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The Effects of Different Carbon Dioxide Concentrations on the Growth of Virulent Anthrax Strains.

Pathogenicity and Immunity Tests on Guinea-pigs and Sheep with Anthrax Variants derived from Virulent Strains.

By MAX STERNE, Section of Bacteriology, Onderstepoort.

Introduction.

In a previous paper (Sterne, 1937) fully virulent anthrax stains were shown to grow smooth mucoid if cultured on 50 per cent. serum agar in an atmosphere of about 65 per cent. carbon dioxide. Such cultures immediately gave rise to rough non-capsule-producing avirulent dissociants. A similar phenomenon has been described by Schaeffer (1936) who found that anthrax strains grew mucoid on coagulated serum and produced rough variants. He cultivated these on serum for several generations and eventually found that they became reduced in virulence or avirulent. The ability to produce capsules was also lost. Sometimes these rough variants still killed and produced capsules. Therefore Schaeffer postulated a stable and an unstable variety of rough. This assumption seems unnecessary in view of the difficulty of ensuring the absolute homogeneity of the selected variants. This difficulty is inherent in all variation work unless single cell isolation technique is used, and Schaeffer's use of coagulated serum rendered his work liable to this source of error. Inspissated serum was tried previously (Sterne 1937) without much success. Fully virulent strains did not grow very mucoid, and moreover were so markedly proteolytic that the surface of the medium became semi-liquid. This rendered the isolation of " pure " variants exceedingly difficult. Stamatin (1934) grew virulent anthrax strains in defibrinated horse blood until mucoid colonies appeared. These produced rough, uncapsuled and avirulent dissociants. Stamatin and Stamatin (1936) produced immunity in rabbits with one of their avirulent strains. Both Stamatin's and Schaeffer's work involved considerable manipulation of their cultures.

Objects.

The results previously obtained with variants developed on serum agar in carbon dioxide 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 serum agar and to examine the influence of the different concentrations on the development of mucoid growth, the production of avirulent roughs and the immunizing power of these roughs.

Technique.

The medium used was 50 per cent. horse serum nutrient agar. This was put up in Mason's tubes (Mason 1933) and partially dried in the incubator for 24 hours. Thereafter, the fluid liberated by syneresis was removed and the tubes were then ready for use. The inoculation was always done on about one square centimetre of surface and incubation carried out in anaerobic jars of 1,900 ml. capacity to which the required amount of carbon dioxide was added. No satisfactory method of keeping the carbon dioxide pressure constant was devised and therefore the results must be interpreted for the particular system used here: that is, each jar of 1,900 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 carbon dioxide tension adjusted. The method of adding carbon dioxide was described in a previous paper.

Mason's tubes were inoculated with the different virulent strains and incubated in various carbon dioxide concentrations. The results are tabulated below.

The following symbols are used to describe the character of the growth:—

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

*S*M denotes a colony as above, but with a more mucoid surface.

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

S*M* denotes a colony with a very mucoid surface, smooth edges and a tendency to flow.

S**M** denotes a colony that can be drawn into long threads when touched with a needle. The growth may be as much as half a centimetre thick.

The extent of development of rough dissociants is shown thus:—

\+ denotes minute rough projections too small to be picked.

++ denotes well developed rough wedges or outgrowths, easy to pick.

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

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

Occasionally, in the tables, the symbols SM or S*M* appear associated with the symbol ++++. This is really in conflict with the scheme, but it signifies a very well developed mucoid centre entirely surrounded by a sharply demarcated rough zone. In these cases it was obvious that the ring of rough growth was due to a running together of rough outgrowths. The tables show that such a combination of symbols is only shown after 48 hours, whereas the colonies at 24 hours showed smooth edges between the rough dissociants.

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 thus *R*S or R*S*.

The virulence tests in Tables I to V were done on guinea-pigs. Three pigs were used for each variant and each received subcutaneously one-third of an agar slope of the particular variant indicated in the table.

Experiments.

1.—*The effect of different carbon dioxide concentrations on virulent strain XXVIII.*

This strain was isolated sixteen years ago and is still very virulent for sheep.

Table I.

