VIRULENCE, IMMUNITY AND BACTERIOLOGICAL VARIATION IN RELATION TO PLAGUE.

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(With Plates IV and V.)

CONTENTS.

IN consequence of an epidemic of plague which occurred in the Gold Coast in 1923 and 1924 the preparation of plague vaccine was started at the Medical Research Institute, Accra. The epidemic did not last long, but a stock of vaccine has been maintained, and the preparation and testing of this has been the starting point of a number of experiments. An account of some of the earlier ones was published in this *Journal* (Burgess, 1927) and since then the work has been continued at intervals on similar lines and, together with certain earlier results not yet reported, forms the subject of the present paper.

I. METHODS AND MATERIAL.

Source of cultures. The cultures of *Bacillus pestis* that were used came from two places, viz. West Africa and South Africa. In the earlier experiments the former were used. They included the two human strains, *L* and *S*, mentioned in the previous paper, and a more recent culture from a Lagos rat for which we are indebted to Dr A. Connal. Subsequently five human strains isolated in South Africa were kindly supplied by Dr J. Harvie Pirie of the South African Institute for Medical Research.

Designation of cultures. Each culture was marked with one or two letters indicating its original source and with a number, which was that of the last passage animal. At each passage therefore the strain received a new number and retained the old letter. To conform to this system the South African cultures, which were distinguished by numbers when received, had to be relabelled and were given the letters P.A. to P.E., the letters P.A., P.C. and P.E. corresponding to Dr Pirie's numbers 11044, 11187 and 11903. The West African culture, *S*, was relabelled G. in the second year's work in order to distinguish recurring numbers.

Nutrient media. For tube and plate cultures trypsinised beef medium was used; 0·5 per cent. anhydrous sodium sulphite, but no sodium chloride, was added and, if a solid medium was required, 3 per cent. agar; the reaction was adjusted to the neutral point of phenyl phthalein and the medium autoclaved. This large amount of sulphite was decided on after comparative tests. Its effect was of the greatest value in plate work. Without it properly spread plates could not be obtained. Cultures were grown at room temperature (about 27° C.).

Some of the agar vaccines were grown on agar prepared as described, but latterly a medium has been used which is simpler to prepare and yields a heavier growth. Instead of making a meat extract the whole of the meat is included in the medium. One pound of beef is minced as finely as possible, and 1½ litres of tap water, 1 per cent. peptone and 3 per cent. agar are added and then after adjusting the reaction the mixture is autoclaved. The idea was derived from the knowledge that media containing particles give the best growth. The medium is of course quite opaque, but transparency is not required. The only difficulty is in adjusting the reaction, for after neutralising a slow change lasting several hours takes place in the direction of acidity, due no doubt to the gradual absorption of alkali by the lumps of meat. Good results, however, have been obtained by neutralising and then adding 40 per cent. excess of alkali. The medium was contained in Roux bottles, was inoculated by flooding with a 2-day broth culture and growth allowed to proceed for 4 days. In consequence of the number of contaminations which occurred after opening the Roux bottles a method of inoculating the agar without removing the plug of the bottle was devised by Mr F. Leeson, laboratory superintendent. A test tube bent at a right angle with a small hole near the lower end was thrust through the plug of the Roux bottle and charged with broth. After sterilisation the broth was inoculated in the ordinary way, and 2 days later the bottle was held upright, when the broth ran out of the tube into the bottle and inoculated the agar. The apparatus is described in Dr Young's (1926–27) *Annual Report of the Medical Research Institute, Gold Coast.* The earlier agar vaccines were standardised to contain 3000 million bacilli per c.c., and the later ones to contain 1·5 mg. per c.c. of dried bacterial substance. From a number of comparative tests these strengths were judged to be equivalent. Sterilisation was effected by means of phenol.

Broth vaccines were grown in the trypsin broth described above and sterilised by phenol.

Counting organisms. Bacilli were counted in an ordinary wet film illuminated by the dark field method. The depth of each field was measured by means of the fine adjustment of the microscope and the area by means of an eyepiece graticule ruled in squares. Further details are given in Dr Young's annual report (1926–27).

Experimental animal. African pouched rats (*Cricetomys gambianus*) were used almost exclusively. They are referred to merely as "rats" in the following

descriptions. They are satisfactory laboratory animals as they are large (average weight $2\frac{1}{4}$ lb.) and stand captivity well, and they are specially suitable for plague work on account of their extreme susceptibility, for after infection with recently passaged cultures (excluding those deliberately attenuated) they always die. Infection was performed by hypodermic or intramuscular injection, and the standard dose (used unless otherwise stated) was 0·01 c.c. of a 2-day broth culture, a quantity which usually contained from 0·5 to 2 million bacilli.

II. Protective inoculation.

Comparison of agar and broth vaccines. A stock of agar-grown vaccine has been maintained since 1926, but it has not been used on human beings because the plague epidemic had subsided before it was ready. A number of tests, however, have been made on rats. In 1926 and 1927 161 rats were given two doses, 0·5 and 1·0 c.c. (0·75 and 1·5 mg. of dried bacterial substance), with a 6- or 7-day interval, and the test dose of virulent culture was given from 6 to 21 days after the last vaccination. There were 17 deaths in the immunisation period, 144 rats were infected and 36 survived, a survival rate (excluding immunisation period deaths) of 25 per cent.

