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On the Glycogen in *Escherichia coli B*; Variations in Molecular Weight during Growth. I

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Molecular weight determinations have been performed on glycogen isolated from *Escherichia coli B* at different conditions of growth.

It was observed that the molecular weight increased when glycogen was accumulated by the cells. A decrease was noted when the glycogen was utilized.

In the experiments [1,2] published earlier it was shown that of the glucose residues incorporated into glycogen during an accumulation of this compound in *E. coli B*, those that were incorporated last were the first to be split off during a subsequent growth period, *i.e.* when the glycogen was utilized by the cells. A possible explanation of this could be that during accumulation the "excess synthesis" of glycogen proceeds by addition of glucose residues to the glycogen molecules that are already present at the beginning of the period. The glucose residues incorporated last would constitute a "peripheral tier" on the molecules, and would in turn be those most easily available during a subsequent utilization.

The purpose of the experiments described in this paper was to test this possibility. Molecular weight determinations by the light-scattering method revealed an increase in the molecular weight of the glycogen during the accumulation period, and a decrease during the utilization. These results seem to confirm the hypothesis, but the possibility that other types of glycogen rebuilding may proceed at the same time cannot be excluded.

METHODS

Molecular weight determination. The molecular weight of glycogen from different animal tissues has been measured by osmotic pressure [3], ultracentrifugation [4,5], and light scattering [6-9]. The values reported usually lie within a range of $1-10 \times 10^6$. The most recent report [9] gives values in the range of 50×10^6. Sedimentation studies on glycogen from *Mycobact. tuberculosis* [10] gave the value 12×10^6 and on glycogen from *Aerobacter aerogenes* [11] 9.2×10^6. The shape of the glycogen molecule has been reported to be spherical [12]. The present paper is the first report on glycogen from *E. coli B*. Light-scattering has been used to characterize the molecule.

Measurements were made in a Brice Phoenix Universal Light Scattering Photometer at a wavelength of 4 358 Å. The solutions to be measured were kept in a semioctagonal glass cell. The measurements were performed at 45°, 90° and 135° with respect to the direction of the direct beam. The molecular weight was calculated according to the formula [13]:

$$\frac{K \cdot c}{R_{90}^{\circ}} = \frac{1}{M \cdot P_{90}^{\circ}} + 2B \cdot c$$

where R_{90}° is the intensity scattered at 90° divided by the intensity of the direct beam, c the concentration of glycogen in g/ml, K a constant which includes the refractive increment of glycogen, M the molecular weight, P_{90}° a particle-scattering factor, and B a constant. The refractive increment used [7] was 0.136 ml/g, giving K a value of 2.94×10^{-7}. The particle-scattering factor can be obtained by using the relation $R_{45}^{\circ}/R_{135}^{\circ} = z = f(P_{90}^{\circ})$. This factor, z, is called the dissymmetry. P_{90}° has been tabulated for different values of z for spherical particles [13].

By measuring the scattering at several different concentrations, it was found that within the concentration range used, $2.5 \times 10^{-6} - 50 \times 10^{-6}$ g/ml, the scattering was independent of the concentration, and the constant B was thus zero. This agrees with light-scattering data on other glycogens [9]. The glycogen concentrations have been determined as described earlier [14].

The molecular weight obtained is a weight-average molecular weight, which is higher than the number-average obtained from, *e.g.*, osmotic pressure measurements. If a specimen is highly polydisperse, which is the case with glycogen, the results will be dominated by the highest molecular weight fraction.

It is possible to calculate the diameter of the molecules from dissymmetry data if the shape is spherical [13]. However, this value is a "z-average", which is even higher than a weight-average. The diameter of the molecules was found to be about 750 Å.

