XXXVIII. FIRST REPORT ON INVESTIGATIONS INTO PLAGUE VACCINES.

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I. INTRODUCTION.

The primary source from which all vaccines are derived is a culture of the organism against infection by which it is desired to protect the animal. The culture may be used as a vaccine either alive or dead. We are concerned here only with the latter. Such a killed culture comprises the organism itself and the products of its metabolism, together with part or the whole of the culture medium in which it has grown. This mixture may be called a *whole vaccine*. By appropriate treatment a "whole vaccine" may be deprived of some or all of such constituents as are not concerned in bringing about, when inoculated into animals, those changes which constitute protection. Such a vaccine may be called a *derived vaccine* in the case when all the immaterial constituents are not removed, the term *antigen* being reserved to designate that substance which alone is concerned in conferring protection. The main object of this investigation is the preparation of a derived vaccine with the hope that the antigen may ultimately be isolated.

Whole vaccines may be prepared in any convenient way provided the contained antigen is not injured. Derived vaccines are prepared by a process of trial and error, using a whole vaccine as the material of investigation. Each preparation must be tested for antigen content by

[1] For a brief period at the beginning of the investigation, the late Dr T. Carlyle Parkinson was associated with Mr Rowland in this investigation. His untimely death from pneumonic plague on February 4th, 1909, clouded the early months of the work. By the loss of Dr Parkinson the Commission has been deprived of the services of an enthusiastic and most promising investigator.

some such method as is described in this report. A continuation of the processes of elimination which converted a whole vaccine into a derived vaccine, if carried sufficiently far, will lead ultimately to the isolation of the antigen.

II. SUMMARY OF PREVIOUS WORK.

That it is possible to immunise an animal against the subsequent inoculation of a living virulent plague culture was first shown by Yersin, Calmette and Borrel[1], who employed a suspension of plague bacilli killed by heating to 50° C. for one hour. Kolle[2] subsequently employed suspensions heated to 65° C. for several hours, while Wyssokowitz and Zabolotny[3] used suspensions heated to 60° C. Haffkine[4] employed heated broth cultures which he grew for some time, believing that some substance was gradually produced by the organisms which went into solution in the broth, and that this substance contributed to the immunising process. Gabritschewsky[5], abandoning the method of killing by heat, employed cultures that had been killed by glycerin, but soon became involved in difficulties due to the poisonous action of the glycerin when injected subcutaneously. The German Plague Commission[6] returned to the use of cultures killed by heat with the extra precaution of the addition of a small proportion of carbolic acid to the vaccine. They observed that if the carbolic was added before the organisms were killed by heat, the immunising value of the vaccine was less than it was when the organisms were heated first and the carbolic added afterwards. They also observed that it was advisable to employ as low a temperature as possible to kill the bacteria, and that at a sufficiently high temperature the immunising value was completely lost. Calmette[7] employed bacilli that had been killed by drying in a desiccator. He grew his bacilli either in broth and obtained them by

[1] Yersin, Calmette and Borrel, *Ann. de l'Inst. Pasteur*, 1897.

[2] Kolle, *Deutsche Med. Woch.*, 1897.

[3] Wyssokowitz and Zabolotny, *Ann. de l'Inst. Pasteur*, 1897.

[4] Haffkine, *Indian Med. Gaz.*, 1897; *Ref. nach Schottelius, Hygien. Rundschau*, 1901, No. 3 ff.

[5] Gabritschewsky, *Ref. Centralbl. f. Bakt.* 1898; Russiches, *Archiv f. Pathol. klin. Med. u. Bakt.*, 1897.

[6] Gaffky, Pfeiffer, Sticker und Dieudonne, *Arbeiten aus dem Kaiserlich. Gesundheitsamt*, 1899, Bd. XVI.

[7] Cited by Tavel, Krumbein u. Glucksman, *Zeits. f. Hygiene*, 1902, Bd. XL. p. 239.

filtration, or on the surface of agar and obtained them by scraping the surface of the agar.

All these vaccines belong to the class of whole vaccines, inasmuch as they consist of the organism together with the culture medium on which it was grown and the products of the metabolism of the organism dissolved in the medium. In the case of Calmette's vaccine some of the products of growth and some of the culture medium were removed.

Strong[1] used avirulent living cultures as vaccines with some success, but as this line of inquiry does not come within the scope of the investigation dealt with in this report no further allusion will be made to it.

As to the vaccinating value of derived vaccines a considerable amount of information exists. Lustig and Galeotti[2] suspended the surface growth from agar in 0·75% NaOH solution, and from the extract thus obtained precipitated by means of acetic acid a flocculent white substance which was of some vaccinating value. This substance could either be dissolved in weak alkalies and used at once, or dried: in the latter form it kept well. 0·36 mg. of this substance immunised a rat and no unfavourable symptoms followed its subcutaneous inoculation. To these authors belong the credit of first producing a vaccinating substance which could be accurately graduated, a great step in advance. Lustig and Galeotti considered their substance to be a nucleo proteid. Tavel, Krumbein and Glucksman[3] grew broth cultures by Haffkine's method and precipitated them, after a month's growth, by ammonium sulphate. The precipitate was extracted with 1% soda, and acetic acid was used to throw down from this extract what the author considered was a nucleo proteid. This was dried in vacuo and preserved in the form of a powder. For use it was dissolved in 1% sodium carbonate.

Another vaccine was recommended by Terni and Bandi[4]. It consisted of a culture of the plague bacillus grown not on an artificial medium, but in the peritoneal cavity of living guinea-pigs. As in the case of other whole vaccines the organisms were killed by heat and the extra precaution was taken of adding a small percentage of carbolic acid.

Another method in which the organisms are grown in the living animal has been proposed by Hueppe and Kikuchi[5]. Under these

[1] Strong, *Philippine Journ. of Science*, 1906, p. 182, and 1907, p. 155.
[2] Lustig and Galeotti, *Deutsche Med. Woch.*, 1897, pp. 23, 227 and 289.
[3] *Zeitsch. f. Hyg. u. Infektionskrankh.*, Leipzig, 1902, XL. p. 239.
[4] Terni and Bandi, *Ibid.*, 1900, XXVI. p. 463; *Rev. d. Hyg.*, Paris, 1900, XXII. p. 62.
[5] Hueppe and Kikuchi, *Cent. f. Bakt. Orig.*, 1905, L. p. 519.

circumstances a class of bodies known as aggressines are produced, which are capable of being employed as vaccinating substances. An investigation of this question will form the subject of a further report.

Still another method depending on the growth of the organism in the living body has been proposed. Klein[1] grows his organisms in the bodies of living guinea-pigs, removes from the dead animals the buboes, spleen, lungs and liver, minces them finely, and dries the minced material in thin layers over sulphuric acid. This treatment kills all the plague bacilli. The dose of this material which he considers contains the specific toxin of the plague bacillus together with "other substances of unknown nature and action" is determined by weight, the immunising dose for a rat being from 10 to 15 mg. S. Wallannah[2] also proposed a similar method.

