## PART TWO.

## SOME CORRELATIONS BETWEEN COLONY VARIATION AND PATHOGENICITY IN STRAINS OF BACILLUS ANTHRACIS.

### I.

### INTRODUCTION.

Bacterial variation, as generally understood, means changes in colony form, physiology and morphology within a species. The changes are sometimes slight, but may be marked enough to give the impression of a new strain.

In the early days of microbiology Naegeli (1877) believed that bacteria formed an extremely labile group with cocci, bacilli and spirilla continually turning into one another. The confusion and exaggerations of this system led to the acceptance of the rigid monomorphic conceptions of Cohn and of Koch who asserted that species were clearly defined and who consigned all deviations from their ideal to the limbo of "involution forms".

Nevertheless, the ideas of Koch and the German school ran counter to many established facts; for Pasteur had already attenuated the anthrax bacillus and shown that modification in virulence was associated with altered colony form. Pleomorphism in microbes such as the diphtheria bacillus was also well known. Gradually evidence accumulated against the strict definitions of the systematists and Massini (1907) showed that a non-lactose-fermenting colony of a coliform organism developed papilli of lactose fermenting bacilli in the presence of that sugar. Baerthlein (1918) experimented with many different bacteria and showed that each produced diverse colony forms. This work was the precursor of the present extensive literature on bacterial variation. Arkwright (1920 and 1921) proved that dysentery, typhoid and paratyphoid bacteria formed

two types of colony, a smooth and a rough, which differed in their agglutination reactions with salt and with specific sera. de Kruif (1921) found a similar smooth to rough (S - R) transformation in a rabbit septicaemia organism and showed that the change was accompanied by a loss of virulence. This was confirmed by Webster (1925). The importance of these observations resulted in intensive investigations and within a few years the general occurrence of this phenomenon was established. Cowan (1923 & 1924) showed that streptococci lost virulence in changing from smooth to rough. Todd (1928) held that the reverse was the case, but Dawson and Olmstead (1934) confirmed Cowan's work. Reimann (1925) showed that pneumococci lost both virulence and type specificity with the S - R change.

We now realize that abrupt changes in bacterial forms and functions occur frequently and involve colony type, morphology, fermentation and serological reactions. As a rule the change is uni-directional and not easily reversed. That is smooth becomes rough; virulent becomes less virulent, capsuled becomes uncapsuled and so on. These changes are now termed dissociations and the changed strains dissociants. The terms mutations and mutants are also used although the term should not be identified with the de Vriesian conception until further evidence becomes available.

It is unnecessary to quote further from the extensive literature now existing on the smooth rough variation, but this transition occurs in almost every bacterium and in the case of pathogenic species is almost always associated with diminished virulence. There is still some doubt in the case of streptococci and up to the present B. anthracis has been considered an exception because here the attenuated strains are smoother than the virulent strains.

The freshly isolated virulent anthrax bacillus produces on nutrient agar colonies with dull flat surfaces

rather like frosted glass. The edges have a very tangled appearance and the extreme roughness of the growth has resulted in the name "Medusa head" being applied to this type of colony.

Many workers have noticed that smooth colony variants could be obtained from rough virulent strains by various methods and that these smooth forms were particularly frequent in attenuated strains. Nungester (1929) gave a comprehensive list of references to the work done up to that time. In 1920 Wagner isolated a smooth strain of anthrax which he claimed was more virulent than the rough form of the same strain. However, as shown by Nungester, Wagner's observations were carried out on so few animals that the sampling error necessarily invalidated his deductions. Nungester himself in 1929 showed that increasing smoothness of the colony structure was paralleled by an increasing loss of virulence in the strain studied.

This change in virulence and colony structure of B. anthracis is not, however, strictly comparable with the smooth to rough variation which occurs in other pathogens. In the typhoid group, for example, the smooth to rough transition is accompanied by a more or less abrupt change in virulence; so that fully virulent colonies, colonies of intermediate virulence and completely avirulent colonies can be found on one culture plate, and these are characterised by their degree of smoothness or roughness. Generally, the smoothest will be the most virulent, and the fully rough forms will be avirulent.