Tube No.	% CO_2.	Type of growth after.				Virulence Test.						
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	Subbed after hrs.	Type of variant subbed.	Death : hours after inoculation. 20	40	60	80	100
A	0	RS	SM	—	—		—					
B	0	R*S*	*SM*	—	—	48	rough edge		† † †			
C	0	RS	RS	—	—							
D	5	*SM* +	SM ++	—	—							
F	10	*SM*	S*M*	—	—							
G	10	*SM*	S*M*	—	—	48	S*M*		† † †			
		+	+++	—	—	48	R (+++)			†	†	/

TABLE I—(*continued*).

Tube No.	% CO_2.	Type of growth after.				Virulence Test.						
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	Subbed after hrs.	Type of variant subbed.	Death : hours after inoculation. 20	40	60	80	100
H	15	SM	SM	—	—	48	SM		† †	†		
		+	++	—	—	48	R (++)					/ / /
I	20	SM	SM	—	—	48	SM		† † †			
			+	—	—	48	R (+)		†	† †		
J.	30	SM	SM	—	—	48	SM	†	† †			
			++	—	—	48	R (++)					/ / /
K	40	SM	SM	—	—	48	SM		† † †			
			++	—	—	48	R (++)					/ / /
L	50	SM ++	SM +++	—	—							
M	50	SM	SM ++	—	—							
N	65	SM	SM	SM	SM	72	SM		† † †			
			+	++	+++	72	R (++)					/ / /
						96	SM		† † †			
						96	R (+++)					/ / /
O	65	SM	SM +	—	—							
P	75	SM	SM +	—	—							

† = Guinea pig, died of anthrax. / = Survived.

Thus mucoid growth was best in carbon dioxide concentrations between 10 and 50 per cent. and dissocation 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.

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 previously.

TABLE II.

Tube No.	% CO_2.	Type of growth after.		Virulence Test.						
		24 hrs.	48 hrs.	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		† ††			
		++	++++	24	R (++)					/ / /
				48	R (++++)					/ / /
C	20	SM ++	R ++++							
D	30	SM	R	24	SM		† † †			
		++	++++	24	R (++)			†	††	
E	30	SM ++	RS ++++							
F	40	SM ++	—							
G	50	SM +	SM ++++							
H	50	SM ++	SM ++++							
I	60	RS +++	RS ++++							
J	65	SM +	SM ++++							
K	70	SM ++	RS ++++							

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 strain was isolated four months previously from a bovine which had died of anthrax.

TABLE III.

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

TABLE III—(*continued*).

Tube No.	% CO_5.	Type of growth after.		Virulence Test.						
		24 hrs.	48 hrs.	Subbed after. Hrs.	Type of variant subbed.	Death : Hours after inoculation. 20	40	60	80	100
K	40	SM	RS							
		+++	++++	48	R (++++)					/ / /
L	50	RS	RS							
		+++	++++	48	R (++++)					/ / /
M	60	RS	RS							
N	60	SM	—							
O	80	RS ++	RS ++							

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 usually 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 previously.

TABLE IV.

Tube No.	% CO_2.	Type of growth after.		Virulence Test.							
		24 hrs.	48 hrs.	Subbed after. Hrs.	Type of variant subbed.	Death : Hours after inoculation. 20	40	60	80	100	200
A	0	—	RS								
B	0	RS	RS								
C	10	SM	SM	24	SM		† † †				
		+++	++++	24	R (+++)						/ / /

TABLE IV—(*continued*).

Tube No.	% CO_2.	Type of growth after.		Virulence Test.								
		24 hrs.	48 hrs.	Subbed after. Hrs.	Type of variant subbed.	Death : Hours after inoculation. 20	40	60	80	100	200	
D	10	*S*M ++	**S**M ++++									
E	20	*S*M +	SM +++									
F	30	*S*M	S*M*	24	*S*M		† † †					
		++	+++	24	R (++)							/ / /
				48	S*M*		† † †					
				48	R (+++)						†	/ /
G	30	*S*M	S*M*	48	S*M*		† † †					
			+++	48	R (+++)							/ / /
H	40	*S*M ++	SM ++++									
I	50	**S**M +	SM ++++									
J	60	RS	*S*M									
K	60	*S*M +	*S*M ++									
L	70	S*M*	S*M*									
		+	++	48	R (++)							† † /
M	75	**S**M	*S*M	48	*S*M		† † †					
			++	48	R (++)							/ / /
N	80	*S*M	**S**M +									

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.—*To test the effects of different carbon dioxide concentrations on virulent strain XXXV.*

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

TABLE V.

