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Variation in *Bacillus anthracis*.

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PART I.

PART II.

Part I.—Some Effects of Carbon Dioxide on the Formation of Capsules and Spores by *B. anthracis*.

THE capsules produced by *B. anthracis* have always been regarded as an important factor in the pathogenesis of anthrax. The chief reason for the assumption is that animals dying of natural anthrax have numerous capsuled bacilli in their blood and tissues, while cultures made from those tissues show only uncapsuled bacilli. There is also experimental evidence which proves that capsuled anthrax bacilli resist phagocytosis both *in vivo* and *in vitro*. Teale (1935). Sobernheim (1931).

After prolonged subcultivation or after attenuation, the anthrax bacillus may produce capsules on artificial media; but generally speaking virulent strains do not produce capsules unless the medium contains a large proportion of serum. The macroscopic appearance of cultures which contain capsuled organisms depends on the number present, and when the majority of bacilli have capsules the growth appears slimy and tenacious. This type of growth is called mucoid.

Nungester (1929) distinguished smooth mucoid and rough mucoid variants. A smooth mucoid culture is very slimy and tenacious and has not the cuneiform structure which the anthrax colony usually shows by transmitted light. Frequently a culture does not develop many capsules in the first 24 hours and only thereafter becomes

mucoid. Such cultures appear rough by transmitted light; but the slimy surface growth, which eventually overlaps the initial rough edges, is then seen to be typically smooth mucoid. From Nungester's description it seems as if this is what he termed a rough mucoid, although it really appears to be smooth mucoid overgrowing a rough or rough-smooth base.

Nungester (1929) demonstrated that smooth mucoid strains of *B. anthracis* developed the mucoid property more profusely when grown in 20 per cent. carbon dioxide, or when grown in association with *B. subtilis* or *B. megatherium*. This, together with the known fact that living tissues which have a high carbon dioxide and low oxygen tension promote capsule formation, suggested that the effect of carbon dioxide on various anthrax strains should be studied.

Technique.

Solid media were used in all the experiments and after inoculation the culture tubes were placed in an anaerobic jar. This was evacuated to a pressure previously decided upon and sufficient carbon dioxide added to bring the total up to atmospheric pressure. The cultures were removed from the jar at one to two days interval and examined. Smears were made and stained with giemsa. Observations were usually carried out for about a week, and the cultures were replaced in the carbon dioxide after each examination. The carbon dioxide concentration was adjusted every day or two, to compensate for the carbon dioxide produced by the growing bacilli. Control tubes of culture were incubated under atmospheric conditions, or in jars containing paraffin gas or hydrogen at the same partial pressure as the carbon dioxide.

Experiments.

Preliminary work indicated that carbon dioxide played a part in capsule formation, and in the experiments described below the effect of different concentrations of carbon dioxide was investigated. Two rough virulent strains, Virulent Boshoff and Virulent Anthrax A. were used.

I. *Experiment in* 85-90 *per cent. carbon dioxide.*

A. (*a*) Two slants virulent Boshoff on nutrient agar pH 7·5: after two days the growth was slight and filmy and smears showed very few capsules.

(*b*) One slant virulent Boshoff on blood agar: the appearance of the culture and smears was the same as on the ordinary agar.

B. (*b*) Two slants virulent Anthrax A on nutrient agar pH 7·5: after two days there was slight growth. No capsules were seen in smears.

(*b*) One slant virulent Anthrax A on blood agar: a few capsules were seen in smears.

II. *Experiment in 75 per cent. carbon dioxide.*

Five slants of virulent Boshoff on nutrient agar pH 7·5: the slants were examined and smears stained every two days for a week. By the fifth day 2-5 per cent. of the bacilli had capsules.

III. *Experiment in 65 to 70 per cent. carbon dioxide.*

A. (*a*) Five slants of virulent Boshoff on nutrient agar pH 7·5: growth was good. Smears showed a fair number of capsules after two days. By the sixth day about 80 per cent. of the bacilli were capsuled.

(*b*) One slant virulent Boshoff on blood agar: capsules developed sooner than on plain agar.

B. (*a*) Two slants virulent Anthrax A on nutrient agar pH 7·5: observations were only made up to the second day. By then about 5 per cent. of the bacilli were capsuled.

(*b*) One slant virulent Anthrax A on blood agar: after two days about 20 per cent. of the bacilli had capsules.

IV. *Experiment in 50 per cent. carbon dioxide.*

A. (*a*) Two slants virulent Boshoff on nutrient agar pH 7·5: after two days about 20 per cent. of the bacilli had capsules.

(*b*) One slant virulent Boshoff on blood agar: after two days all the bacilli showed capsules.

B. Two slants of virulent Anthrax A on nutrient agar pH 7·5 and one slant on blood agar showed about the same appearance as the virulent Boshoff.

V. *Controls grown in air.*

A. (*a*) Seven slants virulent Boshoff on nutrient agar pH 7·5: Very few capsuled bacilli were seen, although smears were made up to the seventh day.

(*b*) Three slants of virulent Boshoff on blood agar: very few capsuled bacilli were found.

B. (*a*) Four slants virulent Anthrax A on nutrient agar pH 7·5: capsuled bacilli were very rarely seen.

(*b*) Three slants virulent Anthrax A on blood agar: capsuled bacilli were very rarely seen.

Discussion.

The two rough virulent strains, V. Boshoff and V. Anthrax A developed far more capsules in the 50 and 70 per cent. carbon dioxide than in air. Concentrations of carbon dioxide above 70 per cent. markedly retarded the growth of the cultures and the production of capsules. Further experiments at different times confirmed this

point. In all subsequent experiments observations were made for a week or more, and concentrations of carbon dioxide between 60 and 70 per cent. were used. The jars were opened and refilled every day to compensate for the carbon dioxide formed by the bacilli.

The formation of capsules might have been due to the reduced oxygen pressure rather than to the increased concentration of carbon dioxide. Another possibility was that desiccation of the media might have hindered the production of capsules in the control slants.

The experiments summarized below were devised to examine these possibilities and the strains used were virulent Boshoff, and a freshly isolated virulent strain called Anthrax 40486.

I. *Experiment with Virulent Boshoff.*

(*a*) Eleven slants of V. Boshoff on nutrient agar pH 7·5 were grown in 65 per cent. carbon dioxide. After about a week all the cultures had slimy surfaces and smears showed that 70 to 100 per cent. of the bacilli had capsules.

(*b*) Three slants of V. Boshoff on nutrient agar pH 7·5 were grown in 65 per cent. paraffin gas.* No capsules were seen, although the cultures were examined for a week.

(*c*) Two slants of V. Boshoff on same media as before. The cultures were incubated in air, but were protected from drying out: at the end of seven days very few capsules had been seen.

(*d*) Six slants of virulent Boshoff on nutrient agar pH 7·5 incubated in air: at the end of seven days only a few capsules had been seen.

II. *Experiments with Anthrax* 40486.

(*a*) Two slants Anthrax 40486 on nutrient agar pH 7·5 grown in 65 per cent. carbon dioxide: after seven days 95 per cent. of the bacilli were capsuled.

(*b*) Two slants Anthrax 40486 in 65 per cent. paraffin gas: no capsules were seen within seven days.

(*c*) Two slants Anthrax 40486 in air showed no capsules during seven days' observation.

Discussion.

This experiment showed that both virulent Boshoff and Anthrax 40486 produced large numbers of capsuled bacilli in 65 per cent. carbon dioxide; but that neither strain developed capsules in paraffin gas; nor did capsules develop if the medium was merely protected from drying.

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* This was the ordinary gas used at Onderstepoort for burners, etc., and was obtained by cracking paraffin.

The conclusion was that the carbon dioxide influenced the production of capsules by the three strains, V. Boshoff, virulent Anthrax A and Anthrax 40486. Subsequently, another virulent stock strain, Pretoria North, and two freshly isolated virulent strains, Drummond and 568 were tested for capsule formation in carbon dioxide. None of these showed capsules when grown on nutrient agar in air, but after growing in 65 per cent. carbon dioxide for four days the surface of the cultures was completely mucoid and all the bacilli capsuled. These experiments were more successful than the earlier ones, because it was then realised that not more than six slants should be placed in an anaerobic jar. If the jar was packed, the partial pressure of the carbon dioxide rapidly became high, and capsule formation was inhibited.

Some Effects of Varying the Hydrogen Ion Concentration.

Experiments were done to see whether change in hydrogen ion concentration produced by the carbon dioxide played any part in the production of capsules. Nungester showed that mucoid variants of *B. anthracis* were more slimy when grown at pH 7·8 than at a lower pH. He suggested that this might be due to an alkaline medium " fixing " more carbon dioxide. Virulent Boshoff was grown in 70 per cent. carbon dioxide on buffered nutrient agar adjusted to different hydrogen ion concentrations. Controls were grown on similar media in air.

I. *Virulent Boshoff at pH* 8·4.

(*a*) Two slants in 70 per cent. carbon dioxide. The final pH was above 7·0. About 80 per cent. of the bacilli showed capsules.

(*b*) Two slants in air showed poor growth. No capsules developed.

II. *At pH* 8·0.

(*a*) Two slants in 70 per cent. carbon dioxide. The final pH was about 6·8. About 60 per cent. of the bacilli showed capsules.

(*b*) Two slants in air showed good growth and very few capsules.

III. *At pH* 7·6 *to* 7·8.

(*a*) Four slants in 70 per cent. carbon dioxide. The final pH was about 6·0 to 6·5. Growth was good and from 60 to 80 per cent. of the bacilli had capsules.

(*b*) Four slants in air showed good growth and no capsules were seen.

IV. *At pH* 6·8.

(*a*) Two slants in 70 per cent. carbon dioxide. The final pH was about 5·0. About 50 per cent. of bacilli showed capsules.

(*b*) Two slants in air. Growth was good. No capsules were seen.

Discussion.

The results were quite clear cut. The final hydrogen ion concentrations in the CO_2 ranged from about 5·0 to 7·2. Capsules were freely developed at all these levels. None of the controls in air showed more than an occasional capsule. Further experiments showed that a culture which showed a few capsules in air at pH 7·5-7·8 would show none at pH 6·8 or lower. It could be concluded that the lowering of the pH by CO_2 did not cause the bacilli to produce capsules.

A point of interest was whether capsule formation in carbon dioxide was due to the selection of a mucoid variant pre-existant in the original rough strain, or whether carbon dioxide actually induced the "rough" bacilli to develop capsules. Accordingly a number of single cells were selected from a freshly isolated rough virulent strain. Cultures from these single cells were mucoid and capsuled in carbon dioxide, but streaks made from the mucoid growth onto agar plates and incubated in air were always rough and uncapsuled. It is probable, therefore, that rough bacilli developed capsules in carbon dioxide, rather than that carbon dioxide selected mucoid variants existing in the original culture.

Carbon Dioxide and Sporulation.

It is generally held that *B. anthracis* requires a high partial pressure of oxygen to sporulate well and the non-production of spores *in vivo* is supposed to be due to the low oxygen tension. While the experiments on capsule development were being done, careful note was taken of the degree of sporulation under the varying conditions. It was found that spores were very rarely formed when cultures were grown in a high partial pressure of carbon dioxide. This held throughout the tests, however long the cultures were kept, and whatever strains were used.

When *B. anthracis* was grown under reduced oxygen pressure, and the difference made up with an inert gas, sporulation and growth of the culture were retarded; but eventually the sporulation was quite as good as sporulation in air.

These observations were very constant. Some strains which sporulated very readily formed a fair number of free spores in carbon dioxide. The number of these was insignificant compared with the number formed in air, or under a high partial pressure of paraffin gas or hydrogen. It seemed therefore as if the pressure of free oxygen did not in itself influence sporulation to any great extent.

This was borne out by an experiment in which three freshly isolated virulent strains were grown as follows.

I. (*a*) On large surfaces of inspissated horse serum in air: the sporulation was very poor.

(*b*) On inspissated serum in 65 per cent. carbon dioxide. There was no sporulation.

II. (*a*) On nutrient agar in air: sporulation complete in all strains.

(*b*) On nutrient agar in 75 per cent. coal gas: sporulation complete in all strains.

(*c*) On nutrient agar in 75 per cent. carbon dioxide: practically no sporulation in any of the strains.

The differences in sporulation on the various media were striking, and the fact that sporulation on serum in air was so poor points to some factor other than the free oxygen as being responsible for stimulating spore formation.

Summary and Conclusions.

(1) A high partial pressure of carbon dioxide stimulated capsule formation in rough virulent anthrax strains.

(2) This stimulation appeared to be independent of the oxygen tension and the hydrogen ion concentration.

(3) A high partial pressure of carbon dioxide markedly inhibited sporulation.

(4) The inhibition of sporulation was shewn to be independent of the oxygen tension.

Part II.—Some Correlations between Colony Variation and Pathogenicity in Strains of *Bacillus Anthracis*.

INTRODUCTION.

A freshly isolated and virulent strain of *B. anthracis* grows well on nutrient agar, and produces a colony with a dull flat surface, rather like frosted glass. The edge has, as a rule, a very tangled appearance, and the colony is extremely rough. The name " Medusa head " has been given to this type of growth.

Pasteur and his co-workers first noted exceptions to this rough development, and since then numerous workers have offered explanations for these variations, or have tried to correlate them with changes in other properties of the organism. It was very soon realized that this change in colony structure always tended towards a smoother appearance of the anthrax colony, and that these smoother colonies appeared more readily in cultures which had undergone some attenuation.

In 1921 Arkwright demonstrated the smooth to rough colony variation in a number of bacterial strains, and he was able to correlate the variation with changes in agglutination reactions. De Kruif (1921) showed that smooth to rough colony variation in a rabbit septicaemia organism was associated with a distinct loss of virulence. A great deal of work has since been done on this type of variation, and it has now been shown that this occurs in practically all bacterial strains. With few exceptions, the direction is from smooth to rough, and in the majority of pathogenic bacteria the change from smooth to rough is associated with a partial or complete loss of virulence.

The anthrax bacillus has always been regarded as an exception to this fairly general rule, because the freshly isolated, virulent bacillus forms a very rough colony. Many workers have observed that smooth colony variants could be obtained from rough virulent anthrax strains by different methods of attenuation. 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 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 avirulent.

However, in the case of anthrax 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 smooth colonies appear amongst the rough. Even the roughs tend towards the smoother type. These facts have been generally conceded; but most authors deny that there is any significant difference in 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 heretofore 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 stabilize these variants after a long period of selection and subculturing and found finally that the rough variant seemed more virulent for rabbits than the smooth. Relatively few rabbits were used and the infecting dose was not large. Moreover, the time that had elapsed since the first isolation of the two variants and the test of their virulence, introduced a factor, whose significance cannot 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 immunization against anthrax to any 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 summarized 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 surprising as it is well known that many fully virulent strains produce few or no capsules in serum. Bail did not 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.

With rare exceptions it has been 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 he did not concern himself with colonial morphology.

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 deals with these findings and their bearing on the problems of colony variation, pathogenicity and immunity in anthrax.

EXPERIMENTAL.

A. Dissociation in Virulent 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 then inoculated with a loopful of virulent stock culture known as Virulent Boshoff. The Erlenmeyer flask was incubated at 37° C. and from time to time the broth was added to the culture to keep the level 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. 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 colony, and this latter could only be maintained by frequent subculturing. 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 to gain further information about 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 occurred in the behaviour of this variant. The growth was always smooth mucoid, and rough variants always commenced to develop after about eighteen hours. At different 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 was apparent that this actively dissociating strain was continuously splitting up into smooth mucoid and into rough variants. The former repeated the dissociation process, whereas the 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 authers 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 examined daily. The growth was a mixture of smooth mucoid and rough elements; the former capsuled and the latter not.

(*b*) The variant picked from a rough outgrowth from the smooth mucoid colony was rough in the carbon dioxide, and showed no capsules 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.

