

WALDEMAR MORDECAI HAFFKINE

(1860–1930)

HAFFKINE INSTITUTE

DIAMOND JUBILEE

1899—1959

SOUVENIR

सत्यमेव जयते

MINISTER FOR FINANCE,
GOVERNMENT OF BOMBAY.

FOREWORD

It was in the year 1899 when plague was raging in Bombay that in the former Government House in Parel was established the Plague Research Laboratory, now known as the Haffkine Institute in honour of Dr. Haffkine, the discoverer of plague vaccine. The birth of the Institute was a memorable milestone in the history of Medical Research in this country. It is therefore with great pride that the citizens of Bombay desire to celebrate the Diamond Jubilee of the event on the date on which Haffkine inoculated himself with the vaccine he had prepared to fight the scourge of plague. This Souvenir is prepared to commemorate the occasion; and as Chairman of the Executive Committee of the Diamond Jubilee Celebration, I consider it a privilege to write this foreword.

It is gratifying to note that Scientists who are foremost in their respective fields in this country as well as abroad, have contributed to the Souvenir Volume. These articles give an idea of the research work done in various fields today. The volume also contains an account of research and of other activities of each of the major departments which have been developed in the Institute during the last sixty years. As against the background of the World progress in medical research the Institute has achieved a measurable success. I take this opportunity to pay tribute on behalf of myself and my Committee to the memory of Dr. Haffkine and to his successors for their notable success; and hope that the Institute will continue to make valuable contributions to medical research and earn the blessings of humanity in time to come.

Jairaj N. Mehta

Chairman,
Executive Committee

31st December 1958

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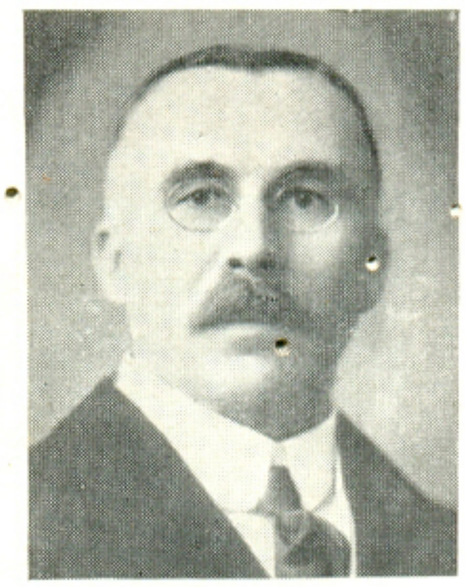
W. B. Bannerman
1904–1911



W. M. Haffkine
1896–1904



W. G. Liston
1911–1923

DIRECTORS OF THE HAFFKINE INSTITUTE 1896 — 1959



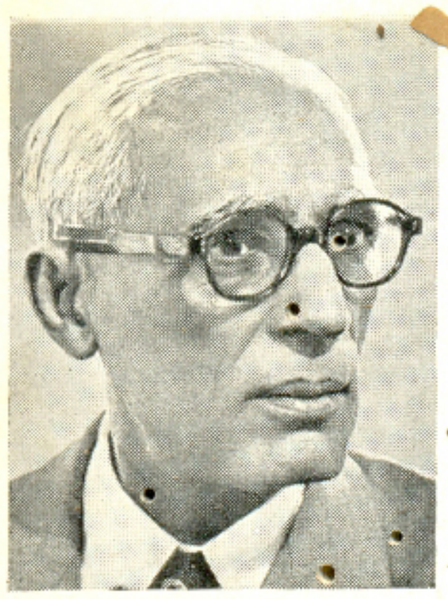
F. P. Mackie
1923–1931



J. Taylor
1931–1932



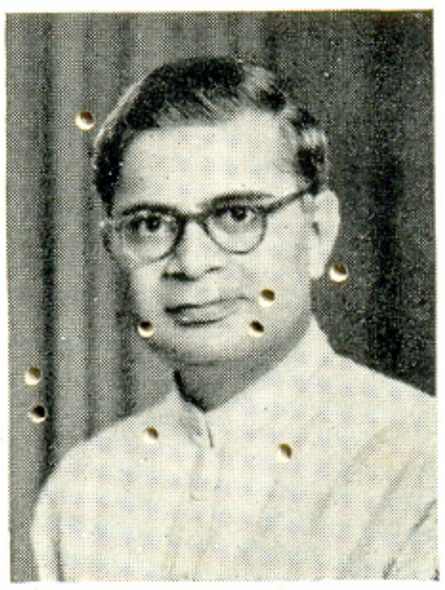
P. M. Wagle
1949–1955



S. S. Sokhey
1932–1949



D. W. Soman
1955–1957



H. I. Jhala
1957–



Stalactite growth of *P. pestis* as observed by Haffkine

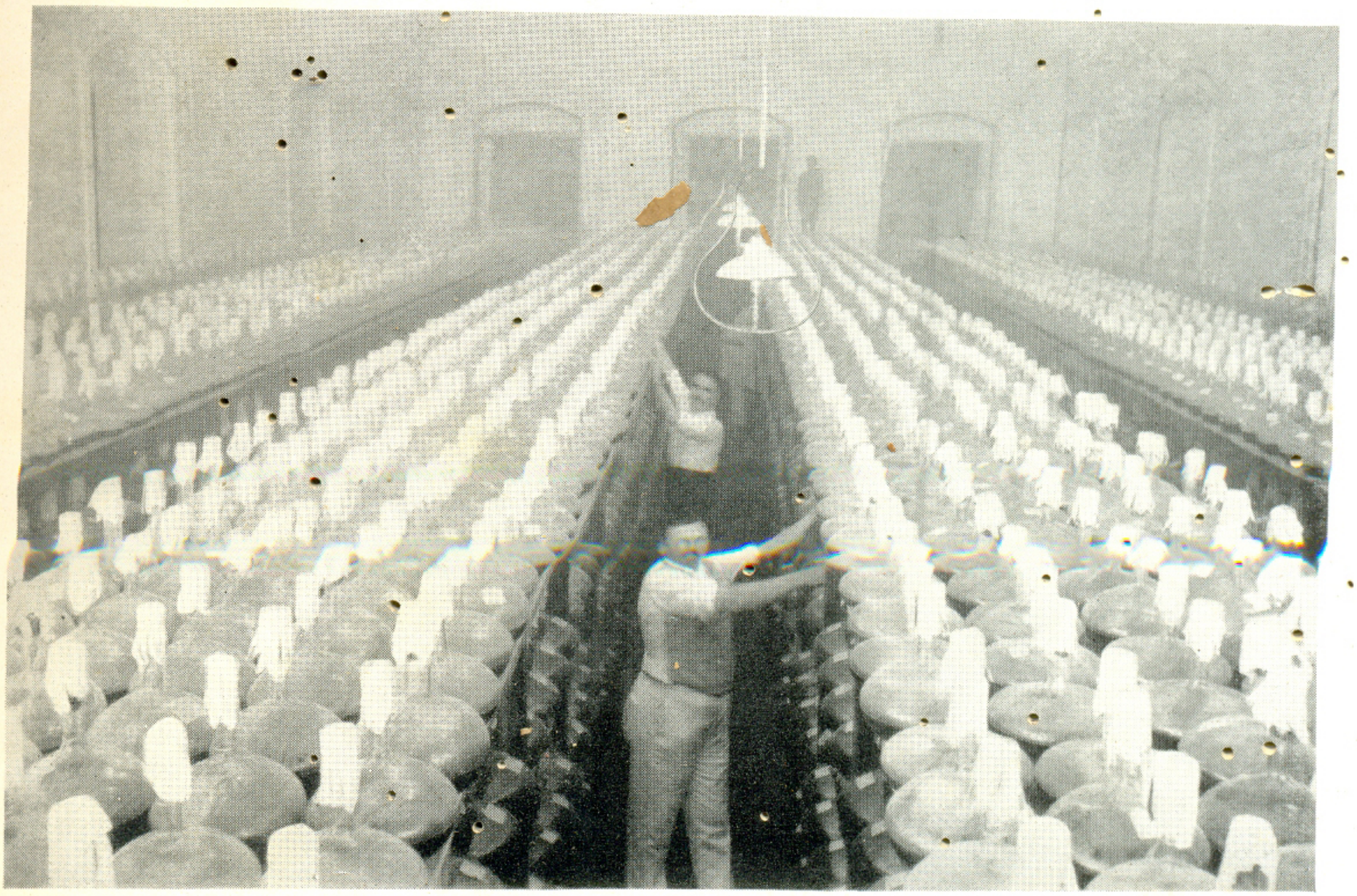
December 1895, where he concluded with *these words:*

"Mr. Chairman and Gentlemen, on the day when I came back from expedition to India, I found my former chief Mons. Pasteur, lying on a bed of death. Whatever might have been his appreciation of the work done in India, there can be only one desire on my part, that all the honour for the results which may possibly come out of my efforts, should be referred to him, to his sacred memory." The words are full of sincerity and humility which this learned scientist possessed. The results obtained by anti-cholera inoculations were so encouraging that tea-planters in Assam invited Haffkine to visit the tea-gardens to undertake the preventive measure and the Government was induced to help him financially. Haffkine returned to Calcutta in 1896 and began his further researches; while he was thus engaged, bubonic plague broke out in an epidemic form in the city of Bombay. Government of India, thereupon, sent Haffkine to Bombay with instructions to investigate the cause of the outbreak and to devise, if possible, some method of dealing with this new and terrible disease.

He reached Bombay on 7th October 1896, and the very next day he started work in the Petit Laboratory of the Grant Medical College. His laboratory consisted of one room and a corridor and a staff of one clerk and three peons. It was here, that the discovery of stalactite growth of plague bacillus was made. In December 1896, Haffkine was successful in protecting rabbits against an inoculation of virulent plague bacilli, while treating them previously with a subcutaneous injection of a broth culture of these organisms sterilised by heat. The rabbits so treated became immune to plague.

On the 10th January 1897 — the day of which the Diamond Jubilee is being celebrated — Haffkine caused himself to be inoculated with 10 c.c. of the plague prophylactic, thus proving in his own person the harmlessness of the fluid. Later on, medical men and prominent citizens of Bombay were publicly inoculated and others were encouraged to submit to it. It was not till the publication of the results of the controlled trials of inoculation of half the prisoners in the *House of Correction at Byculla, Bombay,* where plague had broken out, that the measure became popular. The increased popularity of inoculation involved large-scale manufacture of the prophylactic necessitating removal of his laboratory to the "Cliff" bungalow, Malabar Hill, lent by the Municipality of Bombay. Within a period of 7 months this accommodation also proved to be small and he had to move to another bungalow at Napean Sea Road. At this time, H.H. Sir Sultan Shah, Aga Khan, K.C.I.E. the Head of the Khoja Community, who had been early convinced of the efficacy of the inoculation, fitted up at his own expense "Khushru Lodge" for Haffkine's use. The staff under him was increased and the laboratory remained there for more than a year, but in March 1899, the demands for the vaccine from all parts of the world became so pressing that more accommodation became an urgent necessity. This was found in the Old Government House in Parel which is the present premises of the Haffkine Institute.

This present building in which Haffkine worked has an interesting story. It is supposed to have been built on the site of an old temple of Parli Vaijnath, which gave the name Parel to the adjoining village. On this site the Jesuits built a Monastery and the Chapel sometime between 1596-1693. The exact date is not known, but the present premises of Haffkine Institute was known as Romish Chapel of Jesuits and was in existence in 1673. It must have been built during the period, when the ancient island of Bombay, which consisted of a group of 7 islands referred to by ancient Greek traders as Heptanesia, was in possession of Portuguese since 1534 with the consent of Bahadur Shah — the Mughal Emperor. The Chapel had extensive grounds of its own almost resembling a park with big spreading trees growing all over. From the estates in Bombay, Jesuits maintained a Mission in Agra and Japan, even when Bombay had passed into the hands of the British because according to the Treaty of 1665, the Portuguese were not to be



Incubation room in early days



Old method of vacuum sealing

interfered with in their religious order. In the year 1719, the British confiscated all Jesuit properties after a Maratha invasion, on the plea that the Jesuits had allowed the Marathas to mount their guns aimed at the British from one of their properties at Bandra. The building, thereafter, became the residence of Registrars of Bombay until the demolition of the Fort. The Governors of Bombay used to live in Parel after leaving the Fort. The building was then known as Government House. This area was then considered to be a very aristocratic locality. In 1885, the Governors left this residence and the premises were used as House of Recorders of the Bombay Presidency. In 1895, King Edward VII visited India as Prince of Wales, and stayed on the premises for a week. On such a historical premises Haffkine entered on 10th August 1899.

When Haffkine entered Bombay, the city was no longer a group of islands. They were amalgamated into one in 1775 after *Hornby Vellard* was built. However, it was a very ancient place with a population of little over 8 lakhs living in conditions of great over-crowding, so much so, that in some parts of the town the population per acre was perhaps the highest in the world. Conditions were most insanitary. The premises of the Institute itself had served as a Plague Hospital prior to its occupation by Haffkine. The available medical knowledge and practice were limited; there were a few private dispensaries. The J. J. Hospital was opened in 1842 and Grant Medical College was established in 1845. The syringes were practically unknown to the medical profession, having been only recently introduced in the hospitals. Therefore, the injections were rarely given. Bombay of those days was quite unlike what it is today. It will not be possible for the present generation of Bombayites to conjure up or reconstruct what the city was like sixty years ago. Panic was raging throughout the city as a result of plague and the population had dwindled to 3 lakhs. Strict vigilance exercised by the British plague controlling authorities had excited lot of opposition from religious and orthodox persons and lot of opposition had gathered in the country when Haffkine came to Bombay. A number of official commissions hastened to the afflicted city. They were entrusted with the task of studying the disease. There were the German, Austrian, Egyptian, Russian, Italian, Ceylonese and Turkish Commissions and the Scientific Indian Plague Committee consisting of Manser, Childe, Haukin and Surveyor. Some of these members, like Sticker, Manser and Muller died during the conduct of their investigations. There was a great paucity of doctors who were familiar with the use of a syringe and classes had to be conducted for medical men and others to be trained in the art of inoculating Haffkine's prophylactic.

In 1902, at Malkowal in Punjab, 19 persons who received the prophylactic inoculation developed tetanus after some lapse of time. This incident referred to as Malkowal Disaster gave a great set-back to the plague prophylactic and to Haffkine in particular. Haffkine had changed the method of preparing the Plague Vaccine without consulting the Government and while this method was the correct one as we know today, the change of procedure threw a suspicion on its having possible connection with the Malkowal Disaster. The Commission of Enquiry was formed which took some time to investigate the details of what had transpired and Haffkine was relieved of the charge on 30th April 1904. The Commission consisted of the Chief Justice of Bombay, Principal of the Medical College, Calcutta, and Director, Pasteur Institute, Kasauli. They expressed an opinion that carbolic acid was a valuable agent in restraining tetanus growth when added to the plague prophylactic, and they therefore thought that its omission was a grave mistake. The Report of the Commission was sent up for enquiry by two independent authorities and further experiments were conducted at Lister Institute, London. The conclusion of that Institute was that in all probability tetanus germ was present at the time of inoculation in the fluid contained in the bottle, but that it was impossible to determine at what stage or in what way the

bottle became contaminated. During the course of evidence it was shown that a hospital assistant in a hurry dropped on the ground the forceps with which he opened the rubber stopper of the vaccine bottle, and without re-sterilising, used it and went on injecting from the same bottle. A lot of controversy was raised and Haffkine had to go round all the continental laboratories and meet scientists and argue out his case that at the time of despatch and, until the time of receipt, the bottle could not have contained the germs of tetanus. This put a terrible strain on Haffkine, but ultimately he was exonerated by the Secretary of State and was persuaded again to take up an appointment as Director of the Bacteriological Laboratories in Calcutta in 1907. Haffkine was broken-hearted by this time, but he accepted the work. In 1914 he left India to spend rest of his life in Boulogne-Sur-Seine where he was more given to religion, meditation and quiet life, until his death in Lussanne in 1930.

Great as was the genius of Haffkine, he was full of modesty and was very considerate even in his adversaries. One and all acclaim him as a generous, kind and sympathetic master and a good friend indeed. It is a fitting tribute that at the instance of Lt.-Col. F. P. Mackie, I.M.S., the then Director of this Laboratory, the Bombay Government renamed in 1925, the Plague Laboratory started by him in 1899 already renamed as Bombay Bacteriological Laboratory as HAFFKINE INSTITUTE to perpetuate the memory of one who has conferred on India and its people incalculable benefits. When Mons. Haffkine, D.Sc., C.I.E. was informed of this he wrote as follows: "I am very greatly indebted to Col. Mackie for the name given to the Parel Laboratory and to you for the terms in which you have written to me. Very much do I appreciate also your mentioning of the friendly attitude towards me of the other members of the Institute's staff. The work at Bombay absorbed the best years of my life and I need not explain how much I feel everything connected therewith. I wish the Institute prosperity as an active centre of work on behalf of the health organisation of the country and I send blessings to the whole of its staff."

Under the shadow of such blessings of a noble scientist, the Institute has been privileged to observe the Diamond Jubilee with a creditable account of its achievements.

In 1905, the Government decided that the Plague Research Laboratory should act as a provincial laboratory for giving diagnostic help and it was renamed as Bombay Bacteriological Laboratory on that account. Bannerman succeeded Haffkine in 1905 and carried on till 1908. He was followed by Liston who had been working at this Institute for a number of years. He worked out the life-history of *X. cheopis* and found that of all different fleas he used in order to transmit plague in laboratory animals like guinea-pig and rabbits, he found the cheopis as the most effective transmitter or vector of plague. Suggestion of flea as vector was already made by Haffkine in the beginning but was not successfully established. Liston was associated with the Royal Plague Commission in London since 1899. The line of work which Liston followed was along the same lines as the work of Dr. P. L. Simond who published his observations in the Annals of the Pasteur Institute in October 1898. Liston returned to India in 1903 well-equipped with the training. The Indian Plague Commission also held its sittings in Bombay at the Haffkine Institute — and eminent men like Surveyor, Col. R. Rao were associated with it.

The Institute then also undertook a number of researches on subjects like beriberi, leprosy, epizootic lymphangisis, diphtheria, trepanosomiasis, guineaworm disease, tuberculosis, lathyriasis, snake and scorpion bites, malaria, kala-azar and dysentery and many other problems of infectious diseases.

The Department of Antirabic Vaccine production was added in 1922. During the World War I the Institute served as an "Enteric Depot" (for housing cases of enteric) and a training ground for medical men. In 1924, the Pharmacology Department was opened to carry on research on indigenous

drugs but was closed down in 1933 on account of financial stringency. It re-emerged as a full-fledged Department again in 1935.

In 1938, the department of Entomology was organised; in 1940, the Department of Chemotherapy was added. The Department of Antitoxins and Sera was added in 1940 and the Blood Bank in 1942. Biochemistry Section was opened with the help of the funds of the Indian Research Fund Association along with the pharmacological laboratory and the former has continued uninterruptedly since then. The Institute received a lot of financial support at the hands of Indian Research Fund Association (now Indian Council of Medical Research) and the Council of Scientific and Industrial Research.

Amongst the people who have worked at this Institute we have eminent men like Bannerman, Liston, Taylor, Sokhey, Wagle and Soman as Directors; Soparkar, Chitre, Avari, Naidu, Dalal, Sharif, R. Rao, Balfour, Jung, Mhaskar, Gore, Dogra, Tarkhad, Morrison, Anderson, Dixit, Wats, Sanjiv Rao, Ganapathi, Lahiri, Mehta, Gokhale, Bhatnagar, Puduwal, Menezes, Sathe and many others whose blood, sweat, and work have been responsible for the present growth of the Institute. The Institute was fortunate in receiving international collaboration throughout the world; the U.N. Bodies and previous international organisations had always given cordial help. A number of eminent scientists have visited and worked in this Institute and given their best. The Institute has developed a pattern so characteristic of its own. It piloted a number of schemes for its expansion and has had to struggle hard in the face of financial difficulties. Originally started by Government of India, it became a provincial laboratory in 1905. It helped to lay the foundation of medical research in this country and contributed to its moulding, in its very initial stages. Throughout, it has kept antimicrobic and immunologic bias. It has defined its four functions: (1) to serve as a centre of medical research, (2) to serve as a training centre for researchers, (3) to serve as a centre for supply of biologicals needed for diagnosis, prophylaxis or treatment in a form suitable for the conditions prevailing in India, and (4) to serve as a testing laboratory for medical, public health and drug control needs of the country. It has taken a practical view of research, as an intelligent way of solving difficulties of day-to-day life in public health fields. It has its own research programme and it also conducts sponsored research in collaboration with various research organisations in the country. Today, at the age of sixty, it re-dedicates itself to these functions in the service of humanity.

THE PASSING OF THE PRESENT PLAGUE PANDEMIC AND INDIA

SAHIB SINGH SOKHEY

THE present pandemic of plague which spread from Yunnan to Canton and to Hongkong in 1894 reached Bombay in 1896, and in a few years invaded the whole of India. It also spread to many other parts of the world like Australia, South Africa, North and South America, Egypt, several Mediterranean Ports, England and France. This pandemic is now in a quiescent state and is obviously on the way out. Now is just the time to take stock of the whole situation and see what the epidemiology of the pandemic has to teach us and what steps we must take to prevent re-occurrence of pandemics.

The historical records indicate that plague pandemics have a characteristic cyclical course. Starting from one or other of the endemic centres pandemics have spread over the world, raged with varying intensity for a century or more, caused great loss of life and came to an end of their own accord only to begin again after a lapse of relatively long periods of time.

Epidemiology

The oldest writings mention the occurrence of plague in different parts of the world, but no detailed accounts are available until the historical times. However, this much is certain that endemic centres of rodent plague have existed from time immemorial. Greenwood mentions five such centres: West Coast of Arabia, Uganda, Iraq, Central Asia and North India. From one or other of these centres pandemics of plague have started from time to time. Records show that before the present pandemic, Europe was visited by two severe pandemics, one in the sixth and the other in the fourteenth century. The first of these epidemics started in 542 A.D. in the reign of Justinian. Starting in Egypt it had spread along the coast of North Africa on one side, and along Syria and Palestine on the other, and ultimately affected the whole Roman Empire. The important point is that after raging for about 200 years and causing heavy loss of life it subsided on its own.

The second pandemic started about the year 1347. It was first noticed in Crimea, and is believed to have originated in Central Asia, reaching the North East either by way of China or India, or by both. It spread by numerous land routes to Asia Minor, North Africa and the continent of Europe. This pandemic lasted for over three centuries in Europe and caused great loss of life. In two years from 1347-1349 it is said to have killed more than half of the inhabitants of Western Europe. Hacker placed the total loss of life due to this pandemic in Europe at 25 million persons. We have not come across any detailed account of this pandemic in India, but there is no doubt that it raged in India also. *Akbarnamah* mentions a plague epidemic in Delhi during this period. The writer of this account particularly mentions the exodus of rats from the city during the epidemic. This incidentally is the first written account which connected a plague epidemic with rats.

The present pandemic which reached India in 1896 from Hongkong had in a few years invaded the whole of India. By 1904, deaths from plague in the country reached the high figure of 1,143,993 a year. The total recorded mortality due to plague in

from time to time.
the present pandemic, Europe was visited
by two severe pandemics, one in the sixth total recorded mortality

was resumed again in 1931 when we had succeeded in developing a method for preparing a constant Test Infective Dose to be given subcutaneously, constant both in numbers and virulence, 4,000 to 8,000 organisms of strain of *P. pestis* of which 5-10 organisms per white mouse (Haffkine Institute inbred) kill 100 per cent of the mice. Very interesting results have been obtained. In 1931 only 9·3 per cent of the Bombay house rats were found to be susceptible. The percentage of susceptibility diminished year by year: 8·5 per cent, 7·9 per cent, 6·5 per cent, 5·0 and 0·0 per cent, i.e., by 1936 all susceptible races of house rats had died out. This observation was further supported by the daily examination of rats caught in the city for presence of plague infection. About 200,000 rats are examined every year in the Institute. The number of infected rats found diminished year by year from 1931 to 1935 from 748 to 0, yearly figures being: 1931—748, 1932—600, 1933—393, 1934—34, 1935—0. Ever since 1936, no plague infected rats were found in the city till 1947, but a few infected rats were found in 1948, 1949 and 1952 (but their source of infection is not certain) and again none till this year. Because of the large number of rats examined for plague, it may be taken that plague epizootic among rats has almost come to an end. Under the conditions it is interesting to note that rats of susceptible species have again begun to increase from 0 per cent in 1936 to 15·5 per cent in 1942, and 15·0 per cent in 1943, 17·0 per cent in 1944, 26 per cent in 1950 and 10·8 per cent in 1957.

The same idea gets support from a study which covered almost the whole of India. About 50 towns and cities of the states of Bombay, Madras, United Provinces, Central Provinces, Punjab, Hyderabad and Travancore were selected to include places with high, medium and low aggregate death rates per mile from plague. Rats were caught at the selected places and were brought to Bombay and were tested for their susceptibility to plague infection, and it was found that the susceptibility of rats to plague infection was almost inversely proportional to the respective aggregate incidence of plague at the localities, i.e., localities where more serious epidemics had raged, a smaller percentage of rats susceptible to plague infection were found than in localities which had lighter epidemics.

These studies explain how the pandemics occur and disappear from planes and large centres of human habitations of vulnerable areas. However, even when pandemics have come to an end, slow smouldering epizootics may and do continue in some endemic centres and form the starting point of fresh pandemics.

Endemic Centres in India

The present pandemic wave in its march has left some stagnant pools in the form of endemic centres and it has been found that plague radiates from such endemic centres. The extent of the infected areas increases or decreases very gradually showing a cyclic tendency and the duration of the cycle varies in the different regions. Such endemic centres in India are confined to cool and moderately damp areas situated in the sub-mountain regions and high table lands varying in altitude from 2,000 to 4,000 feet. Plague spreads from them to the neighbouring planes. The extent of the areas invaded by plague depends upon the intensity with which it spreads and the momentum it attains during its march. These epidemics in the planes disappear during hot weather only recurring after a lapse of a year or two, so long as there are some susceptible species of rats still existing in that area.

It is a well-known fact now that plague is not a stationary disease, it moves from place to place. Even in an endemic centre, it is never confined to one definite locality; though it persists in certain adjacent areas having more or less identical climatic conditions. In such areas, its progress is very slow and it lingers for longer duration. Consequently, no particular locality can be designated as an endemic centre in the literal sense, but it is possible to define broad areas in which the plague persists always and moves about. Such an area has been defined and studied by the workers at the Haffkine Institute in Belgaum and

India is given as 12½ millions from 1896-1938. To this may be added another 235,286 from 1939-52. During recent years there has been a very marked decline in mortality amounting to no more than 1,841 in 1951 and 1,007 in 1952 and only 68 in 1956.

This pandemic spread to many parts of the world like Australia, South Africa, North and South America, Egypt and several Mediterranean Ports. Cases also occurred in British Isles, Glasgow, Cardiff, Liverpool and London. France was also involved. Though plague reached Europe as in previous pandemics, it is to be noted that, it did not take root there, claimed a few deaths and disappeared, while it raged wildly in China, India and Africa over a long period of time, and after reaching a peak intensity has been progressively declining. It is now apparent that the pandemic is on the way out.

It is important to understand why the present pandemic though it reached Europe did not take root there, while India, Africa and China proved as vulnerable as ever before. By the time the pandemic reached Europe, the cause of the disease had been found to be a bacillus, but nothing was known about its epidemiology. The result was that most of the steps taken by sanitary authorities in Europe to check the spread of the disease were not effective and Greenwood goes so far as to say "indeed the discovery (of the plague bacillus) may have done temporary harm, because people were set to track the dissemination of the germ and at first looked in all the wrong places". Thus, the failure of plague to take root in Europe cannot be explained by any preventive steps deliberately adopted. It, however, emerges that the most important single factor which seems to have been effective in preventing plague from spreading in Europe was that in the intervening period between the end of fourteenth century pandemic and the present one, Europe had experienced great material prosperity and this had led to great improvement in the housing conditions of the people. Better houses broke the association between rodents and human beings. While in India, China and Africa, housing conditions were just the same as in the times of the previous pandemics. Primitive housing made of unbaked mud bricks or wooden structures with thatched roofs continued the close association of rodents and man. But, of course, in addition to this factor the problem is complicated by the existence of permanent endemic centres in these areas.

We have shown above that when a pandemic reaches a vulnerable country, it develops intensity, then gradually declines and ultimately disappears. In India the stage of decline has been reached and the pandemic is on the way out. It reached its peak in 1904, with an annual recorded mortality of 1,143,993; then began a gradual decline and in 1936-38 there were only 18,759 deaths in the whole country. The factors which underlie this very interesting phenomenon have been studied at the Haffkine Institute, and it emerges that when plague invades a vulnerable area, the epizootic which starts among rats continues uninterruptedly with seasonal variations in intensity over a number of years, until all the susceptible races of rats die out, leaving behind only resistant races. While this epizootic is still going on, it gives rise to repeated epidemics in the area when suitable seasonal conditions, moderate temperature (about 27°C) prevail. In Bombay State, the necessary suitable seasonal conditions for epidemics exist in November to March. When epizootics have come to an end, no fresh epidemics take place. The same phenomenon is repeated in all the localities where the pandemic had spread, so that in course of time the pandemic comes to an end. But when the epizootics have come to an end, the susceptible species begin to multiply again and when they form a majority of rodents in course of century or two or more, the stage is set for a fresh pandemic to take root if it reaches the place either from abroad or from a local centre.

This phenomenon is beautifully illustrated by studies on the susceptibility of Bombay city rats from 1894 to the present day at the Haffkine Institute. The Indian Plague Commission in 1904 found 96 per cent of the Bombay house rats (*Rattus rattus rufescens*) susceptible to plague infection. This study

Dharwar districts and it includes the watersheds of the Western Ghats in these two districts. It probably extends into the Mysore State.

From the past records of plague in India, it is reasonable to surmise that there are three endemic centres situated in the sub-mountain region of the Himalayas. The first one includes the sub-mountain regions bordering Gujrat district, Jammu State, and Gurdaspur and Hoshiarpur districts, which has been quiescent since 1937. The second one includes the sub-mountain region bordering Dehra Dun, Saharanpur, Bijnor, Moradabad, Bareilly districts and Rampur State. Third one includes the sub-mountain regions bordering the districts of Basti and Gorakhpur in the United Provinces and districts of Champaran, Muzaffarpur, Dharbhanga and Bhagalpur in Bihar. All these three endemic centres may be parts of one big sub-Himalayan centre.

In the Central India plateau both the northern and southern watersheds contain one endemic centre, or probably more, from which plague radiates both northward and southward. This endemic centre includes the hilly regions of Saugar and Jubbulpore districts in the Bharner Range, Hoshangabad, Betul and Chindwara districts in the Mahadev Range and Mandla, Bilaspur and Balaghat districts in the Maikal Range. This endemic centre is still active.

In the Deccan Plateau there appear to be three such endemic centres. The first one includes the eastern watersheds of Western Ghats in the Belgaum and Dharwar districts and of Mysore Plateau. The second is situated in the watersheds of Balaghat Range and adjoining hills including the parts of the districts of Bhir, Osmanabad, Bidar, Gulburga, Madak, Aturf-i-Balda and Mahbubnagar. The third one includes the watersheds of Shevaroy, Nilgiris, Palni and Cardamom Hills found in the districts of Salem, Coimbatore and Madura. In view of absence of detailed information, it is not possible to say definitely whether these three endemic centres are distinct or parts of one big endemic centre.

Anti-Plague Measures

Though very few cases of plague are occurring now and the present pandemic may come to an end, plague still remains a very serious problem for India because its housing conditions over the large part of the country, the rural areas, are still as they were centuries ago, and are liable to become the centre of a severe infection if and when the next pandemic hits India. We now have a quiescent time almost free from plague, and now it is the time to take all the steps that are necessary to prevent any further pandemic from ever again taking root in India. We have now the means to achieve this objective with certainty. During the sixty years that the pandemic has lasted in our country, very valuable additions to our knowledge have been made. We have now a very comprehensive idea of the epidemiology of the disease, excellent agents for the destruction of fleas and rats, a very potent plague vaccine for increasing human resistance and drugs for successfully treating plague infection. We have also the time to build suitable housing for our people, as the most effective preventive measure. We simply must and can develop our resources to provide our people good houses. Our armamentarium is so complete that we can now say with a degree of certainty that this dreaded disease can be kept completely under control.

Suitable Housing.—In considering plague preventive measures the main thing that requires to be achieved is to break the intimate association between the rat and the man. For this purpose, the most important single measure which would have a permanent effect would be to build suitable houses, and what requires to be done is to draw up a long-term plan to provide better housing. This can be achieved by giving financial and technical assistance to the people. At the same time, organizations such as railways, district local boards, municipalities, port health and military authorities should be made to make their godowns rat-proof. This will have a very salutary effect by setting a good example for private individuals to copy. In this connection the efforts of the authorities in Java are worth mentioning.

They planned cheap rat-proof dwellings and popularized their use in the country by giving both financial and technical aid.

This particular point brings to our mind the fact that a great deal of confusion arises from lack of co-operation between the Public Health and the Public Works Departments. It is essential that there should be a close co-operation between them, and the engineers should be given some form of special training in rat-proof construction.

Determination and Localization of Endemic Centres.—Continuous research must be carried out to accurately define endemic areas such as we have described above where slow smouldering of epizootic goes on among rats for prolonged periods and anti-rat and flea measures must be carried out there continuously to exterminate them and prevent fresh pandemics from developing and taking root. Because during quiescent periods such centres are not necessarily associated with epidemics, they are likely to be ignored. But actually the quiescent period is just the time when full attention should be given to these endemic areas and the rat population exterminated and not allowed to multiply at all as far as possible.

Extermination of Rats and Fleas.—The provision of rat-proof dwellings obviously is a long-term measure, but a great deal can be done on a short-term basis. For this purpose, we give a place of prominence to the extermination of rats and fleas. During recent years, chemical insecticides, such as DDT, Gammexane preparations and calcium cyanide have placed in our hands very valuable weapons for the destruction of rats and fleas. The use of these agents with discretion and care can almost prevent any plague epidemic developing. On the whole DDT is easy to apply and it is relatively inexpensive. If it is periodically applied in proper formulations to the interior of the houses and to rat burrows, good results can be expected. Indian workers have now great deal of experience of the application of DDT and best formulations and suitable methods of application should be selected. However, calcium cyanide is still a very useful agent for applying to food godowns and particularly to keep down the rat population as a whole. The application of this agent is more difficult and has to be carried out with extreme care.

If we are to depend on any of these measures for their successful application, permanent organizations are needed which would study the epidemiology of plague carefully and adequately fumigate dwellings and other buildings before any case of human plague occurs. So far, these excellent measures have been employed very half-heartedly and inefficiently. It seems that conditions for their proper use do not exist at present, neither permanent organizations nor properly trained staff for the purpose. Local bodies still place reliance on old ineffective methods of rat extermination, such as trapping and poison baiting.

Plague Vaccine.—The Haffkine plague vaccine has played a decisive role in the fight against plague in India. The utility of the measure is so well recognised that it has become very popular. Though there has been a progressive decline in the incidence of plague in India, there has been a steady increase in the amount of vaccine used.

In recent years an accurate method for the bioassay of plague vaccine was developed at the Institute and the effect of every factor which goes into the making of the plague vaccine was studied. These studies have resulted in great improvements in the making of this vaccine in recent years, so that it is now much more potent antigenically than ever before and almost entirely atoxic. The great merit of this vaccine is that it suits the Indian conditions. The vaccine is almost a permanent product. It is not adversely affected by storage at room temperature for years and this is important, as the vaccine has to be distributed all over India and facilities for cold storage are none too plentiful if not altogether absent. Under these conditions this vaccine, even when stored for some years in a cool dark place at room temperature, does not deteriorate.

But we want to stress here that the authorities have tended to use vaccine as the sole preventive measure. It does not require to be stated, it is so obvious that the disease being

and the methods of calculation described above are then applied to the logarithms. The mean of the logarithms in each groups of results is calculated and the deviations from these means are squared and added. The sum of these squares is divided by $(n - 1)$ or $\Sigma (n - 1)$, and the result is an estimate of the ' logarithmic variance '. The square root of the logarithmic variance is the standard deviation of the logarithm of the result of a single test.

The calculation of the fiducial limits of error involves the quantity 't', which depends upon the number of degrees of freedom used to estimate 'S'. The appropriate value may be obtained from table I. When the deviations are expressed as percentages, the fiducial limits of error ($P = 0{\cdot}95$) are $(100 \pm ts)$ per cent; this means that the value will lie between $(100 + ts)$ and $(100 - ts)$ in 95 per cent cases. When the deviations are expressed as logarithms, the fiducial limits may be obtained as percentages by taking the antilogarithms of $(2 \pm ts)$.

* An example to find out the ' direct estimate of error ' is being given below;

Estimate of potency from individual assays	d	d^2
0·738	— 0·0758	0·00574
0·766	— 0·0478	0·00228
0·803	— 0·0108	0·00012
0·817	0·0032	0·00001
0·870	0·0562	0·00316
0·889	0·0752	0·00566
Total 4·883	–	0·01697

Mean 0·8138

$$S^2 = \frac{0{\cdot}01697}{5} = 0{\cdot}003394$$

$S = 0{\cdot}05826 = 7{\cdot}16$ per cent of mean

At $P = 0{\cdot}95$ with 5 degrees of freedom, $t = 2{\cdot}57$

Fiducial limits of error $= 100 \pm (2{\cdot}57)(7{\cdot}159)$
$= 100 \pm 18{\cdot}4$
$= 81{\cdot}6$ to $118{\cdot}4$ per cent.

These limits of error apply to any single estimate of potency from an individual assay. If the calculations are performed on the logarithms of the individual potencies, the fiducial limits of error obtained are 83·2 to 120·2 per cent.

Interpretation of Assay Results

There are three methods of interpreting the result of a biological assay.

Method 1. Effective dose is measured for each animal.

Method 2. Response of each animal is measured.

Method 3. Percentage of positive effects is measured.

Method 1, *Effective Dose is Measured for each Animal.*—The assay of prepared digitalis with guinea-pigs is an example of such a test. The extracts of the standard preparation and of the sample being tested are diluted with saline solution until the concentrations of ethyl alcohol are the same and do not exceed 10 per cent v/v. The diluted extract of the standard preparation is injected at a slow uniform rate into the jugular vein of a guinea-pig which has previously been anaesthetised with urethane and whose respiration is maintained artificially. The injection is continued until the heart is arrested. The amount of extract required to produce this effect is taken as the lethal dose. The experiment is repeated on other guinea-pigs of the same strain using litter mates where possible, and an average lethal dose per kg. of body weight is determined. The diluted extract of the sample also is tested in a similar manner using guinea-pigs of the same strain. The injection is given at a rate of 0·5 ml. per minute. The guinea-pigs should weigh between 200 and 600 gm., the weights of the heaviest and lightest animals should not differ by more than 100 gm., and the mean body weight of the animals receiving the sample being tested should not differ by more than 10 per cent from that of the animals receiving the standard preparation. The result can be calculated as follows;

* Example taken from British Pharmacopoeia 1958, p. 899.

primarily that of rats its course cannot be influenced by anything done to human beings. Though plague vaccine gives a high degree of protection and is a valid protective measure, it should not be made the first and the sole line of defence. The eradication of rats and fleas and better housing should be a major effort. In a disease like plague, epidemiology of which is so well understood, it is unjustifiable to make plague vaccine as the sole preventive measure.

Treatment of Plague.—In Streptomycin we have now almost a specific remedy against plague. The work done by the Haffkine Institute in experimental infection and in field trials have proved that Streptomycin, given early enough, can cure 100 per cent of the cases and even if streptomycin is given after blood septicaemia has developed, it can still cure about 90 per cent of the cases. Only about 4-5 g. of the drug are needed in a mild case. The drug is given in an initial dose of 2/3 g. followed by 1/3 g. every four hours until temperature remains normal for 1 or 2 days. The total period of treatment is 3 to 4 days. While in severe cases 6-12 g. of the drug are needed. The drug is given in doses of 2/3 g. every four hours till the temperature remains normal for 2 to 3 days—total period of treatment being 5 to 6 days. Thus, in Streptomycin we have the ideal drug of choice for the treatment of plague.

Organization of Control

The techniques of plague control discussed in the preceding section have reached such a high degree of effectiveness that from the technical point of view the success of anti-plague campaign can now be guaranteed on the sole condition that the campaign is properly organized. The main problem, then, is mainly one of a proper organisation for the carrying out of control measures.

Now that plague epidemics are declining, it permits us to calmly plan and carry through with great deliberation long-term measures which would prevent plague from taking root ever again in India. From the very nature of the infection it cannot be regarded as a disease of importance only to the area actually invaded, at a particular time; it moves from place to place and rapidly invades large areas. The matter cannot be left to the various states. Therefore, the only effective way of organising control is to create a Central Organization directing and controlling the activities of the health authorities in the plague infected areas. Extermination of plague cannot be expected unless anti-plague measures of the whole country are planned and systematically carried out for a number of years in all the areas showing plague infection. If its total extermination is not planned, as an integrated effort, there is always a chance of its reappearing here and there. Even during this quiescent period plague is tenaciously clinging to sub-Himalayan regions, Central India, Deccan and Mysore plateau, and may be a serious danger for some time, unless it is systematically eradicated. Therefore, centrally planned measures should be adopted to attempt first to define these endemic centres and then continuously treat them year after year to see that they are not harbouring any infection.

We definitely suggest that a Central Organization should be formed to direct and control anti-plague measures all over India. This organization should have a department of epidemiology and research and an executive department with extensive trained personnel for carrying out anti-plague measures. The department of epidemiology and research should have a section for each of the endemic centres for studying the progress of plague with a view to forecasting of epidemics and to planning anti-plague measures. The executive department will loan its personnel to State Department of Health for actually carrying out the anti-plague measures. This loaned personnel will act in the closest co-operation with the local health organizations and the central body. We are aware that a great deal of ingenuity would be needed in planning a scheme of centralization acceptable to the states and capable of functioning smoothly. We are, therefore, limiting ourselves to the bare suggestion that a Central Organization is imperative to deal with plague.

STATISTICAL ASPECTS OF BIOLOGICAL STANDARDISATION

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BIOLOGICAL methods of assay are used to detect and measure the concentration of substances by observing their pharmacological effects on living animals or tissues. These methods are generally sensitive and reliable, but are more difficult and less accurate than physical and chemical methods. If sensitive physical or chemical methods of assay are available or can be devised, these could be used in most instances. In fact, many biological methods of assay of hormones and vitamins have been replaced in recent times by physical or chemical methods.

The fundamental basis of all biological assays is the comparison of an unknown sample with a standard preparation of the same substance (pure or very nearly pure) to see how much stronger or how much weaker it is in relation to the standard. Dry and stable standard preparations of various drugs including biological products are now available which bring the estimation of biological properties into the same satisfactory position as the measurement of ordinary length and height of individuals in any given population or the weight of solid substances. A unit of a standard preparation is defined as a definite weight of the substance. When a biological estimation becomes a comparison between two preparations, the method used for the comparison is not of much importance. We get the same potency of a sample whatever method of assay is used. But in some cases *e.g.* in biological assay of digitalis leaf we may find different potencies by different methods. This is because different samples of leaf contain different proportions of several active principles. The relative toxicity of these active principles differs for different species of animals. So the results of comparisons by different methods will not be the same in this case.

Estimates of Error in ' Official ' Methods of Assays

The methods of biological assay recommended by a particular Pharmacopoeia are satisfactory. But these methods may be replaced by other methods if it can be shown that such methods are at least equally accurate and provide a measurement of the same active principles. Calculations of error should be made from the results of each assay even if the official method of assay is used. Methods for the design of assays and the calculation of their errors are, therefore, described below.

The estimates given in some of the assays of the errors of the official tests are of two kinds *viz.* direct or external estimate of error and indirect or internal estimate of error. It is better to use the term ' variance ' instead of ' error '. Internal estimate of the variance is based on the data of a single experiment. The external estimate or direct estimate of the variance, is possible only when there is a sufficient number of repetitions of the experiment under similar conditions.

Each lethal dose is converted to a logarithm. The mean of the logarithms in each group is calculated, the deviations of the individual logarithms from these means are squared and added, and the standard deviation(S) is calculated from the formula $S^2 = \frac{\Sigma d^2}{\Sigma(n-1)}$ where Σd^2 is the sum of the squares of all the observed deviations and $\Sigma(n-1)$ is the sum of two values of $(n-1)$, and is equal to the number of degrees of freedom. In this case the number of degrees of freedom means the number of animals in a group minus one. The result of the test depends upon the difference (M) between these two means. The ratio of the potencies of the solutions compared is the antilogarithm of M. The variance of M is the sum of the variances of the two means and is calculated from the formula $S_M^2 = S^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)$ where n_1 and n_2 are the numbers of animals in each group. The fiducial limits of error of the test can be calculated by the methods given above. The number of degrees of freedom is $(n_1 + n_2 - 2)$. The following example will give a clear idea of the position explained above.

Method 2, *Response of each Animal is Measured.*—In this method of assay the effects of the drugs on individual biological systems, *e.g.* a whole animal, isolated animal tissue, or a culture of bacteria, are determined. These effects may be an increase in weight of animals or organs or a contraction of tissues. Many of the vitamins and hormones like cortisone and posterior pituitary extracts can be assayed by this method. In order to do a biological assay of a particular drug with this method, the following conditions must be fulfilled.

(*a*) The relation between the logarithm of the dose and the effect can be represented by a straight line with sufficient accuracy over the range of doses used when two similar preparations are compared, and the two lines should run parallel to each other.

(*b*) The effects of any one dose are normally distributed.

(*c*) The standard deviation of the effect is independent of the effect itself.

Detailed Calculation of Digitalis Assay on Guinea-pigs—*

	Estimation of individual lethal dose (ml. per kg).	Log	d	d^2
Standard Preparation	1·12	0·0492	−0·0284	0·000807
	1·44	0·1584	0·0808	0·006529
	1·06	0·0253	−0·0523	0·002735
Total		0·2329		0·010071
Mean		0·0776		
Test Preparation	1·24	0·0934	0·0045	0·000020
	1·38	0·1399	0·0510	0·002601
	1·08	0·0334	−0·0555	0·003080
Total		0·2667		0·005701
Mean		0·0889		

$S^2 = \frac{0{\cdot}010071 + 0{\cdot}005701}{(3-1)+(3-1)} = \frac{0{\cdot}015772}{4} = 0{\cdot}003943$

$M = 0{\cdot}0776 - 0{\cdot}0889 = -0{\cdot}0113 = \bar{1}.9887$; Potency ratio is the antilog. $\bar{1}.9887 = 0{\cdot}97$

$S_M^2 = 0{\cdot}003943\,(\frac{1}{3} + \frac{1}{3}) = 0{\cdot}002629$

$S_M = 0{\cdot}0513$

At P=0·95 with 4 degrees of freedom, $t = 2{\cdot}78$

Fiducial limits of error (Percentage) = antilog $(2 + 2{\cdot}78 \times 0{\cdot}0513)$ per cent
= antilog $(2 + 0{\cdot}1426)$ per cent
= antilog $1{\cdot}8574$ to antilog $2{\cdot}1426$ per cent
= 72·0 to 138·9 per cent

* Example taken from British Pharmacopoeia 1958, p. 900.

Normally these should not differ very much. But sometimes it is found that the external or 'direct' estimate is appreciably more than the internal estimate. This is found in some cases of quantal assays and is due to the fact, that the direct estimate of the variance, includes also the variance due to any change in conditions from experiment to experiment, which may be either undetectable or uncontrollable. (The internal variance is due to experimental error, small number of animals used, etc.) A direct estimate, if it is possible—*i.e.* if there is a sufficient number of repetitions of experiment to give a reliable estimate, is therefore always preferable to an internal estimate. For, the direct estimate of the variance represents the actual variation obtained in practice better than the internal estimate of variance. Where the error of each assay can be calculated from internal evidence, this calculation forms an essential part of the experiment and the Pharmacopoeia prescribes limits within which the result should lie. Where a number of assays are made with a particular preparation under similar condition, the error can be calculated by this direct method. Estimates of error are themselves subject to appreciable error unless they are based on a very large number of observations. That is why in the calculation of fiducial limits of error some allowance is made for this fact. Methods for calculating the fiducial limits of error ($P = 0{\cdot}95$) are given below, the limits of error being expressed as percentages of the stated potency of the substance.

When all the results of the assay are estimates of the potency of the same preparation and the errors are small, the deviation (d) of each result is calculated by subtracting from it the mean of all the results. An estimate of the standard deviation (S) is then obtained from formula $S^2 = \frac{\Sigma d^2}{(n-1)}$ where n is the number of results and Σd^2 is the sum of the squares of all values of d. When calculated in this way, S is in the same units as the result of the assay. It can be converted into a percentage if necessary. The quantity S^2 is known as the 'variance', and its reliability depends on the number of degrees of freedom used, which in this case is $(n-1)$. Estimates of error by this method are unreliable unless at least twelve assays have been made with the same preparation. When some of the errors are likely to exceed by 10 per cent, it is generally preferable to use logarithm, which will give a more accurate estimate of the error. For this purpose, the result of each individual test is converted into a logarithm,

Table I.

Values of t ($P=0{\cdot}95$)

Degrees of freedom	t	Degrees of freedom	t
1	12·71	11	2·20
2	4·30	12	2·18
3	3·18	15	2·13
4	2·78	20	2·09
5	2·57	25	2·06
6	2·45	30	2·04
7	2·37	40	2·02
8	2·31	60	2·00
9	2·26	120	1·98
10	2·23	Infinity	1·96

(*d*) The allocation of individuals to the different experimental groups is made by some random process such as the toss of a coin, or the use of a table of random numbers. If, when whole animals are used, factors such as weight and sex are balanced or litter mates are evenly distributed between the groups, the estimate of potency is valid, but the error is likely to be over-estimated by the methods of calculation described here. In all cases the animals used should be as uniform as possible in age, weight, and sex. They should be kept under uniform conditions both before and during the experiment.*

There are many experimental designs by which we can perform assays by this method. The result of the assay depends upon comparison between the test preparation and a standard preparation. If the effects of a series of doses of each of these preparations are plotted vertically against the logarithm of the doses, it will be possible to fit the data with two parallel straight lines, the horizontal distance between these lines providing the result of the assay or potency of the test preparation. Special tests can be applied in each experiment to discover whether the slopes of the lines differ significantly from one another. If these do, then either condition (a) is not fulfilled or there is some qualitative difference between the standard and test preparations. When more than 2 doses are used, the linearity of response itself can be tested statistically.

The simplest possible design is 1 test × 1 standard dose design. Since with one dose the slope of the line cannot be determined, the potency of the test cannot be found or even if it is determined, the error of the estimate cannot be determined, unless we make some assumption regarding the slope from previous experience. This is generally considered to be an unjustifiable assumption. With two doses of either preparations, this assumption need not be made; that is, the slope is determined from the experiment itself. With more complicated designs, the assumptions regarding linearity and parallelism also can be tested statistically from the experiment. However, since the Pharmacopoeia provides various safeguards this is not always essential. Regarding condition (b) + (c) these assumptions are generally justified except in very extraordinary cases:—

	Designs	*Scope of design*
(1)	$1T \times 2S$	Linearity and parallelism assumed and slope determined from Standard.
	$2T \times 1S$	Linearity and parallelism assumed but slope is determined from Test preparation.
(2)	$2T \times 2S$	Linearity assumed; parallelism can be tested.
(3)	$1T \times 3S$	Linearity of Standard can be tested. Linearity and parallelism of Test are inferred if Standard is linear.
	$3T \times 1S$	Linearity of Test can be tested. Linearity and parallelism of Standard are inferred if Test is linear.
(4)	$2T \times 3S$	Linearity of Standard and parallelism can be tested. Therefore only linearity of Test is assumed.
	$3T \times 2S$	Linearity of Test and parallelism can be tested. Therefore only linearity of Standard is assumed.
(5)	$3T \times 3S$	Linearity can be tested for both the Standard and Test preparations and also parallelism.

Where the number of doses for standard and that for test differ, whether the larger number should apply to the standard or the test may be determined by convenience—that is availability or cost of the drug, or the

* It is not possible to verify the fulfilment of the first three conditions in every experiment, and it is generally sufficient to assume it, provided that previous experiments have made it likely that condition (*a*) will be fulfilled. When the effect as initially measured does not fulfil these conditions, it may be replaced by some other quantity calculated from it (such as its square or logarithm) which does fulfil them. If this is not possible, the methods of calculation described here are not reliable.

amount of confidence in the standard or test preparation.

In a (2S × 2T) dose assay, the animals are divided at random into four equal groups. Two groups receive two different doses of the standard preparation and the other two groups receive two different doses of the test preparation. The doses are so chosen that the relation of the large dose to the small dose is the same for the standard and the test preparation and that the effect of each dose is expected to be equal to that of the corresponding dose of the other preparation. The result can be calculated by means of *Table* 2. In this table, S_2 and T_2 are the mean effects of the large doses of the standard and test preparation; S_1 and T_1 are the mean effects of the small doses. The result of the test is calculated from M, which is the logarithm of the potency ratio (T/S), provided that the doses of the standard and test preparation are identical, weight for weight. Otherwise the ratio should be adjusted accordingly.

Assay of Posterior Pituitary Extract.—The method of doing biological assay of Posterior Pituitary Extract by a $(2S \times 2T)$ dose assay will be described here:

A female rat in dioestrus, weighing 120 to 200 gm., is killed and one horn of the *uterus is suspended in a bath containing a* special solution. The bath is maintained at a temperature of 32°C, at this temperature spontaneous contractions are abolished and the preparation maintains its sensitivity. The solution is oxygenated with a mixture *of 95 per cent of oxygen and 5 per cent of* carbon dioxide, and the contractions of the muscle are recorded on a kymograph using an isotonic and linear lever. The contractions produced by the addition to the bath of two doses of the standard preparation suitably diluted with the above solution are recorded. The doses should be such as to produce clearly discriminated submaximal contractions. When the contractions are complete, the solution in the bath is replaced by fresh solution to relax the muscle. The preparation being tested is diluted so as to obtain responses on the addition of the two doses similar to those obtained with the standard preparation. The ratio between the two doses of the preparation being tested should be the same as that between the two doses of the standard preparation. The two doses of standard preparation and the two doses of the preparation

Table 2.

