THE STRUCTURE OF B. ANTHRACIS AND REVERSAL OF THE GRAM REACTION.*

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PLATES 34 TO 38.

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If a small amount of aqueous solution of acriviolet, gentian violet, or acriflavine be added to a thick aqueous suspension of a young culture of *B. anthracis* it will be found that after a longer or shorter interval of time a large proportion, if not all, of the organisms are changed by the exposure from sharply Gram-positive to sharply Gram-negative. The speed with which this change takes place varies with the strain of *B. anthracis* examined. In one strain studied, Gram reversal began in a few minutes and was complete within 2 hours. In other strains 19 hours were required to produce the change and even at the end of this period a few Gram-positive individuals were sometimes still found in the smears. The Gram-negative forms of *Bacillus anthracis* produced by exposure to these dyes are notably smaller in calibre than the Gram-positive forms, the difference in diameter as measured by means of a filar micrometer being in the neighborhood of 40 per cent.

That such changes could be produced in this organism by exposure to aniline dyes was first observed about a year ago. The present communication is concerned with studies made, since that time, of the reversal of Gram reaction, and of the change in calibre, which the dyes caused; and with attempts to interpret the significance of the two phenomena.

* Preliminary report of this work appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 737.

The Gram Stain.

In our early observations as to the effect of aniline dyes on *B. anthracis* the smears were stained by Paltauf's modification of the Gram method.[1] The findings thus obtained were also confirmed by the study of smears stained by the other well known modifications: Claudius',[2] Burke's,[3] Atkin's,[4] and Kopeloff's.[5] After the examination of about 2000 smears, each specimen being stained and examined by two observers, the definite conclusion was reached that Burke's modification was superior to all others. It was therefore, from that time on, exclusively used. The opinion as to its superiority was amply confirmed by the subsequent studies which involved the examination of nearly 12,000 smears. Whenever "Gram stain" is mentioned in this report it is the Burke modification (unless otherwise specified) to which reference is made. With the Burke technic the results are constant and absolutely clear-cut. The colors in the final smears contrast sharply (a bluish black with a pink) and one is never left in doubt as to whether an individual organism is to be called Gram-negative or Gram-positive.

When stained by the Burke technic young cultures of *B. anthracis* are definitely and constantly Gram-positive. An occasional Gram-negative individual may be seen, but these are *only* occasional and negative forms are usually entirely absent. The Gram-negative organisms, like *B. coli*, which are commonly used for controls in experimental work, are equally constant and sharp in their reaction to the Burke stain. If therefore the technic as described by Burke be carefully followed and the precautions observed which it is well known must be borne in mind in using the Gram method, the Burke modification is a method of exactness and from its results trustworthy conclusions can be drawn.

[1] Sharnosky, J., *Proc. New York Path. Soc.*, 1909, lx, N. S., 5.
[2] Claudius, M., *Ann. Inst. Pasteur*, 1897, xi, 332.
[3] Burke, V., *J. Bact.*, 1922, vii, 159.
[4] Atkin, K. N., *J. Bact.*, 1920, iii, 321.
[5] Kopeloff, N., *Lactobacillus acidophilus*, Baltimore, 1926, 204.

Exposure of Organisms to Aniline Dyes.

The technic of the experiments in which reversal of the Gram reaction of *B. anthracis* was produced was the following.

A heavy suspension of 4 hour culture of the organism was made in distilled water. ½ cc. of this suspension was placed in each of two tubes. To one of these tubes an oeseful of 1 per cent aqueous acriviolet was added. Smears were made at the beginning of the experiment and stained by Burke's method. These always showed the organism to be 100 per cent positive except that an occasional stray Gram-negative individual might be found. The tubes were put away at room temperature and smears from each tube examined at various intervals. It was found that Gram-negative forms began very soon to appear in the tube to which acriviolet had been added. In the case of the more rapidly changing forms, like No. 10,[6] reversal of Gram reaction began almost at once and was complete in 2 hours or less (Fig. 1). In the more slowly changing forms like N.Y.B. of H.,[7] 5 per cent of Gram-negative individuals appeared in 2½ hours.[8] At the end of 19 hours smears from the acriviolet tube showed that the organism had changed from a predominantly Gram-positive to a predominantly Gram-negative one. In other experiments reversal proceeded until about 75 per cent of the organisms had become Gram-negative and further change occurred very slowly if at all.

The first observations as to the effect of aniline dyes on *B. anthracis* were made with a strain furnished by the New York Board of Health. In order to determine that the reversal phenomenon was not peculiar to this particular strain of *B. anthracis* the experiment was repeated

[6] The numbers refer to the American Type Culture Collection.

[7] The strain referred to as "N. Y. B. of H." was isolated at the New York Board of Health Laboratories from a shaving brush in January, 1923.

[8] The roughness of a method in which percentages of Gram-positive and Gram-negative forms in a smear are estimated without actual counts is of course perfectly understood. Small variations are, under such conditions, of no significance whatever and no attention was paid to them. But the difference between a smear containing 99 per cent positive forms and one containing only 30 per cent positive forms is of course obvious at a glance and it was only differences of this order which were taken into account. In all cases the smears were stained by two observers and each smear recorded by both; the figures of the two records agreed pretty closely. It was found that the changes produced by the dyes were the same even when varying amounts were added and exactness of dilution was not necessary; "1 oeseful of 1 per cent dye" was therefore a sufficiently accurate measure. The oese used measured 2.33 mm. internal diameter.

with Strains 8, 9, 10, 240, 241, and 242 of the American Type Culture Collection. The authenticity of all of these strains was established by animal inoculation. It was found that the reversal phenomenon was produced in each instance by the exposure of the organism to aniline dyes so that the conclusion seemed warranted that the reaction was characteristic for the genus.

Similar experiments were then undertaken with other members of the spore-bearing, aerobic group. The first group studied was *B. subtilis* of which six strains were examined: one strain, isolated from hay, in our own laboratory and Nos. 102, 465, 466, 243, and 3 A. T. C. These experiments with *Bacillus subtilis* showed that a reversal of the Gram reaction similar to that of *B. anthracis* results from exposure to aniline dyes; but the phenomenon was less striking, since in some of the strains a moderate degree of reversal also occurred in the control tubes.

The definite findings in the case of *B. anthracis* and the suggestive findings in the case of *B. subtilis* suggested that the phenomenon might be common to all sporogenous aerobic[9] bacteria and a study was therefore made of this whole group, the strains being obtained from the American Type Culture Collection. The following organisms were examined.

B. mesentericus	No. 76
" *megatherium*	" 72
" "	" 71
" *mycoides*	" 270
" " *roseus*	" 80
" *adhærens*	" 271
" *niger*	
" *agri*	
" *laterosporus*	
" *simplex*	
" *globigii*	
" *albolactus*	
" *aterrimus*	
" *panis*	
" *prausnitzii*	
" *ruminatus*	
" *flavus*	" 58

[9] Nothing can at present be said about the behavior of anaerobes.

With a number of these organisms, reversal of Gram positivity was produced just as in the case of *B. anthracis*. This was true for example of *B. megatherium* and *B. mycoides*. But in the case of the majority of strains in this group either the results were inconstant or reversal occurred in the control tubes, so that no statement can be made as to the existence of any general relation between reversal phenomenon and the presence of spores in bacteria.

The investigation was then extended by making a study of non-spore-bearing Gram-positive organisms. Experiments were done with the following 50 strains.