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

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. 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. Sometimes the mucoid growth was not persistent due to the rapid proliferation of avirulent rough variants. Strains tested in 0 per cent. and 80 per cent. carbon dioxide did not grow mucoid and produced no avirulent dissociants. The optimum concentrations of carbon dioxide for obtaining avirulent roughs quickly appeared to lie between 10 per cent. and 30 per cent.

In general, mucoid growth was not as profuse as in the experiments described in the previous paper. This was probably due to the use of a different batch of medium.

In this paper and in a previous paper it was shown that all virulent anthrax strains dissociate in an apparently orderly manner from the time they are isolated. In the particular cases investigated there was a continuous variation in the direction of uncapsuled and avirulent types. The regularity of the process makes it difficult not to entertain the hypothesis that this may be a constant feature of growth in anthrax even under natural conditions and that it may have a bearing on epidemiological and immunological problems in nature.

An interesting aspect of the investigation was the variation in the dissociation rates of the different virulent strains. This carries the possibility that there are fundamental differences between virulent strains and that these differences may play a determining role in, for example, the persistence of an infection in the field.

Comparative Immunity Tests on Guinea-pigs and Sheep with Avirulent Variants obtained in Different Concentrations of Carbon Dioxide.

It was shown previously that sheep could be immunized with avirulent rough variants. The present series of experiments was designed to examine the possibility of using avirulent rough variants as vaccines. These dissociants have obvious advantages over the ordinary attenuated strains: they are avirulent; they produce 99 per cent, free-lying spores in 3 to 4 days; and they can be obtained from virulent strains in 24 to 48 hours.

Parallel experiments were done on guinea-pigs and sheep to see if there was a relation between the degree of immunity produced in these species. Sheep have always been used at Onderstepoort as the final laboratory test animal, so that considerable saving would result if preliminary guinea-pig tests could eliminate strains likely to be unsuccessful as vaccines, before the more expensive sheep test was used.

The majority of the strains now tested on sheep produced poor immunity in guinea-pigs. These strains were tested purposely, because it was as important to know the worst results to be expected, as to know the best.

The strains used for immunization were isolated in the experiments noted in Tables I-V, with the exception of strain XXII A_2 which was isolated some months before. The guinea-pigs tested (except with strain XXII A_2) were those which survived in the experiments noted in Tables I to V. Sheep were immunized with 1/300 to 1/500 dilution of a fully sporulated agar slant (1·5 × 7 cm.) of the avirulent strains. This dose was somewhat smaller than that used in the ordinary pasteur II type of vaccine prepared here for issue. For the sake of comparison, tests on the routine Pasteur II type vaccine were done at the same time as the tests on the avirulent strains, and these results are also shown in the tables. The 0·01 dilution of the Pasteur II is slightly more than 1:300 agar slant.

The virulent test dose for guinea-pigs was $\frac{1}{4}$ to $\frac{1}{8}$ of an agar slant (1·5 × 7 cm.) of strain II Ad. This was the smooth mucoid strain used in previous tests (Sterne 1937), but its virulence had since been considerably enhanced by serial passage through guinea-pigs.

It now always killed unprotected guinea-pigs in 15 to 40 hours, but the majority were dead by the 24th hour. In the first experiment (Strain XXII A_2) the test strain II Ad had not yet been exalted.

The test dose for sheep was a sporulated glycerine-saline suspension of strain XXVIII. This had been titrated to kill all sheep at a dilution of 1:150,000 of a medium sized agar slant (1·5 × 7 cm.) and this dose was much more than an M.L.D. Controls were included with all the immunized sheep. The latter received from 10 to 1,000 times the dose given to the controls. The dilution 1:150,000 has been called a Certain Killing Dose (C.K.D.). The stability of the test suspension was unusually good and strain XXVIII has been the only strain encountered here which retained its virulence in glycerine-saline over long periods. Dilutions of the same suspension were used in all the sheep tests.

