mucoid Boshoff strain. This strain had gradually become 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.

TABLE LVIII.

No. of guinea pigs.	Previous inoculations	Each tested with	Death: Hours after inoculation. 20	40	60	80	100	120	140	60	80	200	20
6	One injection rough avirulent strain XX	1/4 slant virulent XVIII			X				X	X X			/ /
9	Controls	ditto		X X X X X X X X X									
6	One injection rough avirulent strain XX	1/3 slant mucoid Boshoff (II Ad.)			X		X X X	X					/
14	Controls	ditto		X X X X X X X X X X X X X	X X								

The guinea pigs which had received one injection of the avirulent variant from strain XX showed much more resistance to anthrax than normal guinea pigs. Three guinea pigs survived the very large dose 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 in air and there appeared culturally identical. However, the variant from the smooth mucoid growth in carbon dioxide was virulent while the sub-culture 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.

(5) Dissociation of virulent Boshoff (Strain II c) 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 same experimental procedure was adopted as before. The growth was smooth mucoid after 24 hours in carbon dioxide and 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 LIX.

No. of guinea pigs	Each injected with	Death: Hours after inoculation					
		20	40	60	80	100	20
6	1/3 slant variant from smooth growth in CO_2		X X X X X	X			
6	1/3 slant variant from rough growth in CO_2				X X	X X	X X

There was a considerable difference in the length of time that the guinea pigs lived. The rough variant took much longer to kill the guinea pigs and in view of the large doses used, the fact that all the guinea pigs inoculated with the smooth variant were dead before the others had started dying 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 second variant were due to contaminat ing '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 onto agar and incubated in air. Both cultures were rough after 24 hours and guinea pigs were inoculated as follows.

TABLE LX.

No. of guinea pigs	Each injected with	Death: Hours after inoculation 20	40	60	80	100	20
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						/ / /

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 1/3 of an agar slant of the rough variant and survived.

Immunity tests with avirulent strain from virulent Boshoff.

The immunity of three guinea pigs which survived in the experiment noted in table LX was tested after six weeks with a heavy suspension of virulent XVIII. Three of the guinea pigs injected with the avirulent variant three weeks later were also tested with virulent XVIII. The other three guinea pigs were tested with the smooth mucoid Boshoff strain which had been exalted by guinea pig passage. (See page 91). The results are summarized below and the massive test doses used should be noted.

TABLE LXI.

No. of guinea pigs.	Previous inoculations	Each tested with	Death: Hours after inoculation. 20	40	60	80	100	20	40	60	80	200
3	1/3 slant avirulent variant of strain II c	1/4 slant virulent XVIII after 6 weeks		X		X			X			
3	ditto	ditto after 3 weeks		X		X	X					
3	ditto	1/4 slant smooth Boshoff after 3 weeks		X								/ /
7	Controls	ditto		X X X X X X X								
2	ditto Rabbits	ditto				X						/
7	Controls	1/4 slant virulent XVIII		X X X X X								
2	ditto Rabbits	ditto			X	X						

The guinea pigs which had received an injection of the rough avirulent variant from strain II c 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 proved 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 the day after its 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 LXII.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation. 10	20	30	40	50	60
3	1/3 slant sub-culture from smooth mucoid growth in CO_2				X X X		
3	1/3 slant sub-culture from rough growth in CO_2						/ / /

Subcultures of these variants were tested again a week later with the following results.

TABLE LXIII.

No. of guinea pigs	Each inoculated with	Death: Hours after inoculation 10	20	30	40	50	60
3	1/3 slant subculture from smooth mucoid growth in CO_2				X X	X	
3	1/3 slant subculture from rough growth in CO_2						

A third pair of variants was picked after strain XXII had grown in the carbon dioxide for 100 hours. Sub-cultures from the smooth mucoid and the rough portions were grown in air for 24 hours and injected as follows.

TABLE LXIV.

