RIBONUCLEIC ACID AND THE GRAM STAIN

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The importance of the gram stain for bacteriology is such that great effort has been spent in attempting to determine its cause and in standardizing and improving the staining procedure. It is the purpose of this paper to demonstrate that one of the components of the "gram reaction" is ribonucleic acid, and to point out the implications of this fact for any theory of mechanism. This study is based upon the brief note of Henry and Stacy (1943) and confirms and extends their work.

Henry and Stacy observed that by treating heat-killed gram-positive cells of *Saccharomyces cerevisiae*, *Clostridium welchii*, and *Streptococcus salivarius*, with a 2 per cent bile solution (at 60 C) they were able to "strip off" an outer surface layer and thus convert these normally gram-positive organisms into gram-negative forms. The material thus extracted contained polysaccharides, protein, and the magnesium salt of ribonucleic acid. This extracted material could be "replated" back upon the gram-negative "skeleton," thus rendering it gram-positive, provided the "skeleton" was maintained in a suitable state of reduction by the use of 0.1 per cent formaldehyde. This extracted material could not be "plated" upon normally gram-negative forms. The relationship of the extracted material to the gram-positive character of the cells was specific, for although extracts of one organism could be "plated" upon another, the magnesium salt of the ribonucleic acid could not be replaced by the sodium salt, nor could desoxyribonucleic acids, nucleosides, or nucleotides replace the magnesium ribonucleate in this function. It was evident that the total reaction resulting in gram positiveness was not a simple one since neither the "cytoskeleton" nor the extracted material were gram-positive except in combination, and recombination of these materials was not possible unless the "cytoskeleton" was maintained in a reduced state.

EXPERIMENTAL

Experiments with bile salts. Henry and Stacy have not yet published the details of their technique; however, these do not appear to be indispensable and the following methods proved satisfactory. Cell suspensions were prepared by removing the organisms from slants or by centrifuging broth cultures. Washing was not necessary. Two techniques were followed:

1. A smear of the organism was prepared and heat-fixed in the usual manner.

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The slide was then suspended in a 2 per cent sodium choleate or bile salts solution held at 60 C in a water bath. At intervals slides were removed, placed in 1 per cent formaldehyde (1 part of 30 per cent formalin in 100 ml of water) for 10 minutes. The slides were dried and stained with the Kopeloff modification of the gram stain (1922); the modification of Hucker and Conn (1923) was also employed with identical results.

2. The cell suspension was heat-killed and treated with an equal volume of 4 per cent bile salts and sufficient formalin to obtain a concentration of 0.1 per cent. The mixture was placed at 60 C and at intervals portions were streaked out on slides, heat-fixed as usual, and stained.

Replating was done by suspending the slides in either the extract or pure magnesium ribonucleate (its concentration seemed to be of little importance). These were removed and stained at intervals. Another method is described in the following paragraph:

The results obtained may be illustrated with *Staphlococcus aureus* using the second procedure. The growth from a 0.5 per cent peptone agar slant (20 hours, 37 C) was removed and suspended in 2 ml of sterile, distilled water in a graduated centrifuge tube. This was placed in boiling water for 10 minutes and the volume adjusted to 2 ml. Two ml of 4 per cent sodium choleate (adjusted to pH 7) was added and 0.1 ml of 4 per cent formaldehyde added (4 ml of 30 per cent formalin diluted to 100 ml and adjusted to pH 7). A smear made from the mixture and stained is shown in figure 1. Virtually all cells are gram-positive. The mixture was placed in a water bath at 60 C, and smears made and stained at intervals After 30 minutes (figure 2) most of the cells appear gram-negative with an occasional one appearing gram-positive. After 60 minutes' exposure (figure 3) no gram-positive cells could be found. Although Henry and Stacy report that the extracts (purified by precipitation with alcohol) can be "replated" on cells rendered negative by bile salts and that the important agent is magnesium ribonucleate, we decided to go a step farther. After 60 minutes, therefore, the cells were centrifuged from the bile salts mixture, resuspended in 1 ml of 0.1 per cent formaldehyde, and 1 ml of 1 per cent magnesium ribonucleate was added. Smears were made at intervals. These showed a progressive increase in the number of gram-positive cells in the suspension until at 30 minutes virtually all the cells had become gram-positive (figure 4). The magnesium ribonucleate was obtained as follows:

Yeast nucleic acid (Eastman) was dissolved in dilute NaOH and precipitated with sulphuric acid. The precipitate was dissolved in 1 per cent magnesium chloride and again precipitated with dilute HCl (mono-magnesium salt). The precipitated magnesium ribonucleate was dissolved in water, adjusted to pH 7, and used in the experiments.