In the case of anthrax, however, the fully virulent culture is very rough. After a period of growth at 42°C a decrease in virulence will probably be shown, and at the same time the appearance of the culture will be smoother. As attenuation proceeds, the original very rough appearance becomes considerably modified and

eventually many smooth colonies may be found amongst the rougher colonies. Even t e roughs tend towards the smoother type. This has been generally conceded; but most authors deny that there is any significant difference in the virulence between the rough and the smooth colonies on the same plate, that is, in colonies of the same "generation", Tchaikowsky (1935). The appearance of smooth colonies is thus, in a sense, an "index" of attenuation. It has not been possible to find a fully virulent rough on plates where smooth colonies appear during attenuation. All types of colonies at a particular level of attenuation appear to be of about the same order of virulence. This is a critical difference between the type of dissociation found in organisms like S. typhi and the type of variation described in anthrax.

Gratia (1924) obtained both rough and smooth anthrax colonies which at first were readily reversible. He was able to stabilise these variants after a long period of selection and subculturing and at length found that the rough variant seemed more virulent for rabbits than the smooth. Relatively few rabbits were used and the infecting dose was not very large. Moreover, the length of time since the first isolation of the two variants and the test of their virulence, introduced a factor, whose significance could not be assessed with any certainty. Tchaikowsky (1935) was unable to demonstrate differences in virulence in variants picked from a culture and tested soon after isolation.

As a consequence of the somewhat equivocal findings by workers on anthrax dissociation, the results have not found much practical application, and at the present time the position of anthrax prophylaxis is very much what it was twenty years ago. The benefits derived from the work on bacterial variation have up to the present not affected the problems of immunisation against anthrax

to any great extent.

In a series of papers in 1915 Bail reported the isolation of a strain of B.anthracis which did not produce capsules in liquid serum. His results may be summarised as follows:-

(1) The parent strain was completely capsuled in liquid serum and was virulent for rabbits and guinea pigs.

(2) A variant was obtained from (1) by heating. This variant produced rare capsules in serum, and was virulent only for mice.

(3) Another variant, obtained in the same way, never produced capsules in serum and was completely avirulent.

Some variants, however, produced no capsules in serum, but were still virulent for guinea pigs and produced typical capsules in vivo. This last result is not really surprising as it is now well known that many fully virulent strains produce few or no capsules in serum. Bail was not able to make observations on colony structure. He concluded that although the ability to produce capsules in serum did not necessarily imply the possession of virulence, yet no strain which had lost the power to produce capsules could be virulent.

It is generally accepted that the rough form of B. anthracis is the virulent phase. Opinions vary as to whether a difference in virulence is present in cultures of mixed R and S types but it is generally conceded that S forms do not arise in fully virulent cultures. Bail's observations on the capsule are of great interest; but his work was not concerned with colonial morphology and this aspect of the problem was not stressed.

In part I of the present paper it was shown that carbon dioxide allowed virulent strains to produce capsules

on ordinary nutrient agar. The work now presented will deal largely with these findings and their bearing on the problems of colony variation, pathogenicity and immunity in anthrax.

## II.

## EXPERIMENTAL.

### A. Dissociation in certain strains after prolonged cultivation in different laboratory media.

#### (1) Dissociation in a virulent strain of anthrax - V. Boshoff.

50 c.c. of nutrient agar pH 7.4 were poured into a 200 c.c. Erlenmeyer flask. A layer of broth ½ cm. deep was run onto the surface, and the medium was then inoculated with a loopful of virulent stock culture known as Virulent Boshoff. This strain was used in the experiments recorded in Part I. The Erlenmeyer flask was incubated at 37°C and from time to time broth was added to the culture in order to keep the level more or less constant. After a month the growth had become thick and viscid. Plates were streaked from this culture at regular intervals, and after about six weeks a few round smooth colonies were seen. The smoothest were picked and streaked on agar plates until, after a few subcultures, the surface of the colonies developed a pronounced slimy appearance and the typical smooth mucoid colony, as described by Nungester, could be recognised. After 24 hours incubation, or even sooner, the smooth mucoid colonies showed rough outgrowths at different points on their periphery. These rough portions were sharply demarcated from the smooth mucoid growth by their flat dull appearance, rough edge, and by their cuneiform structure shown when viewed by transmitted light. Sometimes these rough portions grew as wedges or sectors in the smooth colony and appeared to have originated from a single bacterial