Mean effects		Effect difference due to		
		E (Dose)	F (Preparation)	G (Slope difference)
T_1T_2	S_1S_2	$\frac{1}{2}(T_2-T_1+S_2-S_1)$	$\frac{1}{2}(T_1+T_2-S_1-S_2)$	$T_2-T_1-S_2+S_1$
Twin cross over		$\frac{1}{4}(Y_1-Y_2-Y_3+Y_4)$	$\frac{1}{4}(Y_1+Y_2+Y_3+Y_4)$	....

Log ratio of doses $=I$; Slope $= b = \tan a = \frac{E}{I}$; Log potency ratio $(T/S) = M = F/b$.

No. of doses	Variances			
	V (Variance of each mean)	$V(E)$	$V(F)=A$	$V(G)$
* 2 × 2	$S^2/n=V$	V	V	$4V$
Twin cross over	$S^2/2n$	$V/2$	$V/2$	....

$B = V(b) = V(E)/I^2$

* It is assumed here that "n" (number of animals) is the same for all groups. If the numbers are only slightly different, the average number can be taken to be "n". If the numbers differ appreciably, the above formulae cannot be applied. More complicated formulae will have to be used.

under test should be given in a random order, and at least four responses to each should be recorded. The doses should be added at regular intervals of three to five minutes depending upon the rate of recovery of the muscle.

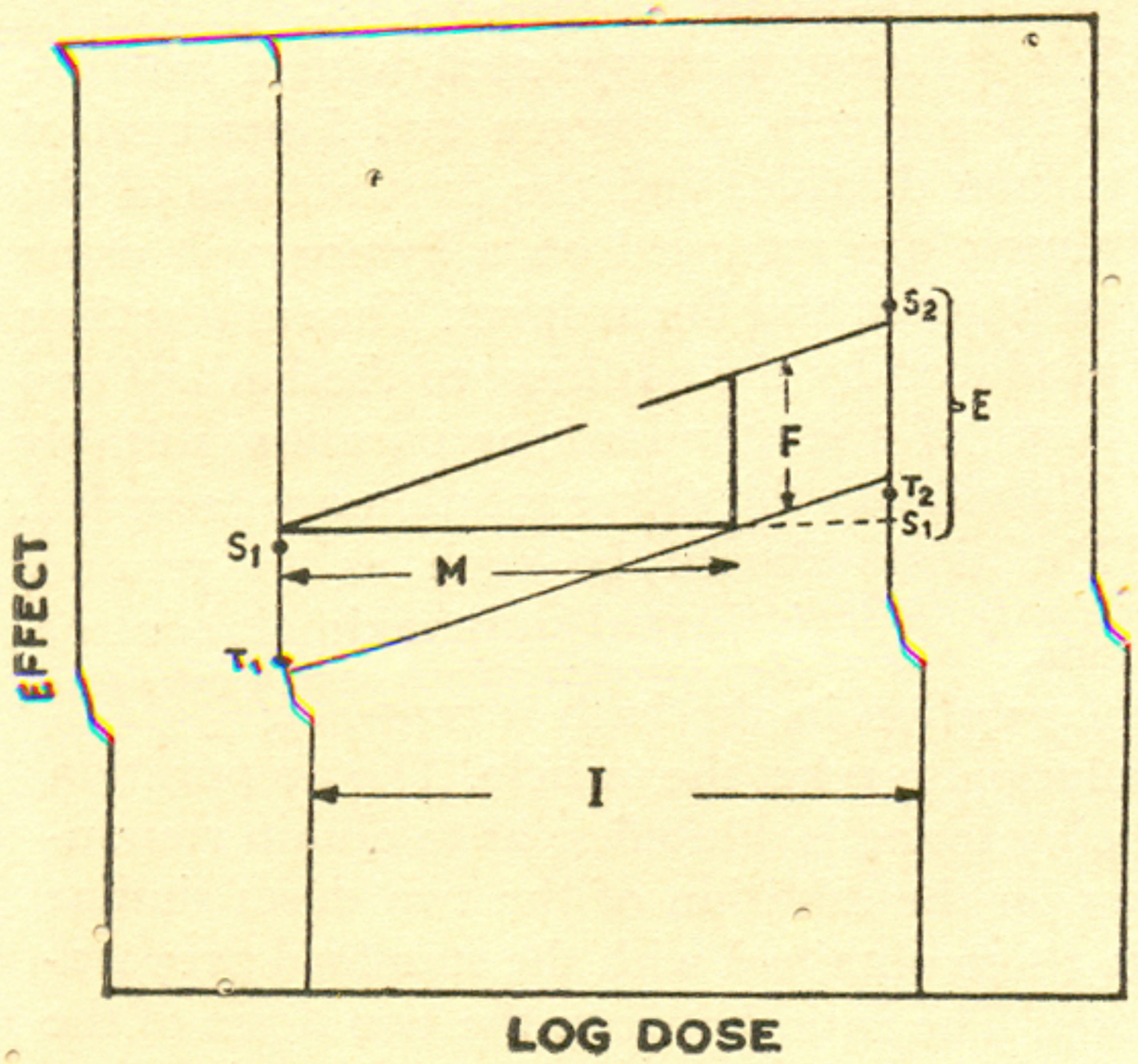

Fig. 1 Theoretical log-dose effect lines to illustrate the calculation of the result of a (2S and 2T) dose assay.

Index of significance of $b = g = bt^2/b^2$

The fiducial limits of the estimate of the potency of the test preparation as a percentage of the estimate itself are given by:

Log (fiducial limits per cent)

$$= 2 \pm \frac{gM}{1-g} \pm \frac{t}{b(1-g)} \sqrt{A(1-g)+BM^2}$$

When g is less than 0·1, it can be neglected and the equation becomes:

Log (fiducial limits per cent) $= 2 \pm \frac{t}{b} \sqrt{A + BM^2}$

When g *is greater than* 1, *the result is valueless.*

The significance of the difference between the two estimates of slope is tested by calculating $G/\sqrt{V(G)}$. If this quantity is greater than the appropriate value of t (*Table* 1), the slopes are significantly different. "Degrees of freedom" for "t" is equal to the number of degrees of freedom for the estimation of S^2.

$S_1 = 19 \quad S_2 = 22{\cdot}7 \quad T_1 = 16 \quad T_2 = 20{\cdot}1$

$I = \log 2 = 0{\cdot}3010$; $E = 3{\cdot}9$; $F = -2{\cdot}8$

$b = 12{\cdot}96$

$M = -0{\cdot}2160 = \bar{1}{\cdot}7840$; Potency ratio is the antilog of $\bar{1}{\cdot}7840$

$$S^2 = \frac{\Sigma d^2}{\Sigma (n-1)} = \frac{45}{12} = 3{\cdot}75$$

$$V = \frac{3{\cdot}75}{4} = 0{\cdot}9375$$

At $P = 0{\cdot}95$ with 12 degrees of freedom, $t = 2{\cdot}18$

$$\frac{G}{\sqrt{V(G)}} = \frac{20{\cdot}1 - 16 - 22{\cdot}7 + 19}{2\sqrt{0{\cdot}9375}} = 0{\cdot}21,$$

which is less than 2·18; therefore the slopes are not significantly different.

$$A = V = 0{\cdot}9375; \quad \frac{B = 0{\cdot}9375}{0{\cdot}3012} = 10{\cdot}35$$

$$g = \frac{10{\cdot}35 \times (2{\cdot}18)^2}{12{\cdot}96^2} = 0{\cdot}293$$

(2 and 2) Dose Assay of Posterior Pituitary Extract.

Response to dose of 0·2 ml. Standard	d	Response to dose of 0·4 ml. Standard	d	Response to dose of 0·2 ml. Test	d	Response to dose of 0·4 ml. Test	d
19·0	0	25·0	−2·3	16·0	0	20·5	−0·4
19·1	−0·1	19·0	3·7	15·5	−0·5	22·0	−1·9
18·5	0·5	21·9	0·8	14·0	2·0	18·0	2·1
19·5	−0·5	25·0	−2·3	18·5	−2·5	19·8	0·3
Mean 19·0		22·7		16·0		20·1	

Table 3.

Arrangement of Doses in Twin Cross-over Test.

	Group of animals			
	1	2	3	4
First part of the test	S_1	S_2	T_1	T_2
Second part of the test	T_2	T_1	S_2	S_1
Mean effect difference $(T-S)$	Υ_1	Υ_2	Υ_3	Υ_4

Log (fiducial limits per cent) $= 2 - \frac{0\cdot063288}{1-0\cdot293} \pm \frac{2\cdot18}{12\cdot96\,(1-\cdot293)} \sqrt{1\cdot1457021}$

$= 2 - 0\cdot0895 \pm 0\cdot2379 \times 1\cdot0704$

$= 1\cdot9105 \pm 0\cdot2546$

$= 1\cdot6559$ to $2\cdot1651$

Antilogarithm of $1\cdot6559$ and $2\cdot1651$ are $45\cdot3$ and $146\cdot2$ respectively.

Fiducial limits of error $= 45\cdot3$ per cent to $146\cdot2$ per cent.

Twin Cross-over Test in Insulin Assay

When the effect of a drug can be measured and observed more than once in the same animal, the experimental design known as a '*twin cross-over test*' is used. The advantage of this arrangement is that it diminishes the error due to the difference between one animal and another, since both drugs are tested on the same animals. It also balances the effect of any difference between the general levels of responses at the two stages of the test. The assay of insulin on rabbits is an example of this. The statistical background of this assay is given below:

The experiment is divided into two parts separated by a sufficient time interval. The animals are divided into four groups and each animal is used twice. Each part of the test may be regarded as a (2 and 2) dose assay carried out as described above, but the experiment is so arranged that the animals which receive the standard drug in one part of the test receive the test drug in the other, and the animals which receive smaller doses in one part of the test receive larger doses in the other. The arrangement of doses is shown in *Table* 3, where S_1 and T_1 refer to the smaller doses of the two preparations and S_2 and T_2 refer to the larger doses. Initial blood sugar in each animal is estimated and then after injecting insulin, five blood sugar estimations are made. The average fall during 5 hours is expressed as a percentage of the initial value.

The effect difference (Υ) is calculated for each animal separately by subtracting the effect of the standard preparation from the effect of the test preparation. The calculations depend entirely on these effect differences and are thus largely independent of the differences between one animal and another. The result of the assay and its error may be calculated by means of *Table* 2, where Υ_1, Υ_2, Υ_3 and Υ_4 are the mean effect differences in the four groups. S^2 is calculated by the formula $S^2 = \frac{\Sigma\, d^2}{\Sigma\,(n-1)}$ from the variations among the effect differences within the four groups of animals; the variance (V) is obtained as in *Table* 2, where n is the average number of animals per group.

Method 3 Percentage of Positive Effects is Measured (Quantal assay).—When the response is measured by the percentage of animals in which the effect is seen, the log dose — response curve is generally sigmoid shaped, which is well approximated by the Normal Distribution Curve. With a percentage response a linear relation is obtained by converting the percentage response into the deviation on the normal frequency curve to which the percentage is equivalent, so that instead of the percentage we take the normal equivalent deviation. In fig. 2, is shown a normal frequency curve and a percentage response curve (dotted).

*Twin Cross-over Test (Insulin Assay)**

Animal	S^2 *1st Day*	T_1 *2nd Day*	$Y = T - S$	d
1	38·2	26·1	— 12·1	1·20
2	52·6	33·8	— 18·8	— 5·50
3	38·4	29·4	— 9·0	4·30
			— 39·9	

$Y_2 = -13{\cdot}3000$

Animal	S_1 *1st Day*	T_2 *2nd Day*		
4	46·9	52·1	5·2	5·33
5	47·9	46·1	— 1·8	— 1·67
6	37·5	33·7	— 3·8	— 3·67
			— 0·4	

$Y_1 = -0{\cdot}1333$

Animal	S_1 *2nd Day*	T_2 *1st Day*		
7	22·3	33·8	11·5	— 4·43
8	24·9	40·1	15·2	— 0·73
9	17·8	38·9	21·1	5·17
			47·8	

$Y_4 = 15{\cdot}9333$

Animal	S_2 *2nd Day*	T_1 *1st Day*		
10	Rabit Convulsed	—	—	—
11	24·5	25·9	1·4	0·60
12	42·6	42·8	0·2	— 0·60
			1·6	

$E = 7{\cdot}075$; $F = 0{\cdot}825$; $I = \log 2 = 0{\cdot}3010$; $b = 23{\cdot}50$; $M = 0\ {\cdot}0351$; Potency ratio $= 1{\cdot}084$

$$S^2 = \frac{142{\cdot}45}{7} = 20{\cdot}35$$

Mean number of animals per group $= \frac{3+3+3+2}{4} = 2{\cdot}75$; $V = \frac{20{\cdot}35}{5{\cdot}5} = 3{\cdot}70$

t with 7 degrees of freedom $= 2{\cdot}365$; $t^2 = 5{\cdot}593$

$A = 1{\cdot}85$; $B = 20{\cdot}42$; $g = 0{\cdot}207$

Log (fiducial limits per cent) =

$$2 + \frac{(0{\cdot}207)\ (0{\cdot}0351)}{0{\cdot}793} \pm \frac{2{\cdot}365}{(23{\cdot}50)\ (0{\cdot}793)} \sqrt{(1{\cdot}85)\ (0{\cdot}793) + (20{\cdot}42)\ (0{\cdot}001232)}$$

$$= 2 + 0{\cdot}0092 \pm 0{\cdot}1269 \sqrt{1{\cdot}467 + 0{\cdot}025}$$

$$= 2{\cdot}0092 \pm 0{\cdot}1269 \sqrt{1{\cdot}492}$$

$$= 2{\cdot}0092 \pm (0{\cdot}1269)\ (1{\cdot}222)$$

$= 1{\cdot}8542$ to $2{\cdot}1642$

antilog of $1{\cdot}8542$ is $71{\cdot}5$ and of $2{\cdot}1642$ is $145{\cdot}9$

Fiducial limits of error = 71·5 to 145·9 per cent.

* Example taken from British Pharmacopoeia 1958, p. 906.

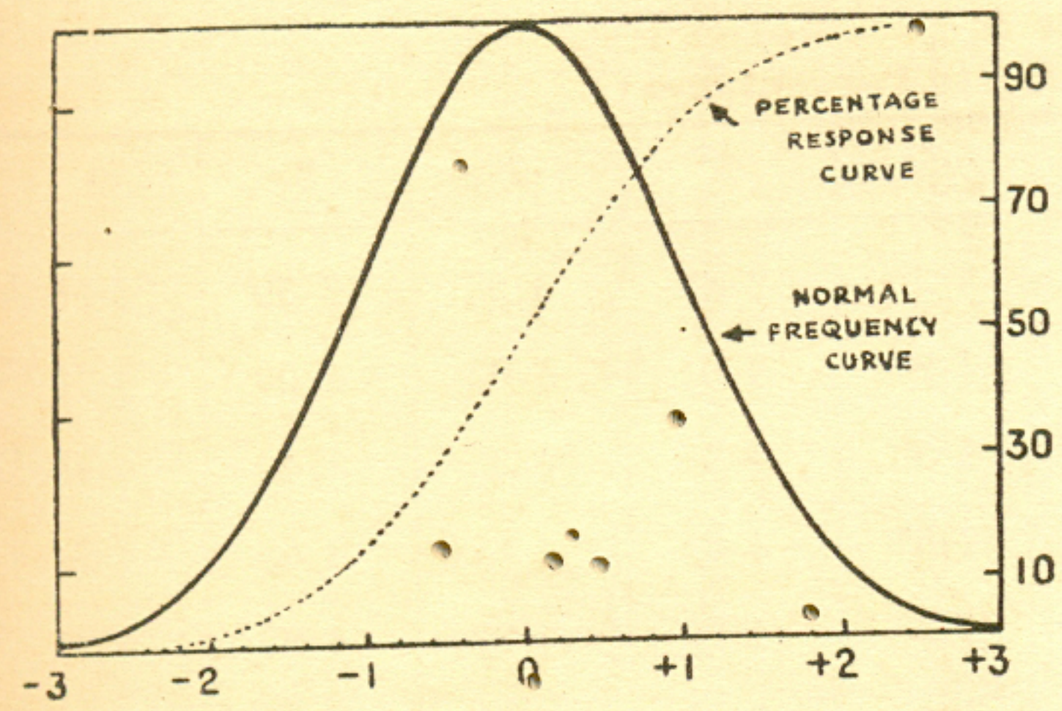

Fig. 2 To show the relation between the percentage response and the normal equivalent deviation.

The *abscissae* of the normal curve are stated in terms of the standard deviation (σ) so that the mean is *O* in the abscissa. Since the deviation of the mean from the mean is *O*, points to the left of the *O*, are negative and those to the right are positive. Now the ordinate 50 per cent on the percentage-response curve corresponds to the mean on the normal curve. Hence we may convert a 50 per cent response to the value *O* which is the deviation on the normal curve to which the percentage is equivalent. Perpendiculars through the points (mean + σ) and (mean − σ) enclose about $\frac{2}{3}$ or, 66·6 per cent of the area of the normal curve. Hence about $\frac{1}{3}$ or, 33·3 per cent of the area is outside these perpendiculars; since it is symmetrically disposed in two parts, the area on the left of the (mean − σ) perpendicular is about 16·6 per cent and that on the right of the (mean + σ) perpendicular is also about 16·6 per cent. Actually a percentage response of 15·9 corresponds to a deviation of − 1 on the normal curve. Similarly, a percentage response of 84·1 corresponds to a deviation of + 1. The ordinates on the normal curve through the abscissa − 2·0 and + 2·0 enclose between them about 95·5 per cent of the total area of the curve, and leave outside them about 4·5 per cent. Thus to the left of the ordinate through − 2·0 is an area corresponding to about 2·25 per cent of the total curve. Actually, the ordinate 2·27 per cent on the percentage-response curve corresponds to the abscissa − 2·0. Similarly, the ordinate 97·72 per cent corresponds to the abscissa + 2·0. The points on the percentage response curve which correspond to points on the abscissa of the frequency curve are given in *Table* 4.

Table 4.

Percentage response curve	Normal equivalent deviation	Probit
2·28 per cent	−2·0	3·0
15·9 ,,	−1·0	4·0
50·0 ,,	−0	5·0
84·1 ,,	+1·0	6·0
97·72 ,,	+2·0	7·0

In order to avoid the negative values, the values for normal equivalent deviation are to be added to 5·0, and then called Probits. Assay of insulin by observing the percentage of mice affected with hypoglycaemic symptoms is done by this method. The method of doing this assay is given below.

In a (2 and 2) dose assay 80 mice are taken and divided into 4 equal groups. Two different doses of the standard preparation of insulin are injected subcutaneously into two different groups of mice. Similarly two different doses of the test preparation of insulin are injected into two other groups of mice. Two different doses of the standard and the test preparations should be the same. The percentage of mice affected with convulsion is then converted by means of *Table* 5 into a probit, which is then taken as a measure of the effect. Difficulties arise if this percentage is 0 or 100 and doses should if possible be chosen to avoid this. If one of the doses is a (2 and 2) dose, assay causing 0 or 100 per cent of responses, the experiment may be interpreted as if it had been originally planned as a (2 and 1) dose assay. The most accurate method of calculating the results of such tests is complicated, but the following method will generally be accurate enough. The calculations are carried out by means of *Table* 2, using the probit in place of the measured effect. The quantity V is taken

Table 5.
(Probits corresponding to Percentages)

	0	1	2	3	4	5	6	7	8	9
0	..	2·67	2·95	3·12	3·25	3·36	3·45	3·52	3·59	3·66
10	3·72	3·77	3·82	3·87	3·92	3·96	4·01	4·05	4·08	4·12
20	4·16	4·19	4·23	4·26	4·29	4·33	4·36	4·39	4·42	4·45
30	4·48	4·50	4·53	4·56	4·59	4·61	4·64	4·67	4·69	4·72
40	4·75	4·77	4·80	4·82	4·85	4·87	4·90	4·92	4·95	4·97
50	5·00	5·03	5·05	5·08	5·10	5·13	5·15	5·18	5·20	5·23
60	5·25	5·28	5·31	5·33	5·36	5·39	5·41	5·44	5·47	5·50
70	5·52	5·55	5·58	5·61	5·64	5·67	5·71	5·74	5·77	5·81
80	5·84	5·88	5·92	5·95	5·99	6·04	6·08	6·13	6·18	6·23
90	6·28	6·34	6·41	6·48	6·55	6·64	6·75	6·88	7·05	7·33
	0·0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9
99	7·33	7·37	7·41	7·46	7·51	7·58	7·65	7·75	7·88	8·09

as equal to the reciprocal of the mean of the four values of wn, where w is the weight factor corresponding to the observed percentages, as obtained from *Table* 6. The number of degrees of freedom corresponding to t in the calculations based on *Table* 1, or in the test for differences between slopes, is infinite.

Table 6.
(Weight Factors corresponding to Probits)

	1	2	3	4	
0·0	0·001	0·015	0·131	0·439	
0·1	0·001	0·019	0·154	0·471	0·9
0·2	0·001	0·025	0·180	0·503	0·8
0·3	0·002	0·031	0·208	0·532	0·7
0·4	0·002	0·040	0·238	0·558	0·6
0·5	0·003	0·050	0·269	0·581	0·5
0·6	0·005	0·062	0·302	0·601	0·4
0·7	0·006	0·076	0·336	0·616	0·3
0·8	0·008	0·092	0·370	0·627	0·2
0·9	0·011	0·110	0·405	0·634	0·1
....	0·015	0·131	0·439	0·637	0·0
	8	7	6	5	

(2 and 2) dose assay of Insulin using mice*

Dose	Convulsions out of 20	Convulsions per cent	Probit	n	w	wn
S_1 0·2 ml. *S.*	4	20	4·16	20	0·490	9·80
S_2 0·3 ml. *S.*	13	65	5·39	20	0·602	12·04
T_1 0·2 ml. *T.*	6	30	4·48	20	0·576	11·52
T_2 0·3 ml. *T.*	14	70	5·52	20	0·576	11·52
						44·88

$$V = 1 \Big/ \frac{44 \cdot 88}{4} = 0 \cdot 0891$$

$I = \log 1 \cdot 5 = 0 \cdot 176$; $E = \frac{1}{2}(5 \cdot 52 - 4 \cdot 48 + 5 \cdot 39 - 4 \cdot 16) = 1 \cdot 135$.

$$b = \frac{1 \cdot 135}{0 \cdot 176} = 6 \cdot 45; \quad F = \tfrac{1}{2}(5 \cdot 52 + 4 \cdot 48 - 5 \cdot 39 - 4 \cdot 16) = 0 \cdot 225$$

$M = \dfrac{0 \cdot 225}{6 \cdot 45} = 0 \cdot 0349$; Potency ratio is the antilog of $0 \cdot 0349 = 1 \cdot 08$

$$A = 0 \cdot 0891; \quad B = \frac{0 \cdot 0891}{0 \cdot 0310} = 2 \cdot 87$$

t with infinite degrees of freedom $= 1 \cdot 96$

$G = -0 \cdot 19$; $V(G) = 4\,(0 \cdot 0891) = 0 \cdot 3564$

$G/\sqrt{V(G)}$ (ignoring sign) $= \dfrac{0 \cdot 19}{0 \cdot 597} = 0 \cdot 32$, which is less than $1 \cdot 96$

Therefore the slopes are not significantly different.

$$g = \frac{(2 \cdot 87)\,(3 \cdot 84)}{41 \cdot 60} = 0 \cdot 265$$

Log (fiducial limits per cent) =

$$2 + \frac{(0 \cdot 265)\,(0 \cdot 0349)}{0 \cdot 735} \pm \frac{1 \cdot 96}{(6 \cdot 45)\,(0 \cdot 735)} \sqrt{(0 \cdot 0891)\,(0 \cdot 735) + (2 \cdot 87)\,(0 \cdot 001218)}$$

$$= 2 \cdot 01258 \pm \frac{1 \cdot 96}{4 \cdot 741} \sqrt{0 \cdot 06898}$$

$$= 2 \cdot 01258 \pm 0 \cdot 10856$$

$= 1 \cdot 9040$ to $2 \cdot 1211$

antilogs of $1 \cdot 9040$ and $2 \cdot 1211$ are respectively $80 \cdot 2$ and $132 \cdot 2$

Fiducial limits of error = 80·2 to 132·2 per cent of estimated Potency.

Conclusion

Shortage of space makes it necessary to limit the consideration of this important subject to an area most commonly required by workers in India in connection with their standardisation studies with samples of digitalis, posterior pituitary extracts and insulin. Accuracy and proper quantitative evaluation of potent substances which are often of a labile and complex nature and whose chemistry has not yet been fully worked out demands that bioassayists in India use more and more precise statistical methods in the final calculation of their laboratory data to avoid errors which might

* Example taken from British Pharmacopoeia 1958, page 908.

vitiate the results and conclusions that might be drawn from such results. Legal enforcement of drug standards in India also makes it obligatory on testing and analytical laboratories to remain abreast with modern developments in this field of biostatistics which has made it possible for bioassayists to interpret quantitative pharmacology in a way not possible before.

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UTILITY OF RESEARCH IN INDIAN INDIGENOUS DRUGS

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IN India, a country with very ancient civilization, a system of medical treatment indigenous to the country had met the needs of the people for many centuries. After the Muslim invasion and the establishment of their rule, the Tibbi or Unani System established itself in many parts of the country. During the centuries that followed, it gradually merged itself with the old Ayurvedic or the Hindu medicine and the combination of these two constituted the indigenous medicine which gave and still gives medical relief to the people. With the advent of the British, the western medicine was brought in and gradually spread over the whole country. As it was the only system recognised by the State all others were relegated to the background during the British rule. In spite of this, the indigenous medicine has flourished and even at the present time it caters to the needs of the major portion of the population, particularly in the rural areas. During this long contact it has imbibed much from the western medicine especially on the treatment side. The term indigenous drugs has thus come to mean drugs drawn from all sources and used in the treatment of diseases in the indigenous medicine in this country.

Since the establishment of our own Government a good deal of interest has been taken in the old Indian indigenous medicine on modern lines. A number of states of the Union have established Departments of Indian Medicine and Hospitals and Dispensaries belonging to them are running side by side with those of the western system for the benefit of the people.

There has of course been much controversy with regard to the intrinsic value of the indigenous medicine in the treatment of diseases but even the western scholars, who have made an inquiry into it, have agreed that there is much of value in it which could be successfully utilized to alleviate the suffering of humanity. It is admitted that surgical side has not been developed much, but belief in the efficacy of its drugs is almost universal. It has even been said that Materia Medica of Indian medicine is more suited to the Indian constitution and temperament. These systems undoubtedly have a vast materia medica which is predominently derived from the vegetable kingdom. Although about two thousand plants growing in India have been considered to have some medicinal property or other, only about five hundred of these are commonly used by the practitioners in different parts of India in their day-to-day practice at the present time. The rest are practically an unexplored field. It is reasonable to presume that all this vast array cannot possess the wonderful properties popularly attributed to them but there is little doubt that there must be many among these which may deserve the reputation they have earned as cures. With a view to determine what these are and what medicinal properties they really possess, a

study of these drugs was begun in India in early part of the 19th century.

Early Studies

The work at first was confined to the collection of available information with regard to various medicinal plants growing in different parts of the country. The earliest contributions were from the writings of Sir William Jones whose memoir entitled *Botanical Observations on Select Plants* is well known. This was followed in 1810 by John Fleming's *Catalogue of Medicinal Plants* and *Ainslie's Materia Medica of Hindoostan* in 1813. In 1840, O'Shaughnessys published *Bengal Dispensatory and Pharmacopoeia* which was the first book of its kind which dealt exclusively with the drugs used in Bengal. The *Pharmacopoeia of India* was published under the able editorship of Waring in 1868 and it signalised a new epoch in establishing and recording the value of drugs used in indigenous medicine according to modern conception. The more important drugs were officially recognised with a view to their eventual adoption in the British Pharmacopoeia. A very comprehensive work on the Indian medicinal plants, the *Pharmacognaphia Indica* was published in 1885 under the joint editorship of Warden and Hooper. The most elaborate work of all, *A Dictionary of the Economic Products of India*, was published in 1895 by Sir George Watt, the Reporter on the Economic Products to the Government of India. This monumental work, compiled with the help of a large team of workers, refers to all the previous work on medicinal plants and other plants of economic importance. Its pages are replete with information of every description regarding the use of barks, roots, flowers, leaves and woods for medicinal and other purposes. A revised edition of this encyclopaedic work under the name of *Wealth of India* is now being published by the Council of Scientific and Industrial Research and a number of volumes has already come out. Later works such as Kirtikar and Basu's *Indian Medicinal Plants* are largely summaries and compilations from the literature in a more systematised and elaborated form. There is no doubt that a considerable amount of botanical investigations into medicinal plants is accomplished. Admirable as all these attempts were, the chemistry and pharmacology of most of the indigenous drugs remained an unexplored field till recent years. The reason for this is not far to seek. Investigation of this nature requires a considerable outlay of money in the form of well-equipped chemical and pharmacological laboratories, while a liberal staff of competent chemists and pharmacologists is another essential prerequisite. These were not available in former days.

Recent Work

Investigation of Indian medicinal plants on scientific lines was started a few decades ago, with the main object of making India self-sufficient and self-supporting by enabling her to utilise drugs produced in the country. This work is now being carried out in India by eminent chemists and pharmacologists in the various University Laboratories and Research Institutes such as School of Tropical Medicine, Calcutta, Central Drug Research Institute, Lucknow, Drug Research Laboratory, Jammu, Haffkine Institute, Bombay, and others. Side by side efforts are being made to discover effective remedies from the claims of Ayurvedic and Unani sources, drugs which may be effective for employment in modern medicine. A good beginning has been made and already a number of indigenous drugs have found their way into the Pharmacopoeia of India 1955 and some even into the British and other pharmacopoeias.

The work has been directed into two main channels.

Pharmacopoeial Drugs and their Substitutes

A large number of drugs recognised in the pharmacopoeias of different countries grows in India and their chemical composition and therapeutic value are known. The majority of these grow wild and in great abundance in many parts of India and certain numbers are even cultivated. Some of these were collected and exported to foreign countries

and came back to us in the form of standardised pharmaceutical preparations and active principles in pure condition probably at a price many times that of the original crude drug. *Atropa accuminata* which is an allied species to *Atropa belladonna* grows in abundance and in a state of nature in the Himalayan ranges from Simla to Kashmir at altitudes of 6,000 to 10,000 feet above the sea level. Large quantities of the root were collected and were previously exported to Europe and America. *Hyoscyamus niger* is a native of the temperate Himalayas at altitudes of 3,000 to 7,000 feet and a drug of good quality can also be grown in the plains of northern India. A number of species of Mentha, Aconite and Juniper grow all over the Himalayas: *Juniperus communis* of standard quality occurs abundantly at higher altitudes in Kashmir, and *Valeriana wallichii* is found in large quantities from Kashmir to Bhutan. A number of varieties of *Artemisia* grow in the Himalayas and santonin-bearing *Artemisia brevifolia* grows abundantly in the north western Himalayan region. A very good quality of *Podophyllum emodi* is met with in the higher shady temperate forests of the Himalayas from Sikkim to Kashmir at altitudes of 7,000 to 9,000 feet above the sea level.

The optimum time of collection, drying and storage of many such drugs, have been worked out in order to produce drugs of standard potency and stability. Recently a good deal of work has been done on the cultivation of these drugs and their quality and yield have been considerably improved in many cases.

In addition to the above a large number of plants grows in India which, though not exactly the same as plants recognised in the pharmacopoeias, possess similar properties and actions and form excellent substitutes. A systematic chemical and pharmacological study of this group of plants has revealed that in the majority of cases the indigenous substitutes are as active as the foreign imported drugs. For example, *Colchicum luteum*, found on the slopes of the western temperate Himalayas forms an excellent substitute for the official *C. autumnale*. *Scilla indica*, which grows extensively on the sea coast and on the drier hills of the lower Himalayas and the Salt Range would make a good substitute for *S. maritima*. *Ferula narthex*, from which a gum resin resembling asafoetida can be obtained, grows in Kashmir; the properties of *Picrasma quassioides* and *Gentiana kurroo* resemble those of *Picrasma excelsa* and *Gentiana lutea* respectively of the British Pharmacopoeia. Several *Dryopteris species* (male fern) growing in the north western Himalayas compare favourably both chemically and pharmacologically with the official drug of British Pharmacopoeia.

Rheum emodi growing wild in the Himalayas forms an excellent substitute for the official drug *Rheum palmatum* which is indigenous to China. The seeds of *Strophanthus wightianus* a climbing shrub growing wild in Malabar forms an excellent substitute for the official drug *S. kombe* which does not grow in India.

Systematic work is now being carried out on these indigenous substitutes for the recognised pharmacopoeial drugs and a number of them have already been included in the Indian Pharmacopoeia.

Introduction of Exotic Plants

There is a large number of pharmacopoeial drugs which are widely used by the medical profession, but which do not naturally grow in the country and are consequently imported. India with such a diversity of soil and climatic conditions can grow every conceivable drug growing anywhere in the world ranging from those growing in hot tropical climates to those growing in dry temperate and very cold climates. These can easily be acclamatised in one part of the country or the other.

A systematic research into the introduction and cultivation of these exotics has resulted in the large-scale cultivation of a number of valuable drug plants which were previously imported from abroad. Examples of such drugs are numerous but a few outstanding ones may be mentioned, such as *Digitalis lanata* and *D. purpurea*, *Ipecaucunha*, *Cinchona*, *Eucalyptus*, etc. now grown in the country. Recent experimental work by the Drug Research Laboratory in the field research

stations in Jammu and Kashmir has shown that useful exotic medicinal plants such as Japanese mint (yielding oil possessing a very high menthol content), *Anethum graveolens* (dill), *Lavender, Glycyrrhiza, Pyrethrum*, etc. grow well in India in suitable places and there is no necessity of importing these drugs.

Drugs Used in the Indigenous Medicine

We have stated that there are about two thousand medicinal plants which are reputed to have medicinal properties of some description or other. It is believed that out of a large number of these used for centuries past and still in use there may be some at least which might deserve the reputation they have earned as cures. Since the period of decay and recompilation of the Indian systems of medicine, many of the effective remedies have been lost and a number of others has come into use. The result is that at the present time almost every plant and shrub growing in the country has some medicinal virtue ascribed to it. In this way remedies have multiplied without proof but by belief and as they hail from all parts of India no one seems to have any correct notion with regard to their uses and properties.

A thorough and complete research into all these drugs could constitute the life-long work of innumerable chemists, pharmacologists and physicians. For this reason, to begin with, it has been considered advisable to take up drugs which enjoy great local reputation. Besides this many of the drugs have been clinically tried by medical men practising western medicine or other systems and favourable opinion has been expressed regarding their efficacy. This would also be helpful in selection of drugs which might be taken up for investigation. A careful examination of the selected drugs should be carried out from every point of view. Their chemical composition should be determined, the pharmacological action of the active principles be worked out by animal experimentation and finally suitable preparations made from the drugs should be tested on patients in the hospitals. It is only by such a thorough enquiry that the real merits of these drugs can be proved and a demand created for them not only in India but in other parts of the world. This laborious work carried out during the past 3 or 4 decades has brought into prominence the merits and qualities of certain drugs and it has been shown that, if brought into general use, they may prove to be very valuable additions to the treatment of diseases.

With development of the latest methods of investigation in chemistry and pharmacology and with the growth of pharmaceutical firms and many chemical laboratories in Universities, significant progress has been recorded during recent years. An institute for research in indigenous medicine has been recently established in Jamnagar under the Central Health Ministry. It may be interesting to note here that several research centres in Great Britain, Switzerland and the United States of America have also taken up intensive study of some Indian indigenous drugs. Such work has brought into prominence the merits and qualities of certain drugs such as *Holarrhena antidysenterica* (Vern. Kurchi), *Rauvolfia serpentina* (Vern. Chota chand), *R. canescens*, *Caesalpinia bonducella* (Vern. Kat karanj), *Adhatoda vasica* (Vern. Vasaka), *Bacopa herba* (Vern. Brahmi), *Daemia extensa* (Vern. Utran), *Cissampelos pareira* (Vern. Akanadi), *Psoralea corylifolia* (Vern. Babchi), *Boerhaavia diffusa* (Vern. Punarnava), *Swertia chirata* (Vern. Chireta), *Andrographis paniculata* (Vern. Kalmegh), *Plantago ovata* (Vern. Ispaghul), *Thevetia neriifolia* (Vern. Pilakaner), *Moringa pterygosperma* (Vern. Soanjna), *Dichroa febrifuga* (Vern. Basak), *Withania somnifera* (Vern. Asvagandha), *Pristemera indica* and a number of others.

Holarrhena (Kurchi) has come to stay as a reliable antidysenteric remedy, particularly in sub-acute and chronic forms of amoebiasis complicated with what has often been described clinically as 'post dysenteric abdomen'. Standardised liquid extract and a preparation of Kurchi Bismuth Iodide have been accepted as recognised remedies in the Indian Pharmacopoeia. *Rauvolfia*, an old drug used empirically by indigenous practitioners for centuries as a sedative and in the treatment of insanity, has been found to be one of the best hypotensive agents and

sedatives in the treatment of insomnia. It is now extensively used in Europe and America as one of the best remedies for the treatment of hypertension and certain neuro-psychiatric disorders. The first pharmacological paper on this sovereign remedy was published as far back as 1933 by R. N. Chopra and B. Mukerji. *R. canescens* an allied species growing wild in India has also been found to contain the alkaloid resperine present in *R. serpentina* and shows similar sedative properties.

Recent work on *Cissampelos pareira* at the Central Drug Research Institute, Lucknow, indicates that from this Indian plant, available in plenty in the lower ranges of the Himalayas, the alkaloid hyatin has been isolated which is as good a smooth muscle relaxant as d-tubecurarine chloride. From *Thevetia neriifolia* a pure glycoside has been obtained having properties and uses similar to Digitalis. In *Andrographis paniculata* and *Swertia chirata*, we have bitters and cholagogues which compare favourably with the best items of their class in foreign pharmacopoeias. The use of *Plantago ovata* has now been accepted all over the world. *Withania somnifera* which finds extensive use in Ayurveda contains alkaloids and has been found to possess mild sedative properties. The paste from roots of *Pristemera indica* is used as a remedy for respiratory troubles. It has been found to contain an antibiotic substance pristimarin which is effective against gram positive organisms and clinical trials indicate that it is effective in throat infections. *Moringa pterygosperma* contains an antibiotic substance pterygospermarin which has been found effective against gram-negative organisms and especially against *Mycobacterium tuberculosis*. *Dichroa febrifuga* grows wild from Bhutan to Khasia Hills. Its roots are used as a cure for malaria in China and as a febrifuge in India. This drug contains number of alkaloids of which febrifugine and isofebrifugine are important. Clinical trials have revealed that the drug has antipyretic action resembling quinine (1·5 times) but no anti-parasitic action.

These are only a few examples out of the 200 or 300 drugs which have so far been scientifically studied. Some of these have shown definite physiological activity and could be brought into use. There must be many others which future research will perhaps reveal to be good remedies.

We have shown elsewhere that a large number of herbs is used in this country — often with considerable benefit — by the indigenous practitioners in the treatment of uterine and menstrual disorders. A number of these herbs is being systematically examined in Jammu and Kashmir laboratories. Besides the drug like *Rauvolfia serpentina* which is already well established, there is a number of other drugs which are reported to be useful in the treatment of hypertension, nervous disorders and for their sedative action. This group may well repay, if studied. Some are sedative expectorants, cardiovascular sedatives, nervine sedatives or general sedatives. All these require careful study. The literature on indigenous medicines also reveals that a large number of vegetable drugs is used with benefit in common disorders of the gastro-intestinal tracts, as stomachics, carminatives and cholagogues. The study of the Materia Medica of indigenous systems also shows that certain plants have the reputation of being effective in the treatment of bowel diseases such as dysentery and cholera. Some other plants are said to be useful in the treatment of prolonged fevers such as the group of enteric fevers, while certain others are reputed to be effective in the treatment of tuberculosis. Others have insecticidal and insect-repellent properties; still others are poisonous to men and animals.

Value of Research in Indigenous Drugs

The question has often been asked: what is the use of research in Indian indigenous drugs? During recent years chemistry has made rapid strides and remarkable progress has been recorded particularly in the field of synthetic chemistry. Chemists have synthesised very potent and effective remedies such as arsenicals and antimalarial compounds for the treatment of protozoal diseases and sulphonamides for the treatment of bacterial diseases. The group of antibiotic drugs has

revolutionised the treatment of many bacterial and rickettsial diseases and even some of the virus diseases are being touched. The importance of research in indigenous drugs has been well emphasized in *The Practitioner* in its issue of December, 1950. Under the heading 'Indigenous Herbs' it says, "The wise and experienced clinician never spurns an 'old wife's tale', until he has good evidence for doing so. The lore of the countryman is built upon the experience of generations often obtained at a price in human lives which no modern research worker would ever dream of considering. It is particularly appropriate at the present moment, when the pharmaceutical companies of the world are emitting an increasing flow of new synthetic drugs, that attention should be turned to the possible remedies that may be found among indigenous herbs of this and other countries."

Synthetic processes for which a chemist requires enormously high degrees of heat and pressure are being quietly carried by nature in plants at ordinary conditions of temperature and pressure. Chemists synthesised such alkaloids as quinine after intensive work extending over half a century whereas cinchona plant does this without difficulty everyday. Many active antibiotics occur in plants and this is yet an unexploited field. In fact we are only at the threshold of work of plant analysis and research. What is in store, nobody but nature knows. The research on these drugs should, therefore, go on for the good of the humanity.

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RABIES AND ANTIRABIES TREATMENT

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RABIES is a disease known from the earliest times. The phenomenon of normally quiet and docile animals suddenly turning vicious and succumbing after a period of violent behaviour was in the ancient times attributed to supernatural agencies.

Rabies is ubiquitous as far as its distribution is concerned. Thus it may occur amongst the wolves in the snowbound regions of the Arctic or amongst the wild or domesticated animals in the subtropical or tropical regions. It is a disease of warm-blooded animals, mostly of canine species such as wolves, jackals and dogs. Man is infected almost always by the bite of an animal suffering from rabies.

That the disease in man is entirely related to the disease in animals was recognized even as early as 100 A.D. and cauterization or surgical removal of the bitten area was recommended. That the disease was transmitted from animal to animal through the inoculation of saliva of the biting beast was proved experimentally by Zinke in 1804 and by Gruner & Sahn-Reifferscheidt in 1813.

It was however Pasteur and his group of workers who first established the etiology of the disease in 1881 and paved the way for an intensive study of the disease. They obtained the infective agent in a relatively pure state from the brain of an animal dead of the disease and transmitted the disease to other animals by inoculating them intracerebrally with the infected brain material. However, the inability to demonstrate the infective agent either microscopically or in culture media used for bacteria led Pasteur to postulate the ultra-microscopic nature of the agent and call it a "virus" from the latin word for poison. Later in 1903 Remlinger demonstrated that the infective agent could pass through the ordinary Berkefeld filters which held back bacteria and thus confirmed Pasteur's hypothesis.

Rabies in Man

The incubation period of rabies may vary from 15 days to 6 months. It is held by some that the incubation period depends upon the distance of the place of the bite from the brain, longer the distance longer the incubation. This is however disputed, the amount of virus introduced and the type of tissue being considered the factors which determine the length of the incubation period. The relatively short incubation period following bites on the face and head is explained by the richer sensory innervation of the area and also by the severe laceration usually caused in such bites. Longer incubation periods are explained by the temporary arrest of multiplication of the virus either at the site of the bite or along the nerve route by which the virus travels to the central nervous system. In experimental infection of animals where the virus is introduced intracerebrally the incubation period is definitely found to depend upon the quantum of virus introduced.

The disease is commonly ushered in by 2 or 3 days of prodromal symptoms, which are usually headache, restlessness, anorexia, and frequent sensation of pain or tingling at the site of the wound, the latter is of early diagnostic significance. Soon the typical symptoms of the disease develop. The patient develops a dread of water as attempts to drink it result in severe spasms of the muscles of deglutition and respiration. This characteristic symptom has given the disease the name " hydrophobia ". Later the spasms occur in reaction to sight or smell of liquids, loud sounds or even strong drafts of wind. Swallowing becomes totally impossible and due to spasms of respiratory muscles dyspnoea may occur. As the disease progresses the patient becomes very excited, noisy and at times maniacal. Between the periods of excitement the patient may be quiet and answer questions rationally. This is the phase of excitement and a patient usually dies during this phase.

If the patient survives this phase, he passes on into a state of stupor or coma. The patient becomes depressed, muscle spasms disappear and swallowing becomes less difficult. Progressive paralysis may follow which in rare cases is of the Landriform type. There is difficulty in closing the eyes or mouth and the voice becomes hoarse due to weakness of muscles of phonation. Very occasionally paralytic symptoms may be the only ones observed. Rabies following bites of vampire bats in Trinidad is of the paralytic type and is known as Trinidad rabies.

The disease is uniformly fatal and there is no single instance proved of recovery from rabies.

Pathogenesis of Rabies

From experimental evidence it is generally accepted that the rabies virus spreads to the central nervous system from the wound through the nerve fibres. It is also conceded that infection does not occur through unbroken skin, though rare cases of such infection have been reported. The wound need not necessarily be from a bite as infections after clawing or scratching by a rabid animal have been recorded.

Transmission of Rabies

Transmission of the disease in nature depends upon the capacity of the virus to reach and multiply in the salivary glands of the rabid animal. This ability to invade the salivary glands varies with different strains and with the different species of animals, all warm-blooded animals being susceptible to the infection.

The large majority of cases follow bites of rabid dogs, jackals and wolves. A normal dog bitten by a rabid animal may develop the disease after an incubation period varying from 10 days to a few months, though a period between 3 to 7 weeks is usual.

Rabies in dogs is of 2 types, 'furious' and 'dumb', depending upon the nature of symptoms and signs in the animal. In the 'furious' type the symptoms of excitement are prolonged while in the 'dumb' type paralytic symptoms develop early. Both types however may manifest in the same dog, a short period of excitation followed by paralysis.

In the prodromal stage the dog may appear to be increasingly nervous and seek dark corners and solitude. It may also refuse food or develop capricious appetite. As the disease progresses the dog becomes very excitable and will bite at the least provocation. If loose it may run about biting any animal that comes in the way. Though swallowing may be difficult there is no symptom of hydrophobia as such. Paralysis of the muscles of phonation results in a characteristic change in its bark. It may also eat any refuse it may come across. If the dog survives this stage it develops progressive paralysis and coma ending in death.

In a rabid animal the virus is demonstrable throughout the central nervous system, the maximum concentration being in the medulla and the thalamus. The sub-maxillary glands also contain a large amount of virus, and to a lesser extent other salivary glands, thus rendering the saliva of a rabid animal infective. It is this fact that is responsible for the transmission of the disease in nature.

Diagnosis

It is a matter of great importance to ascertain whether a dog which has bitten a person was actually rabid or not. If alive, it should be secured, isolated and observed for symptoms of rabies. If dead, the brain should be subjected to both microscopical and biological examination for evidence of rabies. With all due care, the brain is removed and dissected to expose the Ammon's horn of the hippocampus which is removed for examination.

Microscopical Examination

The diagnosis by microscopical examination, for which films, wet or stained, as well as stained sections can be used, depends upon the detection of inclusion bodies known as Negri bodies which are considered pathognomonic of rabies. These specific inclusions are present in the cytoplasm of large neurons and are particularly abundant in Ammon's horn. With appropriate staining they are seen as oval or spherical or elongated eosinophilic bodies of varying sizes. Negri, who first described these inclusions which go by his name, believed that they represented a stage in the development of a protozoan parasite. Later studies however discount this theory and indicate that they are degenerative structures derived from all constituents which may contain virus particles. Negri bodies increase in number as the disease advances, therefore, it is always advisable to quarantine animals on the least suspicion of rabies. Their immediate destruction makes diagnosis by microscopical examination difficult as there may be very few Negri bodies in the brain.

Biological Examination

For this purpose material from the bulb or other basal part of the brain is ground in sterile saline and lightly centrifuged. The supernatant is injected intracerebrally into rabbits, guinea-pigs or albino mice. Treatment of the material with a mixture of penicillin and streptomycin will counteract any bacterial contamination. In the case of rabbits the incubation period will be 15-30 days and in guinea-pigs 10-20 days. In mice it is about 7 days and therefore they are the animals of choice for this purpose as the diagnosis is obtained much earlier than with rabbits or guinea-pigs. The injected animals become paralysed and Negri bodies can be demonstrated in their brain material. Occasionally, specimens which do not show Negri bodies by microscopical examination may prove positive on animal inoculation.

The Rabies Virus

The rabies virus, as existing in nature, is known as " Street Virus ". Characteristic features of infection with " Street Virus " are a long and variable incubation period, development of furious symptoms, and presence of Negri bodies in the brain tissue of the infected animal. Pasteur and his associates in their attempts to develop a strain that could be used for vaccination, were the first to modify the pathogenicity of the virus. By successive serial intracerebral passage of the virus in rabbits, they found that the incubation period which varied from 15-20 days progressively decreased and was stabilized at 6-8 days. They also found that after about 100 passages or so the pathogenicity was so reduced that dogs were not infected by subcutaneous inoculation. This stabilized and modified virus is called " fixed virus " and it is generally accepted that reversion to street virus does not occur. It further differs from the street virus in that no Negri bodies are found and only paralytic symptoms are noted in fixed virus infections. In view of its very low pathogenicity for man and its attenuated affinity for tissues other than the central nervous system, Pasteur and his associates utilized this strain for evolving a vaccine for immunization against rabies.

Antirabies Treatment

Pasteur et al first reported in 1884, successful immunization of dogs against rabies by inoculation with spinal cord material of rabbits infected with fixed virus. He observed that the virulence of the cord could be reduced by drying over potash, and thus obtained fixed virus material of virulence varying from no infectivity to maximum infectivity. He vaccinated dogs with the graded virus beginning with material of no

infectivity. These vaccinated dogs were found to be resistant to experimental infection with street virus. From these experiments he evolved a method of human antirabies treatment.

In 1885, Joseph Meister, a boy aged 9 years was very badly bitten by a rabid dog and was taken to Pasteur. As it appeared that there was little or no chance of the boy escaping rabies and on the plea that something should be done, Pasteur gave the boy daily subcutaneous injections of the graded fixed virus in the abdomen, beginning with a cord that had been dried for 14 days. There were no ill-effects and the boy remained perfectly healthy and later in life became the concierge of the Pasteur Institute, Paris. This spectacular success led more and more people bitten by rabid animals to flock to Pasteur for treatment. Except in a very few cases the success was repeated. The whole medical world, barring just a few, hailed Pasteur's achievement with satisfaction.

The original method evolved by Pasteur has with passage of time undergone several modifications. Thus Hoegyes modified the virulence of fixed virus by dilution instead of by drying.

The most important change was however introduced by Fermi which was further modified by Semple. Before this change the vaccine contained living virus though attenuated in varying degrees by drying or dilution. Fermi's vaccine contained virus inactivated by phenol which was a radical departure from the previous vaccines. Though the efficacy of killed virus vaccines was disputed by some workers, the exhaustive statistical analyses published by McKendrick showed that while killed virus vaccines are in no way inferior to live virus vaccines with regard to immunizing properties, they are less liable to produce serious reactions than live vaccines.

At present there are numerous types of antirabies vaccines in use, and they can be broadly classified as killed or live vaccines. Amongst killed vaccines, the one employed in most parts of the world including India is the Semple's vaccine.

To prepare Semple's vaccine an 8 per cent suspension of fixed virus-infected brain in 1 per cent phenol in saline is incubated at 37°C. for 24 hours after which normal saline is added to make a 5 per cent suspension. As it is possible that at this stage the vaccine may still contain a few particles of live virus it is not issued for use until after a month's storage when it is expected to be almost completely devoid of live virus. In India vaccine is prepared from sheep brain and the strain of fixed virus employed is mostly the classical 'Paris' strain which has been experimentally proved to be more immunogenic than other Indian strains.

The most outstanding advantages of phenolized vaccine are that incidence of serious reactions, such as neuroparalytic accidents, is considerably minimized, and as the vaccine is easily transportable antirabies treatment can be decentralized, thus saving patients from long journeys to Pasteur Institutes. Further, phenol keeps the vaccine sterile and is also known to conserve the immunizing potency of the vaccine for several months.

Indications for Antirabies Treatment

Antirabies treatment should be started immediately a person is bitten or scratched by

(1) An animal showing clinical symptoms of rabies.
(2) An animal proved to be rabid on microscopical examination.
(3) An animal strongly suspected to be rabid though microscopic examination is negative.
(4) A stray animal that has escaped and cannot be identified.

Immediate treatment is also indicated when fresh cuts or abrasions of the skin have come into contact with the saliva of an animal proved to be rabid. In instances where a person is bitten by an animal that can be apprehended and not at all suspected to be rabid, it is recommended in India, to give 2 injections of the class of treatment appropriate to the bite and observe the dog for a period of 10 days. If the animal develops symptoms of rabies during the period of observation, antirabies treatment is at once continued. If however the animal remains

healthy and well during the period, no further treatment is given.

Antirabies Treatment in Man

The first and foremost step to be taken in case of bite by a rabid animal is thorough cauterization of the wound as soon as possible, preferably immediately after the bite. It is believed that the virus remains localized in the wound for some time and then slowly travels to the central nervous system along the nerve routes. Removal or destruction of the virus in the wound of course supplemented by vaccine treatment should therefore give the best results. Several agents including 20 per cent soap solution have been recommended for this purpose, but fuming nitric acid, particularly if applied within 4 hours after the bite, has specific value which is incontestable. Infiltration of the wound and the immediately surrounding area with antirabies serum is also reported as giving good results. It has its limitations with regard to the site of bite and possible sensitivity of the patient to the serum.

Vaccine treatment should also be started as soon as possible. In India antirabies treatment is of three classes depending upon the site and severity of the bite.

CLASS I — Licks or indirect contact with saliva on fresh cuts except on head, face, neck and fingers — 2 c.c. of 5 per cent vaccine daily for 7 days.

CLASS II — Bites on the legs or trunk and licks on fresh cuts on fingers — 5 c.c. of 5 per cent vaccine daily for 14 days.

CLASS III — Deep and multiple bites, and any sort of bite or lick on fresh cuts on the face, head or neck — 10 c.c. of 5 per cent vaccine for 14 days. All jackal and wolf bites which invariably cause extensive laceration are given this class of treatment.

In spite of antirabies treatment with phenolized or any other type of vaccines, a small percentage of cases nevertheless die of rabies. McKendrick's analyses of figures relating to treated persons give a mortality rate of about 0·33 per cent. His analyses also indicate several factors as influencing the mortality rate. They show the mortality rate after treatment amongst non-Europeans as about 3·7 times as great as among Europeans. It also increases more or less in proportion to the number of tooth marks. The mortality rate is in a descending order, depending upon whether the person is bitten on the head, arm, trunk or leg. Intervention of clothing, delay in the commencement of treatment and species of the biting animals are also indicated as influencing the mortality rate.