In addition to the methods of immunising by means of a vaccine alone—however prepared—methods have been proposed which combine the use of a vaccine with that of plague immune serum, that is to say, a serum taken from an animal that has previously been inoculated with some preparation of the plague bacillus or even with the living bacillus. Thus Shiga[3] employed a mixture of immune serum and an emulsion of plague bacilli which had been killed by heat, and Besredka[4] employed a similar mixture but heated his bacilli after the addition of the serum (which addition had caused them to clump together). The mass of organisms which he obtained in this way was washed, made into an emulsion, and used as a vaccine. Gosio[5] made use of the precipitating action of immune serum in the preparation of vaccine on a large scale.

In a later method of Besredka[6] the use of immune serum was abandoned in favour of normal horse serum, organisms which have been treated (after having been killed) with normal serum being stated to be atoxic. In a still later method Besredka[7] starts with an emulsion in salt solution of a 48-hours' growth on agar. The organisms are killed by heating to 60° C. and dried in vacuo. The dry mass of organisms is now ground in an agate mortar with salt and the ground

[1] Klein, *Rep. to Loc. Gov. Board on a new Plague prophylactic*, London, 1906; *Brit. Med. Journal*, 1906, p. 155.

[2] Wallannah, *Ann. de l'Inst. Pasteur*, 1905, IX. p. 589.

[3] Shiga, *Ber. über die Pest in Kobe und Osaka*, Tokio, 1900, p. 54.

[4] Besredka, *Ann. de l'Inst. Pasteur*, 1902, XVI. p. 918.,

[5] Gosio, *Zeitsch. f. Hyg. u. Infektionskrankh.*, Leipzig, 1905, L. p. 519.

[6] Besredka, *Ann. de l'Inst. Pasteur*, 1905, XIX. p. 479.

[7] Besredka, *Ibid.*, 1906, XX. p. 304.

mass made into an emulsion in water. After shaking, standing and centrifuging, a solution is obtained which he considers contains the endotoxine of the plague bacillus.

Some successful attempts have been made to confer immunity by the use of a toxin which under certain circumstances is excreted by the plague bacillus, but as the circumstances which determine this secretion are altogether unknown but little use has been made of it. With our present knowledge, the appearance of a toxin in a fluid culture of the plague bacillus is altogether fortuitous, some cultures will contain it, and others grown under apparently identical conditions will be without any toxic action. Markl[1] obtained some success using this method. He grew his cultures in broth to which a small quantity of serum had been added. He obtained evidence of the existence of toxic substances in solution, especially in old cultures, and considered that a method of immunising which employed both bacilli and free toxin as the vaccinating material was to be preferred, as it rendered the animals (in his opinion) resistant not only to the invasion of living organisms, but also to the toxin produced by them. Dean[2] also obtained evidence of the existence of free toxin in solution in the case of old cultures, and showed that it could be separated from the organisms by filtration. Further reference will be made to this work when the question of antitoxic serum is considered.

A consideration of the work of these various authors suggests that

1. The plague bacillus contains a toxin within its cell substance.
2. The plague bacillus contains a nucleo proteid within its cell snbstance.
3. That occasionally some amount of toxin becomes free in the culture medium, but that the conditions which determine this are not clearly defined.

III. GENERAL METHODS OF PREPARATION OF VACCINES.

It is an axiom that an ideally perfect vaccine is a solution of the antigen in some inert fluid. Starting with a culture of the plague organism, any treatment that we may subject it to in the quest of this perfection must fulfil the following conditions:

[1] Markl, *Ann. de l'Inst. Pasteur*, 1898, xxiv.; *Wien. Med. Woch.*, 1900; *Zeit. für Hyg.*, 1901, xxxvii.

[2] Dean, *Studies in Pathology in celebration of the Quater Centenary of the Univ. Aberdeen*, Aberdeen, 1906.

1. The organism must be killed.
2. The antigen must be injured to the minimal extent.
3. Substances other than antigen must be removed as far as possible.

The methods that are available for killing the organism are restricted to those which do not greatly injure the antigen. Such methods are much fewer than might be supposed. Thus heat, the most convenient of all methods, must be used with caution, for in the case of every antigen that has yet been investigated, heating in the presence of water has a deleterious action. The usual means of minimising this destructive action of hot water is to employ as low a temperature as possible and to repeat the application on several occasions. Speaking generally heat is an agent that is better avoided if possible.

The objections that can be urged against the use of heat as a killing agent apply to most of the chemical means available. Even a comparatively innocent antiseptic may entirely destroy the antigen. Chloroform and toluol do not seem to appreciably damage it, whereas thymol destroys it. (See p. 564.)

There remain certain mechanical and physico-chemical methods which while destroying the life of the bacillus may not greatly injure the antigen:

1. mechanical disruption of the bacilli by grinding; and
2. desiccation.

The present writer in conjunction with the late Dr. A. Macfadyen[1] devised a purely mechanical method of killing the organisms which had the advantage of being conducted at a low temperature, $-180°$ C. The bacilli were brittle and could be ground to a fine state of division at this low temperature, thus facilitating the subsequent solution of the contained substances.

Desiccation of cultures over calcium chloride or sulphuric acid has been used to kill plague bacilli by Calmette and Klein. It is an uncertain process and difficult to accomplish satisfactorily unless a very thin layer be exposed. I have found it unhandy in operating upon large quantities of bacilli and not free from danger. To obviate these disadvantages I have, instead of drying by exposure to dry air in thin layers, mixed the bacterial paste with a suitable amount of anhydrous sulphate of soda. This works very satisfactorily. The hard mass formed can be melted

[1] Macfadyen and Rowland, *Ber. der Deut. chem. Ges.* 1900, xxiii; *Proc. Roy. Soc.*, London, lxxi. p. 77; *Cent. für Bact.*, i., xxxv. p. 415.

above 35° C. and the bacteria were always found to be killed after desiccation in this manner.

The early experiments to be shortly detailed were conducted with plague bacilli killed in this way. Subsequently it was found that preliminary killing with choloroform vapour did not materially influence the yield of antigen whilst considerably reducing the danger of the various manipulations.

IV. METHODS EMPLOYED IN DETERMINING THE DEGREE OF IMMUNITY CONFERRED AND IN INTERPRETING THE EXPERIMENTAL RESULTS.

In an investigation of this kind, which aims at giving quantitatively the comparative value of various toxic and immunising substances derived from the plague bacillus, difficulties are met with, for not only is there a variable resistance amongst the test animals employed, but in addition the virulence of the test culture employed may also vary. We require to be assured that during the progress of the work both these factors have remained reasonably constant.

As a preliminary, a culture was selected from a large number received from Bombay which, when grown on agar at laboratory temperature, appeared to retain a uniform virulence for many successive generations. It has been kept on agar, at the temperature of the laboratory, in the dark. For the purposes of a test it is grown in rat broth, in which medium the plague bacillus grows well without excessive clumping, and as a further precaution some glass beads are included in the culture flask with which clumps can be broken up. Finally, in order to ensure as homogeneous a suspension as possible, the culture is filtered through cotton wool immediately before using. As a test dose $\frac{1}{10}$ c.c. of a 72-hours' broth culture of this organism injected subcutaneously has been employed. This dose does not kill every rat injected with it, and it was found that multiplying the dose by ten or reducing it to $\frac{1}{100}$ c.c. did not greatly influence the proportion destroyed by it. (See Table I.)