It has thus been possible to remove the gram-positive staining character of *Staphlococcus aureus* by exposing the killed cells to bile salts. One can then remove the cells from the bile salts and cause them to become gram-positive by exposure to magnesium ribonucleate obtained from yeast.

Studies with Ribonuclease. Recently Kunitz (1939, 1940) has obtained the

enzyme "ribonuclease" in a pure crystalline form; hence it should be possible, by the use of this enzyme, to remove specifically the ribonucleic acid from the cell surface, and thus, if this is indeed the important compound responsible for the

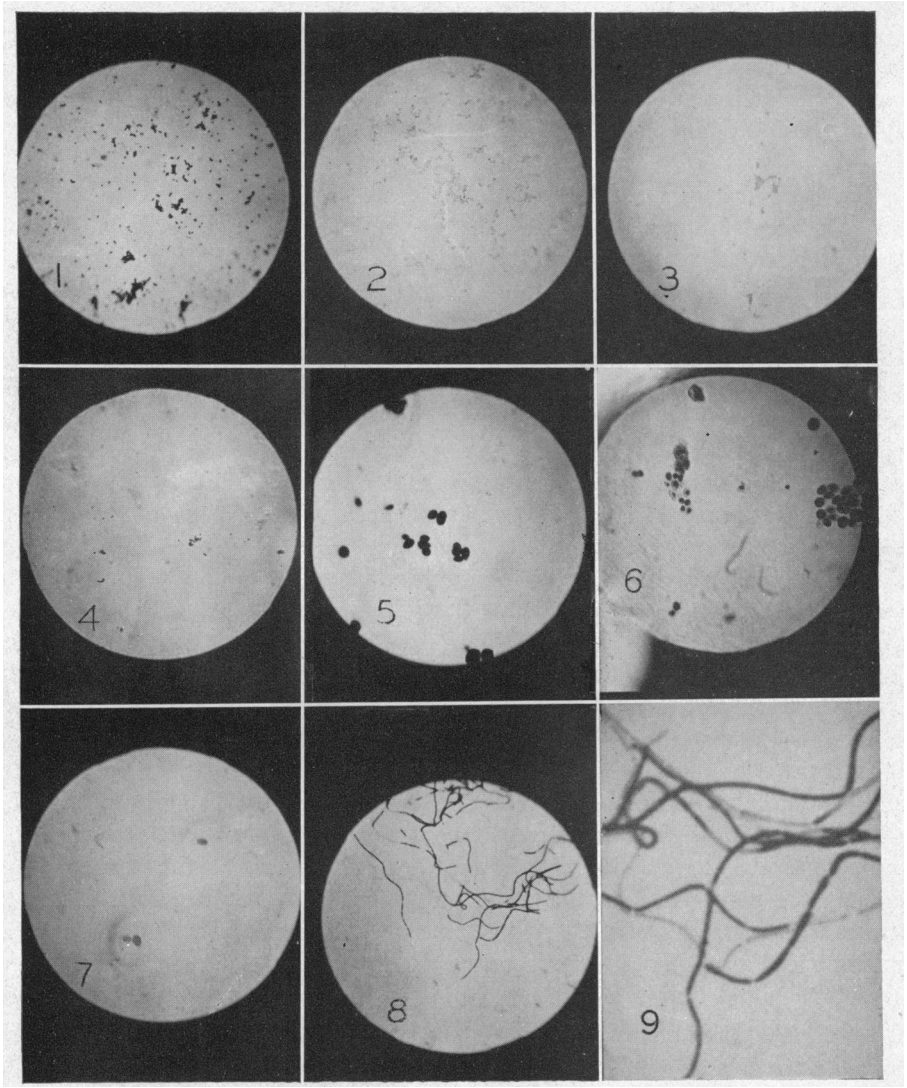

FIG. 1. STAPHLOCOCCUS AUREUS, GRAM STAIN. (SEE TEXT)
FIG. 2. AS IN FIGURE 1, TREATED WITH SODIUM CHOLEATE FOR 30 MINUTES AT 60 C, GRAM STAIN
FIG. 3. AS IN FIGURE 2, AT 60 MINUTES
FIG. 4. AS IN FIGURE 3, "REPLATED" WITH MAGNESIUM RIBONUCLEATE PREPARED FROM YEAST NUCLEIC ACID
FIG. 5. SACCHAROMYCES CEREVISIAE, ORIGINAL, GRAM STAIN
FIG. 6. AS IN FIGURE 5, EXPOSED TO CRYSTALLINE RIBONUCLEASE FOR 30 MINUTES
FIG. 7. AS IN FIGURE 5, EXPOSED TO CRYSTALLINE RIBONUCLEASE FOR 60 MINUTES
FIG. 8. BACILLUS CEREUS, TREATED 60 MINUTES WITH RIBONUCLEASE, GRAM STAIN
FIG. 9. ENLARGEMENT OF CENTRAL PORTION OF FIGURE 8. NOTE SIZE DIFFERENCES BETWEEN GRAM-POSITIVE (DARK) AND GRAM-NEGATIVE (LIGHT) FORMS

gram-positive character of cells, gram-positive cells should be converted into gram-negative forms.

Two preparations of pure crystalline ribonuclease were employed in our studies; one prepared in the laboratory of the McArdle Institute and the other obtained by Dr. V. R. Potter from Dr. M. Kunitz. The latter sample was used in the test reported below, but both samples gave similar results. The nature and mode of action of this enzyme had been discussed elsewhere by Kunitz (1939, 1940), Fankuchen (1941), Allen and Eiler (1941), and Eiler and Allen (1941).

The pure crystalline ribonuclease was dissolved in sterile, distilled water to give a concentration of 100 micrograms per milliliter. The organisms used were from 20-hour cultures, grown in the appropriate liquid medium at suitable temperatures, as shown in the figures. Each culture was distributed in 0.5-ml quantities into sets of 4 sterile, serological test tubes, which were then plugged with sterile corks. Two tubes of each set were then