Haffkine's plague vaccine / by J. Taylor.

Memoir No. 27. *March, 1933.*

INDIAN MEDICAL RESEARCH MEMOIRS

Supplementary Series

TO THE

INDIAN JOURNAL OF MEDICAL RESEARCH

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BY

Lieut.-Colonel J. TAYLOR, D.S.O., M.D., D.P.H., I.M.S.,

Director, Haffkine Institute, Bombay.

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FOREWORD

HAFFKINE'S plague vaccine has now been in use in India for over thirty-four years during which period approximately thirty-six million doses have been issued. The use of the vaccine occupies a primary position amongst the plague preventive measures adopted in India ; its value is accepted and inoculation is relied on as a measure of personal prophylaxis and for the reduction of mortality.

No full account of the development of the vaccine or the results of its use has yet been published and it is considered that such is now due. In the present *Memoir* which has been compiled mainly from the records of the Haffkine Institute, it is intended to present an account of the origin and development of the vaccine, the methods of preparation employed, the statistical evidence of the value of inoculation, and the results of the study of its properties.

There are still many gaps in the experimental evidence in regard to the effect of various factors on the value of the vaccine and these, it is hoped, will be filled as the result of a fresh investigation which has now been entered upon and for which a new and more accurate experimental technique will be employed. It may possibly be found advisable, as the result of later knowledge, to modify the methods of manufacture of the vaccine but, so far, the results of many years' observation and experiment have confirmed the value of the methods originally adopted by Haffkine for the production of a potent plague vaccine and, although changes and improvements have been made in regard to details, the principles laid down by him have not been departed from in any essential.

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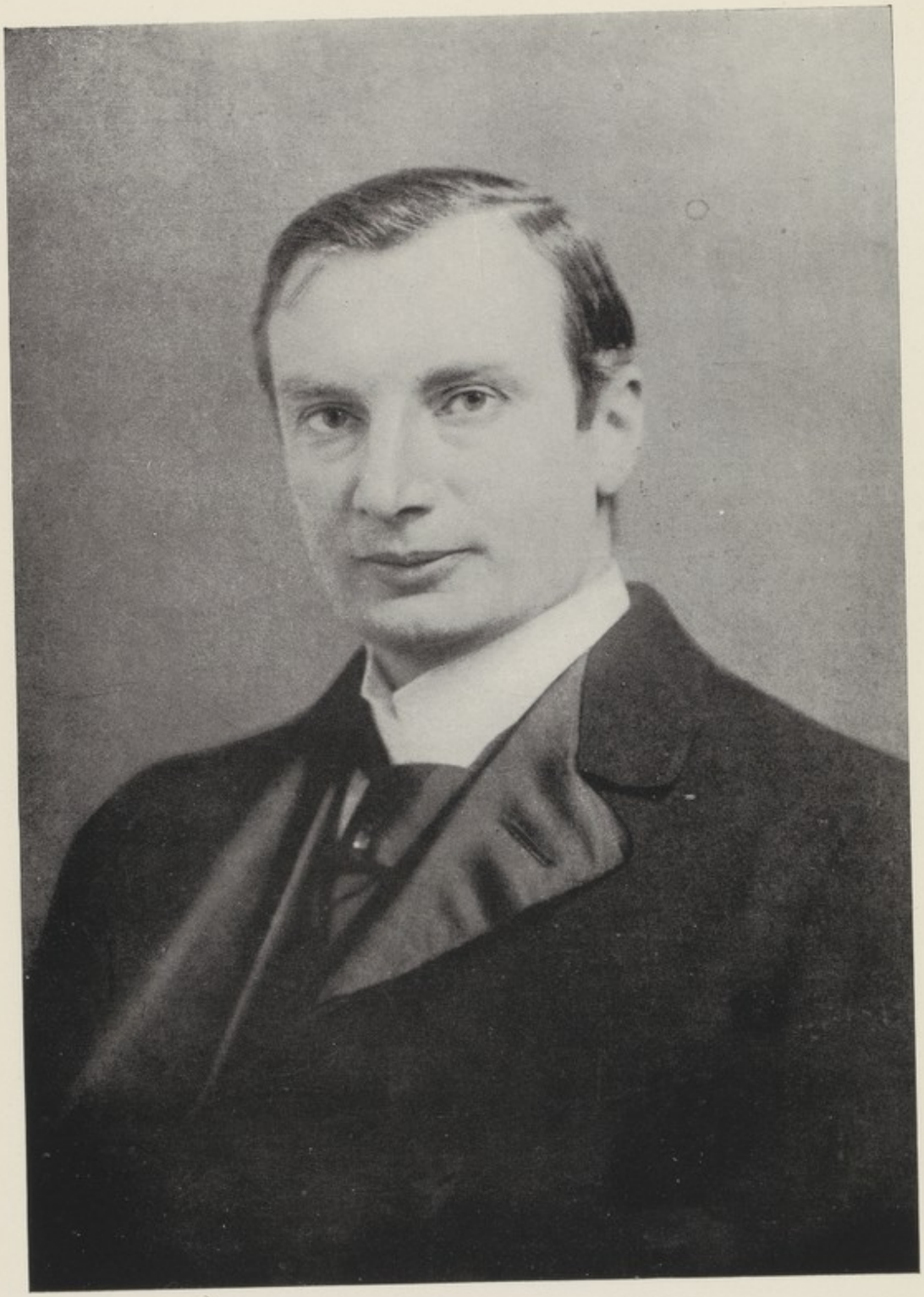

WALDEMAR MORDECAI WOLFF HAFFKINE,
1860-1930.

The Indian Medical Research Memoirs, Memoir No. 27, March, 1933.

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Director, Haffkine Institute, Bombay.

[Received for publication, August 27, 1932.]

CHAPTER I.

THE ORIGIN OF THE VACCINE.

WALDEMAR MORDECAI WOLFF HAFFKINE was in India in 1896 when the outbreak of bubonic plague occurred in Bombay which was the starting point for the spread of the disease throughout India resulting in the subsequent establishment of the endemic and epidemic conditions which have persisted up to the present day.

Prior to coming to India Haffkine had, for eleven years, been working in the Pasteur Institute, Paris, which during that period had been the scene of the brilliant work of Pasteur on the subject of prophylactic inoculation against anthrax, rabies, etc. Haffkine himself, while at the Institute, had studied experimentally the question of prophylactic inoculation against cholera and, in view of the encouraging results he had obtained, desired to test the effect of a cholera vaccine on human beings. The use of anti-cholera vaccine had actually been attempted in Spain by Dr. Ferran, but from the information available it appears that Ferran's vaccine was prepared in a crude manner from broth cultures of the *V. choleræ*, and the details of preparation were not revealed by the author. No statistics as to the results of its use are available. Ferran's work was apparently of short duration and was not continued.

In seeking an opportunity for testing his cholera vaccine Haffkine had at first intended to proceed to Siam where the disease was epidemic, but his work having come to the notice of the Marquis of Dufferin and Ava, at that time British Ambassador in Paris, and formerly Viceroy of India, Lord Dufferin arranged through the India Office that Haffkine should be given an opportunity to try the vaccine in India.

Haffkine accordingly proceeded to India in 1893 as a voluntary worker and commenced the trials of his cholera vaccine in the field. After a period out of India on account of ill-health, Haffkine returned again in 1895 to continue his cholera inoculation work, but this time in the employment of the Home Department of the Government of India.

The first cases of plague were recognized in Bombay in September 1896, and, amongst the measures adopted by the Government of Bombay in connection with the outbreak was the appointment of a Scientific Committee, 'to enquire into the nature and the history of the disease'.

Haffkine's services were lent by the Home Department to the Government of Bombay for employment on this Committee, the other members being:—

Surgeon-Major Manser, I.M.S. (*President*), Surgeon-Captain Childe, I.M.S., Professor Hankin, and Dr. Surveyor.

The terms of reference of this Committee, as given in the memorandum of the Surgeon-General with the Government of Bombay, dated 10th October, 1896, were as follows:—

'The points for investigation will embrace everything on which reliable information may be obtainable as to the mode of origin and nature of the disease, its communicability from one human being to another by infection, contagion, inoculation or otherwise; whether communicable from man to animals or animals to man; whether there is any connection between the disease and grain used as food; whether the grain is sound or otherwise; (*a*) by direct inoculation, or (*b*) when received by ordinary exposure to infection or contagion from a person suffering from the disease, or articles of clothing, bedding, etc., used by the sick; the period during which persons apparently recovered may retain the power of infecting the healthy, etc. The suggestion of any special preventive or curative measures, which may be deduced as the result of these enquiries, and any other matters of practical or professional interest which may occur to the Committee'.

The Committee assigned to each of its members certain lines of inquiry, clinical, epidemiological, pathological, etc., and to Dr. Haffkine was assigned 'The study of the microbe and its relation to the patient and to the animal body; the question of its communicability by infection or contagion between man and man and between man and animals; inoculations, etc.; diagnostic methods; the period during which the patient continues infectious; immunity; and anti-toxine and other treatment'.

Haffkine commenced his work on 8th October, 1896, in the Petit Laboratory of the Grant Medical College, Bombay, the accommodation provided for him consisting of one room and a corridor, and his staff of one Indian clerk and two peons or messengers.

With this limited staff and accommodation, and with a minimum of equipment and without expert technical assistance, he began his studies on plague, and in particular the questions of the production of a prophylactic vaccine and of curative serum.

Haffkine was at no time a prolific writer and, apart from a few papers in the scientific and medical journals and certain lectures and addresses, has left few published accounts of his work. We have not been able to obtain any definite information as to the details of his work during the first few months of his plague investigations or the steps by which he approached the problem of the plague vaccine. We know that amongst his earliest observations was the recognition of the characteristic stalactite growth of *B. pestis* in broth cultures which is described later on in this *Memoir*.

Bannerman (1902) records that in December 1896 Haffkine was successful in protecting rabbits against an inoculation of a virulent culture of *B. pestis* by treating them previously with a subcutaneous inoculation of a broth culture of these organisms sterilized by heating.

Yersin, Calmette, and Borrel (1895) had previously shown that a certain degree of immunity could be conferred on animals by the injection of dead cultures and their findings had been confirmed by Klein.

Having obtained successful results on experimental animals Haffkine next proceeded to demonstrate the harmlessness of the material he had prepared by injecting himself with a dose of 10 c.c., and, being satisfied that it could be safely administered to human beings, announced his results in the following letter addressed to the Secretary to the Government of India, Home Department, dated 16th January, 1897 :—

'I have the honour to communicate the following facts relating to the possibility of protective inoculation against the plague :—

'In the course of the present researches I have found different media which give rich cultures of the plague bacillus, permitting to cultivate them in abundant and concentrated quantities. The virulence of these cultures is shown by the fact that 1 or 2 minims are sufficient to communicate death to the largest rodents.

'The destruction of the bacilli in the culture by delicate processes, such as the addition of essence of mustard, of very weak solutions of carbolic acid, or by desiccation, or by heat, deprives these cultures of their fatal properties, and makes a dose 40 or 50 times bigger than the fatal one quite harmless to the animals.

'But, while depriving the cultures of their noxious properties, the above processes leave to them the powers of protecting the system against fatal infection. Rodents which have had an injection of such cultures (with microbes killed in them), when infected five days after the prophylactic treatment, stand easily a dose of virus which would be fatal to ten other non-protected animals.

'Having established these facts, I caused myself to be inoculated on the 10th of this month to observe the symptoms of the operation in man.

'The injection was done in the flank with 10 cubic centimetres of a culture in which the microbes were killed by heating during one hour at a temperature of 70°C.

'The symptoms produced consisted in pain at the seat of inoculation and in a rise of temperature. The highest point reached was 102°F. eight hours after the injection, which was accompanied by slight headache and feeling of faintness. The temperature got again normal 24 hours later. The bowels remained normal. The pain at the seat of inoculation was mostly felt next morning while getting up from bed. It extended, on the left side, to the region above the Poupart's ligament and, on the right, towards the axilla without reaching it. Since the next day the pain gradually disappeared. A small nodule remained afterwards at the seat of inoculation, but got rapidly absorbed. These symptoms show that the operation is perfectly harmless to man.

'These observations made in the laboratory lead to the conclusion that the inoculation, as described above, will increase the resistance of man against the disease. But the extent of that protection, and the modifications to be introduced

in the method for the object of increasing that protection, can be shown only by observation on man during the epidemic '.

Haffkine had thus, in no longer period than three months, evolved and tested experimentally his vaccine, which in its general lines remained unchanged subsequently, and has undergone no essential variations in principle up to the present day. When it is considered that the incubation period alone of the vaccine was six weeks it will appear evident that, from the very beginning of his work on the subject, Haffkine must have formulated his ideas as to the lines to be followed and adhered to the principles he fixed. There could not have been sufficient opportunities, or time, for testing the various factors which would influence the immunizing value of the vaccine and, as Haffkine himself states, the lines which he followed were based on *a priori* reasoning. The results of many years' subsequent experimental work on the vaccine has shown us that in regard to almost every point in connection with the manufacture of the vaccine, Haffkine had adopted the methods which have been found to produce a vaccine of the highest antigenic value.

Having tested the effect of the vaccine on himself, Haffkine next proceeded to try and popularize inoculation with his vaccine in Bombay. In a short period 77 medical men and prominent citizens were inoculated publicly without ill effects, although the vaccine used at the time undoubtedly gave rise to severe reactions. Haffkine soon reduced the dosage and there were probably few very excessive reactions experienced. Plague was severely epidemic in Bombay at the time and no other protective method of value was then known, the knowledge of the epidemiology of the disease being slight and the importance of rats and fleas in transmission not realized. In the absence of any other effective method of combating plague the possibility of obtaining individual protection by means of Haffkine's vaccine would probably have resulted in its use being taken up to a considerable extent, but to popularize the method an effective demonstration of its value in protecting persons definitely exposed to risk was required. An early opportunity for such a demonstration occurred in the House of Correction, Byculla, when plague broke out in this Jail in the end of January 1897. Half the inmates of the Jail were inoculated by Haffkine and half kept as controls. The results of this test are given in detail in Chapter VIII, and it will be seen that amongst 148 inoculated only two cases of plague occurred, neither of which was fatal, while amongst 172 uninoculated prisoners there were twelve cases with six deaths.

On the publication of the results of this trial there was an immediate demand for inoculation, and it became necessary to provide for manufacture of the vaccine on a wide scale.

The Bombay Municipality placed at the disposal of Haffkine a bungalow on Malabar Hill called ' The Cliff ', for use as a laboratory, and this bungalow was utilized for the purpose from April to November 1897. Haffkine's staff at this time had been increased to two Military Assistant Surgeons, two clerks, three peons and four menials. Major W. B. Bannerman, I.M.S., and two Indian doctors were later added to the establishment of the laboratory. ' The Cliff ' was soon found to afford insufficient accommodation for the purpose and, following a short move to a bungalow in Nepean Sea Road, more extensive accommodation for manufacture

Plate I.

Fig. 1. Haffkine in his Laboratory, Khushru Lodge.

Fig. 2. Decanting the vaccine into small sterilized bottles. Khushru Lodge, 1898.

Fig. 3. Haffkine and his assistants inoculating in the streets of Bombay.

PLATE II.

Old Government House, Parel, Bombay.
(Taken over as Plague Research Laboratory in 1899.)

of the vaccine was provided at Kushru Lodge, Mazagaon, which was placed at Haffkine's disposal by His Highness Sir Sultan Shah, Aga Khan, the head of the Khoja Community, who had taken great interest in the work and supported the use of the vaccine from the beginning.

The manufacture of the vaccine then proceeded at an increasing rate and further additions to the staff were necessary. Four medical men sent out to India by the Secretary of State and four more local medical men were attached besides additional subordinate staff.

Kushru Lodge in its turn proved too small for the purpose of the manufacture of the vaccine on the scale to which the demand had developed. Still more extensive accommodation had to be sought and the choice of buildings fell on the Old Government House, Parel, at the northern end of Bombay Island. This building after being derelict for 15 years had been used as a temporary plague hospital in 1898 for a short period. The building was a very large one and of sound construction and could easily be put in order. Work was commenced on it in March 1899, and the move from Kushru Lodge commenced in July of that year. The building was finally opened in August 1899, and has ever since under the successive names of the ' Plague Research Laboratory ', the ' Bombay Bacteriological Laboratory ' and finally, in 1926, the ' Haffkine Institute ', been the home of the manufacture of the plague prophylactic. The Laboratory, in addition, housed the ' Plague Research Commission ' whose contributions to the epidemiology of plague are well known.

Old Government House, Parel, Bombay.
(Taken over as Plague Research Laboratory in 1899.)

A detailed account of the early methods employed by Haffkine in the manufacture of his vaccine is obtainable from the Minutes of Evidence in the *Report of the Indian Plague Commission* * (1898-1899).

Haffkine first gave evidence before the Commission on the 29th and 30th November, 1898, and the following extracts from his evidence show the methods he employed :—

' The plague prophylactic is not taken from animals as is the case with vaccine lymph, with the material used for inoculation treatment against rabies, or with the serum employed for the curative treatment of diphtheria. It is prepared in an artificial bouillon of the following composition. A certain quantity of minute fragments of mutton is infused in strong hydrochloric acid. The exact proportions of the material is as follows : One and a half kilos of mutton are infused in three litres of water plus two hundred and twenty-five cubic centimetres of hydrochloric acid. As a rule the material is kept in this infusion for two or three days in the cold. Afterwards it is subjected to a high temperature of 130°C. to 140°C., which corresponds to two and a half atmospheres of pressure. It is kept at that temperature for six hours. The material is syphoned off and filtered and the solid residue of mutton is rejected and not employed for inoculation. The liquid is then diluted with a quantity of water which brings the amount I quoted up to four and a half litres. This solution is neutralized by sixty grammes of caustic soda and again

* The Indian Plague Commission was a body appointed to take evidence as to the causation and prevention of Plague which met in Bombay during 1898-1899 and should not be confused with the Plague Research Commission in India which was a working Commission at a later date carrying out an experimental investigation into the epidemiology of Plague.

heated at a temperature equal to the previous one for only half an hour, then filtered again and whatever solid residue is produced by the second heating is again rejected and only the liquid part employed. We call this " Warden's bouillon "*.

' For the cultivation of the plague prophylactic it is mixed with a small quantity of *ghee* or coconut oil, distributed into big flasks, sterilized, and inseminated with a minute quantity of the most virulent microbes we can get hold of. The microbes are specimens obtained in the first case from a plague patient. Their activity is easily maintained by passages through animals according to the hydrophobia or cholera process. The liquid, inseminated as stated above, is kept fermenting. During the first two days scarcely any sign of change is observed. Minute flakes appear underneath the suspended drops of oil or *ghee*. These flakes in the course of 12 to 24 hours grow down in the shape of icicles or stalactites. The liquid remains clear, except for a small quantity of powder-like residue which very early in the process falls to the bottom of the flask. The stalactites in the course of two or three days fill up the upper half or sometimes even the whole volume of the liquid. The least oscillation of the vessel is sufficient to detach the suspended icicles from the drops of *ghee* or oil, and the whole growth in the course of a day or so falls to the bottom of the flask while the liquid appears again perfectly limpid. This stalactite appearance of the growth has an utmost importance, as there are no other microbes up to now known capable of giving the same, and, on the other hand, there is no plague microbe of whatever origin or previous history which up to now has failed to show that characteristic. The stalactite growth offers thus one of the best methods known in bacteriology for identifying the nature of a microbe. After the first growth of stalactites has been brought down by shaking, a new crop of flakes appears underneath the droplets of oil or *ghee*. The same process as described above is repeated again, except that it may be a little slower in development. After another two or three days the flask is again shaken and the second crop brought down. This process is renewed ten or twelve times. The development takes five or six weeks before it is perfectly accomplished. The growth becomes slower and slower until it stops entirely.

' In the course of this process the liquid is modified under the influence of the vegetable microbe in the same way as is modified on an ordinary soil inseminated with the seeds of a plant '.

To preserve the continuity of the description of the processes of manufacture of the plague vaccine, as originally carried out, we may turn to the evidence of Haffkine given on a subsequent date. As regards the temperature of incubation, Haffkine stated that the temperature used was 80°F. to 90° F., but that it did not matter within a degree or two. (This is ordinary room temperature in Bombay).

He stated with regard to sterilization :—

' The method adopted to sterilize the culture is twofold. First the liquid is subjected to a temperature of 65°C. for one hour. I have found from an exact

* Dr. Warden was chemical examiner in Calcutta and had been lent to assist Haffkine in this part of the work.

experiment that it is possible to kill the plague microbe with a temperature of 45°C. and that no very prolonged heating is required for that. I have adopted 65°C. for giving a margin for mistakes from an unexpected fall of temperature. Similarly I have adopted one hour as the period during which the cultures are heated although it is certain that the microbe of plague does not require anything like that time for being killed. The flasks are heated in a big water-tub, the contents of which is, I should say, a hundred times larger than the contents of a flask. Five or six flasks are put simultaneously into such a tub. We begin by heating the water to 70°C.; when the flasks are introduced there is a fall in the temperature. A central flask is put in the middle between the other flasks. Into this central flask a thermometer is placed. The hour of heating begins only when the thermometer placed in the central flask shows 65°C. After the flasks have been heated carbolic acid in the proportion of one-half per cent is introduced into them. I have ascertained that one-quarter per cent of carbolic acid is sufficient to kill the plague bacilli within a comparatively short time. A very elaborate procedure has been adopted to ensure that on no occasion does a flask which has not been heated or has not been carbolized pass into the hands of the decanters'.

The final processes of bottling and testing as originally carried out can be followed from the evidence of Captain C. J. R. Milne, I.M.S., before the Indian Plague Commission. A simple form of syphon was used for decanting, the flow being started by mouth suction and the tube used for this purpose being immediately detached.

A few drops of the vaccine were first run on to the surface of an agar slope as a control for the sterility of the vaccine and the vaccine bottles filled in succession direct from the syphon, a pinch-cock being used to control the flow.

The bottles were of simple type, four to six ounces in capacity and sealed with rubber stoppers. At a later stage the bottles were made with the neck at one side, this shape being adopted to facilitate the taking up of the complete contents into the syringe.

The procedure to be employed in decanting and sterility testing was laid down in 'Rules of Procedure for the Preparation of M. Haffkine's Prophylactic Fluid' given in an appendix to the *Report* of the Commission which need hardly be quoted here.

CHAPTER II.

Haffkine's reasons for adopting the particular type of vaccine.

As has already been stated, Haffkine based his vaccine on considerations derived from *a priori* reasoning, and not on the results of experiment.

In his first short account of the vaccine published simultaneously in June 1897, in the *Indian Medical Gazette* and the *British Medical Journal*, Haffkine (1897) outlined these considerations in the following words :—

'The theoretical conjectures which led to the plan of preparation of the plague prophylactic were the following : The inoculation against cholera, made with bodies of Koch's comma bacilli cultivated on solid media appears to result in a reduction of the susceptibility and of the absolute mortality from the disease, but does not affect the case mortality. In the light of present information this seems to be in connection with the production, in the inoculated individual, of bactericidal powers, but not of anti-toxic, as has been actually demonstrated to be the case by Pfeiffer and Kolle. There is the possibility of the bactericidal power being created by the injection of the bodies of the microbes, or substances enclosed in them, while anti-toxic properties may be communicated by the injection of the metabolic substances secreted or produced in the surrounding media. This conclusion is only a hypothetical one, and taken as a temporary guidance in the work. In the present instance I have decided to make an attempt at effecting both a reduction in susceptibility and of the case mortality by combining in the prophylactic substance large quantities of bodies of microbes together with intensified extra-cellular toxins'.

After describing the method of preparing the vaccine he states that :—

'In quiet position in test-tubes two different substances are obtained : a perfectly limpid fluid and a thick white sediment. Injected into animals they produce : (1) The sediment—a local inflammation and a nodule at the seat of inoculation, accompanied with little fever of general effect, and (2) the fluid—a considerable rise of temperature with general affection, with no local effect'.

It was this toxic supernatant fluid of which Haffkine stressed the importance as a constituent of his vaccine.

Haffkine (1899) discussed the principles of his vaccine at greater length in an address delivered at the Royal Society, London, on 8th June, 1899, and certain of his reasoning with regard to the type of vaccine adopted by him for plague will be seen from the following extract :—

'Immunity against symptoms generated by the products of microbes does not seem to imply necessarily the ridding of the system of such microbes. It is known

now, since the discoveries of Behring and Kitasato, that such a resistance against the products can be originated artificially by gradually treating the system with increasing quantities of toxins. The system reacts by developing anti-toxins, tending to neutralize the effect of the toxins. On the other hand Gamaleia first attracted attention to the fact that it is possible to create in an animal resistance against lethal doses of virulent microbes without that animal getting any resistance against a dose of the products prepared from the microbe in the laboratory.

'One seems justified therefore to consider separately two kinds of immunity, one against the living microbe, which would prevent it from entering the system and causing an attack, and another against the fatality of the symptoms of the disease caused by the products of the microbe when the latter overcomes the initial resistance and does invade the system. In inoculation against cholera, which is done with the bodies of microbes, the first result alone is obtained.

'These considerations come to be strengthened by a set of laboratory experiments by Pfeiffer and Kolle, intended for verifying our Indian results, and in the course of which they detected in the serum of men inoculated with only one dose of cholera vaccine an extremely high protective power, equal to that which, in goats, for instance, could be created only after a very prolonged treatment, extended over five or six months, and including injections with gigantic doses of cholera vaccine. On analysing in detail the properties of that serum they found that it possessed an intense power of destroying the cholera microbes, but exhibited no anti-toxic properties capable of neutralizing the effect of the products of these microbes.

'When, in 1896, I was confronted with the problem of working out a prophylactic treatment against the plague, I determined to put to test the ideas originated by the observations on our cholera patients, and to attempt, in the new preventive inoculation, to obtain at once a lowering of the susceptibility to the disease and a reduction of the case mortality. This I resolved to obtain by treating the system with a combination of bodies of microbes and of concentrated products of them. In giving the above considerations, I beg that they should be considered as temporary, subject to modification or to complete refutation. There may exist already facts unknown to me which are opposed to the guesses implied in them. It was those guesses which led to the results obtained in the plague inoculation; but in giving the reasoning which I passed through while working out the method I am yielding only to a demand to that effect, as I consider that part of my communication unnecessary; the more so that the theoretical conjectures above enumerated are not shared by a number of experimenters, such as Pfeiffer himself, to whose results I owe some of my premises; and the correctness of the composition of the plague prophylactic with regard to the extra-cellular toxins which I have added to it is subjected to theoretical contest. It is certain that no theoretical conjectures conceived by one experimenter are binding or need even be interesting to others. What is obligatory is acceptance of the actual results obtained'.

To what extent Haffkine's theoretical conjectures as to the bactericidal and anti-toxic effects of the vaccine prepared in the method utilized by him were correct will be discussed later, in connection with the results of experimental work directed to ascertain the properties of the different fractions of the vaccine.

CHAPTER III.

Contemporary opinion on Haffkine's plague prophylactic.

Prior to the introduction of Haffkine's plague prophylactic vaccines for immunization of human beings had been used by Ferran and Haffkine in the case of cholera, and by Wright in regard to typhoid fever. The vaccines used for the prophylaxis of these diseases had consisted of young killed cultures of the organisms and were standardized as a rule to fixed dosage of culture. Immunization was attributed to the action of the bacterial substance contained in the dead organisms injected and not to the presence of any substance elaborated or dissolved in the media used for culture.

The methods used in the preparation of Haffkine's plague prophylactic differed from those used for the cholera and typhoid vaccines, and resulted in the production of a fluid in which the immunizing substances were present in solution in the supernatant fluid of the vaccine.

The bacterial bodies present in the broth cultures used for the vaccines after the period of six weeks' incubation consisted of both living and dead organisms, and the greater portion of the sediment was composed of an amorphous debris of disintegrated bacteria. Such a vaccine could not be standardized on the basis of bacterial content.

When Haffkine's vaccine for plague was first introduced it underwent a considerable amount of criticism, chiefly in regard to the value which Haffkine attached to the fluid portion of his prophylactic, and also in regard to the methods of standardization used.

An opportunity arose for a very full discussion of the vaccine when Haffkine gave his evidence before the Indian Plague Commission in 1898, and it was with regard to the value of the fluid portion and the absence of exact standardization that the Commission mainly disagreed with Haffkine. Haffkine stated, and strongly upheld, his opinion that both the sediment and supernatant fluid of his vaccine were of immunizing value but had not, at that time, any experimental evidence in support of this contention. He expressed his opinion to the Commission that while his belief in the value of the supernatant fluid in the vaccine was based on *a priori* reasoning, the success that had been obtained by the use of the vaccine made it inadmissible to modify it. He further contended that even if experimental work on animals were to show that the supernatant fluid did not confer protection he would not be prepared to abandon the method. He stated that he considered that any change in the principles of the vaccine would only be accepted by him on the basis of guarded tests on an isolated group of men with restricted experiments repeated with regard to testing the particular point as to the efficiency of the fluid. Such tests had been projected by him but he had not been able to carry them out.

In his address on the subject of Protective Inoculation before the Royal Society already quoted, Haffkine (1899) admitted the possibility of modifying his vaccine

in the light of future experience but did not find that there was any necessity to do so.

The question of the efficacy of the two main fractions of the vaccine is discussed at length in the *Report of the Indian Plague Commission* in the light of the evidence available at the time.

The following extract from the *Report* will show the views held and some of the available evidence :—

'The utility or otherwise of each separate element in the plague vaccine is manifestly a matter which can only be determined by experiment. We may, therefore, set ourselves to enquire in the case of each element, first, whether any specific toxic effect, and, secondly, whether any protective effect, is produced by the introduction of that element into the system.

'We begin with the consideration of the deposit of plague bacteria which collects in the culture flasks.

'It has been firmly established by numerous experiments in which dead bacterial cultures have been introduced into the organisms of animals—and in this connection we may specially note the experiments which were done by the German Commission on monkeys—that the dead bodies of plague bacilli contain appreciable quantities of poisonous substances. Alterations in the blood, hæmorrhages in the gastric mucous membrane, and necrotic changes in the subcutaneous tissues at the site of the injection and in the liver, are the pathological effects which most often manifest themselves after the injection of plague bacilli.

'It is not open to doubt that the introduction of the toxic elements, which are contained in the bodies of the plague bacilli, ultimately confers upon animals a certain power of resisting plague.

'Yersin, Calmette, and Borrel, in 1895, first brought forward evidence to show that a certain amount of protection could be conferred by inoculations of dead plague bacilli.

'Further, we may note that this fact was confirmed by the experiments of Dr. Klein. We desire particularly to direct attention to the fact that a certain measure of protection was obtained in these experiments of Dr. Klein, for Mr. Haffkine has drawn from these experiments the conclusion that a protective measure may turn out to be effective in man, even when experiments on lower animals do not appear to contain "any elements justifying its application to men."

'The protective efficacy of injections of dead plague bacteria is further established by the many very careful experiments of the German Plague Commission. In particular, it was shown by the German Plague Commission that in the case of the brown monkey (*Macacus radiatus*), and in the case of the rat, a considerable measure of protection can be conferred by these inoculations. But the experiments of the German Commission also show that very slight, if any, protection is conferred by these inoculations on the grey sacred monkey (*Semnopithecus entellus*).

'The protective effect of inoculations of dead bacterial cultures was again confirmed by our own experiments. These were done on guinea-pigs, brown monkeys, and rats.

'It is to be noted that in the many series of experiments which have been referred to above, there is hardly to be found an instance (unless possibly we have such an instance in the case of certain experiments on rats which were performed by Lieutenant Walton, I.M.S.), where a very high degree of immunity has been obtained. In almost every instance, severe symptoms have supervened upon the subsequent test inoculation of living plague bacteria to which the vaccinated animal was subjected. What was achieved in the above experiments was, therefore, merely a modification of the plague attack, and a delayed or diminished mortality. These results (which, be it noted, were obtained by the injection of plague bacilli suspended in a merely watery medium), are in all respects identical with the results which have been obtained on man in cases where Mr. Haffkine's inoculation has not succeeded in averting an attack of plague. Thus the diminished case mortality which Mr. Haffkine imputes, in the case of man, to the effect of the supernatant fluid which forms a constituent of his anti-plague vaccine, is, in the case of animals, obtained in the absence of that supernatant fluid.

'The question as to whether the supernatant fluid of Mr. Haffkine's prophylactic causes, when injected into the organism, the elaboration of substances which modify the severity of a plague attack, is a question which cannot be answered until we have determined whether a specific toxin is produced when the plague bacillus is cultivated in a fluid nutrient medium. It behoves us, therefore, first to consider this question.

'Yersin, Calmette, and Borrel came to the conclusion that there was no specific toxin produced by the plague bacillus when it was cultivated in a liquid nutrient medium.

'The German Plague Commission also investigated the question as to whether specific soluble plague toxins were developed when plague bacilli were cultivated in a liquid nutrient medium. In the first series of experimental injections which were made upon animals by the German Commission with a view of resolving this question, filtrates from 10-day old cultures of plague were employed. The experiments were performed upon rabbits and brown monkeys. In each case, 5 c.c. of the filtrate was injected. No toxic effect was observed to follow from these injections. No appreciable amount of soluble toxins was therefore produced in this instance. In a second series of experiments, the German Commission employed carbolized filtrates from sterilized six-week old cultures. The experiments were in this case done upon two rats. No control experiments were done with carbolized bouillon. The German Commission inferred, from the fact that both rats died, that a small quantity of toxins may possibly, after the expiration of six weeks, escape from the macerated bacterial bodies, and pass into solution in the nutrient fluid.

'Several additional series of experiments on this question were instituted by ourselves. The results of these experiments are incorporated in Tables I, II and IV of Lieutenant Liston's evidence. In the first of these series of experiments the material which was employed was the (carbolized) filtrate from Mr. Haffkine's prophylactic. The animals, which received respectively 2 per cent and 4 per cent of their body-weight of the supernatant fluid, succumbed to its effects. It is manifest that the carbolic acid in the supernatant fluid may be answerable for this result. In the case of the second series of experiments (i.e., of the experiments whose results are incorporated in Table II), the material which was employed consisted of the non-carbolized filtrate from Mr. Haffkine's prophylactic. In this case, with

the exception of an animal which died from intercurrent disease, all the animals survived. A slight amount of necrotic change was observed to occur at the site of injection in the case of the animals which had been injected with doses of supernatant fluid amounting to 4 to 6 per cent of their body-weight. In view of the fact that these necrotic changes did not occur in the control animals injected with similar quantities of Mr. Haffkine's nutrient medium, it is manifest that a small amount of some specific toxin must here have been present in the filtrate from Mr. Haffkine's prophylactic. We are disposed to believe that this small amount of toxin may have been derived from the macerated bodies of the plague bacilli. The material which was employed in the third series of experiments consisted of a filtrate from a culture of virulent plague, which had been grown in ordinary peptone broth. The results of this series of experiments are incorporated in Table IV of Lieutenant Liston's evidence. It was here established that when the plague bacillus is grown in an ordinary peptone broth toxins which are lethal to guinea-pigs may, under certain conditions, be produced. In the experiments in question, the animals succumbed when the amount of the filtrate amounted to or exceeded 5 per cent of their body-weight.

'The first experiments which were made to determine whether any protection was conferred by injections of filtrates from plague cultures were made in 1895 by Yersin, Calmette, and Borrel. These observers arrived at the conclusion that such injections conferred no protection.

'The German Plague Commission re-investigated this question. They experimented upon two monkeys. Each of these monkeys received a subcutaneous injection of 5 c.c. of the filtrate of a 10-day old bouillon culture. Both monkeys succumbed to a subsequent test inoculation of plague. In the case of one of the monkeys no protection, and, in the case of the other, only a very slight amount of protection would appear to have been conferred. This latter effect is explained by the German Commission by the assumption that the filtrate which was used in this case must have contained a certain quantity of substances which were derived from the macerated bodies of the plague bacilli.

'In addition to the experiments which have just been recorded, the German Commission made experiments on monkeys with the supernatant fluid, which was filtered off from a sample of Mr. Haffkine's prophylactic, and they compared the effects of the supernatant fluid with the effects of the bacterial sediment. The results of these experiments led the Commission to the conclusion that the whole protective power of Mr. Haffkine's vaccine was resident in the bacterial sediment, as distinguished from the supernatant fluid.

'The above results are in absolute conformity with the results which were obtained by ourselves in two series of experiments.

'In the series of experiments recorded in Table III of Lieutenant Liston's evidence, it will be seen that the only guinea-pig which had survived the inoculation of the carbolized filtrate from Mr. Haffkine's prophylactic fluid succumbed with extreme rapidity when tested with a living culture of plague. This guinea-pig had obviously not acquired any power of resisting plague.

'The results of the second series of experiments are recorded in Table VI of Lieutenant Liston's evidence. In this series of experiments the prophylactic power of the supernatant fluid of Mr. Haffkine's vaccine was compared with the

prophylactic effect of a freshly-prepared sample of his nutrient medium. It was found that the average period of survival of (*a*) the three guinea-pigs which received only injections of the nutrient medium, and (*b*) of the three guinea-pigs which received comparable quantities of the filtrate from Mr. Haffkine's prophylactic fluid, was in each case $2\frac{1}{3}$ days. It is manifest, therefore, that if there is any specific soluble plague toxin in the filtrate from Mr. Haffkine's prophylactic fluid—and we have seen that this is doubtful—this specific plague toxin must be a toxin which confers absolutely no protection against plague.

