EFFECTS OF THE GRAM STAIN ON MICROSPHERES FROM THERMAL POLYAMINO ACIDS

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ABSTRACT

Fox, Sidney W. (The Florida State University, Tallahassee) and Shuhei Yuyama. Effects of the Gram stain on microspheres from thermal polyamino acids. J. Bacteriol. 85:279-283. 1963.—Microspheres produced from acid proteinoid accept the Gram stain. The stain is negative, but microspheres produced from mixtures containing a sufficient proportion of lysine proteinoid stain positive. Microspheres produced from mixtures containing the appropriate proportions contain individuals which stain positive and others which stain negative.

Microspherical units in the range of size of the cocci arise in a continuum from thermal experiments which also yield pathways suggesting biosynthetic networks of reactions, and polymers having the qualitative composition, molecular size, and many other properties of protein molecules (Fox, 1960). [Seventeen amino acids are recovered from mineral acid hydrolysates of proteinoids, tryptophan being destroyed but having been demonstrated in the intact polymer (Fox and Harada, 1960). Ammonia is also recovered in amounts which require the presence of glutamine or asparagine or both, hence, 18 to 20 amino acids.] Such thermal polyamino acids are referred to as proteinoids. They have been prepared by use of sufficient proportions of aspartic acid and glutamic acid or of lysine in initially dry mixtures of amino acids (Fox and Harada, 1960; Fox, Harada, and Rohlfing, 1962). The resultant polymers are referred to as acid proteinoids or lysine proteinoids, respectively. Microspheres have been made from acid proteinoids (Fox, Harada, and Kendrick, 1959) and, in the present studies, from mixtures of acid proteinoid and lysine proteinoid. Attempts to produce them from the highly soluble lysine proteinoid alone have not been successful.

Because of a number of features of resemblance of the microspheres to bacteria (Fox and Yuyama, Ann. N.Y. Acad. Sci., in press), the possibility that they might accept the Gram stain was examined. The results suggested that at least one concept of the molecular basis for positive and negative Gram stains could also be tested in a morphological model in which many metabolites would not be present to confuse the picture.

Henry and Stacey (1943, 1946) and Henry, Stacey, and Teece (1945) attributed the Gram-positive stain to a nucleoprotein of which the basic protein moiety is one essential part. More recently, Scherrer (1961) observed that Gram-stainability of some cellular structures in Bacillus megaterium can be restored by basic proteins. Salton (1960) and Gale (1959) indicated that gram-negative bacteria have walls composed of polymers of all of the common amino acids. In the gram-positive organisms, lysine is a principal component in a polyamino acid composed usually of three, four, or five amino acids, and with a portion of the aspartic acid and glutamic acid present as amide residues. These facts can be interpreted as indicating that gram-negative bacteria are predominantly composed of acidic proteins having a full roster of amino acids, whereas basic lysine-containing peptides characterize the composition of gram-positive bacteria. The basic hexosamine has been reported in some cases.

Experiments on the staining of synthetic microspheres made from acid proteinoid and from mixtures of acid proteinoid and lysine proteinoid were carried out to test the principal concept. Such experiments are reported in this paper.

MATERIALS AND METHODS

The acid proteinoid was prepared from 10.0 g of DL-aspartic acid, 10.0 g of L-glutamic acid, and 5.0 g of the 16 other common amino acids present in the mixture in equimolar proportion (Fox and Harada, 1960). The lysine proteinoid was prepared from 11.25 g of the same 16-amino acid mixture plus 1.125 g of L-glutamic acid, 1.125 g of DL-aspartic acid, and 12.5 g of free DL-lysine by heating at 185 C for 5 hr and purifying by dialysis. The soluble fraction recovered by lyophilization weighed 2.6 g.

The analysis of the acid proteinoid and of the lysine proteinoid, after 72 hr of hydrolysis of samples with 250 parts of 6 N HCl at 110 C in sealed tubes under nitrogen gas, is given in Table 1. These values were determined on a Phoenix model K-5000 amino acid analyzer.

When 200 mg of lysine proteinoid was dissolved in 10 ml of water, the solution had a pH of 8.2. When 500 mg were dissolved in 10 ml of water, the pH was 8.3.

