





S T U D I E S   O N   V A R I A T I O N   I N   A N T H R A X .

PART ONE.

SOME EFFECTS OF CARBON DIOXIDE ON THE FORMATION OF CAPSULES AND SPORES BY BACILLUS ANTHRACIS.

PART TWO.

SOME CORRELATIONS BETWEEN COLONY VARIATION AND PATHOGENICITY IN STRAINS OF BACILLUS ANTHRACIS.

M A X   S T E R N E   B.V.Sc.

Thesis submitted to the University of South Africa for the degree of Doctor of Science.

D.Y.E.

STUDIES ON VARIATION IN ANTHRAX.

PART ONE.

SOME EFFECTS OF CARBON DIOXIDE ON THE FORMATION OF CAPSULES AND SPORES BY BACILLUS ANTHRACIS.

PART TWO.

SOME CORRELATIONS BETWEEN COLONY VARIATION AND PATHOGENICITY IN STRAINS OF BACILLUS ANTHRACIS.

I.

INTRODUCTION.

II.

EXPERIMENTAL.

	<u>Page.</u>
A. Dissociation in virulent strains after prolonged cultivation.	
(1) Dissociation in virulent "Boshoff" .....	16.
(2) " " " " "Anthrax A." .....	36.
(3) " " " " "568" .....	40.
(4) " " " " Drummond .....	42.
(5) " " " " Pretoria North ....	45.
B. Dissociation in attenuated strains.	
(1) Dissociation in a strain partially attenuated at 42°C - Strain IV .....	48.
(2) Dissociation in a strain attenuated at 42°C - Strain 568 .....	51.
C. Dissociation of anthrax strains in 65 per cent carbon dioxide. ....	52.
(1) Dissociation of virulent "Pretoria North" ..	56.
(2) " " " " "Drummond" .....	58.
(3) " " " " "568" .....	61.
D. Dissociation of virulent anthrax strains on 50 per cent serum agar in 65 per cent carbon dioxide.	63.
(1) Dissociation of virulent Strain XIV .....	66.
(2) " " " " " "Drummond" ..	76.
(3) " " " " " XVIII .....	81.
(4) " " " " " XX .....	88.
(5) " " " " " "Boshoff"...	92.
(6) " " " " " XXII.....	95.
(7) " " " " " XXVI.....	97.

E.	Comparison of immunity produced in rabbits by a number of avirulent variants, and an ordinary vaccine. ....	100.
F.	The effects of different concentrations of carbon dioxide on the dissociation of virulent strains on 50 per cent serum agar .....	103.
	(1) Dissociation of Strain XXVIII .....	104.
	(2) " " " XXXIII .....	107.
	(3) " " " XXIV .....	108.
	(4) " " " XXXIV .....	110.
	(5) " " " XXXV .....	111.
	(6) Comparative immunity tests on guinea pigs and sheep with rough variants from strains 1 - 5.	113.
G.	Dissociation of attenuated anthrax strains on 50 per cent serum agar in 65 per cent carbon dioxide.	124.

## III.

## DISCUSSION.

## IV.

## SUMMARY AND CONCLUSIONS.

## V.

## REFERENCES.

MEG.

PART ONE.

SOME EFFECTS OF CARBON DIOXIDE ON THE FORMATION  
OF CAPSULES AND SPORES BY B. ANTHRACIS.

By MAX STERNE.

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

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

Nungester (1929) distinguished smooth mucoid and rough mucoid variants. A smooth mucoid culture is very slimy and tenacious, and has not the cuneiform structure which the anthrax colony usually shows by transmitted light. Frequently a culture does not develop many capsules in the first 24 hours and only thereafter becomes mucoid. Such cultures then appear rough by transmitted light but the slimy surface growth which eventually overlaps the initial rough edges, is seen to be typically smooth mucoid. From Nungester's description, it seems as if this is what he termed a rough mucoid, although it really appears to be

smooth mucoid overgrowing a rough or rough-smooth base. In the present work some strains grown in carbon dioxide were found to have a tangled medusa-like edge; but both the colony and the medusa-like margin were typically smooth mucoid. This type of colony formation could more properly be called rough mucoid.