'The evidence which has been reviewed above appears to place beyond doubt the fact that the injection of the bacterial sediment does in the case of animals exercise an effect in the direction of the production of immunity. This immunity may be absolute where the test-quantum of plague culture which is introduced into the organism does not exceed a very small minimum. In such cases as this, the plague bacilli are destroyed before they can produce any noticeable symptoms. In other cases, what is achieved is merely a modification of the plague attack. This effect comes under observation wherever the test-quantum of plague culture which is introduced into the system is somewhat larger than the maximum quantity which the system is capable of effectually dealing with.

'Further, the evidence which has been considered above indicates that the filtrate which is derived from the plague culture (the supernatant fluid of Mr. Haffkine's vaccine) does not lead to the elaboration of any substances which are capable of favourably modifying a plague attack. It neither confers upon animals any immunity against attack, nor does it so far as can be seen, operate in the direction of reducing mortality. It would appear, therefore, that the supernatant fluid might with advantage be dispensed with, in the case of man, and it might readily be eliminated from the vaccine by a mere process of decantation. If it was so eliminated, several advantages would be gained. The task of inoculating would be rendered very much less laborious. The reduction of the volume of the vaccinating material would tend to diminish the dangers which might result from the possible contamination of the vaccine. By the elimination of the supernatant fluid there would be eliminated from the vaccinating material a considerable quantity of poisonous substance (peptone) which stands in no known relation to the production of immunity'.

These findings of the Indian Plague Commission have been quoted at considerable length, not for their actual value, but for the purpose of illustrating the difficulties of the earlier workers in regard to the experimental study of vaccine therapy. The finding that the supernatant fluid of Haffkine's vaccine was of no value in conferring immunity and that it could with advantage be dispensed with has been subsequently disproved and, in fact, the most recent work has shown that 100 per cent immunity can be obtained in white mice by a small dose of the supernatant fluid while the sediment of 100 times the quantity is required to confer an equal protection. It would have been more correct to say that in Haffkine's vaccine the sediment could quite well be dispensed with. The incorrect opinions expressed by the Indian Plague Commission could only have been reached as the result of the use of unsuitable experimental methods and, as will be shown in a later section, the difficulties in this regard are only gradually being overcome.

An examination of the experimental evidence on which the Commission had to base its findings shows that the experiments quoted were carried out, as a rule, with insufficient numbers of animals, with animals of very varying degrees of susceptibility to plague infection, and to the toxicity of the vaccine, and without a careful selection of the vaccine dose to be used for immunization and of the *B. pestis* test dose used for infection.

Balfour-Stewart (1900) first provided experimental evidence that the supernatant fluid of the vaccine could confer immunity. In his short series of tests the immunity obtained by the use of the supernatant fluid was greater than that obtained from either the whole vaccine or the sediment alone. Subsequent experimental evidence on this point is given in Chapter XII and it will be seen that it has been abundantly proved that Haffkine was correct in assigning a high value to the supernatant fluid of his vaccine.

Believing, as the members of the Commission did, that the value of the vaccine resided in the sediment only, it is not surprising that Haffkine's method of standardizing his prophylactic according to the reaction obtained in human beings with various doses was subjected to strong criticism. In the light of subsequent confirmation of the fact that the supernatant fluid contains most of the antigenic material, it is obvious that no method of bacterial counting or estimation of the sediment would have been capable of affording a true basis of standardization. A vaccine prepared from an avirulent strain of *B. pestis* may give as heavy a sediment as that from a virulent strain but will not give protection, and will be of much reduced toxicity.

When Haffkine fixed his dosage of the vaccine as the amount of a particular brew necessary to produce a temperature reaction up to 102°F. in the majority of those inoculated he was, in effect, employing a biological method of standardization, but with the irregularity in methods of manufacture employed in the earlier days there resulted a variation in dosage necessary to produce such a reaction which might extend from 2·5 c.c. to 12 c.c.

The Indian Plague Commission, when taking Haffkine's evidence on this point brought out the fact that standardization of dosage in this way by reaction in human beings could rarely be possible at the laboratory as the supply of uninoculated persons on which to carry out tests was limited, and in consequence found that, in practice, the onus of estimating the suitable dose producing the degree of reaction required would rest on the inoculators in the field. The difficulty in carrying out a series of preliminary inoculations and subsequently making temperature observations before proceeding to a large scale use of a brew was obvious and a serious defect in the vaccine.

It was also brought out in the evidence that Haffkine adopted an additional or alternative method of standardization by a rough estimate of opacity when it was not possible to fix the dosage by trials on human beings. He considered the reaction obtained in human beings along with the opacity test for abundance of growth was satisfactory as a practical measure, and preferred these methods to that of weighing the sediment, although, in other evidence before the Commission, it had been shown that in a series of weighings that sediment in different brews had been found to vary by as much as seven or eight hundred per cent.

The Indian Plague Commission in their final report stated their opinion that :—

'In view of the results of these weighings, and further, in view of the fact that no mere estimation of opacity would enable a judgment to be arrived at as to the quantity of dissolved toxins contained in a fluid, should such be present, it seems to us that the routine method of standardization of the vaccine which was employed by Mr. Haffkine must be pronounced to be imperfect. So imperfect is the method of standardization that we have no data for judging what the relation of strength is between the doses of the samples we received and the original dose which Mr. Haffkine has taken as his standard, nor are we able to form any estimate of the quantity of bacterial sediment which was contained in the original "standard dose".'

The difficulties of this kind which arose were largely due to the handicaps under which Haffkine worked in regard to staff, equipment, and facilities for experimental observations. Provided that uniform methods could always be used in the manufacture of the vaccine subsequent experience has shown that a very uniform product could be obtained, and although the criticisms of the Commission were fair in regard to the conditions prevailing at the time they do not constitute any permanent objection to the use of the vaccine on a fixed arbitrary dose arrived at as the result of extensive experience. The question of dosage and standardization will be referred to later.

The medical members of the Commission before whom Haffkine gave his evidence and whose criticisms are included in the extracts given were: Professor T. R. Fraser, Professor A. E. Wright, and Dr. M. A. Ruffer.

CHAPTER IV.

The manufacture of the vaccine: Subsequent developments, 1899–1905.

When the Laboratory was moved to old Government House, Parel, in 1899, Dr. Haffkine was designated Director-in-Chief and Major W. Burney Bannerman, I.M.S., appointed Superintendent. The latter systematized the production of the vaccine and introduced various improvements in manufacture. A detailed account of the methods employed at this time is contained in an address communicated by Bannerman (1902) to the Royal Society of Edinburgh of which the following extract may conveniently be quoted at length :—

'The preparation of the culture medium or bouillon is the first process, and as this differs from the methods employed in European laboratories, it is necessary to describe it somewhat in detail. The necessity for this departure from established custom arose from a consideration of the religious prejudices of the natives of India, certain articles such as beef and pork being abhorrent to the majority of them. Now the peptone of commerce is derived from one or other of these substances, and it could not in consequence be used to enrich the bouillon in the ordinary way. The Government of India therefore deputed the late Colonel Warden, I.M.S., who was then the Chemical Examiner in Calcutta, to try to discover a method of making peptone from goat's flesh, and the process now to be described, though not fundamentally novel, is the result of his labours, modified as experience dictated. The operations connected with this process are carried on in a spacious stone-paved room on the ground floor, fitted up with eight large autoclaves. Lean goat's flesh finely minced is the basis of the bouillon. The meat, placed in glass jars, has 80 c.c. of hydrochloric acid of the B. P. standard added to it for each kilo, and the jars are then immersed up to the neck in water at 70°C. (158°F.), kept warm in a jacketed tub. The jars are left there for a week, by which time the meat has become dissolved so that the contents look like brown porridge. The albumen has become converted into acid albumen with a little albumose and peptone. The fluid is now transferred to large glazed earthenware jars, and water is added to make the bulk equal to three litres of fluid for each kilo of meat originally present. The large jars are now placed in the autoclaves, and kept there for three hours under a steam pressure of three atmospheres, or 44·1 lbs. to the square inch, which gives a temperature of about 143°C. (290°F.).

'The fluid after this treatment is dark-brown and somewhat viscid, containing quantities of undigested fibrous tissue, etc. It is undoubtedly rich in albumoses and peptones but the amount of each has not yet been determined. This fluid is filtered and made up to the original bulk of three litres per kilo. Bone charcoal is now added in the proportion of 150 grammes per litre, and the whole well stirred for a quarter of an hour. This improves the colour of the bouillon and also its cultural properties. The charcoal is then roughly strained out by means of a cloth, and the fluid neutralized by the addition of caustic soda. It is also diluted by the

addition of from two to two and a half volumes of water, for each volume of peptone solution. In this dilution the amount of hydrochloric acid used, when neutralized as above, gives a bouillon containing 0·5 per cent of chloride of sodium or common salt. If the bouillon is required for ordinary laboratory purposes, each volume of the peptone solution is diluted with from three to five volumes of water, and in this case more salt has to be added to get the 0·5 per cent required for the nutriment of bacteria. This mixing and neutralizing is done in a wooden tub holding about 60 litres, for it is quite a common thing for us to produce 50 litres or more of bouillon daily. The neutralizing is in the first place purposely carried too far, for during the heating process the reaction goes back, and a perfectly neutral fluid is necessary for the proper growth of the *Bacillus pestis*. The fluid is then heated for an hour under a pressure of 15 lb. to the square inch (121°C. or 250°F.) to throw down the phosphates, etc., refiltered and distributed in quantities of 2 litres into 4-litre flasks. These flasks are specially made for us in Paris, of pale green glass, flat-bottomed and oblately spheroidal in shape. Two litres of fluid in these flasks form a layer of barely two inches thick, thus affording a very large surface for aeration of the growing bacilli.

'This medium has been found excellent for the growth of all sorts of bacteria, and has proved much cheaper than the ordinary method of making meat extract, and then adding commercial peptone, for not only is the expense of buying peptone avoided—and we used to use as much as twenty-five shillings' worth in a day—but from one kilo of meat we make as much as 9 or 10 litres of bouillon, as against 2 by the old method. This medium is known in the laboratory as "Warden's bouillon", but the process of gradual digestion in the water-bath and treating with charcoal are modifications devised by Dr. F. Maitland Gibson, one of the permanent staff. A similar bouillon was also made by the latter official having for its basis wheat flour, from which the starch has been removed by washing, but it is not at present widely used. It was made to meet the objections of some vegetarian sections of the native community and though not now much in demand, has served the purpose of reconciling some of the leading native practitioners to the use of the prophylactic.

'Into each flask thus made ready for final sterilizing are dropped four drops of coconut oil, which produces an abundance of droplets on the surface, without forming a continuous layer of oil on the bouillon. The necks of the flasks are plugged with sterilized cotton-wool in the ordinary way, and the whole batch of fifty or sixty sent out to the corrugated iron shed containing the big sterilizer. This sterilizer is merely a jacketed clothes disinfector made strong enough to stand a pressure of 15 lb. to the square inch, and with a trolly fitted with shelves on which sixty flasks can be safely placed. The flasks are kept under this pressure for one and a half hours, one hour having in practice been found insufficient to sterilize their contents when working on this wholesale scale.

'The flasks containing the sterilized bouillon with the layer of oil on the surface are now taken to the incubation room to be sown with plague bacilli. It is therefore necessary to explain where the germs come from and how a pure culture of them is produced. It is unfortunately easy at any time of the year to find cases of virulent plague in Bombay or its vicinity, and there has not, therefore, arisen any necessity to have recourse to cultivation in animals to obtain plague microbes of a suitable potency.

PLATE III.

FIG. 2. Stalactite growth in sowing balloon.

FIG. 1. Sowing balloon.

PLATE IV.

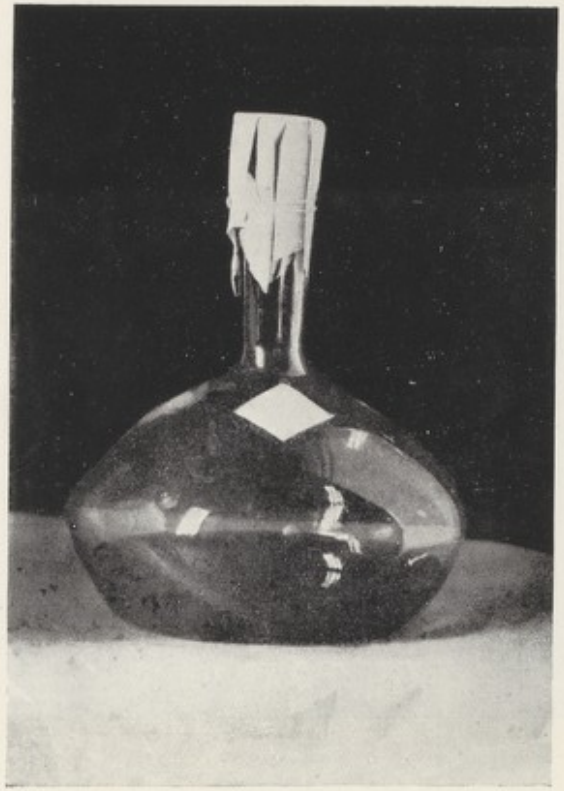


FIG. 1. Uninoculated flask.

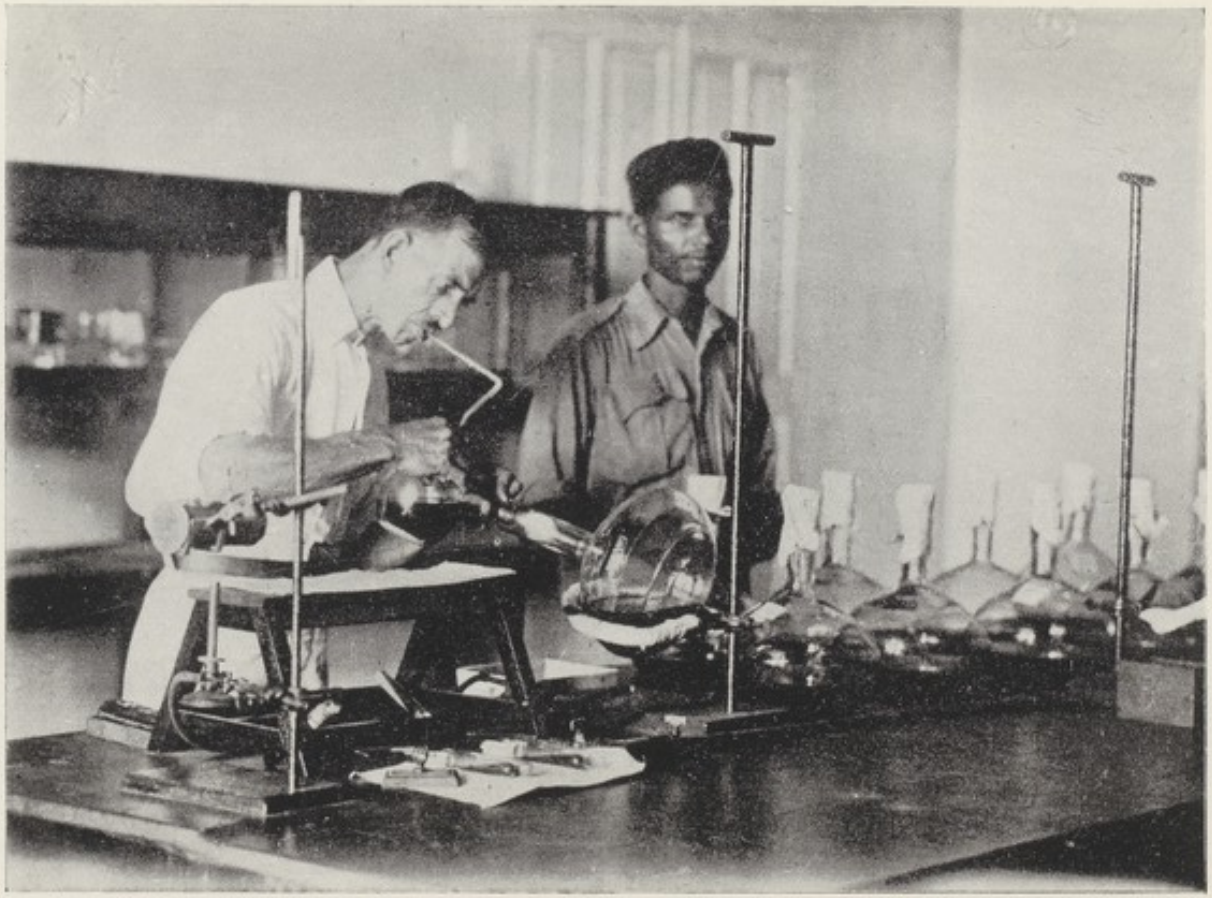
FIG. 2. Flask after incubation.

PLATE V.

FIG. 1. Uninoculated flask.

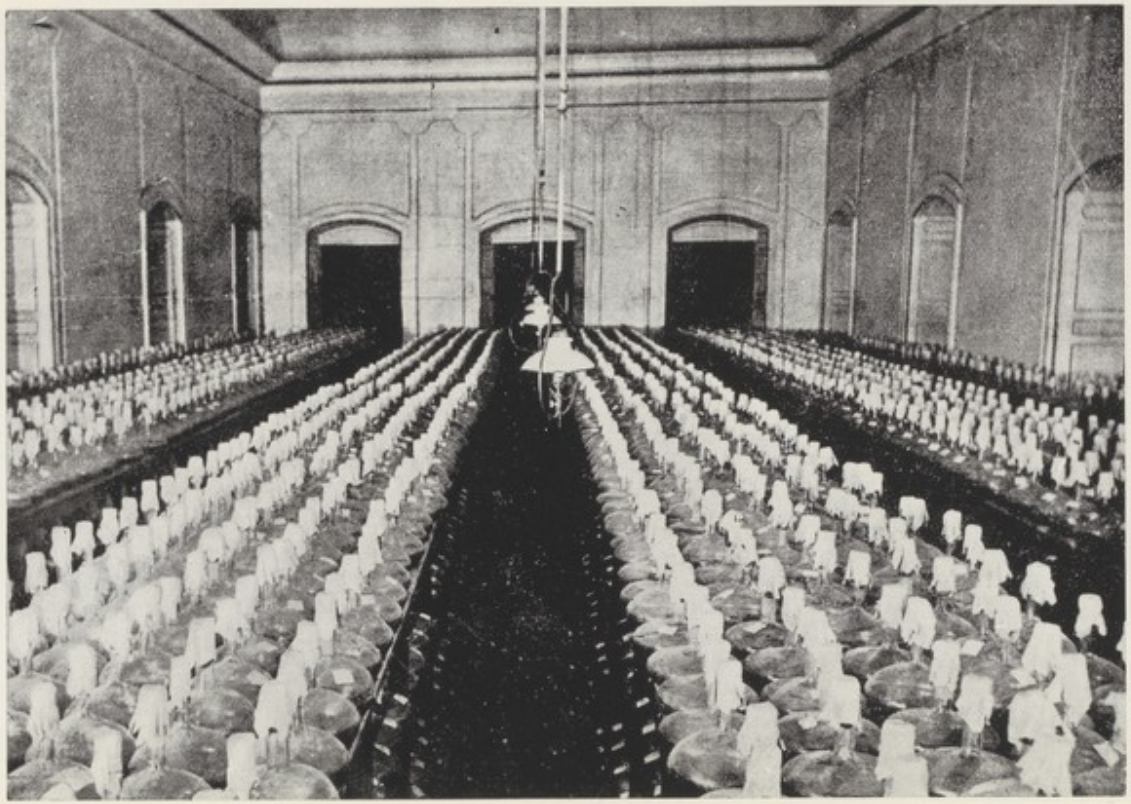
FIG. 2. Flask after incubation.

PLATE V.



Sowing.

PLATE VI.



The incubating room.

'A little of the juice of a plague bubo is aspirated out by means of a sterilized glass tube drawn out to capillary size at one end and known as a Pasteur's pipette. When this juice is withdrawn with suitable precautions, it is found to yield in almost every case a pure culture of plague bacilli when sown on agar jelly. If the resulting culture appears to be pure, then portions of it are removed to small trial flasks of bouillon prepared exactly as the large ones are. These are placed in the dark and kept at a suitable temperature and in forty-eight hours thereafter, if the growth sown in them has been pure, an abundant crop of stalactites makes its appearance in the hitherto perfectly clear bouillon, forming, when viewed by transmitted light, one of the most curious and beautiful sights a bacteriologist could wish to see. The stalactites have been likened to silk threads hanging down into the bouillon from the surface. Having proved the absolute purity of the growth in this way, the operator may proceed with confidence to transfer the contents of these trial flasks to the "*ballons Pasteur*" which are used for sowing the large fermentation flasks.

'This operation is performed in what was originally the chancel of the Portuguese church, and the flasks when sown are at once removed to the body of the church, which, for the past hundred years, has served as the Governor's banqueting-hall. This splendid hall, measuring some eighty feet in length, is now furnished with six rows of stout teak-wood tables running lengthways down the room. Each row of tables serves to accommodate three rows of the large 4-litre flasks. The newly-sown flasks remain in this room for six weeks at a temperature of 26·6°C. (80°F.). For a few months in the cold weather the hall has to be heated by gas stoves, but as a rule this is not necessary. During this six-weeks period the flasks are watched carefully, and any showing signs of contamination are at once removed and destroyed. Every second day the contents of each flask are shaken to break up the stalactites and prevent the formation of growths in masses, which might afterwards block the needle of the injection syringe. Once a week every flask and table is wiped down with corrosive sublimate solution, and the floor scrubbed with the same antiseptic, as a precaution against the countless moulds which flourish in the steamy Bombay climate. At the end of six weeks the growth of stalactites has completely ceased, and though bacilli remain alive in the flasks for months sometimes, yet no perceptible thickening of the fluid takes place after the lapse of this period. The flasks containing the most turbid fluid are chosen, therefore, and sent into an adjoining room, where they are tested for purity. The number of flasks thus sent out daily varies from twenty to thirty, according as the demands for material or other circumstances necessitate. The testing is done by withdrawing a drop of fluid from each flask, with the usual precautions, and smearing this on the surface of a couple of *dry* agar tubes. The requisite dryness of the agar jelly is got by keeping the tubes in a closed jar containing quicklime for a couple of days or so and the agar is ready for use when it has shrunk away a little from the glass at the bottom of the tube. If the agar is thus dried, the fluid containing the bacilli is at once absorbed by it, and two days after, a characteristic growth of plague microbes is found covering the entire surface. Four days after these tubes are sown they are examined by the testing officer, along with the Superintendent, and the result noted in the register kept for the purpose. In case either of the examiners should have any doubt as to the appearance of any of the tubes, a portion of the contents is at once placed in a small test flask and the formation of stalactites watched for. Only when an unanimous verdict is given

by both examiners is a flask passed as fit for use. The flasks, immediately after testing, are passed on into the sterilizing room, where they are taken in hand by one of the native medical men. They are then firmly fixed on heavily-weighted retort stands, and immersed in water kept at 60°C. (140°F.) in a jacketed tub. The flasks are arranged in a ring round the circumference of the tub, and in the centre is placed a similar flask containing as much cold water as the others do prophylactic. By means of a hole in the lid of the tub a thermometer is lowered into this centrally-placed water-flask, and it is assumed that by the time its temperature has reached the desired height, the temperature of the vaccine flasks will be similar. Fifteen minutes later the flasks are removed and at once plunged into a trough containing cold water circulating through it, which rapidly cools them down to the atmospheric temperature, prolonged heating being one of the dangers to be avoided. As soon as the contents of the flasks have been cooled down, carbolic acid is added in the proportion of 1-200 of the bulk of the fluid, or in other words, 0·5 per cent. The vaccine is then ready to be decanted into bottles for distribution. This is done in a long room contrived out of the east verandah of the banqueting room, by converting the arched openings along the side into large windows. This department of the work is presided over by another native medical man, who supervises the seven decanters and is responsible for all that is done in the room. The flasks to be decanted are kept in a sloping position by means of rings attached to large heavily-weighted retort stands, and are well elevated above the tables to admit of the adaptation of a glass syphon. This syphon has a portion of rubber-tubing inserted in its length for the application of a clip to control the flow, and as a means of starting the syphon action. The syphon apparatus is sterilized in a large autoclave under three atmospheres of pressure, the open ends of the tubes having been closed with cotton plugs in the usual way. The only part, therefore, that has to be sterilized by the decanter before he introduces the end into the flask is the outside of the tube, and this he does in a bunsen burner immediately before use. The syphon is started by successively pinching the rubber tubing with a sort of milking action, one finger and thumb grasping it alternately below the other. In this way suction by the mouth is avoided, and the lower end of the tube is in no danger of contamination. The syphon action having been started, the lower end of the tube is sterilized carefully in a bunsen flame, and the fluid filled into the bottles held below. These bottles have been specially made for the purpose, and have the neck flush with one corner, so that no difficulty may arise in extracting the last drops of the contents when the syringe is filled from it. They each hold from 25 to 30 c.c., i.e., five or six doses, and are closed with rubber stoppers. The bottles before being filled are plugged with cotton-wool and sterilized by dry heat at a temperature of 150°C. (300°F.) kept up for two hours. This is done in an oven holding 1,400 bottles at one time, which number usually suffices for a day's work though in times of pressure the stove has to be filled twice a day. The rubber stoppers are sterilized by soaking in a 5 per cent solution of formaline for three days, though by experiment it was found that three hours was sufficient for this purpose.

'Each decanter has supplied to him a tray full of bottles and a dish of stoppers immersed in formaline solution, and he manipulates these latter by means of spring forceps. The wool plug is withdrawn, the neck of the bottle sterilized in the flame of the bunsen burner, the end of the syphon inserted into its mouth, the clip loosened, the bottle filled, and the stopper inserted in a wonderfully short time,

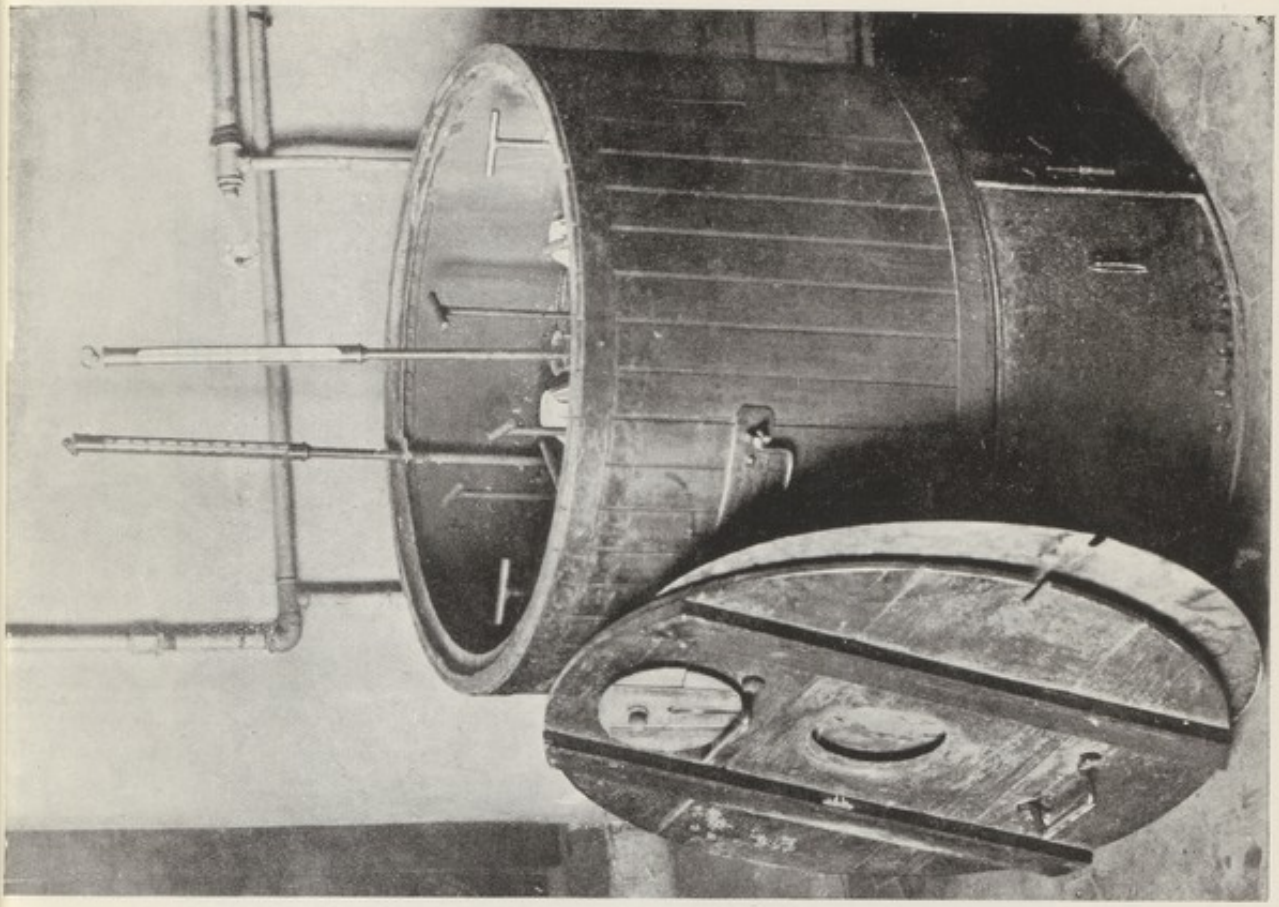


Fig. 2. Sterilizing tub.

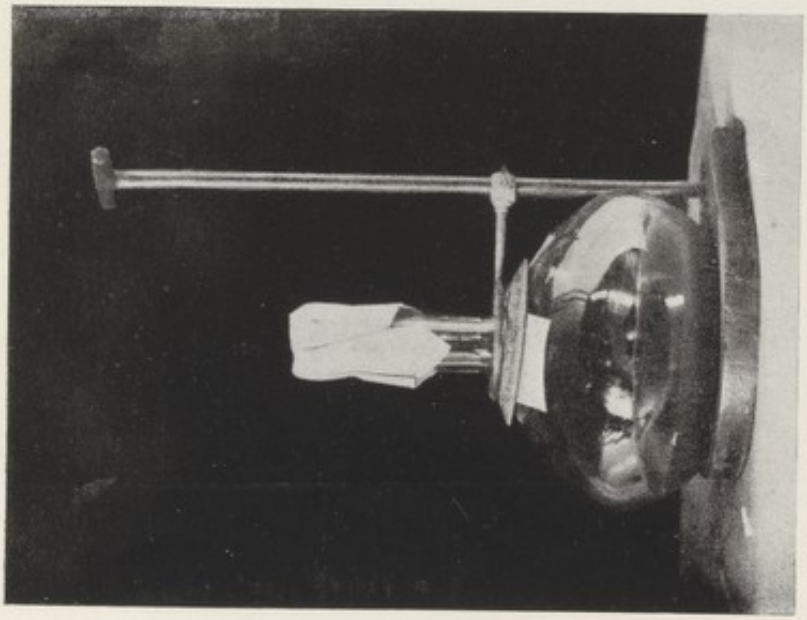
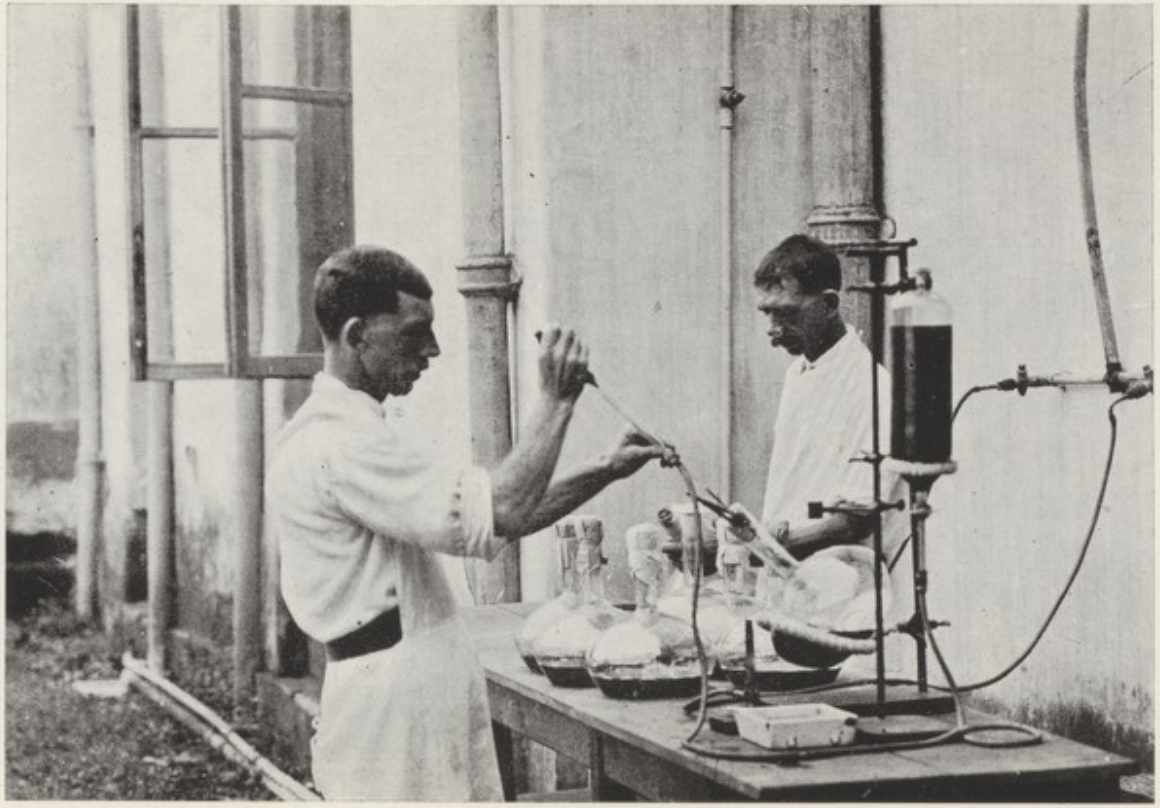


Fig. 1. Flask ready for sterilizing.

PLATE VIII.



Carbolizing.

and without the slightest danger of contamination from the operator's fingers. Before the filling of each bottle the flask from which it is to be filled is shaken round so as to distribute the bacterial sediment equally through the fluid, and thus ensure an equal distribution of this valuable material among all the bottles of the batch. The decanters are trained at first with water, then with flasks of sterile broth, and not till they are able to decant and bottle aseptically a flask of such highly putrescible material, are they allowed to touch the carbolized vaccine, which, of course, is much less liable to contamination than the uncarbolized bouillon. To test the purity of the vaccine at this stage, specimens from the first and the last few drops taken from the flasks are dropped into test-tubes containing 10 c.c. of sterile broth. Four days after, these tubes are examined to see whether any appearance of growth is visible or not, thus serving at once as a test of the sterility of the vaccine and as a check on the skill of the decanter. In this department—as indeed in all the others—a careful record is kept by which the identity of the operator is known and any shortcoming on his part brought home to him. After decanting, the empty flasks and syphons are sent to the cleaning department, while the batch of bottles is labelled with date and number of flask from which they are taken. These bottles are kept separate for ten days, and then from each batch two samples are taken at random for examination. These are opened and a drop from each is placed in a couple of test-tubes containing 10 c.c. of sterile bouillon. If after four days of incubation no sign of growth is apparent, the batch to which it refers is passed into the packing department. Two standard bottles from each brew are sent up to the Superintendent to have the dose fixed. This is at present done by estimating the opacity of a layer of constant thickness, and the results are checked occasionally by actual experiment on human beings. This may not appear a scientific procedure, but it has proved in practice surprisingly accurate, and no other workable method has as yet been devised, though many have been tried.

'Inoculation of animals has hitherto proved unreliable for this purpose, and even if effective would be unmanageable on the scale on which we work. For instance, it is quite usual for the laboratory to turn out twenty-five different brews in a day for weeks on end, each of which would, if tested on animals, require the sacrifice of three lives, or a total of seventy-five animals daily. This would necessitate the employment of two or perhaps three skilled bacteriologists devoting their whole time to this and nothing else, to say nothing of the number of assistants and animal keepers, and the multitude of animals that would be required. The dose having been fixed, the labels can now be filled up for each bottle. Another long label with directions for opening and handling is pasted across the stopper to preserve the contents from the investigations of the curious. The bottles are then packed in pairs in wrappings of corrugated paper and stowed in boxes holding 1,000 doses, for transmission to any part of the world. To give an idea of the care with which these various processes are carried out, I would refer to the number of tests the material is subjected to at every stage of its manufacture. The elaborate precautions taken to ensure the purity of the microbial growth to begin with, the daily supervision of the fermenting flasks, the trial for purity before the sterilization of the vaccine, the double test in the decanting room, and the final trial which takes place ten days after bottling. Each department keeps an accurate record of all operations performed, with the result of the tests, and the whole is copied into a large register forming a history of each brew from the actual making of the

broth to the despatch of the vaccine to the operator. By referring to this register we have been able frequently to convince an inoculator that the appearance of abscesses in his patients has been due to his own shortcoming and not to ours. That the processes are suitable to the conditions of the country and climate is manifest from the results obtained, as the following extract from the half-yearly report to Government for the first part of 1900 will testify. The report is written by Mr. Haffkine, as Director-in-Chief, and is the last that I have access to. Referring to the question of the sterility of the prophylactic, he says: "Of the 2,139 brews manufactured during the first half of the year, 152 were rejected upon the results of the examination made before they had reached the bottling room, and one was rejected subsequently on account of its weakness. The arrangement followed in the laboratory was that the material, bottled and closed with india-rubber stoppers, and made ready for despatch, was kept in the laboratory at least a fortnight after its preparation; and ten days after the bottling, samples of each brew were re-tested for sterility.