Control 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 showed in air, while the bacilli from the rough colonies were quite devoid of capsules even in the carbon dioxide. The stock rough virulent strain (Boshoff) 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 uncapsuled and rough when grown in carbon dioxide, further experiments were undertaken to test the observation thoroughly. A rough variant from a smooth mucoid colony was grown in different concentrations of carbon dioxide, to see if capsule formation could be induced. No capsules could be found in cultures grown in 30 per cent., 40 per cent., 60 per cent., and 70 per cent. carbon dioxide.

A further experiment was done in which the strains were grown on blood agar in 65 per cent. carbon dioxide. This procedure had never yet failed to elicit the formation of capsules in the stock rough strains; but again the rough strain (*b*) failed to produce capsules.

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 previously tested had become capsuled in carbon dioxide, the behaviour of this strain was somewhat exceptional, and therefore its ability to produce capsules *in vivo* was investigated.

Pathogenicity of rough variant from smooth mucoid Boshoff.—

This rough strain which had shown no capsule formation in carbon dioxide was grown on agar slants is air. The growth was washed off after 24 hours, and five guinea-pigs each inoculated with one fifth of the suspension 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 after infection. 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 uncapsuled anthrax bacilli at the inoculation site and in the spleen. 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; none of these bacilli showed any capsules. The deaths were probably due to a toxic effect of the enormous doses injected. The time between infection 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 its inability to produce capsules in the animal body. This variant also failed to produce anthrax in guinea-pigs and mice and the lack of virulence was probably associated with the loss of ability to form capsules, either *in vitro* or *in vivo*.

Comparison of the pathogenicity of the smooth mucoid and the rough variants of V. Boshoff.—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 was given to minimize individual variation.

(*a*) A single cell was picked from a colony of the smooth mucoid variant. The resulting cultures were a mixture of smooth mucoid and rough elements. The smooth mucoid variant was kept going 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	*¼ slant smooth mucoid variant			x x x		
3	¼ slant rough variant					/ / /

x Death from anthrax.
/ Alive after 30 days.
* 1 Slant is the growth on a surface 7 × 2 cms.

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 developed rough outgrowths after 24 hours. A subculture was made from one of the rough wedges and from the mucoid growth. After 24 hours the former was rough, and the latter mostly smooth mucoid. Each variant 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	⅓ slant smooth mucoid variant			x x x				
3	⅓ slant rough variant						x	x x

As the doses given were exceedingly large the difference in survival time between the two lots was most likely significant. It was possible that a few virulent bacilli were carried over when the rough colony was picked and that these were enough 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. Moreover the cultures recovered from all six of the dead guinea-pigs were of the same type; namely, actively dissociating smooth mucoid strains.

The following experiment was carried out to see whether 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 again used.

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.

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

Guinea-pigs were then inoculated as follows:—

TABLE III.

No. of Guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 10	20	30	40	50
3	⅓ slant smooth mucoid strain			x x x		
3	⅓ slant rough variant 2nd sub-culture					/ / /
3	⅓ slant rough variant 3rd sub-culture					/ / /

Therefore it was probable that the deaths after inoculation with the rough variant in Table II were really due to the presence of smooth forms.

The experiments detailed above showed that a rough variant which had arisen in 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 immunization 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 immunization 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 immunizing power was investigated.

Three guinea-pigs which had survived the inoculation 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.

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 and again survived. Four weeks later each of these guinea-pigs, together with three controls, were injected 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	¼ slant smooth mucoid strain					/ / /
Controls	3	¼ slant smooth mucoid strain				x x x	

The experiment was repeated with identical results.

The three inoculated guinea-pigs remained alive.

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

Up to this time no guinea-pig 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 all the immunized guinea-pigs survived, while 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. Thus the 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 with a recently isolated very virulent strain—V. Drummond. This strain was very virulent for sheep. At the same time four guinea-pigs which had undergone the same process of immunization were tested against a large dose of the smooth mucoid strain.

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 xxx		
Controls	4	¼ slant virulent Drummond				x x x x					
3 injections 1 c.c. broth culture rough variant	4	¼ slant smooth mucoid Boshoff								Died after 17 days	/ x x x
Controls	4	¼ slant smooth mucoid Boshoff				x x x x					

Another experiment was then carried out to test the resistance of inoculated guinea-pigs to the virulent Drummond strain. The results were as follows:—

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	$\frac{1}{5}$ Agar slant virulent Drummond				x	x				/
							x				
							x				x
Received 2 doses of rough variant	5	$\frac{1}{5}$ Agar slant virulent Drummond				x					/
					x	x					/
Controls	6	$\frac{1}{5}$ Agar slant virulent Drummond		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 much larger doses of rough variant were used to immunize the guinea-pigs. The test with virulent Drummond showed that all the immunized guinea-pigs were more resistant than the controls, and three out of eleven were completely protected. Three survivors must be regarded as a very good result, when it is considered that a most virulent test strain was used and very large doses administered. Doses of the order of 1/500,000 of the amount given to the guinea-pigs still killed sheep.

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. As this was the first of the rough variants isolated, some immunizing power had possibly been lost. On the other hand, smaller doses were used in the immunization 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 incoulation. 10	20	30	40	50
2 injections rough variant	6	$\frac{1}{5}$ slant smooth mucoid strain					/ / / / / /
2 injections recently isolated rough variant	6	$\frac{1}{5}$ slant smooth mucoid strain					/ / / / / /
Unprotected controls	6	$\frac{1}{5}$ slant smooth mucoid strain				x 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. 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 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 later the twelve immune guinea-pigs were killed and the blood collected.

The protective power of the serum 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	1/5 slant smooth mucoid variant						x		x x
2 c.c. immune serum	3	1/5 slant smooth mucoid variant					x x x			
4 c.c. normal guinea pig serum	6	1/5 slant smooth mucoid variant	x	x x x x x						
Untreated controls	10	1/5 slant smooth mucoid variant	x x x x x x x x x			x				

It was clear that the serum of the immune guinea-pigs gave considerable protection against a very large dose of the smooth mucoid variant. Two guinea-pigs injected with normal serum lived a few hours longer than the controls, but their survival time did not approach that of the guinea-pigs inoculated with the immune serum. One guinea-pig protected with 4 c.c. of immune serum survived and the indication was that larger amounts of immune serum might have given complete protection.

Immunity experiments on sheep and goats.—The immunization of guinea-pigs by ordinary attenuated vaccine strains has always been difficult. Few guinea-pigs survive inoculation with vaccine anthrax strains, and the survivors are rarely immune. As the rough variant had given such good results in the immunization of guinea-pigs, it was decided to test its immunizing power in sheep and goats.

The immunity was tested three weeks later with about 20 M.L.D.'s of a virulent spore suspension. Controls were not included as the test done was in constant use and the minimum fatal dose reasonably well known.

TABLE IX.

Sheep.	Date received rough variant.	Date received virulent suspension.	Result.
39256	14/2/35	8/3/35	/
38217	,,	,,	x anthrax 20/3/35.
39753	,,	,,	/
40103	,,	,,	/
40665	,,	,,	x anthrax 14/3/35.
40690	,,	,,	/
Goats.			
41141	,,	,,	/
41150	,,	,,	/
41157	,,	,,	/

Another test was then performed on goats. Four goats received approximately the same dose of rough variant as those noted in Table IX (1/10th slant).

The suspension was made up in lanolin and oil (Ramon and Staub, 1935). Four other goats received the same amount of rough variant suspended in physiological saline. These goats, together with two controls, were tested six weeks later as follows:—

TABLE X.

Numbers, Goats.	Date inoculated with rough variant in lanolin and oil.	Date inoculated with 20 M.L.D. virulent spores.	Results.
42932	8/5/36	17/6/35	x anthrax 25/6/35.
42934	,,	,,	x anthrax 22/6/35.
42935	,,	,,	/
42941	,,	,,	/
	Date inoculated with rough variant in saline.		
42924	8/5/35	17/6/35	/
42937	,,	,,	/
42939	,,	,,	/
42940	,,	,,	/
Controls, Goats.		10 M.L.D.	
44302	—	17/6/35	x anthrax 20/6/35.
44317	—	,,	x anthrax 21/6/35.

Another experiment was then carried out to examine the immunizing power of two inoculations of the rough variant. Four goats received two inoculations of 1/10th of an agar slant of rough variant. Sixteen days later, they were tested with 200 M.L.D.'s of virulent spore suspension. The results were as follows:—

TABLE XI.

Goats.	1st Dose rough variant.	2nd Dose rough variant.	200 M.L.D.'s virulent spore suspension.	Results.
42928.....	5/7/35	24/7/35	10/8/35	x anthrax 14/8/35
42938.....	,,	,,	,,	/
44314.....	,,	,,	,,	x anthrax 17/8/35.
44320.....	,,	,,	,,	x anthrax 20/8/35.

The immunity produced in sheep and goats by the rough variant was disappointing. Judging from the results in guinea-pigs a higher degree of protection was expected. The dose of the rough variant given to the sheep was, however, smaller than the dose given to the guinea-pigs. Nevertheless the number of bacilli was still larger than that included in most anthrax vaccines.

The importance of dosage is of course well known; but there is a tendency to assume that the *in vivo* multiplication of the attenuated anthrax bacilli inoculated would compensate largely for the relatively small numbers of organisms included in most living anthrax vaccines. The data given below were extracted from the anthrax vaccine book kept at Onderstepoort and give the results of titrations of the vaccines on sheep for the last ten years. The quantities 20 c.c., 0·1 c.c., 0·01 c.c., etc., represent amounts of vaccine before dilution for issue. The 0·1 c.c. amount was approximately 20-40 times a sheep dose of vaccine as finally issued. There was a striking difference in immunizing power between 20 sheep doses and 2,000 sheep doses (20 c.c.) and it is clear from Table XII that the usual dose of vaccine does not produce a maximum result and that the immunizing power is markedly improved by increased dosage.

TABLE XII.—*From Vaccine Book.*

Dose of concentrated vaccine.	No. of sheep.	Large dose virulent spores.	No. of deaths.
20·000 c.c.....	155	,,	2 or 1·3 %.
0·1 c.c.....	145	,,	25 or 17·2 %.
0·01 c.c.....	155	,,	69 or 44·5 %.
0·005 c.c.....	150	,,	67 or 44·7 %.
0·002 c.c.....	90	,,	50 or 55·6 %.
0·001 c.c.....	105	,,	81 or 77·1 %.

The results obtained with the rough variant in guinea-pigs could probably have been duplicated in sheep had comparable doses been employed. Such large doses of rough variant would probably have been perfectly safe, but would be impracticable because of the difficulty of preparing several million doses of such concentrated vaccine annually. An attempt to compensate for this dosage factor by using Ramon's method of incorporating the bacilli in lanolin and oil (Table X) was not encouraging. Too few goats were used for any real conclusion to be drawn from the experiment.

Summary of experiments on variants of V. Boshoff.—(1) An actively dissociating smooth mucoid strain of virulent Boshoff was virulent for rabbits and guinea-pigs.

(2) Rough variants picked from the smooth mucoid strain were avirulent for sheep, goats, guinea-pigs and mice.

(3) The smooth mucoid strain was capsuled and the rough variant was uncapsuled. The latter was stable as regards avirulence and inability to form capsules *in vitro* or *in vivo*.

(4) Two large doses of the rough variant solidly immunized guinea-pigs against the smooth mucoid parent strain and gave them a high degree of resistance to a very virulent field strain.

(5) A moderately large single dose of rough variant produced a fair, but not solid immunity in sheep and goats.

(2) *Dissociation in a virulent anthrax strain—V. Anthrax A.*

This strain was isolated from the original stock virulent Boshoff by the single cell technique. It was grown in Erlenmeyer flasks in a thin layer of broth as with V. Boshoff. As before, an actively dissociating smooth mucoid strain was eventually isolated, and this—like the smooth mucoid Boshoff—continuously threw off rough variants. These rough variants were also unable to produce capsules in carbon dioxide.

Pathogenicity of variants of V. Anthrax A.—A rough colony was picked and subcultured five times to eliminate capsuled bacilli. The smooth mucoid parent strain was also subcultured at the same times as the rough dissociant. The pathogenicity of the variants was then tested on guinea-pigs as follows:—

TABLE XIII.

No. of guinea pigs.	Each inoculated with:	Results.
3.....	1 slant smooth mucoid variant	/ / /
3.....	1 slant rough variant	/ / /

Neither variant possessed any residual virulence for guinea-pigs, although each guinea-pig received a whole slant of the respective strains.

The pathogenicity was then tested on white mice as follows:—

TABLE XIV.

No. of mice.	Each inoculated with:	Death: Hours after inoculation. 30 40 100 200	Remarks.
18	1/25th agar slant smooth mucoid strain	x x x x xx xx xx x xx x x x /	1 mouse died of inter-current infection.
18	1/25th slant rough variant	No deaths from anthrax	4 died of inter-current infections.

All the mice indicated thus X died with a typical anthrax septicaemia. This smooth mucoid strain, therefore, still retained some virulence. This virulence was not however high, as in spite of the large doses used, there was a certain amount of scattering in the times of death.

Immunity tests with variants of V. Anthrax A.—The six guinea-pigs which had survived the inoculation with the smooth mucoid and the rough variants (Table XIII) received two further injections of the same strains and the immunity was then tested as follows:—

TABLE XV.

Previous injections.	No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20 40 60 80
3 injections smooth mucoid V. anthrax A.	2	$\frac{1}{5}$ slant smooth mucoid V. Boshoff	x x
3 injections rough V. anthrax A.	3	$\frac{1}{5}$ slant smooth mucoid V. Boshoff	x x x
Uninoculated controls	4	$\frac{1}{5}$ slant smooth mucoid V. Boshoff	x x x x

All the treated guinea-pigs died, and all but one died at the same time as the controls. Although very large doses had been used for immunization, neither variant was able to immunize guinea-pigs against the smooth mucoid Boshoff strain. Similar experiments using the rough variant from the Boshoff strain had resulted in a solid immunity being established.

Immunity tests on mice.—One lot of four mice each received two injections of the rough variant of V. anthrax A, and another lot of six mice each received one injection. Their immunity was tested twelve days later as follows:—

TABLE XVI.

Previous injections.	No. of mice.	Each inoculated with:	Results. Death: Hours after inoculation. 20	40	60	80	100	120
2 injections rough variant V. anthrax A.	4	1/25th slant smooth mucoid V. anthrax A.		x				
				x				
				x				
					x			
1 injection rough variant V. anthrax A.	6	1/25th slant smooth mucoid V. anthrax A.		x				
			x	x				
			x	x				x
1 injection smooth mucoid V. anthrax A.	1	1/25th slant smooth mucoid V. anthrax A.		x				
Uninoculated controls....	5	1/25th slant smooth mucoid V. anthrax A.	xx					
			xx		x			

There was no evidence that the treated mice were more resistant than the controls. The one mouse which had survived a previous injection of the smooth mucoid strain was not immune to a second dose of the same magnitude.

Summary.—(1) An actively dissociating smooth mucoid strain was isolated from a virulent anthrax strain—V. Anthrax A.

(2) A rough variant, which had lost its ability to produce capsules *in vitro* and *in vivo* was obtained from the smooth mucoid strain.

(3) The smooth mucoid strain was avirulent for guinea-pigs, but moderately virulent for mice.

(4) The rough variant was avirulent for guinea-pigs and for mice.

(5) Neither variant was able to confer an appreciable immunity on guinea-pigs and mice, although several large immunizing doses were given.

(3) *Dissociation in a virulent anthrax strain—V.* 568.

568 was a fully virulent strain newly isolated from an outbreak of anthrax. It was grown in Erlenmeyer flasks in the same way as the two previous strains (1 and 2), except that the broth contained 0·1 per cent. phenol to promote dissociation. Loopfuls of the culture were plated at intervals, and after about six weeks smooth mucoid colonies began to appear in the streaks. These colonies were picked and plated a number of times, but they showed very little tendency to dissociate. Eventually, however, a few rough variants were obtained. These were subcultured several times to fix the type, but it was impossible to eliminate all capsuled bacilli from these rough variants.