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

#### TECHNIQUE.

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

#### EXPERIMENTS.

Preliminary work indicated that carbon dioxide had an influence on capsule formation and in the experiments

described below the effect of different concentrations of carbon dioxide was investigated. Two rough virulent strains, Virulent Boshoff and Virulent Anthrax A. were used.

1. Experiment in 85 - 90 per cent Carbon Dioxide.

A. (a) Two slants virulent Boshoff on nutrient agar pH 7.5 :- after two days the growth was slight and filmy and smears showed very few capsules.

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

B. (a) Two slants virulent Anthrax A on nutrient agar pH 7.5:- after two days there was slight growth. No capsules were seen in smears.

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

II. Experiment in 75 per cent Carbon Dioxide.

Five slants of virulent Boshoff on nutrient agar pH 7.5 :- the slants were examined and smears stained every two days for a week. By the fifth day 2 - 5 per cent of the bacilli had capsules.

III. Experiment in 65 to 70 per cent Carbon Dioxide.

A. (a) Five slants of virulent Boshoff on nutrient agar pH 7.5 :- growth was good. Smears showed a fair number of capsules after two days. By the sixth day about 80 per cent of the bacilli were capsuled.

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

B. (a) Two slants virulent Anthrax A on nutrient agar pH 7.5 :- observations were only made up to the second day. By then about 5 per cent of the bacilli were capsuled.

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

IV. Experiment in 50 per cent Carbon Dioxide.

A. (a) Two slants virulent Boshoff on nutrient agar pH 7.5 :- after two days about 20 per cent of the bacilli had capsules.

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

B. Two slants of virulent Anthrax A on nutrient agar pH 7.5 and one slant on blood agar showed about the same appearance as the virulent Boshoff.

V. Controls grown in air.

A. (a) Seven slants virulent Boshoff on nutrient agar pH 7.5 :- very few capsuled bacilli were seen, although smears were made up to the seventh day.

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

B. (a) Four slants virulent Anthrax A on nutrient agar pH 7.5 :- capsuled bacilli were very rarely seen.

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

DISCUSSION.

The two rough virulent strains, V. Boshoff and V. Anthrax A developed far more capsules in the 50 and 70 per cent carbon dioxide than in air. Concentrations of carbon dioxide above 70 per cent markedly retarded the growth of the cultures and the production of capsules. Further experiments at different times confirmed this point. In all subsequent experiments observations were made for a week or more, and concentrations of carbon dioxide between 60 and 70 per cent used. The jars were opened and refilled every



day to compensate for the carbon dioxide formed by the bacilli.

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

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

#### I. Experiment with Virulent Boshoff.

(a) Eleven slants of V. Boshoff on nutrient agar pH 7.5 were grown in 65 per cent carbon dioxide. After about a week all the cultures had slimy surfaces and smears showed that 70 to 100 per cent of the bacilli had capsules.

(b) Three slants of V. Boshoff on nutrient agar pH 7.5 were grown in 65 per cent paraffin gas. No capsules were seen, although the cultures were examined for a week.

(c) Two slants of V. Boshoff on same media as before. The cultures were incubated in air but were protected from drying out.

At the end of seven days very few capsules had been seen.

(d) Six slants of virulent Boshoff on nutrient agar pH 7.5 incubated in air:-

At the end of seven days only few capsules had been seen.

#### II. Experiment with Anthrax 40486.

(a) Two slants Anthrax 40486 on nutrient agar pH 7.5 grown in 65 per cent carbon dioxide.

After seven days 95 per cent of the bacilli were capsuled.