While McKendrick found little evidence to commend any one type of vaccine in preference to another from the point of view of efficacy, his figures show that the incidence of accidents with live vaccines was about four times as high as with killed vaccines.

Value of Antirabies Treatment

There is a small section of scientific workers which still believes that vaccine treatment of persons bitten by rabid animals is of doubtful value. It is pointed out that a very large proportion of persons treated may not have been at risk at all, that some treated cases may have died of rabies but reported as successfully treated due to inadequate follow-up, and that a proper evaluation of vaccine treatment can be had by comparing mortality rates among treated and untreated persons bitten by proved infective animals.

However, Veeraraghavan has effectively met these arguments. From a carefully conducted study, he has reported that "8·86 per cent of persons bitten by proved infective animals develop the disease in spite of having a complete course of treatment. If the number dying within 15 days after the completion of treatment, who really cannot be classified as failures are excluded the mortality rate would be 3·68 per cent. The death rate among a similar group of persons who refused treatment is 48·11 per cent. If the persons who died during treatment are also classed among the untreated, the mortality rate among the untreated would be 54·17 per cent. It is interesting to note that the mortality rate among the incompletely treated is 17·24 per cent."

It is possible due to various causes that the phenolized vaccine issued may on occasions

be devoid of immunizing potency. It is therefore highly advisable that the vaccine is tested from time to time for immunizing potency. Webster first introduced a mouse immunity test for the purpose which has subsequently been modified by Habel, who has laid down a standard that the minimum requirement of an efficient vaccine should be protection against at least 1000 MLD of virus. He also recommends in this connection periodical titration in mice of the fixed virus used for preparing vaccine.

Mechanism of Protection by Antirabies Treatment

Immunization by injections of antirabies vaccine is accompanied, though not invariably, by the development of serum antibodies.

The exact relationship between protection against the disease and the serum antibodies is not definitely known. Thus a high content of antibodies may be unaccompanied by protection against rabies while a person showing no serum antibodies may be protected against the disease. It is considered possible that antirabic immunity is a tissue immunity the nerve tissue being immunized against the virus. The precise mechanism is thought to be probably an " interference phenomenon " the fixed virus particles, live or inactivated occupying the susceptible cells, thus blocking the entry of street virus into the nervous system.

Reactions after Antirabies Treatment

Antirabies treatment may be accompanied in some individuals with reactions which may vary from minor disorders such as fever and headache to such serious reactions as neuroparalytic accidents. Local reactions such as urticaria, rashes or erythematous patches may occur.

These local reactions usually subside in spite of continuance of treatment. Fever, headache and nausea are sometimes preludes to the serious and sometimes fatal complications known as neuroparalytic accidents and on their occurrence treatment should be stopped at once. This accident usually develops between the 13th and the 15th day of antirabies treatment, and may be of the neuritic type, Landry's type or the dorso-lumbar type.

The neuritic type is usually characterized by facial paralysis. Recovery in two or three weeks is the rule in this type.

The Landry's type sets in with fever and pain in the back. Paralysis of the legs, and retention of urine and faeces follow. The arms become paralysed next. If the paralysis ascends to the face, tongue and other muscles supplied by the bulb the case may terminate fatally. Otherwise recovery is usually rapid and without sequelae.

In the dorso-lumbar type there is slight fever and the legs become paralysed. There may be sphincter disturbances. Fatality rate is very low and most of the cases recover quickly.

There are various theories concerning the etiology of neuroparalytic accidents and there is yet no unanimity of opinion on the subject. However the commonly accepted explanation based on experimental evidence is that the accidents are due to specific sensitization to brain material which functions as an organ-specific instead of a species-specific antigen.

Control Measures

As the domestic dog is the commonest animal responsible for rabies in man, control measures imply control of rabies in dogs. The dog while making the man his best friend seems to have developed an undue hostility to members of his own species. Thus, measures preventing dogs from biting one another particularly when rabies is prevalent in a locality are essential. All stray dogs should be destroyed and owned dogs licensed and muzzling enforced until 6 months have elapsed from the last reported case. Compulsory leashing may also be enforced in place of the irksome muzzling. All rabid animals and stray animals bitten by rabid animals should be destroyed at owners' option or in the alternative isolated for a period of 6 months and given antirabies treatment. Dogs which have had contact with other dogs suspected to be rabid should be destroyed or isolated. Any animal not suspected to be definitely rabid biting a human being

should be under close observation for a period of 10 days. This is to ascertain whether the biting animal was really rabid or not, from the point of view of treatment of the bitten person.

Island countries like Great Britain can in addition enforce a remarkably efficient measure of quarantining for 6 months all dogs imported into the islands.

In countries where the disease is harboured among wild animals or where quarantining of animals is not practicable, prophylactic vaccination of all owned dogs and destruction of all stray dogs are very effective measures in controlling the incidence of the disease. Phenolized vaccine is mostly used for this purpose at present. However a new type of live vaccine employing the virus adapted to chick-embryo (Flury) is also now being tried on a large scale particularly in the United States. Field trials have indicated that this live vaccine while not pathogenic to dogs confers a high degree of immunity.

Recent Advances in Antirabies Treatment

It has been the urge since Pasteur's time among workers in this field to evolve a highly potent and stable vaccine which will at the same time be free from dangerous reactions.

Recently workers in the Central Research Institute, Kasauli, India, reported that phenolized antirabies vaccine prepared in distilled water instead of in physiological saline had quicker immunizing properties with fewer risks of neuroparalytic accidents. Coonoor workers, however, have not been able to confirm these findings and judgment has to be suspended on this matter until more work is carried out.

Another new type of vaccine that has been introduced is the " UV " vaccine where the virus is inactivated by ultraviolet irradiation and merthiolate is used as preservative. This is considered to be a very effective vaccine and is available for clinical use in U.S.A.

A recent advance with high potentialities is the evolution of the strain adapted to growth in chick-embryo. This is known as the Flury Strain of rabies virus. It was originally a strain of street virus propagated by brain passage in chicks. This was subsequently adapted to growth in chick-embryo. It was found that as a result of continuous serial passage in the chick-embryo the strain became so attenuated that it failed to produce encephalitis in adult mice even after intracerebral inoculation. This avirulent strain used as a live vaccine for immunization of dogs appears to have given very satisfactory results. The vaccine is prepared from inoculated chick-embryo from which the heads are removed and is hence almost completely devoid of central nervous system tissue. As neuroparalytic accidents are believed to be due to sensitization to brain material such a vaccine should be considered ideal for treatment in man. However it has been noted that this virus though living does not multiply extraneurally in man, and even vaccines of fairly high titres proved to be of marginal potency from the point of view of antibody protection. Thus only vaccines having the highest virus content possible seem to be suitable for use, though the significance of antibodies in relation to actual protection to rabies is still unsettled. This type of vaccine is actively engaging the attention of several workers and further interesting developments may be expected.

Another recent landmark in this field is the use of hyperimmune antirabies serum in rabies prophylaxis in man. Habel has reported excellent results with the use of antirabies serum as an adjunct to vaccine in experimental rabies infection of animals. Veeraraghavan in Coonoor in a detailed experimental study assessed the value of antirabies serum in antirabies treatment. With local street virus strains as challenge material he found that with serum alone while the incubation was considerably prolonged, there was no saving effect. One very interesting fact elicited in this study was that when the serum was homologous to the strain of challenge virus good results were obtained and that for best results the antirabies serum should be homologous to both the challenge virus as well as the species of animal used. Used as an adjunct to phenolized vaccine, he found that the

combined treatment gave best results when the treatment started as early as possible. With egg-passage vaccine the serum conferred no protection against local virulent street virus strains.

In connection with the use of the combined treatment with antirabies serum and vaccine, Atanasiu has made the interesting observation that the presence of passive antibody in the blood in high concentrations during active immunization, may interfere with the production of active antibodies. Habel has stated that this interference can be overcome by giving a single early dose of serum followed by 14 daily doses of vaccine.

It is only to be expected that these important findings will stimulate further research which will eventually evolve a system of prophylaxis which will be both safe and efficient.

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DIETARY DEFICIENCIES AND LIVER INJURY

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INVESTIGATIONS carried out in South India showed that dietary deficiencies are important factors in the causation of the liver cirrhosis[1]. For a long time little attention was paid to the role of nutritional factors in the causation of the disease, and only during recent years has the subject engaged the attention of both research workers and clinicians. The different clinical types of cirrhosis of the liver met with in India and the etiological factors with special reference to nutritional deficiencies, are discussed by Radhakrishna Rao.[1]

Portal cirrhosis of the liver is commonly seen in the poorer class of people in the third and fourth decades of life. The disease is characterized clinically by emaciation, secondary anemia, subicterus, dry skin, atrophic liver, enlarged spleen, prominence of superficial abdominal veins and ascites. Histologically, the parenchyma of the liver is divided by a collagenous connective tissue network into rounded islands of various sizes containing two or more of the so-called lobules.

From a careful investigation into the personal histories of 120 patients in one series, it became evident that most of the males and all the women and children in the series had never touched alcohol in any form. But some of the male patients occasionally indulged in toddy (fermented palmyra juice) or country-made arrack and rarely brandy on festive occasions. In no case was there a history of continuous alcoholism. Most of the patients were non-vegetarians. Dietary studies revealed an excess of carbohydrate in the diet and a deficiency of protein, fat, and vitamins, especially vitamins A, B complex, and C. In general, the diet contained an excess of chillies.

The disease is insidious in its onset, runs a protracted course and ends fatally. It is not uncommon in children belonging to the poorer classes and the clinical and pathological manifestations in children are similar to those seen in the adults.

In marked contrast to the portal cirrhosis, a progressive and rapidly fatal cirrhosis of the liver is commonly seen in children, between the ages of 1 and 3 years, of middle and rich class vegetarian Hindus. The disease is characterized in the early stages by a progressive, persistent, and painless enlargement of the liver, irregular intermittent fever and constipation, and; in the later stages, by gradual contraction of the liver, jaundice, enlargement of the spleen and ascites. Histologically, the liver shows sub-acute necrosis of the parenchyma, extensive obliterative lesions of the smaller divisions of the hepatic venous tree and replacement fibrosis; attempts at regeneration of the hepatic parenchyma are usually marked.

The disease takes a high toll of life among Indian children and its causation is still not clear. Although the children affected

with the disease belong to the economically better classes, dietary deficiencies are common both in the nursing mother and in the child, on account of the imposed religious restrictions. Protein of animal origin, e.g. meat or fish, is not consumed by the orthodox section of the Hindu community.

Experimental dietary liver disease produced by deficient diets have been known since nearly last three decades. In the early years, most of the researches were carried out on fatty infiltration and liver cirrhosis[2, 3]. In 1935, Weichselbaum[4] had reported production of "haemorrhages throughout the liver" and "clear signs of icterus" by a cystine deficient diet. Subsequently, du Vigneaud and his associates[5] produced liver necrosis in rats on diets deficient in the sulphur containing amino acids. However, the differentiation of these two types of liver disease was not precisely understood till 1942, when it was clearly established by Daft, Sebrell and Lillie[6] that the two diseases were separate entities. By carefully devising the experimental diets, Himsworth and Glynn[7, 8] in England were also able to produce these two distinct lesions independently of each other. The dietary factors causing these two different types of liver injury are summarised in *Table* 1.

Our present knowledge and all the current trends in research on the subject have been excellently reviewed in the monograph based on the symposium on "Nutritional Factors and Liver Diseases" held by the Section of Biology of the New York Academy of Sciences[9].

In view of the topical importance of the problem, experimental investigations were undertaken in our laboratory since 1944 to study the role of nutritional factors in the production of liver cirrhosis and related lesions. Some of our observations made in this connection are briefly reviewed in the following pages.

Effect of Protein Level and Vitamin B_2 Complex Deficiency on the Liver of Rat

In these experiments, casein was fed at four levels viz. 18, 10, 8 and 5 per cent in the diet in the absence and presence of vitamin B_2 complex.

The growth and survival of the rats depended largely on the amount of casein in the diets. On the 5 per cent casein diet, the animals did not survive for more than 2 to 3 months. In the other groups, the average life of the animals increased as the casein in the diet was raised.

The relationship between liver weight and body weight of the rats was found to

Table 1.

Dietary Factors in Liver Injury.

Dietary factor	Cirrhosis	Necrosis
Protein	Protects	Protects
Methionine	Protects	Protects
Cystine	Enhances	Protects
Choline	Protects	Enhances
Vitamin E	Without effect	Protects
Betaine	Protects	Enhances
Vitamin B_{12}	Protects	Enhances
Folic acid/citrovorum factor	Protects	Enhances
Factor 3 of Schwarz	Not known	Protects

vary according to the level of casein in the diets. In general, as the percentage of casein in the diet was decreased, the average liver weight per 100 g. body weight was increased.

The estimation of water, fat and protein content of the liver showed that the increase in the weight of the livers in the low protein groups was not due to accumulation of fat alone; for there were significantly larger amounts of water and protein present as compared to normal liver, except in the 5 per cent group which showed slight increase in the water and fat content. The results are given in *Table* 2.

In animals receiving 18 and 10 per cent casein diets respectively, the liver parenchyma showed moderate to severe fatty infiltration, particularly around the central veins. Traces of ceroid pigment were also visible in these areas. In the 8 per cent and 5 per cent groups, extensive fatty infiltration, more marked around the central veins, was seen. An interesting feature in the 8 per cent protein group was the appearance of replacement fibrosis, patchy in distribution around the central veins. In certain cases, this was more marked and there was a tendency for the formation of pseudolobules. Animals receiving 5 per cent protein diet did not show any marked replacement fibrosis. None of the animals in any group showed massive necrosis. On the other hand, animals receiving 18 and 10 per cent casein diets with supplements of yeast showed normal structure of the hepatic parenchyma, while those receiving 8 and 5 per cent casein showed slight fatty infiltration around the central veins. Fibrosis of the liver was altogether absent in these groups of animals even after prolonged feeding of the diets.

Effect of Restricted Intake of Protein (casein) in an otherwise Adequate Diet on the Histological and Biochemical Changes in the Liver of Rat

An interesting feature of the previous experiment was the absence of massive necrosis of the liver on protein deficient diets. Himsworth and Glynn[8] however have claimed to have produced massive hepatic necrosis and its sequelae in rats by limiting the intake of casein between 200-500 mg. per rat per day. They found that the amount of fat, carbohydrate, minerals or vitamins in the diet did not have any influence on the production of hepatic necrosis.

Table 2.

Proximate Composition of Liver.

(Values are expressed in g./100 g. body Wt.)

	Group No.	Casein in Basal Diet Per cent	LIVER			
			Weight	Moisture	Total Lipids	Protein
Deficient in B_2 complex	I	18	4·54	3·10	0·47	0·87
	III	10	8·45	5·32	1·45	1·30
	V	8	8·00	5·15	1·16	1·25
	VII	5	6·31	4·05	1·15	0·94
Receiving Yeast supplement	II	18	3·85	2·75	0·17	0·79
	IV	10	3·82	2·81	0·19	0·73
	VI	8	3·10	2·22	0·14	0·61
	VIII	5	5·09	3·72	0·36	0·77

Table 3.

Effect of Protein level in the Diet on the Liver and Plasma proteins.

Group	Daily casein intake g.	Gain in weight g. (150 days.)	Liver	Water	Fat	Protein	Liver choline mg./g. of fresh liver tissue	Total Plasma Protein g./100 cc plasma
			(g./100 g. body weight.)					
(*a*)	1·2	132·5	3·24	2·28	0·17	0·65	2·14	6·83
(*b*)	0·6	112·0	2·87	2·04	0·17	0·55	1·89	6·11
(*c*)	0·4	103·0	3·16	2·19	0·31	0·51	1·43	5·52
(*d*)	0·2	50·2	4·16	2·81	0·48	0·65	1·55	4·81

In a somewhat similar experiment in our laboratory, the growth of the animals paralleled the daily casein intake. The animals receiving a maximum of 1·2, 0·6 and 0·4 g. casein daily were quite healthy and normal and there was no sign of vitamin deficiency in any case, whereas those receiving 0·2 g. casein showed considerable emaciation, loss of hair over the body and in general appeared weaker. There was no edema or ascites in any case. Liver weight per 100 g. body weight showed a definite increase as the daily intake of casein was reduced. The amount of liver fat per 100 g. body weight also showed an appreciable increase as the intake of casein was progressively reduced (*Table* 3).

The total plasma protein concentration shows a definite decline as the daily intake of casein is reduced and the liver choline content also shows a similar fall.

On microscopical examination, the liver in animals which received 1·2 and 0·6 g. of casein per rat per day showed no pathological changes. Animals which received 0·4 and 0·2 g. of casein per rat per day, on the other hand, showed slight fatty change of the hepatic parenchyma around the central veins. But, in marked contrast to the animals in the low protein groups which were deprived of vitamins of B_2 complex, none of the animals in this group showed marked fatty change in the hepatic parenchyma around the central veins, accumulation of ceroid pigment or diffuse hepatic fibrosis.

Effect of Choline and Methionine on the Experimentally produced Hepatic Lesions in Rat

Divergent opinions have been expressed on the value of choline and methionine in the prevention of cirrhosis of liver in rats. Radhakrishna Rao, Datta and Krishnan[10] have shown that daily supplement of 4 mg. of choline or 20 mg. of methionine is not sufficient to completely prevent the fatty change and the subsequent liver injury. However, 8 mg. of choline supplement daily resulted in almost normal livers when the fat content of the diet was 30 per cent.

Clinical Experiments

In addition to our experimental work, clinical treatment of decompensated portal cirrhosis cases with high protein, high caloric diets with vitamin supplements formed an important part of our studies on the 'cirrhosis of the liver' and this investigation was carried out in collaboration with Dr. N. J. Modi, in the medical unit of the Goculdas Tejpal Hospital, Bombay.

Twenty patients were treated with high protein diets which included skimmed milk powder, egg protein, fresh milk and casein hydrolysates. Vitamin supplements were given in the form of yeast tablets with injections of vitamin B_2 complex and liver extract

in some cases. The majority of the patients came from the poorer classes and their dietary histories revealed gross deficiency in calories, protein and vitamin intake. Ten out of the twenty cases gave history of having had attacks of malaria. Nine cases were addicted to alcohol. Past history of dysentery was present in four cases and that of jaundice in two cases.

The presenting symptom of all patients was enlargement of abdomen, varying in duration from 15 days to two years. All the cases showed evidence of fluid in the abdomen. Spleen was enlarged in eight cases and liver was found enlarged in four cases only. The level of serum proteins was estimated before, during and after treatment as this afforded an objective evidence of the course of the disease.

A common observation in all the patients before treatment was the fall in albumin and rise in globulin fraction of the serum proteins thus showing an altered albumin/globulin ratio from the normal. In the globulin fraction, the euglobulin showed marked increase (*Table* 4).

The period of observation during treatment was on an average 5 to 6 months. The results of therapy at the hospital have been quite encouraging. Patients treated with high protein diet showed definite clinical improvement. Ascites, edema and other manifestations of the disease disappeared in many instances. Clinical improvement was attended with a change in the serum protein level towards normal values.

Since liver is the main source of synthesis and supply of plasma phospholipids, it may be that in cirrhosis of the liver, there would be a corresponding decline in the plasma phospholipids according to the course and severity of the disease. However, analyses of the sera of cirrhotic patients do not show any significant difference in the major phospholipid component choline from the normals and other patients (*Table* 5). This limits the diagnostic significance of the choline content of the plasma in cirrhosis of liver[11].

Experimental Hepatic Necrosis

Several workers have studied the hepatic lesions resulting from feeding deficient diets to experimental animals and often produced fibrosis in the liver similar to that of human 'cirrhosis'. Himsworth and Glynn[8] could produce massive hepatic necrosis in rats by low protein diets. Subsequently it was shown that deficiency of cystine in such diets was largely the causative factor[12]. In the meantime, Schwarz[13, 14], Gyorgy[15] and others indicated the importance of tocopherol content of the dietary fat on the incidence of liver necrosis in animals.

In the early part of the study, attempts made to produce experimental hepatic necrosis in rats using Himsworth's 'yeast necrogenic diet' were not met with success. In these experiments, effect of tocopherol as also comparative effect of lard and hydrogenated vegetable fat (Dalda) was investigated. In one series of rats receiving

Table 4.

Pattern of Serum Proteins in cases of Cirrhosis.

Subjects	No.	Total protein	Albumin	Globulin	Euglobulin	Pseudo-globulin
		(g./100 c.c Serum)				
Normals	22	6·90	4·57	2·33	0·46	1·87
Adult Cirrhosis	32	6·12	2·34	3·78	1·36	2·42
Juvenile Cirrhosis ..	29	6·43	2·80	3·63	0·92	2·71
Other diseases	16	5·57	2·26	3·31	1·38	1·93

Table 5

Choline content of the Serum in normals, Cirrhosis of the liver, and Anaemia.

Description	Number of Cases	Choline in mg. per 100 c.c.
Normal subjects	17	19·77 ± 3·75 (13·48 — 26·90)
Adult cirrhosis	31	18·27 ± 7·64 (8·53 — 35·46)
Infantile biliary cirrhosis	7	16·38 ± 6·33 (9·54 — 25·07)
Anaemia and malnutrition	15	14·60 ± 2·85 (9·50 — 20·19)

tocopherol with necrogenic diet, glutathione was also estimated in livers since it plays an important role in the oxidation-reduction metabolism. The tocopherol supplement appeared to have no influence in controlling the level of glutathione in the livers of rats on such diets.

Radhakrishna Rao[16] had pointed out the different factors including strain variation of the rats which might be responsible for these discrepancies. In view of this, the earlier tests were repeated using the Haffkine Institute inbred strain and the Wistar strain of rats obtained through the courtesy of the Tata Memorial Hospital, Bombay. Both strains of rats including control groups were kept on test diets for 150 days; however, no necrosis was produced.

It was considered that the inability to produce massive necrosis with yeast diets might possibly have been due to the presence of small amounts of cystine in the yeast used for the experiments. In order to eliminate this factor, a fresh experiment was planned in which hydrogen peroxide treated yeast was used. The animals were on this diet for over a period of 60 days, yet none of the animals which died or killed showed any signs of hepatic necrosis, both macroscopically and microscopically.

In the meantime, McLean and Beveridge[17] suggested that experimental hepatic necrosis can be produced in rats only at an optimum level of yeast, both higher and lower levels of yeast disfavouring the production of the hepatic lesions. In order to ascertain whether there is any optimum level of dietary yeast which produced necrosis in the livers of experimental animals, fresh experiments were carried out, in which young male albino rats were fed 'yeast necrogenic diet' with yeast levels varying from 20 to 60 per cent. The animals were killed 60 days onwards at regular intervals and the livers studied histologically.

The liver sections of animals fed 60 per cent yeast showed normal lobular pattern, the parenchymal cells showed normal histological appearances. Occasionally there were traces of fatty infiltration of the parenchymal cells. When the yeast level was at 50 and 40 per cent the liver sections showed similarly occasional fatty change in the parenchymal cells and vacuolation of the cytoplasm. At 30, 25 and 20 per cent yeast levels, the liver sections showed massive haemorrhagic necrosis of the parenchymal cells. The sinusoids were dilated and engorged with blood. In severe cases, only very small patchy islands of parenchymal cells were seen around the portal tracts.

The production of massive hepatic necrosis (*Figs.* 1 and 2) regularly in rats at lower levels of yeast feeding viz. 25 and 20 per cent

FIGURE 1

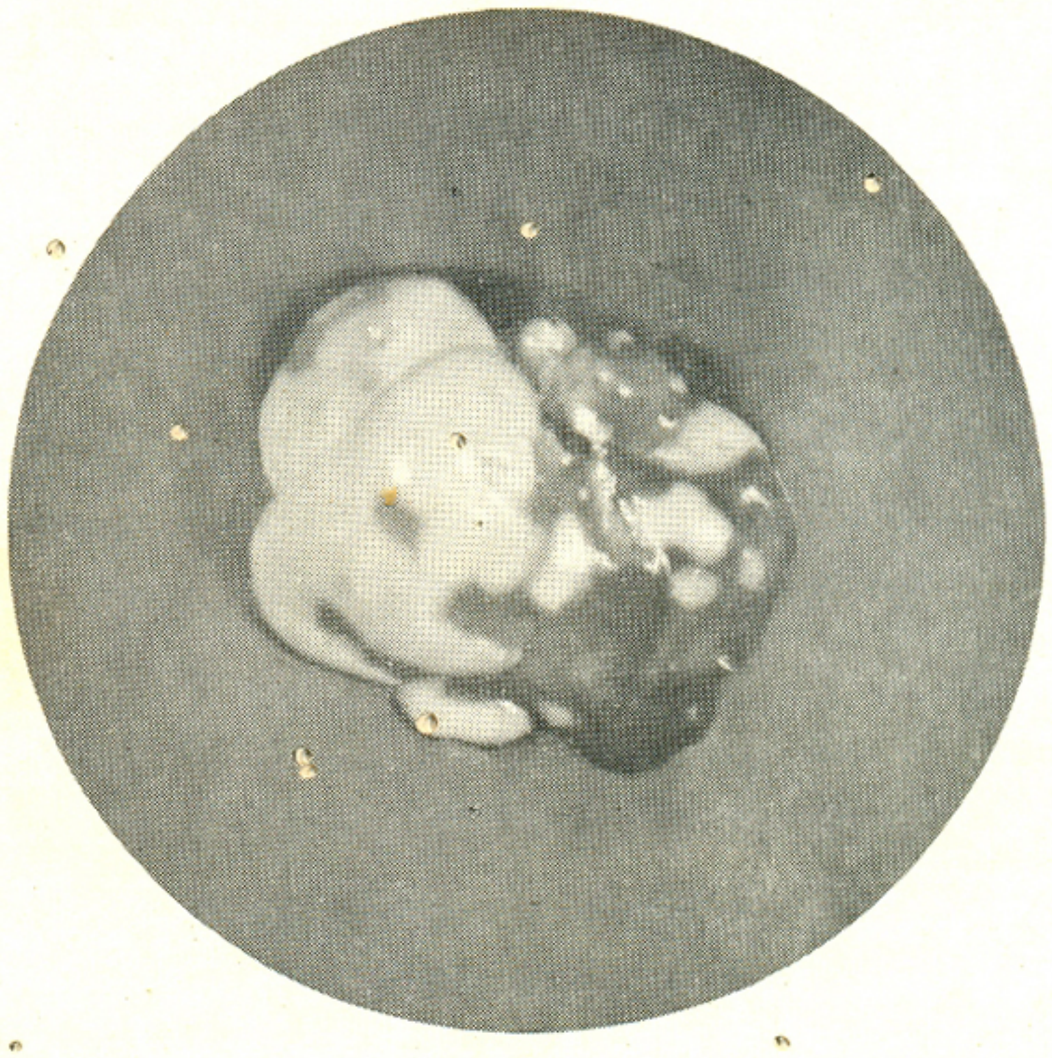
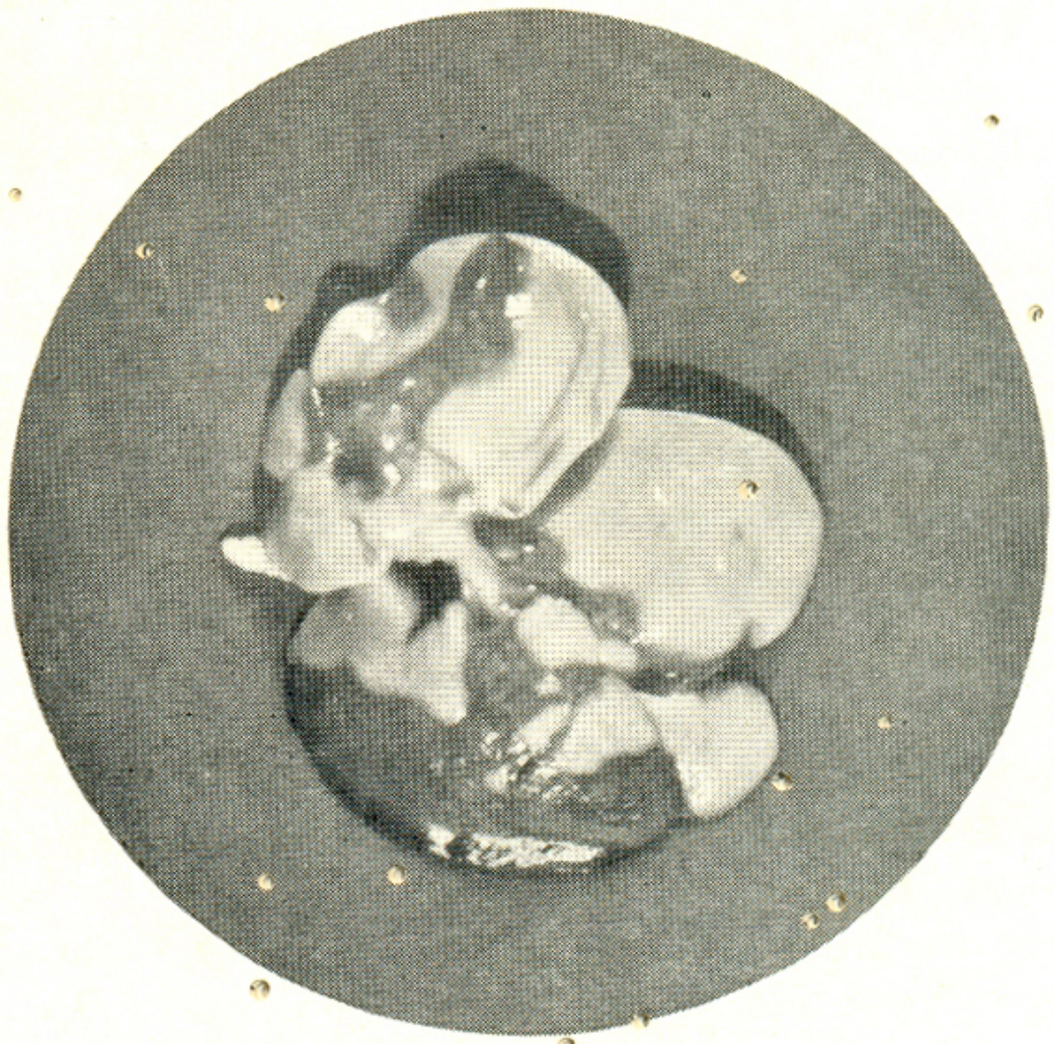


FIGURE 2



Dorsal and ventral views of liver in an experimental rat fed ' yeast necrogenic diet '

was subsequently confirmed[18]. These findings are in agreement with those reported by Himsworth and Glynn[8].

To investigate the possible metabolic changes taking place in livers at the onset of the disease, some biochemical estimations were undertaken. To begin with, water, nitrogen and alkaline phosphatase levels of livers — necrotic and normal — were determined.

The rats maintained on both the 'yeast necrogenic diet' as well as the control diet grew at suboptimal rate, the average increase in weight at the end of two months being 34 g. and 53 g. respectively. The rats on the test diet developed hepatic necrosis between the period 60-165 days and was uniformly found in all the animals fed the test diet. It was observed that the necrotic livers were heavier than the control, the average liver weight per 100 g. body weight being 5 g. in the former while it was 3 g. in the latter. This is evident from the water content values which are 78·4 per cent and 69·5 per cent in the necrotic and the non-necrotic livers respectively. The estimations of nitrogen showed about 19 per cent decrease in the necrotic livers as compared to the control. The alkaline phosphatase activity of the necrotic livers showed considerable increase in comparison to that of the livers from the control animals. The average phosphorus liberated in two hours by one gram of the necrotic liver was 5·5 mg., while it was 1·1 mg. in case of the non-necrotic liver.

Similar experiment to produce massive hepatic necrosis in young mice was carried out. The animals were kept on test diet for 180 days and sacrificed thereafter. The livers appeared to be normal both macroscopically and histologically.

Influence of Fermented Foods on Fatty Infiltration of the Liver in Rat

It is generally known that the people who take low protein high carbohydrate diets take numerous foods and beverages which undergo fermentation at some stage or other in their preparation, and it has been widely speculated that micro-organisms might contribute appreciably to a restricted dietary[19, 20]. Fermented foods may also lessen the strain put on the digestive enzymes of the body, by dismutating the macromolecules of the diet to more assimilable forms; thus improving the efficiency of utilisation of foods in digestive disturbances as found, for example, in Kwashiorkor. Indian dietaries include fermented foods in one way or the other.

Experiments were, therefore, carried out in which the effect of ingestion of *idli* (fermented preparation of black gram and rice) at 50 and 100 per cent replacement of dietary casein protein on the prevention of experimentally produced fatty infiltration of the liver by high fat low protein diet was observed.

These tests clearly indicated the beneficial effects of *idli* in preventing the deposition of fat in the livers, particularly when it was fed at 50 per cent replacement (*Table* 6). Similar effect was not so pronounced in the 'curative' groups. This probably suggests that the period (60 days) for which the

Table 6.

Effect of Idli and the Corresponding Unfermented preparation on the fatty infiltration of Liver in Rats when fed at 50 per cent replacement of Casein Protein on dry weight basis in High Fat Low Protein Diet (preventive tests only)

Group	No. of rats	No. of Days	Gain in weight g.	g. per 100 g. of the body Wt.		
				Liver Wt.	Liver fat	Liver protein
Control	6	60	32·9	11·06	3·3	1·14
'Unfermented preparation' ..	12	60	43·0	8·57	1·83	1·01
Idli (Fermented preparation) ..	12	60	58·0	6·55	1·02	0·84

animals were kept on *idli* in the latter case, was perhaps not enough to clear off the already deposited fat.

Histological examination of liver sections of control animals showed moderately severe fatty infiltration leading to fibrosis in few cases. Rats on unfermented preparation showed fatty infiltration but no fibrosis of the liver. In the case of *idli* group, liver sections showed normal lobular pattern with only occasional fatty infiltration of the parenchymal cells here and there. Thus, the histological findings are in good agreement with the biochemical observations. Further work on the lipotropic property of *idli* is being pursued in our laboratory.

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SEROLOGICAL STUDIES IN PLAGUE —ANTIGENIC STRUCTURE, SERODIAGNOSIS AND SEROTHERAPY

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VERY early in the development of our knowledge about infection and resistance, the investigators began to study the reactions which occur when blood, serum or other body fluids are allowed to react in the test tube with bacteria or their products and it was soon realized that persons after recovery from an infectious disease or even during their suffering develop certain properties in their body fluids which are capable of giving specific reactions with the corresponding infection (microbes) or their products which were termed as bacteriolysis, agglutination, precipitation, complement fixation reactions etc. These properties could also be produced artificially in man and animals by using live, attenuated or killed organisms or their extracts. These are the serological methods which, since their discoveries in latter part of the 19th century have been extensively used for diagnostic, therapeutic, and preventive purposes in most of the infectious diseases including plague.

The most effective application of these principles and the specificity of reactions largely depend upon the detailed and accurate knowledge of the antigenic structure of the particular organism. Thus before dealing with the serodiagnosis and serotherapy of plague it would be worthwhile to briefly review the advancement in knowledge of its antigenic structure.

Antigenic Structure of *Pasteurella pestis*

The search for the specific substance which is responsible for stimulating the protective mechanism in the animal system resulted in the finding that microbes often contain multiple antigens which chemically may be proteins, polysaccharides, lipoids or a combination of these substances. Only one or a combination of them are involved or capable of inducing protective antibodies in a particular microbic infection. The plague workers were fortunate in having some laboratory animals, particularly white albino mice and guinea pigs, to be highly susceptible to plague infection which permitted to test the specificity of antigens of plague bacillus in a convenient manner. The results thus obtained are fairly applicable to human beings as well. It may be worthwhile to mention here that the plague workers prior to 1940 were facing certain difficulties in regard to the physical, chemical, serological and immunological properties of plague bacillus and its dissociants and related organism like *P. pseudotuberculosis*, due to divergence of observations made by them. The particular difficulty was in its serological behaviour and its differentiation with *P. pseudotuberculosis*. In fact, the success of field epidemiology and of the study of reservoirs during the interepidemic period largely depended upon the efficiency of methods for identifying and differentiating the two organisms.

Investigations made by various workers from time to time (Barber, 1912; Sokhey *et al*, 1937; Schutze, 1939; Bhatnagar, 1940; Jawetz and Meyer, 1944; Seal, 1950-53; Devignat, 1951; Burrows *et al*, 1956; Girard, 1952) have greatly clarified the position. The plague bacillus is now primarily divided into (1) virulent and (2) avirulent (*a*) protective and (*b*) non-protective strains based on the animal experiment. The virulence deteriorate quickly on artificial subculture, even spontaneously and under natural conditions. The author has recently shown that the plague bacillus isolated from live rats during interepidemic period and from partially immune rats are of low virulence or some time practically avirulent. Such low virulent organisms have also been reported by Pirie (1936), Meyer *et al* (1937) and Macchiavello (1942).

Serologically, Seal (1951) was able to differentiate virulent from the avirulent, non-protective plague and pseudo-tuberculosis organisms but no differentiation could be made between the virulent and avirulent protective plague strains except by animal test and possibly by the spectrographic readings of the specific soluble proteins isolated from the two organisms in a quartz ultraviolent spectrograph (Seal, 1947). Englesberg, Chen, Levy, Foster and Meyer (1954) stated that their studies eliminated the possibility of the existence of an antigen solely in virulent strains and concluded that virulence was determined by the quantitative relationship between envelope and toxin production. Recently, however, Burrows and Bacon (1956) by a special technique have discovered two additional antigens called V and W which can differentiate virulent from the non-virulent plague strains being present in the former and absent in the latter. The V antigen is relative to the property in virulent organism of resisting phagocytosis by the mouse polymorphs.

Previously, Schutze (1932) enunciated the concept of 'envelope' and 'somatic' antigens of plague bacillus on serological methods, and this was generally accepted by all subsequent workers but some of them assumed a third antigenic factor being present in the virulent organism and wanted to call the envelope antigen by the term capsular antigen. This antigen is thermolabile but fully protective against plague infection as shown by Kurauchi & Homma (1936) and other workers, while the somatic antigen is thermostable and shares immunogenic characteristics with *P. pseudotuberculosis* which according to Bhatnagar (1940) possess both group and type specific somatic antigens. Summarising the above findings the serological antigenic structure of plague and pseudotuberculosis organisms stands as follows:

Organism	*Antigens*				
P. pestis virulent.	Envelope	—	V. & W.	—	and common rough somatic.
P. pestis avirulent protective.	Envelope	—	....	—	and common rough somatic.
P. pestis avirulent non-protective.	Little or no envelope.	—	....	—	and common rough somatic.
P. pseudotuberculosis.	Flagellar	—	Smooth Somatic (*a*) group specific. (*b*) type specific.	—	and common rough somatic.

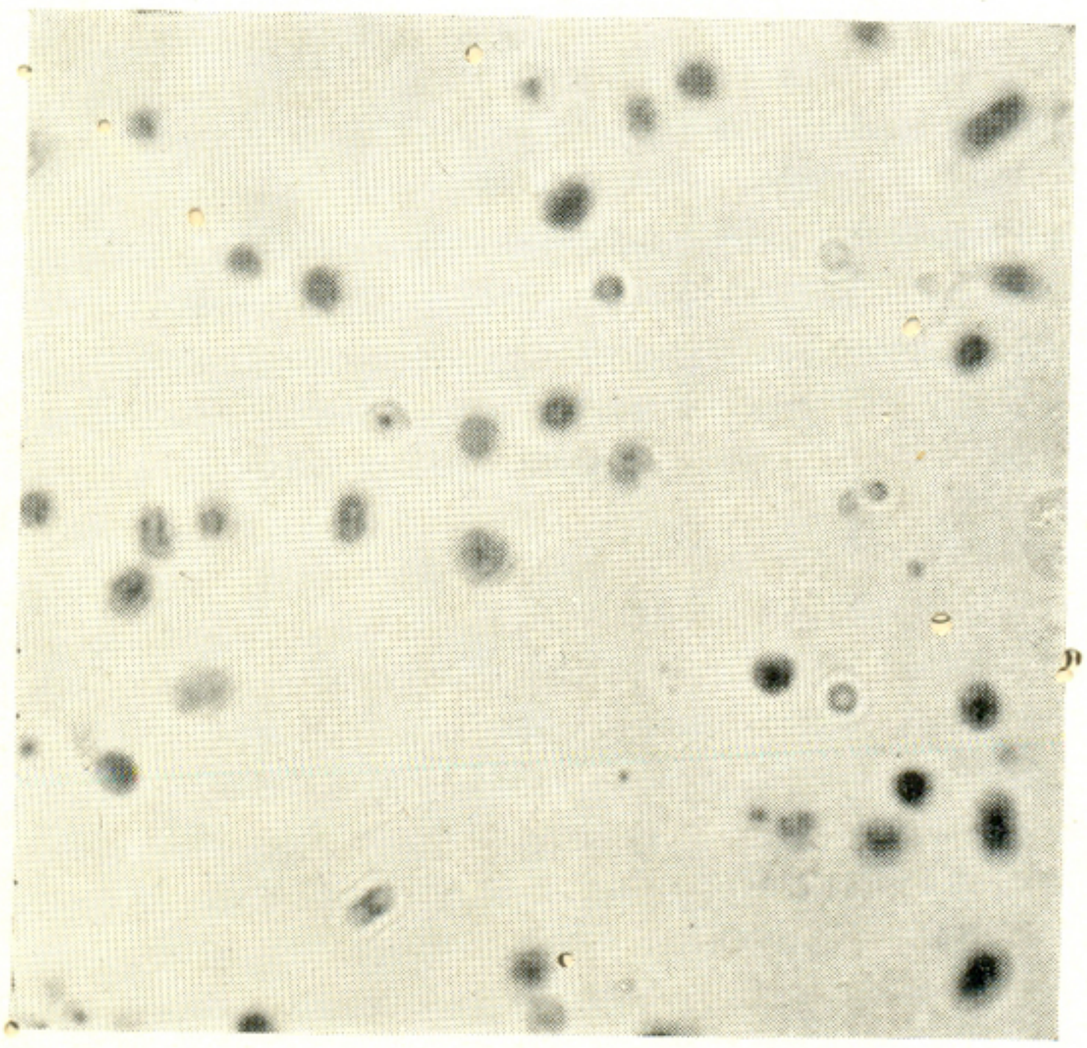
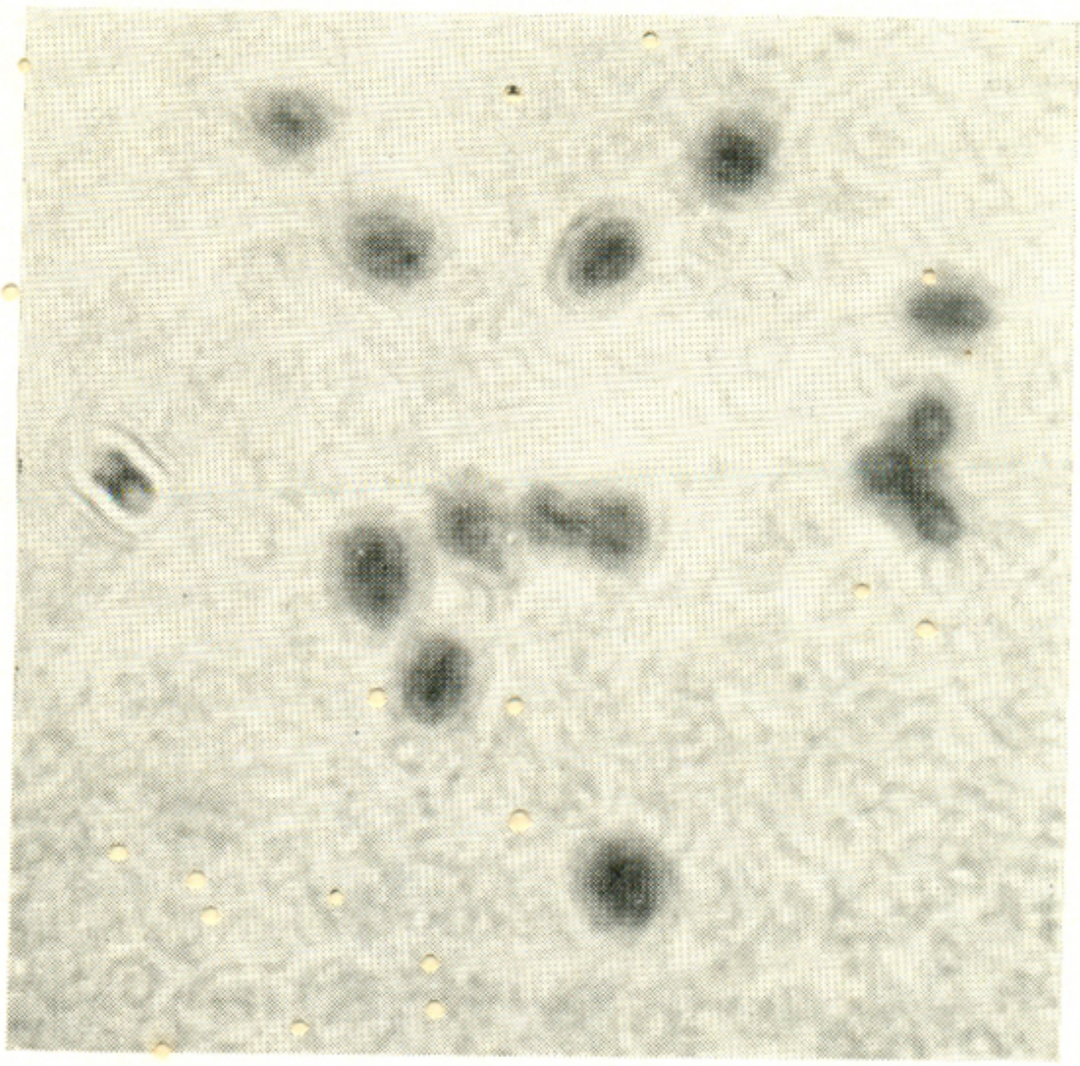


FIGURE 1 : Showing the capsule and the core of the organism and an unstained hallow around

FIGURE 2 : Showing the envelope and the core of the organism differentially stained by a special technique



Envelope and Capsular Antigen

As mentioned above, little controversy still exists in regard to the term "envelope" antigen. Kurauchi and Homma (1936) called it capsular antigen or the specific immunising fraction and Chertnik (1940) 'membrane antigen'. On the other hand, Rowland (1914) had postulated that the capsule was present under certain conditions only and that the slimy envelope, which was ordinarily not visible in unstained preparations could be demonstrated by the India-ink method. But in 1940 Sokhey concluded that the plague bacilli possessed a capsule under all circumstances and the envelope seemed to be only an unstained capsule plus a halo which was produced by a peculiar settling down of the particles of India-ink at a distance from the capsule due to the operation of physical forces. Again in 1951 Amies refuted this and with the aid of dark-field examination tried to establish that the so-called envelope of *P. pestis* was nothing more than a partially well-developed bacterial capsule. These contradictory observations seriously raised the question, whether the terms 'envelope' and 'capsular' are interchangeable or they are really different entities.

Seal carried out some intensive study on this problem and finally has been able to find out a clue to the existence of both envelope and capsule in a fully developed virulent *P. pestis* by an ingenious method of staining procedure (to be published shortly). The result is shown in the figures.

The difference is easily seen in photos shown above. Fig. 1 shows that the picture after the usual and Fig. 2 after the special method of staining. The latter clearly shows three distinct layers—the outer first an almost unstained envelope substance like membrane, the middle second thinly stained layer which is ordinarily considered as envelope or capsule and an inner third deeply stained core in the centre. It thus appears that the capsule is constantly present but its extent may vary according to the environment in which it is grown or placed, as postulated by Rowland and many others and lately by Burrows and Bacon (1956). Thus grown in a suitable environment the envelope is present in virulent as well as in avirulent protective plague strains like Tjiwidej, EV and 53/H av etc. but it is absent in avirulent non-protective and pseudo-tuberculosis strains and necessarily it is not associated with virulence.

Toxin of the Plague Bacillus and Enzyme Studies

Most workers are convinced that the plague bacillus has an endotoxin as originally suggested by Rowland (1910) and is associated with the soluble protein of the bacillus set free in broth culture by disintegration or extractable from the bacillary suspension. A few workers also suggested that the toxin of *P. pestis* might represent a mixture of metabolic products of living bacteria and of endotoxic substance set free after disintegration. There is no doubt that clinically the patients suffering from plague infection show various degrees of toxaemia. However, the work of Girard (1941) and Girard and Sandor (1947) shows that unlike the endotoxins of other gram-negative organisms, plague endotoxin is similar to exotoxin, being thermolabile and convertible into toxoid. Various methods have been used by different workers to isolate this endotoxin viz., old autolysed culture in broth containing normal horse serum, nucleoprotein extracts, freezing and thawing, precipitating water-soluble fraction at 0·55—0·67 saturation of $(NH_4)_2SO_4$ etc.

According to Jawetz and Meyer (1944) the agent at work might be of an enzyme nature. In this connection Rochenmacher (1949) claimed that the catalase activity of virulent strains was greater than that of avirulent growths. This brings us to the question of whether virulence was associated with the enzymic make up of the organisms. A group of workers under Dr. Shrivastava, namely Sagar, Agarwalla, Srikantan and Saxena (1956, 1957) have studied (1) deamination of aminoacids, (2) alkaline phosphatase activity, (3) oxidation metabolism, (4) transamination reaction and (5) dehydrogenases in both virulent and avirulent strains of *P. pestis*. In addition to

the above they also studied the action of certain antibiotics and sulpha drugs on the oxidative metabolism and transaminase reactions. In all these activities the optimum temperature was found to be 37·5°C and cells grown in broth showed greater deaminase activity than those grown on solid medium, whereas the maximum phosphatase activity was obtained in casein or papain digest agar. No correlation was found between these two enzymic activities and virulence. Similarly the rate of consumption of oxygen was also not found connected with it.

A comparison of the transaminase activity using L-alanine, DL-aspartic acid and amino donors showed no general correlation between virulence and transamination, though all the strains were capable of catalyzing these reactions significantly. Inhibition studies proved achromycin, aureomycin and terramycin to be potent inhibitors of the succinate dehydrogenase in *P. pestis*, whereas chloromycetin was moderately effective and dihydrostreptomycin and neomycin were almost without any action. On the whole, it appears that enzymic studies did not give any clue to the difference in virulent and avirulent strains but might yield interesting results.

Reverting back to the question of endotoxin (Baker et al 1952) Fraction II of specific soluble protein of plague bacillus (isolated between 0·4 to 0·67 saturation of ammonium sulphate) contained the toxic fraction but the identity of this substance was not clear. A very useful work has been recently done by Ajl, Reedal, Durrum and Warren (1955) followed by further work by these workers (Ajl, Rust, Hunter, Woebke and Bent, 1958) and by Spivack and Karler (1958). While the former group used casein hydrolysate-mineral-glucose (CHMG) medium and the avirulent strains Tjiwidej, the latter workers employed either agar-grown acetone-killed dried bacilli or 6-8-day shake culture, autolysates of the virulent strain 195/P and the attenuated toxic strain EV76. Partial purification was initially achieved by fractionation with ammonium sulphate between 0·35 to 0·70 saturation and final purification by continuous flow paper electrophoresis or by electrochromatographic methods alone. In the former case the final product had an intraperitoneal LD_{50} of 0·7 μg for 16-20 g. mice and intravenous Ld_{50} of 0·2 μg. In the latter case the intravenous LD_{50} varied from 0·1 to 0·3 μg for toxin isolated from either virulent strain or the attenuated strain EV76. From the experimental results it appears that even this relatively pure toxin molecule may still contain portions, which are not necessary for toxicity. The toxin could be denatured by physical or chemical agent with decrease or eventual disappearance of toxicity, but the formalin-treated toxin retained its ability to react with its specific antiserum, while the enzyme-hydrolysis with trypsin, chymotrypsin and papain did not markedly affect the toxic or the serologic activity of the toxin even though a considerable liberation of free amino-acids occurred.

Chemical Antigenic Structure

In regard to the study of chemical antigenic structure the concerted attempts have been made during the last two decades. Among the earlier workers were included Lusting and Galeotti (1900), Rowland (1900), Brooks (1912) and Morison et al (1924). Their work sufficiently indicated that the plague bacillus was composed of at least two varieties of proteins one was soluble in distilled water or saline and contained the immunizing and toxic substance and the other was insoluble in water and salt solution and had no specific immunizing properties. Following the early attempts of Shrivastava (1937) several workers, namely Seal (1943, 1953, 1956), Baker et al (1947, 1952), Amies (1951) and Bhagavan et al (1955, 1957) have greatly advanced our knowledge on the chemical antigenic structure of the organism, using different methods for isolation and purification. Jawatz and Meyer (1943) proposed that an antigenically complete strain contained an envelope antigen, a somatic antigen, a virulent factor (vi) and an antigen (avi) responsible for the immunigenic efficiency of some strains and that the difference with other dissociants was only quantitative. Later, Baker et al (1947) isolated three antigens IA, IB and II from

water soluble portion of the acetone dried plague cultures from hormone agar solid medium and the water insoluble portion as residue. The first two fractions were capable of inducing immunity in mice, white rats and monkeys but not in guineapigs and the immune serum thus produced was capable of neutralising plague toxin. The fraction II obtained from the supernatant left over after extracting the fractions IA and IB contained plague toxin. Except for the carbohydrate moeity fraction IA could not be differentiated from IB, which, however, was shown serologically related to Fraction II.

The author obtained five fractions — A, B, A & B combined, fraction obtained by raising the concentration from 0·5—1·0 saturation of Na_2SO_4 and the residue from the supernatant of plague vaccine prepared in enriched casein hydrolysate broth. Antigen A was found to be specific for virulent and avirulent protective plague strains and did not react with pseudo-tuberculosis and was fully protective to mice and rats in amounts equivalent to the yield from the dose of vaccine giving the same degree of protection. Antigen B was non-protective and acted as a common somatic link between the plague and pseudo-tuberculosis organisms. A polysaccharide yielding osazone resembling that of arabinose (Seal, 1951) was found associated with fraction A but was absent in fraction B and avirulent protective plague as well as pseudo-tuberculosis strains.

The ethanol purified fraction of Amies was single protein and is supposed to be highly immunogenic but was devoid of the carbohydrate moeity. Bhagavan et al, on the other hand, utilized the gel diffusion precipitation test. According to them the supernatant of plague vaccine consists of 3 antigens in large amounts and 4 antigens in small amounts. Of the 3 fractions A, B and C, A & B are highly antigenic and protective for mice while fraction C is inactive. Fraction A did not precipitate with anti-pseudo-tuberculosis serum, while fractions B and C reacted strongly.