Pathogenicity of variants of strain 568.—Three guinea-pigs each received 1/10th of an agar slant of smooth mucoid 568.

Three guinea-pigs each received 1/5th of an agar slant of rough 568.

All remained alive.

Mice were then injected as follows:—

TABLE XVII.

No. of mice.	Each inoculated with:	Death: Hours after injection. 20	40	60	80	100	
14	1/20th slant smooth mucoid 568 subcutaneously		xxx		x		x
13	1/20th slant rough 568 subcutaneously		x	x x	x	x x	

Both variants were thus avirulent for guinea-pigs and both were weakly virulent for mice. There was no demonstrable difference in virulence between the smooth mucoid and the rough variants, although both were very much attenuated compared with the original strain 568.

Immunity tests with variants of strain 568.—Three guinea-pigs each received two inoculations of the smooth mucoid strain at fourteen days interval. Three other guinea-pigs received two injections of the rough variant. About 2/3rds of a slant were given to each guinea-pig on each occasion. Two guinea-pigs which had received the smooth mucoid strain died of an intercurrent infection. The four remaining guinea-pigs together with three controls were tested with 1/5th agar slant smooth mucoid Boshoff ten days later.

TABLE XVIII.

Previous injections.	No. of guinea pigs.	Each inoculated with $\frac{1}{5}$ slant smooth mucoid Boshoff.	Death: Hours after inoculation. 20	40	60
2 injections smooth mucoid 568	1	Ditto.		x	
2 injections rough 568	3	Ditto.		x x x	
Uninoculated controls	3	Ditto.		x x x	

Two large doses of these variants of 568 were thus not able to confer any added resistance on the guinea-pigs tested.

Summary.—(1) A smooth mucoid variant was isolated from a virulent anthrax strain—568.

(2) This smooth variant was fairly stable, and only rarely threw off rough variants.

(3) The rough variants showed traces of capsuled bacilli and were not pure roughs.

(4) Neither variant was virulent for guinea-pigs, but both were slightly virulent for mice.

(5) Two large doses of either variant did not give guinea-pigs any demonstrable resistance to the smooth mucoid variant of V. Boshoff.

(4) *Dissociation in a virulent anthrax strain—V. Drummond.*

This strain had recently been isolated from a severe minor epidemic of anthrax, and was fully virulent and very rough. Dissociation was induced in Erlenmeyer flasks in the manner described for strain 568. After six weeks incubation a stable smooth mucoid strain was isolated and this occasionally gave rise to rough flat outgrowths containing only uncapsuled bacilli. Two rough variants were selected and one was subcultured four times and the other seven times to eliminate the smooth bacilli if possible. At the same time the smooth mucoid parent was also subcultured an equal number of times.

Pathogenicity tests of variants of V. Drummond.—Three guinea-pigs were inoculated with 1/3rd slant smooth mucoid variant. One guinea-pig died of an intercurrent infection.

Three guinea-pigs were each inoculated with 1/3rd slant of the 4th subculture of a rough variant. One guinea-pig died of an intercurrent infection.

As none of the variants were pathogenic for guinea-pigs, a test was performed on mice. The results are given below:—

TABLE XIX.

No. of mice.	Inoculated with (each):	Death: Hours after inoculation. 20	40	60	80
12	1/50th slant smooth mucoid Drummond	x			
		x			
		x	x		
		x	x		
		x	x		
		x	x	x	/
16	1/50th slant sub-culture: Rough variant	No deaths.			

The smooth mucoid variant was thus virulent for mice, while the rough variant which had arisen from the smooth mucoid was avirulent.

Immunity test with variants of V. Drummond.—The five guinea-pigs which survived the virulence test, together with three uninoculated controls, each received 1/6th of a slant of the smooth mucoid variant from virulent Boshoff.

TABLE XX.

Previous inoculations.	No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60
One inoculation ⅓ slant smooth mucoid Drummond	2	1/6th slant smooth mucoid Boshoff		x	
				x	
⅓ slant rough Drummond	2	1/6th slant smooth mucoid Boshoff		x	
				x	
Uninoculated controls	3	1/6th slant smooth mucoid Boshoff		x	
				x	x

There was no evidence of increased resistance to the test dose. In experiments not mentioned in this paper, single injections of the rough variant from Boshoff increased the survival time of guinea-pigs up to ten days, when approximately the same test dose was used.

Summary.—(1) A smooth mucoid variant which threw off rough variants fairly readily was obtained from Virulent Drummond.

(2) The rough variants did not produce capsules *in vitro* or *in vivo*.

(3) Neither variant was virulent for guinea pigs. The smooth mucoid variant was virulent for mice, while the rough variants were avirulent.

(4) Neither variant protected guinea pigs against the smooth mucoid Boshoff strain.

(5) *Dissociation in a virulent anthrax strain—V. Pretoria North.*

This strain was an old stock virulent strain called Pretoria North. It was grown in sterile unheated horse serum and after ten days incubation streaks made onto agar showed a few smooth colonies amongst the rough. A smooth colony was picked and subcultured daily for about two weeks, until the smooth mucoid characteristic was established. Rough variants were difficult to obtain and it was a month after isolation before a rough variant was picked which seemed free from capsuled bacilli. When this rough variant had been subcultured five times, guinea pigs were inoculated as follows:—

Three guinea-pigs each received 1/3rd agar slant of smooth mucoid variant; one died of anthrax and one of gas gangrene.

Three guinea pigs each received 1/3rd agar slant of rough variant. One guinea pig died of gas gangrene.

The virulence was then tested on mice as follows:—

Six mice received a subcutaneous injection of 1/20th slant smooth mucoid Pretoria North. One mouse died and showed a few anthrax bacilli in the spleen. One died of an intercurrent infection.

Six mice received a subcutaneous injection of 1/20th slant rough variant Pretoria North. During the following ten days, four of these mice died, but none showed anthrax.

Immunity tests with variants of Pretoria North Strain.—The guinea pigs which had survived inoculation with the rough variant, together with four controls, each received ¼ agar slant smooth mucoid variant from the virulent Boshoff strain. The results were as follows:—

TABLE XXI.

Each previously inoculated with :	No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	80	100	120	140
½ slant rough Pret. North	2	¼ slant smooth mucoid Boshoff							/ x
Uninoculated controls	4	Ditto.		x x x x					

It seemed as if the previous injection of the Pretoria North variant considerably increased the resistance of the guinea pigs to a large test dose of smooth mucoid Boshoff. Another experiment was therefore carried out to test this observation.

Six guinea pigs were each injected twice with ½ agar slant of the rough variant from the Pretoria North strain. None of these animals showed any ill effects. Two weeks after the second injection the resistance of these six guinea-pigs was tested as follows:—

TABLE XXII.

Each previously inoculated with :	No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	80	100	120	140	160
2 injections ½ slant rough Pretoria North	3	¼ slant smooth mucoid Boshoff								/ / /
Uninoculated controls	3	¼ slant smooth mucoid Boshoff		x x x						
2 injections ½ slant rough Pretoria North	3	1/6th slant virulent Drum-mond			x			x		x
Uninoculated controls	3	1/6th slant virulent Drum-mond		x x x						

Thus two injections of large amounts of the rough variant from the smooth mucoid Pretoria North variant immunized guinea-pigs solidly against a massive dose of smooth mucoid Boshoff. Considerable resistance was shown also to a large test dose of virulent Drummond.

Summary.—(1) A slowly dissociating smooth mucoid variant was isolated from the virulent " Pretoria North " strain, and a rough variant was isolated from the smooth mucoid strain.

(2) The smooth mucoid strain possessed a slight degree of virulence for guinea pigs and mice, whereas the rough variant was completely avirulent.

(3) One inoculation of the rough variant considerably increased the resistance of guinea pigs to the smooth mucoid Boshoff strain, while two injections immunized solidly against this strain.

(4) Two injections of the avirulent rough increased the resistance of guinea pigs to a large test dose of V. Drummond; but did not immunize solidly against it.

B. Dissociation in Attenuated Anthrax Strains.

(1) *Dissociation in a strain partially attenuated at 42° C.*

This strain was isolated six months previously from a pig which had died of anthrax naturally acquired, and was known as strain IV. It was incubated at 42° C. in broth, and after sixteen days the cultures were sealed and stocked at room temperature. Subcultures made two months later showed smooth mucoid growth, although the mucoid characteristic was not particularly marked. Rough wedges were sometimes seen in the smooth colonies, but the dissociation was not very active. A somewhat rough variant was picked from the smooth strain; smears from it showed no capsules, although a few developed when the variant was grown in 65 per cent. carbon dioxide. It was not therefore a pure rough.

Pathogenicity tests with variants of strain IV.

Table XXIII.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 40	80	120	160	200	240	280	320
6	⅓ slant smooth strain from strain IV		x						
			x	x					
			x	x	x				
3	⅓ slant 6th subculture rough variant from smooth								/
									/
									/
3	⅓ slant 3rd subculture rough variant								/
									/
						x			

The smooth strain thus proved virulent whereas the rough variants were practically avirulent. The virulence of the smooth strain was not very great, since in spite of the large doses given, the first deaths occurred at about the 90th hour. The rough variant produced a few capsules in carbon dioxide and its very slight virulence was possibly due to a small dose of smooth bacilli included with it.

Immunity tests with variants of strain IV.—Fourteen days after the experiment noted in table XXIII the five guinea pigs which survived received a second dose of the rough variants. One guinea-pig died of an intercurrent infection. Two weeks after the second injection, the four remaining guinea-pigs, together with four controls, were tested with the smooth mucoid strain of Boshoff. The results were as follows:—

TABLE XXIV.

Each previously inoculated with:	No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation.							
			20	40	60	80	100	120	140	160
2 injections rough variant from smooth strain IV	2	$\frac{1}{5}$ agar slant smooth mucoid Boshoff					x			/
2 injections rough variant	2	$\frac{1}{5}$ agar slant smooth mucoid Boshoff					x			x
Uninoculated controls	4	$\frac{1}{5}$ agar slant smooth mucoid Boshoff		x x x x						

Therefore two injections of the rough variants from Strain IV gave guinea pigs a much increased resistance to the smooth mucoid Boshoff strain, but a solid immunity was not established.

Summary.—(1) A smooth variant was isolated from a partly attenuated anthrax strain and rough variants were isolated from this smooth strain.

(2) The smooth variant was moderately virulent for guinea pigs, whereas the rough variant was practically avirulent. The slight residual virulence of the rough variant was prabably due to contaminating smooth forms

(3) Guinea-pigs which received two large injections of the rough variants were much more resistant (Table XXIV) to a large dose of smooth mucoid Boshoff than were uninoculated controls.

(2) *Dissociation in a strain of anthrax attenuated at* 42° *C.*

Strain 568 was grown in broth at 42° C. until 1 c.c. of the broth culture failed to kill a rabbit. This took 42 days. A loopful of the broth culture was streaked onto a Mason's tube (Mason 1933) of nutrient agar. This type of culture tube has a flat bottom and is more convenient than a Petrie plate. Rough and rough-smooth colonies grew in the streak. One of each type was selected and subcultured until the differentiation was more complete, and by the tenth subculture the RS colony type had become slightly smooth mucoid. A virulence test was then carried out as follows:—

Three guinea pigs were each inoculated with $\frac{1}{3}$rd of a slant of the smooth variant.

Six mice were each inoculated with 1/20th of a slant of the smooth variant.

Three guinea pigs were each inoculated with $\frac{1}{3}$rd of a slant of the rough variant.

Six mice were each inoculated with 1/20th of a slant of the rough variant.

All the mice and all the guinea pigs survived.

Immunity test with variants of attenuated strain.—The immunity of the six surviving guinea pigs was tested as follows: –

TABLE XXV.

Each previously inoculated with :	No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	80
$\frac{1}{3}$ slant smooth variant	3	$\frac{1}{4}$ slant smooth mucoid Boshoff	x	x x		
$\frac{1}{3}$ slant rough variant	3	$\frac{1}{4}$ slant smooth mucoid Boshoff		x x	x	
Uninoculated controls	4	$\frac{1}{4}$ slant smooth mucoid Boshoff		x x x x		

Therefore inoculated guinea pigs were not more resistant than the controls.

Summary.—(1) A smooth variant and a rough variant were obtained from an attenuated anthrax strain.

(2) Neither variant was virulent for guinea pigs or mice, and neither variant gave guinea pigs any protection against a test dose of smooth mucoid Boshoff.

C. DISSOCIATION OF VIRULENT ANTHRAX STRAINS IN CARBON DIOXIDE.

In none of the foregoing experiments was the rough dissociant from a smooth or smooth mucoid colony the more virulent of the two strains. In most cases the rough variant showed a marked decrease in virulence as compared with its smooth or smooth mucoid parent, and frequently the rough variant was completely avirulent although the smooth mucoid parent strain had retained its virulence to a greater or lesser extent. At one end of the scale there was an almost fully virulent smooth mucoid strain V. Boshoff which gave rise abruptly to a completely avirulent rough dissociant; while at the other end of the scale were cases where both the smooth and the rough variants were avirulent. In no case, however, was the rough variant more virulent than the smooth parent. Smooth or smooth mucoid variants did not appear in fully virulent strains until these had been subjected to one or another of certain rather

lengthy procedures. (Prolonged storage, attenuation at 42° C., prolonged cultivation, etc.). These procedures undoubtedly affected the pathogenicity of the strains to a considerable degree. It was also clear that none of the smooth or smooth mucoid strains or variants could be considered fully virulent, so that in the experiments which have been presented, the S➤R dissociations observed were dissociations occurring in strains that were already to some extent attenuated.

At the same time it was abundantly clear that the further dissociation of the smooth variants resulted in an abrupt change of colony form and virulence, quite comparable with the S➤R variations in other strains of micro-organisms. The question therefore was whether the S➤R dissociation in smooth mucoid strains of anthrax had a more general applicability to variation in *B. anthracis*, or whether this S➤R change was limited to the relatively attenuated smooth strains which have already been discussed.

There was never much doubt as to the extreme roughness of the fully virulent anthrax strain, and there could also be little doubt that subcultivation and attenuation resulted in a less rough strain, and in general a less virulent strain. It was therefore difficult to see what part the S➤R dissociation as seen in the smooth mucoid strains could play in the general behaviour of the *Bacillus anthracis*. As things were, it was not legitimate to extend the results obtained here and to state categorically that the S➤R change in anthrax was associated with a change from the more virulent to the less virulent state, as such a statement would have conflicted with numerous observations by many workers on the problem. It was not improbable that the present series of observations was actually of limited applicability and had no wider bearing on anthrax dissociation. Nevertheless the results were clear cut and showed so close an analogy with dissociation phenomena as seen in other pathogenic micro-organisms that there seemed a strong likelihood of these observations having a greater generality that the actual experiments suggested.

The work of Felix et al (1934) on the virulence antigen of *S, typhi* suggested a possible relationship between the S➤R dissociations as seen here in much subcultured strains of anthrax and the very rough appearance of the fully virulent strains. Felix and his co-workers noticed that if a fully virulent smooth typhoid strain was grown at a temperature of 25° C. it became quite rough and at the same time lost virulence. When, however a subculture from this rough avirulent strain was grown at 37° C. there was an immediate return to the smooth colony structure and full virulence. Thus this particular type of S➤R variation was conditioned by a simple environmental factor and was fully and immediately reversible. The change, appareutly, had not affected the genetic structure of the organism in any way.

In the light of the author's experience with smooth mucoid strains of anthrax and their rough variants, it was thought possible that the ordinary rough appearance of a virulent anthrax strain grown under the usual laboratory conditions was the expression of a somewhat similar mechanism to that operating in the case of *S. typhi*

when grown at 25° C. The assumption was that the rough virulent anthrax strain would be smooth or smooth mucoid if a suitable environment offered, but that the usual conditions under which anthrax was grown in the laboratory was an unfavourable environment analogous to that obtaining in the case of *S. typhi* grown at 25° C.

