GLYCOGEN OF ENTERIC BACTERIA

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In the course of an investigation of the use of infrared spectrophotometry for bacterial identification (Stevenson and Bolduan, 1952; Levine et al., 1953b), it has been found that a characteristic set of absorption bands appears in the spectra of *Enterobacteriaceae* and other bacteria under certain cultural conditions. Since this set of bands is common to many organisms, it is important in identification studies. Further work has revealed that these bands are due to a glycogen-like storage polysaccharide. The effects of age, temperature, inoculum density, and medium constituents on the content of this polysaccharide in enteric bacteria, as revealed by infrared spectrophotometry, have been studied, and the results are presented in this paper.

Isolations of glycogen-like polysaccharides have been reported from *Mycobacterium tuberculosis* (Chargaff and Moore, 1944), *Neisseria perflava* (Hehre and Hamilton, 1948), and *Bacillus megaterium* (Barry et al., 1952). We have isolated a similar product (which will be referred to as glycogen for brevity) from enteric bacteria and have determined some of its chemical and physical properties.

METHODS

Diffuse surface cultures were obtained by inoculating agar plates with a dense suspension of organisms. For infrared observations the resulting growth was scraped from the surface with a rubber policeman, spread on a disk of silver chloride, and dried in room air. A Perkin-Elmer model 21 double-beam recording spectrophotometer was employed, with a blank silver chloride disk in the reference beam. Samples of approximately two mg dry weight in the

area of the beam were adequate. Smaller samples, two or three isolated colonies, were analyzed by using a special holder in the microcell position (Stevenson and Levine, 1953).

Infrared spectra of mixtures represent a summation of the absorptions of individual components. The presence of glycogen in bacteria thus can be detected by the appearance in the bacterial spectrum of absorption bands characteristic of glycogen (Levine et al., 1953a). The spectrograms in figure 1 demonstrate that the strong glycogen bands at 8.7 and 9.75 μ are particularly useful in this regard since they do not coincide with absorption maxima of other bacterial components. The weaker glycogen bands at 10.75, 11.8, 13.2, and 14.2 μ may appear also in the bacterial spectrum, but only when the glycogen concentration is fairly high. It is apparent that the degree of prominence of the 8.7 and 9.75 μ bands can be used as a measure of glycogen concentration in the cells.

In order to use infrared spectra for glycogen analysis, a series of standard curves was required. Two methods were explored: (1) The organism under study is grown under a variety of conditions known to result in different glycogen concentrations. Spectra are recorded and chemical analyses for glycogen performed for each specimen. (2) Bacteria (or bacterial lysate) with little or no glycogen content are mixed with pure glycogen to produce mixtures of known composition whose spectra then can be recorded.