autoclaved for 20 minutes at 121 C to obtain heat-killed cells. To one of the tubes containing heat-killed cells and to one of the tubes containing living cells, 0.5 ml of the enzyme solution was added at zero time; to the remaining two tubes of each set, 0.5 ml of sterile water was added to provide similar cells under similar conditions except for the absence of the enzyme. All tubes were placed in a water bath at 45 C, and smears were made from them at intervals. Each slide had a smear of each of the 4 conditions to which a given organism was subjected, and the gram-staining procedure was applied to the whole slide; thus the cells from the four conditions were all subjected to an identical gram-staining procedure. All of the slides were subjected to a carefully standardized procedure in which the time for decolorization was purposely shortened. The procedure was as follows: The smear was air-dried and heat-fixed; crystal violet (Hucker modification containing ammonium oxalate) was applied for 1 minute and washed off with distilled water; iodine (Gram's modification of Lugol's solution) was applied for 1 minute and washed off; the smear was then decolorized for 20 seconds in 95 per cent ethyl alcohol, washed, blotted dry, and counterstained with 0.25 per cent safranin for 30 seconds. The percentage (to the nearest 5 per cent) of gram-negative cells in the smear was then calculated by observing several fields and tabulating the gram-positive and gram-negative cells present. The results are presented in table 1. It will be noted that the pure crystalline ribonuclease removed the gram-positive character from the heat-killed cells of all of the organisms tested, but that all of the other conditions had no effect. Figures 5, 6, and 7 show 3 stages in the conversion of cells of *Saccharomyces cerevisiae* from gram-positive to their gram-negative form. Figure 5 shows the gram-positive character of the yeast cells at zero hour, figure 6 shows the variable appearance of these cells after exposure to ribonuclease for 30 minutes, figure 7 shows the completely gram-negative appearance of the cells after exposure to the enzyme for 1 hour. In view of the specific character of the action of the ribonuclease, there is little doubt that ribonucleic acid is *the* important factor in the gram-positive character of these organisms.