An alternative hypothesis was that the rough virulent anthrax strain was virulent by virtue of rare highly pathogenic smooth organisms present amongst avirulent bacilli. This supposition was easily disproved by making single cell isolations from rough virulent strains. These isolations were in all cases as rough and as virulent as the parent strain.

The problem, therefore, was to devise an environment in which the rough virulent strain would appear smooth or smooth mucoid. Such conditions would enable the problem of dissociation to be studied in less artificial circumstances than before: that is to say circumstances which involved long continued cultivation under unfavourable conditions.

It was shown in Part I of this paper that rough virulent anthrax strains developed capsules and tended to be smooth mucoid when grown in certain concentrations of carbon dioxide. The assumption was made that this was the " real " appearance of the virulent strain. Therefore the fully virulent rough strains were grown in carbon dioxide to see whether they would become mucoid and to see then whether rough variants would occur naturally in these cultures.

Technique.—A Mason's tube (Mason, 1933), of nutrient agar pH 7·4 was inoculated with a virulent strain of anthrax. The inoculum was spread over a small patch of the medium (2 sq. cm.) at about the centre of the agar surface. This procedure was adopted to enable the growing edge of the culture to be kept under observation for some time. After inoculation the culture tubes were incubated in an atmosphere of 65 per cent. carbon dioxide and observed daily. This part of the experiment was carried out as described in Part I.

(1) *Dissociation of virulent Pretoria North in* 65 *per cent. carbon dioxide.*

The rough virulent Pretoria North strain was grown in 65 per cent. carbon dioxide according to the technique stated above. The growth became smooth mucoid and spread gradually. On the fifteenth day a flat, dry, sharply demarcated rough outgrowth appeared at a spot on the periphery of the colony. The rough outgrowth from the smooth mucoid culture was subcultured onto agar in a Mason's tube. At the same time another tube was inoculated from the smooth mucoid part of the culture and a third tube was inoculated with a rough virulent strain—V. Drummond. The three cultures were incubated in 65 per cent. carbon dioxide. The first tube showed only rough growth and no capsules. The other two tubes showed patches of smooth mucoid growth with numerous capsuled bacilli. Each strain was then subcultured onto agar and incubated in air. All

grew rough and uncapsuled and were morphologically indistinguishable from one another. Thus the fact that the first strain (the rough variant) differed from the other two strains was not detectable in cultures grown under ordinary atmospheric conditions; but this difference immediately became patent when the strains were grown in carbon dioxide.

Pathogenicity of variants of Pretoria North grown in CO_2.—The rough variant isolated from the strain which grew smooth mucoid in carbon dioxide was grown on agar in air. At the same time a subculture from the smooth mucoid growth and a subculture from the original stock virulent Pretoria North strain were grown in air. All three cultures appeared rough and morphologically indistinguishable from one another. Guinea pigs were then inoculated as follows:—

TABLE XXVI.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation.							
		20	40	60	80	100	120	140	160
3	⅓ slant original stock V. Pretoria North		x x x						
3	⅓ slant subculture from smooth mucoid in CO_2		x x x						
15	⅓ slant subculture from rough variant picked in CO_2			x x x	x x x		x x x x	x x	/ / /

Three weeks later the surviving guinea pigs, together with six uninoculated controls each received $^1/_5$th of an agar slant of the smooth mucoid variant of the Boshoff strain.

2 Inoculated guinea pigs were dead by the 40th hour.

1 Inoculated guinea pig survived.

6 Controls were all dead by the 40th hour.

Summary.—(1) A rough virulent strain of anthrax grew smooth mucoid in carbon dioxide and gave rise to a rough variant.

(2) Both the strain which was smooth mucoid in carbon dioxide and its rough variant were rough in air; but the rough variant was distinctly less virulent for guinea pigs.

(3) The rough variant was still able to produce capsules *in vivo* although these had not been detectable in carbon dioxide (Table XXVI).

(4) Guinea pigs which survived an inoculation of the variant which was rough in carbon dioxide were hardly more resistant than uninoculated controls to an injection of the smooth mucoid Boshoff strain.

(2) *Dissociation of the virulent Drummond strain in carbon dioxide.*

The experiment was repeated using " Virulent Drummond "; a very rough strain. After a week in carbon dioxide the growth was almost completely smooth mucoid and a week later a rough outgrowth was picked which failed to produce capsules in carbon dioxide. This was now termed the " rough " variant and a subculture was grown in air. At the same time a subculture from the smooth mucoid portion of the growth in carbon dioxide was incubated in air and this was termed the " smooth " variant. Both variants appeared quite rough after growing in air. These two cultures were then injected into guinea pigs with the following results.

TABLE XXVII.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	80
3	⅓ slant rough variant		xxx		
3	⅓ slant smooth varian		x x x		

The " rough " variant seemed the less virulent, and in view of the large doses administered the difference in survival time was probably significant. As the guinea pigs were inoculated with the first subcultures of the variants it was possible that the rough variant might have been avirulent, but mixed with some virulent elements. The presence of a few virulent elements would explain both the virulence of the rough variant and also the fact that it took significantly longer to kill than the " smooth " variant. To test this three guinea pigs were inoculated with ⅓rd of a slant of the smooth variant and another three with ⅓ of a slant isolated from the blood of one of the guinea pigs which died after being injected with the rough variant in the previous test. If the rough variant was a mixture of an avirulent strain and a virulent strain, then the strain re-isolated from the guinea pig should have been fully virulent. The results of the test were as follows: —

TABLE XXVIII.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	
3	⅓ slant rough strain isolated from guinea pig				xxx
3	⅓ slant smooth strain		xxx		

Thus passage through a guinea pig did not enhance the virulence of the rough variant, and therefore the virulence of the rough was probably not due to a mixture of avirulent and virulent elements.

Each strain was then subcultured a number of times in rapid succession; the " smooth " strain nine and the " rough " strain seven times. Guinea pigs were then inoculated as follows:—

TABLE XXIX.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 40	80	120	160
3	⅓ slant of 7th subculture of rough variant			x x	x
2	⅓ slant 9th subculture of smooth variant	x x			

Again the guinea pigs inoculated with the rough variant took much longer to die than those which received the smooth variant. In all three tests, therefore, none of the guinea pigs inoculated with the rough strain commenced to die until some time after all those inoculated with the " smooth " variant had died. In view of the large doses given the results were probably significant. As far as could be judged from the small number of animals used, the rough strain appeared fairly stable at the reduced level of virulence.

Neither a passage through a guinea pig nor a period of rapid subculturing affected the relative virulence of the two strains to any extent. Although the rough variant produced capsules *in vivo* these were not detectable in the cultures grown either in carbon dioxide or in air.

Summary.—(1) A rough virulent strain grown in carbon dioxide became smooth mucoid.

(2) A rough variant was isolated from this smooth mucoid grown in carbon dioxide.

(3) When grown in air, subcultures from both the smooth mucoid growth and from the rough growth in carbon dioxide were rough and morphologically indistinguishable from one another.

(4) The rough variant obtained from the smooth mucoid in carbon dioxide took a significantly longer time to kill guinea-pigs than the subculture from the mucoid growth in carbon dioxide.

(3) *Dissociation of virulent anthrax strain* 568 *in carbon dioxide.*

After seven days in carbon dioxide differentiation into mucoid and non-mucoid growth was not marked. However, cultures were made from the more mucoid and non-mucoid portions. Each variant was subcultured three times in four days and then injected into guinea-pigs as follows:—

TABLE XXX.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60
3	⅓ slant rough variant		xx	x
3	⅓ slant smooth varian	x x	x	

The difference in virulence was slight and it was impossible to say whether it was significant or not. Because of the large doses given, and bceause the original stock rough was very virulent and always killed guinea pigs in from 30-40 hours, it is possible that the rough variant was slightly less virulent than the smooth.

After 14 days the " smooth " variant and the " rough " variant used in Table XXX were again subcultured onto agar in air and injected into guinea pigs. The results were as follows: —

TABLE XXXI.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60
3	⅓ slant rough variant		x x x	
3	⅓ slant smooth variant		x x x	

The difference in virulence was again slight; but again the rough variant seemed a little less virulent. The slight differences noted in Tables XXX and XXXI did not warrant any conclusion as to the relative pathogenicity of the variants.

Summary.—(1) A virulent strain of anthrax developed a patchy smooth mucoid growth in carbon dioxide.

(2) It was not possible to demonstrate a significant difference in virulence between the smooth mucoid and the non-mucoid portions of the culture.

D. Dissociation of Virulent Strains of Anthrax on Serum Agar in Carbon Dioxide.

In the previous series of experiments (C) a certain amount of success was obtained in an attempt to pick relatively attenuated variants from virulent strains grown in carbon dioxide. However, this technique did not yield completely non-pathogenic strains, and it was realized that the inability to produce a detectable number of capsules in carbon dioxide was not an index of complete lack of ability to produce capsules *in vivo* because the less virulent strains which appeared rough and uncapsuled in carbon dioxide, were able to produce capsules *in vivo*. It was realized that the carbon dioxide

was not as good an environment for the production of capsules as at first thought, and did not approach *in vivo* conditions in this respect. For although fully virulent rough strains became fairly mucoid and formed large numbers of capsules in the carbon dioxide, some strains were not able to produce capsules although still virulent.

It was previously established that the complete avirulence of some rough strains was associated with the loss of ability to develop capsules and that these rough strains were derived from smooth mucoid strains; therefore a medium was sought in which the fully virulent strain would grow completely smooth mucoid, and in which rough variants, if present, could be picked with a fair degree of certainty that their roughness indicated inability to become capsuled, and possibly avirulence.

According to Bail (1915) the virulent anthrax bacillus regularly produced capsules in liquid serum. A liquid medium was, however, of no use in the study of colony variation, and it was also found that capsule production by different virulent strains in serum varied considerably. A number of experiments were carried out in which virulent strains were grown on inspissated serum. It was found that about 20-30 per cent. of the organisms developed capsules, but that the strains were not regularly smooth mucoid. Moreover, the liquefaction of the serum by the bacilli complicated the observation of the growth to a considerable extent. The opacity of the inspissated serum also made the medium unsatisfactory. Further experiments were carried out in which the strains were grown on 50 per cent. horse serum agar. All the virulent strains were rough, but most of the cultures had rather a shiny surface, and by the 4th day about 20 per cent. of the virulent bacilli showed capsules. Laboratory strains with any tendency to produce mucoid growth on ordinary agar, developed a richly mucoid structure on the 50 per cent. serum agar and all the bacilli of such strains developed capsules. Strains like the rough avirulent Boshoff remained rough and uncapsuled on this medium. The 50 per cent. serum agar was also firm and transparent. Rough virulent strains did not, however, grow mucoid on this medium, although they showed an increased number of capsules. Since virulent strains tended to develop a larger number of capsules on the serum agar, the effect of a combination of this medium and an atmosphere of 65 per cent. carbon dioxide was investigated.

Several rough virulent strains were streaked on 50 per cent. unheated horse serum agar contained in Mason's tubes. The tubes were placed in an atmosphere of 65 per cent. carbon dioxide immediately after inoculation, and incubated at 37° C. After 24 hours all these virulent strains were entirely smooth mucoid and showed no sign of a rough structure; but when the smooth mucoid cultures were subcultured and incubated in air they again showed the normal very rough appearance of the virulent strains. The facility and regularity with which these results could be repeated suggested that this technique fulfilled the desired requirements. Moreover, fairly wide divergences in the carbon dioxide concentration did not materially affect the results. Accordingly, several experiments were carried out on the same lines as those performed in Sectoin C, except that 50 per cent. serum agar was used instead of nutrient agar.

(1) *Dissociation of strain XIV on 50 per cent. serum agar in 65 per cent. carbon dioxide.*

This strain was isolated a month previously from a bovine naturally infected with anthrax, and had not been used in any experimental work. Culturally it had the characteristic very rough appearance of a recently isolated virulent strain. A loopful was streaked over a small area on 50 per cent. serum agar and incubated in 65 per cent. carbon dioxide. The growth was completely smooth mucoid after 24 hours. Two days later two rough sectors were present in the smooth mucoid colony. These were dull, flat and had the typical cut glass appearance of a rough anthrax strain. There was a sharp division between these rough sectors and the surrounding mucoid culture.

The smooth mucoid growth and the rough outgrowths were each streaked onto a tube of 50 per cent, serum agar and incubated in 65 per cent. carbon dioxide. The subculture from the smooth mucoid growth was again completely smooth mucoid, and all the bacilli were capsuled; whereas the subculture from a rough wedge was rough and the bacilli showed no capsules. Each variant was then streaked on ordinary agar and incubated in air. Both grew rough on this medium and were morphologically indistinguishable from each other. They were subcultured once more on agar in air and each was again rough. The virulence was then tested on guinea pigs as follows:—

Three guinea pigs each received $\frac{1}{4}$ slant of the rough strain derived from the smooth mucoid growth in carbon dioxide. One died of anthrax after six days.

Three guinea pigs each received $\frac{1}{4}$ slant of the rough strain derived from the rough growth in carbon dioxide. All remained alive.

Both strains seemed to have become practically avirulent, although the variant derived from the smooth mucoid growth in carbon dioxide killed a guinea pig in six days. The virulence of these strains was investigated in somewhat greater detail, because of the known pathogenicity of the parent strain XIV.

The two strains, the one from the smooth mucoid and the other from the rough growth in carbon dioxide, were each streaked on 10 per cent. serum agar and incubated at 37° C. in air. After 24 hours the growth of each strain was rough and guinea pigs were injected as follows:—

Three guinea pigs inoculated with $\frac{1}{3}$rd slant of strain originally isolated from smooth mucoid growth in carbon dioxide—all survived.

The guinea pigs inoculated with $\frac{1}{3}$rd slant of strain originally isolated from rough growth in carbon dioxide—all survived.

This experiment confirmed the previous one. The possibility was considered that the loss of virulence might be due to the strain which had been mucoid in carbon dioxide losing this property during the course of the experiments. Therefore each of the strains was again grown on 50 per cent, serum agar in carbon dioxide, three weeks after the first isolation of the variants. The variant originally

isolated from the smooth mucoid growth in carbon dioxide was again smooth mucoid and the variant originally rough in carbon dioxide was again rough and uncapsuled. Thus neither of these variants had altered as regards its colony morphology on 50 per cent, serum agar in carbon dioxide.

A further test of the virulence of the two strains was then performed as follows: each variant was grown on 50 per cent. serum agar in carbon dioxide. After 24 hours the one was smooth mucoid and the other rough. They were washed off and injected into guinea pigs.

Three guinea pigs each received 1/5th slant of smooth mucoid growth in carbon dioxide. All survived.

Three guinea pigs each received 1/5th slant of rough growth in carbon dioxide. All survived.

Three guinea pigs were each inoculated with 1/3rd slant of an agar slant of a culture made from the only guinea pig which died of anthrax in this series of experiments with strain XIV. All these guinea pigs survived.

It was clear, therefore, that this strain had almost completely lost virulence for guinea pigs. Neither the variant from the smooth mucoid growth in carbon dioxide, nor the variant from the rough growth in carbon dioxide was virulent for guinea pigs. The direct inoculation of the smooth mucoid growth harvested from 50 per cent. serum agar in carbon dioxide was also avirulent, and one passage through a guinea pig failed to restore the virulence of this strain.

To see if there was any difference in virulence between the strains, experiments were carried out in mice. The combined results of two experiments are summarized in Table XXXII.

TABLE XXXII.

No. of mice.	Each inoculated with:	Death: Hours after inoculation.						
		20	40	60	80	100	120	
22	1/24th agar slant of variant from smooth mucoid in carbon dioxide							/
								/
			x					/
			x			x		/
			x			x		/
			x	x	x	x		/
		x	x	x	x	x		/
								x
22	1/24th slant variant from rough in carbon dioxide	All survived.						