No. of guinea pigs.	Each inoculated with	Death: Hours after inoculation 20	40	60	80	100
3	1/3 slant subculture picked from smooth growth in CO_2 after 100 hours.		X X X			
3	1/3 slant subculture picked from rough growth in CO_2 after 100 hours.			X		//

In the experiments noted in tables LXII and LXIII there was a clear cut difference in the behaviour of the two variants. In that noted in table LXVI there was also a clear cut difference, but one guinea pig inoculated with the variant from the rough growth in carbon dioxide died of anthrax. There was a possibility (inherent in all the experiments) that a few virulent forms could have been carried over. A culture was made from the heart's blood of the guinea pig that died and after 24 hours three guinea pigs each received 1/3 of a slant of this culture. All three guinea pigs were dead by the 40th hour. It was very likely therefore that the rough variant had contained a few virulent forms. It was unlikely that a strain which in large doses killed one out of three guinea pigs rather slowly, could have full virulence restored by one passage, unless the slight remaining virulence was due to rare fully virulent bacilli.

Immunity tests with avirulent variant of strain XXII.

These tests can be compared directly with those carried out with the variants of strain IIc (LXI) as they were performed at the same time, with the same virulent

strains and with the same set of controls. The table summarizes the r esults of the immunity test carried out 4 to 5 weeks after the inoculation of the avirulent strain.

TABLE LXV.

No. of guinea pigs.	Previous inoculations	Each tested with	Death: Hours after inoculation. 10	20	30	40	50	60	70	80	90
3	1/3 agar slant rough avirulent strain XXII	1/4 slant virulent XVIII						X	X		/
2	1/3 slant rough variant (Table LXI)	ditto									/ X
7	Controls	ditto			X X X	X X X					
Rabbits 2	Controls	ditto						X			X
guinea pigs 3	1/3 slant rough avirulent strain XXII	1/4 slant smooth mucoid Boshoff				X					/ /
7	Controls	ditto			X X X	X X X					
rabbits 2	Controls	ditto									/ X

Again a newly isolated virulent strain dissociated on serum agar in carbon dioxide. The variants were culturally identical when grown in air, but the variant derived from smooth mucoid growth in carbon dioxide was virulent, while that 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 LXVI.

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

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

TABLE LXVII.

No. of guinea pigs	Each inoculated with	Each tested with	Death: Hours after inoculation 20	40	60
7	1/3 slant rough variant strain XXVI	1/4 slant II Ad.		X X X X X	X /
6	Controls	ditto		X X X X X X	

Thus the guinea pigs immunised 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.

This was done at the same time as the test on guinea pigs. Six sheep were each injected with 1 c.c. of a 1:2000 dilution of a Mason's tube. This was about 2/3 the usual dose of vaccine and contained about 200,000 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 LXVIII.

Sheep No.	15.5.36 Each immunised with	5.6.36 each tested with	Death: Hours after inoculation 20	40	60	80	100	200	60	300
46770	1/500 slant strain XXVI	1 c.c. 1:25 dilution Batch XX virulent								/
45726										/
45802					X					
45804									X (negative for anthrax)	
45443		"							X	
45824		"								/
	Batch 97									
45799	20.0 c.c.	"			/					
45823	20.0 c.c.	"			/					
45811	0.1 c.c.	"			X					
45800	0.1 c.c.	"			X					
45403	0.01 c.c.	"			/					
45355	0.01 c.c.	"			X					
45810	0.01 c.c.	"			X					
45714	0.005 c.c.	"			X					
45791	0.005 c.c.	"			X					
45829	0.005 c.c.	"			X					
44988	Control	"			X					

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 vaccines, so that the conditions were slightly weighted against it. This variant was tested on sheep because it was the weakest immuniser 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 as to 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 dioxide.

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 culturally they were also very rough; quite as rough indeed as 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 immunised 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 immunised with the ordinary attenuated laboratory vaccine. Strain XVIII gave particularly good results.

E.
COMPARISON OF IMMUNITY PRODUCED IN RABBITS BY A NUMBER OF AVIRULENT VARIANTS AND AN ORDINARY ATTENUATED ANTHRAX STRAIN.

In the experiment noted in table LIII 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 immunising

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 "immunised" 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 immunising abilities could perhaps be made. Guinea pigs could not be used, although their very uniform susceptibility to virulent cultures made them suitable subjects, because they could not withstand the attenuated strain XVII. 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. This had been subcultured three times during the intervening period.

(b) The avirulent variant of strain VII (Drummond) obtained on serum agar in carbon dioxide four months previously and subcultured four times in the intervening period. 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.

