open. Alcohol evaporates, and the material can now be mounted in glycerol jelly.

Flukes, Embryo and Hydra Staining

Fixatives

Worms	Carnoy, Gilson's
Embryos	Zenker's or Bouin's
Hydra	Formolacetic or saturated mercuric chloride

Staining Acetic 95/5

A number of stains are available.

Grenacher borax carmine (Galigher, 1934*)*

Carmine	3.0 g
Borax	4.0 g
Distilled water	100.0 ml

Boil until carmine dissolves. Then allow it to stand for some time and then add 100.0 ml of alcohol.

Procedure

1. Bring down material to 50 per cent alcohol.
2. Transfer to Borax carmine solution for 4 hrs or overnight
3. Add conc. hydrochloric acid dropwise to carmine until precipitate has settled overnight.
4. Add equal volume of 3 per cent HCl in 70 per cent alcohol and mix thoroughly. Draw precipitated carmine with pipette. Repeat it until most of the carmine is removed.
5. Add acid alcohol so that tissue is destained.
6. Then transfer specimens to 80 per cent alcohol for 1 hr.
7. Dehydrate.
8. Clear in a mixture of absolute alcohol and creosote and then into pure creosote or xylene.
9. Mount.

Mayer's carmalum (Cowdry, 1952)

Stock (Carmine stock)

Carmine	1.0 g
Ammonium alum	10.0 g
Distilled water	200 ml

When dissolved, filter and add 1.0 ml of formalin.

Working solution

Carmine stock	5.0 ml
Glacial acetic acid	0.4 ml
Distilled water	100.0 ml

Procedure

1. Stain for 2 days. No destaining is necessary.
2. Dehydration procedure is same as above.

Haematein (Kornhauser, 1930)

Stock

Haematein	500 mg
95 per cent alcohol	10.0 ml
Potassium aluminium sulphate	500.0 ml
(5 per cent aqueous)	

Put haematein in a mortar and add alcohol and grind it and then add it to potassium aluminium sulphate. Haematein stain is recommended for flat worms. Hitherto alum cochineal was popular but alum carmine is better.

Procedure

1. Stain for 10–15 hrs.
2. Place in 70 per cent alcohol.
3. Destain with acid alcohol.
4. Blue in alkaline alcohol or ammonia or sodium bicarbonate in 70 per cent alcohol.
5. Dehydrate, clear and mount.

For small organisms, alum haematoxylin is used. Celestine blue (Demke, 1952) and trichrome stain (Chubb, 1963) are also popular.

Cochineal haematoxylin

Alum cochineal	3 parts
Potash alum	30.0 g
Cochineal	30.0 g
Distilled water	100.0 ml
Delafield haematoxylin	1 part
Distilled water	25 parts

Let it stand for few hours. Filter and use.

Procedure

1. Wash with 50 per cent alcohol after fixation.
2. Wash in distilled water for 10 min.

3. Keep in staining solution overnight.
4. Place in 70 per cent alcohol.
5. Differentiate in acid alcohol (alcohol 99 : 1 Acid) until internal organization is clear.
6. Transfer to 70 per cent alcohol.
7. Blue in a solution of 70 per cent alcohol with few drops of lithium bicarbonate.
8. Dehydrate.
9. Clear in absolute alcohol + cedar wood oil (1 : 1) for 1 hr.
10. Clear in pure cedar wood oil.
11. Mount in synthetic dye.

STAINING

Protozoa

Chen (1944) Cover Glass Method

Fixation

Most favourite fixative is Schaudin's at 50–56°C for 15 min. Other recommended fixatives are Bouins, Champy's Flemmings or Worcester for fixing *Stentor, Sporostomum* and *Vorticella*. Consult Merton (1932) also.

Amoeba and *Paramecuim* must be centrifuged. Pour off the supernatant and then add Schaudin's fixative or any other fixative. Amoeba settles down at the bottom of the culture dishes. Decant most of the culture medium and pour Schaudin's (50–60°C) over the organism. After it cools down, pour an equal volume of 85 per cent alcohol. Transfer the solution to a centrifuge tube for centrifugation. Pour off the fixative, wash several times with alcohol; after each wash, centrifuge.

Procedure

1. After washing in 70 per cent alcohol, wash with 80–85 per cent for 15 min.
2. Take a cover glass and smear albumen on it. Keep the albuminized side up on slide. Place few drops of alcohol containing organisms with a pipette in the centre of the cover glass.
3. Alcohol will begin to evaporate, and specimens are in contact with albumen. When the centre of the cover glass is still moist add few drops of 95 per cent alcohol on the specimen and slowly keep the cover glass in Petri dish containing 95 per cent alcohol.
4. Transfer the cover glass carefully to absolute alcohol and later flood it with 1 per cent celloidin. Excess celloidin is drained off. Place in 70–80 per cent alcohol till it is stained.
5. Stain with haematoxylin, carmine or Feulgen staining depending on the study to be carried out.
6. Dehydrate, clear and put some mounting medium on the slide and place the cover glass with specimen on the medium.
7. Subbed cover glasses are good.

Smyth (1944) mentioned the following method. After fixation, usual procedure of passing through graded series of alcohols up to absolute alcohol is followed. Then a drop of this is put on an albuminized slide. Place the slide in absolute alcohol. After water, flood with 1 per cent celloidin. Blot excess celloidin and air-dry. Later, it can be stained with alum carmine, haematoxylin and Fuelgen.

Agrell (1958) recommends the following method. Place minute embryos on albuminized slides. Allow them to dry, and during this process, they become flattened. Dip into absolute alcohol and then into fixative. Place them in 95 per cent alcohol vapour for 1 min. Then fix. This coagulates the embryos and attaches them.

Paramaecium can be preserved intact with normal shape without contraction by adding copper sulphate or acetate.

Merton method (Kirby, 1947)

1. Put a drop containing *Paramecia* on albuminized slides for 30 sec.
2. Put an equal amount of 1 per cent copper sulphate for 7–8 min. or 3 per cent copper acetate for 45 sec.
3. Draw off part of flint and suspend slide over 2 per cent osmic acid for 46 sec.
4. Now add saturated solution of mercuric chloride on the drop of the organism for 10 min.
5. Dip in a solution containing 70 per cent alcohol and small amount of iodine for 10 min.
6. Wash with distilled water.
7. Stain.

Prescott and Carrier method (1964)

1. Place a drop of few amoebae on subbed slides.
2. Now place a drop of fixative (70 per cent alcohol or acetic alcohol) on to a cover glass and place it on *Amoebae*.
3. Freeze in liquid nitrogen for 15 sec. Take off cover glass. If liquid nitrogen is not available, fix in 50 per cent aqueous nitric acid and freeze in dry ice.
4. Rinse the slide in 95 per cent alcohol, and air-dry.

Staining of Protozoa

A number of staining methods have been described.

1. To stain fibrillar elements, use iron haematoxylin and the fixative should contain chromium. Warm the stain at 50°C and de-stain with 10 per cent H_2O_2 (Kidder, 1933).
2. To stain flagella, Giemsa stain is used (Rothenbacher and Hitchcock, 1962) and also Loefler's stain (Kirby, 1947).
3. In ciliates to stain cilia, basal granules and connecting filaments, Gelei osmium-toluidine blue method is adopted (Kirby, 1947, Pitelka, 1945).
4. For silver line system, the best techniques of Chatton and Lwoff (1930, 1935, 1936), Frankel and Heckman (1968), Gelei (1932, 35) and Klein (1926) are adopted. Also *See* Corliss (1953) for silver method. Protozoans are excellent subjects for vital staining and enzyme techniques.
5. Micro and macronuclei can be differentiated by Safranin–fast green method of Schiff *et al.* (1967).

Borror (1968) *Nigrosin Method*

Solution preparation

Solution 1

Saturated aqueous mercuric chloride	10.0 ml
Glacial acetic acid	2.0 ml
Formaldehyde	2.0 ml
Tertiary butanol	10.0 ml

Solution 2

Formaldehyde	20.0 ml
Nigrosin (water solution)	4.0 g
Distilled water	100.0 ml

Solution 3

Working solution

Solution 1	12 parts or 120 cc
Solution 2	1 part or 10 cc

Procedure

1. Place a cluster of organisms on a slide.
2. Add a drop of solution 3 for 3 to 4 sec.
3. Wash.
4. Dehydrate, clear and mount.

Result

Ciliary organelles	Black

Sectioning Protozoa

Stone and Cameron (1964) *modification of Kimball and Perdue* (1955) *agar method*

1. Select a glass tubing which is sealed at one end and then pour melted agar into it; chill until agar hardens.
2. Pipette out thick concentration of organisms on top of agar and then pipette fixative.
3. Specimens settle on agar. Then draw the fixative. Add some more agar to which eosin is added so that small organisms could be easily located. Chill agar.
4. With pipette, siphon water with force to loosen the block.
5. Dehydrate, clear and embed block of agar.
6. When block is sectioned, cells could be located because of eosin colour.

Dry Mounts

Radiolarians, foraminiferans require opaque type of slide mounting. Glue these dry specimens on a black background and cover it with a cover glass and some supporting ring around them. Gray (1964b) recommends the following method. Take two cardboard pieces of the size of a slide. Paint with black paint or cover with a strip of black paper. Make a 5/8 of an inch hole on one cardboard piece. Make 7/8 of inch square on the other cardboard and stick the two pieces by dry mounting tissue. Now place a drop of gum in the centre and with a brush pick up a specimen and place it in the centre of a drop of gum. Breathe on it because moisture is necessary to make it adhere. When the specimens have adhered cover the hole. Depression slides can also be used.

Animal parasites Haematoxylin/eosin method is good for sections of tissue parasitized by protozoa or helminths. PAS method is excellent since protozoa and helminth worms are strongly PAS-positive due to stored glycogen. Methenamine silver method is very effective for flagellates. The scolices and the hooks are best shown with haematoxylin background. Kinney *et al.* (1971) used acid-fast staining to stain hooklets.

Intestinal Protozoa Smear Technique (Concentrated Smears)

Arensbarger and Markell (1960)

Procedure

1. Take 1 ml of faeces in a tube and add 15 times its volume of water. Mix well and strain through two gauze layers of gauze in a funnel. Collect in a small centrifuge tube. Add 1 ml of ether, then shake the tube and fill with water.
2. Centrifuge (2500 rpm) and decant supernatant fluid.
3. Add 2–3 ml of normal saline and shake. Allow it to settle. Fill tube with normal saline up to 1 cm.
4. Decant the supernatant. A small trace of the original faecal matter is taken with stick (applicator stick) and mixed well with sediment at bottom of tube.
5. Transfer the material with an applicator stick on to a clean slide. Make a smear. Immediately fix in Schaudin's.
6. Stain with one of the following.

Goldman (1949) *smears*

Fixation

Schaudin's for 15 min.

Reagents required

Haematoxylin

Ferric ammonium sulphate

Glacial acetic acid

Concentrated sulphuric acid.

Preparation

Stock solution 1 Haematoxylin

10 per cent haematoxylin in 95 per cent alcohol	1.0 ml
(10 g/ 100 ml 95 per cent alcohol)	
95 per cent ethyl alcohol	99.0 ml

Stock solution 2 Alum solution

Ferric ammonium sulphate	4.0 g
Glacial acetic acid	1.0 ml
Concentrated sulphuric acid	0.12 ml
Distilled water	100.0 ml

Working Solution

Equal parts of stock 1 and stock 2 solutions. A purple colour is seen, but within a short time it turns brown. Then filter. When the solution turns greenish black it should be discarded.

1. Treat slides with 70 per cent alcohol and later with iodine alcohol.
2. Wash in 70 per cent alcohol till brown colour disappears.
3. Stain in haematoxylin for 3–5 min.
4. Wash in running water for 30 min.
5. Dehydrate, clear and mount.

Result

Protozoa	Black nuclear stain

Kohn stain – combination of both fixative and stain (Faust et al., 1970*)*

Reagents required

Methanol

Glacial acetic acid

Phenol

Phosphotungstic acid

Chlorazol black E

Preparation of reagent

Solution 1 Basic solution

90 per cent alcohol	170.0 ml
Methanol	160.0 ml
Glacial acetic acid	20.0 ml
Phenol	12.0 ml
1 per cent phosphotungstic acid	12.0 ml
Distilled water	618.0 ml

Take 5 g of chlorazol black E in a mortar and grind it. Add small quantity of solution 1. Add some more solution to the paste. Allow to settle and pour off supernatant into a container. Continue adding solution 1 gradually till the paste

turns into a solution. Add the remaining solution 1 and allow it to ripen for 4–6 weeks. Filter and store it. This solution is also available commercially.

Procedure

1. Treat sections with solution 1 for 2–3 hrs. Time varies with dilution, i.e., stain in positive basic solution, 1 : 1 overnight, 2 : 1 for 4 hrs, 1 : 2 overnight and 1 : 3 overnight.
2. Dehydrate in 95 per cent alcohol for 10–15 sec.
3. Dehydrate, clear and mount.

Result

Protozoa	Grey-green, grey or black
Cysts	Grey-green
Nuclei	Dark-green

Kessel (1925) *and Chen* (1944*a*) *Smear* (*modified*)

Fixation

Schaudin's at 40°C for 15 min.

Reagents required

Ferric ammonium sulphate
Haematoxylin
Lugol's iodine
Sodium thiosulphate
Lithium carbonate

Preparation

Solution 1 Iron alum

Ferric ammonium sulphate	4.0 g
Distilled water	100.0 ml
Haematoxylin stock	

Solution 2

Haematoxylin	1.0 ml
Absolute ethyl alcohol	100.0 ml

Allow it to ripen for several months. There are methods to hasten the process. But it is always better to use only ripened stain for a length of time.

Solution 3 Working solutions

Solution 2 (Haematoxylin stocks)	0.5 ml
Distilled water	99.5 ml

Add few drops of saturated lithium carbonate.

Procedure

1. Place slides in 70 per cent alcohol (from fixatives) for 3 min.
2. Treat with a Lugol's iodine solution for 3 min.
3. Wash.
4. Decolorize in 5 per cent sodium thiosulphate for 2 min.
5. Wash in running water.
6. Mordant in solution 1 (iron alum) at 40°C for 15 min.
7. Wash.
8. Place in solution 3 haematoxylin working solution at 40°C for 15 min.
9. Wash.
10. Destain in 2 per cent iron alum (2 g / 100 ml) and cool.
11. Wash, dehydrate, clear and mount.

Results

Nuclei—Chromatoidal bodies stain blue-black.

Lawless' Rapid methods (1953)

Fixation

Schaudin's

Reagents required

Chromatrope—2 R
Light green
Fast green
Phosphotungstic acid
Glacial acetic acid

Preparations

Solution 1 Staining solution

Chromatrope 2 R	600 mg
Light green yellowish	150 mg
Fast green	150 mg
Phosphotungstic acid	700 mg
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Add glacial acetic acid to three dyes and later add phosphotungstic acid. Allow it to stand for 30 min. before use.