It was clear that the variant derived from the smooth mucoid growth in carbon dioxide was moderately virulent for mice whereas the " rough " variant was avirulent.

Immunity tests with variants of strain XIV.—The resistance of the guinea pigs which survived the inoculation of the variants of strain XIV obtained in carbon dioxide was tested by inoculating them with a large dose of smooth mucoid Boshoff. No immunity was demonstrable in either group (see Table XXXIII).

TABLE XXXIII.

No. of guinea pigs.	Previously immunized with :	Immunity tested with :	Death : Hours after inoculation.					
			20	40	60	80	100	120
6	¼ agar slant strain XIV derived from mucoid growth in CO_2	⅙ serum agar slant smooth mucoid Boshoff starin		x				
				x	x			
				x	x	x		
9	¼ agar slant strain XIV from rough growth in CO_2	⅙ serum agar slant smooth mucoid Boshoff strain		x				
				x				
				x		x		
				x	x	x	x	
9	Uninoculated controls	⅙ serum agar slant smooth mucoid Boshoff strain		x	x			
				x	x			
				x	x			
				x	x	x		

Summary.—A rough virulent strain was grown on 50 per cent. serum agar in carbon dioxide and a smooth mucoid growth resulted. After a day rough dissociants appeared. The smooth mucoid and the rough variant were sown separately onto plain agar in air and both grew rough. Neither culture was virulent for guinea pigs, but the strain derived from the smooth mucoid culture in carbon dioxide was moderately virulent for mice, the other strain being completely avirulent. Guinea pigs which had received a large single dose of these variants were not resistant to a large test dose of the smooth mucoid Boshoff strain.

The whole experiment with virulent strain XIV was then repeated. After 24 hours in carbon dioxide the growth was again smooth mucoid and a day later rough sectors and outgrowths appeared in the smooth colony. Smears made from the smooth mucoid growth showed 100 per cent. capsuled bacilli, smears from the rough growth showed about 5 per cent. capsuled bacilli. Each type of growth was streaked onto ordinary agar and incubated in air at 37° C. Under these conditions both variants were very rough. Each was then subcultured twice onto agar in air and guinea-pigs were injected as shown in Table XXXIV. The total time between the seeding of the virulent strain XIV on to serum agar and the inoculation of the guinea pigs was four days. That is—after one day the growth was smooth mucoid; after the second day the variants were streaked on plain agar; on the 3rd and the 4th day the variants were subcultured, and the guinea pigs were inoculated on the 5th day.

TABLE XXXIV.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60
3	¼ agar slant variant from smooth mucoid in CO_2		x x x	
3	¼ slant variant from rough in CO_2			/ / /

A week later the test was repeated.

TABLE XXXV.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60
3	¼ slant variant from smooth mucoid in CO_2		x x x	
3	¼ slant from rough in CO_2			/ / /

Each variant was then kept on agar slants in a refrigerator for two months, and then subcultured. These subcultures were both rough. Guinea pigs were inoculated with these cultures as shown below. At the same time each variant was grown on 50 per cent. serum agar in 65 per cent. carbon dioxide. The variant which had previously been smooth mucoid under these conditions was again smooth mucoid and capsuled; the other was again rough and uncapsuled.

TABLE XXXVI.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60
3	⅕ slant variant from smooth mucoid in CO_2		x x	x
3	¼ slant variant from rough in CO_2			/ / /

Thus it was possible to isolate an avirulent rough uncapsuled variant from a smooth mucoid virulent strain, in carbon dioxide, although both strains were very rough when grown in air. These strains were unchanged after two months storage, both as regards their colony morphology in air and in carbon dioxide and as regards their relative virulence (Table XXXVI).

Immunity tests with variants of strain XIV.—Three guinea pigs which survived an inoculation with the rough variant (Table XXXIV) received a second large inoculation of the same strain 18 days later. Two weeks later an immunity test was carried out on these three guinea pigs and on three guinea pigs which had received one inoculation of the rough variant (Table XXXV). The smooth mucoid Boshoff strain was again used as the test organism.

TABLE XXXVII.

No. of guinea pigs.	Previous inoculations.	Each inoculated with :	Death : Hours after inoculation. 20	30	40	50	60	
3	*Two* injections rough variant strain XIV	$\frac{1}{5}$ slant smooth mucoid Boshoff						/ / /
3	*One* injection rough variant strain XIV	$\frac{1}{5}$ slant smooth mucoid Boshoff						/ / /
6	Uninoculated controls	$\frac{1}{5}$ slant smooth mucoid Boshoff			x x x x	x x x		

All the guinea pigs previously inoculated with the rough variant survived. Moreover, this was the first occasion on which one injection of an immunizing strain protected solidly against the Boshoff strain. There was evidence that the virulence of the Boshoff strain had decreased since the early experiments; but up to the present no guinea pigs inoculated with this strain had survived, unless previously immunized.

Six weeks later nine guinea pigs were each inoculated with $\frac{1}{4}$ of an agar slant of the avirulent variant of strain XIV and their immunity tested after three weeks. Three test strains were used. (1) Smooth mucoid Boshoff, which had been used in the majority of the tests, but was now losing virulence. (2) Virulent XVIII recently isolated and virulent. (3) Virulent Boshoff—old stock strain—virulent for rabbits and sheep, but had decreased in virulence since its isolation many years ago and was now less virulent than XVIII.

TABLE XXXVIII.

No. of guinea pigs.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 20	40	60	80	100	120	140	160
3	¼ slant avirulent rough XIV	1 slant smooth mucoid Boshoff					x			/ /
6	Uninoculated controls	1 slant smooth mucoid Boshoff			x x x	x x x				
3	¼ slant avirulent rough XIV	1/12th slant XVIII			x x					x
6	Uninoculated controls	1/12th slant XVIII		x x x x x x						
3	¼ slant avirulent rough XIV	⅕ slant virulent Boshoff				x		x x		
3	Uninoculated controls	⅕ slant virulent Boshoff		x x x						

It seemed that the smooth mucoid Boshoff strain had decreased further in virulence since the last test, as the controls took considerably longer to die than before. The fact that two out of three immunized guinea pigs survived must be considered in relation to this. It was probable that the immunizing strain had lost some potency, since in Table XXXVII none of the immunized guinea pigs died although the test culture seemed more virulent than in the present experiment. For the rest, the immunized guinea pigs all survived considerably longer than the controls although tested with very large doses of virulent strains.

Summary.—Strain XIV grew smooth mucoid on serum agar in carbon dioxide and readily produced rough variants. Subsultures from the smooth variant onto agar in air were rough and in one case virulent. Subcultures from the rough variant were also rough in air but were completely avirulent for guinea pigs. Guinea pigs which received one inoculation of this avirulent strain showed a considerable resistance to a subsequent inoculation of virulent culture. It should be noted that in the first experiment where both the smooth mucoid and the rough variant of XIV were avirulent, neither produced an appreciable immunity. (Table XXXIII).

Dissociation of virulent strain XIV on 50 *per cent. serum* agar.—The successful dissociation of strain XIV on serum agar in carbon dioxide suggested that 50 per cent. serum agar alone might be used. Strain XIV was therefore streaked onto a small patch on the surface of 50 per cent. serum agar and incubated in air at 37° C. After 24 hours the growth was rough. After 48 hours the colony was rough and rather opaque, but one spot near the periphery of the growth appeared flatter and more translucent. Each type of growth was streaked onto agar slants and incubated in air. After two more subcultures guinea pigs were inoculated as follows:—

TABLE XXXIX.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60
3	¼ slant from opaque centre of colony on scrum agar		x	x x
3	¼ slant from translucent wedge at margin of colony on serum agar		x x x	

The experiment was repeated; again the colony on serum agar showed an opaque centre, and a sharply demarcated more translucent wedge at the margin. Each type of growth was injected into guinea pigs as follows:—

TABLE XL.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60
3	¼ slant isolated from opaque centre..		x x x	
3	¼ slant isolated from translucent margin		x x	x

A further attempt was made to obtain variants differing in virulence. In this case growth was allowed to proceed for seven days; there was then a fairly clear-cut differentiation into a translucent centre which projected in a number of rays to the margin of the colony, and rough, flat and dull sectors between. Each type of growth was injected into guinea pigs as follows:—

TABLE XLI.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60	80
3	¼ slant subculture from translucent centre part of culture	x	x	x	
3	¼ slant subculture from rough part of culture		x x	x	

Thus strain XIV did not show a homogeneous colony morphology on the serum agar: the differences were fairly sharp, but the extent to which these differences constituted dissociation was doubtful. No difference in virulence could be detected, nor did the different types of growth retain their characteristics on subcultivation. Smears from the cultures on serum agar showed a few capsuled organisms, although none could be found on plain agar. These capsules were not restricted to any particular area of the colony. When subcultures from the different types of growth were grown on 50 per cent. serum agar in 65 per cent. carbon dioxide, all developed a highly mucoid structure and all the bacilli showed capsules after 24 hours. Therefore the serum medium alone, without CO_2, was not suitable for differentiating variants.

(2) *Dissociation of virulent Drummond strain (Strain VII) on* 50 *per cent. serum agar in carbon dioxide.*

This strain was used in some of the earlier experiments on dissociation. It was still very virulent and doses of the order of one hundred thousandth of an agar slant killed sheep in twenty-four hours. However, the strain was not very stable, and suspensions in glycerine-saline lost virulence fairly rapidly. The same technique was adopted as in the experiments with strain XIV. After 24 hours incubation in carbon dioxide the growth was smooth mucoid. Twenty-four hours later a few rough outgrowths appeared at the periphery of the smooth growth and on the third day the rough growth had extended somewhat.

Each type of growth was streaked onto an agar slant in air and incubated. Both variants grew very rough. Two further subcultures were made on agar in air on successive days and guinea pigs were then inoculated with large doses of each variant, as shown in the table below. At the same time each variant was streaked onto the surface of a tube of 50 per cent. serum agar and incubated in 65 per cent. CO_2. *Both* variants grew smooth mucoid in the carbon dioxide.

TABLE XLII.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60
3	$\frac{1}{3}$ slant variant from smooth in CO_2	x x	x x	
3	$\frac{1}{3}$ slant variant from rough in CO_2	x x	 x	

The test was repeated two days later with the next subcultures of the two variants.

50 per cent. serum agar slants were inoculated at the same time and incubated in 65 per cent. carbon dioxide. Both variants were smooth mucoid and developed capsules.

The result of the second guinea pig test was as follows:—

TABLE XLIII.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40
3	$\frac{1}{4}$ slant variant from smooth in CO_2		x x x
3	$\frac{1}{4}$ slant variant from rough in CO_2		x x x

Both variants were virulent for guinea pigs and when streaked onto serum agar in carbon dioxide, both grew smooth mucoid and developed capsules. Thus, although the variants were originally selected because of a difference in colony morphology and capsule formation in carbon dioxide, this difference has ceased to exist when the guinea pigs were inoculated; so the fact that both strains proved virulent was not surprising. The rough variant originally selected was probably contaminated with capsuled forms at the time of picking.

The experiment was then repeated, and the very rough virulent Drummond was grown on serum agar in carbon dioxide as before. The growth was smooth mucoid after 24 hours and the following day rough outgrowths were present but no attempt was made to pick them until two days later. The culture was then clearly divisible into smooth mucoid and rough portions. Each variant was streaked onto

agar and incubated in air. After 24 hours both were very rough and guinea pigs were then inoculated as follows. At the same time each variant was streaked onto serum agar and incubated in carbon dioxide. The strain originally obtained from the smooth mucoid growth in carbon dioxide was again smooth mucoid and capsuled, while the strain from the rough variant in carbon dioxide was again rough and showed no capsules.

TABLE XLIV.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60	80
3	⅓ slant variant from smooth growth in CO_2		x x x		
3	⅓ slant variant from rough growth in CO_2				/ / /

Six weeks later subcultures were made from stored tubes of the two variants (*a*) onto agar slants in air, (*b*) onto serum agar incubated in 65 per cent. carbon dioxide. After twenty-four hours the former (*a*) were both rough, and guinea pigs were injected with the cultures as shown below (Table XLV). In the case of (*b*) the variant which had originally been smooth mucoid in carbon dioxide was again smooth mucoid and capsuled, while the other variant was rough and uncapsuled.

TABLE XLV.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60
3	¼ slant variant from smooth mucoid growth in CO_2	x	x x	
3	⅓ slant variant from rough growth in CO_2			/ / /

Summary.—The virulent Drummond strain grew smooth mucoid on 50 per cent. serum agar in 65 per cent. carbon dioxide and almost immediately commenced to throw off rough variants. When the smooth mucoid growth and the rough growth were grown on ordinary nutrient agar in air, both were very rough. When guinea pigs were injected both variants proved virulent. The two variants were then again grown on serum agar in carbon dioxide and *both* proved to be smooth mucoid. In the light of previous work with similar variants

it seemed highly probable that a considerable number of smooth elements had been picked when the rough variant was transplanted. In the second experiment (Tables XLIV and XLV) the growth in carbon dioxide was allowed to develop for two days longer before the variants were picked in order to allow a very clear differentiation between the smooth mucoid and the rough growth. The subculture from the smooth mucoid growth was virulent, while the subculture from the rough growth was avirulent. The latter result tended to confirm the opinion that the virulence of the " rough " variant previously isolated was due to contamination with smooth elements. When the virulent strain and the avirulent strain were tested after a period of six weeks (both had been kept sealed in the interim) neither the appearance of the growths on serum agar in carbon dioxide, nor the relative pathogenicities had changed.

Immunity tests with variants of Virulent Drummond.—A number of guinea-pigs inoculated with the avirulent variant were tested about a month later with the virulent strain XVIII and with the smooth mucoid Boshoff strain. The virulent test strains were those used to investigate the immunity conferred by the variant of strain XIV, and the tests were carried out at the same time so the results are strictly comparable with those shown in Table XXXVIII. The same controls were used in each case, and are included in the present table to facilitate comparison.

TABLE XLVI.

No. of guinea pigs.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 20	40	60	80	100	120	140	160
3	Two injections avirulent Drummond	1/12th slant XVIII (virulent)			x				x x	
3	One injection avirulent Drummond	1/12th slant XVIII (virulent)			x					x /
6	Untreated controls	1/12th slant XVIII (virulent)		x x x x x x						
3	One injection avirulent Drummond	1 slant smooth mucoid Boshoff								/ / /
6	Untreated controls	1 slant smooth mucoid Boshoff			x x x	x x x				

The three guinea pigs which had received an injection of the avirulent strain six weeks after the original test (see Table XLV) were inoculated with virulent XVIII after three weeks.

TABLE XLVII.

No. of guinea pigs.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 40	80	100	140	180	200
3	One injection avirulent rough Drummond	¼ slant virulent XVIII		x				/ x
9	Controls	¼ slant virulent XVIII	x x x x x x x x x					

The table shows that the rough strain had retained its power to immunize after six weeks storage. The immunity conferred by the single injection of this variant was relatively good. Nine controls were dead in from 30-40 hours. Two of the immunized guinea-pigs lived considerably longer that the controls and one survived. The test dose was a very large one and a fairly recently isolated virulent strain was used.

The results detailed in Table XLVI show that guinea pigs immunized with the rough variant of virulent Drummond were more resistant than uninoculated animals to a large test dose of virulent XVIII. Here too there was one survivor. When a less virulent strain, smooth mucoid Boshoff, was used to test the inoculated guinea pigs, all the immunized animals survived. The smooth mucoid Boshoff strain was not fully virulent and took a longer time to kill the controls than strain XVIII; nevertheless all the controls died and in no experiment of the series has any non-immunized guinea pig survived a test dose of the smooth mucoid Boshoff strain. It was certain that the three guinea pigs which received one immunizing injection resisted an inoculation of many killing doses of this test strain.

