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VARIATION OF *BACILLUS ANTHRACIS* WITH SPECIAL REFERENCE TO THE NON-CAPSULATED AVIRULENT VARIANT

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(With Plates 15—18)

INTRODUCTION

During my investigation of the taxonomic relationship between *Bacillus anthracis* and the saprophytic aerobic sporing bacilli it was considered advisable to investigate the variability of *B. anthracis* itself before attempting to compare it with other species.

Numerous observations on the variation of *B. anthracis* have already been recorded (Preisz, 1908, 1911; Nungester, 1929). The most important, however, was that made by Sterne (1937 *a, b*) on the variation in capsule production and virulence. Sterne, following up the observations of Nungester (1929) and Ivánovics (1937) on the effect of CO_2 on capsule production by *B. anthracis*, found that *B. anthracis*, although generally producing rough, dull and flat colonies on ordinary nutrient agar in air, would invariably produce a smooth mucoid growth when grown on 50 % serum agar in the presence of air containing 65 % CO_2. He later showed that on further incubation the smooth mucoid growth usually gave rise to a rough outgrowth. Subcultures from the outgrowth gave a variant which differed from the parent culture in its inability to produce capsules and mucoid growth under these special conditions and in its loss of virulence. Similar dissociation has previously been observed by Stamatin (1934), Stamatin & Stamatin (1936) and Schaeffer (1936) in cultures grown on defibrinated horse blood and coagulated serum. Since these cultural conditions were not optimal for the production of capsules by *B. anthracis* the results obtained by the earlier workers were not as definite as those obtained by Sterne. Unfortunately, apart from making use of the variant as a new vaccine for anthrax immunization, this important dissociation has not been further investigated by Sterne and was very little known by other workers.

This paper describes further study of this important dissociation, the object being to find out whether it occurs only under the special cultural conditions used by Sterne or also in ordinary laboratory cultures and in nature, and how readily and regularly. Special attention has been given to the implication of the variation in the taxonomy of *B. anthracis*.

METHODS AND MATERIALS

Source of cultures. A large collection of authentic cultures and freshly isolated strains of *B. anthracis* was used in this investigation. They include: seven type cultures from the National Collection of Type Cultures; twenty-four strains from Dr Tomlinson, Public Health Laboratory, Bradford; five strains from the Ministry

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of Agriculture's Veterinary Laboratory; three strains from the late Dr Bruce White; about 100 cultures freshly isolated from hair and wool imported from all over the world; and four strains freshly isolated from infected cattle and pigs.

Seed cultures. The blood of a guinea-pig dead of experimental infection was used as seed culture for normal capsulated cells. In the study of bacterial variation it is essential to start cultures from single cells. The surest way of achieving this aim is by fishing a single cell under the microscope, but this method is not very practicable when a large number of cells has to be examined. A less reliable but more convenient way is by picking single colonies. But for an organism like *B. anthracis*, which generally grows in long chains, each colony is more likely to arise from a chain of cells than from a single cell. This fact was not fully realized in most of the earlier work on variation of *B. anthracis*. In the present investigation this difficulty was partly overcome by using free spore suspensions instead of young vegetative cells as seed cultures and by diluting the inoculum sufficiently. These modifications have greatly facilitated the detection of the dissociation and made possible some interesting findings which were not disclosed by Sterne.

Capsule staining. In addition to McFadyean polychrome methylene blue, toluidine blue, Giemsa's stain and Wright's stain were found satisfactory for the staining of the capsule. Toluidine blue is one of the metachromatic dyes often used by histologists (Lison, 1935). The writer found that it also stained the capsule of *B. anthracis* metachromatically. The metachromatic reaction given by the dye was more marked and consistent than that obtained with methylene blue. The method consists of staining a heat-fixed smear with a 0·1 % toluidine blue solution in 1 % alcohol for 5 min.

EXPERIMENTAL OBSERVATION

The effect of cultural conditions on capsule production

A number of cultures of *B. anthracis* were made on nutrient agar plates containing 0–50 % fresh horse serum. One of each was incubated in ordinary atmosphere and the rest in McIntosh and Filde's anaerobic jars in which 5–50 % of the air was replaced by CO_2. The appearance of the colonies and the degree of capsule formation under the various cultural conditions are compared in Pl. 15, figs. 1 and 2.

Both CO_2 and serum favour the production of capsules and the smooth mucoid colonies, but it is not necessary to use such a high concentration as was used by Sterne. 5–10 % of each is sufficient.

Gladstone (1946) obtained encapsulated *B. anthracis* by growing it in C.C.Y. medium (Gladstone & Fildes, 1940) aerated with CO_2. He further showed that the presence of bicarbonate in the medium had a favourable effect. The above experiment was therefore repeated using bicarbonate buffered C.C.Y. agar in 5–50 % CO_2. The growth, although containing a large proportion of encapsulated cells, is not so uniformly mucoid and characteristic as on serum agar.