The enzymatic approach to the problem of the gram reaction has been some-

what neglected, but in view of the fact that only recently have pure enzymes been available, there is a surprising number of articles on the subject. Hottinger (1916) observed that certain "ferments" removed the gram-positive character of some organisms. Avery and Cullen (1923) observed that filtrates of autolysed pneumococcus cells of types I and II contained an enzyme which could remove the gram-positive character of heat-killed pneumococcus cells, regardless of type, and which had the same action on *Streptococcus viridans*. However, they reported that this preparation was inactive against *Staphylococcus aureus* and *hemolytic streptococci*. Dubos (1937a, 1937b) and Dubos and MacLeod (1937) obtained nucleases from various animal tissues which were capable of rendering gram-positive pneumococci gram-negative. Further, Thompson and Dubos

TABLE 1

Conversion of gram-positive organisms to the gram-negative form by the action of crystalline ribonuclease

Data recorded are percentages of gram-negative forms present

ORGANISM (20 HOURS)	GROWTH CONDITIONS		HEATED CELLS EXPOSED TO RIBONUCLEASE AT 45 C				NONHEATED CELLS EXPOSED TO RIBONUCLEASE AT 45 C				HEATED CELLS WITHOUT RIBONUCLEASE (IN WATER AT 45 C)				NONHEATED CELL WITHOUT RIBONUCLEASE (IN WATER AT 45 C)			
			(Time in minutes)															
	Medium	Temp.	0	15	30	60	0	15	30	60	0	15	30	60	0	15	30	60
		C																
S. liquefaciens	A	37	0	95	100	100	0	0	0	0	0	0	0	0	0	0	0	0
S. thermophilus	A	37	0	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	B	37	0	10	10	40	0	0	0	0	0	0	0	5	0	0	0	0
B. subtilis	B	37	10	90	100	100	10	20	20	20	15	25	40	40	10	20	20	20
B. cereus	B	30	0	0	10	30	0	0	0	0	0	0	0	0	0	0	0	0
L. bulgaricus	A	45	0	100	100	100	0	0	0	0	0	0	0	5	0	0	0	0
S. cerevisiae	A	30	0	0	10	90	0	0	0	0	0	0	0	0	0	0	0	0
Clostridum	C	30	0			100	0			0	0			0	0			0

A = Carrot liver broth.
B = Beef-extract peptone broth.
C = Saline suspension of cells from nutrient agar, grown under anaerobic conditions.

(1938), working with an enzyme from autolysed pneumococcus cells, attempted to determine the chemical changes which occurred when the pneumococcus cells were changed to the gram-negative form. They were interested in this problem chiefly as an attempt to explain the action of this enzyme in removing the antigenic character of the pneumococcus capsule. They also knew, however, that the change from a gram-positive state to a gram-negative state indicated the loss of this antigenic property. They found that the material liberated upon the action of this enzyme could be separated into two fractions, one containing nucleoprotein, the other a ribonucleic acid. In addition Kunitz (1939) reported that a sample of his crystalline ribonuclease was used by Dubos and was found to effect the gram reaction of the pneumococcus cell in a manner similar to that of

the impure enzyme preparations of Dubos. There is thus ample evidence for the pneumococci that a ribonucleic acid is associated with their gram-positive character.

THE THEORY OF THE MECHANISM OF THE GRAM STAIN

There are three types of theories concerning the mechanism of the gram stain as follows:

1. Those based on a morphological interpretation which propose that there exists a gram-positive "ectoplasmic" layer about gram-positive organisms which is absent from gram-negative ones, and that the protoplasm of all bacteria (beneath the outer layer in gram-positive forms) is gram-negative (Gutstein, 1925; Churchman, 1927, 1929).

2. Those based on a membrane permeability interpretation in which the cell membrane of a gram-positive organism is thought to be permeable to the dye and to the iodine but not to the dye-iodine complex formed inside the cell (Benians, 1912, 1920; Burke and Barnes, 1929).

3. Those based on colloidal chemical interpretation in which there is a supposed relationship between the isoelectric point of the cell protoplasm and its affinity for the basic dye used. Gram-positive organisms have an isoelectric point at a lower pH value than the gram-negative organisms, and hence the basic dye has greater affinity for gram-positive organisms and the latter are not as easily decolorized by the action of alcohol (Stearn and Stearn, 1924a, 1924b).

It seems obvious that the present studies, as do those of Henry and Stacy, favor the first type of theory. It is, therefore, desirable to examine the evidence that the gram-positive character of gram-positive bacteria resides in the "ectoplasmic" surface layer and to re-examine the objections which have been raised against this conception.