In practice there is a good amount of uniformity in the findings of the above workers on the basic antigenic structure of plague bacillus and its dissociants in spite of the apparent diversity. Probably the difference noted was due mainly to the materials and methods used. The PD of the fractions A & B of Bhagavan et al were 8 and 8·3 micrograms against a challenge dose of 160 MLD., that of Baker et al 12 to 22 microgram against 100 MLD. and that of Seal 0·6 to 2·5 microgram against 13 MLD.

The author recently tested his antigen A and Baker et al's antigen I (combined IA and IB) against a challenge dose of 5,000 virulent *P. pestis* (195/P) in *R. rattus* and *B. bengalensis*, caught from the city area of Calcutta. The former proved somewhat better than the latter so far as the test rodents were concerned. The results are given in Table 1.

Table 1.
Results of immunisation with different protein fractions of *P. pestis*.

Antigen	Immunising dose*	Animals immunised	
		R. rattus survivals	*R. bengalensis* survivals
Antigen A (Seal)	0·1 mg.	19/20	18/20
Antigen I (Baker et al)	0·1 mg.	16/20	14/20
Antiplague vaccine prepared in enriched C.H. broth containing 1,000 million organisms per ml.	0·4 ml.	10/20	9/20

* Given in 2 doses at week's interval.
N.B.—Challenge dose = 5,000 virulent *p. pestis* 195/P.

It appears that fractions A of Seal, IA and IB of Baker et al, A of Bhagavan et al and the fraction of Amies are all obtained from the envelope and/or capsular antigen of *P. pestis*, as all of them have suggested. All these fractions are equally specific and highly protective against virulent plague infection. In fact, Baker et al themselves suggested that Seal's antigen A is a mixture of IA and IB of theirs, IA having the carbohydrate moeity as in Seal's antigen A and IB and Amies's fraction being the same as IA without the carbohydrate moeity. Amies had therefore commented that not much importance should be given to this apparent discrepancies. What is needed is to evolve a standard technique for mass production of this specific antigen for human immunisation.

Importance of the growth medium

The author (1950) had shown that the nature and the standardisation of the medium was an important factor as the yield and quality of the specific antigen depended upon the nutrition of plague bacillus and also on the temperature of incubation. According to him the protein-free enriched casein hydrolysate broth is superior to the solid media containing agar. It yields all the soluble antigens including the toxin while the agar medium may not give the maximum yield of all antigens and the suspension is required to be made agar free, before extracting the antigen.

Serodiagnosis

Agglutination.—The problem of identification of *P. pestis* of various types and of the serodiagnosis of plague infection in man or animal by means of agglutination test was fraught with difficulties for three main reasons, viz., (*i*) plague strains often form unstable suspension in normal saline, (*ii*) the antiplague serum raised against the whole organism reacts with both plague and pseudo-tuberculosis organism and (*iii*) the agglutination titre of the same organisms incubated at different temperatures differ against the same antiserum.

Different workers in the past tried and suggested various methods but none were found fully satisfactory. The author made an extensive review of this problem in 1951 and reinvestigated the issue by carrying out straight and cross agglutinations and absorption and cross-absorption of various agglutinins raised by him against antigens grown in different media and prepared in different ways, and evolved techniques which gave quite satisfactory results. In this connection he found that the preparation of antigen suspension, antiserum and the actual technique of the test were all of equal importance.

Antigen Suspension.—The best and stable antigen suspension is prepared in the following manner: The organism is grown on 5 per cent rabbit-blood agar for 24-48 hours (preferably 24 hours) at 37°C and the culture is withdrawn with a loop without disturbing the agar surface and slowly emulsified in normal saline upto No. 2 opacity of Brown's tube for plague and No. 3 for pseudo-tuberculosis organism, and the suspension shaken by gentle strokes over the palm of the hand. If 48 hours culture is taken, the emulsion so made as above may be better filtered through a light piece of sterile cotton wool. This forms a good stable suspension and is used in live state usually, but a drop of formalin might be added also. It should, however, be mentioned that strains of all categories do not always make an ideal suspension, as TRU, PM and pseudo-tuberculosis strains which usually form rugose colonies even on blood agar. This difficulty can, to a large extent, be overcome by using cultures of 18 to 24 hours only.

Agglutinating serum.—For the production of agglutinating serum in rabbits against the whole organisms, live suspension prepared as above is used, initially by treating with formalin, followed by live organism and careful dosing, irrespective of the type of organism—virulent or avirulent. Antisera against specific soluble proteins are also used for agglutination test. This antiserum may either be produced by injecting purified protein antigen A of Seal or IA of Baker et al or by the water-extracted protein solution of virulent plague strain obtained by suspending the organism in distilled water at concentration of 500 million organisms per ml. and incubating it for 14 days and then making it free from bacterial debris by filtration.

Technique of Agglutination Test.—To various dilutions of antisera placed in uniform bore Felix tubes equal amount of suspension is added and each tube gently shaken. The mixture is incubated at 37°C overnight and the readings taken next morning (A preliminary reading may be taken 2 hours after incubation). The highest dilution at which the agglutination is visible to the naked eye is recorded provided the normal serum and normal saline controls are negative.

The agglutination reaction is of two types, *viz.* (1) floccular or woolly and (2) granular. The former is related to the surface and protective antigen which give comparatively low titre agglutination and the latter is related to the somatic antigen which generally gives high titre agglutination.

Specificity of Agglutination Test.—By using three antisera, namely (1) against fraction A or water-extractable protein of virulent *P. pestis*, (2) against *P. pestis* suspension boiled for ½ hour and (3) *P. pseudotuberculosis* antiserum absorbed with *P. pestis* live or boiled, a serological distinction between *P. pseudotuberculosis* and virulent and avirulent protective plague strains can be made, the last antiserum being useful in distinguishing pseudotuberculosis strains from avirulent non-protective plague strains which is often found in chronic or mitigated plague in rats and as such need to be differentiated from the former. The serological relationship between the above organisms is shown in Table II.

For diagnosis of suspected cases of plague or retrospective diagnosis of human plague, slide agglutination might give quick results but the tube agglutination test as described earlier is the best. The author had the opportunity of testing 26 bacteriologically positive cases, 56 clinically suspected cases and 55 non-plague cases in 1949 in connection with the Calcutta epidemic. The results are given in Table III.

It will be seen that the agglutination test gives a fairly reliable result. It may be mentioned that this was obtained in spite of sulphonamide or streptomycin treatment. No positive serum was tested earlier than 7 days after the onset of symptoms.

Haemagglutination.—Haemagglutination may be performed as an indirect method of agglutination by absorbing the organisms to erythrocytes of human, sheep or guinea-pig. In the opinion of Amies (1951) this test was unlikely to be of much value as a diagnostic procedure because of low development of immunity in plague but perhaps it might be useful in retrospective diagnosis of plague

Table II.

Serological relationship between *P. pestis* and *P. pseudotuberculosis*.

Antisera produced against	*P. pestis*		*P. pseudotuberculosis*
	Virulent and avirulent protective	Avirulent non-protective	
(1) Virulent *P. pestis*.	+	+	+
(2) Virulent *P. pestis* and absorbed with P. pseudotuberculosis.	+	0	0
(3) *P. pestis* boiled for ½ hour.	0	+	+
(4) *P. pseudotuberculosis*.	0	+	+
(5) Water-extractable protein of *P. pestis* (virulent).	+	0	0
(6) P. pseudotuberculosis and absorbed with *P. pestis* boiled.	0	0	+

Table III.

Results of agglutination test in bacteriologically positive and suspected human cases of plague 1949.

Nature of cases	No. tested	Date of collection	Date of examination	Positive	Per cent	Agglutination titre
1. Bacteriologically positive.	26	7–40 days after onset.	26 to 133 days after onset.	23 (3 doubtful).	88·4	1/10 to 1/200
2. Suspected cases bacteriologically negative or not done.	56	6–36 days after onset.	24–134 days after onset.	5	9·0	1/10 to 1/100
3. Non-plague cases.	55	....	....	2 (both inoculated.)	3·6	1/25 and 1/50

to confirm clinical diagnosis. Chen (1952), however, noted that the antigen responsible for haemagglutination though present in old broth culture of *P. pestis* as well as in the extracts of killed and dried plague bacilli, was absent in the protein fraction obtained by precipitation with ammonium sulphate, the nature of the substance being a polysaccharide.

Precipitation.—In the serodiagnosis of plague infection, precipitation test could not be made popular for want of proper antigen. The isolation of specific soluble protein fractions by various workers described above opened up the prospect of utilizing this method for (*i*) serodiagnosis of plague infection in human beings and rats, (*ii*) identification of plague strains and (*iii*) quantitative estimation of potency of antiplague serum. Recent work of Ajl et al (1958) and of Spivack and Karler (1958), on plague toxin has the potentiality of estimating the antitoxic value of an antiplague serum.

Technique of Precipitation Test.—For serodiagnosis of plague infection by precipitation test, the following method was utilized by the author. The specific protein antigen A diluted to contain 0·1 mg./ml. (1 : 10,000) is distributed in Felix tube in 0·5 ml. amounts. Patient's sera, diluted 1 : 10 upwards are added in 0·5 ml. amounts. The tubes are shaken and incubated at 37°C for 2 hours. A preliminary reading is taken and the tubes are placed in the refrigerator for the final reading next morning. The highest dilution showing visible precipitates against a reflected light is taken as the titre of the reaction.

Ring Precipitation Test.—For quicker diagnosis, ring precipitation test may also be done in the following manner: The antigen A 1/1000 dilution is taken in Dreyer's tube and the patients' undiluted serum or 1 : 2 to 1 : 10 dilution are added on the top of the antigen by means of capillary pipette. A precipitating ring develops in $\frac{1}{2}$ hour at room temperature. The filtrate of casein hydrolysate broth inoculated with the unknown organism or the water extractable filtrate of distilled water suspension of the unknown organism may also be used for both ring and tube precipitation test.

For the Rapid Diagnosis of Rodent Plague.—Cambosu (1938), Tumansky (1939), Menezes (1941) and Larson et al (1951) used precipitation test for diagnosis of rodent plague. The antigen is prepared from the tissue suspensions of animals which died of suspected plague and sterilized by diethyl ether and is tested against a high titre antiplague serum. Precipitation test with antigens prepared from such tissues kept at 37°C for as long as 14 weeks also showed positive result.

Quantitative Precipitation Test.—The method of quantitative precipitation test with the specific soluble substance has been utilized by the author (1943, 1954) and by Silverman et al (1952) for estimating the potency of therapeutic antiplague sera, by Heidelberger's technique through estimation of precipitated antibody nitrogen, at the zone of equivalence. The test is performed at $0 \pm 4°C$ and incubated at the same temperature for 48 hours. The author has also suggested a tentative mouse-protective Unit after statistical evaluation of the data so that the method can be adopted as an official method of standardisation of antiplague serum whenever such a need is felt. The advantage of this method is that it is done in vitro and the result can be obtained within 48 hours whereas the biological method developed by Sokhey for testing the potency of antiplague sera is time-consuming and is often subject to variation due to strain virulence, mouse susceptibility and facilities available in the Laboratory concerned.

Complement Fixation Test.—Several investigators (Moses, 1909; Damperoff, 1910; Joltrain, 1920; Simard, 1921; Dickie, 1926; Mitin, 1937 and others) employed complement fixation test with either bacillary suspension or extracts as antigen, but the results were not always satisfactory. Greval and Dalal (1933) observed specific graded results using filtered supernatant of Haffkine plague vaccine against antiplague sera produced in sheep, buffalo or bullock, but Wats and Wagle (1935) did not find this technique sensitive enough. The latter used phagelysed growth of *P. pestis* in papain-digest broth with no better results.

The isolation of specific soluble fractions by the author and Baker et al and others greatly facilitated the performance of complement fixation test. With the help of this antigen and a high titre antiserum against this antigen in rabbits, it has been possible for the author (1953) and Chen et al (1952) to:

(1) determine the evidence of plague infection in the tissue extracts of animals died of suspected plague, and

(2) detection of antibodies to fraction A or I in the sera of human convalescents, and immunized man and animal.

According to Chen and his co-workers this method of complement fixation test, besides being of value for retrospective diagnosis of human plague, it is apt to be of importance for the field diagnosis of wild rodent plague especially "when the isolation of *P. pestis* or the interpretation of the pathological lesion at autopsy is rendered impossible by contamination or decomposition."

Using the specific antigen it has also been possible to differentiate between *P. pestis* and *P. pseudotuberculosis*, and to detect the antigenic deterioration of plague strains by cross complement-fixation test.

Technique of Complement Fixation Test.—Antigen A is diluted to 1 : 4000 or 1 : 5000 in 1 per cent NaCl solution. The test sera are inactivated by heating in water bath at 54°C for 10 minutes. Fresh complement is obtained by bleeding guinea-pigs each day and is used after preliminary titration.

Antibody Titration.—In a series of tubes are placed 0·25 ml. of various dilutions (1 : 4 to 1 : 200) of inactivated antiserum, followed by 0·25 ml. of 1 : 4000 to 1 : 5000 dilution of antigen solution and 0·25 ml. of $1\frac{1}{2}$ - 2 M.H.D. of complement. The contents are then mixed well by shaking and the tubes incubated at $0 \pm 3°C$ for half an hour and at room temperature for half hour and then at 37°C for half hour. The sensitized cells, prepared by the method of Greval (1946) were then added in 0·25 ml. amounts, the tubes shaken and incubated at 37°C for half hour. After a preliminary reading the tubes are left in the refrigerator overnight for the final reading next morning. Each series of test is to be accompanied by serum, antigen, serum and antigen and antigen and sensitized cells control. Complement fixation is indicated by ++++ or '4' and complete haemolysis by 'O' and partial fixation by +++ or '3', ++ or '2' and + or '1' according to the shades of haemolysis, better estimated after the tubes are held at $0 \pm 3°C$ when the haemolysed cells settle at the bottom.

For testing antibody in human sera higher antigen dilution to the extent of 1 : 10,000 to 1 : 20,000 is sometimes necessary. The sera of 30 convalescent patients of confirmed plague infection tested by this technique in 1949 gave 83 per cent positive results.

Flocculation.—Advantage has also been taken of the specific plague proteins in developing a flocculation technique for estimating the potency of antiplague sera. Based on constant serum and variable antigen method between Antigen A and the various horse and rabbit antiplague sera, the author succeeded in establishing a flocculation test, the flocculation appearing in 5 to 15 minutes at 45°C. A preliminary assessment of L_f values of the antiplague sera compared very favourably with the corresponding observed precipitation Nitrogen values ($r = 0 \cdot 9843$ — highly significant). It was also proposed that 1 L_f unit to be equivalent to 1 mg. of specific protein, calculated from the optimum proportion figures of antigen and antiserum at which the first flocculation was observed. This flocculation method can now serve as the rough and ready in vitro test for the titration of antiplague serum. Perhaps this technique would also be useful in titrating the antitoxic potency of such sera.

Among other workers Girard (1941) tried to develop a diagnostic procedure by mixing 5 drops of patient's serum with 1 ml. of plague endotoxin. Flocculation took place in positive cases. Readings were taken after 30 minutes, 3 hours and 24 hours.

Serotherapy

Considerable difficulties were initially encountered in developing a potent and effective antiplague serum. The method of preparation was greatly improved between 1936 and 1939 at the Haffkine Institute, but by that time the sulpha drug was about to come. Even so, the serotherapy could not make much mark as differences existed in the potency of sera prepared by different methods in different countries and also due to the loss of potency for want of storage facilities and to the absence of a standard method of assay of those sera. Further, the observations made by workers in different countries from time to time could not be compared due to different kinds of recording and preservation of data or inadequacy of the number of persons treated and of equivalent control.

The criterion of success of serotherapy, according to Sokhey et al (1952) was the results of treating cases of bacteraemia. While better results were obtained in treating milder forms of bubonic cases, the success was poor in septicaemic cases and very disappointing with primary pneumonic cases, except for the occasional success reported by Wu Lien Teh (1926), De Moura et al (1936), Joltrain (1936) and a few others. In the earlier years of the last plague pandemic in India, Choksey reported about 60 per cent of success in non-septicaemic cases. This was, however, the only treatment of any worth available at that time. According to Meyer et al (1952), the pooled experience of the recent use of antiplague serum in the eastern hemisphere gives a fatality rate of 45·13 per cent in 3,840 treated cases against 83·5 per cent in 1,726 untreated cases. In the western hemisphere the fatality rate in 19,540 treated cases was 32·75 per cent against 74·07 per cent in 213 untreated cases. The latter data suffers from the defect of a small number of controls.

The most effective antiplague sera available recently are the ones produced against avirulent EV Madagascar strain and the Haffkine Institute plague serum. These have been used on a fairly large scale with better results. Girard (1941) and Le Gall (1943) reported about 60-65 per cent cure with the EV antiplague serum and Sokhey et al (1941 — 1948) 76·5 per cent overall cure in 157 cases treated with Haffkine antiplague serum only, but taking only 71 bacteraemic cases the rate of cure was only 49·3 per cent while only one death was registered among the remaining 86 cases. In another report of Sokhey quoted by Meyer (1952), the case fatality rate in 163 serum treated patients was 26·6 per cent against 63·7 per cent in 135 untreated patients during the same outbreak (1948). The summary of the results is given in Table IV.

Table IV

Summary of the reported results of treatment with antiplague serum (Meyer et al 1952.)

Area of place	Serum-treated cases		Untreated cases	
	Number	Fatality rate	Number	Fatality rate
1. Eastern hemisphere (India, Indonesia, Japan, Middle East, Africa and Madagascar).	3,840	45·13	1,726	83·5
2. Western hemisphere (North America, Argentina, Brazil and Peru).	19,540	32·75	213	74·07
3. Haffkine Institute (1941–1949).	320 (71 bacteraemic cases.)	25 (50·7)	135	63·7

The initial daily adult dose of the present serum varied between 40 ml. advocated by Sokhey et al in bubonic cases and 100 ml. by Lobe and Silvette (1941) given partly intravenously and partly intramuscularly. Le Gall (1943) used 40-60 ml of the serum prepared by EV strain. The serum was administered generally for two consecutive days for mild cases and up to five days in serious cases. It all depended upon the strength and quality of the serum. If the serum is potent enough, the serotherapy certainly influences the outcome favourably, particularly if the disease is not too far advanced.

Combined Sero and Chemotherapy.—Before the advent of the newer sulpha drugs and the antibiotics, plague workers were advocating combined sero and chemotherapy, particularly Sokhey and Wagle (1946) in whose hands the combined treatment yielded better results. Those who have handled the Haffkine Institute antiplague serum must agree that at least in one respect the serum can establish its utility. It reduces the toxic condition of the patient within 24-48 hours depending upon the intensity of toxic manifestation. The same was the experience of Wang (1942). Comparative results of combined sero and chemotherapy and chemotherapy or serotherapy alone in simple and plague septicaemic cases are shown in Table V A and B.

In both septicaemic cases and all cases combined, some superiority of combined treatment is noticed in the data given above.

It is time that in the overall experience of treatment with sulphadrugs like sulphadiazine and sulphamerazine and with antibiotics the results are even better than what is shown above. Early application of a good sulphadrug is generally enough but in serious cases, antibiotics and in pneumonic and septicaemic (toxic) cases, antitoxic serum with antibiotics should be the treatment of choice.

The author finally wishes to suggest that since there is no demand for serotherapy now, only antitoxic serum may be prepared against specific plague proteins and toxin in rabbits and the concentrated serum globulin may be preserved by lyophilization for emergency use.

Production of Antiplague Serum.—In 1931, Naidu et al used virulent plague bacilli to prepare antiserum in buffaloes and reported encouraging results in bubonic cases at Hyderabad. But it was not defined how many bacteraemic cases could actually be cured. In 1936 Sokhey & Girard utilized horses for serum production, as also Pirie & Grasset in 1938. The mouse protection dose of the new Haffkine antiplague horse serum was 0·05 ml. as against 0·30 ml. of buffalo serum of Naidu et al and 0·50 ml. of Lister and Pasteur Institute's sera (Sokhey, 1936).

Sokhey & Dixit (1940) claimed 83 per cent cure of experimental plague infection in mice. In 1941 the workers at the Hooper Foundation, San Francisco, carried out a comparative trial of sera produced in horse, rabbit and guinea-pig on mice according to a standard technique and came to the conclusion that, to be effective, antiserum should be administered early (Meyer et al 1952). The author (1942, 1943, 1953) noted better results with rabbit antiplague serum than with horse serum. This is supported by the observation of Meyer et al (1952) mentioned above. The most effective antiserum according to the author's experience was, however, the rabbit antiplague serum produced by injecting specific plague protein antigen isolated by him or Baker et al. The method of preparing antiplague serum at the Haffkine Institute at that time was as follows:—

The first three weekly injections consisted of live avirulent plague culture on agar slope at 37°C followed by three doses of the same culture intravenously and then by alternate subcutaneous and intravenous injection of live virulent plague culture at 37°C beginning with 3 ml. and rising up to 20-25 ml. of strength equivalent to Brown's capacity tube No. 10. Subcutaneous injection of 20 ml. Haffkine vaccine filtrate followed each two of these injections. The immunity level was kept up by injecting live EV or Tjs strains at intervals.

Table V

A. Results of treatment of septicaemic cases with combined sero and chemotherapy as against chemo or serotherapy alone:

Drugs and/or antiserum	Mild septicaemic cases			Severe septicaemic cases			All septicaemic cases		
	Cases	Survivors	Per cent Survived	Cases	Survivors	Per cent Survived	Cases	Survivors	Per cent Survived
Antiplague serum	29	22	75·8	42	13	38·0	71	35	49·3
Sulphathiazole	27	23	85·2	104	53	50·1	131	76	58·0
Sulphathiazole + antiserum	4	4	100·0	21	13	69·1	25	17	68·0

B. Results of treatment of all cases of plague with combined sero and chemotherapy against chemo or serotherapy alone:

Drug and/or antisera	Number of cases treated	Number survived	Percentage of patients survived
Antiplague serum	157	120	76·5
Sulphathiazole	345	266	77·1
Sulphathiazole and serum	60	48	80·1

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HUMAN HAEMOGLOBIN AND ITS VARIANTS

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THE impact of haemoglobin and its variations on contemporary medical sciences has indeed been remarkable. Ten years ago haemoglobin was considered to be more or less the same substance from one person to the other. Today at least twelve distinct variants of haemoglobin are known. The implications of these different haemoglobins have interested not only the haematologists and biochemists but also the anthropologists and the geneticists. Many excellent review articles bearing on the various aspects of abnormal haemoglobins and haemoglobinopathy have appeared during the last three years. [9, 19, 22, 40, 42] The present communication has been designed to give a brief outline of the problem with reference to the conditions prevailing in India.

Haemoglobin Molecule

Haemoglobin is a conjugate of the protein, globin, and the pigment unit, haem. Its molecular weight is 68,000. The haem was shown to be Ferroprotoporphyrin IX and its synthesis was accomplished by Fischer and Zeile in 1929.[31] Haem is thus a metal complex consisting of an iron atom in the centre of a porphyrin structure. The iron atom in haem has a co-ordination valence of six. Four are attached to the four pyrrole nitrogens. The fifth bond is supposed to be attached to a nitrogen of the imidazole group of the histidine in the globin, while the sixth one is held by an oxygen molecule. The amino acid composition of globin is now known. The amino acids of human globins in order of decreasing proportions are leucine, valine, aspartic acid, alanine, lysine, histidine, phenylalanine, glutamic acid, threonine, proline, glycine, tyrosine, arginine, tryptophane, methionine and cystine.[41] Globin constitutes about 96 per cent of the molecule, while the percentage of iron is 0·366. In a haemoglobin molecule four haems are attached to the globin.

Haemoglobin is present not only in man and animal but also in protozoa, in yeasts, in moulds and in the roots of leguminous plants. The haem-proteins, to use a more general term, are universally concerned in the respiratory activities of living cells. The oxygen capacity of a gram of haemoglobin is 1·34 ml. Haemoglobin unites with molecular oxygen in a reversible fashion and this is the basis of respiratory function of blood which has been studied intensively both from physiological and pathological aspects. In spite of these intensive studies, adequate and precise information on certain basic questions pertaining to its structure, its homogeneity, its relation to other components of red cells are not available. In short, final characterisation of the haemoglobin is yet to be achieved.

In the formation of haemoglobin, the biosynthesis of porphyrin and protein moieties are independent processes. The cleavage of haem from globin and their recombination *in vitro* was demonstrated by Hill and Holden

though there was slight difference between the reformed and original haemoglobins in the position of the Soret band and of the protein-tryptophan band. Jope, Jope and O'Brien were recently successful in obtaining crystalline methhaemoglobin and reduced haemoglobin by recombining native globin and haematin.[31]

The haem is identical in all species. The molecular weight of globin is variable in invertebrates; in vertebrates it has a uniform value. The nature of globin, however, varies from animal to animal. In the same species, there may be more than one type of haemoglobin (Hb.) with variable globin structure. In man, difference between foetal (F) and adult (A) types of haemoglobin is now well known. With the discovery of Hb. S and with the demonstration of its role in the causation of various clinical and haematological signs, Pauling very aptly advanced the concept of 'molecular disease'.[28] Sickle cell anaemia, first described in 1910 and since regarded as an instance of morphological aberration of erythrocytes, has now been proved to be due to an abnormal haemoglobin (Hb. S) which differs from Hb. A in many essential points. Two outstanding and easily demonstrable differences are in electrophoretic mobility and solubility. Recent work of Ingram[18] has unravelled yet another difference in the amino-acid composition of the two haemoglobins, A and S.

Anomalies in the Haem

Reversible oxygenation of Hb. is under physiological conditions independent of iron atoms which are present in bivalent ferrous form. Oxidation of ferrous haemoglobin to ferric haemoglobin (methhaemoglobin), will interfere with the easy exchange of loosely bound oxygen. Similarly when ferrous haemoglobin combines with carbon monoxide, carbon dioxide or hydrogen sulphide, oxygen dissociation curve would change with considerable handicap to the reversible oxygenation process. Clinical conditions of methhaemoglobinaemia and sulphhaemoglobinaemia result from such interference of normal oxygenation process. These disorders are mostly acquired due in main to the toxic effects of drugs like phenacetin, acetanilide, antipyrin and sulphonamides. Of considerable interest is the rare hereditary form of methhaemoglobinaemia where the normal mechanism for the reduction of methhaemoglobin is lacking. The exact cause of this anomaly is not known. In air, haemoglobin undergoes a slow spontaneous oxidation to methhaemoglobin; in the red cell, it is, however, maintained in the reduced state by an enzymatic system which utilises glucose as substrate. Reduced pyridine nucleotides generated from oxidation of glucose appears to reduce the methhaemoglobin. Rate of reduction of methhaemoglobin in the red cells of this disorder is lower than in normal cells. The disorder may be due to an inherited deficiency of the enzyme diaphorase which is normally concerned in the reduction of methhaemoglobin. This condition is also temporarily reversible by methylene blue and ascorbic acid. Horlen and Weber, however, encountered one family with hereditary methhaemoglobinaemia where the globin moiety was abnormal and where treatment with methylene blue or ascorbic acid failed to effect any significant reduction of ferric ion.[13] This was the first evidence of a hereditary disorder involving haemoglobin molecule.

In this context, it must be mentioned that in deficiencies involving iron, copper and pyridoxine, the production of haem is decreased.[32]

Anomalies in the Globin: Anomaly of Haemoglobin Synthesis

While the changes in haem are usually reversible, anomalies of globin synthesis represent hereditary defects of an irreversible type. Known disorders of this group result either from a block in the production of normal haemoglobin (as in thalassaemia) or from the production of an 'abnormal' haemoglobin (*viz.*, Hb.S, C, D, E.....Q). Sometime, these two factors combine to produce a disorder due to genetic interaction between thalassaemia and one of the abnormal haemoglobins giving rise to conditions

like Hb. E-thalassaemia disease or Hb. S-thalassaemia disease.

Normal Haemoglobins.—The knowledge of the existence of two different types of haemoglobin could be traced as early as 1866 when Korber demonstrated the difference between the haemoglobins of the new born infant and the adult. He showed that foetal haemoglobin was alkali resistant whereas adult haemoglobin could be rapidly denatured by alkali. These two haemoglobins, now known as Hb.F and Hb.A respectively, are considered to be physiological. Under normal conditions, Hb.F is almost completely replaced by Hb.A within the first year of post-natal life. Persistence of high level of Hb.F beyond one year of age is a pathological phenomenon seen in hereditary haemolytic disorders due to some form of defect in the normal synthesis of haemoglobin. Apart from the difference in alkali resistance, Hb.F has also been shown to differ from Hb.A. in (*i*) crystallography, (*ii*) solubility, (*iii*) amino-acid composition, (*iv*) immunological specificity, (*v*) rate of spreading in surface films, (*vi*) electrophoretic mobility, (*vii*) spectrophotometric differences in the ultraviolet and in (*viii*) oxygen dissociation curves.[3, 4, 34, 40]

Both these haemoglobins are under genetic control. It appears that a separate locus is involved in the production of Hb.F which is active (with the suppression of normal loci) under condition of relative tissue anoxia as present in foetal life. The biochemical evidence also favours the theory of separate loci for Hb.A and F.[22, 38, 42]

Abnormal Haemoglobins.—The conception that in addition to two physiological haemoglobins enumerated above, there are abnormal or pathological variants, is of comparatively recent origin being brought out, as already mentioned, by Pauling and his associates in 1949. The abnormal Hb.S was found to the extent of approximately 70-98 per cent in sickle cell anaemia and 30-40 per cent in sickle cell trait. Studies on human haemoglobins in general population and in hereditary haemolytic disorders have since led to the recognition in quick succession of a series of abnormal haemoglobins, designated in the order of their discovery as haemoglobins C, D, E, G, H, I, J, K, and L.[1, 22, 38] The letter B was originally assigned to sickle cell haemoglobin known later and at present known as Hb.S. Methhaemoglobin of Horlen and Weber is recognised as Hb.M. Two new haemoglobins described from Liberia were originally designated as Liberian I and Liberian II. It appears that Liberian II is the same as Hb.K. Liberian I has been called Hb.N. Hbs.O and P refer respectively to abnormal haemoglobins described from Indonesia ("Buginese-X") and Texas ("Galveston type"). The label Hb.Q has been proposed for a new haemoglobin found in a Chinese along with Hb.H.[39] Fessas and Papaspyrou (1957) described a new "fast" haemoglobin in an infant shortly after birth.[15] This haemoglobin, however, disappeared later on. It is therefore not certain whether this may be included in this group of genetically transmitted haemoglobin variants. Lastly mention must be made of the "Bart's" haemoglobin recently described by Ager and Lehmann in an infant in the St. Bartholomew's (Bart's) Hospital,[1] and of haemoglobin "Norfolk" in an English family.[1(a)]

Genetical Aspects of Abnormal Haemoglobins.—Excepting haemoglobin H on which presently available data are insufficient, all the other haemoglobins appear to be inherited as simple Mendelian characters. Although it is likely that all the variants of haemoglobin A are allelomorphs, direct proof of this assumption has so far been obtained for haemoglobin A, S and C only. Recent studies of Schwartz *et al* show that haemoglobin G is perhaps a normal variant rather than an abnormal type of haemoglobin. The common mode of inheritance in abnormal haemoglobins is attributed to the presence of specific gene which in heterozygous state does not usually cause any significant handicap to the person concerned. In homozygous state these may, however, produce various grades of haemolytic anaemia. The heterozygous condition is also known as 'trait', the corresponding homozygous condition being known as 'disease'. The terms haemoglobinosis and

haemoglobinopathy have respectively been proposed for these two conditions.[22]

Thalassaemia or Cooley's anaemia represents an inherited defect of haemoglobin synthesis closely related to haemoglobinopathies. In this condition, production of haemoglobin A is suppressed while that of F is significantly increased. No abnormal haemoglobin has so far been incriminated as the cause of thalassaemia. The homozygous form of thalassaemia results in severe anaemia, whereas heterozygous condition may be asymptomatic or usually productive of mild anaemia. The gene responsible for thalassaemia and that for Hb.A appears to be under independent genetic control. Instances of thalassaemia gene interacting with that of known abnormal haemoglobins-S, C, D, E, G, and H — usually manifest as haemolytic anaemias of varying degree.

Geographical Distribution of Abnormal Haemoglobins.—During recent years there have been comprehensive reviews depicting the geographical distribution, haematological features and various other aspects of thalassaemia and of abnormal haemoglobin.[22, 42] Certain parts of Asia and Africa appear to be rich reservoirs of abnormal haemoglobins. Hb.S is found in African Negroes (between the Sahara desert and Zambesi river) and their descendants in other parts of the world, in some Mediterranean races, in Eti-Turks, in Veddoids in India and in Arabia. Hb.C is found mostly in West Africans and in their descendants elsewhere. Hb.D first discovered in a white American family, has later on been reported from Britain, North Africa and in Indians (Punjabis and Gujratis). Hb.E first reported in a child whose father was partly of Indian origin has since been found in Thailand, Burma, North Eastern Malaya, Indonesia, Assam, Bengal, Nepal, Ceylon and recently in an Eti-Turk. Hb.G has so far been reported in two Negro families and one Italian family. Hb.H has been found in Chinese, Philippinos, Thais and one Malayian, in two Greek families, and in a Transjordanian Arab. Hb.I first seen in an American Negro has since been reported from Algiers. Hb.J first described in an American Negro family has since been described in West Africa (at first designated Liberia I which due to demonstrable difference from Hb.J has now been labelled as Hb.N), in French Canadians, in Indonesians and in Indians. Hb.K has been encountered in Liberia (also known as Liberia II), in Kabyles of North Africa and recently in an Indian. A single instance of Hb.L has so far been reported in an Indian (Punjabi Hindu). The distributions of Hbs.O, P and Q are still to be worked out. Thalassaemia once thought to be restricted to Mediterranean races, now appears to be much more widely spread, its incidence in the old world ranging from Western Europe and West Africa in the West to China and Philippines in the East.

Haemoglobinopathy in India

Earliest instance of haemoglobinopathy in India was first described in a 2½-year-old Bengali boy from Calcutta. On clinical and haematological evidence, this appeared to be a case of homozygous thalassaemia (Cooley's anaemia), but in the absence of characterisation studies which were unknown at that time, it is not possible to state whether there was any associated abnormal haemoglobin. Since 1938, similar cases have been described from different parts of India, particularly from Bengal, Uttar Pradesh, Punjab, Bombay and Nagpur.[13, 14] Characterisation studies in these cases have recently shown that many of the so-called thalassaemics represent instances of interaction between thalassaemia gene with haemoglobin E and S.[6, 7, 8, 27] The actual incidence of thalassaemia in different parts of India is not known. In a series of 700 unrelated Bengali Hindus, the incidence of thalassaemia trait is estimated to be 3·7 per cent.

The first record of the incidence of sickle cell trait in India was documented by Lehmann and Cutbush in the aboriginal tribes of Nilgiri hills of South India in 1952.[23] In the same year Dunlop and Mozumder reported cases of sickle cell trait and presumptive cases of sickle cell anaemia among the tea garden labourers

of Upper Assam originating from the tribal population of Orissa and Bihar. Instances of S haemoglobin have also been recorded amongst the tribal population of Uttar Pradesh and Western India and in Mahars and Kunbis of Madhya Pradesh.[24, 35, 36, 37] A high incidence of 55 per cent has been recorded in Parjah Kondhs. Thus haemoglobin S has been found mostly in the aboriginal tribes.

Haemoglobin E has so far been described in Bengalis, Assamese and Nepalese. Incidence of Hb.E in a series of 700 unrelated Bengali Hindus was estimated to be approximately 3·9 per cent, an incidence similar to that of heterozygous thalassaemia. The incidence of Hb.E in India thus appears to be lower than that in Burma, and Thailand, where the incidence approximates 15 per cent and 13·6 per cent respectively.[7, 10, 11] Haemoglobin D has been recorded to the extent of about 3 per cent in Sikhs from Punjab and 1 per cent in Gujratis. Instances of J, K and L have recently been reported in Indians. No instance of Hb.C, G, H, and I have so far been encountered in Indians.[5]

Presently available data do not permit any more comprehensive account on the haemoglobinopathies in India. These, however, indicate that India is a reservoir for abnormal haemoglobins and that haemoglobinopathy, in one form or other, is responsible for mortality and morbidity not only in paediatric but also in general medical practice. Recent advances in therapeutics and public health measures while decreasing the incidence of commoner causes of anaemia, *viz.*, anaemias due to deficiency, malaria, kala-azar and other infections and infestations, are bringing into light increasing numbers of hereditary haemolytic disorders manifesting as instances of refractory anaemias. Extensive survey work in different states and in different communities are obviously indicated to find out the nature and extent of prevalence of abnormal haemoglobins and their impact on population in general. While collecting such data, attention should be given to assess the possibility of the heterozygotes possessing special resistance protecting them against the prevalent infections, infestations and other unfavourable environmental influence to which their normal counterparts are ordinarily more easily susceptible. This study is particularly indicated in view of the contention which now appears fairly well established that in Africans sickle cell heterozygotes are unduly resistant to infection with *Plasmodium falciparum*. In India, there is scope for evaluating the theory of balanced polymorphism as has been advanced for the perpetuation of genes for abnormal haemoglobins.[38]

Salient Features of Homozygous Thalassaemia and HB. E-Thalassaemia as seen in Indians (from an Analysis of 260 Cases)[5, 30]

Clinical features.—A group of seven patients is shown in figure 1. These usually conformed to a common pattern manifesting as instances of refractory anaemia, splenomegaly of obscure origin and sometimes as unexplained jaundice. Age at the time of diagnosis varied from one month to 24 years. The degree of anaemia and consequent symptoms varied greatly depending mainly on the degree of anomaly in the synthesis of haemoglobin. Analysis of the available history with reference to the mode of onset and natural evolution of the disease showed that in the severest form, significant degree of anaemia had been detected as early as the first month of life. Usual symtomytology during the first year of life was that of anaemia, splenomegaly, failure of normal growth and development and susceptibility to respiratory and gastro-intestinal infections. At this stage the disease had been very often mistaken for rickets; the question of Rh incompatibility and congenital syphilis had also been raised. During the 2nd to 4th year of life, icteric tinge of conjunctiva was usually discernible and this along with splenomegaly and febrile episodes had been attributed to chronic malaria. Anti-malarial therapy, as expected, did not in any way influence the course of the disease which was then not infrequently diagnosed as kala-azar and treated as such. In cases with significant degree of jaundice, diagnosis of biliary

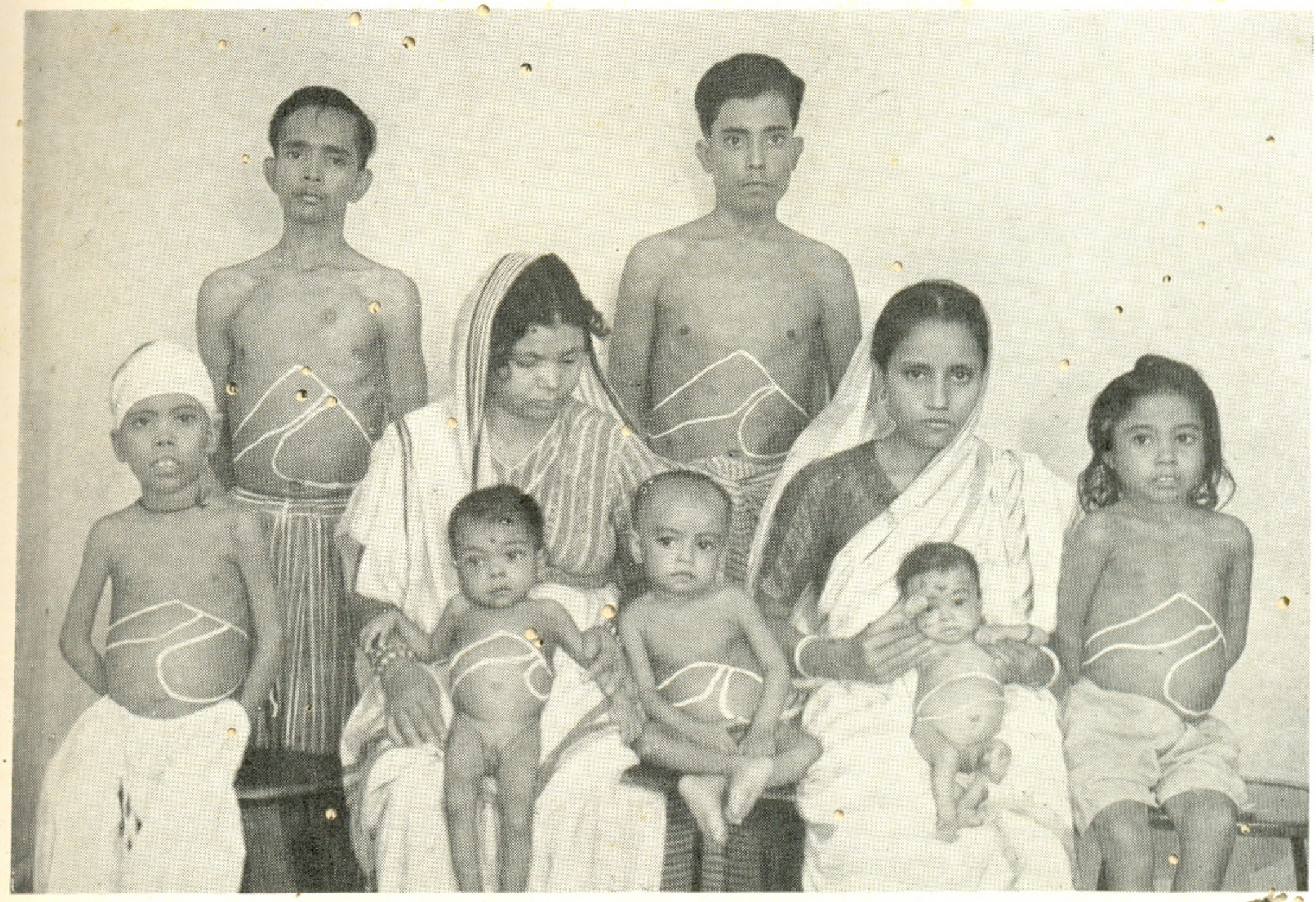

FIGURE 1 : A group of seven patients with [illegible]-thalassaemia in different ages. The patients belong to seven different families. The youngest child has homozygous thalassaemia ; others have Hb. E-thalassaemia. (From the *Bulletin Calcutta School Trop. Med.*, *6* : 28, 1958).

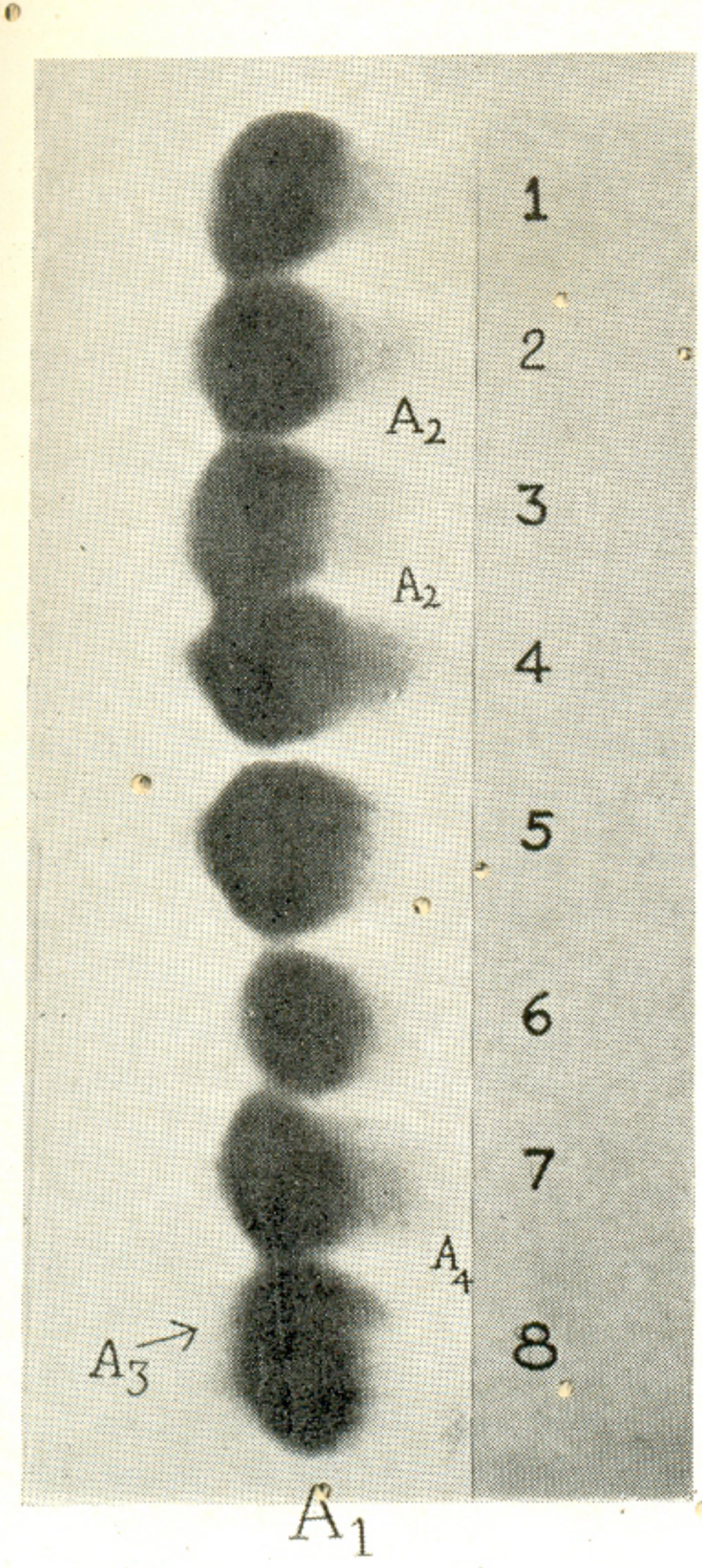

FIGURE 4 : Electrophoretograms on paper at pH 8·6, stained with Bromophenol blue. Minor components of A (A_1, A_2, A_3, A_4) are shown. Increased proportion of A_2 component in samples 1, 2, 3, and 4, obtained from thalassaemia heterozygotes.

cirrhosis had been entertained. Still later, these cases were usually dubbed as Banti's syndrome, splenic anaemia and idiopathic splenomegaly. Sometimes, the disease first detected in the second or third decade of life, had passed as an instance of infective hepatitis.

In milder forms of the disease, the anaemia was usually moderate in degree. Jaundice, splenomegaly and the usual haematological signs of haemolytic anaemia were, however, present. Some of the patients with the mildest form of the disease were carrying on normal routine pursuits of life with but slight symptoms in the form of chronic fatigue and weakness interfering with sustained work.

Erythrocytic Morphology.—In all the cases, the overall picture was characteristic of thalassaemia. There was, however, significant variation from case to case regarding particular features like the number of target cells, spherocytes and stippled cells. Target cells were seen in greater numbers in the milder forms of the disease where normoblastosis was slight or absent. Target cells were relatively scanty in infants or in younger children suffering from severe forms of the disease; normoblastosis of fair degree was usual in this situation. Inclusion bodies as described from Thailand were not seen. Erythrocytic morphology *per se* provided no definite clue in the differentiation of homozygous thalassaemia from Hb.E-thalassaemia disease.

Bone Marrow.—In general, bone marrow was greatly hypercellular with extreme normoblastic hyperplasia. Stippled normoblasts or sideroblasts were present in all cases. Bone marrow haemosiderin content was usually abundant. There was no significant change in granulopoiesis except relative decrease of granulocytic elements. Megakaryocytes were also relatively reduced but there was no significant qualitative change. 'Foam' cells were conspicuous in a few cases.

Foetal Haemoglobin.—As measured by the alkali denaturation technique, the foetal haemoglobin level was always high. The mean value was 37·37 per cent and the range varied from 6·58 to 82·95 per cent. In practice, high level of foetal haemoglobin always provided a valuable confirmatory evidence in favour of the diagnosis. The exact significance of the persistence of foetal haemoglobin in hereditary anaemias is not known. It is possible that foetal haemoglobin persists to compensate for the congenital anaemia. There was no significant correlation of the foetal haemoglobin level with the degree of anaemia, reticulocytosis, bilirubinaemia, plasma haemoglobin level, erythrocytic fragility or of any other haematological data. In thalassaemia trait, without any significant anaemia, the foetal haemoglobin was usually normal.

Plasma Haemoglobin.—As measured by a modified method of Bing and Baker, the plasma haemoglobin level was almost always increased. The mean value was 16·98 mg. and the range of variation was from 4·3 to 56·50 mg. per cent. The value 4·3 mg. was obtained in only one patient. The minimum level in the remaining cases was 7·7 mg.

In the present series, there was no significant correlation of the plasma haemoglobin level with the age of the patient, degree of anaemia, reticulocytosis, leptocytosis, erythrocytic fragility, bilirubinaemia or foetal haemoglobin level.

High level of plasma haemoglobin indicates that intravascular haemolysis contributes significantly to the haemolytic component. Red cells in this condition are presumably unable to withstand even the normal circulatory trauma and are destroyed readily not only in the spleen and other reticulo-endothelial organs but also in open circulation.

Erythrocytic Fragility.—Osmotic fragility was decreased in all cases. The mechanical fragility, as measured by a modified method of Shen *et al* was either normal or increased. There was, however, no significant correlation of the mechanical fragility with the age of the patient, degree of anaemia, leptocytosis, reticulocytosis, or plasma haemoglobin level. In general, higher degree of bilirubinaemia was observed in the patients whose erythrocytes were more susceptible to mechanical trauma.

Results indicate that in this disorder osmotically resistant red cells are at least, as susceptible to mechanical trauma as normal red cells, if not more. The differential behaviour of erythrocytes when tested for osmotic and mechanical fragilities suggests that *in vivo* haemolysis is mediated through forces which are perhaps more mechanical than osmotic. Association of higher degree of hyperbilirubinaemia in patients with comparatively increased mechanical fragility tends to corroborate this view.

Serum Iron and Iron Binding Capacity of Serum.—The serum iron level was normal or increased, ranging from 70·0 to 355·0/μg per cent (mean 170·0/μg per cent). The unsaturated iron binding capacity was greatly decreased.

Cytochemical Study.—PAS positive material was found in the cytoplasm of a number of normoblasts.

Immunological Investigations.—In 9 out of 69 cases, Coombs' test was positive. In the positive group one patient was in apparent haemolytic crisis; the other 8 cases showed no evidence of associated infection and none of them had ever received any blood transfusion. The 'warm' and 'cold' haemoagglutination tests performed on patient's serum with 'auto' and 'iso' red cells were, however, uniformly negative in all these cases.

Intra-erythrocytic Haemoglobin Crystals.—During routine sickling test carried out with 2 per cent solution of sodium metabisulphite,[12] intraerythrocytic crystals of varying shape, size and numbers were seen in Hb. E-thalassaemia disease and in AE subjects. The phenomenon was particularly seen 6-8 hours after the preparation for sickling had been made. These crystals resembled very closely those described by Diggs *et al* in Hb.C diseases.

Two points emerge from these findings, viz., (*i*) intra-erythrocytic crystals are by no means specific of Hb.C; (*ii*) the unwary might mistake these crystals as sickled cells.[30]

Radiologic Investigation.—Characteristic "hair-on-end" appearance of skull bones was only seen in 10·0 per cent of cases. The more commonly observed radiological abnormalities in skull bones were loss of density at places with increased porosity and coarse mottlings due to osteosclerosis.

Response to Therapy.—Response to any form of therapy was in general unsatisfactory. None of the haematinics were of any use. Cobalt chloride in 50 to 100 mg. daily dose given orally appeared to cause some amount of non-specific erythrocytosis, the effect of which did not, however, last and wore off soon after withdrawal of therapy. Periodic blood transfusion was the only sure method of maintaining the patients at a comfortable clinical and haematologic level. Splenectomy was done on 28 patients.

Observations on Blood Transfusion.—Following blood transfusion, foetal haemoglobin level falls and remains low suggesting a suppressive effect of blood transfusion on endogenous erythropoietic activity. In three children aged 6 months, 6 months and 2 years respectively, exchange transfusion was given; in two of them exchange transfusion was followed by splenectomy on the 6th and 7th day after transfusion. These three cases were followed to study the effect of exchange transfusion and splenectomy on endogenous erythropoiesis. The details have been published. If foetal haemoglobin may be regarded as a natural tag of endogenous erythropoietic activity, the data would support the contention that transfusion reduces erythropoietic activity.

Observations on Splenectomy.—Splenectomy was done on 28 patients, consisting of both homozygous thalassaemia and Hb. E-thalassaemia, with ages varying from 6 months to 24 years. The haematologic status of these patients was variable with haemoglobin ranging from 1·78 to 8 gm., reticulocyte from 2 to 32 per cent, foetal haemoglobin from 17 to 78 per cent, plasma bilirubin from 0·4 to 3 mg., plasma haemoglobin from 7·5 to 88 mg., and serum iron from 90 to 354 μg per cent. These cases have so far been followed from approximately 6 months to 7 years. Partial improvement was seen in 25 patients. Signs of improvement were slight elevation of Hb. and R.B.C. levels, less transfusion requirements and decreased incidence of 'crisis'.

In two patients, aged 9 months and 3 years, there was no significant improvement and they died 7 and 9 months after splenectomy. A male patient aged 24 years with Hb. E-thalassaemia disease developed high fever and chest pain after splenectomy and died on the third post-operative day.

Methods of Study in the Characterisation of Abnormal Haemoglobins and Haemoglobinopathy. [19,26,40,42]

It is evident that characterisation of haemoglobin molecule needs a co-ordinated study from clinical, haematological, biochemical, and genetic aspects. Brief reference of the implications and values of the standard methods are, therefore, indicated.

Clinical and Haematological

In the study of a hereditary disorder, the value of comprehensive clinical examination including examination of family members for the evidence of a similar disorder cannot be over-emphasized. The type of anaemia, the characteristic changes of erythrocytic morphology and the osmotic resistance of red cells show a typical composite pattern which continues to be a useful screening procedure. Iron deficiency anaemia is, however, known to show a similar picture. The possibility of iron deficiency should therefore be excluded by careful history and if necessary by determination of serum iron and bone marrow haemosiderin contents. In doubtful cases a therapeutic trial of iron may also be given.