As a result of this observation, the standard conditions finally adopted for the study of capsule formation by *B. anthracis* throughout this work were growth on a nutrient agar plate containing 10 % horse serum in an atmosphere of 10 % CO_2 and 90 % air.

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The production of the non-capsulated variant on serum agar in the presence of CO_2

To be sure that the starting organisms were typical virulent capsulated *B. anthracis*, the blood of a guinea-pig which had died of experimental anthrax was used. Immediately after death, a loopful of the heart blood was suspended in 10 ml. of sterile saline. The suspension was further diluted with saline and one drop of it was evenly spread on a 10% serum agar plate. The plate was incubated in 10% CO_2. After 16 hr. incubation the colonies all appeared smooth, mucoid and glistening. Twenty-four hours later, small, rough, dull, irregular outgrowths began to appear in some of them. On further incubation more and more smooth colonies threw off rough outgrowths and the earlier outgrowths rapidly increased in size. The cells which made up the rough outgrowths differed from those in the mucoid central growth in lacking capsules. The capsulated cells in the mucoid central growth, when transplanted on fresh serum agar, developed mucoid colonies and underwent dissociation into capsulated and non-capsulated variants again. They thus behaved like the parent cell. The non-capsulated variants isolated from the outgrowths, on the other hand, were very stable. They never grew mucoid colonies and capsules again. They appeared to have lost permanently the ability to produce capsules. The dissociation pattern is shown in Pl. 15, fig. 3. Over 140 strains of *B. anthracis* obtained from all over the world have been found to undergo the same pattern of dissociation and from all of them non-capsulated variants have been isolated.

The detection of the non-capsulated variant in laboratory stock cultures of Bacillus anthracis

The next thing was to see if the dissociation also occurred under cultural conditions generally used in the laboratory for growing *B. anthracis*. A number of type cultures of *B. anthracis* were requested from different laboratories. They were usually received on nutrient agar slopes. On arrival the original cultures were held in the incubator at 37° C. for 3 days to allow full sporulation. The whole growths were then washed off with sterile saline and thoroughly mixed. After standing overnight for the large clumps to settle down, the supernatants, which consisted mainly of individual spores, were further diluted. The diluted spore suspensions were then plated on 10% serum agar and grown in 10% CO_2. After 16 hr. incubation nearly all the cultures examined produced two kinds of colony; a smooth mucoid colony of well-encapsulated cells and a rough, dull, flat colony of non-capsulated cells (Pl. 16, figs. 4 and 5). This showed that all the cultures received already contained the non-capsulated variant. The percentage of the variant cells present in the various cultures differed considerably (Table 1). Strain 282 contained as many as 32·4% but the majority of the cultures contained from 0·1 to 1%.

The possible occurrence of the non-capsulated variant in nature

A matter of much taxonomical and epidemiological interest is whether the dissociation will occur in nature. One indirect way of investigating this problem is to see if this non-capsulated variant of *B. anthracis* can be found in soil or animal

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products from anthrax-endemic districts. Investigation of this kind was handicapped by the occurrence in nature of many saprophytic bacilli, which could hardly be differentiated from *B. anthracis* by known methods. Because of this gap in our knowledge this investigation was impossible until a fairly satisfactory method for the differentiation of *B. anthracis* from the saprophytic species had been developed by the extensive work to be presented in subsequent papers.

Table 1. *Percentage of non-capsulated variant present in stock cultures of* Bacillus anthracis

Strain	No. of capsulated cells found	No. of non-capsulated cells found	Percentage of non-capsulated cells
282	250	120	32·4
2620	470	89	15·9
Tunny	385	35	8·3
2041	805	50	5·8
2047	1250	58	4·5
1506	940	21	2·2
281	550	11	2·0
Ellis	445	5	1·1
Vollum	600	2	0·33
8380	700	1	0·14

Table 2. *Immunizing power of anthrax-like organism isolated from goat hair against anthrax*

Organism used for immunization:	No. of guinea-pigs tested	No. of guinea-pigs survived a challenged dose of 100 M.L.D. of *B. anthracis*
Non-capsulated variant of a typical *B. anthracis*	6	5
Anthrax-like organism isolated from goat hair	6	5
Typical *B. cereus*	6	0
Control	3	0

The guinea-pigs were vaccinated with two doses of 1 ml. each of a suspension of the organism of density equivalent to Brown's tube no. 1 in 10-day intervals. Two weeks later they were challenged with 100 M.L.D. of a virulent culture of *B. anthracis*.