Evidence that the material responsible for the gram reaction resides in the outer layer of the cell. The data of Churchman (1927, 1929), of Henry and Stacy (1943) and of this paper as previously presented all favor the conception of an "ectoplasmic layer." Churchman, for example, observed a 25 per cent decrease in cell size upon the conversion of *Bacillus anthracis* from the gram-positive to the gram-negative state by the action of acriviolet. He also observed decreases in cell size and in their weight upon converting certain gram-positive organisms to the gram-negative form by water at 52 C. Henry and Stacy observed a "stippled" effect (similar to that shown in figure 6), which they attribute to partial removal of ribonucleic acid at the cell surface. The fact that one can convert gram-negative forms derived from gram-positive bacteria by bile salt treatment or ribonuclease back into gram-positive forms by suspending in magnesium ribonucleate is also presumptive evidence that the material occurs at the surface.

Further evidence may now be cited. It is a common experience, especially when staining lactobacilli, to observe a result such as is shown in figure 8 and figure 9, the latter being an enlargement of the central portion of figure 8 (*Bacillus cereus*). Here it is evident that the gram-positive (darker) cells of the chain appear larger than the gram-negative cells. One may note that the cells lie so

close together that they must have been subject to the same staining conditions, yet some are positive and some negative; thus the effect is not due to faulty staining. But before accepting this as evidence of an "ectoplasmic" layer of gram-positive material one must consider the possibility that the gram reagents themselves are the cause of the increased size of the positive cells; that one is observing only the precipitated "dye-iodine" complex and not any material pre-existing at the cell surface.

We realized that we had available a method, denied previous investigators, of solving this problem. If such an ectoplasmic layer existed it should be possible specifically to remove the ribonucleic acid with ribonuclease (leaving proteins and carbohydrates, which are extracted by the Henry and Stacy method) thus preventing any "precipitation" of the "dye-iodine" complex. We could then outline the cell with nigrosin and determine whether the cell (which appears smaller on counterstaining since only the "skeleton" stains) was actually smaller when so treated than one which still contained the ribonucleate and permitted such dye "precipitation." A culture of *Bacillus cereus* was therefore prepared and treated with the enzyme, ribonuclease, as in table 1 for 60 minutes. At this time both gram-negative and gram-positive staining forms were present. Smears were made and stained in the usual manner until the point of decolorization with alcohol. At this point they were lightly coated with nigrosin and air-dried. It is of interest that gram-positive forms retained the violet against nigrosin while gram-negative forms were decolorized, which indicates a chemical combination in the gram-positive form rather than an adsorption. Since the cell length would normally be subject to considerable variation, the cell width was measured with a calibrated filar micrometer. The measurement of the width of 10 cells of each form yielded the following results: gram-positive forms, $2.00 \pm 0.08\ \mu$; gram-negative forms, $1.90 \pm 0.12\ \mu$. The cells which do not have a "dye-iodine-complex precipitate" (those appearing gram-negative) have thus the same width as those assumed to possess such a "precipitate." The apparent increased size of gram-positive cells (as shown, for example, in figures 8 and 9) is, therefore, not due to a precipitation of a "dye complex." It should be noted that without the nigrosin treatment the cell width is apparently decreased in the gram-negative form from 30 to 50 per cent.

Re-examination of evidence unfavorable to surface layer theory. We recognize that a tremendous wealth of information is available on the gram stain and that the "ectoplasmic" theory of Churchman has been all but discarded in recent years (cf., for example, Knaysi, 1944). It is therefore desirable to examine the evidence which caused investigators to discount this theory.

Examination of the literature reveals much controversy over points the explanation of which would be obvious if it were granted that the "ectoplasmic" layer was ribonucleic acid. Three, however, are not so obviously explained. These are:

1. The observation that the mere breaking of the cell wall caused the loss of the gram-positive character of the protoplasm (Burke and Barnes, 1929; Churchman, 1929; Knaysi, 1944).

2. The observation that *Bacillus cereus* lost its gram-positive character without decreasing in size (Stearn and Stearn, 1929).

3. The alleged conversion of gram-negative organisms into gram-positive forms by the use of extremely alkaline conditions (Stearn and Stearn, 1924a, 1924b).