'"In this way it was detected that out of 1,987 brews passed into the bottling room as sterile, 4, or about 1 in 500, got contaminated subsequently, and were rejected; the 1,983 others had remained sterile, and were declared fit for distribution to operators.

'"These details warrant the presumption that no contaminated bottles are issued from the laboratory.

'"The above results were obtained by an effective supervision over each part of the preparation, and by the duties being distributed amongst an adequate staff of officers".

'The above remarks refer only to the operations as carried on in the new laboratory, which was not in existence when the Indian Plague Commission visited Bombay. This fact should be kept in mind by any one studying the elaborate report of that Commission'.

This description of the methods of manufacture shows certain minor changes from Haffkine's original procedure. The temperature of sterilization had been reduced from 65°C. to 60°C. and shortly after was further reduced to 55°C. The use of oil on the surface of the broth had been continued in the new Laboratory but was soon discarded. Improvements had been made in the manufacture of the broth, and the methods of decanting and sterility testing revised. The proportion of contaminated brews was greatly reduced, and the testing of each brew reduced the risk of issue of contaminated vaccine. As will be seen later, during periods of pressure of work and in consequence of certain temporary alterations in technique this standard was not always maintained.

In the *Report* on the working of the Laboratory for the years 1896-1902 Haffkine mentions an alteration in the method of preparing the vaccine which he had introduced, probably just after the address which has been quoted was written. This method consisted in enriching the vaccine by emulsifying the surface growth of 300 square centimetres of a four-day agar culture in 400 c.c. of a two months' broth culture. Bannerman refers to this method in the *Report of the Plague Research Laboratory* for 1905. He states: 'This process consists in adding to the six weeks' old broth culture of plague which constitutes the original or "Standard" prophylactic, a large number of fresh plague microbes grown for four days only on agar plates. A great increase in thickness is thus produced by the addition

PLATE IX.

PLATE IX.

Inoculation in a Punjab village, 1900.

PLATE X.

Packing the original type of rubber-stoppered bottles.

of these fresh bacillary bodies and to all appearance a stronger prophylactic is obtained. *A priori* one would be inclined to agree with the advocates of this process that the addition of fresh microbes must increase the effectiveness of the prophylactic, but judged by the result of experiments on animals this is not the case. From experiments on guinea-pigs, rats and two species of monkeys, it was found that the standard vaccine was at least ten times more efficient than the enriched vaccine made from the same strain of germs when the latter was given in the proportionately smaller amount prescribed according to its thickness'.

Bannerman gives an account of the results obtained by the use of this enriched vaccine on human beings and states that the results bear out the conclusions arrived at by animal experiments, the enriched prophylactic being found to be less effective than that made by the standard method. It should be noted, however, that further experimental work on this point in 1925 showed that enrichment did materially increase the efficiency of the vaccine but the additional manipulations required and the added risk of contamination in the process, was not considered to justify the use of the method. Enrichment was discontinued after a short period.

The standard method of manufacture of the vaccine described by Bannerman was not always adhered to, or the use of broth prepared by the standard method with the addition of enrichment with agar cultures. For a period during 1902 when the demands for the vaccine were more than the Laboratory could possibly cope with, Haffkine departed from his principles and manufactured a vaccine which was simply an agar culture washed off with water and sterilized by heat. The use of carbolic acid was dispensed with and a second sterilization was carried out by immersing the bottles in water at 60°F. This agar-water vaccine was manufactured in connection with an inoculation campaign which had been projected on a very large scale in the Punjab, and for which it was found impossible to manufacture a sufficient quantity of the standard type of vaccine in time, owing to delays and difficulties in regard to obtaining the necessary equipment.

In October 1902 nineteen persons in the village of Mulkowal, inoculated with the contents of one bottle of the prophylactic prepared in this way, developed tetanus and died. Inoculation largely ceased in the Province as a result of this occurrence and a very serious view was naturally taken of the matter. A special Commission was appointed to investigate the occurrence but did not report until 1906 when their findings were published by the Home Department of the Government of India. The bottle responsible for the deaths was one of five bottled from the same flask on 19th September, 1902, and no ill effects had followed the use of the other bottles of this brew. The finding of the Commission was that the bulk of the evidence pointed to the contamination of the bottle having occurred before it was opened at Mulkowal. In dealing with the possibilities of contamination during the process of manufacture the Commission narrowed the chances down to three possibilities: (*a*) the bottle may have been insufficiently sterilized before it came into the decanters' hands, (*b*) the decanting may have been done with defective precautions, (*c*) the final sterilization may have loosened the stopper with the result that specific contamination entered, either in the cold bath, or afterwards before the bottle was opened at Mulkowal. The Commission could not determine between these possibilities. The report was referred to the Governing Body of the Lister Institute for opinion and the Institute agreed with the findings of the

Packing the original type of rubber-stoppered bottles.

Commission. Haffkine took exception to some of the conclusions and further reference was made to the Lister Institute who, in reply, stated : ' The governing body of this Institute thinks that Mr. Haffkine has ground for his main contention that the Lister Institute, which is of the opinion that the probabilities were in favour of the view that the tetanus impurity was previously in the fluid, did not feel justified in asserting this to be a proved fact in view of the possibilities of contamination at Mulkowal '.

While these were the official findings in regard to this case, Haffkine at all times contested the possibility of the contamination having occurred during the process of manufacture in his laboratory. It came out in the evidence taken by the Commission that during the process of inoculation at Mulkowal the forceps used for removing the rubber stoppers from the bottles had fallen to the ground and had not been re-sterilized before use. It also appeared that the instructions for conducting inoculation had been altered in regard to sterility technique. Haffkine held that if the bottle had been contaminated at the Laboratory the development of *Cl. tetani* in the period of 41 days which elapsed before it was used would have produced a strong disagreeable odour which, according to Dr. Elliot, under whose direction it was opened, was not observed. He also considered that, if contaminated at the Laboratory, the number of tetanus bacilli and the amount of toxin which would have been present would have resulted in a fulminating type of case with very early symptoms. The cases were not of this type. Haffkine on his return to Europe put his case before many eminent bacteriologists and received a wide measure of support to his claim for exoneration from responsibility for this disaster. The case for Haffkine was put forward very strongly by Ross and others in a letter published in the *Journal of Tropical Medicine and Hygiene* (1st August, 1907).

The departure of Haffkine from the principles which he had originally laid down for the manufacture of the vaccine was considered by him to be justifiable in view of the necessity of meeting the needs of the Punjab inoculation campaign and, in giving evidence before the Commission of Inquiry in the cause of the Mulkowal disaster, he cited the opinion of the Indian Plague Commission that the supernatant fluid was superfluous. He stated that the ordinary form of his prophylactic had two constituents and that all that was done as a temporary measure was that one of them was, under the urgency of the case, temporarily eliminated, and that the eliminated constituent was one which most authorities considered useless.

The omission of the use of carbolic acid in the vaccine was severely criticized and it appears that other officers on the staff of the Laboratory at the time had expressed their opinion as to the inadvisability of such omission.

The addition of a second sterilization by the immersion of the bottles in hot water after filling had introduced the additional risk of loosening the stoppers. Much of the vaccine issued at the time was definitely contaminated, and amongst the instructions issued to inoculators was, to notice whether there was any explosive noise on opening the bottle, as well as to observe the presence of any putrescent odour.

Haffkine stated that whereas one out of 60 or 70 brews was formerly tested for sterility one out of every three or four brews was tested at this time, but

occasionally non-sterile brews went out. Haffkine (1903) in a letter to the Commission of Inquiry referred to his evidence before the Indian Plague Commission (*Report* **3,** p. 645) as to the possibility that harmless organisms might be present in the prophylactic without danger and drew a distinction between non-sterility and dangerous contamination—a point of view which, in the light of modern practice in the preparation of vaccines, seems very curious indeed.

It is unnecessary to enlarge further on the subject of this disaster which unfortunately threw a shadow over Haffkine's life and seriously affected his career. The older method of preparation was at once reverted to.

During the period under consideration the use of seed material obtained from human sources had at first been continued but later animal passages, by cutaneous inoculation of rats or guinea-pigs was employed, a change of strain from a human source being introduced when available.

In 1905 the method of preparing the broth medium used for the vaccine was further improved by Dr. Maitland Gibson. The digestion of the meat-acid mixture was carried out at 70°C. for three days only followed by subsequent heating at 100°C. for one hour. A lighter broth was obtained in this way which did not require decolorization with animal charcoal and which gave better growth.

No other changes of importance in the methods of manufacture were made at this period and the method of bottling by syphonage into rubber-stoppered bottles was still being employed.

CHAPTER V.

The manufacture of the vaccine: Subsequent developments, 1905–1923.

In 1905 an important advance in manufacturing methods was made by the introduction of a method of bottling which had been devised by Dr. Maynard, and of which a first short description was given by Bannerman in his report for that year.

Dr. Maynard had been on the staff of the Laboratory during the period in which an attempt had been made to carry out the production of the vaccine on a very vast scale for requirements in connection with a special inoculation campaign in the Punjab. He had seen the difficulty of obtaining a sterile vaccine with the method of bottling by syphonage which was used, and the serious defects due to the use of rubber-stoppered bottles as containers. The risks of contamination by exposure of the vaccine to the air and to the methods of handling, and the imperfection of sealing had been shown by the number of contaminated bottles detected by operators in the field, and the occurrence of the Mulkowal tetanus cases made a more reliable method essential, if issue of the vaccine was to be continued with definite assurance of safety. Maynard set himself to devise a suitable technique and introduced his vacuum method which has been used in India since 1905 not only for Haffkine's plague prophylactic but for other vaccines manufactured in bulk.

A description of the method will not be out of place here in view of the fact that the process originated in the Institute and that, in describing it, the account of the methods of procedure and the accompanying photographs will illustrate some of the manufacturing processes which are employed for the preparation of the plague vaccine. The following are the details of the method :—

The phials used for bottling the vaccine are of the shape shown in Plate XIV. The bulb of the size now used has a capacity of 28·5 c.c. to contain six doses of 4 c.c. with a small margin for use in cooling the syringe and for wastage. The thin neck is 4½ inches in length. (Phials of similar shape in capacity down to 5 c.c. may be used). The phial is opened by cutting off the extreme tip of the neck by means of a glass-cutting knife. The apparatus shown in Plate XI is used to vacuumize the phials. This consists of a vacuum pump, an air drying chamber, and a vacuum chamber with gauge to record the negative pressure. The air drying chamber is not essential. From the vacuum chamber pressure tubing leads to each operator. Each tube terminates in a metal capsule of special construction with a small aperture in the end which is covered inside by a rubber disk with a minute perforation in the centre. When the pressure gauge on the vacuum chamber shows a sufficient negative pressure, the tip of the long neck of the phial is inserted into the aperture of the capsule and penetrates the rubber disk. The phial is immediately vacuumized and, before withdrawing it, the point of the stem is sealed by fusing it in the blow-pipe flame.

PLATE XI.

Vacuumizing the phials.

Plate XII.

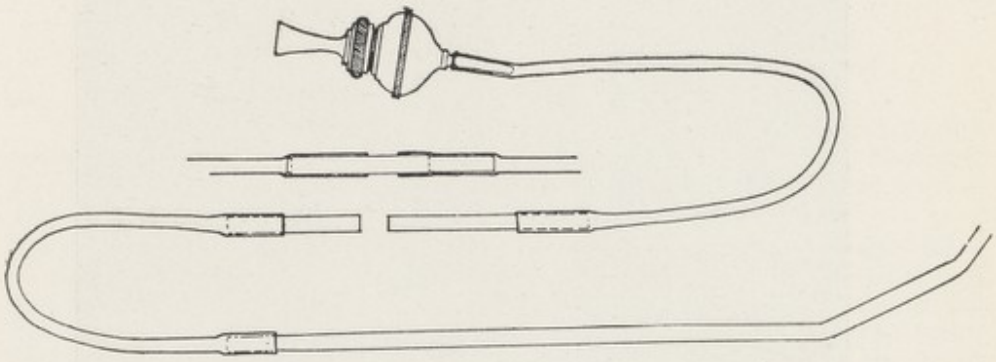


Fig. 1. The parts of the syphon with the filling capsule.

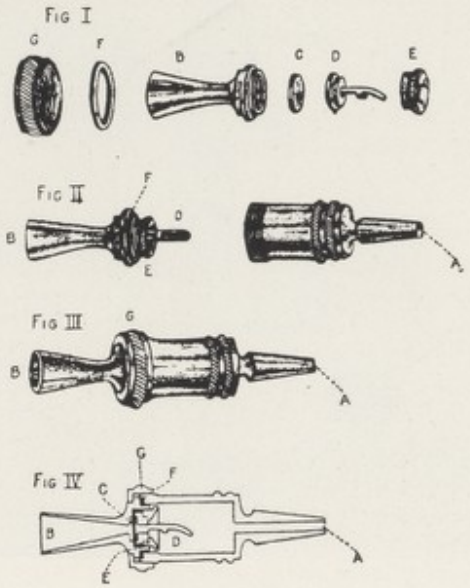
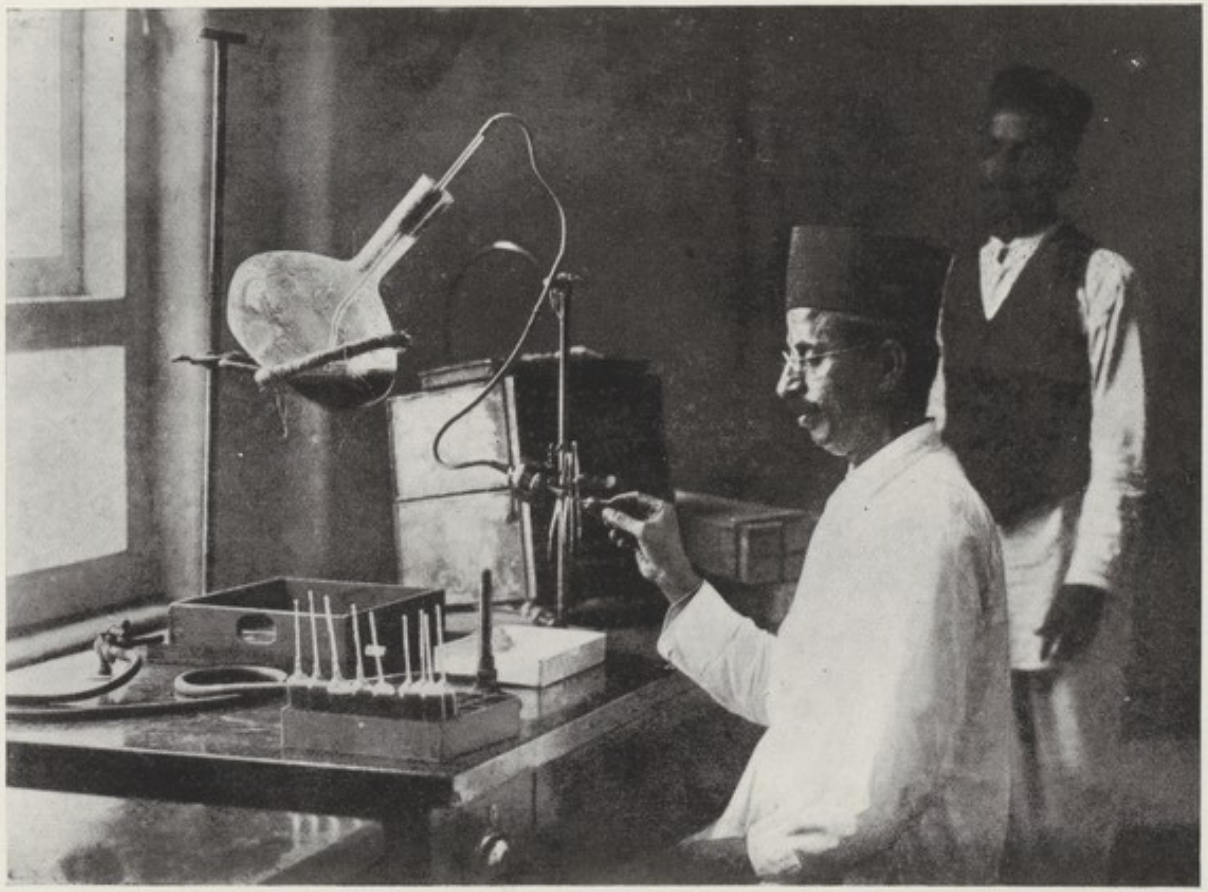


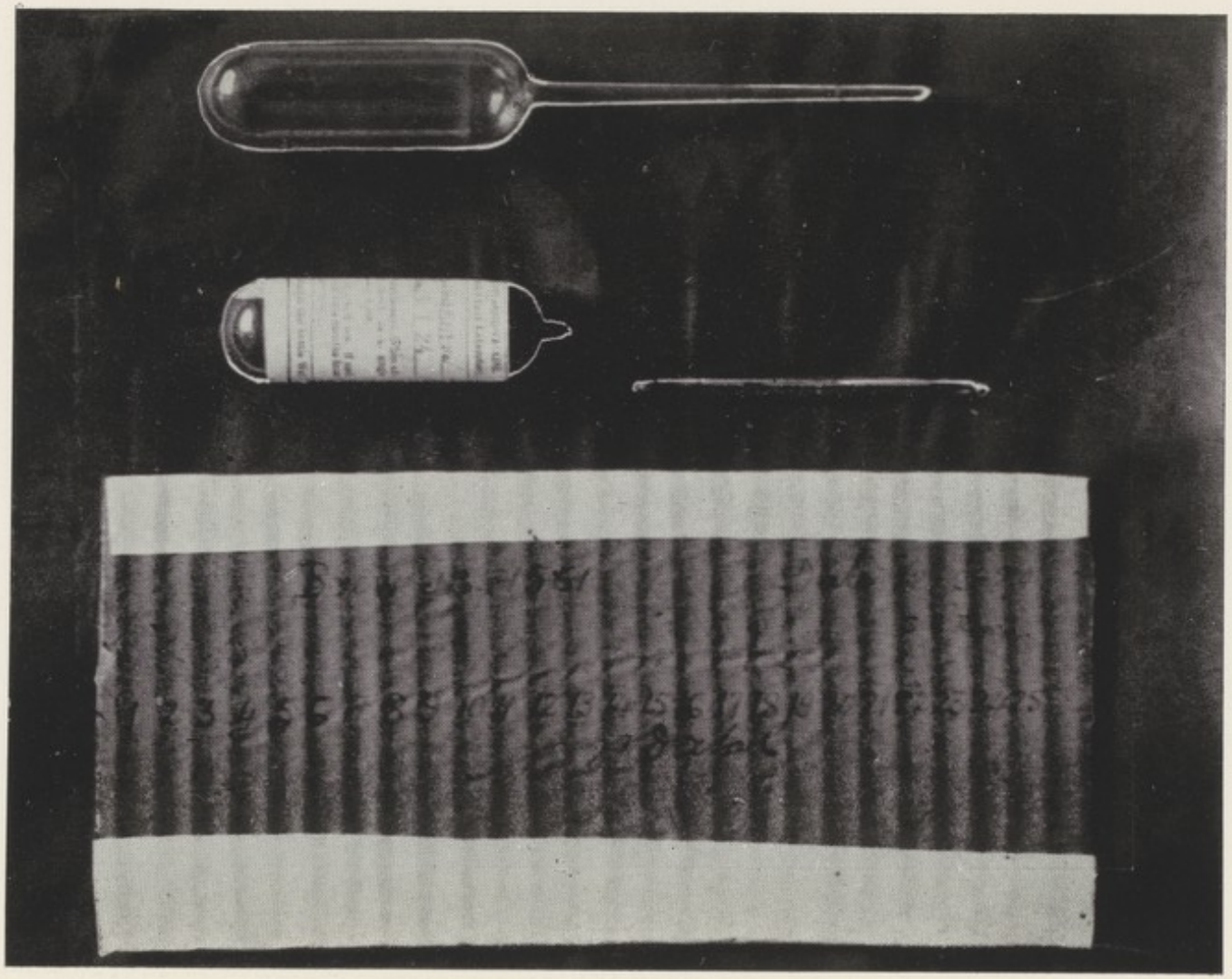
Fig. 2. The parts of the filling capsule.

PLATE XIII.



Bottling by Maynard's method.

PLATE XIV.



Stages of phial in filling and sealing. The neck of each phial is kept in the hollows of the corrugated paper.

These vacuumized phials are then sterilized by dry heat at 160°C. in special metal boxes and are ready for filling. The remaining portion of Maynard's apparatus consists of a syphon (Plate XII, fig. 1) with a filling capsule attached. The syphon is in two portions, (*a*) a glass tube long enough to pass right through the flask used for the growth of the vaccine and bent so as to reach the bottom of the flask when in position for syphoning and to this is attached a rubber tube and a female ferrule, and (*b*) a filling capsule attached by a rubber tube to a male ferrule. These two portions of the syphon are prepared by soaking in carbolic lotion and then after covering the ends with paper sterilized in the steam disinfector in special boxes. The glass tube of the first portion is flamed and introduced into the flask to be decanted, with the usual aseptic precautions, and the two portions joined up by connecting the ferrules flamed to red heat. The filling capsule attached in this way to the outer end of the syphon is fixed in a burette clip.

The layout of the apparatus is shown in Plate XIII. The filling capsule is composed of the parts shown in Plate XII, fig. 2. The tip of a phial is introduced into the capsule and through the rubber diaphragm. It then enters the small curved portion shown separately in the figure and when the capsule is pressed forward and rotated the tip breaks off. The vacuumized capsule sucks up the contents of the flask. The first phial only serves to fill the syphon tube with the vaccine. The second phial fills to about two-thirds of its volume. These two are rejected and the subsequent phials are filled completely. After filling each ampoule is immediately sealed at the tip in the blow-pipe flame.

The remainder of the process consists in filling the neck of each phial with the vaccine by giving it a vigorous shake, next removing some of the vaccine from the base of the neck by a short spin in a slow hand centrifuge and finally sealing off the neck close to the capsule. A strip of corrugated paper is prepared and numbered for each brew, and the necks of the phials are arranged in succession in the grooves of the corrugated paper and numbered. An ' office copy ' of each phial is thus retained, and can be referred to in case of any complaint as to the vaccine. These stages are indicated in Plate XIV.

The introduction of this method of bottling was a great advance in regard to the manufacture of the vaccine and relieved the staff of many of their difficulties and anxieties as to the production of a safe and sterile fluid.

Revised methods of sterility testing were also introduced adapted to the new method of bottling. From the flask containing a litre of the vaccine about 35 phials are filled by one syphoning operation. Of these the 5th and the last are selected for sterility testing carried out in a special department of the Laboratory by technicians employed on this work only. The flask to be tested is opened by breaking the neck and by means of a pipette a few drops are placed on an agar slope and in a tube of acid-digest broth. In addition, a tube of glucose broth sealed with vaselin is inoculated for the purpose of anaerobic test. Six tests are thus put up for each flask and finally examined, after incubation, by a responsible officer who certifies their sterility in a register kept for the purpose. This procedure has been continued up to the present time.

Stages of phial in filling and sealing. The neck of each phial is kept in the hollows of the corrugated paper.

At this phase of development of the vaccine the formation of a typical stalactite test was still relied on as an indication of the purity of the seed material, and the

naked eye appearance of cultures on agar for determining the purity of the final product before sterilization. The only changes in the methods of making the vaccine, apart from improvements in bottling and testing methods, which were made during the period now being dealt with, were in regard to the period of incubation employed. The changes made and the reasons for their adoption are dealt with in detail in Chapter XIII. An attempt was at first made to reduce the toxicity of the vaccine, and minimize the reaction on inoculation, by increasing the period of incubation to four months, but subsequent experimental investigation of the immunizing value of the vaccine showed that vaccines of such long incubation were of less protective value. The incubation period was again reduced and storage of the vaccine for a certain period depended on to reduce its toxicity.

In 1911, in connection with the work of the Plague Research Commission whose headquarters were at the Laboratory, it was found that the Bombay rats had developed a high degree of immunity to plague. Their susceptibility was compared with that of *R. rattus* trapped in Madras City which had been almost entirely free from epidemic and epizootic plague. The Madras rats were found to be highly susceptible and suitable for cutaneous passage of the *B. pestis*. Since that time these rats have been regularly imported into Bombay and used for the passage of the organism for the purpose of maintaining the virulence of the strain to be used for seed purposes. No intermediate cultures are made on artificial media.

From the introduction of Maynard's method of bottling, with the exception of the minor changes which have been indicated, the methods of preparing the vaccine underwent little change during a period of nearly twenty years.

PLATE XV.

PLATE XV.

Final sterility testing.

CHAPTER VI.

Manufacture of the vaccine: Recent developments, 1923–1932.

From 1923 onwards the work in connection with the vaccine entered into a new phase by the institution of an extended experimental inquiry into its properties, and into the value of the methods employed in manufacture, in order to place our knowledge on all points on a sounder basis than had formerly existed, and also to ascertain whether further improvements might be made. For many years the details of the processes of manufacture employed had been based on assumptions as to the value of different procedures which rested on a somewhat slender basis of experimental evidence. A re-determination of all points of importance was carried out including amongst others—(*a*) the relative value of the sediment and supernatant fluid, (*b*) the relationship between virulence and immunity, (*c*) the relation of toxicity to immunity, (*d*) the effect of the period of incubation employed, and (*e*) the effect of storage, etc. The results of this work are incorporated in subsequent chapters dealing with the questions at issue. The findings on the whole confirmed our previous views on most points but were of great value as providing more accurate knowledge on which improvements might be based or more exact methods employed.

No immediate changes in the methods of preparing the vaccine were first introduced as a result of this work. Along with other experimental inquiries carried out at this time a regular testing of a long series of successive brews of vaccine was instituted in order to determine the immunity produced as ascertained by animal experiments. For this purpose a technique long in use was employed, consisting of the inoculation of a fixed dose of vaccine into susceptible rats followed by the subsequent administration of an infecting dose of a weighed quantity of the spleen of a passage animal. The result showed that while a certain average standard of immunity was reached very considerable variations occurred in the immunity shown with different brews of the vaccine. It had been hoped that this method of testing immunity would have afforded a basis for standardization of the vaccine, but the differences indicated by the results of successive tests of vaccine, prepared under as far as possible exactly the same conditions, suggested that either the methods of manufacture employed permitted a wide variation in the potency of the vaccine, or that the experimental methods used were not capable of furnishing accurate results. Opinions varied as to the value of the method of testing and an attempt to introduce it into the routine of manufacture would have resulted in the discarding of a large proportion of the vaccine if the average immunity shown in the course of the experiments had been taken as a standard. It was obviously necessary to determine (*a*) whether the methods employed in the processes of manufacture could be more accurately standardized, and (*b*) to investigate the value of the experimental methods themselves.

Variations in the potency of the vaccine might possibly occur in relation to (*a*) the strain of *B. pestis* used, (*b*) the composition of the nutrient medium, and (*c*) the period and temperature of the incubation employed in manufacture.

The first important advance made was in regard to obtaining greater regularity in strains used for seed purposes. Dr. Goré, Assistant Director of the Institute, had, during the years 1926 and 1927, been engaged in a study of the isolation of the *B. pestis* from passage animals, and in the course of this work had found the occasional presence of contaminants associated with *B. pestis* in the heart blood of the rats used for maintaining the strain.

The method which had always been in use for preparing the seed material, consisting in the inoculation of the heart blood into broth, had frequently resulted in the seed material being declared to be impure as a result of the observations of the stalactite test, and vaccine under incubation had also in many cases to be rejected at different stages or at the final purity test. Dr. Goré's observations of these contaminants showed that the majority of them would be detected by the stalactite test of the original baloon prepared for seeding purposes, but that others might escape detection by this test when incubation was carried out at 80°F., and, in addition, those whose colony characteristics on solid media were found to resemble very closely that of *B. pestis* might not be detected at all by the final purity test on agar. These observations indicated serious defects in former technique and Dr. Goré proceeded to devise a new method of isolation of the organism for seed purposes, and for the testing of purity of strains. The details of the work carried out and the methods evolved will be reserved for Chapter X, but for the continuity of the account of the changes taking place at this phase a short summary will be given here.

It was found that when small quantities of high dilutions of heart blood of septicemic animals were placed on blood agar, a growth of discrete colonies of *B. pestis* was obtained on this medium in one or other dilution while the same quantity would produce no growth on ordinary agar. Quantities sufficient to produce growth on agar resulted in the formation usually of small clumps of colonies. A single colony selected from blood agar was taken for the purpose of preparing seed material and submitted to a somewhat elaborate series of tests for purity, which have been found to be necessary for the detection of the types of contaminants which had been observed in heart blood of passage animals, or for the detection of other organisms of the pasteurella group. The introduction of this method of obtaining a pure seed resulted in an elimination of waste of material on account of contaminations, and also the avoidance in delay in manufacture due to impure seed, and the frequent discarding of vaccine found contaminated which had been depended upon to be available for issue.

The technique of single colony isolation on blood agar was applied also by Major S. S. Sokhey, I.M.S., for the purpose of preparing test doses consisting of dilutions of standard cultures of a known content of *B. pestis* for use in animal experiments. It was found that on adopting a regular procedure of culture the dilutions prepared showed regularly the presence of a number of organisms within a comparatively narrow range, and the regularity of the growth of *B. pestis* on blood agar enabled checks of the bacterial content of the emulsions to be made by plating the dilutions. The accuracy of this method was further confirmed by direct counting under the microscope. The highest of the series of 10th dilutions which are prepared from the cultures used for test purposes shows an average number of 5 to 10 bacilli per c.c. and this number of organisms has been found to kill 100 per cent of white mice with considerable regularity.

This method of preparing suspensions for use in experimental work provided a means of testing the virulence of the material used for the production of the vaccine and, as the process of preparing the cultures for the seed material and the cultures and dilutions for the test proceed on exactly parallel lines, all experimental work by means of the test virus acts as a check on the virulence of the strain used for the vaccine.

On the basis of the work which has been described the new method of preparing seed material was taken into use in 1928 and we can now be sure that all vaccine is prepared from a pure strain of *B. pestis* of standard tested virulence. This improvement in technique probably represents the most marked advance which has been attained in the manufacture of the vaccine since Haffkine's time.

In regard to virulence there are still certain difficulties. It has been found that, especially during the hot weather, the organism in the passage animal may show a loss of virulence, the reason of which has not been so far ascertained. The methods employed of testing virulence within a very narrow range enabled such changes to be observed and minor changes in virulence would probably pass unnoticed unless such accurate testing was employed. The occurrence of loss of virulence is at once detected and the manufacture of the vaccine from such strains can be avoided. The standards of virulence shown by our experience of the strain used at the Haffkine Institute would appear to be possibly of a much higher level than those used by workers elsewhere, if it is possible to judge by the doses used in animal experiments. This high virulence of the Bombay strains may play an important part in regard to the potency of the vaccine which is prepared.

The next subject of importance which was investigated at this period was the question of the standardization of the medium used in the production of the vaccine. The acid digest broth originally introduced by Warden, and subsequently improved by Gibson, has always been employed. The reasons given for the adoption of this type of medium for use in India in the first instance, which are described in Bannerman's account of the earlier methods of manufacture quoted in Chapter IV still hold, and this medium has been found to give a better growth of *B. pestis* at the temperature of 80°F. used than any other which can conveniently be employed. There appears to be no reason for changing the medium, but the methods of preparation used do not ensure that it will always be a uniform composition.

Major S. S. Sokhey, I.M.S., undertook an inquiry into the composition of the broth for the purpose of obtaining some basis of standardization and, as a result of his work, selected the figure of total nitrogen content. The average figure found in a series of separate brews was in the vicinity of 230 mg. of nitrogen per 100 c.c., and when, from the original concentrated broth, a series of dilutions were prepared and their nitrogen contents standardized at figures above and below this, the resulting growth in the media was found to be best at the figure given. As the result of this finding the medium is now standardized to 230 mg. of nitrogen per 100 c.c. at the time of final dilution.

The effect of initial reaction of the broth on the total growth obtained, and on the potency of the vaccine, was also studied by means of the estimation of the viable organisms present and by tests of its immunizing value. Maximum growth was found to occur when initial reaction was in the vicinity of neutrality. The immunity tests were carried out by means of a series of vaccines prepared by adjusting the

reactions over a certain range with differences of pH 0·2. Using the older methods of testing the results tended to indicate that best results were obtained with a vaccine of original pH 6·8, but in this series the results did not go in any regular sequence and further confirmation as to the value of the particular reaction which is most suitable is required. pH 6·8 was actually adopted as the standard reaction, but later work suggests that a more alkaline reaction may be advisable. Further work is continuing on this point.

The period of incubation required to produce a potent vaccine has been investigated at considerable length. The older work on this subject and the details of experiments at this period are given in detail in Chapter XIII. It has always been an accepted principle, originally laid down by Haffkine, to aim at obtaining the maximum growth which the medium will sustain. The present observations show that after a period of 4 weeks the number of viable organisms present and the increase in the sediment which occurs can represent only a small addition in antigenic substance produced, in proportion to the total obtained in the earlier period. Such addition would probably be equivalent in immunizing value to such slight variation in the dose as may be given in the process of routine inoculations. The question also arises as to the value for immunization purposes of late additions of bacterial substance to the prophylactic, in view of the known reduction in virulence which occurs on prolonged incubation, and to the lesser value of the immunization with material which is not virulent.

During a considerable number of years there has been a tendency to select shorter incubation periods. The original Haffkine's period was six weeks and at one time incubation was prolonged to a period of four months, later reduced as the result of experimental observations of loss in value of vaccine of too longer incubation, and fixed at two months for a number of years. A subsequent reduction took place to six weeks, which still involved a period of two months being necessary for the production of vaccine from start to finish. Any further reduction which could be made would be of very great value in regard to meeting sudden demands for the vaccine, and the investigations which were now carried showed that a period of four weeks would furnish a vaccine of the highest potency.

The immunization experiments which were carried out in regard to this point were conducted both by means of the older technique and the newer methods. One series, using the older technique, showed best results with vaccine of five weeks incubation, but comparatively little difference was shown in the case of vaccine incubated at periods from four to six weeks. Tests by various methods showed that four weeks vaccine was of high potency and that a dose of 0·03 c.c. would produce a 100 per cent immunity in white mice against a dose of *B. pestis* which killed all controls. When the vaccine was used fresh a dose as low as 0·007 c.c. proved effective. Four weeks was finally adopted as the period of incubation to be employed in 1929.

The temperature of incubation of the vaccine has only recently been the subject of special study and no changes in respect to this factor have been made. The average Bombay room temperature for the greater part of the year is fortunately close to the optimum for *B. pestis*, and increase in temperature up to 37°C. results in diminished growth. Accepting the principle of obtaining maximum growth the temperature employed (approximately 27°C.) meets this requirement but there are indications that, especially in the hot weather, a more exact temperature regulation

may be advisable, in the incubating room, than is now obtained. A new point has been raised by Schutze (1932) by his observations on the antigenic fractions of the *B. pestis* and the influence of temperature on their development which is now being investigated.

The above account of the work which was done, and of the improvements and alterations in procedure introduced during this period, shows the following important points: (*a*) the improved method of obtaining seed material from passage animals by the selection of single colonies of cultures tested in a reliable manner for purity and controlled for virulence, (*b*) the standardization of the chemical composition and the reaction of broth on the basis of suitability of the production of potent vaccine, (*c*) the reduction of the period of incubation to four weeks on the basis of the observations as to comparative growths and tests of value, and (*d*) the introduction of a reliable method of final purity testing.

Of these procedures the methods of preparation and testing of seed material and the final purity have involved considerable additions to the work of manufacture, and occupy the whole time of one member of the staff. The other alterations make little difference to the work with the exception of the regular estimation of nitrogen content of the broth which is carried out in the Biochemical Section.

The work in regard to the preparation of standard test material for experimental purposes has been mentioned in relation to its usefulness as a check on the virulence of the organism used tor seed. The preparation of this test material was undertaken in connection with the efforts made to devise an experimental technique which would yield comparative results of a regular type in successive experiments and make possible the introduction of biological standardization. The work which has been done on this subject is described in Chapter IX. For prolonged periods at a time the experimental work by the new method devised has shown very satisfactory results and has enabled many points to be settled with a high degree of accuracy. The method being in the experimental stage, certain defects, especially those due to loss of virulence, have not yet been overcome—but there appears a possibility that a reliable method of biological standardization may eventually be obtained.

This phase of work on the plague prophylactic has shown that improvements are possible and have been attained but, at the same time, the lines on which manufacture has been carried out have not departed from Haffkine's principles in any of the essentials.

CHAPTER VII.

Summary of the present methods of manufacture.

The developments and changes which have occurred during the last 35 years in the methods of manufacture of Haffkine's plague prophylactic have resulted in the adoption of a technique which now varies considerably from the original one employed. It will be suitable to summarize the methods now in use. The procedures are as follows :—

Strain of B. pestis *used.*—The strain is obtained originally from a human case of plague with septicaemia, by blood culture. The organism is maintained in continuous passage in susceptible *R. rattus* by cutaneous inoculation of the spleen of one animal on the abraded skin of the next in series, without intermediate culture on artificial media. Death should occur within 3 to 4 days. The maintenance of high virulence by the strain is ascertained by tests on white mice for which a dose of 5 to 10 organisms should prove fatal.

