Bull. Org. mond. Santé
Bull. World Hlth Org. } 1952, **5**, 165-226

PLAGUE STUDIES *

3. Problems in Immunology

R. POLLITZER, M.D.
Division of Epidemiology, World Health Organization

Manuscript received in July 1951

VIRULENCE OF THE PLAGUE BACILLUS

Since immunological terms like " virulence ", " virulent " or " avirulent " are often used rather indiscriminately with reference to bacilli, it is necessary to begin the present disquisition by defining in what sense they are applied here. In doing so, the apt distinction drawn by Jawetz & Meyer [77] between virulent and avirulent plague bacilli may be quoted :

> By a virulent plague bacillus, we mean one that will multiply greatly and finally bring about the death of a given susceptible host animal when introduced in numbers not large enough to be toxic without multiplication. An avirulent plague bacillus may then be defined as one not being able to cause the death of the same susceptible host animal unless introduced in numbers sufficient to produce toxic death without multiplication.

Thus, as these authors point out, mere invasiveness cannot be made the criterion of virulence, this term indicating the capacity of the organisms to multiply in the tissues of the host to a dangerous extent. At the same time a sharp distinction has to be made between virulence and toxicity. Since, as justly maintained by Jawetz & Meyer, " death from plague in final analysis is always due to a toxaemia produced by decomposition products of the plague bacillus ", one might consider the virulence of *Pasteurella pestis* as the means of bringing about a fatal termination of the disease which, however, is ultimately due to the toxicity of the organisms.

Generally speaking, the virulence of the plague bacillus is high. In a classical experiment Barber [5] was able to show that six out of nine guinea-pigs and two out of twelve monkeys which had been inoculated with single plague bacilli succumbed to the infection. Identical results have been obtained with guinea-pigs by Otten [122] while, as will be discussed below, infective doses consisting of not more than six to twelve plague bacilli have been found suitable for standard virulence tests with mice.

It may be further maintained that on the whole the virulence of *P. pestis* is remarkably stable. Petrie,[131] summarizing the data of several workers,

* This is the third of a series of studies which, when complete, will form a manual on plague and which will be published in separate editions in English and in French in the Monograph Series of the World Health Organization. — ED.

found that out of 152 laboratory strains of diverse origin, some of them many years old, 12 only showed marked impairment or absence of virulence while the remaining 92% were virulent. The interesting experiments of Francis with a 20-year-old plague strain, referred to previously,[a] serve as a corollary for these observations.

The fact remains, however, that not only may the virulence of plague cultures be abated by artificial means but the cultures may become avirulent spontaneously, sometimes even without being kept in the laboratory for prolonged periods (Revenstorf [144]).

It has been claimed also by a few observers that plague strains of low virulence may become prevalent under natural conditions. The evidence brought forward in this respect in connexion with the supposed existence of " chronic " rat plague will be discussed in a later study; but it may be noted here that Pirie [133] referred in 1936 to a perceptible loss of virulence shown by plague strains recently isolated in the Union of South Africa from human as well as rodent sources. Likewise, Macchiavello [101] maintained that the plague cultures obtained in north-east Brazil were often less virulent than those met with in other countries and were apt to undergo a total loss of virulence when exposed to adverse conditions in the laboratory. Meyer et al.,[112] studying an instance of chronic plague meningitis in California, had the impression that the recent cases of plague in the western states merely reflected the behaviour of an agent which caused mild infections with a tendency to latency.

Measurement of Virulence of Strains

The following methods used in the past for measuring the virulence of plague strains, though now of historical interest rather than of actual importance, may be mentioned :

(1) The procedure recommended by Kolle & Martini [87] consisted of rubbing an appropriate test dose into a carefully-shaved area of standard size on a guinea-pig's abdomen. The same procedure was also used for some time in the Haffkine Institute where, however, rats instead of guinea-pigs served as test animals (Sokhey [167]).

(2) Kolle & Krumbein [86] employed a syringe needle of standard calibre which was dipped into a bacterial suspension of definite concentration and then introduced under the skin near the root of a rat's tail.

(3) Rowland,[149] who also used rats as test animals, worked with a syringe so arranged that one turn of a screw caused delivery of 0.1 ml of the culture to be examined.

(4) An alternative method adopted in the Haffkine Institute consisted of the subcutaneous administration to rats of an infective dose containing 0.003 mg of the spleen of a rat that had died of acute plague. As was to be expected, it gave grossly inconsistent results (Sokhey [167]).

An exact method of determining the virulence of plague strains was introduced by Sokhey,[167] who had previously devised a method of counting

[a] Pollitzer, R. (1952) *Bull. World Hlth Org.* 5, 101

the number of viable plague bacilli present in broth cultures. The principle of this procedure was to make progressive dilutions, each one-tenth the strength of the preceding one, from the growths to be tested and to spread 0.05 ml of the diluted fluids on blood-agar slants which were then incubated for 48 hours. Using adequate dilutions, it was thus possible to obtain growth of suitably low numbers of colonies.

Sokhey had also established that the strain of white mice inbred in the Haffkine Institute was highly susceptible to plague, ten or even less highly virulent organisms being sufficient to cause a 100% mortality.

To determine the virulence of a plague strain, 0.5 ml of a broth subculture was implanted into a tube containing 9.5 ml of nutrient broth and this second subculture was incubated at 25°-27°C for 48 hours, care being taken to keep the tube in a vertical position and free from jolts or jars.

At the termination of the incubation period progressive tenth dilutions of the growth were made and used on the one hand for subcutaneous infection of mice, and on the other for determination of the number of viable plague bacilli with the aid of the method described above. Experience had shown that, in the case of highly virulent strains, 0.2 ml of the 10^{-7} dilution contained the minimal lethal dose (m.l.d.) consisting of 6-12 organisms. This and the 10^{-6} and the 10^{-8} dilutions were used, therefore, for injecting batches of five to ten mice with doses of 0.2 ml per animal.

Results were expressed as the smallest number of organisms of a given strain of *P. pestis* which, when administered subcutaneously, killed approximately 100% of the animals within three to eleven days.

The superiority of Sokhey's new test over the method of rat infection formerly used in the Haffkine Institute is shown as follows :

Strain	*Old test*		*New test*		
	number of animals		*number of organisms given*	*number of animals*	
	infected	*died* [a]		*infected*	*died* [a]
A	2	2 (4.2)	4	5	5 (7.8)
			40	5	5 (5.6)
B	2	2 (4.0)	8	5	1 (7.0)
			80	5	4 (10.0)

[a] The figures in brackets indicate the average number of days elapsing between infection and death.

As will be noted, the two strains appeared to be equally virulent when the old method was used, but showed a marked difference in their virulence when tested with the aid of the procedure recommended by Sokhey.

Gokhale,[61] studying the oxygen uptake of three virulent and four avirulent plague strains with the aid of Warburg's manometric technique, came to the conclusion that the enzymatic properties of both types were identical. However, as noted in the preceding section of these studies, Rockenmacher [147] found the catalase activity of virulent strains to be greater than that of avirulent growths and suggested that tests measuring this activity might be used for screening the virulence of plague cultures in

vitro. So far no practical advantage seems to have been taken of this recommendation.

Maintenance of Virulence of Strains

The problem of maintaining the virulence of plague cultures was exhaustively dealt with by Sokhey.[167]

Passage through susceptible animals

Sokhey noted that the method of passage through susceptible animals, which had been almost universally used hitherto for this purpose, possessed serious drawbacks. While tests with highly susceptible animals gave satisfactory results, the virulence of the strains was likely to be impaired if the passage animals happened to be resistant to plague. Yet no method was available to determine beforehand their receptivity to the infection. Worse still, the use of massive doses for the infection of the passage animals was apt to give a wrong impression of the virulence of the strains used, as is shown thus :

Number of organisms per house-rat (subcutaneously)	*Number of rats inoculated*	*Number of deaths*	*Number of days between inoculation and death*
64	24	0	—
64,500	25	1	17.40
132,250,000	25	25	3.4 (average)

As will be seen, the strain in question, though actually of very low virulence, would have appeared fully satisfactory, had reliance been placed upon tests with massive inocula.

Storage at low temperatures

Bearing in mind that storage at low temperatures was helpful for preserving the virulence of plague cultures, Sokhey recommended replacing the method of animal passage by the following procedure :

Primary cultures from human cases with severe septicaemia were obtained by plating venous blood on agar slopes. After four days' growth at room temperature (26°–32°C), they were tested for purity with the aid of the cultural and biochemical methods recommended by Gore (Taylor [184]). After a culture had been found pure, its virulence was measured quantitatively; if found highly virulent, that is, if 6 to 12 plague bacilli per Haffkine-Institute-inbred mouse killed not less than 80% of the animals used in an average period of about seven days, large numbers of subcultures on 5% rabbit-blood agar slopes were made from the primary culture, and the tubes were sealed on the flame and stored in a refrigerator at $4° \pm 2°$C. A tube was removed from time to time to measure the virulence. It was found that cultures stored in this manner retained their virulence unimpaired for at least three years.

Drying from the frozen state

Still better results could be obtained by drying from the frozen state; by this method " virulence is maintained much longer, probably indefinitely " as was stated by Sokhey[170] in 1947. According to Sokhey (personal communication), the following procedure is actually used in the Haffkine Institute for freeze-drying plague cultures :

The strains in question are grown for 48 hours on blood-agar slants at 28°C. Three parts of a suspension prepared from such growths are then mixed with one part of a 20% solution of gum acacia which had been adjusted to pH 7.0–7.2 and, after distribution in tubes, had been sterilized for half an hour at 120°C.

The mixture of the culture suspensions and the gum acacia solution is well emulsified, distributed in suitable containers and mechanically shaken to ensure an even distribution of the bacilli in the menstruum. After distribution in quantities of 0.1 ml in tubes of a suitable size (115 mm × 8 mm) the dispensed emulsions are rapidly frozen at -30° to -50°C. The tubes are then attached to the manifolds of a cryochem apparatus and the vacuum is brought up to 200 in five minutes. To avoid the possibility of thawing, the tubes are kept immersed in methyl acetone at -30°C for about 30 minutes. The process of desiccation is continued for 24 hours; the growths are then sealed under vacuum and tested with a vacuum tester. The dried growths are stored at a temperature ranging from 0° to +1°C.

For regeneration, a small quantity of broth (about 5 ml per tube of dried culture) is added; the mass is emulsified, transferred to a sterile tube, and incubated at 37°C overnight. The material is then planted on agar slopes.

As has been shown by comparative tests, gum acacia solution, among all the emulsifiers tried, yielded the highest survival rate (51.0%) of *P. pestis*. The virulence of plague cultures preserved as described above was recently found to be unabated after five years.

Mitigation of Virulence of Strains

In order to evaluate the methods used for attenuating the virulence of plague strains, it is necessary to determine first in what manner the transition from the virulent to the avirulent state takes place. Theoretically there are two possibilities—either that all bacterial cells composing a virulent plague culture and possessing an equal degree of virulence undergo some change which renders them uniformly avirulent or that, virulent and avirulent bacilli being both pre-existent in the original growth, the loss of virulence is due to a process of dissociation by which the avirulent elements become preponderant or even solely present. Actually there is no reason to doubt the validity of the latter assumption which, as pointed out by Jawetz & Meyer,[77] is strongly supported by the fact that Otten[127] was able to obtain

avirulent subcultures by single-colony picking from virulent growths of *P. pestis.*

Discussing the various methods for attenuating the virulence of plague strains, Jawetz & Meyer [77] aptly distinguished between (*a*) the above-mentioned procedure of Otten which took advantage of a process of "natural" dissociation and (*b*) "enforced" dissociation effected by exposing the strains to adverse environmental conditions.

Most important among the latter procedures were :

(1) Repeated subcultivation at weekly intervals, a method used successfully by many workers, more recently by Girard & Robic,[58] Pirie & Grasset,[135, 136] Sokhey,[167] Macchiavello,[101] and Hsue.[75]

(2) Prolonged cultivation of virulent plague strains in broth to which alcohol had been added. Hetsch,[74] when introducing this method, recommended the use of gradually increased alcohol concentrations (0.5%–5%) and incubation at high temperatures (41°–43°C). Donskow & Lochov [30] claimed to have obtained avirulent variants within a month through cultivation in broth containing 10% alcohol, and within a few days when using an alcohol concentration of 15%. Jawetz & Meyer [77] started with hormone broth containing 0.5% alcohol, incubating the growths for three weeks at 32°C. Subcultures were then made in 3% alcohol broth and kept for three weeks at 32°C, and for a further four weeks at 4°–6°C.

A third procedure, originally used by Burgess,[17] was to attenuate the virulence of plague strains with the aid of passage through immune animals. To judge from the experiments made by Burgess himself and by Jawetz & Meyer,[77] it was apparently far more difficult to obtain permanent results with this method than with those mentioned above.

Russian workers (Pokrovskaya; [137, 138] Korobkova [89]) were able to obtain avirulent variants of *P. pestis* through bacteriophage action. Otten,[127] while confirming that avirulent strains of high antigenic value could be produced with the aid of this method, found it rather laborious and time-consuming.

As has been noted previously,[b] bubbling of air through broth cultures in the apparatus devised by Devignat led to an impairment of the virulence of the strains. Devignat [26] took practical advantage of this procedure to render three virulent plague strains avirulent and also to abate a slight increase in virulence of the EV strain (initials of the child from whom it was obtained) used by him for inoculation.

Describing the methods for the laboratory diagnosis of rat plague, Petragnani [130] mentioned, without giving details, that the virulence of plague strains could be attenuated through cultivation on bile media or media prepared with "vegetable" materials.

[b] Pollitzer, R. (1952) *Bull. World Hlth Org.* 5, 84

TOXIN OF THE PLAGUE BACILLUS

Nature of Toxin

Petrie,[131] analysing problems of plague immunity in 1929, pointed out that workers on this subject somewhat loosely applied the terms antigen, endotoxin, immunizing substance, and vaccine, almost as if they were interchangeable. He himself, though certain that *P. pestis* possessed a specific endotoxin which was associated with the soluble protein of the bacillus and which was set free in broth cultures by disintegration of the bacilli after their death or in bacillary suspensions by extraction methods, stated it as his belief that no principal difference existed between the antigenic substances present in (*a*) old autolysed broth cultures, (*b*) the purer products obtained by extracting the bacillary bodies, and (*c*) " whole " vaccines, whether prepared by Haffkine's or other methods. Petrie emphasized in particular that all preparations used for active immunization against plague contained toxin, toxoid, or a mixture of these antigens.

Although a few workers like Markl,[105, 106, 107] Kossel & Overbeck,[93] and Dieudonné & Otto [29] suggested that the toxin of *P. pestis* might represent a mixture of metabolic products of the living bacteria and of endotoxic substances set free after their disintegration, most experts are convinced that the plague bacillus has an endotoxin only, as originally postulated by Rowland [149] and Besredka (quoted by Girard & Sandor [60]).

However, Girard and some other French workers stressed that the " endotoxin " of *P. pestis* differed in some ways from the typical endotoxins of other bacterial species and resembled in other respects the exotoxins. Thus Girard [50] and Girard & Sandor [60] noted that the plague bacillus did not possess the glucidolipoid complex found in the endotoxins of numerous other Gram-negative micro-organisms. On the other hand, as summarized by Ramon, Girard & Richou,[143] the plague endotoxin was similar to the exotoxins insofar as it consisted, like these, of proteins, could be easily transformed into toxoid, and was rather thermolabile.

Hence, although there is no reason to revise the concept that the plague toxin is an endotoxin, it will be noted that in this as in many other respects *P. pestis* shows peculiar features.

Preparation of Toxin

The methods used by earlier workers for obtaining plague toxin consisted either of the filtration of old broth cultures containing autolysed plague bacilli or of extraction processes. The following procedures deserve mention :

Filtration

Petrie,[131] modifying a method devised by Markl, [105, 106, 107] prepared toxic extracts thus : a medium of ordinary peptone broth (pH 7.0–7.3),

with or without the addition of 1% normal horse serum, was distributed into flasks in such a manner as to ensure good access of air to the cultures which were kept at room temperature (10°–15°C) for about two months. At the end of this time toluene was added to each flask and the flasks were put aside for a few days to allow the toluene to act. Seitz filtration was then used.

Extraction

Nucleoprotein extracts were first prepared in 1897 by Lustig & Galeotti[100] who, treating suspensions of *P. pestis* with 1% potassium hydroxide solution and then slightly overneutralizing with 0.5% acetic acid, obtained the nucleoprotein in the form of a white flocculent precipitate. This substance conferred immunity against plague on rats if used in doses of 0.36 mg.

Rowland[149] prepared toxic nucleoproteins by treating agar-grown plague cultures, which had been killed with chloroform, with anhydrous sodium sulfate. The extracts thus obtained were fatal to rats within 18 hours when administered in doses of 0.05–0.1 mg; doses of 0.001–0.01 mg afforded substantial protection against plague infection. Simple digestion of chloroform-killed plague cultures in saline yielded solutions which possessed the same chemical properties as the extracts obtained with sodium sulfate and could be rendered toxic and immunogenic if care were taken to remove the chloroform which formed a loose combination with the nucleoproteins (Rowland[150]).

Recently Girard[47, 48] obtained toxic extracts by three times freezing and thawing suspensions of plague bacilli according to the method of Grasset & Gory,[66] then centrifuging and filtering. The filtrate, if administered in doses of 0.05–0.2 ml, killed mice within 6 to 36 hours.

Jawetz & Meyer[77] prepared toxic extracts in the following manner: plague bacilli grown at 37°C were

" suspended in buffered saline (pH 7.4) and adjusted to a density of 20 billion [20 milliard] organisms per ml. These suspensions were incubated for 48 hours at 37°C and then left for 24 hours at 4°–6°C in the refrigerator. The bulk of the cellular material was then centrifuged off (2,000 r.p.m. for 90 minutes), and the supernatant fluid filtered through W Berkefeld candles and frozen in small pyrex bottles at −76°C. At this temperature no deterioration of the toxic power took place over a period of at least three months."

As will be mentioned later (page 175), Baker et al.[4] were able to obtain from acetone-dried plague bacilli, through extraction with neutral salt solutions, a water-soluble and a water-insoluble antigenic fraction. The former was shown to contain at least three antigenic compounds including a toxic fraction (fraction II), soluble in ammonium sulfate at 0.33 saturation and at pH 7.0–7.5, and almost completely precipitated when the ammonium sulfate concentration was raised to 0.55–0.67 saturation.

Baker and co-workers pointed out that it had not been possible to isolate this toxin fraction in a state approaching chemical or immunological purity, all preparations being " contaminated " with the immunogenic fraction I in sufficient quantities to produce antibodies to the latter in rabbits. However, Baker and his colleagues claimed to have succeeded in preparing toxins free of fraction I by serological techniques. They observed that plague bacilli grown at room temperature were fully toxic, but extracts of such bacilli contained very little fraction I. It proved possible to remove the residual fraction I by absorption with either fraction IA or IB antisera. The resulting absorbed extract showed no decrease in toxicity, and produced antisera in rabbits capable of neutralizing plague toxin. However, this antiserum was devoid of protective value for mice, did not agglutinate antigenically-complete plague bacilli to significant titre, and did not react with either fraction IA or IB except to a very slight degree in sensitive ring-tests. Its value in inducing immunity in mice and guinea-pigs has not as yet been investigated.

Bacteriophage action

Girard [55] reported recently that he could obtain toxic lysates by adding bacteriophage to three-day-old cultures of his EV strain and a virulent plague strain and filtering after seven hours when incomplete lysis of the bacilli had taken place. Mice infected intraperitoneally with 0.2 ml, or subcutaneously with 0.5 ml, of these lysates succumbed 10–18 hours later.

Girard was unable to obtain toxic filtrates from broth cultures of *P. pestis* incubated at 28°C before the 6th–9th day ; bacteriophage action seemed thus greatly to accelerate the normal process of bacteriolysis.