The microspheres described in Tables 2 and 3 and Fig. 1 and 2 were prepared by boiling the indicated number of mg in 5.0 ml of aqueous solution for 60 sec and allowing the hot decanted solution to cool spontaneously without disturbance.

In the experiments of Table 3, the microspheres were made in the manner described and then transferred to buffer solutions prepared from 0.2 mM NaHPO4 and 0.1 mM citric acid, except that at pH 3.7 and 2.3, the components were 0.1 mM HCl and 0.1 mM citric acid. The pH values given are the measured values of the solutions with microspheres suspended in them.

Examination of the stains was controlled by reference to stains of the gram-positive *Sarcina flava* and the gram-negative *Escherichia coli*. These were cultures in active growth, kindly supplied by Loretta Ellias of the Department of Biological Sciences. The hues observed in the tests were only slightly different from those in the microspheres of corresponding sign.

The details of the Gram stain follow. Each slide was prepared by spreading a thin film of egg white-glycerol adhesive on it; a drop of the suspension being tested was placed on this film and allowed to stand until dry. A few drops of crystal violet solution (2.0 g of the dye in 20 ml of 95% ethanol, mixed with 0.8 g of ammonium oxalate in 80 ml of water) were added and allowed to stand for 60 sec. This was flushed with water and then covered with Gram’s iodine stain (1.0 g of iodine and 2.0 g of potassium iodide in 300 ml of water) for 60 sec. The iodide solution was flushed with water. The preparation was decolorized by the dropwise addition of 1 to 2 ml of ethanol, this step of the process being rigidly held to 20 sec. The preparation was immediately flushed with water, and treated with a few drops of Safranin solution (10 ml of a 2.5% solution of Safranin 0 in 95% ethanol with 90 ml of water), and allowed to stand for 60 sec. The preparation was again flushed with water and allowed to dry. The results were then observed.

RESULTS

Microspheres of either acid proteinoid or acid proteinoid mixed with lysine proteinoid accept the Gram stain (Fig. 1 and 2). These responses
are of interest in view of the observation that most animal and plant cells stain negatively, and the gram-positive characteristic is abundant only among the yeasts, the bacteria, and the molds (Bartholomew and Mittwer, 1952).

In all tests, all spherules were stained. The original hues of the Gram stain, corresponding respectively to counterstained pink units and to deep purple spherules, are presented in black-and-white photomicrographs (Fig. 1 and 2). The microspheres prepared from acid proteinoid only are gram-negative (Fig. 1). The sign of the Gram stain changes to positive with increasing proportions of lysine proteinoid (Table 2). The gram-positive microspheres are shown in Fig. 2. In Table 2 are also seen the results at borderline proportions, which yield both gram-positive and gram-negative spherules in the same mixture.

To test the effect of pH as determined by independently buffered liquids instead of by the proteinoids, additional experiments were performed (Table 3). The microspheres described were transferred to the solutions of pH designated. The results of repeating the experiments of Tables 2 and 3 with a different preparation of lysine proteinoid were in the same order, but all of the proportions yielding a given result were shifted slightly in the same direction.

Microspheres made from acid proteinoid alone were much more soluble at a given pH than those made from mixed proteinoids. As Table 3 shows, microspheres of mixed composition remained undissolved at pH 8.6, whereas the spherules produced from acid proteinoid alone would dissolve at lower pH values.

**Discussion**

The fact that the microspheres accept the Gram stain indicates a degree of compositional kinship to microbes. As cell models, these are of interest because of size, shape, composition, stainability,
TABLE 2. Variation in Gram stain with composition of polymers used in preparation

