It was clear that the serum of the immune guinea pigs gave a considerable degree of protection against a very large dose of the smooth mucoid variant. One control lived longer than the other nine, and longer than any non-immune guinea pig which had received a similar dose of smooth variant ever lived. In subsequent experiments no similar control ever lived as long. 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 might have given complete protection.

Immunity experiments on sheep and goats.

The immunisation of guinea pigs with 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 immunisation of guinea pigs it was decided to test its immunising power in sheep and goats.

Six sheep and three goats each received about one tenth of an agar slant of the rough variant. None of these animals showed any rise of temperature after the inoculation. The immunity was tested three weeks later by the subcutaneous administration of a virulent spore suspension. The dose lay between five and fifty lethal doses. This test dose was the same as that used over a period of several years in testing the immunity produced by routine anthrax vaccines. For this reason controls were considered unnecessary and were not included.

TABLE IX.

Sheep	Date received rough variant	Date received virulent emulsion	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 experiment was then performed on goats. Four goats received approximately the same dose of rough variant as those noted in table IX (1/10 slant). The suspension was made up in lanolin and oil according to the method of Ramon and Staub (1935). These authors claim that by the use of this vehicle they were able to produce a high grade immunity in sheep with one dose of Pasteur I vaccine. The lanolin and oil are supposed to prevent phagocytosis of the bacilli and to retain them at the inoculation site for long periods, thus maintaining a prolonged antigenic stimulus. Four other goats received the same amount of rough variant suspended in physiological saline. Six weeks later the goats were inoculated with twice the amount of virulent spores given to the animals noted in table IX. Two controls received half the above dose.

Table X records the results.

TABLE X.

Goats	Date inoculated with rough variant in lanolin & oil	Date inoculated with virulent spores	Results.
42932	8.5.35	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	"	/
42937	"	"	/
42939	"	"	/
42940	"	"	/
Controls Goats		Half amount virulent spores	
44302	-	17.6.35	X anthrax 20.6.35
44317	-	"	X anthrax 21.6.35

Another experiment was then carried out to examine the immunising power of two inoculations of the rough variant. Four goats received two inoculations each consisting of 1/10 of an agar slant of rough-variant with twenty day intervals between the inoculations. Sixteen days later, they were tested with a dose of virulent spore suspension ten times as large as that noted in table IX and twenty times as large as that used in the routine testing of immunity produced by anthrax "goat" vaccine. The results were as follows:-

TABLE XI.

Goats	1st Dose rough variant	2nd Dose rough variant	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 far higher 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 vaccine 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 immunising power between 20 sheep doses and 2000 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 immunising 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 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 and laboratory animals, including mice.

(3) The smooth mucoid strain was capsuled and the rough variant 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 immunised 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 - Virulent 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 on agar, exactly as in the previous experiments 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 in order to eliminate capsuled bacilli. This was easily accomplished. 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, three groups of twelve being used. As the technique and the results were essentially the same, the details are presented in the same table. Doses of the order of 1/25 of an agar slant were given to each mouse, and the injections made subcutaneously.

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 intercurrent infection.
18	1/25th slant rough variant	No deaths from Anthrax.				4 died of intercurrent infections.

All the mice indicated thus X died with a typical anthrax septicaemia. This smooth mucoid strain, therefore, still retained some virulence for mice. The virulence was not very high, as in spite of the relatively large doses used, there was a certain amount of scattering in the times of death. No mouse which received the rough variant showed evidence of anthrax.

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 strain as had first been administered. About the same amounts were given as in the previous experiment. One of the guinea pigs

inoculated with the smooth mucoid variant of V. anthrax A died of an intercurrent infection. Nine days after the last injection of these avirulent strains, the five remaining guinea pigs, together with four controls, each received one fifth of an agar slant of the smooth mucoid virulent Boshoff strain. This latter strain was the one used as a test strain in the earlier experiments on guinea pigs.

The results are summarized below:-

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	1/5 slant smooth mucoid V.Boshoff		X X		
3 injections rough V. Anthrax A	3	ditto		X X	X	
Uninoculated controls	4	ditto		X X X X		

All the treated guinea pigs died, and all but one died at the same time as the controls. Very large doses had been used for immunisation, so that it could be assumed that neither variant was able to immunise the guinea pigs against the smooth mucoid Boshoff strain. Similar experiments using the rough variant from the Boshoff strain had resulted in a solid immunity.

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. All the injections were of the order of 1/25 of an agar slant of bacilli. Twelve

days after the last injection these mice, together with five controls, were each inoculated with 1/25 slant of the smooth mucoid V. Anthrax A. This had previously proved virulent for mice (table XIV). The one mouse which had survived a previous inoculation of the smooth mucoid strain (table XIV) received the same injection. The results are tabulated below.