It is of interest to note in this connexion that, although almost without exception pseudotuberculosis strains do not yield toxic filtrates or extracts, Lazarus & Nozova [97] were able to demonstrate the presence of toxic lysates when subjecting 8-hour-old cultures of two *P. pseudotuberculosis* strains of American origin to bacteriophage action. Girard,[55] who confirmed this result, ascribed the much more rapid appearance of toxic substances in the lysates from these two strains than in those obtained from plague bacilli to the considerably quicker growth of *P. pseudotuberculosis.*

Plague toxoid

The transformation of the plague toxin into toxoid (anatoxin) is easily effected by adding 3.4 °/₀₀ formalin to plague cultures and incubating at 37°C for a few days. As maintained by Girard,[55] the toxoid thus formed is not capable of protecting mice or rats against the plague toxin. It is interesting to add that, according to MacConkey,[102] the toxin in old autolysed solutions of plague nucleoprotein had undergone a partial change into toxoid, but that this comparatively atoxic substance stimulated the production of plague antitoxin in the horse.

Heat Resistance of Toxin

The problem of heat resistance of the plague toxin is rather involved. As pointed out by Petrie,[131] the time factor was of marked influence as well as the temperature so that storage under a comparatively low temperature for prolonged periods had the same effect as short exposure (half an hour to one hour) to temperatures within the limits of 37°–70°C. Petrie emphasized that according to his experience toxin solutions or extracts, in order to retain their toxicity, had to be kept constantly at temperatures not higher than 3°C.

Some recent observations on the upper limits of the heat resistance of plague toxin showed that it became inactivated by exposure for four hours to 55°C (Meyer, quoted by Girard [55]), for one hour to 65°C, or for half an hour to 80°C (Gheltenkoff [40]).

Susceptibility of Experimental Animals

Valuable data on the susceptibility of laboratory animals to plague endotoxin assembled by Petrie [131] may be summarized as follows :

Mice

Mice are highly susceptible to the toxin, subcutaneous or intraperitoneal administration usually killing the animals within 18 hours. By measuring the toxicity of broth toxins from a large number of plague strains by injecting graduated doses intravenously into mice, Petrie obtained the following results :

Number of toxins tested	*Average m.l.d.* * *(ml)*
53	0.013 (1/76)
26	0.0095 (1/105)

* The average m.l.d. of 17 broth toxins prepared from Shiga-dysentery strains was 0.0053 (1/190) ml.

White rats

White rats usually died after 48 hours when the toxin had been given subcutaneously, and 24 hours after intraperitoneal administration. The lethal dose of broth toxins was 0.05–0.5 ml per 100 g of body-weight, smaller amounts being needed for intraperitoneal than for subcutaneous administration. Rowland's nucleoprotein killed the animals in doses of 0.1 mg.

Guinea-pigs and rabbits

These animals withstood large doses of the toxin, a dose of 27 mg of plague nucleoprotein (that is, a quantity 270 times the m.l.d. for white rats) failing to cause death in guinea-pigs. However, some of the animals, though not showing acute symptoms, developed a condition of marasmus some weeks later.

Goats and horses

These animals were found susceptible to the toxin and died from toxaemia if given excessive doses.

Jawetz & Meyer,[77] while finding mice highly sensitive to plague toxin, noted that guinea-pigs could withstand at least 5,000 lethal mouse-toxin doses. Tame rats and ground-squirrels (*Citellus beecheyi beecheyi*) combined, in the experience of these workers, a relatively low susceptibility to plague infection with a high resistance to the endotoxin.

ANTIGENIC STRUCTURE OF THE PLAGUE BACILLUS

The antigenic structure of *P. pestis* has been studied by chemical as well as serological methods. Moreover, some workers determined the antigenic make-up of this micro-organism by noting differences in the immunogenic qualities of its antigens for different rodent species. According to the methods of investigation used, the different findings concerning the antigenic structure of *P. pestis* are here grouped under the following subheadings :

Water-Soluble and Water-Insoluble Fractions

In the opinion of Rowland,[149] which was shared by subsequent observers such as Morison et al,[117] the plague bacillus appeared to be built up by two varieties of protein, one soluble in distilled water or saline and containing immunizing and toxic substances, the other insoluble and possessing no antigenic or toxic properties. Noting that, according to the observations of Rowland,[149] the plague bacilli retained their shape after extraction of the soluble protein, though appearing fragile and easily breaking up, Petrie [131] drew the inference that the cell membrane was composed of the insoluble fraction.

Baker et al.[4] were able to obtain from alcohol suspensions of a virulent plague strain, grown for three days at 37°C with the aid of acetone precipitation at -70°C, repeated washing with acetone, and drying in vacuo, a bacterial powder of high antigenicity and toxicity (see page 172). Extraction of this substance with neutral salt solutions yielded a water-soluble and a water-insoluble antigenic component, the former being toxic and immunogenic for mice and rats but of little activity for guinea-pigs, and the latter, while inert as far as mice and rats were concerned, proving of high immunogenic value for guinea-pigs when tested as an alum precipitate.

Soluble fraction

As shown by saturation or precipitation with ammonium sulfate at various concentrations, the water-soluble fraction contained at least three

antigenic compounds, called fraction IA, IB, and II by the authors who thus described the properties of these substances :

Both fractions IA and IB gave rise in rabbits to potent antisera which apparently agglutinated all plague strains tested, with the exception of one avirulent growth thought to be devoid of an " envelope " by Schütze,[156] but were incapable of neutralizing plague toxin. Both fractions induced immunity in mice as well as in white rats and monkeys (Meyer [111]) but not in guinea-pigs, and represented, as assumed by Baker and his co-workers, the " envelope " of the plague bacillus.

The relationship between fractions IA and IB could not be exactly defined. They were almost identical serologically, but fraction IB, which could be obtained in crystalline form, possessed a slightly lower immunogenic value. Baker and his colleagues suggested that the normal antigen present in the bacterial cells might be fraction IA which contained a carbohydrate component besides protein, while the carbohydrate-free fraction IB was an artefact formed during the death and treatment of the bacilli. As shown by serological studies, fraction I (IA and IB) was formed by all virulent strains tested as well as by a salt-stable avirulent strain whereas salt-instable avirulent growths produced only traces. However, incubation at 37° C seemed necessary to produce these antigens in quantity, plague bacilli grown at room temperature yielding only relatively small amounts.

It is interesting to note in this connexion that according to Bhatnagar [10] " it is the envelope substance which bestows salt stability on a suspension of the plague bacillus ".

These findings are in accord with the observations on the " envelope " antigen made by Schütze [158] and by Wats et al.[187] when comparing plague strains grown at 37°C and 26° or 27°C respectively (see page 210).

The properties of the toxic fraction II have already been dealt with in the present study (page 172).

Insoluble fraction

Only small amounts of protein could be extracted from this with the aid of mild alkalis. Anhydrous phenol, liquified with 10–15% acetone, dissolved about 25% of the water-insoluble fraction but both the phenol-soluble fraction and the insoluble residue contained the antigen protecting guinea-pigs. In contrast to fraction I, the water-insoluble antigen did not produce specific protective antibodies in the serum of monkeys or man (Meyer [111]).

As will be noted, the valuable studies of Baker and co-workers have contributed much to the knowledge of the antigenic structure of the plague bacillus. It is interesting to note that they bear out Otten's contention (see page 179) regarding the existence of separate "guinea-pig" and "rat" antigens.

It should be noted that Shrivastava,[163] in the course of an earlier study on the antigenic structure of *P. pestis*, had been able to isolate the following fractions from the supernatant fluid of Haffkine vaccine :

(*a*) an active protein fraction by saturating the fluid with sodium sulfate;

(*b*) a nucleoprotein fraction by precipitation in the cold with dilute hydrochloric acid;

(*c*) an active polysaccharide fraction with the aid of alcohol precipitation in the presence of sodium acetate.

Further work in this direction was done by Seal & Mukherji,[161] who were able to isolate, by precipitation with sodium sulfate in different concentrations, a specific soluble substance of the plague bacillus which proved highly active serologically.

" Envelope " and Somatic Antigens

Working with serological methods, Schütze [156] reached the conclusion that the plague bacillus possessed two antigens, one contained in its " envelope " and the other in its somatic portion. This concept of the existence of an " envelope " and a somatic antigen has been accepted by practically all subsequent workers but, as discussed below, some of them assumed that in addition to these two a third antigenic factor was present in virulent plague bacilli. Since the belief in the existence of an " envelope " is not shared by all observers, the term " envelope antigen " is not very felicitous. Apart from the fact, however, that it has been widely accepted, it would be difficult to replace it. To speak of a " capsular " antigen or, following Chertnik,[20] of a " membrane " antigen, seems not permissible in view of the findings of Rowland [149] recorded above.

Schütze [156] stated that the " envelope " antigen was thermolabile, becoming haptenized (incapable of giving rise to antibodies) after exposure to 100°C for 15 minutes, and was destroyed after one hour at that temperature. According to further experiments of this author,[157] heating for 15 minutes at 80°C was sufficient for haptenizing the " envelope " antigen. The somatic antigen was more heat-stable, still provoking antibody formation in the rabbit after one hour at 100°C and reacting in vitro even after three hours' exposure to the same temperature.

The conclusions reached by Schütze [156] when comparing the antigenic structure of the plague and pseudotuberculosis bacillus may be summarized as follows :

Species		*Antigen*	
P. pseudotuberculosis	Flagellar	Smooth somatic	Common rough somatic
P. pestis	" Envelope "	—	

Bhatnagar [10] postulated that the pseudotuberculosis bacillus possessed both group- and type-specific somatic antigens.

Studying the antigenic structure of the plague bacillus with the aid of chemical procedures, Kurauchi & Homma [94] were in agreement with Schütze that this micro-organism possessed two independent antigens— a somatic antigen which shared immunogenic characteristics with the pseudotuberculosis bacillus, and a capsular antigen. Considering that the latter alone was of importance in conferring immunity against plague infection, they called it the " specific immunizing fraction ".

The two workers extracted this substance by a procedure not specified in their report, purified it " par la méthode à l'acide-acétone ", and concentrated it. They found it contained 1.84% nitrogen, 8% ash, and 40% reducing substances.

Experimenting with this substance, Kurauchi & Homma found it to confer a stronger immunity when administered twice to guinea-pigs and rats than ordinary plague vaccine. Three monkeys which received two injections also withstood a challenge infection with virulent plague bacilli but a fourth, which had received one dose only, succumbed. It was found, however, that after absorption of the specific soluble substance by metallic salts satisfactory results could be obtained with a single injection.

As stated in the 1950 report of the South African Institute for Medical Research,[179] Amies was able to obtain, with the aid of a purification method based on iso-electric precipitation, from the " envelope " substance of *P. pestis* a highly antigenic substance which appeared to be a protein of relatively simple constitution. This antigen, if administered in one or two doses of 5 μg, was sufficient to protect experimental animals—*Rattus (Mastomys) natalensis*—against 500 m.l.d. of highly virulent plague bacilli and it is added that " the high immunogenic activity and freedom from toxicity of these preparations would seem to recommend their use for human prophylaxis ".

Serological Properties and Virulence

Schütze,[158] as well as Wats et al. [187] and Jawetz & Meyer,[77, 78] emphasized that it was impossible to distinguish with the aid of serological tests between virulent and avirulent, smooth or rough plague bacilli. Jawetz & Meyer [79] also stated that a factor which enhanced spreading and capillary permeability had been found in extracts of both virulent and avirulent *P. pestis*.

Russian observers (Fadeeva;[31] Gheltenkoff & Khvorostukina;[42] Korobkova[91]) postulated that virulent plague bacilli possessed an antigen similar to the Vi antigen in the salmonellae, but Jawetz & Meyer [77] were unable to confirm this claim. Before dealing with the explanation they gave for the antigenic difference between virulent and avirulent plague strains, it is necessary to set forth the opinion held by Otten regarding the antigenic structure of *P. pestis*.

Otten, [124] comparing the immunizing power of his avirulent "Tjiwidej" strain with the potency of other avirulent plague strains, came to the conclusion that

" most of these strains possess an antigen especially active in guinea-pigs but producing less or even slight effect in rats. The strain 'Tjiwidej' on the other hand, is the best antigen for rats but must be put behind others as regards guinea-pigs. This difference is of a qualitative nature, as it cannot be overcome by increasing the dose ".

In a subsequent paper [127] Otten maintained that this difference remained manifest when tests were made with heat-stable (somatic) antigens of these

various strains after the heat-labile (" envelope ") antigens had been rendered inactive through heating. Otten postulated, therefore, that the somatic antigen was composed of a " guinea-pig " antigen and a " rat " antigen, present in different proportions in the various strains.

Jawetz & Meyer,[77] while considering Otten's hypothesis as insufficient, were also disinclined to believe that different immunity mechanisms, due to variations in the resistance to plague toxin, might be at work. They assumed that some antigenic constituent, chemical group, or property, present in most live vaccines, but often absent in " killed " ones, might explain the divergent results obtained in different animal species. Mice might not be in need of this factor, thus becoming easily immunized with killed vaccines. It was, however, essential for guinea-pigs which, therefore, could be protected by specially-prepared killed vaccines only—for instance, the sugar vaccine of Minervin et al.[115] and Haffkine vaccine (Sokhey;[168] Sokhey & Maurice [178]).

Generally speaking Jawetz & Meyer assumed that there was

" little doubt of the presence of some property or chemical group responsible for virulence in the plague bacillus, since some natural dissociants seem to be identical with virulent organisms in all respects except for a complete loss of virulence ".

They stated in a later paper [78] that the agent at work might be of an enzymic nature. Rockenmacher's [147] claim that the catalase activity of virulent strains is greater than that of avirulent growths is of great interest in this connexion.

NATURAL RESISTANCE TO PLAGUE

Although, as will be discussed in a later study, most animals other than rodents are insusceptible to plague, the immunological aspects of their resistance seem to have been fully studied only in the case of birds.

Birds

The natural resistance of birds to plague cannot be considered as absolute. Thus Giaxa & Gosio (quoted by Dieudonné & Otto [29]) found starving pigeons and sparrows susceptible and, as observed by Albrecht & Ghon [2] and others, intravenous or intraperitoneal administration of large doses of highly virulent plague bacilli to normal birds occasionally produced fatal infections. However, these exceptions do not invalidate the rule that under ordinary circumstances birds are resistant to plague.

London [99] ascribed this resistance to the presence of specific humoral antibodies in normal birds, but both Flu [34] and Hoessli (quoted by Meyer [111]) failed to confirm this claim. As maintained by Meyer,[111] no credence should be given to the assumption that the insusceptibility of

chickens or other birds was due to their high body-temperature (42°C). Thus, in the opinion of this author, " phagocytic destruction of plague bacilli by fixed macrophages in all probability constitutes an important, if not the determining, factor in the natural resistance of birds to plague infection ".

It is interesting to note that this insusceptibility to plague is not shared by chick embryos (Buddingh & Womack;[16] Jawetz & Meyer[78]). Jawetz & Meyer observed in this connexion that, while virulent plague bacilli introduced in small numbers multiplied rapidly and caused the death of chick embryos within four to eight days, small numbers of avirulent bacilli failed to proliferate freely. Another interesting point was that a potent plague serum was unable to protect the embryos against *P. pestis* because, as these workers assumed, a cellular defence mechanism, without which the serum could not exert its protective action, was still absent.

Rodents

Although plague is primarily a disease of the rodents, even these animals are by no means uniformly susceptible to the infection. Not a few of the numerous species composing the order *Rodentia* have been found to be rather, or even completely resistant to experimental infection with *P. pestis*. More important still, marked differences in plague susceptibility, due either to the existence of differently receptive races or to seasonal influences, have been found even in the species most concerned in the perpetuation of the infection.

While the mechanism underlying this racial or seasonal resistance seems not yet to have been fully elucidated, all available evidence tends to show that, as in the case of the birds, so also in naturally plague-resistant rodents a principal role is played by cellular defence factors.

Proof for this contention seems to have been furnished by Bhatnagar & Shrivastava[11] who worked with Bombay rats (*Rattus rattus*) so resistant to the infection that 91% of the animals survived subcutaneous injection with $3\text{–}4 \times 10^3$ virulent plague bacilli. Through a continued study of the blood picture of the infected animals, the two observers were able to show that during the first 48 hours after infection the polymorphonuclear leucocytes reacted against the invaders. Then an increasing number of clasmatocytes appeared in the blood and obviously continued the fight until on the seventh day, simultaneously with the disappearance of these cells and the appearance of young polymorphonuclears, the condition of the animals improved.

Similarly Meyer[111] found that in the comparatively resistant cotton rat (*Sigmodon hispidus hispidus*) the reticulo-endothelial cells of the liver were instrumental in removing *P. pestis*. It is also noteworthy that, as shown by Larson (quoted by Meyer[111]), the fatal termination of plague infection in monkeys was preceded by a drop in lymphocytes and monocytes.

An interesting observation on the seasonal changes in plague susceptibility was that according to Meyer [111] guinea-pigs surviving from litters, other members of which had been fully amenable to infection during the summer, became quite resistant during the winter. Washed peritoneal-exudate cells of such animals were able to destroy in vitro several hundred plague bacilli within 3–24 hours.

Plague in hibernating wild rodents will be dealt with in a later study, but it should be mentioned here that according to Gaiski,[38] who devoted much effort to a study of this problem, the resistance of such animals during hibernation was due not to immunological factors but to bacteriophage action.

Man

No convincing evidence is available to show that a natural immunity to insect-borne plague exists in man. In particular there is no secure foundation for the belief that certain races are less susceptible to this type of the infection than others, the differences observed in this respect being, in all probability, due to greater or lessened chances for infection and not to racial differences in susceptibility. The existence of so-called healthy carriers of bubonic plague, postulated by a few observers, must not necessarily invalidate the rule that no natural resistance to insect-borne plague exists in man, because such individuals, having probably passed through an unnoticed attack of pestis minor, were presumably convalescent and not healthy carriers.

It cannot be claimed, however, that the same explanation holds true in the case of the carriers of pneumonic plague observed upon several occasions or, more generally speaking, of persons who did not contract this highly infectious form of the disease in spite of taking no precautions when in prolonged close contact with pneumonic-plague patients, for instance, in wards (Wu Lien-teh [189]). While it is possible, therefore, that instances of a resistance to pneumonic-plague infection exist, they are of such rare occurrence as to be of no practical importance.

MECHANISM OF ACTIVE IMMUNITY TO PLAGUE

Earlier views on the mechanism of active immunity to plague may thus be summarized :

Infecting white rats by the intraperitoneal route, Markl [108] found that lysis and phagocytosis of the bacilli competed with the rate of multiplication of those remaining free in the exudate.

Similar results were obtained by Rowland [151] with intraperitoneally- and subcutaneously-infected rats. Finding that virulent bacilli multiplied slowly in the body of vaccinated rats, and that avirulent bacilli did likewise

even in normal rats while virulent infection progressed rapidly in the latter, he reached the conclusion that the essential factor in plague immunity was that which affected the multiplication of the bacillus.

Commenting upon these findings of Markl and Rowland, Petrie [131] stated that the part taken by phagocytosis in the defence mechanism could be explained on the basis of an opsonic action. He noted in this connexion that Douglas (quoted by Brooks [15]) had found a good opsonic response in man after inoculation with Haffkine vaccine while Brooks [15] had the same result when injecting white rats with the soluble protein of *P. pestis*. However, the bacteriolytic part of the defence mechanism could not be easily explained, since most workers failed to demonstrate a bactericidal element in immune serum.

Malone et al.,[104] estimating the bactericidal power of rat-blood and afterwards infecting these animals with a standard dose of plague bacilli, found that the results obtained by these two methods did not tally, artificially immunized rats having a better chance for surviving after the challenge infection than the moderately-resistant Bombay rats or the fully-susceptible Madras rats, regardless of their haemobactericidal power. Malone and his co-workers assumed, therefore, either that the blood of immune animals possessed in vivo some antibacterial quality which could not be demonstrated in the laboratory, or that part of their immunity was derived from sources outside the blood stream (" tissue " or " antiblastic " immunity).