Lately a broth vaccine has been tried and of a batch of 16 rats, which were treated with doses of 0·5 and 1·0 c.c. with 7 days' interval and an equal interval before infection, nine survived, a survival rate of 56 per cent. This vaccine had been allowed to grow for 10 weeks, but the period is probably longer than necessary, for a sample tested after only 10 days' growth protected two of eight rats and therefore at this early stage had definite immunising power.

Control animals were used in all vaccine tests and all died; 75 were used in the tests detailed above, their average survival period being about 2·5 days.

The comparison is much in favour of broth vaccine, but it has not been possible to adjust the doses to a standard of comparison. A standard based on toxicity is desirable, but the relative toxicity has not been ascertained, for deaths in the immunisation period occurred at most irregular intervals and seemed to be due rather to frequent handling than to the effect of the vaccine. Moreover, very large doses of vaccine produced no ill effects; for example, four rats each received 4 c.c. (6 mg.) of agar vaccine in one dose and showed no sign of illness. They were infected a week later and three died in from 2·5 to 2·7 days and only one survived, showing that the large dose of vaccine had produced but little immunity.

Spleen pulp vaccine. Hindle's (1929) success in protecting monkeys against experimental yellow fever by using sterilised infected spleen as a vaccine suggested a trial of a similar method for plague. Rowland's (1915) insistence on the importance of using a "body strain" for the preparation of plague vaccine, and the fact that the organs of animals which have died of acute plague contain enormous numbers of bacilli, lent support to the idea.

The so-called *spleen pulp vaccine* was prepared in the following way. Rats

were infected with virulent culture and as soon as possible after death the spleen of each was excised, passed through a small mincer and the pulp weighed. Three times as much 1 per cent. aqueous phenol was then added with constant stirring, the emulsion was shaken for half an hour with glass beads, left at room temperature for 24 hours and then kept in the ice chest. Just before administration it was shaken, left a few seconds for the larger lumps to settle and injected hypodermically, using a rather large needle. The results obtained are as follows:

Six rats received a single dose of 0·125 grm. of spleen pulp and one survived the test dose. Survival rate, 17 per cent.

Fourteen rats received a single dose of 0·25 grm. of spleen pulp and eight survived the test dose. Survival rate, 57 per cent.

Eight rats received two doses of 0·125 grm. and 0·25 grm. with a 4-day interval and six survived. Survival rate, 75 per cent.

The test doses were given 6 or 7 days after the final vaccination. There were no deaths during the immunisation period.

In addition to the above, four rats were treated with spleen pulp and four with liver pulp vaccine in five doses of 0·075 grm. with intervals of 2 days. One died during the immunisation period and is excluded, and of the seven remaining five survived, a survival rate of 71 per cent.

Thus spleen pulp vaccine gave rather better results than broth vaccine and much better results than agar vaccine, the respective survival rates with two doses of vaccine being 75, 57 and 25 per cent. The numbers of bacilli in the spleen pulp and agar vaccine were judged to be about equal, and a count of bacilli in a spleen section, 5μ thick, indicated that the bacillary content of the vaccine was 4500 million per c.c. against 3000 million of the agar vaccine. The superiority of the former therefore can scarcely be attributed to a larger dose of bacilli, especially as great increase in the dose of agar vaccine has not been found to produce much increase in immunity. Its superiority might perhaps be attributed to the presence of antibody, but as all the spleens (ten in number) were from animals which had died very acutely (average survival period about 2·25 days) it is unlikely that there was much antibody present. The experiments therefore support the view that plague bacilli, taken straight from the body after acute death, have greater immunising power than those grown on artificial medium; in other words they emphasise the value of a body strain. But considering the disadvantages the results were disappointing and the method does not appear likely to acquire practical value, for the immunity obtained, though great, was not complete and the vaccine produced undesirable local effects; at best a nodule consisting of encapsuled spleen pulp persisted until the autopsy and in other cases an ulcer or a cold abscess developed.

III. Factors affecting virulence.

Attenuation by passage through immunised animals. In the previous paper two experiments were described, in which the virulence of *B. pestis* was reduced by passage through a partially immunised animal. These experiments have been repeated and confirmed on several occasions, advantage being taken of opportunities arising in the routine testing of vaccines. The results are given in Table I. In order to make this clear an outline of the method may be given. A number of rats are vaccinated or otherwise immunised and after a suitable interval they receive a test dose of virulent culture. A number of untreated

Table I.