Procedure

1. A portion of stool is fixed (1 part stool + 3 parts fixative) for 15 min.–1 hour. Decant excess fixatives, keep it in a vial, shake vigorously and cover the vial with gauze. Now take out the moist residue and spread it on a slide. Dry it in air and transfer the slide to iodine alcohol. These smears can be stored.
2. Decolorize in 70 per cent alcohol giving 2 changes for 1 min. in each.
3. Stain in solution for 1–10 min.
4. Differentiate in acetic acid alcohol (1 drop acetic acid / 10 ml alcohol) for 20 sec.
5. Dehydrate, clear and mount.

Result

Background	Green
Cysts	Black-green
Engulfed red cells	Either green, red or black.
Helminth eggs	Red

Remarks

If the cysts appear refractory to the stain, it means that fixation is incomplete. Bouin's fixative is preferred for karyosomes (Hajian, 1961), Phloxine-toluidine blue is used for *Leishmania*, intestinal protozoa and *microfilariae* (Tamilnson and Grocott, 1944), whereas PAS combined with haematoxylin and light green was recommended by Silva (1961) and carmine by Mariweather (1934).

10

METHODS FOR SPECIAL ORGANS

Soon after the death of an animal, autolysis sets in. It is advisable to fix the tissue within an hour or two after death. Suitable general fixatives are

1. Mercuric chloride–Formaldehyde
2. 5 per cent formaldehyde in 0.9 per cent sodium chloride
3. Zenker's fluid
4. Helly's fluid

Suitable stains are

- Ehrlisch haematoxylin/eosin
- Heidenhain's iron haematoxylin/eosin
- Masson's trichrome
- Van Gieson
- Ponceau-S

To get accurate cytological details, tissues should be fixed immediately after death while the body is still warm. Sometimes injection of the fixative avoids autolysis.

ALIMENTARY CANAL

Good fixatives for the mucosa of alimentary canal are

1. Susa
2. 5 per cent formaldehyde in 0.9 per cent sodium chloride
3. Mercuric chloride–formaldehyde
4. Zenker's fluid

For the intestine, fixative should be injected with a syringe. Stomach, oesophagus and pharynx are cut open and put in fixatives. For cytological work, Flemming's fluid or Helly's fixative are useful. Dehydration is as in normal procedure. But clearing is done with benzene or cedar wood oil instead of xylene. Paraffin embedding is quite good but vacuum embedding is preferred. Standard histological techniques can be applied. Oxyntic cells of gastric mucosa are well shown with Mann's methylene blue/eosin or by Masson's acid fuchsin–aniline blue method.

Masson's Stain

Solution 1 Acid fuchsin stain

Acid fuchsin	1.0 g
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Solution 2 Phosphomolybdic acid

Phosphomolybdic acid	1.0 g
Distilled water	100.0 ml

Solution 3 Saturated solution of aniline blue in 2.5 per cent acetic acid

Boil 100 ml water and add 2–3 g of aniline blue and when saturated, add 2.5 ml of glacial acetic acid, cool and filter.

Procedure

1. Hydrate slides to water.
2. Stain nuclei with iron haematoxylin.
3. Transfer to solution 1 Masson's acid fuchsin for 5 min.
4. Rinse in distilled water.
5. Transfer to solution 2 for 5 min.
6. Flood slide with 5 to 6 drops of solution 3 for 30 sec.
7. Rinse in distilled water.
8. Transfer to 1 per cent aqueous acetic acid for 5 min.
9. Dehydrate.
10. Clear in xylene or toluene.
11. Mount in Canada balsam.

Result

Nuclei	Black
Cytoplasm	Red
Collagen fibres	Blue

Fat absorption could be studied in material fixed in Flemming's or stained with Sudan black. Mitochondria could be demonstrated by Heidenhain's iron haematoxylin or Metzner's method (fixation Helly). For Golgi, Cajal's or Da Fano methods are good.

Cartilage

The best fixatives are Susa and 5 per cent formaldehyde. Embedding is done in paraffin or celloidin. Suitable stains are Ehrlisch's haematoxylin/eosin and Harris haematoxylin/eosin.

EYE

It is a composite organ containing many tissue layers, so the sooner the eye is fixed after death, the better. Complete eye is fixed in formal saline or Zenker's fluid for 24 hrs. This is wrapped in a thin gutta percha membrane and frozen. When it is completely frozen, it is bisected with a sharp knife. As usual, dehydrate and embed the specimen in celloidin. Take 15–25 μ thick sections. For staining, standard methods are applied for the connective tissue myelin neuroglia. Retina could be stained with Heidenhain's iron haemetoxylin, fat-soluble stains and PAS technique.

EAR

External ear is elastic, internal ear or cochlea requires special treatment. Fix the material within an hour after death. Inter-vascular injection is preferred. Fix the material in 5 per cent aqueous solution of trichloro acetic acid for 3 days. This fixative also acts as a decalcifier. Fixed material can be directly transferred to absolute alcohol to which a little iodine has been added. Embed in paraffin. Zenker's fluid also serves as a fixative (one or two days). Post-treatment in iodine alcohol, usual dehydration, clearing and embedding are all routine procedures.

The stains used are Ehrlisch's haematoxylin/eosin and Weigert's iron haematoxylin/eosin.

PANCREAS

For general micro-anatomical features, fixatives like Susa or Zenker's fluid could be used. Formalin or Zenker-formalin will give good results. As far as pancreas is concerned, autolysis sets in rapidly so it is better to fix the tissue as soon as possible.

For routine examination, haematoxylin and eosin staining is quite adequate but for alpha (α), beta (β) and Δ cells, islets of Langerhans and zymogen cells lining the alveoli, special methods are required. β-cell granules are important in the formation of insulin and zymogen granules for pancreatic enzymes.

Mitochondria are demonstrated by Champy's technique, β-cells stain pale orange-brown with trichrome stain, negative to PAS and take purple colour with aldehyde fuchsin. Cell granules take a red stain with trichrome stain. Δ cells stain with aniline blue and light green. Zymogen granules are acidophilic and PAS-positive.

PITUITARY GLAND

Pituitary gland is situated at the base of the brain and is separated into two main parts a) pars anterior and b) pars nervosa.

1. Pars nervosa is composed of nervous tissue and can be demonstrated by the usual method.
2. Pars anterior comprises two types of cells.
   i. Chromophobe cells which do not have any stainable cells and form at least 50 per cent of the cells.
   ii. Chromophil cells, which in turn are divided into two types based on their staining affinities.
      a) α (alpha) or acidophilic cells which constitute at least 40 per cent and
      b) β (beta) cells or basophil cells which form the remaining part of cells.

α-cells are PAS-negative and take a red shade when stained with any trichrome stain. b-cells are PAS-positive and take a red shade.

Slidder's Orange—fuchsin

Reagents required

Celestine blue

Ferric ammonium sulphate

Glycerine

Orange G

Phosphotungstic acid

Preparations (Celestine blue solution)

Solution 1

Celestine blue B	500 mg
Ferric ammonium sulphate	5.0 g
Glycerine	14.0 ml
Distilled water	100.0 ml

Dissolve iron alum in water and add the celestine blue for 5 min., cool, filter and then add glycerine.

Solution 2 Orange G solution

95 per cent alcohol	100 ml
Orange G	500–700 mg
Phosphotungstic acid	2.0 g

Dissolve phosphotungstic acid in alcohol and saturate solution with orange G.

Procedure

1. Hydrate slides to water.
2. Stain in solution 1 (celestine blue) for 5 min.
3. Rinse in water.
4. Transfer to Mayer's haemalum for 5 min.
5. Wash, differentiate in acid alcohol.

6. Wash in distilled water.
7. Rinse in 95 per cent alcohol and stain in solution 2 for 2 min.
8. Stain in 0.5 per cent acid fuchsin (in 0.5 per cent acetic acid) for 5 min.
9. Rinse in distilled water.
10. Treat with 1 per cent phosphotungstic acid for 5 min.
11. Rinse in distilled water.
12. Transfer to 1.5 per cent light green for 2 min.
13. Rinse in distilled water.
14. Dehydrate, clear and mount.

Result

Nuclei	Blue-black
Acidophils	Orange-yellow
Basophils	Reddish purple
Chromophobe cells	Grey
Erythrocytes	Yellow
Connective tissue	Green

SUPRARENAL GLANDS

Suprarenal glands are situated at the upper part of each kidney. These glands are vulnerable to autolysis and as such they must be fixed within one or two hours after death.

The medulla contains chromaffin tissue which possesses the property of turning dark brown after prolonged treatment with chromic acid. This is due to the reduction of chromic salts by the adrenaline. Fixation of adrenals is done in a fixative with non-acid dichromate containing fluid. Suitable general fixatives for routine anatomical details are Zenker, Helly's, Susa, formalin, etc. Staining can be done with haematoxylin/eosin or Weigert's, and Van Gieson stains used for connective tissue (See Chapter 8) may also give good results.

CHROMAFFIN TISSUE

Fresh material is fixed in Regaud's post-chromation. Frozen sections of such tissue show the typical brown colour of the cells of medulla. Either frozen or paraffin sections can be stained with azure-eosin stain producing a characteristic yellowish green colour. A greenish colour is obtained by the suprarenal tissue (medullary) with Vulpian reaction. Treat the tissue with fresh dilute solution of ferric chloride. It will impart a green colour to the tissue.

Chromaffin tissue gives a greenish blue colour with Schmorl's ferric chloride–ferric cyanide test and greyish red colour with PAS.

ADRENALINE

This tissue is demonstrated by osmium tetroxide method. Thin slices of suprarenal is fixed in vapour of 2 per cent osmium tetroxide for 2–3 hrs at 37°C. The tissue is rapidly dehydrated and embedded in paraffin wax. In this tissue, globules of fat which are blackened by osmium tetroxide are removed from sections with turpentine. The black granules which remain after turpentine treatment are adrenaline.

TEETH

General technique is as for bone. Ground teeth sections are similarly prepared. Cutting of thin slices before grinding with a saw may be difficult. A metal wheel, the edges of which are impregnated, with diamond dust, should be used. Very thin cut sections may be directly mounted in H.S.R. or DPX.

Fixation may be in any general fixative, formalin being the best. Staining methods are same as that for bone.

LUNGS

Lung substance requires special treatment because the air inside has to be removed before it can be satisfactorily embedded. Animals are killed by a blow on the head or by anaesthesia. Best way to kill the animal is to open a large blood vessel which should be rapidly performed.

Trachea should be exposed without opening the thoracic cavity. Insert a cannula and inject the fixative with a syringe. Lungs, heart and trachea are dissected and placed in fixative.

Fixation

Susa

Mercuric chloride–formaldehyde

5 per cent formaldehyde in 0.9 per cent

- Sodium chloride
- Bouin's fluid
- Zenker's fluid

Staining is by routine methods like Heidenhain's iron haematoxylin/eosin, Van Gieson and Orcein.

Alveolar Epithelium

Thick sections (35–40 μ) are required. Heidenhain's iron haematoxylin and eosin can be used.

SKIN

Skin has two layers, the outer epidermis and inner dermis.

- Epidermis has 2 layers. The malpighian layer which has connection with dermis and the outer horny layer consisting of dead cells.

- Dermis consists of connective tissue and contains the hair follicle, sweat glands and sebaceous glands.

Skin exhibits certain difficulties in processing and cutting sections, routine fixatives like formalin, Zenker or Bouin's fluid can be used. Pieces of skin are first stretched on a core board, otherwise they will curl. Immerse the cork into a fixative.

Skin becomes hardened during processing so it is better to go in for celloidin embedding. Since skin is a dense tissue, it should be left in the embedding medium for longer periods than normal. Haematoxylin and eosin is sufficient for normal histological study of the structures, Alcian blue and Alcian blue/PAS techniques are recommended for connective tissue/polysaccharides and eleidin can be demonstrated by picro-nigrosin technique.

Picro-nigrosin technique for Eleidin

1. Cut frozen sections.
2. Wash in water.
3. Transfer to saturated aqueous picric acid for 5 min.
4. Dip in distilled water.
5. Transfer to stain in aqueous nigrin for 1 min.
6. Wash in distilled water.
7. Rinse in 96 per cent alcohol.
8. Clear in origanum.
9. Mount in Canada balsam.

Result

Eleidin	Black
Other elements	Yellow

BONE MARROW AND BLOOD-FORMING ORGANS

Bone marrow can be studied in the form of

1) films which are made and stained by the usual methods for blood films.
2) Impression smears—by pressing the unfixed material on a slide and fixed in Schaudin's fluid and stained in the same way as for blood films.
3) Needle biopsis—by fixing in Susa or Cappell, Hutchinson and Harvey–dichromate. If bone marrow has small spicules of bone, it may be decalcified in Gooding and Stewart's fluid.

Gooding and Stewart's fluid

Formic acid	5–25.0 ml
Formalin	5.0 ml
Distilled water	100.0 ml

Cappell, Hutchinson and Harvey–Smith method

Fix pieces of bone marrow in fresh prepared solution

Zenker's fluid	45.0 ml
Formalin	5.0 ml
Formol saline	50.0 ml

Fix for 20 min. Add 400 ml of distilled water to the container and allow the pieces to settle. Remove the supernatant fluid and wash the pieces in distilled water (2 changes). Remove water and add 100 ml of 70 per cent alcohol, leave the pieces for 24 hrs.

Then dehydrate, clear in toluene. Keep the pieces in a flat-bottomed tube and pour molten wax—leave it for 2 hrs with two changes of cool wax, free the block by breaking the dish and cut the sections.

Histochemical Staining

Leishman's stain

Leishman's stain (powder)	15 mg
Methyl alcohol (pour)	100.0 ml

Grind the powder in a mortar and pour methyl alcohol and then pour the alcohol in a bottle, now add more alcohol into the mortar and repeat the process.

1. Hydrate slides to water.
2. Incubate in pH 6.8 phosphate buffer at 56°C for 30 min.
3. Transfer to Leishman's stain which is diluted 1 : 3 with pH 6.8 buffer for 30–60 min.
4. Dip in pH buffer.
5. Differentiate in acetic acid (1 : 1,0000).
6. Blot the section.
7. Flood with xylol.
8. Mount in DPX.

Results

Nuclei	Bluish red
Acidophil granules	Pink to red
Basophil granules	Blue
Red blood cells	Salmon pink

Maximow's Stain

Reagents required

Eosin

Azure

Preparation

Stock solution A	1 : 1000 eosin
Stock solution B	1 : 1000 azure II

Working solution

Add 10 ml solution A to 100 ml of 6.8 pH phosphate buffer and then add 10 ml of solution B.

Procedure

1. Dehydrate slides to water.
2. Stain in Ehrlisch's haematoxylin for 10 min.
3. Incubate in pH 6.8 buffer.
4. Transfer to working solution.
5. Differentiate, clear and mount as in previous case.