Preparation of seed material.—Two or three drops of blood are removed by heart puncture after an aseptic post-mortem and transferred to 1 c.c. of citrated saline solution. From this a series of tenth dilutions from 1 in 10 to 1 in 10,000 is prepared, by means of standard platinum loops, and a 1 mm. loopful of each dilution is placed on a rabbit-blood-agar slope. The cultures are incubated at 37°C. for 48 hours. Any contaminating organisms will show growth in 24 hours but colonies of *B. pestis* will not be visible until after 48 hours. One or other of the tubes will contain discrete colonies at this period of incubation. A tube providing discrete colonies is selected and kept for a further period of 48 hours at room temperature unless the material is urgently required. If the purity tests which have meanwhile been carried out are satisfactory, a single colony is picked off into a peptone-water tube and within half an hour 0·5 c.c. to 1 c.c. of this suspension is inoculated into a seeding balloon containing about 700 c.c. of acid-digest broth. The balloon is allowed to incubate at room temperature for four days.

Purity of seed.—At the same time as the blood-agar cultures are made a 5 mm. loopful of the original blood suspension is inoculated into a peptone-water tube. From this culture the purity tests described in detail in Chapter X are carried out, viz., (*a*) tests for nitrite formation, H_2S and indol, (*b*) differential growth on blood-agar and standard agar, (*c*) fermentation tests with glucose, mannite, laevulose, lactose, saccharose and dulcite. These tests are also applied to the peptone-water culture used in preparing the seeding balloon and to the balloon before and after sowing.

The medium.—The acid-digest broth described in Chapter XI is used. This is standardized to contain 230 mg. of nitrogen per 100 c.c., and to a reaction of pH 6·8. The broth is distributed in special-shaped five-litre flasks, one litre being placed in each. The same broth is used for the sowing balloons.

PLATE XVI.

Haffkine Institute, 1932.

Sowing.—If the suspension used to inoculate the sowing balloons is found to be pure they are used after four days at room temperature to inoculate the flasks. The flasks used and the method of sowing have been described in Chapter IV. A first sample from the flask is taken for purity test immediately before sowing, and the flask returned for purity testing at the end of the operation. Up to 200 flasks are sown at one time from a balloon.

Incubation.—This is carried out in a darkened room at 80°F. A thermographic record is kept and shows that throughout the year the temperature does not vary by more than 1°F. above and below this temperature except in the hot weather. The period of incubation is four weeks.

Final purity testing.—At the end of incubation a sample is taken from each flask by means of a long pipette with rubber-tube and glass mouthpiece. The surface of an agar slope is completely covered with several drops of the vaccine and a small quantity sent for special purity testing. The test applied is the inoculation of a 1 mm. loopful on blood-agar and on standard agar. If no growth occurs on standard agar after 24 hours at 37°C., and typical growth on blood agar at 48 hours and the appearances of the growth on the heavily seeded agar tube are characteristic the flask is passed as pure.

Sterilization.—After taking the purity test samples sterilization is carried out as previously described by heating the flasks at 55°C. for 15 minutes in tubs in which the temperature is automatically controlled, cooling, and then adding 5 c.c. of pure carbolic acid to each flask containing one litre of broth culture to produce a concentration of 0·5 per cent.

Bottling and sealing.—When the contents of a flask have been declared pure by the final test the vaccine is bottled by Maynard's process. The standard phials are of 28·5 c.c. capacity.

Sterility testing.—The average number of bottles filled from each flask is thirty-five. Of these the fifth and the last filled are selected for sterility testing. The selected phials are incubated at 37°C. for four days and the following tests made: (*a*) culture on standard agar, (*b*) culture on nutrient broth, and (*c*) culture on glucose broth (covered with melted vaseline, for detection of anaerobic organisms). If these tests are sterile after four days' incubation, the phials belonging to the particular brew are prepared for issue by sealing off their necks close at the body of the phial and the necks with the small amount of vaccine retained in them kept for reference in case of any complaint in regard to the vaccine.

Dosage.—Four c.c. is fixed as the adult dose and, if used within three months of manufacture, of which the date is given on the phial, the dose is reduced to 3 c.c.

Standardization.—The regularity of the processes of manufacture are depended on for the production of a vaccine of uniform composition. Biological standardization is under consideration.

PART II

STATISTICS OF INOCULATION

CHAPTER VIII.

Statistics of anti-plague inoculation.

Although over 30 million doses of anti-plague vaccine have been issued in India, the conditions under which inoculation is usually carried out have not permitted of any corresponding mass of figures of the results of inoculation being available which would be of value for assessing the degree of protection conferred.

Inoculation in the districts is carried out partly by local staff and partly by special inoculation staff who complete as many inoculations as possible in one area and then move on to the next. The inoculation staff do not remain to observe the results of their work, and information as to the subsequent incidence of plague attacks and deaths has frequently to be based on the reports of non-medical persons. Reliable information is not usually available on which to calculate the average populations of uninoculated and inoculated persons exposed to risk of infection during the period of an epidemic.

The greater proportion of the figures relating to inoculation supplied to the Haffkine Institute by the Public Health Authorities in different parts of India, who carry out the work, are of only limited statistical value and cannot pretend to any high degree of accuracy.

In the case of certain outbreaks of plague, however, special efforts have been made to observe carefully all occurrences in connection with the incidence of plague attacks and deaths, and it has been possible to check with a considerable degree of accuracy the incidence of the disease in the inoculated and uninoculated groups and also, from daily and weekly figures, to arrive at a fair estimate of the average populations of the two groups. These figures will form a basis for the estimation of the value of inoculation which will have a considerable degree of reliability.

We will therefore in an analysis of the statistics of inoculation present the available figures separately for two classes of returns :—

Class 1.—Unchecked figures of plague attacks and deaths amongst inoculated and uninoculated persons with calculations of the incidence of the disease based on crude populations and not on average populations exposed to risk.

Class 2.—Detailed statistics of the incidence of plague in inoculated and uninoculated persons along with the figures necessary for the calculation of the average numbers exposed to risk on which the relative incidence of plague in the two groups can be calculated.

Inoculation statistics—Class 1.

The figures for this group will be dealt with first, and on account of their comparatively limited value, at short length only. The information supplied in regard to plague inoculation campaigns in this class usually consists of the following figures :—

(*a*) Total inoculated population in the area.
(*b*) Total uninoculated population.
(*c*) Number of plague attacks and deaths amongst inoculated.
(*d*) Number of plague attacks and deaths amongst uninoculated.

The inoculated population given will be the figure of total inoculations carried out and will be based on inoculations possibly spread over a period of weeks or even months. The final figure may be many times the number present at the earlier stages when the epidemic may have been at its height.

The uninoculated population will be given as the total population figure as estimated at the previous decennial census minus (*a*). This may present considerable errors and will not allow for evacuation and migration from the infected area during an epidemic. As during the period over which inoculation is spread there is a gradually increasing figure for the inoculated population and a falling one for the uninoculated, final calculations of the incidence of plague based on the figures taken at the end of the inoculation campaign will unduly favour the inoculated and will differ very considerably from calculations based on average populations.

With these comments as to the value of figures of this type the following figures for different periods are given :—

(1) The figures for 1905–1912 during which the manufacture of the vaccine was less systematized than later on are shown in Table I. These figures may be summarized as follows :—

Total inoculated population	162,049
Total uninoculated population	649,771
Inoculated attacks	0·95 per cent.
Uninoculated attacks	4·59 ,,
Inoculated deaths	0·33 ,,
Uninoculated deaths	3·72 ,,
Relative incidence of attacks in inoculated and uninoculated	1 : 4·8
Relative incidence of deaths in inoculated and uninoculated	1 : 11·3
Inoculated case mortality	35 per cent.
Uninoculated case mortality	81 ,,

(2) Figures for the years 1930 and 1931 during which the manufacture of the vaccine had been further systematized are shown in Table II and may be summarized as follows :—

Total inoculated population	106,128
Total uninoculated population	304,824
Inoculated attacks	0·19 per cent.
Uninoculated attacks	1·63 ,,
Inoculated deaths	0·05 ,,
Uninoculated deaths	1·13 ,,
Relative incidence of attacks in inoculated and uninoculated	1 : 8·5
Relative incidence of deaths in inoculated and uninoculated	1 : 22·6
Inoculated case mortality	26 per cent.
Uninoculated case mortality	69 ,,

So far as any reliance can be placed on these figures it would appear that the protective value of the vaccine now manufactured has doubled as compared with that formerly prepared.

TABLE I.

TABLE I.

Inoculation statistics—Class I, 1905–1912.

Year.	Place or Community.	Inoculated.					Uninoculated.				
		Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.
1905	Nagpur	1,477	25	8	1·69	0·54	124,673	5,327	5,075	4·27	4·07
1906	Labour staff, Bombay Municipality.	7,182	14	13	0·19	0·18	418	28	26	6·70	6·20
	Karachi Municipal staff ..	1,245	22	4	1·76	0·32	60	8	5	13·33	8·33
	Dheds of Anand ..	252	3	1	1·2	0·6	179	13	8	7·2	4·5
	Dheds of Chikoda ..	104	6	1	5·8	0·95	146	9	8	6·16	5·5
	Dheds of Samarka ..	228	2	..	0·88	..	105	8	6	7·6	5·7
	Dheds of Gamri ..	68	..	..	..	..	238	11	8	4·6	3·4
	Dheds of Adas ..	93	1	..	1·09	..	74	3	2	4·05	2·6
	Adas village	340	1	..	0·29	..	3,294	100	66	3·0	2·0
	Dheds of Nagaski ..	88	4	1	4·5	1·13	50	11	8	22·0	16·0
	Valaram village ..	78	5	1	6·4	1·3	96	23	17	24·0	17·7
	Samarka village ..	54	2	1	3·7	1·8	32	12	8	37·4	25·0
	Adas village	24	1	..	4·2	..	44	22	16	50·0	36·4
	Nagaon Kartha ..	140	9	2	6·4	1·4	300	40	27	13·3	9·0
	Dudhondi	204	5	3	2·4	1·4	1,462	92	75	5·3	5·1
	Ghogaon	66	2	1	3·03	1·5	377	39	25	10·3	6·6
	Uplavi	283	5	..	1·7	..	556	50	26	8·9	4·6
	Tupari	63	..	..	..	..	380	4	2	1·05	0·52
	Nagarali	42	..	..	..	..	949	43	22	4·5	2·3
1907	Chaloda	602	20	5	3·3	0·8	796	98	49	12·3	6·1
	Saroda	176	3	1	1·7	0·5	194	22	13	11·3	6·7
	Chamars of Dholka ..	178	14	3	7·8	1·6	260	87	42	33·7	15·7
1908	Kolligal villages ..	20,108	407	150	2·02	0·74	31,305	1,180	921	3·76	2·94
	Sarda	631	..	..	..	..	350	15	10	4·28	2·85
	Pithapur	172	..	..	..	..	1,000	147	123	14·7	12·3
	Karachi Municipal Sweeper staff.	1,114	6	3	0·54	0·27	92	13	11	14·13	12·0
	Kavalapur	848	5	..	0·59	..	2,152	142	76	6·59	3·53
	Divraj Monje ..	244	2	1	0·82	0·41	627	61	36	9·72	5·74
1909	Villages of Azamgarh Dist.	2,818	53	22	1·88	0·77	13,830	484	456	3·49	3·29
	Nagpur	22,347	45	24	0·2	0·1	105,387	7,859	7,269	7·4	6·90
	Nasirabad Cantonment ..	7,803	21	12	0·26	0·15	1,500	269	196	17·9	13·06
	Bushaval	894	8	5	0·89	0·56	4,632	517	507	11·2	10·9

TABLE I—*concld.*

Year.	Place or Community.	Inoculated.					Uninoculated.				
		Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.
	Dharampur	1,027	24	6	2·3	0·58	2,500	209	157	8·0	6·28
	Kholapur City ..	4,118	5	1	0·12	0·02	50,255	1,132	893	2·25	1·77
	Sadra	689	1	1	0·01	0·01	600	25	18	4·16	3·0
	25 villages in Gujranwala Dist.	2,595	41	18	1·58	0·69	19,628	1,013	600	5·16	3·05
	Chak	408	15	8	3·6	1·96	99	65	49	65·6	49·49
	45 villages in Amritgar Dist.	5,846	90	17	1·53	0·29	44,243	2,513	1,651	5·60	3·73
	Neemuch	3,350	39	6	1·16	0·23	6,000	382	220	6·36	3·66
1911	Gadag	2,742	20	3	0·73	0·10	30,258	1,695	1,150	5·60	3·80
	Gadag villages ..	1,508	17	9	1·12	0·59	7,934	635	439	8·0	5·53
	Mirzapur villages ..	364	9	1	2·47	0·27	385	52	43	13·5	11·17
	Ramdurg	1,117	4	2	0·35	0·18	3,500	331	255	9·45	7·28
	Matigira	2,363	4	..	0·17	..	376	15	13	4·0	3·46
	Warora	991	15	7	1·51	0·70	7,076	392	297	5·54	4·19
	Chindwara	1,907	17	5	0·88	0·25	10,965	470	435	4·28	3·97
	Yeotmal	3,119	27	6	0·86	0·19	7,426	407	367	5·4	4·94
	Harikar	2,138	92	18	4·3	0·84	1,783	283	203	15·4	11·38
	Palghat	10,794	2	1	0·02	0·01	33,383	168	127	0·5	0·38
	Calicut	2,416	..	..	..	..	72,565	22	22	0·03	0·03
	Palni	13,825	89	63	0·64	0·45	3,343	179	167	5·30	4·99
	Bijapur	3,590	28	8	7·79	2·22	6,410	1,228	857	19·15	13·36
	Chinnapatna	1,673	71	10	4·0	0·59	1,447	166	120	11·47	8·28
	Clorpet	888	9	1	1·01	0·11	2,463	192	120	7·79	4·87
	Tyamagondlu ..	521	18	6	3·47	1·13	704	86	70	12·21	9·94
	Harikar	2,138	92	18	3·47	0·84	1,783	283	203	15·87	11·38
	Kadlipet	238	2	1	0·83	0·42	651	11	10	1·69	1·53
	Mercara	2,911	2	1	0·07	0·03	3,359	80	44	2·38	1·31
	Hyderabad (Deccan) ..	3,071	47	21	1·53	0·68	2,988	204	204	6·82	6·82
1912	Bassapur	63	1	..	1·50	..	114	17	10	14·91	8·77
	Nichanki	252	3	1	1·18	0·79	376	25	15	6·65	4·0
	Kittur	980	13	6	1·32	0·61	1,196	63	45	5·27	3·76
	Gadag	6,479	39	6	0·6	0·09	23,942	599	367	2·50	1·11
	Indian troops, Jubbulpur	3,681	26	13	0·7	0·35	254	27	19	10·63	7·48
	Balaram Cantonment ..	4,383	5	1	0·11	0·02	5,307	149	127	2·8	2·39
	Totals and percentages ..	162,049	1,540	529	0·95	0·33	649,771	29,845	24,157	4·59	3·72

TABLE II.

Inoculation statistics—Class I, 1930–1931.

	Inoculated.					Uninoculated.				
	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.
1930.										
Malegaon	4,406	10	1	0·23	0·02	19,099	1,368	975	7·11	4·06
Nasik	11,268	7	3	0·06	0·03	26,921	187	136	0·69	0·50
Sankaraparam ..	910	3	2	0·31	0·21	2,127	10	6	0·48	0·48
Pannaipuram ..	1,642	15	1	0·91	0·06	4,113	136	30	3·30	0·73
Pettipuram ..	688	..	..	..	..	3,496	57	38	1·88	1·11
Thivaram ..	2,774	5	..	0·17	..	3,941	253	113	6·41	2·86
Kombai Union ..	3,506	2	..	0·05	..	3,622	107	26	2·67	0·71
Kumbam „ ..	4,823	2	..	0·04	..	11,876	193	115	1·62	0·97
Pudupetty ..	2,245	16	3	0·71	0·13	1,315	110	43	8·36	3·04
Gudalur	4,696	..	..	..	..	9,912	129	79	1·30	0·79
Gummalapuram ..	640	1	1	0·15	0·15	360	18	(?)	5·0	(?)
Bevanatham ..	143	..	..	..	..	747	10	4	1·33	0·53
Mallasandiram (N. A.)	409	..	..	..	..	394	20	6	5·07	1·52
Tirupatam ..	1,594	5	2	0·31	0·13	14,681	126	94	0·85	0·64
Denkanikota ..	2,211	2	1	0·09	0·05	2,344	53	26	2·26	1·11
Dhawendram ..	122	..	..	..	..	398	10	4	2·51	1·01
Arasakuppam ..	327	..	..	..	..	1,062	26	19	2·45	1·75
Mallasindaram (S.) ..	369	..	..	..	..	131	19	6	14·5	4·58
Ichangur	139	..	..	..	..	61	10	1	16·3	1·63
Sarapalli	97	..	..	..	..	1,139	21	5	1·84	0·44
Muthali	1,026	..	..	..	..	174	15	11	8·62	6·32
Mugalapalli ..	444	..	..	..	..	156	10	(?)	7·63	..
Mornapalli ..	189	..	..	..	..	211	20	5	9·48	2·32
Benkari	1,557	2	2	0·13	0·13	697	65	31	9·33	4·45
Thimmsandiram ..	72	..	..	..	..	211	11	5	5·21	2·32
Totals and percentages ..	162,049	1,540	529	0·95	0·33	649,771	29,845	24,157	4·59	3·72

TABLE II—*concld.*

	Inoculated.					Uninoculated.				
	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.	Population.	Attacks.	Deaths.	Attacks per cent.	Deaths per cent.
Kodnaichickandoddi ..	746	..	..	..	..	54	16	10	29·63	18·52
Mathagiri	744	..	..	..	..	1,256	10	4	0·79	0·32
Panchakshipuram ..	336	..	..	..	..	164	17	10	10·37	6·09
Muthuganki ..	396	..	..	..	..	104	12	7	11·54	6·73
Kottur	5,534	18	7	0·32	0·12	838	77	59	9·19	7·04
Harpanhalli ..	5,071	9	3	0·18	0·06	2,393	16	8	0·66	0·33
1931.										
Ogji	236	1	1	0·42	0·42	2,764	76	47	2·75	1·70
Kottur	5,534	15	7	0·27	0·12	838	79	61	9·46	7·28
Madagondapalli ..	282	..	..	..	..	1,376	12	4	0·87	0·29
Mokopa	218	..	..	..	..	4,678	100	40	2·14	0·86
Kelod	100	..	..	..	..	5,685	60	27	1·06	0·47
Dundiraj	409	..	..	..	..	7,573	220	37	0·29	0·36
Deori	506	..	..	..	..	4,863	49	22	1·0	0·45
Hoshangabad ..	2,356	..	..	..	..	9,692	31	7	0·32	0·07
Mandalay ..	18,263	16	5	0·08	0·03	113,974	1,279	1,151	1·12	1·01
Kumbum Union ..	2,460	..	..	..	..	14,096	55	29	0·39	0·19
Anunanthanpatty ..	2,748	48	3	1·74	0·11	613	79	43	12·7	7·0
Kombay Union ..	2,421	..	..	..	..	4,747	55	15	1·16	0·32
Chimnamanum Union	4,788	5	4	0·10	0·02	7,315	106	56	1·45	0·76
Uthamapalayam ..	5,568	6	1	0·10	0·02	3,819	105	59	2·75	1·78
Kamayakavandanpatty	922	..	..	..	..	4,201	217	157	5·40	3·74
Narakathanpatty ..	1,577	..	..	..	..	2,855	45	6	1·57	0·28
Uttangi	996	13	6	1·31	0·60	1,528	33	24	2·16	1·57
Total and averages	106,128	201	53	0·19	0·05	304,824	5,388	3,463	1·63	1·13

Inoculation statistics—Class 2.

The statistics belonging to this class, which we have collected from all available records, form our main basis for the estimation of the value of prophylactic inoculation with Haffkine's vaccine and will be presented at considerable length. The figures given cover the period from the first introduction of the vaccine in 1897 up to 1919 and include certain of the original results obtained by Haffkine.

Only those inoculation campaigns have been included in this class for which it is possible to calculate the average populations exposed to risk or in which definite fixed populations of inoculated and uninoculated in certain communities have been selected for observation.

We have selected the series which follows with regard to the comparative reliability of the figures and have, from such evidence as is available, expressed our opinion as to reliability in connection with each inoculation campaign the figures of which are given.

Details of carefully observed anti-plague inoculation campaigns for which reliable statistics are available.

(1) *House of Correction, Byculla*, 1897.

The outbreak of plague in this jail in January 1897 afforded Dr. Haffkine his first opportunity of testing the efficacy of his vaccine in a population definitely exposed to risk.

Approximately half of the jail inmates accepted inoculation. The risks of inoculated and uninoculated were apparently identical.

Table III shows the populations of inoculated and uninoculated and the occurrence of plague amongst them. From the figures given therein the following calculations have been made:—

	Inoculated.	Uninoculated.
Average daily population ..	147	172
Attacks	2	12
Deaths	*nil*	6
Attack rate per 1,000 ..	13·6	69·7
Death „ „ „ ..	*nil*	34·8
Case mortality	*nil*	50 per cent.

The success of this small initial trial of the vaccine was of the greatest importance at the time as it encouraged the use of inoculation in Bombay where plague was epidemic and from which the subsequent spread took place resulting in the infection of all the provinces of India. The figures are reliable.

TABLE III.

House of Correction, Byculla, 1897.

Statement showing inoculated and uninoculated populations, plague attacks and deaths.

Day.	Population.		Inoculated.		Uninoculated.	
	Inoculated.	Uninoculated.	Attacks.	Deaths.	Attacks.	Deaths.
1st	151	177	1	..	2	1
2nd	150	172	..	..	1	1
3rd	146	173	..	..	1	1
5th	146	171	..	..	1	1
6th	146	169	..	..	2	1
7th	146	169	1	..	5	1
Average ..	147	172	..	..	..	..
Totals ..	..	..	2	..	12	6

(2) *Lanowli, July* 1897.

An outbreak occurred in this small town in July 1897, and was most severe in C and D wards. A very careful record was kept of the inoculated and uninoculated populations from day to day and also the occurrence of plague attacks and deaths in these two groups. The details are shown in Table IV. The following calculations have been made as to the incidence of plague :—

	Inoculated.	Uninoculated.
Average daily population ..	323	377
Attacks	14	78
Deaths	7	57
Attack rate per 1,000 ..	43	207
Death „ „ „ ..	21	151
Case mortality	50 per cent.	72 per cent.

The inoculation campaign was accurately observed and the figures are reliable.

TABLE IV.

Lanowli, 1897.

Statement showing daily inoculated and uninoculated population, plague attacks and deaths.

Date.	Population.		Inoculated.		Uninoculated.	
	Inoculated.	Uninoculated.	Attacks.	Deaths.	Attacks.	Deaths.
24th July ..	45	711	..	..	4	4
25th „ ..	116	636	..	..	5	5
26th „ ..	126	621	..	..	4	3
27th „ ..	175	568	..	..	2	2
28th „ ..	197	544	..	..	3	3
29th „ ..	266	472	..	..	2	1
30th „ ..	276	466	..	..	6	4
31st „ ..	300	430	1	1	3	2
1st August ..	328	398	3	2	8	6
2nd „ ..	342	373	3	1	8	6
3rd „ ..	363	341	1	..	1	1
4th „ ..	366	336	1	..	3	2
5th „ ..	368	331	1	..	1	..
6th „ ..	367	329	..	..	3	1
8th „ ..	370	323	..	..	1	..
9th „ ..	370	322	..	..	1	1
10th „ ..	371	320	1	..	1	..
11th „ ..	370	319	..	..	1	1
12th „ ..	370	318	1	1	1	..
13th „ ..	370	316	..	..	1	1
14th „ ..	370	315	..	..	1	..
17th „ ..	370	314	..	..	1	..
19th „ ..	369	313	1	1	1	1
20th „ ..	369	312	..	..	1	..
22nd „ ..	368	311	..	..	6	5
23rd „ ..	368	305	1	1	..	..
26th „ ..	368	305	..	..	1	1
3rd September ..	368	304	..	..	1	1
4th „ ..	368	303	..	..	1	..
6th „ ..	368	302	..	..	1	1
7th „ ..	368	301	..	..	3	3
13th „ ..	368	298	..	..	1	1
23rd „ ..	368	297	..	..	1	1
Average ..	323	377	..	..	..	..
TOTALS ..	..	..	14	7	78	57

The figures are omitted for days on which no plague attacks or deaths were registered.

(3) *Umerkhadi Jail, Bombay,* 1898.

Three cases of plague occurred in the jail at the end of December 1897. Haffkine inoculated as nearly as possible half of the prisoners on 1st January, 1898. Every second man from each class of prisoner was inoculated and the number of subsequent releases was almost equal amongst inoculated and uninoculated. The results were as follows :—

	Inoculated.	Uninoculated.
Average daily population ..	147	127
Attacks	3	10
Deaths	..	6
Attack rate per 1,000 ..	20	79
Death „ „ „ ..	*nil*	47
Case mortality	*nil*	60 per cent.

The figures are accurate and the risks of inoculated and uninoculated were similar.

(4) *Andhera Village,* 1898.

The inoculation campaign in this village although not on a large scale was one of the most carefully controlled and observed which has been recorded. Out of a population of 1,031 on 5th January, 1898, 76 persons died of plague between that date and 12th February.

On 12th February Dr. Haffkine and Major Bannerman, I.M.S., visited the village and inoculated 513 persons. Each household was called out in turn and half of the inmates were inoculated, care being taken to inoculate half the men, half the women and half the children in the family and an equal number of the sickly and the strong.

The evenness of the resulting inoculated and uninoculated populations will be seen in the following table which shows the distribution of inoculated persons in 28 houses which subsequently had plague cases :—

Ages.	Inoculated.	Uninoculated.
5 years and under ..	Males 4, Females 9 = 13	Males 5, Females 5 = 10
6 years to 59 years ..	Males 34, Females 20 = 54	Males 18, Females 33 = 51
60 years and over ..	Males 3, Female 1 = 4	Male 1, Females 2 = 3
TOTALS ..	71	64

The results were investigated on 4th April, 1898, by Surgeon-General R. Harvey, Director-General, Indian Medical Service, Dr. Haffkine, Major Bannerman,

I.M.S., and Captain Dyson, I.M.S., each house in which a plague case had occurred being visited.

Plague had continued for 42 days after the inoculations were performed and affected 28 families. The following were the facts elicited by the above investigators :—

Amongst the inoculated.	*Amongst the uninoculated.*
(*a*) No deaths from other causes than plague.	(*a*) One child aged 1 year died of bronchitis.
(*b*) No deaths during the first 3 days after inoculation. The first plague death occurred 8 days after inoculation.	(*b*) Three died of plague during the first 3 days after inoculation and are omitted from the calculations.
(*c*) From 15th February till the end of the epidemic there were 8 attacks of plague with 3 deaths.	(*c*) From 15th February till the end of the epidemic there were 27 attacks with 26 deaths.

The details with regard to the houses in which plague occurred are given in Table V.

From these figures the following calculations have been made :—

	Inoculated.	Uninoculated.
Average population ..	71	64
Attacks	8	27
Deaths	3	26
Attack rate	11·3 per cent	42·2 per cent
Death rate	4·2 „	40·6 „
Case mortality	37 „	96 „

The statistics are of a high degree of reliability.

TABLE V.

Andhera Village, 1898.

Statement showing the incidence of plague in houses where cases occurred.

House number.	INOCULATED.			UNINOCULATED.		
	Number of inoculated in family.	Attacks.	Deaths.	Number of uninoculated in family.	Attacks.	Deaths.
8	4	..	..	1	1	1
63	3	..	..	2	1	1
67	3	..	..	2	1	1
24	1	..	..	1	1	1
Carried over ..	11	..	..	6	4	4

TABLE V—*concld.*

House number.	INOCULATED.			UNINOCULATED.		
	Number of inoculated in family.	Attacks.	Deaths.	Number of uninoculated in family.	Attacks.	Deaths.
Brought forward ..	11	..	..	6	4	4
1	2	..	..	2	1	1
15	2	1	..	3	..	..
20	3	1	..	4	1	1
29	3	..	..	2	1	1
39	4	1	1	3	1	1
42	1	..	..	2	1	..
48	5	..	..	3	1	1
49	1	..	..	5	1	1
7	1	1	..	1	1	1
8	2	1	..	1	..	..
10	4	1	..	1	..	..
12	2	..	..	2	1	1
13	2	..	..	1	1	1
18	3	..	..	2	2	2
26	1	..	..	2	2	2
30	1	..	..	3	1	1
31	4	1	1	2	..	..
34	2	..	..	3	1	1
35	1	1	1	1	1	1
53	2	..	..	2	1	1
80	4	..	..	4	1	1
84	5	..	..	5	2	2
89	2	..	..	1	1	1
90	3	..	..	3	1	1
TOTALS ..	71	8	3	64	27	26

(5) *Hubli*, 1898.

The inoculation campaign in this town of about 50,000 inhabitants of whom 38,712 were inoculated was up to the time the largest carried out under conditions permitting of fairly accurate observation. Inoculation was

carried out from 11th May to 27th September, 1898, and the details are given in Table VI :—

TABLE VI.

Hubli, 1898.

Dates.	Population as per weekly census.	Number not inoculated.	Number inoculated.	Plague deaths amongst uninoculated.	Plague deaths amongst inoculated.
11th May to 14th June	Fell from 50,000 to 47,427	44,573	2,854	47	1
Week ending 21st June ..	47,082	41,494	5,588	22	3
28th June	47,485	39,042	8,443	29	1
5th July	46,537	36,020	10,517	55	6
12th „	46,518	33,255	13,263	34	6
19th „	45,240	29,716	15,524	82	7
26th „	43,809	24,112	19,697	100	15
2nd August ..	43,707	21,031	22,676	140	16
9th „ ..	42,768	15,584	27,184	272	19
16th „ ..	40,441	10,685	29,756	386	61
23rd „ ..	39,400	6,367	33,033	371	41
30th „ ..	38,210	4,094	34,116	328	28
6th September ..	38,382	2,731	35,469	227	34
13th „ ..	38,408	1,116	37,292	138	46
20th „ ..	39,142	937	28,205	106	35
27th „ ..	39,315	603	38,712	58	20

Discarding the figures for the period from 11th May to 14th June which are only approximate, the incidence of plague is calculated as follows :—

	Inoculated.	Uninoculated.
Average weekly population ..	24,631	17,786
Plague deaths	338	2,348
Death rate per 1,000 ..	13	132

The figures are not absolutely reliable, but the Indian Plague Commission did not consider that the defects in the statistics were sufficient to vitiate deductions expressed in percentages.

(6) *Southern Mahratta Railway Employees, Hubli*, 1898.

In connection with the Hubli outbreak special statistics are available with regard to the employees of the Southern Mahratta Railway who were living in barracks in the railway yard at Hubli under the supervision of their British Officials who formed a special committee for the purpose of dealing with the plague

outbreak and had their Medical Officer as their adviser. A most careful record was made of all occurrences. Table VII shows the relative figures and from this the following calculations are made :—

	Inoculated.	Uninoculated.
Average population ..	1,260	760
Attacks	11	35
Deaths	2	21
Attack rate per 1,000 ..	8	46
Death „ „ „ ..	1	27
Case mortality	19 per cent	60 per cent

The original report also gives the following figures :—

	Numbers.	Attacks.	Deaths.
Twice inoculated ..	990	6 (0·6 per cent)	1 (0·1 per cent)
Once inoculated ..	270	5 (1·8 „ „)	1 (0·3 „ „)
Not inoculated ..	760	35 (4·6 „ „)	21 (2·7 „ „)

The statistics given for this inoculation campaign are considered to be highly reliable and the supervision was strict throughout.

Table VII.

Southern Mahratta Railway Employees, Hubli, 1898.

Statement showing numbers of inoculated and uninoculated, with plague cases and deaths in each group.

Dates.	Inoculated.					Uninoculated.			Total population.
	Once.	Twice.	Total.	Attacks.	Deaths.	Population.	Attacks.	Deaths.	
11th June ..	41	0	41	..	..	1,794	2	2	1,835
29th „ ..	439	235	674	..	..	1,243	2	1	1,917
5th July ..	385	471	856	..	..	1,063	2	2	1,919
9th „ ..	365	552	917	..	..	1,063	1	1	1,980
11th „ ..	365	552	917	..	..	1,129	1	1	2,046
13th „ ..	337	597	934	..	..	1,125	2	2	2,059
14th „ ..	335	603	938	..	..	1,120	1	1	2,058
19th „ ..	344	821	1,165	..	..	880	2	1	2,045
20th „ ..	344	821	1,165	..	..	880	2	2	2,045
Carried over ..				..	..	..	15	13	..

TABLE VII—*concld.*

Dates.	Inoculated.					Uninoculated.			Total population.
	Once.	Twice.	Total.	Attacks.	Deaths.	Population.	Attacks.	Deaths.	
Brought forward..	..	..	..	..	..	..	15	13	..
21st July ..	344	821	1,165	1	..	881	2	2	2,046
22nd „ ..	344	821	1,165	..	..	882	1	1	2,047
23rd „ ..	344	821	1,165	..	..	886	2	1	2,051
4th August ..	297	880	1,177	..	..	862	3	..	2,039
5th „ ..	297	880	1,177	..	..	827	2	..	2,004
6th „ ..	297	880	1,177	..	..	827	1	..	2,004
9th „ ..	297	880	1,177	..	..	834	2	..	2,011
10th „ ..	215	965	1,180	1	..	831	..	..	2,011
12th „ ..	215	968	1,183	..	..	829	2	..	2,012
21st „ ..	245	1,132	1,377	2	..	619	2	..	1,996
22nd „ ..	234	1,146	1,380	..	..	618	1	..	1,998
23rd „ ..	151	1,231	1,382	1	..	615	..	..	1,997
24th „ ..	224	1,253	1,477	1	..	520	..	..	1,997
25th „ ..	210	1,272	1,482	..	..	516	1	..	1,998
26th „ ..	287	1,277	1,564	2	1	418	..	..	1,982
30th „ ..	292	1,354	1,646	1	1	333	..	..	1,979
1st September ..	342	1,375	1,717	1	..	261	1	..	1,878
21st „ ..	64	1,697	1,761	..	..	102	1	..	1,868
6th October ..	134	1,713	1,847	1	..	31	..	..	1,878
9th „ ..	84	1,723	1,807	..	..	65	1	..	1,872
Totals of attacks and deaths ..				11	2	..	37	23	..

Two of the deaths amongst the uninoculated in the railway yard occurred amongst persons who were not permanent residents, and these deaths have been deducted in calculations of relative incidence of plague.

(7) *Belgaum Cantonment*, 1899.

The epidemic lasted from May to September and during this period out of a total population of 9,543 there were 8,749 inoculated. The figures for inoculated

and uninoculated populations are given in Table VIII. From these the following calculations are made :—

	Inoculated.	Uninoculated.
Average weekly population ..	4,842	4,558
Attacks	78	506
Deaths	40	346
Attack rate per mille ..	16	111
Death „ „ „ ..	8	75
Case mortality	51 per cent	68 per cent

The figures have a considerable degree of reliability.

TABLE VIII.

Belgaum Cantonment, 1899.

Statement showing weekly inoculated and uninoculated populations with plague attacks and deaths in each group.

Week ending	Inoculated.	Uninoculated.	Inoculated.		Uninoculated.	
			Cases.	Deaths.	Cases.	Deaths.
12th May ..	1,230	8,313	14	8	34	19
19th „ ..	2,128	7,415	10	4	17	6
26th „ ..	2,579	6,964	5	3	31	19
2nd June ..	2,810	6,733	3	..	27	21
9th „ ..	3,009	6,534	10	3	32	21
16th „ ..	3,174	6,369	1	3	45	26
23rd „ ..	3,248	6,295	2	..	34	19
30th „ ..	3,361	6,182	1	2	33	22
7th July ..	3,671	5,872	3	1	47	34
14th „ ..	4,319	5,224	4	1	71	50
21st „ ..	5,470	4,073	6	4	51	33
28th „ ..	6,724	2,819	4	3	30	36
4th August ..	7,869	1,674	4	2	24	15
11th „ ..	8,182	870	12	3	18	15

TABLE VIII—*concld.*

Week ending	Inoculated.	Uninoculated.	Inoculated.		Uninoculated.	
			Cases.	Deaths.	Cases.	Deaths.
18th August ..	8,048	626	5	3	6	6
25th „ ..	Not available.	Two cases and deaths occurred.				
1st September ..	8,590	375	3	..	1	1
8th „ ..	8,749	315	..	..	5	3
Average weekly population.	4,842	4,558	..	..	..	..
Plague attacks and deaths			78	40	506	346

(8) *Villages in Ahmednagar District*, 1899.

An inoculation campaign was carried out in the plague infected villages of this district under the supervision of Mr. F. G. H. Anderson, I.C.S., First Assistant Collector, who took special trouble to observe all occurrences and used a special method of calculation of populations exposed to risk.

As soon as plague appeared in a village a house-to-house census was made and thereafter a roll call was instituted morning and evening. The inoculated were granted certain concessions such as not being turned out into camp if cases occurred in the block or street in which they resided. The inoculated were in this way probably exposed to infection for a longer period than the uninoculated. Mr. Anderson, in reporting on the inoculation campaign, showed that concealment of cases and deaths amongst the inoculated was very unlikely. With his system of roll calls he maintained that there were no appreciable errors in the statistics finally presented. He based his calculations on 'units', the 'unit' being—one day's residence of one person. The attack rate and death rate were calculated by dividing the number of attacks and deaths into the number of units in each class. The details for the villages are given in Table IX.

The results of the series may be summarized as follows:—

	Inoculated.	Uninoculated.
Total units	144,117	509,085
Attacks	70	563
Deaths	31	415
Unit attack rate	1 in 2,059	1 in 904
Unit death rate	1 in 4,648	1 in 1,226
Case mortality	44 per cent	73 per cent

These statistics have a very high degree of reliability.