a	b		c	d	e	f	g	h
				Virulence test before passage	Virulence test after passage			
	Passage animal						No.	s.p. of
How treated	Culture from		s.p. days	All died Av. s.p.	Av. s.p. days	No. tested	sur- vived	those dying	Culture no.
Agar vaccine (2 doses)	Liver	{	7·75 to 16·0	2·35	8·7	48	11	6·3	} Various*
	Liver		2·5 to 5·6	2·35	2·5	31	0	—	
	Site	{	8·75	2·5	2·5	8	0	—	G. 138*
Spleen pulp vaccine (2 doses)	Liver	⎧	5·7	2·5	3·0	8	0	—	P.C. 133
	Site	⎨	15·0	2·5	2·5	8	0	—	P.C. 128
	Liver	⎬	10·0	2·5	7·5	6	1	5·6	P.A. 166
	Site		,,	2·5	2·8	6	0	—	,,
	Site	⎩	Survived reinfected	2·5	17·0	4	4	—	P.A. 164
Agar vaccine (1 dose)	Liver	{	7·7	3·1	3·2	6	0	—	P.E. 232
	Lumbar gland		,,	3·1	2·6	6	0	—	,,
Avirulent living culture	Liver	⎧	10·6	2·1	13·8	6	4	7·5	P.E. 266
	Site, fringed col.	⎨	,,	2·1	15·4	6	5	7·7	,,
	Site, irreg. col.	⎩	,,	2·1	17·0	6	6	—	,,
Spleen pulp vaccine (5 doses)	Site	⎧	12·5	2·5	7·7	6	1	5·8	P.A. 326
	Lumbar gland	⎪	,,	2·5	8·5 .	6	2	4·4	,,
	Spleen	⎨	,,	2·5	7·5	6	1	5·5	,,
	Liver	⎪	,,	2·5	7·0	6	0	—	,,
	Heart blood	⎩	,,	2·5	12·0	6	3	7·2	,,

* West African cultures.

rats (controls) are infected at the same time and in the same way and their average survival period (s.p.) gives the *virulence before passage*, shown in column *d*. Of the vaccinated rats some survive and some die and plague cultures may be obtained from the latter. A vaccinated or otherwise immunised rat from which a plague culture is recovered is the *passage animal* of the table. The virulence of the culture thus obtained is then ascertained by injecting it into a number of untreated rats and this experiment is the *virulence test after passage*. In the table columns *d* and *e* give the essential details, viz. the average survival period of the test animals (an inverse measure of the virulence of the culture) before and after passage. Before passage every culture killed all the

animals infected; the calculation of the average survival period was therefore a simple matter. From four to eight rats were used in each test. Passage reduced the virulence of cultures in certain cases and there were survivors among the animals of the *after passage* tests. As described in the previous paper a survival period of 17 days is allotted to these survivors for the purpose of calculation. The figures in column *e* are therefore somewhat arbitrary and consequently further details of the *after passage* tests have been given in columns *f, g* and *h*. Column *i* gives the designation of the culture after passage, and therefore the number of passage animal. Since in the earlier experiments all the cultures with one exception were obtained from the liver at autopsy and all the passage animals were treated with agar-grown vaccine, it is unnecessary to give details of each experiment separately and the results obtained with liver cultures have been collected and are shown in the first two lines of the table.

The exception mentioned was that of a culture obtained from the abscess which had formed at the site of infection (culture G. 138). The result in this case was unexpected and gave rise to the idea that the region of the body from which a culture is obtained may have something to do with its virulence. In the later experiments, therefore, cultures were obtained from different regions of the body, and in addition variations were made in the method of immunising the passage animal. It is consequently necessary to give individual details of the later experiments.

It was found that reduction in virulence only occurred when the passage animal lived a relatively long time, as shown in column *c*. It will be seen that the West African cultures recovered from the livers of vaccinated animals, which died of plague from 7·75 to 16 days after infection, had their killing times increased from about 2·5 days to 8·4 days, there being no survivors before passage and 20 per cent. survivors after passage, whereas in cases where the passage animal survived 7·7 days or less there was no alteration in virulence.

In the exceptional case mentioned (culture G. 138) pus was found at autopsy at the site of infection. This showed no organisms other than plague bacilli in films and yielded a pure culture. Although the animal had survived as long as 8·75 days, the culture was still fully virulent. This suggested that organisms remaining or multiplying at the place of introduction remain unchanged, or at any rate are not transformed as rapidly as those wandering into the general circulation. Support for this idea was obtained in the case of cultures P.C. 128 and P.A. 166. The latter is of special interest, for site and liver cultures were both tested and the liver culture showed well-marked loss of virulence, while the site culture was unchanged. At death, therefore, the animal harboured two bacterial strains differing in virulence.

It will be seen that experiments with cultures P.A. 164, P.E. 266 and P.A. 326 appear to contradict these results, as the site cultures showed considerable diminution in virulence. The conditions of the experiment, therefore,

must be further considered. Of course, in making cultures from an abscess when there is septicaemia, one may get organisms from the neighbouring tissues and so get a mixture of systemic and localised strains, but this does not matter in the present case as the character of the more virulent strain will determine the result of the virulence test. The chief difference noted was that in rats 164, 266 and 326 the abscess was small and indefinite and that in rats 138, 128 and 166 large and well defined. Assuming that the change in virulence is due to the action of antibodies which appear in the general circulation as a result in the first place of vaccination and subsequently and chiefly as a result of the test infection, it is conceivable that these antibodies will have more ready access to organisms lying in a small quantity of pus not encapsuled than to those embedded in a large quantity of pus inside a firm abscess wall, and consequently strains obtained from a large well-defined abscess are more likely to have retained their virulence than others.