Method of inoculation. Many observers have used the cutaneous method of inoculating (*i.e.* rubbing a culture into a portion of recently shaved skin) rather than the sub-cutaneous, but as experiments made on this point led to the conclusion that the sub-cutaneous method was more constant in its results, it was accordingly adopted.

TABLE I.

Strain	Dose c.c.	Rat	Result
No. 1	1·0	1	+
	1·0	2	0
	0·1	3	+
	0·1	4	+
	0·01	5	+
	0·01	6	0
	0·001	7	+
	0·001	8	0
No. 2	1·0	1	+
	1·0	2	+
	0·1	3	+
	0·1	4	0
	0·01	5	0
	0·01	6	0
	0·001	7	+
	0·001	8	0
No. 3	1·0	1	+
	1·0	2	+
	0·1	3	0
	0·1	4	0
	0·01	5	+
	0·01	6	0
	0·001	7	+
	0·001	8	0

+ = animal died. 0 = animal survived.

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It was then necessary to determine—using a large number of animals—how many rats this culture would kill. One hundred rats, taken at random out of the stock, were inoculated with the dose of culture mentioned above. Of this 100, 22 survived.

TABLE II.

Inoculation of 100 *rats for statistical error.*

+ = died of plague. 0 = recovered.

Rat	Result	Rat	Result	Rat	Result	Rat	Result
1	+	26	+	51	+	76	0
2	+	27	+	52	0	77	+
3	+	28	+	53	+	78	+
4	+	29	+	54	+	79	+
5	+	30	0	55	+	80	+
6	+	31	+	56	+	81	+
7	+	32	+	57	0	82	+
8	+	33	+	58	0	83	+
9	+	34	+	59	+	84	0
10	0	35	+	60	+	85	+
11	+	36	0	61	+	86	+
12	+	37	+	62	+	87	+
13	+	38	+	63	+	88	0
14	0	39	+	64	0	89	0
15	+	40	+	65	+	90	+
16	0	41	+	66	0	91	+
17	+	42	+	67	+	92	0
18	0	43	+	68	0	93	+
19	+	44	+	69	+	94	+
20	+	45	+	70	0	95	+
21	+	46	0	71	+	96	+
22	0	47	+	72	0	97	+
23	+	48	0	73	+	98	+
24	+	49	+	74	+	99	+
25	+	50	0	75	+	100	+

Having in this experiment used a large number of rats the chances are some twenty to one that we should not get a lower percentage mortality than 70 or a higher mortality than 86 in the population from which the sample 100 was drawn.

It would obviously be inconvenient to have to repeat this control series of 100 rats every time an experiment was made, but a small test series of ten rats could be and was employed on every occasion.

The next question arises, How many of a test series of 10 should die to enable it to be said that neither the natural immunity of the rats

used nor the virulence of the culture has varied from that found previously in the series of 100? This is a statistical problem of greater complexity than I am capable of dealing with, but my colleague Mr Greenwood, Statistician to the Lister Institute, has been good enough to come to my assistance, and I wish here to express to him my gratitude. Mr Greenwood informs me that the question may be answered in the following way:

If p be the number of deaths and n the whole number of animals used in the first experiment, if $q = n - p$, $\bar{p} = \frac{p}{n}$ and $\bar{q} = \frac{q}{n}$, then the most probable number of deaths to occur in a second sample of m rats is[1]

$$m\bar{p} + \frac{m}{n+2}(\bar{q} - \bar{p}).$$

Using this formula we conclude that on the basis of a preliminary sample of 100 having given 78 deaths, we should expect a subsequent random sample of 10 to yield 7·745 deaths. Now we have 19 such samples of ten controls each, and we require to know how their yields compare with the expectation. The mortality in these controls is shown in Table III below:

TABLE III.

Date	Rats		Date	Rats	
	Survived	Died of plague		Survived	Died of plague
March 24	1	9	July 29	2	8
31	2	8	Oct. 8	4	6
April 5	0	10	18	2	8
21	3	7	29	5	5
May 26	1	9	Nov. 11	3	7
June 5	0	10	23	1	9
19	3	7	30	0	10
July 9	1	9	Dec. 15	5	5
17	0	10	Jan. 21	3	7
25	1	9		37	153

The approved way of testing agreement[2] is to form the sum of $\frac{(\text{Expected number} - \text{Actual number})^2}{\text{Expected number}}$ for the whole number of trials and to compare the sum with its tabled values.

Applying this test we have χ^2 (the sum in question) $= 6{\cdot}294$ and looking up the table we have $P = {\cdot}994$. Which means that if 7·745 is

[1] Pearson, *Phil. Mag.*, 1907, pp. 365—378.

[2] Pearson, *Ibid.*, 1900, pp. 157—175; Elderton, *Biometrika*, I. p. 155. 1902.

really the most probable value, we should, owing to the unavoidable errors of random sampling, get no better agreement than actually found 99 times in every 100 repetitions of the series.

Some other tests could be and have been employed by Mr Greenwood, who is responsible for the previous calculation. The result of these analyses is, in his opinion, sufficiently definite to support the following statement:

The variations in the mortality among the controls are perfectly consistent with the results yielded by the preliminary inoculation of 100 *rats. They do not need to be explained by* (1) *a change in the natural immunity of rats, or* (2) *any alterations in the virulence of the culture.* This is a most important and satisfactory assurance.

We have now to consider what amount of diminution in mortality must occur in the groups of rats injected with various products of the plague bacillus before such fall in mortality can be safely regarded as causally related to the treatment they have received; in other words, it is necessary to ascertain what chance variation may be reasonably expected in groups of the size employed—15 to 50 animals.

Having ascertained the mortality (p) in a sample of n individuals (in our case 100) the probable mortality in a second series of m individuals can be calculated[1] from the formula

$$p \frac{m}{n} \pm \cdot 6745\, m \sqrt{\frac{p}{n} \times \frac{n-p}{n} \left(\frac{1}{m} + \frac{1}{n}\right)}.$$

The fraction preceded by the signs $\pm$ is called the probable error of the calculated value. The chances are equal that the number will fall within or without this range. An allowance of merely the probable error does not however afford any security and it is advisable to have as margin some multiple of this.

The chances against a mere accidental variation accounting for an unusual mortality in the second sample rapidly grows as the range allowed is increased, as may be seen from the following approximate table:

	Chances against deviation occurring[2]
Margin of probable error	2 to 1
,, twice probable error	10 to 1
,, three times probable error ...	43 to 1
,, four times ,, ,, ...	285 to 1

[1] Todhunter, *History of the Theory of Probability*, Chapter on Laplace; Pearson, 1907, *op. cit.*

[2] These are the chances against a deviation occurring in a given direction; the chance against, *e.g.* a positive deviation of twice the probable error or more is 10 to 1; the chance against a deviation of this order, but either positive or negative, is 4·5 to 1.