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

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

#### DISCUSSION.

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

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

#### SOME EFFECTS OF VARYING THE HYDROGEN ION CONCENTRATION.

Experiments were then performed to see whether change in hydrogen ion concentration produced by the carbon dioxide influenced the production of capsules. Nungester showed that mucoid variants of B. anthracis were more slimy when grown at pH 7.8 than at lower pH levels and suggested

that this might be due to an alkaline medium 'fixing' more carbon dioxide. Virulent Boshoff was grown in 70 per cent carbon dioxide on buffered nutrient agar adjusted to different hydrogen ion concentrations. Controls were grown on similar media in air.

#### I. Virulent Boshoff at pH 8.4.

(a) Two slants in 70 per cent carbon dioxide. The final pH was about 7.0. About 80 per cent of the bacilli showed capsules.

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

#### II. At pH 8.0.

(a) Two slants in 70 per cent carbon dioxide. The final pH was about 6.8. About 60 per cent of the bacilli showed capsules.

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

#### III. At pH 7.6 to 7.8.

(a) Four slants in 70 per cent carbon dioxide. The final pH was about 6.0 to 6.5. Growth was good and from 60 to 80 per cent of the bacilli had capsules.

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

#### IV. At pH 6.8.

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

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

#### DISCUSSION.

The results were quite clear cut. The final hydrogen ion concentrations in the CO<sub>2</sub> ranged from about

5 to 7.2. Capsules were freely developed at all these levels. None of the controls in air showed more than an occasional capsule. Further experiments showed that a culture which showed a few capsules in air at pH 7.5 - 7.8 would show none at pH 6.8 or lower. It could be concluded that the lowering of the pH by CO<sub>2</sub> is not the determining factor in causing the bacilli to become capsuled.

A point of interest was whether capsule formation in carbon dioxide was due to the selection of a mucoid variant pre-existent in the original rough strain, or whether carbon dioxide actually induced the 'rough' bacilli to develop capsules. The experiments summarized below were carried out to investigate this.

Six virulent strains of anthrax were grown in 60 per cent carbon dioxide on nutrient agar until the surface of the culture was covered by a thick mucoid growth. Streaks were made from the mucoid onto agar plates and incubated in air. These subcultures were always rough. Numerous repetitions of the experiment gave similar results.

The results might have been due to the 'rough' bacilli developing capsules in carbon dioxide and losing them in air. On the other hand it was possible that the carbon dioxide favoured mucoid variants, while ordinary conditions favoured rough variants. Some of the experiments were repeated using single cell isolations from the new Drummond strain. These single cell cultures gave the same results in carbon dioxide and in air, as the original strain.

As it is reasonably certain that most strains vary continually, and that the macroscopic appearance of a culture is an expression of the dominant variant, the question of the carbon dioxide acted by selecting a particular variant or by inducing the 'rough' bacilli to become capsuled, could be answered only by direct observation.

Carbon Dioxide and Sporulation.

It is generally held that B. anthracis requires a high partial pressure of oxygen to sporulate well.

This theory is used to explain the fact that spores are not formed in vivo. While the experiments on capsule development were being done careful note was taken of the degree of sporulation under the different experimental conditions. It was found that spores very rarely developed when cultures were grown in a high partial pressure of carbon dioxide. This held throughout the tests, however long the cultures were held, and whatever strains were used.

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

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

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

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

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

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

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

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

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

#### SUMMARY AND CONCLUSIONS.

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

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

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

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

#### BIBLIOGRAPHY.

NUNGESTER, W.J. (1929). Dissociation of *B. anthracis*.

J. Infect. Dis. 44. 2. p. 73. 1929.

SOBERNHEIM, G. (1931). Handbuch der Pathogenen Mikroorganismen. Kolle, W., Kraus, R. & Uhlenhuth, P. Vol. 3. 2. pp. 1041-1174.

TEALE, F.H. (1935). Circulating antibody in immunity.

J. of Immun. 28. 2.