<table>
<thead>
<tr>
<th>Proportion of lysine proteinoid to acid proteinoid</th>
<th>Microspheres produced in 0.17 M NaCl</th>
<th>Microspheres produced in 0.17 M KCl</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/100</td>
<td>---*</td>
<td>---</td>
<td>3.2</td>
</tr>
<tr>
<td>5/100</td>
<td>---</td>
<td>---</td>
<td>3.4</td>
</tr>
<tr>
<td>10/100</td>
<td>+++</td>
<td>+++</td>
<td>3.5</td>
</tr>
<tr>
<td>25/100</td>
<td>**+</td>
<td>**+</td>
<td>3.2</td>
</tr>
<tr>
<td>35/100</td>
<td>**</td>
<td>**</td>
<td>3.5</td>
</tr>
<tr>
<td>40/100</td>
<td>++</td>
<td>++</td>
<td>4.2</td>
</tr>
<tr>
<td>45/100</td>
<td>**+</td>
<td>**+</td>
<td>4.2</td>
</tr>
<tr>
<td>50/100</td>
<td>**</td>
<td>**</td>
<td>4.2</td>
</tr>
<tr>
<td>60/100</td>
<td>**</td>
<td>**</td>
<td>4.0</td>
</tr>
<tr>
<td>70/100</td>
<td>++</td>
<td>++</td>
<td>4.2</td>
</tr>
<tr>
<td>100/100</td>
<td>None</td>
<td>None</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* Results expressed as: --- = intensely gram-negative; ± = mixed gram-negative and gram-positive; + = faintly gram-positive; ++ = intensely gram-positive.

TABLE 3. Effect on Gram stain of adding microspheres to buffered solutions

<table>
<thead>
<tr>
<th>Ratio of lysine proteinoid to acid proteinoid</th>
<th>Suspension pH</th>
<th>Sign of stain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20/100</td>
<td>8.2</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>---</td>
</tr>
<tr>
<td>50/100</td>
<td>3.8</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>++</td>
</tr>
<tr>
<td>70/100</td>
<td>3.7</td>
<td>±</td>
</tr>
</tbody>
</table>

* Signs: --- = intensely gram-negative; ± = mixed gram-negative and gram-positive; + = faintly gram-positive; ++ = intensely gram-positive.

when those descriptions are interpreted in terms of acidic and basic biopolymers. The regulation of pH by added buffer (Table 2) influences the sign of the stain, but only by modification, as is true for bacteria (Stearn and Stearn, 1924). The compositional similarities agree with the emphasis of Stearn and Stearn (1924) on the function of bacterial protein and with the emphasis of Gale (1959) and Salton (1960) and others in ascribing negativeness to a compositionally complete protein and positiveness to a basic type of natural polyamino acid. The polymer which imparts positiveness to the microspheres is not, however, composed of fewer amino acids, but rather of a more basic combination. This emphasis on basic polymers is also consistent with the findings of Scherrer (1961) and in part with the emphasis of Henry and Stacey (1943, 1946). The possible relationship of these interpretations to suggested contributions of ribonucleic acid and the magnesium salt is, however, not clear. The role of ribonucleic acid in the compositional influence on the Gram reaction is open to question in view of the findings of Lamanna and Malette (1950) that the reaction is unaffected by removal of ribonucleic acid (RNA) by chloroform. One hypothesis, however, is that the RNA may function to maintain basic protein in the cell, in a proper orientation (Chelton and Jones, 1959).

Aside from the intrinsic significance of these findings, the results suggest that some bacterial phenomena can be studied in relatively uncomplicated organized synthetic units.

The solubility properties are also parallel to those of bacteria, gram-negative bacteria being more soluble in alkali than are the gram-positive type (Lamanna and Malette, 1959).

The finding that certain compositions yield mixtures of positive and negative microspheres is probably due to variations in the formed units and emphasizes the fact that, in production of microspheres in experiments in this laboratory, individual differences of many kinds are often observed. Such observations are significant in interpretation of abiogenic processes. The production of thermal polyamino acids and of microspheres are now believed to represent inexorable processes over a wide range of conditions (Fox and Yuyama, in press) which probably occurred at many places and many times. On this picture may be superimposed the factor that

responses to salt, birefringence, and modes of association (Fox, 1960; Fox and Yuyama, in press). Their attractiveness as precell models is accordingly enhanced, when viewed in association with other phenomena emerging from thermal experiments (Fox, 1960; Fox and Yuyama, in press).

Without metabolic products to complicate the picture, the sign of the Gram stain is seen to be a function of the composition of the units. These compositional variations seem to be in general accord with the descriptions in the literature

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numbers of individual variations exist in these units within a single preparation. Under these circumstances, the likelihood of a chance emergence of a precellular unit with the necessary biochemical apparatus for replication, in spontaneous processes on the primitive earth, is enhanced over the possibilities which have been visualized prior to the experiments.

Acknowledgments

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Literature Cited


