FURTHER STUDIES ON THE GRAM STAIN

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In 1886 Christian Gram devised a staining technique for differentiating bacterial species. The nature of the action of the stain upon bacteria has been of considerable interest, and many theories have been proposed concerning the mechanism of the staining reaction. One theory is that of Jobling and Petersen (1914), who are of the opinion that gram-positive bacteria are richer in unsaturated fatty acids than are other bacteria. They argue that the addition of iodine at the double bonds forms an alcohol-insoluble combination with the unsaturated fatty acids in the "ectoplasm" and thus renders the cell wall impermeable to alcohol. This impermeability prevents the removal of the dye that has been absorbed. Another theory is that of Benians (1912). He believes that the effect of the iodine is to dissociate the dye from its adsorption compound with the tissues to form a large molecular body. The iodine and the dye combine. This dye-iodine complex diffuses out of gram-negative bacteria upon alcohol application but does not pass out of gram-positive bacteria.

The importance of proteins in bacterial chemistry, as related to the gram stain, was the basis of the theory of Stearn and Stearn (1928). They demonstrated that the proteins of gram-positive bacteria exhibit an isoelectric point further on the acid side of neutrality than do the proteins of gram-negative bacteria. In either case the isoelectric point is on the acid side; thus both types of organisms are stained with basic dyes. However, the gram-positive group will hold the stain more securely since the iodine shifts the pH further to the acid side and therefore increases the strength of the original staining. Thus, after treatment with iodine, the alcohol does not wash out the violet stain as readily from gram-positive bacteria.

The "modern" conception of the gram-staining mechanism was proposed by Churchman (1929). He concluded that the protoplasm of all bacteria is gram-negative and that the gram-positive bacteria are covered by an envelope of gram-positive material. This hypothesis led to more recent work. Henry and Stacy (1943) demonstrated that by using bile salts they could strip off the gram-positive outer coat from a number of bacterial species. By this treatment they were able to separate the gram-negative skeleton, which retained the shape of the organism. The gram-positive character could be restored by replating the extract back on the gram-negative skeleton. Although extract from the gram-positive cells contained carbohydrates and proteins, the most abundant constituent was magnesium ribonucleate. Henry and Stacy (1943) were also able to restore to the gram-positive state organisms rendered gram-negative as a result of bile salt treatment, by putting them into a neutral solution of the magnesium salt of pure ribonucleic acid. It is noteworthy that organisms that are normally gram-negative are not affected by this treatment and also that desoxyribonucleic

acid cannot replace ribonucleic acid in replating gram positiveness. This experiment of Henry and Stacy suggests that the gram-positive character resides in a ribonucleate complex and that the gram-negative bacteria do not contain this material.

Bartholomew and Umbreit (1944) confirmed Henry and Stacy's results and extended them further. They converted gram-positive bacteria to gram-negative forms by digestion with the enzyme ribonuclease. They were also able to replace the bacterial ribonucleate with magnesium ribonucleate prepared from yeast and thus replated the gram positiveness.

This communication reports the plating of gram positiveness upon normal gram-negative *Escherichia coli* through the action of desoxyribonucleic acid.

MATERIALS AND METHODS

The strains of *E. coli* were isolated from culture plates obtained from stool specimens of patients. The bacteria were grown in nutrient broth at a pH of 8.0.

The desoxyribonucleic acid was prepared by a modification of the method of Mirsky and Pollister (1942). The preparation when dried was light brown in color and in solution gave the most viscous solution of any preparation made in this laboratory to date, suggesting a high degree of polymerization. Solutions of desoxyribonucleic acid were prepared in sterile saline or distilled water and contained 6 mg of the acid per milliliter (of solution). All incubation was done at 37 C. Centrifugation of the bacteria was conducted at room temperature at 2,000 rpm for 30 minutes. The method of staining employed was that of Hucker and Conn (1947). All preparations were stained upon the same slide to ensure equality of conditions. The saline used in this work contained 0.85 grams of NaCl per 100 ml of distilled water.

Two 100-ml flasks of nutrient broth were each inoculated with a loopful of *E. coli* and incubated for 18 hours at 37 C. The bacteria from each portion were then centrifuged and washed 3 times each, one portion with saline, the other portion with distilled water. The portion which was washed with distilled water was suspended in 5 ml of distilled water. The portion washed with saline was suspended in 5 ml of saline. Four tubes were set up: two tubes (no. 1 and no. 2) were employed for the distilled water bacterial suspension; the remaining two tubes (no. 3 and no. 4) were used for the saline bacterial suspension. The suspensions were then treated in the following manner: To tube no. 1, 1 ml of bacteria in distilled water and 1 ml of distilled water were added. This tube served as a distilled water control. To tube no. 2, 1 ml of bacteria in distilled water plus 1 ml of desoxyribonucleic acid were added. To tube no. 3, a 1-ml portion of bacteria in saline and 1 ml of saline were added. This tube served as a saline control. Tube no. 4 contained 1 ml of bacteria in saline and 1 ml of desoxyribonucleic acid solution.

RESULTS

All control tubes contained only gram-negative bacteria. However, the organisms in the tubes containing the desoxyribonucleic acid were gram-positive

with only occasional gram-negative cells. After these gram-positive cells were washed, a further change in the staining characteristics was seen. The bacteria previously suspended in distilled water when washed 5 times with distilled water were then found to be gram-negative. However, if they were washed with saline the positiveness persisted. The same observations were noted in the bacteria originally suspended in saline; when washed with water they became gram-negative, but if washed with saline they remained gram-positive. These results are summarized in table 1.

This experiment indicates that gram positiveness can be imposed upon normal gram-negative *Escherichia coli* by the presence in the system of a highly viscous desoxyribonucleic acid. It is of importance to note that the changing of the gram-staining characteristics of *E. coli* is produced only by certain preparations of desoxyribonucleic acid, since it was shown in the course of our investigation that samples of desoxyribonucleic acid giving a less viscous solution have failed to produce the gram-staining changes.

TABLE 1

Changes in the gram stain of E. coli incubated with desoxyribonucleic acid

	TUBE 1	TUBE 2		TUBE 3	TUBE 4	
	Dist. H_2O + Bact.	Dist. H_2O + Bact. + DRNA		Saline + Bact.	Saline + Bact. + DRNA	
Gram stain.....	−	+		−	+	
		AFTER REPEATED WASHING			AFTER REPEATED WASHING	
		With H_2O	With saline		With H_2O	With saline
Gram stain.....	−	−	+	−	−	+

Desoxyribonucleic acid is soluble in distilled water but only slightly soluble in 0.85 per cent saline. This suggests a possible mechanism by which the positiveness produced upon the *E. coli* is retained after washing with saline. Since the distilled water dissolves the added desoxyribonucleic acid, it apparently removes the acid from the bacteria and thus destroys the positiveness. However, since the saline cannot dissolve the added desoxyribonucleic acid from the bacteria, the positiveness remains. Thus, it seems that the desoxyribonucleic acid combines with the *E. coli*. This combination is soluble in distilled water and insoluble in saline.

SUMMARY

Gram positiveness can be imposed upon normal gram-negative *Escherichia coli* by the addition of a highly viscous desoxyribonucleic acid.

The gram positiveness cannot be washed away with physiological saline but can be removed with distilled water.

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