The sickling test carried out with one of the reducing agents offers specific information on the presence or absence of Hb.S. The fallacy of false sickling should be remembered. There is need for repeated tests with more than one method. With the advent of paper electrophoresis, the fallacy of false sickling in a doubtful case can, however, be easily clarified.

Physicochemical Methods

Moving Boundary Electrophoresis.—With this method, Pauling demonstrated the classical difference in the mobilities of Hb.S and Hb.A and thus ushered in the new era in the study of haemoglobin. It is still the most sensitive and dependable technique for the separation, quantitative method and characterisation of haemoglobins. The technical details for the study of haemoglobin with this method are not, however, simple and facilities for such study are not also available at all centres.

Filter-paper Electrophoresis.—This simple technique has been used most universally. Many new haemoglobins have been discovered with this method. Details of techniques with appropriate modifications for improving limitations have been discussed by many workers. Relative positions of haemoglobins on paper electrophoresis at pH 8·6 are shown in figure 2.

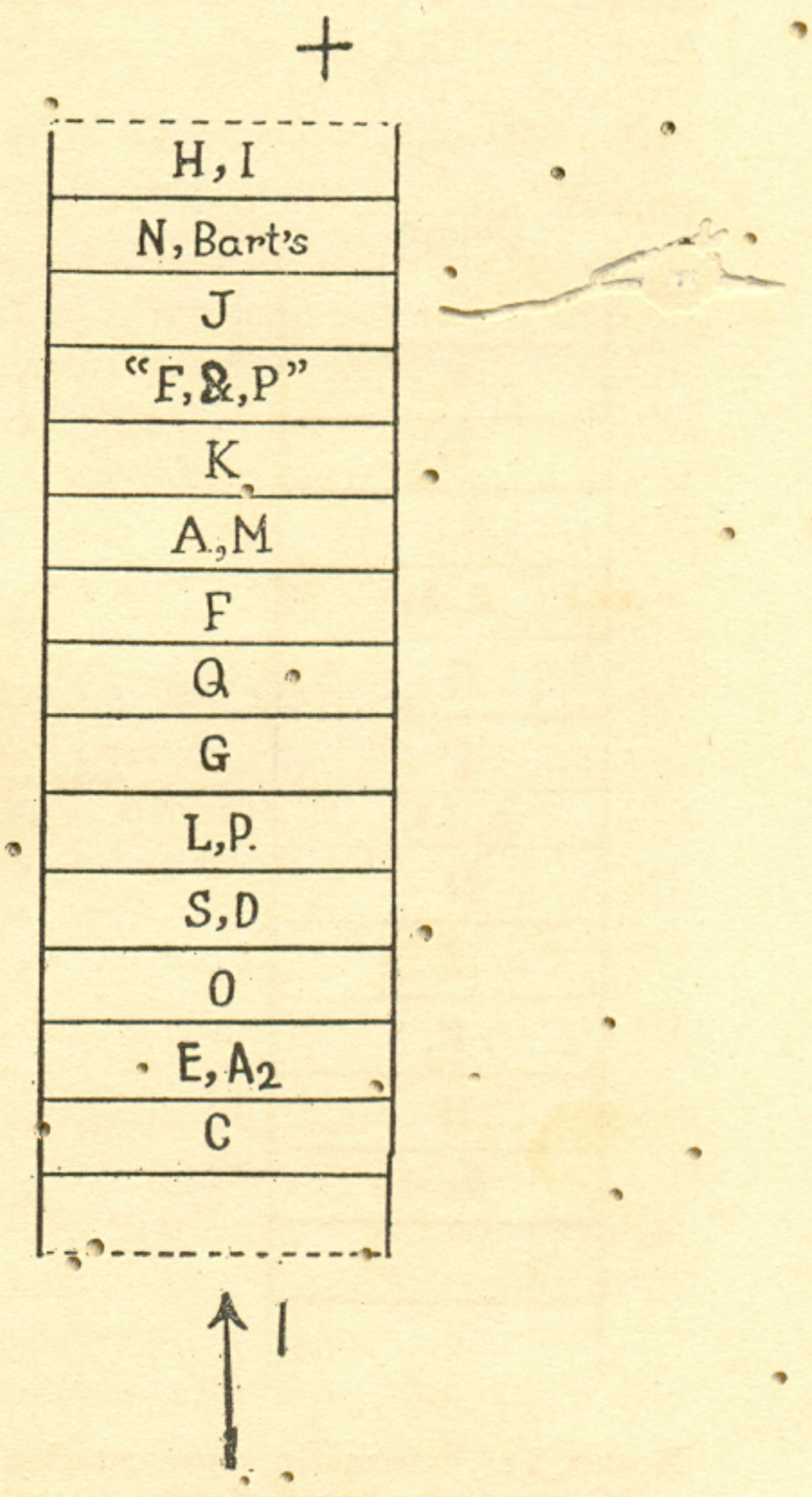

FIGURE 2 : Schematic representation of the relative positions of the haemoglobins in paper electrophoresis at pH 8·6. The haemoglobins with similar mobilities can be differentiated by other methods. Hbs. H and I can be differentiated by electrophoresis at pH 6·5. (F & P = Hb. of Fessas and Papaspyrou.)

Use of agar plate and starch block has yielded additional information not obtainable by the conventional method on paper.[20, 21, 25]

For routine study in survey and clinical work it continues to be the standard method. For the detection of minor components, for precise quantitation, for characterisation of new haemoglobins and for the differentiation of haemoglobins with identical mobilities, the method must be supplemented by other procedures.

Chromatography.—Application of the chromatographic method to the separation of haemoglobins has provided a valuable tool for the characterisation of new haemoglobins. A schematic representation of the positions of different known haemoglobins on ion exchange chromatography at pH 6 is shown in figure 3. Use of cation exchange resin Amberlite IRC-50 offers possibilities.[1, 26, 29]

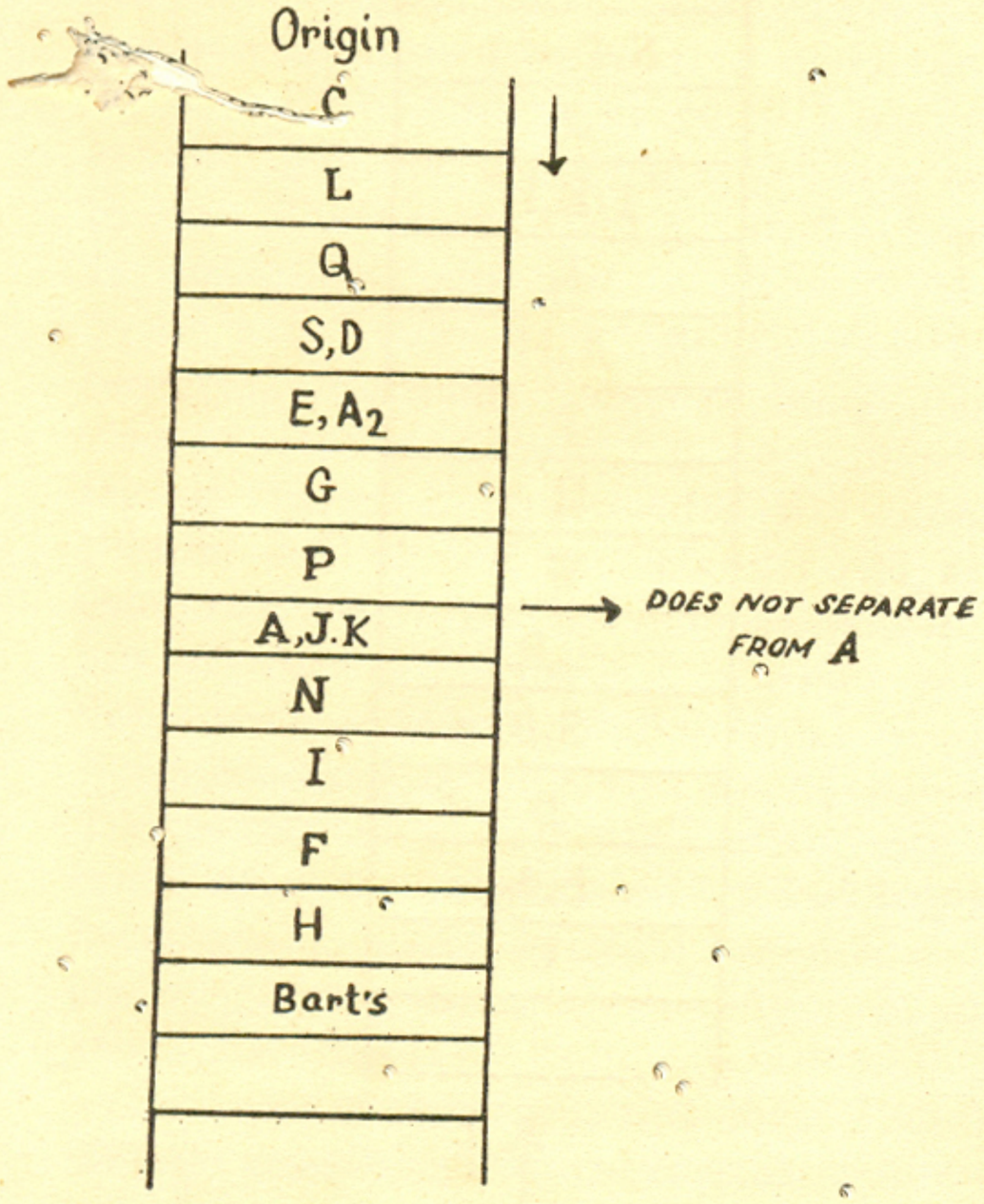

FIGURE 3 : Schematic representation of the relative positions of the haemoglobins in column chromatography.

Solubility.—Although oxyhaemoglobins of A and S have more or less similar mobilities, ferrohaemoglobin S has very low solubility, quite unlike any other haemoglobin. Solubility study in phosphate buffers (in 2·24 M and 2·58 M concentration) is essential to differentiate Hb.S from Hb.D which behave identically in electrophoresis and chromatography. In the homozygous state, C has the highest ferrohaemoglobin solubility followed in order by A, G and S. Solubility of EE is higher than AA. Solubility of ferrohaemoglobin from the homozygous thalassaemia and from Cord blood (AF) is higher than AA. In the heterozygous state, AC is most soluble. AD and AE have similar solubilities which do not differ significantly from AA. Solubility of ferrohaemoglobin from thalassaemia heterozygotes does not differ from that of AA.

Solubility is now recognised to be a standard procedure for characterisation of haemoglobin. Description of any haemoglobin is not complete without data on solubility.

Chemical Methods

Spectrophotometry.—The absorption spectra of the abnormal haemoglobins do not differ from that of Hb.A. Hb.F is however a notable exception with an unique ultraviolet spectrum with a " tryptophan notch " at 289·8 mμ. In Hbs. A, S, C, D and E the tryptophan absorption is denoted by an unresolved shoulder at 291·0 mμ. This difference can be utilized for approximate quantitation of Hb.F in a mixture.[19]

The conversion of oxyhaemoglobin to ferrohaemoglobin results in the appearance of an absorption peak at 630 mμ. The early stages of denaturation in haemoglobin samples can also be detected by slight shifts in the Soret band.

Alkali Denaturation.—This is at present the main practical method for the identification and quantitation of Hb.F.[4, 34, 40] The method of Singer which measures the percentage of Hb. resisting denaturation after one minute's exposure to an alkaline medium at pH 12·7 is now widely used. Other methods utilize a lower pH, taking multiple readings with a view to estimating the rate of denaturation. The breaks in the curves so obtained have been interpreted as indicative of the

denatured material. If there are several fractions of Hb.F and Hb.A with differential pattern of resistance to alkali, these cannot be distinguished by Singer's method.

When denatured Hb. is precipitated with ammonium sulphate, some of the undenatured material is liable to be included in the precipitate through absorption and co-precipitation. This phenomenon produces significant errors in the estimation of samples with low percentages of Hb.F. Undenatured Hb.F may be carried down by large masses of denatured alkali-labile Hb.A giving rise to unduly low values for the alkali resistant fraction. The error decreases as the proportion of Hb.F rises in the mixture.

In routine studies, the Singer's method is still very useful. It may, however, be remembered that the alkali-resistant fraction in this method is in fact a mixture of resistant Hb.F and dissolved denatured Hb. and the precipitate is a mixture of denatured Hb. and a small amount of absorbed undenatured Hb.F. These considerations are of great significance when searching for a small amount of Hb.A in the presence of SF or EF in such conditions as Hb.S-thalassaemia or Hb.E-thalassaemia.[19]

Amino-acid Composition.—For locating the precise difference in the globin moiety of the various haemoglobin variants, careful analysis of amino-acid compositions has been done by different investigators. Proter and Singer compared Hb.F with Hb.A and found that the former contained only half the number of valine terminal residues.[19] Schroeder, Kay and Well reported small difference between Hb.S and Hb.A;[33] this finding was confirmed by Huisman *et al* who also showed that Hb.C in respect of basic amino-acid contents was different from Hb.A and AS.[17] Works of Huisman *et al* are not in agreement with those of Scheinberg *et al* who pointed out that Hbs. A, S and C differ in their content of free carboxylic acid groups.[19]

Anderson *et al* suggested that the difference between the various haemoglobins is perhaps due to different small molecules complexing reversibly with the same haemoglobin.[2] This 'reversible-complex' hypothesis is not supported by experimental observations.

Recent work of Ingram has proved that difference of Hb.S from Hb.A is due to the substitution of a molecule of valine for a molecule of glutamic acid in one of the polypeptide chains. In Hb.C, Lysine appears instead of glutamic acid in the same position in the peptide chain as is incriminated in case of Hb.S. Ingram has also shown a difference between Hb.A_2 and Hb.E in amino-acid sequence.[1, 1(a)]

Immunologic Techniques

These have been of value in detecting Hb.F in different haemoglobinopathic states. Antigenic specificity of Hb.A and Hb.F was first observed by Darrow *et al.* In confirmation of Darrow's observations, it has been further shown that Hb. showing the antigenic specificity of Hb.F is present in sickle cell anaemia and Cooley's anaemia. With a precipitin ring test Chernoff reported the presence of minute amounts of Hb.F in normal individuals.

Antigenic specificity of other haemoglobins has not so far been established.

Genetic Methods

Genetic data provide valuable information pertaining to the precise genotype with reference to Hb. genes. Certain clinical states (Hb. E-thalassaemia and Hb.E disease; Hb. S-thalassaemia and Sickle cell anaemia) having more or less similar electrophoretic pattern can only be differentiated by complete investigation of parents' blood.

Heterogeneity of Haemoglobin A

Along with the discovery of new haemoglobin evidences have accumulated pointing to the fact that normal adult haemoglobin (Hb.A) is not a homogenous substance. Kunkel and Wallenius using starch block electrophoresis demonstrated three components in Hb.A.[21] The main component is Hb.A_1 which constitutes more than 90 per cent. Hb.A_2 which at pH 8·6 has the same mobility as Hb. E constitutes 1-3 per cent and Hb.A_3 (which moves slightly faster than Hb.A_1, but does not always separate out from it) about

2 per cent. In thalassaemia trait, the A_2 component is significantly raised and has been found to the extent of 10 to 15 per cent. The exact significance of raised A_2 component in thalassaemia trait is not yet known. In practice, this increased A_2 component has proved very useful in the diagnosis of thalassaemia trait. In homozygous thalassaemia, the A_2 component is also raised but not to the same extent as in thalassaemia trait. In homozygous thalassaemia Hb.A content is usually very low. Therefore when expressed as percentage of haemoglobin A, the proportion of A_2 component in this condition must be considered quite high. The interrelationship between Hb.A_2 and Hb.E is yet to be found out. Ingram has found some difference between these two haemoglobins so far as amino-acid sequence is concerned.

Recently a fourth component (A_4) has been reported. This moves slower than A_3 and contributes about 1 per cent or less. Like A_2, A_4 also appears to be increased in thalassaemia trait.[20,25] These different components of A are demonstrable on starch block, as also on thick paper (Fig. 4).

The various minor components of Hb.A are distinguished by electrophoretic means. At present these represent mostly electrophoretic entities. But with chromatography some of these components may be separated.

Conclusion

Recent knowledge regarding the biosynthesis of haemoglobin and the existence of a series of abnormal haemoglobins have elucidated to some extent the pathogenesis of hereditary haemolytic anaemias. New knowledge has also brought new problems. Comprehensive investigations are underway to unravel the structures and ultra-structures with a view to characterise the abnormal haemoglobins as fully as possible. The possibility that the gene-controlled defect in haemoglobin synthesis may be an expression of some primary and fundamental disturbance involving earlier phases of a complex enzymatic process cannot be overlooked. Information in this direction is yet to emerge.

In spite of all these recent advances, treatment in severe forms of haemoglobinopathy remains very unsatisfactory. There is no specific remedy which can improve the hereditary defect in haemoglobin synthesis. Therapeutic limitation continues to provide a challenge to the investigative spirit of the workers concerned.

The geographical and racial distribution of abnormal haemoglobins and the complexities of various genetic interactions have provided exciting and intriguing problems to the anthropologists and the geneticists.

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GOOD LABORATORY ANIMALS—ONE OF THE BASIC REQUIREMENTS OF MEDICAL AND BIOLOGICAL RESEARCH

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RECORDS of animal dissection and experimentation go back some two thousand five hundred years, to Alcmaeon of Croton and, shortly after, Hippocrates of Cos, who is generally regarded as the father of scientific medicine. These, and many of the ancients since then, showed a zeal for enquiry which led them to appeal to the animal in their study of living processes, and to the bodies of man and animals as subjects for dissection. Animal experimentation, of a sort, probably goes back much further, if we may include the use of dogs, or of slaves, for the detection of poison in the food of kings and pharoahs, but this was motivated not so much by a spirit of scientific enquiry as by a desire for self-preservation.

It is, indeed, to the ancient Greek civilization that the world owes the origination of the inductive method of enquiry, and on which the whole corpus of occidental philosophy has been raised, leading by well-recognized steps to the birth of "modern" scientific thought in the sixteenth century. The older civilizations of the East, reaching maturity before the Greek, did not feel the need to adopt a way of thought which was quite foreign to them. They had developed systems of deductive reasoning in which there was no place for the experimental outlook that every scientist of today regards as his birthright, and it is not surprising that in neither the Indian nor the Chinese history of those times is there any record of animal experimentation. Perhaps they had had their era of free enquiry, and had left no record of it. Indeed, out of the development of Greek-inspired thought in the West there developed systems, fully comparable with the oriental systems, which inhibited further free enquiry with its appeal to experiment and direct observation. Not even the Arab civilization of a thousand years ago was able to break away from systematic thought. Only the West achieved a renascence of inquisitiveness, which eventually went all round the world.

There is a story of an ancient monastery in which the monks were divided on the question of the number of teeth possessed by a horse. The controversy raged for years, one party claiming twenty-six teeth, and the other thirty-two. A young novice, having eventually completed his seven years' initiation, during which he had been under a vow of silence, asked leave to join in the discussion. After due consideration of his presumptuous request, his superiors granted him permission. He pointed out that, whereas some authorities appeared to favour the lower number, others of equal antiquity and

standing supported the higher. Would it not be reasonable, he asked, to go to the monastery stables, and look at a horse's mouth ?

At this, both parties in the controversy turned on their brother and, beating him soundly for daring to suggest that the answers to all questions could not be found in ancient writings, chased him out into the wilderness where, being crippled by his beating, he lived to a ripe old age on the alms of the people.

Such an event could have taken place anywhere from Paris to Peking, from Leningrad to Leopoldville. It could have been in any century, including the middle of the twentieth ; for we see even today the shackling of scientific enquiry by political and other preconceptions.

The Last Two Centuries

There was an outburst of new and independent thinking in Europe in the sixteenth century, when the foundation of modern science was laid, and it was not until then that anything like orderly experimentation took place. (What had gone before, long before, was in the nature of casual curiosity, without much conscious purpose in it.) But this outburst, in the biological field, was short-lived. Names like Vesalius, Fallopius, Servetus, Harvey and de Graaf stand out as great men born two centuries or more before their time. It was not until the early nineteenth century that, in Europe, schools of physiology grew up, in which the experimental method was developed in such a way as to yield a continuing harvest of knowledge that has led us to the medicine of the present day. As the century drew to a close, modern biology had become a world-wide discipline, well established in Europe and North America. In the penultimate year of the century, in the Haffkine Institute, it staked its claim to a proper share of India's vast and traditional intellectual potential. It is one of the happy accidents of history that India, a country old in wisdom but young in science, should be the marriage bed of a liaison between the two that in little more than half a century is proving its fertility.

Use of Animals Today

Turning more specifically to animal experiments, which are among the building blocks of medical and biological research, we find laboratories engaged in their utilization from Jammu to Trivandrum, and from Bombay to Dibrugarh, the length and breadth of India. Indeed, every country in the world today that supports universities and medical schools uses laboratory animals. Since science has succeeded in overcoming the limitation of systematic philosophy and of exclusively deductive reasoning, it has become more truly international than any other human activity. It is not surprising, therefore, that when we study the use of laboratory animals in different countries, we find much the same picture, and the same problems everywhere.

How to get the right sort of animal, in the right numbers, in the right place and at the right time, is the problem that is met wherever medical research is being undertaken. The *right sort of animal* implies a correct choice of species, and of strain within a species, in a state of health up to the level required by the purpose for which it is to be used. It involves consideration of all aspects of quality; freedom from disease, in a sound state of nutrition, of a more or less clearly defined genetic constitution, and so on. To have the *right numbers* of animals available implies a fore-knowledge of needs, and it is a commonplace that scientists find it extremely difficult to estimate their requirements in advance. Nevertheless, it must be done, if we are to avoid the non-availability of animals holding up research on the one hand, or on the other the wastage of animals provided but not used. In countries where distances are great, this can be a real difficulty, for conditions of transportation may be such as to lead to serious deterioration in the quality of the animals *en route* to the laboratory. And, of course, the animals must be ready at the *right time* that is, when they are wanted.

In a large laboratory in the United Kingdom, which breeds nearly all its own requirements of small laboratory animals, it has been found that of every hundred

animals used in the course of a year, twenty-five are required in the period April-June, and twenty-five during the period October-December. This is, of course, the average rate of consumption for the whole year. But in the period January-March, the requirement is thirty-five per cent, considerably above the average, and this at a time when, in a north temperate climate, breeding is at its lowest; while in the period July-September the requirement is only fifteen, when breeding is at its height but universities are on long vacation and most people wish to take their annual leave.

A good deal of planning, therefore, is necessary if supply and demand are to keep in step. It has been frequently pointed out that animals have no storage life; they must be ready when they are needed, and they must be used when they are ready. Whoever is responsible for planning animal supplies must have some accurate data on which to base his programme, and this sort of information was never systematically collected until recently.

The first country to conduct surveys of demand and supply was Great Britain. Some partial or sample surveys were conducted in the immediate post-war years, but in 1953 a complete survey was conducted of the production and use of animals in the whole country during the year 1952. Since then other surveys have been carried out, and in 1956-7-8 many countries have been covered, including India, with the help of funds from UNESCO and at the instigation of ICLA (International Committee on Laboratory Animals). The Indian survey was made by Mrs. B. K. Batra, of the Indian Cancer Research Centre, Bombay, and is a most comprehensive document which should be studied by anyone wishing to improve the supply and use of laboratory animals in India. It is a first-class investigation, by a scientist, of a problem which even today is not everywhere considered to be strictly scientific.

Other surveys, in other countries, have also been completed, and a consolidated report of the first five has already been published by ICLA. More will be added, in the form of supplements to the first consolidated report, as they become available. As might be expected, there are great similarities between the various countries. For example, the proportion of the total number of animals that is represented by mice is nearly always between 60 and 70 per cent. This is an enormous preponderance of one species, and it will be found in all countries, where there is an active pharmaceutical industry for drug research and testing, use mice in large numbers. The proportions of other species are also comparable from one country to another, with infrequent exceptions. Indeed, such exceptions will immediately lead one to enquire into the reason for a typical specific utilization.

Most countries that have conducted surveys have drawn attention to the lack of any central source of information on laboratory animals, which they feel is an essential beginning to solving their animal problems. They point also to inadequate animal house facilities, to poor animal house labour, and other things. It is, perhaps, useful to quote Mrs. Batra's recommendations, taken from her Indian survey. She recommends:—

"1. The creation of a co-ordinating body to function as a Laboratory Animals Information Service which would (*a*) collect and maintain data on animals and their uses and (*b*) expedite procedure for the supply of animals.

"2. The production of a standard inexpensive diet which meets the nutritional requirements of laboratory animals.

"3. The establishment of a standard for adequate facilities for housing and maintaining laboratory animals.

"4. A training programme for (*a*) research workers and (*b*) animal house personnel.

"5. The establishment of a central institute to initiate the programme of training, and breed adequate numbers of quality animals to supply to the different institutions.

"6. Till such time as such a training and supply centre is established, recognized research laboratories should undertake the responsibility".

Now these proposals or recommendations are modest enough, and indeed it will come as a surprise to many that it was necessary to make them at all. Many men might assume that in such a highly organized field as medical research this particular aspect had received its proper share of attention, and this would be a reasonable, but erroneous, assumption. But there are two points about the recommendations that merit emphasis. The first is that they are based on a detailed factual survey, not on *a priori* argument, and they cannot be pushed on one side as so much political dogma. The observations have been made, the data presented, and the conclusions drawn. An unsatisfactory situation in all its detail has been brought to light, and to differ from the proposals put forward for its correction is to assume an obligation to suggest a better remedy.

The second point is that Mrs. Batra's recommendations are very similar to those suggested in other countries. The situation in India is not peculiar, it is universal; and any differences between countries are differences of detailed emphasis at the most.

The Price of Poor Animals

All research is costly. It consumes raw materials, scientific and technical man-power, and laboratory facilities. It can only pay if it is brought to a successful conclusion, by advancing the frontiers of knowledge in some desired direction. If an investigation is frustrated at any point in its prosecution, so that there is no conclusion, the only value remaining to it is as an intellectual excercise, and this is seldom sufficient to justify the effort put into it. The further from the commencement of the investigation the breakdown occurs, the greater the loss.

It is, however, a regrettable fact that even today many investigations are frustrated, or their conclusions rendered unsound, because the animals used fail at some point to react as good animals should, or even fail to survive the period of the investigation. Premature death of animals through intercurrent infection, or nutritional deficiency, or a meaningless response due to these causes, or to wrong choice of species or strains, or delay in the investigation through non-availability of animals, are all aspects of frustration. Some examples may illustrate these points.

Hoyland[1] compared the number of guinea-pigs bred in his laboratory for diagnostic tests over an eight year period. In 1949 it was necessary to use 5,131 guinea-pigs for 2,617 diagnostic tests, the number dying from intercurrent causes being 189, which included those that died before being used and those that died during the period of the test. In 1956, 1,848 guinea-pigs were used for 2,422 investigations (it was then possible to use a single animal, on occasion, for two tests) and only 27 died from intercurrent causes. The cost of guinea-pigs purchased had nearly doubled during that period, but even so their better quality represented a net cash saving in purchase price for a nearly equal volume of work, apart from a very laudable saving in animal life and a further cash saving in housing and feeding over long periods.

In this case the animals were purchased from outside, but even more avoidable extravagance can occur when a laboratory breeds poor animals inefficiently; for it is unfortunately true that laboratories have demonstrated that they can breed just as bad animals as any commercial breeder, and at even greater cost, if they do not practise good methods. An animal breeding station attached to a university department succeeded in supplying some 20 per cent of the department's total requirement of mice, the remainder being purchased. The cost of breeding was some five times the cost per mouse of those purchased, and the quality of both was of the same order. And yet the facilities in the breeding station were perfectly adequate, if properly used, to meet the total requirements at little greater cost than that required to produce 20 per cent of requirements; while the quality should have been, indeed could be, superior. An even worse example of wasted facilities has been reported to the author, of an institute maintaining a stock of some four thousand breeding mice; no small holding, by any

standards. In a good year the total output was not more than seven thousand mice for experiment, which is a poor utilization of the reproductive potential of that most prolific of mammals.

And then there is the waste of human effort. Scientists, few of whom would regard themselves as being over-paid, are nevertheless expensive both to produce and to maintain, and they have to be supported by trained technicians and ancillary personnel in proportion to their numbers. Their really productive years are normally few, often dismally so, but during the best of them their intensity of work and effort is great. When their experiments are vitiated or brought to nought through the use of unsatisfactory animals the loss is a double one. Their immediate efforts are partially or completely wasted, and instead of being able to proceed to further profitable fields they find themselves going out in much the same place as they came in. No business would prosper if it squandered its skilled man-power in such a way; no university department, no publicly or privately supported laboratory can afford to be complacent in such circumstances.

The Price of Poor Facilities

It is not uncommon, in the world of biological research, to find well-equipped laboratories supported by quite inadequate animal houses, often added as an afterthought in new buildings, when money was running short, or resulting from the conversion of existing premises essentially unsuitable for the purpose. Everyone, including members of finance committees, likes to see well-lit and ventilated laboratories, with a sufficiency of apparatus and equipment of good quality maintained in sparkling condition. They are an encouragement to good work; indeed, in these days, little is to be expected of string-and sealing-wax methods.

Why is it, then, that the same outlook does not govern the provision of good facilities in the animal house, which is, after all, but a department of the total research establishment? Perhaps an analogy may illuminate the situation. The following quotation is taken from Guy's "Pocket Cyclopaedia", published by G. Woodfall in 1929.

"... Considerable repugnance has always been manifested, by the illiterate and uninformed, to the dissection of the dead; but it is sincerely hoped that, as knowledge becomes more diffused, and the conviction of its absolute necessity more generally prevails, such prejudices will in time be done away; and that while anatomy be pursued with decency and privacy, and some restrictions removed which now prevent the obtaining of subjects, anatomical dissection will be rendered at once, and forever, legitimate, respectable and meritorious."

The anatomists have won their battle. Well-intentioned people bequeath their bodies for dissection, and medical schools no longer have frequent cause to complain either of the shortage of subjects or of the state of their dissecting rooms. But the "considerable repugnance" still attaches, in the minds of many, to the use of animals for experiment, and is reflected in the unwillingness so often seen to provide suitable facilities for breeding and housing the "subjects". With so much human poverty, malnutrition and bad housing throughout the world, it may seem an impertinence to demand conditions for housing and feeding animals better than those of the generality of the population. Mrs. Batra in her report refers to the difficulty of using diets containing milk or milk products when there is not enough milk for human beings in need of it.

But this is to take a very narrow view. The more practical question to ask is this: Is it better to have a thousand animals poorly housed and to feed them inadequately, in the hope that some of them will be useful; or to house a hundred animals excellently and feed them well, so that all, virtually, will make their contribution? That is something like the hard headed arithmetic of the case. The animal house is not the "poor relation" of the laboratory. It has, or should be regarded as having equal rights with the laboratory to proper facilities, or its provision should not be undertaken. As well do your

measuring in spans and cubits, your weighing in pinches and handfuls, as house your animals in pest-infested shacks. It is probably a wise plan, when constructing new biological laboratories, to build the animal house first, while the money for it still flows, so that the breeding programme can get into its stride by the time the laboratories proper are completed.

The Price of Poor Technicians

The argument reaches its climax when we come to the staff of the animal house, for the most important single factor in the production and care of good animals is the quality of the labour employed in looking after them. And here I would like to intrude a personal observation. In the autumn of 1955 I had the privilege of visiting many laboratories and animal houses in India, and I came away with one outstanding impression. It was that India had no lack of men who were capable of becoming highly skilled animal technicians. In a country where 80 per cent of the population is rural and grows up in close, often intimate, touch with domestic animals, it is hardly surprising that so many of them should have an intuitive understanding of animals. This is a tremendous advantage, and represents a capital asset not possessed by more urbanized countries. To what extent has it been exploited?

I have seen, in India, more skilful handling of monkeys than in any other laboratories I have visited anywhere. I have seen Indian animal technicians, to a large extent self-educated, of a standard or ability that compares favourably with that I have worked with in the United Kingdom. Here animal technicians have had their own association since 1950, which conducts courses of instruction, holds examinations, and publishes a quarterly journal. But I have also seen badly cared for animals in Indian animal houses, where even the poor facilities were not a sufficient excuse, and much better conditions could have been achieved merely by the choice of better trained technicians.

Once again Mrs. Batra draws attention in her report to the need for animal technician training. But there must be some incentive for a young man to seek this sort of training, especially during the period, which one hopes is transient, when the work is considered for one reason or another to be undignified. Nobody willingly becomes an animal technician, it seems, without having a strong interest in, and love for, animals, because the work is arduous, exacting and sometimes unpleasant. No man of promise is likely to remain one if he cannot, by his work, support his family from his earnings. A recognition of the importance of the animal technician should carry with it a recognition of his entitlement to a proper reward.

Trends Towards Improvement

To see that a problem exists is not difficult, but the nature of the problem may be harder to define. This is partly because each worker is likely to see that aspect of it that affects his own work in higher relief than other aspects. And until the problem has been defined, that is, until an accurate diagnosis based upon an appreciation of its aetiology has been made, it is not very profitable to talk about a remedy.

Now, before the physician can make a diagnosis he has to seek the answers to a number of questions, and he does this by taking a history and making a physical examination. In the case of laboratory animals, some of the relevant questions are as follows. Is there a numerical shortage of animals, or of certain species? Is the deficiency in quality rather than quantity? Are enough animals available in one place but not finding their way to where they are wanted, through lack of information? What are the particular defects of quality? Do the laboratories receive good animals but allow them to deteriorate while in their care? Are the present sources, supplying animals that are in some respects unsatisfactory, capable of being improved? If not, what alternative sources should be developed or encouraged? What is the likely direction of future trends in demand, as to species and strains, both in numbers and in quality? Is diet a problem? Is animal care as good as it should be? Do animal houses need to be improved? Is there a need

for the regular exchange of information between animal-using laboratories?

Some of these questions are a matter of history taking, but the physician must always put his own interpretation on the patient's complaints, and follow up with his eyes, his ears and his hands, and also have a recourse to special examinations. So in the present case, before a sound diagnosis of animal problems could be attempted, detailed information had to be sought, and the result was the survey already referred to, whose recommendations have been quoted above. These recommendations are, unlike the arguments about the number of teeth in a horse's mouth, based on factual observations, and it is interesting to compare them with any recommendations that can be found, which preceded the survey. This is not the place to make such a comparison in detail, but those who wish to do so should study, first Mrs. Batra's report and, afterwards, those parts of "Medical Research in the Second Five Year Plan" (ICMR 1955) that refer to laboratory animals; in particular, two of the appendices, by Dr. V. R. Khanolkar and the present author respectively. These two articles were written quite independently, the first by a man whose tremendous knowledge of the scope and prospects of Indian medical research gave him an almost intuitive understanding of what needed to be done; and the second by one who had studied the problem in his own country and tried to work out certain general principles, but who at that time had almost no detailed knowledge of conditions in Indian laboratories.

The astonishing — and reassuring — fact emerges that the proposals put forward for consideration in 1955, and which every medical research worker in India has had three years to consider, are almost line by line endorsed by the survey carried out in 1957-8. This almost defeats the foregoing argument that policies must be based on facts, not conjectures; almost, but not quite, because the failure so far to give effect to the 1955 recommendations cannot be entirely attributed to financial stringency. In the absence of facts, it was legitimate to doubt the validity of the 1955 recommendations, which were nearly all contained in Dr. Khanolkar's article, because they were based on opinion. The survey had to be done to convince the sceptics that the opinion was sound. Moreover, if the arguments about the price of having poor animals, poor facilities and poor technicians are accepted, the cost of delay in implementing the recommendations would have gone some way at least towards meeting the cost of implementing the 1955 proposals.

Briefly, the recommendation was that there should be established at a suitable location in India, a biological institute whose function would be to maintain colonies of laboratory animals of high quality in order to supply laboratories with breeding stock. Subsidiary functions were to supply the technical knowledge for breeding the animals satisfactorily, to train animal house curators and technicians, to conduct research into *ad hoc* problems of laboratory animal management and, not the least, to act as a general information centre serving all laboratories in the country. It was — it is — an ambitious programme, but it does not involve a vast expenditure and, as has already been suggested, it would be likely to pay for itself, indirectly, by raising the standard of laboratory animals and thus the value of the work for which they are employed.

LAIS, ICLA and the Future

The International Committee on Laboratory Animals (ICLA) was formed in December, 1956, under the sponsorship of IUBS (International Union of Biological Science) and UNESCO, with further support from CIOMS (Council for International Organisations of Medical Sciences) and IUPS (International Unit of Physiological Science)[2]. Details of its activities will be found in ICLA Bulletins published half-yearly from September, 1957.

With the generous support of UNESCO, surveys of the production and use of laboratory animals in many countries have been, and are being, carried out, and it was as part of this programme that the Indian survey, already referred to, was initiated.

Among ICLA's many aims is to act as an exchange for information about laboratory animals in different countries, and to stimulate interest in the various aspects of this subject.

The Indian survey was followed, in India, by the setting up of LAIS — Laboratory Animals Information Service — which published its first Bulletin in April, 1958. A second Bulletin appeared in October, 1958, and half-yearly publication is now planned. The establishment of this service was made possible by a further grant from UNESCO, but it is unlikely that this will suffice to cover the cost of more than the first two bulletins; the continuation of the service will have to depend on national support. It is hard to believe that this will not be forthcoming, in view of the importance to medical research of this sort of work. It might in fact be regarded as the initiation of an important part of the programme of the proposed biological institute, and it will presumably be included among that institute's tasks when the institute comes into being. It is, however, a cause of considerable satisfaction that in India there is already the germ of a national laboratory animals organization. India and Japan are the first two Asiatic countries to witness this development, and in this they are in advance of several European countries.

Indeed, India was represented, by Mrs. Batra, on a committee set up in 1956 by IUBS to look into laboratory animal problems; the work of this committee, together with similar work initiated independently by UNESCO, was later taken over by ICLA. India can therefore, claim to have been concerned in this field of work, on the international side, from the beginning, and naturally occupies a leading place in it in South Asia today. The achievement of this pre-eminence is not solely due to her outstanding importance as the major country of South Asia, but is also due to her very active prosecution, going back for many decades, of medical research; and the Haffkine Institute, of course, has a proud and famous record here. It is a great honour to be asked to contribute to this Diamond Jubilee Souvenir, and to write on a subject in which India herself is setting a leading example in her part of the world.

One may legitimately expect the proposed biological institute, if it materialises, in anything like the form envisaged in the 1955 ICMR Report, to be a model, attracting widespread interest within and without India. If I were asked to indicate an aspect of laboratory animal management in which we might expect such an institute to make an early major contribution to practical knowledge, I would suggest the field of nutrition: the devising of suitable feeding regimes for the common laboratory species. On the basis of work already carried out in many Indian laboratories, I would expect to see developed methods of utilizing local cereals, pulses and other inexpensive foods to provide complete diets in suitably presentable forms, and to establish a pattern for imitation in other countries where the raw materials of the diets commonly used in America and the West are difficult to obtain or prohibitively expensive. Research laboratories are being established in many new places all over the world, in Africa, South America, Indonesia, and the countries of the Far East, and this is a problem that will be acute wherever new animal houses are built. A model solution of it will be an important practical step in realizing the research programmes in their new laboratories, and no country is in a better position to make it than India.

If there is one lesson that emerges from the detailed study of laboratory animals throughout the world, it is that medical research is going to demand animals of a greatly superior quality in the future than it has used in the past; and, therefore, arrangements must be made to provide them. Sixty years of medical research, an international reputation and a future no less promising than the achievements of the past, constitute a record of which any institute in any country can be justifiably proud. There is no reason why the Haffkine Institute should not continue its outstanding contribution to medical knowledge by playing a prominent part in implementing or

abetting the creation of a biological institute, in whatever form and under whatever name it finally emerges. Medical research is everywhere clamouring for animals of a quality that until recently was not considered necessary, and even more recently considered unattainable. But today we know that really good animals are needed and can be had, and that the cost of their provision is not prohibitive. We are also confident that they will pay substantial dividends in medical research. The world needs the contribution of Indian genius to the solution of the problem of finding them.

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DEPARTMENT OF VACCINE

H. I. JHALA

THE Vaccine Department of this Institute is as old as the Institute itself. The very first activity of its founder was directed towards development of a plague prophylactic at this Institute. Later many other vaccines were added.

Plague Vaccine

Haffkine Institute is the birth place of plague vaccine and it is but natural that the first systematic studies in plague vaccine were also initiated at the Institute. The lack of a suitable method for the assay of the protective power of a plague vaccine had handicapped all further work in the improvement of it. Sokhey's work on the biological assay of plague vaccine has contributed not only to assessing of the protective power of the plague vaccine but also to solution of quite a number of other problems connected with it, resulting in the development of a vaccine with a high immunising value and a low toxicity.

It was realised that to work out a standardized method for measuring the protective power of the vaccine, it was imperative that the infective dose used to challenge the immunised animals must be constant both as regards the number of viable organisms contained in it and the virulence of the organisms. In order to solve these problems it was necessary to solve the following subsidiary problems with which the main problems were intimately associated. They were (1) the solid medium of choice for the growth of *P. pestis*, (2) method of determining the number of viable organisms in broth cultures, (3) experimental animal of choice for plague work, (4) method of measuring the virulence of plague cultures, (5) method of maintaining the virulence of plague cultures.

The main problems facing the production of plague vaccine at that time were (1) existence of *P. pestis* strains of different antigenic types, (2) suitability of using virulent and avirulent strains, (3) the advantages or disadvantages of liquid or solid media for growth, (4) the most suitable temperature of incubation, (5) the most suitable period of incubation and (6) method of killing the organisms.

The existence of Different Antigenic Types of *P. pestis*

The work carried out at the Institute on the mouse protective values of plague vaccines prepared from different strains has clearly shown that the vaccine prepared from one strain can give as good protection against both homologous as well as heterologous strains. Vaccines prepared from strains isolated in places like Java and Madagascar, which are far apart, show that they give as good a protection as vaccines made from Indian strains when tested against infective doses of Indian strains. In other words, *P. pestis* does not exhibit any strain specificity, a finding of fundamental importance for vaccine production.

Suitability of Virulent and Avirulent Strains for Vaccine Preparation

Haffkine, believed that for the production of a potent plague vaccine, the strain employed must be a highly virulent one. Later the German Plague Commission and other subsequent workers also corroborated the views held by Haffkine. But Schutze on the basis of his experimental work contented that superiority of virulent strains over avirulent strains could not be demonstrated. Extensive work carried out at this Institute with vaccines made from strains of different degrees of virulence and tested by the standardized mouse protection test, has clearly indicated that an avirulent strain has to possess sufficient amount of residual virulence, to produce a vaccine of good immunising value.

Agar Vaccines and Broth Vaccines

Haffkine advocated the use of vaccines grown in the liquid medium as he believed that the vaccine grown in liquid medium was superior to that grown on solid medium. Later the German Plague Commission recommended the vaccine which is grown on agar surface and killed by heat. But in India we continued to use Haffkine vaccine as it was very much simpler to prepare in very large quantities and also because Haffkine vaccine had given proof of its excellent immunising value in the various field trials. Experimental work carried out at the Institute has shown that agar vaccine has got as good immunising properties as the Haffkine vaccine and possesses the great merit that it is very much less toxic than the Haffkine vaccine. It is this finding that led us to the search of the Casein Hydrolysate Plague Vaccine which is discussed separately.

Temperature of Incubation of Plague Cultures

The experimental work carried out has shown that Haffkine vaccine grown at 28°C was as good as agar vaccine grown at 37°C. Agar vaccines grown at 28°C and broth vaccines grown at 37°C possessed very poor protective values.

Period of Incubation

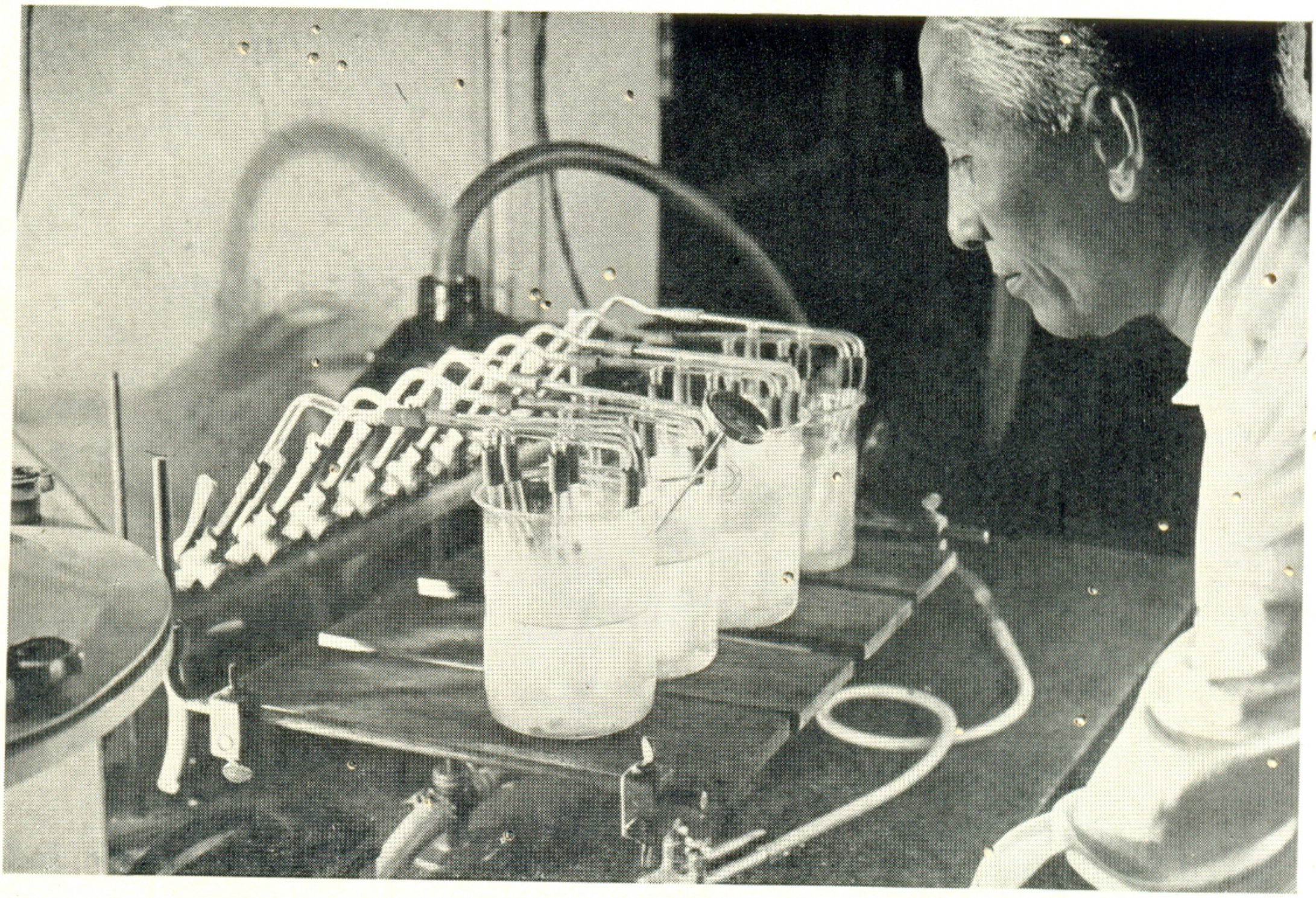
Haffkine incubated his cultures for 5 to 6 weeks. This period varied from time to time until on the basis of mouse protection tests it was found that there was no particular advantage in incubating the cultures beyond four weeks for preparing Haffkine Plague Vaccine. The Haffkine vaccine prepared at the Institute till it was replaced by the Casein Hydrolysate Plague Vaccine used to be incubated for four weeks.

Method of Killing the Cultures

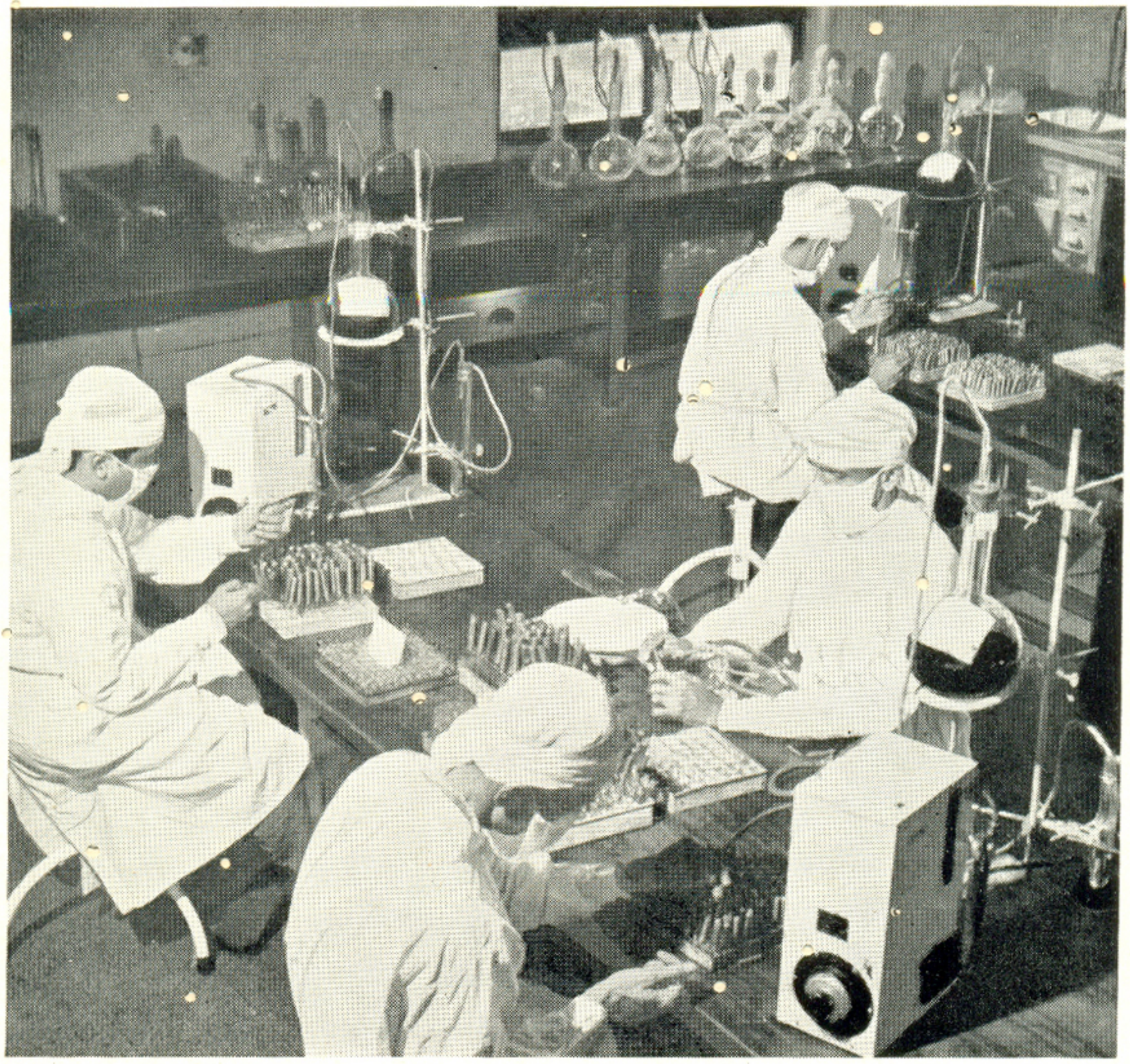
Haffkine killed the cultures by heating at 65°C for 1 hour. He later reduced this temperature to 55°C for 15 minutes. Since then different workers have tried different sterilising agents *viz.*, formalin, alcohol, acetone, chloroform and phenol. But the different workers employed different methods for assessing the potency of the vaccines and hence the results were not comparable. Therefore, it was thought necessary to make a reappraisal of the effect of the different killing agents by the standardized mouse protective test developed at the Institute. The work carried out at the Institute has shown that the nature of the different killing agents used for sterilising the cultures does not in any way affect the protective power of the vaccine, if used in quantities sufficient to kill the cultures and no more.

Casein Hydrolysate Plague Vaccine

Inspite of the considerable amount of work done to standardize and improve the Haffkine vaccine, it still suffered from very serious defects. The medium, mutton digest broth used for preparing the vaccine, contained a good deal of non-specific protein matter, which naturally was very undesirable. The vaccine also was highly toxic and therefore it was very difficult to popularise Haffkine vaccine. On the other hand, the agar vaccine is free from both these defects. Therefore, the choice before the Institute was either to improve the vaccine grown in the liquid medium by eliminating the defects or change over to agar vaccine. The casein hydrolysate medium of Mueller and Johnson, which is free from precipitable protein and



Lyophilisation



Aseptic filling of vaccines

gave good growth of *P. pestis* appeared to solve the problem. However, good deal of experimental work was necessary to standardize and further improve it to make the medium suitable for the production of plague vaccine.

The problems regarding the temperature of incubation, period of incubation, killing agent to sterilize the vaccine, keeping qualities of the vaccine were studied *de novo* and the preparation of the casein hydrolysate plague vaccine was fully standardized. The vaccine is at present prepared by incubating the cultures in the modified casein hydrolysate medium at 32°C for two weeks. The culture is killed and detoxicated by the addition of 0·07 per cent formalin and preserved by the addition of 1·5 mg. of phenyl mercuric nitrate per 100 ml. of the vaccine. This vaccine has got the great advantages that it possesses high immunising value, low toxicity, and low cost. Thus a problem awaiting solution for a very long time was ultimately solved.

Lag Phase in the Growth Cycle of *P. pestis*

A significant contribution was made in the study of lag phase in bacterial growth cycle. A number of postulation have been advanced, to explain the lag phase *viz.*, (1) age of the culture, (2) size of the inoculum, (3) composition of the medium, (4) temperature of incubation and so on. Wilson and Miles have brought out a new concept that lag phase is not a phase of rest but a phase of intense growth activity.

Our studies have conclusively proved that none of these concepts is correct. Lag phase is mainly due to the lack of nutritional requirements. A medium which supplies the nutritional requirements showed no lag phase. At least this was the case with *P. pestis*. Perhaps it may be with other bacteria also.

Capsule of the Plague Bacillus

A significant contribution was made on the study of the antigenic structure of the plague bacillus. It was shown that the plague bacillus possessed a regular capsule and not an envelop as shown by the other workers.

Optimum Temperature and pH for the Growth of *P. pestis*

Paucity of data based on quantitative measurements on the optimum temperature and optimum pH values, for the growth of *P. pestis* led to the investigation of this problem again. It was shown on the basis of statistically evaluated data that the optimum temperature for the growth of *P. pestis* was 27° to 28°C and the optimum pH was 7·2 to 7·6.