Table IX.

Villages in Ahmednagar District, 1899.

Village.	Occurrences.	Inoculated.				Uninoculated.			
		Units.	Attacks.	Deaths.	Death rate 1—	Units.	Attacks.	Deaths.	Death rate 1—
Mangaon ..	9th May to 14th June, disease spreading slowly and becomes epidemic. Inoculation begins. 37 days.	24	..	..	..	37,945	11	8	4,743
	15th June to 31st August. Inoculation resorted to freely. 37 days.	18,940	4	2	9,470	59,937	40	28	2,140
	1st to 30th September. Inoculation general, 30 days.	22,119	7	4	5,530	7,364	23	12	613
	Total 145 days ..	41,083	11	6	6,847	105,246	74	48	2,192
Vakodi ..	3rd July to 15th August. No inoculation. 44 days.	..	..	..	..	38,663	14	10	3,865
	After 15 cases in 2 days people take to inoculation. 16th August to 5th September. 21 days.	2,218	..	..	..	15,860	36	28	566
	6th September to 12th October. Inoculation carried on. 37 days.	26,116	8	2	13,090	5,992	4	1	5,968
	Total 102 days ..	28,334	8	2	13,090	60,515	54	39	1,551
Nepti ..	6th July to 13th August. Inoculation declined. 39 days.	66	..	..	..	43,848	50	38	1,154
	14th August to 13th September. Epidemic dies out through evacuation. 31 days.	7,377	6	2	3,687	26,500	19	16	1,656
	Total 70 davs ..	7,443	6	2	3,721	70,348	69	54	1,302

	TOTAL 70 days ..	7,443	6	2	3,721	70,348	69	54	1,302
Valunj ..	Little inoculation, disease very virulent, 1st to 28th August. 28 days.	497	3	2	249	17,923	53	49	365
	9th August to end of epidemic. 14th October inoculation. 47 days.	20,920	5	5	4,184	7,362	24	24	306
	TOTAL 85 days ..	21,417	8	7	3,060	25,295	77	73	345
Nimblak ..	25th August to 13th October. Little inoculation done. No fair trial. 50 days.	2,269	9	2	1,135	37,329	56	34	1,098
Bhimgar ..	From 7th September to 19th October. 43 days.	31,338	7	5	6,269	148,300	59	47	3,155
Nagardevala ..	24th September to 7th October. Plague increasing. No inoculation. 14 days.	..	..	..	..	15,460	30	24	644
	8th October to 13th. Inoculation started.	1,384	..	..	..	5,105	25	21	243
	TOTAL ..	1,384	..	..	..	20,565	55	45	457
Durevadi ..	11th September to 13th October. Inoculation started on first day cases discovered. 33 days.	10,396	18	5	2,079	6,852	47	33	206
Hingangaon ..	Plague in vicinity. 21 persons inoculated on 31st. Two developed plague in 48 hours and died, 1 recovered. Little other inoculation done.	453	3	2	226	34,635	62	42	824

Two of the deaths in Hingangaon occurred in persons probably incubating plague at the time of inoculation.

(9) *Jewish Community in Aden*, 1900.

An epidemic occurred in Aden from 11th March to 13th June. The following cases occurred :—

Month.	Cases in total population.	Cases in Jews alone.
March ..	32	13
April ..	183	89
May ..	144	11
June ..	12	0
	371	113

The incidence amongst Jews was very high. The Jewish community lived in a fairly compact group in one part of Aden and for the purpose of observing the effect of inoculation a special census was taken by house-to-house visitation early in the outbreak. The total Jewish population at the time was 2,614. A register of all occurrences was kept with great care and all deaths were certified by a Medical Officer. The details of population and of attacks and deaths are given in Table X. From these figures the following calculations have been made :—

	Inoculated.	Uninoculated.
Average daily strength ..	1,190	982
Attacks	23	83
Deaths	8	65
Attack rate per 1,000 ..	19	84
Death „ „ „ ..	6	66
Case mortality	34 per cent	78 per cent

The figures are highly reliable.

TABLE X.

Jewish Community, Aden, 1900.

Statement showing daily inoculated and uninoculated populations with plague attacks and deaths in each group.

Date.	Number of inoculated present.	Plague attacks.	Plague deaths.	Number of uninoculated present.	Plague attacks.	Plague deaths.
1900.						
24th March ..	2	..	..	2,602	1	..
25th „ ..	2	..	..	2,602	..	1
26th „ ..	6	..	..	2,597	1	..
27th „ ..	6	..	..	2,595	2	2
28th „ ..	41	..	..	2,553	..	2
29th „ ..	41	..	..	2,551	2	..

TABLE X—*contd.*

Date.	Number of inoculated present.	Plague attacks.	Plague deaths.	Number of uninoculated present.	Plague attacks.	Plague deaths.
30th March ..	56	..	..	2,536	..	..
31st „ ..	56	..	..	2,536	..	..
1st April ..	95	..	..	2,497	..	..
2nd „ ..	130	..	..	2,467	1	..
3rd „ ..	150	..	..	2,439	2	1
4th „ ..	245	..	..	2,341	3	2
5th „ ..	358	..	..	2,227	..	..
6th „ ..	463	..	..	2,118	4	..
7th „ ..	465	..	..	2,114	1	..
8th „ ..	579	..	..	1,995	5	2
9th „ ..	728	..	..	1,844	1	3
10th „ ..	975	..	..	1,593	4	2
11th „ ..	1,105	2	..	1,457	4	3
12th „ ..	1,196	1	..	1,341	2	3
13th „ ..	1,401	..	..	1,131	5	..
14th „ ..	1,398	3	1	1,124	7	4
15th „ ..	1,398	3	..	1,119	3	..
16th „ ..	1,467	1	1	981	3	6
17th „ ..	1,461	..	..	976	2	5
18th „ ..	1,460	1	..	975	1	1
19th „ ..	1,457	..	..	969	4	3
20th „ ..	1,441	1	..	966	3	2
21st „ ..	1,440	1	..	965	1	5
22nd „ ..	1,439	..	1	957	4	2
23rd „ ..	1,614	..	..	781	..	..
24th „ ..	1,622	4	1	720	2	2
25th „ ..	1,652	2	1	673	3	2
26th „ ..	1,685	1	..	631	..	..
27th „ ..	1,694	..	..	603	..	1
28th „ ..	1,689	1	2	601	1	1
29th „ ..	1,651	..	..	588	1	..
30th „ ..	1,642	..	..	558	1	..
1st May ..	1,586	..	..	503	2	1
2nd „ ..	1,610	1	..	475	1	1
3rd „ ..	1,545	..	..	454	..	..
4th „ ..	1,545	..	..	428	..	..
5th „ ..	1,545	..	..	426	1	..
6th „ ..	1,484	..	..	400	1	1
7th „ ..	1,489	..	..	368	1	..
8th „ ..	1,504	..	1	353	..	1
9th „ ..	1,492	..	..	346	..	1
10th „ ..	1,488	..	..	341	..	..
11th „ ..	1,489	..	..	332	..	1
12th „ ..	1,489	..	..	332	..	1
13th „ ..	1,494	..	..	327	..	..
14th „ ..	1,503	..	..	318	..	..
15th „ ..	1,490	..	..	296	1	..
16th „ ..	1,487	..	..	288	..	..
17th „ ..	1,493	..	..	282	..	..
18th „ ..	1,510	..	..	259	..	1
19th „ ..	1,509	..	..	258	1	..

TABLE X—*concld.*

Date.	Number of inoculated present.	Plague attacks.	Plague deaths.	Number of uninoculated present.	Plague attacks.	Plague deaths.
20th May ..	1,500	1	..	252	..	..
21st „ ..	1,511	..	..	222	..	1
22nd „ ..	1,522	..	..	211	..	..
23rd „ ..	1,522	..	..	201	..	..
24th „ ..	1,525	..	..	197	..	..
25th „ ..	1,536	..	..	186	..	..
26th „ ..	1,536	..	..	186	..	..
27th „ ..	1,531	..	..	186	..	..
28th „ ..	1,534	..	..	179	..	..
29th „ ..	1,535	..	..	175	..	..
30th „ ..	1,536	..	..	174	..	..
31st „ ..	1,539	..	..	170	1	..
1st June ..	1,553	..	..	151	..	..
2nd „ ..	1,553	..	..	151	..	1
TOTALS ..	..	23	8	..	83	65
Daily average ..	1,190	..	..	982	..	..

(10) *Nawashahr, Punjab*, 1900.

Plague was discovered in this village on 26th January, 1900. On that date according to the Patwari's list of inhabitants the population was 4,809. The village was cordoned immediately after plague was declared so that there was probably little change in the population during the epidemic except from plague. The inoculated and uninoculated populations could thus be calculated with a considerable degree of accuracy. The details of inoculations and of attacks and deaths from plague were carefully registered and are given in Table XI. From this table the following calculations have been made :—

	Inoculated.	Uninoculated.
Average daily population ..	2,648	2,121
Attacks	9	41
Deaths	5	32
Attack rate per 10,000 ..	34	193
Death „ „ „ ..	19	151
Case mortality	55 per cent	78 per cent

The figures are reliable and of statistical value.

TABLE XI.

Nawashahr, 1900.

Statement showing inoculated and uninoculated populations, along with plague attacks and deaths.

Date.	Inoculated population.	Uninoculated population.	Inoculated.		Uninoculated.	
			Attacks.	Deaths.	Attacks.	Deaths.
26th January ..	435	4,364	..	..	4	2
27th ,, ..	435	4,360	..	..	..	..
28th ,, ..	435	4,360	..	..	1	..
29th ,, ..	580	4,214	..	..	1	..
30th ,, ..	745	4,048	..	..	1	1
31st ,, ..	769	4,023	..	..	..	2
1st February ..	769	4,023	..	..	..	..
2nd ,, ..	861	3,931	..	..	2	1
3rd ,, ..	1,265	3,525	..	..	..	..
4th ,, ..	1,571	3,219	..	..	3	1
5th ,, ..	1,657	3,130	..	..	1	..
6th ,, ..	1,782	3,004	..	..	..	..
7th ,, ..	1,782	3,004	..	..	..	..
8th ,, ..	2,087	2,699	..	..	1	1
9th ,, ..	2,189	2,596	..	..	1	1
10th ,, ..	2,189	2,595	..	..	1	..
11th ,, ..	2,189	2,594	..	..	..	..
12th ,, ..	2,363	2,420	..	..	..	1
13th ,, ..	2,582	2,201	..	..	..	1
14th ,, ..	2,582	2,201	..	..	..	..
15th ,, ..	2,582	2,201	..	..	1	..
16th ,, ..	2,582	2,200	..	..	..	..
17th ,, ..	2,582	2,200	..	..	1	1
18th ,, ..	2,582	2,199	..	..	3	1
19th ,, ..	2,646	2,132	..	..	1	2
20th ,, ..	2,784	1,993	1	..	4	..
21st ,, ..	2,836	1,936	..	..	2	..
22nd ,, ..	2,836	1,934	..	..	..	..
23rd ,, ..	2,998	1,772	..	..	1	..
24th ,, ..	2,998	1,771	..	..	1	..
25th ,, ..	2,998	1,770	..	..	..	4
26th ,, ..	3,086	1,682	1	..	..	1
27th ,, ..	3,085	1,682	..	1	1	..
28th ,, ..	3,085	1,681	..	..	..	1
1st March ..	3,085	1,681	..	..	..	..
2nd ,, ..	3,085	1,681	2	..	..	..
3rd ,, ..	3,196	1,568	1	..	1	..
4th ,, ..	3,195	1,567	..	1	1	1
5th ,, ..	3,195	1,567	..	1	1	..
6th ,, ..	3,195	1,567	..	..	..	..
7th ,, ..	3,195	1,567	..	..	2	..
8th ,, ..	3,195	1,567	..	..	..	..
9th ,, ..	3,195	1,566	1	..	..	1
10th ,, ..	3,281	1,478	..	..	..	..
11th ,, ..	3,285	1,473	..	1	1	..

TABLE XI—*concld.*

Date.	Inoculated population.	Uninoculated population.	Inoculated.		Uninoculated.	
			Attacks.	Deaths.	Attacks.	Deaths.
12th March ..	3,285	1,473	..	..	..	2
13th „ ..	3,285	1,471	..	..	..	..
14th „ ..	3,285	1,471	..	..	..	1
15th „ ..	3,285	1,471	..	..	..	..
16th „ ..	3,285	1,471	..	..	..	..
17th „ ..	3,314	1,441	..	..	..	..
18th „ ..	3,314	1,441	..	..	..	2
19th „ ..	3,314	1,441	..	..	..	..
20th „ ..	3,314	1,441	..	..	..	1
21st „ ..	3,314	1,441	..	..	..	..
22nd „ ..	3,314	1,441	..	..	..	..
23rd „ ..	3,314	1,438	2	..	3	..
24th „ ..	3,312	1,438	..	..	..	..
25th „ ..	3,312	1,438	..	..	..	..
26th „ ..	3,312	1,438	..	..	..	1
27th „ ..	3,312	1,438	..	..	..	..
28th „ ..	3,312	1,436	..	..	..	..
29th „ ..	3,312	1,438	..	1	..	1
30th „ ..	3,312	1,438	..	..	..	..
31st „ ..	3,312	1,438	..	..	..	..
1st April ..	3,312	1,438	..	..	..	1
2nd „ ..	3,312	1,438	..	..	..	..
3rd „ ..	3,312	1,438	1	..	..	..
Daily average population.	2,648	2,121	..	..	..	..
Plague attacks and deaths			9	5	41	32

(11) *Kahma, Punjab,* 1900.

This village was in close proximity to another in which plague had occurred and as a precautionary measure 690 persons were inoculated in the first week of March 1900. An additional number of 176 were inoculated on 15th March and 3 cases occurred on that date. From the commencement of the outbreak the inoculated and uninoculated populations were approximately the same. The occurrences are shown in Table XII. The calculations from these figures are :—

	Inoculated.	Uninoculated.
Average daily population ..	863	889
Attacks	*nil*	10
Deaths	*nil*	5
Attack rate per 10,000 ..	*nil*	113
Death „ „ „ ..	*nil*	56
Case mortality	*nil*	50 per cent

The figures are reliable.

TABLE XII.

Kahma, 1900.

Statement showing inoculated and uninoculated populations together with plague attacks and deaths.

Date.	Inoculated population.	Uninoculated population.	Inoculated.		Uninoculated.	
			Attacks.	Deaths.	Attacks.	Deaths.
15th March ..	863	896	..	..	3	..
16th ,, ..	863	893	..	..	..	..
17th ,, ..	863	893	..	..	..	..
18th ,, ..	863	893	..	..	..	..
19th ,, ..	863	893	..	..	..	1
20th ,, ..	863	893	..	..	..	..
21st ,, ..	863	893	..	..	..	..
22nd ,, ..	863	893	..	..	..	..
23rd ,, ..	863	893	..	..	2	..
24th ,, ..	863	891	..	..	1	..
25th ,, ..	863	890	..	..	2	..
26th ,, ..	863	888	..	..	1	2
27th ,, ..	863	887	..	..	..	1
28th ,, ..	863	887	..	..	..	..
29th ,, ..	863	887	..	..	..	..
30th ,, ..	863	887	..	..	..	..
31st ,, ..	863	887	..	..	..	..
1st April ..	863	887	..	..	..	..
2nd ,, ..	863	887	..	..	..	..
3rd ,, ..	863	887	..	..	..	..
4th ,, ..	863	887	..	..	..	..
5th ,, ..	863	887	..	..	..	..
6th ,, ..	863	887	..	..	..	..
7th ,, ..	863	887	..	..	..	..
8th ,, ..	863	887	..	..	..	..
9th ,, ..	863	887	..	..	..	..
10th ,, ..	863	887	..	..	..	..
11th ,, ..	863	887	..	..	..	..
12th ,, ..	863	887	..	..	..	..
13th ,, ..	863	887	..	..	1	1
Daily average population.	863	889	..	..	..	..
Plague attacks and deaths			..	..	10	5

(12) *Khatkar Kalan, Punjab,* 1900.

Precautionary inoculation was carried out in this village during the first fortnight of March 1900, and 1,043 persons out of a total of 1,384 were inoculated. Plague was discovered on 26th March. The progress of events is shown in Table XIII. It is recorded that the entire population lived under exactly similar conditions throughout the epidemic. The first case was a Brahmin who had refused

inoculation although the other members of his family had been done. This case was fatal. He lived in one of a group of three very small houses opening into a common courtyard. The second uninoculated case occurred in another of these three houses and was fatal. This person was the only uninoculated individual in his family. The third case occurred in the remaining house of the group. The patient was an inoculated woman and recovered.

From the table the following calculations have been made :—

	Inoculated.	Uninoculated.
Average daily population ..	1,040	338
Attacks	6	6
Deaths	2	5
Attack rate per 10,000 ..	58	178
Death „ „ „ ..	19	148
Case mortality	33 per cent	83 per cent

The figures are reliable.

TABLE XIII.

Khatkar Kalan, 1900.

Statement shows inoculated and uninoculated populations together with plague attacks and deaths.

Date.	Inoculated population.	Uninoculated population.	Inoculated.		Uninoculated.	
			Attacks.	Deaths.	Attacks.	Deaths.
23rd March ..	1,043	341	..	..	1	..
24th „ ..	1,043	340	..	..	..	..
25th „ ..	1,043	340	..	..	..	..
26th „ ..	1,043	340	..	..	1	..
27th „ ..	1,043	339	2	..	..	..
28th „ ..	1,041	339	..	..	..	1
29th „ ..	1,041	339	..	..	..	..
30th „ ..	1,041	339	1	..	2	1
31st „ ..	1,040	337	..	..	1	..
1st April ..	1,040	336	1	..	..	..
2nd „ ..	1,039	336	..	..	..	..
3rd „ ..	1,039	336	..	..	..	1
4th „ ..	1,039	336	..	..	..	..
5th „ ..	1,039	336	..	..	..	1
6th „ ..	1,039	336	1	..	..	..
7th „ ..	1,038	336	..	1	1	..
8th „ ..	1,038	335	..	..	..	..
9th „ ..	1,038	335	1	1	..	1
TOTALS (18 days) ..	18,727	6,076	6	2	6	5
Average daily population.	1,040	338	..	..	..	..

(13) *Salem*, 1910.

The inoculation campaign was observed and reported on by Mr. L. E. Buckley, I.C.S., the Collector of the District. The following is an extract from his report :—

'The first attack was discovered on 11th August, 1910, when the estimated population was 73,000. Between that date and the end of March 1911, when the population was estimated at about 60,000, there were 2,127 attacks and 1,721 deaths from plague of which 1,693 attacks and 1,495 deaths were among non-inoculates and 434 attacks and 226 deaths were among inoculates. The total numbers inoculated in the town were 52,440. The total number of inoculates residing in the town at the end of March 1911 was, however, higher than this by 1,500 or more, as many refugees from Salem who were inoculated in Rasipur and other villages returned at the beginning of 1911.

'These figures show that of every 100 persons attacked 80·9 died. The percentage among non-inoculates was 88·3 while among inoculated it was 52·1 or roughly speaking of every 10 non-inoculates attacked only one survived, while of every 10 inoculated attacked five survived. The value of inoculation as giving an increased chance of recovery from attack has been clearly demonstrated.

'It is less easy to be certain of the degree of protection from attack afforded by inoculation, owing to the variations of the factors to be considered.

'(*a*) The population of the town estimated at 73,000 in August 1910 commenced to decrease rapidly at the end of September and fell to about 30,000 in the third week of November. In December it began to rise again till it was about 60,000 at the end of March.

'(*b*) It was not until the disease had been established for nearly six weeks that the people could be induced to think of inoculation and it was not till at least two months after the commencement of the outbreak that the numbers of inoculates became considerable. After that the numbers increased rapidly.

'I have attempted to exhibit the history of the attack as plainly as possible in the following statement (*see* Table XIV). In this statement the following items may be considered reliably accurate :—

(1) Population of town at commencement of outbreak.

(2) Population of town at beginning of March 1911.

(3) Total number of inoculates.

(4) Numbers of attacks and deaths.

'The following items are based on estimates : in framing such estimate figures have been adopted which are unfavourable to inoculation rather than those which are favourable.

'(*a*) The estimated population in each week between August 1910 and March 1911. The lowest figures, in November, I have put at 30,000 to be on

the safe side, though the estimate made after some inquiry at the time was 25,000. The adoption of the higher figure reduces enormously the percentage of attacks among the uninoculated during the worst weeks.

'(*b*) The number of inoculates in the town in each week. The great majority of those who become inoculated did so in order to be able to stay in the town; a small number became inoculated in order that they might pass in and out on business without having to take passports and a still smaller number left the town for various reasons after being inoculated.

'If the allowance of 5 per cent for absentees seems low, any suspected deficiency in the allowance is probably more than compensated for by the weekly population of the town being over-estimated especially in the middle of the outbreak.

'As a consequence of (*a*) and (*b*) the weekly numbers of non-inoculates are unreliable. It is fairly certain, however, that they are overstated and it is not improbable that at the worst period of the outbreak they are placed at double what they ought to be.

'The figures given show that the weekly percentage of attacks amongst inoculates varied from 1/2 to 1/30th of the weekly percentage of attacks amongst non-inoculates. Taking the figures for November and December when the outbreak was at its worst and the number of inoculates for the greater part of the time largely exceeded the number of non-inoculates, it will be seen that the percentage of attacks amongst inoculates was not more than 1/6th of the attacks amongst the non-inoculated. In other words the risk of infection, if inoculated, was reduced to 1/6th of the risk amongst the non-inoculated, but considering that the number of non-inoculates is probably much overstated the risk may have been reduced to anything as low as 1/12th.

'Working these conclusions out with the percentages of deaths amongst attacks given in paragraph 2, the risk of death from plague was reduced amongst the inoculated to 1/10th of the risk amongst the non-inoculated and possibly to anything as low as 1/20th.

'The accompanying statement has been prepared to show more clearly and concisely the advantages of the inoculation campaign among the people of Salem Town'.

Table XV from Mr. Buckley's report shows the calculated attack and death rates based on average populations comparable with the figures which have been given for other inoculation campaigns.

The case mortalities were :—

Inoculated	52 per cent.
Uninoculated	88 ,,

The figures have a fair degree of reliability and it will be seen from Mr. Buckley's account of the methods of calculation that such inaccuracies as exist probably result in over-estimation of the uninoculated population with consequent reduction in the calculated incidence of plague amongst them.

Table XIV.

Table XIV.

Salem, 1910–1911.

Statement showing the weekly plague attacks and deaths and other particulars among the uninoculated and inoculated in Salem town from the commencement to the end of the outbreak (11*th August*, 1910 *to* 31*st March*, 1911).

Weeks.	Population estimated.	Total inoculated.	Inoculates in town estimated at 95 per cent of column 3 up to 31st December, 1910, and cent. per cent thereafter.	Total uninoculated.	Inoculated.		Uninoculated.		Total.		Percentage of attacks of column 6 to column 4.	Percentage of attacks of column 7 to column 5.	Remarks.
					Attacks.	Deaths.	Attacks.	Deaths.	Attacks.	Deaths.			
1	2	3	4	5	6		7		8		9	10	11
August 1910.—													
11th August, 1910, to 17th August, 1910 ..	73,000	..	..	73,000	..	..	31	25	31	25	..	0·03	
18th „ „ to 24th „ „ ..	73,000	..	..	73,000	..	..	18	14	18	14	..	0·02	
25th „ „ to 31st „ „ ..	73,000	..	..	73,000	..	..	28	25	28	25	..	0·04	
Totals ..				..	..	..	77	64	77	64			
September 1910.—													
1st September, 1910, to 8th September, 1910	71,000	..	..	71,000	..	..	52	33	52	33	..	0·07	Inoculation commenced after 20th September, 1910.
9th „ „ to 16th „ „	69,000	..	..	69,000	..	..	86	71	86	71	..	0·12	
17th „ „ to 23rd „ „	65,000	193	185	64,800	..	..	86	79	86	79	..	0·13	
24th „ „ to 30th „ „	63,000	1,252	1,190	61,700	..	..	75	67	75	67	..	0·12	
Totals ..				..	..	..	299	250	299	250			

TABLE XIV—*concluded.*

Weeks.	Population estimated.	Total inoculated.	Inoculates in town estimated at 95 per cent of column 3 up to 31st December, 1910, and cent per cent thereafter.	Total uninoculated.	Inoculated.		Uninoculated.		Total.		Percentage of attacks of column 6 to column 4.	Percentage of attacks of column 7 to column 5.	Remarks.
					Attacks.	Deaths.	Attacks.	Deaths.	Attacks.	Deaths.			
1	2	3	4	5	6		7		8		9	10	11
October 1910.—													
1st October, 1910, to 8th October 1910 ..	58,000	3,546	3,370	54,500	2	2	71	64	73	66	0·05	0·13	
9th „ „ to 16th „ „ ..	55,000	6,078	5,774	49,000	1	1	59	59	60	60	0·02	0·12	
17th „ „ to 23rd „ „ ..	50,000	7,639	7,257	42,400	3	1	53	52	56	53	0·04	0·12	
24th „ „ to 31st „ „ ..	48,000	10,720	10,184	37,300	8	7	87	80	95	87	0·07	0·23	
			TOTALS ..		14	11	217	255	284	266			
November 1910.—													
1st November, 1910, to 8th November, 1910	45,000	13,333	12,670	31,700	18	11	118	105	136	116	0·14	0·37	
9th „ „ to 16th „ „	35,000	15,469	14,696	19,500	49	25	129	116	178	141	0·33	0·65	
17th „ „ to 23rd „ „	30,000	19,448	18,476	10,550	48	26	191	150	239	176	0·26	1·8	
24th „ „ to 30th „ „	30,000	20,304	19,290	9,700	48	24	147	137	195	161	0·25	1·5	
			TOTALS ..		163	86	585	508	748	594			
December 1910.—													
1st December, 1910, to 8th December, 1910	31,000	22,260	21,147	8,740	44	10	135	122	179	131	0·20	1·5	
9th „ „ to 16th „ „	32,000	24,205	22,995	7,800	45	18	100	83	146	101	0·19	1·3	
17th „ „ to 24th „ „	33,000	25,913	24,617	7,080	50	26	85	78	135	104	0·20	1·2	
25th „ „ to 31st „ „	34,000	27,874	26,480	6,130	17	11	59	56	76	67	0·06	0·96	
			TOTALS ..		156	65	379	338	535	403			

	TOTALS ..	156	65	379	338	535	403						

January 1911.—													
1st January, 1911, to 8th January, 1911 ..	35,000	30,371	30,371	4,630	40	18	23	24	63	42	0·13	0·49	
9th „ „ to 16th „ „ ..	36,000	32,497	32,497	3,500	30	23	26	19	56	42	0·09	0·74	
17th „ „ to 21st „ „ ..	39,000	35,882	35,882	4,120	10	5	26	27	36	32	0·02	0·63	
25th „ „ to 31st „ „ ..	44,000	39,103	39,103	4,900	13	13	3	5	16	18	0·03	0·06	
TOTALS ..					93	59	78	75	171	134			
February 1911.—													
1st February, 1911, to 7th February, 1911 ..	46,000	42,409	42,409	3,590	7	4	..	..	7	4	0·01	..	
8th „ „ to 15th „ „ ..	49,000	44,621	44,621	4,400	..	..	1	2	1	2	..	0·02	
16th „ „ to 22nd „ „ ..	53,000	46,243	46,243	6,800	..	..	2	1	2	1	..	0·03	
23rd „ „ to 28th „ „ ..	56,000	48,514	48,514	7,500	..	..	1	1	1	1	..	0·01	
TOTALS ..					7	4	4	4	11	8			
March 1911.—													
1st March, 1911, to 8th March, 1911 ..	58,500	50,022	50,022	8,500	..	..	..	..	..	..	..	..	
9th „ „ to 16th „ „ ..	60,000	51,215	51,215	8,790	1	..	..	..	1	..	0·002	..	
17th „ „ to 24th „ „ ..	60,000	52,069	52,069	7,930	..	1	1	1	1	2	..	0·01	
25th „ „ to 31st „ „ ..	60,500	52,440	52,440	8,060	..	..	..	..	..	..	..	..	
TOTALS ..					1	1	1	1	2	2			
GRAND TOTALS ..	..	..	..	..	434	226	1,693	1,495	2,127	1,721			
Percentage of mortality ..	..	..	..	..	..	52·07	..	88·3	..	80·9			

TABLE XV.

Statement showing attacks and deaths from plague among the inoculated and uninoculated of Salem town from the commencement on 11th August, 1910 to the end of 31st March, 1911.

Number of weeks and months.	Total inoculated.	Total uninoculated.	Inoculated.		Uninoculated.	
			Attacks.	Deaths.	Attacks.	Deaths.
3 weeks August ..	..	219,000	..	..	77	64
4 „ September ..	1,375	266,500	..	..	299	250
4 „ October ..	27,585	183,200	14	11	270	255
4 „ November ..	65,132	71,450	163	86	585	508
4 „ December ..	95,239	29,750	156	65	379	338
4 „ January ..	137,853	17,150	93	59	78	75
4 „ February ..	181,787	22,290	7	4	4	4
4 „ March ..	205,746	33,280	1	1	1	1
31 weeks TOTAL	714,717	842,620	434	226	1,693	1,495
Average for the period* ..	27,489	27,181	434	226	1,693	1,495
Ratio per 1,000 of the average strength for the period	..	..	15·8	8·23	62·3	55·00

* Derived by dividing the aggregates by the number of weeks during the period : total number of inoculated by 26 (number of weeks) and total number of uninoculated by 31.

(14) *Coimbatore*, 1913–1914.

An Honorary Plague Officer was appointed to be in charge of anti-plague operations during this campaign and assisted in preparing the statistics relative to inoculation. These are shown in Table XVI, reproduced from the original report.

The Honorary Plague Officer in commenting on the statistics writes as follows :—

'The estimated population of the Coimbatore Municipality was 47,007 at the commencement of the epidemic in 1913. Deducting 17 deaths from plague which occurred prior to the commencement of the inoculation campaign, the estimated population on 3rd September, 1913 was taken as 46,990.

'The conditions which affected the weekly population during the course of the epidemic and which have to be taken into account are four, viz.

(1) Reduction by deaths.
(2) Reduction by compulsory evacuation.
(3) Increase by reoccupation.
(4) Increase by extension of municipal limits.

Each of these conditions have been taken into account in arriving at the weekly population in the accompanying table (*see* Table XVI).

'With regard to these conditions (1) reduction by deaths can be more or less relied upon as correct; (2) reduction by compulsory evacuation is necessarily treated as a rough and therefore inaccurate estimate. The actual number of persons who left the town week by week cannot possibly be correctly stated. The figure quoted in the tables are based upon the systematic and compulsory evacuation of houses and whole streets which was periodically enforced, and the number of persons so evicted has been calculated on the population of each street or block as recorded in the 1911 census. This has been the only possible way of arriving at the variations of the population of the town week by week. (3) Increase by re-occupation has been shown in the 10th week of the inoculation campaign when houses were allowed to be re-occupied by their owners who had previously been compulsorily evicted. These figures are of course not accurate; they are but an average or rough estimate. (4) The increase by extension of the municipal area shown in the 10th week was due to the inclusion of three important hamlets which were infected with plague and where inoculation was also carried on.

'It is interesting to note that the number of inoculated nearly equalled the number of uninoculated in the 11th week of the campaign.

'The average weekly inoculated population may be stated as approximately 26,558 while the similar uninoculated population may be placed at 22,604. The ratio per mille of attacks and deaths calculated on the average population shows that among the inoculated the figures are 5·64 attacks and 2·1 deaths while among the uninoculated the figures are 31·28 attacks and 21·76 deaths. The number of attacks among the uninoculated was therefore five and a half times greater than among the inoculated, while the deaths among the uninoculated were ten times more numerous than among the inoculated'.

For convenience of reference the figures are here stated as in the reports of other inoculation campaigns.

	Inoculated.	Uninoculated.
Average weekly population ..	26,559	22,604
Attacks	150	707
Deaths	56	492
Attack rate per 1,000 ..	56	312
Death „ „ „ ..	21	117
Case mortality	37 per cent	70 per cent.

The figures are reliable allowing for the difficulties which have been stated in regard to the calculations of population.

Table XVI.

Statement showing attacks and deaths from plague among inoculated and uninoculated persons in Coimbatore Town from the commencement of the inoculation campaign, 3rd September, 1913 to the end of the epidemic, 4th April, 1914.

The total population of the old town .. 47,007
Deaths prior to the commencement of inoculation 17
} The total population after deducting the deaths at the commencement of inoculation .. .. 46,990.

Month.	Week.	Population at the commencement of the week.	Increased by		Decreased by		Total number of inoculated.	Total number of uninoculated.	Inoculated.		Uninoculated.		Deducting the number of attacks and deaths from the inoculated within the incubation period of 7 days.	
			Re-occupation.	Extension of municipal limit.	Compulsory evacuation.	Deaths during weeks among inoculated and uninoculated.			Attacks.	Deaths.	Attacks.	Deaths.	Attacks.	Deaths.
September 1913 ..	1st	46,990	..	..	..	7	309	46,674	..	..	6	7	..	..
	2nd	46,983	..	..	..	10	4,445	42,528	..	..	14	10	..	..
	3rd	46,973	..	..	..	12	7,065	39,896	4	1	22	11	..	..
	4th	46,961	..	..	1,500	20	8,610	36,831	2	2	20	18	1	..
October „ ..	5th	45,441	..	..	2,000	9	10,190	33,242	5	..	17	9	4	..
	6th	43,432	..	..	500	17	11,575	31,340	4	1	21	16	3	1
	7th	42,915	..	..	1,500	21	13,956	27,438	1	..	29	21	..	..
	8th	41,394	..	..	1,000	47	17,179	23,168	8	3	53	44	6	1
November „ ..	9th	40,347	..	..	500	64	19,655	20,128	7	6	82	58	6	6
	10th	39,783	3,500	11,000	1,000	38	24,818	28,427	10	3	42	35	9	1

	10th	39,783	3,500	11,000	1,000	38	24,818	28,427	10	3	42	35	9	1
	11th	53,245	..	..	..	26	26,906	26,313	5	4	30	22	5	3
	12th	53,219	..	..	1,000	36	27,987	24,196	4	2	57	34	3	2
	13th	52,183	..	..	..	44	29,213	22,926	10	3	67	41	9	3
December „ ..	14th	52,139	..	..	..	38	30,712	21,389	7	2	62	36	6	3
	15th	52,101	..	..	..	29	32,490	19,582	11	2	37	27	8	1
	16th	52,072	..	..	..	20	34,492	17,560	12	..	34	20	10	..
	17th	52,052	..	..	..	18	35,656	16,378	8	3	23	15	7	1
January 1914 ..	18th	52,034	..	..	..	17	36,803	15,214	8	3	24	14	6	3
	19th	52,017	..	..	..	18	37,697	14,302	9	7	18	11	7	7
	20th	51,999	..	..	..	17	38,592	13,390	14	6	18	11	13	6
	21st	51,982	..	..	..	12	40,509	11,461	5	2	6	10	5	2
	22nd	51,970	..	..	..	9	42,280	9,681	4	1	13	8	2	1
February „ ..	23rd	51,961	..	..	..	10	43,596	8,355	4	..	8	10	1	..
	24th	51,951	..	..	..	7	44,360	7,584	6	3	3	4	5	2
	25th	51,944	..	..	..	2	44,853	7,089	2	2	1	..	2	2
TOTALS (25 weeks)	..	..	..	..	..	..	663,948	565,092	150	56	707	492	118	45
Weekly average population for the period.							26,559	22,604	..	..	..	..	..	..
Ratio per mille of the average strength for the period.							..	..	5·64	2·1	31·28	21·76	4·44	1·69

(15) *Villages in Dharwar District*, 1914.

The statistics with regard to these villages have been dealt with in a special manner so as to provide almost equal proportions of inoculated and uninoculated persons living under conditions which entailed an equal definite risk for the same period. In analysing the figures only those households have been dealt with in which both inoculated and uninoculated individuals were living and in which plague cases occurred in one or other group. Households in which all were inoculated or all uninoculated are excluded and the observations were made from a date subsequent to the day on which the last person was inoculated in the house. The incidence of plague in the inoculated and uninoculated is summarized in Table XVII. No calculations as to average population are necessary for the reasons stated above. The facts may be summarized as follows :—

	Inoculated.	Uninoculated.
Population	311	344
Attacks	34	116
Deaths	7	84
Attack rate per 1,000 ..	109	337
Death „ „ „ ..	22	244
Case mortality	20 per cent	72 per cent

The figures are of the highest degree of reliability and the method of observation employed reaches the level of a careful laboratory experiment. We consider this evidence of the highest value.

TABLE XVII.

Villages in Dharwar District.

Summary of inoculations and incidence of plague in 131 *houses in which plague occurred.*

Number of houses.	Number of inhabitants.	Age and sex distribution.	INOCULATED.									UNINOCULATED.							
			Sex.		Age periods.							Sex.		Age periods.					
			Male.	Female.	1—10	11—20	21—30	31—40	41—50	51 and over.	Total.	Male.	Female.	1—10	11—20	21—30	31—40	41—50	51 and over.	Total.
		Number ..	237	74	87	86	76	34	23	5	311	146	198	74	82	67	41	32	48	344
131	655	Attacks ..	26	8	9	13	9	1	2	..	34	41	75	19	40	24	9	11	13	166
		Deaths ..	3	4	2	2	3	..	..	..	7	29	55	14	30	18	5	6	11	84
Percentage attacks ..			10	10·8	10	15	12	3	9	..	10·9	28	37	25	48	35	22	34	27	33·7
Percentage deaths ..			1·2	5·4	2·3	2·3	4	..	..	..	2·2	19·8	27·7	19	36·6	27	12·2	19	23	24·4

(16) *Baghdad*, 1919.