In the course of the present investigation several hundreds of goat hair specimens, imported from various parts of the world, have been examined for the presence of anthrax spores. Many of these specimens were found to contain virulent capsulated anthrax bacilli and from a few of them was isolated a non-capsulated organism resembling typical *B. anthracis* in all respects except for being non-capsulated. The facts that it possesses a good immunizing power against anthrax, which is known to be possessed only by *B. anthracis* (Table 2), and that it has so far been found only in material from anthrax districts strongly suggest its being a non-capsulated variant of *B. anthracis* rather than a saprophytic species. This observation shows that the dissociation is likely to take place in nature. This belief was further supported by the detection of the non-capsulated variant in sterile soil experimentally contaminated with anthrax-infected blood which

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contained only the capsulated organism. This view, however, needs to be confirmed by much more field investigation in anthrax endemic areas.

The rate of production of the non-capsulated variant and the effect of cultural conditions

The proportion of variant in a culture may not reflect the rate of dissociation directly, because it also depends on the culture age at which dissociation begins and the relative growth rate of parent and variant cells.

In the following paragraphs, the overall rate of production of the non-capsulated variant in cultures grown under different conditions will be discussed, and not the actual rate of dissociation.

Table 3. *Percentage of colonies showing visible outgrowths of the non-capsulated variant on* 10% *serum agar plates grown in* 10% CO_2

Total no. of colonies on each plate	Time of incubation (days)	Colonies showing visible dissociation	
		No.	%
Plate 1 (8)	1	0	0
	2	1	12·5
	3	2	25·0
	5	8	100·0
Plate 2 (112)	1	0	0
	2	10	8·9
	3	13	11·5
	5	45	40·0
Plate 3 (370)	1	0	0
	2	10	2·7
	3	25	6·7
	5	38	10·2

Let us first see how readily the variant appeared on serum agar in the presence of CO_2. 10% serum agar plates were inoculated with serial dilutions of anthrax-infected blood and grown in 10% CO_2. Table 3 gives the percentage of colonies which produced visible outgrowth of the non-capsulated variant during incubation. The percentage of colonies which showed visible dissociation was highest in the plate containing fewest colonies, in which more space and nutrients were available for the variants to produce a visible growth. A large proportion of the colonies which showed no visible dissociation, especially those over three days old, were found on subculturing to have contained small numbers of the variant (Table 4).

The same anthrax blood was inoculated on ordinary nutrient agar plates and grown in air. After 5 days' incubation some of the colonies were removed from the plates and each was suspended in 10 ml. of sterile saline. The suspensions were then plated on serum agar and incubated in air containing 10% CO_2 to see if they contained any non-capsulated variants. Table 4 shows that the variant was present in all colonies examined in the proportion of about 0·02 to 0·12%.

The fact that the non-capsulated variant was found in cultures grown on ordinary nutrient agar in air shows that dissociation is not induced by the presence of CO_2 and serum. These particular cultural conditions, however, play two im-

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portant roles in the dissociation. (1) They provide the necessary raw material with which the cells able to produce capsules do so, thus making possible the recognition of a variant which is genetically incapable of producing capsules. (2) The normal cells, probably owing to the presence of the hydrophylic mucoid capsule, no longer grow in long filaments but in rather short chains so that the growth is less spreading. The non-capsulated variants, on the other hand, still retain the characteristic long chain growth and are soon able to grow beyond the

Table 4. *The presence of non-capsulated variants in colonies showing no visible dissociation*

Medium	Age of colony (hr.)	Size of colony (mm.)	No. of cells examined: Capsulated	No. of cells examined: Non-capsulated	Percentage of non-capsulated cells
Serum agar	14	0·5	3000	0	—
	14	0·5	2840	0	—
	14	0·5	2500	0	—
	72	2	3200	12	0·37
	72	2	2500	13	0·52
	72	2	3400	9	0·27
Plain agar	72	6	6800	2	0·03
	72	6	6000	6	0·10
	72	6	5000	3	0·06

Table 5. *The over-all rate of production of the non-capsulated variant in cultures grown on different media*

Cultural condition	Age of culture (days)	No. of capsulated cells found	No. of non-capsulated cells found	Percentage of non-capsulated cells
10% serum agar plate in 10% CO_2	1	8900	1	0·011
	3	3500	730	20·5
	5	3400	1420	42·5
Plain agar plate in air	1	8500	1	0·012
	3	6000	3	0·05
	5	5400	4	0·07

mother colony to the surrounding medium where they have a better supply of nutrient without the competition of the dominant normal cells. Their rate of further multiplication is thus much higher than that of the normal cells which are now living in the exhausted part of the medium. Because of this favourable effect, the over-all proportion of the variant found in cultures grown on serum agar in CO_2 was higher than in cultures grown on ordinary agar in air (Table 5).

Owing to a number of technical difficulties it was not possible to make any accurate estimation of the rate of dissociation. However, from the readiness with which the variant occurred in the culture the dissociation appeared to be a fairly frequent one, probably with a dissociation rate between 0·01 and 0·1%.