Three times the probable error is usually regarded as safe, and in the immunisation experiments detailed below after setting out the mortality which actually occurred in the different series of treated animals, an additional column has been added giving the mortality which might be expected to occur once in 44 times calculated on the mortality amongst the 100 controls, by mere chance distribution of extra resistant rats. As will be seen, the mortality in the different series treated either with a vaccine composed of the whole bacillus or of one particular constituent of the bacillus reduced the mortality considerably beyond the margin allowed on the above basis. Moreover, as the experience of seven successive series of experiments was uniform, the probability against any chance distribution becomes enormously increased and the results can be relied upon as bearing with certainty the interpretation placed upon them, viz., that the major part of the diminished mortality is accounted for by the prophylactic injections.

V. PREPARATION AND INVESTIGATION AS TO CHEMICAL AND ANTIGENIC PROPERTIES OF PRODUCTS OBTAINED FROM THE PLAGUE BACILLUS.

The object of the research being to ascertain the best method of preparing an immunisator against plague, it was considered that the most likely means to arrive at this goal would be to ascertain if possible to which constituent or constituents of the bacillus the antigenic property was due, and to isolate as far as might be practicable, the active constituent. If this could be accomplished it would then be possible to investigate the development of immunity and to study the effect of varying conditions upon the more or less isolated antigen with greater exactitude. Having arrived at this knowledge one would be in a position to devise the best procedure for its extraction from the bacillus with a minimum of damage and to decide the most satisfactory course to pursue in the practical immunisation against plague.

At the outset, however, one is confronted with the problem of killing the bacilli without undue destruction of antigen. For the first stage of this investigation it is not essential that the method should be that least prejudicial to the antigen. It is however essential to choose one which leaves a considerable amount of antigen undestroyed and one which is convenient and yields consistent results. After some pre-

liminary trials, killing by chloroform was selected as satisfactorily fulfilling these conditions[1].

An attempt has been made in this investigation to employ quantitative methods. Thus it will be noted that the dose of organisms or product therefrom used in any of the experiments is expressed in terms of weight. The weight given—in milligrammes—is the weight of dry substance contained in the dose given. In the case of organisms this is determined by centrifuging a known volume of emulsion, washing the deposited organisms, drying in the oven at 105° C. and weighing the residue. In the case of a protein solution, the weight is the dry weight of the protein in solution, after precipitation and washing free from salts and non-precipitable substances.

The additional labour involved in employing these methods is considerable, but the advantages are well worth it. Such units as a "loopful," "half an agar slope," are far from satisfactory. By substituting milligrammes in the dry condition we secure the following advantages:

(1) the possibility of repeating an experiment under the original conditions;

(2) the ability to compare results obtained at one period of the work with those obtained at another; and

(3) the power of selecting from a number of different preparations of the organism the one which exhibits in the greatest degree the properties we are in search of.

Another method that was found invaluable in the course of the work was the determination of the total nitrogen content of any preparation (by Kjeldahl's method). In attempting to resolve into products a material of the complexity of a bacterial body, the distribution of the nitrogen before and after the use of any solution, extraction or disintegration method often throws considerable light on what is happening.

Choice of experimental animals. The susceptibility of animals to plague infection varies considerably. Of laboratory animals the rat, mouse and guinea-pig are most easily infected, the rabbit less so. The facility with which these animals can be protected against infection also varies. Thus Yersin, Calmette and Borrel[2] succeeded in immunising

[1] The statement by Pick (Kraus and Levaditi, *Handbuch der Technik und Methodik der Immunitätsforschung*, Bd. 1. p. 353) to the effect that chloroform destroyed the antigen of the plague bacillus and was therefore not available for the preparation of a vaccine has not been confirmed.

[2] Yersin, Calmette and Borrel, *Ann. de l'Inst. Pasteur*, 1895.

rabbits but not guinea-pigs. Wyssokowitz and Zabolotny[1] immunised monkeys, and Haffkine's fluid can immunise man. The German Plague Commission[2] employed monkeys and rats as experimental animals and succeeded in immunising both.

The most difficult animal to immunise is the guinea-pig, and success has only followed in the case of those observers who have employed the method of living cultures. Chief amongst such is Strong[3], who has succeeded with these animals and with man. MacConkey[4] has shown that guinea-pigs and rats may be protected against plague by inoculation with the bacillus of pseudo-tubercle of rodents.

The choice of animal for the purposes of this investigation, bearing in mind the results obtained by the observers just quoted, fell on rats, and, for the purposes of the first experiments, it was decided to restrict the observations to the effects of a single inoculation, followed fourteen days later by a test inoculation of living virulent organisms. It was also thought best to employ as the index of immunity the survival or death of the animal, and to leave till later the investigation of the mechanism of the immunity conferred.

There are additional reasons for using rats as the test animals. In both man and rats the effects of the disease are explicable on the assumption of a considerable degree of susceptibility to a toxin formed from the bacillus. Thus in both animals death often occurs whilst the bacilli are still confined, or almost confined, to the lymphatic glands, that is to say, before a general septicaemia has arisen. Before the advent of death there are abundant evidences of a profound intoxication, chief amongst which is a pronounced cardiac failure. If a rat, in the earlier stages of the disease, be roughly handled, and so provoked to struggle, sudden collapse and death will often follow; the same phenomenon is often observed in man. There are thus great resemblances between plague stricken men and rats. For all these reasons rats appeared the most suitable animal on which to work out the protective power of the various bacterial products investigated.

The possibility that the toxic substances to which rats are sensitive may not be equally reactive with other animals has not been lost sight of. The results obtained and described in this report apply only to rats, and are now being extended to guinea-pigs and rabbits.

[1] Wyssokowitz and Zabolotny, *Ann. de l'Inst. Pasteur*, 1897.
[2] German Plague Commission, *loc. cit.*
[3] Strong, *Philippine Journ. of Science*, 1907, II. 3.
[4] *Journal of Hygiene*, VIII. (1908), 335.

Culture Medium. For the purposes of this investigation, in which large quantities of organisms as free as possible from the products of metabolism were required for chemical examination, a solid medium was essential. The quantity of growth obtained on a solid medium is much greater than that obtained in a liquid one. The question of the advantages gained by the addition of other substances to the ordinary media was not investigated, as it was found that quite a sufficient growth was obtained on a medium having the following composition: 1 % Lemco, 2 % peptone, 3 % agar. The choice of Lemco in place of beef broth was determined by the possibilities it afforded of securing a medium of constant composition. The use of Roux flasks offers many advantages over plate or dish methods of propagating.

Substance A.