cell or group of cells; at other times the rough growth commenced as a small projection from the otherwise unbroken contour of the parent colony. The rough offshoot tended to overgrow the more slowly developing parent mucoid, and this latter could only be maintained by subculturing every one or two days. These subcultures behaved exactly like the original strain: that is, they commenced as smooth mucoid colonies and very soon showed rough sectors and outgrowths.

In order to eliminate the possibility that mixed colony types had been present from the outset, several single cells were isolated from the smooth mucoid part of the growth. The majority of these grew and were typically smooth mucoid for the first twenty-four hours. Thereafter rough outgrowths developed just as in the strains which had not been "single-celled". It was plain therefore that the smooth mucoid strain was not a mixture of strains, but that active dissociation was occurring.

Trend of dissociation.

A large number of subcultures were made in order to gain further information as to the direction of the dissociation. The smooth mucoid part of the colony was subinoculated every second day for about eighteen months. During this time no change was noticed in the behaviour of this variant. The character of the growth was always smooth mucoid, and rough variants always commenced to develop after about eighteen hours. At various times rough outgrowths were picked and cultured separately. These always grew as pure roughs. Some of these roughs were subcultured at frequent intervals for some months but showed no change in colony morphology. Other roughs were allowed to stand after a couple of subcultures. When subcultured and examined after several months, the character of the growth was unchanged. Further experiments confirmed the great stability of the colony morphology of this variant.

It could be concluded that this actively dissociating strain was continuously splitting up into smooth mucoid and into rough variants. The former continued and repeated the dissociation process, whereas the latter rough variant was stable and reproduced only its own type. A similar type of variation was described for a strain of S.aertrycke by Deskowitz and Shapiro in 1935. These authors gave a mathematical treatment of the variation rates.

The bacilli from the smooth mucoid growth were all surrounded by typical anthrax capsules, whereas the organisms from the rough variant possessed no trace of an envelope. In view of the results obtained in Part I, an experiment was performed to test the behaviour of these variants in carbon dioxide (see Part I for technique).

Growth of variants of V. Boshoff in 65 per cent carbon dioxide.

(a) The smooth mucoid variant was streaked on an agar slant and incubated for six days, and the culture examined daily. The growth was a mixture of smooth mucoid and rough elements; the former were capsuled and the latter not.

(b) The rough variant picked from a rough outgrowth in the smooth mucoid colony was rough in the carbon dioxide, and no capsules could be seen in stained smears.

(c) The original stock strain of rough virulent Boshoff was used as a control. This grew more and more mucoid until 90 per cent of the bacilli showed capsules.

Controls in 65 per cent paraffin gas.

(a) The smooth mucoid variant was streaked on an agar slant and developed as a mixture of smooth mucoid growth showing capsuled bacilli, and rough patches containing only uncapsuled organisms.

(b) The rough variant picked from an outgrowth

in a smooth mucoid colony was rough and showed no capsules.

(c) The subculture from the original virulent Boshoff grew rough and showed no capsules.

The behaviour of the rough variant (b) picked from the smooth mucoid strain was unexpected. Up to that time all the rough strains tested had grown capsules and become mucoid when incubated in carbon dioxide. The smooth mucoid strain showed the same clearcut dissociation in carbon dioxide and in paraffin gas as it had always shown in air, and the bacilli from the rough colonies were quite devoid of capsules even in the carbon dioxide. The stock rough virulent strain (Boshoff) which was used as a control (c), showed large numbers of capsuled bacilli after incubation in carbon dioxide.