The stability of the non-capsulated variant

So far as capsule formation and pathogenicity were concerned, the variant appeared to be extremely stable. Many cultures have been kept in the laboratory

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for more than 4 years without showing any sign of reversion. During this period, hundreds of subcultures have been made on serum agar and grown in the presence of CO_2, but not a single mucoid colony has ever been found. Methods similar to those used for the conversion of R form pneumococcus into S form, namely cultivation in the presence of extracts of capsulated organisms and repeated animal passage also failed to convert the non-capsulated variant of *B. anthracis* back to its normal capsulated form.

Comparison of other characteristics of the variant and the normal cell

Pathogenicity. The pathogenicity of the non-capsulated variant has been compared thoroughly with that of its parent cell by Sterne. He found that the loss of the ability to produce a capsule was accompanied by a loss of pathogenicity. This was confirmed in the present investigation. Large numbers of the variant have been produced during the course of the study, and none of them was virulent

Table 6. *The immunizing power of the non-capsulated variants of* Bacillus anthracis

Strains used for immunization		No. of guinea-pigs tested	No. of guinea-pigs which survived a challenge dose of 100 M.L.D.
Non-capsulated variant of a virulent strain, Ellis		10	10
Attenuated strain 'Pasteur Vaccine 1'	Capsulated	7*	1
	Non-capsulated	10	0
Atypical slight virulent strain '5328'	Capsulated	10	8
	Non-capsulated	10	0
Control, not vaccinated		10	0

* The other three died from the vaccine.

enough to kill guinea-pigs or mice. The organism could, however, germinate, survive and possibly proliferate at the site of inoculation for 2 or 3 weeks, causing considerable local swelling. The persistence of the organism at the site of inoculation and its failure to spread to other parts of the body account for its high immunizing power and non-virulence.

Immunizing power. Although the variant was no longer virulent, Sterne found it a good immunizing agent. A direct comparison of the immunizing power of the variant with that of its virulent capsulated mother cell is not possible unless the latter is of such low virulence that it can be used as living vaccine. Sterne has tried to produce the variant from the attenuated strain without success. He found that the attenuated strains, although producing mucoid growths, were so stable that no outgrowth of the non-capsulated variant could be obtained. In the present investigation, by using the improved technique, it was possible to obtain variants from a number of attenuated strains, including the two Pasteur strains. Table 6 shows a comparison of the immunizing power of some of these attenuated strains with their respective non-capsulated variants and also the immunizing power of non-capsulated variants derived from fully virulent and attenuated strains.

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The results showed that non-capsulated variants derived from virulent strains mostly possessed high immunizing power, while those derived from attenuated strains were completely ineffective. The normal capsulated cells of the attenuated strains were, however, capable of conferring a certain degree of immunity on guinea-pigs. The effect of the capsule on the immunizing power of the attenuated strains is not clear; but it possibly prolongs the survival and proliferation of the organisms in guinea-pigs, since the growth of *B. anthracis* in the tissue was found to be necessary for the production of immunity (Munne, 1934; Gladstone, 1946).

Antigenic structure. Apart from its loss of capsular antigen, the non-capsulated variant appears to have the same antigenic structure as the capsulated mother cell. Thus, antigens extracted from vegetative cells of both strains grown in ordinary medium in air were essentially similar, since in such cultures the potentially capsulated cells also produced little capsular material. Spores of the non-capsulated variant were likewise agglutinated by serum against spores of the capsulated cell and *vice versa.* This work will be dealt with more fully in a subsequent paper.

Morphological, cultural and biochemical characteristics. The failure of the non-capsulated variant to produce capsules *in vivo* or on serum agar in the presence of CO_2 appeared to be the only difference between the variant and its capsulated mother cell. The other morphological and physiological characteristics of the variant were exactly the same as those of the capsulated cells from which they arose.

DISCUSSION

It must be emphasized that the variation described above is concerned mainly with the potential ability of the cell to produce a capsule and with the associated change of colony appearance on serum agar in the presence of CO_2. This variation is different from the more widely known colony variation of *B. anthracis* described in the text-books. The latter refers to the variation of colonies grown on plain agar in air and has been extensively studied by numerous workers since the early days of bacteriology (Preisz, 1908, 1911; Wagner, 1920; Nungester, 1929; Sterne, 1938). It is hoped in this discussion to bring together these two kinds of variation so as to present a general picture of the range of variations which have been observed in cultures of *B. anthracis*.