	No.		No.
Sarcina lutea	272 A.T.C.	*Actinomyces maduræ*	552
" *rosea*	188	" *bovis*	549
" *aurantiaca*	146	" *gypsoides*	550
" *flava*	147	*Staphylococcus epidermidis*	155
Micrococcus auranticus	387	" *albus*	251
" *luteus*	379	" *citreus*	395
" *freudenreichii*	407	" *aureus*	477
" *flavus*	140	" *candicans*	154
" *conglomeratus*	401	" *tetragenus*	159
" *flavoroseus*	397	*Bacillus xerosis*	373
" *cereus*	394	" *diphtheriæ*	475
" *varians*	399	" *hoffmanii*	371
" *ureæ*	408	*Staphylococcus pyogenes*	160
Rhodococcus ruber	534	*Streptococcus lactis*	558
" *rhodochrous*	184	*Lactobacillus casei*	334
" *roseus*	185	*Staphylococcus* (No. 33)	Torrey
" *cinnabareus*	514	" (Richards)	"
Actinomyces asteroides	322	" (No. 77)	"
" *hominis*	551	" (isolated by Churchman)	

B. diphtheriæ	N. Y. B. of H.
" "	" " " " "
" "	" " " " "
B. hoffmanii	" " " " "
Pneumococcus Type 1	" " " " "
" " 2	" " " " "
" " 3	" " " " "
" " 1	Rockefeller Institute
" " 2	" "
" " 3	" "
B. tuberculosis bovine	N. Y. B. of H.
" " "	" " " " "

This study of the behavior toward aniline dyes of non-spore-bearing Gram-positive bacteria showed, that in striking contrast to the results produced in the case of *B. anthracis* and other members of the spore-bearing group, the dyes mentioned were without any effect on the Gram positivity of the majority of the non-spore bearers. The experiments with certain of these organisms (about 15 strains) were, however, unsatisfactory and from them no conclusions could be drawn. Pneumococcus for example proved too fragile for this type of experiment and we were unable to get constant results whether we worked with blood agar cultures or with the peritoneal fluid from injected mice. Nor were we able to draw positive conclusions from the experiments with *Streptococcus lactis*, nor with *Actinomyces hominis* or *Actinomyces maduræ*. But for the majority of the organisms (about 35 strains) in this group the dye was absolutely without effect on the Gram reaction, even after many days exposure and even when the tube was heated in order to make the test more severe. The difference between the behavior to aniline dyes of *B. anthracis* and a number of the Gram-positive non-spore bearers was well shown by an experiment in which a suspension containing both *B. anthracis* and *M. freudenreichii* was used. Both of these organisms are definitely Gram-positive and the smears made at the beginning of the experiment showed nothing but Gram-positive forms. Smears made (at the end of 19 hours) from the tube to which acriviolet had been added showed that none of the individuals of *M. freudenreichii* had been changed but that about 95 per cent of the individuals of *B. anthracis* had become Gram-negative. The smears from the control tube at this time showed all the organisms of both strains to be still Gram-positive (see Fig. 2).

It seems clear from the experiments just described, in which the bacterial field was pretty completely covered so far as the Gram-positive aerobic forms are concerned, that two types of organisms may be distinguished, one of which exhibits reversibility of Gram reaction when exposed to acriviolet and the other of which remains Gram-positive in spite of long exposure to the dye. The organisms which exhibit the reversal phenomenon in clear-cut fashion belong in the spore-bearing group; the organisms which resist this effect of the

dye belong in the non-spore-bearing group.[10] This fact is sufficiently striking and may be of significance. There are, however, numerous exceptions in both groups, so that one cannot definitely relate the reversal phenomenon to the presence or absence of sporogenic material.

Other Dyes.

It has been said that the reversal phenomenon is brought about by exposure to acriviolet and this dye did appear to be more effective than any other in producing the result. But exposure to other dyes, notably acriflavine, gentian violet (both Grübler, and Coleman and Bell), and ethyl violet also resulted in a reversal of Gram reaction and a diminution of calibre.

Other Substances than Dyes.

Since the dyes are known to have a bacteriostatic effect upon certain of the organisms which exhibit reversal phenomenon experiments were done with other substances, not dyes, also capable of producing bacteriostasis. In these experiments both *Bacillus anthracis* and *Staphylococcus aureus* were exposed to the action of alcohol, formalin, and Zenker's fluid. These substances produced no effect on the Gram reaction of either organism.

Relation of Reversibility to Acidity.

The dyes used, though basic or neutral in name, have a pH between 3 and 4[11] and this fact raises the question also suggested by the work of Deussen[12] (who noted the effect of acids on the Gram reaction—but not on the size—of certain Gram-positive organisms), whether the phenomenon here reported might not be due to the slight acidity

[10] Deussen (Deussen, E., *Z. Hyg. u. Infectionskrankh.*, 1918, lxxxv, 235) noted a similar difference in the effect of certain substances on the Gram reaction of different Gram-positive organisms.

[11] The potentiometer readings are as follows:

1 per cent aqueous gentian violet..... pH 3.5
" " " " acriflavine..... pH 3.5

[12] Deussen, E., *Z. Hyg. u. Infectionskrankh.*, 1918, lxxxv, 235. This author regarded the phenomenon as a purely chemical one and thought the effect of acids to be in direct proportion to their degree of dissociation.

(it is very slight) of the bacterial suspension resulting from the addition of the small amount of 1 per cent acriviolet or gentian violet used.[13] Since the result produced by these dyes was not produced by acid fuchsin (pH 2.55) it seemed unlikely that acidity of dye was the whole explanation of the phenomenon. That the addition of strong acids, at least, did not produce reversal of Gram reaction nor diminution of calibre was easily shown by the following experiment.

Three tubes each containing ½ cc. bacterial suspension (*B. anthracis* N. Y. B. of H.) were prepared. To the first was added 1 oeseful of 1 per cent acriviolet, to the second 1 oeseful of 10 per cent HCl, to the third 1 oeseful of concentrated HCl. Complete reversal took place in the acriviolet tube within 19 hours. In the acid tubes no change was observed at the end of 19 hours, and only slight change at the end of 48 hours.

A second experiment to test this point was done by adding to a series of tubes containing the bacterial suspensions 1 oeseful of water which had been brought to pH 1.2, 1.8, 2.4, 3, 3.8, and 7. In none of these tubes did reversal occur, although complete reversal took place in the control tube containing dye. This experiment is open to the objection that the acid used was not buffered and therefore its action may not have been continuous. In the dyes, on the other hand, acid may be present in just the right proportion and it may be so buffered as to act continuously. Efforts to test this point out by buffering with KCl and with KH phthalate, the acid added to the bacterial suspensions were defeated because it was found that the buffers themselves led to some reversal. The point must, therefore, be left for the present in abeyance.

Modifying Factors.

The question of the relation of the reversal phenomenon to the age of the culture was investigated. This was done by making bacterial suspensions of agar growths, at intervals of 1 hour, from 1 hour up to 7. Though the results of these experiments were not absolutely constant, there is reason for believing that very young cultures are more resistant to the reversing effect of acriviolet than older cultures.

The question of the nature of fluid in which the suspension is made and its relation to the reversal phenomenon was studied by making

[13] The pH of the distilled water used in this laboratory is 6.0. Potentiometer readings of ½ cc. of this water containing 1 oese 1 per cent acriviolet showed a pH of 5.3+.

suspensions in distilled water, in tap water, and in physiological saline, and adding dye to these tubes. Reversal proceeded more rapidly in the water tubes than in saline.

Reduction in Calibre of Bacteria by Exposure to Dyes.

Reference thus far has been made only to the change in Gram reaction produced by exposure to acriviolet. But another equally striking, if not more striking, change has also been observed; for it is noted in the smears of *B. anthracis* which have been exposed to acriviolet that the Gram-negative forms are much smaller in transverse diameter than the Gram-positive forms. This difference in size is so striking, the organisms stained with safranine being quite slender and those stained with methyl violet quite stout, that one might easily conclude that the smears contained a mixture of two different organisms, one a robust Gram-positive and the other a slender Gram-negative (see Fig. 1, *B*). In smears made when the change is about half complete these pink and bluish black segments may alternate in the same chain giving a most striking appearance (see Fig. 1, *B*, and Fig. 5, *A–D*). It will be noted that when spores are present they are entirely contained within the Gram-negative forms. This is apparent in many of the illustrations; but particular attention is called to the organisms marked *a* Fig. 1, *B*, and *a* Fig. 3, *A*.