As this was the first time that a rough strain (b) had remained completely uncapsuled and rough when placed in carbon dioxide, further experiments were undertaken to test the observation more thoroughly. A rough variant which had originated in a smooth mucoid colony was grown in different concentrations of carbon dioxide, to see whether any capsule formation could be induced. No trace of a capsule could be found in cultures grown in 30%, 40%, 60%, and 70% carbon dioxide.

A further experiment was then performed in which the strains were grown on blood agar in 65% carbon dioxide. This procedure had never failed to elicit the formation of capsules in the stock rough strains. The following strains were used:-

(a) The rough variant which had failed to produce capsules in the previous experiments: the result was again the same, and the growth remained rough.

(b) A rough variant picked from a smooth mucoid colony and immediately streaked onto the blood agar: one or

two capsules were seen after the first and second days, but none after that.

(c) A stock rough strain of virulent Boshoff used as a control: the number of capsules incresed daily until about three-quarters of the bacilli were capsuled.

Controls were grown on blood agar in air at the same time. Control for (a) was rough and showed no capsules,

(b) was rough and showed a few capsules in the first 48 hours,

(c) was rough and showed a few capsules.

Subsequent subcultures of strain (b) never showed capsules in carbon dioxide or in air, so that the few seen in the above experiment were probably carried over from the parent mucoid strain.

It was concluded that the rough offshoot from the smooth mucoid strain had lost the ability to produce capsules in carbon dioxide. As all rough strains tested previously had become capsuled in carbon dioxide, the behaviour of this strain was somewhat exceptional, and therefore the ability of this strain to produce capsules in vivo was investigated.

Pathogenicity of rough variant from smooth mucoid Boshoff.

This rough strain which had shown no trace of capsule formation in carbon dioxide was grown on agar slants in air. The growth was washed off after 24 hours, and five guinea pigs were each inoculated with one fifth of the emulsion subcutaneously. Four of these guinea pigs survived this large dose, while one died of an intercurrent infection.

Twenty white mice each received subcutaneously one fifth of a large agar slant of the rough variant. With one exception all died in 2 - 6 days. There was a fair amount of oedema and infiltration at the site of inoculation, and the spleen was somewhat swollen. The mice that died

first showed numerous anthrax bacilli at the inoculation site and in the spleen, those in the latter being mostly phagocyted. There was no evidence of multiplication, as most of the bacilli contained spores and appeared to be a portion of the original inoculum. The mice which died on the sixth day also showed numerous bacilli in the spleen; but a certain amount of multiplication appeared to have occurred, because spore containing bacilli were rarer, and some chain formation had taken place. Many involution forms were seen. None of the bacilli from these mice showed any capsules. The deaths of the mice were due probably to a toxic effect of the relatively enormous doses which had been injected. The time between injection and death was long enough for capsules to form had the bacilli still been able to produce them.

Another batch of mice was inoculated with the same strain. In this experiment doses of the order of 1/50 of an agar slant were used, and all the mice survived.

Thus the inability of the rough variant to form capsules in carbon dioxide was paralleled by the loss of its power to produce capsules in the animal body. This variant also failed to produce anthrax in guinea pigs and mice. This lack of virulence was very likely associated with the loss of ability to form capsules, <u>either in vitro</u> or <u>in vivo.</u>

## <u>Comparison of the pathogenicity of the smooth mucoid and the rough variants of V. Boshoff.</u>

A series of experiments was undertaken to compare the virulence of the smooth mucoid strain and the rough variant isolated from it. Guinea pigs were used in the tests because of their fairly uniform susceptibility, and large doses of culture were given to minimize individual variation.

(a) A single cell was picked from a colony of the smooth mucoid variant. The resulting cultures were mixtures of smooth mucoid and rough elements. The smooth mucoid variant was maintained by frequent subculture. A rough colony was picked from the dissociating smooth mucoid, and after two subcultures, pathogenicity tests were carried out as follows:

TABLE I.

| Number of Guinea pigs. | Each inoculated with | Death: Hours after inoculation. 10 | 20 | 30 | 40 | 50 |
|---|---|---|---|---|---|---|
| 3 | 1/4 slant smooth mucoid variant | | | X<br>X<br>X | | |
| 3 | 1/4 slant rough variant | | | | | /<br>/<br>/ |

X Death from Anthrax.
/ Alive after 14 days.