Let us start with a typical virulent capsulated *B. anthracis*, say, from the infected blood. If this organism is grown on ordinary plain agar in air, it generally gives rise to the well-known rough 'medusa-head' colony characterized by a flat dull ground-glass surface and a curled edge. But after cultivation in the laboratory for some time, colonies different from the typical form are frequently encountered. Apart from the 'medusa-head' colony (usually designated as R) there are raised opaque colonies with smoother edge (S), colonies of smooth mucoid appearance (SM) and colonies intermediate between these three types. The change from the rough growth to the smooth or mucoid growth can be accelerated by prolonged cultivation in the presence of weak antiseptic or at higher temperature (Preisz, 1908, 1911; Nungester, 1929).

The production of the rough 'medusa-head' colony by *B. anthracis* on nutrient agar in air is due largely to its long chain growth and to its hydrophobic cell

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surface. Any change of these two characteristics of the organisms will therefore have a great influence on the appearance of the colony. Thus the growth of the S variant in shorter chains is responsible for its smoother colony, while the ability of the SM variant to produce the hydrophilic capsule in air accounts for its mucoid growth. These colony variants have been widely studied. It is well-known that unlike other bacteria, cultures of *B. anthracis* with rough colonies are usually more virulent than those with smoother colonies.

Although these colony variants produce different types of colonies on nutrient agar in air, they all produce smooth mucoid colonies on serum agar in the presence of CO_2, because these cultural conditions enable the cells with the genetic potentiality of producing capsules to produce them. The mucoid hydrophilic capsule so produced then becomes the factor determining the colony appearance.

During multiplication, the cells genetically able to produce capsules constantly give rise to a small number of variants which are genetically incapable of producing capsules. The non-capsule-producing variant differs from its capsule-producing mother cell in being unable to produce capsules under any cultural conditions or *in vivo*. Thus each of the colony variants R, S and SM can be further dissociated into a potentially capsulated variant and a variant that can never produce a capsule. No matter how rough or smooth their colonies look when they are grown on nutrient agar in air, the potentially capsulated variants always produce smooth mucoid colonies on serum agar in the presence of CO_2. On the other hand, the cells with no capacity for capsule production have the same colony form under both cultural conditions. Thus we have 'capsulated R', 'non-capsulated R', 'capsulated S', 'non-capsulated S' and 'SM' variants, where R, S and SM denote the types of colony produced on plain agar in air, and 'capsulated' and 'non-capsulated' indicate whether or not they are capable of producing capsules and smooth mucoid colonies on serum agar in CO_2. The capsulated 'R' variant represents the normal virulent *B. anthracis*. The Pasteur variants are examples of the capsulated S variant. The non-capsulated R and non-capsulated S are avirulent variants derived from normal virulent R and S cultures of *B. anthracis*. The SM variant is, of course, a capsulated organism but it differs from the usual potentially capsulated cells in being able to produce abundant capsule not only on serum agar in CO_2 but also on plain agar in air.

The whole range of variation in colony appearance and cell structure of *B. anthracis* described above can be best summarized in Pls. 17 and 18. It can be seen that it involves three different types of change. First, there is the variation of colony form on plain agar in air due mainly to the difference of the chain length of the growth, and to the ability of the cells to produce capsules in air. Secondly, there is the change of cell morphology and colony appearance due to change of cultural conditions, and lastly there is the dissociation of potentially non-capsulated variants from normal capsulated cells which can best be observed in cultures grown on serum agar in CO_2.

The writer agrees with Sterne (1937*a*) that the smooth mucoid colony produced by normal virulent capsulated *B. anthracis* on serum agar in CO_2 should be regarded as the normal colony form of *B. anthracis*, equivalent to the smooth colony form

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of other bacteria. The production by the normal capsulated cell of the non-capsulated avirulent variant which gives rise to a rough colony instead of the smooth mucoid colony is equivalent to the usual S-R dissociation of other bacteria, especially pneumococcus, in which case the change from the smooth form to the rough form is also associated with the loss of a visible capsule. In this way the S-R variation of *B. anthracis* no longer differs from that of other bacteria; that is to say, the smooth or mucoid form represents the normal virulent cell and the rough form the avirulent variant.

The finding that *B. anthracis* can give rise with such readiness and regularity to the non-capsulated avirulent variant has opened up a difficult taxonomic problem. According to the existing classification, *B. anthracis* is defined as an aerobic sporing bacillus capable of causing anthrax infection in animals. We have now an organism which is a direct descendant of *B. anthracis*, but owing to loss of its pathogenicity is more likely to be classified as *B. cereus*, a saprophytic species closely related to *B. anthracis*, than as *B. anthracis*. This shows either that the criteria used in the present classification are inadequate and further differential criteria have to be sought, or that these two species are really indistinguishable and should be grouped together as a single species as was recently suggested by Smith, Gordon & Clark (1946). Smith *et al.*, who have contributed so much on the classification of the aerobic sporing bacilli, have suggested in their recent monograph (1946) that *B. anthracis* should be regarded as a pathogenic variety of *B. cereus* and proposed to rename it as *B. cereus* var. *anthracis*. Their suggestion was based on the impression that non-pathogenic *B. anthracis* is indistinguishable from *B. cereus*.