Figure 1 shows a standard series of spectra produced by the second method. The glycogen content of an unknown specimen of bacteria can be estimated roughly by visual comparison of the depth and prominence of the 8.7 and 9.75 μ bands with this standard series. The depth of these bands, however, depends not only on glycogen concentration but also on the thickness of the film of bacteria, a factor which is difficult to control. In order to obviate the effect of film thickness, the glycogen band at 9.75 μ was compared with an internal standard—the
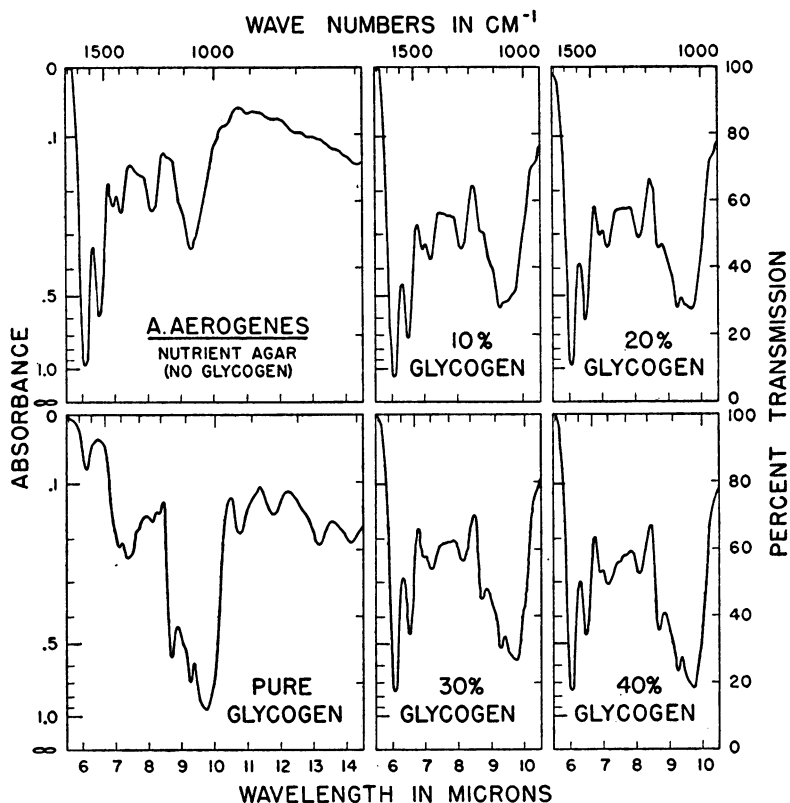
Figure 1. Infrared spectra of *Aerobacter aerogenes*, glycogen, and their mixtures.

protein band at 6.45 to 6.50 μ. The ratio of absorbances (optical densities) at these two wavelengths, obtained from a standard series of spectra, was plotted against glycogen concentration (figure 2). An unknown was analyzed by determining the ratio from the spectrum and reading glycogen concentration from the reference curve.

In setting up a series of standard curves for *Aerobacter aerogenes*, growth from nutrient agar (Difco) was used to represent cells of zero glycogen content. On analysis by the method of Good *et al.* (1933) these cultures appeared to contain one to two per cent of glycogen. On isolation, however, infrared spectra showed that this material was not glycogen (and not agar) even though it simulated glycogen in the Good-Kramer-Somogyi analytical procedure.

Washed cultures were used for chemical analyses and isolations. Dry weights were determined on aliquot samples by heating at 103 C overnight. Glycogen analyses were performed by the Good-Kramer-Somogyi method
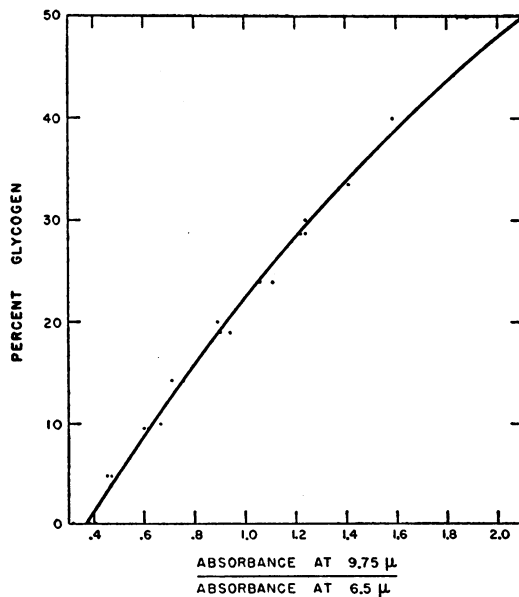

Figure 2. Relation between absorbance ratios (determined from spectra) and glycogen concentrations in cultures of *Aerobacter aerogenes*.

using the iodometric method of Somogyi (1945) for reducing sugars.

Glycogen was isolated by digesting the cultures with 30 per cent KOH for three hours in a boiling water bath, followed by several precipitations with 1.1 volumes of 95 per cent ethanol. Glycogen could not be extracted from intact bacterial cells with water, even at boiling temperatures. Isolations have been accomplished, however, after sonic disintegration of the cells (10 kc magnetostrictive oscillator), centrifugal removal of cell debris, and repeated precipitations with one volume of 95 per cent ethanol. The aqueous solution then was freed of protein traces by several treatments with ¼ volume chloroform and ⅟₁₀ volume isoamyl alcohol (Sevag et al., 1938) in a cold Waring blendor for 1 to 2 minutes. This was followed by dialysis and reprecipitation with ethanol.