Biological Assay of Typhoid Vaccine

A modified method has been developed for the biological assay of typhoid vaccine. The method has been in use at the Institute for the last 15 years and has proved valuable for the assay of different batches of vaccines and for the fixation of a working standard for passing the vaccine.

Immunisation with Combined Vaccines

Opinion is still divided about the advisability of giving mixed antigens for immunisation. The experimental work has shown that the mixed antigens react as if they are given independently without producing either synergism or antagonism.

Casein Hydrolysate Cholera Vaccine

Faced with an acute shortage of the supplies of agar in India on account of the outbreak of war with Japan, investigations were undertaken to find out the possibility of preparing a cholera vaccine in a liquid medium. It was merely a matter of finding a suitable liquid medium for the growth of cholera vibrios. The modified casein hydrolysate medium of Mueller and Johnson was found to be an excellent medium. The vaccine prepared in this medium by growing the vibrios at 37°C for 3 days and killed by the addition of 0·08 per cent formalin and preserved by adding phenyl mercuric nitrate has been found to possess high immunising value and is easier and cheaper for preparation of large quantities.

Method of Biological Assay of Cholera Vaccine

To assess the protective value of the casein hydrolysate cholera vaccine a mouse protection test was developed. The test has been found very useful for determining the protective values of different batches of vaccine produced in the laboratory and has thus supplied a long felt need. The method has been found to be more accurate than the N. I. H. method.

Antigenic Structure of Vibrio Cholera

Studies carried out here on the 0 antigenic structure of *V. cholerae* based on mouse protective tests showed that it bore no relation whatsoever to the 0 antigenic structure of *V. cholerae* based on serological determinations. This method has thus helped materially to select out suitable strains for vaccine production which the serological methods failed to give.

DEPARTMENT OF CLINICAL PATHOLOGY AND DIAGNOSTIC REAGENTS

M. V. SANT

FROM its very commencement, the Institute has been carrying out a certain amount of clinical diagnostic work. In June 1905, pursuant to a directive from the Government of India, the Institute was made the Provincial Pathological Laboratory. The Diagnostic section so started, has now developed into the present Department of Clinical Pathology and Diagnostic Reagents, fully equipped to take up all higher types of routine work, training of technicians and researches.

The research problems tackled have been on the various aspects of major diseases like typhoid, cholera, tuberculosis, sprue, maternal mortality, etc. These have been studied in detail by workers of the Institute and research fellows sent by various scientific bodies.

Typhoid Fever

Investigations in connection with typhoid fever have been as old as the existence of the Institute. Owing to the endemic nature of the disease, it has continuously remained a problem for workers from time to time. Problems regarding (*i*) suitability of laboratory tests, (*ii*) superiority of one medium over another, (*iii*) nature and (*iv*) distribution of types etc., were some of the topics that engaged the attention of various workers.

Reviewing the value of haemoculture and Triple Widal Test in diagnosis of enteric group of fevers, it was concluded that haemoculture was the only sure method of diagnosing enteric group of infections. Comparing the results of haemocultures from week to week it was observed that bacteremia in typhoid was not of a short duration and that positive haemoculture could be obtained even as late as in the fourth week. The clot culture method had advantages over whole blood culture, in that, serum was available for Triple Widal Test and clot for the culture; and the positive results appeared more and earlier with clot culture than with whole blood culture. Single Triple Widal Test had very little diagnostic significance in an endemically infected population.

With the introduction of chloramphenicol in the treatment of enteric fevers, the problem of early diagnosis still became difficult. A study of cases under treatment of chloramphenicol showed that the drug interfered with the blood cultures and Triple Widal Tests.

The value of D. E. C. medium in the isolation of intestinal pathogens as compared to MacConkey and Litmus Lactose Bile Salt Agar medium was worked out and reported. Later on, comparative studies on use of glucose broth, Hartley's broth and bile broth were made and it was observed that bile broth offered higher positive isolations from blood cultures of suspected enteric fever cases.

The value of Vi agglutination Reaction in the diagnosis of enteric fever as compared to routine Triple Widal Test and the haemoculture, was worked out. It was observed that Vi agglutination test, by itself, was found

to have little diagnostic value as compared with combined method of clot culture and Triple Widal Tests.

The problem of handling large number of contaminated clot cultures was studied. The use of B.S.A. medium in addition to D.E.C. medium had the advantage of giving higher percentage of positive isolations.

Typhus

In a place where typhoid fever has been an endemic disease, the possibility of typhus fever as one of the probabilities in the differential diagnosis, has always drawn the attention of clinicians.

Ever since Weil and Felix reported on the minimum diagnostic titres for proteus OX 19, OX K and OX 2, the use of Weil Felix reaction has become a part of the extensive Widal Test in routine diagnosis of continuous fever of 3-4 days duration.

Various workers like MacKenzic, McCony Brockbank and others worked on fixing the minimum diagnostic titre for typhus infection in non-endemic areas.

Similar studies were undertaken here and analysis of 1,263 sera from pyrexial and apyrexial cases showed the titre of 1 : 125 to be presumptive and 1 : 250 or over confirmatory in the diagnosis of typhus. It was also stressed that typhoid and paratyphoid infection did not influence Weil Felix titres and also did not give rise to anamnestic reaction.

Further studies on incidence, distribution and laboratory methods of diagnosis of scrub typhus were continued. Out of 1,578 sera from pyrexial cases, 16 were serologically diagnosed and 8 by demonstration of causal rickettsiae in mice and rabbits. Cases usually occurred in months of September, October and November and it was also shown that even a metropolitan city like Bombay is not free from this infection.

Relapsing Fever

Investigations on relapsing fever had been started since 1900. During a severe epidemic of this disease among some children in the Nasik Mission Settlement in 1907, the valuable discovery of body-louse as the vector of the infection, was made. This fact was subsequently confirmed by another independent worker Ed. Sergent from the Pasteur Institute of Paris.

The spirilla of this fever was observed to have three types of motility; viz. darting, vibrating or thrilling, and undulating or swaying.

Bugs fed on infected monkeys yielded spirilla regularly upto the fourth day and in some up to seven days. Infected bugs transmitted the infection to a healthy monkey.

Experiments conducted showed that the attack of spirillum fever in monkeys and other animals produced immunity by formation of anti-substances and in the case of monkeys such protection lasted for about two months.

Diphtheria

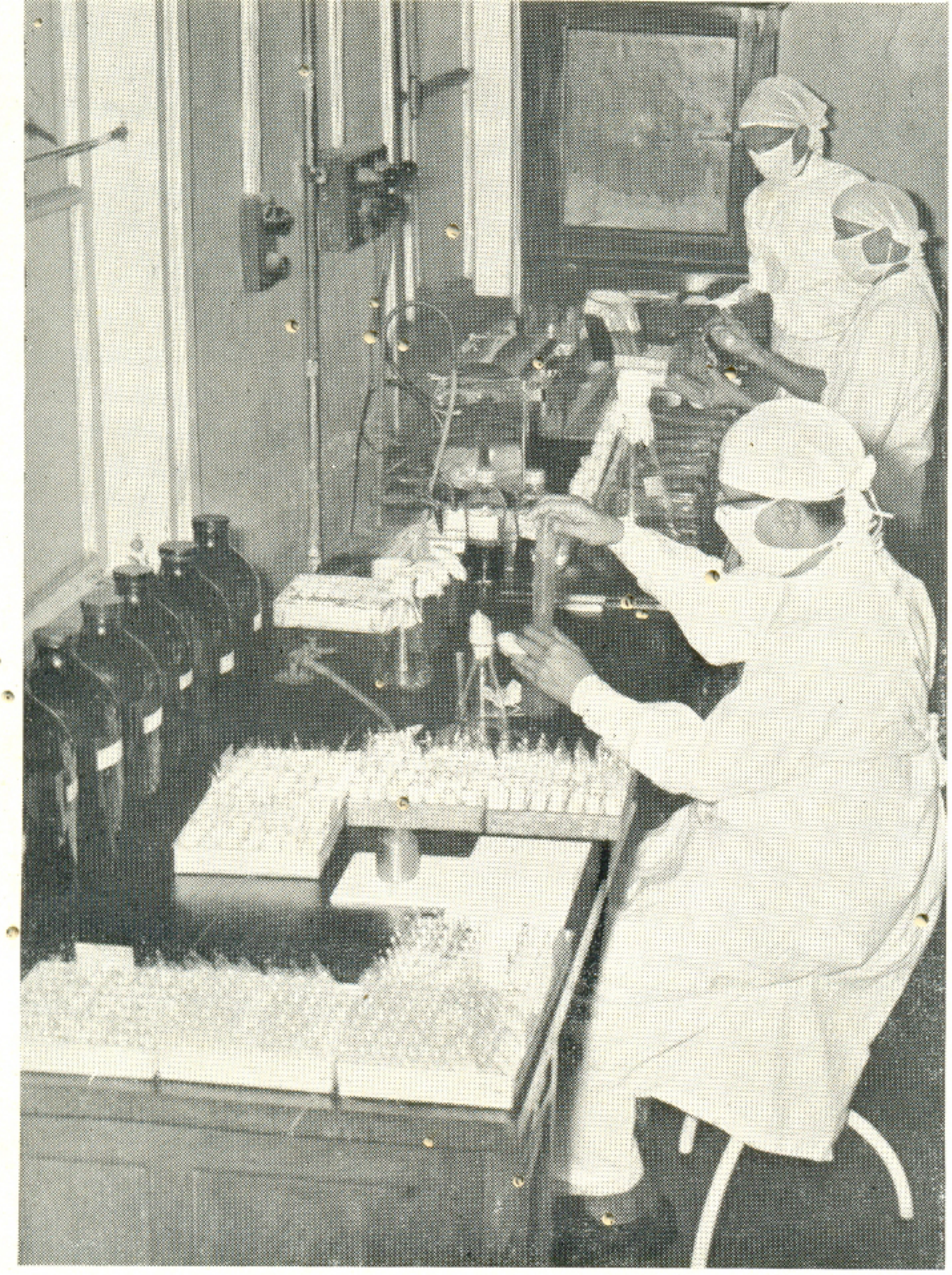
Since Anderson and others first introduced Tellurite medium in the diagnosis of diphtheria, the recognition of the three types has become almost a routine procedure in public health laboratories.

A comparative study of Loeffler's medium and Neill's medium for primary isolation of *C. diphtheria* showed that 92 per cent of strains could be isolated from both media, 2·3 per cent on Loeffler's medium only and 4 per cent on Neill's medium alone. Thus a combination of two media was recommended for routine use.

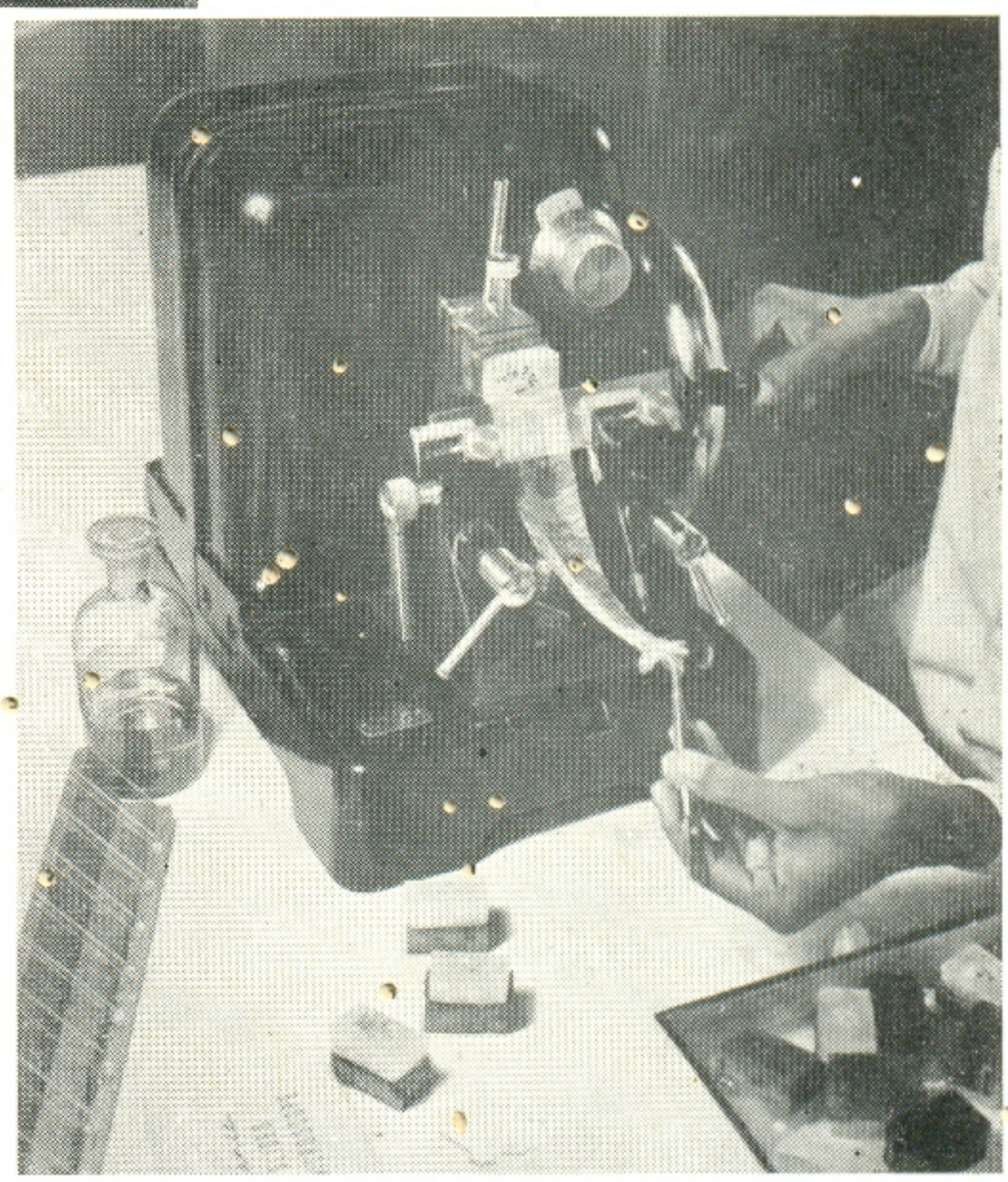
Out of 500 throat swabs examined from suspected diphtheria cases, 175 strains of *C. diphtheria* were isolated. These were classified as mitis (167), gravis (7) and intermedius (1).

Virulence tests on guinea pigs by subcutaneous or intradermal routes showed that 89 per cent of them were virulent.

With the introduction of *in vitro* methods for determining the toxigenicity of *C. diphtheria* strain in 1933 by Elek and Ouchterlony and the use of chick embryos by Evans in 1951, a study of 35 strains of *C. diphtheria* was undertaken. It was observed that toxigenicity could be demonstrated by all the three methods. However, the plate method and the chick embryo method showed a distinct advantage over the guinea pig test as both were economical and simpler to perform.



Diagnostic Reagents Section



Micro-section in the diagnosis of tumours

Tuberculosis

Studies on tuberculosis (*i*) both the human and animal types, (*ii*) nature of the tubercle bacillus, (*iii*) allergic reactions, and (*iv*) improvements in the methods of cultivation, have been carried out at this Institute from 1913 onwards.

A preliminary enquiry into the prevalence of tuberculosis among Bombay cattle by (*a*) macroscopic examination of carcasses at the slaughter house and (*b*) examination of milk samples for presence of tubercle bacilli microscopically and by animal inoculation techniques, gave negative results. A case of bovine tubercle bacillus infecting a human being was reported. Continuing the studies further, it was found that 4 per cent of swine were suffering due to infection by the human type of bacilli (10 strains) and also by the bovine type (1 strain), the nature of infection being indicative of the locality.

Experiments on the tubercle bacillus showed that a filtrable phase existed. When filtrates of tuberculous material were serially passaged into experimental animals, pure cultures were obtained which, however, differed in virulence from their parent strains.

The Vernes Risorcin serum test was studied to find out the specificity of this test for tuberculosis. However, results proved unsatisfactory as it gave positive results for various conditions other than tuberculosis.

Continuing studies on tuberculosis, it was reported that nature of allergic reactions in tuberculosis supported the Maurice Nicolle and Wolf Eisne hypothesis regarding toxicity of the sensitized skin cells of the tuberculous animal. It was observed that the blood serum of a tubercular animal, when mixed with tuberculin, on inoculation into a normal healthy animal, failed to produce the same reaction as when produced by inoculating a mixture of healthy skin of a tubercular animal and tuberculin.

Improvements in the methods of cultivation of tubercle bacilli were also introduced. An easy method of transplanting tubercle bacilli from solid to liquid culture media was developed, by which, any culture, dry, moist, or slimy, could be transplanted from solid media directly on the surface of fluid media when growth begins to appear and spread. This is also highly satisfactory in obtaining large numbers of broth cultures of the organisms.

While isolating tubercle bacilli from routine laboratory specimens such as sputa and body fluids, a comparative study on the concentration methods such as TSP and Petroff's NaOH method was undertaken. As regards positive isolations, TSP method was found to be the inferior one, though it showed a better inhibitory action on contaminants.

Cholera

Investigations in connection with epidemics of cholera were undertaken since 1906 when an outbreak in Umer Khadi jail was reported.

Vibrios isolated from various sources in Bombay from fresh or old stools, wells, tanks and sewage were systematically studied. Guinea-pig inoculation tests and complement fixation tests were done on these strains. It was observed that these vibrios differed in their haemolytic and agglutinating properties. During subsequent outbreaks in 1943-45, work on serological subtyping of the strains was undertaken. It was noted that out of 164 strains isolated, 161 belonged to Ogawa subtype, 3 Inaba, and none of intermediate type.

Sprue

The considerable and apparently increasing incidence of sprue in Bombay led to an investigation into the nature and the causation of this serious and widespread malady. The researches of Ashford and others had focussed attention on the presence of yeasts (monilia) in the intestinal tracts of sprue cases and their probable causative influence. Hence, the role of these yeasts was studied in its different aspects.

Fresh stool samples from cases of sprue were subcultured on the special sprue medium. The yeasts isolated were divided into two groups as maltose fermenters and maltose non-fermenters, because, it was noted that all those so-called " Pathogenic " yeasts

believed to be concerned with sprue, thrush, and such like conditions, came under the maltose fermenters, whilst, the so-called 'Wild-yeasts' were almost invariably maltose non-fermenters. It was found that this key reaction of maltose fermenters was constant and unaffected by prolonged subculture or passage through animals.

Monilia conforming in all respects to *M. psilosis* (Ashford) as described in the literature were found present in 40 per cent of sprue cases, in 37 per cent of other intestinal diseases, 38 per cent in other diseases, 46·2 per cent in healthy men, 60 per cent in healthy animals and 44·4 per cent in experimental animals. Thus, the yeasts studied did not show any causative relation to sprue.

Animal experiments were carried out with freshly isolated strains of *M. psilosis* (Ashford) from clinical cases of sprue. A large number of monkeys, guinea-pigs, white mice and rabbits were used.

When *M. psilosis* (Ashford) and other species of monilia were injected into animals by the peritoneal route, a small proportion died of peritonitis with or without a generalized monilia septicaemia. A large number showed a mild degree of plastic peritonitis and recovered. Many recovered without any signs at all. These conditions were there whether one or repeated doses of monilia were given.

Feeding experiments were also negative when healthy animals were used. The faeces of healthy animals frequently showed the presence of monilia which were indistinguishable from *M. psilosis*.

No exaltation of virulence was detected as a result of passage of *M. psilosis* through a series of animals by intraperitoneal infection. No sprue could be produced in monkeys and other laboratory animals.

In some of these experiments, monkeys were fed on a vitamin C deficient diet, hoping this would induce susceptibility to sprue infection. No sprue could be produced in these animals; but it was found that avitaminosis in itself produced an increased susceptibility to diseases of the intestinal tract. So, the association of bowel diseases with vitamin C deficiency was studied in detail. Monkeys which were fed on a dietary deficient or lacking in vitamin C became anaemic, lost weight rapidly and generally suffered from a terminal dysentery which ended in death. This scorbutic condition could be checked by the administration of orange juice or early return to normal dietary. These changes appeared in all animals kept in a diet deficient in vitamin C whether given infective sprue material or not.

Summarizing the experience of eight autopsies, it was observed that "sprue is primarily a disease of the intestinal tract, which, if progresses, results ultimately in degeneration and destruction of the absorbing and secretory tissues and the production of a condition of slow progressive starvation. The absorption of toxins from the damaged mucosa perhaps associated with actual bacterial invasion, appears to be an important factor in the progressive anaemia and other late manifestations of the disease".

Reference in literature on the efficiency of liver in sprue were scattered and very few. Therefore, a study was undertaken to see if modern functional tests would throw any light on the problem. The four functional tests selected were (*a*) Levulose Tolerance test, (*b*) Van den Berg reaction, (*c*) Nitrogen partition of blood and (*d*) Brom Sulphalein test of Rosenthal and White.

Thirteen cases of well marked sprue were studied. In all these it was found that the liver was not affected to such an extent as to show impairment by the liver function tests adopted.

Ever since sprue has been described as a distinct entity by Hillary, bulky, gaseous and pale-coloured stools have been recognized as a cardinal feature of the disease. Almost every observer has considered this bulk to be due largely to increased excretion of fat. It was suggested by some observers that this so-called fatty stools would be due to disturbance in the secretion of pancreatic juice. The results reported by different workers were contradictory. Hence the problem was tackled by (*a*) analysis of fat in faeces, and (*b*) direct examination of duodenal contents for the presence of distase, trypsin and lipase.

These examinations conducted on five sprue patients did not show any variation from normal persons to suggest any impairment of normal external secretion of pancreas in sprue.

A bacteriological study, by blood culture, during life, of thirty cases of sprue, yielded an unknown bacillus in two. The rest were sterile both to bacteria and to yeasts.

Protozoa such as amoebae and flagellates were rare in sprue faeces, probably as a result of their high acidity.

Ninety-two specimens of sprue faeces were closely studied for intestinal bacteria. In a complete analysis of 51 cases out of these, not once was a recognized pathogen isolated, though an atypical strain resembling *B. dysenteriae* (Flexner) was found once.

The condition of blood in sprue has constituted a problem of continuous difficulty to physicians investigating tropical diseases and for four years various members of the staff of the Institute were engaged in collecting haematological data from sprue cases. An analysis of these data of 67 cases showed that the red corpuscles averaged 3,242,000 per c.mm., the haemoglobin 65·5 per cent and the colour index 1·0 for the whole series.

A number of workers, particularly Ashford and his colleagues, of the Porto Rico School, had advocated the fungoid theory of sprue with *Monilia psilosis* (Ashford) as the true causative agent. It was also held by them that the extracts of this yeast yielded reliable antigens for serological reactions which were enthusiastically advocated both in clinical diagnosis and in the assay of the progress or efficacy of treatment.

The serological studies carried out showed that rabbits inoculated intravenously with saline suspensions of *M. psilosis* yielded sera of high grade titre when tested *in vitro* by means of the complement fixation reaction using aqueous extracts of the monilia as antigen. Identical antigens when used against human sera derived from sprue patients failed to show positive reactions in 15 out of 17 cases tested. Thus no confirmation of the claims of Ashford-Martiner circulating monilia antibody in the sera of sprue cases was obtained.

Infantile Diarrhoea

Diarrhoeas in infants and children under two years of age, constitute a well-recognized, much-dreaded disease entity. Investigations carried out during an outbreak of diarrhoea in a local Hospital for children, in September-October 1905, gave *B. enteritidis sporogenes* as the main causative organism, present in nine out of eleven stool samples received for study.

Another outbreak in 1906 in a Hospital in the city also showed *B. enteritidis* group as the important one causing severe outbreaks of diarrhoea. *B. dysenteriae* (flexner) was found only once. A thorough knowledge of the bacterial flora of the intestinal tract both in normal and diseased infants and children is an essential prerequisite for the diagnosis and treatment of diarrhoeas.

A recent survey of the intestinal tract bacterial flora of infants and children yielded the following results. (*See page 90*)

Bilharziasis

The studies were started on bilharziasis as far back as 1920 and continued upto 1927. They were carried out in goats and water buffaloes and monkeys. The latter were shown to have a species immunity. Water buffaloes were found to be a definite host of *S. spindalis*. Serological studies carried out showed the presence of a group antigen.

Guinea Worm Infection

The distribution of guinea worm infection in India was studied in 1914. Data were collected and it was found that the most affected part was western part of Madras Presidency, next in order were, Deccan, Northern Circles, Carnatac, Gujarat, West coast and Indus Valley. The survey was not complete as details about army and prisoners were only collected. It was suggested that the infection was due to " step wells " and could be easily prevented.

Anaemias in Pregnancy and Maternal Mortality

It has long been known that severe anaemias occur during pregnancy but it is only since the beginning of the present century that a distinction has been made between the

Age	*Samples*	*No. of samples*	*Predominant organisms*
0-10 hours	Meconium	16	Nil
10-48 hours	Meconium	19	Enterococci and Micrococci
	Stool	6	
48-96 hours	Stool	25	Enteroçocci and colon group
5 days (breast-fed)	Stool	40	*L. bifidus* and colon group
5 days (bottle-fed)	Stool	10	*L. acidophilus* and colon group
9-24 months	Stool	14	Colon group and enterococci

The bacteriological findings in 150 cases of infantile diarrhoeas were as under.

Name of organism	Salmo-nella	Shigella	Proteus morgani	Proteus mirabilis	Pseud-aerogi	Para-colon	Staphy-lococci	Strepto-cocci	Others
No. of times isolated	8	7	5	51	7	13	9	1	25
Per cent	5·33	4·66	3·33	34	4·66	8·66	6	6	16·66

anaemias of pregnancy and the pernicious anaemia.

In view of the frequency with which this disease occurs in India, a detailed investigation on 150 cases in Bombay were carried out. Pyrexia was present in 83·3 per cent of the patients, oedema in every case, diarrhoea in 38 per cent, spleen-enlargement in 18 per cent, vomiting in 40 per cent, sore-tongue in 31 per cent, epistaxis in 7 per cent and bleeding from gums in one case. Pulse was invariably rapid in all cases. Age-group showed, 61·2 per cent between 20 and 30 years, 22·6 per cent between 30 and 40, 3·6 per cent under 20 years and 2·6 per cent over 40 years. Blood pictures showed few variations.

An investigation undertaken to throw more light on the condition of Indian women during child-birth, on the causes of their mortality and on the diseases from which they are most prone to suffer, involved a study and analysis of 11,343 cases.

Maternal mortality rate was proved to be 21·5 per thousand as against 9 in British Hospitals. Anaemia and Osteomalacia (unknown or rare in the British Hospitals) were serious causes of disease and death.

A preliminary study of the diets of patients suffering from anaemia of pregnancy suggested that certain dietetic faults might be causatively related to this disease. With a view to investigating this possibility further, a large-scale dietetic and hygienic survey of different classes of women in Bombay city was carried out.

The data collected showed that class A (healthy Hindu Women) was well-fed and not anaemic. Class B (hospital cases of women) was relatively to A, both quantitatively and qualitatively ill-fed and also anaemic. The anaemia was slight and due to

a parallel reduction to both the number of red cells and the percentage of haemoglobin. The diet of class B was different in (*a*) calories, (*b*) both total and animal protein and fat, (*c*) all fresh fruit and vegetables, (*d*) in vitamins A and C, and (*e*) in salts. The amount of Vit. B present varied in the different groups. This relative deficiency in Vit. A and C or some factor associated with this deficiency appeared to be concerned in the etiology of 'pernicious anaemia of pregnancy' and that the proportion of fat and Vit. B in the diet may play some part in determining the incidence of this disease in a population generally short of Vit. A and C.

The discovery of the efficiency of liver in the treatment of pernicious anaemia suggested a new line of treatment. The pregnant cases responded more slowly than others; when the condition was severe, large doses equivalent to 600 gms. of fresh liver daily were needed for rapid response.

Treatment with vitamin A and C proved a complete failure. Animal experiments proved the deficiency of these two vitamins was not concerned in the production of such macrocytic anaemias. These latter experiments did, however, strongly suggest that some deficiency of vitamin B complex was significant as a causative factor and that vit. B had curative properties. Experiments with patients were began with Marmite, a form of yeast extract. Marmite was found to be a curative agent as potent as liver extract.

The nature of the haemopoietic factor in Marmite was investigated. For this, clinical trials with different preparations like yeast powder, watery yeast extract, Marmite, insoluble and soluble fractions of Marmite, alkaline autoclaved Marmite, alcoholic extract of Marmite and acid clay vitamin B were carried out. Only Marmite was found to be effective. The trials with soluble and insoluble fractions showed that the active principle was water-soluble and also alcohol-soluble.

The haemopoietic principle in Marmite was found heat-stable-after autoclaving for 5 hours at pH 5·5, at 120°C and 20 lbs. pressure—the preparation was as active as before; in fact, the cures with this autoclaved Marmite were so spetacular that the tendency was to give this preparation rather than untreated Marmite.

Marmite is an autolysed yeast extract made from brewer's yeast. Salt is added during the process of manufacture. Dry yeast in large quantities was not found effective. These suggested that the active principle in Marmite may be a protein break-down product.

Endometrial Biopsy

The diagnostic value of endometrial biopsy in many gynecological conditions is being recognised and the histological finding accepted as more dependable now than at any time before.

An analysis of 1217 samples of endometrial biopsy material showed that the incidence of chronic endometritis to be 53·92 per cent and tuberculous endometritis to be 5·55 per cent. It was observed that this higher incidence existed amongst poorer class due to mal-nutrition, insanitary condition of life and repeated child birth.

Paul Bunnell Test

The value of Paul Bunnell Test in the diagnosis of glandular fever was stressed by the authors in 1932 by the accidental discovery of heterophile antibodies in the form of an agglutinin for sheep's red cells, in patient's blood serum. Since then, various workers reported their observations on its diagnostic value. A study to determine the level of heterophile antibodies in the local population and in case of pyrexias of 3-4 days duration was undertaken here in 1947. Out of 600 sera tested only two showed a titre of 1 : 80 from amongst pyrexial cases. Fifteen out of 64 sera from suspected glandular fever cases showed positive Paul Bunnell Tests.

Epidemic Dropsy

An epidemic of "Mysterious disease" (epidemic dropsy) prevailing in Nadiad district was investigated in 1953.

Clinical examination showed that people of all socio-economic levels experienced malaise, vague upper abdominal discomforts, passed frequent and semi liquid stools

and developed edema of the feet. In addition an erythematous rash, particularly over the edematous parts was noticed. Routine and cultural examinations of blood and urine did not indicate any bacteriological factor. Cultural examination of stools showed organisms of proteus and paracolon group only. Subsequent investigations of samples of edible oils etc., showed the presence of Argemone oil in some of them, particularly in ground nut oil.

Hook Worm

A survey to find out the incidence of hookworm in Kaira district was undertaken in 1954-55. In all, 10,699 samples of stool were examined from the district, of which, 3,447 (35 per cent) showed hook worm ova. Blood samples of 2,340 cases out of 3,447 positive cases were examined for evidence of anemia. Four hundred and twenty-five cases showed evidence of severe anaemia.

Leprosy

Efforts to infect laboratory animals (white mouse, rat, guinea pig and monkey) with leprosy virus from material obtained from human lesion showed that (*a*) simple subcutaneous and intraperitoneal injections of human leprosy material failed to produce any obvious disease in the mice, rat, guinea pig and monkey, (*b*) six weeks after 4-6 intraperitoneal injections, white mice developed minute seedlike nodules in the gastro splenic omentum and these showed acid fast bacilli.

Further work on analysis of over 100 leprous sera tested for complement fixation showed the following: (*a*) in case of cutaneous leprosy with massive acid fast bacillary invasion of the skin, one observed a strong complement fixation reaction in 100 per cent, (*b*) in the neural type, where no bacilli were demonstrable in the skin, a negative complement fixation in 100 per cent cases was obtained, and (*c*) in case of tuberculoid type, the reaction gave a doubtful result. Histological studies on rat leprosy also confirmed these findings.

Biological Test for Pregnancy

Ever since Zondek reported the immature female white rat to be much more sensitive to prolan (A. P. L. hormone) than the immature female mouse, the rat has been tried as an experimental animal for pregnancy hormone tests. Various workers reported on modifications which reduced the test period from 48 hours to 24 hours. A method has been devised in rats by which the period could be reduced to 3 hours only by selecting the intraperitoneal route of inoculation.

DEPARTMENT OF VIRUS DISEASES INCLUDING RABIES

H. I. JHALA

THE Department of Virus Diseases including Rabies was started primarily for the production of antirabic vaccine in 1922. Today, it consists of a production unit, an Out Patient's Section for antirabic treatment, Yellow Fever Inoculation Section and a research section. Since 1952 it is functioning as a W.H.O. centre in India and actively participating in the influenza programme of that organisation.

The research work conducted here falls under the following main headings : (*i*) experimental studies on preparation and testing of potency of virus vaccines, and standardization of methods employed, (*ii*) development of new and simple techniques, for the detection, identification, and study of viruses, and (*iii*) epidemiological surveys and field trials.

Experimental Studies on Preparation and Testing of Potency of Viral Vaccines

Rabies.—A single dose canine rabies vaccine was introduced in 1948 to replace the 5 per cent Semple's vaccine which had to be administered in multiple doses. The single dose canine vaccine has proved to be far superior and is in use at present.

The Semple's vaccine prepared in Waring Blender contains a substantial amount of brain tissue which is not completely broken down, therefore, mechanical maceration by various methods was tried. An electrical ball mill was found to be most suitable for breaking up the brain substances. Vaccine prepared in this manner was lyophilized and inactivated with phenol. Phenol inactivated the virus quicker in this preparation than in Semple's vaccine. Chloroform treated freeze dried vaccine prepared by this method gave a homogeneous suspension when reconstituted and its potency was comparable to Semple's vaccine. The effect of storage, action of phenol, and inactivation with chloroform, on Semple's vaccine, were studied. It was observed that the vaccine retained its potency for a period of 10 months which exceeds the six months period laid down for the Semple's vaccine. The potency tests carried out using phenolised and chloroform inactivated vaccine indicated that the immunizing property of the latter was superior to a small degree, and did not justify the use of chloroform in preference to phenol.

Avianised rabies vaccine has been prepared using the Flury strain and is under test. Adaptation of locally isolated rabies street virus to chick embryos was started in 1957. Nine strains were taken up for adaptation but only three have been adapted to eggs so far. It was observed that serial passages in mice upto the 10th passage facilitated the mechanism of adaptation and strains which did not initially take to the chick embryo were successfully adapted after further passages in mice. It is expected that this study will yield a suitable strain for preparation of avianised vaccine.

Influenza.—Influenza vaccine has been prepared by modifying the chick cell adsorption elution and methyl alcohol precipitation methods. In the preparation of vaccine by the chick cell adsorption elution method it was possible to reduce the adsorption time to two and half hours under controlled conditions, instead of overnight adsorption as recommended in the standard method. It has been observed that this gives a relatively better yield of virus, besides the additional advantage of finishing the whole processing in one day.

Complement Fixation Test in Rabies.—Biological tests on rabies brain specimens received at this Institute for detection of Rabies virus were examined microscopically as well as by mouse inoculation. It was observed that 18·8 per cent of specimens found to be negative by microscopic examination were positive when inoculated intracerebrally in mice. It is therefore necessary to carry out a biological test before pronouncing a specimen negative.

In the course of development of complement fixation test in rabies, 101 brain specimens and 49 salivary glands specimens were examined by the direct smear examination, mouse inoculation and complement fixation test. It was possible to obtain correct results by the complement fixation test in 96 per cent brain specimens and 89 per cent of the salivary glands. No false positive results were observed. Considering the ease with which this test can be performed and the short time required for obtaining the results it can be recommended as a diagnostic test. Specimens giving a negative complement fixation test may be subjected to mouse inoculation for confirmation.

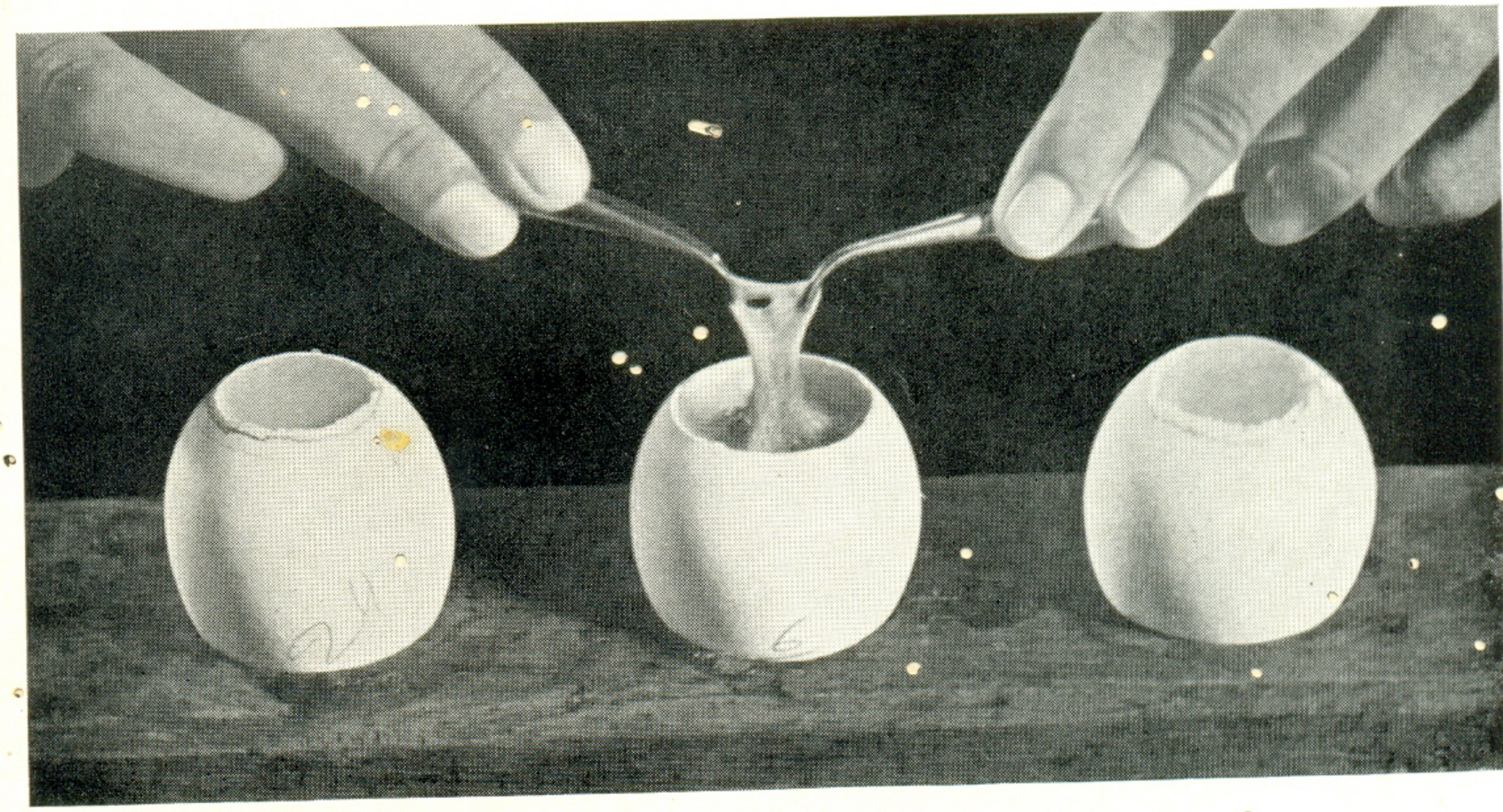
An investigation on salivary glands collected from positively diagnosed rabid animals, conducted over a period of five years, has revealed the presence of virus in the glands in 70 per cent of the animals only. Virus could not be detected in the glands of all rabid animals. This may prove to be of additional advantage to the clinician in treating persons bitten by rabid animals.

Diagnosis of Influenza.—Over 400 samples have been tested in this department for the presence of influenza virus. Thirty-three strains have been isolated and studied so far. In controlled studies carried out, no strain of the virus appears to have been missed by cutting down of the 5-day incubation period employed.

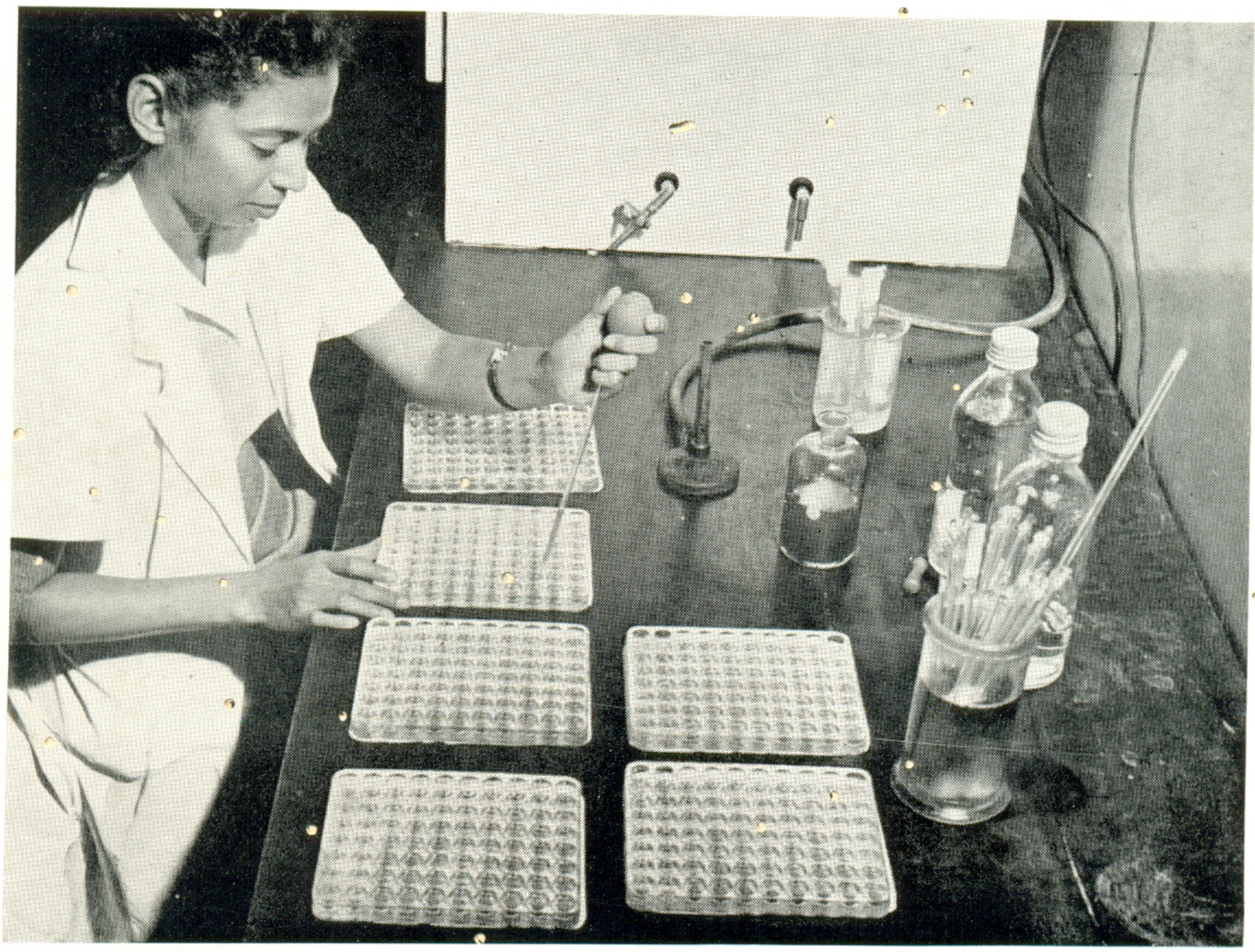
Complement Fixation Test in Influenza.—The test has been extensively used to study the antigenic relationship of *Influenza B* strains and demonstration of antibody rise in convalescent sera. The titre obtained in cases of Asian flu were low but significant for diagnostic purposes. Both viral and soluble antigens were used for the complement fixation test.

Haemagglutination Test.—It has been utilised to demonstrate a four-fold or higher rise in the antihemagglutinin titre in sera of patients and vaccinated human beings. Typing of strains is also done by the Haemagglutination Inhibition test. Thirty-three strains isolated here have been successfully typed by this method. In a recent study of antigenic relationship of Asian flu strains, isolated here, to other influenza virus strains isolated earlier, it has been shown that the Asian strains are quite distinct from the strains isolated so far and that they are quite closely related within their group.

Laboratory Diagnosis of Small-pox.—This work was carried out with a view to assess the reliability of the various tests that are available for diagnosis and their application to aid the diagnosis of doubtful cases of modified small-pox. One hundred and sixty-one cases of small-pox, at different stages, from the pre-eruptive to the crusting stages, were investigated. The specimens were subjected to microscopic examination, egg inoculation, and complement fixation test. The results obtained with the three different tests were compared. The clinical follow up showed that the results obtained in the laboratory were correct. Each method was found to be quite dependable. The complement fixation test was carried out by using anti-vaccinial serum prepared in rabbits and vesicular fluids or blood as the antigen. Blood samples collected from patients in the early pre-eruptive stage and a few haemorrhagic cases were also tested



Harvesting of yolk-sac membranes of the developing chick embryo



Test for identification of influenza viruses

by egg inoculation and the complement fixation test upto the 5th day of illness. The presence of the virus and viral antigen in blood after the first three days of illness was found to be of grave significance in the prognosis of the patient. All such cases turned out to be fatal.

A presumptive diagnosis based on microscopic examination can be given in a few hours or after a complement fixation test is done in 24 hours time. This has been found to be a reliable test. Isolation of the virus by egg inoculation takes 4-5 days and the results can be made available only after the virus isolated on the chorioallantoic membrane has been identified.

Epidemiological Surveys and Field Trials

Typhus.—A survey of incidence of typhus in Bombay city and suburbs has revealed that *Tsutsugamushi disease (Scrub typhus) is* fast on the decline. Examination of 9,958 sera gave a positive Weil Felix reaction in 4·4 per cent only. A few (22) strains were also isolated in mice from human beings but specimens from rodents and mice were found to be negative.

The distribution of murine typhus in field and domestic rodents was studied by the complement fixation test. About 24 per cent of 739 sera tested were found to be positive. Two strains of Typhus isolated in guinea-pigs during the course of this investigation were proved to be of murine type.

Q Fever.—Over a thousand samples of sera collected from animals and human beings in Bombay state were tested by the complement fixation test. About 10 per cent of animal sera and 3 per cent of human sera gave a positive complement fixation test. As is evident from the result, Q fever occurs sporadically in this area in a clinically unrecognized form.

Influenza Vaccine.—Since the vaccine was made available after the Asian flu had assumed epidemic proportions in this city, it was not possible to study the protective value of the vaccine in clinical field trials. However, antihaemagglutinin response was studied in human volunteers. Controlled laboratory experiments in animals and the antihaemagglutinin response in human beings suggest that the vaccine is effective in about 70 per cent cases.

Rabies.—An assessment of antibody level in human beings receiving antirabic treatment was carried out. It was observed that the titre of antibodies in patients is proportional to the quantity of vaccine administered. The work on confirming these findings is being continued.

DEPARTMENT OF PHARMACOLOGY

N. K. DUTTA

THE history of the establishment of the Department of Pharmacology dates back to the year 1924, when a small unit was added to this Institute through the generosity of the Indian Research Fund Association now known as the Indian Council of Medical Research. The object of this unit was to explore therapeutic possibilities of Indian indigenous drugs, so that what was good of the old system could be brought to light and incorporated in the modern scientific method of the West. While this objective is not lost sight of even today, the unit has developed into a full-fledged laboratory to cover its manifold activities in the fields of research and drug testing. Research on drugs has been directed to (*i*) the studies on Indian medicinal plants, (*ii*) chemotherapeutic investigations, (*iii*) general pharmacological studies, (*iv*) analytical problems and (*v*) some pharmaceutical investigations.

Indian Medicinal Plants

Holarrhena antidysenterica, *Butea frondosa* and *Gymnema sylvestre* were the few plants to be taken up for study quite a long time ago. The seeds and the bark of *Holarrhena antidysenterica* were investigated chemically, pharmacologically and clinically. The seeds were found to contain 29·36 per cent fixed oil and 0·025 per cent alkaloids as the main constituents. The pharmacological examination of the seeds showed that they have low toxicity, no antiseptic properties and no astringent action, but they affect the respiratory centre. Clinically good results were obtained in cases of amoebic and non-amoebic dysentery and also in cases of diarrhoea. The bark yielded 7·56 per cent gum and 0·22 per cent alkaloids among other minor components. The powdered bark proved satisfactory for clinical applications to dysentery and diarrhoea cases. The alkaloid conessine isolated from the bark, when subjected to pharmacological investigation, appeared to paralyse the respiratory centre in toxic doses. It showed a true vasoconstrictor action and a weakening effect on the heart. It also proved to be efficacious in clinical trials. The fixed oil from the seeds was found to be non-toxic and effective in the treatment of diarrhoea. The fixed oil from the seeds and the gum from the bark were proved to be valuable as anti-dysentery drugs but the bark or the seeds themselves were superior to them clinically.

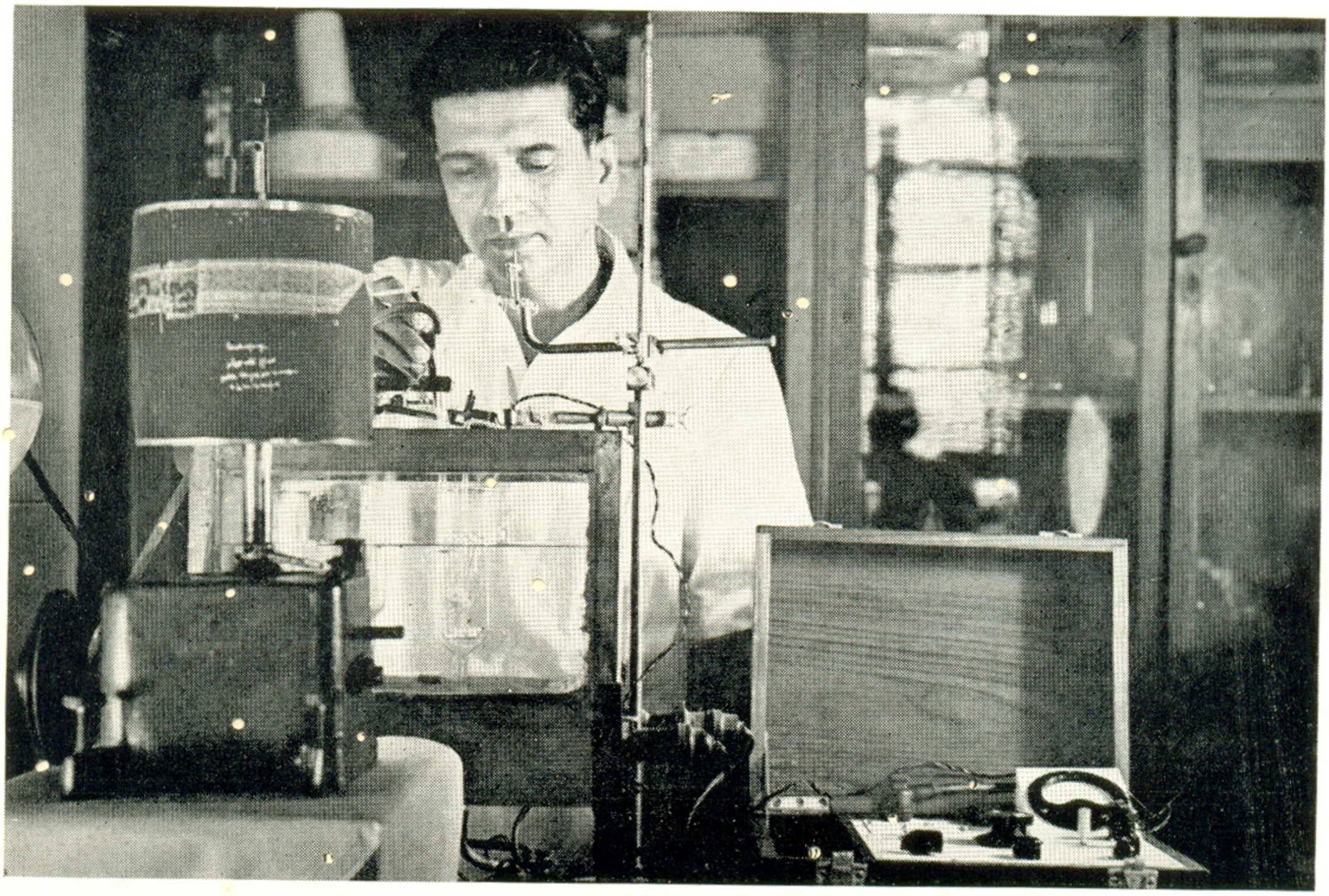
The fixed oil from the seeds of *Holarrhena antidysenterica* did not exhibit any marked anthelmintic properties, but the alcohol-soluble fraction of *Butea frondosa* was shown after clinical trials to possess such action.

Wrightia tomentosa proved to be inert in the treatment of dysentery, whereas *Wrightia tinctoria*, *Garcinia mangostana* and *Alstonia scholaris* were found to possess anti-diarrhoeal properties.

Chemical examination of the leaves of *Gymnema sylvestre* revealed the presence of anthraquinone derivatives and the absence of an insulin-like substance capable of destroying glucose *in vitro*. Yet, an oral administration of a decoction of leaves or a hypodermic injection of extracts in rabbits brought about significant lowering of the blood sugar content. The drug was found to have purgative properties and it could also stimulate heart muscle giving rise to increase in blood pressure. Although there was no



Intra-intestinal inoculation of *V. cholerae* in the infant rabbit



Study of curarising drugs on rat diaphragm

evidence to show that the drug can directly affect the carbohydrate metabolism, it can decrease the content of blood sugar probably indirectly by an increase in the insulin secretion of the pancreas.

The absence of any marked cardio-tonic activity of the bark of *Terminalia arjuna*, which was claimed to be a potent cardiac stimulant, initiated a detailed comparative study of the pharmacology of the Indian species of the genus *Terminalia*. Fifteen out of the twenty Indian species could be obtained for this work. Healthy and well-fed dogs, monkeys, rabbits, guinea-pigs and at times frogs were employed as experimental animals. Alcoholic extracts and in some cases decoctions or hot infusions were prepared and after dilution with Ringer solution they were used for administration. The barks of the two species *T. manii* and *T. travancorensis* were almost incapable of any pharmacological action on the heart. *T. arjuna*, *T. belerica* and *T. pallida* proved to be mild diuretics while *T. bialata*, *T. coriacea* and *T. pyrifolia* were shown to have marked cardio-tonic properties. The barks of *T. catappa*, *T. chebula*, *T. citrina*, *T. myriocarpa*, *T. oliveri*, *T. paniculata* and *T. tomentosa* were found to possess both diuretic and cardio-tonic properties.