Each group of rabbits received an injection of one of the above strains and one group of nine rabbits was retained as a control and received no immunising injection. The results of the test are summarised in the following table. One four-hundredth of an agar slant of test strain XX killed sheep.

TABLE LXIX.

Rabbits	Each inoculated with	Each tested with	Death: Hours after inoculation 20 40 50 60 80 100 200
1	1/4 slant avirulent XVIII	1/5 agar slant virulent strain XX 6 weeks later.	/
2			/
3			/
4			X
5			X
6	Died of intercurrent infection before test.		
1	1/4 slant avirulent VII.	1/5 agar slant virulent strain XX 6 weeks later	/
2			X
3			X
4			X
5			X
6			X
1	1/4 slant avirulent XXII	ditto	/
2			/
3			/
4			/
5			/
6	Died of intercurrent infection before test		
1	1/4 slant attenuated XVII	ditto	/
2			/
3			/
4			/
5			/
6			/
1	Control	ditto	X
2			X
3			X
4			X
5			X
6			X
7			X
8			X
9			X

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 LXV) had shown 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.

F.
THE EFFECTS OF DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE ON THE DISSOCIATION OF VIRULENT B.ANTHRACIS ON 50 PER CENT SERUM AGAR.

Pathogenicity and immunity tests on guinea pigs and sheep with dissociants from virulent anthrax strains.

The results noted in section D were encouraging enough to warrant more extensive and critical experiments. These were carried out to discover the effects of varying the carbon dioxide concentration on virulent strains grown on 50 per cent serum agar and to examine the influence of the different concentrations on the development of mucoid growth, the production of avirulent roughs and the immunising power of these roughs.

The technique adopted was described in section D and the following symbols are used to describe the character of the growth.

SM denotes a colony with a rough edge and slightly mucoid or moist surface.

SM denotes a colony as above, but with a more mucoid surface.

SM denotes a colony with smooth edge and completely mucoid surface.

SM denotes a colony with a very mucoid surface and a pronounced tendency to flow.

SM denotes a colony that can be drawn into long threads. The growth may be almost half a centimetre thick.

The extent of rough growth is shown thus:

ɻ denotes minute rough projections too small to be picked.

ɻɻ denotes well defined rough wedges or outgrowths fairly easy to pick.

ɻɻɻ denotes extensive well defined rough projections from the smooth edge of the colony.

ɻɻɻɻ denotes a broad ring of rough growth entirely surrounding the mucoid centre.

R denotes completely rough growth.

S denotes completely smooth growth.

RS denotes intermediate or rough-smooth growth and the relative preponderance of one or the other factor is indicated by bars, thus RS or RS.

Mason's tubes were inoculated with the various strains and grown in different carbon dioxide concentrations as shown in the following tables.

The relative virulence of a number of the variants obtained was tested on guinea pigs. The variants were first streaked on nutrient agar slants and incubated in air for 24 hours. All grew as rough as the original virulent strains, whether they were derived from the mucoid or the rough growth in carbon dioxide. Three guinea pigs were used in each test and each received 1/4 agar slant of the 24 hour culture from the variant in carbon dioxide.

(1)
The effect of different carbon dioxide concentrations on strain XXVIII.

Strain XXVIII was isolated sixteen years ago and is very virulent for sheep.

Table LXX.

TABLE LXX.

Tube No.	% CO_2	Type of growth after 24 hrs.	48 hrs.	72 hrs.	96 hrs.	Virulence Test: Subbed after hrs.	Type of variant subbed	Death: hours after inoculation 20	40	60	80	100
A	0	RS	SM	-	-		-					
B	0	RS	SM	-	-	48	rough edge		X X X			
C	0	RS	RS	-	-	-	-					
D	5	SM I	SM II	-	-							
F	10	SM	SM									
G	10	SM	SM	-	-	48	SM		X X X			
		I	III	-	-	48	R(III)			X	X	/
H	15	SM	SM	-	-	48	SM		X X	X		
		I	II	-	-	48	R(II)					/ / /
I	20	SM	SM	-	-	48	SM		X X X			
			I	-	-	48	R(I)		X	X X		
J	30	SM	SM	-	-	48	SM	X	X X			
			II			48	R(II)					/ / /
K	40	SM	SM			48	SM		X X X			
			II			48	R(II)					/ / /
L	50	SM II	SM III	-	-							
M	50	SM	SM II	-	-							