RESULTS

Formation of glycogen in surface cultures as revealed by infrared spectrophotometry. Among the many cultures of *Salmonella, Shigella, Aerobacter,* and *Escherichia* which have been grown on numerous sugars, polyhydroxy alcohols, and organic acids, marked strain variation has been noted in the occurrence of glycogen and in its rate of accumulation. Generally the organisms studied produced no glycogen after growth on Difco nutrient agar (with agar content increased to 2.2 per cent for ease in harvesting). The addition of a utilizable carbohydrate to this medium (before autoclaving) usually stimulated glycogen synthesis. However, the examination of a single specimen was inconclusive since the characteristic glycogen bands may not be present early and may disappear rapidly. This was particularly true for strains of *A. aerogenes* grown at 37 C in which glycogen reached a peak between 16 and 24 hours and markedly diminished or disappeared at 32 to 48 hours (table 1). The disappearance of glycogen from surface cultures of *Escherichia coli* and *Salmonella* was much slower and may be correlated with the persistently low pH maintained by such cultures on sugar containing media. *A. aerogenes* cultures also produce acid but return to neutrality rapidly, perhaps in this way avoiding adverse effects of low pH on glycogenolytic enzymes.

The amount of glycogen formed by the organisms studied usually was increased when

incubation was done at 15 to 20 C instead of 37 C. However, it took longer to reach peak concentrations (2 to 3 days) and longer for the glycogen to start diminishing. Results with two strains are presented in table 1. These figures cannot be taken as representing the general behavior of *A. aerogenes* and *Salmonella montevideo* because of the marked variability in glycogen synthesizing powers among different strains. The two strains chosen were picked because of consistently high glycogen production. The highest levels of glycogen were observed in some strains of *S. montevideo* after growth on one per cent mannose at 15 to 20 C. Absorbance

TABLE 1

Effect of time and temperature of incubation on glycogen levels

TIME AND TEMPERATURE	ABSORBANCE AT 9.75 μ* ABSORBANCE AT 6.5 μ	
	Aerobacter aerogenes, glucose agar	Salmonella montevideo, mannose agar
37 C, 10 hr	0.74	—
16 hr	0.92	1.51
24 hr	0.66	1.07
32 hr	0.60	1.06
48 hr	0.41	1.04
15 to 20 C, 1 day	0.82	1.26
2 days	1.48	2.10
3 days	1.30	2.10
4 days	1.21	—
7 days	0.95	1.47

* Absorbance ratios can be converted to glycogen concentration by use of reference curve.

ratios of 2.1, corresponding to 50 per cent glycogen, frequently were observed. In order to confirm this, glycogen was isolated from 0.851 grams of organisms. The yield of crude glycogen was 0.495 grams. This was found to be 83.2 per cent pure according to reducing activity after hydrolysis, giving a corrected yield of 48.4 per cent. The *S. montevideo* strain, unlike most enteric organisms, was capable of accumulating some glycogen after growth on nutrient agar *without* added carbohydrate.

These two organisms and a strain of *E. coli* have been examined after growth on nutrient agar (Difco) supplemented by addition of either glucose, mannose, galactose, fructose, lactose,

or sodium succinate at 37 C and at 15 to 20 C. Glycogen was formed in variable amounts, and there was no consistent relationship in glycogen forming ability among the 3 strains when grown on different substrates. Low incubation temperature consistently increased the glycogen synthesizing ability of the *A. aerogenes* and *S. montevideo* strains, but the extent of the increase varied with both substrate and organism. On the average, the *E. coli* strain produced much less glycogen, and glycogen synthesis was not always favored by low temperatures.

The use of sugars in one per cent concentration produced maximal glycogen levels. Concentrations of 0.5 per cent usually resulted in much lowered yields. Concentrations of two per cent or higher produced little or no increase in glycogen and often inhibited its synthesis completely.