(Where survivors were not tested for immunity after 2 weeks they were kept a full month before discharge).

The cultures recovered from the dead guinea pigs were all smooth mucoid and showed the same tendency as the original culture to throw off rough variants.

As very large infecting doses were used, the results were probably significant, and in this instance the smooth mucoid variant was virulent while the rough dissociant was avirulent. This result confirmed the findings of the previous experiment.

(b) A culture of B.anthracis was isolated from the heart's blood of one of the guinea pigs which died in the last experiment. This grew smooth mucoid, and rough outgrowths developed after 24 hours. One of the rough wedges was touched with a needle and this was streaked over an agar slant. At the same time another agar slant was

streaked from the smooth mucoid growth. After 24 hours the former was rough, and the latter mostly smooth mucoid. Each slant was then injected into guinea pigs as follows:-

TABLE II.

| No. of guinea pigs. | Each inoculated with | Death: Hours after inoculation. 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|---|---|
| 3 | 1/3 slant smooth mucoid variant. | | | X<br>X<br>X | | | | |
| 3 | 1/3 slant rough variant | | | | | | X | <br>X<br>X |

All three guinea pigs in the first group were dead by the 33rd hour, while the first death in the second group was at the 60th hour. As the doses given were exceedingly large the difference in survival time between the two lots was probably significant. The chances are that a few virulent bacilli were carried over when the rough colony was picked and that these were sufficient to kill the guinea pigs in the "rough" group. Thus the lengthened survival time was probably due to the small dose of virulent elements administered. In the experiment described on page 20 , capsuled bacilli could be found in the first culture of the rough variant after picking. If, as seemed likely, the capsuled bacilli formed the virulent part of the strain, the results shown in Table II were to be expected. Additional force was lent to this suggestion by the fact that cultures recovered from all six of the dead guinea pigs were of the same type; namely, an actively dissociating smooth mucoid strain .

The following experiment was carried out to test further the supposition that the deaths of the guinea pigs inoculated with the rough variant were due to contaminating virulent forms. The smooth mucoid strain of the previous

experiment was also used here.

A rough wedge in the smooth colony was picked and cultured on agar. Two further subcultures were made at short intervals.

Another rough wedge was picked and cultured on agar. One further subculture was made.

Both these variants appeared rough on the first slant made.

The smooth mucoid parent strain was subcultured whenever the rough variants were transferred.

Guinea pigs were then inoculated, as it was thought that one or both of the rough variants might now have lost any contaminating smooth elements.

TABLE III.

| No. of Guinea Pigs. | Each inoculated with | Death: Hours after inoculation. 10 | 20 | 30 | 40 | 50 |
|---|---|---|---|---|---|---|
| 3 | 1/3 slant smooth mucoid strain. | | | X<br>X<br>X | | |
| 3 | 1/3 slant rough variant 2nd subculture | | | | | /<br>/<br>/ |
| 3 | 1/3 slant rough variant 3rd subculture. | | | | | /<br>/<br>/ |

This experiment bore out the supposition that the deaths resulting from inoculation with the rough variant in table II were really due to the presence of smooth forms.

The experiments tabulated above showed that a rough variant from a smooth mucoid parent strain was avirulent, whereas the smooth mucoid strain retained its virulence. The complete avirulence of the rough strain was not definitely established until it had been subcultured two or three times after first being picked. This

observation lent force to the hypothesis that the smooth strain varied continuously, and that its rough variant divided true to type.

Immunity tests with rough variant.

Bail (1915) reported the immunisation of guinea pigs with a rough strain which did not produce capsules. A number of workers have however asserted that some degree of capsule formation in vivo was a prerequisite of immunisation against anthrax. Munne (1934) definitely affirmed this at the 12th International Veterinary Congress. The rough strain noted above seemed in many respects similar to Bail's strain and for this reason its immunising power was investigated.