Tube No.	% CO_2	Type of growth after 24 hrs.	48 hrs.	72 hrs.	96 hrs.	Virulence Test: Subbed after hrs.	Type of variant subbed	Death: hours after inoculation. 20	40	60	80	100
N	65	SM	SM	SM	SM	72	SM		X X X			
			ı	ıı	ııı	72	R(ıı)					/ / /
						96	SM		X X X			
						96	R(ııı)					/ / /
O	65	SM	SM ı	-	-							
P	75	SM	SM ı	-	-							

Thus mucoid growth was best in carbon dioxide concentrations between 10 and 50 per cent and dissociation was most active over approximately the same range. Subcultures from smooth mucoid growth in 10 to 65 per cent carbon dioxide were virulent whereas roughs obtained in the same concentrations were reduced in virulence or avirulent. A subculture from the rough edge of B in 0 per cent CO_2 was fully virulent. This confirmed previous observations.

In this and some of the following experiments the symbols SM or SM and ıııı occur together. According to the scheme drawn up at the start this cannot be, but it signifies a colony with a profusely mucoid centre and a broad sharply demarcated ring of rough around it. It would have been misleading to call the growth SM because the mucoid nature was too marked. The utilization of more symbols to include these cases would make the scheme too involved.

(2)

To test the effects of different carbon dioxide concentrations on virulent strain XXXIII.

This strain was isolated from a goat which died of anthrax, naturally acquired, six weeks before.

TABLE LXXI.

Tube No.	% CO_2	Type of growth after 24 hrs.	Type of growth after 48 hrs.	Virulence Test. Subbed after hrs.	Type of variant subbed	Death: Hours after inoculation. 20 40 60 80 100
A	5	RS	-			
B	10	SM	SM	24	SM	X X X
		ıı	ıııı	24	R(ıı)	/ / /
				48	R(ıııı)	/ / /
C	20	SM ıı	R ıııı			
D	30	SM	R	24	SM	X X X
		ıı	ıııı	24	R(ıı)	X X X
E	30	SM ıı	RS ıııı			
F	40	SM ıı	-			
G	50	SM ı	SM ıııı			
H	50	SM ıı	SM ıııı			
I	60	RS ııı	RS ıııı			
J	65	SM ı	SM ıııı			
K	70	SM ıı	RS ıııı			

Mucoid growth was best maintained in carbon dioxide concentrations between 20 and 50 per cent, but even at best was not marked. By the second day the mucoid character had almost disappeared. The guinea pig tests showed that this was due to the rapid development of rough avirulent variants swamping the mucoid growth.

(3)

The effect of different concentrations of carbon dioxide on strain XXIV.

This was isolated four months ago from a bovine which had died of anthrax.

TABLE LXXII.

Tube No.	% CO_2	Type of growth after 24 hrs.	Type of growth after 48 hrs.	Virulence Test. Subbed after	Type of variant subbed	Death: Hours after inoculation 20	40	60	80	100
A	0	RS	RS							
B	5	SM ıı ı								
C	10	SM ıı ı	SM ıı ı							
D	20	SM	RS	24 hrs	SM	X X X				
		ıı ı	ıı ı	24 hrs	R(ıı ı)	All guinea pigs died in a week showed marked oedema but no capsuled bacilli. Test repeated with first subculture (below).				
				1st subculture of R(ıı ı)		/ / /				
E	20	SM ıı ı								
F	20	SM ıı ı	RS ıı ı							
G	30	SM ıı ı								
H	30	SM	RS	24 hrs	SM	X X X				
		ıı ı	ıı ı	24 hrs	R(ıı ı)	X X	/			

Tube No.	% CO_2	Type of growth after 24 hrs.	Type of growth after 48 hrs.	Virulence Test. Subbed after	Type of variant subbed	Death: Hours after inoculation 20 40 60 80 100
I	30	SM ııı				
J	30	SM ııı	RS ıııı	48 hrs	R(ıııı)	/ / /
K	40	SM ııı	RS ıııı	48 hrs	R(ıııı)	/ / /
L	50	RS ıııı	RS ıııı	48 hrs	R(ıııı)	/ / /
M	60	RS	RS			
N	60	SM				
O	80	RS ıı	RS ıı			