None of the various drugs commonly used in India as anthelmintics could actually fulfill their claims. *Carum copticum* and *Artemisia maritima*, although the source of powerful anthelmintics thymol and santonin respectively, themselves did not exhibit any marked activity. Attempts at correlation of chemical composition with therapeutic properties led to the discovery of new anthelmintic drugs like oil of sassafras, oil of cloves, oil of anise and oil of wintergreen.

Evaluation of indigenous medicinal plants as snake-bite remedies was done on an extensive scale and can be regarded as almost exhaustive. Dogs weighing 10 kg. on an average were used as the experimental animals. The venoms of cobra and daboia were injected subcutaneously over the muscles of the thigh. Depending on the weight of the animal, 60 to 120 ml. of a hot or cold extract of the drug in the form of a pulp or dry powder were introduced slowly by means of a stomach tube. Drugs specified to be used externally were employed as a viscous fluid extracts instilled into eyes or nostrils at intervals of 15 to 30 minutes. Local application of a drug was effected by frequently rubbing it in a powdered form over a 4 square-inch area around the site of inoculation. As far as possible dosage prescribed in the literature was strictly followed or it was calculated on the basis of the therapeutic data available. Examination of 314 individual plants and 184 combinations of the same, indicated that practically none had therapeutic effect in animals which had received a lethal dose of the snake-venoms. The fact that the list of drugs tried covered almost all the possible remedies enjoying reputation in Indian systems of medicine makes this investigation a significant contribution to clinical practice although the results could not be positive.

A similar work of exhaustive nature was carried out in regard to indigenous medicines commonly used as remedies against scorpion-bite. The studies in properties and toxicity of scorpion venom pointed out that in no case a scorpion sting could be fatal for human beings. Tests were performed with 195 individual plants and 33 combinations prepared out of these with a conclusion that none of them could exert any preventive, therapeutic or antidotal effect.

Roots of *Curcuma longa* Linn., *Berberis asiatica* Roxb., and *Cissampelos pareira* Linn. were examined pharmacologically and therapeutically in cases of corneal ulcers and conjunctivitis, but the results were contrary to claims in Indian sytems of medicine.

Similar claims for insecticidal activity in the case of fresh leaves of *Adhatoda vasica* Nees, *Anona squamosa* Linn., and *Lantana camara* Linn., and capsules and leaves of *Datura alba* Nees., and *Uraria crinita* Desa, could not be proved experimentally when tried against mosquitoes.

Wagatea spicata Dalzell (N. O. Leguminose) was investigated chemically and pharmacologically and concentration of an alcoholic extract of the roots of the plant yielded a crystalline substance similar in physical and chemical properties to *vakerine*,

a crystalline substance previously isolated from *Caesalpinia digyna* Rottler belonging to the same natural order and having the same vernacular name. A sterol-glycoside was also obtained from light petroleum ether extract of the alcoholic extractive after removal of *vakerine*. The pharmacological examination of vakerine indicated no action on blood pressure and respiration and no antagonising effect against histamine and acetyl choline. The roots are known as a remedy against pulmonary tuberculosis. *Vakerine* could not, however, inhibit the growth of *E. coli*, *S. aureus* and *E. typhi*.

Chemotherapeutic Investigations

Chemotherapy of plague, malaria and cholera were investigated using either compounds synthesised in the department or already known synthetic drugs.

Chemotherapy of plague was the subject of experimental investigation in respect of some sulphonamides and twelve halogenomercuriphenols, nine of which were synthesised. *In vitro* studies of these compounds against *P. pestis* revealed that chloro derivatives are more active than the corresponding bromo, iodo and fluoro derivatives, the para compounds being more potent than the ortho or di-derivatives. The content of mercury in these compound did not have any direct relationship with toxicity. A systematic experimental work on rabbits gave a general indication that no definite therapeutic efficiency could be claimed for these synthetic drugs although prolongation of life and diminution in septicaemia were noted.

A number of heterocyclic and other derivatives of sulphanilamide were prepared. Out of these, six compounds were tested for their value in plague infection, employing a method developed for ascertaining the curative value of anti-plague sera. 2-N′-sulphanilamidothiazol proved to be very promising in these experiments on plague-infected white mice as it could be compared fairly well with a good anti-plague serum. Similar experiments on sulphathiazole and sulphapyridine in which these sulphonamides were administered immediately or 48 to 72 hours after the introduction of plague in white mice showed that about 80 per cent of the animals could be saved from death. Sulphathiazole was found to be much more effective than sulphapyridine in its curative action in plague-infected mice.

A part of the work on synthesis of two new series of compounds structurally related to quinine and cinchonine was carried out in the department. Pharmacological examination of both the series of compounds revealed that they were effective against paramaecia. The series of compounds which resembled cinchonine proved to be without any effect in avian malaria.

Rhesus monkeys infected with *Plasmodium knowlesi* were employed as experimental animals in another series of experiments in which sulphathiazole and its newly synthesised derivatives were tried for their therapeutic value in malaria. An oral treatment of infected monkeys with sulphathiazole given 0·5 g. twice a day for three days resulted in complete cure. No toxic effect was observed during this oral administration.

One of the synthesised derivatives of sulphathiazole, viz. 2-N′-sulphanilamido-5-isopropylthiazole was found to afford a radical cure with only 0·5 g. given orally without producing any untoward side-effects.

These studies in chemotherapy of malaria were extended to a significant investigation into malarial immunity in the Rhesus monkey. The experimental work involved replacement of a major part of the blood of immune, normal and infected monkeys with normal and immune blood as the case might be and subjecting the animals to further infection. From the experimental findings it was inferred that the cellular agencies probably act more significantly and directly on malarial parasites, while the humoral agencies exert an indirect effect through stimulation of the cells.

A significant contribution to chemotherapy of cholera and a stride towards progress in the field of pharmacology is the evolution of a biological method for testing of drugs with any probable action against cholera. Experimental investigation into susceptibility of rabbits of various ages to *V. cholerae* concluded with a finding that infant rabbits (less than 15 days old) were the most susceptible

and hence the most suitable experimental animals. The disease in the rabbits is characterised by severe diarrhoea haemo-concentration, dehydration and cramps in the muscle. The picture closely resembles that of human cholera. Three conditions need to be fulfilled, if experimental cholera with 100 per cent mortality is to be expected : the animal should be below 16 days old, the inoculating vibrio should be rabbit passaged and the organisms should be given intra-intestinally.

Oxalated heart blood of such infant rabbits infected intra-intestinally with 10,000 V. cholerae per 100 g. body weight was studied biochemically in respect of cell volume, urea and non-protein nitrogen. The results of biochemical studies of blood and of the post-mortem examination of experimental animals showed that the disease very closely resembles cholera in human beings. This certainly gives promise as an important tool in the hands of pharmacologists who can now assess the correct value of drugs claimed to be efficacious in the treatment of cholera. Application of this technique to various drugs has led to interesting conclusions. Thus, it has been found that chloramphenicol, chlortetracycline, oxytetracycline, sulphaguanidine and formaldehyde sulphathiazole have a different degree of preventive or protective action without any curative effect. Novobiocin, a recently introduced antibiotic, however, gave promising results. It was found to possess curative action against cholera in infant rabbits, if it were administered before the onset of diarrhoea.

General Pharmacological Investigations

Plasmoquine was subjected to very thorough investigations in regard to its toxicity to animals and bacteria and action of the circulatory system, on blood, respiration, digestion, nervous and the reproductive systems. The drug has no marked action against bacteria and protozoa and hence its malarial paraciticidal effect should be considered as more specific. Although it has no antipyretic action, its combination with quinine has synergistic effects. The most noteworthy effects of the drug were the fatty degeneration of the liver and a moderate stimulation of the uterus. It was also observed that placenta prevented large quantities of the drug from reaching the foetus, thus allowing its use during pregnancy. It was shown to stimulate oxygen-uptake of tissues like blood, spleen, liver, brain, and cell-free liver extract of a guinea-pig and the probable cause of this phenomenon was traced out to be an enzymatic reaction. A study of the fate of plasmoquin in the body led to the conclusion that the drug is destroyed by enzymelike substances in the liver and some other tissues like brain.

A similar study of placental transmission was undertaken in respect of 4:4′ diaminodiphenylsulphone which has become a drug of choice for treatment of leprosy. The fact that the drug which is rather known to be toxic is given to pregnant lepers aroused interest in the problem as to whether it passes from maternal to foetal circulation with any adverse effects. Rabbits fed with 100 mg. per kg. of the drug from the day of mating to that of the delivery were kept under observation. No untoward symptoms were noticed during the period of pregnancy and even the litters after birth were found to be normal, in spite of the experimental result that the concentration of the drug in the blood of the mother rabbits was almost the same as that obtained for their young ones.

The pharmacological properties of some flavones like 7-hydroxyflavone, chrysin, genkwarin and 4-methylumbelliferone were investigated. It was observed that they do not possess any anthelmintic, antiseptic or germicidal properties. Intravenous administration of the same in dogs was observed to depress their blood pressure.

Importance of acetylcholine in human physiology suggested an important problem regarding the role played by choline esterase in blood in regulating physiological functions. The content of choline esterase in blood was found to be constant for an individual and remained unaffected under different physiological conditions like starvation, loss of sleep and rise in temperature. Even drugs like morphine, strychnine and acetylcholine cause no change in blood

esterase values. This could not, however, indicate anything regarding possible changes in the esterase values of different tissues under various physiological conditions. Formation of acetylcholine by tissues was the subject of another investigation which showed that it is formed at all parts of the nervous system which are rich in nerve cells and exhibit this distinguishing property.

It is known that histamine plays an important role in the secretion of gastric juice from stomach, but how it does this is still a matter of speculation. Previous investigations have demonstrated that substances, such as atropine, pethidine, procaine, quinidine and diphenhydramine hydrochloride show certain pharmacological properties in common with one another. One of these is their ability to antagonise the action of histamine. A study of the effect of these substances on the gastric secretion in cats induced by histamine showed that none of these substances significantly altered the secretagogue action of histamine on the gastric parietal cells of the stomach.

It has long been recognised that the physiological effects of an intravenous injection of snake venom bear much resemblance to the acute effects of histamine or anaphylaxis in human beings. Experiments performed here showed that the venoms of cobra (*Naja naja*), krait (*Bungaras coeruleus*) and saw-scaled viper (*Echis carinatus*) liberate histamine from the isolated rat diaphragm (skeletal muscle), cobra venom being the most active and the saw-scaled viper venom the least active in this respect. Anti-histamine drugs like antazoline, tripelennamine or promethazine did not reduce the toxic effect of cobra venom on rats and guinea-pigs, the survival period being not significantly altered when envenomed animals were treated with these drugs.

Protein hydrolysate, commonly used for treatment of hypoproteinemia and similar conditions, is conventionally prepared from casein, a milk protein. Manufacture of sera and anti-toxins from horse blood carried out in the Institute gives rise to the by-product fibrin which does not find any use at present. Possibilities of utilisation of fibrin for preparing protein hydrolysate for parenteral administration were explored experimentally. A hydrolysate prepared by partial acid digestion of horse fibrin was found to contain higher amounts of some important essential amino-acids like tryptophane as compared with the contents of casein hydrolysate. The higher content of tryptophane was considered to be the probable reason for a better recovery in adult protein-depleted rats when fibrin hydrolysate was substituted for casein hydrolysate. Clinical study showed that fibrin hydrolysate is non-toxic and devoid of any antigenic properties.

Studies on Analytical Methods

Development of new analytical methods or modification of existing ones for achieving more reliability has remained one important aspect of investigations carried out. It has manifested itself not only after analysis of drugs became the routine work of the department but also before that when, during the course of certain investigations, estimation of drugs like plasmoquine or adrenaline in certain tissues had to be carried out.

An incidental observation that chromates, di-chromates and complex acids of tungsten and molybdenum develop purple to blue colour with plasmoquine led to the development of methods for detection and determination of plasmoquine and the aforesaid metallic elements. The ease with which adrenaline is oxidised suggested an enzymic method for estimation of adrenaline from suprarenal glands. Oxidase prepared from the seeds of *Dolichos lablab* was employed to oxidise adrenaline at a pH between 4·4 to 7·6 to give a red colour the intensity of which was found to be proportional to the concentration of the drug. The results of estimation of adrenaline by the colorimetric method compared favourably with those of the biological assay based on blood-pressure changes.

Another very sensitive and accurate method was also worked out for estimation of plasmoquine in a wide variety of tissues of animals. Application of the Folin's phenol reagent to plasmoquine resulted in a blue colour, which developed in about half an hour and remained stable for about 3 hours, strictly followed Beer's law even upto a

concentration of 1 part per million of plasmoquine. Recovery of the drug by this method was sufficiently accurate from a number of tissues and biological fluids except urine of different experimental animals.

A modification of the Fujiwara reaction for detection of chloroform was worked out for estimation of trihalogen anaesthetics in blood and tissues of animals. The volatile anaesthetic compounds are air-distilled into ice-cooled pyridine and estimated colorimetrically.

Detection and determination of saccharin in common beverages like tea or in pharmaceutical preparations was felt necessary for helping public health measures to check replacement of sugar with saccharin in preparation of beverages. Kastle's reaction for detection of saccharin was fully investigated and finally developed into a quantitative method for determination of the synthetic sweetening agent. The colorimetric method developed was found to be sensitive, accurate and capable of yielding results closely comparable with those obtained after following the British Pharmaceutical Codex method. This has placed in the hands of the analyst a tool that will enable him to help the public health authorities to check adulteration of sugar.

Colorimetric determination of folic acid in pharmaceutical preparations presented some serious and apparently inexplicable difficulties for the analyst. A thorough investigation into the nature and causes of interference with such determination by commonly employed therapeutic and other ingredients of pharmaceutical specialities led to some important conclusions. Effect of iron slats and l-ascorbic acid on the reliability of the colorimetric assay of folic acid suggested some minor as well as major changes in the assay procedure to be adopted for routine evaluation of pharmaceutical preparations.

Pharmaceutical Investigations

Failure of a large number of samples analysed in the department provoked the interest of analysts in the instability of some vitamins incorporated in pharmaceutical preparations. Thiamine hydrochloride or mononitrate and folic acid have been subjected to such investigations of their stability on storage. Copper sulphate and iron salts like ferrous sulphate and ferric ammonium citrate were found to exert a very deleterious influence on the stability of solutions of thiamine mononitrate or thiamine hydrochloride in water or dilute alcohol stored at room temperature for 10 months. The loss on storage ranged from 10 to 90 per cent of the original strength of solutions investigated. The presence of riboflavin, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate and folic acid was observed to be beneficial in regard to retention of thiamine in aqueous alcoholic solutions. While vitamin A acetate and liver extract do not affect the course of deterioration, ascorbic acid and yeast extract have adverse influence on the stability of the vitamin.

A similar study of the stability of folic acid in pharmaceutical preparations could not be very simple as the methods for estimation of folic acid proved to be unreliable under certain conditions. The method for assaying folic acid was chosen and thoroughly examined as to its faults when employed in the presence of commonly occurring ingredients of complex pharmaceutical specialities. Ways and means of avoiding the descrepancies observed among the results of determination of folic acid in the presence of interfering factors were devised. Temperature, light, vehicles, hydrogen-ion concentration and a representative variety of substances incorporated along with folic acid in pharmaceutical preparations were the aspects of the experimental investigation into the reasons for instability of folic acid on storage. Aqueous solutions of folic acid could retain their initial strength within the pH range of 5·0 to 8·0. The pH range of 6·0 to 6·6 was found to be the optimum for retention of folic acid stored in buffered vehicles. The study revealed that thiamine, riboflavin, choline, ascorbic acid, manganese sulphate and calcium hypophosphite were main among the substances responsible for instability of folic acid during storage. A significant observation that ascorbic acid prevents the decomposition of folic acid caused by riboflavin may serve as a clue for formulation of multivitamin preparations containing folic acid.

BIOCHEMISTRY SECTION

R. N. JOSHI

IN 1923, at a meeting of the Scientific Advisory Board of the Indian Research Fund Association, a request was made for the establishment of a Biochemical Unit at the Haffkine Institute. This was one of the first reflections of the growing awareness of the subject in the country. Accordingly, a unit was set up at the Institute in 1924.

"The Biochemistry of Sprue" was the first problem taken up for study from chemical, medical and biochemical points of view. The problem of the so-called disease was approached from the biochemical side with respect to the fat metabolism of the intestinal contents, the secretion of digestive juices, fat, protein and calcium contents of the blood. The functional efficiency of the stomach, liver, pancreas and kidney was also taken into consideration while studying this problem. These researches threw much light on the causation and treatment of sprue. A certain amount of evidence was made available favouring the view that sprue was primarily a metabolic disease characterised by inability to utilise fats in normal way.

Researches on Haffkine's plague prophylactic were carried out with a view to finding out a new method for the measurement of the growth of *P. pestis* in liquid medium. As difficulties were encountered with the opacity method and the method of counting colonies, an effort was made to devise an alternative method. With this object in view carbon dioxide, given out by the organisms during their growth, was measured and compared with the weight of the organisms grown.

The effect of amino-acids on the growth of *P. pestis* was also studied. A 2·5 per cent solution of Glycocoll prevented the growth of the organism altogether, while 1 per cent solution gave poor growth. The amino-acid content of the broth was found to be about 0·15 — 0·20 per cent and did not rise above 0·5 per cent during several weeks' growth. Glycocoll was not found to be an injurious factor.

Studies on the Isolation of the antigenic factor from Haffkine's plague prophylactic revealed that filtrates of the vaccine were found to possess almost as high immunising value as the original prophylactic. A heat coagulable protein, most probably an albumin, was present in the filtrate but not in the original broth. This substance when precipitated with the proteoses present in the broth and redissolved in slightly alkaline saline gave a high immunity value.

The unit then undertook studies on basal metabolic rate of Indians. Standard conditions were worked out. The Basal Metabolic Rate was found to be 10 to 23 per cent below the Du Bois standards. It was noticed in this connection that the level of protein metabolism was low, urinary excretion of nitrogen being between five and seven grammes per day. Thus it was concluded that the average rate for Indians was about ten per cent lower than that for Europeans.

Researches on anemia had revealed the great prevalence of anemia resembling



Measurement of the oxygen consumption of living cells

pernicious anemia among men and women in Bombay. It was shown that anemia of sprue and of pregnancy had great resemblance to pernicious anemia and it was believed that the anemia of pregnancy was not a distinct entity, but merely an aggravated condition of anemia commonly prevalent in non-pregnant women. It was considered that deficiency of some substance normally present in the liver of healthy animals was probably a common factor in the causation of pernicious anemia, sprue and anemia of pregnancy.

The work of establishing normal standards for haemoglobin and cellular contents of healthy Indian males were completed. Normal standards for various substances usually investigated in diagnostic medicine in the chemical analysis of blood and urine such as non-protein nitrogen, urea, uric acid, creatinine, phosphates, cholesterols, fatty acids, plasma proteins, fibrin, albumin, globulin, were also worked out.

It was shown that oxidation of solutions containing glucose and ammonia in concentrations similar to those present in blood gave rise to the formation of urea. It is possible that the same reaction takes place in the body and that hyperglycemia is a protective mechanism against the excessive formation of ammonia, which is thereby converted into urea. Experiments were carried out using ammonium hydroxide and other ammonium salts. Among these salts ammonium hydroxide definitely produces hyperglycemia.

A valuable investigation of studying by chemical methods the composition of standard broth used for the preparation of Haffkine plague vaccine was carried out. This work was taken up with special reference to the fractions used up by *P. pestis* in its growth and the substances elaborated by this organism during growth. The bearing of this work on plague vaccine preparation was basic in nature. It helped to solve certain outstanding problems in this connection, namely, whether it is possible to reduce the present large dose and the toxicity of the broth vaccine and whether an agar vaccine can be used instead of a broth vaccine.

The unit then worked on "Anti-Plague Serum" in close co-operation with the Plague Enquiry. An important step in the preparation of a therapeutic serum is the process of concentration. This had to be studied afresh for each batch of serum. The first problem tackled was to determine the critical zones for the precipitation of the various fractions of globulin (Euglobulin, Pseudeglobulin I and II) in the normal sera of the animals used for the production of anti-plague serum. It was found that in the case of anti-plague serum, as opposed to antitoxin of tetanus and diphtheria, the curative principle was found to be present in all the three fractions of the globulin.

Study on shrinkage of red blood cells due to potassium oxalate revealed that the use of neutral potassium oxalate as an anti-coagulant in blood causes a shrinkage of the red blood corpuscles. Consequently the value obtained for the volume of packed cells in blood samples where potassium oxalate is used is not real.

For estimation of vitamins A and D great stress is always laid on the rearing of a standard stock of white rats on a diet which should allow the animals to reach the standard limits of weight and have free reproduction with sufficient reserves of the vitamins concerned. It was also noted that a colony stock diet found suitable in one laboratory may not necessarily be suitable in another. Keeping this in view a preliminary study of four stock diets was made, *viz.*, (1) Smith and Chick, (2) Dog Chow, (3) Sherman and Smith and (4) Coward, Cambden and Lee. To meet the local conditions certain modifications were necessary. It was found that the diet No. (4) was the best for the purpose and its consumption value was as high as others.

Work on determination of "A haemoglobin constant" was then undertaken. A study of 121 men and 101 women between 18—30 years of age belonging to the state of Bombay (medical students and nurses) gave 15·37 gm. for men and 12·99 gm. for women as values for haemoglobin content per 100 c.c. of blood. The haemoglobin

determinations were made by Van Slyke oxygen capacity method. Contrary to all expectations these averages tallied exactly with similar haemoglobin averages worked out in America for American subjects in spite of the dissimilar dietetic and climatic conditions. This observation points to the possibility of there being a physiological constant for haemoglobin. Further investigations indicated that there is a close relationship between the surface area of the body and the total quantity of haemoglobin.

The power of the kidney to eliminate urea from the blood stream is employed as a measure of renal function for clinical purpose. Moller, McIntosh and Van Slyke's blood urea clearance test, worked out on normal American subjects, is usually employed for this purpose. It was noticed in the course of the clinical work that in the case of normal Indians, while their blood urea content was within the same limits as that of American normal subjects, the urinary urea content, on the other hand, was about half. This raises an interesting issue whether the formula on which the urea clearance test is based applies to Indian subjects. Further work in that direction established following averages for maximum and standard clearance in normal Indians. The average values obtained are 44 c.c. for maximum clearance and 34 c.c. for standard clearance. The corresponding averages for Americans were 75 c.c. and 54 c.c. respectively. This is one of the most useful sets of observations for clinical work.

The unit then undertook the work of studying "Respiration of the *Plague Bacillus*". Oxidation of a series of substrates including carbohydrates, amino-acids and organic acids was studied by means of Warburg Manometric technique using different virulent and avirulent strains. Effect of some drugs on the respiration of the bacillus was also studied.

The object of the study of the oxidation by virulent and avirulent strains of *P. pestis* was to find out whether the enzymatic properties of the bacterial cells are destroyed or get modified when the organism passes from the virulent to the avirulent state. Results indicated that the cells of the plague bacillus in the virulent and avirulent strains have the same enzymatic properties.

A modified "one-piece" uranyl reagent has been developed for the estimation of sodium in serum and other biological fluids. A simple micro-chemical method has been developed for the accurate determination of serum sodium. The main feature of this method is that the sodium and the serum proteins in 0·1 ml. of the serum are precipitated simultaneously, the precipitation being complete and quantitative even at room temperature within less than 15 minutes. The presence of potassium, calcium, iron and phosphates upto certain concentrations do not materially affect the accuracy. This simple procedure is applicable to most of the body fluids except urine. With a little modification it can also be applied for urine. The phosphates in urine are removed first with calcium oxide and then the method is applied.

The work on fractionation of serum proteins by chemical method was also undertaken. It was observed that the so-called "Howe" albumin fraction obtained by fractionation with 22–23 per cent sodium sulphate contains an appreciable portion of the alpha globulins. The results of 23 selected cases of hypoalbuminemia re-emphasised the fact that the use of 22·4 per cent sodium sulphate as recommended by Howe for fractionation of serum proteins leads to albumin values higher than the true values and conceals the real picture in many pathological conditions. Hence for fractionation of serum proteins 27·4 per cent sodium sulphate is recommended.

DEPARTMENT OF ENTOMOLOGY

P. J. DEORAS

WORK has been done at this Institute on the various aspects of plague epidemiology since the theory was developed that rats and fleas are involved in the transmission of plague. To canalise these studies a full fledged Department of Entomology was started in 1938. Since the inception of this department it has worked on plague epidemiology and the general aspects of medical entomology. In 1951 the work on snakes was incorporated therein. The lines of work in this department therefore consist of plague epidemiology, rat ectoparasites, rodents, insecticides, ecology of insects of medical importance and snakes.

Ecology of Plague

The family Muridae, in the Barsi Taluka where work was done in the field, is represented by *Rattus rattus rufescens, Mus dubius, Bandicota malabarica, Tatera indica, Bandicota bengalensis, Millardia meltada, Leggada booduga* and *Leggadilla platythrix*. In the Dharwar Taluka there are two additional species i.e., *R. rattus rufescens* and *R. rattus wroughtoni*. On the whole the former sub-species is more common than the latter; but comparatively the latter sub-species is more abundant in a village than in a town. *B. bengalensis* which forms about 40 per cent of the entire rodent population of the Bombay City was almost absent from the human habitations in villages and towns of both the talukas, and is only found in the open fields.

The examination of the ectoparasites of both the wild and domestic rodents has led to the conclusion that *X. cheopis* is predominantly found on the house-rat and musk shrew, and *X. astia* is occasionally found on them. On the other hand, *X. astia* is exclusively found on the wild rodents. *T. indica* harbours the largest number of fleas, with a flea index varying from 8·6 to 53. *B. bengalensis* is next to it with a flea index seldom exceeding 6. *M. meltada* has the least number of fleas with a flea index less than one. Other ectoparasites like lice and mites are specific to their hosts. It was found that the proportion of *X. astia* increases on domestic rats collected from houses bordering the fields. It was absent from domestic rats from the centre of the town; but it predominated on domestic rats in farm houses. Similarly the presence of other ectoparasites of wild rodents like *P. stephensi, L. echidninus* and *L. nutalli* in very small numbers on domestic rats, especially during the rainy season, showed their close association with wild rodents. Some of the collections of *M. meltada* which, as a rule, does not harbour fleas had a few specimens of *X. cheopis*, showing thereby that the rodents of this species either sometimes venture into the villages or they pick up fleas scattered by the domestic rats that leave a village for their extramural excursions. There is, however, some evidence to show that domestic rats that leave a village during the epizootic scatter fleas on their way. In villages Devgaon and Saundra, when the epizootic was in full swing the rats collected from the houses in the villages showed an unusually high flea index of about 20, while those trapped from the huts set up as the

result of evacuation had very few fleas. The occasional presence of a very few specimens of fleas that cannot establish themselves on rodents of a locality tends to the conclusion that scattering of fleas through grain merchandize, human agency, etc. was going on in the nature, and possibly it played a part in the dispersal of plague.

The increase in the flea index of the domestic rats is governed by three important factors, which work independently of one another. Firstly the climatic factor controls the increase and decrease of fleas on rats. There was a considerable fall in the gross flea index of all the non-infected villages from 15th March to 15th July, when the atmospheric climate was hot and dry. In April the flea index was less than half of what it was in February. Secondly, the storage of grains in the premises increases the flea population, on account of the abundance of food for the rats and the flea larvae. Thirdly, the existence of plague infection in the premises increases the flea population enormously.

There is another factor which plays a very important part in the ecology of plague. The duration of life of the infected fleas is very short, especially in the warm and dry months, and they can only be transported through very short distances accompanied by a high rate of mortality amongst them. It is probably for this reason the progress of plague is very slow and uncertain.

From the standpoint of plague control, an immediate destruction of the tremendous flea population in a plague infected premises is essential, so as to prevent its dispersal. In this connection the destruction of musk shrews is very desirable. They may be very useful animals otherwise; but they play a very important part in the dissemination of plague amongst the house rats of a locality, by acting as a passive carrier of infected fleas. Their wandering habits from house to house are well known. Even they undertake long excursions from village to village and they have actually been caught along with their young ones from burrows in the fields about a mile and a half away from any human habitation.

The total flea population of a premises is composed of three components: (1) those found on the body of a host, (2) those found in its lair or nest and (3) free wandering fleas. These three components are subjected in nature to three different sets of climatic conditions: those of (1) the room, (2) the burrow and (3) the atmosphere. The climatic conditions of the atmosphere undergo considerable diurnal and seasonal fluctuations, those of the rooms less, and those of the burrows the least. The intensity of flea infestation of wandering rats depends upon climatic conditions of a room, which account for the seasonal variation in their flea index. The micro-climate of a rat-burrow, especially a deep one, undergoes only slight seasonal variations, and consequently the breeding of fleas in them goes on throughout the year. It is for this reason that after a few showers flea index of house rats suddenly rises by the middle of July. The free wandering fleas are controlled by the climatic conditions of a room and those of the atmosphere. During cool and wet months, these are abundant in rooms and can remain on the human body for a long time, whilst they cannot do so during hot and dry months. Epidemiologically free wandering fleas are important. The disappearance of plague during hot and dry months is due to the behaviour of adult fleas towards climatic conditions rather than to their decrease in population. During these months the infection is only confined to burrows, and it moves from burrow to burrow without causing any severe plague epizootic and epidemic. It is suggested that burrows in some of the grain cellars well protected against heat which are found in the Barsi Taluka and elsewhere enable the infection to tide over the unfavourable season.

Infection amongst wild rodents has been detected in the Barsi Taluka on eight different occasions. In seven cases *T. indica* was involved. In all instances the wild rodents were caught very near the infected villages at the height of infection in them. The maximum distance from the village, from which the infected fleas were recovered from *T. indica*, was four furlongs. The fact

that *T. indica* is experimentally susceptible to plague and is infested with *X. astia* to the exclusion of other fleas militates against the idea that this species serves as carrier of plague during the off-season. But, so far, there is nothing to prevent this species from playing some part in dissemination of plague in nature. It has a migratory habit and lives in colonies of about 10 or more individuals and harbours excessive numbers of fleas throughout the year. These factors favour the dissemination of plague through them.

In Bombay State, wild rodents do not play any part in the perpetuation and transmission of plague; only domestic rats are involved. During the off-season the infection runs the course of a slow sub-terranean enzootic in some suitable places. The end of the hot and dry off-season is marked by the onset of the rainy season, which operates in two ways: first, it increases the rat population indoors; secondly, it lowers the temperature and raises the humidity, which leads to an increase of the flea population and permits the fleas to leave the rat burrows and to attack human beings.

Both the survey of the villages and the study of progress during the past few years tend to the conclusion that big grain trade centres are chiefly responsible for the spread of infection. A large population of the rodents in these centres is highly immune to plague, so much so, that the imported infection only smoulders amongst the sparsely scattered susceptible rodents, as long as possible, without flaring up into a severe epidemic. The comparative susceptibility of the domestic rodent population of a place plays a very important part in the dissemination of plague.

The idea that plague is more a rural than an urban problem appears to be fallacious. The disease may not break out severely in the commercial towns; but still these towns play an important role in dispersing plague to villages, where it flares up owing to the higher susceptibility of their rat populations.

There are two types of plague epizootics. In warm table lands and plains, the infection is often severe, leading to a very heavy mortality among rats, which results in the disappearance of the disease within a short time. In the cooler regions comprising the watersheds of the Western Ghats, plague infection is slow-spreading and persists for a long time owing to lower rat mortality; these areas, which have moderately moist and cool climatic conditions most of the year, are considered to constitute an endemic plague centre.

Collection of Rat Ectoparasites in Bombay City

The collection of ectoparasites of Bombay rats was undertaken. The flea parasites found were — *X.cheopis, X.astia, C.felis* and *X.brasilliensis*. Of these *X.cheopis* predominates. Two species of ticks the bigger *R.sanguineus* and the smaller *R.tricuspis* and two species of mites *Dermanyssus muris* and *Laelaps echidinus* and one species of louse *Polyplax spinulosus* were recorded. A certain amount of specificity on rats has been observed wherein *X.cheopis* is found more on *Rattus rattus* while *X.astia* is more on *Bandicotta bengalensis*. In the sex ratio, the males of *X.cheopis* are recorded more than females in the collections while it is just the reverse in *X.astia*. The sex ratio of the fleas observed during different months in the field was compared with similar ratio in emergence, in the breeding of fleas in the laboratory both for *X.cheopis* and *X.astia*. There is an indication that during certain months the sex ratio may be equal and immediately after that the total number of fleas collected is seen to increase. There is specificity of fleas on rats. *X.cheopis* is more on *R.rattus* and *X.astia* on *B.bengalensis*. The former rat is a house rat and the latter that of the field. These studies are in conformity with the results obtained in the field.

The observations on collection of rats and their ectoparasites from one ward in the heart of the city (Bombay) and one in the suburbs were made recently. Areas selected were Kamathipura and Mahim. The total number of rats examined were 2,057 and number of fleas recovered were 3,060.

The following points emerge out of the observations on these collections: (*i*) there

were extremely few *Rattus norvegicus* collected from the suburban areas as compared with those from the city area; (*ii*) in the total collections more female rats were caught than males; (*iii*) the sex ratio and the percentage of fleas collected in these two wards were markedly different from each other and (*iv*) four specimens of *X.brasilliensis* have been recovered (one male from *R.rattus* in the city and three females from *B.bengalensis* in suburban areas). These were not heard of recently.

Ectoparasites of Laboratory Animals at the Haffkine Institute

The following animals were selected for observing the ectoparasites:—

White mouse, white rat, guinea-pig, rabbit and poultry. The ectoparasites recovered from the above animals are mainly lice and mites and only one specimen of flea *X.astia*.

Lice — *Polyplax spinulosa*; *Neumidilipeurus tropicalis*; *Menacanthus pallidulus*; *Gyropus ovalis*; *Gliricola porcelli*.

Mites — *Cheyletiella paasitivorax*; *Listrophorus gibbus*; *Chirodiscoides caviae*; *Megninia sp.* *Pterolichus obtusus*.

Morphological Studies on Rat Fleas

E.gallinacea is the smallest and thinnest flea as compared to others. The mandibular teeth of *X.cheopis* are spread over 2/3 portion of the mandible which helps in making a deep incision. Labrum epipharynx is shorter as compared to other fleas. Antennae of *X.cheopis* are more flagellated. Legs of *X.cheopis* are shorter in comparison to those of *C.felis* and *C.canis* but claws of *X.cheopis* are better developed.

The pharynx is more oval in *X.cheopis*, which helps in having a sustained power to suck blood. Proventriculus has eight types of teeth arranged in definite pattern to perform the act of straining and grinding and also it acts as a valve to prevent the outflow of blood. Rectal papillae of *X. cheopis* are broad and can reabsorb moisture efficiently; hence *P.pestis* may get a better chance to develop in rectum of *X.cheopis*.

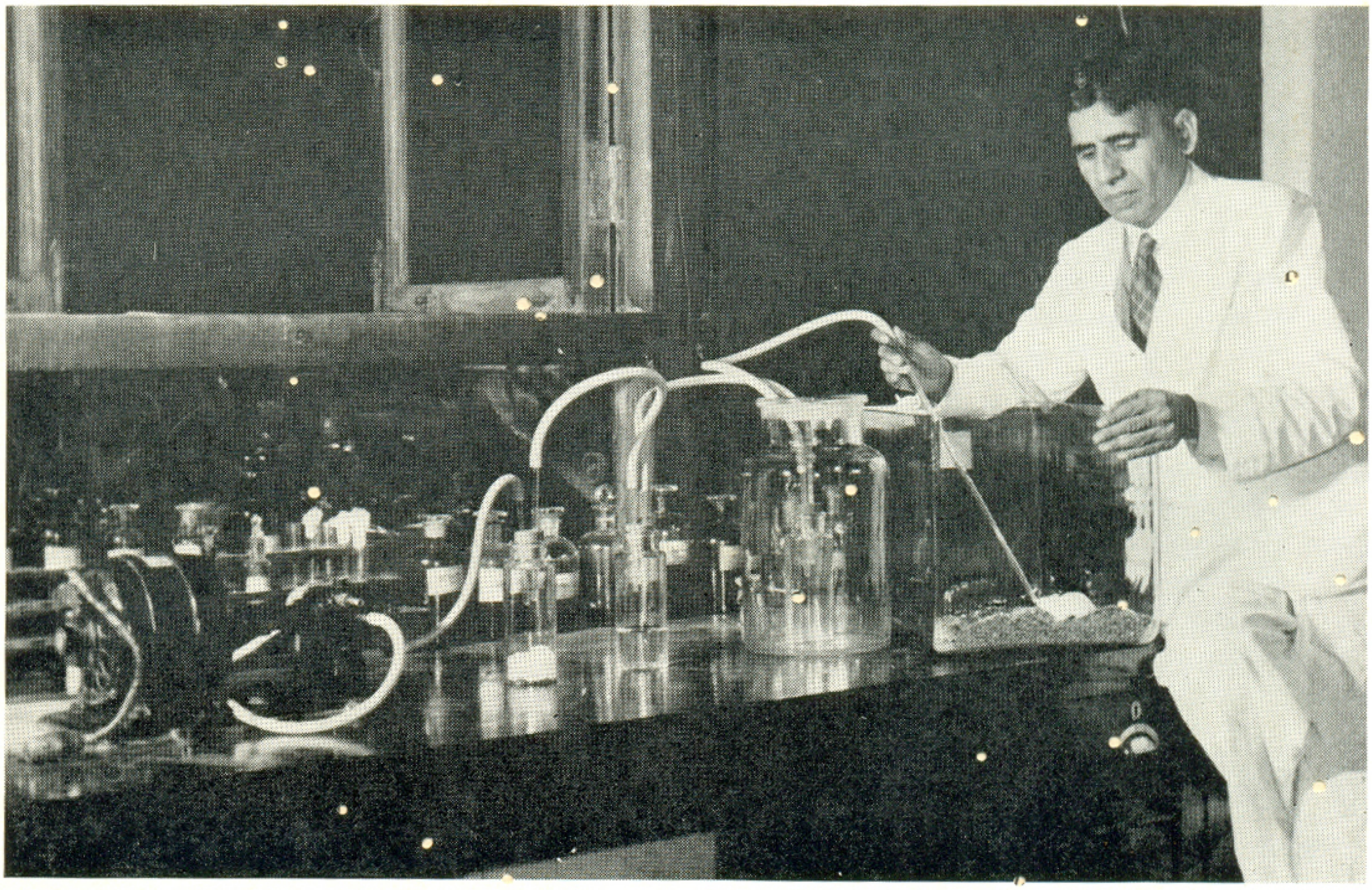
R.rattus is found in large numbers in Bombay and it harbours *X.cheopis* more in numbers. It seems that *R.rattus* and *X. cheopis* are the ideal combination in the Bombay region.

Proventriculus in Rat Fleas.—In live specimens it is found to be actively pulsating while the flea is sucking the blood and also after the suction of the blood. In *X.cheopis*, this organ was counted pulsating 90 beats per minute soon after the blood had been taken. Due to the peristaltic movements of the gut, the ingested blood is seen to pass through this organ again and again. It bulges sideways, then becomes normal and expands in linear direction. The sideway expansion and relaxation are very fast and this has been observed well in *X.cheopis*.

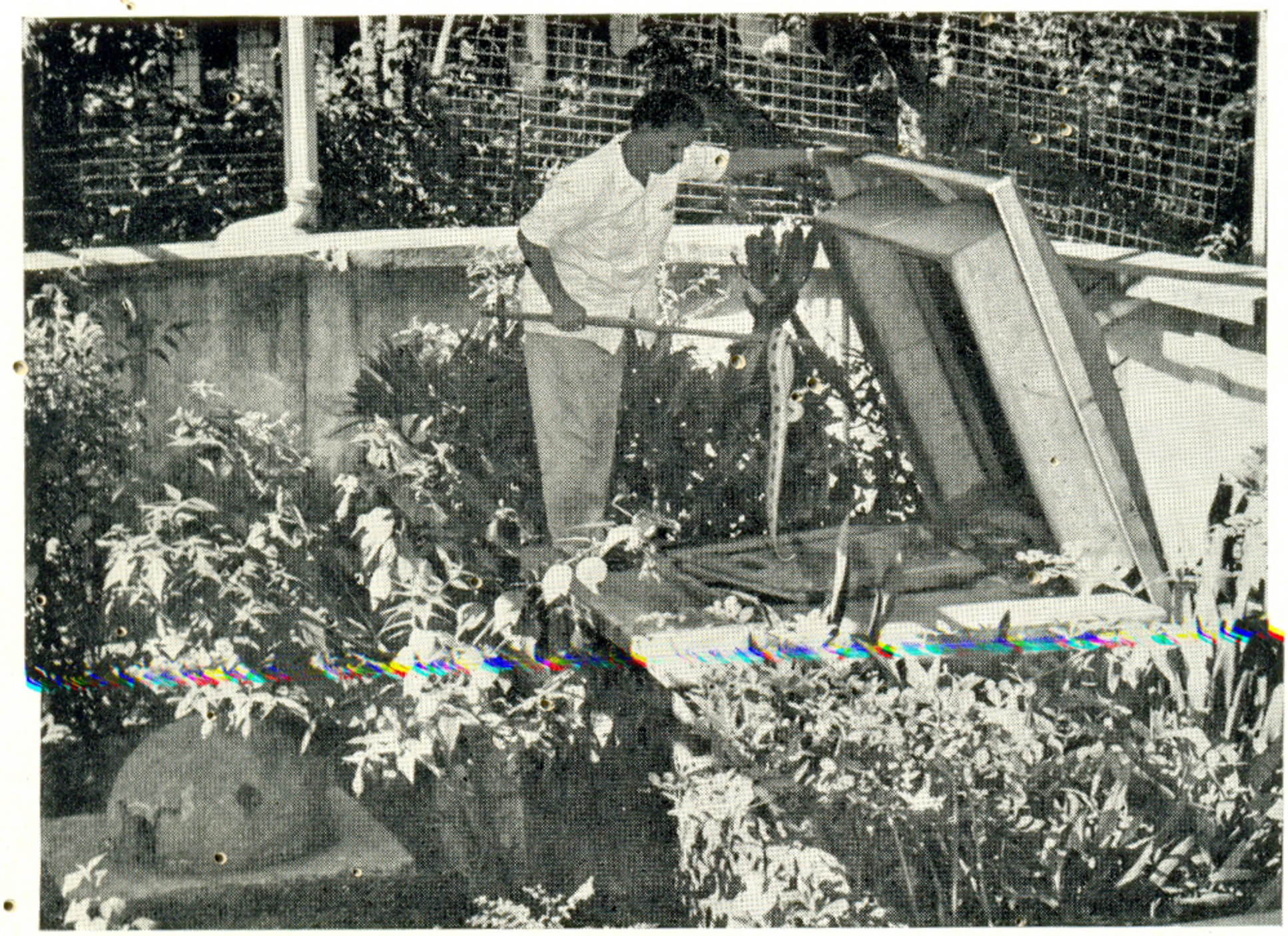
In sections one finds the proventriculus narrower in the anterior region and broader posteriorly. Inside the proventriculus, a number of hollow, delicate, and hexagonal as well as round teeth are found.

These teeth could be seen projecting backward and entering into the lumen of the mid-gut. There are 3 sets according to their location and shape. They are arranged in a spiral manner in whorls of 14 to 15. The teeth are different in sizes and shapes but are all broad at the bases and narrow at the apices.

Three types of teeth could be classified according to the structure of their apices; the blunt rounded, the pointed forcipate and the conical pointed. The anterior blunt teeth do not meet in the centre, thus leaving a lumen. The forcipate middle rows of teeth meet in the centre, thus leaving a lumen. The forcipate middle rows of teeth meet in the centre, blocking the lumen. The posterior conical teeth which are the biggest project towards the mid-gut end of the proventriculus. In relation to the apices and the length, the teeth are arranged in such a way that the blunt and short teeth are at the anterior region, the medium sized forcipate teeth are in the middle region and the longest conical pointed teeth are at the posterior end. Of all the teeth, the forcipate teeth are greatest in number. Third row of conical teeth shows distinct denticles on their two lateral margins. These denticles vary in number in different flea species. It was observed that the younger fleas have



Collection of fleas for the study of transmission of plague



Snake Farm

slightly less number of teeth as compared with those of the adult ones. It is observed that *C.canis* has maximum number of teeth while *E. gallinacia* shows the least number; *X.astia, C.felis, X.cheopis* and *X.brasilliensis* lie in between the series. The same gradation holds good for the length, i.e., the longest being in *C.canis* and the shortest in *E.gallinacea*. It is also seen that the number of teeth is nearly double in females as compared with the males. The shape of different teeth is more or less the same in all the species. The size of the proventriculus in females is bigger than in males in nearly all the species.

Male Genitalia in Indian Fleas.—The anatomy and homologies of the external male genitalia of *C. felis orientis* are revised in the light of the existing knowledge of the insect male genitalia. The abdomen of the adult male flea consists of ten segments, and the last one may contain elements of the eleventh segment. The complicated structure forming the so-called 'penis' is the copulatory apparatus, as it includes the harpagones in addition to the intromittent organs. The male genitalia, specially the copulatory apparatus, is highly musculated.

Nutritional Requirements of the Younger Stages of the Indian Rat Fleas

With a view to determine the comparative nutritional value of different foods for the flea larvae, recently hatched larvae of *X. cheopis, X. brasiliensis* and *X. astia* were reared on highly milled wheat flour devoid of bran, dried horse-blood, and mixtures of equal parts of flour and horse-blood, and blood and yeast, at 25°C and 80 per cent relative humidity, and changes in the rates of their growth were noted. Larvae of all the three species cannot breed successfully on blood alone. Less than 50 per cent larvae of these species bred successfully on flour alone, but the adult fleas were of unusually small size and less sclerotized. The larvae of *X. astia* bred successfully in 60-112 days with a larval life of 44-80 days and those of the other two species in 41-75 days with a larval life of 24-59 days. The rate of growth amongst the larvae was not uniform and after 41 days all stages of development from a first larval instar to an adult were found. The larvae died in different stages of growth. The marked disparity in the growth of larvae clearly suggests some favourable change in the food, which is responsible for the successful breeding of some of the larvae. The only apparent change noticed was the presence of fungus growth on the dead larvae. This fungus growth had evidently served as an accessory food for the other larvae. It is therefore, evident the wheat flour by itself has not got sufficient nutritive value for these larvae, and the association of micro-organisms like yeast, bacteria or fungi with food are essential. The successful breeding of the flea larvae on flour in the presence of micro-organisms enables them to establish themselves at places far away from their original home. The larval life of *X. astia* was much longer than that of the other two species and the mortality rate was very high.

The addition of blood to the wheat flour shortened the larval life considerably, being 13-24 days in *X. cheopis* and *X. brasiliensis* and 23-37 days in *X. astia*. Almost all the larvae grew and many of them died in the resting stage. The percentage of successful breeding of the larvae was low, when compared with those fed on flour, being 20·1 to 26 per cent in *X. astia* and *X. cheopis* and 10 per cent in the *X. brasiliensis*. The absence of cocoon-formation was responsible for the high rate of mortality in the pupal and resting larval stages. The adults were highly sclerotized and bigger than those reared on flour. The nutritive value of the mixture of blood and flour is more than that of flour alone. As the mixture was less hygroscopic, it caused a high rate of mortality.

The addition of dry medicinal yeast to blood gave almost a hundred per cent success in rearing of fleas within 19-32 days with a larval life of 8-14 days. The addition of yeast increased the hygroscopic power of the food and did away the necessity of the growth of micro-organisms in the food to ensure successful breeding.

It is experimentally proved that blood is not so essential for the successful breeding

of the larvae of these fleas as it is in the case of *Nosopsyllus fasciatus*. The larvae can breed successfully without blood, but better results are obtained when blood forms the integral part of the food. In addition, it is found that association of some micro-organisms with the food ensures a more successful breeding of the larvae.

Effect of Temperature and Humidity on the Growth of Early Stages in Rat Flea

About 20,000 newly hatched larvae of three species of Xenopsylla were subjected to temperatures from 11° to 36·5°C and relative humidity of 40 to 100 per cent. Larval food comprising blood and yeast underwent diverse changes at different humidities, which affected flea breeding materially. It appeared that at low effective humidities much food was consumed by larvae in order to gain more metabolic water so as to compensate for an excessive water loss through evaporation. The food increased in volume at optimum and high humidities. The ill effect of high humidities on early stages of fleas was solely due to the food; larvae and pupae died owing to the formation of a crust and a compact mass, and when the food and sand mixture became soggy; this could be avoided by disturbing the mixture so as to allow free access of air.

The effective range of humidity for larvae varies according to temperature, and this range of effective percentage of humidities is wider at 27°C and gradually narrows down as the effective temperatures depart from this temperature which should not happen if a particular value of saturation deficiency is essential for the growth of the larvae. It is experimentally proved that the successful development of a flea larva depends on the water content of the food and that of the atmosphere. Effective humidity as a rule does not affect the rate of growth of the larva except when it is near the lower fatal limit, when the rate of growth is definitely retarded.

Failures and successes in rearing of adults at hundred per cent humidity led to the conclusion that excessive moisture by itself is not inimical to the growth of both the larvae and the pupae. Its injurious effect is due to the formation of a crust impermeable to air on the surface of the soil, the growth of fungi and putrefaction that sets in a soil rich in organic material. These factors prevent the supply of air necessary to larvae and pupae which die of suffocation. This brings us to the importance of the soil as a factor that should influence the increase and decrease of the flea population, and especially so, when we know that fleas are soil insects as all the early stages of fleas live in the soil and even a greater portion of the adult life is spent in the soil.

Hibernation, astivation and diapause do not occur in the larval or pupae stage of the three species.

Pulicidal Effect of BHC, DDT, and Pyrethrum

BHC—Benzene hexachloride was tried in the form of an emulsion and dust on the adult rat-fleas of *X. cheopis* and *X. astia*. A 5 ml. of diluted kerosene soap emulsion containing 0·004 g. of BHC when sprayed on and thoroughly mixed with 1,000 g. of sand in a jar, i.e., a concentration of 1 in 250,000 killed 100 per cent adult fleas in an experiment tried upto 28 days.

Powder.—Both the jar and burrow experiments were conducted with a powder containing 20 per cent of BHC with 2·6 per cent of "gammaxane". In the jar experiments, 0·026 g. of gammaxane, when mixed with 1,000 g. of sand, killed 100 per cent adult fleas up to 48 days. In the burrow experiments, when dusted with 50 g. of this powder, about 100 per cent of the adult fleas were killed upto 65 days. This was tried after dusting the burrow at a place about 9½ feet away from its entrance.

Smoke.—BHC Smoke Generators, No. 2-2 oz. containing the smoke generating mixture with about 3 per cent of BHC were used for ascertaining their pulicidal value in the artificial W-shaped rat burrows. When smoked with one such generator, about 100 per cent rat fleas, *Xenopsylla cheopis*, were killed in an experiment tried after 45 days of smoking the burrow at a distance of 20 feet 4 inches from the entrance.

DDT—Solution, Emulsion.—Experiments in jars were tried with sprays. Each jar containing 1,000 g. of sand, was at first sprayed with a measured quantity of the solution or of the diluted emulsion, and then shaken in such a way that its entire quantity was thoroughly mixed with the sand. About 0·08 g. of DDT in kerosene oil gave 100 per cent kill of adult rat-fleas of *Xenopsylla cheopis* and *X. astia* in the experiments tried up to 120 days after spraying. Even 0·017 g. of DDT in the form of kerosene soap emulsion killed 100 per cent rat-fleas, so far, upto 49 days.

Powder.—10 per cent DDT in pyrophylite, was used for experiments with dusting. Both jar and burrow experiments were tried with it.

In the jar experiments, a concentration of 1 in 10,000 killed 100 per cent rat-fleas up to 396 days after dusting the DDT powder. In the control experiments the mortality rate varied from 3 to 33 per cent.

The minimum lethal dose for the adult fleas of *X. cheopis* was 0·005 mg. of DDT per gram of sand. Even a concentration of 1 in 200,000 gave a fairly long residual effect on adult fleas; but higher concentrations only helped in the early abolition of their jumping power.

When the artificial burrows were dusted with 37-79 g. of 10 per cent DDT powder, the results were not so consistent as in the jar experiments. In an individual experiment, the percentages of mortality on the total and the recovered fleas respectively were 58-100 and 97·8-100 within 428 days tried in the first arm up to a distance of about 5 feet, 68-96 and 81·3-100 upto 425 days in the second arm up to a distance of about $10\frac{1}{2}$ feet, 50-100 and 54·4-100 within 397-420 days in the third arm up to a distance of about $15\frac{1}{2}$ feet, and 30-96 and 33·3-100 within 230-336 days in the fourth arm up to a distance of about $20\frac{1}{2}$ feet.

In order to ascertain the effect of DDT on different stages of rat-fleas, 1 or 5 g. of the 10 per cent DDT dust were thoroughly mixed with 615 to 965 g. of debris of about one year old breeding jars, containing many thousands of adults, cocoons, larvae and eggs of *X. astia*. All the adults and larvae of the existing generation were killed within 24 hours. Only recently emerged adults and hatched larvae were seen alive, which ultimately died.

Pyrethrum.—Sixty-nine pounds of pyrethrum flowers were treated with petroleum ether to yield 1346·26 g. of Pyrethrum extract. The pyrethrum content of the extract varied from 17·05 per cent to 30·37 per cent, on the basis of the weight of the extract and from 0·68 per cent to 1·19 per cent on the basis of the weight of the powder.

The powders made from the extracts were tested at different concentrations with laboratory bred *X. cheopis*. One mg. per sq. feet of the concentration gave the best results. When analysed statistically these results show that pyrethrum as such does have a residual effect comparable to DDT in the same concentrations. Stabilisers like hydroquinone and resorcinol showed chemically some check in the deterioration of pyrethrum extract, but in biological tests the results were not significant. Boosters like piperonyl butoxide, sesame oil, castor oil, and diesel oil were used; the first one gave very significant results in the improvement of knock-down effect of deteriorated pyrethrins.

Field trials of the powders were done in some villages of Madras State and in Satara District of Bombay State. Pyrethrum powder as such was found to give a knock-down effect in the rat burrows upto 76 days after treatment; when mixed with piperonyl butoxide and tested on a comparative basis with 10 per cent DDT, pyrethrum powder gave a lower flea-index than DDT in 76 days. This powder had shown in Uttar Pradesh a knock-down effect upto 105 days.