The inoculation campaign in Baghdad is the largest which has been carried out under conditions providing for strict control and accurate observation. The present writer was at the time Medical Officer of Health of Baghdad, and was provided with a well-trained sanitary staff. The city was in Military occupation and control. The sanitary staff included the members of a British Sanitary Section who were, in civil life, sanitary inspectors by profession and were qualified men. Each of these was in charge of a ward of the city and saw all deaths and had them medically confirmed if necessary. Burial was not permitted without a certificate. Certificates were given to all inoculated persons and these were prized and retained by them, and could be inspected in the case of an attack or death from plague. Inoculation was a very popular measure.

Anti-plague inoculation was commenced in Baghdad East (the left bank of the Tigris) on 19th January, 1919, and in addition to 5,540 inoculations which had been done in December 1918, a further 51,457 were carried out between that date and 12th May bringing the total inoculated in Baghdad East up to 56,997.

In Baghdad West inoculation was carried on between 15th March and 30th May, 25,561 in all being done representing 73 per cent of the population.

The total thus inoculated in the whole city reached 82,558 or about 50 per cent of the population.

The progress of inoculation and the attacks and deaths from plague are shown in the Tables XVIII and XIX.

The relative incidence of plague in the inoculated and uninoculated populations was as follows :—

Baghdad East.

	Inoculated.	Uninoculated.
Average weekly population ..	42,752	87,048
Attacks	48	580
Deaths	22	431
Attack rate per 10,000 ..	11	66
Death „ „ „ ..	5	49
Case mortality	45 per cent	74 per cent

Baghdad West.

	Inoculated.	Uninoculated.
Average weekly population ..	20,703	14,497
Attacks	17	226
Deaths	14	207
Attack rate per 10,000 ..	8	156
Death „ „ „ ..	6	142
Case mortality	82 per cent	91 per cent

Baghdad—whole city.

	Inoculated.	Uninoculated.
Average weekly population ..	56,555	108,445
Attacks	65	806
Deaths	36	638
Attack rate per 10,000 ..	11	74
Death „ „ „ ..	6	58
Case mortality	55 per cent	79 per cent

The figures are of a high degree of reliability.

Table XVIII.

Baghdad, 1919.

Weekly inoculated and uninoculated population.

Week ending	Baghdad East.		Baghdad West.		Whole City.	
	Inoculated population.	Uninoculated population.	Inoculated population.	Uninoculated population.	Inoculated population.	Uninoculated population.
24th January ..	7,985	121,815	..	..	7,985	157,015
31st „ ..	16,115	113,685	..	..	16,115	148,885
7th February	20,595	10,205	..	..	20,595	144,405
14th „ ..	23,239	106,561	..	..	23,239	141,761
21st „ ..	27,156	102,644	..	..	27,156	137,844
28th „ ..	29,512	100,288	..	..	29,512	135,488
7th March ..	31,795	98,005	..	..	31,795	133,205
14th „ ..	34,095	95,705	..	..	34,095	130,905
21st „ ..	34,095	95,705	4,681	30,519	38,776	126,224
28th „ ..	38,659	91,141	9,043	26,157	47,702	117,298
4th April ..	44,561	85,239	12,766	22,434	57,327	107,673
11th „ ..	47,930	81,870	16,374	18,826	64,304	100,696
18th „ ..	50,849	78,951	18,059	17,141	68,908	96,092
25th „ ..	52,159	77,641	21,108	14,092	73,267	91,733
2nd May ..	54,720	75,080	22,489	12,713	77,209	87,793
9th „ ..	56,641	73,159	24,075	11,125	80,716	84,284
16th „ ..	56,997	72,803	24,481	10,719	81,478	83,522
23rd „ ..	56,997	72,803	24,808	10,392	81,805	83,195
30th „ ..	56,997	72,803	25,561	9,639	82,558	82,442
6th June ..	56,997	72,803	25,561	9,639	82,558	82,442
13th „ ..	56,997	72,803	25,561	9,639	82,558	82,442
20th „ ..	56,997	72,803	25,561	9,639	82,558	82,442
27th „ ..	56,997	72,803	25,561	9,639	82,558	82,442
4th July ..	56,997	72,803	25,561	9,639	82,558	82,442
Totals ..	1,026,022	2,089,118	331,250	231,952	1,357,332	2,602,670
Averages ..	42,752	87,048	20,703	14,497	56,555	108,445

TABLE XIX.

Baghdad, 1919.

Weekly plague cases and deaths.

Week ending	Baghdad East.						Baghdad West.						Whole City.					
	Total cases.	Total deaths.	Uninoculated cases.	Uninoculated deaths.	Inoculated cases.	Inoculated deaths.	Total cases.	Total deaths.	Uninoculated cases.	Uninoculated deaths.	Inoculated cases.	Inoculated deaths.	Total cases.	Total deaths.	Uninoculated cases.	Uninoculated deaths.	Inoculated cases.	Inoculated deaths.
24th January ..	5	2	5	2	..	..	..	..	..	..	..	..	5	2	5	2	..	..
31st „ ..	3	3	3	3	..	..	..	..	..	..	..	..	3	3	3	3	..	..
7th February	2	1	1	1	1	..	..	..	..	..	..	..	2	1	1	1	1	..
14th „ ..	8	6	7	6	1	..	..	..	..	..	..	..	8	6	7	6	1	..
21st „ ..	13	7	13	7	..	..	..	..	..	..	..	..	13	7	13	7	..	..
28th „ ..	6	5	6	5	..	..	..	..	..	..	..	..	6	5	6	5	..	..
7th March ..	12	11	11	10	1	1	..	..	..	..	..	..	12	11	11	10	1	1
14th „ ..	14	11	13	11	1	..	4	4	4	4	..	..	18	15	17	15	1	..
21st „ ..	17	12	16	11	1	1	5	4	5	4	..	..	22	16	21	15	1	1
28th „ ..	33	29	31	19	2	1	11	9	11	9	..	..	44	29	42	28	2	1
4th April ..	32	26	32	26	..	..	10	8	9	8	1	..	42	34	41	34	1	..
11th „ ..	44	28	37	24	7	4	8	7	8	7	..	..	52	35	45	31	7	4
18th „ ..	43	33	43	33	..	..	15	14	12	11	3	3	58	47	55	44	3	3
25th „ ..	47	28	40	25	7	3	13	13	12	12	1	1	60	41	52	37	8	4
2nd May ..	50	31	44	28	6	3	35	29	31	27	4	2	85	60	75	55	10	5
9th „ ..	40	26	37	25	3	1	22	22	22	22	..	..	62	48	59	47	3	1
16th „ ..	37	28	33	26	4	2	24	23	22	21	2	2	61	51	55	47	6	4
23rd „ ..	38	31	34	29	4	2	44	42	39	37	5	5	82	73	73	66	9	7
30th „ ..	66	50	64	49	2	1	28	25	27	24	1	1	94	75	91	73	3	2
6th June ..	40	32	38	31	2	1	10	9	10	9	..	..	50	41	48	40	2	1
13th „ ..	29	23	28	23	1	..	11	10	11	10	..	..	40	33	39	33	1	..
20th „ ..	27	20	23	18	4	2	3	2	3	2	..	..	30	22	26	20	4	2
27th „ ..	13	12	12	12	1	..	..	..	..	..	..	..	13	12	12	12	1	..
4th July ..	9	7	9	7	..	..	..	..	..	..	..	..	9	7	9	7	..	..
TOTALS ..	628	453	580	431	48	22	243	221	226	207	17	14	871	674	806	638	65	36

Summary of statistics of inoculation campaigns.

The series of inoculation campaigns described above provide figures of a fair degree of reliability suitable for the form of statistical analysis which has been applied to them.

It will be convenient to summarize here in one table (Table XX) the appropriate figures of campaigns for which comparable statistics are available.

The figures given deal with average daily or weekly populations exposed to risk and show the numbers of attacks and deaths amongst the inoculated and uninoculated, except in the case of Hubli for which the figure of attacks is not available.

The following calculations have been made from the combined table :—

(*a*) *Excluding Hubli.*

	Inoculated.	Uninoculated.
Average population ..	121,134	168,638
Attacks	803	4,014
Deaths	385	3,194
Attack rate per 10,000 ..	66	232
Death „ „ „ ..	31	189
Case mortality	48 per cent	79 per cent

(*b*) *Including Hubli.*

	Inoculated.	Uninoculated.
Average population ..	145,765	186,424
Deaths	723	5,542
Death rate per 10,000 ..	49	297

The ratio of plague attacks and deaths amongst the uninoculated as compared with the inoculated is shown in Table XXI.

The combined figures indicate that the incidence of plague attacks was approximately four times as high amongst the uninoculated as amongst the inoculated and the death rate six or seven times greater.

The ratios given in Table XXI (to the nearest full figure) show the attack ratio to have varied from 4 to 17 times, and the death ratio (excluding small outbreaks in which no deaths occurred amongst the inoculated) from 7 to 11 times.

The case of the Dharwar villages is of special interest. A very high figure is shown for the total incidence of plague. This is due to the fact that only houses have been included in this series in which at least one case of plague has occurred. The definite risk of plague infection is thus evident and as the statistics for these villages deal with houses in which observations were only made after the completion of inoculations, and with subsequent fixed populations exposed to risk, the conditions for the trial of the efficacy of anti-plague inoculation are ideal. In this series the relative attack incidence amongst uninoculated as compared with inoculated was 3 to 1 and the mortality ratio 11 to 1.

TABLE XX.

Combined summary of figures of outbreaks for which comparable reliable figures are available.

Outbreak.	Average populations.		Inoculated.		Uninoculated.	
	Inoculated.	Uninoculated.	Attacks.	Deaths.	Attacks.	Deaths.
House of Correction, Byculla.	147	172	2	..	12	6
Lanowli	323	377	14	7	78	57
Umerkhadi Jail ..	147	127	3	..	10	6
Undhera	71	64	8	3	27	26
Hubli	24,631	17,786	Not available.	338	Not available.	2,348
S. M. R., Hubli ..	1,260	760	11	2	35	21
Belgaum	4,842	4,558	78	40	506	346
Jewish Community, Aden.	1,190	982	23	8	83	65
Nawashahr ..	2,648	2,141	9	5	41	32
Kahma	863	889	..	..	10	5
Khatkar Kalan ..	1,040	338	6	2	6	5
Salem	27,489	27,181	434	226	1,693	1,595
Coimbatore ..	26,559	22,604	150	56	707	492
Baghdad	56,555	108,445	65	36	806	638
Average population ..	147,765	186,424	..	..	..	..
Attacks and deaths ..	..	..	803*	723	4,014*	5,542

*Excluding Hubli.

Throughout the whole series a figure which we consider to be of a fairly reliable nature is that of case mortality. Over the whole series the ratios for inoculated and uninoculated were 48 per cent and 79 per cent respectively. From Table XXI it will be seen that, excluding groups without fatal cases, the mortality varied from 20 per cent to 55 per cent amongst inoculated and from 50 per cent to 96 per cent amongst uninoculated. The figure of 96 per cent refers to one of the very carefully observed campaigns. In every instance the case mortality amongst the inoculated was considerably lower than amongst the uninoculated.

A consideration of the evidence available from these figures would indicate that inoculation with Haffkine's plague prophylactic gives roughly a fourfold protection against attacks and an eightfold protection against death.

The figures deal with a total average population of over 300,000 exposed to risk with the occurrence of over 5,000 deaths and should be sufficiently large to form a basis for the estimation of the value of inoculation as over 40 per cent of the average population were inoculated.

The statistics include figures for large cities, smaller towns and villages, institutions, and populations living under special conditions of housing and observation,

and may be taken to be fairly representative of average conditions in India and other Eastern countries.

In regard to certain of the inoculation campaigns, as for example those in jails and selected communities, a high degree of reliability can be assured ; while in regard to the remainder, subject to such defects as have been mentioned in individual instances, the figures are fairly reliable, but are probably as accurate as we are likely to obtain under the conditions prevailing in India and with the resources usually at our disposal.

TABLE XXI.

Showing ratio of attacks and deaths, case mortality and comparative incidence of plague attacks and deaths amongst inoculated and uninoculated.

Outbreak.	RATIO PER 10,000 OF POPULATION.				RATIO OF UNINOCULATED TO INOCULATED.		PERCENTAGE OF CASE MORTALITY.	
	INOCULATED.		UNINOCULATED.					
	Attacks.	Deaths.	Attacks.	Deaths.	Attacks.	Deaths.	Inoculated.	Uninoculated.
House of Correction, Byculla.	130	..	700	350	5 to 1	..	..	50
Lanowli.. ..	430	210	2,070	1,510	5 to 1	7 to 1	50	72
Umerkhadi Jail ..	200	..	790	470	4 to 1	..	..	60
Undhera ..	1,130	420	4,220	4,060	4 to 1	10 to 1	37	96
Hubli	Not available.	130	Not available.	1,320	Not available.	10 to 1	Not available.	
S. M. R., Hubli ..	180	30	460	270	3 to 1	9 to 1	20	60
Belgaum ..	160	80	1,110	750	7 to 1	9 to 1	51	68
Jewish Community, Aden.	190	60	840	660	4 to 1	11 to 1	34	78
Nawashahr ..	34	19	193	151	6 to 1	8 to 1	55	78
Kahma	..	..	113	56	..	..	..	50
Khatkar Kalan ..	58	19	178	148	3 to 1	7 to 1	33	83
Salem	158	82	623	550	4 to 1	7 to 1	52	88
Coimbatore ..	56	21	312	217	5 to 1	10 to 1	37	70
Dharwar villages ..	1,090	220	3,370	2,440	3 to 1	11 to 1	20	72
Baghdad ..	11	6	74	58	7 to 1	9 to 1	55	79

PART III

NOTES ON THE VACCINE AND ITS PROPERTIES

CHAPTER IX.

Experimental methods.

The natural occurrence of plague in animals suitable for laboratory use, and the susceptibility of many species to artificial infection, provides facilities for the investigation of the effect of immunization procedures which are hardly obtainable in regard to any other kind of human disease. When Yersin (1895) first carried out his work on immunization against plague knowledge of the experimental methods suitable for investigation was in its infancy, and during the earlier period of study of the plague vaccine the methods employed were undoubtedly defective. The results obtained in regard to different points were very discordant in the hands of different observers. The defects in the methods used may be illustrated by the fact that, on a consideration of the experimental evidence before them, the Indian Plague Commission arrived at conclusions with regard to the relative value of the sediment and supernatant fluid of Haffkine's vaccine which were exactly the opposite of the truth, as shown by subsequent work. While certain of the experimental results obtained during the earlier years have provided information on which has been based many improvements in manufacture, the methods employed were lacking in accuracy and not capable of producing consistent results in a series of successive experiments. The methods were not capable of showing quantitative differences in value due to variations in procedure adopted for preparing the vaccine, while at the same time providing useful information on broader lines.

The difficulties of earlier workers and the variations in their results have been found to be largely due to the selection of unsuitable types of experimental animals, and to the use of unsuitable and inaccurately measured doses of *B. pestis* for tests. Recent work carried out at the Haffkine Institute has shown the importance of these points and also demonstrated that it is possible to employ an experimental technique of considerable accuracy and of differential value, and that repeated experiments may be made to show consistent results not obtained by the use of older experimental methods. Solid immunity in plague is rarely obtainable and the results of our tests with animals possessing some degree of natural immunity show this. As has been indicated in previous chapters a natural immunity has, after a long series of years of epizootic plague, developed in the rats of Bombay city. A comparative test of the susceptibility of these rats to infection with different doses of *B. pestis* gave the following results :—

Rats used.	Number of *B. pestis* injected.	Number of rats inoculated.	Number of plague deaths.	Percentage of plague deaths.
Bombay city ..	64	24	0	0
Madras „ ..	64	4	3	75
Bombay „ ..	64,500	25	1	4
Madras „ ..	64,500	4	4	100
Bombay „ ..	32,250,000	25	25	100
Madras „ ..	32,250,000	4	4	100

It will be seen that while the Bombay rats showed complete immunity against the smallest dose used, a considerable increase in the number of organisms injected resulted in a high mortality. It is obvious that if we are to attempt to demonstrate immunity the dosage of the test virus employed must be within certain limits, and if possible an accurately measured one which will kill a 100 per cent of controls. In much of the earlier work no attempt was made to fix an exact dose of *B. pestis*, based on the number of organisms injected, as a test of immunity, although definitely killing doses of virulent strains were used. Under the circumstances successive experiments, including those carried out at this Institute, were not directly comparable and were found to show considerable differences in results. An example of the variations which occurred is given in Chapter XVII, in regard to the tests of the value of successive brews of the plague vaccine in which wide variations were indicated, and the results of repeated tests of the same brew did not tally. Later work by revised methods showed a distinct regularity in the action of the vaccine. It is unnecessary to describe in detail the methods of test doses used for experiment by different workers at different times. These include preparations of agar slopes, quantities of broth cultures, platinum loopfuls of cultures, cutaneous inoculations with cultures or passage animals' spleens, insertion of a needle dipped into broth cultures, weighed doses of plague-rat's spleen, etc.

In this Institute, for many years, the infecting dose used consisted of an emulsion of a spleen of the passage rat given in a dose equivalent to 0·006 to 0·003 g. of original spleen. The method provided a great deal of useful information and, although lacking in accuracy, so far as the dosage represents any consistent number of organisms, the results obtained by its use have formed the basis of many of the improvements which have been made in the vaccine from time to time. The method, however, failed to be of sufficient accuracy and regularity to permit of its being adopted in connection with a technique for biological standardization of the vaccine, and for the study in exact detail of the effect of variations in methods of preparation, or the changes which take place in the vaccine at various stages of its manufacture, which had been undertaken in the hope of further improving the methods. The reason for the failure in accuracy of this method and the irregularity in results obtained by it will be found in the great variations which occur in the bacterial content of suspensions prepared from successive passage animal spleens. One such series examined showed that the suspensions equivalent to 0·003 g. of spleen gave counts per c.c. of 120, 5,435, 250, 15,600, 26,000, 46,000, 32,000. Other experimental work has shown that if a carefully measured quantity of *B. pestis* is given to test animals and to controls in high doses but of such strength that immunized animals will be able to show some degree of resistance against it, less regular results will be obtained than when the infecting dose consists of a known multiple of the minimum lethal dose. These observations with regard to the importance of certain dose limits used in testing immunity probably explain how the earlier differences in opinion as to the value of the vaccine and its fractions arose.

Before going on to describe the experimental methods now being tried, the question of the suitability of different animals for the purpose may be considered. The animals which have been used for testing plague vaccines have been of varying suitability and include monkeys of several species, rabbits, guinea-pigs, trapped wild rats of several species, and laboratory-bred albino rats and mice.

Monkeys were used for some of the experimental work of the German, Austrian and Russian Plague Commissions. The species employed included *Macacus sinicus*, *Macacus radiatus* and *Semnopithicus entellus*. Of these species *Macacus radiatus* was the least susceptible and the results obtained with all species were irregular. Strong (1907) used *Cynomolgus philippinensis* and noted that its susceptibility was very irregular. He used a method of infection, consisting of thrusting under the skin a hypodermic needle dipped in a broth suspension of *B. pestis* and later a measured loopful of culture which he considered to represent many times the minimal lethal dose. Strong believed the variations in his results with monkeys to be largely due to the difference in individual susceptibility.

Monkeys are, in our opinion, unsuitable for most types of plague experimental work. It is difficult to determine a suitable infecting dose, results are irregular, and to carry out work on a sufficient scale the expense is prohibitive in comparison with the cost of other more suitable animals. We have recently used monkeys for work in connection with work on anti-plague serum and although some results of value were obtained with them the variation in their response to the infecting doses of *B. pestis* used for test proved them to be, on the whole, unsatisfactory.

Rabbits have been used to a considerable extent for plague experimental work and were the animals used by Yersin, Calmette, and Borrel (1895) in their first demonstration of the fact that immunity against plague could be obtained by bacterial vaccines. Haffkine used rabbits in much of his original work but the subsequent experimental work on plague carried out at the Institute by the Plague Research Commission in their epidemiological studies and the Institute staff on the subject of the vaccine was conducted on such a scale that a supply of rabbits sufficient to meet the requirements was impossible. Five thousand or more animals are used annually for experimental purposes and for this reason it has not been possible to work with rabbits. Our recent experience of these animals for plague experimental work is based mainly on their use for trials of anti-plague serum and for chemotherapeutical experiments, for which they were selected on account of their suitability for intravenous injections. It was found in the course of this work that there were considerable differences in the susceptibility of different breeds of rabbits. The ordinary stock animal used in the Institute is of mixed breed, the rabbits being of all colours and with a Belgian hare cross. These were more resistant to plague than a pure strain of blue English rabbits bred from imported stock. The English rabbits were found to be very susceptible to plague and in the case of 80 rabbits given a dose of *B. pestis* varying from 18 to 75 organisms 100 per cent mortality occurred. In the case of the mixed breed much larger doses are required to produce 100 per cent mortality, escapes occurring with a dose of 1,000 organisms and even with 10,000 bacilli subcutaneously. Rabbits are, on the whole, unsatisfactory for immunity experiments on account of the variability of their response to the infecting dose given for final test.

Guinea-pigs have been largely used for plague experimental work, but do not respond well to immunization, and are not suitable for testing the toxicity of plague vaccine. A dose of Haffkine's plague prophylactic which kills up to 30 per cent of *R. rattus* has no toxic effect on these animals.

Schutze (1925) reports that immunity of 50 per cent and 44 per cent respectively was obtained in successive experiments with guinea-pigs immunized with Lister Institute vaccine and tested with considerable doses of broth cultures of

B. pestis. Comparing the immunizing value of different vaccines including Haffkine's vaccine he obtained results with guinea-pigs and rats which did not correspond. We do not, on the whole, consider guinea-pigs to be very suitable for investigation of the effect of anti-plague immunization.

Trapped wild rats have been the most extensively used animals in plague experimental work in India. At the Haffkine Institute 1,500 or more are received daily for examination and destruction, the Institute carrying out this work for the municipality in connection with their routine rat trapping. The bulk of the rats caught are *R. rattus* and *R. decumanus*. The former only are used for experimental work, being easier to handle. Several hundred thousand have been used for different types of experiment but, as has already been mentioned, the development of a degree of immunity to plague amongst the rats of Bombay city has rendered these local rats unsatisfactory for tests of the protective value of the vaccine, and susceptible rats from Madras city have now to be imported for experimental purposes. Since 1911 a continuous supply of these Madras rats has been kept up, those trapped in Madras being sent to us in travelling cages containing 80 to 100 each.

These rats have been invaluable for many years, but their use has several disadvantages. There is a considerable mortality in transport, the rats are of unknown ages and of variable states of nutrition ; they are liable to injury in handling which may lead to irregular experimental results. They are very susceptible to the toxic action of Haffkine's plague prophylactic and show a toxic mortality from a dose of 0·5 c.c. of the vaccine which may be as high as 30 per cent. Starting an experiment with different batches of rats of equal numbers submitted to various doses or fractions of a vaccine, the mortality from handling and toxic action will result in only irregular groups being available for final testing.

The percentage immunity obtainable in trapped *R. rattus* after inoculation with Haffkine's vaccine is not high and varies from 10 per cent to 60 per cent against different methods of infection.

Naidu and Jung (1929) in summarizing the results with 5,193 immunized *R. rattus* and 1,052 controls show an average immunity of 32·3 per cent against a survival rate of 6·4 per cent in controls.

Even when a carefully measured dose of *B. pestis* is used the results obtained are not as regular as would be desirable. An example of the range of variation in the results of experimental work with these animals is shown by the following series of tests and repeat tests of the protection conferred by the plague vaccine.

The results quoted by Naidu and Jung (1929) show an even greater variation when the infecting dose used was a measured quantity of plague-rat spleen.

Used in sufficient numbers, and with large series of controls, *R. rattus* is of value in determining on broad lines many points with regard to the value of vaccines prepared by different methods, but the results obtained with the wild rat are not sufficiently regular to permit of it being used for biological standardization of the vaccine. They are not fully suitable for elucidating the effect of variations in the methods of manufacturing the vaccine, or estimating the changes in toxicity and immunizing value and in other respects, which the vaccine undergoes during the various stages of its incubation, with the degree of accuracy we would desire.

Variation in response of R. rattus *to immunization.*

Vaccine number.	Number of rats infected.	Number of organisms in infecting dose.	Percentage of immunity.	Percentage of mortality of controls.
333	26	1,000	42	90
333	23	725	35	100
257	29	1,000	55	90
257	29	1,065	52	90
257	27	690	30	90
257	27	725	37	100
241	27	527	25	80
241	29	690	51	90
241	25	725	28	100
279	25	527	28	80
279	30	690	31	90
286	27	605	37	70
743	30	605	37	70
743	27	615	45	100
757	27	605	31	70
292	30	737	23	70
292	28	490	29	80
290	30	737	28	70
290	25	490	52	80
314	30	737	23	70
314	25	490	44	80
296	29	810	48	100

The laboratory-bred albino rat has been used to some extent for experimental work on the plague vaccine but we do not favour its use. Its susceptibility is less than that of wild *R. rattus* and we have found it irregular in its response to infection with various doses of *B. pestis*.

In one series of tests with a dose of 58 *B. pestis* a 66 per cent mortality was obtained with albino rats while with ten times the dose of the same material only 33 per cent mortality occurred. Schutze (1925) obtained a higher degree of immunity with these rats both with Haffkine's vaccine and with other vaccines than we obtain with the trapped rats, but his controls with the same strain showed a percentage survival of 50 per cent and 22 per cent respectively in successive experiments.

In another series of experiments Schutze (1932) obtained a high degree of protection in white rats by means of a Haffkine type of vaccine against doses of 600,000 *B. pestis* or even larger dosage. In our experience the wild *R. rattus* cannot be protected against such a dose of a virulent strain. This resistance of the white rat must be allowed for in interpreting results and they are probably unsuitable for differential work.

Recent improvements in experimental technique.

The irregular results obtained in the attempts made to standardize the Haffkine vaccine biologically by testing its immunizing value in trapped Madras *R. rattus* against an infecting dose of plague-rat spleen emulsion, during the years 1923 to 1928, showed the necessity of overhauling the experimental methods used.

A prolonged investigation for this purpose was undertaken by Sokhey and la Frenais and has resulted in the introduction of a revised technique which has given reliable and regular results over a considerable series of tests, but which still presents certain difficulties.

Attention was first directed to the question of the infecting dose of *B. pestis* used for the final test of immunity. A method was sought for preparing test material which would represent an exact measured dose of *B. pestis* of regular virulence, and which could be reproduced with a fair degree of accuracy for use in successive experiments. The work which had been carried out on the methods of preparing seed material from single colonies on blood-agar provided the basis for a procedure which was devised for the preparation of standard culture dilutions in a regular manner at a fixed remove from the passage animal source. The procedure at first adopted consisted in isolating the *B. pestis* as described in Chapter X and after 48 hours at 37°C. and a subsequent four days at room temperature a single colony picked off the blood-agar was inoculated into 10 c.c. of acid-digest broth of standard composition. This was incubated at 37°C. for exactly 48 hours and 0·5 c.c. of the culture then transferred to a second 10 c.c. broth tube which was incubated for a similar period. From the second broth tube a series of tenth dilutions was prepared. 1·0 c.c. of dilution 1 in 1,000,000 of the culture prepared in this way was found to give with remarkable regularity a colony count of 50 to 100 organisms. The strain of *B. pestis* used is one of which a dose of 7 to 10 bacilli will kill 80 per cent to 100 per cent of white mice or Madras *R. rattus* with certainty, and will usually kill 100 per cent of these animals. Ten times this dose has been selected for use as a test of immunity.

The tendency for *B. pestis* to form short chains during its growth in broth introduces a certain difficulty in obtaining accurate counts by plating but in practice the method has been found to give regular results.

The introduction of the use of blood-agar has made possible a fair degree of accuracy in estimating the number of *B. pestis* by colony culture, no such result being obtainable on ordinary agar on which no growth at all may appear when quantities of plague suspensions are inoculated on to it which will give definite growth in the richer medium.

The regularity of the results obtained by the method of preparing the culture dilutions and the counts for them is shown by the fact that the fourth dilution has been found on repeated tests to give within very narrow limits ten times the number of colonies shown in the fifth dilution.

The colony counts have been checked against direct counts under the microscope using the Petroff-Hausser Bacteria Counter.

The following are the comparative results obtained by the two methods with three successive cultures :—

	Colony counts. Millions per c.c.	Direct counts. Millions per c.c.
1st Culture ..	52	56
2nd „ ..	78	85
3rd „ ..	79	95

The differences are probably explainable by each organism of a chain being separately counted while a chain will probably produce only one colony. The microscopical method does not of course distinguish viable organisms.

In using test doses prepared in this way the final dilution was used for infecting as soon as prepared, and the count results obtained later in confirmation of the strength of the dose.

With the use of this method of preparing a standard fixed dose of *B. pestis* a considerable improvement in the regularity of the experimental results was obtained, but at the same time there was still some degree of variation. One difficulty experienced was the lower virulence occasionally found of the strain obtained from a particular passage rat, in comparison with that of the strains obtained from those in the series preceding and following it. In the efforts made to improve the technique further the suitability of other experimental animals for the maintenance of the passage strain and for the experimental tests was considered.

The laboratory-bred albino mouse was selected for trial and was found to possess a very regular susceptibility to infection with *B. pestis*, to be immunizable to a very high degree, and to show differential results in response to immunization with varying doses and different types of vaccine, which were of a quantitative character.

In contrast with the wild *R. rattus* which requires a dose of Haffkine's vaccine which is capable of causing toxic mortality in a certain proportion of those used, to produce any considerable degree of immunity, and with which the average immunity obtained is only in the vicinity of 30 per cent to 40 per cent, the laboratory-bred white mouse will show 100 per cent immunity with great regularity against the test dose used, when immunized with non-toxic doses of the vaccine.

The suitable dose for white mice of 20 to 25 grammes weight has been found to be 0·03 c.c. of the standard Haffkine vaccine and full immunity is obtained by the use of this dose. With fresh vaccine full protection is obtained by doses down to 0·007 c.c. in certain series of experiments. The use of the graded doses from 0·03 c.c. downwards shows differential results in the cases of weaker vaccines.

Albino mice have been selected for future work on the plague vaccine on account of the regularity of the results obtained with them. In subsequent sections of this *Memoir* in which the first results of the experimental work in hand are detailed, these were the animals used.

The method of preparation of the infecting dose has been revised in regard to certain points. The primary culture on blood agar is kept at room temperature (27°C.) only and the colony selected for inoculation into broth is picked off on the sixth day. Both the first and the second 48 hours broth cultures are also kept at this temperature. The period of incubation used and the temperature now employed result in somewhat higher counts than were obtained when incubation at 37°C. was used. The same dilutions as formerly are used for test, as the adoption of a dose ten times that of the killing one in the first instance was arbitrary.

CHAPTER X.

The organism.

Preparation of seed material and determination of purity and virulence.

The manufacture of the plague vaccine from passage strains introduces difficulties which are not experienced in the case of vaccines prepared from organisms maintained in culture. When cultures are made from the heart blood of passage rats, for seed purposes, there is always a risk of other organisms which have secondarily invaded the blood stream being present along with *B. pestis*, and the experience has been that these contaminants are very insidious and difficult to detect unless special methods are employed.

Haffkine relied on the typical appearance of the growth of the culture in broth, with the formation of stalactites and the clearing of the rest of the medium, as has already been described, as an indication of purity, and considered that only *B. pestis* was capable of furnishing this characteristic type of growth. This test was carried out at 80°F. The stalactite test was the sole test of purity of the seed material used in Haffkine's time and for many years after. Of the balloons inoculated with the heart blood of passage animals for the purpose of this test, and for subsequent use for the sowing of the vaccine flasks, a considerable proportion—as much as 60 per cent at times—had to be rejected as impure, with a consequent delay in the manufacture of the vaccine. In addition, during the period of incubation many flasks were rejected as showing obvious signs of contamination and there was a further rejection of brews at final testing of purity. Haffkine also relied on naked-eye appearances as a guide to purity of his vaccine at final testing at the end of the incubation period. A characteristic ground-glass appearance of the surface growth on agar when seen by reflected light through the depth of the agar was considered by him to be a reliable test. The Indian Plague Commission in regard to this point stated their opinion that : ' We do not think this method of controlling the purity of a plague culture is one that ought to be generally adopted. We are, however, far from disputing that the character referred to may, in the hands of a bacteriologist who has the special experience of Mr. Haffkine, constitute a trustworthy index of the purity of the plague culture '.

This test continued to be used as a routine for many years and, while obvious contaminations were frequently detected by it, our personal experience has been that great difficulty was frequently experienced in deciding whether or not to pass certain cultures as pure. While many brews were correctly rejected as impure on this test in the light of later experience it appears by no means improbable that vaccine was occasionally passed in which the growth was not pure *B. pestis*. The difficulties and delays due to use of material liable to contamination, under the special conditions of animal cultures adopted, interfered greatly with the regular production of the vaccine and the rejections of doubtful material were a large source of expense. It became necessary to revise the methods used and a special

study of the contaminants obtained from animal cultures was undertaken for the purpose by Dr. Goré, for many years Assistant Director of the Haffkine Institute, on the results of whose work a new technique for purity testing has been based.

Dr. Goré studied in detail those organisms which were found along with *B. pestis* in cultures from the heart blood of passage animals which gave a surface growth on agar indistinguishable from or markedly resembling *B. pestis*. Organisms with these characteristics were found in about 5 per cent of heart blood cultures and consisted of Gram negative bacilli, Gram positive bacilli, and Gram positive cocci. The greater proportion of contaminants belonged to the *coli* group and would have been detected in the old stalactite test by the production of a uniform turbidity in broth culture in 24 hours, which would not subsequently clear.

Certain of the other organisms which have been found as contaminants, if present in small numbers along with *B. pestis*, would not multiply sufficiently at 80°F. (the temperature at which the stalactite test is carried out) to interfere appreciably with the appearance of the stalactite growth, and such material might be used for seed purposes. The final test of purity by naked-eye examination would not suffice for the detection of these contaminants if their growth characteristics on agar resembled those of *B. pestis*. With the possibilities of the occurrence of mixed growths from heart blood cultures it became evident that it was desirable to work with a single colony cultures. The inoculation on to the surface of ordinary agar of a sufficient amount of material to ensure growth and at the same time to attempt to obtain separate colonies results in an irregular growth in which the colonies are of variable size and appearance, and from which it is not always possible to distinguish a colony of *B. pestis* from that of some of the possible contaminants found.

A more reliable method had to be found and for this purpose blood-agar cultures were tried. It had been noticed that when blood cultures were made from human cases of plague by spreading blood taken by vene-puncture on the surface of agar, a very regular development of discrete colonies was obtained. When blood from human cases was spread on the surface of a variety of different media the development of the colonies was markedly regular. This suggested that the presence of blood was especially favourable to the growth of *B. pestis* and responsible for the regular growth. Dr. Goré tested this point by comparative cultures on blood-agar and the acid-digest agar used as a routine for the vaccine, and found that, when a series of dilutions of a broth culture was placed on the surface of these two media the growths obtained were very different. On the blood-agar a growth of discrete colonies was obtained in dilutions down to 1 in 100,000 and a progressively larger number of colonies developed in lower dilutions, the growth becoming confluent at a dilution of 1 in 100 in 48 hours at 37°C. On the acid-digest agar no growth occurred in dilutions below 1 in 100, very rare colonies at that dilution, and a few at 1 in 10 (Plate XVII). Repeated tests confirmed this finding.

Other organisms, including the usual contaminants obtained from passage animals heart blood, were found to grow equally well on either medium at 37°C. in 24 hours.

The regular character of the growth of *B. pestis* on blood-agar is well shown in Plate XVIII. A definite quantitative difference in the growth obtained from different amounts and dilutions is shown in contrast with the failure of growth on acid-digest agar and standard agar.

The application of this test of differential growth proved to be a very useful means of distinguishing *B. pestis* from contaminating organisms and has been shown to differentiate all contaminants which have been come across in the course of cultures from passage animals, and also all others which we have had an opportunity of testing including *B. pseudo-tuberculosis rodentium*.

For the purpose of this test Dr. Goré (1929) has introduced a special type of rabbit-blood agar slope consisting of a layer of blood-agar placed on the surface of an ordinary agar slope, which results in a very marked saving of the expensive portion of the medium.

The method for obtaining a single colony culture and determining the purity of the material adopted as a routine is as follows: Blood is taken from the heart of the passage animal by puncture with a pipette after aseptic post-mortem. One or two drops are placed in 1 c.c. of citrated saline solution. From this a series of dilutions—1 in 10, 1 in 100, 1 in 1,000, and 1 in 10,000—are made by means of standard platinum loops.

A 1 mm. loopful of each dilution is placed on the surface of the superimposed blood-agar, only two tubes being employed and the area of each being divided into two sections by a grease pencil line on the glass. At the same time a 5 mm. loopful of the blood suspension is placed in a peptone-water tube for special tests.

From the peptone-water suspension a 5 mm. loopful is diluted in 10 c.c. saline solution and of this dilution a 5 mm. loopful is put on standard agar and a 1 mm. loopful on blood-agar for a differential test of growth.

The original blood-agar slopes inoculated with the dilutions of heart-blood suspension are incubated at 37°C. for 48 hours at the end of which period small colonies, discrete in one or other dilution, will be present.