Rat 164 was treated rather differently from the rest, for not only had it been vaccinated, but it had also received the test infection, from which it had recovered, before the culture under experiment was injected. The test culture was injected into the left thigh, 17 days later a large dose of the same strain was injected into the right thigh and 7 days after this the animal, which had shown no symptoms, was killed. At autopsy the only abnormality found was a little streak of pus at the site of the last injection, and from it a culture was made. In this case passage was through an animal which had survived virulent infection and was therefore completely immune, and the plague bacilli were introduced into tissues already impregnated with strong antibodies and had therefore no opportunity of sheltering inside an abscess wall. It is not surprising, therefore, that the strain underwent an unusually profound modification, its virulence being reduced from maximum to zero by a sojourn of 7 days in the tissues of the animal.

Rat 266 was immunised, not by vaccine but by the injection of living culture of reduced virulence (0·1 c.c. of a 2-day broth culture). It was one of a batch of four, two of which survived and two died of plague. It will be noted that the change in virulence was very marked. The site culture was plated out and different colonies selected. Further details will be given in the section on *bacterial variation*.

In the case of rat 326 cultures were made from different organs. All showed considerable diminution in virulence, but the differences between them were not large enough to be significant.

Restoration of virulence. In the previous paper, an experiment is described which suggests that the virulence of a strain attenuated by passage through an immune animal is less easily restored than that of a strain attenuated by long cultivation. With the latter it has been found that a single passage through a normal rat will restore full virulence, but in the experiment referred to three successive passages were made and the virulence was not increased. This experiment has been repeated with culture P.A. 326 (lumbar gland),

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using batches of four rats for each passage and choosing the shortest lived one for further passage. Table II gives the average survival periods (allotting 17 days to survivors) and the proportion of survivors after each passage.

Table II.

	Average s.p. days	Proportion of survivors
Culture P.A. 326	8·6	2/6
After 1st passage	5·0	0/4
,, 2nd ,,	5·3	0/4
,, 3rd ,,	9·5	1/4
,, 4th ,,	4·2	0/4
,, 5th ,,	2·5	0/4
,, 6th ,,	2·5	0/4
,, 7th ,,	3·0	0/4

Thus virulence was fully restored, but not until after the fifth passage. The process was complete but slow, and it does seem that attenuation by passage is a more stable change than attenuation by cultivation.

Attenuation in culture. Recent observations on the retention of virulence in culture are not in accord with the earlier ones recorded in the previous paper, for cultures at room temperature now tend to lose virulence in a few months. This applies both to West African and South African strains. Some experiences with the latter are worth mentioning. Four of the five cultures were virulent when received, a month later one showed marked attenuation, killing only two of seven rats and two others had become quite avirulent in 4 months, while the remaining one was unaltered. All four were from human cases and had never been put through animals. The three which lost virulence rapidly had been isolated 2½ years before reaching the Gold Coast, and the one, which retained virulence, only 7 months before. Virulence could be easily maintained by frequent passage through rats, but quickly declined. It is remarkable that three strains should have retained virulence for 2½ years in South Africa and then lost it in a few months in the Gold Coast. The higher temperature may have had something to do with it and in this connection an experiment may be mentioned, in which two West African strains were each kept in parallel culture at room temperature (about 27° C.) and 37° C. respectively, sub-cultures into broth being made every two or three days for 2 months. At the end of this period both room temperature cultures were fully virulent, while the 37° cultures had become much attenuated, one killing two of six rats and the other only one of six. This indicates that the higher temperature acts adversely upon virulence, an effect not due to more rapid growth, for growth was slower at the higher temperature. The experiment also suggests that strains which have been isolated a long time are of less stable virulence than those recently isolated from human epidemic cases. The condition of stable virulence may have an important relation to the occurrence of epidemics, either as cause or effect, and it may also be an important factor in the choice of a strain for vaccine preparation, for an old strain, though virulent at first, may become attenuated in the period of cultivation required by a broth vaccine.

IV. Bacteriological variation.

When it was found that alteration in virulence occurred with some regularity, a study of cultures was made in the hope of finding that changes in virulence were associated with changes in the character of cultures. In the previous paper it was suggested that the relatively avirulent strain obtained by passage through a partially immune animal was a rough variant, and a good deal of work has been done with cultures to test this hypothesis and to determine the character of other avirulent strains. It must be admitted that work on these lines has been very disappointing; the results have been indefinite and the hypothesis, in so far as it implies a sudden transformation of a bacterial strain into one with modified but more or less stable characters, has not been established.

As is well known, *B. pestis* shows much diversity both in the form of individual bacilli and of colonies. It is true that some colonies were found to have a certain amount of stability in that they retained their distinctive appearances in a general way on subculture or even after animal passage, so that by selecting instances a case for mutation might be made out, but taken altogether the experiments showed that different types of colony might have the same virulence, and strains differing widely in virulence might give rise to the same type of colony. Further, the variations observed appear to be the result rather of a gradual change than of a sudden mutation, for between extreme types of colony it was the rule to find all intermediate grades, although very rarely plates did show sharply divided types. Nor, if virulence be taken as the criterion, was there any better evidence in favour of a sudden change, for, as is the common experience, cultures of all degrees of virulence were encountered (see Table I, col. *e*), and when tests were repeated at intervals of a few months, the change was found to be gradual. Gradual diminution in virulence in a culture can scarcely be attributed to an increasing proportion of avirulent forms, for the survival period of an animal infected with plague depends only in minor degree on the size of the dose, and so long as any of the virulent form remains the virulence will remain high.