After 4 days' incubation at 32° C. the bacilli were scraped from the surface of the agar by means of a glass rod and suspended in salt solution; the emulsion of living organisms thus obtained was washed in salt solution to remove culture fluid and products of growth, and suspended in salt solution. The nitrogen in a given volume of the resulting emulsion was determined by Kjeldahl's method. The organisms were then killed by chloroform and allowed to soak in chloroform water for 2 hours at 18° C. At the end of this time the emulsion was centrifuged and the clear fluid examined. It contained proteid in solution, and a determination of the nitrogen in the fluid showed that 29·5 % of the nitrogen in the emulsion had gone into solution. On adding acetic acid and boiling a precipitate was obtained, and it was found that this precipitate accounted for 17·6 % of the emulsion nitrogen, the balance of 11·9 % not being precipitated. On adding tannic acid a precipitate was obtained and it was found that the non-precipitable nitrogen accounted for 11·8 % of the emulsion nitrogen.

Schematically the distribution of the nitrogen in a chloroform emulsion may be represented:

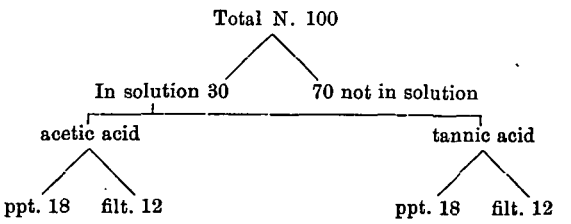

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TABLE IV.

Chloroform killed. Water extracted.

Dose mg.	Rat	Result*
1·4	1	+ 1 day
	2	+ 1 day
0·14	3	0
	4	0

Chloroform killed. Extracted with dilute sodium sulphate solution. Density 1046.

2·0	1	+ 2 days
	2	+ 2 days
1·0	3	0
	4	0
0·5	5	+ 2 days
	6	+ 2 days

Chloroform killed. Extracted with dilute solution sodium sulphate. Density 1051.

1·0	1	0
	2	0
0·5	3	0
	4	0

Toluol killed. Extracted with dilute NaCl.

1·0	1	+ 1 day
	2	+ 2 days
0·5	3	+ 3 days
	4	0

Toluol killed. Extracted with dilute sodium sulphate. Density 1051.

1·0	1	+ 1 day
	2	+ 1 day
0·5	3	+ 1 day
	4	+ 1 day
0·25	5	+ 1 day
	6	+ 2 days

When precipitated from its solutions by acetic acid and re-dissolved in dilute alkali the toxicity was:

2·5	1	+ 1 day
	2	+ 3 days
0·84	3	+ 3 days
	4	0
0·42	5	+ 2 days
	6	+ 2 days

* Henceforth the sign + signifies died and 0 survived.

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This proteid substance which goes into solution on the death of the bacillus by chloroform accounts, therefore, for 18 % of the total nitrogen of the organism. It is precipitated from its solution by acetic acid. The precipitate re-dissolves in dilute alkali with facility. A solution gives Mollisch's as well as the tryptophane, biuret, and xanthoproteic reactions. It contains much combined phosphorus, and 16 to 18 % nitrogen. It corresponds to a nucleo-protein, and is here called "*Substance A.*"

Inoculated into rats it is toxic, but its toxicity is very variable, the lethal dose of different preparations varying from ·25 to 2 mg. Table IV gives some idea of this variation. The organisms were killed with chloroform and in two cases with toluol. Dilute sodium sulphate or salt solutions were used for extraction of the substance A. The variation in the relation of nucleo-protein content to toxicity suggests that this protein is not the toxic substance, but that the latter is closely associated with it.

The association of the toxic substance with the nucleo-proteid appears to be a close one.

Immunising Properties. Of 49 rats inoculated with ·01 mg. of the nucleo-proteid of solution A 34 succumbed to a subsequent inoculation of living plague bacilli (mortality 70 %).

Of 30 rats inoculated with ·01 mg. of another preparation of the nucleo-proteid A precipitated by acetic acid and dissolved in weak alkali 20 succumbed to the subsequent inoculation (mortality 51 %).

Of 43 rats inoculated with ·001 mg. of the same material 29 succumbed to the subsequent inoculation (mortality 68 %).

Allowing three times the probable error, in accordance with the principles discussed on p. 546 above, a mortality as low as 64, 61 and 62 respectively might be expected to occur once in 44 times. Only the second series show any diminution of mortality beyond this range. Hence it is concluded that preparations of the nucleo-proteid from solution A may give indications of immunising properties in doses of ·01 mg., but the different preparations are not consistent in this respect.

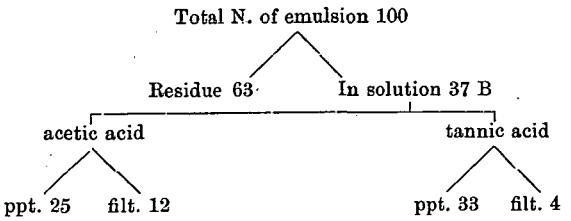
Substance B.

The organisms that have been extracted with water or dilute saline solutions can by appropriate treatment be made to yield a further substance in solution ("*Substance B*"). This treatment, the reason for which may not appear quite obvious, originated, as explained above, in the course of an examination into the various means available for killing

the plague organism without injuring its antigen. Amongst such means dehydration was investigated, and amongst other dehydration methods the use of anhydrous sodium sulphate suggested itself. It was soon found that this reagent was of the greatest utility and that it could be employed with advantage even on cultures already killed. The method found to be the most useful is as follows:—

200 Roux flasks containing neutral agar (1 % Lemco, 2 % peptone, 3 % agar) are inoculated into the condensation water, incubated for 24 hours at 32° C. and the condensation water swept over the surface of the agar by tilting the bottle, by which means a uniform surface inoculation is obtained. Incubation at 32° C. is continued for 4 days. About 1 c.c. of chloroform is then introduced into each flask and the flasks left in the hot room till the next morning. About 10 c.c. of dilute salt solution are squirted into each flask and the surface growth emulsified with a glass rod. The emulsion is drawn by a syphon arrangement into a large flask. This flask contains, therefore, dead bacilli, dilute culture fluid, products of growth and the substance A which we have seen goes into solution on the death of the organisms. The contents of the flask are centrifugalised in a special form of machine (vide Appendix), and the pasty mass of organisms obtained is re-emulsified in salt solution. This emulsion is passed through fine linen to remove chance lumps of agar, and again centrifugalised, the washing in salt solution being repeated if thought necessary. The paste of organisms finally obtained contains about 80 % of water and 20 % solids (dried at 105° C.). This paste is thoroughly mixed in a mortar with finely powdered anhydrous sulphate of soda. Pounding is continued until a dry powder is obtained which is set aside in an ice chest until the next day. The powder is then warmed to 37° C. when it becomes a semi-fluid mass, well stirred, and again set in the ice chest. This alternate cooling and heating is repeated several times. Finally sufficient water is added to dissolve the sulphate of soda, and to make a saturated solution at 37° C. The bacterial bodies are filtered off through hardened paper at 37° C. and suspended in water, whereupon solution of a second part of the bodies takes place. This I call "*Substance B.*"

The nitrogen distribution in this process is as follows. Calling the total nitrogen of the washed emulsion before sulphating 100, 37 parts will be found in solution in the dilute sulphate solution after the process. Of this 37, 25 are precipitated by acetic acid and 33 by tannic acid.



Chemical Properties of Solution B.