Three guinea pigs which had survived the injection of rough variant (Table III) were each inoculated with one sixth of an agar slant of the smooth mucoid variant two weeks later.

One guinea pig died of anthrax in three days and one in seven days. The third survived. Unfortunately, controls had not been included in the test, so that it was not known for certain whether the virulence of the test strain had become less or whether these guinea pigs were particularly resistant.

The other three guinea pigs which had survived one injection of rough variant (table III) were given another one third slant of the same variant two weeks later. All three animals survived. Four weeks later these guinea pigs, together with three controls, were tested as follows.

TABLE IV.

| | No. of guinea pigs. | Each inoculated with | Death: Hours after inoculation. 10 | 20 | 30 | 40 | 50 | |
|---|---|---|---|---|---|---|---|---|
| Two previous injections R.variant | 3 | 1/6 large slant smooth mucoid strain | | | | | | / / / |
| Controls | 3 | ditto | | | | X X X | | |

The experiment was repeated as follows: three guinea pigs were each inoculated with one third of an agar slant of the rough variant used above. The injection was repeated twice at ten day intervals. Two weeks after the last injection these three guinea pigs and three uninoculated controls received subcutaneously one sixth of an agar slant of the smooth mucoid variant.

The three inoculated guinea pigs remained alive.

The three control guinea pigs died before the 40th hour.

Up to this time no guinea pig had lived after inoculation with the smooth mucoid variant, even when quite small doses were given. In the immunity tests shown above, very large amounts of the virulent strain were injected, and where more than one protecting injection had been given, all the immunised guinea pigs survived, and all the controls died. In every case the controls died within minutes of each other. This was further evidence of the magnitude and virulence of the infecting dose. It seemed reasonable to assume that guinea pigs which had received two or more injections of the rough variant were solidly immune to a very large test dose of the smooth mucoid strain.

The immunity produced by the rough variant was then exposed to a more severe test. Guinea pigs received 1 c.c. of a broth culture of rough variant at ten day

intervals. Three doses were given, and two weeks after the third dose the immunity of five of these guinea pigs was tested by the inoculation of a large dose of virulent Drummond. This strain had recently been isolated from cattle in an extensive natural outbreak of anthrax, and was very virulent for sheep and rabbits. Four controls were included. At the same time four guinea pigs which had undergone the same process of immunisation were tested against a large dose of the smooth mucoid strain. Four controls were also included in this experiment.

TABLE V.

| Previous History. | No. of guinea pigs | Each inoculated with | Death: Hours after injection. 10 | 20 | 30 | 40 | 60 | 80 | 100 | 200 | 250 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 injections 1 c.c.broth culture rough variant. | 5 | ¼ slant virulent Drummond | | | | | X | | X<br>X X X | | |
| Controls | 4 | ditto | | | X<br>X<br>X<br>X | | | | | | |
| 3 injections 1 c.c. broth culture rough variant | 4 | ¼ slant smooth mucoid Boshoff | | | | | | | X 17 days<br>X 17 days | X<br>X | |
| Controls | 4 | ditto | | | X<br>X<br>X<br>X | | | | | | |

Another experiment was then carried out to test the resistance of inoculated guinea pigs to the virulent Drummond strain. Six guinea pigs were each given a large dose of rough variant (about half an agar slant). This was repeated on two more occasions. Another batch of five guinea pigs each received two inoculations of the rough variant, with a three weeks interval between the injections. Three weeks after the last injection these guinea pigs and six unprotected controls each received one fifth of an agar slant of virulent Drummond.