(3) *Dissociation of virulent anthrax strain XVIII on* 50 *per cent. serum agar in* 65 *per cent. carbon dioxide.*

This strain was isolated from the skin of a goat which had died of naturally acquired anthrax. On the following day some of the virulent culture was streaked on a portion of a 50 per cent. serum

agar surface and incubated in 65 per cent. carbon dioxide. After 24 hours the growth was smooth mucoid and by the following day rough outgrowths appeared at the edge of the colony. Agar slants were streaked from the smooth mucoid and from the rough parts of the colony. These were incubated at 37° C. in air and both grew very rough. Three guinea pigs were then inoculated with each variant. All the guinea pigs died at about the same time so that the variants showed no difference in virulence.

The original strain XVIII (now about a week old) was again streaked onto 50 per cent. serum agar and incubated in 65 per cent. carbon dioxide. After 24 hours the growth was smooth mucoid and on the following day rough outgrowths appeared. The smooth mucoid growth and a rough outgrowth were each streaked onto 50 per cent. serum agar and incubated in 65 per cent. carbon dioxide. After 24 hours the former showed a mixture of smooth mucoid and rough growth and the latter was entirely rough. The smooth mucoid and the rough were again each streaked onto serum agar in carbon dioxide and again after 24 hours the former was smooth mucoid and capsuled and the latter rough and uncapsuled. Each type was then streaked on plain agar in air and after 24 hours *both* were very rough. Guinea-pigs were inoculated with the cultures grown on agar in air. Both these cultures were rough and morphologically indistinguishable from one another, although the parent cultures in carbon dioxide differed so markedly. The result of the guinea pig test was as follows:—

TABLE XLVIII.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 20	40	60	80
3	¼ slant variant from smooth mucoid growth in carbon dioxide		x x x		
3	¼ slant variant from rough growth in CO_2				/ / /

Thus there was a clear cut difference in virulence between the variants. Six more guinea pigs were then each inoculated with ¼ slant of the " rough " variant and all survived.

After six weeks' storage each variant was streaked on an agar slant and both grew rough. At the same time each was streaked on 50 per cent. serum agar and incubated in 65 per cent. carbon dioxide. The variant originally derived from the smooth mucoid growth in carbon dioxide was smooth mucoid and capsuled, whereas the other variant was again rough and uncapsuled. The subcultures on agar grown in air were then tested on guinea pigs as follows:—

TABLE XLIX.

No. of guinea pigs.	Each inoculated with :	Death : Hours after inoculation. 20	40	60	80
3	⅓ slant variant from smooth mucoid growth in carbon dioxide		x x x		
3	⅓ slant variant from rough growth in CO_2				/ / /

This confirmed the previous test and showed that the distinctive characteristics of the variants had been retained in cultures stored for six weeks.

Immunity tests with variants of strain XVIII.—The immunity of a number of guinea pigs previously inoculated with the rough avirulent variant of strain XVIII was tested about six weeks later. The virulent strains were those used in the tests on strain VII and XIV (Tables XXXVIII and XLVI), and the controls were also the same.

TABLE L.

No. of guinea pigs.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 20	40	60	80	100	140	180	200	240	280
3	Two injections avirulent rough XVIII	1/12th slant virulent XVIII			x	x	x					
3	One injection avirulent rough XVIII	1/12th slant virulent XVIII										/ / /
6	Controls	1/12th slant virulent XVIII		x x x x x x								
3	One injection avirulent rough XVIII	1 slant smooth mucoid Boshoff										/ / /
6	Controls	1 slant smooth mucoid Boshoff			x x x	x x x						

The tests on the immunity produced by the rough avirulent variant of strain XVIII were strictly comparable with the tests on strains XIV and VII, since they were carried out at the same time and with the same virulent test strains. Three of the six guinea-pigs tested against a large dose of virulent XVIII survived and the other three guinea pigs lived considerably longer than the controls. This was a severe test since strain XVIII was a fully virulent and fairly recently isolated strain. The three pigs tested against the less virulent smooth mucoid Boshoff strain all survived. When the results are compared with those obtained with strains XIV and VII (Tables XXXVIII and XLVI) it seems as if the rough variant of XVIII gave the best immunity.

The three guinea pigs which survived an injection of the rough avirulent variant of strain XVIII six weeks after the others (Table XLIX) were tested four weeks later with virulent XVIII.

TABLE LI.

No. of guinea pigs.	Previous Inoculations.	Each injected with :	Death : Hours after inoculation. 40	80	120	160	200	240	280	320	360	400
3	1 injection avirulent rough XVIII	¼ slant virulent XVIII					x			x		/
9	Controls	¼ slant virulent XVIII	x x x x x x x x x									

The guinea pigs immunized with the rough strain which had been kept for six weeks showed a high degree of resistance to virulent XVIII. One survived, one lasted more than a week and one a fortnight. All the controls died in 30-40 hours. This result must be compared with Table XLVII as the tests were done together. There seemed little difference in the immunity produced by the two strains, but again strain XVIII appeared a little better.

Summary.—As in the case of strains XIV and VII, strain XVIII dissociated when grown on serum agar in carbon dioxide. The parent growth in carbon dioxide was smooth mucoid and gave rise to rough sectors. Subcultures from each type of growth repeated the parent type when grown under carbon dioxide on serum agar, but both were very rough when grown in air. The variant from the smooth mucoid growth in carbon dioxide was virulent, while the " rough " variant was avirulent. A single large injection of the rough variant conferred a high degree of resistance on guinea-pigs.

Immunity Tests on Sheep.—The outcome of the immunity tests on guinea pigs warranted a trial on sheep. To assess the value of rough strain XVIII as an immunizing agent a comparison was made with an ordinary vaccine strain known as Batch 88. This batch had given good results in the laboratory titration but had proved too virulent for field use. Six sheep were inoculated subcutaneously with B. 88 at double the strength used in the field tests. At the same time eight sheep received a subcutaneous injection of the rough variant of strain XVIII. The latter dose contained about twice as many bacilli as the dose of Batch 88. The immunity of the sheep was tested after five weeks with a large dose of virulent organisms. The test dose was 1·0 c.c. of a $^{1}/_{100}$th dilution of a dense spore suspension of Strain XX. This strain was isolated from a natural case of anthrax in a bovine seven weeks previously and had since been kept in a refrigerator. The actual M.L.D. of the test dose was not determined, as this would have involved the sacrifice of too many sheep, but the fate of the controls showed that the sheep received a large multiple of the M.L.D. The results of the experiment are summarized in the following table.

TABLE LII.

Sheep Nos.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 20	40	60	80	100	120	140
45405	1/100th slant rough XVIII	1·0 c.c. 1/100th dilution vir. XX							/
45408	,,	,,							/
45410	,,	,,							/
45411	,,	,,							/
45417	,,	,,							/
45426	,,	,,							/
45431	,,	,,							/
45442	,,	,,							/
45607	1 c.c. 1/100th di-luiton batch 88	1·0 c.c. 1/100th dilution vir. XX		x					
45449	,,	,,		x					
45402	,,	,,		x					
45434	,,	,,		x					
45407	,,	,,		x					
45412	,,	,,		x					
44989	Controls.	1·0 c.c. 1/100th dilution vir. XX			x				
45003	,,	,,			x				
45413	,,	,,		x					
45377	,,	,,					x		
45416	Controls.	1·0 c.c. 1/10,000th dil. strain XX			x				
45375	,,	,,			x				

The results show clearly that the avirulent variant obtained in carbon dioxide from strain XVIII was a much better immunizing agent than an ordinary vaccine strain. The vaccine strain B. 88 was chosen because it had yielded good results in an earlier titration, although it failed to stand up to the present more severe test. The avirulent XVIII, however, seemed to possess immunizing properties of a much higher order. This was to be expected from the results obtained in the experiments with guinea pigs. When the experiment was carried out it was thought that equivalent doses of the avirulent variant and Batch 88 had been given. A recalculation of the dilution later showed that a larger dose of the avirulent strain had been administered. The results therefore would tend to flatter the avirulent strain; but it is very unlikely that the relatively small difference in dosage (about 750,000 as against 300,000 organisms) would have influenced the results as markedly as the table showed. See Table XII for a consideration of this point.

(4) *Dissociation of virulent anthrax strain XX on* 50 *per cent. serum agar in* 65 *per cent. carbon dioxide.*

This strain was tested four days after it had been isolated from a natural case of anthrax. After 24 hours in carbon dioxide the growth was mainly smooth mucoid, but a few rough outgrowths had appeared at the edge of the colony. Each variant was then streaked onto agar and incubated in air. After 24 hours both were very rough and guinea pigs were injected as follows:—

TABLE LIII.

No. of guinea pigs.	Each injected with:	Death: Hours after inoculation.			
		10	30	50	70
3	$\frac{1}{5}$ slant variant from smooth growth in CO_2		x x	 x	
3	$\frac{1}{5}$ slant variant from rough growth in CO_2		x	x x	

There was too little difference in the times of death to form an opinion as to the relative virulence of the two strains.

After strain XX had been incubated in carbon dioxide for a further 24 hours, the cleavage into smooth mucoid and rough portions was more marked. Subcultures were again made from each variant and grown in air. Again these were very rough and guinea pigs were inoculated as follows:—

TABLE LIV.

No. of guinea pigs.	Each injected with :	Death : Hours after inoculation. 10	30	50	70
6	$\frac{1}{5}$ slant variant from smooth growth in CO_2 (48 hours)		x x x x x x		
6	$\frac{1}{5}$ slant variant from rough growth in CO_2 (48 hours)			x x	x x xx

In this case all the guinea pigs inoculated with the subculture from the smooth growth in carbon dioxide were dead before those inoculated with the rough variant had commenced to die. In view of the large doses given, the difference was probably real. A culture was made from a guinea-pig inoculated with the less virulent strain and three guinea-pigs inoculated with it. These died within 40 hours. Thus there was an abrupt return to full virulence after one passage. It is probable, therefore, that in selecting the rough variant some virulent bacilli were carried over, and that these were the killing agents. The retarded deaths were very likely due to the small dose of virulent bacilli in a predominantly avirulent culture.

The experiment was repeated a week later. After 24 hours in carbon dioxide the growth was smooth mucoid with one or two rough sectors. After 48 hours the rough portions had increased in size; each variant was streaked onto agar and incubated in air for 24 hours. Both cultures were then very rough, and guinea pigs were inoculated as follows:—

TABLE LV.

No. of guinea pigs.	Each injected with :	Death : Hours after inoculation. 10	20	30	40	50	60
6	$\frac{1}{3}$ slant variant from smooth growth in CO_2 (48 hours)				x x x x x x		
6	$\frac{1}{3}$ slant variant from rough growth in CO_2 (48 hours)						/ / / / / /

After strain XX had grown for another 24 hours in carbon dioxide variants were again picked (72 hours incubation in CO_2). After 24 hours growth in air both variants were very rough and guinea pigs were injected as follows:—

TABLE LVI.

No. of guinea pigs.	Each injected with:	Death: Hours after inoculation. 10	20	30	40	50	60
6	⅓ slant variant from smooth growth in CO_2 (72 hours)				x x x x x x		
6	⅓ slant variant from rough growth in CO_2 (72 hours)						/ / / / / /

Immunity tests with rough avirulent variant from strain XX.—The immunity of the guinea pigs which had received the avirulent rough variant was tested a month after inoculation. Six of them were tested against virulent XVIII. This test was done together with the second test on the avirulent variants of strains XVIII and VII so that the results can be compared with those in Tables XLVII and LI. The other six immunized guinea pigs were tested with the smooth mucoid Boshoff strain. This strain had gradually been growing less mucoid and had lost considerable virulence. Before the present experiment it was passaged from guinea pig to guinea pig (without intermediate culturing) seven times and was then again able to kill all guinea pigs within 40 hours. This strain received the number II Ad. It was also virulent for rabbits but was not tested on sheep.

The guinea pigs which had received one injection of the avirulent variant from strain XX showed a much higher resistance to anthrax than normal guinea pigs. Three guinea pigs survived the very large doses of virulent culture.

Summary.—The recently isolated virulent strain XX dissociated freely on 50 per cent. serum agar in 65 per cent. carbon dioxide. The smooth mucoid and the rough variant obtained under these conditions were both rough when grown in air and then appeared culturally identical. However, the variant from the smooth mucoid growth in carbon dioxide was virulent while the subculture from the rough growth in carbon dioxide was avirulent for guinea pigs, although very large doses were given.

Guinea pigs inoculated with the avirulent rough variant showed considerable resistance to very large doses of virulent test strains.

TABLE LVII.

No. of guinea pigs.	Previous inoculations.	Each injected with :	Death : Hours after inoculation. 20	40	60	80	100	120	140	160	180	200
6	1 injection rough avirulent strain XX	¼ slant virulent XVIII			x				x	x x		/ /
9	Controls	¼ slant virulent XVIII		x x x x x x x x x								
6	1 injection rough avirulent strain XX	⅓ slant mucoid Boshoff (II Ad.)			x	x x x		x				/
14	Controls	⅓ slant mucoid Boshoff (II Ad.)		x x x x x x x x x x xx xx								

(5) *Dissociation of virulent Boshoff (strain IIc) on 50 per cent. serum agar in 65 per cent. carbon dioxide.*

This strain had been used as a virulent test strain for a number of years. It still killed sheep in fairly high dilutions, but was not as virulent as the freshly isolated strains. The growth was smooth mucoid after 24 hours in carbon dioxide; after 48 hours rough outgrowths appeared at the periphery of the colony. Subcultures

from the smooth mucoid growth and from the rough growth were both rough when incubated in air and each variant was injected into guinea-pigs as follows:—

TABLE LVIII.

No. of guinea pigs.	Each injected with:	Death: Hours after inoculation. 20	40	60	80	100	120
6	⅓ slant variant from smooth growth in CO_2		x x x x x	x			
6	⅓ slant variant from rough growth in CO_2				x x	x x	x x

There were considerable differences in the survival times. The rough variant took much longer to kill, and in view of the large doses used, the fact was probably significant. A culture was made from one of the guinea-pigs which had received the rough variant and died on the 5th day. This was rough after 24 hours and was injected into three guinea-pigs. All three were dead by the 40th hour. Therefore it was very likely that the deaths with the "rough" variant were due to contaminating "smooth" bacilli.

The whole experiment was then repeated and after 24 hours in carbon dioxide virulent Boshoff was smooth mucoid, but showed some rough outgrowths at the periphery of the colony. Growth was allowed to continue for five days and subcultures were then made from the smooth and from the rough growth and incubated in air. Both cultures were rough after 24 hours and guinea-pigs were inoculated as follows:—

TABLE LIX.

No. of guinea pigs.	Each injected with:	Death: Hours after inoculation. 20	40	60	80	100	120
3	⅓ slant variant from smooth growth in CO_2		x x x				
3	⅓ slant variant from rough growth in CO_2						/ / /

The subculture from the smooth mucoid growth in carbon dioxide proved virulent, while the culture from the rough growth in carbon dioxide was avirulent. Three weeks later six guinea pigs were each inoculated with ⅓rd of an agar slant of the rough variant and survived.

Immunity tests with avirulent strain from virulent Boshoff.— The immunity of the nine guinea pigs which had received an injection of the avirulent variant was tested with massive doses of virulent strain XVIII and the exalted smooth mucoid Boshoff strain (II Ad). The results are shown in the following table.

TABLE LX.