Entomological Survey of Kurla Area

In 1952, myriads of 'mosquito-like' insects were reported to have invaded the Kurla area in suburban Bombay.

The area was divided into 5 zones, and from each zone a daily collection of all types of insects was made by 50 sweeps of a handnet per day. In all 28,127 insects were collected in the course of 12 months from the various zones. The collection was found on exami-

nation to contain specimens belonging to 50 families of different orders of insects. The most dominant group numerically was the *Chironomidae,* followed by *Ephydridae, Ceratopogonidae, Sepsidae* and *Jassidae.* The following Table contains the names of the Diptera collected.

Order—DIPTERA

Family	Specific names of insects
Ceratopogonidae	*Stilobezzia notata* Meij.
Chironomidae	*Nilodorum sp.*
Psychodidae	*Telinatoscopus albipunctatus Will.*
	Psychoda alternata Say.
Tipulidae	*Conosia irrorata* Wied.
Culicidae	*Anopheles* (Myzomyia) *subpictus Grassi Culex* (*Culex*) *fatigans* Wied.
Dolichopodidae	*Chrysosoma sp.*
Syrphidae	*Xanthogramma* (*Ischiodon*) *scutellare* Fb.
Calliphoridae	*Chrysomyia megacephala* Fb.
Muscidae	*Musca domestica* Linn.
	Musca (*Philaematomyia*) *crassirostris* Stein.
	Musca (*Ptilolepis*) *inferior* Stein
	Orthellia indica R.-D.
	Stomoxys calcitrans L.
	Atherigona laeta Wied.
	Lispe kowarti Beck.
	Lispe leucospila Wied.
	Lispe sp. assimilis Wied.
Trypetidae	*Rhabdochaeta asteria* Hend.
Otitidae	*Chrysomyza aenea* F.
Sepsidae	*Saltelliseps niveipennis* Beck.
	Saltelliseps niveipennis var. *robusta* Duda.
Chloropidae	*Pachylophus rufescens* Meij.
	Hippelates minor Meij.
	Gaurax sp. indet.
Diopsidae	*Sphyracephalahearseyana* Westw.
Ephydridae	*Psilopa mentita* Cress.
	Scatella sp. indet.
	Octhera sp. indet.
	Notiphila sp. indet.
Borboridae	*Leptocera sp.*

Rodenticidal Value of Dethdiet Red Squill, Rodine, Ratex, etc.

Experiments to test the rat poisons, Dethdiet Red Squill and Rodine were carried out on a comparative basis with barium carbonate. The poisons were given in the form of baits of 1 g. each. One part of any of the first two poisons with 9 parts of the base, maize flour, was equally effective for *Rattus rattus, Rattus norvegicus* and *B. bengalensis* as 1 part of barium carbonate with 4 parts of the base, there being no statistical difference in the mortality rates with the three poisons.

Ratex.—The trial on a comparative basis with barium carbonate was undertaken. Poison was administered in stipulated doses with Bajra flour, and the baits prepared were kept for a period of 17 hours at a time. The following table represents the efficacy of these two rat poisons.

Antu.—Experiments to test the rat poison alphal-naphthylthiourea, generally known as "*Antu*", "*Chemical 109*" or "*Rat Poison 109*", were carried out, in a concentration of 10 mg. per g. of the base of Bajri flour. It was very effective for *Rattus*

Name of rats	*The percentage of Rat Mortality*		
	Percentage mortality with Ratex	Percentage mortality with Barium Carbonate	Remarks
Rattus rattus	16·7	83·3	*Rattus rattus* showed disinclination to eat Ratex bait. In spite of complete pills being swallowed a few of these rats did not die.
Bandicota bengalensis	83·3	100	Ratex pills became spongy during rains and very hard during dry spells within 17 hours.
Rattus norvegicus	25	100	

norvegicus with a mortality rate of 94·1 per cent; but in this concentration it was ineffective for *R. rattus rufescens* and *B. bengalensis*.

Dethmor.—The baits were made by mixing Dethmor with " Wari " (*Paniceum maliaceum*) in the proportion of 1 : 19.

Laboratory experiments were done with *R. rattus*, *R. norvegicus* and *B. bengalensis*. Weighed rats were kept separately and fed on the poison baits in the first experiment and with poison baits and normal food in the second experiment. It was observed that *R. rattus* consumed a total of 40 g. of the bait and died on the 6th day; *B. bengalensis* consumed 45 g. and died on the 4th day, while *R. norvegicus* consumed 30 g. and died on the 3rd day. *Rattus rattus* and *B. bengalensis* had consumed $\frac{1}{3}$ of their body weight of the bait before they died, while *R. norvegicus* consumed only 1/6 before it died. Along with the normal food in the second experiment, the amount of bait consumed was nearly the same as before but the deaths took place after 12, 8 and 6 days respectively.

The field experiments were done for 21 days over 28·9 acres of the Institute area. The maximum amount of baits consumed in a single day was 212 g. in one cage, while the total amount of baits eaten during the entire period was 53 lbs. The rats began to die after the 5th day and the total number of rats dead during the period that showed typical internal haemorrhage were 33, of which 17 were *R. rattus*, 4 *B. bengalensis*, 8 *Bandicota malabarica*, 3 *Mus musculus* and 1 *Suncus sp*.

Venom Tolerance in Some Poisonous Snakes

Seven-day old Russell's viper snake killed a white mice weighing 20 g. in 20 minutes, and a fifteen-day old one killed the same in 4 minutes. As these young snakes were biting each other without any ill effect, experiments were done to see the amount of tolerance in these snakes and adult *Echis carinatus* to four kinds of adult snake venoms. The venom solutions were prepared from lyophilised venom which was then injected subcutaneously into 3 to 4 week old Russell's viper young ones weighing about 8 to 12 g. The mouse lethal doses were worked out on groups of mice weighing 20 g. and the dose desired to be given was contained in 0·5 ml. of the venom solution. The results of the experiments indicate that *Russell's vipers*' young ones could tolerate upto 5 mg. of *cobra, krait* and *Russell's viper* venoms and only 2 mg. of *Echis* venom. *Echis carinatus* weighing about 12 to 14 g. were seen to tolerate upto 25 mg. of cobra, Russell's viper and krait venom and upto 37·5 mg. of *Echis* venom.

It has been observed in a number of experiments that 0·25 ml. of *Russell's viper* serum neutralised nearly 2 M.L.D. of *Russell's viper* venom (0·5 mg. of *R. viper* venom). On this basis a normal *Russell's viper* snake with about 100 ml. of the total blood may tolerate 85 mg. of *Russell's viper* venom.

Further work on Russell's viper serum has shown that this completely inhibited the anti-lecithinase activity of *Russell's viper* venom when tested on rabbit blood. This serum also shows a trypsin inhibiting activity which is about 3/4th that of human serum.

The Influence of Snake Venom and Sera on Rabbit Erythrocytes

It has been previously noted by a number of workers that the cobra venom would cause haemolysis of the red blood corpuscles of certain mammals and the serum of this snake inhibited this haemolysis. This was tested on rabbit blood which was centrifuged and the blood cells washed in saline. These cells were then diluted to the original volume of the blood and the concentration of venom added on a M.L.D. basis. The results are given on page 114.

Food Habits of Echis Carinatus

These observations were made at Deogad (Bombay State) for one month in November 1954, and February 1955. Specified number of stones were turned each day in specified beat to reveal any *Echis* lying there. All the animals found alive under the stones were

Venom	*Serum*	*Result*
R. viper	R. viper	Complete inhibition
R. viper	Cobra	Partial inhibition
R. viper	Krait	,,
Cobra	Cobra	,,
Cobra	R. viper	No inhibition
Cobra	Krait	,,
Krait	Cobra	,,
Krait	Krait	Partial inhibition

recorded. The live *Echis,* when recovered, were killed and dissected in the field and the entire gut contents fixed and preserved in formalin. The gut contents were examined and the material indentified. The animals recovered under the stones were frogs, scorpions, spiders, centipeds, millipeds, annilid worms, nematodes, some snakes and a large number of other insects. The common insects were *Dermaptera, Orthoptera, Hymenoptera, Isoptera, Coleoptera, Hemiptera* and occassionally *Lepidoptera.* The most common insects being *Forficula* and ants.

It has been possible to keep a number of *Echis* alive in the field station in cages on a diet of insects only.

Output of Venom when Snakes kept in Natural Environment

Cobra, Krait and Russels's viper were kept in a snake farm in order to study the effect of natural environment on the output of venom and their longevity. The results show that these snakes live longer and give better yield of venoms than those kept under captivity. The output of venom is shown in the Table.

Average Yield of Venom in g. per Snake.

Year	COBRA			
	Farm		*Captivity*	
	Male	Female	Male	Female
1957	0·1893	0·1168	0·1464	0·1405
1958	0·1786	0·0972	0·2000	0·0926
	R. VIPER			
1957	0·1071	0·0988	0·0822	0·0988
1958	0·1233	0·1157	0·0695	0·0870
	KRAIT			
1957	0·0276	0·0066	0·0122	0·0063
1958	0·0101	0·0052	0·0133	0·0069

DEPARTMENT OF CHEMOTHERAPY

C. V. DELIWALA

THE Department of Chemotherapy was organised in the year 1940 at Haffkine Institute to apply the powerful tool of synthetic organic chemistry towards the treatment of infectious diseases, and secondly to investigate the possibilities of manufacturing some of the important and life-saving drugs.

Within a short period of few years the department was able to contribute a number of papers on the synthesis and testing of a series of derivatives of sulphonamides, to establish the efficacy of sulphathiazole as a curative agent in the treatment of plague and take out six patents for the preparations of sulpha-drugs and intermediates. The novel method worked out by the department for the preparation of sulphathiazole has the advantage of dispensing with the use of a complicated equipment, the use of anhydrous sulphonyl-chloride and 2-aminothiazole and also that of pyridine as condensing agent and solvent. On the manufacturing side the department took up the preparation of organo-mercuric compounds for use as preservatives in the manufacture of vaccines and also started the manufacture of an antiseptic solution called *Pemon*.

With the confidence gained in the batch-wise laboratory preparation of sulphathiazole and other drugs, it was decided to take up the study of the preparation of these on pilot plant scale and towards this end necessary equipments such as reactors, filter-presses, distillation assemblies in glass-lined steel and stainless-steel were obtained and the pilot-plant for sulphathiazole was set up between 1945-46. A new section for the concentration of vitamin A from shark-liver oil was also opened. An efficient process for the saponification of oil and solvent extraction of vitamin A was worked out for a large scale operation and the department itself designed the necessary equipment and got it fabricated locally.

In the field of research, attention was given to anti-malarials and in particular the synthesis of derivatives of quinoline, thiazole, guanidine and biguanide. Due to the important part played by the thiazole ring system in sulphathiazole and vitamin B_1, a detailed study of the chemistry of thiazole system was taken up. With the grants from I.C.M.R. (formerly known as Indian Research Fund Association) a unit for the study of penicillin and streptomycin was established in the department, which took up the work of finding out the most suitable media for their growth, the practicable methods of extraction, purification and other related problems.

A detailed pilot plant study on the problem of manufacture of proguanil was conducted and on the basis of the results obtained it was found that it would be quite easy to manufacture this anti-malarial at a low cost.

During 1947-48, hydnocarpyl and phthalyl derivatives of sulphonamides, 4:4′-diaminodiphenylsulphone, and 4-nitro-4′-aminodiphenylsulphone were synthesised for possible use against tuberculosis and leprosy. A limited number of mono and di-substituted derivatives of diaminodiphenylsulphone and a series of p-aminophenyl-thiazolylsulphones were also synthesised. In the field of chemotherapy of malaria various quinoline derivatives with substituted biguanides were

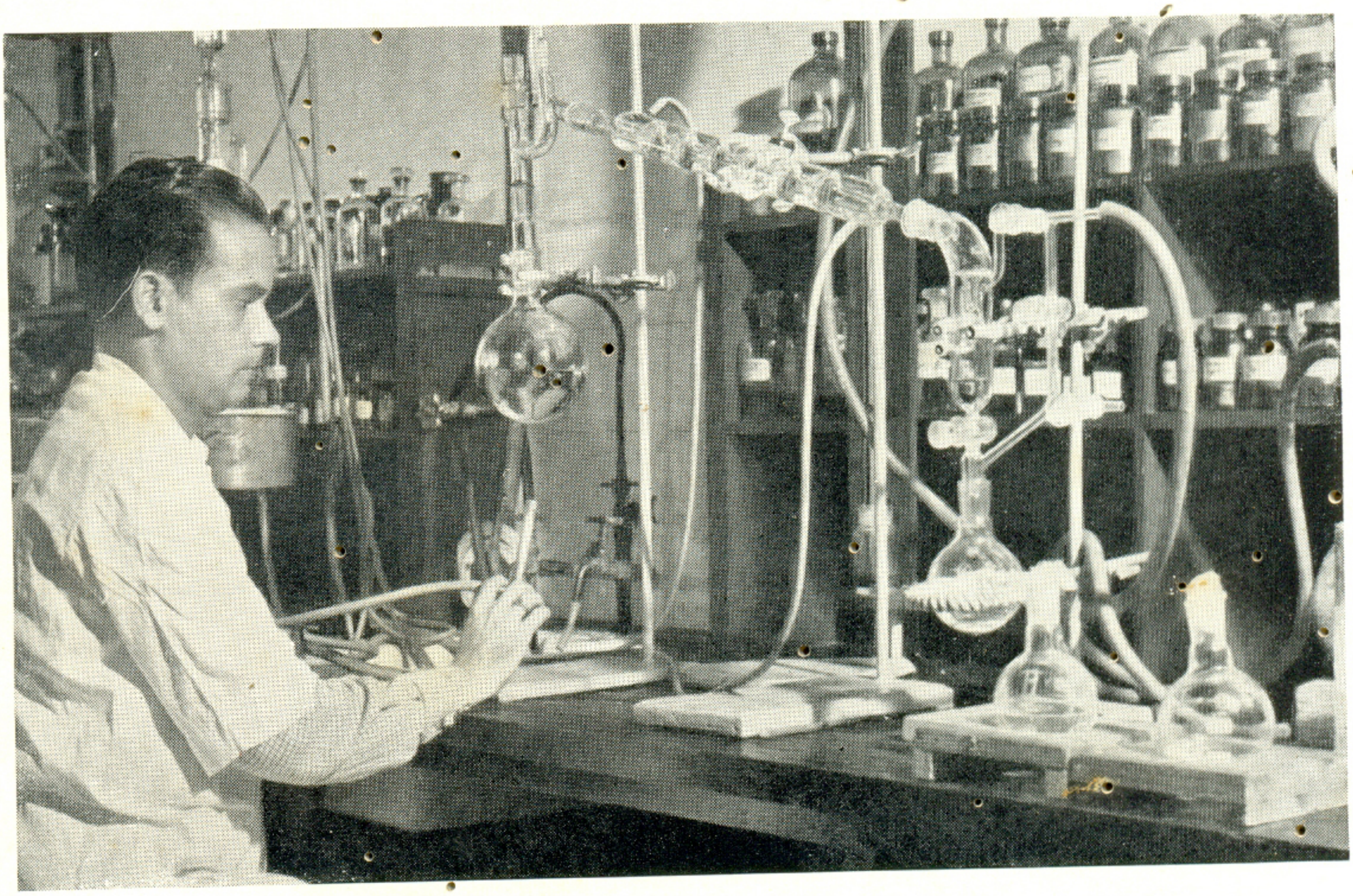
synthesised on the model of proguanil. Improved methods for the preparation of 5:5′-dichlorosalicyl were worked out in view of the claimed activity of this compound. *In vivo* tests showed this compound to be ineffective against *P. pestis*. A series of interesting heterocyclic ring systems in imidazole and pyrazine were obtained starting with chlorosalicil, benzil, 2-2′ -dichlorobenzil, anisil, etc. A total number of 28 compounds were synthesised and a few representative ones were subjected to *in vivo* trials against *P. pestis*. Although these compounds were found to be less toxic than the parent chlorosalicil, they also failed to exhibit any curative action. A series of N^4-acyl-N^1-substituted sulphanilhydroxamides were also synthesised in view of the reported effectiveness of N^4-caproylsulphanylhydroxamide in streptococcal and tubercular infections. Also a series of orthophthalic and ortho-toluic acid derivatives of well-known sulphonamides and diaminodiphenylsulphones were prepared. During the period 1949-1951 various N^1-aryl-N^2 -(p-alkylguanido)-phenyl-guanidines, guanidylphenylaminoquinolines and thiasolylaminoquinolines were sythesised as a part of anti-malarial research programme. Work was also done on the synthesis of pteridines substituted with various aryl and aliphatic groups to see if any of these compounds would act as antimetabolites of folic acid. Five different methods for synthesis of p-nitrosalicylic acid were studied with regard to their suitability for commercial manufacture of para-aminosalicylic acid. Of these, the one involving the use of phenylacetic acid as starting material was found interesting and practicable. This process was, therefore, patented. A number of new derivatives of para-aminosalicylic acid was synthesised. In particular several N-acyl-derivatives were prepared to see if these would be more suitable for the treatment of tubercular infection of the alimentary canal.

Numerous N^4-acyl-derivatives of the various potent sulphonamides such as sulphathiazole, sulphadiazine and sulphamerazine were synthesised expecting them to undergo slow but progressive hydrolysis into the parent active drug *in vivo* over an extended period of time in the gastrointestinal tract, without giving rise to unduly high concentration of the drug in circulating blood and thus to eliminate the toxicity usually encountered with the parent drugs. A few selected number of N^4-acyl-derivatives of sulphadiazine were tested by *in vivo* experiments to find the concentration of the derivatives in the different organs and tissues. For the estimation of the acyl-sulphadiazine as well as the free drug, the original method of Bratton and Marshall had to be modified in view of the difficultly hydrolysable nature of the N^4-acyl-derivatives. It was observed that the deacylation takes place both in the gastrointestinal tract as well as in liver. Whereas N^4-acetyl compound was deacetylated to a very small extent, the next two homologues viz. propinyl and butyryl were deacylated to a great extent, and as the chain-length increased further, the deacylation decreased and finally the N^4-palmityl derivative got deacylated almost to a negligible extent. As far as the absorption of the acyl-derivative as such was concerned, the acetyl compound was absorbed to the maximum extent and with the increase in length of the chain the absorption decreased. Chlorinated acetyl derivatives were found to get hydrolysed more than the unchlorinated ones.

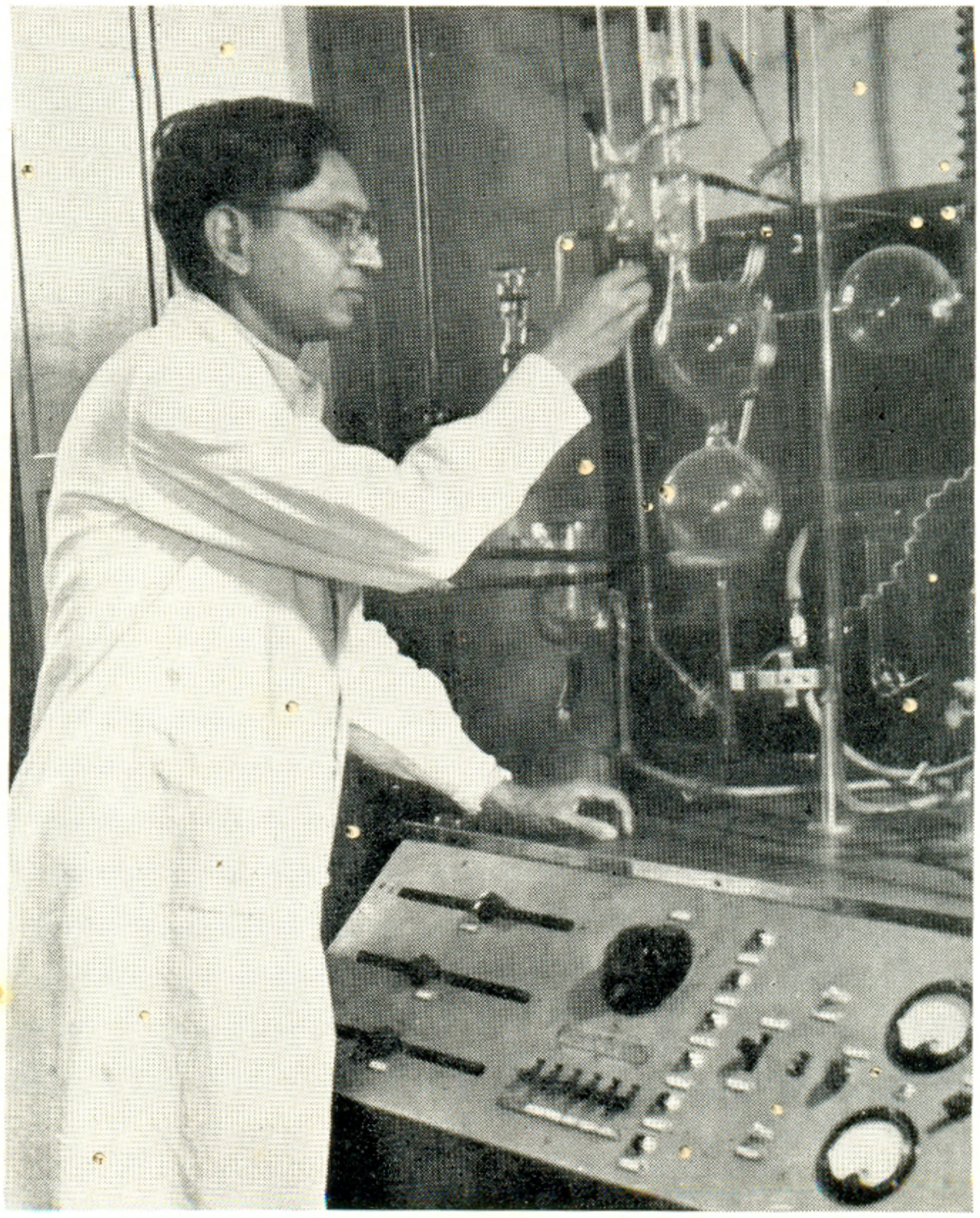
A number of N^1-(6-benzimidazolyl) sulphonamides having a variety of alkyl and aryl radicals at 2-position of the benzimidazole were synthesised.

Pyrimidine portion of the thiamin molecule was successfully synthesised by lithium-aluminium hydride reduction of the alkly ester of 2-methyl-4-amino-pyrimidine-5-carboxylic acid which afforded 2-methyl-4-amino-5-hydroxy-methylpyrimidine in good yield. This compound on treatment with hydrobromic acid afforded the corresponding 5-bromomethyl compound which on condensation with 4-methyl-5-beta-hydroxy-ethyl-thiazole yielded thiamin bromide hydrobromide.

An economical process for the synthesis of the pyrimidine part of thiamin in better yields was developed wherein acrylonitrile



Fractional distillation — a stage in the synthesis of drugs



Molecular distillation of Shark Liver Oil

was used as an intermediate in place of cyanchydrine which had been used by Chelinstev and Behevolenskaya.

2-Amino-4-m e t h y l-5-betahydroxyethyl-thiazole and 2-mercapto-4-methyl-5-betahydroxyethyl thiazole were synthesised by employing thiourea and ammonium dithiocarbamate. The amino group or the mercapto group in position-2 of the thiazole ring was successfully replaced by hydrogen to get 4-methyl-5-hydroxy-ethyl thiazole in good yield.

About 50 compounds related to thiamin were synthesised wherein the thiazole moiety contains substituents in position 2, 4 or 5 and the pyrimidine portion having a methyl or ethyl substituent in position 2. It is proposed to study whether any of these compounds show anti-vitamin activity.

A unit for the screening of anti-malarial compounds was established in the department with a grant from I.C.M.R. A large number of compounds were screened against *Plasmodium berghii* during the years 1951 to 1953.

In recent years work is being conducted on the synthesis of compounds analogous to chloramphenicol. During this work two new processes for the synthesis of p-nitroacetophenone, an important intermediate for the preparation of chloramphenicol, have been worked out and patented. In the first process p-nitrobenzoyl chloride is condensed with a beta-ketonic ester such as ethyl acetoacetate whereas in the second process p-nitrobenzoyl chloride is condensed with acetylacetone. Both the processes, which are easy to operate and eliminate the use of sodium metal and the consequent risky operation, proceed in the presence of anhydrous alkalies in the presence of a non-polar solvent.

DEPARTMENT OF ANTITOXINS AND SERA

A. K. HAZRA

THIS department started in 1940 with a view to undertake research on modern lines and to exploit the available scientific knowledge to produce various therapeutic and prophylactic biologicals. It is but natural that at a time when the department started, more stress was laid on perfecting the production of various antitoxins which were so urgently required and were in short supplies.

The main problems during the war were to standardize methods suitable to our country for the large scale production of various antitoxins. Below is presented an account of the work carried out in the department since its beginning.

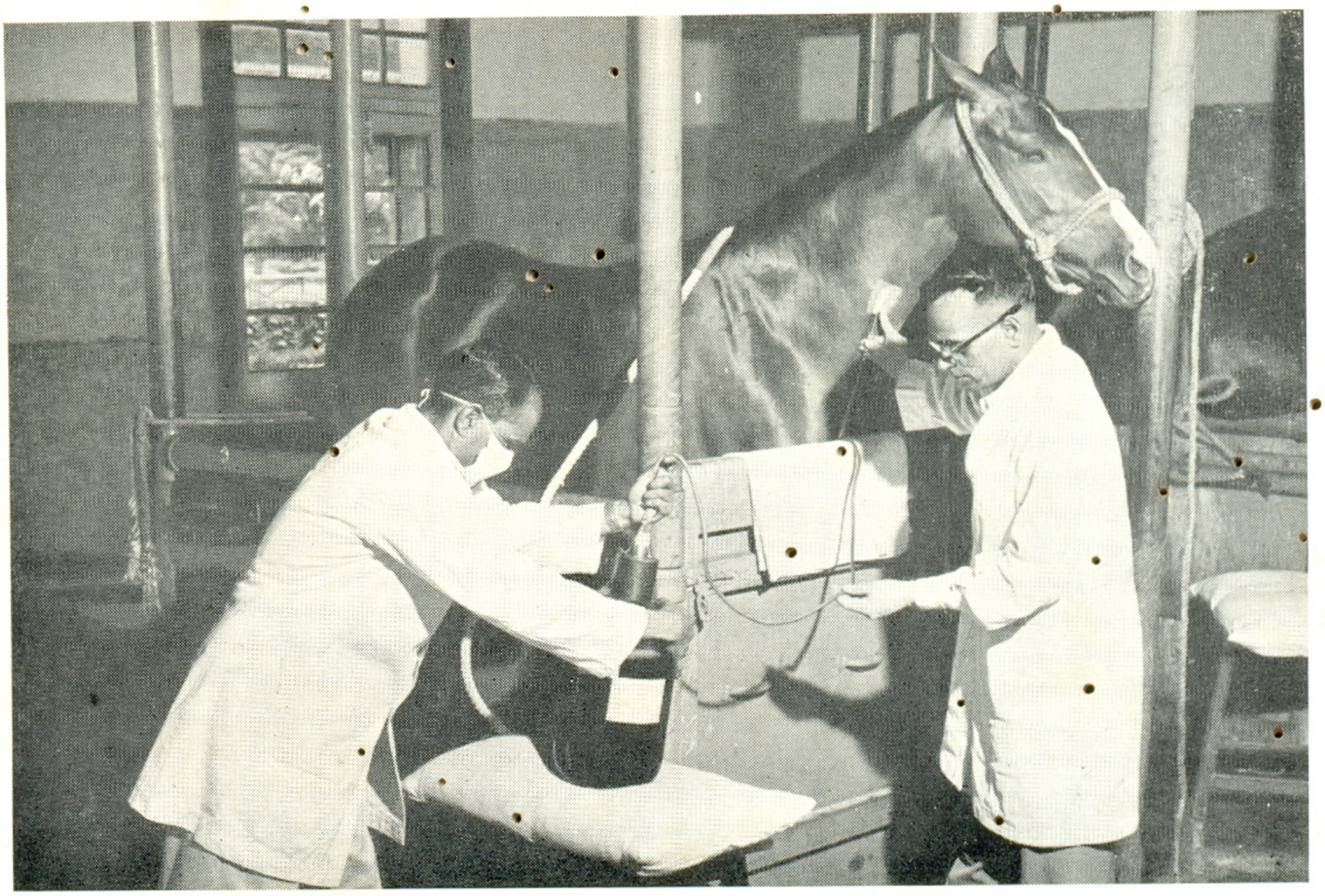
The problems of preparation and utilization of various antigens for hyperimmunization of horses for different therapeutic antitoxins presented difficulties.

The work on antigen for obtaining antiplague serum was the one for which no definite experimental data was available. Antisera containing high agglutinating titre was found to contain little antitoxic properties to combat the toxaemias met in advanced cases of plague. It was, therefore, realised early in our attempts that attenuated cultures or killed cultures, although potent antigens for obtaining good agglutinins, the clinical course of the disease in human or animal cases was unaffected if the treatment was started late after the onset of disease. Organic iodine, formalin, and such other additions to modify the antigen failed to yield an ideal immunizing agent. The course of immunization consisted in employing modified antigens in the initial stages followed by gradually increasing doses till virulent live cultures of *Pasteurella pestis* were tolerated intravenously. The antiserum thus obtained was given a field trial and was found to be efficacious in advanced cases of plague where antibiotics alone failed to check the course of the disease.

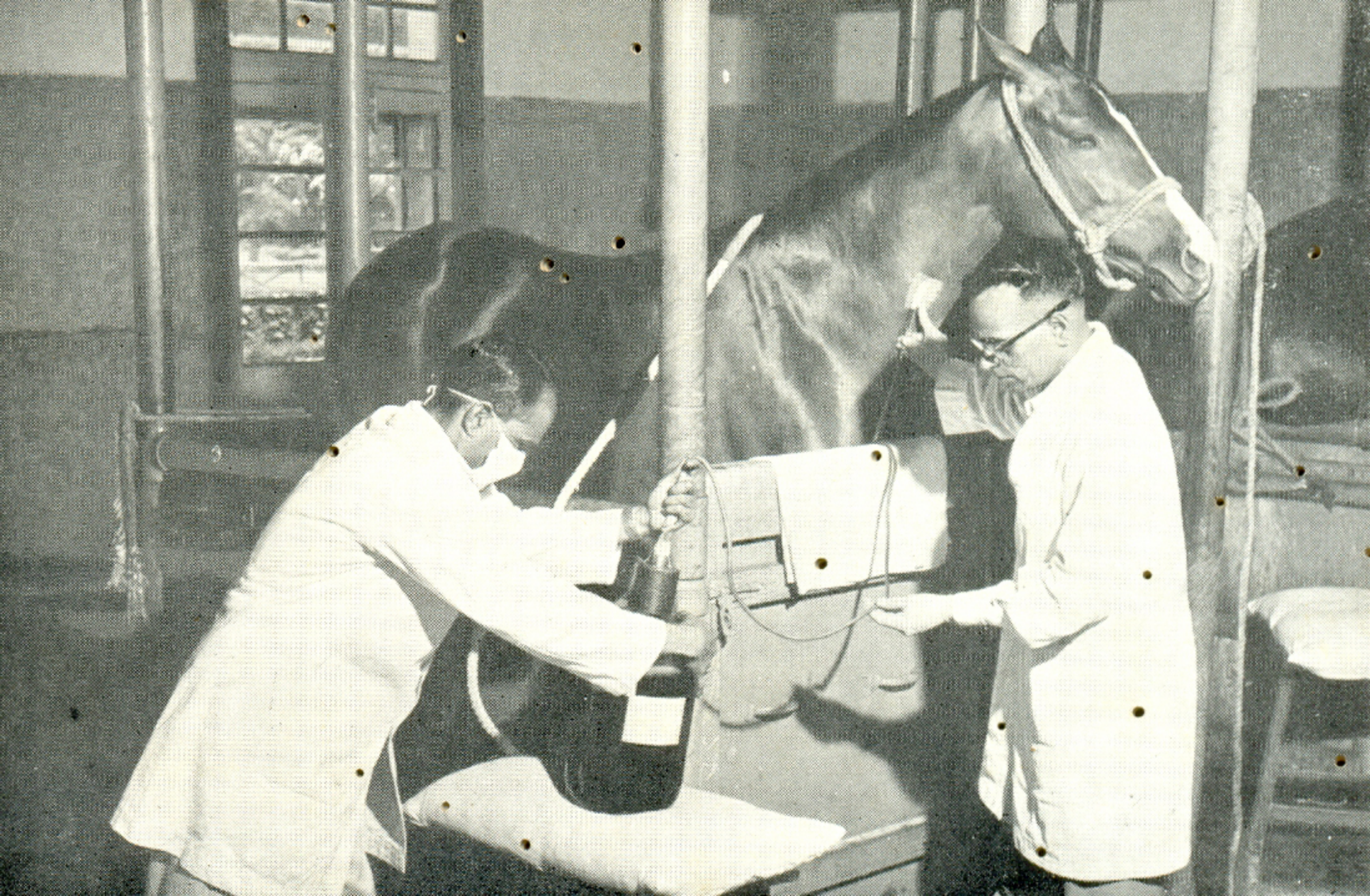
The antiserum thus obtained was concentrated by the usual salting out methods for separation of globulin fractions. It was noted during clinical trials that the solution of pseudoglobulins thus obtained was less efficacious than the natural antisera. This may be due to the fact that antitoxic bodies in case of plague antisera are not wholly confined to pseudoglobulin fraction. Researches in this connection have been undertaken and though final confirmations are not yet available, it is evident from the results so far worked out that euglobulin fraction also plays an important role in developing antibodies in case of plague cultures used for production of antisera.

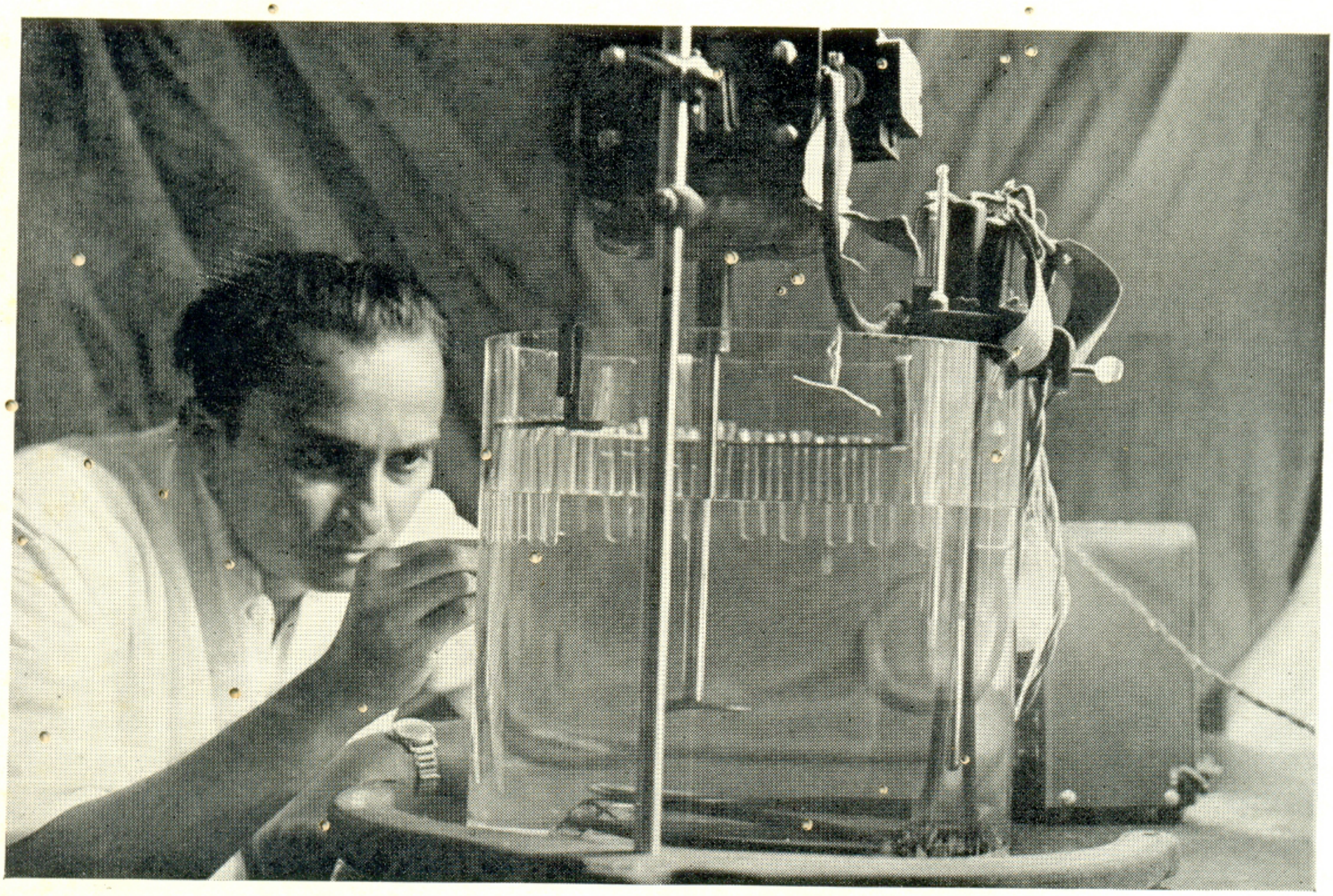
The analysis of *P. pestis* antigens was carried out using the gel diffusion technique and electrophoresis. It was shown that the heat-stable antigen of *P. pestis* was common to *P. pseudotuberculosis*. The soluble antigen of *P. pestis* has been fractionated using ethylene and diethylene glycol for extraction. An antigenic fraction responsible for protection of mice against virulent plague infection was obtained in a purer form by this method.

The antigens used for the production of antitoxic sera against tetanus toxin had not presented much difficulty excepting for the fact that during the war-period availability of peptone and other agents was rather difficult. It was, therefore, found necessary



Bleeding of horses for the manufacture of antitoxins





Titration of diphtheria antisera by flocculation with diphtheria toxin

to work out alternative media with agents locally available. The main efforts were directed towards perfecting a medium with the utilization of available hog stomach. It was observed that in spite of a uniform method for the preparation of media, the results obtained in terms of toxin yield were variable beyond the expected limits of biological variations. It was felt that the animals usually slaughtered for food purposes were not always physiologically perfect. This, it appeared, affected the yield of the enzyme and other constituents as well as the quality of media. The same difficulty was experienced in obtaining beef of the optimal quality. In the case of veal, the only satisfactory solution appeared to be that the selection, slaughter and collection should be done under direct supervision.

The preparation of antigen for human immunization against tetanus undertaken on conventional lines presented problems of varying nature under local conditions. Some of the then available peptones used for the preparation of toxins were found to yield toxoids resulting in sensitization of the subject to varying extent. This formed a problem inasmuch as peptones found to be suitable for yields of potent toxins were those that were prone to give sensitization effect. Various methods with different preparations were tested and finally the medium was standardized to yield toxins of adequate potency for the preparation of toxoids. A preparation was ultimately obtained that gave adequate protective immunity but no reaction whatsoever.

With the production of tetanus toxoid for human prophylaxis attention was directed towards the development of a simple and more direct method for the standardization of the toxoid. A mouse protection test for the assay of tetanus toxoid was worked out. The method depends upon the measurement of tolerance of a group of 30 mice actively immunized with half the human dose of the toxoid under test to 20 C.L.D. of a potent tetanus toxin, four weeks after the immunization. The results were regular and reproducible and agreed well with the results obtained by the standard method of testing the sera of guinea pigs immunized with toxoid for their antibody content.

A comparative study of two different methods for human prophylaxis against tetanus was carried out. The new method consisted in giving 3 doses of 1·0 c.c. each of formal tetanus toxoid at the interval of 3 weeks to a group of 23 volunteers. The second or the control group was injected with 2 doses of 1·0 c.c. each of the toxoid at an interval of 6 weeks. It was observed that the former group of volunteers receiving 3 doses at the interval of 3 weeks showed better antibody response than the latter.

The problem of production of a potent toxin of the gas-gangrene group of organisms was faced in the earlier stages when it was needed for hyperimmunization of horses. A medium consisting of veal and liver digest without addition of any commercial peptones was developed. A highly potent Perfringens (Welchii) toxin (type A) could be produced in the medium. Further studies on the toxin produced in this medium showed that the Perfringens type A toxin consisted of 2 antigens each of which was characterized by a different type of haemotoxin.

In the preparation of diphtheria antitoxin the production of suitable diphtheria toxin for immunization presented problems of interest. It has been the common experience of all workers on the subject that the yield of diphtheria toxin of adequate potency depends on various factors, which have yet not been fully understood. With us the problem had been still more complicated on account of the non-availability of materials during earlier stages of the war.

The studies revealed that in both the veal infusion peptone broth and hog stomach autolysate-veal infusion medium, the yields of potent toxin were low and often irregular. Optimal conditions with special reference to the concentration of iron, sugar in the medium and the surface area volume ratio have been worked out which give a much high and regular yield of potent toxin than that reported earlier.

The changes associated with the process of conversion of toxin to toxoid with formalin were studied. A close correlation has been

shown to exist between the loss of toxicity of diphtheria toxin during the process of conversion of toxin to toxoid and its resistance to the action of heat and to its digestion with crystalline trypsin.

With a view to lessening the sensitising reaction due to non-specific protein present in the crude diphtheria toxoid, it was precipitated with 1 per cent solution of alum and the precipitate suspended in borate buffer to give the standard A.P.T. Optimum conditions regarding the concentration of alum, pH, etc., had to be worked out.

It had been observed that in routine immunization of horses for antitoxin production the veal infusion toxin and toxoid gave better response. Hence the composition of the crude diphtheria toxin was studied in greater detail. It was shown that the crude veal infusion peptone diphtheria toxoid contains a non-specific synergistic factor having the property of enhancing the antigenicity of the purified diphtheria toxoid. The synergistic factor was isolated from the dialysable impurities of the crude toxoid absorbed by activated charcoal and eluted with alkali. This preparation was given a trial in human volunteers which showed encouraging results. It was observed that purified toxoid in combination with this synergistic factor was immunogenic and caused less allergic reactions.

Of late this department started the preparation of P.T.A.P. following the method of Holt with certain modifications. This new toxoid fulfilled the need of a reliable and accurately reproducible prophylactic of high potency and stability free from any local reaction in inoculated persons. In the preparation of this toxoid the preliminary concentration of the crude diphtheria toxoid is carried out by means of ammonium alum and this concentrated and partially purified toxoid is then subjected to further purification by means of ammonium sulphate fractionation method. The concentrated and purified toxoid is finally adsorbed on aluminium phosphate gel.

In the initial stages, experimental work for the immunization of horses for tetanus antitoxin production was undertaken. The addition of non-specific adjuvant to help induce better immunity response presented problems in view of the fact that all the available horses were advanced in age and previously used by the army. They suffered from local reactions and cicatrization of tissues with agents such as Tapioca, etc. It was found that to overcome this difficulty and to utilise the animal for longer courses of injections and bleedings, only non-specific agent which proved useful was calcium chloride, which had to be included in the later stages of the treatment. The immunity response showed an appreciable upward trend, particularly in older animals, with the administration of adrenaline chloride solution at suitable stages of the treatment.

Antivenin

Soon after the final undertaking on the part of this department to produce antitoxins for plague, tetanus, diphtheria, gas-gangrene etc. it was felt that a Polyvalent Antivenom Serum against common poisonous snakes of our country, viz. *N. naja*, *B. coeruleus*, *V. russellii*, and *E. carinatus* was a necessity which could no longer be postponed.

Choice of Antigens for Immunization—The well-known zoological differences in the snake presented a problem of preparing an antigen of adequate components to yield a polyvalent antivenin. Fortunately, a stock of dried venom collected during the last 30 years or more, was available at the Institute. The venoms of various species were pooled separately and thus it was possible to obtain a fairly representative antigen for venoms of respective species. In recent years the physiological actions rather than the immunological behaviour or aspects of the venoms have been studied more fundamentally. It was, therefore, considered desirable to make preliminary studies with a mixture of venoms for its behaviour as an antigen. It was observed that for the production of antivenin of a real polyvalent character all the four common venoms had to be included. The antisera against venoms of *N. naja* and *V. russellii* showed no evidence of any neutralizing effect against the venoms of other

two commonly prevalent poisonous snakes. (*B. caeruleus* and *E. carinatus*).

The next problem encountered was the form of venoms to be administered for optimal immunity response for production of antivenin. There were hardly any data available for the use of any modified venom in preference to non-modified ones for the immunization of horses to obtain antivenom serum. The studies on the immunological aspects of venoms gave evidence in favour of non-modified venoms as perfect antigens for the purpose. The usual formalization process of antigens for detoxication did not serve the purpose as the antigenic behavior was not affected. It was found that with progressive loss of toxicity of venoms, while in contact with formalin, the antigenic properties of this modified venom also were affected adversely. The other methods tried for the modification of venoms with a view to minimise toxic effects were not found helpful. It was decided to use non-modified venom to obtain the desired results, as was evident from the studies with modified and non-modified venoms on rabbits. The necessity for the modification of venoms was apparent since by virtue of the presence of various enzymes they were found to cause very severe local reactions thereby hindering administration of progressively increasing doses necessary for the production of antisera. The reactions and tissue necrosis caused by venoms resulted in their non-absorption.

The studies on rabbits showed that in the earlier stages of immunization not only smaller quantities of venom but also dilution of venom with normal saline or distilled water improved the toleration of venom administration. It has been found possible to inject a mixture of all the four non-modified venoms in horses in doses of 40 mg. of total venom in 1 in 100 solution with a little or no adverse reaction. The initial dosages if administered in high dilutions are not only well tolerated but also evoke a very encouraging immunity response. During the preliminary studies it was observed that diluted venoms were well tolerated by the animal and smaller quantities were sufficient for complete immunization of horses. Thus it has been possible to economise very considerably the use of venoms, a factor which is of considerable importance for large scale production of antivenin, in view of the difficult position of the availability of venoms.

Recently biochemical techniques for studies of venoms have been in progress. These studies have resulted in detecting cross reacting antigens of different venoms. Studies on proteases of venoms have been undertaken and cobra venom is found to contain five proteases while viper venom contains two. Heparin inhibition of protease in venoms is not found to be of high order.

In connection with the studies on standardization of the polyvalent antivenin, it was found that Anderson Cains method which is in common use, consists in injecting mixtures of fixed amount of antivenin and varying lethal doses of venom in pigeons not only gave unsatisfactory results, often difficult to repeat. It also entailed the use of venoms in larger quantities. Intradermal methods on lines similar to diphtheria antitoxin assay were found to be equally unworkable. The Ipsen Assay method in accordance with suggestions of Permanent Commission on Biological Standardization of the League of Nations was tried for assaying the polyvalent serum with suitable modifications for the respective assays against the four venoms stated above. This method, which gives a final neutralization line deviating a little from the preliminary neutralization line in the case of the three venoms of cobra, viper and krait, shows marked divergence with the venom of *E. carinatus*. This could be due to some characteristic behaviour of *Echis* venom in lower dilutions or due to inhibitive effects of the natural defence of the mice used for the test.

The production of polyvalent antivenin and perfection of a method for the assay of its neutralizing properties against the four venoms by using a mouse protection test was achieved during the earlier years. However, search was continued for *in vitro* method for the same purpose. Studies on flocculation of cobra venom with antivenin

showed that more than one zone of flocculation occurred in the venom-antivenin reaction and one of these zones gave an optimal flocculation volume which corresponded well with the neutralization results obtained by mouse protection tests. A toxic factor could be isolated from the venom-antivenin floccules of this zone.

The electrophoretic analysis of polyvalent antivenin showed that a definite increase in the β-globulin fraction, but no distinct peak of globulin was observed as in the case of diphtheria antitoxin obtained from hyperimmunized horses.

The antigenic composition of the venoms of the four poisonous varieties was studied with the help of Ochterloney's gel-diffusion technique. It was observed that there were three common or cross-reacting antigens between the venoms of cobra and krait which belong to the same family of *elapidae*. Similarly, the presence of five common antigens was observed between the venoms of Russell's viper and saw-scaled viper. There was also a faint indication of one common antigen between the Russell's viper venom and cobra venom.

The LD_{50} of all the four venoms was also determined both by subcutaneous and intravenous routes. The ratio between subcutaneous to intravenous LD_{50} was 1 in the case of cobra venom while those for krait, Russell's viper and saw-scaled viper were 10·5, 26 and 14 respectively.

Enzymic make-up of the proteases of the venoms of cobra and Russell's viper was studied. Five proteases were detected in cobra venom and the presence of two proteases was demonstrated in Russell's viper venom. One of these proteases of Russell's viper venom is active at an optimum pH of 3·6 on heat denatured haemoglobin and its activity is inhibited by heparin. The other has an optimum pH of 9·0 and is only slightly affected by heparin. It was also observed that 200 I.U. of heparin could protect mice against 1·5 C.L.Ds of Russell's viper venom when the two were

towns and vast rural areas in our country has practically resulted in eliminating the use of these vital products for therapy. This problem was more acute especially in the case of antivenin which finds more use in rural areas. Hence all the efforts were directed towards overcoming this difficulty. The easiest solution to the problem was to issue the serum in lyophilized form. However, a great hurdle was met with when attempts in this direction were made.

It was observed that the lyophilised concentrated pseudoglobulin did not dissolve well in contrast to natural immune horse serum when it was reconstituted before injection. A very large portion of the dried product remained undissolved on addition of water in the beginning and dissolved very slowly on keeping. With a view to overcome this drawback of the lyophilised product, extraction of liquid serum with ether alone and ether in presence of bile salts was attempted and it was observed that the latter improved the solubility of the lyophilised product. Further work in this direction has shown that improvement in solubility is practically all due to the presence of bile salts and ether extraction plays a very small role. These investigations thus have paved the way of distributing sera in lyophilised form.

Methods of Purification of Antitoxins

The purification and concentration of antitoxins had to be achieved by a method which was a compromise between the maximum yield and the minimum of possible untoward reactions. The aim was to meet the heavy demand for these products during war when supply from outside was not easily available. The ammonium sulphate precipitation was selected as a method of choice. The method adopted varies from the conventional one. Firstly ammonium sulphate precipitation of globulin fractions is carried out at lower temperatures and dialysis is done at room temperature. In our modified procedure no heat-treatment is given during th[illegible]

The dialysis of globulin fraction precipitated with half saturation was carried out at 4°C in the beginning which has now been replaced by dialysis at room temperature resulting in saving of time without affecting the overall recovery of the antitoxin or its quality. Though the method has given satisfactory results for a large-scale manufacture, it gives a product containing some impurities which may be responsible for certain allergic reactions. The other methods generally adopted for the purification depend upon the use of some enzymes like pepsin, trypsin etc. These methods which give a purer product and antitoxic globulin of lower molecular weight cause less sensitizing reactions. However they give a comparatively lower yield when applied on a large scale. Attempts are being made to evolve a method to obtain higher yields. However, recoveries of more than 50 per cent of the total antitoxins were not possible in our hands with the existing methods.

The product obtained by ammonium sulphate fractionation method adopted in the department, when analysed electrophotometrically, always shows a peak of globulin which is of no significant importance from the point of view of antitoxic properties of the serum. The studies on the effect of extraction of lipoids from diphtheria antitoxin globulin on its flocculating ability and combining power were carried out. It was observed that pseudoglobulin fraction of the diphtheria antitoxin (obtained at half saturation with ammonium sulphate and dried to 1 per cent moisture content) when extracted with ether-alcohol mixture gave a product with no loss in antitoxin content, although the flocculation time was slightly prolonged. However, there was an increase in the purity of the antitoxic globulin varying from 11 per cent to 40 per cent in different samples probably due to removal of globulin as shown by electrophoresis.

Allergic manifestation in a small number of cases receiving equine antisera, seldom leading to grave results, is too well-known, and the manufacture and issue of antitoxins on a large scale for prophylactic or therapeutic use is always attended with this problem of "Serum reactions". With a view to minimising these untoward reactions an anti-allergic serum in horse was prepared by injecting intravenously gradually decreasing doses of human serum. This serum which showed anti-allergic properties was mixed with the horse diphtheria antitoxin and was administered for therapeutic use in the Infectious Diseases Hospital along with the normal antitoxin to a control group. The incidence of allergic reactions in the group treated with antitoxin mixed with antiallergic serum was found to be less than in the control group. Though this study needs trials on a larger scale to arrive at significant results, the preliminary results obtained seem to be encouraging.

The standardization of diphtheria antitoxin by Ramon's flocculation method presented an unusual problem in the case of certain horse sera inasmuch as the same sera showed either no flocculation in reasonable time, or did so after an unusually long period. In this connection some work was undertaken to detect easily the optimal flocculating tube and see if the time of flocculation could be reduced. The use of Balsam Tolu in Ramon's flocculation was found to facilitate the detection of the optimal flocculation in diphtheria toxin antitoxin reaction.

With the advance of immunological techniques specially with reference to gel diffusion, Oudin and Ouchterloney's techniques for studies of precipitation reaction in semi-solid media have been worked out in greater details for their use in determining the purity of proteins, or polysaccharide antigens, or detection of common or cross-reacting antigens.

With the evolution of diphtheria prophylactics such as A.P.T., N.A.F.T. and P.T.A.P., investigations regarding the incidence of naturally occurring antibodies in adult population of Bombay city divided into three groups according to the socio-economic status were undertaken. It was observed that the natural antibody was found to be more in economically poorest group which was probably due to their unhealthy environment resulting in greater exposure to infection and consequent antibody production.

The proteolytic activity of rabbit brain thromboplastin has been measured. The presence of one proteolytic enzyme active at pH 3·2 was demonstrated and this was found to be competitively inhibited by heparin.

The action of crystalline trypsin on native heat-denatured and formalised egg albumin was measured. It was observed that trypsin did not act on native as well as formalized egg albumin either before and after boiling in the case of the latter. Heat-denatured albumin was however readily acted upon by crystalline trypsin. The chromatographic analysis of the amino-acids released by the action of trypsin showed that arginine was released first followed by lysine, glutamic acid, tyrosine or tryptophane and leucine. Almost all the amino-acids could be detected in a 32 hour digest.

The studies on lens proteins were undertaken in collaboration with workers from a sister institution in the city. By making use of electrophoresis and gel diffusion techniques it was shown that lens proteins differed antigenically from serum proteins. However, aqueous and vitreous humors contained a mixture of both the lens and serum proteins.

The electrophoretic pattern of sera from Kala-azar patients was studied. It was observed that the amount of γ-globulin increased even during earlier stages of the disease.

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OF

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