THE CHEMICAL MECHANISM OF BACTERIAL BEHAVIOR

II. A NEW THEORY OF THE GRAM REACTION¹

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I

Throughout the large number of suggested modifications in technique for Gram staining a few conditions have always, whether intentionally or not, been kept constant. The first and most important is the character of the primary stain. Many individual dyes have been suggested, for example pararosaniline and the basic rosaniline dyes in general, pyronine, rhodamine and safranine. All the dyes which give satisfaction—all in the above list—are basic in character. That these are lipoid soluble has been deemed important.

Another condition invariably adhered to is the use of a mild oxidizing agent as mordant. This was noted and discussed in a preceding paper.

Still another observation upon which all workers are agreed is that of the decolorizing power of acids for the primary stain. Most papers do not differentiate between the action of acid on basic and acid dyes. We have shown that acid dye stains are less easily decolorized in the presence of acids than in neutral solution, but, as was stated above, the primary stains used are all basic so that this observation is quite general. In the same way workers are agreed that the addition of mild alkali such as NaHCO_3 will render results much more clear cut.

This section may be summed up by stating that good differential results, so far as technique is concerned, can be obtained by

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employing a deep colored basic primary stain, "mordanted" by a mild oxidizing agent in slightly alkaline solution.

II

It is not the function of this section of the paper to propose a definite theory of the Gram reaction, but it seems to the authors that it is quite possible to eliminate one of the two current explanations. Inasmuch as there are more than two possible explanations, elimination of one does not mean sanction of the other without modification.

The two current explanations are the so-called "physical" and the "chemical." It is not clear who suggested the latter explanation, but it has been attributed to Unna who is thought to have made the assertion that "para-rosaniline dyes with iodine and bacterial protein form a relatively insoluble compound." In recent years, however, led perhaps by the colloid chemists, there has been a distinct tendency to shy away from such chemical explanations, and the physical explanation, based on certain postulates as to diffusion and permeability commonly current, seems to be at present the favorite.

According to this idea the dye squeezes through the pores in the cell membrane followed by the mordant molecule, and there, on the inside of the cell, these unite to form a large molecule which cannot squeeze out in the case of Gram-positive organisms. Other organisms either do not permit the dye to enter or else do not hinder the escape of the large dye-mordant molecule. Whatever the explanation of the Gram reaction may be, the above suggestion seems out of the question. It is perhaps best championed by T. H. C. Benians (1920) in a very interesting paper. He divides organisms into three groups: (1) Gram-positive which absorb the dye, (2) Gram-negative which absorb the dye, and (3) Gram-negative (*B. coli*) which do not absorb the dye. The present authors have shown (Stearn and Stearn, 1923) that *Bact. coli* will carry down basic dye when the acidity of the solution has been sufficiently lowered. A striking confirmation of results published in the article referred to above is shown in table 1. The fuchsin was obtained from a bottle labeled "acid fuchsin,"

and there was great surprise that it should behave in just the same way as the basic gentian violet, combining with the *Bact. coli* more in alkaline solution than in acid. This led to the discovery that the dye used contained no trace of sulphur, and thus could not be acid fuchsin, which is a sulphonic acid derivative of ordinary fuchsin. We were dealing with a basic dye which was acting in a perfectly normal manner.

Even a Gram-positive organism, such as *Staphylococcus pyogenes-aureus*, will fail to carry down a basic dye when the solution is sufficiently acid. The following experiment, following the technique by which Benians arrived at the classification given above, shows this.

Experiment. Into a tube containing 2 cc. 1:40,000 parts gentian violet and HCl to a pH of 1.2, twenty-four hours growth of the *Staphylococcus* was washed, shaken and thrown down in a centri-

TABLE 1

CULTURE	FINAL pH	APPEARANCE OF LIQUID
<i>Bact. coli</i>	9.0	colorless
<i>Bact. coli</i>	8.5	slight pink
<i>Bact. coli</i>	8.45	slight pink
<i>Bact. coli</i>	4.75	deep red

fuge. The liquid remained deeply colored and the bacterial growth was only lightly stained. Another tube with dye at a pH of about 3.0 was partially decolorized by another culture of the same organisms, which organisms in turn became blue in color. At a higher pH (5.5) the bacterial growth practically removed the color from the solution.