Solution B is a clear yellowish liquid containing about 5 % sulphate of soda in solution. It is faintly alkaline in reaction and holds a nucleo-proteid dissolved in it. When first prepared it is limpid and transparent, but deposits a light cloudy precipitate slowly on standing. On boiling a precipitate is obtained, as also on the addition of acetic acid; the latter precipitate is only thrown down when the solution is distinctly acid. This precipitate is not soluble in reasonable excess of the acid but is very soluble in dilute alkali. The acetic acid precipitate contains organic phosphorus in considerable amount. The filtrate from the acetic acid precipitate also contains some phosphorus. The precipitate contains about 16 % of nitrogen.

On dialysis no precipitation occurs. The dialysed solution gives the following reactions. No precipitate is formed on heating, but after the addition of sodium sulphate to the extent of 5 % the formation of a considerable precipitate occurs on heating. Acetic acid causes a precipitation of all proteins. 90 % alcohol causes no precipitate even on standing, the addition of acetic acid to such a mixture causes an immediate precipitate, which settles in flocculi. The solution gives the biuret (slowly), xanthoproteic, glyoxylic and Mollisch's reaction. It also gives Millon's reaction. The filtrate from a portion that has been precipitated by acetic acid gives no biuret, Millon, glyoxylic or Mollisch's reactions but gives a slight xanthoproteic colouration. From these reactions the conclusion is justified that solution B also contains a nucleo-proteid. This conclusion was confirmed by the recognition of adenin and guanin nitrates as decomposition products. A portion of the precipitate obtained on the addition of acid (acetic) was placed in nitric acid of a density of 1·2 in the ice chest. After a lapse of some days crystals corresponding in appearance to the nitrates of guanin and adenin were recognised microscopically. It has not yet been possible to spare sufficient quantity of the substance to make a more thorough analysis.

Toxic Properties of Solution B.

The following experiments show that when inoculated into rats this substance is weight for weight considerably more toxic than substance A.

Dose mg.	Rat	Result
2·0	1	+ 1 day
	2	+ 1 day
	3	+ 1 day
0·2	4	+ 1 day
	5	+ 1 day
	6	+ 1 day
0·02	7	+ 6 days
	8	+ 7 days
	9	0
0·002	10	+ 9 days
	11	0
	12	0

Lethal dose of the same preparation kept 41 days under toluol at the temperature of the laboratory.

2·0	1	+ 1 day
	2	+ 1 day
0·2	3	+ 5 days
	4	0
0·02	5	+ 2 days
	6	0

Lethal dose of the same sample as the last after heating to 55° C. for 30 minutes.

2·0	1	+ 1 day
	2	+ 2 days
0·2	3	0
	4	0
0·02	5	+ 4 days
	6	0

Lethal dose of the same preparation as the first after keeping for three months under toluol at the temperature of the laboratory.

2·0	1	+ 1 day
	2	+ 1 day
	3	+ 1 day
0·2	4	+ 1 day
	5	+ 1 day
	6	+ 2 days
0·02	7	0
	8	0
	9	0

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Lethal dose of another preparation of B.

Dose mg.	Rat	Result
1·0	1	+ 1 day
	2	+ 1 day
	3	+ 1 day
0·1	4	+ 1 day
	5	+ 1 day
	6	+ 1 day
0·01	7	0
	8	0
	9	0

These experiments show that the lethal dose of solution B corresponds to about one-tenth of a milligramme of contained nucleo-proteid; that the solution can be kept under toluol at room temperature for a considerable time without much loss of toxicity; and that heating to 55° C. diminishes toxic power.

The effects observed post-mortem in rats after a fatal dose of this solution are: inflammatory oedema at and about the seat of inoculation and congestion of all the organs of the body, the appearances recalling those observed in rats after death from plague infection but without the buboes.

Effects of Dialysis on Toxicity of Solution B.

Samples of solution B of which 0·1 mg. was a fatal dose were thoroughly dialysed against distilled water. The table below assesses the toxicity of a dialysed solution freshly dialysed, and after 20 and 41 days' keeping under toluol at the temperature of the laboratory.

	Fresh		20 days		41 days	
Dose mg.	Rat	Result	Rat	Result	Rat	Result
7·2	1	+ 1 day	1	+ 1 day	1	+ 1 day
	2	+ 1 day	2	+ 1 day	2	+ 1 day
2·4	3	+ 1 day	3	+ 1 day	3	+ 1 day
	4	+ 1 day	4	0	4	+ 2 days
1·2	5	+ 1 day	5	+ 1 day	5	+ 2 days
	6	+ 1 day	6	0	6	+ 3 days
0·72	7	+ 1 day	7	+ 2 days	7	+ 3 days
	8	0	8	0	8	0
0·24	9	0	9	0	9	0
	10	0	10	0	10	0

Dialysis deprived solution B of some of its toxic power and the toxicity fell still further on keeping. The toxic substance did not pass through the dialysing medium for on evaporating down in vacuo at 15° C. several litres of the dialysate, it was devoid of toxicity.

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Toxicity of the acetic acid precipitate from B.

Dose mg.	Rat	Result
1·8	1	+ 1 day
	2	+ 1 day
0·9	3	+ 1 day
	4	+ 3 days
0·18	5	+ 1 day
	6	0

Precipitation by acetic acid thus causes a fall in toxicity. That no toxic substance was left unprecipitated was shown by evaporating a large quantity of the filtrate at 25° C. and failing to find any toxic effect on inoculation into rats. Thus dialysis or precipitation diminishes the toxicity of B. An explanation of this result is not at present obvious.

The effect of salting out solution B.

A solution was prepared containing one lethal dose in one-tenth of a cubic centimetre and sufficient sodium sulphate added to bring the density to 1200. The precipitate was filtered off and dissolved in a volume of water equal to that of the solution before the addition of the salt. The table gives the results of the inoculation into rats of the filtrate and dissolved precipitate.

	Precipitate		Filtrate	
Dose c.c.	Rat	Result	Rat	Result
1·0	1	+ 1 day	1	0
	2	+ 1 day	2	+ 2 days
0·8	3	+ 1 day	3	0
	4	+ 1 day	4	0
0·6	5	+ 1 day	5	+ 2 days
	6	+ 1 day	6	0
0·4	7	+ 1 day	7	0
	8	+ 1 day	8	0
0·2	9	+ 1 day	9	+ 2 days
	10	0	10	0
0·1	11	+ 1 day	11	0
	12	+ 1 day	12	0

Thus sodium sulphate in the strength mentioned (27 grammes of salt to 100 c.c. of water) precipitates the proteins and with them the toxic substance.

This experiment was repeated, the strength of the solution being

19 grammes of salt to 100 c.c. of the solution (density 1150) with these results;

Dose c.c.	Precipitate		Filtrate	
	Rat	Result	Rat	Result
1·0	1	+ 1 day	1	+ 1 day
	2	+ 2 days	2	+ 1 day
0·8	3	+ 1 day	3	+ 1 day
	4	+ 2 days	4	+ 2 days
0·6	5	+ 1 day	5	+ 1 day
	6	+ 2 days	6	+ 1 day
0·4	7	+ 1 day	7	+ 2 days
	8	+ 3 days	8	+ 1 day
0·2	9	+ 5 days	9	0
	10	0	10	0
0·1	11	0	11	0
	12	0	12	0

Sodium sulphate of the strength mentioned distributes the proteins into two portions with an approximately equal distribution of the toxin.