Although the writer agrees with Smith *et al.* that *B. anthracis* is closely related to *B. cereus* he feels that much more work is needed before one can decide whether they should be reclassified into a single species. The mere fact that they are not distinguishable by the criteria used hitherto for their separation does not necessarily mean that they are identical, because our present knowledge of these organisms is so incomplete, being confined to morphology and certain physiological activities. It is for this reason that an extensive comparative study of *B. anthracis* with the saprophytic aerobic sporing bacilli was made by the writer and will be presented in subsequent papers in which the taxonomic relationship of *B. anthracis* with the saprophytic species will be more fully discussed.

SUMMARY

Normal virulent *B. anthracis* all possess the genetical potentiality of producing capsules, but the capsule is only produced *in vivo* or under special cultural conditions suitable for capsule formation, especially in the presence of CO_2.

During multiplication the potentially capsulated cells regularly produce a small number of a stable non-capsulated avirulent variant. All laboratory stock cultures of *B. anthracis* examined were found to contain from 0·14 to 32·4% of this avirulent variant. The variant has also been demonstrated in hair and wool from anthrax endemic areas, which suggests that the dissociation may also occur in nature.

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Both the normal potentially capsulated cell and the variant incapable of producing capsules grow on plain agar in air as rough colonies with apparently non-capsulated cells. But when they are grown on serum agar in the presence of CO_2, the normal potentially capsulated cell gives rise to a smooth mucoid colony with fully capsulated cells, while the variant still produces a rough colony with non-capsulated cells. If we regard the smooth mucoid colony produced by normal virulent *B. anthracis* on serum agar in CO_2 as the normal colony form of *B. anthracis*, then the dissociation of the avirulent non-capsulated variant with the rough colony form falls in line with the usual S-R dissociation of other bacteria.

The more widely known variation of *B. anthracis*, which had long been held as an exception to the usual S-R variation of other bacteria, refers to colonies grown on plain agar in air. This variation is different from the one just described and is not so clear-cut and significant. Its underlying cause is not quite clear except that the chain length of the growth appears to play some part.

A summarized picture showing the whole range of variation in colony appearance and cell structure of *B. anthracis* is presented.

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EXPLANATION OF PLATES 15, 16

Plate 15

Fig. 1. Colonies of *B. anthracis* grown on nutrient agar containing various amounts of horse serum incubated in air with various concentrations of CO_2, 24 hr. culture. × 5.

Fig. 2. Morphology of *B. anthracis* grown on nutrient agar containing various amounts of horse serum incubated in air with various concentrations of CO_2, 24 hr. culture, stained with 0·1 % toluidine blue. × 1000.

Fig. 3. The production of a non-capsulated variant by *B. anthracis*. The left-hand side of the diagram shows how a rough outgrowth of a non-capsulated variant is produced from the smooth mucoid colony of the normal capsulated *B. anthracis* grown on 10 % serum agar in air containing 10 % CO_2. The right-hand side compares the morphology of the cells which made up the smooth mucoid colony and the rough outgrowth.

Plate 16

Fig. 4. The detection of the non-capsulated variant in stock culture of *B. anthracis* by cultivation on 10 % serum agar in air containing 10 % CO_2. Note the marked difference between the smooth, raised and shining colonies produced by the capsulated cells and the rough, flat and dull colonies produced by the non-capsulated variants. About four-fifths of the natural size, photographed with reflected light.

Fig. 5. As fig. 4, but photographed with transmitted light. × 5.

Fig. 6. The marked difference between the normal capsulated cell and the non-capsulated variant grown on 10 % serum agar in 10 % CO_2, 24 hr. culture, stained with 0·1 % toluidine blue. × 1000.

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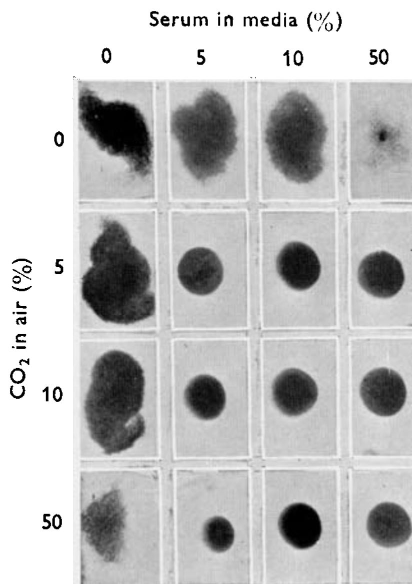

Fig. 1.