Results

Same as for Leishman's

May-Grunwald–Giemsa Technique

1. Hydrate slides in water.
2. Transfer to pH 6.8 buffer for 30 min. 37°C.
3. Transfer to May-Grunwald–Giemsa stain for 15 minutes.
4. Rinse in buffer solution.
5. Transfer to Giemsa stain with dilution of 1 : 10 with pH 6.8 for 15 min.
6. Differentiate in buffer.
7. Differentiate in a mixture of glycerin–ether.
8. Again differentiate in buffer.
9. Dehydrate, clear in xylol and mount in DPX.

Results

The result are the same as for Leishman's stain.

BONE

Bone should be decalcified and then embedded in celloidin. Paraffin embedding can also be done.

Fixation

Muller's fixative or formol saline

Staining

Schmorl's Picro-thionine Method

1. Wash sections in water.
2. Transfer to saturated aqueous solution of thionine which contains 0.880 ammonia for 100 ml of stain.
3. Rinse in water.
4. Transfer to saturated aqueous phosphotungstic or phosphomolybdic acid for 30 sec.

5. Wash.
6. Differentiate in 70 per cent alcohol.
7. Dehydrate, clear and mount.

Result

Ground substance	Yellow to brown
Lacunae and canaliculi	Dark brown
Cells	Red
Ground substance	Purple

Schmorl's Thionine-phosphotungstic Acid Method

Fixation

Muller's fluid or formol saline

Avoid mercury-containing fixative

Procedure

1. Bring sections to water.
2. Transfer to alkaline thionine for 30 min.
3. Rinse in water.
4. Place in saturated aqueous PTA (phosphotungstic acid) or PMA (phosphomolybdic acid) for few seconds.
5. Wash in water.
6. Treat with 10 per cent ammonia in distilled water.
7. Differentiate in 90 per cent alcohol.
8. Dehydrate, clear and mount.

Results

Ground substance	Pale green
Lacunae	Blue or blue-black

OVARY

General fixatives like Susa, Zenker's and Bouin's fluid. For cytological observations, Helly's or Flemming's without acetic acid is preferable. Staining is by Heidenhain's iron haematoxylin and Da Fano technique for Golgi elements. As far as ovary is concerned, paraffin embedding is preferred over celloidin embedding. Dehydration is as usual but clearing is in cedar wood oil or benzene. Good results are obtained with Masson's trichrome or Heidenhain's Azan technique.

Phloxine–Methylene Blue Method

Fixation

Any general fixative

Reagents required

Phloxine
Glacial acetic acid
Methyl blue
Azure B
Borax

Preparation of reagents

Solution 1 Phloxine solution

Phloxine	500 mg
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

Should be filtered before use.

Solution 2 Methylene blue azure

Methylene blue	250 mg
Azure B	250 mg
Borax	250 mg
Distilled water	100 ml

Procedure

1. Hydrate slides to water.
2. Place in solution 1 for 2 min.
3. Rinse in distilled water for 1 min.
4. Transfer to solution 2 for 1 min.
5. If required, destain in 0.2 per cent acetic acid.
6. Dehydrate, clear and mount.

Result

Nuclei	Blue
Plasma cell cytoplasm	Blue
Other elements	Rose or Red

TESTIS

General fixatives are Susa, Zenker's, Bouin's. If chromosomes are to be studied Sanfelices fluid is good.

Solution 1

40 per cent formaldehyde	128.0 ml
Acetic acid	16.0 ml

Solution 2

Chromium trioxide	1 g
Distilled water	100.0 ml

Solution 3

Working solution

Solution 1	9 ml
Solution 2	15 ml

Fixation time is 12–24 hrs. Best stain for chromosome study is crystal violet-iodine.

Preparation

Solution 1

Crystal violet	1 g
Distilled water	100.0 ml

Solution 2

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100.0 ml

Procedure

1. Hydrate slides to water.
2. Place in solution 1, crystal violet stain, for 10 min.
3. Rinse in distilled water.
4. Transfer to solution 2, Lugol's iodine, for 10 min.
5. Rinse in distilled water.
6. Dehydrate rapidly, clear in xylene and mount in DPX.

For mitochondrial study, fix in Helly's and stain with Heidenhain's haematoxylin and eosin.

SPLEEN AND LYMPH GLANDS

Smears may be made from spleen and lymph gland and stained with Leishman's or Giemsa's stain for sectional purposes. Zenker's or Helly's or formal saline may be used. Paraffin embedding standard histological stains can be used. Trabiculae and capsules are well-demonstrated by Van Gieson or Mallory stain.

EMBRYOS

Fixation is done in Susa, 4 per cent formaldehyde, Zenker's, etc. Embryos are likely to shrink. To avoid this, dehydration should be carried out very rapidly. Dioxane gives good results. Xylene is to be avoided

as clearing agent. Another important point to be noticed is to not transfer the material directly from xylene to paraffin. Embryos should be kept in a mixture of clearing agent and paraffin.

Hill's method

1. Place the dehydrated material in cedar wood oil overnight and then transfer to benzene for a few hours.
2. Transfer to benzene–paraffin mixture and keep overnight.
3. Embed in paraffin.

HAIR

For whole mounts, place hairs in a small tube filled with equal volumes of ether and absolute alcohol. Shake well to remove sebaceous matter and debris, clear in benzene, partially dry on filter paper and mount in balsam.

For sections

1. Treat as above with absolute alcohol and ether, place in 6 vol. of 2 per cent alcohol and 4 volumes of 5 per cent ammonia.
2. Soak in 10 per cent potassium hydroxide at 50°C for 1 to 2 min., wash for few min. in 5 per cent sulphuric acid and absolute alcohol.
3. Clear in benzene for 10–15 min.
4. Dry with filter paper.
5. Impregnate with paraffin wax for 15 min. and block.
6. Cut sections 8–10 μ in thickness.

LIVER

Fix in Susa, formol saline, or Zenker's fluid. For staining, Heidenhain's iron haematoxylin can be used and Atlamann–Metzner method after Helly's fixation. For bile canaliculi, fix in formol saline—staining by Heidenhain's iron haematoxylin.

Liver areas attached to alimentary tract should be avoided immediately after death as in these regions hydrogen sulphide is released from the intestine and haemoglobin breaks down to form iron sulphide.

KIDNEY

For cytological work on renal epithelium, kidney should be absolutely fresh. In Susa, formol saline, Zenker's fluid are suitable fixatives.

Masson's method of staining is excellent after fixation in Susa, Altmann–Metzner technique after Helly's fixation for mitochondria, Golgi elements by Da Fano method.

THYROID

Thyroid has the secretory epithelial cells and extracellular amorphous colloid. This colloid is a stored secretion and is made up of mainly proteins and carbohydrates. Some proteins are of basic type, Sakaguchi's reaction for argunine, ferric ferricyanide reaction for sulphydryl groups. It is PAS-positive, and may be containing mucopolysaccharides. AFA is used for fixation.

Acetic acid	1 part
Formalin	5 parts
Absolute alcohol	15 parts

Fix for 24 hrs.

BONE

Bone can be decalcified by removing calcium salts using any of the decalcifying methods (*See* chapter on decalcification). Best results could be obtained by celloidin embedding. Paraffin embedding will also give good result. Sometimes decalcification impairs the bone material. In such cases frozen un-decalcified sections are tried. Bone is fixed in formol saline for more than 3 hrs before freezing. Von Kosa's silver staining procedure counterstained with Van Gieson is usually preferred. Sometimes ground bone is made use for sections. First the bone is cut by saw. Smear carborundum on a glass plate and now rub both sides of the cut pieces, wash, dehydrate and air-dry. Then mount on a glass slide with Canada balsam.

Histochemical Methods

Schmorl's Picrothionine Method

Fixation

Muller's or formol saline. Mercuric chloride-containing fixatives are not favoured.

Reagents required

Thionine

Ammonia

Picric acid

Preparation

Solution 1

Saturated thionine solution	100.0 ml
0.880 ammonia	1 drop

Solution deteriorates very quickly with addition of ammonia.

Solution 2

Saturated picric acid

Procedure

1. If frozen sections are used, wash in distilled water for 10 min.
2. Immerse slides in solution 1 for 15 min.
3. Rinse in distilled water.
4. Keep the slides in solution 2 for 1 min.
5. Rinse in 70 per cent alcohol for 10 min. until no excess stain comes out.
6. Dehydrate, clear and mount.

Result

Ground substance	Yellow
Ground substance of cartilage	Purple
Cells	Red

Schmorl's Thionine–PTA Method

Fixation

Muller's

Formol saline

Reagents

Thionine (alkaline)

PTA

Ammonia

Preparation

Solution 1

Saturated solution of alkaline thionine

Solution 2

Saturated solution of PTA (Phosphotungstic acid)

Solution 3

10 per cent ammonia in 100 ml distilled water

Procedure

1. Hydrate slides in water.
2. Place slides in solution 1 for 30 min.
3. Rinse in distilled water.
4. Place slides in solution 2 for 3 sec.
5. Wash in water till sections turn sky blue.
6. Transfer to solution 3 for 5 min.

7. Differentiate in several changes of 90 per cent alcohol. If section is overstained, differentiate in acid alcohol (one per cent).
8. Dehydrate, clear and mount.

Result

Ground substance	Pale green
Lacunae and fibrillae	Blue black

BONE MARROW

Cappell-Huthcinson and Harvey–Smith Method

Fixation

Fixation is carried out in a fluid containing

Zenker's	45.0 ml
Formalin	5.0 ml
Formol saline	50.0 ml

1. Take the fluid in a flask and fix the bone marrow. Fix for 30 min., add 500 ml of distilled water to the flask and fragments will settle.
2. Siphon out the supernatant fluid.
3. Wash the fragments for 10 min.
4. Pipette out supernatant water and add 60 per cent alcohol for 15 min.
5. Siphon out and add 80 per cent alcohol for 15 minutes, 95 per cent alcohol for 15 min. and 100 per cent alcohol for 15 min.
6. Pipette out and add toluol for 15 min.
7. Slowly with a pipette transfer the particles to a test tube and add liquid wax and leave for 2 hrs (with 2 changes).
8. Cool wax breaks the tube and cut sections.

Now the sections are ready for staining.

Maximow's Stain

Reagents

Eosin

Azure II

Haematoxylin solution (Ehrlisch)

Preparation

Solution 1

Eosin	1.0 g
Distilled water	1000.0 ml

Soltuion 2

Azure II	1.0 g
Distilled water	1000.0 ml

Solution 3

Phosphate buffer (6.8 pH)	100 ml
Solution 1	10.0 ml
Solution 2	10.0 ml

Solution 4

Ehrlisch haematoxylin

Procedures

1. Hydrate slides to water.
2. Place slides in solution 4 for 10 min.
3. Transfer to phosphate buffer (pH 6.8) for 30 min at 56°C.
4. Place in solution 3 for 30 min to 1 day.
5. Differentiate in acid alcohol (1 : 10,000 acetic acid).
6. Dehydrate, clear and mount.

Result

RBC	Pink
Nuclei	Bluish red
Acidophil granules	Pink to red
Basophil granules	Blue

Leishman's Stain

Reagents required

Leishman's stain

Methyl alcohol

Acetic acid

Preparation

Solution 1

Leishman's stain	150 mg
Methyl alcohol	100.0 ml

Grind the stain powder in a mortar with alcohol. Add alcohol till all the powder dissolves. Leave the stain overnight in a bath adjusted to 37°C.

Solution 2

Acetic acid	1.0 ml
Distilled water	10,000.0 ml

Procedure

1. Hydrate slides in water.
2. Incubate in phosphate buffer (pH 6.8) for 3 min. at 56°C.
3. Place the slides in solution 1 which is diluted with phosphate buffer at 1 : 3 ratio for 60 min.
4. Differentiate in buffer.
5. Rinse in solution 2.
6. Put the section and air-dry.
7. Flood section with xylol and mount in DPX.

Results

Same as with Maximow's.

SOME FIXATIVES AND STAINS

Muller's fluid

Potassium dichromate	2.5 g
Sodium sulphate	1.0 g
Distilled water	100.0 ml

Regaud's fluid

3% potassium dichromate	80 ml (3 g/100 ml water)
40% formaldehyde	20 ml

Prepare before use. It is a deep penetrant but never hardens tissue. Fixation time is 24 hrs. Post-chromation in 3% potassium dichromate.

San Felice's fluid

1% chromic acid	80.0 ml
40% formaldehyde	20.0 ml
Glacial acetic acid	5.0 ml

Mix before use. Fixation time is 4–6 hrs. Good for chromosomes and mitochondria. Wash in running water for 6–12 hrs.

Flemming's fluid

1% chromic acid (1 g/100.0 ml water)	15.0 ml
2% osmic acid (2 g/ 100.0 ml water)	4.0 ml
Glacial acetic acid	1.0 ml

Mix before use. This acts slowly. Fixation time is 12–24 hrs.

Gilson's fluid

Concentrated nitric acid	15.0 ml
Glacial acetic acid	4.0 ml

Mercuric chloride	20.0 ml
60% ethyl alcohol	100.0 ml
Distilled water	880.0 ml

Fixation time is 24 hrs.

Schaudinn's fluid

Mercuric chloride (saturated)	66.0 ml
95% alcohol	33.0 ml
Glacial acetic acid	5–10.0 ml

Fixation time is 20 min.

Worcester fluid

Mercuric chloride	14.0 g
Distilled water	200.0 ml
40 per cent formaldehyde	22.5 ml
Acetic acid	25.0 ml

SOME STAINS APPLIED FOR TISSUES

Harris Haematoxylin

Haematoxylin 2.5 g	
Absolute alcohol	50.0 ml
Ammonia or potassium	50.0 ml
Distilled water	500.0 ml
Mercuric oxide	1.5 g
Glacial acetic acid	20.0 ml

Dissolve separately the haematoxylin in absolute alcohol and alum in water using heat if necessary. Mix the two solutions. Heat the mixture to boiling and then add mercuric oxide. It is better to add glacial acetic acid to get precise results.

Mallory's Rapid One-step Method

Fixation

Any general fixative.

Preparation of reagent

Distilled water	200 ml

Dissolve each of below before adding mixture.

Phosphotungstic acid	1.0 g
Orange G	2.0 g

Aniline blue	1.0 g
Acid fuchsin	3.0 g

This solution lasts for several months.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Keep the sections in staining solution for 5 min.
3. Rapidly wash the slides.
4. Dehydrate rapidly, clear and mount.

Results

Collagen	Blue
Ground substance of bone	Yellow
Elastin	Pink or Yellow

Masson's Trichrome Staining

Fixation

Any general fixative.