On slowly adding sodium sulphate to solution B of the same protein strength as used in the last two experiments, the first signs of precipitation took place at density 1140 (18 grammes to 100 c.c.). The precipitation limits of the proteid by sodium sulphate are thus density 1140 —density 1200.

Filtration of Solution B.

An experiment was made to determine what loss of toxicity occurred on filtration through a close porcelain filter. For this purpose a small Chamberland F. was chosen. Filtration took place rapidly without necessitating much lowering of pressure. The table following gives the toxicity of the solution before and after filtration. No certain loss was observed: the first portions of the filtrate were not rejected. Representing the total nitrogen in the solution before filtration as 100, the total nitrogen in an equal volume of the filtrate was 87, a loss of 13 %.

Toxicity of B before and after filtration.

Dose c.c.	Before		After	
	Rat	Result	Rat	Result
1·0	1	+ 1 day	1	+ 1 day
	2	+ 1 day	2	+ 1 day
0·8	3	+ 1 day	3	+ 1 day
	4	+ 1 day	4	+ 1 day
0·6	5	+ 1 day	5	+ 1 day
	6	+ 1 day	6	+ 1 day
0·4	7	+ 1 day	7	+ 1 day
	8	+ 1 day	8	+ 2 days
0·2	9	+ 1 day	9	+ 1 day
	10	0	10	+ 1 day
0·1	11	+ 1 day	11	0
	12	0	12	0

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Immunising value of Solution B.

It has been shown that solution B possesses marked and constant toxic properties and that the toxicity is closely associated with the presence of a nucleo-proteid in solution. It will now be shown that it can be used as a highly efficient vaccine.

The rats in the series of experiments below were each injected with the material in a single dose of the amount mentioned. Fourteen days later their resistance was tested by the subcutaneous inoculation of $\frac{1}{10}$th c.c. of the broth culture of plague (see p. 542):

Series	Dose mg.	No. of rats	Mortality	% mortality	Minimum % mortality which might be expected once in 43 times (See p. 546 above)
1	0·02	23	2	9	59
2	0·02	29	5	17	61
3	0·01	18	1	6	60
4	0·003	20	4	20	59
5	0·001	15	2	13	55
6	0·0001	24	9	38	59

The effect of heating the solution before use (55° C.) for 30 minutes is brought out by the results of the next series of experiments:

7	0·02	20	6	30	59

This series should be compared with series 1 which was vaccinated with the same solution but not heated.

Series 2 was vaccinated with a solution that had been kept for three months at the temperature of the laboratory.

The fall in mortality after the test inoculation with plague which occurred in those series of rats which had previously been treated with ·0001 to ·02 milligrammes of the protein contained in solution B, is far in excess of any error due to chance (see p. 546 above), and shows that a single injection of even minute quantities had conferred upon them a substantial immunity.

The greater part of the antigen evidently passes into solution after submitting the organisms to the sulphating process. It will be seen later that the residue if sufficiently washed possesses no immunising properties.

The degree of protection obtained with the substance in solution after treatment of the bacilli with anhydrous sulphate of soda compares very favourably with that obtained by the injection of the whole bacillus killed with chloroform and subsequently washed with water. A preminary series of experiments indicated that ·03 mg. of such washed

bacilli was the optimum protective dose. The results of two series of rats injected with this dose are given below:

Series	Dose mg.	No. of rats	Mortality	% mortality	Minimum % mortality which might be expected once in 43 times (See p. 546 above)
1	0·03	34	9	27	61
2	0·03	28	7	25	60

The mortality in these series after ·03 mg. of bacillus was considerably greater than that obtained with rats protected by ·001 mg. of the proteins in solution B.

The Residue of Sulphated Bacilli after Extraction of the Constituents soluble in Saline.

On the completion of the sulphating process, after the final extraction of the sulphated bacteria with water, there remains a residue which consists of the bodies of the bacteria from which those substances contained in solutions A and B have been dissolved out. Examined unstained with the microscope, using dark ground illumination, these extracted bodies do not appear to differ materially from the unextracted organisms. They are however profoundly modified. In the first place, they will take no stain except hot and strong fuchsin; and secondly, their physical characters are changed. Whereas a fresh bacillus examined on a dark ground in water will stand fairly violent treatment and remain intact, a body of a sulphated bacillus is very fragile and slight pressure on the cover glass causes its disintegration. It was at first thought that the effect of the repeated dehydrations of the sulphate process was to rupture the organism and thus allow of the escape of its contents, but this is apparently not the case. Indeed one of the ideas which originated the process was that by means of the strains set up in the alternating fusion and crystallisation the organism might be mechanically ruptured. It would appear however that the treatment modifies the consistence and permeability of the bacterial envelope. Whatever the explanation of the process may be, there is no doubt that a profound alteration in the organism is brought about, and that subsequent to this treatment the proteins dissolved out carry with them a larger proportion of toxic and immunising substances.

The residue consists of material insoluble in water or saline solution. About 17 % is soluble in 1 % Na_2CO_3 and a further quantity in caustic alkali. It contains 10—11 % of nitrogen and some phosphorus in organic

combination, but the amount has not yet been estimated. The residue accounts for 45 % of the total nitrogen of the bacillus. It possesses very small toxicity for rats and the more completely it has been extracted the less poisonous it is. In one case 22 mg. killed one out of two rats, and 7 mg. failed to kill either of two rats. Corresponding with this absence of toxicity there is an absence of immunising power as may be seen by the following experiments:

Series	Dose mg.	No. of rats	Mortality	% mortality	Minimum % mortality which might be expected once in 43 times (See p. 546 above)
8	0·1	39	27	70	62
9	0·03	42	36	86	62
10	0·01	45	38	85	63
11	0·001	44	35	80	63

Even 1000 times the quantity which in the case of solution B afforded a substantial protection, failed to indicate any advantage over the control series.

Another way in which the influence of the sulphating process in facilitating the dissolving out of the toxic substances in the bacilli is strikingly demonstrated is by comparing the toxicity of the residue after

(1) simple extraction with water,

(2) sulphating and subsequent extraction.

This is shown in the experiments below, from which it will be seen that the former leaves a highly toxic remainder, whereas the latter exhibited no toxicity within the limits investigated.

Toxicity of an emulsion killed by chloroform, after extraction by water.

Dose mg.	Rat	Result
2·7	1	+ 1 day
	2	+ 2 days
1·4	3	+ 3 days
	4	0
0·7	5	+ 2 days
	6	0
0·3	7	+ 3 days
	8	0
0·1	9	0
	10	0

Toxicity of an emulsion killed by chloroform, after extraction by water, sulphating, and again thoroughly extracting in dilute saline.