The progress of glycogen synthesis as observed in the spectra of diffuse surface cultures of *A. aerogenes* at 15 to 20 C was compared with the rate of depletion of glucose in the medium (quantitative Benedict method). Maximum glycogen levels in the organisms coincided approximately with maximum dry weight yield of bacteria and with complete depletion of glucose in the medium. This point was reached in 32 hours on 0.5 per cent glucose agar and in 2 to 3 days on one per cent glucose agar. The peak glycogen level reached on 0.5 per cent glucose agar was less than the peak level on one per cent glucose agar, and in fact approximated the level reached on the richer agar when an equivalent amount of sugar had been utilized. The surface pH remained low until all the free glucose had disappeared; then it returned to neutrality. With further incubation the pH rose slightly (7.5 to 7.8) while the glycogen concentrations and dry weight yield of organisms fell. These last findings are apparently analogous to the drop in turbidity and polysaccharide content of broth cultures after the log phase of growth (Dagley and Dawes, 1949).

The density of the inoculum affected the rate of glycogen synthesis. Plates inoculated with dilute suspensions had a slower rate of glucose loss from the medium, and the cultures showed slower increases in weight and in glycogen concentration. After 2 to 3 days at 15 to 20 C, however, the density of inoculum had little or no effect on the results. Spectra of well separated colonies have shown low glycogen levels which

appeared slowly as compared to diffuse surface growths under identical conditions.

The addition of 0.0002 per cent of sodium azide to one per cent glucose agar sufficed to prevent glycogen synthesis by *A. aerogenes* grown at 37 C. Sodium iodoacetate, 0.001 per cent, caused partial inhibition while 0.0013 per cent caused complete inhibition of glycogen synthesis without preventing growth. When *A. aerogenes* was grown on a different brand of nutrient agar (instead of the Difco product) with added glucose, no glycogen synthesis was observed and growth was poor (only one batch was tested).

Various enteric organisms were grown on several selective media, some of which resulted in bizarre bacterial spectra. On SS agar the spectra indicated the synthesis of large amounts of glycogen by most *Salmonella* and *Shigella* strains after incubation at either 37 C or 15 to 20 C. The basic nutrients of this medium are sodium citrate and a peptone, the combination of which permits only minimal glycogen synthesis. A systematic evaluation of the other components revealed that bile salts and sodium thiosulfate added to the basic nutrients had all the glycogen promoting activity of the complete SS medium. Lactate was as effective a carbon source as citrate, and thiosulfate was omitted with only partial loss of activity. Attempts to evaluate the effect of bile salts on glycogen synthesis from sugars were made difficult by the appearance of an artifact in the spectra. The low pH attendant upon glycolysis caused precipitation of bile acids in the bacterial growth and in the agar. The crystals inadvertently harvested with the bacteria contributed to the spectrogram. Artifacts of this sort also must be anticipated if the agar surface is not smooth due to the presence of particulate matter (such as calcium carbonate added as a buffer).

Properties of isolated glycogen. Glycogen was prepared from surface cultures of *A. aerogenes* (two days' incubation at 15 to 20 C on one per cent glucose-nutrient agar, Difco) by potassium hydroxide digestion and by ethanol precipitation of sonic lysates. Both materials were highly opalescent in aqueous solution, much more so than two commercial glycogen preparations (Mann and Pfanstiehl). They gave a strong reddish-brown color with iodine and had no reducing activity before hydrolysis. Treatment

with saliva rapidly abolished the opalescence and iodine color and caused the appearance of reducing activity. The optical rotation of 0.5 per cent solutions in 0.2 M NaCl in a one decimeter tube could not be determined accurately because of the strong opalescence. Readings varied from +0.65 to +1.60°, and the average values corresponded to a specific rotation of roughly +200°. The infrared spectra were identical with the spectra of the commercial glycogens. Analytical values for the KOH and sonic lysate preparations were respectively: nitrogen, 0.04 per cent and 0.085 per cent; phosphorus, 0.14 per cent and 0.04 per cent; reducing sugars after hydrolysis, 93 per cent and 91.5 per cent of theoretical. Paper chromatography of a hydrolyzate revealed a single component, the Rf value of which was identical with that of glucose. The sonic lysate preparation became water insoluble after several weeks in a vacuum desiccator over "drierite".