Strain XXIV never produced completely mucoid growth. The centre of the culture showed a slightly mucoid surface in carbon dioxide concentrations from 5 to 40 per cent, but usually this was gone at 48 hours. No other virulent strain had behaved in this way and therefore attempts were made to obtain more profuse mucoid growth by using different proportions of serum or whole blood. All were unsuccessful. However, the protocols of the guinea pig tests show that the rough growth was not due to an inability of the virulent strain to become mucoid, but rather to the rapid proliferation of avirulent rough variants; because subcultures from the edges of the rough colonies were mostly avirulent.

(4)

To test the effect of different carbon dioxide concentrations on virulent strain XXXIV.

This strain was isolated from the hide of a bovine seven days ago.

TABLE LXXIII.

Tube No.	% CO_2	Type of growth after 24 hrs.	Type of growth after 48 hrs.	Virulence Test: Subbed after hrs.	Virulence Test: Type of variant subbed	Virulence Test: Death: Hours after inoculation (20 40 60 80 100 200)
A	0	-	RS			
B	0	RS	RS			
C	10	SM	SM	24	SM	X X X
		ııı	ıııı	24	R((ııı)	/ / /
D	10	SM ıı	SM ıııı			
E	20	SM ı	SM ııı			
F	30	SM	SM	24	SM	X X X
		ıı	ııı	24	R(ıı)	/ / /
				48	SM	X X X
				48	R(ııı)	X / /
G	30	SM	SM	48	SM	X X X
			ııı	48	R(ııı)	/ / /
H	40	SM ıı	SM ıııı			
I	50	SM ı	SM ıııı			
J	60	RS	SM			
K	60	SM ı	SM ıı			

Tube No.	% CO_2	Type of growth after 24 hrs	Type of growth after 48 hrs	Virulence Test: Subbed after hrs.	Type of variant subbed	Death: Hours after inoculation 20 40 60 80 100 200
L	70	SM	SM			
		ı	ıı	48	R(ıı)	X X /
M	75	SM	SM	48	SM	X X X
			ıı	48	R(ıı)	/ / /
N	80	SM	SM ı			

This strain was more mucoid in carbon dioxide concentrations between 20 and 70 per cent and rough dissociants were more freely produced in 10 to 50 per cent concentrations. All the smooth mucoid variants tested were virulent for guinea pigs and the roughs avirulent or reduced in virulence.

(5)

<u>To test effects of different carbon dioxide concentrations on virulent strain XXXV.</u>

This strain was isolated two days ago from a bovine dead from naturally acquired anthrax.

<u>TABLE LXXIV.</u>

Tube No.	% CO_2	Type of growth after 24 hrs	Type of growth after 48 hrs	Virulence Test: Subbed after hrs.	Type of variant subbed	Death: Hours after inoculation 20 40 60 80 100
A	10	SM	SM			
		ı	ıııı	48	R(ıııı)	/ / /
B	20	SM	SM			
			ıı	48	R(ıı)	/ / /
C	30	SM	SM	48	SM	X X X
			ıı	48	R(ıı)	/ / /
D	60	RS	RS ı			
E	80	RS	RS	48	rough edge	X X X

Thus mucoid growth was good in carbon dioxide concentrations between 10 and 30 per cent. In 60 and 80 per cent there was no mucoid development nor production of rough dissociants. Avirulent roughs were obtained in all the cultures grown in concentrations up to 30 per cent, whereas the non-mucoid culture in 80 per cent CO_2 had lost no virulence. Culture B was rather interesting. The avirulent variant grew as a thin rough film (phantom colony, Nungester 1929) and within this film/rough non-phantom colonies developed. This process recurred with every subculture of the phantom, but the rough colony did not dissociate further. Neither variant killed.

Discussion.

Five virulent strains were grown in different carbon dioxide concentrations and examined for their ability to grow mucoid and to produce rough avirulent dissociants. Mucoid growth was found to depend partly on the strain and partly on the carbon dioxide concentration. Where it was not persistent, this was due to the rapid proliferation of avirulent rough variants. However, strains tested at 0 per cent and 80 per cent concentrations did not grow mucoid and produced no avirulent dissociants. The optimum carbon dioxide concentrations for obtaining avirulent roughs quickly appeared to lie between 10 and 30 per cent.