Role of Phagocytosis

Dealing with the behaviour of *P. pestis* in normal and immune experimental animals (guinea-pigs and mice), Jawetz & Meyer [80] found that if smaller inocula were given to immune animals, the bacilli were frequently fixed at the site of infection and slowly destroyed there. Administration of large numbers of virulent bacilli led to their rapid distribution in actively-immunized as well as in normal animals, but in the case of the former this was followed in a few days by the disappearance of the micro-organisms first from the blood, then from the liver and spleen, and afterwards from the lymph-nodes and site of infection.

Continuing their studies, Jawetz & Meyer [79] were able to confirm the above-mentioned opinion of Petrie as well as the results of recent work by Joukov-Verejnikov & Fadeeva [83] by showing that the serum of animals immune to plague was unable to destroy or lyse *P. pestis* either in vitro or in vivo in the absence of phagocytic cells. Whole blood of plague-immune animals could destroy a much greater number of plague bacilli than blood of normal animals, this activity being found to be primarily inherent in the plasma which probably either made the bacilli more easily digestible for the phagocytes or conferred specific qualities upon the latter.

Assessing their results as a whole, Jawetz & Meyer [79] found that the problem of plague immunity was rather complex, and that " in all probability different mechanisms may be responsible for a greater or lesser share in the total picture of immunity in the various laboratory animals ".

In a recent comprehensive survey of the problems of plague immunity, Meyer [111] pointed out that, as shown by the investigations of Burroughs,[18] the intact skin was capable of barring the entrance of plague bacilli to a considerable degree. Since, however, infection through flea-bites led to a direct invasion of the capillaries, a further defence mechanism, furnished by the leucocytes and auxiliary elements, had to come into play.

In order to study the factors involved, Meyer and his co-workers made ample use of cytograms, a method originally devised by Rowland [151] and also used by Pokrovskaya & Kaganova,[139] to whose work reference is made below. Experimenting with different animals, particularly with monkeys and mice, Meyer and colleagues used the following technique :

" An area of the ear or sternum is inoculated with plague bacilli and a fine needle is inserted into the area. Samples of the tissue fluid are aspirated at regular intervals and suitably-stained smears are made for cytological examinations ".

As stated by Meyer, during the first two hours after infection, when in normal as well as in moderately-immune animals the injected area became markedly oedematous, the films prepared from either normal or immune animals did not differ strikingly. Then, however, marked differences began to appear, as shown by the following summary of Meyer's principal findings:

(1) Between two and eight hours after injection the plague bacilli became agglutinated in immune but not in normal animals. Many single organisms were swollen and indistinct in the immune animals and, in contrast to the non-immune, the number of the bacilli was definitely reduced.

(2) With the arrival of polymorphonuclear leucocytes between four and six hours after, phagocytosis became pronounced in the immune animals while only occasional cells of the non-immune ingested a few bacilli. By the 12th hour phagocytosis dominated the microscopic picture in the immune animals while in the non-immune animals not more than 10% of the leucocytes were able to ingest plague bacilli in the early stages of the infection. Thus, while in the exudate of the immune host phagocytosis competed successfully with the rate of multiplication of the plague bacilli, in the non-immune animals the bacilli multiplied extracellularly without hindrance and phagocytosis practically ceased by the 24th hour.

(3) Within 24–36 hours the serous tissue fluid in the immune animals became converted into a dense cellular exudate. Simultaneously the leucocytes clumped and the exudate condensed, thus aiding fixation of the bacilli. The few micro-organisms which did not become phagocytized were ultimately taken up by polyblasts of the macrophage series.

Summing up the problem of plague immunity in general, Meyer considered that

" phagocytosis is the most important mechanism which animals and man use in guarding against and disposing of a plague infection. The mesenchymal tissue cells are responsible not only for cellular immunity, but for humoral defense as well. Their cytoplasm, modified by effective contact with antigen, develops adaptive enzymes and thus circulating antibodies . . . which in turn remove the antiphagocytic property of the slimy ' envelope ' of the plague bacillus. . . . The effectiveness of the mechanism

and consequently the fate of the host are determined by the balance between bacterial multiplication and the efficacy of the clearing mechanism ".

" Organ " and Local Immunity

Some evidence exists that under certain circumstances even in immune animals the lungs are less resistant to plague infection than organs like the liver and spleen.

It should be noted in this connexion that, as established by Batzaroff [8] and confirmed by other workers such as Girard [53] and Jawetz & Meyer,[80] animals partly immune to plague, and therefore succumbing to a challenge infection after prolonged illness, regularly showed marked lung involvement in the absence of liver and spleen lesions.

Discussing these findings, Jawetz & Meyer [80] drew attention to the observation of Sprunt & Camalier [180] that the resistance of the lung to bacterial infections was lowered through circulating toxins. Experimental animals like guinea-pigs, monkeys, and also mice, if partially immune, could withstand the original impact of the infection but at the same time plague bacilli persisted and slowly multiplied at the site of the infection and in the regional lymph-nodes, and disintegration of part of the micro-organisms led to the liberation and circulation of endotoxins. The latter led to the establishment of a locus minoris resistentiae (site of least resistance) in the lungs so that pneumonic foci could become established even as the result of a minor bacterial invasion. That no foci of the infection developed in the liver and spleen was probably due to an enhanced cellular activity which not only prevented multiplication of the plague bacilli but also resulted in an increasing toxin resistance.

The explanation given by Pokrovskaya & Kaganova [139] for the low resistance of the lungs to plague infection was that the cells of this organ were lacking in phagocytic power. Discussing the pathogenesis of two human cases of chronic meningitis and encephalitis observed in California, Jawetz & Meyer [80] similarly assumed that the bacilli reaching the brain early in the disease could persist and slowly multiply there because they were protected from phagocytic action.

Petragnani [130] claimed that a local immunity could be created through instillation of avirulent plague bacilli into one eye of guinea-pigs. Such animals survived if some weeks later virulent bacilli were instilled into the same eye but succumbed to generalized plague if the unprotected eye were infected.

Anti-infectious and Antitoxic Immunity

A distinction between an anti-infectious and an antitoxic immunity was made by some observers.

In the experience of Jawetz & Meyer,[79] a high antitoxic resistance did not by itself protect against plague infection whilst a high anti-infectious

immunity was invariably accompanied by a certain degree of toxin resistance. This was in agreement with the results previously obtained by Markl,[108] but not with those of Rowland [151] who claimed that anti-infectious immunity was conferred by the administration of plague toxin or toxoid.

In the opinion of Otten, antitoxic immunity could be more easily produced than anti-infectious immunity but was of little value under natural conditions where the invasive power of the plague bacilli was the decisive factor. Jawetz & Meyer,[79] while admitting that in anti-infectious immunity the defence was primarily directed against bacterial multiplication and not against liberated endotoxin, considered it as likely

" that active or passive antitoxic immunity can temporarily ward off the effects of products of bacterial disintegration and thereby permit the development of an efficient defense mechanism directed against the multiplying bacilli ".

As established by these workers,[77] fairly good antitoxic immunity could be produced in mice by either live avirulent or formalin-killed vaccines, provided a restimulating injection was administered in addition to the initial dose.

ACTIVE IMMUNITY TO PLAGUE

It is certain that human beings as well as rodents, if surviving a plague attack, are apt to be resistant to the infection. However, owing to the fact that until recently instances of recovery from severe forms of the disease were infrequent in man, little information is available on the solidity and duration of this naturally-produced state of immunity.

As pointed out by Dieudonné & Otto,[29] the fact that people who had recovered from plague were unlikely to contract this infection again, had long been known. For instance, attempts were sometimes made in the past to employ preferably persons who had survived an attack of the disease (the so-called " mortis ") as attendants in the plague wards. Dieudonné & Otto also noted that this empirical knowledge had been confirmed in recent times by the demonstration of specific antibodies in the serum of convalescents. At the same time, however, they were careful to point to some instances of re-infection of individuals who had previously had bacteriologically-confirmed attacks of plague and maintained in general that the immunity to this disease, acquired under natural conditions, was evidently relative in degree and limited in duration. Meyer [110] has recently endorsed this opinion.

Attempts to protect human beings against plague, in the same way as variolation was practised in the case of smallpox, were made at an early date.

The Hungarian physician Weszpremi, who seems to have been the first to use this method (1755), was followed by Samoilovski in Russia who, having become infected with pus from a bubo in 1781, contracted the

disease in a slight form and recommended, therefore, protection against plague by means of a tampon moistened with such pus which was applied to the arm with the aid of a bandage. As was to be expected, the use of this method led sometimes to disastrous results so that it soon came into discredit (Dieudonné & Otto [29]).

The modern history of plague vaccination may be said to have begun in 1895 when Yersin, Calmette & Borrel [190] showed that rabbits could be immunized against this infection by repeated inoculation with suspensions from agar cultures killed by exposure to 58°C for one hour. About a year later Haffkine [71, 72] obtained identical results when using broth cultures of *P. pestis* sterilized by heating.

Since that time large-scale advantage has been taken of anti-plague inoculation not only with killed vaccines grown either in broth or on solid media but, recently, also with live avirulent bacilli. The possibility of using extracts prepared from *P. pestis* by chemical methods for the same purpose has also received attention.

Killed Plague Vaccines

Evaluation of potency

Although, as testified by an enormous literature, numerous methods or modifications of methods have been recommended for the preparation of killed vaccines, until recently the value of these procedures could not be properly assessed. It was next to impossible to arrange for actual tests in the field on a sufficiently large scale and, moreover, most difficult adequately to interpret the effects of these methods in man. It would seem at first glance that tests made in the laboratory ought to have given less doubtful results, but no exact methods were available for this purpose and great uncertainty existed regarding the proper choice of test animals.

Discussing the latter problem, Taylor [184] considered both the lower monkey species used in plague work and the rabbit as unsuitable for testing vaccines on account of the variability of their response to challenge doses.

Guinea-pigs also appeared unsuitable for vaccine work, both on account of their resistance to toxins and because they did not respond well to immunization.

Trapped wild rats (*R. rattus*), which were largely used in the Haffkine Institute, were by no means ideal. In this connexion Taylor pointed out the necessity of transporting susceptible rats from Madras to Bombay, which resulted in a considerable mortality. Moreover, the animals proved very susceptible to the toxic action of the vaccine, frequently 20% to 30% succumbing when the usual test dose of 0.5 ml was administered. The average immunity of the rats, on the other hand, was only in the vicinity of 30% to 40%. Therefore wild rats, though useful for tests on broad lines, were unsuitable for biological standardization or the elucidation of finer differences between the vaccines tested.

Laboratory-bred albino rats were found less susceptible than the wild *R. rattus* and irregular in their response to infection with various doses.

However, as announced by Taylor, Sokhey had recently established that the strain of white mice inbred in the Haffkine Institute had proved excellent. These animals were as a rule fully protected when receiving 0.03 ml of Haffkine vaccine while 10 times this amount was required to produce toxin deaths. At the same time their susceptibility to the challenge infection was very regular.

The great value of the white mouse for testing plague vaccines has been recently endorsed by Meyer et al. [114] who reached the conclusions that

(1) the lyophilic envelope fraction IB' antigen is probably essential in the maximum protection of man;

(2) since this antigen is equally indispensable in the protection of mice against plague, it is deemed advisable to use mice rather than guinea-pigs to measure the immunogenic potency of a plague vaccine.

The method of biological standardization of plague vaccines introduced by Sokhey to replace the former unsatisfactory procedures was based upon the use of the method for measuring the virulence described earlier (page 166); it consisted in determining the minimum quantity of a plague vaccine that would save a white mouse challenged seven days after administration of the second dose of the vaccine under test. As described by Sokhey & Maurice [177] the procedure was as follows :

> The quantity of the vaccine to be administered was divided in two equal doses and given subcutaneously with a seven-day interval. Five mice were used for each quantity of vaccine and four to five quantitites were used at a time. The minimum quantity that saved at least three out of five mice during a 25-day period of observation, commencing with the day on which they had been given the challenge dose, was taken as the protective dose of that particular vaccine. The results for all the vaccine quantities used had to be consistent among themselves and the result of duplicate tests had to tally. The mortality of the control animals had to be 100%.

Again, discussing the method of biological standardization, Sokhey & Maurice [178] stated that they had obtained good results with Haffkine vaccine in guinea-pigs as well as in white mice, as shown thus :

Number of animals	*Total vaccine dose (ml)* [a]	*Challenge dose (number of organisms)*	*Number of deaths*
5	0.80	1 million	1
5	1.25	1 million	0
5 (controls)	—	1 million	5

[a] Given in two equal doses at seven-day intervals.

It should be noted that satisfactory results with guinea-pigs have been recently reported also by Wayson et al.[188] and by Meyer [110] who worked with vaccines treated with alum or other synergists.

Comparing the efficacy of killed vaccines for white mice (body-weight 25–30 g) and white rats (200–270 g), Sokhey & Habbu [173] found that, contrary to the statements made by some other observers, the latter animals

could be equally-well protected by Haffkine broth vaccine grown at 28°C and agar vaccine prepared from cultures incubated at 37°C :

Vaccine	*Mouse-protective dose (ml)*	*Rat-protective dose (ml)*
Haffkine vaccine (28° C), four week's growth	0.004	0.030
Agar vaccine (37° C), 1,000 million organisms per ml	0.004	0.028

Relation of virulence to potency

Summarizing the literature available up to 1936, Pollitzer [140] stated that the necessity of using the most virulent strains possible for the preparation of plague vaccines had been stressed by pioneers in this field such as Haffkine and the German Plague Commission as well as by all subsequent workers. However, some recent observations have thrown doubt upon the absolute validity of this claim. Thus Schütze,[158] experimenting with Haffkine-type vaccines prepared from virulent and avirulent plague strains, came to the conclusion that " virulent cultures do not result in more potent vaccines for either rats or mice than do avirulent ones ". Likewise Sokhey & Habbu [173] obtained the following results when comparing two heat-killed vaccines (A and B) prepared from strains showing marked differences in virulence :

Vaccine	*Mouse m.l.d. (number of organisms)*	*Mouse-protective dose (number of organisms)*
A	5	6,500,000
B	300,000,000	5,300,000

Sokhey & Habbu concluded that these experiments had not been carried to a logical end, but the observations so far made showed that even considerable differences in the virulence of strains did not make any difference to the protective power of a heat-killed vaccine.

Assessing the comparative value of virulent and avirulent plague strains for the manufacture of killed vaccines, it must be kept in mind, however, that while the antigenic value of the former is and remains equal under proper conditions of storage, avirulent growths show great variations in potency (Sokhey [168]) and, as will be discussed later (page 200), even the antigenic value of one and the same strain may deteriorate.

Relation of toxicity to potency

The relation of the toxicity to the potency of the vaccines was thus summarized by Pollitzer [140] in 1936 :

Haffkine was convinced that a definite degree of toxic reaction was desirable and necessary for effective immunization; in fact the dosage of his prophylactic fluid for man was originally determined on the basis of toxicity. Rowland, though pointing out that the toxicity of a vaccine could be abated without interfering with the antigenic properties, was in accord with Haffkine's views. Taylor, summarizing the subsequent experiences of the Haffkine Institute, admitted some influence of storage on the toxicity as against the potency, but also insisted upon a close relationship between these two

properties not only in the case of Haffkine's fluid but in that of all effective plague vaccines. Petrie, discussing the theoretical aspects of this problem, stated that there was no clear proof of the existence of an independent antigen apart from the toxin and the opsonic antigen which conferred an active immunity against the living culture. The rapid induction of immunity conferred by the nucleoprotein seemed, in Petrie's opinion, to bear a relation to the rapid absorption of plague toxin or the rapid opsonic response rather than to the slower action of a bacterial antigen of the thermostable type.

Considering this evidence, Pollitzer stressed the fact that, as shown by some of the above-mentioned and also by other observers, the toxicity of plague vaccines could be abated without any considerable loss in potency. Thus, as shown by Schütze [156] and confirmed by Sokhey & Maurice,[176] incubation of broth vaccines at 37°C instead of 27°C reduced their toxicity without interfering with their antigenic properties.

Measuring the toxicity of broth- and agar-vaccines by determining the doses which killed 50% of the white mice used (body-weight 26–27 g), Sokhey & Habbu [173] obtained the following results:

Vaccine	*Toxic doses (ml)*
Haffkine vaccine (28°C), four weeks' growth	0.2
Agar vaccine (37°C), 1,000 million organisms per ml	1.0

Sokhey & Habbu concluded from this and confirmatory tests that agar vaccine was much less toxic than broth vaccine. At the same time, however, they recorded encouraging results when using formalin instead of heat to sterilize the vaccine obtained from four-week growths in casein hydrolysate.

Method of sterilization	*Toxic dose for mice (LD_{50})*
Heat (54°C) + phenol (0.5%)	0.2
Formalin (0.05%) + phenylmercuric nitrate (1 mg per 100 ml)	0.4
Formalin (0.075%) + phenylmercuric nitrate (1 mg per 100 ml)	0.5

Since, as will be discussed later (see page 192), the new casein-hydrolysate vaccine was an excellent prophylactic, these observations support the view that it is possible to reduce the toxicity of plague vaccines without biasing their antigenic value.

Comparative potency of supernatant fluid and solid portion of vaccines

While some early observers like the German Plague Commission [39] were of the opinion that the antigenic potency of Haffkine vaccine was vested in the solid portion containing the body-substances of the bacilli, most workers were agreed that since the antigenic fraction of *P. pestis* responsible for the production of immunity apparently passes into solution, the whole value of this vaccine resides in the fluid portion (Taylor [184]).

Dealing again with this problem recently, Sokhey & Habbu [171] confirmed that, in the case of broth vaccines, the total protective power resided in the supernatant fluid, regardless of whether incubation at 27°C or 37°C

had been used. They likewise observed that when 48- or 72-hour agar growths incubated at 37°C were suspended in water and the bacilli were left in this for three to seven days, the total immunogenic power was contained in the clear fluid separated from the solid part of the suspension. However, in the case of growths incubated at 27°C, the clear fluid obtained in the same manner had very poor protective power.

Sokhey & Habbu suggested that it might be advantageous to use only the supernatant fluid of broth vaccines grown at 27°C or of suspensions of agar cultures incubated at 37°C for inoculation because such vaccines would be less toxic.

Methods of killing vaccines

Heat. The pioneers of antiplague inoculation resorted to heat to render their vaccines innocuous and this practice has been followed until recently by most workers.

Haffkine first sterilized his broth-vaccine brews by exposing them for one hour to 70°C but soon reduced the temperature to 50°–55°C and the period of heating to 15 minutes. While this period remained the standard in the Haffkine Institute, temperatures of 60°–64°C were used for the preparation of broth vaccine after the departure of Haffkine in 1905 and until Sokhey demonstrated that they exerted a most deleterious effect on the potency of the brews. The method of applying a temperature of 55°C for 15 minutes (Taylor;[184] Sokhey[167]) was therefore once more adopted.

Antiseptics. While sterilization by heat remained until recently the standard method for the preparation of Haffkine vaccine, other workers began to use antiseptics for the same purpose. Thus mere phenolization at room temperature was employed for the manufacture of the agar-grown Lister Institute vaccine and was also utilized by Burgess.[17] Row and Nikanorov (quoted by Pollitzer[140]) worked with glycerol but their products, which were destined for the treatment of plague patients rather than for prophylaxis, had the drawback of requiring prolonged storage before use.

Formalin, which had previously been used by Batchelder[7] to prepare plague antigens for laboratory purposes, was actually used for the manufacture of plague vaccines by French workers as well as in the USA (Meyer[110]) during the second World War, and has also been adopted for the production of the new casein-hydrolysate vaccine in the Haffkine Institute (Sokhey & Habbu[174]).

Comparing different methods of killing vaccines, Sokhey & Habbu[172] recorded the following results :

Method	*Mouse protective dose*	
	agar vaccine	*broth vaccine*
Heating at 54°C	0.0065	0.006
Merthiolate (1/500,000)	0.0068	0.006
Sulfathiazole (1%)	0.0070	—
Alcohol (Felix)	0.0160	—

Types of killed vaccines

(1) *Haffkine's vaccine.* Bearing in mind that, owing to the introduction of the new casein-hydrolysate vaccine, the methods of manufacturing the classical Haffkine fluid have become a matter of historical rather than of actual interest, it is proposed to deal here only with those principles involved in its preparation which remain important for future work.