Examination in the analytical ultracentrifuge revealed that both preparations were of very large particle size and were polydisperse. The apparent average particle weight based on sedimentation rate, diffusion constant, and partial specific volume was found to be 9.2×10^6 for a 0.5 per cent solution of KOH-glycogen in 0.2 M NaCl. It was of interest to determine whether glycogen existed within the cell in the form of such a large and polydisperse particle or whether degradation or aggregation resulted from the purification procedure. The nearest approach we could make to this problem was to break the cell wall and examine the lysate immediately in the analytical ultracentrifuge (Schachman et al., 1952). Grinding the cells in the cold with finely powdered pyrex glass proved to be very adequate. A lysate prepared from A. aerogenes grown on glucose agar at 15 to 20 C and thus containing much glycogen had a major component which sedimented at a rate comparable to that of the isolated glycogen and without marked difference in polydispersity. That this component was actually glycogen was made very likely by the failure to find anything similar in a lysate of the same organism grown without glucose in the medium, and thus lacking glycogen. Identical findings were obtained with sonic lysates as shown in figure 3. The ultracentrifuge pattern of the glycogen purified from a sonic lysate was very similar in sedimentation

rate and polydispersity to the glycogen component of the crude lysate. Although the glycogen peak of the sonic lysate appeared to be somewhat more polydisperse than the glycogen of the glass powder lysate, the degree of polydispersity could not be increased by lengthening the sonic exposure from three-quarters of an hour to over 3 hours.
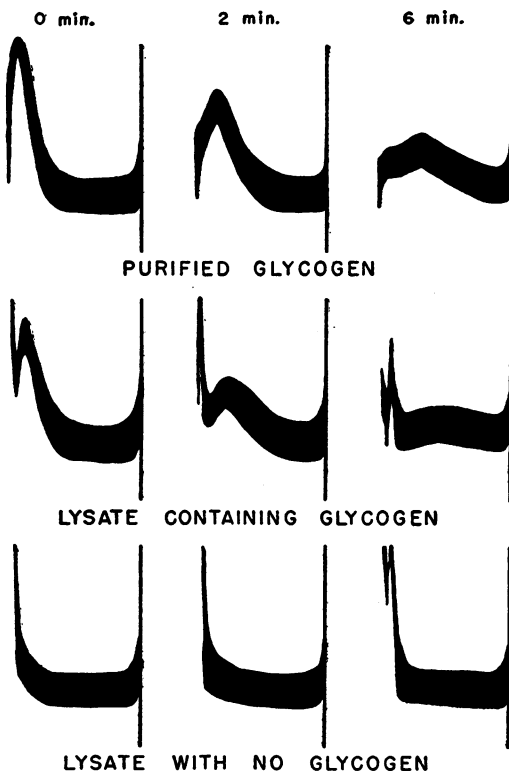

Figure 3. Ultracentrifuge patterns of pure glycogen, of a lysate of Aerobacter aerogenes grown on glucose agar, and of a lysate of A. aerogenes grown on plain nutrient agar. Sedimentation proceeds to the right. The time after reaching 23,150 rpm (39,000 G) is indicated.

Glycogens prepared from E. coli and S. montevideo corresponded to the A. aerogenes preparation in iodine color, opalescence, optical activity, and infrared absorption.

DISCUSSION

The results presented emphasize the widespread distribution of glycogen among enteric bacteria and the marked variability in glycogen

storing ability. The formation of large amounts of glycogen in surface cultures is undoubtedly due to the high degree of efficiency in carbohydrate utilization under strongly aerobic conditions, making much of the carbohydrate available for synthesis to storage polysaccharide. We have not observed comparable levels of glycogen in broth cultures.