Large numbers of measurements of the Gram-positive and Gram-negative forms were made, a Zeiss filar micrometer being used for the purpose. These measurements showed the Gram-positive forms to be practically always larger in transverse diameter than the Gram-negative forms. The difference in size is of course not absolutely constant in degree, varying from .123μ to .962μ; but in only one instance has a Gram-negative segment of a bacterial chain been seen which was larger than the adjacent Gram-positive segment, and in only the most occasional instances were they of the same calibre. Comparative measurements of 25 Gram-positive and 25 Gram-negative adjacent segments from a smear of *B. anthracis* No. 10 stained by Burke showed: average diameter of the Gram-positive forms 1.2236μ, of the Gram-negative forms, .7091μ, a difference of .5145μ or 42+ per cent.

In the complete study 292 organisms were measured. The average diameter of the Gram-positive forms was 1.112μ; of the Gram-negative forms .686μ. This gave an average difference of .426μ or 38.3 per cent.

When measurements were made of specimens which had been stained by the

Paltauf modification, the same general facts came to light although the difference between the Gram-negative and Gram-positive forms was not quite so marked as in specimens stained by Burke's method (see Fig. 3, *A* and *B*, and Fig. 5, *D*). In this set of observations, measurements were made of 50 organisms. The average transverse diameter of the Gram-negative forms was .311432μ smaller than that of the Gram-positive forms, a reduction of 29.74 per cent in diameter.

Exposure to acriviolet therefore not only causes *Bacillus anthracis* to change its Gram reaction from sharply Gram-positive to sharply Gram-negative; it also leads to a reduction, by as much as 40 per cent or even more, in its transverse diameter. Whether a similar change in longitudinal measurement also occurs it is impossible to say with certainty since the variation in length of the individuals in a given smear of *B. anthracis* is usually so great as to make comparative measurements impossible. But Gram-positive material may be shown to exist between the Gram-negative segments of a chain of *B. anthracis* and when this is removed from the ends of the organisms by excessive decolorization of lightly stained specimens diminution in length must occur (see Fig. 4, *A*, *a*, *b*, and *c*, and *C*, *a* and *b*).

Significance of Reversal.

What is the significance of the change in Gram reaction and the diminution in size produced by the exposure of *B. anthracis* to acriviolet? It is clearly not a specific reaction since it is produced also by substances other than dyes and indeed occurs in a few individuals even in the control tubes. Since acriviolet is known to have a marked bacteriostatic effect on *B. anthracis*, the reversal produced by this dye might be thought to be purely a death phenomenon. This, however, seems unlikely to be the case since other organisms (like *M. freudenreichii*) which are killed by acriviolet do not exhibit reversal on exposure to this dye, and since—if the vegetative forms of *B. anthracis* be killed in other ways (as by prolonged boiling or exposure to bichloride of mercury)—reversal is not produced. Since a certain amount of reversal usually occurs sooner or later in the control tubes to which no dye has been added, it is not unlikely that the dye simply promotes or hastens a change in the bacteria, caused by degeneration or lysis (dependent perhaps on change of reaction), which would occur without any dye. Bacteria are no longer thought of as entities

with permanent characteristics, but biological units which change constantly. It is now well known that in their cycle of growth they both lose and acquire characteristics; the Gram reaction is one of the characteristics which may change during growth or be changed by environment. No one has ever demonstrated an anatomical basis which might explain this change of Gram reaction; in the case of *B. anthracis* the finer structure of the organism as revealed by these studies appears to offer such an anatomical basis.

But what is the cause of the change in size? Three explanations suggest themselves. It might be thought that the change in size was apparent rather than real and that it resulted from the heaping up of methyl violet on the surface of the Gram-positive segments, a heaping up which did not occur in the Gram-negative segments whose diameter appeared therefore to be less. It is possible of course that the apparent size of the stained bacteria is greater than the actual size of the living organisms but I am inclined to think that the difference in size produced by staining is not great. In any case the suggestion that the observed difference in size between Gram-negative and Gram-positive segments is not real but entirely dependent on accumulations of stain in some segments which do not occur in others is excluded by the fact that the difference in size is to be observed (under the limitation noted below) also in hanging drop specimens—which have not been stained at all—as well as in specimens stained by safranine alone, by fuchsin alone, by Burke's methyl violet alone, and by saturated alcoholic gentian violet alone (Fig. 3, *C* and *D*).

The second explanation which suggests itself is that the difference in size between adjacent Gram-positive and Gram-negative segments is due to shrinkage. It is conceivable that when dye is added to an aqueous suspension of *B. anthracis* osmotic forces, acting through the bacterial membrane, extract material (possibly water) from certain segments, whose size is in this way diminished. Since the weighing experiments were done with desiccated specimens, dehydration could not be the cause of the loss of weight in the Gram-negative segments.

If dehydration be brought about by exposing the bacteria to 95 per cent, followed by absolute alcohol no such picture is obtained as follows exposure to the

dyes. A slight diminution in diameter does occur, as the following measurements show, but the Gram reaction is not reversed.

Diameter control organisms (15 measured)	1.06 μ
" after 48 hours in 95 per cent alcohol	1.00 μ
" " 24 " additional absolute alcohol	.85 μ
Total loss of diameter	.21 μ
	20 per cent

If a bacterial suspension be evaporated to dryness and complete desiccation thus produced smears for examination of the stony material which results can only be obtained after grinding it in a mortar. This vigorous treatment destroys bacterial morphology and no satisfactory observations can be made on the debris.

A third explanation of the phenomenon is that the difference in size observed in the hanging drop as well as in stained specimens is a real difference and that it is to be explained by the fact that *B. anthracis* is composed of two parts, an outer layer which is Gram-positive, and an inner core which is Gram-negative. According to this explanation, when the proper dye is added to a suspension of *B. anthracis* the outer Gram-positive coat is destroyed and only the inner Gram-negative core remains. Ectoplasm and endoplasm might be the most correct terms to apply to these portions of the bacterial structure if they had not already been used by Zettnow[14] in a somewhat different sense. Cortex and medulla are convenient descriptive terms although usually applied to an organ rather than an organism. Adopting these terms tentatively we may say—if the suggested hypothesis as to difference in size is correct—that the Gram-positive cortex of *B. anthracis* is rather easily destroyed by lysis and by degeneration; that it undoubtedly often disappears during bacterial growth (which accounts for the occasional presence of Gram-negative forms of *B. anthracis* in smears from cultures and their frequency in old cultures); that the cortex is susceptible to the action of distilled water, so that it will disappear in this medium after prolonged exposure; that it is notably sensitive to the action of certain dyes; and that when the cortex is thus destroyed the slender Gram-negative medulla comes into view.

[14] Zettnow, *Z. Hyg. u. Infectionskrankh.*, 1899, xxx, 1; 1918, lxxxv, 17.

A good deal of evidence has been gathered for the correctness of this hypothesis, but it cannot perhaps be said that the case is absolutely established. Final proof would be afforded if cross-sections of bacteria could be obtained and if these, when stained by Burke's method, exhibited a Gram-negative core and a Gram-positive periphery. This proof, though we have endeavored to obtain it, cannot now be presented at least in convincing form. Numerous efforts have been made by varying technic to get satisfactory bacterial sections but, largely because of technical difficulties, without success. In the preparations examined a few objects were seen which strongly suggested cross-sections of bacteria, pink in the centre and blue at the periphery. But in examining these preparations, with the high-power and strong illumination required, color distortions occur—particularly in looking at minute objects—and these may easily lead one astray. Further attempts of this kind are now in course.