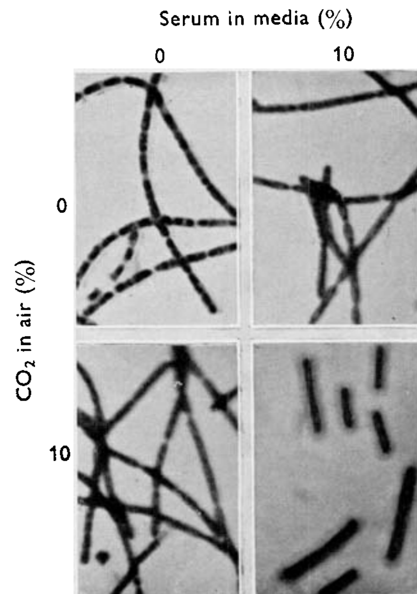

Fig. 2.

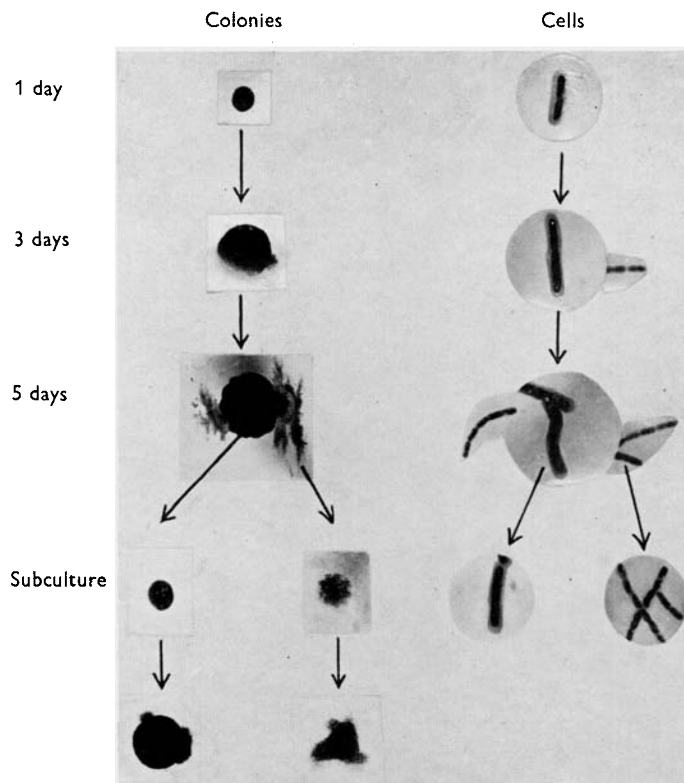

Fig. 3.

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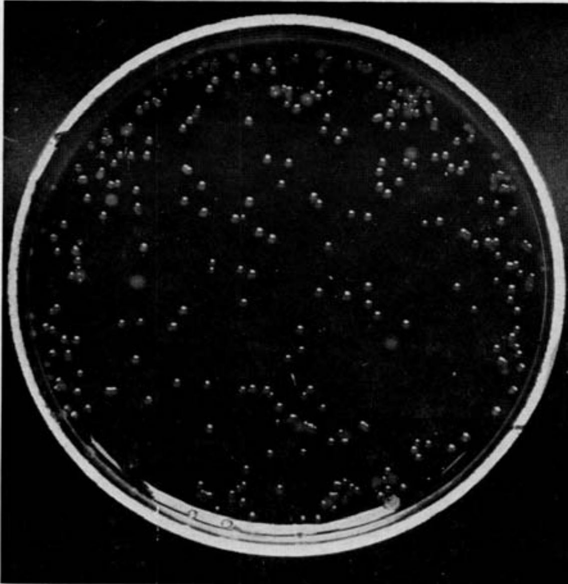

Fig. 4.

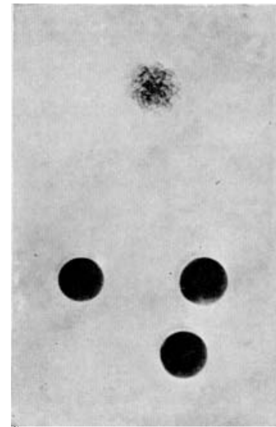

Fig. 5.

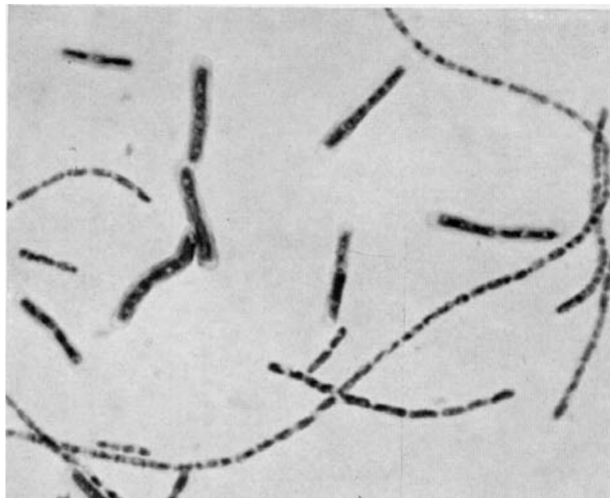

Fig. 6.