For the first of these, there is no adequate explanation. As a matter of fact, the observation that the breaking of the cell wall results in a loss of the gram-positive character has been difficult to interpret on any basis other than permeability. The electron microscope photographs of Mudd *et al.* (1941), especially figure 1 of that paper, show that the rigid structures interpretable as cell wall fragments are slightly narrower and somewhat less opaque than the cells from which they were derived. This would lead one to believe (but, of course, does not constitute proof) that upon breaking the cell wall one may also break the combination of ribonucleic acid with the cell surface. It will be recalled that the studies of Henry and Stacy showed that the ribonucleate material was bound to the cell surface, and that on removal the gram-negative cell body had to be kept in a reduced condition if a recombination of the two was to be accomplished. Destruction of the cell wall with consequent diffusion of the protoplasm outside the cell could well serve to break the linkage of the ribonucleate to the central core and to prevent the recombination. The nature of the linkage of the ribonucleate to the central core will be discussed later.

The explanation of the second of these objections is included in the report of Henry and Stacy, who found that the outer layer of gram-positive organisms was not solely ribonucleic acid but that it also contained polysaccharides and protein. We have shown that there is no difference in size between a gram-positive cell and one which has been converted into its gram-negative form by the action of ribonuclease. In view of these observations it would seem possible that, in the course of the removal of ribonucleic acid from a cell by a specific (in the case of Stearn and Stearn, an internal) ribonuclease, a stage might be reached in which the outer portion of the cell would lose its gram-positive character but still contain enough protein and other material to be visible in counterstaining; as the length of exposure to the conditions is increased the ability to take the counter stain is eventually lost. Indeed the data reported by Ingram (1939) on *Bacillus cereus* demonstrate this effect nicely.

The third objection, that of the alleged conversion of gram-negative organisms into gram-positive forms by the staining at a very alkaline pH, required more careful consideration. It is indeed correct that if a gram stain is applied to a smear of *Escherichia coli* at a pH of 13, violet organisms are obtained. But it is characteristic of the gram stain that gram-positive organisms retain the violet color only when treated with iodine. *Escherichia coli* will retain the violet color with or without the application of iodine, hence the observations of Stearn and Stearn do not bear upon the gram stain as such. They show that at a pH of 13 crystal violet unites with the protein of a gram-negative organism and that this combination cannot be destroyed by alcohol. There is, therefore, no question but that pH influences staining reactions. That this relationship is specifically

the cause of the gram stain is, however, an unjustifiable extension of the isoelectric point concept. Similar conclusions have been reached by Burke and Barnes (1929), Burke and Gibson (1932), and Winslow and Upton (1926).

With respect to gram-negative cells, two viewpoints are possible. First, it may be considered that gram-negative cells do not possess ribonucleate in the outer layer. Second, such a layer may be postulated, but the combination of the ribonucleate and the surface protein may be of a different nature than it is in gram-positive organisms. Indeed, both the surface protein or the ribonucleate may differ. To our minds there is considerable evidence that the first viewpoint is correct, but we know of no critical evidence that definitely eliminates the second possibility. It also appears that volutin (ribonucleate) within the cell may, under some circumstances, give gram-positive reactions. We feel that this type of reaction accounts for the positive granules sometimes seen in otherwise negative cells. We do not regard it as necessary for the ribonucleate to be at the cell surface before a gram-positive reaction is obtained but only that it be in a particular kind of combination with a particular kind of protein, the nature of which it is our purpose to investigate in the following section. This viewpoint, we feel, effectively answers the "inner wall" argument of the Stearns (1930).

The Nature of the Gram Reaction. Conceived as a chemical reaction, the gram stain is influenced by a number of factors. These are:

1. A cell of a particular structure involving a surface protein containing arginine (Henry and Stacy, 1943) bound in some manner to magnesium ribonucleate. When separated, both the ribonucleate and the surface protein are gram-negative and cannot be recombined unless the latter is kept in a "reduced" state.

2. The gram reagents themselves: crystal violet, iodine, the extracting agent, and the counterstain. It is generally agreed (Conn, 1940) that the extracting agent and the counterstain are not specific and are replaceable with a wide variety of materials.

3. The reaction between the first two factors does not occur (or the products of the reaction are destroyed) at an acid reaction.

In an effort to learn something of the nature of the chemical reaction involved in the gram stain we have briefly studied the specificity of the crystal violet and the iodine in the reaction.