Perhaps as strong evidence as could be desired for the hypothesis that *B. anthracis* consists of a Gram-positive cortex and a Gram-negative medulla (short of that which bacterial sections would furnish), is provided by the picture obtained when smears from a young culture of this organism are stained by a modified Burke technic in which duration of exposure to dye and mordant is greatly shortened, and duration of exposure to decolorizer greatly increased. In specimens of this kind one finds individual bacteria in all stages of decolorization. A few of them may have retained their Gram positivity. But many are entirely Gram-negative, while still others show the remains of Gram-positive material clinging to the surface in the form of granules or lumps or plaques among which the pink Gram-negative medulla can be seen shining through. The appearance is exactly that of a banana, the skin of which had been in places removed, partially exposing the fruit within (see Fig. 4, *A*). Such a picture fits in well with the hypothesis under discussion.* Whether in specimens which have thus been partially decolorized bacterial substance is actually dissolved away from the surface of the organism by the

* A. T. Henrici (*J. Med. Research*, 1914, xxx, 409) has described similar partial decolorizations of yeast cells but appears not to have drawn any conclusions as to the significance of these observations beyond their bearing on the Gram reaction.

decolorizer, or whether merely the mordanted methyl violet is dissolved away leaving the now unstained and invisible bacterial surface material behind, has not yet been definitely proven. The former explanation seems perhaps more likely to be the correct one. If it is, then specimens which have been partially decolorized ought—if restained by the ordinary Burke technic—to look just as they did before the restaining, since the surface material, capable of retaining the methyl violet, has been in large part removed by the partial decolorization. Such a result is illustrated in Fig. 4, *C*. The only difference between this picture, which represents a specimen which has been partially decolorized and then restained by Burke and the picture shown in Fig. 4, *A*,—which represents a specimen that has been partially decolorized but not restained—is that the Gram-negative material in Fig. 4, *C*, has a slight purplish tint. If such a result had been always obtained when this experiment was done the matter would be clear enough. But sometimes the partially decolorized organisms, when restained, retained the Gram stain throughout.

Two critical experiments could be done to determine whether the change of size, in the bacteria which have been exposed to dye, actually rested on the anatomical structure of *B. anthracis* as we have suggested. One ought to be able to show an actual loss of weight in the bacteria which have been subjected to the dye, as compared with the controls; the demonstration of such a loss of weight, provided the specimens be desiccated before weighing, would prove that apparent diminution in size was real and would eliminate shrinkage as an explanation of the phenomena. One ought also to be able to demonstrate the presence of protein or hydrolytic products of protein in the acriviolet to which the bacteria had been exposed. These two tests were made and since both gave definite results agreeing with the hypothesis they provided additional testimony that the explanation suggested was correct.

Weighing Experiments.

The weight of the desiccated centrifugate from a heavy aqueous suspension of *B. anthracis* was determined and compared with the weight of a similar centrifugate from a suspension to which gentian violet had been added and in which reversal of Gram and diminution

in calibre had been produced. Definite loss of weight was shown to have occurred in the gentian violet suspension. These experiments gave the following results.

	Control	*Gentian violet tube*
Weight of tube plus dried centrifugate...	11.350 gm.	11.8250 gm.
" " empty tube.....	11.324 "	11.8114 "
" " bacteria.....	.027 "	.0136 "
	.0136 "	
Loss of weight in gentian violet tube.....	.0134 " = 49.6 per cent	

If it be assumed that all the gentian violet dissolved in the tube went to the bottom with the bacteria during centrifugation the figures become (since the amount of gentian violet used weighed .001 gm.):

Weight of bacteria in control tube.....	.027 gm.
" " " " gentian violet tube.....	.0126 "
Loss of weight in gentian violet tube.....	.0144 " = 53.3 per cent

This experiment was repeated by identical technic except that on this occasion it was necessary to allow the tube, to which gentian violet had been added, to stand only 3 hours in order to produce a complete reversal of Gram reaction. The results of this experiment were as follows:

	Control	*Gentian violet tube*
Weight of tube plus dried centrifugate...	11.4535 gm.	11.0547 gm.
" " empty tube.....	11.3336 "	11.0274 "
" " bacteria.....	.1199 "	.0273 "
	.0273 "	
Loss of weight in gentian violet tube....	.0926 " = 77.2 per cent	

The experiment was again repeated in another way. Instead of centrifugating the specimens they were passed through previously weighed Berkefeld N 5 filters, which were then dried and weighed. The results were as follows:

	Control	*Gentian violet tube*
Weight of filter plus dried bacteria.....	64.1370 gm.	74.9680 gm.
" " " alone.....	61.5384 "	73.3028 "
" " bacteria.....	2.5986 "	1.6652 "
	1.6652 "	
Loss of weight in gentian violet suspension	.9334 " = 35.9 + per cent	

Protein Tests of the Filtrate.

If the suggestion is correct that the difference in diameter between the Gram-positive and Gram-negative forms of *B. anthracis*, is due to the solution of an outer Gram-positive coat, the presence of this protein—or protein-like—material ought to be demonstrable in the filtrate of a suspension of *B. anthracis* to which dye has been added. Such a demonstration was made. For the purpose the ninhydrin test was used. This test is not specific for proteins[15–17] so that it can only be said—not that the presence of protein was demonstrated—but that ninhydrin-positive bodies were present in the filtrates from suspensions of *B. anthracis* which had been exposed to dyes. These experiments were done as follows:

Heavy aqueous suspensions of *B. anthracis* were centrifugated, washed three times in saline, and filtered through unused Berkefeld filters into glassware which had been cleansed with aqua regia. Filtrates were tested by ninhydrin, biuret, and Heller tests. The control filtrates were negative. The filtrates from suspensions to which dye had been added gave a positive ninhydrin test, and, when concentrated by evaporation, a positive biuret.

It seems clear therefore that material, probably but not certainly protein in nature, is dissolved away by gentian violet, since its presence can be determined in the filtrate from suspensions of *B. anthracis* to which the dye has been added, the controls (made with saline in order to avoid bacteriolysis) being negative by the biuret and ninhydrin tests. Experiments are now under way to determine the nature of the material present in the gentian violet filtrate and to see whether it has any toxic, immunizing, or other important properties.

Many observers have noticed the occurrence, in cultures of *B. anthracis* (and indeed of other organisms), of bizarre forms. Some of these bear a remote similarity to those produced by exposure to dyes. But they have usually been dismissed as "involution forms" resulting from plasmolysis or plasmoptysis (*e.g.* Gotschlich),[18] or as

[15] Ruhemann, S., *J. Chem. Soc.*, 1910, xcvii, 2030; 1911, xcix, 798.

[16] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500–506; 1914, lxvii, 56.

[17] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxv, 350.

[18] Gotschlich, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, i, 45.