All the guinea-pigs which survived in Tables I-V were tested for immunity, but, for the sake of brevity, only the tests with a bearing on the sheep experiments have been given in the following tables. The guinea-pig immunity tests, however, gave no indication that there was any relation between the immunizing power of a strain and the CO_2 concentration in which it developed.

TABLE VI.

Guinea Pigs.	Immunized with.	Tested 3 weeks later with.	Death : Hours after inoculation. 20	40	60	80	100	40	80
5	rough avirulent XXII A_2	$\frac{1}{4}$ slant II Ad.	†	†	†				†(12 days)
5	Controls	ditto		† † † † †					

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

TABLE VI—(*continued*).

Sheep.	Immunized with.	Each tested 3 weeks later with.	Death : Days after inoculation. 1	2	3	4	5	6	7	8
45,825	1 c.c. $\frac{1}{3000}$ agar slant XXII A_2	10 C.K.D. XXVIII		†						
46,056					†					
45,988		,,		†						
45,742	,,	,,								/
46,014	,,	,,								/
45,965	,,	,,					†			
45,796	Controls.	,,			†					
45,807		,,		†						
46,731		1 C.K.D.				†				
45,915		,,		†						

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

TABLE VII.

Avirulent strain XXVIII K was obtained after 48 hours in 40 per cent. CO_2 in the experiment noted in Table 1.

No. of guinea pigs.	Immunized 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 (40% CO_2)	$\frac{1}{8}$ agar slant II Ad. (each)				††					†
6	Controls	ditto		†† † †		† †					

TABLE VII—(*continued*).

Sheep.	Each immunized with.	Tested 3 weeks later with.	Death : Days after inoculation. 1 2 3 4 5 6 7 8 9 10 11 12
46,699	1 c.c. $\frac{1}{500}$	10 C.K.D.	/
45,868	agar slant	strain	†
46,113	XXVIII K	XXVIII	/
45,908	,,	,,	/
46,060	,,	,,	/
46,077	,,	,,	/
46,688	1 c.c. $\frac{1}{300}$	,,	†
45,952	agar slant	,,	/
47,052	strain	,,	/
46,109	XXII A_2	,,	/
45,906	isolated	,,	/
46,019	4 months previously.	,,	/
	Vaccine Batch 5		
45,913	20·0 c.c.	,,	/
46,027	20·0 c.c.	,,	/
46,143	0·1 c.c.	,,	/
45,953	0·1 c.c.	,,	†
45,945	0·1 c.c.	,,	/
46,991	0·1 c.c.	,,	/
45,938	0·01 c.c.	,,	/
46,106	0·01 c.c.	,,	/
46,063	0·01 c.c.	,,	/
46,053	0·01 c.c.	,,	/

NOTE.—The 0·01 c.c. dose B. 5 is equivalent to 1/300 agar slant.

46,083	Controls	10 C.K.D.	†
46,690	,,	strain XXVIII	†
		1 C.K.D.	
45,997	,,	,,	†
47,007	,,	,,	†

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 that produced by an avirulent rough strain (XXII A_2) isolated some months previously and with that produced by an ordinary vaccine batch. Thus, this relatively poor avirulent strain gave satisfactory immunity when tested on sheep. Controls all died.

TABLE VIII.

Avirulent strain XXXIII B was obtained after 24 hours in 10 per cent. CO_2 and avirulent strain XXXIV F after 24 hours in 30 per cent. CO_2 (see Tables II and IV).

