Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 10, *Number* 2, *April*, 1938.

Printed in the Union of South Africa by the Government Printer, Pretoria.

Variation in the Colony Form of the Anthrax Bacillus.

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THE number of variant forms which can develop in cultures of the anthrax bacillus seems to be very large. (Gratia 1924; Nungester 1929; Stamatin 1934, 1935a, 1935b; Januschke 1935.) Some changes in colony form are so abrupt and occur so regularly that they appear, at first sight, to be stages of a life cycle or a life sequence. For example, all virulent strains investigated here (Sterne 1937a and 1937b) have produced a smooth mucoid growth on serum agar in an atmosphere containing certain concentrations of carbon dioxide; and all have, under such conditions, rapidly and continuously produced avirulent daughter strains. Nevertheless, the majority of variant colony types occur in a more haphazard manner so that it is usually impossible to predict, with any assurance, the changes that will occur in a culture subjected to certain conditions.

The results summarized below were obtained during the course of experiments done to see whether any "trends" could be recognized in the dissociation pattern of *B. anthracis*.

EXPERIMENTS.

Dissociation of Strain XXII.

This strain was grown on 50 per cent. horse serum agar in 20 per cent. carbon dioxide immediately after its isolation from an animal which had died of anthrax during a natural outbreak of the disease. From the resulting smooth mucoid culture a rough, avirulent, and uncapsulated variant was obtained. This was used in further experiments on dissociation because its inability to produce capsules considerably simplified observation and moreover, dissociation as far as it affects the capsule was discussed in a previous paper (Sterne 1937 a). See also Stamatin (1934) and Schaefer (1936).

Dissociation of the Uncapsulated Variant of Strain XXII.—Two nutrient broth and two serum broth tubes were inoculated with the uncapsulated strain. One of each lot was incubated at 37° C. and the other at 42° C. At intervals a loopful of each culture was streaked on nutrient agar and the form of the developing colonies examined.

The following symbols are used to denote the type of growth:

R = Rough.
S = Smooth.
M = Mucoid.
P = Phantom.
T = Transparent, flat and spreading.
O = Opaque and raised.

The degree of sporulation is indicated thus:—

\- No spores.
\+ + Occasional free-lying spores.
\+ + + + 90 to 100 per cent. free-lying spores.

The following table shows the main types of colonies obtained

TABLE I.

Loopful of broth culture streaked after	Types of colonies which grew in streaks made from uncapsulated avirulent strain grown in			
	Broth at		Serum broth at	
	37 C.	42 C.	37 C.	42 C.
7 days.....	R++++	R++++	R++++	R− RO++
14 days.....	RT− RO++++	RT− RO++++	R++++	RT− RO+
21 days.....	RT− RO++++	RT− RO++	R P	R
28 days.....	RT− RSO++++	RT− RSO++++	RT++ RSO+++ P	R− RO+++ P
50 days.....	R− RSO+++	No growth	R RO++++	

It seems that the rough uncapsulated parent strain split into two main strains, the one having a transparent, flat rough colony and the other a more raised, opaque and somewhat smoother colony. The bacilli in the former did not sporulate or sporulated rarely, whereas the bacilli in the latter sporulated readily.

Further Dissociation of Variants Noted in Table I.—Some of the variants noted above were studied in more detail to see whether further dissociation would occur freely. A rough, transparent non-sporulating colony and an opaque sporulating colony (14 days, column 1, Table I) were seeded into separate tubes of nutrient broth and the cultures which developed streaked at intervals on nutrient agar.

TABLE II.

Loopfuls of broth culture streaked after	Types of colonies which grew in streaks made from broth cultures of	
	Rough transparent non-sporulating variant (RT−).	Rough opaque sporulating variant (RO++++).
1 day.....	RT−	RSO++++
18 days.....	RT	RT− RO++++
25 days.....	RT−	RT− RSO++++
50 days.....	RT−	RT−

Other rough opaque and rough transparent colonies were grown in broth for periods of 180 days and streaks made from these cultures showed almost the same appearance as streaks made in the experiment noted in Table II. Thus it seems that the RO variant dissociated into RO and RT types while the RT variant did not produce RO forms. This dissociation of the RO colony sometimes occurred as " sector " variation—i.e. sectors of the RT type developed in RO colonies.

It must not be supposed that only these two sorts of colonies appeared during the course of the experiments. Although the pattern of variation was mainly that indicated above yet subsidiary " trends " were frequently seen. The RT colonies, in which the bacilli grew in chains, sometimes showed sectors in which the bacilli grew singly. Such sectors, while still rough, transparent and non-sporulating, could be clearly differentiated from the long chained parent strain. The differences persisted after the variants had been sub-cultured. Occasionally, smooth looking colonies which resembled slightly smooth mucoid anthrax strains were noted. The bacilli, however, were neither capsulated nor virulent.