The confused results obtained from a study of colonies is probably due in part to the number of factors which determine their form. The intrinsic character of the strain is undoubtedly a factor, but accidental conditions, such as the degree of crowding of colonies, the composition and moisture content of the medium and the proximity of contaminating colonies, also have some influence.

In describing plague colonies the only practicable method is to select extreme types between which intermediate forms occur. To cover all the forms met with in the course of this work it is necessary to mention three types (illustrated in Plates IV and V) which are as follows:

(a) *The small fringed type.* This is the typical form (Pl. IV, fig. 2, *A* and *B*).

(*b*) *The simple round type*, distinguished from (*a*) chiefly by the absence of fringe (Pl. IV, fig. 6, *A*).

(*c*) *The large irregular type* (Fig. 1, *A* and Fig. 9).

The classification applies to colonies 3 or 4 days old on agar. The types are not new and it is unnecessary to give detailed descriptions. The *small fringed* type is well known. It has a hemispherical centre with a mat surface and a wide and very delicate fringe. In young colonies (*e.g.* 24 hours old) the centre is often irregular and wrinkled. The type is described by Dieudonné and Otto (1928). The *simple round* type is slightly larger and more opaque than the small fringed type and has little or no fringe, but the difference is not very striking. It is mentioned because it was the most marked example of variation that could be found in the West African strains, and because importance has been attributed by certain authors to the width of fringe. The *large irregular* type is characterised by dirty yellow colour, irregular or knobby surface and a steep edge running down to a narrow thick irregular fringe. The colony shown in Pl. IV, fig. 3, by Dieudonné and Otto (1928) is probably of this type, but it is only 24 hours old and its characters are not very pronounced. It is rather difficult to apply the classification to types described by other authors. Markl (1914) describes three types, but these do not correspond to those mentioned above, but appear to be mature and immature forms of the *small fringed* type. Bezsonova, Semekoz and Kotelnikov (1927) illustrate a number of forms, some of which bear a dim resemblance to the types mentioned above, but their *sunflower* form, which has a thick fringe with regular radial markings, has not been encountered in the course of this work.

As regards the forms of bacilli found in the different colonies, all that can be said is that in old cultures the average length of the bacilli is greater than in freshly isolated ones. In most cultures, beside the ordinary bacilli, there are a number of longish filaments. As a culture ages both elements tend to become longer. The bacilli average perhaps 2μ in length in recent cultures and 3μ or 4μ in old ones, while the filaments, which measure perhaps 6μ to 10μ in recent cultures, may attain a length of anything up to 100μ in old ones (Pl. IV, figs. 3, 4 and 5). When, however, an old avirulent culture tends to produce *small fringed* colonies, these may furnish long bacilli. Bacillary length and colonial irregularity are characters dependent on the age of the culture, but they are not interdependent and one may exist without the other.

The relationship of the different types of colony to states of virulence is very vague. All that can be said is that the *small fringed* type is more likely to predominate in virulent than in avirulent strains, whereas the *large irregular* form is more readily found in avirulent strains. However, nearly every plate shows diverse colonies, and virulent colonies which have grown more rapidly than the rest as a result, for example, of particles in the medium or of the proximity of contaminating colonies (Drennan and Teague, 1917) tend to the *large irregular* form, while plates of avirulent strains nearly always show some

small fringed colonies. Indeed, very old avirulent strains which, as often happens, have lost their capacity for rapid growth may produce only small round colonies with or without fringe, and no irregular ones. In fact rapidity of growth, whether due to vigour of the strain or suitability of the medium, appears to be a very important factor in determining the form of colony. As regards the *simple round* type our experiments indicate that size of fringe is no indication of the degree of virulence and, moreover, that this character depends on the composition of the medium, the addition of sulphite, for example, promoting the growth of fringes. Also different strains appear to differ in their tendency to produce *large irregular* colonies. The South African strains used had a much greater tendency to produce this type than had the West African ones, but the difference had no significance as regards virulence.

Attempts were made with four West African cultures to isolate the *small fringed* and the *simple round* types as distinct strains. Colonies of each type were picked out and plated and beginning with this pair of plates successive plates were inoculated, picking out colonies of extreme type for each inoculation. It was found that the first pair of plates showed distinct differences, the parent type of colony predominating in each, but the difference did not increase in the succeeding plates and even tended to become less. One of the cultures was studied after passage through rats, pairs of colonies from the first and second pairs of plates being selected for this purpose. Four batches, each of six rats, were infected. The colonies definitely retained their character after passage. Pl. IV, fig. 6, is a photograph of a plate made directly from one of the animals infected with a *simple round* colony.