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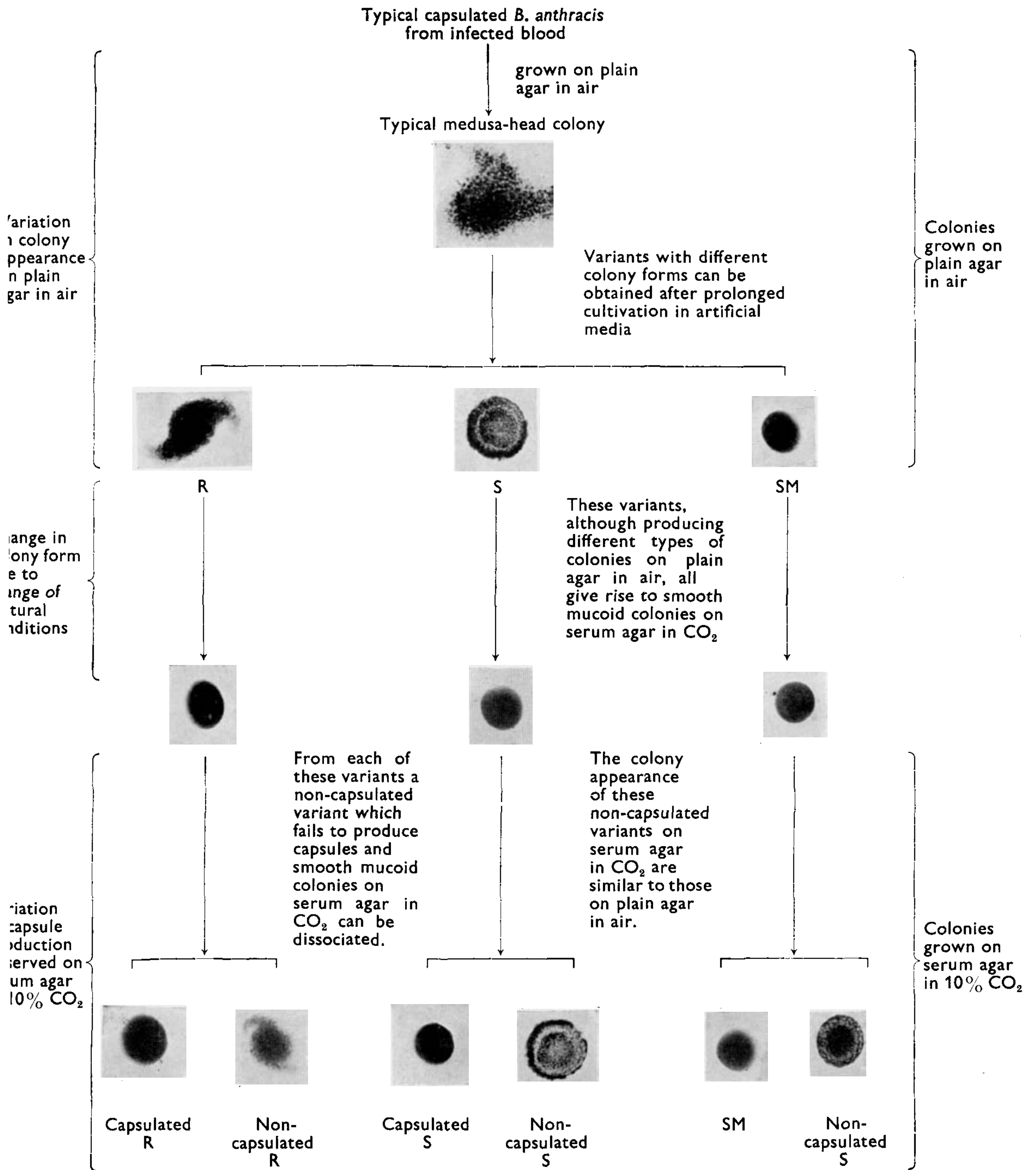

Fig. 7. Summary of colonial variation of *B. anthracis*.

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	Typical	Variants			
	Capsulated R	Capsulated S	Capsulated SM	Non-capsulated R	Non-capsulated S
Occurrence	Usual form	Rare variant	Rare variant	Frequent variant	Rare variant
Colonies grown on plain agar in air					
Colonies grown on serum agar in 10% CO_2					
Cells grown on plain agar in air					
Cells grown on serum agar in 10% CO_2					
Growth in plain broth in air					
Growth in serum broth in 10% CO_2					
Growth in gelatin stab in air					
Pathogenicity	+	±	±	–	–

Fig. 8. Comparison of the different variants of *B. anthracis*.

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