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 the smooth mucoid grown in carbon dioxide.

(3) When grown in air, subcultures from both the smooth mucoid 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 two days incubation in carbon dioxide the growth generally was rough, but the centre portion was opaque and the opacity extended to the edge of the culture in a number of radiating lines. These projected as opaque rough outgrowths from the edge of the colony. Small smooth mucoid patches commenced to grow in the opaque areas. Between the opaque areas were rough areas which were more translucent and showed a more marked cuneiform structure than did the opaque parts of the culture. The denser and the less dense parts were sharply demarcated from each other.

The smooth mucoid patches gradually extended, but were restricted to the opaque areas and did not tend to overlap the more translucent parts of the culture. After strain 568 had been grown in carbon dioxide for seven days, subcultures were made (a) from the mixture of mucoid and opaque portion of the growth and (b) from the more translucent rough part of the culture. (a) was termed the smooth variant and (b) the rough variant. Both variants were rough when grown in air and the colonies were then morphologically indistinguishable from one another. 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	1/3 slant rough variant		X X	X
3	1/3 slant smooth variant	X	X X	

The difference in virulence between the variants was slight and it was impossible to say whether it was significent. Because of the large doses given, and because 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 the experiment noted in table XXX were again subcultured onto agar in air and guinea pigs were again injected. The results were as follows:-

TABLE XXXI.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation. 20	40	60
3	1/3 slant rough variant		X X	X
3	1/3 slant smooth variant		X X X	

The difference in virulence was very slight; but again the rough variant seemed a little less virulent. The slight differences noted in tables XXX and XXXI did not really 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 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, although rough and uncapsuled in carbon dioxide, were able to produce capsules *in vivo*. Thus the carbon dioxide was not as good an environment

for the production of capsules as at first thought, and certainly 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 in a completely smooth mucoid form, 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 little 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 was a factor which 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 serum agar. All the virulent strains were rough, but most of the cultures had rather a shiny surface, and by the fourth day about 20 per cent of the virulent bacilli showed capsules. Laboratory strains with any tendency at all to the production of mucoid growth on ordinary agar, developed a richly mucoid structure on the

50 per cent serum agar and all the bacilli or such strains developed capsules. Strains like the rough avirulent Boshoff remained rough and uncapsuled on this medium. The 50 per cent serum agar had also a satisfactory consistency and transparency. Rough virulent strains did not, however, grow mucoid on this medium, although they showed an increased number of capsules. Since these 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 sub-cultured 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 Section C, except that 50 per cent serum agar was used instead of nutrient agar.

The technique eventually adopted was as follows. The medium used was 50 per cent horse serum nutrient agar. This was put up in Mason's tubes and partially dried in the incubator for 24 hours. Thereafter, the fluid liberated by hysteresis was removed and the tubes were ready for use. The inoculation was always done on about one square cm. of surface and incubation carried out in anaerobic jars of 1900 ml. capacity to which the requisite amount of carbon dioxide was added, as described in Part I. No satisfactory

method for keeping the carbon dioxide pressure constant was devised, therefore the results must be interpreted for the particular system used here; that is, each jar of 1900 ml. contained one Mason's tube inoculated on one square centimetre of surface. Whenever incubation lasted more than 24 hours the jars were opened daily and the CO_2 tension adjusted.

(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 and showed no trace of roughness. Two days later 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 again on agar in air and each was again rough. The virulence was then tested on guinea pigs as follows:-

Three guinea pigs each received 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 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 1/3 slant of strain originally isolated from smooth mucoid growth in carbon dioxide - all survived.

Three guinea pigs inoculated with 1/3 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 s train 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 incarbon 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 which was 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 1/5 slant of smooth mucoid growth in carbon dioxide. All survived.

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

Three guinea pigs were each inoculated with 1/3 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 killed 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	1/24 agar slant of variant from smooth mucoid in carbon dioxide	X		X X X X X	X X	X X X X	X	/ / / / / X
22	1/24 slant variant from rough in carbon dioxide	All survived.						