(*a*) *Incubation temperature:* The incubation temperature of about 27°C originally recommended by Haffkine has been found best for the preparation of fluid plague vaccines by most subsequent workers. As confirmed by the exact tests of Sokhey,[166] broth vaccines grown at about 27°C were more potent than those incubated at 37°C because growth of *P. pestis* was maximal at or near the former temperature. Further, as stated by Taylor,[184] incubation at 27°C was also beneficial insofar as it restrained the development of many other micro-organisms including the pasteurellae tested. It was likewise a convenience that during the greater part of the year the average room-temperature at Bombay was about 27°C.

The lesser toxicity of broth vaccines grown at 37°C noted before is a point deserving great attention but, as has been found by Sokhey & Habbu,[173] the use of formalin instead of heat for manufacture of such vaccines seems to go a long way towards restoring a balance in favour of incubation at 27–28°C.

(*b*) *Period of incubation:* Though Haffkine had recommended incubating his vaccine for six weeks, this practice was not strictly adhered to by his successors and finally an incubation period of four weeks was made the standard.

(*c*) *Storage:* It is generally agreed that storage of the Haffkine vaccine, while reducing its toxicity (Stevenson & Kapadia [182]) did not exert an unfavourable influence on its potency. Taylor [184] noted in this connexion that after a slight drop in potency which occurred during the first month, the vaccine, even if stored at room temperature, retained a high immunizing value for a year and lost but little of its potency during the following six months. Sokhey & Habbu [173] even found that a batch of the vaccine which had been lying at room temperature for about 10 years still showed a very high protective power. They also made the following observations comparing the potency of two lots of broth vaccines, grown for four weeks at 28°C and heat-killed, with that of two lots of heat-killed agar vaccines, grown at 37°C and stored at different temperatures :

Media	*Storage*		*Potency*
	temperature (°C)	*period (weeks)*	*(mouse-protective dose) (ml)*
Broth	0		0.0052
,,	45	8	0.0040
Agar	0		0.0035
,,	45	6	0.0060

It will be noted that, in contrast to agar vaccine, broth vaccine kept well even if stored at 45°C.

(2) *Casein-hydrolysate direct vaccine.* The preparation and properties of the new casein-hydrolysate plague vaccine were thus described by Sokhey & Habbu [174] (see also Sokhey, Habbu & Bharucha [175]) :

A very much improved plague vaccine with a consistently high protective value can be prepared by the use of Mueller & Johnson's casein hydrolysate. The medium is so prepared that controlled digestion produces a fluid which is entirely free of proteins and is perfectly limpid with a light-yellow tinge. It is adjusted to contain 270 mg of nitrogen per 100 ml.

The medium is distributed in quantities of 1 litre in modified Haffkine flasks of three-litre capacity and is seeded with specially selected strains of *P. pestis* preserved by drying from the frozen state. The growths are incubated at 28°C for two weeks. Killing is done by the addition of 0.1% formalin, and phenylmercuric nitrate is used as the preservative.

The mouse-protective dose of this vaccine is 0.004 ml. It is much less toxic than the previous vaccine, since 0.6 ml, as opposed to 0.2 ml, is needed to kill a mouse. The keeping qualities of the new vaccine are very good; stored at 37°C for 18 months, it does not show any loss of protective power.

(3) *Agar-grown vaccines.* The technique originally adopted by the German Plague Commission [39] for the manufacture of agar-grown plague vaccine consisted in washing off the growth of two-day-old virulent cultures with broth or saline, heating the collected washings for one to two hours at 65°C and afterwards adding carbolic acid so as to obtain a concentration of 0.5%.

The modifications of this procedure introduced by subsequent workers may be summarized thus :

(*a*) *Media :* While mos tof the earlier workers did not specify the composition of the solid media they used, Burgess [17] recommended a " whole meat " agar as being simple to prepare and yielding abundant growth. Special media preferred by recent workers were glycerol agar (Barreto [6]), hormone or hormone-sulfite agar, used for the preparation of the US " Army vaccine " during the second World War, and blood tryptose beef-heart agar, used by Wayson et al.[188] for obtaining vaccines for experimental purposes.

(*b*) *Temperature and period of incubation :* The earlier workers do not seem to have been particular as to the temperature of incubation, apparently using temperatures ranging from 27°C to 30°C. Schütze [156] seems to have been the first who insisted on incubation at 37°C because he assumed that in this way growths rich in " envelope " antigen resulted. Sokhey [166] also found that agar vaccines grown at 37°C were far more potent than those obtained at 27°C, when the mouse-protective dose was 0.1 ml as against 0.002–0.004 ml in the case of the former. However, he did not ascribe

this difference to the presence or absence of an " envelope " but pointed out that the plague bacilli grown at 37°C were bigger and that, consequently, the actual bulk of agar vaccine obtained at this temperature was greater than that of 27°C vaccine containing the same number of organisms. Sokhey assumed, however, that this was probably not the only factor involved.

An incubation temperature of 37°C was also found preferable by other recent workers such as Gracian.[63] The US Army vaccine seems to have been grown at 30°C but Wayson et al.[188] incubated the vaccines they prepared for comparison with it at 39°C.

The periods of incubation adopted by the various workers ranged from 40 to 72 hours.

(*c*) *Standardization :* Besides the usual methods of standardization (actual counting or opacity tests) some special procedures were utilized. Burgess,[17] who formerly adjusted his plague vaccine to contain 3,000 million bacilli per ml (the standard previously adopted for the Lister Institute vaccine), afterwards used a standard of 1.5 mg of dried bacterial substance per ml and considered both these strengths as equivalent.

A more expedient method originally devised by Japanese workers was to weigh the sediment obtained by centrifuging the vaccine and then to dilute the latter so that each ml contained 6 mg of the sediment (Pollitzer [140]).

Sokhey [169] used agar vaccines adjusted to contain 1,500 million plague bacilli per ml so as to give them the potency possessed by broth vaccine incubated at 27°C for four weeks. A lower standard (1,000 million) was adopted by Barreto,[6] a higher for the Army vaccine (2,000 million per ml).

Comparing agar- and broth-grown plague vaccines, it has to be noted that the former can be manufactured more easily and expeditiously than the latter. This difference is of great importance in emergencies, when it will be possible quickly to prepare agar-grown vaccines from freshly-isolated local strains. In areas where plague occurs perennially, however, stocks of broth-grown vaccine, which in contrast to agar-grown vaccine possesses excellent keeping qualities, can be prepared beforehand.

Sokhey & Habbu,[173] while considering the protective power of Haffkine vaccine grown for four weeks at 28°C roughly to equal that of agar vaccines obtained through cultivation at 37°C, admitted that the former was more toxic. It was on account of this drawback that new research work was started which led to the introduction of the casein-hydrolysate vaccine. As has been noted, this combines high potency with low toxicity.

(4) *Pseudotuberculosis vaccine.* As a result of previous work by Rowland and others (see Pollitzer [140]), a vaccine prepared from formalin-killed pseudotuberculosis bacilli (containing 4,000 million per ml) was introduced for human antiplague inoculation in Madagascar (Boyé [13, 14]). According to Boyé this product protected about 51 % of domestic mice against infection with a tenfold m.l.d. of plague bacilli.

(5) *Lipo-vaccine.* A lipo-vaccine, also introduced by French workers, was prepared according to Boyé,[13, 14] in the following manner :

Plague bacilli were grown for 36 hours at a temperature of 34°C on agar of pH 7.4, then washed off with saline containing 5 ml of formalin per litre. After storage at 37°C for ten days, 12.5 g of centrifuged and dried bacilli were suspended by a special process in an oily excipient.

Pons & Advier,[142] challenging grey mice with 200 virulent *P. pestis* ten days after prophylactic inoculation, found the lipo-vaccine to give a 70% survival as compared with 51.5% in animals protected with aqueous vaccine and 20% in the controls. Almost equally good results were obtained when both plague and pseudotuberculosis bacilli (6,000 million of each species per ml) were used for preparation of the lipo-vaccine.

(6) *Sugar vaccine.* As summarized by Pollitzer,[140] Minervin et al.[115] obtained remarkably good results when protecting sisels with heat-killed suspensions of avirulent plague bacilli in a saccharose solution. Korobkova et al.[92] worked with a similar vaccine which they called AD vaccine (vaccin " adénaturé "). However, in a subsequent report, Korobkova [91] recommended using virulent instead of avirulent plague bacilli for the preparation of this vaccine and also stressed the necessity of sterilizing it without the application of heat so as not to damage the heat-labile Vi antigen of *P. pestis* supposed by her to exist. Consequently, she added to a concentrated suspension of virulent plague bacilli grown at 37°C a double volume of 80% saccharose solution and let the mixture stand at room temperature for 20–25 days to effect sterilization.

Korobkova found that two injections of this vaccine protected 82–100% of white mice against a challenge infection with highly virulent plague bacilli and that protection was also afforded to 50–85% of guinea-pigs which had been given three vaccine doses. A sugar vaccine prepared with the avirulent EV strain was less potent. The addition of 0.05% agar to the sugar vaccines increased their protective power.

(7) *Precipitated vaccines.* The procedures adopted by Wayson et al.[188] for the preparation of plague vaccines with the aid of precipitation methods were as follows :

(*a*) *Alcohol-precipitated vaccine :* Two volumes of 95% ethanol were added to the phenolized saline suspensions of virulent plague bacilli grown on blood tryptose beef-heart agar for 40 hours at 39°C, and the mixtures were centrifuged after having been held at 5°C overnight. The supernatant was discarded, and the sediment was washed in saline, centrifuged, and resuspended in saline and merthiolate (1/7,500) to make up the volume of the original phenolized saline suspension, the bacterial content of which had been standardized.

(*b*) *Alcohol- and alum-precipitated vaccine :* Two volumes of 95% ethanol were added to the phenolized saline suspension as above and held overnight at 5°C; 2.7 ml of a 10% sodium bicarbonate solution and 25 ml

of a 4% potassium alum solution were added per 100 ml of volume, and the mixtures were allowed to stand at 5°C for five hours. The supernatant fluid was removed by centrifuging and the sediment resuspended in saline and held at 5°C for 40 hours. Final treatment was the same as in case of the alcohol-precipitated vaccine.

Results in guinea-pigs immunized with these vaccines and three weeks later either exposed from time to time during a month to the bites of infected fleas or challenged by subcutaneous infection with a large dose of virulent plague bacilli were as follows :

Vaccine	*Flea-infected guinea-pigs*			*Subcutaneously infected guinea-pigs*	
	tested	*clinical plague*	*died*	*tested*	*died*
Alcohol-precipitated	8	8	0	10	2
Alcohol-alum-precipitated (divided dose)				10	4
Alcohol-alum-precipitated (single dose)	5	4	0	10	3
" Army "	8	7	3	10	6
Typhoid	8	7	6	10	9
—	15 [a]	13 [a]	11 [a]	12 [a]	9 [a]

[a] Controls

Wayson and his collaborators expressed the opinion that alcohol- and alum-precipitated vaccines were not superior to those obtained through alcohol precipitation alone.

Meyer [111] obtained the following results when challenging guinea-pigs which had been immunized with ordinary or precipitated vaccines by various routes :

Agent used for killing organisms	*Adjuvant*	*Number infected*	*Survival*	
			number	*percentage*
Phenol	None	29	5	17.2
	Alum	30	13	43.3
	" Falba " [a]	28	16	57.1
		30 [b]	0 [b]	0 [b]
Alcohol	None	30	7	23.3
	Alum	30	17	56.7
	" Falba "	30	24	80.0
		30 [b]	0 [b]	0 [b]
Formalin	None	40	10	25.0
	Alum	37	20	54.0
	" Falba "	37	33	89.7
		40 [b]	0 [b]	0 [b]

[a] " Falba " is a mixture of oxycholesterin and lanolin
[b] Control animals

It will be noted that remarkably good results were obtained with formalin-killed and Falba-precipitated vaccines and to a slightly lesser degree also with those killed by alcohol and precipitated with the aid of Falba.

(8) *Chemically-prepared extracts of* P. pestis. As has been noted before (page 178), Kurauchi & Homma [94] were able to immunize guinea-pigs, rats, and monkeys satisfactorily against plague infection with their " specific soluble substance ", particularly if this had been absorbed by metallic salts.

Meyer et al.[114] recorded the results, given in table I, which they obtained with the various fractions of *P. pestis* separated by them with the aid of chemical procedures.

TABLE I. RESULTS OF DOSING DIFFERENT ANIMALS WITH VARIOUS FRACTIONS OF PASTEURELLA PESTIS

Antigen	Guinea-pigs		White rats		Cotton rats	Monkeys
	Dosage (mg)	Result [a]	Dosage (mg)	Result [a]	Result [a]	Result [a]
Fraction 1A	1.5	0/20	—	—	—	—
,, + alum	1.5	0/20	—	—	—	—
Fraction 1B	1.5	0/19	0.07	14/19	1/9	10/10
,, + alum	1.5	1/20	0.07	16/18	6/10	—
Insoluble residue	2.5	10/20	0.35	7/20	0/10	5/10
,, ,, + alum	2.5	16/20	0.35	8/19	—	—
Insoluble residue	12.5	13/20	—	—	—	—
,, ,, + alum	12.5	19/20	—	—	—	—
Formalin-killed suspension. .	1.5	2/10	0.35	17/19	5/10	8/10
,, ,, + alum	1.5	8/10	0.35	18/18	9/10	—

[a] The first figure in these columns indicates the number of survivors after challenge infection, the second the number of animals used.

It will be noted that the best results in guinea-pigs were obtained with alum-precipitated preparations of the water-insoluble residue but that a formalin-killed and alum-precipitated vaccine also proved quite satisfactory. In white rats, on the other hand, good success was obtained with fraction IB as well as with formalin-killed suspensions of *P. pestis* while the insoluble residue gave indifferent results.

The vaccines obtained with the aid of bacteriophage will be dealt with later on.

Live Avirulent Vaccines

Although some of the pioneers, particularly Kolle & Otto,[88] had shown that experimental animals, including guinea-pigs, could be rendered immune to virulent plague infection through prophylactic administration of *P. pestis* strains, the virulence of which had been lost spontaneously or had been abolished by artificial means, Strong [183] in Manila seems to have been the first modern worker who took practical advantage of live avirulent plague

bacilli to immunize 900 human beings. Since the disease was not present in Manila at the time, the actual value of the method could not be assessed, but it was significant that the serum of those inoculated was found to contain agglutinins and was also apt to protect experimental animals against challenge infection with *P. pestis*.

However, the history of inoculation with live avirulent plague vaccine may be said to have really begun in 1934 when Girard & Robic [57] and Otten [123] paved the way for the large-scale use of this prophylactic in Madagascar and Java respectively. The great success obtained in these two areas led to the introduction of this type of plague vaccination into other countries, such as Argentina (Savino [154]), the Belgian Congo (Devignat [26]), Brazil (Goobar [62]), French West Africa (Rotman [148]), Tunisia (Magrou [103]), and the Union of South Africa (Grasset [64]).

Girard & Robic's EV strain

The properties of the strain described by Girard & Robic,[58] and much used in Madagascar and elsewhere for human inoculation, are as follows :

The EV strain had become rather avirulent while being subjected for five years to monthly agar subculture at 16°-20°C, after that time proving innocuous to guinea-pigs and rabbits when administered percutaneously, subcutaneously, conjunctivally, or by feeding. Subcutaneous injection of guinea-pigs was apt to produce a local induration which disappeared in about a week without involving the regional lymph-nodes (Girard & Robic [57]). Intraperitoneal injection of one-third of a slant, or more, of this strain was apt to produce in guinea-pigs or rabbits a fatal peritonitis terminating in septicaemia, but the bacilli isolated from the blood were avirulent. If lesser, though still considerable, doses were given intraperitoneally, and the animals were sacrificed from the 5th to the 15th day after injection, nodules were found to be present in the spleen, and more rarely in the liver, which did not contain the bacilli. This process was accompanied by spleen hypertrophy which disappeared about the 20th day after injection.

Girard & Robic laid great stress upon these reactions because they assumed that the antigenic potency of their strain was due to preservation of some of its virulence and toxicity. In a later publication, Girard & Radaody-Ralarosy [56] also recorded that, in contrast to non-immunogenic avirulent strains, the EV strain possessed invasive powers, subcutaneous administration of a dose of 1,000 million leading to the appearance of the bacilli after 40 hours in the blood, towards the 4th day in the spleen, and somewhat later in the liver. The bacilli disappeared in the same order within two days after injection from the blood, and within 11 and 13 days, respectively, from the spleen and liver.

At the same time, however, prophylactic administration of the EV strain protected guinea-pigs against challenge with enormous doses of

virulent plague bacilli by various routes, including the bite of infected fleas and intratracheal infection.

Whilst *R. rattus* behaved in much the same way as guinea-pigs, mice and white rats, being toxin-sensitive, could not tolerate large doses of the EV strain. They could, however, be satisfactorily immunized with smaller doses.

Otten's avirulent strains

For his early work Otten [124-127] used the Tjiwidej strain, named after the place where it had been isolated from a rat in 1929. This strain had lost its virulence spontaneously after having been kept in serum-agar stab cultures at 5°C for four months.

Subcutaneous administration of even 5 ml. of a broth culture of the Tjiwidej strain or of suspensions prepared from whole agar slants proved harmless for guinea-pigs and *R. rattus diardii*. At the same time, the strain even in single doses proved highly immunogenic for these rodent species which Otten considered most susceptible to plague.

While fresh suspensions of the Tjiwidej strain seemed to possess little toxicity if administered subcutaneously, rats inoculated intraperitoneally were apt to succumb to toxaemia, apparently because this mode of administration led to a rapid liberation of endotoxin. For the same reason, 35% of rats which had been injected subcutaneously with suspensions of the Tjiwidej strain heated to 60°C succumbed within 48 hours to toxaemia. Similar results were obtained by Anchezar [3] when heating suspensions of the EV strain for half an hour at 58°C.

The Tjiwidej, like the EV strain, produced a nodule at the site of subcutaneous injection (Otten; [127] Savino & Anchezar [155]). Although, according to the latter authors, no macroscopically visible reaction was produced in the spleen, Girard & Robic [58] could prove its presence through histological examination. The Tjiwidej strain possessed invasive powers, Otten [127] finding live bacilli present for periods of up to one week at the site of subcutaneous injection as well as in the organs, particularly in the spleen.

As has been mentioned before, Otten,[127] experimenting with a number of other plague strains, was able to isolate avirulent variants through single-colony picking. He emphasized most appropriately that in this way alone it was possible to produce an irreversible loss of virulence.

One of the above-mentioned strains, which had been originally isolated during the 1920-1 pneumonic epidemic at Harbin, yielded an avirulent variant possessing a higher immunogenic power for guinea-pigs than the Tjiwidej strain, which in its turn protected rats particularly well. Consequently mixtures of these two strains were prepared for human inoculation but, as the Harbin strain was rather toxic, it had to be used in a smaller dosage. When inoculation work was resumed after the second World War, it was decided at the Netherlands meeting on tropical hygiene, 1948

(Nederlandsche Vereeniging voor tropische Geneeskunde) [121] to dispense with this strain and use the Tjiwidej strain alone.

Comparing the latter with their own strain, Girard & Robic [58] thought the Tjiwidej strain to be more attenuated. They agreed with Otten that it protected rats better than guinea-pigs, while the EV strain had reverse properties.

Strains used in the Union of South Africa

As stated by Grasset,[64] the live plague vaccine at first used in the Union of South Africa was so prepared as to contain equal proportions of the EV and Tjiwidej strains with a total concentration of 1,000 million organisms per ml. Later, however, in place of the Tjiwidej strain an old strain of Rowland's, labelled K/120, was utilized in conjunction with the EV strain. The strain K/120 was highly immunogenic for *R. (Mastomys) coucha* as well as for guinea-pigs and rats, while the EV strain did not protect the latter or multimammate mice quite so well if virulent South African strains were used for challenge infection (Grasset [65]).