TABLE XVI.

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

There was little evidence that the treated mice were more resistant than the controls. The times of death from an injection with the smooth mucoid V. anthrax A in table XIV showed as much scattering as the deaths of the 'immunised' mice in the above table. Thus any immunity as the result of injections of the rough variant could only have been a low grade one. 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 immunising 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 (I and II), 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 sub-cultured 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 1/10 of an agar slant of smooth mucoid 568.

Three guinea pigs each received 1/5 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	200
14	1/20 slant smooth mucoid 568 subcutaneously		X	X X	X		X /
13	1/20 slant rough 568 subcutaneously.		X	X X	X	X	/

died
One mouse in each group/of an intercurrent infection.

Thus both variants were 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 test 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 2/3 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 1/5 agar slant smooth mucoid Boshoff ten days later.

TABLE XVIII.

Previous injections	No. of guinea pigs.	Each inoculated with 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	

Thus two large doses of these variants of 568 did not confer any added resistance on the guinea pigs tested. The treated guinea pigs all died at the same time as the controls.

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. From about the third week onwards smooth mucoid colonies grew in streaks made

from the Erlenmeyer flask, but attempts to stabilize them failed. In four to five weeks smooth mucoid colonies were obtained which showed a high degree of stability. They produced a few rough outgrowths, but it was found impossible to separate these from the smooth mucoid elements. After a few days incubation these roughs showed a medusa head structure but were covered by a mucoid layer, full of capsuled bacilli. After six weeks incubation of the Erlenmeyer flask cultures, a smooth mucoid strain easy to maintain was isolated and this occasionally gave rise to rough flat outgrowths containing only uncapsuled bacilli. Two rough variants were selected and one 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 each inoculated with 1/3 slant smooth mucoid variant. One guinea pig died of an intercurrent infection.

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

Three guinea pigs were each inoculated with 1/3 slant of the 7th subculture of a rough variant. Two of these died of gas gangrene.

As none of the variants was pathogenic for guinea pigs, another test was performed on mice. Twelve mice were each inoculated with 1/50 of a slant of the smooth mucoid variant, four mice with 1/50 slant of the 9th subculture of one rough variant, eight mice with the twelfth subculture; and lastly, four mice with the sixth subculture of another rough variant. The results are given below:-

TABLE XIX.

No.of mice	Inoculated with (each)	Death: Hours after inoculation. 20	40	60	80	100
12	1/50 slant smooth mucoid Drummond	X X X X X X	X X X	X		/
8	1/50 slant 12th sub-culture: rough.	No deaths				
4	1/50 slant 9th sub-culture: rough.	No deaths				
4	1/50 slant 6th sub-culture: rough.	No deaths.				

Thus the smooth mucoid variant was virulent for mice, while the rough variants which had arisen from the smooth mucoid were avirulent.

Immunity Test with variants of V. Drummond.

The five guinea pigs which had survived the virulence test, together with three uninoculated controls, each received one sixth 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 1/3 slant smooth mucoid Drummond	2	1/6 Slant smooth mucoid Boshoff		X X	
1/3 slant rough Drummond	3	ditto		X X	X
Uninoculated controls	3	ditto		X X	X

There was thus no evidence of any 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.

The strain used was an old stock virulent strain called "Pretoria North". A layer of meat broth agar was run into the bottom of an Erlenmeyer flask. When set, a layer of sterile unheated horse serum was poured on top of the agar to a depth of 0.5 cm. This medium was inoculated with the virulent Pretoria North. Sterile serum was occasionally added to compensate for evaporation. After ten days incubation at 37°C 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 from this smooth mucoid culture, and it was a month after isolation before a rough variant was picked which seemed free from capsuled bacilli.

When this variant had been subcultured five times, guinea pigs were inoculated as follows:-

Pathogenicity of variants of Pretoria North Strain.

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

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

It was doubtful whether the guinea pig supposed to have died from anthrax really did so. Very few bacilli were seen in the spleen and these might easily have been part of the original inoculum.

The virulence was then tested on mice as follows:-

Six mice received a subcutaneous injection of 1/20 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 1/20 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 three guinea pigs which had survived the virulence test, together with four controls, each received 1/4 agar slant smooth mucoid variant from the virulent Boshoff strain.

TABLE XXI.