Preparation of reagents

Solution 1 Iron alum

Ferric ammonium sulphate	4.0 g
Distilled water	100.0 ml

Solution 2

Haematoxylin

Solution 3 Acid fuchsin

Acid fuchsin	1.0 g
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

Solution 4 Ponceau de-xylidine

Ponceau de-xylidine	1.0 ml
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

Solution 5 Fast green

Fast green	2.0 g
Distilled water	100.0 ml
Glacial acetic acid	2.0 ml

Solution 6 Phosphotungstic acid

Phosphotungstic acid	1.0 g
Distilled water	100.0 ml

Solution 7 Acidic water

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides in water.
2. Mordant in solution 1 (Iron alum) for 30 min.
3. Wash in running water for 5 min.
4. Keep the slides in solution 2 (haematoxylin) (De la Field).
5. Wash in water for 5 min.
6. Differentiate in saturated picric acid.
7. Wash in running water for 10 min.
8. Transfer the slides to solution 3 (acid fuchsin).
9. Rinse in distilled water for 5 min.
10. Keep slides in solution 4 (Ponceau de-xylidine for 5minutes).
11. Rinse in distilled water.
12. Differentiate in solution 6.
13. Transfer directly to solution 5 (Fast green).
14. Differentiate in solution 7 (acidic water).
15. Dehydrate in absolute alcohol giving 2 changes.
16. Clear and mount.

Results

Nuclei	Deep blue to black
Cytoplasmic elements	Varying shades of red
Muscle	Red
Collagen	Green

Azan

Preparation of reagents

Solution 1 Azocaramine

Azocaramine G	1.0 g
Distilled water	100.0 ml
Glacial acetic acid	1.0 ml

Solution 2 Aniline alcohol

Aniline oil	1.0 ml
90% alcohol	100.0 ml

Solution 3 Acid alcohol

Glacial acetic acid	1.0 ml
95% alcohol	100.0 ml

Solution 4 Phosphotungstic acid

Phosphotungstic acid	5.0 g
Distilled water	100.0 ml

Solution 5 Aniline blue stain

Aniline blue	0.5 g
Orange G	2.0 g
Oxalic acid	2.0 g
Distilled water	100.0 ml

Procedure

1. Hydrate slides in water.
2. Stain in solution 1 at 56°C for 1 hr.
3. Rinse in distilled water.
4. Differentiate in solution 2 for few sec.
5. Rinse in distilled water.
6. Differentiate in solution 3 for few sec.
7. Rinse in distilled water.
8. Keep in solution 4 for 1 hr.
9. Wash in distilled water.
10. Place in solution 5 for 30 min.
11. Wash in water.
12. Dehydrate, clear and mount.

Result

Nuclei	Brilliant red
Collagen and reticulin	Blue
Muscle	Red
Cytoplasm	Orange-red
Chromo bodies	Light grey

Picro Ponceau De-xylidine

Preparation of reagents

Solution 1 Haematoxylin (Ehrlisch or De la Field)

Solution 2 Picro-ponceau

Ponceau S (1% aqueous)	10.0 ml
Saturated picric acid	86.0 ml
1% aqueous acetic acid	4.0 ml

Procedure

1. Hydrate slides in water.
2. Overstain in solution 1 (haemotoxylin).
3. Wash in running water.
4. Place slides in solution 2 (Picro-ponceau).
5. Rinse in distilled water.
6. Dip several times in 70% alcohol.
7. Dehydrate, clear and mount.

Result

Nuclei	Bluish black
Collagen and reticulin fibres	Red
Elastic fibres, erythrocytes, epithelia	Yellow

Dafano Method

Fixation

Cobalt nitrate 1.0 g	
Distilled water	100.0 ml
Formalin	15.0 ml

Fix for 3–18 hrs.

Preparation of reagent

Solution 1 Ramony Cajal's developer

Hydroquinone	2.0 g
Formalin	6.0 ml
Distilled water	100.0 ml
Sodium sulphate (anhydrous)	0.15 mg

Solution 2 Gold chloride (stock)

Gold chloride 1% (1 g /100.0 ml distilled water)

Distilled water	40.0 ml
Stock solution	1.0 ml
Distilled water	80–90 ml

Procedure

1. Rinse blocks of the tissue in distilled water.
2. Impregnate in 1.5% silver nitrate (1.5 g/100.0 ml water) for 2 days.
3. Rinse in distilled water.
4. Cut blocks into thin slices of 2 mm thickness.
5. Reduce in solution 1 for 5 hrs.
6. Wash in distilled water.
7. Dehydrate, infiltrate and embed.
8. Take 6–7 μ thick sections and deparaffinize and hydrate sections.
9. Tone in solution 2 (gold chloride) for 2 hrs.
10. Rinse in distilled water and fix in 5% sodium thiosulphate for 3 minutes.
11. Wash in running water.
12. Counterstain in haematoxylin or carmalum.
13. Dehydrate clear and mount.

Result

Golgi	Black
Cytoplasm	Grey
Mitochondria	Dark grey or black

Altman's Method

Fixation

It is done in read's solution. Change daily for four days. Mordant in 3% postassium dichromate (8 days) with a change in every two days. Wash in running water overnight and dehydrate and embed.

Preparation of reagents

Solution 1

Prepare saturated solution of aniline in distilled water. Shake well and then filter. Add 10 g of acid fuchsin to 100.0 ml of filtrate. Allow it to stand for 24 hrs. This lasts for one month.

Solution 2 Methyl green

Methyl green	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Keep the slides in 1% potassium permanganate (1.0 g in 100 ml distilled water).
3. Rinse in distilled water.
4. Bleach in 5% oxalic acid (5 g/100.0 ml distilled water).
5. Rinse in distilled water for 1–2 min.
6. Dry the slides with paper and then flush solution 1. Heat gently until aniline fume smelling seizes.
7. Drain most of the stain.
8. Flush solution 2 (methyl green) for 5 sec.
9. Drain off stain, rinse briefly in 95% alcohol.
10. Dehydrate in absolute alcohol, clear and mount.

Results

Mitochondria	Bright red

Schmorl's Ferric–ferricyanide Method

Fixation

10% formalin buffered with 3% calcium acetate for 1–3 days.

Preparation of reagents

Ferric–ferricyanide solution

Potassium ferricyanide (1% aqueous)	10.0 ml
Ferric chloride	75.0 ml
Distilled water	5.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Keep in freshly prepared ferric–ferricyanide solution.
3. Rinse in three changes of distilled water.
4. If desired, counterstain with safranin.
5. Dehydrate, clear and mount.

Result

Enterochromaffin granules	Dark blue
Nuclei	Red

Champy Kull's Method

Preparation of reagent

Solution 1

Saturated solution of acid fuchsin in (14%) aniline water. 15–10 ml of aniline oil and 500. 0 ml of distilled water –filter.

Solution 2 Toludine blue

Toluidine blue	0.500 mg
Distilled water	100.0 ml

Solution 3 Aurantia

Aurantia	0.500 mg
70% alcohol	100.0 ml

Procedure

1. Hydrate slides to water.
2. Flood slides with solution 1, heat the slide.
3. Rinse in distilled water.
4. Flood with solution 2 for 3 min.
5. Rinse in distilled water.
6. Flood the slides with solution 3 for 2 min.
7. Rinse in absolute alcohol.
8. Clear in xylene and mount.

Results

Mitochondria	Red
Nuclei	Blue
Cytoplasm	Yellow

11

INVERTEBRATE STAINING METHODS

Trematode whole Mounts (Gower, 1939)

Fixation

FAA 70 per cent

Reagents required

Carmine

Acetic acid

Potassium chlorate

Conc. hydrochloric acid

Preparation of reagents

Solution 1 Acidified carmine

Carmine	10.0 g
45 per cent acetic acid	100.0 ml

Boil, cool and filter. This residue is acidified carmine.

Solution 2 Working solution

Solution 1	1.0 g
Alum	10.0 g
Distilled water	200.0 ml
Potassium chlorate crystals	100.0 g
Conc. HCl	0.1 ml
70 per cent alcohol	100.0 ml

Add HCl to potassium chlorate in a closed dish.

Procedure

1. Hydrate specimen in water.
2. Place the specimen in a dish containing solution 2 for 36 hrs. Time schedule depends on the size of the specimen.
3. Wash in water.
4. Destain in acid alcohol.
5. Dehydrate, clear in cedar wood oil.
6. Mount.

Result

All principal organs take a deep rose shade, parenchyma, cytoplasm and muscle remain unstained.

Demonstration of Nervous System in whole Mounts of Trematodes and Cestodes (Kishore *et al.*, 1988a, 1988b, 1988c, 1988d, 1990)

Fixation

10 per cent neutral formalin. Specimens were flattened between 2 slides and fixed for 6 hrs.

Reagents required

5-Bromoindoxyl acetate

Tris buffer

Potassium ferrocyanide

Potassium ferricyanide

Calcium chloride

Preparation of reagents

Solution 1 Incubating medium

1–2 per cent of 5-bromoindoxyl acetate	0.1 ml	
0.1 M tris buffer (pH 6.8)		2.0 ml
0.05 M potassium ferrocyanide	1.0 ml	
0.05 M potassium ferricyanide	1.0 ml	
0.1 M calcium chloride	1.0 ml	
Distilled water		5.0 ml

Mix bromoindoxyl in 0.1 ml alcohol and then add the following in the order given above.

Procedure

1. After fixing for 6 hrs, bring the specimen to water.
2. Place in a dish containing solution 1 (incubating medium) for 12 hrs at room temperature.
3. Wash in distilled water.
4. Dehydrate through 70 per cent, 90 per cent, 95 per cent and absolute alcohol.

5. Clear in creosote.
6. Mount in DPX.

Result

Nervous system Deep indigo blue

Egg shell in Trematodes and Cestodes (Smyth, 1951)

Fixation

0.5 per cent formol saline

Reagents required

Acidified carmine
Alum
Malachite green
Orange G

Preparation of reagents

Solution 1

Acidified carmine	1.0 g	
Alum		10.0 g
Distilled water		200.0 ml

Dissolve by heating, cool and filter.

Solution 2

Malachite green	500 mg
Distilled water	100.0 ml

Solution 3

Orange G	1.0 g
Absolute alcohol	99.0 ml

Procedure

1. Hydrate slides to water.
2. Place them in solution 1 for 2 hrs.
3. Rinse in water.
4. Transfer to solution 2 for 2 min.
5. Wash and dehydrate.
6. Counterstain in solution 3 for 1 sec.
7. Rinse in absolute alcohol.
8. Clear in xylene and mount.

Result

Egg shell	Green or greenish blue
Nuclei	Red
Cytoplasm	Pink

Staining Spines of Trematodes (Echinostomes) by Azure-I Schiff Reaction (Kasten, 1957) Modified by Hanumantha Rao and Murthy (1972)

Fixation

Unfixed

Reagents required

Azure-I

Schiff reagent (*See* Chapter 8)

Procedure

1. Place the cercaria on a clean slide.
2. Add few drops of Azure-I Schiff reagent (final pH 4–4.3) for10 min.
3. Rinse in water.
4. Rinse in absolute alcohol.
5. Clear in xylene and mount.

Result

Echinostome spines	Blue

Gomori's Aldehyde Fuchsin for Neurosecretory Cells in the Trematodes: (Shyamasundari and Hanumantha Rao, 1975a; Shyamasundari, 1985)

Fixation

10 per cent formalin or Susa

Reagents required

Basic fuchsin

Conc. Hydrochloric acid

Paraldehyde

Potassium permanganate

Oxalic acid

Preparation of reagents

Solution 1 Aldehyde fuchsin

Basic fuchsin	500 mg

Boiling water	100.0 ml
Conc. HCl	1.0 ml
Paraldehyde	1.0 ml

Add basic fuchsin to 100.0 ml of boiling water, boil, cool and filter. Add 1.0 ml of conc. HCl and 1.0 ml of paraldehyde. Leave the bottle stoppered in a dark chamber till red colour of the fuchsin disappears and a violet colour is retained.

Solution 2

Potassium permanganate	300 mg
Distilled water	100.0 ml
Conc. H_2SO_4	0.3 ml

Solution 3

Oxalic acid	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Oxidize in solution 2 for 1–2 min.
3. Wash.
4. Bleach in solution 3 till tobacco colour of permanganate is lost.
5. Wash in running water.
6. Stain in solution 1 for 10–30 min.
7. Differentiate in 95 per cent alcohol.
8. Dehydrate, clear and mount.

Result

Neurosecretory cells with neurosecretory substance appear purple.

Mucosubstance appears purple.

Demonstration of Phospholipids in Mehlis' Gland of Trematodes (*Fasciola hepatica*) by Applying Acid Haematein Method (Hanumantha Rao, 1959)

Fixation

Formol calcium for 24 hrs followed by post-chromation with 2 per cent potassium dichromate for 24 hrs at room temperature and 24 hrs at 60°C.

Reagents required

Haematein

Sodium iodate

Potassium ferricyanide

Sodium tetraformate

Preparation of reagents

Solution 1 Acid haematein solution

Haematein	550 mg
1 per cent sodium iodate	1.0 ml
Distilled water	49.0 ml

Solution 2 Differentiator

Potassium ferricyanide	250 mg
Sodium tetraformate	250 mg
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in solution 1 (acid haematein) at 60°C for 5 hrs.
3. Rinse in distilled water.
4. Transfer to solution 2 for differentiation for 10 hrs at 37°C.
5. Wash.
6. Mount in glycerine jelly.

Result

Phospholipids Dark blue

[Also refer Hanumantha Rao (1960, 1963)].

Application of Copper Phthalocyanin to *Fasciola* Mehlis' Gland for the Demonstration of Phospholipids (Hanumantha Rao, 1959)

Fixation

Fix in formol calcium. Postchromate as in previous procedure.

Reagents required

Luxol fast blue G
Lithium carbonate
Neutral red

Preparation of reagents

Solution 1 Copper phthalocyanin

Luxol fast blue G	10 mg
95 per cent alcohol	100.0 ml

Solution 2

Lithium carbonate	50 mg
Distilled water	100.0 ml

Solution 3

Neutral red	1.0 g
Distilled water	100.0 ml

Procedure

1. Fix the material (control) in weak Bouin's. After usual procedure, cut 8 μ thick sections and keep them in pyridine for 12 hrs. Then wash.
2. Bring both sections, i.e., formol calcium-fixed and control to absolute alcohol.
3. Place both sections in solution 1 for 6–18 hrs at 60°C.
4. Rinse in 70 per cent alcohol and wash in water.
5. Differentiate in solution 2 for 30 min.
6. Rinse in water.
7. Counterstain in solution 3 for 10 min.
8. Rinse in water.
9. Dehydrate, clear and mount in Canada balsam.

Result

Phospholipids stain blue.

Control sections show no phospholipid activity and remain unstained.

Catechol Technique for Vitellaria in Trematodes and Cestodes (Johri and Smyth, 1956)

Fixation

70 per cent alcohol or 10 per cent formalin—70 per cent alcohol is preferable.

Reagents required

Catechol

Preparation of reagents

Solution 1

Catechol	100 mg
Distilled water	100.0 ml

This must be freshly prepared before use.

Procedure

1. Deparaffinize and bring slides to water.
2. Transfer to solution 1 for 60–90 min. at 37°C or 4–5 hrs at room temperature.
3. Wash in distilled water for 15 min.
4. Dehydrate, clear and mount.