TABLE VI.

| Previous History of Guinea Pigs. | No. of guinea pigs. | Each inoculated with | Death: Hours after injection 10 | 30 | 50 | 70 | 90 | 110 | 130 | 150 |
|---|---|---|---|---|---|---|---|---|---|---|
| Received 3 doses of rough variant | 6 | 1/5 Agarslant virulent Drummond | | | | X | | | | |
| | | | | | | | X | | | / |
| | | | | | | | X | | | |
| | | | | | | | X | | | X |
| Received 2 doses of rough variant | 5 | ditto | | | | | | | | / |
| | | | | | X | X | | | | |
| | | | | | | X | | | | / |
| Controls | 6 | ditto | | X | | | | | | |
| | | | | X | | | | | | |
| | | | | X | | | | | | |
| | | | | X | | | | | | |
| | | | | X | | | | | | |
| | | | | X | | | | | | |

The results recorded in table V show that inoculation with the rough variant gave some protection against virulent Drummond. Although none of the guinea pigs actually survived, one may accept the results as significant in view of the very large amounts of culture injected. A greater degree of protection was given against the smooth mucoid strain although this was not as complete as in previous experiments.

In table VI very much larger doses of rough variant were used to immunize the guinea pigs. The test with virulent Drummond shewed that all the immunised guinea pigs were more resistant than the controls, and three out of eleven were completely protected. There was no significant difference in the immunity produced by two or by three injections of the rough variants, although the small number of guinea pigs used does not allow of definite conclusions being drawn. Three survivors must be regarded as a very good result, when it is considered that a most virulent strain was used and very large doses administered. (Doses of the order of 1/1000th of those given to the guinea pigs killed sheep in 24 to 36 hours, while doses in the

neighbourhood of 1 in 500,000 of the dose given to guinea pigs still killed sheep. It was rather surprising that doses which killed sheep in 24 - 36 hours, rarely killed guinea pigs before the 40th hour).

Reference to table V shows that three injections of the rough variant failed to protect the guinea pigs solidly against the smooth virulent strain although in the earlier experiments two injections had given a solid immunity against it As this was the first of the rough variants isolated, some immunising power had possibly been lost. On the other hand, smaller doses had been used in the immunisation process for this experiment. It was important to know whether there had been any marked diminution of the antigenic power of the rough variant, and the following experiment was carried out to ascertain this.

Six guinea pigs were each inoculated twice at fortnightly intervals, with the rough variant used in the previous experiment. Six other guinea pigs each received two inoculations of a much more recently isolated rough variant. This strain had been subcultured about seven times altogether whereas the original rough had been in constant use for months. Two weeks after the last injection of the rough variant both batches of guinea pigs, together with six controls, were tested against the smooth mucoid strain.

TABLE VII.

| Previous History. | No. of guinea pigs. | Each inoculated with. | Death: Hours after inoculation. 10 | 20 | 30 | 40 | 50 | |
|---|---|---|---|---|---|---|---|---|
| 2 injections rough variant | 6 | 1/5 slant smooth mucoid strain | | | | | | / / / / / / |
| 2 injections recently isolated rough variant | 6 | ditto | | | | | | / / / / / / |
| Unprotected controls | 6 | ditto | | | | X X X X | X X | |

There was thus no difference in the degree of protection afforded by the rough variant isolated about six months previously, and by the rough variant recently isolated. Two large inoculations of either protected solidly. The failure to protect solidly against the smooth variant in table V could possibly be attributed to the smaller doses of rough variant which those guinea pigs had received. On the other hand it was possible that the virulence of the smooth mucoid test strain had decreased because the controls (table VII) took slightly longer to die than in the previous experiments.

Three weeks after the test dose the twelve immune guinea pigs were killed and the blood collected. After the serum had separated its protective power was ascertained by injecting guinea pigs intraperitoneally and administering a test dose of culture 24 hours later.

TABLE VIII.

| Previous treatment | No. of guinea pigs | Each inoculated with | Death: Hours after injection. 20 | 40 | 60 | 80 | 100 | 150 | 200 | |
|---|---|---|---|---|---|---|---|---|---|---|
| 4 c.c. immune serum. | 3 | 1/5 slant smooth mucoid variant | | | | | X | | | / x |
| 3 c.c. immune serum. | 3 | ditto | | | | | | X | | X X |
| 2 c.c. immune serum | 3 | ditto | | | | | X X X | | | |
| 4 c.c. normal serum | 6 | ditto | | X X X X | X X | | | | | |
| Untreated controls | 10 | ditto | | X X X X X X X X X X | | | X | | | |