The differences observed in the dissociation rates of the virulent strains are very interesting and may explain partly the rapid loss of virulence undergone by some stock strains and not others. In general the mucoid growth was not as profuse as with the strains used in section D. This was possibly due to the use of a different batch of medium.

(6)

Comparative immunity tests on guinea pigs and sheep with avirulent dissociants obtained in different concentrations of carbon dioxide.

It was found previously (Section D) that sheep could be immunised with avirulent rough dissociants. These possess obvious advantages over the ordinary vaccines in that they are avirulent, they produce 99 per cent free-lying spores in 3 - 4 days, and they can be obtained from virulent strains in 24 hours. More extensive experiments were now carried out to see whether these variants could be used successfully as vaccines.

Merino sheep and guinea pigs were tested in parallel experiments in case strong correlation existed between the immunity produced in these animals. At Onderstepoort, sheep have always been used in the final titrations of anthrax vaccines, and considerable saving would result if preliminary guinea pig experiments could eliminate unsatisfactory strains.

Most of the strains used here produced indifferent immunity in the preliminary guinea pig experiments. However, these were tried on sheep because, from the standpoint of vaccine production, it is as important to know the worst as to know the best results to expect. All the surviving guinea pigs noted in tables LXX to LXXIV received 1/4 to 1/8 slant (1.5 x 7 cm.) of a culture of smooth mucoid "Boshoff". This strain (II ad) was used in section D, but had since been passaged a number of times and now killed the majority of unprotected guinea pigs in 24 hours. Thus the test was severer than those applied previously. For brevity, only the guinea pig tests with a bearing on the subsequent sheep experiments are tabulated, but there was no indication of any correlation between the immunising power of the avirulent strains and the CO_2 concentrations in which they developed.

Sheep were immunised with avirulent variants from the five strains noted in tables LXX to LXXIV. Strain XXII A_2

however, was obtained four months before the first test on sheep (table LXXV) and eight months before the last test (table LXXIX). This strain was introduced into the experiments to get an idea of the keeping qualities of an avirulent suspension.

The immunising dose for sheep was 1/300 to 1/400 of a sporulated culture from a slant 1.5 x 7 cm. This dose was somewhat less than that in the ordinary Onderstepoort spore vaccine (Pasteur II type). When possible, the immunity tests on the ordinary vaccine were done at the same time for comparison and these results are also tabulated. The 0.01 c.c. dose in the routine titration is slightly greater than the immunising dose of the avirulent dissociants.

The virulent test dose was a sporulated glycerine - saline suspension of strain XXVIII. The equivalent of one in one hundred and fifty thousandth of an agar slant (1.5 x 7 cm) of this strain never failed to kill sheep. This dilution probably contained several lethal doses and is termed a Certain Killing Dose (C.K.D.) Non-immune sheep were included in all the experiments and received from one tenth to one thousandth of the dose given to the treated sheep. The suspension of strain XXVIII held its virulence unusually well and dilutions of the same suspension were used in all the tests now reported. This suspension still retains its virulence at the present time.

TABLE LXXV.

Guinea Pigs	Immunised with	Tested 3 weeks later with	Death: Hours after inoculation 20	40	60	80	100	40	80	
5	rough avirulent XXII A_2	1/4 slant II Ad.	X	X	X					/ X (12 days)
5	Controls	ditto		X X X X X						

Strain II Ad. had not been exalted for this experiment.

Sheep	Immunised with	Each tested 3 weeks later with	Death: Days after infection 1	2	3	4	5	6	7	8	
45825	1 c.c. $\frac{1}{300}$ agar slant XXII A_2	10 C.K.D. XXVIII		X							
46056					X						
45988		"		X							
45742	"	"									/
46014	"	"									/
45965	"	"					X				
	Controls										
45796		"			X						
45807		"		X							
46731		1 C.K.D.				X					
45915		"		X							

Strain XXII A_2 was isolated four months ago (Section D). The immunity produced in sheep was not good, although two of the sheep survived this fairly severe test dose.

TABLE LXXVI.