The question which was first studied was, Can one replace crystal violet with other dyes of similar structure? Of course, the mixture of pararosanilines called "gentian violet" contains crystal violet, hence data on its action are not pertinent. Using both gram-positive and gram-negative organisms, we were unable to find any dye which would replace crystal violet in the gram stain. Two facts eliminated all of the dyes tested:

1. Failure to decolorize on the application of alcohol, even though no iodine had been applied (methylene blue, safranine, carbol fuchsin, Congo red).

2. Failure to be retained by gram-positive organisms after application of iodine (eosin, erythrosine, malachite green; and methyl green; the latter differs from crystal violet by only one methyl group).

We have therefore concluded that but a slight modification in the structure of

the crystal violet will render it useless for the gram stain. This, of course, does not deny that dyes exist which may be suitable substitutes for crystal violet (cf. Scales, 1922; Conn, 1940; Knaysi, 1944), but it does point out that some part of the structure of crystal violet is specific for the gram stain.

With respect to substitutes for iodine, there is much conflicting literature. Stearn and Stearn (1924a, 1924b) claim that iodine is replaceable by oxidizing agents whereas Burke and Barnes (1929) claim that iodine is replaceable only by agents which form a precipitate with crystal violet. With *Bacillus graveolens* as a gram-positive and *Escherichia coli* as a gram-negative organism, smears stained with crystal violet were exposed to a variety of materials for five minutes, decolorized with alcohol, and counterstained with safranine. The following materials had no effect: KI (0.1 M), KBr (0.1 M), KCl (0.1 M), $KMnO_4$ (0.02 M), $K_2Cr_2O_7$ (0.02 M), H_2O_2 (1.0 %), $K_3Fe(CN)_6$ (0.02 M), Ce $(SO_4)_2$ (0.1 M). Of the materials which permitted some color retention by the bacillus, mercuric chloride (0.1 %) was by far the best, although stannous chloride (1 %), pyrogallol (1%), and freshly prepared hydroquinone (1%) had some effect. It is readily admitted, however, that none of these materials would be a suitable substitute for iodine if the quality of the results obtained was considered.

As the gram-positive reaction is observed only when the "reduced" cell protein and the magnesium ribonucleate are combined, and as crystal violet is irreplaceable and iodine practically so, it appears that the gram-positive character must reside in the interaction of all of these components. It is obvious that contemporary knowledge of reactions involving a protein combined with a ribonucleate treated with crystal violet and iodine is insufficient to allow the formulation of a straightforward chemical mechanism. Certain very suggestive implications are, however, available.

It is known that the bond between ribonucleic acids and their associated proteins is of a different nature (Mirsky, 1943) from that of the desoxyribose nucleoproteins which appear to be salts. The exact nature of the combination of the former is not clear, but it somehow involves basic groups, especially arginine. There is thus presumptive evidence that a bond of this sort is present in the gram-positive bacteria. There is also some indication that sulfhydryl groups are involved in the actual combination and that these react during the staining reaction. The indications are as follows:

1. When ribonucleic acid is removed by bile salts, it will no longer unite with the cell protein if the latter has been oxidized with air. Sulfhydryl groups are autoxidizable with air when exposed on the protein surface.

2. The recombination is possible, however, even on exposure to air, if the "skeletons" are treated with formaldehyde. The latter unites in low concentration with sulfhydryl groups and prevents their autoxidation.

3. Iodine is essential to the gram reaction, and it is the only known material which reacts with sulfhydryl groups of proteins even when these are located deep within the protein molecule. Furthermore, while the relations (if any) between hydroquinone, stannous chloride, or pyrogallol and sulfhydryl groups are not known, mercuric chloride, which can partially replace iodine, does react with sulfhydryl (Fildes, 1940).

In considering the data as a whole one can scarcely fail to conclude that the gram-positive character of a cell resides in an outer layer of material surrounding a gram-negative core. The important material in this outer layer is magnesium ribonucleate. The ribonucleate in combination with the cell protein is responsible for the gram-positive characteristics, and crystal violet and iodine react chemically with this combination.

SUMMARY

The data contained in this paper are capable of supporting the following conclusions:

Ribonucleic acid exists in the outer layer of gram-positive organisms, and in combination with magnesium and the cell protein is responsible for the gram-positive staining reaction.

The apparent difference in size between gram-positive and gram-negative forms of the same organism is due, not to the precipitation of the gram reagents, but to the visibility of the outer layer.

Indications point to the involvement of sulfhydryl groups in the over-all gram reaction.

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