Each previously inoculated with	No. of guinea pigs	Each inoculated with	Death : Hours after inoculation. 20	40	60	80	100	120	140
1/3 slant smooth mucoid Pret.North	1	1/4 slant smooth mucoid Boshoff		X					
1/3 slant rough Pret.North	2	ditto							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 guine 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 at fourteen days interval with 1/2 agar slant of the rough variant from the Pretoria North strain. None of these animals showed any ill effects, so that the avirulence of the variant could be assumed. Two weeks after the second injection of the rough variant 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	ditto		X X X						
2 injections ½ slant rough Pretoria North	3	1/6 slant virulent Drummond			X			X	X	
Uninoculated controls	3	ditto		X X X						

The results showed that two injections of large amounts of the rough variant from the smooth mucoid Pretoria North variant immunised guinea pigs solidly against a massive dose of smooth mucoid Boshoff. Considerable resistance was shown also to a large test dose of virulent Drummond. This strain was still very virulent for sheep and rabbits.

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 immunised solidly against this strain.

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

B.

DISSOCIATION IN ATTENUATED STRAINS.

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

This strain, known as strain IV, was isolated six months previously from a pig which had died of naturally acquired anthrax. It was incubated at 42°C in broth, and subcultures were tested at intervals for virulence. After sixteen days the cultures were sealed and stored at room temperature for two months. The virulence was not tested immediately before storage. Subcultures made from the broth tubes after storage showed smooth mucoid growth, although the mucoid characteristic was not particularly marked. Rough wedges were sometimes seen in the smooth colonies, but

dissociation was not very active.

After the first subculture of the smooth strain, the mucoid characteristic was lost, and henceforward the strain was smooth. Smears showed a few bacilli with capsules. A somewhat rough variant was picked from the smooth strain; and smears 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.

In one subculture of this rough variant a faint film like outgrowth developed. On subculture this grew as a faint dull film and was exceptionally rough by transmitted light. After 48 hours condensations formed in this film and these developed into intermediate or rough-smooth (RS) anthrax colonies. Smears from the film like growth showed only B.anthracis. This corresponded to the rough phantom variant of Nungester (1929). One of the rough condensations was picked and subcultured a few times and the growth remained rough to rough-smooth.

Pathogenicity tests with variants of strain IV.

Six guinea pigs received 1/3 of a slant of the smooth variant of strain IV.

Three guinea pigs received 1/3 of a slant of the rough variant from the smooth strain.

Three guinea pigs received 1/3 of a slant of the rough variant from the rough phantom strain.

The results are given in the table below:-

TABLE XXIII.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation 40	80	120	160	200	240	280	320
6	1/3 slant smooth strain from strain IV		X X X	 X X	 X				
3	1/3 slant 6th subculture rough variant from smooth								/ / /
3	1/3 slant 3rd subculture rough variant from rough phantom.					X			/ /

The smooth strain was thus proved virulent whereas the rough variants were practically avirulent. There was a trace of virulence in the rough variant as shown by the death of one guinea pig in the third group. 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. As the pure smooth strain was not very virulent, it was very likely that a small dose of smooth in the rough strain would kill only an occasional animal. Had the smooth bacilli been very virulent, a few included in the rough variant would probably have killed almost as well as the pure smooth strain; in which case there would not have been a marked difference in virulence between the strains.

Immunity tests with variants of strain IV.

Fourteen days after the experiment noted in table XXIII the five guinea pigs which had survived received a second large dose of the same rough variant. 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 test dose was one fifth of an agar slant and 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	1/5 agar slant smooth mucoid Boshoff					X			/
2 injections rough from rough phantom Strain IV.	2	ditto					X			X
Uninoculated controls	4	ditto		X X X X						

Two injections of the rough variants from Strain IV gave guinea pigs a much increased resistance to the smooth mucoid Boshoff strain. 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 rough variant showed a few capsules in carbon dioxide and this might be the explanation of the slight residual virulence.

(3) Guinea pigs which had received two large injections of the rough were much more resistant (table XXIV) to a large dose of smooth mucoid Boshoff than were uninoculated controls, although a solid immunity was not established.

(2) Dissociation in an attenuated strain of anthrax.

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. Rough and rough-smooth colonies grew in the streak. One of each type was selected and subcultured until the differentiation was more complete. 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 1/3 of a slant of the smooth variant.

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

Three guinea pigs were each inoculated with 1/3 of a slant of the rough variant.

Six mice were each inoculated with 1/20 of a slant

of the rough variant.

All the mice and all the guinea pigs survived.

Immunity test with variants of attenuated strain.

The six guinea pigs which had survived the virulence test together with four controls each received 1/4 slant of the smooth mucoid Boshoff strain. The results were as follows:-

TABLE XXV.