Avirulent strain XXVIII K was obtained after 48 hours in 40 per cent CO_2 in the experiment noted in table LXX.

No. of guinea pigs.	Immunised with (each)	Tested 3 weeks later with	Death: Hours after inoculation 10	20	30	40	50	60	70	80	90
3	avirulent R. from XXVIII K (%)	1/8 agar slant II Ad. (each)				XX					X
6	Controls	ditto		X	X X X	X X					

Sheep	Each immunised with	Tested 3 weeks later with	Death: Days after inoculation 1	2	3	4	5	6	7	8	9	10	11	12
46699	1 c.c. $\frac{1}{500}$ agar slant XXVIII K	10 C.K.D. strain XXVIII			X									/
45868														
46113														/
45908														/
46060	"	"												/
46077	"	"												/
46688	1 c.c. $\frac{1}{300}$ agar slant strain XXII A_2 isolated 4 months ago.	"									X			
45952		"												/
47052		"												/
46109		"												/
45906		"												/
46019		"												/
	Vaccine Batch 5													
45913	20 c.c.	"												/
46027	20 c.c.	"												/
46143	0.1 c.c.	"												/
45953	0.1 c.c.	"				X								
45945	0.1 c.c.	"												/
46991	0.1 c.c.	"												/
45938	0.01 c.c.	"												/
46106	0.01 c.c.	"												/
46063	0.01 c.c.	"												/
46053	0.01 c.c.	"												/

Note: The 0.01 c.c. dose B.5 is equivalent to 1/300 agar slant.

46083	Controls	10 C.K.D. strain XXVIII									X			
46690	"			X										
		1 C.K.D.												
45997	"	"					X							
47007	"	"			X									

There was only a slight indication of immunity in the guinea pigs to the large test dose given them.

The same strain tested on sheep however showed a relatively sound degree of immunity (XXVIII K). The immunity in sheep was compared with an avirulent rough strain (XXII A_2) isolated some months ago and an ordinary vaccine batch. Thus, this relatively poor avirulent strain gave satisfactory immunity when tested on sheep. Controls all died.

Table LXXVII.

Avirulent strain XXXIII B was obtained after 24 hours in 10 per cent CO_2 and avirulent strain XXXIII F after 24 hours in 30 per cent CO_2 (see table LXXI).

No. of guinea pigs.	immunised with	Tested 3 weeks later with (each)	Death: Hours after inoculation. 20	40	60	80	100	60	200
3	XXXIII B (24 hrs) (10% CO_2)	1/6 agar slant II Ad.	X					X	X
3	XXXIV F 24 hrs. (30% CO_2)	ditto	X		X		X		
6	Controls	ditto	X X X X X X						

Sheep	Each immunised with	Each tested with 3 weeks later	Death: 1	2	Days after inoculation 3	4	5	6	7	8
47014	1/350 agar	100 C.K.D.								/
47045	slant	XXVIII								/
46987	R variant				X					
45963	XXXIII B	"								/
46692		"								/
46054	"	"	This sheep was very poor and had pneumonia for a week. Blood and spleen smears negative.							X
46748	Ditto	"								/
46121	XXXIV F	"								/
46028	"	"								/
47016	"	"		X						
46726	"	"								/
46756	"	"								/
	Batch 6									
46984	20 c.c.	"								/
44677	20 c.c.	"								/
45964	0.1 c.c.	"								/
45935	0.1 c.c.	"								/
46994	0.1 c.c.	"								/
47012	0.1 c.c.	"								/
45980	0.01 c.c.	"								/
46998	0.01 c.c.	"								/
46981	0.01 c.c.	"								/
46996	0.01 c.c.	"								/
	Controls									
45931	"	"			X					
46051	"	"		X						
		1 C.K.D.								
45852	"	"			X					
46136	"	"		X						

The two avirulent strains tested above showed only a moderate degree of protection in guinea pigs, but a very satisfactory protection in sheep.

TABLE LXXVIII.

Avirulent strain XXIV D was obtained after 24 hours in 20 per cent CO_2 (see table LXXII).