If contaminants are present these will be well developed in 24 hours. The cultures are kept for a further 48 hours at room temperature (80°F.) when the colonies of *B. pestis* will be well developed and characteristic. (It must be remembered that *B. pestis* grows best at 80°F. and that incubation at body temperature acts in restraint of growth of this organism). From these cultures a single colony is easily picked off. The purity of the material has meantime been tested by three methods: (*a*) special tests on the peptone-water culture, (*b*) fermentation tests, and (*c*) differential growth, all of which are of definite value.

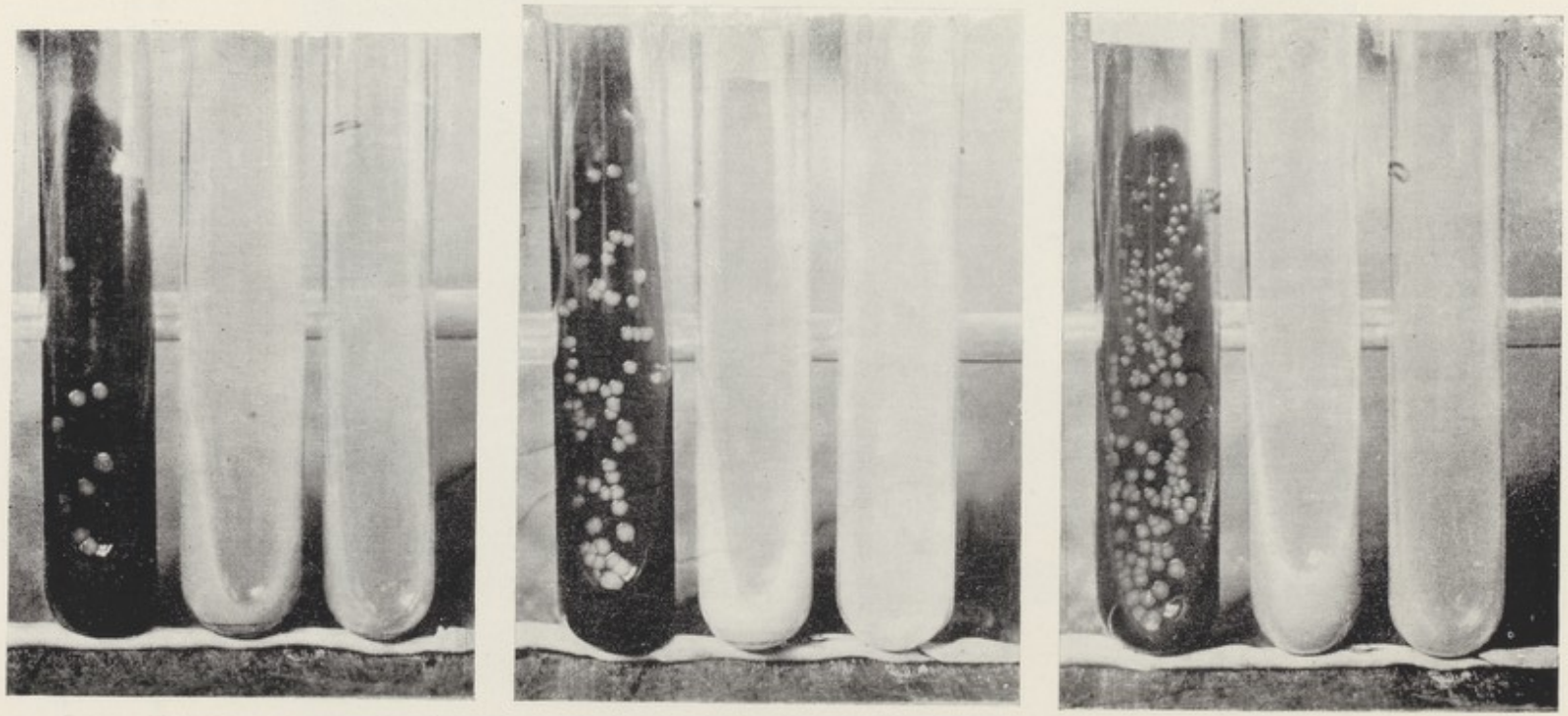
The special tests are those for the production of H_2S, nitrite and indol, *B. pestis* producing nitrite but giving negative results with the other two tests. These tests are carried out by the special technique elaborated by Dr. Goré and all are performed with the one peptone-water culture.

(*a*) *H_2S test.*—The peptone-water tube has a plug of white surgical cotton-wool. This is moistened with a 5 per cent solution of lead acetate before incubating. If H_2S is produced the plug will show a brownish discoloration after 24 hours incubation.

PLATE XVII

Plate XVII.

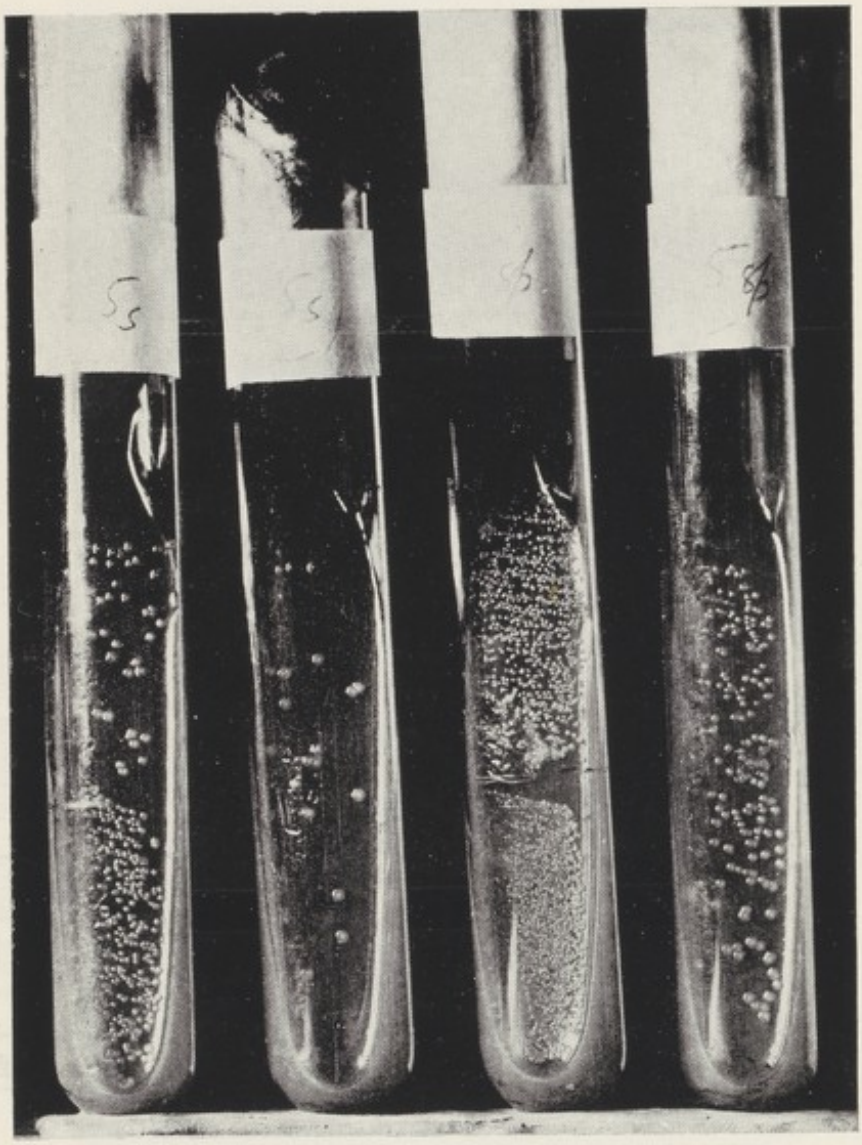
Relative growth of B. pestis *on blood-agar and other media.*



1 2 3

Fig. 1. 2 mm. loopful 1 in 100,000 dilution of broth culture of blood of human plague case.
Fig. 2. 2 mm. loopful 1 in 10,000 do. do. do. do. do. do. do. do.
Fig. 3. 3 mm. do. do. do. do. do. do. do. do. do. do. do. do.
Growth of rabbit-blood agar; no growth on acid-digest agar or standard agar with same quantities.

PLATE XVIII.



1 2 3 4

Regularity of growth of B. pestis *on blood-agar.*

Tubes Nos. 1 and 2. Successive tenth dilutions of passage animal heart blood on rabbit-blood agar: 2 dilutions on Tube 1, 1 dilution on Tube 2.

Tubes Nos. 3 and 4. Ditto, with spleen dilutions.

(*b*) *Nitrite test.*—A 5 mm. loopful is taken from the 24-hour culture and mixed with a 2 mm. loopful of Ilosvay's reagent on an opal glass plate. If nitrite is present a pink colour will develop within a minute or less.

(*c*) *Indol test.*—The outer end of the cotton-wool plug of the 24-hour culture is moistened with Böhme's reagents and the plug inverted in the tube. At the end of a further 24 hours incubation the development of a pink colour on the plug will indicate the production of indol. If by these tests a positive result is given for nitrite and a negative result for H_2S and indol the peptone-water culture is used to inoculate fermentation tubes.

The fermentation tests put up will give the following results with *B. pestis* :—

Glucose	..	..	.. acid.
Mannite	..	..	.. acid.
Laevulose	..	..	.. acid.
Lactose	..	..	.. *nil.*
Saccharose	..	..	.. *nil.*
Dulcite	..	..	.. *nil.*

The presence along with *B. pestis* of contaminating organisms which do not ferment lactose, saccharose or dulcite will not be detected by these tests. An occasional contaminant which has been found is an organism of the *pasteurella* group which ferments saccharose.

The third test, that of differential growth on blood-agar and standard agar, will, if pure, show discrete colonies on the former medium after 48 hours incubation at 37°C. and no growth on the standard agar.

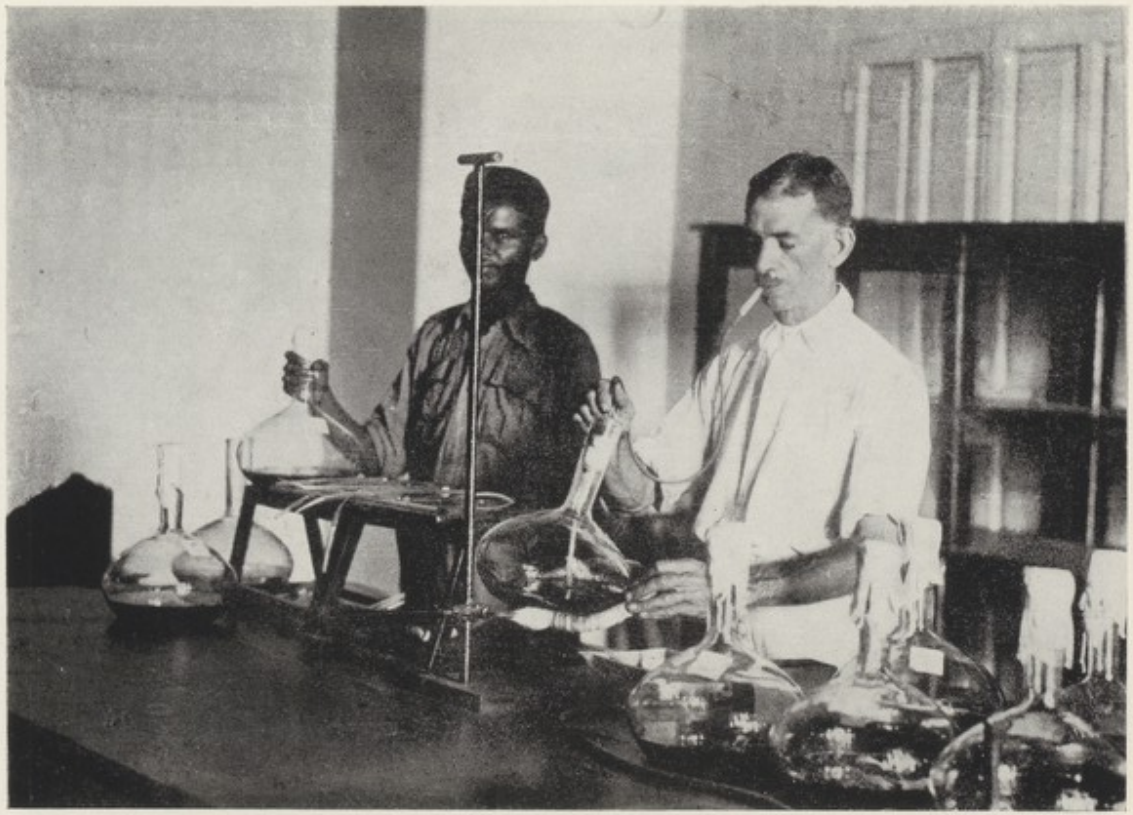
The material having passed all these tests a single colony picked off from one of the original dilutions on blood-agar and inoculated into a peptone-water tube. About 0·5 to 1 c.c. of this suspension is then inoculated into the balloon of acid-digest broth to be used for seeding purposes (Plate III) and kept at 80°F. The peptone-water suspension used for this purpose is submitted to the purity tests and the balloon kept for 3 or 4 days until growth is well developed. The flasks are then sown from the balloon and samples taken for purity tests before and after sowing. If these purity tests for the balloon should show evidence of contamination the flasks sown would be rejected from the incubating room, but with the precautions taken by use of the methods described such rejections do not now occur. Formerly a large number of brews had to be destroyed for obvious contamination during the early stages of incubation.

A final purity test is made from the contents of each flask at the termination of the period of incubation. In the testing room several drops of the culture are poured over an agar slope so as to produce an even and complete surface growth. A small quantity in a test-tube is also sent to the purity testing department. From this latter sample a 1 mm. loopful is placed on blood-agar and a similar quantity on standard agar. As a measure of economy each tube of agar or blood-agar is used to accommodate the cultures from five different brews the area available for growth being divided up by a

series of grease pencil marks on the glass (Plate XX). The brew is passed as pure if no growth occurs on the standard agar in 24 hours at 37°C., while a confluent growth occurs on blood-agar at 48 hours or later, and the heavily seeded agar slope prepared in the testing room shows characteristic appearances. These tests detect the presence of contaminants which develop during the course of incubation in a small proportion of flasks and, without the use of the differential growth test, it has been our experience that the naked-eye examination of the growth in the heavily seeded tube would fail to detect the presence of contamination or mixed growth in many instances. In the case of any doubtful colonies the other purity tests may be applied.

The virulence of the passage strain is controlled by the means of tests on white mice which are inoculated with standard dilutions of cultures prepared in a parallel method to the seed material and a dose of 5 to 10 organisms in a dilution prepared in the manner described in Chapter IX is ordinarily fatal for these animals.

PLATE XIX.



Purity testing. A sample is removed by suction.

PLATE XX.

1 2 3 4

Final purity tests.

Differential test on standard and rabbit-blood agar. Five tests on each pair of tubes.

Incubated at 37°C. for 24 hours. Tubes Nos. 1 and 2 show no visible growth at 24-hours, cultures pure plague. Tubes Nos. 3 and 4 show growth in 2 out of 5 sections—contamination indicated of two flasks of vaccine. (Plague colonies will be seen on the blood-agar only with the quantities used after 48 hours).

CHAPTER XI.

The medium.

Preparation of the medium.—The acid-digest broth, originally introduced by Warden and subsequently improved by Maitland Gibson, has always been used for the manufacture of the vaccine. The reasons for the adoption of a medium of this type for use in India given by Bannerman, which have been quoted in Chapter IV, still hold. Religious prejudices preclude the use of beef, or beef products such as peptone, and the economy which the method of preparation presents, in a laboratory in which up to 10,000 litres may be required annually, is an important consideration. The changes which have been made in the manufacture of the broth from time to time have been well summarized by Naidu and Jung (1927) and have also been indicated in previous chapters. The present method of preparation adopted is as follows :—

The medium is prepared in large bulk, about 200 litres usually being made at a time. A sufficient quantity of goat's flesh is obtained and fat, connective tissue, and gristle removed. The flesh is then put through an electrical mincing machine and minced very fine. To each kilo of minced flesh 80 c.c. of pure hydrochloric acid is added and the mixture placed in large earthenware jars and allowed to macerate in a water-bath at 70°C. for three days. During this process 75 per cent of the available albumen is converted into albumose and peptone. At the end of the three days two litres of boiling water are added for each original kilo of minced flesh and the mixture neutralized to litmus by the addition of 40 per cent KOH solution. The neutralized mixture is then heated at 100°C. in the steam sterilizer without pressure, for one hour, and subsequently filtered through textile filter-paper to remove the heavy precipitate which formed on neutralization. A sample of the filtrate is taken for the estimation of total nitrogen content of the broth and, according to the results of this test, a final dilution with water is made to produce a concentration equivalent to 230 mg. of nitrogen per 100 c.c. The medium is then standardized to pH 6·8 with phenol red as the indicator and passed through special cloth filters. Filtration through filter-paper is made when the broth is finally distributed into flasks for sterilization.

The medium prepared in this way is used not only for the growth of the vaccine but also for general culture work and as a basis for the preparation of agar media used for plague work.

Comparative tests carried out with vaccines prepared with other media such as tryptic-digest, papaya-digest, casein-digest broth and hormone broth have shown that these present no advantages over the easily prepared acid-digest broth ordinarily used. The addition of glucose to the medium is found to render vaccine prepared from it of little or no value.

Tubes Nos. 3 and 4 show growth in 2 out of 5 sections. Contamination indicated by [illegible]
(Plague colonies will be seen on the blood-agar only with the quantities used after 48 hours).

Standardization of the medium.—A medium prepared by the older methods cannot be expected to show the exact composition which is obtained in the case of those prepared by the use of weighed quantities of ingredients such as peptone, meat extract, etc., but in practice it has been found that the variations which occur in the acid-digest broth are within comparatively narrow limits. It has only recently been decided to adopt a method of standardization for the medium and the figure selected as a basis was the total nitrogen content. The examination of a series of successive brews prepared in the usual manner showed that the average total nitrogen content was approximately 230 mg. per 100 c.c. As the result of a comparison of the amount of growth of *B. pestis* occurring in broth of this standard with growth in media adjusted to figures considerably above and below 230 mg., it was shown that the intermediate figure gave the heaviest growth. This was found to occur both with virulent and avirulent cultures. Two hundred and thirty mg. of nitrogen per 100 c.c. has now been fixed on as the standard and the medium for the vaccine is adjusted to this level at final dilution.

Reaction of the medium.—Bannerman (1908) studied the question of the reaction of the broth and came to the conclusion that a reaction slightly on the acid side to phenolphthalein was the most favourable. D'Aunoy (1923) showed that the *B. pestis* had a wide range of growth with an optimum at pH 6·2 to 7·0. With the introduction of the more accurate methods of standardization of media it was found that the older methods employed resulted in variations in reaction of different brews. Examining 100 samples Naidu and Jung (1927*a*) found pH values ranging between 6·6 and 7·4, and in 85 per cent the reaction was between pH 6·6 and 7·0. A special broth was prepared by these workers and portions adjusted to reactions from pH 6·0 to pH 7·2 with intervals of 0·2. Vaccine was made from the media with these differences and tested for potency. The results of the series were irregular but highest protection was given by the vaccine at initial reaction pH 6·8. The proportionate growth of *B. pestis* in media of a corresponding range of reaction was tested by estimation of viable organisms after 48 hours incubation at 27°C. The results were :—

Initial pH.	Millions per c.c.	Initial pH.	Millions per c.c.
5·8	30	6·8	62
6·0	30	7·0	72
6·2	50	7·2	55
6·4	44	7·4	60
6·6	60		

The favourable initial reaction would appear to be pH 6·8 to pH 7·0 and the former reaction is now employed.

During the course of incubation the reaction of the vaccine becomes increasingly alkaline. This had been noted by Bannerman and was further studied by Naidu and Jung (1927*a*) who found a rapid increase in alkalinity during the first three weeks of incubation, a continued rise to the end of the fifth week, and thereafter irregular variations from time to time. The reaction of the vaccine was not found to vary during storage for prolonged periods.

A recent series of tests of changes in reaction carried out by exact methods have given the following results :—

Period of incubation.	At 27°C.		At 37°C.	
	pH.	c.c. N/10 acid or alkali required to neutralize 100 c.c.	pH.	c.c. N/10 acid or alkali required to neutralize 10 c.c.
Before incubating ..	6·8+	1·4	6·8	1·4
3 days.. ..	6·8+	2·0	6·8	2·8
1 week ..	7·4—	3·5	6·8	2·8
2 weeks ..	8·2—	12·0	7·0—	..
3 weeks ..	8·4—	20·0	7·6—	4·3
4 weeks ..	8·4	25·0	7·8	8·0
6 weeks ..	8·4+	27·5	8·0—	10·5
8 weeks ..	8·4+	27·3	7·8+	8·3
12 weeks ..	8·3+	26·8	7·8—	7·4

A comparison of these findings with the results of estimations of growth at various stages given in Chapter XIII will show that the marked change of reaction corresponds with the heavy growth which occurs in the first two weeks, and the subsequent slighter increase in alkalinity, eventually reaching a fixed level, follows the course of the lessened growth which is found in the later weeks of incubation.

CHAPTER XII.

The relative value of the constituents of the vaccine.

The differences in opinion which were at first held as to the value of the separate constituents of Haffkine's plague prophylactic have been fully discussed in an earlier chapter. The method employed by Haffkine for the manufacture of his vaccine resulted in the production of a fluid which differed in many respects from the simpler bacterial suspensions and cultures which had, up to the time of its introduction, been used for the prophylaxis of other bacterial diseases. The protection conferred by vaccines such as those employed for cholera and typhoid fever was attributed to the bacterial bodies present and Haffkine's contention as to the value of the supernatant fluid in his vaccine was not at first generally accepted, especially as he had no definite experimental evidence on this point to begin with. The opinion of the Indian Plague Commission that the supernatant fluid was of no value for immunization and might with advantage be dispensed with has already been quoted along with the evidence on which this finding was based.

The question of the relative value of the sediment and the supernatant fluid in immunization is one which is obviously capable of demonstration without difficulty provided suitable experimental methods are used, and work on this subject has since shown very definitely that the finding of the Commission was exactly the opposite of the true facts. Balfour Stewart (1900) first demonstrated that the supernatant fluid was of immunizing value equal to that of the whole prophylactic and further work on a large scale has confirmed this. Morison, Naidu, and Avari (1924) in a series of protection experiments on *R. rattus* obtained the following results against an infecting dose of plague-rat spleen :—

Protecting dose.	Number of rats used.	Deaths.	Percentage of mortality.
Nil (controls)	184	176	95·65
Broth (0·5 to 2 c.c.) ..	41	39	95·1
Sediment of 0·5 c.c. ..	42	38	90·4
Supernatant fluid of 0·5 c.c. ..	209	111	53·11
Whole prophylactic 0·5 c.c. ..	169	94	55·6

These figures are the combined results of a series of tests with vaccines of different periods of maturity after manufacture.

Naidu, Malone, and Avari (1926) gave the following results of further tests in regard to the value of the constituents :—

Protective dose.	Number of brews tested.	Number of rats used.	Deaths.	Percentage of survival.
(1)				
Nil (controls) ..	..	482	450	8·7
Whole prophylactic ..	18	327	212	35·2
Supernatant fluid ..	18	355	214	39·8
Sediment	14	207	178	14·0
(2)				
Nil (controls) ..	..	184	178	3·3
Whole prophylactic ..	11	168	106	37·0
Supernatant fluid ..	12	223	142	36·4
Sediment	4	42	38	9·6

These figures show that the immunizing value of the supernatant fluid is practically equal to that of the whole prophylactic and that a dose of the sediment of a similar quantity of vaccine produces little or no immunity. The tests have been repeated by means of the revised experimental technique with white mice and standardized infecting doses of *B. pestis* and in every instance when 100 per cent immunity was obtained with the whole vaccine in these animals a similar degree of immunity was reached by the use of the supernatant fluid of the same quantity.

A vaccine of which 0·03 c.c. showed 100 per cent protection in white mice gave the following results with large quantities of sediment :—

Vaccine dose.	Infecting dose.	Number of mice used.	Plague deaths.
Sediment of 0·3 c.c. ..	106 *B. pestis.*	5	2
„ „ 3·0 c.c. ..	„ „	5	..
„ „ 10·0 c.c. ..	„ „	3	..

Only slight protection was shown by the sediment of ten times the amount of whole vaccine or supernatant fluid which gave full immunity. One hundred and three hundred times the dose protected fully. These quantities did not produce any toxic deaths.

The whole value of Haffkine's vaccine resides in the fluid portion, the antigenic fraction of the *B. pestis* responsible for the production of immunity apparently passing into solution. The solubility of the antigenic material is well shown by the work of Flu (1929) who obtained high protection with simple watery extracts of the bacillus. The sediment in the doses used has no protective value. It is possible, and some of the observations made support the idea, that a simple bacterial suspension containing an equivalent total of antigenic material may be of equal value, but such suspensions are likely to be highly toxic.

CHAPTER XIII.

Period and temperature of incubation.

The period of incubation of the vaccine originally employed by Haffkine was six weeks. He fixed this period on the basis of his observations of the stalactite growth and, shaking down successive crops of stalactites, noted that they no longer began to form after the end of six weeks. Bannerman (1908) later on considered that the cessation of growth was due to the increasing alkalinity of the culture during incubation and found that, after neutralization, growth again commenced. The six weeks period was not always adhered to in making the vaccine and when the first large demands occurred a shorter incubation period was frequently adopted to accelerate production. An examination of old registers of manufacture, dealing with a period of many years, shows great irregularity in this respect and it would appear that the principle adopted was to incubate for six weeks as the normal period but (*a*) when there was a heavy demand for vaccine the incubation period might be reduced to as short a period as three weeks, while (*b*) if there was little demand and a large amount of bottled vaccine in stock the flasks which had been sown might be left to grow in the incubation room for periods even up to nine months. The possible effect of these variations in incubation period on the potency of the vaccine does not appear to have been sufficiently realized in the earlier years of manufacture and this point had not been the subject of experimental study. Subsequent work has shown that a very prolonged incubation materially reduces the potency of the vaccine. Stevenson and Kapadia (1924), in a delayed publication on the results of experimental study of this question, carried out in 1911, found that the autolytic changes which occur during incubation result in a diminution of its antigenic value which commences somewhere between the 47th day and the third month of incubation. Attention was drawn to the question of the period of incubation by reports as to the severity of the reaction after inoculation experienced with certain brews in 1909. On examining the details with regard to the brews complained of the severe reactions were found to be associated with a short period of incubation. To test this point the members of the staff of the Laboratory who were due to undergo their annual inoculation were divided into three groups and inoculated with brews of different periods of incubation or periods of storage after manufacture. The following are the details of the test :—

Vaccine used.	Number of persons.	Number whose temperatures were				Local reaction.
		Below 100°F.	100°F. to 101°F.	101°F. to 102°F.	Above 102°F.	
Incubated 2 months Used immediately	29	3 (10·3 per cent)	10 (34·7 per cent)	10 (34·7 per cent)	6 (20·6 per cent)	Very severe.
Incubated 2 months Stored 4 months	39	10 (34·6 per cent)	17 (43·6 per cent)	8 (20·3 per cent)	4 (10·3 per cent)	Severe.
Incubated 4 months Used immediately	26	7 (26·9 per cent)	9 (34·6 per cent)	6 (23·1 per cent)	4 (15·4 per cent)	Slight.

In the *Annual Report of the Laboratory* (1909) the comment of the Director on this test is as follows :—

'From personal observation of these men it was quite evident to those who were inoculated with the vaccine which had been incubated for the long period of four months suffered least and next to them were those who received the vaccine of short incubation period but which had been kept after sterilization for four months, while those who had the highest temperatures and most severe local reaction were the first group the members of which had been vaccinated with vaccine of short incubation and which had not been kept at all. It is true that the figures are too small to satisfy a statistician but the experience was sufficient to satisfy both the experimenter and those experimented on.'

As a result of this experience a rule was made that all vaccine should be incubated for four months before being sterilized and bottled. This procedure, although effective as a method of reducing the reaction after inoculation, was not based on any considerations as to the effect of such prolonged incubation on the immunizing value of the vaccine, and no work on this point had been done up to that time.

In 1911 reports of severe reactions were again received, and the reactions in this case shown to be associated with the use of freshly bottled vaccine. It became necessary on this account to determine the effect of the two separate factors, (*a*) period of incubation, and (*b*) period of storage.

Investigations into these points were carried out by Stevenson and Kapadia (1924). The experiments were done with the susceptible Madras rats, selected doses of vaccines of different periods of incubation and storage being given and a measured infecting dose of plague-rat spleen administered at a later period. A series of brews of vaccine prepared by incubation for periods varying from six weeks to eight months were tested for potency after periods of storage between ten days and two years. The results showed that the highest degree of immunity was obtained with vaccines of short incubation period, from six to eight weeks, and that, even after storage for over a year from the date of manufacture, such vaccines gave results superior to those prepared by more prolonged incubation. These findings indicated that mitigation of the reaction on inoculation would best be obtained by storage of the vaccine for some period before issue, and that, when prolonged incubation was employed in order to attain this result, the lessened reaction would be obtained at the expense of immunizing value. Stevenson and Kapadia considered that length of storage did not diminish the potency of the vaccine as rapidly as it did its toxicity.

On the basis of the results of this work the incubation period which was to be employed was changed to two months and a policy was adopted of storing the vaccine for several months before issue. If it became necessary to issue fresh vaccine instructions were given for the reduction of the adult dose to 3 c.c. when used within three months of manufacture.

Haffkine's method of preparing the vaccine was designed to produce the maximum amount of growth of *B. pestis* in the medium used. The observations which have been quoted as to the reduction in potency on prolonged incubation suggested the advisability of determining the rate of growth of the organism and the shortest period of incubation which was really necessary to produce a vaccine of high immunizing value. Further information on this point has been obtained by estimations

of the total growth which occurs at various periods by comparative tests of the immunizing value of vaccines made from cultures incubated for different periods. Two methods have been employed in estimating the rate of growth : (*a*) the counting of viable organisms by plating dilutions on blood-agar, and (*b*) the estimation of dry weight of sediment.

Counting by plating presents considerable difficulties after the first few days of incubation owing to the clumping of the organisms which occurs. The results obtained by such estimations are very irregular but give a general indication of the proportionate growth at different stages. The following are examples of the progress of growth observed :—

Period of incubation.	Viable organisms in millions per c.c.		
	(1)	(2)	(3)
3 days	177	59	Not done
1 week	310	330	370
2 weeks	65	175	525
3 „	29·5	65·5	71
4 „	11	67	32
5 „	Not done	11	Not done
6 „	Not done	7·3	0·2

A series of counts made on vaccines of six weeks or longer incubation have given the following figures of viable organisms in millions per c.c. :—

Six weeks.— 2·0, 2·1, 1·8, 3·0, 4·2, 2·4, 3·9, 1·7.
Seven weeks.—2·0, 2·2, 2·3.
Eight weeks.—1·5, 1·3, 1·2.

The results obtained by the estimation of dried sediment are somewhat more regular, but with the quantities which it is possible to deal with by separation in a high-speed centrifuge the slightest clumping may lead to variations at final weighing.

The following figures of two estimations are sufficiently regular to give an indication of the growth occurring :—

Period of incubation.	Mg. of dry sediment per 100 c.c.	
	(1)	(2)
3 days	9	6
1 week	19	38
2 weeks	60	36
3 „	65	64
4 „	67	63
6 „	66	64
8 „	66	..

Differences in the progress of the growth in the earlier stages are suggested in these two examples but by the end of the third week the total sediments correspond closely. No perceptible addition to the sediment is shown after the fourth week but it is possible that a certain amount of antigenic substance may continue to pass into solution at this period.

The results, both of the counts of viable organisms and of the estimations of sediment, indicate that such extra growth as may occur after the fourth week can produce only a small fractional addition to the total bacterial substance developed in the vaccine cultures. This would suggest that so far as quantity of antigenic substance in the vaccine is concerned there is no special advantage to be gained by more prolonged incubation.

Naidu, Malone, and Avari (1926) carried out an extensive series of tests of potency on forty brews of vaccine prepared by incubation for periods varying from one to twenty weeks, using the older technique for estimating the immunity produced in *R. rattus*. They found that there was a progressive increase in immunizing value up to a period of 29 to 35 days incubation after which, until the end of the 13th week, the value of the vaccine remained fairly constant. Further incubation showed the commencement of a drop in potency. The toxicity of the vaccine, which has been found to be closely related to its immunizing value, was highest when a period of 28 days had been used for incubation, and a distinct drop in toxicity occurred after twelve weeks incubation.

As the result of these observations on growth and potency of the vaccine the incubation period which had been reduced from two months to six weeks was further reduced to four weeks. A series of checks of the potency of the vaccine carried out on white mice with vaccine incubated for 28 days gave the results shown in Table XXII, 100 per cent protection being obtained with the standard dose of 0·03 c.c. in these animals. Equal protection was obtained when one-half and one-quarter of this amount of vaccine was used within a month of manufacture.

TABLE XXII.

Tests of immunizing value of successive brews of vaccine, incubated for four weeks.

Number and age of brew.	Dose of vaccine.	Infecting dose.	Number of mice used.	Toxic deaths.	Plague deaths.
No. 7643, age 23 days ..	0·03 c.c.	78 *B. pestis*	4	1	*nil*
	0·015 c.c.	,,	4	*nil*	*nil*
	0·007 c.c.	,,	4	*nil*	*nil*
Toxicity	0·3 c.c.	*nil*	3	2	..
No. 7853, age 18 days ..	0·03 c.c.	78 *B. pestis*	4	*nil*	*nil*
	0·015 c.c.	,,	4	*nil*	*nil*
	0·007 c.c.	,,	4	*nil*	*nil*
Toxicity	0·3 c.c.	*nil*	3	3	..

TABLE XXII—*concld.*

Number and age of brew.	Dose of vaccine.	Infecting dose.	Number of mice used.	Toxic deaths.	Plague deaths.
No. 7946, age 13 days ..	0·03 c.c.	78 *B. pestis*	4	*nil*	*nil*
	0·015 c.c.	,,	4	*nil*	*nil*
	0·007 c.c.	,,	4	*nil*	*nil*
	0·3 c.c.	*nil*	3	2	..
Controls	*nil*	78 *B. pestis*	3	..	3
Supernatant fluid of vaccines.					
No. 7643	0·015 c.c.	78 *B. pestis*	4	*nil*	*nil*
No. 7853	0·015 c.c.	,,	4	*nil*	*nil*

The temperature of incubation (80°F.) adopted by Haffkine for the growth of cultures for his vaccine fulfils the requirement of producing maximum growth. This temperature is, as previously stated, average room temperature in Bombay during the greater part of the year, and an incubating room was easily arranged for in which only the slightest daily variations of temperature would occur except in the hot weather.

A temperature of 80°F. (approximately 27°C.) has been found to be the optimum one for growth of *B. pestis* while restraining the growth of many other organisms, including those of the *pasteurella* group, which have been tested. At 37°C. a much less rapid growth of *B. pestis* takes place as is shown by the following comparative figures of viable organisms present, and sediment formed, at various stages when the same strain of *B. pestis* was used :—

Period of incubation.	Viable organisms in millions per c.c.		Mg. of dry sediment per 100 c.c.	
	at 27°C.	at 37°C.	at 27°C.	at 37°C.
1 week	370	90	19	6
2 weeks	525	56	60	14
3 ,, ...	71	31	65	21
4 ,, ...	32	10	67	15 (?)
6 ,, ...	0·2	0·3	66	25

On solid media *B. pestis* also shows slower growth at 37°C., this point being a useful one for the purpose of differentiation.

An important point in regard to the temperature of incubation employed has recently been raised by Schutze (1932) as the result of his observations on the

antigenic fractions of *B. pestis*. Schutze considers that a heat-labile antigen, associated with the presence of a proportion of organisms showing the formation of a gelatinous envelope, is the main factor in immunization. He finds that envelope-formation is more marked at 37°C. than at 27°C. and his tests of the immunity produced by vaccines prepared at these temperatures gave better results when the higher temperature of incubation was employed. Preliminary observations on this point at the Haffkine Institute have not so far corroborated Schutze's findings and there are differences as to the strains used and the experimental methods adopted which make direct comparison of results difficult. The question is obviously one of the highest importance especially in view of the lower toxicity of the vaccine incubated at 37°C. noted by Schutze. Further work will be required to settle this question.

In a previous chapter the loss of virulence which occurs during incubation in a fluid medium at 37°C. has been shown and, although the conditions of growth were not exactly the same as in the case of vaccine under incubation, the results of the tests would suggest that some degree of loss might be expected if vaccine was incubated at this temperature. Such loss in virulence might have an influence on the potency of the vaccine. In the present state of our knowledge it appears advisable to adhere to the use of a temperature of 27°C. for incubation.

CHAPTER XIV.

The relation of virulence to potency.

Haffkine laid down as a principle that the vaccine should always be made from virulent strains of *B. pestis*. He obtained fresh strains as frequently as possible from human cases and, in the earlier days in Bombay, had no difficulty in doing this. When human fresh strains were not available he advocated the maintenance of the virulence of cultures by the use of passage animals. In giving evidence before the Indian Plague Commission, Haffkine showed that owing to laboratory difficulties he was not always able to carry out such passages and attributed the less satisfactory results obtained by the use of certain brews of vaccine to the use of culture strains. It does not appear that Haffkine's advocacy of the use of virulent strains was based on experimental evidence.