Coupled with Benian's results this experiment indicates that any such classification is arbitrary. Under certain conditions Gram-positive bacteria will take up basic dye, under other conditions they will not: under certain conditions Gram-negative bacteria will take up basic dye, while under other conditions they will not. These conditions were not specifically controlled in Benian's work.

The principal criticism of this physical explanation comes, however, from the following considerations:

A. The dye-iodine molecule cannot be of an order of magnitude much larger in size than the dye itself. If we consider the dimensions as molecular and not colloidal, then calculations of molecular volumes will show that it would require either the straight addition of over fourteen atoms of iodine, or the substitution of sixteen atoms for hydrogen atoms to merely double the size of the molecule, assuming the reaction to go in a manner similar to that in which eosin is produced from fluorescein by bromination. That is, the atomic volume of iodine in its compounds is less than 7 per cent of the molecular volume of gentian violet calculated from either Traube's (1899) or Kopp's (1842; 1855) values. Actually there are only twelve possible places where iodine could substitute in this way, and the likelihood of more than four atoms going in is very small. The maximum increase in molecular size, then, would be about 75 per cent, while 20 per cent would very probably more nearly represent it. If it be assumed that the free dye is not molecularly dispersed but in a colloidal state, then it is hard to explain the action of the iodine. Certainly it is not mere coagulation of the colloid, since the precipitate is a product different from the original dye. It may also be pointed out that if such large molecules are really essential, it would be difficult to reconcile many statements that the mordant, though important, is not absolutely necessary, and also that certain substances, e.g., H_2O_2 , which we have shown in the preceding paper to have the properties of mordants do not precipitate gentian violet.

We have found in this laboratory that the dye-iodine precipitate, while still basic in character, is less so than the original dye. It was found to be more difficultly soluble in HCl and seemed to be an oxidation product of the original dye since such reducing agents as stannous chloride tended to yield some of the original dye again. The effect of the mordant on the dye seems to be mild oxidation, and it is believed by the authors that this effect is only a secondary matter and of little or no significance in the Gram reaction. The real function of the mordant, it is thought, lies in its action on the bacterial matter. This will be discussed in a subsequent section.

B. In the light of recent researches one can no longer consistently view the process of diffusion through a semi-permeable membrane in the light of a "differential screening" or a "selective filtration." From such a point of view it would be difficult indeed to explain Kahlenberg's (1906; 1921) experiments in which he succeeded in dialyzing a colloid from a crystalloid through a membrane permeable only to the former. By using pyridine as solvent and vulcanized rubber as membrane one can dialyze camphor from silver nitrate, naphthalene from lithium chloride, sulphur from cane sugar, etc. One crystalloid can be separated from another, one colloid from another, a crystalloid from a colloid or colloid from a crystalloid by proper choice of solvent and membrane. There is absolutely no relation, in selective permeability, between size of molecule and diffusion. For example colloidal metallic soaps such as copper oleate can be dialyzed from lithium chloride, the former going through the membrane.

It has been shown that under pressure (West, 1923) water can be made to pass through glass. One of the authors has shown that this phenomenon also depends on the "membrane" which in this case is the type of glass. A good grade of non-sol glass which is highly insoluble in water will prevent diffusion of water at pressures under which water readily penetrates the more soluble grades of glass found in cheaper ware. In such experiments thickness of glass does not seem important, since the laboratory ware used was quite thin, while the cheaper glass through which water passes more easily is in general thicker.

The significant factor in determining permeability is mutual solubility—not size of particle. With a hydrocarbon membrane such as rubber, materials like naphthalene and camphor, which readily dissolve in it, will pass through, leaving behind salts such as silver nitrate, lithium chloride and other crystalloids like sugar, etc. The mechanism is a matter of solution in the membrane on that side where the concentration is high, and what might be thought of as evaporation from the membrane on the other side into a more dilute environment.

C. Finally, from the physical theory of the Gram reaction one should expect either fairly specific differences, i.e., constancy of

Gram character, or else all organisms should be about equally variable, this variability being measured by a hypothetical elasticity of membrane pores. Czalewski (1896) states that even among Gram-positive organisms there are many gradations of "positiveness." Spirilla as a class are very easily decolorized, Rauschbrand bacillus is less so but is still easily decolorized, while *B. tumescens* is very retentive of the primary stain. There is no very sharp line between the two types of organisms, Gram-positive and Gram-negative, and slight changes in environment—sometimes merely the age of a culture and the effect of change in media due to growth—seem to alter the Gram character.