Continuous Dissociation of a Phantom Variant.—The phantom growth which developed from strain XXII (21 days, column 3, Table 1) was streaked on nutrient agar. After 24 hours a faint lustreless film appeared which showed, under low magnification, a number of minute slightly raised condensations. After a further 24 hours these condensations were plainly visible as raised rough opaque colonies scattered in the film. The phantom growth was subcultured two or three times a week for seven months and always commenced as a thin film with minute condensations which later developed into rough colonies. The rough colonies remained rough on subculture.

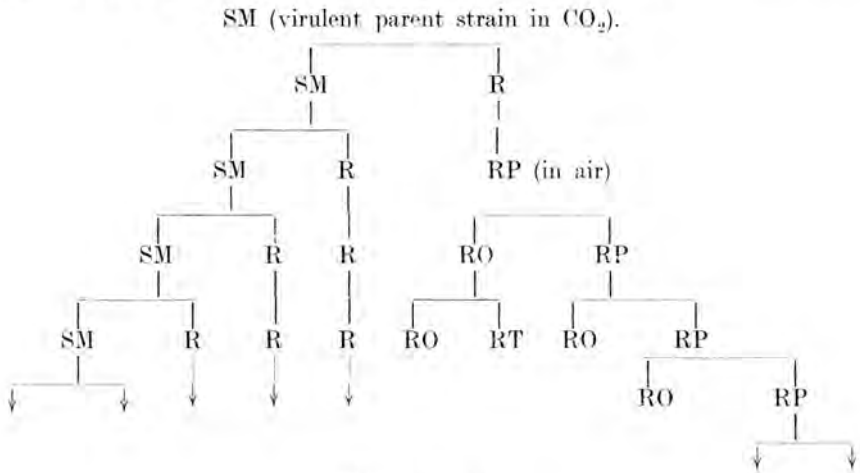
This seemed to be therefore, a clearcut example of continuous variation where the unstable rough phantom continuously reproduced itself and also threw off a relatively stable rough non-phantom daughter strain.

Dissociation of Strain XXXV.

This virulent strain was grown on 50 per cent. serum agar in 20 per cent. carbon dioxide immediately after its isolation. Under these conditions the growth was, as is the rule, smooth mucoid. After 48 hours a rough variant sector was selected and streaked on nutrient agar. This subculture appeared as a thin film dotted with small opaque rough colonies. Selection and subculture of the latter yielded only rough opaque colonies which in their turn gave rise to the rough transparent type. The peculiarity of strain XXXV was the rapidity with which different sets of dissociating colonies were established.

Schematic Representation of Dissociation in Strain XXXV.

All the SM variants were virulent and all others avirulent. The SM—>R change is naturally only recognizable in carbon dioxide. All the other changes were under ordinary conditions.



Discussion.

Sufficient has been done to show that there are a large number of factors involved in dissociation phenomena and that even relatively debased strains possess great variability. The examples of continuous variation given above and more particularly the example of strain XXXV, emphasize the fluidity of bacterial characteristics and show that a diversity of colony forms can be quickly obtained from apparently homogeneous cultures. Deskowitz and Shapiro (1935) and Deskowitz (1937) suggested that there was only a quantitative difference between unstable (continuous) variation and ordinary variation. The results obtained above with the moderately unstable RO strain support their contention.

Anthrax cultures often show a marked heterogeneity (interesting examples are given by Stamatin 1935a and 1935b) and such bacterial populations are, in general, very sensitive to environmental changes. This sensitivity is partly owing to the absence or rarity of reverse mutations so that an equilibrium during growth is only exceptionally established. As a rule a stable state can only be maintained by active intervention, as for example by frequent subculturing or the use of special media.

During the present work smooth contoured variants sometimes arose from rough parent strains and it seemed that such occurrences were more frequent in " debased " strains (variants which formed neither capsules nor spores and were neither virulent nor able to elicit immunity). Such late appearing smooth forms, although resembling superficially the smooth precursors of the rough variants were in reality entirely different and lacked properties characteristic of the early smooth strains (capsules, spores, virulence). It must be remembered that " rough " and " smooth " are vague, elastic terms and may include a variety of colony types. Such a fortuitous resemblance, the result of a common smooth appearance, must not be allowed to imply either a reversion or a cyclical change.

A consideration of the variants described here and of the wide variety of forms described by other authors emphasizes the difficulty of postulating any cyclical variation in the anthrax bacilli. Most strains, apparently, undergo degradation on artificial media. Capsulated and virulent strains become uncapsulated and avirulent; sporulating strains become non-sporulating. It is impossible to say whether these and the many other variants described are important stages in a life history or reactions to an artificial environment.

Summary.

1. Two virulent anthrax strains incubated in carbon dioxide gave rise to uncapsulated avirulent variants and from the latter a number of other variants were isolated and studied.

2. There was a tendency for sporogenous variants of the uncapsulated strain to produce asporogenous types.

3. On different occasions highly unstable rough phantom variants developed from the uncapsulated strains and gave rise continuously to rough non-phantom daughter strains. These in their turn showed further dissociation.

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