Each previously inoculated with	No.of guinea pigs	Each inoculated with.	Death: Hours after inoculation 20	40	60	80
1/3 slant smooth variant	3	1/4 slant smooth mucoid Boshoff	X	X X		
1/3 slant rough variant	3	ditto		X X	X	
Uninoculated controls	4	ditto		X X X X		

The 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 ANTHRAX STRAINS IN CARBON DIOXIDE.

In none of the foregoing experiments had the rough dissociant from a smooth or smooth mucoid colony been 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 variation in other strains of micro-organisms. The question to be decided, 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 has never been 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 that it 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 than 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 dissociation 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 30°C it became quite rough and at the same time became completely avirulent. 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, apparently, 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 30°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 B.typhosus grown

at 30°C. An obvious difficulty was that the "rough" typhosus in Felix's experiment was avirulent while the rough anthrax was virulent. If, however, the virulence depended on the organism regaining the "normal" or smooth form *in vivo* (as has been shown to occur in the case of anthrax) the difference in behaviour between the two organisms could be ascribed to the *B. anthracis* remaining viable for a sufficient length of time to enable this change to take place; whereas the rough *S. typhi* could possibly be more easily eliminated before reaching the virulent stage in sufficient numbers to overcome the defences of the body. A number of experiments showed that rough avirulent anthrax variants persisted in guinea pigs and mice and remained viable for many days in spite of active phagocytosis.

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. Thus the theory was untenable, unless it was assumed that the rough bacilli - which were assumed to be avirulent - gave rise quite freely to virulent smooth forms. The experiments which had been carried out with rough variants from smooth mucoid strains told against this point of view unless the rough virulent bacillus was a"pseudo-rough" in the sense that S. typhi grown at 30°C was a rough. In this case of course the problem was again as stated in the first hypothesis.

The problem, therefore, was to devise an environment in which the rough virulent anthrax 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 circumstances involving long continued cultivation under unfavourable conditions.

It has been 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 therefore made (by analogy with S. typhi) 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 whether rough variants would occur naturally in these cultures.

Technique.

A Mason's tube 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 enabled the growing edge of the culture to be kept under observation for some time, since variants were more readily obtained from the edge of the culture than from the centre. 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.

A 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, rough outgrowth appeared at a spot on the periphery of the colony and was sharply demarcated from the surrounding mucoid culture.

The following experiment was then carried out:-

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	1/3 slant original stock V. Pretoria North.		X X X						
3	1/3 slant subculture from smooth mucoid in CO_2		X X X						
15	1/3 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 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". This strain was always very rough, but after a week in the carbon dioxide the growth was almost completely smooth mucoid. During this time rough outgrowths occasionally appeared at the edges of the advancing growth, but these always developed a mucoid surface. If the rough outgrowths were picked and incubated in carbon dioxide, these subcultures tended to be smooth mucoid. After the original tube had been two weeks in carbon dioxide some rough flat outgrowths were seen which when picked and incubated in carbon dioxide failed to develop any mucoid characteristics. This was now

termed the "rough" variant and a subculture was made and grown in air. At the same time the smooth mucoid portion of the growth in carbon dioxide was subcultured and incubated in air. This latter was termed the "smooth" variant. Both variants appeared quite rough in the subcultures incubated in air. These were then injected into guinea pigs without further subculturing.

TABLE XXVII.

No. of guinea pigs.	Each inoculated with	Death: Hours after inoculation. 20	40	60	80
3	1/3 slant "rough" variant			XXX	
3	1/3 slant "smooth" variant	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 from 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 fact that the rough variant was virulent and also the fact that it took a significantly longer time to kill than did the "smooth" variant. To test this, three guinea pigs were inoculated with 1/3 of a slant of the smooth variant and three guinea pigs with 1/3 of a slant isolated from the blood of one of the guinea pigs which had died from the injection with the rough variant in the previous test. If the rough variant had been a mixture of an avirulent strain and a virulent strain, it is probable that the strain re-isolated from the guinea pig would be fully virulent. The results of this test were as follows.

TABLE XXVIII.

No. of guinea pigs.	Each inoculated with	Death: Hours after inoculation. 20	40	60
3	1/3 slant rough strain isolated from guinea pig			X X X
3	1/3 slant "smooth" strain		X X X	

Passage through a guinea pig did not enhance the virulence of the rough variant because the survival time of the guinea pigs inoculated with it was still longer than the survival time of those inoculated with the "smooth" strain. On this result one may assume that 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 times 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	1/3 slant of 7th subculture of rough variant		X X	X	
2	1/3 slant 9th subculture of "smooth" variant	X X			

Again the guinea pigs inoculated with the rough variant took a much longer time 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