The example (P.E. 266) shown in Table I may now be mentioned. Plates were inoculated directly from the liver and from the abscess at the site of infection of an immunised rat. On the liver plate the colonies were fairly uniform and were of the fringed type, but on the site plate they were of two distinct types, viz. *small fringed* and *large irregular*. Each kind of colony (viz. *liver fringed*, *site fringed* and *site irregular*) was then plated out again. All the colonies of this second generation retained the parent character except that on the *site fringed* plate there were, in addition to numerous *small fringed* colonies (the parent type), a very few *large irregular* ones, which, though not quite of extreme type, presented such a striking contrast that they were suspected of being contaminations. Films from each, however, showed typical plague bacilli, the only difference being that those from the small fringed colony were more varied in size. Photographs of the colonies are reproduced in Pl. V, figs. 7, 8 and 9. The broth cultures used for the virulence tests were obtained from these colonies. It will be seen from Table I that the liver culture killed two of six rats, the fringed site culture one of six and the large irregular site culture none. If a conclusion can be drawn from such small numbers it would be that the fringed colonies are the more virulent, but it must be noted that, though all types of colony were represented, the virulence which was of a low order did not vary much in the three cases.

The conclusion is that the wide variations observed in the form of plague colonies are due probably to a number of factors of which virulence is only one, and that, although with loss of virulence the average colony does become larger, more opaque and more irregular, the change is of little significance in individual cases as it is masked by other factors.

Salt stability. In the case of the Salmonella group alteration of salt stability is regarded as an important indication of mutation and with this in view a number of salt stability tests were made with different strains of *B. pestis*, but the results have been no more definite than those obtained from a study of colonies. About sixty tests were made with twenty-nine different cultures. Ten salt concentrations were used, viz. 3·36, 2·52, 1·68, 1·26, 0·84, 0·63, 0·42, 0·31, 0·21 and 0·16 per cent. The emulsions were prepared by rubbing off agar cultures into 0·1 per cent. formalin, shaking for 20 minutes with glass beads, allowing to settle for 1 or 2 hours and pipetting off. The concentrated emulsion thus obtained was diluted to a certain opacity, usually McFarland tube no. 6, which contains barium sulphate equivalent to 0·06 per cent. of barium chloride. A considerable dilution, *e.g.* twenty to fifty times, was necessary. The emulsion was stored in the ice chest but was usually used within a few days of preparation. In the majority of cases it was sufficiently stable, but in a few, in which very old cultures were used, a good deal of the solid matter settled and the supernatant fluid was used. Equal quantities of emulsion and double strength salt solution were quickly mixed, placed in a water bath at 37° C. for 4 hours and left at room temperature overnight before the final reading was taken. The highest dilution, in which complete sedimentation occurred leaving the supernatant fluid quite clear, was taken as the reading.

The tests indicated that a lowered virulence was associated in a general way with lowered salt stability, but the average change in the latter was not very great and individual fluctuations were very marked, for not only did strains of the same virulence give diverse results, but different growths of the same strain showed some variation. As an instance of the irregularities met with it may be mentioned that three cultures, one virulent and the others partly virulent, did not agglutinate at all; the emulsions, though weaker than the standard, were fresh and their *p*H value was between 7 and 8 and no explanation of the failure was found, but on retesting two of the cultures with stronger emulsions agglutination occurred. Excluding these failures it was found that the majority of virulent strains agglutinated in 1·26 per cent. salt and the majority of partly avirulent or completely avirulent ones in 0·84 per cent. salt. Table III gives the results in more detail.

Table III.

	Number of cultures agglutinated					
Salt percentages	2·52	1·68	1·26	0·84	0·63	0·42
Virulent cultures	1	1	10	8	.	.
Partly avirulent	.	.	5	10	1	1
Avirulent ...	.	2	2	8	2	2

There is a general trend towards loss of salt stability with diminishing virulence, but the variations in each class of culture are so great as to obscure the difference between individuals of each class. The partially avirulent class is made up chiefly of cultures, the virulence of which was reduced by passage through immunised animals, and it cannot be said that salt stability tests furnished much evidence for these strains being rough variants.

V. SUMMARY AND CONCLUSIONS.

1. African pouched rats (*Cricetomys gambianus*) are very suitable animals for plague experiments on account of their tolerance of captivity and their extreme susceptibility. After inoculation with small doses of plague culture of ordinary virulence they always die.

2. Broth-grown vaccine was much more efficient as a prophylactic than agar-grown vaccine. When two doses were given a survival rate of 56 per cent. was obtained with the former and 25 per cent. with the latter.

3. A vaccine composed of carbolised spleen pulp from animals, which had died of acute plague, was rather more efficient than broth vaccine, giving a survival rate of 75 per cent. But it is not likely to be of practical value, as preparation is difficult, there are undesirable local effects and its superiority over broth vaccine is not very great. Its efficiency is attributed to the fact that the organisms which it contains are a true "body strain." The method of preparation is similar to that of Hindle's yellow fever prophylactic.

4. A number of experiments are described in which virulence was abolished or reduced by passage through immune or partially immune rats. This occurred only when the passage animal showed a considerable degree of immunity, as indicated by the length of the survival period after the test infection. Cultures made from the abscess at the site of infection were less often attenuated than those from the liver of an immunised animal, but this was observed only in the case of large well-defined abscesses. It is suggested that a firm abscess wall protects organisms inside it against the action of antibodies appearing in the general circulation.