No. of guinea pigs.	Immunized 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)	$\frac{1}{6}$ agar slant II Ad.	†						††
3	XXXIV F 24 hrs. (30% CO_2)	ditto	†	†		†			
6	Controls	ditto	† † † † † †						

Sheep.	Each immunized with.	Each tested with 3 weeks later.	Death : Days after inoculation. 1	2	3	4	5	6	7	8	
47,014	$\frac{1}{350}$ agar	100 C.K.D.									/
47,045	slant	XXVIII			†						/
46,987	R variant				†						
45,963	XXXIII B	,,									/
46,692		,,									/
46,054	,,	,,	This sheep was very poor and had pneumonia for a week. Blood and spleen smears negative.							†	
46,748	ditto	,,									/
46,121	XXXIV F	,,									/
46,028	,,	,,									/
47,016	,,	,,		†							
46,726	,,	,,									/
46,756	,,	,,									/
	Batch 6										
46,984	20·0 c.c.	,,									/
44,677	20·0 c.c.	,,									/
45,964	0·1 c.c.	,,									/
45,935	0·1 c.c.	,,									/
46,994	0·1 c.c.	,,									/
47,012	0·1 c.c.	,,									/
45,980	0·01 c.c.	,,									/
46,998	0·01 c.c.	,,									/
46,981	0·01 c.c.	,,									/
46,996	0·01 c.c.	,,									/
	Controls										
45,931	,,	,,			†						
46,051	,,	,, 1 C.K.D.		†							
45,852	,,	,,		†							
46,136	,,	,,		†							

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

Table IX.

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

Guinea pigs.	Each immunized with.	Each tested with.	Death : Hours after inoculation. 20	40	60	80	100
3	$\frac{1}{3}$ slant XXIV D	$\frac{1}{4}$ slant II Ad.				†	† /
6	Controls	,,	†				
			†				
			†	†			
			†	†			

Sheep.	Each immunized with.	Each tested with.	Death : Days after inoculation. 1	2	3	4	5	6	7	8
45,871	$\frac{1}{300}$ slant	100 C.K.D.								/
45,902	sporulated	XXVIII								/
45,886	culture	,,								/
46,725	XXIV D	,,								/
45,909	,,	,,								/
45,944	,,	,,								/
45,859	,,	,,								/
46,740	,,	,,								/
45,971	,,	,,								/
46,072	,,	,,								/
46,110	,,	,,								/
45,922	,,	,,								/
Controls		1 C.K.D.								
45,876		,,			†					
45,885		,,		†						
46,038		,,		†						

This avirulent strain produced a high degree of resistance in guinea-pigs and immunized 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 to the virulent dose. The majority showed no reaction at all.

TABLE X.

Avirulent strain XXXIV G was obtained after 24 hours in 30 per cent. CO_2; XXXIV M after 48 hours in 75 per cent. CO_2; XXXV A after 48 hours in 10 per cent. CO_2; XXXV B after 48 hours in 20 per cent. CO_2 (see Tables IV and V).

No. of guinea pigs.	Immunized with.	Each tested with.	Death: Hours after inoculation. 20	40	60	80
3	XXXIV G rough 24 hours 30% CO_2	$\frac{1}{8}$ slant II Ad.				/ / /
3	XXXIV M rough 48 hours 75% CO_2	,,			†	/ /
2	XXXV A rough 48 hours 10% CO_2	,,	†	†		
3	XXXV B rough 48 hours 20% CO_2	,,	†††			
12	Controls	,,	† ††† ††† †††	† †		

Sheep.	Each immunized with.	Tested 3 weeks later with.	Death: Days after infection. 1	2	3	4	5	6	7	8
46,075	1 c.c. $\frac{1}{400}$ agar slant XXXIV G	1,000 C.K.D. strain XXVIII								/
45,847	,,	,,								/
45,878	,,	,,								/
45,846	,,	,,								/
46,114	,,	,,								/
46,024	,,	,,								/
46,673	1 c.c. $\frac{1}{400}$ agar slant XXXIV M	,,								/
45,922	,,	,,								/
46,089	,,	,,		†						
46,055	,,	,,								/
46,671	,,	,,								/
45,854	,,	,,		†						
45,883	1 c.c. $\frac{1}{400}$ agar slant XXXV A	,,						†		
45,960	,,	,,				†				
46,091	,,	,,					†			
46,714	,,	,,		†						
45,896	,,	,,								/
46,095	,,	,,				†				

TABLE X—(*continued*).