Guinea pigs	Each immunised with	Each tested with	Death: Hours after inoculation 20	40	60	80	100	
3	1/3 slant XXIV D	1/4 slant II Ad.				X	X	/
6	controls	"	X					
			X					
			X	X				
			X	X				

Sheep	Each immunised with	Each tested with	Death: Days after inoculation. 1	2	3	4	5	6	7	8	
45871	1/300 slant	100 C.K.D.									/
45902	sporulated	"									/
45886	culture	"									/
46725	XXIV D	"									/
45909	"	"									/
45944	"	"									/
45859	"	"									/
46740	"	"									/
45971	"	"									/
46072	"	"									/
46110	"	"									/
45922	"	"									/
Controls		1 C.K.D.									
45876		"			X						
45885		"		X							
46038		"		X							

This avirulent strain produced a high degree of resistance in guinea pigs and immunised sheep solidly against a large test dose of virulent culture. Sheep were not considered "solidly" immune unless all survived and none showed more than a slight transient temperature reaction. The majority showed no reaction at all.

TABLE LXXIX.

Avirulent strain XXXIV G was obtained after 24 hours in 30% CO_2
XXXIV M after 48 hours in 75% CO_2
XXXV A after 48 hours in 10% CO_2
XXXV B after 48 hours in 20% CO_2
(see tables LXXIII and LXXIV).

No. of guinea pigs.	Immunised with	Each tested with	Death: Hours after inoculation 20	40	60	80	
3	XXXIV G rough 24 hours 30% CO_2	1/8 slant II Ad.					/ / /
3	XXXIV M rough 48 hours 75% CO_2	"				X	/ /
2	XXXV A rough 48 hours 10% CO_2	"	X	X			
3	XXXV B rough 48 hours 20% CO_2	"	XXX				
12	Controls	"	X XXX XXX XXX	 X X			

Sheep	Each immunised with	Tested 3 weeks later with	Death: Days after infection. 1	2	3	4	5	6	7	8	
46075	1 c.c. 1/400 agar slant XXXIV G	1000 C.K.D. strain XXVIII									/
45847	"	"									/
45878	"	"									/
45846	"	"									/
46114	"	"									/
46024	"	"									/
46673	1 c.c. 1/400 agar slant XXXIV M	"									/
45922	"	"									/
46089	"	"		X							
46055	"	"									/
46671	"	"									/
45854	"	"		X							
45883	1 c.c. 1/400 agar slant XXXV A	"						X			
45960	"	"				X					
46091	"	"					X				
46714	"	"		X							
45896	"	"									/
46095	"	"				X					

Sheep	Each immunised with	Tested 3 weeks later with	Death: Days after infection 1	2	3	4	5	6	7	8	
46015	1 c.c. $\frac{1}{500}$	1000 C.K.D.									/
46757	slant	strain									/
46008	XXXV B	XXVIII									/
46094	"	"			X						
46044	"	"									/
45983	"	"		X							
45855	1 c.c. $\frac{1}{300}$	"									/
46040	slant	"									/
46058	batch 7	"									/
46741	"	"									/
46142	"	"									/
46010	"	"									/
46765	1 c.c. 1/30	"									/
46138	slant rough	"									/
45851	avirulent	"									/
46724	XXII A_2	"									/
46764	"	"									/
46674	"	"									/
	Controls										
46105		10 C.K.D.		X							
46747		"		X							
45873		1 C.K.D.			X						
45863		"			X						

In this experiment, two avirulent rough strains which gave very good immunity in guinea pigs and two avirulent rough strains which gave very poor immunity in guinea pigs were compared with a rough avirulent strain ten times as concentrated, and an ordinary vaccine (Pasteur II) type. The concentrated suspension of the avirulent rough strain XXII A_2 was prepared f our months ago from an avirulent strain isolatec 8 months ago.(See table LXXVI for t est on this strain at $\frac{1}{300}$ dilution). Four uninoculated controls were included.

Strain XXXIV G immunised guinea pigs and sheep solidly. XXXIV M increased the resistance of guinea pigs considerably but was only moderately effective in sheep. Strain XXXV A gave a poor result in both guinea pigs and sheep. XXXV B gave a poor result in guinea pigs and immunised the sheep fairly well.

The concentrated strain XXII A_2 and the Pasteur type vaccine both produced a solid immunity in sheep.

It should be noted that the test dose used in this experiment was particularly severe.