Result

Vitellaria	Reddish brown

Diazo Technique using Fast Red Salt B for Vitellaria in Trematodes and Cestodes (Johri and Smyth, 1956)

Fixation

70 per cent alcohol for 4 days

Reagents required

Fast Red salt B

Preparation of the reagent

Solution 1

Fast Red Salt B	1.0 g
Distilled water	100.0 ml

This solution should be freshly prepared and filtered before use.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place sides in solution 1 for 10–40 min.
3. Wash in distilled water for 10–15 min.
4. Dehydrate, clear and mount.

Result

Vitellaria, vitelline ducts	Orange to red

Malachite Green Method for Egg Shell in Trematodes and Cestodes (Smyth, 1951)

Fixation

0.5 to 10 per cent formalin

Reagents required

Carmine

Alum

Malachite green

Orange G

Preparation of reagents

Solution 1

Acidified carmine	1.0 g (*See* same Chapter)
Alum	10.0 g
Distilled water	200.0 ml

Solution 2

Malachite green	500 mg
Distilled water	100.0 ml

Solution 3

Orange G	1.0 g
Absolute alcohol	99.0 ml

Procedure

1. Deparaffinize and hydrate to water.
2. Stain in solution 1 for 2 hrs.
3. Rinse in water.
4. Stain in solution 2 for 2 mins.
5. Dehydrate in 70 per cent and 90 per cent alcohol.
6. Differentiate in absolute alcohol.
7. Counterstain in solution 3 for 1 sec.
8. Rinse in absolute alcohol, clear and mount.

Result

Egg shell material	Green or greenish blue

Demonstration of Disulphides in the Neurosecretory Cells of the Trematodes by Applying Performic Acid/Alcian Blue Technique (Shyamasundari and Hanumantha Rao, 1975a)

Fixation

Formalin, Carnoy, etc.

Reagents required

Formic acid

Hydrogen peroxide

Sulphuric acid

Alcian blue

Preparation of reagents

Solution 1 Oxidizing solution

98 per cent performic acid	40.0 ml
100 per cent of hydrogen peroxide	4.0 ml
Sulphuric acid	0.5 ml

Solution 2 Staining solution

Alcian blue	1.0 g

98 per cent sulphuric acid	2.7 ml
Distilled water	47.2 ml

Procedure

1. Deparaffinize and hydrate slides in water.
2. Treat with solution 1 for 5 min.
3. Wash in tap water.
4. Dry sections.
5. Rinse in tap water.
6. Transfer to solution 2 (alcian blue) for 1 hr.
7. Wash in running water.
8. Counterstain if desired.
9. Wash.
10. Dehydrate, clear and mount.

Result

Sites with disulphide	Dark blue

Demonstration of Calcium by Alizarin Red S Method in Crustacean Intermoult Cuticle (Rama Devi *et al.*, 1991)

Fixation

Any general fixative, preferably neutral fixatives. Avoid acid fixatives.

Reagents required

Sodium alizarin sulphonate

Hydrochloric acid

Preparation of reagents

Solution 1 Alizarin Red S

Sodium alizarin sulphonate	500 mg
Distilled water	45.0 ml

Mix the solution thoroughly and add 5 ml of ammonia (28 per cent ammonia 1 part and 99 parts of distilled water). Stir well while adding and the final pH should be 6.3–6.5. Adjust it with buffers.

Solution 2 Differentiator

Hydrochloric acid	0.1 ml
95 per cent alcohol	99.9 ml

Procedure

1. Dewax and hydrate slides to water.
2. Treat with solution 1 for 2 min.

3. Wash in distilled water for 10 sec.
4. Rinse in solution 2 for 10 sec.
5. Rinse in 95 per cent and 100 per cent alcohols.
6. Clear in xylene and mount in cedar wood oil.

Result

Calcium sites	Orange-red

Demonstration of Lipase with Gomori's Method in Crustacean Cuticle (Erri Babu *et al.*, 1985)

Fixation

Fresh frozen

Reagents required

Tween 60 or 80
Tris buffer
Calcium chloride
Lead nitrate
Ammonium sulphide
Haematoxylin

Preparation of reagents

Solution 1

2 per cent tween 60 or 80	5.0 ml
0.2 M tris buffer pH 7.2	20.0 ml
4 per cent calcium chloride	5.0 ml
Distilled water	20.0 ml

Solution 2

Lead nitrate	2.0 g
Distilled water	00.0 ml

Solution 3

Ammonium sulphide	1.0 g
Distilled water	100.0ml

Procedure

1. Unfixed sections or chilled acetone-fixed sections for 24 hrs.
2. Dehydrate in 2 changes of acetone (room temperature).
3. Clear in benzene giving 2 changes for 45 min each for 2 hrs each.

4. Embed in paraffin.
5. Cut 5 μ sections and float on warm water at 35°C.
6. Bring them to wash.
7. Incubate in solution 1 at 37°C for 6–24 hrs.
8. Rinse in distilled water.
9. Transfer to solution 2 for 10–15 min.
10. Treat with solution 3 for 1 min.
11. Stain nuclei with haematoxylin.
12. Mount in glycerine jelly or dehydrate in dichloroethylene and mount in Gurr's medium.

Results

Sites of lipase activity	Brown
Nuclei	Blue

Demonstration of Keratin with Rhodamine B in the Spermatophore Wall Capsule of Crustaceans (Hanumantha Rao *et al.*, 1989)

Fixation

Any general fixative

Reagents required

Toluidine blue

Rhodamine B

McIlvaine's buffer

Na_2HPO_4 and citric acid

Preparation of reagents

Solution 1

Toluidine blue	100 mg
Distilled water	100.0 ml

Solution 2

0.2 M Na_2HPO_4 (pH 3.6)	64.4 ml
0.1 M citric acid	135.6 ml

Solution 3

Solution 2	100.0 ml
Rhodamine B	100 mg

Procedure

1. Deparaffinize and hydrate slides to water.
2. Immerse slides in solution 1 for 10 min.

3. Wash in distilled water.
4. Transfer to solution 3 for 10 min.
5. Wash rapidly.
6. Dehydrate very rapidly, clear and mount.

Result

Sites of keratin activity — Rose-red

Demonstration of Elastin in the Stomodaeum of the Amphipod Crustacean with Weigerts Resorcin Fuchsin (Shyamasundari and Hanumantha Rao, 1973)

Fixative

Any general fixative

Reagents required

Basic fuchsin
Resorcin
Ferric chloride
Hydrochloric acid

Preparation of reagents

Solution 1

Basic fuchsin	2.0 g
Resorcin	4.0 g
Distilled water	200.0 ml
29 per cent ferric chloride (29 g/100 ml water)	25.0 ml
95 per cent alcohol	200.0 ml
Hydrochloric acid	4.0 ml

Boil water and add fuchsin and resorcin. While boiling, add 25.0 ml ferric chloride. Boil for 5 min., cool and filter. Discard the filtrate and dry the precipitate in the filter paper. After thorough drying, bring the powder to another dish and add 200.0 ml of 95 per cent alcohol. Heat and take out the filter paper. When the precipitate is dissolved, cool, filter and add 95 per cent alcohol to make it to 200.0 ml. Add 4.0 ml of HCl.

Procedure

1. Dewax and bring slides to water.
2. Stain in solution 1 for 20–60 min.
3. Differentiate in 95 per cent alcohol.
4. Wash in tap water.
5. If desired, counterstain in haematoxylin.
6. Dehydrate and mount.

Result

Elastin lining	Dark blue or black

Also refer Trinadh Babu *et al.* (1989a)

Simultaneous Demonstration of Neurosecretory and Mucous Substances in the Amphipod (Crustaceans) Tissue Section (Shyamasundari and Hanumantha Rao, 1975b)

Fixation

Methanol, formaldehyde, acetic acid, Bouin's or Susa

Reagents required

Basic fuchsin
Paraldehyde
Hydrochloric acid
Potassium permanganate
Oxalic acid
Mercurochrome

Preparation of reagents

Solutions 1, 2 and 3 are as for Gomori's aldehyde fuchsin for neurosecretory cells.

Solution 4

Mercurochrome	500 mg
Distilled water	100.0 ml

Procedure

1. Dewax 2 sets of slides and hydrate in water.
2. Oxidize in solution 2 for 2 min.
3. Wash.
4. Bleach in solution 3 for 3 min.
5. Wash in running water.
6. Stain in solution 1 for 10–30 min.
7. Differentiate in 95 per cent alcohol.
8. A) Dehydrate one set, clear and mount.
   B) Counterstain another set with solution 4 for 10 min., wash, dehydrate and mount.

Result

Neurosecretory substance	Purple (8A)
Mucosubstances	Purple
Neurosecretory substance	Brick red (8B)
Mucosubstances	Purple

Demonstration of Disulphides in the Neurosecretory Cells of Crustaceans with Performic Acid/ Alcian Blue Technique (Shyamasundari, 1977)

Fixation

Any general fixative, formol

Reagents required

Performic acid

Sulphuric acid

Alcian blue

Preparation of reagents

Solution 1

Alcian blue	3.0 g
2N sulphuric acid	100.0 ml (pH 0.2–0.3)

Solution 2

98 per cent formic acid	40.0 ml
30 per cent H_2O	4.0 ml
Conc. H_2SO_4	0.5 ml

Allow the mixture to stand for 1 hr.

Procedure

1. Dewax and hydrate slides to water.
2. Blot.
3. Immerse sections in solution 2 for 5 min.
4. Wash gently in tap water.
5. Rinse in 70 per cent alcohol and blot.
6. Transfer to solution 1 at room temperature for 1 hr.
7. Wash in distilled water.
8. Counterstain if desired.
9. Dehydrate, clear and mount.

Result

Sites containing cystine	Deep blue

Also refer: Erri Babu *et al.* (1979, 1980a, 1980b; Jalaja Kumari *et al.*, 1980; Trinadha Babu *et al.*, 1989a; Lalitha *et al.*, 1993)

Application of Alcian Blue/Safranin for the Demonstration of Strongly Acidic Mucosubstance in the Crustacean Tegumental Glands (Shyamasundari, 1979)

Fixation

Cetylpyridinium chloride in 10 per cent formalin

Reagents required

Alcian blue

Safranin

Preparation of reagents

Solution 1

Alcian blue	500 mg
3 per cent acetic acid	100.0 ml

Solution 2

Safranin	250 mg
0.125N HCl	100.0 ml

Procedure

1. Deparaffinize and hydrate slides in water.
2. Stain in solution 1 for 30 min.
3. Wash in distilled water.
4. Transfer to solution 2 for 30 sec.
5. Dehydrate rapidly, clear and mount.

Result

Most strongly acidic substances	Red

Hale's Colloidal Iron Method for Acid Mucosubstances in Crustacean Tissue (Shyamasundari, 1979)

Reagents required

Ferric chloride

Hydrochloric acid

Potassium ferrocyanide

Acetic acid

Preparation of reagents

Solution 1 Stock colloidal iron solution

29 per cent ferric chloride (29 g/100 ml water)	2.2 ml
Distilled water	125.0 ml

Boil distilled water. When it is boiling, add ferric chlroride and stir. The solution is dark red. At this point remove it from heat and cool.

Solution 2

Glacial acetic acid	5.0 ml

Solution 1	20.0 ml
Distilled water	15.0 ml

Solution 3 Acid ferrocyanide mixture

Potassium ferrocyanide	2.0 g
Conc. hydrochloric acid	2.0 ml
Distilled water	98.0 ml

First add potassium ferrocyanide to water and dissolve. Add hydrochloric acid.

Procedure

1. Deparaffinize and hydrate slides to water.
2. Rapidly rinse in 12 per cent acetic acid.
3. Transfer to solution 2 for 1 hr.
4. Again rinse in 12 per cent acetic acid.
5. Transfer to solution 3.
6. Wash in distilled water.
7. Dehydrate, clear and mount.

Result

Acid mucosubstances	Bright blue

[Also refer for crustacean tissue. Shyamasundari and Hanumantha Rao (1975a, 1975b): Shyamasundari (1977); Trinadha Babu *et al.* (1989a, 1989b, 1989c, 1989d, 1991, 1993); Lalitha *et al.* (1993a, 1993b, 1996); Kameswaramma *et al.* (1987, 1990); Rama Devi *et al.* (1987, 1991)].

Demonstration of Sulphated and Carboxylated Mucosubstances in the Oesophageal Glands of Crustaceans by the Application of Alcian Blue/Alcian Yellow Technique (Shyamasundari, 1979)

Fixation

Susa or cetylpyridinium chloride

Reagents required

Alcian blue 8GX

Alcian yellow

Hydrochloric acid

Neutral red

Preparation of reagents

Solution 1

Alcian blue 8GX	1.0 g
N/5 hydrochloric acid	100.0 ml

Solution 2

Alcian yellow	1.0 g
3 per cent acetic acid	100.0 ml

Solution 3

0.5 per cent neutral red

Procedure

1. Deparaffinize and take down slides to water.
2. Rinse in N/5 hydrochloric acid.
3. Treat with solution 1 for 5–15 min.
4. Rinse in N/5 hydrochloric acid.
5. Transfer to solution 2 for 5–10 min.
6. Wash in water.
7. Counterstain with solution 3 for a few seconds.
8. Wash.
9. Dehydrate, clear and mount.

Result

Sites of sulphated mucins	Blue
Sites of carboxylated mucins	Yellow
Mixtures	Green

Demonstration of Elastin Type Protein in the Seminal Receptacle Wall by the Application of Spirit Blue Technique (Raghu, 1993)

Fixation

Helly's fluid

Reagents required

Potassium permanganate

Sodium metabisulphite

Concentrated hydrochloric acid

Spirit blue

Aniline oil

Phosphotungstic acid

Picrofuchsin

Acid fuchsin

Preparation of reagents

Solution 1 Potassium permanganate

0.5 per cent potassium permanganate	100.0 ml
(500 mg in 100 ml water)	
Concentrated sulphuric acid	3 drops

Solution 2 Sodium metabisulphite

1 per cent sodium metabisulphite	100.0 ml
Concentrated hydrochloric acid	1 drop

Solution 3 Spirit blue

Spirit blue (aniline blue alcoholic soluble)	750 mg
70 per cent ethyl alchol	100.0 ml
Aniline oil	2 drops

Solution 4 Phosphotungstic acid

Phosphotungstic acid	5.0 g
Distilled water	100.0 ml

Solution 5 Picric acid–acid fuchsin

Saturated alcoholic picric acid	100.0 ml
1 per cent acid fuchsin	5.0 ml
(1 g/100 ml water)	

Procedure

1. Deparaffinize and hydrate slides in water.
2. Oxidize in solution 1 for 1 min.
3. Wash.
4. Bleach in solution 2 for 30 sec.
5. Transfer to solution 3 for 2 min.
6. Wash in distilled water.
7. Dip in 70 per cent alcohol.
8. Treat with solution 4 for 5 min.
9. Wash in tap water.
10. Place in solution 5 for 5 sec.
11. Wash, dehydrate, clear and mount.