It was clear therefore, 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 had 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 immunised with	Immunity tested with	Death: Hours after inoculation 20	40	60	80	100	120
6	1/4 agar slant strain XIV derived from mucoid growth in CO_2	1/6 serum agar slant smooth mucoid Boshoff strain.		X X X X X X				
9	1/4 agar slant strain XIV from rough growth in CO_2	ditto		X X X X X X X		X	X	
9	uninoculated controls	ditto		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 rough strain being completely avirulent. Guinea pigs which had received a large single dose of these variants were not more resistant to a large test

dose of the smooth mucoid Boshoff strain than uninoculated controls.

The whole experiment with the original virulent strain XIV was then repeated from the beginning. 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, and 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. Both variants were very rough under these conditions. Each was then subcultured twice more onto agar in air and then 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	1/4 agar slant variant from smooth mucoid in CO_2		X X X	
3	1/4 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	1/4 slant variant from smooth mucoid in CO_2		X X X	
3	1/4 slant from rough in CO_2			/ / /

Each variant was then kept on agar slants in a refrigerator and subcultured two months later. The subculture 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 originally been smooth mucoid under these conditions was again smooth mucoid and capsuled. The other was again rough an uncapsuled.

TABLE XXXVI.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation 20	40	60
3	1/5 slant variant from smooth mucoid in CO_2		X X	X
3	1/4 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 had 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	1/5 slant smooth mucoid Boshoff						/ / /
3	One injection rough variant strain XIV	ditto						/ / /
6	Uninoculated controls	ditto			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 immunising strain sufficed to protect 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 immunised.

Six weeks later nine guinea pigs were each inoculated with 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 definitely virulent for rabbits. (3) Virulent Boshoff - old stock virulent strain - virulent for rabbits and sheep, but less virulent than when isolated 15 years ago and less virulent than strain 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	1/4 slant avirulent rough XIV.	1 slant smooth mucoid Boshoff					X			/ /
6	Uninoculated controls	ditto			X X X	X X X				
3	1/4 slant avirulent rough XIV	1/12 slant XVIII			X X					X
6	Uninoculated controls	ditto	X X X X X X							
3	1/4 slant avirulent rough XIV	1/5 slant virulent Boshoff				X		X X		
3	Uninoculated controls	ditto	X X X							

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

Summary.

Strain XIV grew smooth mucoid on serum agar in carbon dioxide and readily produced rough variants. Subcultures from the smooth variant onto agar in air were rough and in the one case virulent. Subcultures from the rough varian

were also rough in air but were completely avirulent for guinea pigs. Guinea pigs which had 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).

(1) 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 in a similar way. Strain XIV was therefore streaked on to 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 at one spot near the periphery 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	Deaths: Hours after inoculation 20	40	60
3	1/4 slant from opaque centre of colony on serum agar		X	X X
3	1/4 slant from translucent wedge at margin of colony on serum agar		X X X	

The experiment was repeated from the beginning. 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 streaked on agar slants, and after a further subculture, guinea pigs were inoculated as follows.

TABLE XL.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation 20	40	60
3	1/4 slant isolated from opaque centre		X X	 X
3	1/4 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 two types of growth: a translucent centre which projected in a number of rays to the margin of the colony with rough, flat and dull sectors between. Each type of growth was streaked onto agar slants and injected into guinea pigs after 24 hours.

TABLE XLI.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation 20	40	60	80
3	1/4 slant sub-culture from translucent centre part of culture		X X	X	
3	1/4 slant sub-culture from rough part of culture		X X X		

It was apparent that strain XIV did not show a homogeneous colony morphology on the serum agar. The difference was 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 made from the culture on the 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 was not suitable for experiments on rapid dissociation.

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

This strain had been 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 previous series of 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	1/3 slant variant from smooth in CO_2	X	X X	
3	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 under these conditions.