A culture attenuated by passage through an immunised rat was restored to normal virulence by successive passages through normal rats, but the process was slower than in the case of cultures attenuated by long cultivation, five passages being required in the former case and only one in the latter.

5. Cultures used in the later work, unlike those used in the earlier work, lost virulence rapidly when kept at room temperature. Experiments and observations are described which suggest that old strains, even when subjected to rat passage, are less stable as regards virulence than those recently isolated from human epidemic cases, and that the high temperature of the Gold Coast has an adverse effect upon virulence.

6. Attempts to isolate stable variants of *B. pestis* were not successful. Colonies vary much in form. Three types of colony are mentioned, but all intermediate grades were observed. Certain old avirulent strains had a greater

tendency to produce large irregular colonies than recent strains, but the relation was so loose that the form of colony gave little indication of the degree of virulence. In old avirulent strains the bacilli tend to become longer and filaments of great length may be present. Salt stability tends to become less as a strain grows old and avirulent, but the change is not very marked and is apt to be masked by individual fluctuations.

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DESCRIPTION OF PLATES IV AND V.

All the colonies of *Bacillus pestis* illustrated herein were grown for 4 days at room temperature (about 27° C.). Sodium sulphite was added to the medium in all cases except that of Fig. 6.

The photographs of colonies were taken by transmitted light. This was arranged with slight obliquity and its aperture cut down to half or two-thirds of that of the objective (Zeiss *mikrotar*).

Magnifications: Figs. 1 and 2, × 10; Figs. 3–5, × 1000; Figs. 6–9, × 20.

PLATE IV.

Figs. 1 and 2 (× 10) show avirulent and virulent plague bacillus colonies. The two strains were plated at the same time on plates poured from the same batch of medium and the colonies were photographed on the same photographic plate.

Fig. 1. Strain P.C. 0 (avirulent). This had been about 3 years in culture and had never been put through an animal. It was quite avirulent to rats. *A* is a typical *large irregular* colony. *B* corresponds to the *simple round* type in size, narrowness of fringe and evenness of outline, but differs in that the centre shows concentric ridges.

Fig. 2. Strain P.E. 531 (virulent). Its virulence had been maintained by frequent rat passages. *A* and *B* are typical *small fringed* colonies.
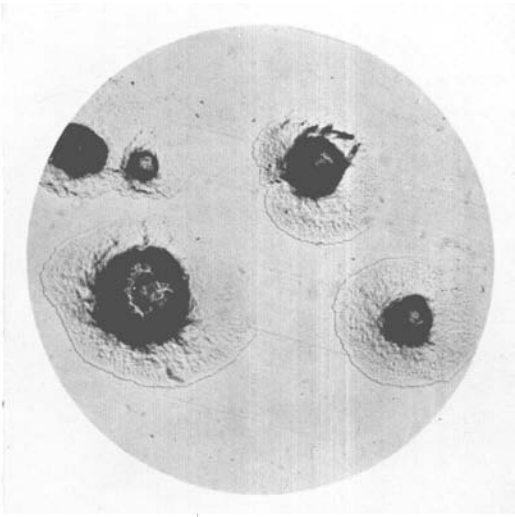
Figs. 3, 4 and 5 (× 1000) are photographs of bacilli taken from the colonies shown in Figs. 1 and 2, and stained with carbol fuchsin.

Fig. 3 is from a small colony of Fig. 1 and the field was chosen to show the long filaments.
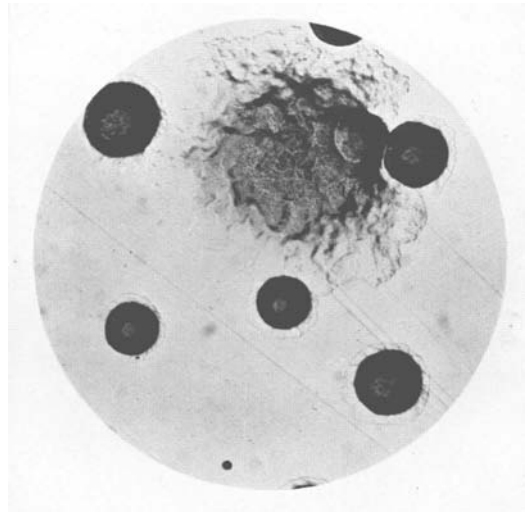
Fig. 4 is an average field from a small fringed colony.