Strains used in other countries

While the EV strain alone was used in Argentina, the Belgian Congo, Brazil, French West Africa, and Tunisia, Korobkova [90] established that guinea-pigs were immunized particularly well by a combination of this and her strain 46-S, obtained from a virulent plague growth through bacteriophage action. A single injection of a mixture of both strains or an initial inoculation with 46-S followed one week later by EV inoculation protected guinea-pigs against challenge infection eight months later. Only 78% of the animals inoculated with EV vaccine alone survived, however, if challenged after the same interval of time.

Views on immunity mechanism

Strong considered the process of immunization produced by the administration of live avirulent plague bacilli as a true vaccination, the organisms multiplying in the tissues and their successive generations stimulating the production of corresponding groups of antibodies (Otten [127]).

As has been stated before, in the opinion of Girard & Robic, [58] the EV strain was immunogenic because it had not altogether lost its virulence and toxicity, possessing, in contrast to non-immunogenic avirulent strains, invasive powers. In fact, in a later communication Girard [54] maintained that " les souches dites ' avirulentes ' et vaccinantes ne sont que des souches de virulence *affaiblie* (et non atténuée, ce qui signifierait qu'elles sont définitivement fixées dans cet état) ".

According to Girard, virulence had to be defined in relation to the animals, the mode of inoculation, and the dosage used. Thus 10,000 EV organisms, while innocuous if introduced subcutaneously or intraperitoneally, killed a guinea-pig when given intracerebrally. Girard postulated

that the immunity engendered by inoculation with live avirulent plague strains was of a cellular rather than a humoral category, and maintained that this assumption was supported by the work of Pokrovskaya & Kaganova [139] who claimed to have enhanced the defence mechanism of the lung against plague infection through the administration of live avirulent vaccines by inhalation. As stated by Girard, the antigenic properties with which the immunizing power of the avirulent strains was associated could become degraded; the factors known to bring about this process were frequent subcultivation at 37°C and temperature variations to which the strains were subjected during transport or storage. Robic [146] had shown that the virulence of the strains could be restored to a certain degree in the laboratory, but in the opinion of Girard this had never been observed under natural conditions.

In connexion with this last-mentioned claim, attention should be drawn to Devignat's recent observation [26] of a slight and temporary increase in virulence of the EV strain he used for vaccine preparation. He states that :

(*a*) A guinea-pig inoculated subcutaneously with 2 ml of a batch of EV vaccine died five days later " avec autopsie typique ", yielding cultures of the strain from its organs.

(*b*) The vaccine batches available at the time produced more marked local and general reactions in man than previous lots. As noted before (page 170), Devignat successfully used his method of bubbling air through broth cultures of the strain to suppress this slight increase in virulence.

Otten,[127] refuting the view that the immunizing action of live avirulent plague strains was associated with their invasive powers, ascribed the superiority of live over killed vaccines to differences in the antigenic properties which, while deteriorating during preparation of the latter vaccines, remained intact in the former. Jawetz & Meyer [77, 80] also maintained that the immunogenic activity of avirulent plague strains was a function of their antigenic make-up, and not of their invasive or pathogenic power.

Finding a close correlation to exist between a high fraction IB content of avirulent plague strains and their power in small doses of protecting mice against challenge infection as well as of stimulating the formation of antibodies in monkeys and man, Meyer et al.[114] concluded that the immunogenic activity of avirulent plague bacilli depended in part upon their antigenic make-up. At the same time, however, these workers, sharing the views of Strong, maintained that

" there is every reason to believe that the high degree of immunity is dependent upon persistence of the antigenic agent in susceptible cells of the body, and not merely on the intactness of the antigenic components ".

Preservation of antigenic potency

To preserve the antigenic potency of avirulent plague strains destined for vaccine manufacture, Girard [54] stressed the necessity of keeping them

constantly at a temperature of 2°-4°C and of subcultivating them not more frequently than once a year. He added that it had been possible in this way to keep the EV strain intact for 14 years.

Storage

As maintained by Otten[126] and Girard,[54] live avirulent plague vaccines cannot be stored for prolonged periods but must be used within a maximum of one month.

Otten[127] noted in this connexion that, if kept at 5°C, his vaccine remained potent for several weeks. Even at room temperature (23°C) the potency decreased only slightly within this time but the number of bacteria diminished and simultaneously the toxicity of the vaccine increased. Tumansky[185] even maintained that live avirulent plague vaccines did not deteriorate for 16 months if kept at −13°C.

Grasset,[65] while in agreement with these views, stated that lyophilization of live avirulent plague vaccines considerably increased their keeping properties. Experimenting with multimammate mice, he found that lyophilized samples, even if kept in the refrigerator for two years, lost little of their potency. Cultures from these samples showed some delay in growth, but subcultures developed well.

It should be noted, however, that lyophilization failed to give good results in the hands of Girard.[49, 54] He also stated that lysis of the avirulent plague bacilli in normal saline occurred very slowly so that even after two years viable organisms could be found in suspensions with an original bacterial content of 1,000 million. Girard considered it as essential, however, to inoculate with a maximum of viable avirulent bacilli which necessitated rapid use of the live vaccines.

Comparative Potency of Killed and Live Avirulent Vaccines

The laboratory evidence available in regard to the comparative value of killed and live avirulent vaccines, with which alone we are concerned at the present juncture, has been the subject of much, and sometimes even acrimonious, debate.

To compare the results obtained with either killed or live vaccines by numerous investigators would be most difficult, not only because they worked with differently susceptible species or races of animals and challenged them with differently sized infective doses, but mainly because they used methods of different accuracy to standardize the dose.

It would be expected that the results of comparative tests made by the same worker at the same time would be of great value for assessing the merits of killed and live vaccines respectively. Unfortunately, however, the results obtained by such comparative tests are quite often not really comparable : the workers interested in killed vaccines matched these against

live avirulent preparations, the potency of which was not as high as that of the live vaccines actually used in areas like Java and Madagascar; while the advocates of live vaccination tested their products against killed ones less carefully manufactured and standardized than the vaccines now issued by, for instance, the Haffkine Institute.

For these reasons it seems advisable, instead of comparing the experiments made by individual workers, to weigh on the one hand the total evidence available in regard to the killed vaccines, and on the other that brought forward in favour of the live avirulent vaccines. If this is done, no doubt can exist in an impartial mind that, if carefully prepared and fully potent products are used in proper dosages and adequate challenge tests are made, equally good laboratory results can be obtained with both categories of plague vaccines. It follows that the question as to which of them should be used for man cannot be decided through experiments with laboratory animals but must be settled according to observations made in the prophylaxis of human plague—a problem which will receive full attention in a later study.

Onset and Duration of Active Immunity to Plague

Rowland [151] established that immunity produced in white rats by plague nucleoprotein evolved rapidly, being distinctly evident after 24 hours and reaching its maximum on the third day. Stevenson [181] (see also Stevenson & Kapadia [182]), inoculating a large number of Madras rats with Haffkine's vaccine, found that immunity began to develop within a few hours and rose till the second or third day. On the other hand it is interesting to note that according to the German Plague Commission [39] immunity in monkeys appeared much later, being slight on the fifth and reaching its maximum on the seventh day. As pointed out by Petrie,[131] these results were consistent with the observations of Brooks on the development of opsonins : in the white rat the maximum response occurred on the first or second day, and in man on the fifth or sixth day.

In the case of the live avirulent plague vaccines, immunity became apparent, according to Girard & Robic,[59] five to ten days after inoculation; Otten,[127] however, states that it appeared after five to seven days and became maximal after two to three weeks.

The statements made by a few observers that, in experimental animals as well as in man, plague inoculation was followed by a " negative phase " during which the inoculated individuals were particularly susceptible to the infection, will be dealt with in connexion with the problems of plague prophylaxis. As will be shown, fears of this kind are unwarranted.

Rowland [151] found, in the course of the above-mentioned experiments, that the immunity conferred by his nucleoprotein was unimpaired at the end of three months and still appreciable at the end of five months. Dieudonné & Otto,[29] dealing with this problem in a general manner, stated that

no exact information existed as to the duration of the immunity produced in animals by inoculation with killed cultures, but took it to last for months; thus, according to Kolle, rats protected by a single injection still showed after five months a marked immunity, even when infected intraperitoneally.

These observations have been corroborated by experiments made with live avirulent plague vaccines. Otten [124] stated that the immunity produced in guinea-pigs by administration of the Tjiwidej strain was still fairly satisfactory after six months but had markedly decreased after the ninth month. The EV vaccine was found to produce in these animals an immunity lasting for about a year or even longer (Girard [54]).

PASSIVE IMMUNITY

Even since Yersin, Calmette & Borrel [190] had demonstrated in 1895 that the serum of rabbits treated with killed *P. pestis* cultures was apt to prevent or even cure infection in normal animals, numerous workers have endeavoured to produce immune sera suitable for the treatment and prophylaxis of human plague. The results of these labours will be appreciated in a later study devoted to the clinical aspects of the disease. It is appropriate here, however, to describe the methods of producing the sera and of assessing their value, and to define their properties.

Production of Immune Sera

Animals used

While horses were the usual, they were by no means the only, animals chosen for the production of plague immune sera, several workers preferring other smaller or larger domestic animals such as goats, sheep, calves, mules, bullocks, and buffaloes.

Attention was also paid to the possibility of using the rabbit which, as noted above, was the animal chosen by Yersin and his co-workers for their original experiments, and which was continually used to produce immune sera for laboratory needs. Naidu et al.,[119, 120] though obtaining excellent experimental results with rabbit sera, considered the yield from this animal insufficient for actual serum production. However, the use of rabbits for this purpose was again recommended by Korobkova [89] and by Jawetz & Meyer.[79] In view of the fully-satisfactory experimental results of the latter, a concentrated rabbit immune serum for the treatment of human plague was actually produced in the USA (Meyer,[110, 111]).

Although the rabbit, in contrast to the horse, has the advantage, experimentally, of being susceptible to infection with *P. pestis*, in view of the great general suitability of the latter animal for serum manufacture it is not surprising to find that most workers continue to use it for the large-scale production of plague immune sera. Particularly noteworthy in this

connexion is the new horse-produced plague serum of the Haffkine Institute, the efficacy of which is shown by the following comparative results obtained by Sokhey : [165]

Types of sera	*Mouse-protective dose (ml)*
New Haffkine	0.05
Buffalo (Naidu et al.)	0.30
Pasteur Institute	0.50
Lister Institute	0.50
Commercial product	No protection with 0.5

Antigens used

A whole series of antigens or combinations of different antigens has been used by the various workers bent on the production of potent plague immune sera.

Generally speaking, the pioneers in this field used either killed plague cultures alone, or first these and then live virulent bacilli; some, however, completed the process of immunization by administering also toxic filtrates of plague cultures or similar products to their animals. Some workers relied on the latter alone; for instance, nucleoproteins were used by Lustig and others, broth filtrates by Markl and Dean (Pollitzer [140]).

Recently, live avirulent plague bacilli were used in place of the above-mentioned antigens (Girard ; [45, 46] Robic ; [145] Pirie & Grasset ; [135, 136] Schütze; [159] Savino & Anchezar; [155] Jawetz & Meyer; [79] Korobkova [89]).

Using a combination of the above-mentioned antigens, Sokhey [165] prepared the new Haffkine serum by immunizing horses first with living avirulent plague cultures, then with live virulent growths, and finally with filtrates of broth cultures which had been incubated for three weeks at 27°C.

The following special methods of immunization also deserve mention :

(*a*) Joukov-Verejnikov et al.[84] reported good results in guinea-pigs with sera obtained by immunization of horses with lysates of " envelope " cultures of *P. pestis* or with isolated " envelope " substances.

(*b*) Girard & Sandor [60] found that the serum of horses which had been hyperimmunized with plague anatoxin (toxoid) gave experimental results identical with those obtained using sera which had been prepared with the aid of live avirulent but toxic plague bacilli.

(*c*) Meyer [110] obtained potent antisera by the immunization of rabbits and monkeys with the crystalline fraction IB plague antigen.

Concentration of sera

As reported by Russell,[152] fractionation of the sera prepared by Naidu and his colleagues with different concentrations of sodium sulfate had shown that the protective power was vested in the globulin fractions and not in the albumin fraction. While it would seem that no large-scale practical advantage was taken of these findings, actual use has been made

of concentrated plague immune sera introduced by Pirie & Grasset.[134, 135] Their original procedure consisted of two fractional precipitations by means of sodium sulfate and isolation of the pseudoglobulin fraction which, in their opinion, contained the majority of the plague antibodies. However, they afterwards used a one-process method to obtain the " euglobulin-pseudoglobulin constituents " which gave more-satisfactory results.

Meyer,[110, 111] experimenting with rabbit immune sera, found that the antibodies were mainly contained in the gamma-globulins which were consequently isolated for the preparation of concentrated serum.

Methods for Assessing the Potency of Immune Sera

Although, as summarized by Jawetz & Meyer,[79] tests for measuring the potency of plague immune sera had been proposed by numerous investigators, most of the earlier methods were unsatisfactory because, at best, they permitted merely a rough evaluation of the results.

An accurate and fully-reproducible biological test introduced by Sokhey [165, 169] was based upon the use of the highly and uniformly susceptible Haffkine-Institute-inbred white mouse and of the standard infective dose as used also for the evaluation of plague vaccines. The principle of the test was to determine the minimum of a given serum which would protect half the number of mice used against the standard infective dose given simultaneously. Details of the test were as follows (Sokhey [169]) : for any given serum five graduated doses were decided upon, after a preliminary test, so that the 50% end-point protective dose fell about the middle of the selected series. For each dose a batch of five mice was used. Both the serum and the standard test infective dose were given at the same time subcutaneously, but in different parts of the abdominal wall.

Any mouse dying during an observation period of 30 days was examined for evidence of plague infection by smear and cultural examination. At the end of the observation period all survivors were killed and likewise examined. The same held good of the ten controls used for each test which, given the infective dose only, were usually dead by the ninth day.

To avoid inaccurate results, each determination was made twice. From the results of these two determinations the 50% end-point was calculated according to the method of Reed & Muench or another suitable method. A potent antiplague serum was required to have a minimum mouse-protective dose of not more than 0.05 ml.

The principle of a " curative test " introduced by Sokhey at the same time was to determine the minimum quantity of a given serum which would save a plague-infected mouse when serum administration was begun after the development of bacteraemia (which with the standard infective dose used became apparent within 72 hours). Essential features of the test were :

For any given serum five graded doses were decided upon so that the 50% end-point curative dose fell about the middle of the selected series.

For each dose a batch of five or six mice was used. The animals were first given the standard test infective dose and 72 hours afterwards the serum in four equal portions, the first being administered intravenously and the remaining three subcutaneously at 24-hour intervals. This determination was done twice to avoid inaccurate results and the mice were observed as described above. On the results of the two determinations, the 50% end-point was calculated. A suitable plague serum was required to possess a minimum mouse curative dose of 0.4 ml or less.

Following up earlier work by MacConkey [102] and others, Jawetz & Meyer [79] proposed a mouse-protective test based upon the use of a standard serum. They defined as the provisional standard unit the amount of serum which would protect 50% of mice from death when 1,000 average lethal doses (a.l.d.)—about 2,000-5,000 plague bacilli—were injected intra-abdominally 60 minutes after administration of 0.5 ml of serum dilutions by the same route. The potency-determination of the unknown plague immune sera was carried out similarly to the assay of antipneumococcal and antipertussis sera.

It should be noted that Meyer & Foster [113] utilized a similar mouse-protection test for the measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics.

Together with their mouse-protective test, Jawetz & Meyer [79] recommended a technique for a toxin-antitoxin neutralization test, using for this purpose the toxin prepared according to the method described earlier in this study (see page 172). For performance of the test saline dilutions of the sera in quantities of 0.5 ml were injected intra-abdominally into white mice, followed 30 minutes later by 0.3 ml of the toxic filtrates diluted in saline solutions. For purposes of comparison an arbitrary scale was chosen, indicating the relative capacity of a given serum to protect mice against a standard dilution of the test batch of toxin. It was found most advantageous to test three twofold dilutions (1:4, 1:8, and 1:16) of serum (ten mice each) against a filtrate dilution of 1:5 (approximately 10 a.l.d.). The mice were observed for 48 hours and the deaths recorded. The results were expressed as the number of mice surviving over the total number of mice used per serum.

Properties of Immune Sera

Schütze,[157] Bhatnagar,[10] and Gheltenkoff [41] concluded from their serological studies that plague immune sera contained two kinds of antibodies, corresponding to the " envelope " and somatic antigens of *P. pestis*.

In order to study the antibody content of plague sera by immunochemical procedures, Girard & Sandor [60] used a method of serum fractionation devised by Sandor, with the aid of which it was possible to distinguish between the bacterial antibodies contained mainly in the euglobulins, and the antitoxins present in the pseudoglobulins.

Investigating two sera, prepared with toxic live avirulent plague bacilli and with plague toxoid respectively, Girard & Sandor were able to separate three fractions, namely : (*a*) euglobulin I, which showed no antitoxic activity, but possessed antibacterial, agglutinating, and protecting properties; (*b*) euglobulin II, a comparatively inert lipo-protein fraction ; and (*c*) pseudoglobulins, representing the antitoxin and endowed with but feeble precipitating, agglutinating, and protective properties.

Studying this matter further, Sandor et al.[153] found that the sera obtained from horses, which had been immunized intravenously with either live plague bacilli or toxoid, contained large amounts of euglobulin I. However, in sera produced through subcutaneous administration of plague toxoid, this euglobulin was largely replaced by another fraction, called euglobulin IIA by the authors. In contrast to the above-mentioned sera, those produced by the subcutaneous route possessed no flocculating or agglutinating properties. While the protective action exerted by all the sera in mice experiments appeared to be vested in the pseudoglobulins as well as in the euglobulins, euglobulin I, if present in considerable quantities, was definitely more active than the pseudoglobulins.

The classification of plague immune sera has been the subject of much debate. As was mentioned on page 183, the serum of plague-immune animals is unable to destroy *P. pestis* in vitro or in vivo in the absence of phagocytic cells. It must likewise be noted that plague immune sera, even if obtained by administration of plague toxins (broth filtrates or the like) or by additional immunization with such toxins, possess only moderate antitoxic value. Petrie [131] maintained in this connexion that, for no obvious reason, plague antitoxin in high concentration is not easily produced in horses. Gheltenkoff,[40] however, believed that this was true of some of the horses only, while others produced sera of a satisfactory antitoxic titre. A careful selection of suitable animals was therefore essential.

Be this as it might, it is generally agreed that plague immune serum, fitting into neither the class of bactericidal nor that of antitoxic sera, belongs, like the anthrax and rinderpest sera, to the group of " anti-infectious " sera. Meyer,[111] while stating that it is not exactly known to what the anti-infectious properties of plague immune serum are due, drew attention to the hypothesis of Petrie and of Jawetz & Meyer [79] that it probably produces significant opsonization.

SERODIAGNOSTIC METHODS

Agglutination

Preparation of agglutinating sera

According to the usual procedure for the preparation of agglutinating sera, most plague workers used rabbits for this purpose and as a rule immunized them by the intravenous route.

Of the different antigens recommended by the various workers in this field, the following deserve mention :

To obtain an agglutinating serum Batchelder [7] used a normal saline solution containing 0.25% formalin to wash off the growth of plague bacilli on hormone agar containing 0.025% of 10% sodium sulfate. The resulting suspension, which became sterile when kept for seven to eight hours at room temperature, was well tolerated by rabbits even if large doses were given intravenously and produced, when administered twice, a serum with a titre ranging from 1:1280 to 1:2560.

To produce sera against virulent *P. pestis* and also against pseudotuberculosis bacilli for his serological studies, Bhatnagar [10] resorted to the use of silver nitrate as employed by Rainsford for the manufacture of TAB vaccine.