"teratological forms,"[19] or as "degeneration forms." In his beautiful studies of the morphology of *B. anthracis* Preisz[20] pictures, without apparently paying much attention to them, bizarre forms of this kind. He worked with old cultures, the organisms were not stained by Gram, but vitally stained with fuchsin, and nothing is said of their Gram reaction. In Fig. 7, Table 3, of his 1909 publication stout and slender segments are represented. Nothing is said about them. The specimen was not stained by Gram. It was made from a 2 days old culture which had been exposed to a mixture of dog and guinea pig serum (5:1), and Preisz appears to have paid little attention to the question of size. He made no measurements. Preisz' chief interest lay in the capsule which he regarded as a degeneration product of the cell membrane. Zettnow,[14] who was interested chiefly in bacterial chromatin, worked solely with the Romanowski stain. He described the bacillus of anthrax as composed of two parts: an ectoplasm, which remains colorless by the Romanowski and all ordinary staining methods, and an entoplasm which stains blue by Romanowski, and which contains chromatin (stained red by Romanowski). "A large number of bacteria consist entirely of chromatin. Even in those which stain both blue and red, chromatin strongly predominates. In exceptional cases in very young cultures the plasma occurs in larger amounts than the chromatin but as growth proceeds the relation is reversed." The ectoplasm, compared to the entoplasm, is said to be relatively poor in water content and is more concentrated, as is clear from its greater resistance to stains, to plasmolysis, and to destructive influences. In contradistinction to *B. anthracis*, cocci and sarcinæ take only one color, the blue (entoplasm). Zettnow made no studies by the Gram method. He made no measurements. In our own work smears of organisms which had been exposed to acriviolet until partial reversal had occurred were stained by numerous polychrome methods, including Romanowski. No selective staining, such as that described by Zettnow, was observed. From all these data it seems clear that there is no relation between the picture obtained by Zettnow and that described in this communication. Zettnow's ectoplasm is said to remain colorless

[19] Maassen, A., *Arb. k. Gsndhtsamte*, 1904, xxi, 385.

[20] Preisz, H., *Centr. Bakt., 1. Abt., Orig.*, 1903–04, xxxv, 657; 1909, xlix, 341.

in all ordinary staining methods. Since both cortex and medulla stain readily both would appear to lie within the entoplasm.

Gram Stability and Gram Instability.

The fact that the material responsible for the Gram positivity of *B. anthracis* appears to be confined to the surface of this organism and that the centre is Gram-negative, made it seem likely that the Gram positivity of *B. anthracis* would be relatively unstable as compared with that, for example, of *M. freudenreichii*, an organism in which no distinction between Gram-positive surface and Gram-negative interior had been demonstrable. It had been found, indeed, by preliminary observations that,—although young cultures of *B. anthracis* were completely and definitely Gram-positive (when stained by the Burke method), provided the technic was accurately followed—an increasing proportion of individuals in a given specimen became Gram-negative if the time of exposure to dye and mordant was diminished and the time of exposure to decolorizer increased. A systematic study was therefore made to see what the effect on different Gram-positive organisms would be, of variations in time of exposure to stain, to mordant, and to decolorizer. It was found that for many organisms (*M. freudenreichii* may be cited as a typical example) variations in time of exposures made, within wide limits, practically no difference in the results, *M. freudenreichii* being always 99 per cent to 100 per cent positive. In the case of other Gram-positive organisms (*B. anthracis* for example), wide variations in the result could be obtained by varying the technic as regards time of exposure.[21]

The facts are well represented in Fig. 4, *B*, in which are illustrated the results of an experiment done with a suspension containing a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii*. The smear has been stained by a modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes). *B. anthracis* has been largely decolorized: *M. freudenreichii* unaffected.

When investigated in two ways therefore—by the results of exposure to acriviolet (see Fig. 2) or by modifications of staining tech-

[21] Churchman, J. W., *Stain Technol.*, 1927, ii, 21.

nic in the direction of diminished time of exposure to dye and mordant and prolonged time of exposure to decolorizer (see Fig. 4, *B*)—two Gram-positive organisms are seen to behave quite differently, as regards their Gram reaction. Light is thus thrown on the mechanism of the Gram reaction and the fact emerges that this mechanism is not necessarily the same in the case of bacteria of different species even though their behavior to the standard technic is identical. This distinction between stable and unstable Gram-positives appears to be a useful one. It is suggested that in recording the Gram reaction of new species, some such set of comparative tests as here described be carried out so that the stability of the reaction be known.

Examination of Hanging Drop Specimens.

Attention has been called to the facts that the difference in size between Gram-positive and Gram-negative segments of *B. anthracis* is demonstrable in unstained hanging drop specimens, made from suspensions which have been exposed to acriviolet or gentian violet and in smears stained with a single dye like methyl violet. From these facts—represented in Fig. 3, *C* and *D*,—one can only conclude that the reduction in calibre is real and not the result of an artefact.

The experiment of May 25, from which Fig. 3, *D*, was made, may be cited. A suspension of a 3 hour culture of *B. anthracis* No. 10 was made and gentian violet added. At the end of 2 hours smears stained by Burke, showed that the organism had changed from 100 per cent Gram-positive to 50 per cent Gram-positive and beautiful instances of great variation in size between adjacent bacterial segments were numerous. A hanging drop specimen was made. The organisms had picked up enough of the gentian violet to which they had been exposed to be clearly visible and the marked differences in size represented in the figure were readily made out. It was noticeable that the stout segments were always more deeply stained than the slender.

Observations of this sort left no doubt as to the reality of the difference in size between adjacent bacterial segments. But throughout the experiments with hanging drop specimens I was struck by a curious fact: If specimens of *B. anthracis* which had been

treated with acriviolet were stained by Burke or by Paltauf and the stout and slender forms measured, a difference of diameter—often as high as 60 per cent—was readily made out. If, however, specimens from the same tube were stained with a single dye or examined in the hanging drop this difference in size was sometimes by no means so readily observed. This observation led us at first to the conclusion that the difference in diameter which we had seen in the smears stained by Burke was apparent and not real. The demonstration, however, of an actual loss of weight, of a clear difference of size in many of the hanging drop specimens, of the presence of protein (or related substance) in the filtrate from bacterial suspensions containing acriviolet, and of the presence of cortex and medulla, by methods of partial decolorization, all pointed to a real and not an apparent loss of calibre.

The fact that the difference in size between Gram-positive and Gram-negative segments is not as a rule so obvious, in the fresh specimen and in those stained with a single stain, as it is in the specimen stained by Burke, is to be explained partly by the fact that the Burke stain gives a contrast of color which at once attracts the eye and at the same time emphasizes the difference in size. This contrast is absent in the single stains and change in size unless very marked is easily overlooked even when it is present. Another reason for the apparent discrepancy is that the Gram-negative segments—that is to say, the smaller segments—often stain (when only single stains are used) very lightly, in some cases not at all; and these ghosts easily escape detection. In the fresh specimen the slender segments are translucent, often nearly invisible, and it is probable that many of them are overlooked. It may also be the case that the Burke stain somewhat exaggerates the degree of the difference in size, which is none the less real.

It results from all this that one must have for examination in the fresh or with single stains, specimens in which the difference in size is quite marked indeed. In specimens stained by Burke on the other hand the difference in size is very striking even when it is not great in degree.

SUMMARY.

1. The addition of small amounts of aqueous gentian violet, acriflavine, or acriviolet to suspensions of young cultures of *B. anthracis* reverses their Gram reaction, and diminishes their diameter about 40 per cent.

2. The time required for these changes varies with the strain of *B. anthracis* examined.

3. These changes are accompanied by a loss of weight.

4. Ninhydrin-positive substances are demonstrable in the filtrate from suspensions of *B. anthracis* to which dyes have been added.

5. Similar changes are produced by these dyes in many, but not in all, of the sporogenic aerobes.

6. Non-spore bearers are for the most part unaffected in these ways by the dyes, although to this statement there are a number of exceptions.

7. The change in size produced by the dyes is demonstrable in hanging drop specimens as well as in stained smears, but not with equal constancy.

8. Partial decolorizations of *B. anthracis* are described, which are produced by modifications of the Burke technic in which time of exposure to dye is shortened and time of exposure to decolorizer lengthened.

9. The explanation for these phenomena which accords with all the known facts is that they depend on the existence in *B. anthracis* of a Gram-positive cortex and a Gram-negative medulla. Positive proof of the correctness of this explanation must await the evidence furnished by cross-sections of bacteria.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Camera lucida drawing. Magnification × 4200. This plate shows two successive stages in the change produced in the Gram reaction and in the calibre of *B. anthracis* (A. T. C. No. 10) by exposure to acriviolet. Smears stained by Burke's method.