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	1/4 slant variant from smooth in CO_2		X X X
3	1/4 slant variant from rough R in CO_2		X X X

Both variants were virulent for guinea pigs and when the two variants were streaked onto serum agar in carbon dioxide, both grew smooth mucoid and developed capsules. Thus, although the variants had originally been selected because of a difference in colony morphology and capsule formation in carbon dioxide, this difference had 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 from the beginning 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. Agar slants were streaked from the smooth mucoid and from the rough parts of the culture and incubated in air. Both were very rough after 24 hours. The variants were subcultured again the following

day. These subcultures were again rough and were used to inoculate guinea pigs as shown in the table below. 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	1/3 slant variant from smooth growth in CO_2		X X X		
3	1/3 slant variant from rough growth in CO_2				/ / /

A week later six guinea pigs each received 1/3 of a slant of the variant from the rough growth in carbon dioxide and all survived. The three guinea pigs which had first survived the rough variant (table XLIV), each received another large injection of the same strain two weeks later. None died.

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	1/4 slant variant from smooth mucoid growth in CO_2	X	X X	
3	1/3 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 each grown on ordinary nutrient agar in air, the colony form in both cases was 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 most likely that a considerable number of smooth elements had been picked when transplanting the rough variant. 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 proved virulent, and the subculture from the rough growth avirulent. This 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.

Three guinea pigs which had received one inoculation of the avirulent strain received a second inoculation 18 days later. The resistance of these three guinea pigs and of another six which had received one injection of the avirulent rough strain was tested a month later with the virulent strain XVIII and the smooth mucoid Boshoff strain. The virulent test strains were the same used to investigate the immunity conferred by the variant of strain XIV, and the tests were carried out at the same time so that 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 tested with	Death: Hours after inoculation. 20	40	60	80	100	120	140	160
3	Two injections avirulent Drummond	1/12 slant XVIII (virulent)				X			X X	
3	One injection avirulent Drummond	ditto				X				/ X
6	Untreated controls	ditto		X X X X X X						
3	One injection avirulent Drummond	1 slant smooth mucoid Boshoff								/ / /
6	Untreated controls	ditto			X X X	X X X				

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

TABLE XLVII.

No. of guinea pigs	Previous inoculations	Each tested with	Death: Hours after inoculation 40	80	100	140	180	200
3	One injection avirulent rough Drummond	1/4 slant virulent XVIII.		X				/ X
9	Controls	ditto	X X X X X X X X X					

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

In table XLVI it was shown that guinea pigs immunised 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 test strain was used all the immunised 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 other experiment of the series has any non-immunised guinea pig survived a test dose of the smooth mucoid Boshoff strain. It was certain that the three guinea pigs which had received one immunising 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. The owner

of the goat had himself become infected. On the day after the strain was isolated 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. After a further 48 hours 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 were very rough after twenty-four hours. Each variant was then subcultured on two successive days and tested as follows.

TABLE XLVIII.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation. 20	40	60	80
3	1/3 slant sub-culture from smooth growth in CO_2		X		
		X	X		
3	1/3 slant sub-culture from rough growth in CO_2		X		
			X		
			X		

Thus there was no difference in virulence between the variants. 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 nutrient agar in air and both grew very rough. At the same time each type was again streaked on serum agar in carbon dioxide and again the former

was smooth mucoid and the latter rough. Guinea pigs were inoculated with the cultures grown on agar in air. 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 XLIX.

No. of guinea pigs.	Each inoculated with	Death: Hours after inoculation 20	40	60	80
3	1/4 slant variant from smooth mucoid growth in carbon dioxide.		X X X		
3	1/4 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 1/4 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 isolated 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 L.

No. of guinea pigs.	Each inoculated with	Death: Hours after inoculation. 20	40	60	80
3	1/3 slant variant from smooth mucoid growth in carbon dioxide.		X X X		
3	1/3 slant variant from rough growth in CO_2				

This experiment 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 guinea pigs which survived in the experiment noted in table XLIX received a second injection of the 'rough' variant three weeks later. Twenty-five days later the immunity was tested as shown in the table below. The immunity of six guinea pigs which had received one injection of the rough variant six weeks previously was tested at the same time. The virulent strains were those used in the tests on strain VII and XIV (tables XXXVIII and XLIII), and the controls were also the same.