As noted already, Baker et al.[4] obtained immune sera with good agglutinating properties when administering their fraction IA and IB antigens to rabbits.

When preparing agglutinating sera with live plague bacilli, most workers preferred strains which had lost their virulence spontaneously or which had been rendered avirulent artificially. It would seem, however, that Wats et al.[187] used virulent strains to complete the immunization of rabbits which had been previously inoculated subcutaneously and then intravenously with graduated doses of plague cultures heat-killed by exposure to 55°C for 30 minutes. Greval & Dalal [68] reported satisfactory results when making agglutination tests with the therapeutic sera produced in the Haffkine Institute with virulent plague cultures.

Preparation of suspensions

The main difficulty in carrying out agglutination tests with plague bacilli is to obtain suitably uniform suspensions of the organisms. Though numerous procedures have been devised to overcome this impasse (see summary by Pollitzer,[140] and also Ciantini [21]), Wats et al.,[187] again studying this problem, found none of them fully satisfactory and therefore recommended the following method :

Roux bottles, containing meat-digest agar (pH 6.8) were sown with a thick suspension of plague bacilli and incubated for four days at 27°C or 37°C according to the nature of the agglutinable antigen wanted. The growth was then washed off with 20–30 ml of distilled water containing 1% carbolic acid. The washings were poured into a sterile test-tube containing glass beads and kept at room temperature (27°–29°C) for six hours with an initial shaking by hand for five minutes and an occasional shaking during the first two hours. The supernatant homogeneous layer was then pipetted off and left in the refrigerator overnight, so as to allow coarse particles to settle down. The stable portion obtained from the suspension was centrifuged and after the deposit had been washed in saline twice, it was re-

suspended in normal saline containing 0.25% carbolic acid; 0.05% formalin was added if the suspension was produced in bulk for stock purposes.

It would seem that the simpler procedures recommended by Bhatnagar [10] and Seal [160] are not as reliable as the method described above.

As stated previously,[c] Devignat [25] had found that bubbling of air through broth cultures produced a rapid and homogeneous growth of *P. pestis*. Recently studying the problem of agglutination,[27] he used suspensions obtained with the aid of this method, stabilizing them by adding one drop of formalin per 20 ml of the growths.

Rapid agglutination tests

In addition to tube tests carried out in the classical manner, several workers recommended slide agglutination tests for the rapid diagnosis of human plague. Panja & Gupta [128, 129] used the sera of patients in dilutions of 1:3 or 1:4. A mixture of plague bacilli obtained from a few different agar or blood-agar cultures incubated for one to two days at 37°C was distributed into drops of the serum dilutions and the results were read after one minute. Positive results were obtained in 15 out of 17 cases with a positive blood culture, usually on the seventh day after onset of the disease, sometimes earlier. The two negative cases had been tested on the third and fourth day respectively. In 11 bacteriologically negative cases, agglutination proved positive within 4 to 14 days after onset; 75 normal sera yielded negative results.

It should also be noted that recently Tumansky [186] recommended Noble's rapid method of tube agglutination (see Pollitzer [140]) for the identification of plague cultures and for testing the sera of plague-suspect rodents with the aid of known strains of *P. pestis*.

A method recommended by Menezes [109] for the diagnosis of rat plague likewise deserves mention : 250 mg of the spleen or liver of the suspect rats were suspended in 20 ml of normal saline and mixed with equal amounts of 1:10, 1:20, and 1:40 dilutions of immune sera raised against 37°C growths of *P. pestis*. The tubes were then incubated in a water-bath at 40°C and readings were taken after two to three hours. A positive reaction was manifested by a heavy deposit consisting of agglutinated plague bacilli.

Menezes admitted that the test was negative when plague bacilli were scanty in the organs of the rats, but stressed on the other hand that plague livers or spleens which had become putrid or had been kept for a month or even longer in a desiccated state or in glycerol, still gave a positive result.

Types of agglutination

While the existence of serologically-different races of *P. pestis* has been unanimously denied, attention has been drawn to the occurrence of two distinct types of agglutination. Wats et al.[187] maintained that these differ-

[c] Pollitzer, R. (1952) *Bull. World Hlth Org.* **5**, 84

ences depended " on the agglutinable antigen and not on the type of antisera employed ". Bhatnagar,[10] on the other hand, ascribed them to the presence of two antibodies in plague immune sera.

Carrying out agglutination tests with plague bacilli cultivated at room temperature (27°–29°C) and at 37°C respectively, Wats and his co-workers obtained different results, as follows :

Room-temperature growths	*37°C growths*
Agglutination slow	Agglutination rapid
Flakes small and uniform	Flakes larger and of varying size
Sediment compact	Sediment voluminous
Sediment not easily dislodged, small flakes seen in a clear fluid on shaking; clumps take time to reform and settle.	Sediment easily dislodged, uniformly distributed on shaking ; clumps reform quickly (10 minutes) and settle in large masses.

Wats and his colleagues made the following interesting findings when carrying out comparative absorption tests :

(*a*) 37°C growths were capable of absorbing all agglutinins from the sera obtained by immunizing animals with either 37°C or room-temperature growths.

(*b*) Room-temperature growths, on the contrary, were capable of removing all antibodies only from sera raised against them and not from sera prepared with the aid of 37°C growths.

(*c*) 37°C growths, when heated at 100°C for one hour, reacted more or less like room-temperature growths.

Obviously, therefore, the 37°C growth of *P. pestis* possessed an additional antigen which was destroyed by heat and was absent in cultures incubated at room temperature.

Bhatnagar,[10] studying the agglutination of plague bacilli possessing an " envelope " and of plague and pseudotuberculosis strains which were devoid of an " envelope ", with the aid of sera raised against virulent, avirulent immunogenic, and avirulent non-immunogenic plague strains, also noted the presence of two types of agglutination, as follows :

" Envelope " agglutination	*Somatic agglutination*
Forms slowly	Forms slowly
Settles slowly	Settles slowly
Supernatant remains clear	Supernatant remains clear
Sediment voluminous, flakes large, woolly in character and varying in size	Sediment scanty, flakes small, uniform and gritty in character
In lower dilutions sediment easily dislodged and producing a shimmer in the serum-suspension mixture : in higher dilutions definite woolly particles visible.	Sediment easily dislodged and becoming similar to saline control on shaking in all the dilutions of serum.

It will be noted that there was a great deal of resemblance between Bhatnagar's somatic agglutination and that of room-temperature growths

observed by Wats and co-workers on the one hand, and between the " envelope " agglutination and that of 37°C growths on the other.

Specificity of agglutination tests

Summarizing the evidence available up to 1936, Pollitzer [140] reached the conclusion that agglutination tests with plague and pseudotuberculosis immune sera as practised up to then formed no exact means of differentiating between these two bacterial species. He added, however, " that by a judicious application of the newer knowledge upon the antigenic structure of these two germs a satisfactory method might be evolved ".

It is important to note in this connexion that, according to subsequent observations by Bhatnagar,[10] (*a*) pseudotuberculosis immune sera did not react with plague bacilli and (*b*) pure " envelope " sera, obtained through absorption of plague immune sera with *P. pseudotuberculosis* strains, no longer agglutinated pseudotuberculosis bacilli. Further investigations to confirm the general validity of these claims and to prove the practicability of the second procedure which, according to Bhatnagar, was fraught with difficulties, would be most desirable.

Scope of agglutination tests

Advantage may be taken of agglutination to test unknown strains with a plague serum of established potency and to examine the sera of suspects with the aid of known cultures. As has been noted, Panja & Gupta [128, 129] obtained good results with rapid slide tests and the value of agglutination for the diagnosis or retrospective diagnosis of human plague has also been endorsed by other recent observers, particularly by Favarel.[32, 33] This worker came to the opinion that, except in the case of recently inoculated individuals, agglutination even at low titres was significant. Agglutinins appeared as a rule about the seventh day of illness. Two persons who had been cured showed negative reactions after nine and ten months respectively.

It is particularly noteworthy that, as recorded by Favarel and also by Huang et al.[76] and Greval,[67] treatment with sulfonamides or streptomycin, though apt to lead to negative bacteriological results, did not adversely affect those of agglutination tests.

Haemagglutination

As recently established in the South African Institute for Medical Research,[179] guinea-pig erythrocytes sensitized through absorption of purified " envelope " antigen of *P. pestis* are agglutinated by human and animal immune sera.

The results of such tests were easy to read, but the technique was more time-consuming than the precipitin reaction. The diagnostic value of the test was limited on account of the slow development of plague immunity

but the method appeared to be useful " for confirming retrospectively a diagnosis made on clinical grounds ".

Precipitation

While the opinions held by earlier workers (see Pollitzer [140] for summary) regarding the value of precipitin tests for the diagnosis and differential diagnosis of plague had been divergent, this method was recently recommended by Cambosu [19] for the rapid recognition of rat plague.

Flocculation

Girard [49] established that, especially in the case of patients with suppurating buboes of long standing, good diagnostic results could be obtained by mixing five drops of the patient's serum with 1 ml of plague endotoxin (filtrates of broth cultures or preferably extracts obtained by repeated freezing and thawing); flocculation took place in positive cases. Readings were taken after 30 minutes, 3 hours, and 24 hours.

At the same time Girard was unable to confirm the claim of Gheltenkoff[41] that flocculation tests were useful for standardizing plague immune sera.

Haemolysins

The evidence available on the reactions produced by *P. pestis* in blood-containing media is not only somewhat scanty but most contradictory.

Korobkova [91] stated in this connexion that both plague and pseudotuberculosis bacilli were endowed with haemolytic properties, a clear zone appearing within 48-72 hours round colonies on agar plates which contained 5% of defibrinated rabbit, guinea-pig, or horse blood. The same phenomenon was observed on plates containing sheep blood but, in contrast to the above-mentioned media, the sheep erythrocytes remained intact. In liquid media containing 2% of blood, haemolysis became complete in five days.

As noted by Korobkova, filtrates of 12-day-old plague cultures caused feeble lysis of sheep erythrocytes. The haemolytic filtrates caused the death of white mice but not of rabbits. The haemolysins present in plague cultures were not neutralized by plague immune or antihaemolytic sera and possessed no immunogenic properties.

In contrast to the opinion of Bielonowski,[12] Korobkova maintained that there was no significant relation between the haemolytic power and the virulence of plague strains. She found, however, that smooth avirulent plague strains produced haemolysis more slowly than virulent growths, while rough avirulent strains reacted like *P. pseudotuberculosis*, lysing rabbit, guinea-pig, and horse erythrocytes even more rapidly than virulent plague strains.

In marked variance with these statements, Colichon [22] maintained that pseudotuberculosis strains as well as the pasteurellae sensu stricto failed to produce haemolysis on blood-agar plates. Although admixture of human blood was most suitable to show up this difference between *P. pestis* and the above-mentioned micro-organisms, Colichon stated that it was permissible to use instead horse, rabbit, guinea-pig or grey-rat blood.

Wagle & Habbu (unpublished observations), cultivating five laboratory strains of *P. pestis* and two pseudotuberculosis strains at 28°C and 37°C respectively on blood-agar slopes prepared with human, guinea-pig, horse, and rabbit blood, were unable to confirm the findings of Colichon, haemolysis being absent in the case of both micro-organisms. Repeated passage of the plague strains through susceptible experimental animals (*Gunomys kok*) did not alter their behaviour on the blood-agar slopes.

Complement Fixation

Summarizing the literature available up to 1936, Pollitzer [140] stated that the use of complement-fixation tests for the purposes of plague laboratory work had been recommended by several investigators.

Thus Damperoff [24] tested plague immune sera with the aid of this method, using either bacillary suspensions or extracts as antigens. He found fairly constant and reliable results but could not establish a correlation between the complement-fixing titre and the curative value of the sera in question.

Moses [118] found in 25 out of 38 plague patients complement-fixing antibodies and twice demonstrated plague antigens; in most of these instances the blood had been taken on the fifth day of illness. In the experience of Shchastny,[162] however, antibodies were present not earlier than in the second week.

The method of complement fixation for the diagnosis of human plague was again recommended by Joltrain [81] and Simard.[164] The former reported positive results in clinically uncertain cases, in some of which agglutination tests had been negative. No false positives were seen. Dickie [28] confirmed the usefulness of the method but thought it of greater positive than negative value.

Grysez & Wagon (quoted by Dieudonné & Otto [29]) testing old plague-infected tissues, stated that complement was specifically deviated even when there was advanced putrefaction. However, Piras [132] could not confirm this claim for he found the reaction to be positive up to the sixth day only and therefore considered it inferior to animal experiments.

The usefulness of complement-fixation tests for the diagnosis of human plague was again noted by Joltrain [82] in 1936—particularly in the case of convalescents or of patients with pestis minor.

Mitin,[116] trying out 40 different antigens, found it most suitable to suspend plague bacilli grown on agar in distilled water and to shake the

suspension for two to four days in the dark. After centrifugation, the supernatant fluid was pipetted off and phenol was added so as to obtain a concentration of 0.5%.

Using his 40 antigens to make complement-fixation tests with the sera of immunized and non-immunized animals as well as with those of inoculated and non-inoculated human beings, Mitin obtained satisfactory results. He reached the conclusion that the method could not be used to diagnose sisel plague because the sera of these rodents had anticomplementary properties. However, Kuznetsova & Dobrokhtova [95] claimed afterwards that the reaction was useful to assess the incidence of plague among the sisels.

The question as to whether complement-fixation tests are useful for differentiation between plague and pseudotuberculosis bacilli has been answered in different ways by different observers. To judge from the investigations of Zlatogorov & Mogilevskaya as well as from those of Boquet & Dujardin-Beaumetz (quoted by Pollitzer [140]), complement-fixation tests were of value in distinguishing between plague and pseudotuberculosis strains; it should also be noted that Damperoff [24] obtained negative results when making such tests with pseudotuberculosis bacilli and plague sera. However, Shchastny,[162] Mitin,[116] and recently Haas [70] found that pseudotuberculosis bacilli merely gave less marked reactions than *P. pestis* when tested with plague sera, and the extensive studies of Greval & Dalal [68] led to the same result.

As far as the diagnosis of human infections is concerned, it is of no vital importance that complement-fixation tests are unreliable, or at least not fully reliable, for differentiating between plague and pseudotuberculosis, because cases of the latter disease in man are rare, invariably solitary, and usually show peculiar clinical features distinct from those of plague. One cannot fail to note, however, that complement-fixation tests, while more laborious than agglutination tests, do not seem to possess superior diagnostic value.

BACTERIOPHAGE INVESTIGATIONS

As summarized by Harvey [73] in a comprehensive survey of the bacteriophage problem with particular reference to plague and cholera, and confirmed by further investigations, in the case of plague, phages have been isolated from rather varied sources, such as rat faeces and the stools of convalescents; rat lymph-nodes and serum; rat fleas; buboes or blood from plague patients or convalescents; sewage; and canal water. It is of special interest that positive findings were not invariably restricted to localities where the infection was present.

Thus Girard [43, 44] detected an active lytic principle to *P. pestis* in rats from a former plague focus in Antananarivo, Madagascar, and postulated

that a causal connexion might exist between the presence of phages in the rodents and the disappearance of the infection.

Lépine & Bilfinger,[98] examining rats at Athens at a time when an enzootic seemed to be absent, found the serum of two out of 217 animals to be lytic for *P. pestis*.

Flu & Flu [37] (see also Flu [35]) repeatedly isolated from sewage and canal water at Leyden, Netherlands, phages which were active against plague and pseudotuberculosis bacilli as well as against *Escherichia coli* and shigellae. Flu & Flu assumed that the phage strains had originated from rat intestines where they had been adapted to *E. coli*.

Although, as in the case of other infectious diseases, far-reaching claims have been made regarding the role played by bacteriophages in the development of a natural immunity against plague, it seems altogether unlikely that the influence they might exert in this direction is of general importance. Discussing this problem, Harvey [73] stressed that Avari in India had been unable to find phages active against *P. pestis* in either Bombay or Madras rats.

The question of to what extent the action exerted by bacteriophages on plague growths is specific in nature and how far, consequently, observations made with the aid of these lytic agents are of differential-diagnostic importance, has been the subject of much discussion.

The early workers, though sometimes finding that the action of their phages was restricted to those plague strains to which they were adapted, admitted the existence of polyvalent strains possessing lytic powers for all plague growths. However, they were unanimously of the opinion that the action of plague bacteriophages was specific as far as other bacterial species, in particular *P. pseudotuberculosis*, were concerned. Advier [1] established in this connexion that an agar surface treated with a plague phage ceased to be suitable for the growth of *P. pestis* but continued to be favourable for the development of other micro-organisms, and recommended this method for the purposes of differential diagnosis.

Bezsonova et al.,[9] working with two bacteriophages of wild-rodent origin, found them to lyse all 214 plague strains tested but to exert no action on 21 pseudotuberculosis strains. They considered, therefore, the use of suitable plague phages as a subsidiary means for the differentiation of the two organisms.

Girard [51] noted that plague phages were able to lyse pseudo-tuberculosis strains to the same titre as pseudotuberculosis phages and later [52] also confirmed the observation of Flu & Flu that both these phages were active against dysentery bacilli, while vice versa certain shigella phages lysed plague and pseudotuberculosis bacilli. Nevertheless he maintained the differential-diagnostic value of bacteriophage examinations made with the aid of broth media, pointing out that the different appearances of

growth shown by plague and dysentery bacilli in the control tubes were so striking as to preclude diagnostic errors.

In a paper published in 1947, Lazarus & Gunnison [96] reached the conclusion that bacteriophage tests did not constitute a means of clearly distinguishing between plague and pseudotuberculosis bacilli. Further studies by Gunnison et al.[69] showed, however, that it was possible to make such a distinction with the aid of bacteriophage tests carried out at 20°C. The technique used by Gunnison and his co-workers was as follows :

Advier's phage strain was adapted on the one hand to an avirulent plague strain (P phage), and on the other to a pseudotuberculosis strain (PTB phage). The plague strains to be tested were first grown for 18-24 hours at a temperature of 18°–20°C in broth. Dry agar plates were then implanted with the growths, each of which was spread over a circular area, 2 cm in diameter, so that one dish could accommodate ten different growths. After these had become dry, they were touched with a suspension of one of the two phages. The plates were then incubated in an inverted position for 48 hours at 20°C and 37°C respectively. Before carrying out the tests proper, each phage was titrated against the culture to which it had become adapted by testing serial tenfold dilutions of the phage in question in the manner described above. The plates used for the titration of P phage were held at room temperature, those of PTB phage were incubated at 37°C. The highest dilutions giving confluent lysis were recorded and for the final tests ten times this amount, the " critical test dilution ", was used.

The results obtained with 45 pseudotuberculosis strains and 52 plague strains (35 of which were virulent) were as follows :

Type of phage	*Results*
PTB phage	Of no diagnostic value, lysing most plague cultures at 20°C or 37°C and not lysing most of the pseudo-tuberculosis cultures at 20° C.
P phage at 37°C	Lysed many pseudotuberculosis strains and failed to lyse a few plague strains.
P phage at 20°C	Showed a specific action for plague strains.

The attempts made to use specific phages for the treatment of human plague will be dealt with in a later study; it may be noted here, however, that generally speaking the results obtained in this direction with experimental animals were negative or most disappointing, the treated animals succumbing more quickly to plague than the controls. As convincingly shown by Pons [141] in the case of guinea-pigs, this phenomenon was due to the liberation of plague endotoxin by the bacteriophage, which thus became a pathogenic instead of a therapeutic factor.

Joukov-Verejnikov & Favarissova,[85] administering bacteriophage simultaneously with plague infection to guinea-pigs, found that the animals so

treated died at the same time as the controls. The two workers maintained that the bacteriophage and the plague bacilli could co-exist in the animal body without influencing each other and, noting that in vitro addition of normal serum to a mixture of these two agents prevented lysis, expressed the opinion that the absence of phage action in vivo might have been due to phenomena of a colloidal nature.