No. of guinea pigs.	Previous inoculations.	Each inoculated with:	Death: Hours after inoculation.									
			20	40	60	80	100	120	140	160	180	200
3	⅓ slant avirulent variant of strain II c	¼ slant virulent XVIII after 6 weeks		x		x			x			
3	⅓ slant avirulent variant of strain II c	¼ slant virulent XVIII after 3 weeks		x		x	x					
3	⅓ slant avirulent variant of strain II c	¼ slant smooth Boshoff after 3 weeks		x								/ /
7	Controls	¼ slant smooth smooth Boshoff		x x x x x x x								
7	Controls	¼ slant virulent XVIII		x x x x x x x								

It is clear that the guinea pigs which had received an injection of the rough avirulent variant from strain IIc were more resistant to a subsequent virulent test dose than uninoculated controls. Thus the results were substantially the same as those obtained with the four avirulent strains previously examined, although the immunity in the present instance was of a lesser degree. Again, therefore, a virulent strain grew smooth mucoid in carbon dioxide on serum agar and produced rough variants which were avirulent and uncapsuled, but were able to confer an immunity to anthrax on guinea pigs.

(6) *Dissociation of virulent strain XXII on 50 per cent. serum agar in 65 per cent carbon dioxide.*

This strain was isolated from a natural case of anthrax, and the experiment was started on the day following isolation. After 24 hours in carbon dioxide the growth was smooth mucoid. After 48 hours rough outgrowths appeared and agar slants were streaked from the smooth mucoid and from the rough parts of the colony. These were incubated in air and both showed the characteristic very rough appearance of newly isolated virulent strains. Guinea-pigs were then inoculated as follows:—

TABLE LXI.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 10	20	30	40	50	60
3	⅓ slant subculture from smooth mucoid growth in CO_2				x x x		
3	⅓ slant subculture from rough growth in CO_2						/ / /

Subcultures of these variants were tested a week later with the following results:—

TABLE LXII.

No. of guinea pigs.	Each inoculated with:	Death: Hours after inoculation. 10	20	30	40	50	60
3	⅓ slant subculture from smooth mucoid growth in CO_2				x x x		
3	⅓ slant subculture from rough growth in CO_2						/ / /

Thus there was a clear-cut difference in virulence between the variants.

Immunity tests with avirulent variant of strain XXII.—These tests can be compared directly with those carried out with the variants of strain IIc (LX) as they were performed at the same time, with

the same virulent strains and with the same set of controls. The results of the immunity test carried out 4 to 5 weeks after the inoculation of the avirulent strain are summarized below.

TABLE LXIII.

No. of guinea pigs.	Previous inoculations.	Each inoculated with :	Death : Hours after inoculation. 10	20	30	40	50	60	70	80	90	
5	⅓ agar slant rough avirulent strain XXII	¼ slant virulent XVIII					x	x			x	/ /
7	Controls	¼ slant virulent XVIII				x x x x x x x						
3	⅓ slant rough avirulent strain XXII	¼ slant smooth mucoid Boshoff				x						/ /
7	Controls	¼ slant smooth mucoid Boshoff			x x x x x x x							

Again it was possible to dissociate a newly isolated virulent strain on serum agar in carbon dioxide. The variants were morphologically identical when grown in air, but the variant derived from smooth mucoid growth in carbon dioxide was virulent, while that derived from the rough dissociants in carbon dioxide was avirulent. The avirulent variants conferred on guinea-pigs a high degree of resistance to large killing doses of virulent culture.

(7) *Dissociation of strain XXVI on* 50 *per cent. serum agar in* 65 *per cent. carbon dioxide.*

This strain was used a week after its isolation from a sheep dead of anthrax naturally acquired. The growth was smooth mucoid after 24 hours in carbon dioxide, but showed one small rough outgrowth. After 72 hours the rough outgrowth had spread considerably. Subcultures from both the smooth mucoid and the rough part of the growth in carbon dioxide grew very rough in air and were injected into guinea pigs after 48 hours.

TABLE LXIV.

No. of guinea pigs.	Each injected with :	Death : Hours after inoculation. 20	40	60
3	⅓ slant subculture from smooth mucoid in CO_2		x x	x
3	⅓ slant subculture from rough in CO_2			/ / /

Five more guinea-pigs were inoculated with the rough avirulent strain. These, together with two survivors noted in Table LXIV were tested with the exalted smooth mucoid Boshoff strain (II Ad.) a month later. (One of the guinea pigs in LXIV had died after 30 days of an intercurrent infection).

TABLE LXV.

No. of guinea pigs.	Each inoculated with :	Each tested with :	Death : Hours after inoculation. 20	40	60
7	⅓ slant rough variant strain XXVI	¼ slant II Ad.		x x x x x	x /
6	Controls	¼ slant II Ad.		x x x x x x	

Thus the guinea pigs immunized with the avirulent strain were slightly more resistant than uninoculated controls. The immunity conferred by this variant was not nearly as good as that produced by other strains in this series, e.g. XVIII and VII.

Immunity test on sheep.—Six sheep were each injected with 1 c.c. of a 1 : 2000 dilution of a Mason's tube. This was about ⅔rds the usual dose of vaccine and contained about 300,000 bacilli per c.c. For comparison the titration of the ordinary vaccine (strain XVII) was included. This titration was done at the same time and with the same test dose. The 0·01 c.c. dose is equivalent to the dilution used for issue and was slightly larger than the dose of strain XXVI given to the six sheep.

TABLE LXVI.

Sheep No.	15/5/36. Each immunised with :	5/6/36. Each tested with :	Death : Hours after inoculation. 20	40	60	80	100	200	260	300
46770	1 c.c., 1 : 2,000, Mason's tube strain XXVI	1 c.c., 1 : 25, dilution Batch XX virulent							/	
45726	,,	,,							/	
45802	,,	,,			x					
45804	,,	,,							x*	
45443	,,	,,							x	
45824	,,	,,							/	
45799	20 c.c. Batch 97..	1 c.c., 1 : 25 dilution Batch XX virulent			/					
45823	20 c.c. Batch 97..	,,			/					
45811	0·1 c.c. Batch 97.	,,			x					
45800	0·1 c.c. Batch 97.	,,			x					
45403	0·01 c.c. Batch 97	,,			/					
45355	0·01 c.c. Batch 97	,,			x					
45810	0·01 c.c. Batch 97	,,			x					
45714	0·005 c.c .Batch 97	,,			x					
45791	0·005 c.c. Batch 97	,,			x					
45829	0·005 c.c. Batch 97				x					
44988	Control.....	,,			x					

* Negative for anthrax.

The sheep inoculated with a small dose of the avirulent strain XXVI developed a better immunity than those inoculated with the ordinary vaccine strain XVII. The dose of the avirulent strain was smaller than that usually issued in the vaccines so that the conditions were slightly weighted against it. This variant was tested on sheep because it was the weakest immunizer of the avirulent variants yet obtained (see test on guinea pigs Table LXVII) and it was thought that this sheep test would give an idea of the immunity to be expected from a weakly protecting avirulent rough. The results were encouraging.

Summary of Part D (1-7).—1. Seven virulent strains of anthrax all grew smooth mucoid on 50 per cent. serum agar in 65 per cent. carbon dioxinde.

2. All seven strains rapidly developed rough dissociants in the carbon dioxide.

3. Subcultures from the smooth mucoid growth were very rough in air and usually retained the virulence of the original strain, whereas the subcultures in air from the rough dissociants were usually avirulent, although their colony morphology was also very rough; quite as rough indeed as that of freshly isolated virulent strains.

4. All the avirulent variants conferred an increased resistance to anthrax on guinea pigs.

5. Tests were performed on two groups of sheep immunized with the avirulent variants of strains XVIII and XXVI respectively. The former had given the best results of the series with guinea pigs and the latter the worst. Both groups showed a higher degree of immunity than did sheep immunized with the ordinary attenuated laboratory vaccine. Strain XVIII gave particularly good results.

E. Dissociation of Attenuated Anthrax Strains on 50 per cent. Serum Agar in 65 per cent. Carbon Dioxide.

In the early part of the work (Parts A-C) it appeared as if rough avirulent variants derived from attenuated or avirulent smooth mucoid strains were not efficient immunizing agents. In the last part of the work (Part D) it was shown that avirulent rough dissociants, obtained from virulent strains growing smooth mucoid in carbon dioxide, were very efficient immunizers, unless the smooth mucoid culture itself became avirulent (Strain XIV Table XXXIII). To test the possibility of correlation between the degree of virulence of the parent strain and the degree of immunizing ability of its avirulent dissociant, the procedure of part D was applied to relatively attenuated anthrax strains. That is, they were grown on serum agar in carbon dioxide.

Three strains were used: the first was isolated from a commercial saponin vaccine and was avirulent for rabbits but killed about half the number of guinea pigs inoculated with it. The second was one of the laboratory vaccine strains of about the same degree of virulence. The third strain was also isolated from a saponin vaccine, but was considerably more virulent, although it did not kill rabbits. All three strains were intermediate rough-smooth and produced a slightly mucoid growth on nutrient agar in air. Their colony morphology was typical of vaccine strains in ordinary use.

Each strain was streaked onto the surface of 50 per cent. serum agar and incubated in 65 per cent. carbon dioxide at 37° C. All three strains were smooth mucoid after 24 hours growth. They were examined daily and the smooth mucoid growth spread steadily, until at the end of eight days the smooth mucoid growth almost covered the surface of the medium. As there was no hope of obtaining variants in this way, the experiment was discontinued. Now and then during the experiment, small cuneiform patches were seen at the edge of the colony, but these were covered by smooth mucoid material and could not be separated from it. There was little chance for the commencing rough variants to establish themselves as they were almost immediately swamped by the more rapidly growing smooth mucoid part of the strain. With virulent strains rough variants appeared early and multiplied more rapidly than the smooth mucoid part of the culture so that they could establish themselves and even encroach on the smooth growth. In the case of the

attenuated cultures the smooth mucoid variant grew more rapidly, and the rough dissociants were not able to establish themselves. Moreover, the rate of dissociation was faster in the virulent than in the attenuated strains.

F. Comparison of Immunity produced in Rabbits by a Number of Avirulent Variants and an ordinary Attenuated Anthrax Strain.

In the experiment noted in Table LII a comparison was made of the immunity produced in sheep by the avirulent variant of strain XVIII and an attenuated strain. (Strong Pasteur II type). In that experiment small immunizing doses were given, as part of the information required was whether the avirulent type could be used as a vaccine. In the present experiment rabbits were " immunized " with large doses of culture. The doses were made large so that the effect produced by each strain would probably be maximal, and in this way a better assessment of the relative immunizing abilities could perhaps be made. Thirty-three rabbits were divided into four groups of six and one group of nine and were treated with the following strains:—

(*a*) The avirulent variant of strain XVIII obtained in carbon dioxide on serum agar four months before.

(*b*) The avirulent variant of strain VII (Drummond) obtained on serum agar in carbon dioxide four months previously. Both (*a*) and (*b*) had produced a good immunity when tested on guinea-pigs some time before.

(*c*) The avirulent variant of strain XXII. This was also obtained on serum agar in carbon dioxide, but had been isolated only six weeks previously. When tested a month ago it had not shewn as good results as (*a*) and (*b*).

(*d*) Vaccine strain XVII. This was the most virulent attenuated strain used for large scale field inoculations. It did not as a rule kill rabbits, but a sufficient dose always killed guinea pigs. There was thus no indication of its ability to immunize small animals.

Each group of rabbits received one injection of one of the above strains and one group of nine rabbits was retained as a control. The results of the test are summarized in the following Table. One four-hundredth of an agar slant of test strain XX killed sheep.

The degree of protection produced by the three avirulent strains varied considerably. Strain VII the earliest isolated gave the least protection and strain XXII the most recently isolated gave the best immunity. However, earlier tests on guinea pigs (Table LXIII) showed that strain XXII was the least effective of the three strains at the time of its isolation, so that the others (VII and XVIII) had undergone considerable deterioration. Strain XVII the ordinary vaccine strain which was virulent for guinea pigs gave rabbits a very good immunity and was in this respect only equalled by strain XXII.

TABLE LXVII.

Rabbits	Each inoculated with :	Each tested with :	Death : Hours after inoculation. 20 40 60 80 100 200
1	¼ slant avirulent XVIII	⅕ agar slant virulent strain XX 6 weeks later	/
2	,,	,,	/
3	,,	,,	/
4	,,	,,	x
5	,,	,,	x
6	Died of inter-current	infection before test	
1	¼ slant avirulent VII	⅕ agar slant virulent strain XX 6 weeks later	/
2	,,	,,	x
3	,,	,,	x
4	,,	,,	x
5	,,	,,	x
6	,,	,,	x
1	¼ slant avirulent XXII	⅕ agar slant virulent strain XX 6 weeks later	/
2	,,	,,	/
3	,,	,,	/
4	,,	,,	/
5	,,	,,	/
6	Died of intercurrent	infection before test.	
1	¼ slant attenuated XVII	⅕ agar slant virulent strain XX 6 weeks later	/
2	,,	,,	/
3	,,	,,	/
4	,,	,,	/
5	,,	,,	/
6	,,	,,	/
1	Control	⅕ agar slant virulent strain XX 6 weeks later	x
2	,,	,,	x
3	,,	,,	x
4	,,	,,	x
5	,,	,,	x
6	,,	,,	x
7	,,	,,	x
8	,,	,,	x
9	,,	,,	x

DISCUSSION.

In sections A and B of this paper experiments are described, where a number of anthrax strains were subjected to lengthy periods of ageing and intermittent growth to obtain smooth or smooth mucoid variants. Five virulent strains, one partly attenuated and one much attenuated strain were used. The first study was made on virulent Boshoff and this strain eventually developed smooth mucoid colonies which were virulent for guinea pigs and rabbits. There was considerable difficulty in keeping this strain mucoid without making subcultures every one to two days, and this difficulty proved due to the rapid production of fast growing rough variants, which overgrew the parent mucoid colony. The smooth mucoid strain continuously threw off clearly defined rough variants, and the same sequence of events occurred after each new subculture and even after subcultures from single cells. Apart from the difference in colony type, the rough dissociant differed from the smooth mucoid parent in its inability to produce capsules in carbon dioxide or in the animal body; and although the smooth mucoid strain was fully virulent for guinea pigs, the rough variant was not able to kill mice. Nevertheless, in spite of its avirulence, the rough variant produced a considerable resistance to anthrax in guinea-pigs, sheep and goats.

If it be assumed that such a smooth mucoid anthrax strain is analogous with the smooth "normal" form met with in other bacteria, then its behaviour bears a close resemblance to the classical S➛R variation accompanied by a loss of virulence found in the majority of pathogenic bacteria. It is unusual, however, for avirulent roughs to possess any marked immunizing power, and Basset (1935) states that avirulent strains do not give any immunity to anthrax. De Kruif (1921), Korobovka and Kotelnikov (1935), Felix and Pitt (1935) have described avirulent immunizing variants of Pasteurella and Salmonella types, and Bail (1915) showed that this held for anthrax, so that the observations made in the present work, although unusual, are not unique.

In the particular case of the Boshoff strain the abrupt transition from smoothness to roughness, and from virulence to avirulence, was accompanied by the loss of the power to develop capsules. Anthrax septicaemia and smooth mucoid colony form are both invariably associated with the capsuled bacillary form, so it is very likely that the loss of the capsule was the determining feature of the change of Boshoff to the rough and avirulent state. It is unwise to press the analogy with other pathogenic bacteria too far, as S➛R variation is not always determined by capsule loss, although an equivalent phenomenon has been described in the case of some streptococci (Dawson and Olmstead, 1935); and many other cases are known. Nevertheless, the clear cut picture of dissociation shown by the Boshoff strain and the very promising immunity conferred by the rough variant encouraged further experimentation and accordingly four more virulent strains and two attenuated strains were examined in experiments similar to those conducted with V. Boshoff. As was

recorded in Sections A and B different methods were adopted to obtain smooth mucoid variants. The ease with which these were got varied considerably, and in the case of virulent strains the procedure was always lengthy.

Once the smooth mucoid or smooth strains had been got, all showed the S➛R dissociation. The rate and the extent of this change varied considerably in the different strains. The Boshoff and Anthrax A mucoids dissociated rapidly and continuously, whereas the virulent 568 mucoid dissociated very rarely and the variants in this case were not well defined. Nevertheless there were two points of general agreement amongst all the strains tested: firstly, the S➛R change was associated with a more or less complete loss of the ability to grow capsules, and secondly, there was a loss of virulence with the shift from the SM to the R form. In the case of strain 568, the variants were about equal in virulence, but as was noted at the time the two variants were not well defined morphologically. For the rest, where a difference in virulence existed the rough variant was always found to be the less virulent.