A, smear made for control at the beginning of the experiment. The organism is completely Gram-positive.

B, smear made from a specimen which has been exposed to acriviolet for 45 minutes. Partial reversal of the Gram reaction has occurred, about 25 per cent

of the individuals being Gram-positive and 75 per cent Gram-negative. Notice the marked difference in calibre between the Gram-negative forms and the Gram-positive, and the fact that the spore is entirely contained in the Gram-negative portion of the organism (*a*). One gets the impression of a smear made from a mixture of two different organisms.

C, smear made from a specimen which has been exposed to acriviolet for 2 hours, showing complete reversal of Gram reaction. Practically every individual is now Gram-negative.

PLATE 35.

FIG. 2. Camera lucida drawing of a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii* (A. T. C.). Magnification × 4200. Stained by Burke's method.

A, smear from the control tube. Both organisms are sharply and completely Gram-positive. One stray Gram-negative individual of *B. anthracis* is seen.

B, smear from specimen which has been exposed to acriviolet. The Gram reaction of *B. anthracis* has been in large part reversed, almost all of the individual organisms being now Gram-negative. All the individual organisms of *M. freudenreichii* are, on the other hand, as strongly Gram-positive as at the start of the experiment. The difference in calibre between Gram-positive and Gram-negative forms is very evident.

PLATE 36.

FIG. 3. Camera lucida drawings. Magnification × 4200.

This plate represents the slight variations in difference of size, between Gram-positive and Gram-negative forms, produced by varying the conditions of the experiment.

A, smear made from a specimen of *B. anthracis* (N. Y. B. of H.) which has been exposed to acriviolet until partial reversal of Gram reaction has occurred, and *stained by Paltauf's modification*. The difference in calibre, between Gram-negative and Gram-positive forms, though definite, is less marked than in *B*. Notice (*a*) the spores within the medulla.

B, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and *stained by Burke's modification*. Compare the difference in calibre with that shown in *A*.

C, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and *stained only with Burke's methyl violet*. The presence of stout and slender forms is evident. The picture obtained in the specimens stained by the Burke technic cannot therefore be an artefact produced by that technic.

D, drawing of individual organisms seen in a hanging drop specimen made from a suspension of *B. anthracis* (A. T. C. No. 10) which has been exposed to gentian violet. The living organisms have taken up enough of the stain to make them clearly visible and the difference in calibre between Gram-positive segments

(which take the stain fairly well) and the Gram-negative segments (which remain pale) is perfectly clear. The difference in size caused by exposure to dyes is therefore real and not an artefact produced by the Burke technic.

PLATE 37.

FIG. 4. Camera lucida drawings. Magnification × 4200.

A, partial decolorization of *B. anthracis* (A. T. C. No. 10) by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 3 minutes).

The Gram-positive material has been partially removed from the surface, persisting only as plaques or lumps or stippling through which the pink Gram-negative rod in the interior of the organism can be seen. Notice (*a*, *b*, *c*) the caps of Gram-positive material at the ends of the bacterial segments.

B, smear of a mixture of *B. anthracis* (A. T. C. No. 10) and *M. freudenreichii* (A. T. C.) which has been stained by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes).

Almost every individual organism of *B. anthracis* has been completely decolorized; but *M. freudenreichii* has remained completely Gram-positive.

C, smear of *B. anthracis* (A. T. C. No. 10) stained by the same modification of Burke's technic as that used in the experiment represented in *A*, and then restained by the ordinary Burke technic. The portions of the organisms which were decolorized by the first process now fail to take the stain in the second and appear as in *A*, except that the pink color has become purplish pink. Notice (*a*, *b*) terminal caps of Gram-positive material.

PLATE 38.

FIG. 5. Photographs of single chains from specimens of *B. anthracis*, which have been exposed to acriviolet, to show the difference in calibre between Gram-negative and Gram-positive segments as seen at various magnifications. On account of the difference in calibre accurate focussing at the higher magnifications is impossible.

A, magnification × 1400. Stained by Burke's modification. Alternating Gram-positive and Gram-negative segments. *B. anthracis* (A. T. C. No. 10).

B, magnification × 1800. A Gram-negative segment with a Gram-positive segment at either end is plainly seen. *B. anthracis* (A. T. C. No. 10) stained by Burke's modification.

C, magnification × 2500. The same chain represented in *B*.

D, magnification × 3200. *B. anthracis* (N. Y. B. of H.) stained by Paltauf's modification. The difference in calibre between Gram-positive and Gram-negative segments, though clear, is less marked than in *A*, *B*, and *C*.

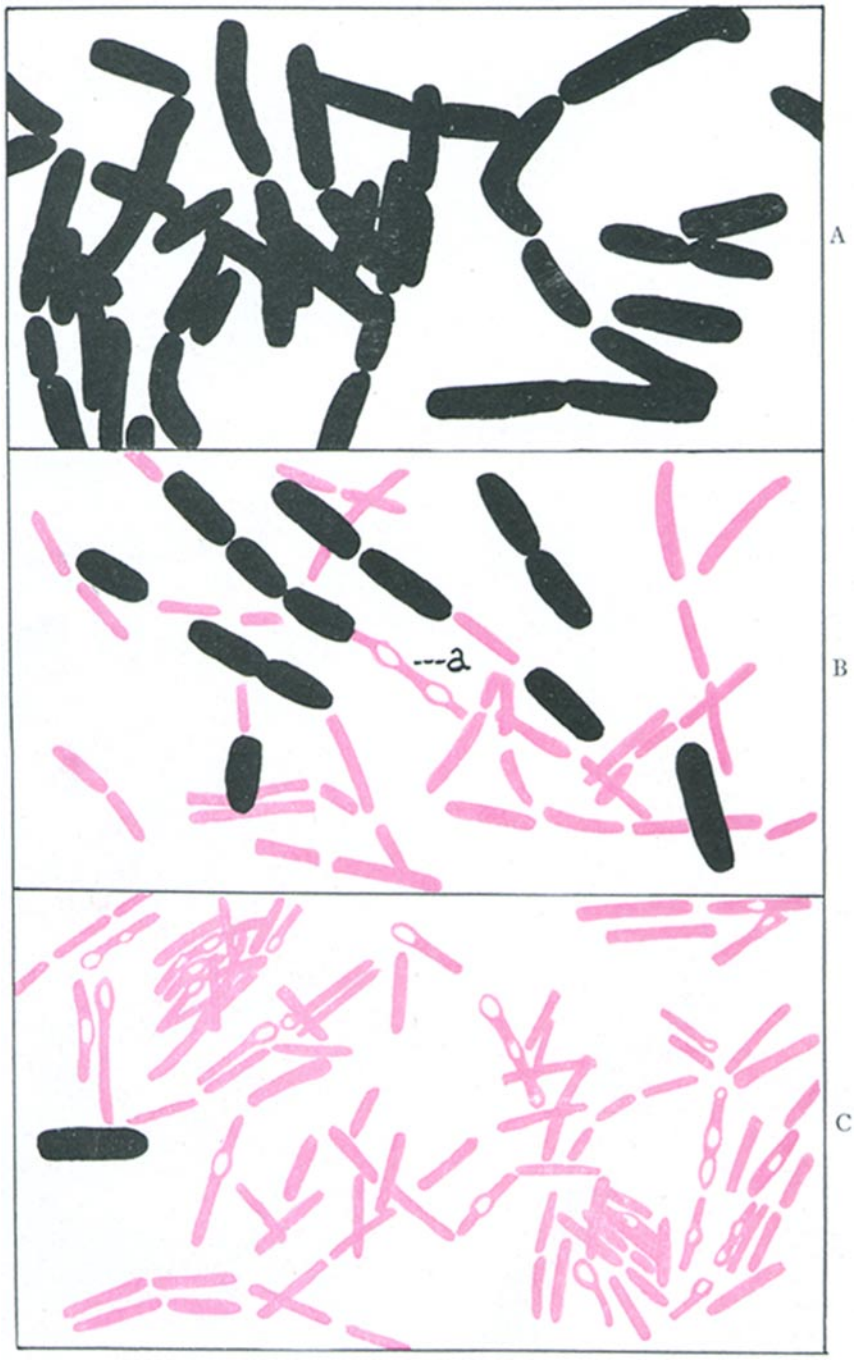

FIG. 1.