Experimenting with different animals (grey and white mice, white rats, and guinea-pigs), Advier [1] found that injection of a mixture of plague bacilli and bacteriophage, if tolerated by the animals, produced a solid immunity against subsequent plague infection. Compton,[23] who had obtained identical results, maintained that the immunity thus produced did not result directly from the action of the bacteriophages but was due to the liberation of immunogenic substances from the bacilli.

To test this hypothesis, Compton used phage preparations treated with formaldehyde. The filtrate obtained by filtering the lysates through L_5 Chamberland candles was incubated for five days to ensure sterility. Then formalin was added in the proportion of 0.4% (0.16% formaldehyde) and the mixture was heated in a vaccine bath at 60°C for one hour. It was then kept in the incubator for 18 days before use.

Compton claimed that better protection in experimental animals resulted from subcutaneous administration of this specific phage-lysed vaccine than from three inoculations with untreated bacteriophage.

Flu, who dealt in numerous publications with the same problem, described in 1933 the preparation of lysate as follows : [36]

> A suspension of a 24-hour-old broth culture of virulent plague bacilli containing 5,000 million organisms per ml was mixed with 2% active phage and incubated at 37°C for 36 hours. The partially-lysed suspension was filtered through cotton-wool, shaken up with an excess of chloroform, and again left for 12 hours at 37° C. The fluid was then carefully decanted from the chloroform, carbolized to 0.5%, allowed to stand for five days at room temperature, and then tested for sterility. Before use the fluid, which was not quite clear, had to be shaken.

Flu stated that even one injection with the vaccine lysate protected 66% of white rats against infection with 10,000 m.l.d. of plague bacilli, whereas three inoculations protected 91% of the animals. He emphasized the necessity of using concentrated suspensions of virulent bacilli for preparation of the lysates, alleging that the failures reported by other investigators were due to the fact that they had worked with products poor in dissolved bacterial substances though rich in phages. Flu's lysate remained potent when kept for four months at room temperature. Lazarus & Gunnison [96] confirmed that phage lysates of *P. pestis* were effective in protecting mice against plague infection, adding that the use of such preparations " offers a number of interesting possibilities for human vaccination ".

SUMMARY

In this study, present knowledge of the virulence, toxin, and antigenic structure of the plague bacillus, *Pasteurella pestis*, is reviewed, and the mechanism of immunization, serodiagnostic methods, and bacteriophage investigations are discussed.

Virulence may be defined as the capacity of an organism to multiply and finally to bring about the death of a susceptible host animal when introduced in numbers not large enough to be toxic without multiplication. The virulence of the plague bacillus is high; although remarkably stable, it may disappear spontaneously, or be experimentally attenuated. Avirulent strains may develop by dissociation from a virulent culture. Various procedures have been proposed for determining the virulence of plague strains. An exact method recommended by Sokhey, based on the enumeration of viable bacilli in a broth culture and the determination of the smallest number of micro-organisms lethal for mice, is described in detail. The virulence of a strain may be maintained by passage through susceptible animals, storage at low temperatures, or freeze-drying. Virulence may be attenuated by repeated subculturing, by adding alcohol to the broth, or by bubbling air through broth cultures.

According to most authors, the plague bacillus possesses a specific endotoxin; it has, however, been shown that plague toxin resembles the exotoxins in many respects. Petrie asserts that similar antigenic substances are found in old broth cultures, in the purer products obtained by extracting the bacillary bodies, and in " whole " vaccines prepared by Haffkine's method or by other techniques. According to Petrie, the various preparations used for active immunization contain toxin, toxoid, or a mixture of these two antigens. Plague toxin may be transformed into toxoid by adding 3-4 $^{o}/_{oo}$ formalin. The preparation of plague toxin by filtration,

RÉSUMÉ

Cette étude résume les connaissances actuelles relatives à la virulence, à la toxine et à la structure antigénique du bacille pesteux. Le mécanisme de l'immunisation, les méthodes de sérodiagnostic et les recherches sur le bactériophage y sont discutés.

La virulence est définie comme la capacité que possède un organisme bactérien de se multiplier au sein d'un animal sensible et d'en provoquer la mort, lorsqu'on l'y introduit en nombre trop faible pour qu'il puisse exercer une action toxique sans multiplication. La virulence du bacille pesteux est élevée. Bien que remarquablement stable, elle peut disparaître spontanément ou être affaiblie expérimentalement. Des souches avirulentes peuvent se développer par dissociation à partir d'une culture virulente. Divers procédés permettant d'évaluer la virulence du bacille pesteux ont été proposés. Une méthode exacte, préconisée par Sokhey, fondée sur le dénombrement des bacilles viables dans une culture sur bouillon et la détermination du plus petit nombre de microorganismes mortels pour la souris, est décrite en détail. La virulence des souches peut être maintenue par passage sur l'animal sensible, conservation à basse température, ou dessiccation sous congélation. Elle peut être affaiblie par de fréquents repiquages, par l'addition d'alcool au bouillon de culture ou le barbotage d'air.

Selon la plupart des auteurs, le bacille pesteux possède une endotoxine spécifique; il a été relevé cependant que la toxine pesteuse possède plusieurs des caractères des exotoxines. Petrie a affirmé que les mêmes substances antigéniques se trouvent dans les vieilles cultures sur bouillon, dans les produits plus purs préparés à partir des bacilles par extraction, ou dans les vaccins « entiers » préparés selon la méthode d'Haffkine ou d'autres procédés. D'après cet auteur, les diverses préparations utilisées pour l'immunisation active contiennent de la toxine, de l'anatoxine ou un mélange de ces deux antigènes. La toxine pesteuse peut être

extraction, and bacteriophage action is described.

By chemical methods of analysis, it has been possible to distinguish in the antigenic structure of *P. pestis* a water-soluble fraction, possessing toxic and immunizing properties, and an insoluble fraction which fails to produce antibodies in the blood of experimental animals or man.

Using serological methods, some authors have reached the conclusion that the plague bacillus possesses two antigens, one contained in its " envelope " and the other in its somatic portion. The term " envelope " has been adopted, although the existence of this formation is not admitted by all workers, and the idea of a capsule or membrane bearing an antigenic fraction is not in accordance with some observations.

Up to now, investigation has not revealed any relation between the serological properties of the plague bacillus and its virulence, although some workers have postulated the existence of a Vi antigen similar to that found in the salmonellae.

The natural resistance of birds to plague, although it cannot be considered absolute, appears to operate in normal circumstances; phagocytosis seems to be the principal defence mechanism. It appears from experiments with chicks, however, that this insusceptibility to plague does not exist in the embryo, presumably because the cellular defence mechanism is undeveloped. Although plague is essentially a disease of rodents, certain species of *Rodentia* are resistant to experimental plague and variations in resistance occur within the same species. In general, it may be said that man has no natural resistance to plague. " Healthy " carriers of plague, mentioned by some authors, may have been immunized by an attack of pestis minor, while the few cases of resistance to pneumonic plague cited in

transformée en anatoxine par addition de 3-4 °/oo de formaline. La toxine pesteuse peut être préparée par filtration, extraction ou action du bactériophage. Diverses méthodes sont décrites.

Les méthodes chimiques appliquées à l'analyse de la structure antigénique du bacille de la peste ont permis de distinguer une fraction soluble dans l'eau — possédant des propriétés toxiques et immunisantes — et une fraction non soluble ne provoquant pas la formation d'anticorps dans le sang des animaux d'expérience ou de l'homme.

Grâce aux méthodes sérologiques, certains auteurs ont établi que le bacille pesteux possède deux antigènes, l'un inhérent à l'« enveloppe », l'autre au corps du bacille (antigène somatique). Le terme « enveloppe » a été adopté bien que l'existence d'une telle formation ne soit pas admise par tous les auteurs et que la notion d'une capsule ou d'une membrane porteuse d'une fraction antigénique soit en désaccord avec certaines observations.

Les recherches faites jusqu'à maintenant n'ont pas prouvé qu'il existe une relation entre les propriétés sérologiques du bacille de la peste et sa virulence, bien que certains auteurs aient admis que le bacille pesteux, comme les salmonellae, possédait un antigène Vi.

Les oiseaux adultes peuvent être considérés comme pratiquement résistants à la peste ; la phagocytose semble être leur principal mécanisme de défense. La résistance n'existe pas encore chez l'embryon (des expériences ont été faites sur l'embryon de poulet), faute de cette défense cellulaire. Bien que la peste soit essentiellement une maladie des rongeurs, un certain nombre d'espèces de *Rodentia* sont réfractaires à la peste expérimentale. La résistance peut varier au sein d'une même espèce. On peut affirmer, d'une façon générale, qu'il n'existe pas chez l'homme de résistance naturelle à la peste. Les « porteurs sains » de germes de la peste, mentionnés dans certains travaux, peuvent avoir été immunisés par une attaque de pestis minor. Les quelques cas de résistance à la peste pneumonique, cités dans la littérature,

the literature may be considered as exceptions which do not invalidate the rule.

All experiments have indicated that the serum of animals immune to plague is unable to lyse *P. pestis*, either in vitro or in vivo, in the absence of phagocytes. Phagocytosis is the most important mechanism involved in the protection of man and animals against plague ; the serum apparently acts through opsonization.

A distinction between an anti-infectious and an antitoxic immunity has been made by some authors. In the opinion of Jawetz & Meyer, a high resistance to the toxin is not necessarily associated with immunity to plague infection; anti-infectious immunity, on the other hand, always presupposes a certain degree of antitoxic resistance.

An attack of plague is apt to confer on survivors a resistance against further infection. Since it is only recently that an appreciable number of recoveries from severe attacks has been reported, little information is available on the duration of naturally produced immunity; it may be assumed, however, that its intensity and duration are limited.

Empirical attempts at immunization against plague were undertaken as far back as the 18th century. Nevertheless, it was not until 1895 that vaccination was established on a scientific basis; the work of Yersin, Calmette & Borrel showed that it was possible to immunize the rabbit by suspensions of heat-killed bacilli. Since then, various immunological methods, using killed vaccines or live, avirulent bacilli, have been recommended.

Sokhey's method of biological standardization of killed vaccines by testing them on mice has replaced other, less suitable, methods. According to Sokhey & Habbu, there is no relation between the virulence of a strain and its antigenic potency; on the other hand, the toxicity of a vaccine may be mitigated without affecting its antigenic potency. The same authors have suggested that the immunological potency of a broth vaccine resides in the fluid

peuvent être des exceptions et n'invalident pas la règle.

Il ressort de toutes les études expérimentales que le sérum des animaux immunisés est incapable de lyser le bacille pesteux in vitro ou in vivo, en l'absence de phagocytes. La phagocytose est le mécanisme le plus important, par lequel les animaux et l'homme se défendent contre l'infection pesteuse; le sérum agirait par ses propriétés opsonisantes.

Dans l'immunité contre la peste, certains auteurs ont distingué une immunité anti-infectieuse et une immunité antitoxique. D'après Jawetz & Meyer, une résistance élevée à la toxine ne protège pas nécessairement de l'infection pesteuse; une immunité anti-infectieuse au contraire comporte toujours une certaine résistance antitoxique.

Il est notoire qu'une attaque de la maladie confère une résistance à une attaque ultérieure. Les cas de guérison à la suite d'une infection grave bien que n'étant pas rares actuellement, n'ont été signalés que depuis peu de temps ; aussi ne possède-t-on que peu de données sur la durée de l'immunité naturelle; on estime cependant que son intensité et sa durée sont limitées.

Des essais empiriques d'immunisation contre la peste ont été entrepris au XVIII[e] siècle déjà. Toutefois, ce n'est qu'à partir de 1895 que la vaccination fut établie sur des bases scientifiques; les travaux de Yersin, Calmette & Borrel montrèrent qu'il était possible d'immuniser le lapin par des suspensions de bacilles tués par la chaleur. Dès lors, divers procédés d'immunisation ont été préconisés au moyen de vaccins tués ou de bacilles avirulents vivants.

La méthode de standardisation biologique des vaccins tués, par épreuve sur la souris, introduite par Sokhey, a remplacé des procédés nettement insuffisants. D'après Sokhey & Habbu, il n'y a pas de relation entre la virulence d'une souche et son pouvoir antigénique; d'autre part, la toxicité du vaccin peut être abaissée sans que son pouvoir antigénique en soit affecté. Les mêmes auteurs ont établi que le pouvoir immunisant d'un vaccin préparé

portion rather than in the sediment. They recommended the use as vaccine of supernatant fluid from broth cultures grown at 27°C and of suspensions of agar cultures incubated at 37°C, since these preparations are less toxic.

Vaccines may be killed either by heat or by formalin. Various types of killed vaccine have been used—Haffkine's vaccine, casein-hydrolysate vaccine, agar-grown vaccine, pseudotuberculosis vaccine, sugar vaccine, vaccines precipitated by alcohol, or by alcohol plus alum, and chemically prepared extracts of *P. pestis*. The methods of preparation of all these vaccines are described.

Since the pioneer work of Girard & Robic and Otten in 1934, in Madagascar and Java respectively, the use of live avirulent vaccines has spread in Africa and South America. Laboratory experiments suggest that these vaccines give results as satisfactory as those of killed vaccines.

Since Yersin, Calmette & Borrel demonstrated that serum from immunized rabbits protected these animals against plague, numerous attempts have been made to produce sera suitable for the prophylaxis and therapy of human plague.

In spite of the advantages offered by the rabbit, most workers continue to use the horse for the production of antiplague serum. Several antigens, singly or in combination, have been used : killed bacilli; live, virulent bacilli; and culture filtrates. Active fractions of the serum have been isolated for prophylactic use, and recent research has shown that it is the gamma-globulin fraction which contains antibodies. Various methods for the evaluation of potency of antiplague sera are described. A method of serum fractionation developed by Girard & Sandor enabled these authors to distinguish the antibodies, which are active against the bacilli, have agglutinating properties, and are found in the euglobulin I fraction, from the antitoxins, which have only feeble agglutinating and protective properties, and are found in the pseudo-globulins.

sur bouillon réside dans le liquide et non dans le sédiment. Ils ont préconisé l'emploi comme vaccin du liquide des cultures sur bouillon à 27° C et de suspensions de cultures sur agar à 37° C, ces préparations étant moins toxiques.

Les vaccins peuvent être tués soit par la chaleur, soit par la formaline. Divers types de vaccins tués ont été utilisés : vaccin de Haffkine, vaccin préparé sur hydrolysat de caséine ou sur agar, bacilles pseudo-tuberculeux, vaccin au saccharose, vaccins précipités par l'alcool ou l'alcool et l'alun, extraits de *P. pestis* obtenus par voie chimique. Les méthodes de préparation de ces vaccins sont décrites.

Depuis 1934, à la suite des essais de Girard & Robic et d'Otten, à Madagascar et à Java respectivement, l'usage de vaccins vivants avirulents s'est répandu en Afrique et en Amérique du Sud. Les expériences de laboratoire semblent montrer que ces vaccins donnent des résultats aussi satisfaisants que les vaccins tués.

Depuis que Yersin, Calmette & Borrel eurent démontré que l'injection de sérum de lapin immunisé protégeait ces animaux contre la peste, de nombreuses tentatives d'immunisation passive de l'homme, à titre prophylactique et thérapeutique, ont été effectuées.

Malgré les avantages que présente le lapin, la plupart des chercheurs continuent à employer le cheval pour la production de sérum antipesteux. Divers antigènes, seuls ou combinés, ont été utilisés : bacilles tués, bacilles virulents vivants, filtrats de cultures. Les fractions actives du sérum ont été isolées et sont utilisées pour l'usage prophylactique. Les recherches les plus récentes ont montré que ce sont les gamma-globulines qui contiennent ces anticorps. Diverses méthodes permettant d'évaluer l'activité des sérums antipesteux sont décrites. Le fractionnement du sérum selon le procédé de Girard & Sandor a permis à ces auteurs de distinguer des anticorps actifs contre les bacilles, doués de propriétés agglutinantes, qui se trouveraient dans la fraction euglobuline I et des antitoxines se trouvant dans les pseudo-globulines, qui n'auraient que de faibles propriétés agglutinantes et protectrices.

The classification of antiplague serum has been the subject of much debate. Since it is neither clearly bactericidal nor clearly antitoxic, it may be classed in the group of anti-infectious sera, to which the anthrax and rinderpest sera belong.

Various methods recommended for the serodiagnosis of plague—agglutination, haemagglutination, precipitation, flocculation, haemolysin tests, and complement fixation—are described.

Antiplague bacteriophages have been isolated from various sources, such as rat faeces, rat lymph-nodes and serum, pus and stools from convalescents, buboes or blood from plague patients or convalescents, sewage, and canal water. Bacteriophages have occasionally been found in localities free from plague. The specificity of antiplague phages is a matter of controversy. From the most recent studies, however, it seems that at 20°C the antiplague bacteriophage exercises a specific and exclusive action on the plague bacillus, and that this feature may even be taken as a criterion for distinguishing *P. pestis* from *P. pseudotuberculosis.*

Attempts at bacteriophage treatment of plague in laboratory animals have given disappointing results. The treated animals died sooner than the controls, because of the liberation of endotoxins; the phage had thus become a pathogenic instead of a therapeutic factor.

La question du classement du sérum antipesteux a été beaucoup discutée. Ce sérum n'étant pas nettement bactéricide ni nettement antitoxique peut être classé dans le groupe des sérums anti-infectieux auquel appartiennent le sérum anti-charbonneux et le sérum antipeste bovine (rinderpest).

Diverses méthodes ont été préconisées pour le diagnostic sérologique de la peste : agglutination, hémo-agglutination, précipitation, floculation, recherche des hémolysines, fixation du complément.

Des bactériophages antipesteux ont été isolés de crottes de rats, de ganglions lymphatiques et de sérum de rats, de pus, de selles de convalescents, de bubons ou de sang de malades ou de convalescents, d'eaux d'égouts et de canaux. On a trouvé des bactériophages dans les localités non infestées de peste. La spécificité du phage antipesteux est controversée. D'après les études les plus récentes, il semble qu'à 20° C le bactériophage antipesteux exerce une action spécifique et exclusive sur le bacille pesteux, et que l'on puisse même recourir à ce caractère pour distinguer *P. pestis* de *P. pseudo-tuberculosis.*

Les essais de traitement de la peste par le bactériophage chez les animaux de laboratoire ont été décevants. La mort des animaux traités est survenue plus rapidement que celle des témoins, par suite de la mise en liberté des endotoxines; le phage a aggravé l'infection plutôt qu'il ne l'a combattue.