TABLE LI.

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

These tests 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 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 guinea pigs tested against the less virulent smooth mucoid Boshoff strain all survived. The six controls all died and up to the present no guinea pig injected with this strain had survived unless previously immunised. When the results are compared with those obtained with strains XIV and VII (tables XXXVIII and XLII) it seemed 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 L) were tested four weeks later with virulent XVIII.

TABLE LII.

No. of guinea pigs	Previous Inoculations	Each tested with	Death: Hours after inoculation 40	80	120	160	200	240	280	320	360	400
3	One injection avirulent rough XVIII	1/4 slant virulent XVIII					X			X		/
9	Controls	ditto	X X X X X X X X X									

The guinea pigs immunised with the rough strain which had been kept for six weeks again showed a high degree of resistance to virulent XVIII. One animal survived, one lived 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 slightly 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 carbondioxide 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 shown to be 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 immunising 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. These inoculations were carried out two months after the rough variant of strain XVIII had first been isolated and the immunity of the sheep was tested five weeks later with a large dose of virulent organisms. The test dose was a 1/100 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 been kept in a refrigerator since. The actual M.L.D. 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 LIII.

Sheep Nos.	Previous inoculations	Each tested with	Death: Hours after inoculation. 20	40	60	80	100	120	140
	1/100 slant rough XVIII	1/100 dilution virulent XX 1 c.c.							
45405	"	"							/
45408	"	"							/
45410	"	"							/
45411	"	"							/
45417	"	"							/
45426	"	"							/
45431	"	"							/
45442	"	"							/
	1 c.c. 1/100 dilution Batch 88								
45607	"	"			X				
45449	"	"		X					
45402	"	"		X					
45434	"	"			X				
45407	"	"		X					
45412	"	"		X					
	Controls								
44989	"	"			X				
45003	"	"			X				
45413	"	"			X				
45377	"	"						X	
		1 c.c. 1/10,000 dilution strain XX							
45416	"	"			X				
45375	"	"			X				

The results show clearly that the avirulent variant obtained in carbon dioxide from strain XVIII was a much better immunising agent than an ordinary vaccine strain. The vaccine strain B. 88 was chosen because it had yielded good results in a previous titration, although it failed to stand up to the present more severe test. The avirulent XVIII, however, seemed

to possess immunising properties of quite a 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 LIV.

No. of guinea pigs	Each injected with	Death: Hours after inoculation 10	30	50	70
3	1/5 slant variant from smooth growth in CO_2		X X	X	
3	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 after 24 hours and guinea pigs were inoculated as follows.

TABLE LV.

No. of guinea pigs	Each injected with	Death: Hours after inoculation 10	30	50	70
6	1/5 slant variant from smooth growth in CO_2 (48 hours)		X X X X X X		
6	1/5 slant variant from rough growth in CO_2 (48 hours).			X X	X X X

In this latter 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 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 were inoculated with this culture. The three 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 had been carried over, and that these were the killing agents. The retarded deaths were probably due to the small dose of virulent bacilli in a predominatingly 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 and 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 LVI.

No. of guinea pigs	Each injected with	Death: Hours after inoculation. 10	20	30	40	50	60
6	1/3 slant variant from smooth growth in CO_2 (48 hours)				X X X X X X		
6	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 each variant was very rough and guinea pigs were injected as follows.

TABLE LVII.

No. of guinea pigs	Each injected with	Death: Hours after inoculation. 10	20	30	40	50	60	70
6	1/3 slant variant from smooth growth in CO_2 (72 hours)				X X X X X X			
6	1/3 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 were tested with 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 LII. The other six immunised guinea pigs were tested with the smooth