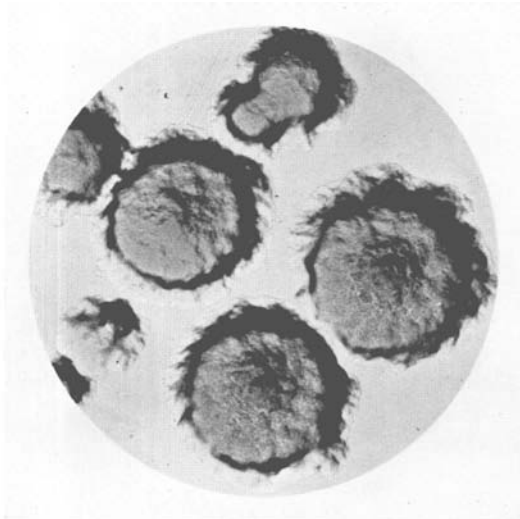*Fig. 5 is an average field from a large irregular colony.*
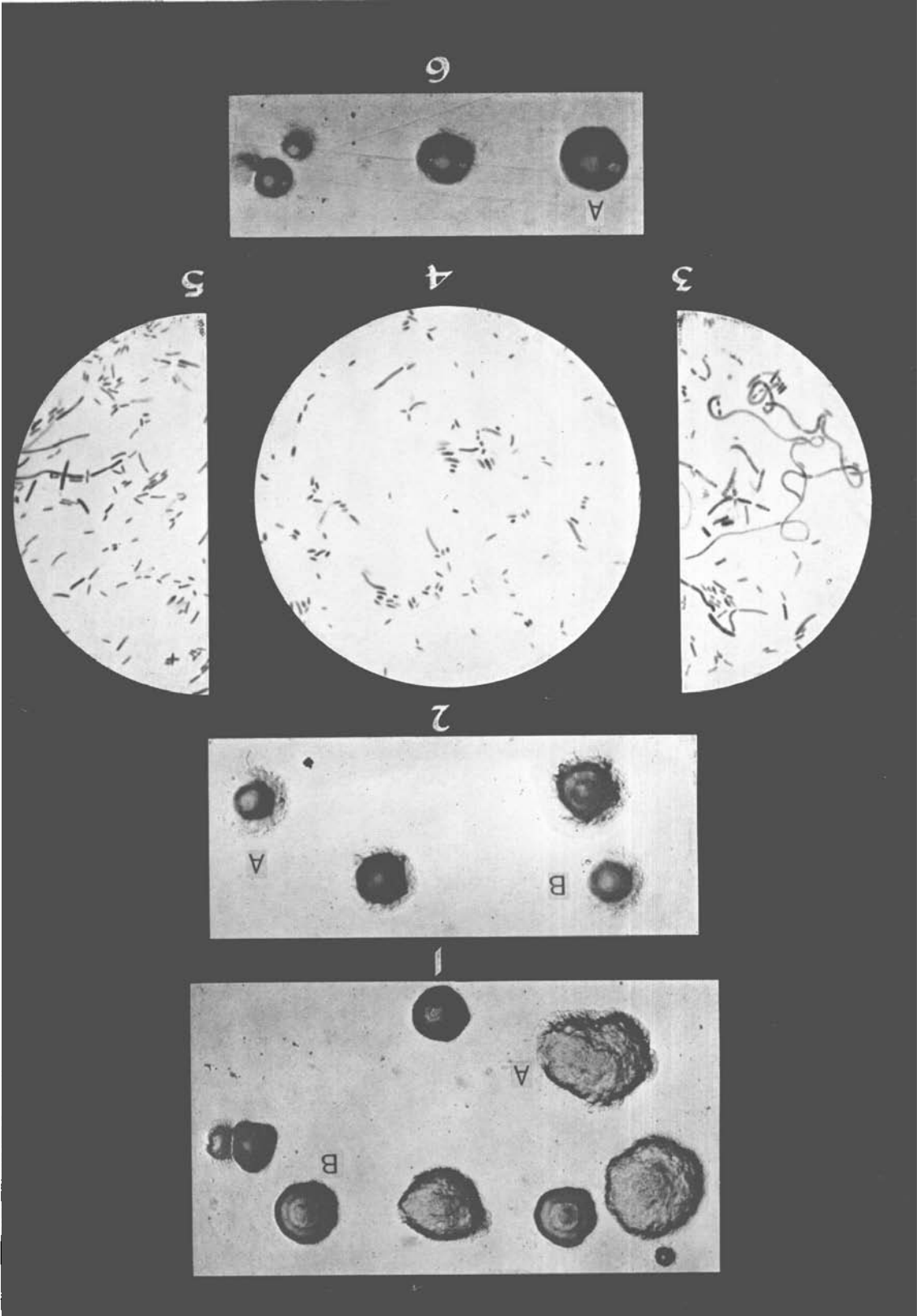
7

8

9

Fig. 6 (× 20) shows a *simple round* colony at *A* (culture G. 392), the medium contained no sulphite and therefore the colonies are small. The plate was made in an attempt to isolate a strain producing only *simple round* colonies.

PLATE V.

Figs. 7, 8 and 9 (× 20). Culture P.F. 266, see Table I.

Fig. 7. First subculture from liver; the parent colony resembled those shown. The colonies differ from the *small fringed* type only in their somewhat larger size and slightly irregular centres. The culture was slightly virulent, killing two of six rats.

Fig. 8. First subculture from site of infection; the parent colony was similar to the small colonies shown. The small colonies differ from the *small fringed* type only in the narrowness of fringe, and the large colony from the *large irregular* type only in the width of fringe. The small colonies were very numerous and the large ones exceedingly scanty. The culture was almost avirulent, killing only one of six rats.

Fig. 9. First subculture from site of infection; parent colony similar to the large ones shown. The photograph shows three typical *large irregular* colonies. The culture was quite avirulent, not killing any of six rats.

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