Result

Elastin	Bright blue
Collagen	Red

Confirmation of Sialomucins by the Application of Neuroaminidase Digestion Technique on the Tegumental Glands of Lobsters (Crustaceans) (Shyamasundari and Hanumantha Rao, 1978)

Fixation

Any general fixative

Reagents required

Neuraminidase
Acetate buffer
Calcium chloride
Alcian blue

Preparation of reagents

Solution 1 Sialidase

Neuraminidase	1.0 ml
Acetate buffer (pH 5.5)	4.0 ml
Calcium chloride	50 mg

Solution 2 Alcian blue solution pH 2.5 (*See* Chapter 8)

Procedure

1. Take 2 test and 2 control sections and dewax and bring them down to water.
2. Place 1 test and 1 control section in solution 1 (neuraminidase solution for 18 hrs at 37°C).
3. Incubate the other two sections in buffer solution at 37°C for 18 hrs.
4. Wash in tap water.
5. Treat all sections with solution 2 (alcian blue) for 5 min.
6. Wash in water.
7. Countertain if desired in Mayer's carmalum.
8. Wash in water.
9. Dehydrate, clear and mount.

Result

Sites of sialomucins remain unstained in sections treated with neuraminidase

Other acid mucins	Blue
Nuclei	Red

Molluscan Tissues Oyster Tissue to Demonstrate Collagen Fibres (Pauley, 1967)

Fixation

Zenker's solution

Reagents required

Ammonium hydroxide
Acid fuchsin
Aniline blue
Orange G
Phosphotungstic acid

Preparation of reagents

Solution 1

Ammonium hydroxide	3 drops
Water	1000 ml

Solution 2

Acid fuchsin	500 mg
Distilled water	100 ml

Solution 3

Aniline blue	500 mg
Orange G	2.0 g
Phosphotungstic acid	1.0 g
Distilled water	100.0 ml

Procedure

1. Deparaffinize slides and hydrate to water.
2. After removing mercury, wash in water.
3. Clear in 5 per cent sodium thiosulphate.
4. Wash in running water.
5. Transfer to Harris haematoxylin for 20 min.
6. Rinse in distilled water.
7. Rinse in 1 per cent acid alcohol.
8. Transfer to solution 1, until sections turn deep blue.
9. Rinse in running water.
10. Transfer to solution 2 for 5 min.
11. Rinse in distilled water.
12. Transfer to solution 3 for 15 min.
13. Rinse in tap water.
14. Pass through 95 per cent alcohol and 100 per cent alcohol.
15. Clear in xylene and mount.

Results

Collagen	Blue
Cartilage	Blue

Epithelium	Orange-red
Nuclei	Reddish blue

Alcian Blue pH 2.5 to Demonstrate Acid Mucopolysaccharides in Pelecypod Foot (Anisa Banu *et al.*, 1979a)

Fixation

1 per cent cetylpyridinium chloride in 10 per cent formalin

Reagents required

Alcian blue 8GX

3 per cent acetic acid

Preparation of reagents

Solution 1

Alcian blue 8 GX	1.0 g (pH 2.5)
3 per cent acetic acid	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Stain in solution 1 for (alcian blue) 30 min. (Requires more time with ageing).
3. Wash in running water.
4. Dehydrate, clear and mount.

Result

Acidic mucopolysaccharides	Deep blue

Alkaline phosphatase activity in the gland cells of the foot of pelecypod.

Calcium Cobalt Method (Anisa Banu *et al.*, 1979b)

Fixation

Cold acetone, paraffin, cold formalin, frozen sections

Reagents required

Sodium glycerophosphate

Diethyl-sodium diethylbarbiturate

Calcium chloride

Magnesium sulphate

Cobalt nitrate

Yellow ammonium sulphide

Preparation of reagents

Solution 1

Cobalt nitrate	2.0 g
Distilled water	100 ml

Solution 2 Incubating medium

3 per cent sodium B glycerophosphate ((3 g/100 ml water))	10.0 ml
2 per cent sodium diethyl barbiturate (2 g/100 ml water)	10.0 ml
Distilled water	5.0 ml
2 per cent calcium chloride (2 g/100 ml water)	20.0 ml
5 per cent magnesium sulphate (5 g/100 ml water)	1.0 ml

Procedure

1. Fix the material in cold acetone (4°C) for 24 hrs.
2. Transfer the blocks to absolute alcohol (change every half an hour).
3. Transfer to ethanol-ether and to 1 per cent celloidin.
4. Clear in benzene.
5. Embed in paraffin wax avoiding high temperatures.
6. Dry slides at 37°C and store at 4°C.
7. Remove wax with light petroleum.
8. Pass to water via absolute acetone.
9. Incubate in solution 2 for 16 hrs at 37°C.
10. Rinse in running water.
11. Place in solution 1 for 5 min.
12. Rinse in distilled water.
13. Treat with dilute solution of yellow ammonium sulphide for 2 min.
14. Wash in waer.
15. Counterstain in 1 per cent eosin for 5 min.
16. Dehydrate, clear and mount.

Result

Alkaline phosphatase activity	Black

Modified Lead Nitrate Method for Acid Phosphatase Activity in the Enzyme Glands of Pelecypods (Anisa Banu *et al.*, 1979b)

Fixation

Cold acetone paraffin; cold formol calcium frozen section

Reagents required

Sodium β-glycerophosphate
Acetate buffer
Lead acetate
Magnesium chloride
Ammoniacal silver nitrate
Sodium thiosulphate

Preparation of reagents

Solution 1 Sodium veronol

Sodium β-glycerophosphate	2.0 g
Distilled water	100.0 ml

Solution 2

0.1 M acetate buffer

Solution 3

Lead acetate	2.0 g
Distilled water	100.0 ml

Solution 4

Magnesium chloride	1.5 g
Distilled water	100.0 ml

Solution 5 Incubating medium

Solution 1	2 vol.
Solution 2	1 vol
Solution 3	1 vol
Solution 4	0.3 vol

Procedure

1. After fixation in cold acetone wash block in distilled water.
2. Mount blocks on cryostat tissue holder and cut 8–10 μ sections.
3. Mount sections on slides smeared with a mixture containing equal volume of gelatin (1 per cent) and 2 per cent formaldehyde. Allow sections to dry for one hour at 37°C.
4. Incubate slides in solution 5 for 2 hrs
5. Develop in ammoniacal silver nitrate solution for 30 min. (add 28 per cent ammonia to 5 per cent silver nitrate).
6. Rinse in 5 per cent sodium thiosulphate for 5 min.
7. Dehydrate, clear and mount in a synthetic medium or mount directly in glycerine jelly.

Result

Acid phosphatase activity	Black

Detection of Neutral Fats in the White Gland in the Pelecypod Foot with Application of Oil Red O Method (Anisa Banu *et al.*, 1980a)

Fixative

Reagents required

Oil red O

Isopropanol

Preparation of reagents

Solution 1 Stock solution

Oil red O	500 mg
98 per cent isopropanol	100.0 ml

Solution 2 Working solution

Stock solution	6.0 ml
Distilled water	4.0 ml

Allow to stand for 24 hrs and then filter.

Procedure

1. Cut frozen sections proceeding as in the case of previous method.
2. Rinse slides in water.
3. Rinse in 60 per cent isopropanol.
4. Transfer to solution 2 for 10–15 min.
5. Differentiation in 60 per cent isopropanol.
6. Wash in water.
7. Counterstain in Mayer's haemalum.
8. Wash in distilled water.
9. Mount in glycerine jelly.

Result

Neutral lipid activity	Red
Nuclei	Blue

Demonstration of Calcium by Applying Alizarin Red S Calcium-cum-excretory Cells of Gastropod Digestive Gland (Umadevi *et al.*, 1981)

Fixation

Neutral formalin

Preparation of reagents

Solution 1

Sodium alizarin sulphate	500 mg
Distilled water	45.0 ml

To this solution add 28 per cent ammonia and stir well (1 part ammonia + 99 parts water). Final pH should be 6.3–6.5.

Solution 2 Differentiator

Hydrochloric acid	0.1 ml
Distilled water	99.9 ml

Procedure

1. Hydrate slides after dewaxing to water.
2. Transfer to solution 1 (Alizarin red S) for 2 min.
3. Wash in distilled water.
4. Differentiate in solution 2 (acid alcohol) for 1 sec.
5. Dehydrate, clear and mount in cedar wood oil.

Result

Sites of calcium deposits	Orange-red

Perl's Prussian Blue Technique to Demonstrate Iron in the Calcium-cum-excretory Cells of Digestive Gland of Snails (Umadevi *et al.*, 1981)

Fixation

Neutral formalin

Reagents required

Potassium ferrocyanide

Hydrochloric acid

Neutral red

Preparation of reagents

Solution 1

Potassium ferrocyanide	2.0 g
Distilled water	100.0 ml

This solution should be prepared afresh.

Solution 2

2 per cent hydrochloric acid	2.0 ml
Distilled water	49.0 ml

Solution 3 Working solution

Solution 1	25.0 ml
Solution 2	25.0 ml

Procedure

1. Dewax and hydrate slides to water.
2. Transfer to freshly prepared solution 3 for 30 min.
3. Wash in water.
4. Counterstain if desired in neutral red.
5. Wash rapidly.
6. Dehydrate, clear and mount.

Results

Sites with ferric alum	Blue
Nuclei	Red

Application of Azure at pH 3.0 and pH 4.0 to Demonstrate Sialic Acid and Hyaluronic Acid Mucin in the Salivary Gland of the Gastropod (Rajalakshmi Bhanu *et al.*, 1981a)

Reagents required

Azure A
Citric acid
Sodium hydrogen phosphate

Preparation of reagents

Solution 1 1 per cent Azure A solution

Azure A	1.0 g
Distilled water	5000.0 ml

Solution 2 10 per cent citric acid

M/10 citric acid

Solution 3

M/5 Na_2HPO_4

Solution 4 Staining solution Azure pH 3.0

Solution 1	48.0 ml
Solution 2	1.65 ml
Solution 3	0.35 ml

Solution 5

Solution 1	48.0 ml
Solution 2	1.25 ml
Solution 3	0.75 ml

Procedure

1. Deparaffinize and hydrate slides to water (2 sets).
2. Stain one set in solution 4 for 30 min. and stain another set in solution 5 for 30 min.
3. Dehydrate in graded series of alcohols.
4. Place in a mixture of xylene and alcohol.
5. Clear in xylene.
6. Mount in caprate or permount.

Result

Sites with sialomucin activity are metachromatic at pH 3.0.

Sites with hyalomucins are metachromatic at pH 4.0.

Cresyl Fast Violet Stain for Oyster Tissue

Fixation

Ice-cold formaldehyde	150.0 ml
1.3 per cent calcium chloride	850.0 ml

Reagents required

Cresyl fast violet

Glacial acetic acid

Preparation of reagents

Solution 1

Cresyl fast violet	5.0 g
Distilled water	500.0 ml
Glacial acetic acid (pH 3.7)	0.5 ml

Procedure

1. Deparaffinize and hydrate to water.
2. Stain in solution 1 for 20 sec.
3. Wash in running water.
4. Dehydrate and clear.
5. Mount in technicon medium.

Result

Fungi	Blue
Nuclei	Dark blue
Bacteria	Dark blue
Cartilage	Pink

Insect Chromosomes (Crosier, 1968)

Reagents required

Sodium chloride
Calcium chloride
Potassium chloride
Sodium bicarbonate
Orcein
Lactic acid
Acetic acid
Sodium acetate

Preparation of reagents

Solution 1

Sodium chloride	14.0 g
Calcium chloride	0.4 g
Potassium chloride	200 mg
Sodium bicarbonate	0.2 ml
Water	1000.0 ml

Solution 2

Colcemid—Ringer 0.05 per cent colcemid (ciba) in Solution 1

Solution 3

Orcein	1.0 g
Lactic acid 85 per cent	28.0 ml
Glacial acetic acid	22.0 ml

Procedure

1. Keep sections in solution 2 at 25°C for 5 hrs.
2. Pass the slides to 1 per cent sodium nitrate for 20 min.
3. Keep sections in acetic methanol (1 : 3) for 30 min.
4. Transfer to a drop of 60 per cent acetic acid on a clean warmed slide and macerate if necessary.
5. Transfer to acetic methanol.
6. Place the slide in acetic ethanol (1 : 3) for 5 min.
7. Rinse in 70 per cent ethanol.
8. Transfer to solution 3, apply coverslip and place in heat for 12 hrs at 50°C.
9. Dehydrate, clear and mount.

Wismar's Quadrachrome Stain for Chitin (Wismar, 1966)

Fixation

Formalin-sublimate-acetic acid for 24 hrs

Formalin 20.0 ml + HgD_2—4 g + acetic acid—5 ml + distilled water—80 ml

Reagents required

Potassium iodide
Iodine
Alcian blue
Acetic acid
Ferric chloride
Woodstain scarlet
Acid fuchsin
Safffron
Sodium thiosulphate

Preparation of reagents

Solution 1 Lugol's iodine

Potassium iodide	6.0 g
Iodine	4.0 ml
Distilled water	100.0 ml

Solution 2

Distilled water	100.0 ml

Solution 2

Alcian blue 8GX	1.0 g
1 per cent acetic acid	100.0 ml

Solution 3 Verhoeff's haematoxylin

5 per cent haematoxylin (5 g/100 ml absolute alcohol)	50.0 ml
10 per cent ferric chloride	20.0 ml
Solution 1	20.0 ml

Solution 4 Wood stain scarlet acid—fuchsin

Wood stain scarlet	100 mg
0.5 per cent acetic acid	100.0 ml

Solution 5

Acid fuchsin	100 mg
0.1 per cent acetic acid	100.0 ml

Solution 6

Solution 4	2 parts
Solution 5	1 part

Solution 7

Extraction of safranin	100.0 ml

Extract 6.0 g of safranin in 100 ml of 100 per cent alcohol at 58–60°C for 48 hrs, fitler, decant and store.

Procedure

1. DepaRaffinize and bring slides to 80 per cent alcohol.
2. Transfer to solution 1 for 5 minutes.
3. Decolorize in 5 per cent sodium thiosulphate for 5 min.
4. Wash in running water.
5. Transfer to solution 2 for 30 min.
6. Wash in running water for 1 min.
7. Transfer to solution 3 for 4 to 6 hrs.
8. Differentiate in 95 per cent ethanol for 3 min.
9. Transfer to solution 6 for 3 min.
10. Transfer to 1 per cent acetic acid solution for 1 min.
11. Differentiate in 5 per cent phosphotungstic acid.
12. Keep in 1 per cent acetic acid for 1 min.
13. Differentiate in 10 per cent iron chloride.
14. Dehydrate in 2 changes of ethanol.
15. Stain in solution 7 for 5 min.
16. Rinse in 2 changes of absolute alcohol.
17. Clear in xylene, and mount.

Result

Chitin	Red
Cytoplasm	Red
Nucleic acid	Purple to black

Aceto-orcein for Insect Chromosomes (Lacour, 1941)

Fixation

Fresh material or acetic alcohol

Reagents required

Orcein

Acetic acid

Preparation of reagents

Solution 1

Orcein	1.0 g
Acetic acid	45.0 ml

Boil 45 ml of acetic acid and add 1 g of orcein. Cool and add 55.0 ml of distilled water.