There was evidence that the power of the rough variants to immunize might be a function of the virulence of the smooth mucoid parent strain. The Pretoria North strain was, however, an exception, but when another attempt was made to examine this strain, the smooth mucoid characteristic had been lost. However, there was no doubt that the ability of the avirulent variant from Boshoff to immunize was not an exceptional case, since the rough variants from strain IV and Pretoria North also increased the resistance of guinea-pigs.

From the point of view of colony dissociation in general, the results obtained were rather unusual. Once the smooth mucoid strains were obtained they appeared to dissociate in the classical manner, that is S➛R, accompanied by a reduction or complete loss of virulence. However, it is well known that fully virulent anthrax strains are very rough, so that dissociation in anthrax appeared to proceed thus: R (virulent)➛Sm (virulent or avirulent)➛R (avirulent). This point was discussed fully in the introduction to section C, and for the reasons stated there the dissociation of virulent strains in carbon dioxide was attempted. It seemed likely that more information on the role of the capsule might be obtained, and the possibility existed that if dissociative processes of virulent strains gave rise directly to avirulent roughs, these might possess a higher immunizing value than those derived from smooth mucoid strains of reduced virulence.

Dissociation of virulent strains in CO_2.—Three rough virulent strains, Pretoria North, Drummond and 568 were grown under a partial pressure of carbon dioxide, and all developed more or less marked smooth mucoid characteristics. The Drummond strain became almost as mucoid as a typical smooth mucoid strain, while 568 showed only a slight tendency to develop this type of growth. All these strains showed some rough variants after seven to fourteen days. When transplantations from the smooth mucoid and from the rough growth were made onto agar in air, the resulting cultures

showed no differences in colonial morphology, but in two of the strains the subcultures from the smooth mucoid growth in carbon dioxide were definitely more virulent than the subcultures from the rough variants. In the case of strain 568 the results were less definite. None of the strains yielded a completely avirulent rough, and although the rough variants were not capsuled in the cultures, they became capsuled in the animal. This meant that although the carbon dioxide favoured capsulation it was not in this respect the equivalent of *in vivo* conditions. Thus the rough colony form in carbon dioxide was not an index of complete avirulence, although it indicated reduced virulence. The procedure was also unsatisfactory in that the dissociation rate was slow, and the strains were subjected to a moderately lengthy period of growth before they could be examined. Again, however, the rough dissociants were significantly less virulent than the smooth parent strains, but in this case the variants could only be distinguished, morphologically, when grown under the special conditions of the experiment.

Virulent strains were then grown on serum agar in carbon dioxide to see if they would develop more fully mucoid characteristics.

Dissociants of virulent strains on serum agar in CO_2.—Seven virulent strains were grown on 50 per cent. serum agar in 65 per cent. carbon dioxide. Six of the strains were fairly recently isolated and three of these were tested immediately after isolation: only one strain had been obtained a long time before. The strains were markedly smooth mucoid when examined after 24 hours growth in CO_2, and all seven showed a great readiness to produce rough uncapsuled variants as soon as 24 hours after inoculation. There was very little difficulty in picking and transplanting these rough variants, as they were sharply demarcated from the original smooth mucoid growth. Transplants from the smooth mucoid growth in carbon dioxide onto ordinary agar in air showed an immediate reversion to the typical very rough growth of the original virulent strains and the same applied to transplants from the rough variants. Therefore these two variants, so clearly to be differentiated on serum agar in carbon dioxide, were indistinguishable from one another when grown under ordinary conditions. If the two variants were again grown on serum agar in carbon dioxide, the one again resumed the dissociating smooth mucoid state, while the other was again rough and devoid of capsules. On testing the pathogenicity of the variants from the different strains the subcultures from the smooth mucoid variants were nearly all virulent, while the subcultures from the rough variant proved mostly avirulent. The virulence tests were done with transplants grown in air and all these were very rough; so that although the colony morphology of the two variants was identical under these conditions, yet there was a sharp difference in pathogenicity between the cultures originating from the smooth mucoid growth and those originating in the rough variants on serum agar in carbon dioxide. The protocols (section D) indicate that the completely avirulent rough variant was not always got at the first attempt. It is readily conceivable that the selection of rough variants from a mixed culture must sometimes result in the carrying over of smooth material. However, rough avirulent strains were obtained in

all seven cases without much difficulty, so their production must be frequent if not usual. Where the rough variant still killed, it again grew mucoid when transplanted onto serum in carbon dioxide.

Now these rough avirulent strains all arose from virulent anthrax strains after a very short period of growth and most of them conferred a high degree of immunity on guinea pigs. The immunity was of a much higher order than that produced with the variants obtained by the earlier methods; so much so that one injection of these latterly isolated variants gave as good an immunity as two injections of the previously isolated strains. Moreover, the immunity produced by the strains isolated in carbon dioxide was tested with strains more virulent than those previously used. The degree of immunity conferred by the different avirulent rough strains obtained in carbon dioxide varied; but the differences were not marked, and all these avirulent roughs effectively immunized guinea pigs. One strain which had given good results in guinea pigs was tried on sheep, and the immunity produced was far better than that given by the ordinary vaccine used. The two strains were compared under identical conditions. (Strain XVIII, Table LII.) The least effective of the avirulent strains was also tried on sheep and gave better results than were expected.

This method of growing virulent strains on serum in carbon dioxide rapidly and consistently yielded avirulent rough variants having the power of conferring immunity to anthrax. From the practical standpoint the method is important because the immunity appears to be of a higher order than that produced by the usual attenuated vaccine strains. The difficulty of protecting guinea pigs and rabbits with ordinary vaccines is well known although rabbits have sometimes been immunized to small test doses. Guinea pigs immunized with Pasteur I strains have shown very little resistance to less attenuated strains. Recently, Mazzucchi (1934), Ramon and Staub (1935), and others have claimed to increase the resistance of rabbits by adding either saponin or lanolin to the inoculum to limit the rate of absorption of the organisms. The test dose, however, was not large. Grajewski (1935) failed to immunize rabbits with several doses of " Carbozoo " vaccine, and Hruska (1934) could not immunize rabbits unless a fairly large number died as a result of vaccination. In the present work, the guinea-pig was used because its uniform susceptibility to anthrax lessened the experimental error. For the same reason doses of bacilli far in excess of those usually employed to test the immunity of small animals were used.

It seems also, that the avirulence of these uncapsuled strains should make anthrax immunization a safe procedure. Some of the variants have been used in the experimental work over a long period and have remained completely innocuous. Munne (1934) stated categorically that capsule formation and multiplication at the site of injection were essential for the production of immunity to anthrax. However, capsule production is not essential, but it is possible that capsules increase the survival time of the bacilli in the animal. In that case the uncapsuled variants will not be as effective immunizers

as can be expected from the experimental work where large doses were used. Against this argument stand the results obtained with sheep (Tables LII and LXVI) where small doses of the avirulent strain were used for immunization.

Sporulation and stability are other factors of importance in vaccine preparation. These properties were not influenced by the dissociation. The sporulation and the stability of the avirulent roughs (although not specifically mentioned in the protocols) appeared to be functions of the degree to which the original virulent strain possessed these properties. Avirulent mutants from freshly isolated and vigorously sporulating virulent strains sporulated vigorously and this property was as stable in the avirulent dissociant as in the original virulent strain. Continued rapid subculturing affects sporulation adversely, and this applied as much to the virulent parent strain as to the non-virulent variant. It was easier to obtain vigorously sporulating avirulent roughs than to obtain vigorously sporulating strains by the usual methods of attenuation. The avirulent roughs obtained in section D 1-7 sporulated like fully virulent strains. This was probably due to the rapidity with which these avirulent strains were obtained from freshly isolated virulent strains.

The ease with which the avirulent immunizing variants were obtained from seven virulent strains makes the questions of sporulation and stability less important. Moreover, the work of Elser, Thomas and Steffen (1935) and of Flosdorf and Mudd (1935) on the preservation of biological products by drying in vacuo from the frozen state may very well solve the problem of conserving the properties of various strains. It would have been interesting to see if there was some relation between the immunizing power of a rough avirulent strain and the virulence of the strain from which it sprang. Attempts to obtain rough dissociants from attenuated strains grown on serum agar in carbon dioxide failed, because although these strains grew very smooth mucoid, they were so stable that no rough variants could be obtained. In this respect the attenuated strains behaved altogether differently from the virulent strains. This rather interesting observation was not investigated further.

General aspects of S→R variation in anthrax.—From the point of view of a dissociation problem the experiments described in this paper show some very interesting features. The normal virulent anthrax strain is very rough, and in the ordinary course smooth mucoid strains appear only in old or in attenuated strains. Some of these mucoid strains were shown to vary continuously (Boshoff and Anthrax A) and the dissociant in these cases was rough, non-mucoid, uncapsuled and avirulent. In this respect the smooth mucoid anthrax strains resembled the " normal " phase of other pathogens. When virulent strains were grown under special conditions they immediately became smooth mucoid and showed a dissociation picture identical with that shown by strains such as the normally smooth mucoid Boshoff or Anthrax A strains. This smooth mucoid growth shown by virulent strains on serum agar in carbon dioxide was not a permanent change and was immediately reversible when the ordinary conditions of aerobic growth were restored. The same

process occurred with single cell isolations of virulent strains so that the change to smooth mucoid and back to rough was conditioned by the environmental change, and was not due to a selective action on particular cells, nor to dissociative change.

It is probable that the " normal " appearance of virulent anthrax is smooth mucoid, since the mucoid bacillus and the *in vivo* bacillus are both invariably capsuled. Therefore the appearance of virulent anthrax strains grown on serum agar in carbon dioxide may very well be the " normal " appearance of the virulent strain. Thus the loss of capsules and of smoothness on the ordinary laboratory media are possibly due to an " unfavourable environment ". The dissociation into avirulent roughs in carbon dioxide appears to be a true dissociation and in this particular case the change is associated with the loss of ability to form capsules. At least, in all cases where the S➛R change with loss of virulence has taken place, the capsule loss has been the constant feature and it is unlikely that this should be merely coincidence.

The rôle of the capsule in anthrax has interested many experimenters. It is universally known that animal anthrax bacilli are all capsuled. On the other hand many weakly virulent or quite avirulent strains are smooth mucoid and fully capsuled, so that the virulence must be independent of the capsule production, or alternatively, the capsules produced by virulent bacilli must differ from those produced by attenuated or avirulent bacilli. Morphologically there is no difference between the capsules seen in anthrax septicaemia, the capsules from virulent strains in carbon dioxide, and the capsules from avirulent smooth mucoid strains. Chemically and immunologically all capsules appear to be the same (Tomcsik and Boden 1934). Thus the capsules from virulent or avirulent strains cannot be distinguished by any known method. However, if the capsule is not associated with the virulence of the anthrax bacillus, it would be difficult to explain the results obtained by Bail, and the results obtained in the present work where the loss of the capsule is closely associated with the S➛R virulent to avirulent mutation.

Any theory of immunity and attenuation in anthrax must include the following observations and must offer a reasonable explanation for them .

(1) The fact that vaccines attenuated at 42° C. retain their ability to produce capsules, but lose virulence, and that the greater the loss of virulence the less the immunity produced.

(2) Virulent strains which have suddenly lost the ability to produce capsules (by mutation) are immediately avirulent, but these completely innocuous strains possess a greater immunizing power than quite virulent vaccine strains attenuated at 42° C.

It is immediately apparent [from (1)] that the capsule cannot be a unique factor determining virulence, although [from (2)] it must be of great importance. Secondly, there is nothing to show that the capsule is at all concerned in the production of immunity.

All virulent strains are, however, able to produce capsules, although capsuled strains are not necessarily virulent. These observations can be reconciled if the following assumptions are made.

(1) That the virulence of an anthrax strain is determined by the capsule and by a factor not associated with the capsule. For convenience this may be called factor (A).

(2) That the immunity to anthrax produced by any strain is due to this same factor or antigen.

According to this scheme two factors must be considered: factor A and the capsule. The variations of virulence and immunizing power in anthrax strains might then occur as follows:—

(1) The A antigen or factor may decrease until none is left, while the faculty of producing capsules remains intact. Such a process will lead to a graded diminution in virulence until the strain becomes avirulent and at the same time the immunizing power will grow less. This is a description of what occurs, for example, in strains attenuated at 42° C. These strains gradually lose virulence, and with this, immunizing power, while the ability to produce capsules remains unimpaired.

(2) The A factor may remain intact and the strain suddenly lose the ability to develop capsules. This will result in an immediate and complete loss of virulence; but the immunizing power should be of a high order. The avirulent rough strains isolated on serum agar in carbon dioxide appear to be examples of this process. It may also happen that the A factor is reduced and the strain also be unable to produce capsules. Such variants will be avirulent, but the immunizing power will decrease as the A factor decreases. A strain may thus become devoid of A factor and ability to form capsules. This will then be avirulent and non-immunizing. Several variants of this type were isolated in the early part of the work.

(3) Another possibility is for a strain gradually to lose the ability to produce capsules, but to retain the A antigen. Such a strain may eventually show slight virulence, but good immunizing power. The Pretoria North strain isolated early in this work (Section A) may be an example. This strain, however, was the only one encountered where slight virulence of the smooth variant was associated with good immunizing properties, and there may be other explanations for this.

(4) A strain may suddenly lose the ability to produce capsules. This may happen at any time and could occur during other processes of attenuation and also in relatively attenuated strains. Such strains would become avirulent. The immunizing power should depend on the amount of residual A antigen and in general this should be less than that present in virulent strains. The strains would then be avirulent and have immunizing power proportional to the virulence of the parent strain. As shown in the earlier part of the discussion, there is evidence that the immunizing power of the rough avirulent variants does depend on the virulence of the smooth mucoid from which they arose. It is also possible that the rarely encountered avirulent vaccine strains possessing slight immunizing power may arise in this way.

It also seems as if changes in virulence which are not referable to loss of the capsule are usually gradual; as for example in ageing strains or during attenuation. The highest degree of immunizing power would probably be possessed by uncapsuled variants from very deadly strains of anthrax, provided that the uncapsuled variant could be obtained quickly by methods which do not greatly affect the hypothetical A factor. The method of dissociation on serum agar in carbon dioxide appears to offer hope of achieving this.

The above statement is no more than a working hypothesis which has proved useful in the present work. It offers an interpretation for a number of observations and is reasonably economical in the number of assumptions made.

SUMMARY AND CONCLUSIONS.

1. Smooth mucoid colonies developed in anthrax cultures held for long periods at 37° C. in a number of different media. Similar colonies developed in strains attenuated at 42° C.

2. All these smooth mucoid colonies yielded rough variants which were usually less virulent than the smooth parent and which in some cases showed a complete and abrupt loss of virulence.

3. The loss of virulence and smoothness was associated with a loss of the ability to produce capsules both *in vitro* and *in vivo*.

4. A number of these rough avirulent daughter strains produced immunity to anthrax in guinea pigs.

5. Fully virulent and freshly isolated anthrax strains always grew smooth mucoid on serum agar in carbon dioxide.

6. These virulent strains rapidly developed rough daughter colonies in the carbon dioxide, in the same way that attenuated smooth mucoid strains developed rough daughters under ordinary conditions (summary 1 and 2).

7. These rough variants obtained on serum agar in carbon dioxide were completely avirulent and uncapsuled *in vitro* and *in vivo*.

8. These rough avirulent dissociants were able to produce a high degree of immunity in guinea pigs and preliminary immunity tests on sheep were very promising.

9. Evidence is brought forward concerning the significance of the capsule in virulence and immunity.

10. The above findings are discussed.

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