III. OUTLINE OF THEORY

The authors have shown that much of the behavior of bacteria toward dyes can be explained by assuming that the chemistry of the bacterial protein is the determining factor. It is significant to note how far the same simple assumption will carry one in understanding the mechanism of the Gram reaction.

Much has been made of the lipoid content of cells. Kendall (1916), from some of the data of Eisenberg, goes so far as to state that the Gram character is determined by the lipoidal content of the cell membrane, and specifically by the unsaturated fatty acids, phosphatids, etc. This means to us that bacterial protein, as might be expected, is not a pure simple protein, but a conjugate protein. An example of such would be a lecithoprotein. Such a substance is a loose combination of a simple protein with a phosphatid such as lecithin which would be stable only between certain pH values. The character of simple proteins has been well worked out by Loeb, but little is known of the physical behavior of the phosphatids. Throughout the ordinary pH range they are said to act as acids and they are much more distinctly acid than are the proteins. Like the proteins they are, however, amphoteric, and can exist in the form of such salts as lecithin chloride in sufficiently acid solution, as well as metallic lecithinates in more nearly neutral or alkaline solutions. Little if any work has been done to determine the isoelectric points of these substances, but their distinctly acidic character

would throw such points at a fairly low pH. Even nucleic acid, which is more acidic by far than the phosphatids, is itself an ampholyte having an isoelectric point at a hydrogen ion concentration of about 2×10^{-1} , or a pH of 0.7 (Fodor, 1920). There is reason to believe, from recent work of Winslow, Falk and Caulfield (1923), that for certain phosphatids this point comes in the neighborhood of a pH of 3. This will be discussed later.

Upon the above assumption of a conjugated protein the predicted behavior of bacteria is as follows:

1. In very acid solutions, whose reaction is on the acid side of the isoelectric points of both protein and phosphatid, both of these substances show a tendency to combine with acids and thus retain, strongly, acid dyes.

2. In solutions of such alkalinity as to be on the alkaline side of both isoelectric points, both of the components of the conjugated protein act as acids combining with bases, and in this range basic dyes are strongly retained.

3. At values of pH between these two isoelectric points the protein is acting as a base and the phosphatid as an acid and it is only through this range that the conjugated protein, as an individual, has any stability. Throughout this range the protein would tend to combine with the phosphatid and thus neither would be completely free to combine with either basic or acid dye appreciably. This range we have termed the "isoelectric range" in the preceding paper.

The experimental results are seen to be in striking accord with this very simple picture. The intermediate pH range will vary from organism to organism depending on the specific protein and phosphatid. For example a conjugate protein consisting of lecithin and such a simple protein as serum albumen whose isoelectric point is itself at a pH of 4.7 would be a substance with a comparatively narrow isoelectric range, and would, through the ordinary pH range, tend to retain basic dye. If for the albumen we would substitute a protein such as gliadin, whose isoelectric point is at a pH of about 9.5, we would then have a substance with a much wider isoelectric range and at ordinary pH values there would be little tendency to retain basic dye. Other fac-

tors remaining constant, the first substance cited would in all probability be found to be Gram-positive and the other Gram-negative. Naturally there will be all sorts of intermediate cases with proteins less alkaline and phosphatids less acid.

Throughout this range of loose combination there may be slight residual retention of color since, with both acid dye and phosphatid available with which to combine, the protein compound will depend on the mass relations of the two as well as their relative acidic strengths. In the same way basic dye would compete with protein to combine with the phosphatid. Thus, while there are fairly distinct limits of pH range at which the intensity of retained color suddenly drops off, some residual staining may be expected inside these limits especially with acid dye at lower pH values and basic dye at higher ones. This retention is enhanced by increasing the concentration of the dye solution, giving the mass effect of the dye more nearly an equal chance with the protein or the phosphatid. In our experiments this residual staining was noted, though there was also a distinct range—quite narrow—through which practically complete decolorization seemed to take place in most cases.