Procedure

1. Dissect out tissues in staining solution.
2. Stain solution strength should be increased (orcein 2 g + 70 per cent acetic acid).
3. Stain for 10 min.
4. Apply coverslip.

Aldehyde Fuchsin for Neurosecretory Products in Insects (Ewen, 1962)

Fixation

Any general fixation (Bouin's: Helly)

Reagents required

Basic fuchsin

Concentrated HCl paraldehyde

Preparation

Solution 1 Aldehyde fuchsin

Solution 2

Potassium permanganate	150 mg
Conc. H_2SO_4	0.1 ml
Distilled water	50.0 ml

Solution 3

Sodium bisulphite	2.5 g
Distilled water	100.0 ml

Solution 4 Acid alcohol

Ethanol	100.0 ml
Conc. HCl	0.5 ml

Solution 5

Phosphotungstic acid	4.0 g
Phosphomolybdic acid	1.0g
Distilled water	100.0 ml

Solution 6

Light green	0.4 g
Orange	1.0 g
Chromotrope 2R	500 mg
Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Procedure

1. Deparaffinize and hydrate slides to water.
2. Place slides in solution 2 for 1 min.
3. Rinse in distilled water.
4. Bleach in solution 3.
5. Differentiate in 50 and 70 per cent alcohol.
6. Stain in solution 1 for 10 min.
7. Differentiate in 95 per cent ethanol.
8. Differentiate in solution 4 for 30 sec.
9. Differentiate in 70 per cent alcohol.
10. Mordant in solution 5 for 10 min.
11. Rinse in distilled water.
12. Counterstain in solution 6 for 1 hr.
13. Differentiate in 0.2 per cent acetic acid in 95 per cent alcohol.
14. Dehydrate, clear and mount.

Result

Cytoplasm	Light green
Neurosecretory substance	Dark purple

Rapid Azan Method for Crustaceans: (Hubschman, 1962)

Fixation

Bouin's picroformalin

Reagents required

Azocarmine
Glacial acetic acid
Phosphotungstic acid
Aniline blue
Orange G

Preparation of reagents

Solution 1

Azocarmine	1.0 g
Distilled water	100.ml

Solution 2

Phosphotungstsic acid	10.0 g
Aniline blue	1.2 g
Orange G	4.4 g
Distilled water	1000.0 ml

Procedure

1. Deparaffinize and hydrate in water.
2. Keep in solution 1 for 15–30 min.
3. Rinse in distilled water.
4. Keep in aniline oil (1 ml in 100 ml alcohol) for 30 min.
5. Wash in water.
6. Transfer to solution 2.
7. Wash.
8. Dehydrate, clear and mount.

Result

Epicuticle	Red
Endocuticle	Blue
Epidermal cells	Yellowish pink
Nuclei	Orange
Hepatopancreas	Pale orange
Oocytes	Grey with red nucleoli
Sperm	Orange

APPENDIX

NORMAL VALUES: HUMAN PHYSIOLOGY

A perusal of the different kinds of symptoms due to parasites leave us in bewilderment. A nematode may simulate the symptoms of six or seven different maladies including those of pregnancy! For various reasons the cause of a malady may remain out of comprehension. As already said the patient's non-communicative attitude adds to the misery. Examination of stools, blood, urine, x-rays, ultrascan method, etc., may greatly aid in diagnosis. Thus it become imperative to know the normal value of parameters concerning the living body (physiology). A kind of ready reckoner is thus provided here. The standard of Diagnostic laboratories differ very greatly. The need for sophisticated approach could be decided by the physician, otherwise in rural areas and even in some urban places an average laboratory would suffice. As per complications, severity and the kind of malady, the physican may ask for a detailed sophisticated investigation. Otherwise, costwise and timewise a simpler clinical investigation can prelude. Today there is no dearth of well trained personal in comparatively smaller laboratories.

Public awareness in matters of medical help has increased greatly thanks to our information technology. In the thorny field of vaccine development there are perpetual controverises. A case in point at present is the vaccine to combat human papilloma virus (HPV). It would appear that close to 70 civil Society groups, public health organizations, medical professionals, etc., have voiced their dissatisfication to the nature of projects concerned with HPV in Andhra Pradesh and Gujarat. However, no vaccine seems to be completely without risk. Pharma companies after considering all aspects continue to back Gardasil for HPV. In fact other medicines and injections (even B Complex) are not entirely without side-effects or even death. Individual susceptibility of sensitivity cannot be ignored altogether. On balance it seems majority may be benefited. There is an old adage. "The Universe" is not in the habit of giving anything for nothing. Of pesticides it has been documented that birds are ultimately affected through pesticide—ridden caterpillars and other food organisms. The proverb "Birds of a feather fly together" is changed to "Birds of a feather die together".

In the case of human schistosomiasis (causing cancer of the Urinary bladder—Egypt), the drug miracil-D is said to precipitate such side effects like nausea, the patient prefers to have the disease rather than treatment. So side-effercts due to drugs are well known in many cases. Unfortunately some of the human problems (tissue specific) have no animal models for drugs and vaccines to be trial tested.

In most cases patients postpone till the symptoms become chronic or severe condition arrives and they are mostly tight-lipped and do not communicate enough with the physician who is left guessing. Language problem may also be there. In such cases and especially with children, literally a veterinarian approach becomes a must. Few years ago, in the ward of Prof. Raghunathan (Principal, Medical College, Viskhapatnam), a 13 year old girl came with anemia and abdominal discomfort. Fortunately from the vomit they isolated trematodes. They were sent to us for identification. She had a massive infection of the echinostom *Artyfechinostomum*[1]. Although silent about her food habits there can be no doubt that she must have eaten raw or undercooked Apple snail *Pila virens* which carries the metacercaria (infective stage).

There are some previous records of difficulties with vaccines. Few years ago the terminology was 'Pandemic Influenza'. Chicken Flu virus emerged suddenly and spread rapidly across the world. This H_5N_1 virus caused great anxiety. Efforts were taken to produce a 'seed strain' that closely matched with the virus. Thus 'Pandemic-like vaccines were the target of companies. Side effects and immune responses have to be meticulously monitered. In the case of 'swine flu' outbreak, a voluntary mass immunization campaign was called off after several cases of "Guiillian-Barre" syndrome, a form of paralysis that can be fatal. In India there has been a succession in flu—First Asian Flu (1957) then chicken flu and now swine flu. What next?

Older records which provide information on scientific ideas or developments in science constitute the foundation. Recording of pioneering ventures is important. Thus 'archives' embracing events of ancient times and modern times remain interesting and stimulating (Jain S.K. 2010. Science archives in India—prospects, problems, procedures and policies. Science letters. 32 (11 & 12) National Academy of Sciences).

CARDIOVASCULAR

1.	Clinical assessment (normal values in tests of cardiovascular autonomic funcition)	From sitting to standing position: (i) Rise in pulse rate: <10/min (ii) Rise in blood pressure: <10 mmHg Heart rate variation in deep breathing (6/min): Maximum-minimum heart rate:> 10/min Breadth-holding test: > 30 s Valsalva ratio: > 1.2 Record blood pressure continuously while the subject is standing for 10–20 min, when the systolic blood pressure should not fall by 20 mmHg or more. Diastolic blood pressure responds to sustained hand grip (for 2–3 min): > 16 mmHg Exercise tolerance test: Heart rate increases rarely over 150/min and rapidly return to normal after ceasing exercise
2.	Resting ECG	Provides accurate information about the state of myocardium
3.	Stress testing	Exercise ECG shows (i) No significant ST depression (ii) Decreased amplitude of 'R' wave in V_5
4.	Circulatory and related Cardiac output measurements	2.5–3.6 L/sq. m of body surface area/min. Circulation time: Arm to lung: 4–8 s Arm to tongue: 10–16 s Ejection fraction, i.e. $\frac{\text{Stroke Volume}}{\text{End-diastolic Volume}} = 0.55 - 0.78$ End-diastolic volume: 75 ± 15 ml/m^2 End-systolic volume: 25 ± 8 ml/ m^2 L.V. Work: Stroke work index: 30–110 (g.m.)/ m^2 Systolic time intervals: (PEP; LVET; QS_2) Pre-ejection period (PEP): 131 millisec Left ventricular ejection time (LVET): 4.13 millisec Total electromechanical systole (QS_2): 546 millisec

$$\text{Ratio of } \frac{\text{PET}}{\text{lvet}} = 0.35 \pm 0.04$$

(A) Flows:

$$\text{Cardaic index} \left[\frac{\text{Cardiac output}}{\text{Body surface area}} \right] = 2.4 - 3.8 \text{ / L / min / sq.m}$$

(B) Pressures

Right atrium: 2–6 mmHg
Left atrium: 2–12 mmHg
Systolic (peak): 15–30 mmHg
End-diastolic: 2–7 mmHg

Pulmonary artery

Systolic (peak): 15–30 mmHg
End-diastolic: 9–7 mmHg

Left ventricle

Systolic (peak): 100–140 mmHg
End-diastolic: 3–12 mmHg
(on exercise: 12 mmHg)

Arterial (systemic)

Systolic (peak): 100–140 mmHg
End-diastolic: 60–90 mmHg
Mean: 700–105 mmHg

(C) Resistances

Systemic vascular resistance: 770–1500 (dyn.s)/cm^5

Pulmonary vascular resistance: 20–120 (dyn.s)/cm^5

Total Pulmonary Resistance: 100–300 (dyn.s)/cm^5

(D) Oxygen consumption: 110–150 L/min/sq.m

Arterio-venous oxygen difference: 30–50 ml/L

5. Haemodynamic studies
6. Other special investigations (apart from cardiac catheterisation studies vide supra)

Echocardiography: For measuring dimensions of chambers and aorta; assessing movements of septum, values and left ventricular wall; and function

Doppler echocardiography (measures blood flow velocity directly)
Angiography
Radionuclide Angiography
(i) Blood pool scanning and Peak LV Filling Rate (PFR) = 2.5 – 4.2 EDV/s
Time to PFR = 58–161 millisec (derived from LV time activity curve which was obtained from equilibrium radionuclide angiography)
(ii) Myocardial scanning

ENDOCRINAL

1. Pituitary function
   (a) Basal function tests
      (i) Measurement of pituitary hormones, serum ACTH (corticotrophin) < 80 pg/mL (< 18 pmol/L)
      Growth Hormone: <5 µg/L (< 5 ug/L) (after administering 100 g of glucose orally)
      TSH; PSH; LH (vide infra)
      Prolactin: 2–15 µg/L (2–15 µg/L)
      ADH (Vasopressin): 0.9–4.6 pmol/L
      Oxytocin: 1.25–5 ng/L (1–4 pmol/L)
      (ii) Measurement of target organ secretion (thyroid, adrenal, gonads)-(Vide infra).
   (b) Dynamic Test: (Insulin hypoglycaemia test): A fall of blood sugar to <40 mg% after administering insulin i.v. (0.1–0.2 U/kg) stimulates hypothalamine pituitary axis. Then serum cortisol, growth hormone, ACTH and blood sugar are measured every ½ h for 2h.
2. Adrenal function
   (a) Basal function tests
      (i) Measurement of adrenal steroids (serum)
      Cortisol (hydrocortisone)
      8 A.M. 5–25 µg/100 mL (140–690 nmol/L)
      4 P.M. 3–12 µg/100 mL (80–330 nmol/L)
      (Cricadian rhythm—cortisol secretion more in the morning)

II-Deoxycortisol: <1 μg/100 mL(< 30 nmol/L)
Aldosterone: < 8 ng/100 mL (< 220 pmol/L)
Dehydrocepiandrosterone (DHEA 0.2–.9 μg/100mL (77–31 nmol/L)
Dehydroepiandrosterone sulphate (DHEA sulphate) 50–250 μg/100 mL (1.3–6.7 μmol/L)
17-Hydroxyprogesterone
Women: 0.02–0.1 μg/100 mL (0.6–3 nmol/L) (higher in luteal phase)
Men: 0.006–0.3 μg/100 mL (0.2–9 nmol/L)

(ii) Measurement of adreno steroids (urine)

Cortisol (free): 20–100 μg/24 h (55–275 nmol/2 h)
17-Hydroxycorticosteroids: 2–10 mg/24 h (5.5–28 μmol/24 h)
Aldosterone: 5–19 μg/24 h (14–53 nmol/24 h)
17 oxogenic steroids
Men: 10–19 mg/24 h
17-Oxesteroids (ketosteroids)
Men: 7–25 mg/24 h (24–88 μmol/24 h)
Women: 4–15 mg/24 h (14–52 μmol/24 hf)

(iii) Measurement of urinary catecholamines

Free catecholamines: < 100 μg/24 h (< 590 nmol/24 h); Epinephrine: < 50μg/24 h (< 275 nmol/24 h)
Vanillylamandelic acid (VMA): (<8 mg/24 h (< 40 μmol/24 h)

(b) Dynamic Tests

(i) Dexamethasone suppression test

1 mg of Dexamethasone orally given at 10 P.M. Serum cortisol estimated of 8 A.M. and it should be < 5 μg/100 ml. in normal persons.

(ii) Insulin hypoglycaemia test (Vide Supra.)

(iii) Metyrapone Test

Administration of metyrapone blocks cortisol formation by adrenal under normal conditions. In turn ACTH is released which results in increased adrenocortical steroid production other than cortisol which is indicated by increased urinary 17-hydroxycorticosteroids.

(iv) Tetracosactrin (synacthen) test

ACTH or its synthetic analogue (synacthen) is given (250 mg i.m.) and serum cortisol response is observed. The initial level should be over 5 μg/100 ml and after 30 min over 20 μg/100 mL and the difference between them not less than 7 μg/100 mL in normal person.

3. Testicular function

(a) Hormonal

Serum testosterone (Secretion more in the morning)
Men: 300–1000 ng/100 mL (10–35 nmol/L)
Women: <100 ng/100 mL (< 3.5 nmol/L)
Prepubertal (both sexes): 5–20 ng/100 mL (0.17–0.7 nmol/L)
Androstenedione
Men: 80–130 mg/100 ml (3–5 nmol/L)
Women: 100–200 ng/100 mL (3.5–7 nmol/L)
Etiocholanolone: < 1.2 μg/100 mL
Gonadotropohins
LH (ICSH) – Secretes androgens and oestrogens
Men: 5–20 mIU/mL (5–20 IU/L)
Women: 5–25 mIU/mL (5–25 IU/L)—much higher at ovulation period and postmenopausal period.
FSH (spermatogenesis)
Men: 50–20 mIU/mL (5–20 IU/L)
Women: 5–20 mIU/mL (5–20 IU/L)—Higher at ovulation and postmenopausal period.

(b) Reproductive—Seminal analysis (vide Appendix-I)

(c) Stimulation test with Gonadotrophic Releasing Hormone (GnRH) or clomiphene or HCG—may be necessary if the hormonal levels are on the border line.