The glycogen level at the moment of observation depends, of course, on a balance between synthesis and utilization. The balance favors synthesis during the early phases of growth and favors utilization in the late phases. Iodoacetate, azide, and high sugar concentrations probably result in low glycogen levels by interfering with its synthesis. Differing rates of utilization may account for the rapid disappearance of glycogen from cultures of *A. aerogenes* and its persistence in cultures of *E. coli* and *Salmonella*. The low pH maintained by the latter organisms may be responsible for a low rate of utilization (by the inhibition of glycogenolytic enzymes) and hence the persistence of glycogen. Since *A. aerogenes* cultures return to neutrality rapidly, the utilization and disappearance of glycogen continue rapidly.

The increased glycogen concentrations due to low temperature incubation, use of dense inoculum, or presence of bile salts in the medium may be due to effects on synthesis or utilization or both. The influence of bile salts may be associated with the effect on respiration reported for anionic detergents (Baker *et al.*, 1941).

The physical state of glycogen is a matter of some importance. Unfortunately, the process of isolation and purification may induce changes. The ultracentrifugal analysis of crude lysates takes us closer to the actual state in the bacterial cell and may avoid some artifacts. Identification of the glycogen peak in such ultracentrifugal patterns is thus of significance. The large particle size and polydispersity of the glycogen found both in crude lysates and in purified preparations may indicate the true state of intracellular glycogen unless the lysing process is capable of altering its physical properties.

The infrared spectrophotometric method for determining glycogen in bacteria has the advantage of speed, simplicity, and small sample requirement. In fact, with microscopic methods the sample requirements would be in the microgram range. Samples need only be dried on silver chloride disks without chemical manipulation to be ready for rapid automatic spectral recording. However, the results cannot be rigorously quantitative because dried films of uneven thickness constitute an inhomogeneously dispersed system which has been shown not to follow Beer's law (Jones, 1952). Losses due to scatter and reflection fortunately operate in the opposite direction with some resulting compensation of errors. The attempt to compensate for the absorption of the silver chloride disk by the use of a blank disk in the reference beam does not rest on firm theoretical foundations. The two disks may not have the same transparency, and even if they do, the energy losses due to reflection from the disk surfaces are influenced by a coating film (of sample) on only one of them. More important deviations are caused by other cell components which absorb at the characteristic glycogen wavelength, 9.75 μ. A correction would be impossible since these substances may vary independently. Capsules or slime, when present, may mask glycogen completely. We have been able to detect glycogen in the spectra of such samples only after the removal of the interfering polysaccharide by a brief washing for loose slime or vigorous aqueous extraction for capsules. The spectra of starch and dextrin are extremely similar to that of glycogen and would require isolation and chemical testing for differentiation. The infrared method for glycogen should be applied where interfering polysaccharides are absent and where relative values for glycogen are more important than absolute values.

Some increase in accuracy might be attained by the incorporation of a foreign substance as internal standard, or by the control of optical path length in sodium chloride cells (bacteria in mineral oil suspension), silver chloride cells (aqueous suspension), or potassium bromide disks (dry, finely ground bacteria). Since the inherent qualities of this type of analysis permit only semiquantitative interpretations, it is doubtful if these more complicated procedures are of sufficient value to displace the simple, rapid, dry film method.

SUMMARY

An infrared spectrophotometric method for the semiquantitative determination of glycogen in bacteria is described. Through use of this method, glycogen has been found to be widespread among enteric bacteria grown on the surface of carbohydrate containing agar media. The glycogen content is influenced strongly by the content of the medium, age of culture, temperature of incubation, and density of inoculum. Glycogen levels as high as 48 per cent of dry weight have been found.

The glycogens of *Aerobacter aerogenes*, *Escherichia coli*, and *Salmonella montevideo* have been isolated and studied. Glycogen in the pure state and in crude bacterial lysates has been found by ultracentrifugal analysis to be polydisperse and of very large particle size.

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