22·0	1	+ 2 days
	2	0
7·0	3	0
	4	0
3·0	5	0
	6	0
1·0	7	0
	8	0
0·1	9	0
	10	0

The bulk of the antigen passes into solution only after the treatment of the bacillus with anhydrous sulphate of soda. It is unlikely that this solution contains only the immunising substances, but I may be permitted to point out here the advantages to be anticipated by the use of an antigen in solution:

(i) Ease of standardization.
(ii) Greater accuracy of dosage.
(iii) Sterilization by filtration.
(iv) Smallness of dose.
(v) Absence of associated substances of no vaccinating value, which may be undesirable and, at any rate, must be dealt with by the organism before the contained antigen is liberated.

It is now my pleasant duty to thank those who have helped me to attain these results. In the first place I desire to record the loyal assistance of my two laboratory attendants, H. Bray and J. Whittingham; and to express my thanks to my colleagues at the Lister Institute, Dr A. Harden, F.R.S., Dr A. T. MacConkey, Dr A. B. Green and Miss H. Chick, D.Sc., each of whom has at one time or another helped me with advice on technical points. It is difficult to express in a few lines my indebtedness to Dr C. J. Martin, F.R.S., Director of the Institute, whose counsel and practical help have been continually at my disposal throughout the investigation.

Conclusions.

1. Washing the living organism with chloroform water, while killing the cell, removes a certain amount of a nucleo-protein (substance A) but only traces of the substances which are toxic and immunising for rats.

2. Organisms which have been so treated are toxic for rats and possess immunising power for rats.

3. By appropriate treatment (sulphating process) a further protein substance (substance B) can be dissolved out by water or weak salt solution.

4. This further substance also consists largely of nucleo-protein but contrasts with substance A in respect of its greater and more constant toxicity and of the extent of immunity which it is capable of conferring on rats.

5. Organisms which have had this second substance removed from them are no longer toxic or capable of conferring immunity on rats.

6. There is an intimate association between the toxicity and immunising value of the solution obtained after the sulphating process and both these properties are closely related to the presence of a nucleo-protein, but there is reason to believe they may be found to be independent of it.

APPENDIX.

In order not to interrupt the main argument of this paper by laboratory details they are collected here.

Strain of organisms. For all the experiments, with the exception of testing for immunity, the culture employed was the stock culture of the Lister Institute. It is the culture employed by the Institute in the preparation of Haffkine's prophylactic, and for the preparation of Yersin's serum. It is not very virulent, but is capable of having its virulence raised by passage. Tested for sugar reactions in Durham's tubes it gave

	Acid	Gas
Glucose	+	0
Lactose	0	0
Cane sugar	0	0
Dulcite	0	0
Adonite	0	0
Inulin	0	0
Mannite	+	0
Litmus milk	No change of colour.	

Centrifuge. This was specially designed for washing bacteria and obtaining a paste of organisms from an emulsion. It consists of a steel bowl rotating on its vertical axis at a speed of about 7,000 revolutions per minute. The interior is divided by a vane bisecting the cylinder in a vertical plane passing through the axis of revolution and not reaching quite to the circumference. This vane is removable, and serves to ensure rotation of the fluid at the same rate as the bowl in which it is contained. The bowl is provided with a screw-down lid which makes a water tight joint by means of a rubber ring. Such a bowl deposits on the side after 15 minutes all the organisms in an emulsion in the form of a stiff paste. This paste can easily be removed, after lifting out the vane, by a spatula. As obtained in this way the paste commonly contained from 10—20 per cent. dry matter at 105° C.

Method of inoculation. When working with living cultures of virulent plague and large number of rats, precautions are necessary. A technique was devised which secures maximum immunity against infection both to operator and assistant. An all-glass syringe was fitted on a stand which could be firmly clamped to the table. The nozzle of the syringe was connected by some two feet of small sized pressure tubing with a needle. The piston of the syringe was arranged to be advanced by means of a screw, which was of such a pitch that one turn of the handle expelled one-tenth of a cubic centimetre of a living culture. The rat was held by an assistant, his left hand grasping the neck and forelegs, his right hand holding the right hind leg and the tail. The left hand of the operator holds the left hind leg of the rat. The operator with his right hand pushes the needle through the skin *and muscles* of the left thigh of the rat until the point of the needle impinges under the skin on the other side of the thigh, the skin being raised by the needle but not perforated. One turn of the handle of the syringe now introduces 0·1 c.c. of culture, pure subcutaneously, on the back of the flank. The area around the point of insertion of the needle is now flooded with lysol and the needle withdrawn into the little pool of lysol left, ready for the next rat. The celerity with which all this can be performed is surprising. Generally about three rats per minute received their dose. No regurgitation is possible owing to the muscles interposed in the track of the needle.

Note on Preservatives. During the numerous and lengthy experimental manipulations of the bacilliary emulsions or proteid fluids obtained from bacilli it is difficult to altogether avoid contamination with aerial organisms, so that the addition of some preservative is advisable. It was found that the best all round preservative for this purpose was toluol. Thymol was tried, but as it was found to destroy the vaccinating value of an emulsion its use was abandoned. This point is brought out in the following tables which compare the vaccinating value of two emulsions, one preserved with toluol and the other with thymol.

Toluol preserved emulsion.

Series	Dose mg.	No. of rats	No. died	% mortality
I	0·03	34	9	27
II	0·03	28	7	25

Thymol preserved emulsion.

III	0·03	36	24	67

Coincidentally with this loss of immunizing power there is a considerable fall in toxicity, as the following table shows:

Toluol			Thymol		
Dose mg.	Rat	Result	Dose	Rat	Result
3·0	1	+ 2 days	6·0	1	+ 1 day
	2	+ 2 days		2	0
1·5	3	+ 2 days	4·0	3	+ 4 days
	4	0		4	0
0·75	5	+ 2 days	2·0	5	0
	6	0		6	0
0·3	7	+ 2 days	1·0	7	+ 4 days
	8	0		8	0
0·15	9	0	0·5	9	0
	10	0		10	0

On the Presence of Free Proteid in Cultures of Plague Bacilli.

In the light of the knowledge gained in the course of this work, the following observations on the presence of free proteid in broth cultures of the plague bacillus are interesting.

In a young (4 days) culture no coagulable proteid can be found, but after 14 days' growth a considerable quantity was observed, in one case in which the amount was estimated as much as 14 milligrammes per 100 c.c. of culture. In the case of a 13 months' old culture a much larger quantity was found, but the amount was not determined. This proteid material presumably came from the bodies of the bacilli. It should be remembered that in a culture of any organism there are always present a certain proportion of dead individuals and that this proportion increases as time goes on. It would appear then that on the natural death of the bacillus there is an extrusion of some of the contents of the bacterial body. Now it has been shown that there is a close association between the toxic substance contained within the plague bacillus and a nucleo-proteid, and many observers have recorded the fact that the filtrates of old cultures are toxic.

Putting these facts together the conclusion is reached, that the toxic substance obtained with the aid of the extraction methods described in this report is probably identical with that obtained in the filtrates of Dean[1] and Markl[2].

[1] Dean, *loc. cit.*

[2] Markl, *loc. cit.*

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