REFERENCES

1. Advier, M. (1933) *Bull. Soc. Path. exot.* **26**, 94
2. Albrecht, H. & Ghon, A. (1900) *Denkschr. Akad. Wiss. Wien*, 66
3. Anchezar, B. V. (1938) *Rev. Inst. bact. B. Aires*, **8**, 196
4. Baker, E. E., Sommer, H., Foster, L. E., Meyer, E. & Meyer, K. F. (1947) *Proc. Soc. exp. Biol., N.Y.* **64**, 139
5. Barber, M. A. (1912) *Philipp. J. Sci.* **7**, section B, 251
6. Barreto, J. de Barros (1938) *Arch. Hyg., Rio de J.* **8**, 347
7. Batchelder, A. P. (1929) *J. infect. Dis.* **44**, 403
8. Batzaroff (1899) *Ann. Inst. Pasteur*, **13**, 385
9. Bezsonova, A., Molodtsova, P. F., Mosolova, O. N., Osolinker, B. E., Krainova, A. N., Pronin, B. A., Timofeeva, R. I., Shaposhnikov, M. S., Sokolinskaia, A. G., Kashkina, N. I., Petrunina, A. M., Goriunova, K. A. & Temiakova, A. S. (1938) *Rev. Microbiol., Saratov*, **17**, 228

10. Bhatnagar, S. S. (1940) *Indian J. med. Res.* **28**, 17
11. Bhatnagar, S. S. & Shrivastava, D. L. (1946) *J. Hyg., Camb.* **44**, 307
12. Bielonowski (1904) *Arch. Sci. biol., St Pétersb.* (Quoted by Pollitzer, 1936)
13. Boyé (1932) *Bull. Off. int. Hyg. publ.* **24**, 1610
14. Boyé (1933) *Bull. Off. int. Hyg. publ.* **25**, 1933
15. Brooks, R. St. J. (1914) *J. Hyg., Camb.* **13**, plague suppl. III, 412
16. Buddingh, G. J. & Womack, F. C., jr. (1941) *J. exp. Med.* **74**, 213
17. Burgess, A. S. (1930) *J. Hyg., Camb.* **30**, 165
18. Burroughs, A. L. (1947) *J. Hyg., Camb.* **45**, 371
19. Cambosu, G. (1938) *Igiene mod.* **31**, 193
20. Chertnik, N. L. (1940) *Rev. Microbiol., Saratov*, **19**, 439
21. Ciantini, F. (1938) *Boll. Ist. sieroter. Milano*, **17**, 129
22. Colichon, H. (1942) *Rev. méd. peruana*, **14**, 117
23. Compton, A. (1930) *Ann. Inst. Pasteur*, **45**, 754
24. Damperoff (1910) *Zbl. Bakt. (1. Abt., Orig.)* **55**, No. 2 (Quoted by Pollitzer, 1936)
25. Devignat, R. (1942) *Rec. Trav. Sci. méd. Congo belge*, No. 1, p. 145
26. Devignat, R. (1949) *Bull. Soc. Path. exot.* **42**, 43
27. Devignat, R. (1951) *Rev. Immunol.* **15**, 173
28. Dickie, W. M. (1926) *Plague in California 1900-1925. Plague pathology and bacteriology.* Abstracted in : *Trop. Dis. Bull.* 1928, **25**, 314
29. Dieudonné, A. & Otto, R. (1928) In : Kolle, W., Kraus, R. & Uhlenhuth, P. *Handbuch der pathogenen Mikroorganismen*, 3. Aufl. Jena, **4**, 179
30. Donskov, G. D. & Lochov, M. D. (1936) *Rev. Microbiol., Saratov*, **15**, 187
31. Fadeeva, T. (1939) *Rev. Microbiol., Saratov*, **18**, 44
32. Favarel, R. (1948) *Arch. Inst. Pasteur Tananarive*, p. 10
33. Favarel, R. (1949) *Bull. Soc. Path. exot.* **42**, 335
34. Flu, P. C. (1919) *Meded. geneesk. Lab. Weltevr.*, 3rd ser. A, p. 116
35. Flu, P. C. (1927) *C. R. Soc. Biol., Paris*, **96**, 1148
36. Flu, P. C. (1933) *Bull. Soc. Path. exot.* **26**, 796
37. Flu, P. C. & Flu, H. (1946) *Ant. Leeuwenhoek (J. Microbiol. & Serol.)*, **11**, 195
38. Gaiski, N. A. (1944) *J. Microbiol., Moscou*, No. 3, p. 5
39. German Plague Commission (1899) *Arb. GesundhAmt., Berl.*, p. 16
40. Gheltenkoff, A. (1938) *Rev. Microbiol., Saratov*, **17**, 272
41. Gheltenkoff, A. (1940) *Rev. Microbiol., Saratov*, **19**, 31
42. Gheltenkoff, A. & Khvorostukina, M. (1940) *Rev. Microbiol., Saratov*, **19**, 194
43. Girard, G. (1934) *Bull. Soc. Path. exot.* **27**, 415
44. Girard, G. (1934) *C. R. Soc. Biol., Paris*, **115**, 1219
45. Girard, G. (1936) *Ann. Méd. Pharm. colon.* **34**, 235
46. Girard, G. (1936) *Quart. Bull. Hlth Org. L. o. N.* **5**, 103
47. Girard, G. (1937) *Ann. Méd.* **42**, 478
48. Girard, G. (1941) *Ann. Inst. Pasteur*, **67**, 365
49. Girard, G. (1939) *Arch. Inst. Pasteur Tananarive*, pp. 11, 32
50. Girard, G. (1941) *C. R. Soc. Biol., Paris*, **135**, 1577
51. Girard, G. (1942) *Ann. Inst. Pasteur*, **68**, 476
52. Girard, G. (1943) *Ann. Inst. Pasteur*, **69**, 52
53. Girard, G. (1943) *Bull. Soc. Path. exot.* **36**, 218
54. Girard, G. (1948) *Proceedings of the Fourth International Congresses on Tropical Medicine and Malaria, Washington*, 1948, **1**, 257
55. Girard, G. (1950) *Ann. Inst. Pasteur*, **79**, 33
56. Girard, G. & Radaody-Ralarosy, P. (1940) *C. R. Soc. Biol., Paris*, **133**, 580
57. Girard, G. & Robic, J. (1934) *Bull. Acad. Méd., Paris*, **111**, 939
58. Girard, G. & Robic, J. (1936) *Bull. Off. int. Hyg. publ.* **28**, 1078

59. Girard, G. & Robic, J. (1938) *Acta Conventus Tertii de Tropicis Atque Malariae Morbis*, **1**, 335
60. Girard, G. & Sandor, G. (1947) *C. R. Acad. Sci., Paris*, **224**, 1078
61. Gokhale, S. K. (1948) *Report of the Haffkine Institute for the years 1944-1946*, p. 75
62. Goobar, J. K. (1943) *Bol. Dep. Hig. Prov. Córdoba*, **2**, 3
63. Gracian, M. (1941) *Rev. Sanid. Hig. publ., Madr.* **15**, 240
64. Grasset, E. (1941) *S. Afr. med. J.* **15**, 373
65. Grasset, E. (1946) *Trans. R. Soc. trop. Med. Hyg.* **40**, 275
66. Grasset, E. & Gory, M. (1927) *C. R. Soc. Biol., Paris*, **96**, 180
67. Greval, S. D. S. (1948) *Indian med. Gaz.* **83**, 137
68. Greval, S. D. S. & Dalal, N. P. (1933) *Indian J. med. Res.* **21**, 283
69. Gunnison, J. B., Larson, A. & Lazarus, A. S. (1951) *J. infect. Dis.* **88**, 254
70. Haas, V. H. (1938) *Publ. Hlth Rep., Wash.* **53**, 1033
71. Haffkine, W. M. (1897) *Brit. med. J.* **2**, 1461
72. Haffkine, W. M. (1897) *Indian med. Gaz.* **32**, 201
73. Harvey, W. F. (1933) *Trop. Dis. Bull.* **30**, 331, 411
74. Hetsch, H. (1904) *Z. Hyg. InfektKr.* **48**, 442
75. Hsue, L. T. (1942) *Chin. med. J.* **61**, 161
76. Huang, C. H., Huang, C. Y., Chu, L. W. & Huang, T. F. (1948) *Amer. J. trop. Med.* **28**, 361
77. Jawetz, E. & Meyer, K. F. (1943) *J. infect. Dis.* **73**, 124
78. Jawetz, E. & Meyer, K. F. (1944) *Amer. J. Path.* **20**, 457
79. Jawetz, E. & Meyer, K. F. (1944) *J. Immunol.* **49**, 1, 15
80. Jawetz, E. & Meyer, K. F. (1944) *J. infect. Dis.* **74**, 1
81. Joltrain, E. (1920) *C. R. Acad. Sci., Paris*, **171**, 413
82. Joltrain, E. (1936) *Bull. Acad. Méd., Paris*, **116**, 601
83. Joukov-Verejnikov, N. N. & Fadeeva, T. (1937) *Rev. Microbiol., Saratov*, **16**, 54
84. Joukov-Verejnikov, N. N., Fadeeva, T., Lipatova, T. & Khvorostukina, M. (1935) *Rev. Microbiol., Saratov*, **14**, 149
85. Joukov-Verejnikov, N. N. & Favarissova, B. Y. (1935) *Rev. Microbiol., Saratov*, **14**, 119
86. Kolle, W. & Krumbein, F. (1909) In : Kraus, R. & Levaditi, C., eds. *Handbuch der Technik und Methodik der Immunitäts-Forschung*, **2**, 463
87. Kolle, W. & Martini, E. (1902) *Dtsch. med. Wschr.* **28**, 1
88. Kolle, W. & Otto, R. (1903) *Dtsch. med. Wschr.* **29**, 493
89. Korobkova, E. I. (1937) *Rev. Microbiol., Saratov*, **16**, 1, 265
90. Korobkova, E. I. (1939) *Rev. Microbiol., Saratov*, **18**, 3
91. Korobkova, E. I. (1940) *Rev. Microbiol., Saratov*, **19**, 3, 450
92. Korobkova, E. I., Favarissova, B. Y. & Kolesnikova, Z. I. (1938) *Rev. Microbiol., Saratov*, **17**, 249
93. Kossel, H. & Overbeck (1901) *Arb. GesundhAmt., Berl.* **18**, 114
94. Kurauchi, K. & Homma, H. (1936) *Bull. Off. int. Hyg. publ.* **28**, 1088
95. Kuznetsova, V. & Dobrokhtova, M. (1938) *Rev. Microbiol., Saratov*, **17**, 91
96. Lazarus, A. S. & Gunnison, J. B. (1947) *J. Bact.* **53**, 705
97. Lazarus, A. S. & Nozova, M. M. (1948) *J. Bact.* **56**, 187
98. Lépine, P. & Bilfinger, F. (1934) *C. R. Soc. Biol., Paris*, **115**, 131
99. London, M. E.-S. (1898) *Arch. Sci. biol., St Pétersb.* **6**, 67
100. Lustig, A. & Galeotti, G. (1897) *Dtsch. med. Wschr.* **23**, 227, 289
101. Macchiavello, A. (1942) *Arch. Hyg., Rio de J.* **12**, 33
102. MacConkey, A. T. (1912) *J. Hyg., Camb.* **12**, plague suppl. II, 387
103. Magrou, E. (1946) *Rev. Méd. nav.* **1**, 105
104. Malone, R. H., Avari, C. R. & Naidu, B. P. B. (1925) *Indian J. med. Res.* **13**, 121
105. Markl, G. (1898) *Zbl. Bakt. (1. Abt.)* **24**, 641, 728 (Quoted by Pollitzer, 1936)

106. Markl, G. (1900) *Wien. med. Wschr.* (Quoted by Pollitzer, 1936)
107. Markl, G. (1901) *Z. Hyg. InfektKr.* **37**, 401 (Quoted by Pollitzer, 1936)
108. Markl, G. (1903) *Z. Hyg. InfektKr.* **42**, 244
109. Menezes, J. P. (1941) *Annual report of the Haffkine Institute for 1939*, p. 37
110. Meyer, K. F. (1947) *Ann. N. Y. Acad. Sci.* **48**, 429
111. Meyer, K. F. (1950) *J. Immunol.* **64**, 139
112. Meyer, K. F., Connor, C. L., Smyth, F. S. & Eddie, B. (1937) *Arch. intern. Med.* **59**, 967
113. Meyer, K. F. & Foster, L. E. (1948) *Stanford med. Bull.* **6**, 75
114. Meyer, K. F., Foster, L. E., Baker, E. E., Sommer, H. & Larson, A. (1948) *Proceedings of the Fourth International Congresses on Tropical Medicine and Malaria, Washington, 1948*, **1**, 264
115. Minervin, S. M., Stupnitzki, P. N. & Tinker, J. S. (1935) *Zbl. Bakt. (1. Abt., Orig.)* **133**, 170
116. Mitin, S. V. (1937) *Rev. Microbiol., Saratov*, **16**, 40
117. Morison, J., Naidu, B. P. B. & Avari, C. R. (1924) *Indian J. med. Res.* **12**, 313
118. Moses, A. (1909) *Mem. Inst. Osw. Cruz.* **1**, 109
119. Naidu, B. P. B., Jung, S. & Kamakaka, K. H. (1930) *Indian J. med. Res.* **17**, 1259
120. Naidu, B. P. B. & Mackie, F. P. (1931) *Lancet*, **2**, 893
121. Nederlandsche Vereeniging voor tropische Geneeskunde (1948) *Ned. Tijdschr. Geneesk.* **92**, 1566
122. Otten, L. (1933) *Meded. Dienst Volksgezondh. Ned.-Ind.* **22**, 131
123. Otten, L. (1934) *Conference of the Netherlands' Association for Tropical Medicine, Amsterdam, 25 March 1934* (Quoted by Otten, 1936)
124. Otten, L. (1936) *Indian J. med. Res.* **24**, 73
125. Otten, L. (1938) *Meded. Dienst Volksgezondh. Ned.-Ind.* **27**, 111
126. Otten, L. (1940) *Geneesk. Tijdschr. Ned.-Ind.* **80**, 2878
127. Otten, L. (1941) *Meded. Dienst Volksgezondh. Ned.-Ind.* **30**, 61
128. Panja, G. & Gupta, S. K. (1948) *Indian med. Gaz.* **83**, 148
129. Panja, G. & Gupta, S. K. (1949) *Indian med. Gaz.* **84**, 383
130. Petragnani, G. (1937) *Bull. Off. int. Hyg. publ.* **29**, 2522
131. Petrie, G. F. (1929) In : Great Britain, Medical Research Council. *A system of bacteriology in relation to medicine*, London, **3**, 137
132. Piras, L. (1913) *Zbl. Bakt. (1. Abt., Orig.)* **71**, 69
133. Pirie, J. H. H. (193–) In : South African Institute for Medical Research. *Annual report for the year ended 31st December, 1936*, Johannesburg, p. 13
134. Pirie, J. H. H. & Grasset, E. (1935) *Brit. J. exp. Path.* **16**, 126
135. Pirie, J. H. H. & Grasset, E. (1938) *S. Afr. med. J.* **12**, 294
136. Pirie, J. H. H. & Grasset, E. (1941) *S. Afr. med. J.* **15**, 275
137. Pokrovskaya, M. (1934) *Rev. Microbiol., Saratov*, **13**, 3
138. Pokrovskaya, M. (1935) *Rev. Microbiol., Saratov*, **14**, 376
139. Pokrovskaya, M. & Kaganova (1945) *Medicine, USSR.* (Society for Cultural Relations with Foreign Countries, No. 10, p. 3)
140. Pollitzer, R. (1936) Immunology. In : Wu Lien-teh, Chun, J. W. H., Pollitzer, R. & Wu, C. Y. *Plague : A manual for medical and public health workers*, Shanghai
141. Pons, R. (1933) *C. R. Soc. Biol., Paris*, **114**, 1066
142. Pons, R. & Advier, M. (1933) *Ann. Med. Pharm. colon.* **31**, 5
143. Ramon, G., Girard, G. & Richou, R. (1947) *C. R. Acad. Sci., Paris*, **224**, 1259
144. Revenstorf (1909) *Zbl. Bakt. (1. Abt., Orig.)* **52** (Quoted by Pollitzer, 1936)
145. Robic, J. (1937) *Bull. Soc. Path. exot.* **30**, 204
146. Robic, J. (1941) *Arch. Inst. Pasteur Tananarive*, p. 10 (Quoted by Girard, 1948)
147. Rockenmacher, M. (1949) *Proc. Soc. exp. Biol., N.Y.* **71**, 99
148. Rotman, C. M. H. (1945) *J. R. nav. med. Serv.* **31**, 155

149. Rowland, S. (1910) *J. Hyg., Camb.* **10**, 536
150. Rowland, S. (1911) *J. Hyg., Camb.* **11**, plague suppl. I, 11, 20
151. Rowland, S. (1912) *J. Hyg., Camb.* **12**, plague suppl. II, 340, 344, 350, 358, 367
152. Russell, A. J. H. (1935) *Far Eastern Association of Tropical Medicine. Transactions of the Ninth Congress...* 1934, **2**, 725
153. Sandor, G., Girard, G. & Skrobisz, C. (1948) *Ann. Inst. Pasteur*, **74**, 516
154. Savino, E. (1943) *Bol. sanit. (B. Aires)* **7**, 103
155. Savino, E. & Anchezar, B. (1939) *Rev. Inst. bact. B. Aires*, **9**, 122
156. Schütze, H. (1932) *Brit. J. exp. Path.* **13**, 284, 289
157. Schütze, H. (1934) *Brit. J. exp. Path.* **15**, 200
158. Schütze, H. (1939) *Brit. J. exp. Path.* **20**, 235
159. Schütze, H. (1939) *Lancet*, **1**, 266
160. Seal, S. C. (1943) *Report of the Haffkine Institute for the years 1940-1941*, p. 47
161. Seal, S. C. & Mukherji, S. P. (1943) *Report of the Haffkine Institute for the years 1940-1941*, p. 48
162. Shchastny, S. M. (1912) *Plague in Odessa in 1910.* Quoted by Bezsonova, A. (1929) *Rev. Microbiol., Saratov*, **8**, 327
163. Shrivastava, D. L. (1939) *Report of the Haffkine Institute for the year 1938*, p. 40
164. Simard (1921) *Bull. Off. int. Hyg. publ.* **13**, 964
165. Sokhey, S. S. (1936) *Bull. Off. int. Hyg. publ.* **28**, 1097
166. Sokhey, S. S. (1937) *Report of the Haffkine Institute for the year 1936*, p. 31
167. Sokhey, S. S. (1939) *Indian J. med. Res.* **27**, 313, 331, 341, 355, 363
168. Sokhey, S. S. (1939) *Report of the Haffkine Institute for the year 1937*, p. 29
169. Sokhey, S. S. (1939) *Report of the Haffkine Institute for the year 1938*, pp. 30, 32
170. Sokhey, S. S. (1947) *Biological assay of plague vaccine* (Unpublished working document WHO.IC/BS/24)
171. Sokhey, S. S. & Habbu, M. K. (1941) *Annual report of the Haffkine Institute for 1939*, p. 33
172. Sokhey, S. S. & Habbu, M. K. (1943) *Report of the Haffkine Institute for the years 1940-1941*, p. 45
173. Sokhey, S. S. & Habbu, M. K. (1945) *Report of the Haffkine Institute for the years 1942 and 1943*, p. 37
174. Sokhey, S. S. & Habbu, M. K. (1946) *Report of the Haffkine Institute for the years 1944-1946*, p. 56
175. Sokhey, S. S., Habbu, M. K. & Bharucha, K. H. (1950) *Bull. World Hlth Org.* **3**, 25
176. Sokhey, S. S. & Maurice, H. (1934) *Report of the Twelfth Conference of Medical Research Workers, Calcutta*, p. 154 (Quoted by Pollitzer, 1936)
177. Sokhey, S. S. & Maurice, H. (1935) *Bull. Off. int. Hyg. publ.* **27**, 1534
178. Sokhey, S. S. & Maurice, H. (1937) *Bull. Off. int. Hyg. publ.* **29**, 505
179. South African Institute for Medical Research (195-) *Annual report for the year ended 31st December, 1950*, Johannesburg, p. 26
180. Sprunt, D. H. & Camalier, W. (1942) *Arch. Path.* **34**, 801
181. Stevenson, W. D. H. (1912) *Proc. Second All-India Sanit. Conf.* **3**, 94 (Quoted by Pollitzer, 1936)
182. Stevenson, W. D. H. & Kapadia, R. J. (1925) *Indian J. med. Res.* **12**, 553
183. Strong, R. P. (1907) *Philipp. J. Sci.* **2**, section B, 155
184. Taylor, J. (1933) *Indian med. Res. Mem.* No. 27
185. Tumansky, V. M. (1938) *Rev. Microbiol., Saratov*, **17**, 261
186. Tumansky, V. M. (1939) *Rev. Microbiol., Saratov*, **18**, 244
187. Wats, R. C., Wagle, P. M. & Puduval, T. K. (1939) *Indian J. med. Res.* **27**, 373
188. Wayson, N. E., McMahon, M. C. & Prince, F. M. (1946) *Publ. Hlth Rep., Wash.* **61**, 1511
189. Wu Lien-teh (1926) *A treatise on pneumonic plague*, Geneva (League of Nations publication C.H. 474)
190. Yersin, A., Calmette, A. & Borrel (1895) *Ann. Inst. Pasteur*, **9**, 589