Sheep.	Each immunized with.	Tested 3 weeks later with.	Death : Days after infection. 1	2	3	4	5	6	7	8
46,015	1 c.c. $\frac{1}{300}$	1,000 C.K.D.								/
46,757	slant	strain								/
46,008	XXXV B	XXVIII								/
46,094	,,	,,			†					
46,044	,,	,,								/
45,983	,,	,,		†						
45,855	1 c.c. $\frac{1}{300}$	,,								/
46,040	slant	,,								/
46,058	batch 7	,,								/
46,741	,,	,,								/
46,142	,,	,,								/
46,010	,,	,,								/
46,765	1 c.c. 1/30	,,								/
46,138	slant rough	,,								/
45,851	avirulent	,,								/
46,724	XXII A_2	,,								/
46,764	,,	,,								/
46,674	,,	,,								/
	Controls									
46,105		10 C.K.D.		†						
46,747		,,		†						
45,873		1 C.K.D.			†					
45,863		,,			†					

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 four month previously from an avirulent strain isolated 8 months previously. (See Tables VI and VII for tests on this strain at $\frac{1}{300}$ dilution.) Four uninoculated controls were included.

Strain XXXIV G immunized 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 immunized the sheep fairly well.

The concentrated strain XXII A_2 and the Pasteur type vaccine batch 7 both produced a solid immunity in sheep.

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

DISCUSSION.

Most of the avirulent variants produced a high degree of resistance in sheep and two of them, as judged by the tests, gave a solid immunity. The successful variants were those that showed

the highest protective power in guinea-pigs. Avirulent dissociants can be isolated easily and rapidly, so that for practical purposes only those giving sound results in guinea-pigs need be tried on sheep. Nevertheless, even strains which produced a relatively poor immunity in guinea-pigs were comparatively successful when tried on sheep.

However, considerable variation existed between the degrees of immunity produced by the different strains. The reason is unknown but the conditions under which the variants can be obtained are as yet ill-defined and the problem may be solved by a refinement in technique and a more rigid standardisation of the medium. This is the more likely because differences in growth were noted with different batches of media and sera.

The immunity produced by the avirulent dissociants was compared with that given by two batches of an ordinary vaccine strain (Pasteur II type). This gave as good an immunity as the best of the roughs. However, this Pasteur type vaccine was exceptional in that it elicited a better immunity than any similar vaccine prepared here for some time. Moreover, its virulence was on the borderline of safety. The experiments were slightly weighted against the avirulent strains as far as dosage was concerned.

The experiment with strain XXII A_2 noted in Table X introduced an interesting possibility. The usual dose of this strain did not produce a strong immunity in guinea-pigs or sheep, but when ten times this concentration was given the sheep acquired a solid immunity, although the glycerine-saline suspension of strain XXII A_2 was then four months old.

This tenfold strength contained about 3,000,000 spores per dose and it is quite feasible to manufacture vaccines containing this number of organisms. Therefore, it appears probable that larger doses of potent avirulent strains may give maximal immunity to anthrax. It has been shown that large doses of the ordinary vaccines improve immunity, but this increased dosage carries with it an increased risk, whereas almost any dose of the avirulent strains can be tolerated.

The immunity tests with the avirulent rough variants must be considered very satisfactory and indicate, as far as laboratory experiments can, that domestic animals can be easily and safely immunized against anthrax with avirulent strains. This is particularly important for goats, horses and wild animals in zoological gardens (Neitz, 1936). These animals are highly susceptible to the use of any but weak, poorly immunizing Pasteur I type vaccines, which means that these animals are practically non-immunizable. Potent avirulent strains should prove very useful in such cases. In laboratory tests not quoted here, one C.K.D. of strain XXVIII killed almost every goat treated with the Pasteur I type of vaccine, whereas it was shown (Sterne, 1937) that a weak avirulent strain produced fair immunity in goats. The terms Pasteur I and Pasteur II type vaccines refer only to the relative virulence of the attenuated strains.

SUMMARY.

(1) Virulent anthrax strains grew mucoid and produced avirulent rough dissociants on serum agar, in a number of different carbon dioxide concentrations.

(2) A relatively small dose of avirulent spores immunized sheep against anthrax.

(3) The immunizing power of the avirulent rough strains was not associated with the carbon dioxide concentration in which they developed.

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