4. Ovarian function

(a) Hormonal

(i) Serum values

oestriol < 0.2 μg/100 mL
oestradiol—Women: 20–6 pg/mL (70–220 pmol/L) (raised at ovulation)
Men: < 50 pg/mL (< 180 pmol/L)
Progesterone-Women: > 5 ng/mL (> 16 nmol/L)
Men, and preovulatory, postmenopausal and prepubertal females: 2 ng/ml (6.4 nmol/L)
FSH & JH: (Vide supra)

(i) Urine values
Oestrogens (oestradiol)
Men: < 50 pg/mL (< 180 pmol/L)
Women: 20–60 pg/mL (70 –220 pmol/L)
Pregnanetriol: 0.2–3.5 mg/24 h

(a) Basal body temperature—Useful to determine the ovulatory cycles. A rise of about 0.5°–1°F is suggestive of porogesterone secretion by corpus luteum following ovulation. This biphasic temperature pattern is normal.

5. Thyroid function

(a) Basal function tests
(i) Hormones

(b) Thyroid stimulation hormone: 0.4–5 u U/mL (0. 5–5 m U/L)
Triiodothyronine (T_3): 70–190 ng/100 mL (1.1–2.9 nmol/L)
Thyroxine (T_4): 5–12 µg/100 mL (64–154 nmol/L)
Free thyroxine: 0.8–2.4 µg/100 mL (10.2 –3.6 nmol/L)
Free thyroxine index: 1–4 µg/100 mL
Effective thyroxine ratio: 0.58–1.1%
Protein bound iodine—Indicates amount of circulating inorganic
Iodine mostly in the form of thyroxine (3–8 µg/100 mL).
Thyoid binding globulin : 7–17 mg/L
T_3 (resin) uptake: 25–35 % (The amount of T_3 not bound to protein and removed on the resin is measured)
Reverse triiodothyronine (rT_3): 10–40 ng/100 mL (0.15–0.61 nmol/L)

(ii) Uptake: Studies radioactive iodine ($I_3 - I_1$) uptake: 5–35 % in 24 h.

(iii) Thyroid scanning:
A dose of radio-iodide or technetium is administered and the activity over the gland is mapped. (Normal: 5 × 2 cm)

(c) Dynamic tests of homostatic control

(i) T_3 suppression test: When (40 µg t.d.s) is given for one week, pituitary TSH is suppressed and consequently thyroid uptake of radio-iodine in a normal person.

(ii) TSH stimulation test: When TSH is given (5–10 units i.m.) daily for 3 days, there is no or little increase in the uptake of radio-iodine if hypothyroidism is due to thyroid failure.

(iii) TRH test: TRH (200 μg) given i.v. and TSH measured before and after one hour. In hypothyroidism, TSH response is exaggerated (rise is > 2 MU/L) whereas in hyperthyroidism the TSH response is contrary (X_2 MU/L). However in, hypothyroidism due to pituitary disease, there may be reduced response.

(iv) Perchlorate discharge test: $13I_1$ is given and after four hours when the counts are stable perchlorate is given orally and uptake measurements are done hourly. 10–15 % reduction in counts is normal

6. Parathyroid function

(a) Basal function tests

(i) Hormones

Parathromone—150–300 pg/mL

(ii) Biochemical

Serum calcium and urinary calcium

Serum phosphate and urinary phosphate

Serum alkaline phosphate

(b) Dynamic test

(i) Parathyroid hormone infusion test—Measures tubular response to the hormone regarding reabsorption of phosphate.

7. Pancreas (endocrine)

(a) Hormones

Glucagon: 50–100 pg/mL (14–29 pmol/L)

Insulin (fasting): 6–26 u/mL (43–186 pmol/L)

(b) Sugar tolerance test : After giving 50 g of glucose orally, blood sugar should not be > 180 mg/100 mL (> 10 mmol/L) within two hours.

GASTROINTESTINAL

1. Oesophageal
   (i) Resting pressure
   Upper oesophageal sphincter (UES): + 40 mmHg: Lower oesophageal sphincter (LES): + 15 mmHg
   (ii) Swallowing pressure: Increases more than twice (> 60 mm/Hg) in the UES and less than twice (< 30 mmHg) in the LES
2. Gastric
   (i) Intragastric pressure: + 5 mmHg
   (ii) Gastric juice
   Volume: 2–3 L/d
   Reaction: pH 1.6–1.8
   $\frac{\text{Basal acid output}}{\text{Maximum acid output}}$ Ratio is 0.6
   (iii) Serum gastrin 60–200 pg/ml
3. Intestinal
   (A) Absorption tests
      (i) D-xylase: 25 g of xylase given orally after fasting overnight. Serum should contain 25–40 mg/100 ml after one hour and urine collected after five hours should contain 5.8 g.
      (ii) Vitamin-A: After 200000 units of Vitamin A in oil given orally after fasting, the serum level should rise to twice that of fasting level in 3–5 h.
      (iii) Vitamin B_{12}: B_{12} labelled with cobalt^{58} is given orally together with a further dose of B_{12} labelled with cobalt^{57} bound to intrinsic factor and a dose of B_{12} 1000 μg s.c. also given at the beginning of the test. 24 h urine collection is then assayed, when more than 10 % of the dose should be excreted within 24 h.
   (B) Colonic flora: Normal jejunum contains 10^1–10^3 organisms. (Any contamination/overgrowth is indicated by breath hydrogen test, i.e. end-expiratory breath samples, at intervals of 30 min for two hours, show a rise of > 20 ppm above base line as measured by breath hydrogen metre, after an overnight fast, on administering 50 g of glucose orally in 200 ml of water.)
   (C) Stool

HEPATOBILIARY AND PANCREATIC (EXOCRINE)

1. Liver

(A) Excretory

(i) Biliary

Serum bilirubin: Total: 0.3–1 mg/100 ml (5.1–17) μmol/L

Direct: 0.1–0.3 mg/dl (1.7–5.1 μmol/L)

Indirect:0.2–0.7 mg/dl (3.4–1.2 μmol/L)

Urinary bilirubin: Absent normally

Urinary urobilinogen: 1–3.5 mg/24h (1.7–5.9 μmol/24h)

Faecal stercobilinogen: 40–280 mg/24h (68–470 μmol/24h)

(ii) Bromsulphthalein excretion: 5 mg/kg i.v. when given < 5% of dose is retained in serum after 45 min (not useful in the presence of jaundice)

(iii) Cholesterol (serum): 150–250 mg/100 ml. (3.9–6.5 mmol/L)

(iv) Enzymes: Serum alkaline phosphatase:
21–91 IU/L (0.4–1.5 μmol/L)

(B) Integrity of hepatocytes

Enzymes

(i) Aspartate aminotransferase (ALT, SGPT): 10–40 Karmen units/ml
(100–300 mmols/L)

(ii) Alanine aminotransferase(ALT, SGPT): 10–40 Karmen units/ml (50–430 mmols/L)

(C) Other enzymes

(i) Gamma-glutamyltranspeptidase: 4–60 U/L

(ii) 5-Nucleotidase: 0.3–2.6 Bodansky units 100 ml (27–233 nmol/L) (useful to confirm isolated rise in serum alkaline phosphatase of suspected liver origin)

(D) Metabolic function

(a) Proteins

(i) Albumin: 3.5–5 g/dl

(ii) Globulin: 2–3.5 g/dl

(iii) Flocculation tests

Thymol turbidity: 0–4 U

Zinc sulphate turbidity: 4–12 U)

(iv) Prothrombin: 60–100%

(v) Ammonia (test for hepatic detoxification) 80–110 mg/dl

(b) Fats

Cholesterol esters (about 60% of cholesterol is esterified normally by the liver)

90–150 mg/100 ml (2.3–3.9 mmol/L)

(c) Carbohydrates: Galactose tolerance (2.5 g/kg in 50% solution when given i.v., disappears from blood in two hours): no longer used

2. Gallbladder

(A) Oral cholecystography: The gallbladder ahould contract to one half of its original size at least, after a fatty meal (not undertaken in jaundice).

(B) Cholangiography: Useful to determine normal patency of the bile duct

3. Pancreas (Exocrine)

(A) Examination of the urine amylase (distase) levels: Normally 35–260 somogyi units/h

(B) Examination of the stool

(i) Faecal fat content should not exceed 10% of the fat intake

(ii) Faecal nitrogen does not exceed 1.5 g representing 10 g of protein

(iii) Microscopy: Partially digested few muscle fibres present

(C) Examination of the blood

(a) Enzymes

(i) Serum amylase: 60–180 somogyi units/100 ml (13–53 mmol/L)

(ii) Serum lipase: 0.2–1.5 U

(iii) Serum alkaline phosphatase: 21–91 IU/L (0.4–1.5 mmol/L)

(b) Coagulation

(i) Serum calcium: 9–10.5 mg/dl (2.2–2.6 mmol/L)

(ii) Prothrombin: 60–100%

(iii) Antithrombin III: 80–120%

(D) Study of duodenal juice

(i) Yellow or yellowish green bile stain

(ii) pH>8

(iii) Pancreatic enzyme content

(iv) Secretory tests

(a) Secretin test (1 U/kg body weight) /Pancreozymin Cholecystokinin i.v. (seldom employed)

(i) Volume of pancreatic juice: > 2 ml/kg in 80 min

(ii) Bicarbonate output: >10 mEq in 30 min

(iii) Bicarbonate concentration: > 80 mEq/L

Pancreatic enzyme content increased

(b) Lundh test meal (liquid form of carbohydrate, protein, fat): Duodenal contents assayed for concentration of pancreatic enzymes

(E) Bentiromide test: 500 mg of bentiromide orally given and p-aminobenzoic acid measured in

(i) Plasma > 3.6(±1.1) μg/ml at 90 min

(ii) Urine > 50% recovered in 6 h

(F) Radiology: ERCP (Endoscopic Retrograde Cholangio Pancreatography); Pancreatic cytology and pancreatic juice analysis can also be done besides visualizing biliary tree and pancreatic duct.

PULMOANARY

1. Ventilatory

(A) Spirometry

	Women	Men
Forced Vital Capacity (FVC)	≥ 3 L	≥ 4 L
Forced Expiratory Volume (FEV_1) in one second	> 2 L	> 3 L
$FEV_1/FVC = FEV_1$ %	> 60 %	> 70 %

Maximal mid-expiratory flow (MMF): > 1.6 L/s >2 L/per second

Maximal expiratory flow rate (MEFR): > 3 L/s >3.5 L/per second

Pulmonary ventilation (Respiratory minute volume): 6 L/per minute

Alveolar ventilation: 4.2 L/min

Maximal voluntary ventilation (MVV): 125–170 L/min

	(B)	Lung Volumes	Women	Men
		Total lung capacity (TC) (IRV + TV + ERV + RV)	4.2 L	6 L
		Vital capacity (VC) (IRV + TV + ERV)	3.1 L	4.8 L
		Inspiratory capacity (IC) (IRV + TV)	2.4 L	3.8 L
		Functional residual capacity (FRC): (ERV + RV)	1.8 L	2.2 L
		Inspiratory reserve volume (IRV)	1.9 L	3.3 L
		Expiratory reserve volume (ERV)	0.7 L	1.0 L
		Tidal volume (TV)	0.5 L	0.5 L
		Residual volume (RV)	1.1 L	1.2 L
		Peak flow with Wright's Flow meter	300–500 L	450–700 L

2. Gas exchange

(A) Arterial blood gases

Arterial oxygen tension (PaO_2): 80–100 mmHg (11–13 kPa)

Arterial oxygen saturation (SaO_2): 95–99%

Alveolar-arterial oxygen difference (A-aDO_2:= ≤ 20 mm Hg

Arterial carbon dioxide tension ($PaCO_2$): = 35–45 mm Hg (4.7–6 kPa)

Arterial bicarbonate (HCO_3): 21–28 mmol/L)

Arterial blood pH: 7.35–7.45

(N.B. 7.6 mm Hg = 1 kPa)

(B) Diffusing capacity for carbon monoxide uptake (DL CO)—Gas exchanging capacity or transfer factor. At rest: 19 ± 3.9/mL CO/min/mmHg; On exercise: 27 ± 3.9 ml CO/min mmHg (N.B. Reduced diffusing capacity causes reduced oxygenation of arterial blood as in emphysema.

RENAL

1. Glomerular

(A) Clearance tests for measuring Glomerular Filtration Rate (GFR)

Creatinine clearance test: 75–125 ml/min

Insulin clearance test: 100–150 ml/min

Urea clearance test: 60–100 ml/min

(N.B: Normal GFR in men is about 125 ml/min, or 180 L/day, and 10% lower in women as against normal urine volume of 1 L/day i.e. 99% of filtrate is reabsorbed normally)

(B) Filtration Fraction (FF) $= \frac{\text{GFR}}{\text{RPF}} = 17\%$ to 21% (Renal plasma flow)

(C) Urine volume: 800–2500 ml/day

(D) Substances depending on filtration for their excretion

Urea: 15–40 mg/100 ml (2.5–6.7 μmol/L)

Creatinine: 0.7–1.5 mg/100 ml (62–133 μmol/L)

2. Tubular function

(A) Urine specific gravity (for assessing distal tubular function)

Water concentration Test: > 1025 (after 12 h of water restriction)

Water dilution Test: < 1003 (after 12 h of liberal water intake)

(B) Phenolsulphonephthalein (PSP) excretion test for assessing proximal tubule—Proximal tubular transport measured by the 15 minute excretion determination and also serves as a clinical measurement of rental plasma flow 6 mg parenterally.

> 25% excreted in 15 min

> 40% excreted in one hour

> 60% excreted in two hours

(C) Urea concentration test

Urine urea concentration: > 2 g/100 ml

(D) Tubular reabsorption of phosphorous: 79–94%

3. Both glomerular and tubular

(A) Urine

Specific gravity: 1002–1028

pH: 4.6–8

Acid load test: Ammonium chloride given (0.1 g per kg body weight) when pH should become < 5.3

Osmolality: 350–1000 moSm/kg

Protein excretion: < 150 mg in 24 h

Other chemical constituents (inorganic, e.g. sodium and organic, e.g. urea)

Deposits: Under high objective (1/6 in)

Cells: Pus cells (2–4), RBC (1–2) epithelial cells (occasional)

Casts: Hyaline (occasional)

(B) Blood

$$\text{Osmolality} = 2\text{Na mEq/L} + \frac{\text{BUN mg/dl}}{2.8} + \frac{\text{Glucose/dl}}{18}$$
$$= 285 - 305 \text{ moSm/kg}$$

Anion gap = $Na-(HCO_3 + Cl) = 10 \pm 2$ mEq/L or mmol/L

$$\frac{\text{Urine osmolality}}{\text{Serum osmolality}} => 3$$

Bicarbonate: 21–28 mEq/L (21–28 mmol/L) mEq/L

(C) Effective renal plasma flow

Para-aminohippuric acid clearance: 490–820 ml/min

(D) Radiology: Intravenous pyelogram

(E) Isotope clearance procedures

(F) Isotope renography (renogram)

(G) Renal scintigraphy (scan)

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