Function of mordant

In the paper just preceding it has been shown that any mild oxidizing agent can be used as a satisfactory mordant providing its own pH is not such as to vitiate results. Most of these tend to precipitate the dye, but not all, and there seems very good reason for believing that the real function of the mordant lies in its action on the bacterial matter. The data presented in this preceding paper show pretty distinctly that the general behavior of Gram-positive and Gram-negative organisms is the same, the difference lying for the most part in the limits of the isoelectric range. Anything which will change this range in one type of bacteria and not in another will tend to amplify any original distinction which may be only very slight. From a chemical point of view Jobling and Peterson (1914) have shown that the "lipoid extracted from Gram-positive organisms differs from that extracted from Gram-negative organisms in that the former

contains a much larger proportion of unsaturated acids which have a great affinity for iodine." These would also have an affinity for any other oxidizing agent. This, then, seems to be the point of attack for the mordant. By this mild oxidation the acid character of the phosphatid is intensified just as oxidation tends in general to increase acid properties where it is not vigorous enough to completely destroy.

Table 2 shows the effect of mild oxidation on the ionization constants of a few typical saturated and unsaturated acids. In each case the acids are paired, the oxidation product of any acid

TABLE 2

SATURATED ACIDS		UNSATURATED ACIDS	
Acid	$K \times 10^5$	Acid	$K \times 10^5$
Acetic.....	1.8	Crotonic.....	2.0
Hydroxy-acetic.....	15.0	Alpha-hydroxy-butyric.....	12.0
Propionic.....	1.3	Fumaric.....	93.0
Lactic.....	13.8	Hydroxy-fumaric.....	276.0
Butyric.....	1.5	Maleic.....	117.0
Alpha-hydroxy-butyric.....	12.0	Hydroxy-maleic.....	250.0
Beta-hydroxy-butyric.....	3.1		
Isobutyric.....	1.4		
Hydroxy-isobutyric.....	10.0		

Where the oxidation substitutes or introduces a halogen the effect is even more marked.

being given just below the acid itself. The increase in acid properties by oxidation is quite general. For example, alcohols, which have practically no acid properties, are oxidized to aldehydes which are distinctly though weakly acidic, and these in turn can be oxidized to the organic acids.

The action of the mordant in ordinary differential bacterial staining is selective in that it acts selectively on those cells with a high concentration of unsaturated material. If a more vigorous oxidizing agent were used some of the organisms now classified as Gram-negative would be then classified as positive as was brought out in table 3 of the preceding paper.

It should be noted in passing that Eisenberg (1912) states that it has been found that oxidation destroys the Gram-positive character. In the same paper he reports the use of 5 per cent chromic acid on his bacteria to oxidize aniline to aniline black in contact with the organisms and reports excellent results.

The partially oxidized phosphatid of the Gram-positive organism is thus rendered slightly more acid in character and its affinity for basic dye increased as well as its differentiation from Gram-negative organisms made more marked.

IV. DISCUSSION

The above theory explains many of the anomalies often met with. Sheppe and Constable (1923) found that addition of hydriodic acid to a pH of about 3 rendered Gram-positive organisms amphophile, and more acid made them Gram-negative. Winslow, Falk and Caulfield (1923) find that at this pH the Gram-positive organism *B. cereus* changes cataphoretic migration direction. Burke (1922) found that lactic acid rendered Gram-positive organisms Gram-negative, and both he and Sheppe and Constable agree that the addition of alkali restores the normal reaction.

For many organisms which are not strongly Gram positive, as for example *B. diphtheriae*, the age of the culture is important. These cases of change of Gram character with age are found to be true of organisms which are normally only weakly Gram-positive and are cultured in fermenting media which become acid in reaction as growth proceeds.

Media which permit synthesis of lipoid material seem to have a tendency toward strengthening Gram-positive characteristics. This is in accord with our idea of mordanting. It is much more difficult to bring about the change from Gram-negative to Gram-positive by regulation of the pH than the reverse change, due to the fact that it is more difficult to work with solutions which range from pH 10.0 to 13.0 than the corresponding ones on the acid side, i.e., 1.0 to 4.0.

Robbins (1923; 1924) has shown that in the case of potato tuber tissue as well as the mycelium of *Rhizopus nigricans* and

Fusarium lycopersici their behavior toward dyes, growth rate, etc., could be explained by assuming an amphoteric character with isoelectric points at pH values of 6.0, 5.0, and 5.5 respectively. After discussing the status of the question he states that "the evidence at hand indicates that the conception of the living cell as an amphoteric colloid is worthy of serious consideration."

A final paper in this series will present a brief consideration of bacteriostasis from the point of view of our theory showing how it may be used to explain and correlate many seemingly unrelated phenomena.

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