The Commission in their *Report*, when dealing with this subject, quoted the finding of the German Plague Commission that no protection was obtained in monkeys by the use of vaccines made from avirulent strains and stated their opinion that, until evidence was obtained as to the possibility of immunity being produced by the use of vaccines prepared from avirulent strains, the use of virulent strains should be adhered to. Liston at the same period had shown that virulence was easily maintained by animal passage.

Bannerman in the *Annual Report of the Laboratory* for 1905 recorded his observation that growth on agar for 23 days at Bombay room temperature (27°C.) lowered the virulence of a strain of *B. pestis* 250 times, and laid down that, for seed purposes, the material used should be prepared by one remove from the animal source to broth. This procedure was employed in Bombay until the recent introduction of the method of single colony culture from blood-agar.

Strong (1907) obtained a high degree of immunity in experimental animals with living attenuated cultures but in the course of his work found that animals which survived an infecting dose of a virulent strain of *B. pestis* showed themselves to be highly immune to a subsequent infection, while those which had received an injection of an avirulent strain (not an immunizing dose) showed no immunity when tested. Using two strains, one of which was of very low virulence and the other of only slightly higher virulence, he found that the survivors of those inoculated with the latter strain showed distinctly higher immunity.

The effect of virulence of the strain used in the preparation of the vaccine on the immunity produced was tested by Naidu, Malone, and Avari (1926) by preparing a vaccine from an old strain kept in agar culture, a comparison being made with the results obtained with a vaccine made from the same strain raised to higher virulence. The original strain had not entirely lost virulence and produced a mortality of 20 per cent in rats when given in a fairly heavy dose standardized to a fixed opacity of a suspension. After forty-one successive passages in rats a corresponding dose of the culture made from the strain enhanced in virulence in this way produced 86·6 per cent mortality. The results obtained with vaccines

prepared from the avirulent and virulent cultures are extracted from the original paper :—

Material.	Number of experiments.	Number of rats used.	Deaths in 7 days following inoculation.	Survivors.	Total deaths in 15 days following infection.	(Survivors) percentage immunity.
Old culture, 5 years ..	2	50	1 (2 per cent)	49	40	18·4
Old culture raised to virulence.	2	50	6 (12 per cent)	44	24	45·5
Untreated controls ..	..	..	..	20	18	10·0

The higher toxicity and immunizing value of the vaccine prepared from the virulent culture is demonstrated by these figures. The infecting dose in this experiment was a suspension of weighed quantity of plague-rat spleen. A later test of this point has recently been carried out by a different technique and with a more accurate measurement of dosage and virulence. The strain selected for experiment was one which was found to kill 100 per cent of white mice in a dose of 55 organisms. A series of fifty-seven subcultures on blood broth was made at the end of which a dose of twelve and a half million bacilli failed to kill. Vaccines were then made from the virulent strain maintained in animal passage and from the avirulent culture strain. The results of tests of their immunizing value were as follows :—

Vaccine.	Dose.	Infecting dose.	Number of white mice used.	Plague deaths.
From avirulent culture	0·03 c.c.	55 *B. pestis*	5	4
	0·06 c.c.	,, ,,	5	4
	0·5 c.c.	,, ,,	5	2
From virulent passage strain.	0·03 c.c.	,, ,,	4	..
	0·03 c.c. of supernatant fluid.	,, ,,	5	..
Controls	..	,, ,,	2	2

Full protection was obtained by the use of the vaccine prepared by use of the virulent strain and little or no protection with that from the avirulent culture even when a much higher dose was given. Burgess (1927) has also found that a much greater protection was given by the use of vaccines made from virulent strains.

It has always been the practice in the Haffkine Institute to use strains of the highest virulence attainable for the vaccine but it is only in recent years that this has been measured with any degree of accuracy. In Chapter IX the standard of virulence which the strains employed reach has been indicated and strains of lesser virulence are not now used for manufacture. The regular tests of virulence which are made ensure the suitability of the seed material in this respect. In addition to

Bannerman's finding as to loss of virulence in culture subsequent observations made on this point, especially since the introduction of accurate methods of testing have shown the degree to which it occurs on different media and at different temperatures.

The results of a recent series of tests of the effect on virulence of growth of *B. pestis* in broth, agar, and blood-agar at room temperature (27°C.) and at 37°C. are given in Tables XXIII, XXIV and XXV.

Commencing with a strain of which four organisms produced 100 per cent mortality in white mice and 45 organisms (the smallest dose used) gave a similar mortality in *R. rattus* the virulence at successive stages of weekly subculture was estimated by the use of varying doses. In the case of the cultures in broth it was shown that only a slight loss of virulence had occurred after the 12th subculture at room temperature but after the 16th subculture a considerable loss was shown. At 37C. the broth cultures failed to kill after the 4th subculture in doses which produced 100 per cent mortality with that kept at room temperature. At later periods of subculture at this temperature doses several thousand times the original killing dose could still be made to infect. The cultures on agar at room temperature showed a great loss of virulence by the 11th subculture and were avirulent in many times the original dose by the 14th. At 37°C. there was a marked loss in virulence by the 4th subculture, several times the killing dose of the unincubated culture not infecting. In blood-agar at room temperature virulence was maintained fairly well up to the 20th subculture although there were indications of slight loss of virulence at this stage. At 37°C. the blood-agar cultures showed marked loss of virulence at the 6th subculture and were entirely avirulent in subsequent tests in doses many thousand times that of the original fatal number of organisms.

TABLE XXIII.

Effect of growth in broth on virulence of B. pestis.

Original virulence of culture: 4 B. pestis *killed* 100 *per cent white mice.* 45 B. pestis *killed* 100 *per cent Madras* R. rattus. *Subcultures made at weekly intervals and incubated at* (a) *room temperature, and* (b) 37°*C.*

Tested at	Cultures at room temperature.			Cultures at 37°C.		
	Dose of *B. pestis.*	Animals used.	Plague deaths.	Dose of *B. pestis.*	Animals used.	Plague deaths.
31 days (4th subculture) ..	4	3 mice	3	4	3 mice	..
	45	5 *rattus*	5	43	5 *rattus*	..
80 days (12th subculture)	6	3 mice	2	45,000	5 *rattus*	3
	650	3 mice	3	..	..	..
	65	5 *rattus*	4	..	..	..
	650	5 *rattus*	5	..	..	..
116 days (16th subculture)	8	4 mice	2	75,000	5 *rattus*	4
	800	4 mice	3	750,000	5 *rattus*	5
	8,000	5 *rattus*	2	..	..	..

TABLE XXIV.

Effect of growth on agar on virulence of B. pestis.

Original virulence of culture: 4 B. pestis *killed* 100 *per cent white mice.* 44 B. pestis *killed* 100 *per cent Madras* R. rattus. *Subcultures made at weekly intervals and incubated at* (a) *room temperature, and* (b) 37°*C.*

Tested at	Cultures at room temperature.			Cultures at 37°C.		
	Dose of *B. pestis.*	Animals used.	Plague deaths.	Dose of *B. pestis.*	Animals used.	Plague deaths.
32 days (4th subculture) ..	2	2 mice	2	50	2 mice	..
	20	4 *rattus*	2	101	4 *rattus*	..
	205	4 *rattus*	4	1,010	4 *rattus*	..
54 days (7th subculture) ..	68	10 *rattus*	9	..	..	..
81 days (11th subculture)	7	3 mice	1	..	..	..
	7	5 *rattus*	..	..	..	..
	67	5 *rattus*	2	..	..	..
	670	5 *rattus*	1	..	..	..
102 days (14th subculture)	500	5 mice	1	..	..	..
	2,000	10 *rattus*	..	..	..	..

TABLE XXV.

Effect of growth on blood-agar on virulence of B. pestis.

Original virulence of culture: 3 B. pestis *killed* 100 *per cent white mice. Subcultures made at weekly intervals and incubated at* (a) *room temperature, and* (b) 37°*C.*

Tested at	Cultures at room temperature.			Cultures at 37°C.		
	Dose of *B. pestis.*	Animals used.	Plague deaths.	Dose of *B. pestis.*	Animals used.	Plague deaths.
45 days (6th subculture) ..	6	3 mice	3	5	3 mice	2
	6	5 *rattus*	4	5	5 *rattus*	..
	60	5 *rattus*	5	54	5 *rattus*	3
79 days (11th subculture) ..	7	3 mice	3	92	3 mice	..
	7	4 *rattus*	3	92	5 *rattus*	..
	75	4 *rattus*	4	925	5 *rattus*	..
112 days (16th subculture)	12	3 mice	3	14,450	5 *rattus*	..
	12	5 *rattus*	3	144,500	5 *rattus*	..
	121	5 *rattus*	2	..	..	..
	1,210	5 *rattus*	4	..	..	..
142 days (20th subculture)	7	3 mice	2	..	..	..
	7	5 *rattus*	..	..	..	..
	75	5 *rattus*	4	..	..	..

The question of the retention of virulence of *B. pestis* in culture has been the subject of a special investigation by Francis (1932) who refers to the earlier experiences of McCoy and Wilson who found cultures to be virulent after periods up to 10 years under certain conditions of storage. Their results indicate that virulence of some degree may be retained by storage at a low temperature without subculture. Francis' own experiments on this point confirm these findings, a culture kept at 10°C. for nine years without transfer being found virulent at the end of that period while a corresponding strain subcultured on agar at three-monthly intervals had lost its virulence. In regard to this work there is no indication of a quantitative test having been used based on a dosage of definite bacterial content.

The question of the suitability of culture strains of *B. pestis* for the preparation of anti-plague vaccine, in relation to maintenance of virulence, hardly arises in the Haffkine Institute where, with a very large output of vaccine, seed material is required twice weekly throughout the year, and continual animal passage is justifiable and represents little extra work and expense. A laboratory engaged in only occasional manufacture might have to consider the use of strains kept on artificial media and the observations on maintenance of virulence which have been quoted indicate the method which might be employed. If stored at low temperature without transfer cultures would probably, after one or two animal passages, be of suitable virulence for use for seed. Estimation of the virulence in the manner which has been described would be advisable.

It is not possible to lay down an exact measure for the virulence which the organism must possess for the purpose of producing a vaccine of the highest potency but, as the result of certain comparisons which have been made, there are indications that the high standard of virulence of the strains used in Bombay may be responsible in some degree for the efficacy of the vaccine as shown by the results of inoculation. A direct comparison of the virulence of the strains used in the Haffkine Institute with those used elsewhere cannot be made owing to the likelihood of changes in virulence in transport, but the results of experimental work and the comparison of dosage used in different countries for test purposes might suggest certain differences. Less than 100 organisms is always a killing dose for *R. rattus* with the strains used in Bombay. Rowland (1910) used three million organisms as a test dose of what he considered to be a virulent strain, in his work on nucleoproteid vaccines, to obtain 90 per cent mortality in white rats. Schutze (1932) used doses of 37,000,000 and 600,000 in recent experiments for test. Rowland found on one occasion a sudden increase in virulence of his strain to a level at which he considered no protection could possibly be obtained. It is a matter for speculation as to whether his strain had not then become of the order of the virulence commonly maintained in Bombay.

The importance of the use of virulent strains of *B. pestis* for the preparation of the vaccine has been demonstrated and it would appear sound that strains of the highest virulence obtainable should be used for this purpose. The maintenance of virulence is best obtained by the use of continuous animal passages.

CHAPTER XV.

The relation of toxicity to potency.

Haffkine's plague vaccine is toxic for man and animals in varying degree in the doses required for immunization. The supernatant fluid of the vaccine is both the toxic and the immunizing fraction. The doses used for man were originally fixed on the basis of toxicity and Haffkine considered that a definite degree of toxic reaction was desirable and necessary for effective immunization. In the case of experimental animals the dose of the vaccine required to immunize may be found to cause fatal toxic effect in a certain percentage of the animals of one species used, while full immunity may be produced in other species without evidence of any toxic action. The differences in this respect are well brought out by Flu (1929) who has summarized the results of experiments with several species as under :—

Species.	Number of animals.	Percentage of mortality before infection.	Number of survivors tested.	Percentage of survival.	Controls.	
					Number.	Deaths from plague.
Guinea-pigs ..	38	13·3	33	63·6	12	12
Wild rats ..	48	45·7	25	28·0	22	22
Rabbits ..	10	10	9	100·0	2	1
Monkeys ..	20	0	20	85·0	4	2
White rats ..	30	10	21	100·0	30	30

The wild *R. rattus* which has chiefly been used for experimental work with the vaccine in Bombay is extremely susceptible to its toxic action. As the results of many years' experience the effective immunizing dose for these animals has been fixed at 0·5 c.c. Naidu and Jung (1929*a*) have specially studied this point and fixed $\frac{1}{8}$th to $\frac{1}{16}$th of the human dose as suitable. De Smidt (1927) accepts the same figure on the basis of other observations. When this dose of 0·5 c.c. is used for the immunization of trapped *R. rattus* a certain proportion die from the toxic effect of the vaccine and show characteristic changes in the liver and spleen, somewhat similar to those found in acute plague but without the presence of *B. pestis*. This toxic mortality is frequently in the neighbourhood of 20 per cent to 30 per cent. Even with a dose producing such a degree of mortality the immunity obtainable in wild *R. rattus* is of a low order.

If lesser doses than 0·5 c.c. of the vaccine are used a still lower degree of immunity is found on subsequent testing. In an extensive series of tests Morison, Naidu, and Avari (1924) comparing the toxic and immunizing effects of 0·5 c.c. of the vaccine, and of the supernatant fluid and sediment of the same quantity, noted the occurrence of the following percentage of deaths due to toxic action :—

Whole prophylactic	35·04 per cent	(274 rats used).
Supernatant fluid	19·78 ,,	(273 ,, ,,).
Sediment	6·6 ,,	(45 ,, ,,).

Forty-five control rats given 0·5 c.c., 1 c.c. and 2 c.c. of broth and kept under the same conditions showed average mortality of 8·8 per cent. (Irregular mortality is frequently experienced in trapped rats used for experiments especially after handling).

The survivors of these experiments when given an infecting dose of plague-rat spleen showed recovery rates of 44 per cent, 47 per cent and 10 per cent respectively as compared with 5 per cent in controls. Naidu, Malone, and Avari (1926) using a dose of 0·5 c.c. of the whole vaccine for the immunization of 1,015 *R. rattus* found a mortality of 23·3 per cent to occur within seven days of inoculation. The survivors showed 33·9 per cent immunity. Repeating the tests with the fractions of the vaccine, the toxic deaths occurring were :—

Whole prophylactic ..	..	28·2	per cent (456 rats used).
Supernatant fluid ..	..	21·4	,, (452 ,, ,,).
Sediment	..	8	,, (225 ,, ,,).

When subsequently infected the relative immunity of the survivors was respectively 35·2 per cent, 39·8 per cent and 14 per cent as compared with 8·7 per cent for controls with the dosage given.

Naidu and Jung (1929*a*) compared the protective value of doses of 0·25 c.c. and 0·5 c.c. of ten successive brews of the vaccine and found that the lower dose resulted in the production of a lesser degree of immunity, the respective figures for the groups of 300 each given 0·25 c.c. and 0·5 c.c. being 28·1 per cent and 37·0 per cent; the toxic mortality in this series was low. The same tests repeated with guinea-pigs showed no perceptible immunity as the result ot the inoculation of 0·25 c.c. while some degree of immunity was produced with 0·5 c.c. No toxic mortality occurred in the guinea-pigs as the result of these doses.

White mice differ from wild *R. rattus* in their susceptibility to the toxic action of the vaccine and can be fully immunized by a dose equivalent to that used for *R. rattus*, in proportion to their body-weight, without the occurrence of any toxic effect. The dose usually required to immunize white mice is 0·03 c.c. and this will as a rule give 100 per cent survival against the standard test dose of *B. pestis*. Ten times this dose is needed to produce toxic deaths. The following is an example of a recent test of immunizing and toxic effect in these animals :—

Vaccine.	Dose.	Infecting dose.	Mice used.	Toxic deaths.	Plague deaths.
Brew 5998 ..	0·03 c.c.	103 *B. pestis*	5	..	..
,, 7112 ..	,,	,,	5	..	1
,, 7293 ..	,,	,,	5	..	..
,, 7298 ..	0·3 c.c.	*nil*	4	1	..
,, 7112 ..	,,	,,	4	2	..
,, 7293 ..	,,	,,	3	3	..
Controls ..	..	103 *B. pestis*	3	..	3

These differences in susceptibility to the toxic action of immunizing doses of the vaccine make it necessary to consider the question of the relationship of toxicity to immunity from another point of view. In Chapter V it has been shown

that the toxic action, as indicated by the reaction occurring on inoculation into human beings, is most severe when the vaccine has been brewed for a short period and when it is used immediately after preparation. Stevenson and Kapadia's results indicate that the conditions which result in high toxicity are those which also produce the most potent vaccine. They assign the highest value to vaccine prepared by a short period of incubation, which will be most toxic to start with, and find that such vaccine, although its toxicity diminishes with storage, remains potent for a prolonged period.

A recent detailed investigation of the effect of the period of incubation on the toxicity and potency of the vaccine was carried out by Naidu, Malone, and Avari with vaccines especially prepared for the purpose. A series of brews made from four different strains of *B. pestis* were used for the manufacture of the vaccine after incubation at periods from one week and upwards, and their toxicity and potency tested on *R. rattus*. It was found that the highest toxic mortality was produced by that incubated up to four weeks. The toxicity on more prolonged incubation dropped somewhat, but continued fairly high until the end of three months' incubation, thereafter dropping considerably. The immunizing value was highest with vaccine of five weeks' incubation period

Naidu, Malone, and Avari have summarized the figures relating to tests made with vaccines prepared by six weeks' incubation (approximately) and kept in storage for varying periods up to eighteen months. The results with *R. rattus* show a certain degree of reduction of toxicity after the first month of storage but throughout the rest of the period a high average toxicity is maintained resulting in a mortality of about 20 per cent, and accompanied by maintenance of potency, which only begins to diminish somewhat after a year of storage. The results of these observations indicate that toxicity and potency are closely related although they do not run exactly parallel under the conditions of test.

An interesting sidelight was thrown on the question of the relationship of toxicity to immunity in the course of tests of vaccines consisting of suspensions of agar-grown cultures prepared in other Institutes. The tests were done on white mice by an exact standard technique and gave the following results :—

Vaccine.	Dose.	Number of mice used.	Toxic deaths.	Infecting dose.	Plague deaths.
L. I. ..	0·1 c.c.	4	..	86 *B. pestis*	..
	0·03 c.c.	4	..	,,	..
	1·0 c.c.	3	3	*nil*	..
P. I. ..	0·1 c.c.	4	..	86 *B. pestis*	4
	0·3 c.c.	4	..	,,	4
	1·0 c.c.	3	..	*nil*	..
Controls ..	..	5	..	86 *B. pestis*	5

The L. I. vaccine was a much heavier suspension and was both toxic and of high potency. The P. I. vaccine under the conditions of test was non-toxic and did not protect.

Rowland (1910) working with 'derived' antigens of the *B. pestis* found that soluble substance extracted by two methods was both toxic and of immunizing value while the remaining bacillary substance was non-toxic and of no value. Vaccines prepared from avirulent strains are non-toxic and of little protective value. Naidu, Malone, and Avari tested this point with vaccines prepared from a strain of low virulence and the same strain made virulent by a series of animal passages. The comparative results were :—

Vaccine.	Percentage of deaths within 7 days of inoculation.	Percentage of survival after infection.
Avirulent ..	2	18
Virulent ..	12	45
Controls ..	..	10

Other tests with white mice have shown the low toxicity of avirulent vaccines.

Although white mice are easily immunized by doses of the vaccine which are non-toxic for them the comparative value of vaccines prepared in different ways has been found to be the same for these animals as for others. Vaccines prepared from virulent strains, by short incubation, or used freshly, which have been shown to be the most toxic for other animals, are found to be of the highest immunizing value for white mice.

The results of animal experiment indicate a close relationship between the toxicity and the potency of the vaccine and, so far, not only the Haffkine vaccine but other vaccines for plague, which have been proved to be effective, have also been found to cause definite reaction of a toxic nature when used in dosage for man suitable for immunization.

CHAPTER XVI.

The effect of storage on potency.

The question of the effect of storage on the potency of the vaccine is one of importance in view of the possibility, which has to be considered, of deterioration which might occur as the result of exposure to high temperatures in India. The keeping properties of the vaccine are of special interest to Public Health authorities in relation to the stocks they require to keep in hand and to the laboratory in regard to the policy to be adopted in production. The potency of vaccine kept for various periods at room temperature was tested on rats by Stevenson and Kapadia (1925) in 1911 and it was found that vaccine prepared by a short period of incubation maintained a high degree of potency for over a year from the date of manufacture. Vaccines for which over three months' incubation had been employed showed a lower protective value after prolonged storage.

A detailed investigation into the effect of storage on potency was carried out by Naidu, Malone, and Avari with vaccines of the short period of incubation which has been adopted for some years. Their results were as follows :—

Stored for days.	Number of experiments.	Number of brews used.	Number of rats used.	Deaths in 7 days after inoculation.	Survivors.	Deaths after infection.	Percentage of immunity.
1—29 ..	18	10	442	150 (33·9 per cent)	292	181	38·1
31—56 ..	14	9	248	49 (19·7 ,, ,,)	199	133	33·2
63—89 ..	10	6	170	41 (24 ,, ,,)	129	73	43·5
99—180 ..	11	7	191	40 (20·9 ,, ,,)	151	91	39·8
185—336 ..	23	12	396	80 (20·2 ,, ,,)	316	206	34·9
364—546 ..	10	7	259	50 (20·9 ,, ,,)	189	132	30·2

The figures of percentage immunity show the irregularities incident on the variations in dosage of *B. pestis* which may occur when a plague-rat spleen emulsion is used for infection, but their general level shows a satisfactory retention of potency up to the end of a year from date of manufacture and only a slight fall up to eighteen months. The standard of immunity indicated is very similar to that which has been observed in routine testing of recently prepared vaccine by the same experimental technique.

A special test of the effect of storage on the vaccine was undertaken in 1927. A series of brews, all made from the same strain of *B. pestis*, by incubation for 64 days was prepared and finally sterilized and bottled on 17th January. The potency of the brews was tested on 11th February and a survival rate of 47·6 per cent was

TABLE XXVI.

Test of potency of vaccine stored in Jullundur and in Haffkine Institute, Bombay, for various periods after manufacture.

Box number.	Brew number.	Date of test.	Rats used.	Toxic deaths.	Percentage.	Survivors tested.	Plague deaths.	Percentage survivors.
Jullundur 1 ..	65	13th May	60	8	13·3	52	23	55·7
Haffkine 1 ..	„	„	60	12	20·0	48	22	54·1
						10	10	0·0 Controls.
Jullundur 2 ..	65	13th June	59	2	3·3	57	32	43·8
Haffkine 2 ..	..	„	60	1	1·6	59	31	54·1
						10	10	0·0 Controls.
Jullundur 3 ..	66	8th July	59	2	3·3	57	31	45·6
Haffkine 3 ..	..	„	60	3	5·0	57	33	42·1
						10	10	0·0 Controls.
Jullundur 4	66	12th August	60	3	5·0	57	30	47·3
Haffkine 4 ..	..	„	60	1	1·6	58	30	48·2
						10	9	10·0 Controls.
Jullundur 5 ..	73	6th September	59	5	8·4	53	23	56·6
Haffkine 5 ..	..	„	60	3	5·0	57	31	45·6
						10	10	0·0 Controls.
Jullundur 6 ..	76	14th October	60	1	1·6	59	29	50·8
Haffkine 6 ..	..	„	60	0	0·0	60	34	43·3
						10	9	10·0 Controls.
Jullundur 7 ..	76	11th November	59	4	6·7	55	18	67·2
Haffkine 7 ..	..	„	57	5	8·7	52	17	67·3
						10	10	0·0 Controls.

obtained in rats inoculated as compared with 10 per cent in controls. Half of each brew was kept in the Haffkine Institute, Bombay, at room temperature, and half at the Public Health Equipment Depot at Jullundur, in order to test the effect of storage under the extreme hot weather conditions of the Punjab on its potency. Bottles of the vaccine were returned from Jullundur at intervals and their potency tested on rats in comparison with bottles of the same brews which had been retained in Bombay. The results of the tests are shown in Table XXVI.

It will be seen that the immunizing value of the vaccine after ten months' storage, including a complete hot weather in the plains of the Punjab at room temperature, was at least equal to that found one month after manufacture. (Allowance has to be made for the possibility of variation in the test doses used which do not render successive tests exactly comparable). The conditions of storage represent the maximum temperatures which the vaccine is likely to be submitted to in storage in India and the shade temperatures in Jullundur during the hot weather show daily maximums frequently over 110°F.

The value of the vaccine after a period of storage is well shown by the results obtained with a vaccine brewed for six weeks, and stored for nine months before use, during an inoculation campaign in villages in the Bijapur district. The statistics of this inoculation campaign are of special value as they deal only with households in which both inoculated and uninoculated persons were present and with occurrences only after the date of inoculation. The following are the figures given for these villages :—

Name of village.	Inoculated.	Uninoculated.	Inoculated.		Uninoculated.	
			Cases.	Deaths.	Cases.	Deaths.
Ilkal	41	38	9	..	37	36
Herur	41	41	3	1	9	7
Gombal ..	42	42	3	2	20	19
Totals ..	124	121	15	3	66	62

In approximately equal numbers of inoculated and uninoculated persons in the same households the respective deaths from plague were 62 and 3.

The vaccine had remained of high immunizing value for a period of nine months from the date of manufacture.

These observations indicate that, after a slight drop in potency which occurs during the first month, the vaccine retains a high immunizing value for a year with little indication of loss of potency. A further period of six months storage results in only a slight diminution in value. The tests of potency were carried out with vaccine kept at room temperature and there does not appear to be any necessity to employ cold storage.

CHAPTER XVII.

Standardization of the vaccine.

When Haffkine introduced his plague prophylactic the prolonged period of incubation employed resulted in a product of complex composition to which methods of standardization based on bacterial content were not applicable. The vaccine would contain living and dead *B. pestis*, a debris of macerated and broken up organisms, dissolved bacterial substance and possibly the products of the metabolism of the organism. As Haffkine considered that the supernatant fluid was an essential element of his vaccine any method which he could adopt for standardization would require to be based on the composition, or effect, of the fluid portion of the vaccine, as well as on the sediment. His opinion as to the value of the supernatant fluid has been fully confirmed.

No method of bacterial counting is applicable to the vaccine for standardization. In the course of incubation the growth of the *B. pestis* is at first rapid and the number of viable organisms reaches its maximum within a period of two or three weeks, thereafter falling considerably and being greatly reduced by the termination of the six weeks period of incubation employed by Haffkine. Estimation of the number of organisms present by means of dilution, plating, and colony counting, would show a much lower count at the end of six weeks when the vaccine would have reached a high potency, than would be found after one week of incubation at which period the vaccine is not effective. Microscopical counting is also not applicable as the sediment in cultures eventually becomes composed mainly of fragments of the bacillary bodies in various stages of disintegration.

Biochemical methods for estimating particular fractions in the supernatant fluid which might have had a definite relationship to its immunizing properties had not in Haffkine's time been developed to the extent that they have been subsequently and such methods were not available for standardization. Haffkine considered that immunization was only obtained with his vaccine when a definite strong reaction occurred after inoculation. Instead of adopting a direct method of estimating any component of the vaccine he used a dose of the vaccine in accordance with its toxicity. In effect, he used as large a dose of the vaccine as possible, and one producing a toxic reaction without such severe effects as would result in extreme unpopularity of inoculation. The vaccine as at first prepared by Haffkine was far from being a regular product. Variations in the strains of *B. pestis* used, and in the different batches of broth, and in period of incubation, according to demand, resulted in the toxicity and probably the potency of different brews varying widely. Haffkine fixed a standard consisting of a rise of temperature to 102°F. after the inoculation of 5 c.c. of the vaccine in the majority of adults

treated, and obtained this degree of reaction with his strongest brews. In the case of brews of lower toxicity he prescribed multiples of this dose according to the reaction shown. The supply of patients at the Laboratory on which these tests could be made, was not reliable and could not be maintained, with the result that the dosage had, as a rule, to be fixed by inoculators as the result of their own experience. On account of this difficulty Haffkine adopted an additional standardization method in the form of a rough measurement of opacity of the vaccine and considered this, along with the human tests, to be satisfactory. When trials on human beings could not be carried out this was the only test employed. His opinions on the value of these methods are given in Chapter III.

The amount of sediment present in the vaccine will be to a considerable extent an indication of the amount of growth which has taken place and consequently might form an index of the amount of antigenic substance which had been available to pass into solution. There are a number of factors which prevent it from forming a reliable basis for standardization but if exact methods were followed in preparing the vaccine and strains of constant immunizing value were used, estimation of the weight of sediment might prove to be in some degree an indication of potency. The method does not take into consideration the effect of virulence on the value of the vaccine. Along with the work which has been done in recent years on the systematization of manufacture possible methods of standardization have been investigated, and standardization by weighing the sediment or estimating the opacity has been rejected as a suitable method on account of certain fallacies and difficulties. When Haffkine's laboratory was transferred to the Old Government House, the better conditions available for the manufacture of the vaccine, and the improvements in staff and equipment, enabled a product of a more regular character to be turned out. As the result of experience of several million inoculations the dose of the vaccine became fixed at 4 c.c. for adults, this amount giving a fairly regular degree of reaction. This dosage was still based on toxicity, the largest dose being given which could be tolerated by the average patient and which did not produce serious toxic symptoms in any. While general principles had been laid down for the manufacture of the vaccine variations in the method of production were permitted for a number of years in regard to points which were not considered essential, and vaccine was turned out of greater or lesser toxicity and immunizing value as affected by differences in the period of incubation employed.

The absence of any method of standardization during a period of years when such variations were allowed must have resulted in the issue of vaccine which was irregular in its potency and effect.

In the *Annual Report of the Bombay Bacteriological Laboratory* for 1905 an account of a biological method suggested for the standardization of the vaccine is given, the details of which had been worked out by Liston. Using a dilution and plating method, for counting the organisms present in the suspension of *B. pestis* used as the infecting dose, Liston fixed a standard unit for the vaccine consisting of ' the maximum quantity of a vaccine which will in 48 hours, protect a guinea-pig weighing 250 to 300 grammes against at least 100 times the lethal dose of a plague culture isolated from the body of an animal not more than one week

previously'. This was intended to be used as an indication of the safe amount to be used in the case of persons living in infected households before the question of the production of a negative phase was settled. Subsequent work has shown the fallacies in counting *B. pestis* by growth of colonies on ordinary agar and the method is very unreliable. The test does not appear to have been used to any extent. The experimental work carried on in regard to the effect of the period of incubation of the vaccine and of storage on potency and toxicity eventually resulted in the adoption of more regular methods of manufacture and in time each factor concerned in the production of the vaccine has been studied in detail and systematized.

A biochemical study of the changes in the vaccine culture has been carried out during the last four years and an attempt made to find a chemical index of potency. Estimations of the protein fractions on a filtrate from the vaccine ('supernatant fluid') were carried out by removal of the heat coagulable protein followed by fractional precipitation of the remaining proteins with sodium sulphate. A series of observations at different stages of incubation indicated a progressive increase in the heat coagulable fraction which followed a course more or less in correspondence with the development of potency which has been observed in immunization experiments with the vaccine. An equal development of this fraction in vaccines prepared from avirulent cultures which are without protective value indicates that the estimation of heat coagulable protein is not a sufficient index of potency to enable this to be adopted as a method of standardization.

The question of the adoption of a nitrogen figure as an indication of potency of the vaccine has been considered but this has also not been found satisfactory or capable of practical application.

De Smidt (1929) adopted a combined method of standardization, partly by chemical means, with a vaccine of the Haffkine type which he prepared in Kenya, consisting of an estimation of the protein content along with an opacity estimation of the sediment. The figures he fixed as indicating a vaccine of satisfactory composition were: (1) 40 to 50 mg. dissolved protein per cent precipitated by Esbach's method; and (2) 0·45 to 0·55 mg. of sediment per c.c. He obtained protection in rats with vaccine reaching this standard, of a degree very similar to that obtained with the vaccine prepared and tested by us in Bombay, but a much lesser degree of protection with brews considerably below this standard.

In recent years the question of biological standardization has been the subject of a large amount of study and in the chapter on Experimental Methods (Chapter IX) we have discussed the lines of work and some of the results obtained. Using a fixed dose of vaccine for the immunization of susceptible trapped *R. rattus* and a subsequent infecting test dose of plague-rat spleen it was found that over a large series of experiments the immunity obtained in rats which survived the toxic effect of the vaccine averaged in the neighbourhood of 30 per cent, but in successive tests and repeat tests there were very marked irregularities. Some of these results have been quoted in Chapters XII and XIII.

Naidu and Jung (1929) gave the results of tests of 103 brews done during the years 1926 and 1927, each brew consisting of 120 to 260 flasks. The following were the results obtained in 1926 :—

Brew.	Percentage of immunity.	Brew.	Percentage of immunity.	Brew.	Percentage of immunity.	Brew.	Percentage of immunity.
444	15·4	460	45·0	476	25·0	492	34·6
445	44·0	461	17·3	477	25·4	493	25·0
446	40·9	462	35·0	478	18·8	24	29·6
447	52·9	463	31·5	479	23·5	26	34·4
448	44·0	464	27·2	480	17·8	27	18·5
449	46·0	465	26·9	481	22·0	28	25·9
450	36·7	466	20·0	482	25·0	30	33·3
451	18·9	467	36·0	484	17·2	32	28·8
452	19·6	468	42·8	485	20·0	33	34·6
453	36·5	469	49·0	486	30·7	40	30·0
454	48·0	470	28·8	487	17·3	41	27·5
455	41·6	471	22·6	488	50·0	43	44·4
456	31·0	472	18·0	489	18·5	44	29·1
457	40·7	473	30·1	490	33·3	45	31·0
458	44·8	474	48·1	491	27·2	49	40·1
459	36·3	475	21·4	..	..	50	28·5

The immunity obtained in the tests done in 1926 varied from 15·4 per cent to 52·9 per cent and in 1927 from 23 per cent to 64 per cent. The 1927 tests were on the whole more regular and out of 40 brews only 9 fell below 30 per cent.

Naidu and Jung gave the following figures for the results obtained in rats immunized with Haffkine's vaccine over a series of years :—

	Number infected.	Deaths.	Percentage of immunity.	Years.
Uninoculated	482	450	6·7	..
Inoculated	778	515	33·9	1923–25
Uninoculated	360	336	6·6	1926
Inoculated	2,895	2,030	29·8	..
Uninoculated	210	198	5·7	1927 (10 months)
Inoculated	1,520	968	36·3	..
Uninoculated	1,052	984	6·4	Total
Inoculated	5,193	3,513	32·3	1923–27

The general value of the test is shown, but the irregularity of the results with individual brews renders it unsatisfactory. The results obtained with repeat tests with the same brew also showed wide variations.

This test was actually employed for a period as a means of standardizing the vaccine and its use resulted in a considerable number of brews not being passed for issue. As the method of preparation of the vaccine had by that time been regularized

in all details it did not appear likely that such variations in potency as were suggested by the tests could really have occurred, and it became necessary to overhaul the methods of testing used.

The irregularity in the bacterial content of plague-rat spleen emulsions which have been used for test purposes and the defects which trapped *R. rattus* are subject to for use as experimental animals have already been shown. The revised technique for testing immunity to plague after inoculation, by the use of white mice and standardized infecting doses of *B. pestis* of known bacterial content, which has been described in Chapter IX appears likely to be suitable for use for the purpose of biological standardization. The method has given consistent results in a long series of tests and the ability to produce a standard infection will enable a direct comparison to be made of the value of successive brews of the vaccine.

In interpreting the results of the tests the principle laid down has been that after immunization with 0·03 c.c. of the vaccine its potency is considered satisfactory if all test mice survive. The occasional death of a single mouse in a group is permitted. Examples of such tests are shown in Table XXII. The method of testing is not yet perfected on account of the change in virulence of the *B. pestis* which occasionally occurs in the passage strain especially in the hot weather. When this difficulty can be overcome the method of testing should be capable of application for routine standardization.

In practice the methods of manufacture used are designed to furnish a product of as regular a composition and value as possible, the procedures adopted to attain this result being : (*a*) the use of an animal strain of high virulence, (*b*) the isolation and preparation of seed material under strictly regular conditions, (*c*) the use of a medium of fixed composition and reaction, (*d*) incubation at regular temperature for a fixed period. A uniform growth of an organism of uniformly high virulence is obtained in this way and, as has been shown by the results of the latest biological method of testing, the immunizing value of the vaccine is constant.

A question which arises is whether the dosage, which has remained fixed at the level of 4 c.c. laid down for many years, is a correct one for human beings. Complaints of excessive reaction are occasionally received in regard to the use of vaccine within a short period after its preparation but not with regard to older vaccine. This has always been the case and on the score of reaction there does not appear to be any reason for reducing the dosage. It has not been possible to obtain statistics of the relative protective value of the vaccine in human beings with different doses and it does not appear likely that we shall be able to test this effectively. In Kenya the vaccine of the Haffkine type prepared is used in doses of 2 c.c. for adults, and de Smidt (1929) in comparing the results of a small series of plague cases in inoculated individuals with certain of the Indian figures in which the larger dose was used finds that the survival rate was very similar in the two groups. It is possible that 2 c.c. may be a sufficient immunizing dose but we have no opportunity of determining this.

In summing up this chapter we may state our opinion that the plague vaccine, as prepared at the Haffkine Institute, by the use of standardized methods of manufacture is a product of regular composition and strength, and that biological methods of testing show it to be of regular and high immunizing value.

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