(Churchman: *B. anthracis* and Gram reaction.)

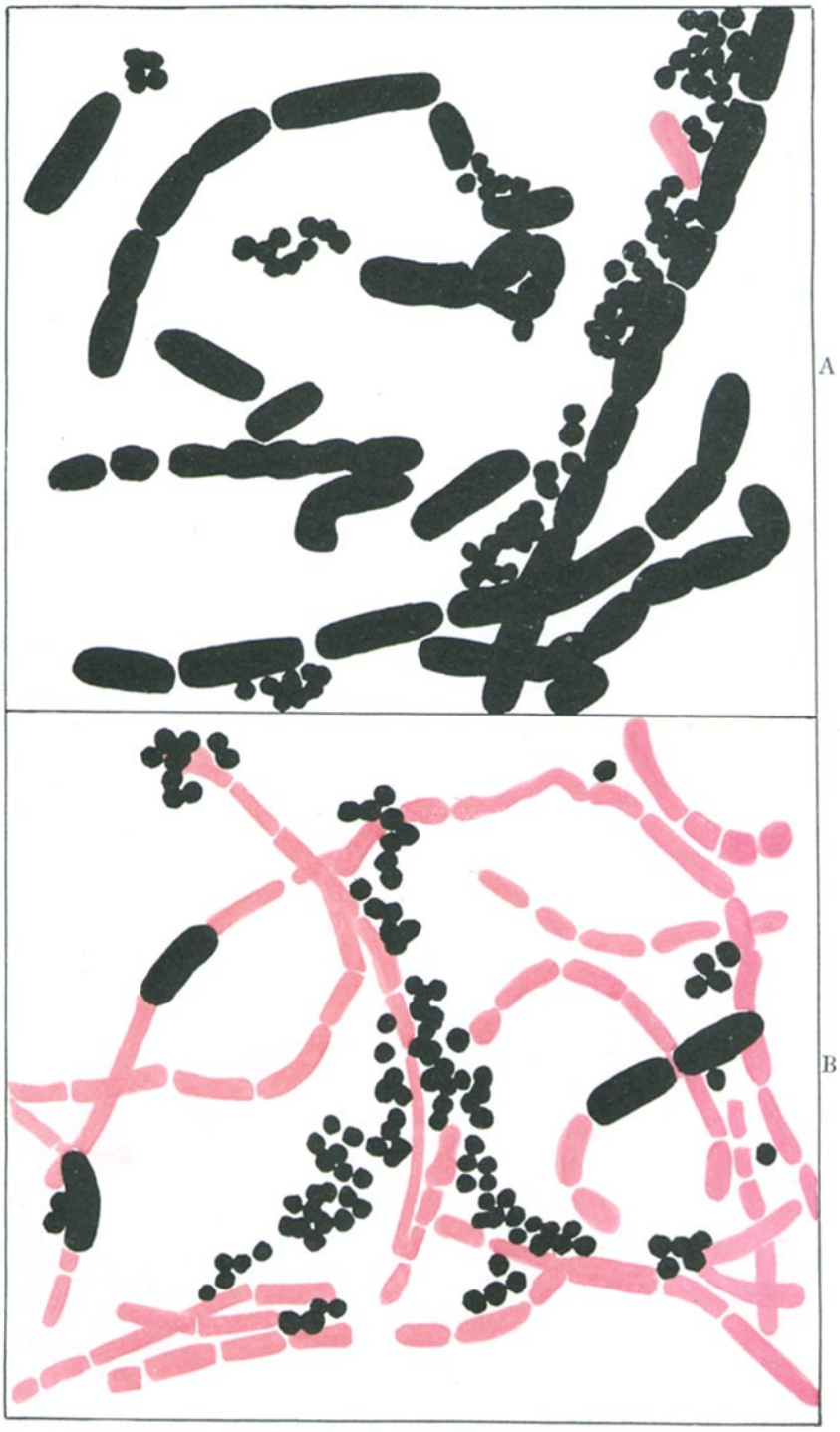

FIG. 2.

(Churchman: *B. anthracis* and Gram reaction.)

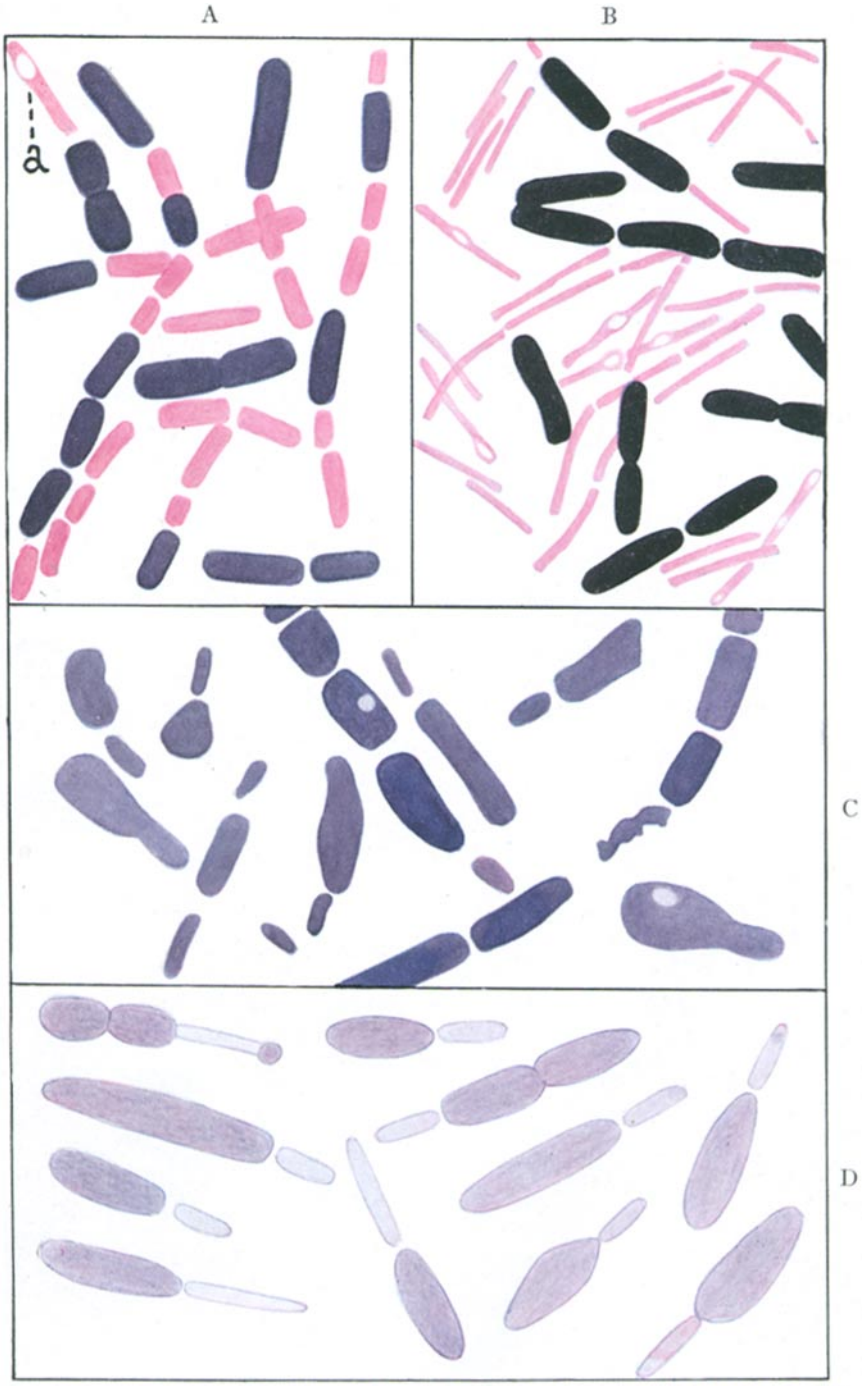

FIG. 3.

(Churchman: *B. anthracis* and Gram reaction.)

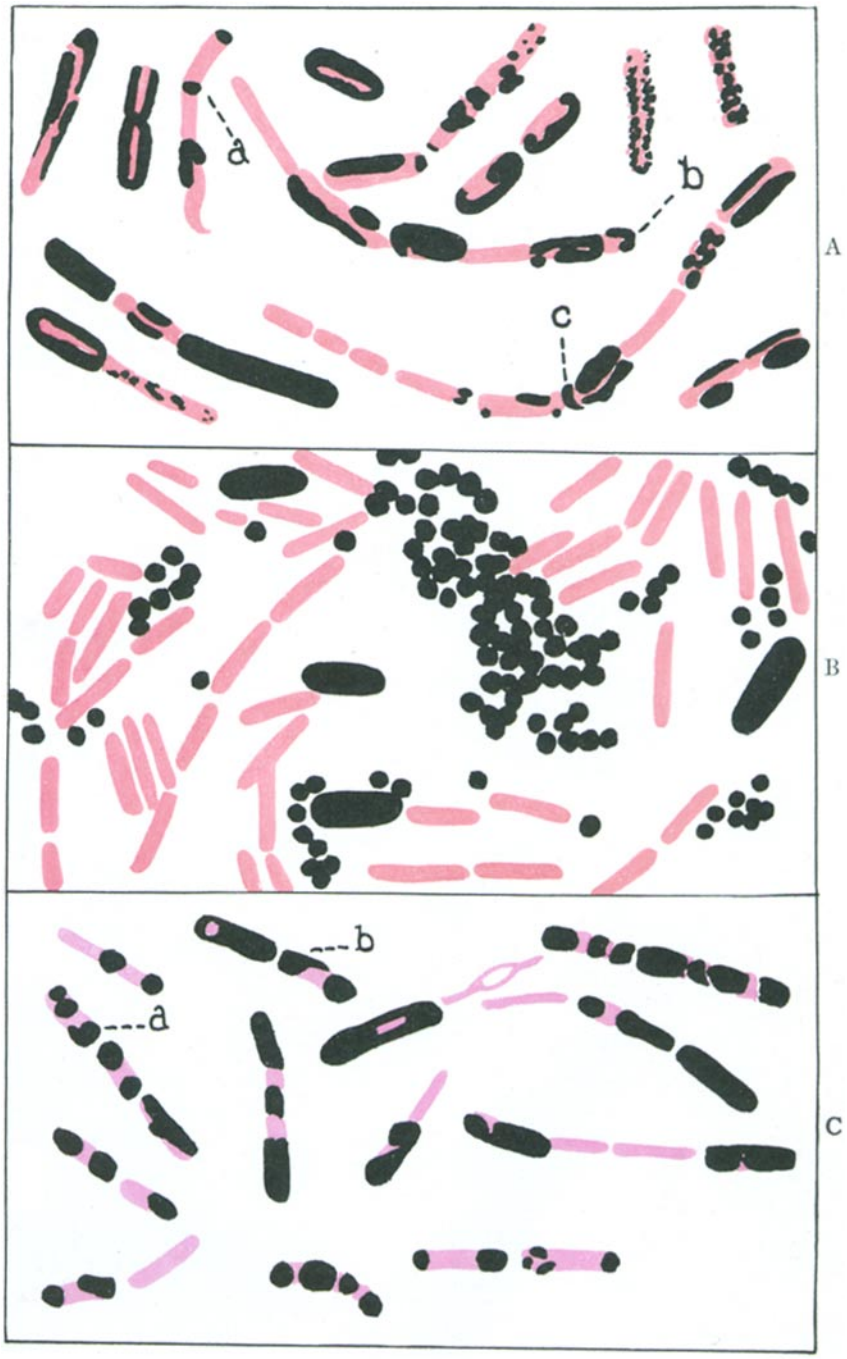

FIG. 4.

(Churchman: *B. anthracis* and Gram reaction.)

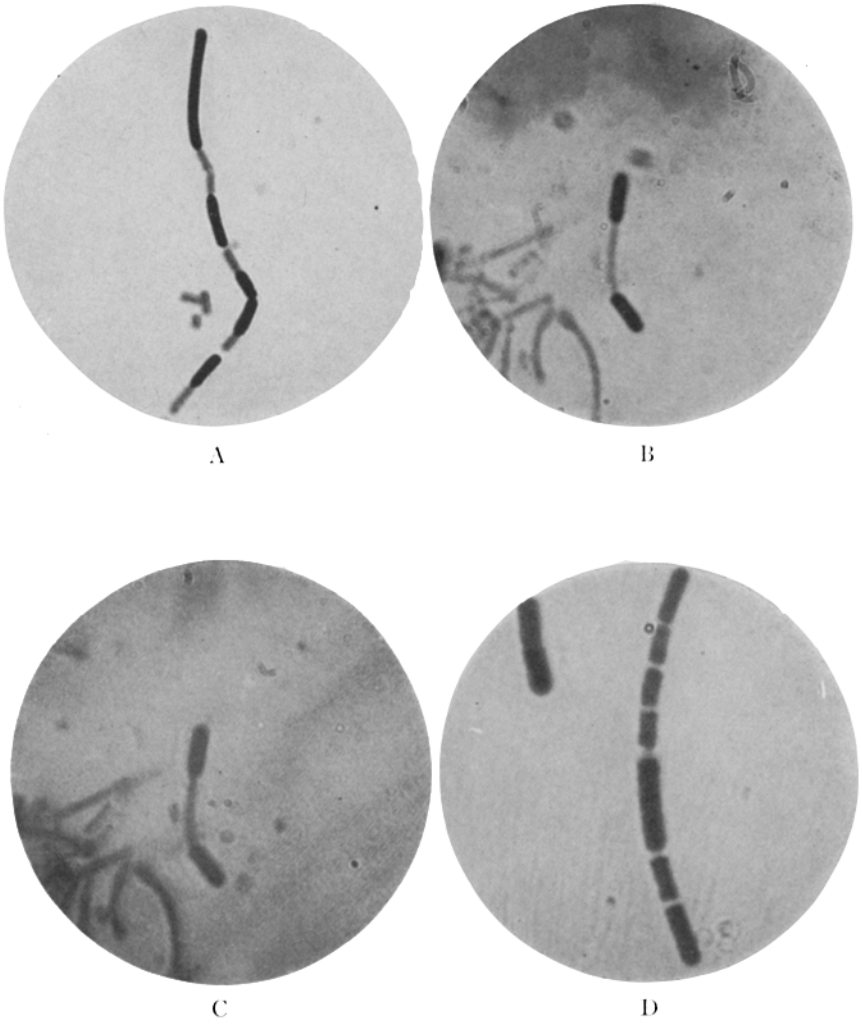

FIG. 5.