The glycogen specimens were quantitatively dissolved in an 0.1 M dust-free sodium chloride solution. The dust had been removed by filtration through a membrane filter with a pore diameter of 200—400 Å. When filtration or high speed centrifugation was used to take away dust from glycogen solutions, a large amount of glycogen was also removed. These solutions were therefore centrifuged in a small laboratory centrifuge in order to remove the largest extraneous particles. The amount of glycogen which sedimented during this centrifugation was within the limits of detection by the method of analysis. After this procedure, there conceivably may still remain some contamination of the glycogen specimens by dust; however this dust does not seem to disturb the measurements to any great extent. For one thing, the scattering from glycogen is very strong. Also, the dust would be expected to give high dissymmetries, but the measurements did not show excessively high values (1.60—2.50), or change if the solutions were kept overnight, when some of the dust ought to have settled. Finally, there was a high degree of reproducibility in the measurements, as shown in Table 1. Stetten *et al.*[9] also came to the conclusion that dust does not disturb the measurements.

Microbiological methods. Freeze-dried cultures of *Escherichia coli B* were cultivated in Friedlein's synthetic medium. Ammonium chloride was the sole nitrogen source and sodium lactate the carbon source. In the deficient media, ammonium chloride was used in 1.3×10^{-3} M (expt. B) or 1.0×10^{-3} M concentrations (expts .C and D). The cultivation methods and harvesting procedures have been reported earlier [14,15].

A typical nitrogen starvation experiment (expt. D) is presented graphically in Fig. 1. The amount of cells inoculated corresponded to about 20 mg of dry cells per litre of culture. The inoculum consisted of a suspension of cells grown for 16—18 h in Friedlein's medium, and washed once in nitrogen-free medium. Three h after the inoculation the dry weight of cells per litre of culture was about 90 mg. During the subsequent 90 min there was only a small increase; this period we call the starvation phase. This phase was interrupted by adding ammonium chloride to the culture. After this addition there was a second growth period. The glycogen per dry weight of cells in one litre of culture is recorded in the same figure. After collecting the cells of each sample by centrifugation, they were freeze-dried, and stored at −20°C until the material was weighed in for analysis.

Chemical methods. The glycogen was isolated by alkaline hydrolysis of the cells (30 % KOH, 100°C, 3 h), followed by precipitation with 66 % ethanol [14]. No shorter time

Table 1. Experiment A. Reproducibility of the methods used. For further details, see the text.

Sample	Molecular weight	Dissymmetry z
I	55.4×10^6	1.85
II	53.4	2.01
III	53.1	1.96
IV	50.0	1.93
V	51.6	1.92
VI	56.1	1.95
VII	54.9	1.99
Average	53.5×10^6	1.94

Table 2. Experiments B, C, and D.

Experiment	I Time after inoculation (min)	II Dry weight of cells per litre of culture (mg)	III Glycogen per litre of culture (mg)	IV II−III	V Molecular weight
B	120	39.8	2.6	37.2	27.8×10^6
	150	51.6	2.5	49.1	23.9
	300	174.0	26.0	148.0	78.2
	330	181.7	32.7	149.0	73.8
	330 addition of ammonium chloride				
	360	233.3	31.3	202.0	64.2
	405	313.8	23.8	290.0	82.2
	450	390.8	22.9	367.9	65.8
	480	454.3	20.0	434.3	66.6
C	270	122.6	27.0	95.6	79.1×10^6
	300	126.1	32.5	93.6	61.7
	330	134.5	36.2	98.3	72.6
	330 addition of ammonium chloride				
	420	217.1	41.8	175.3	53.7
	510	392.1	31.6	360.5	61.2
D	130	53.6	2.3	51.3	30.2×10^6
	150	70.1	3.1	67.0	25.7
	180	95.1	5.7	89.4	25.8
	270	119.1	20.8	98.3	52.3
	270 addition of ammonium chloride				
	360	257.8	18.5	239.3	76.6
	450	436.0	11.3	424.7	47.5
	540	562.8	9.6	553.2	37.4
	600	696.0	11.9	684.1	52.6

could be taken for the alkaline hydrolysis, nor could lower concentrations of the KOH be used if a low-nitrogen preparation was to be achieved. It was found necessary to perform the alkaline hydrolysis in new Pyrex tubes, since otherwise material was extracted from the glass, making it impossible to dissolve the glycogen completely. After the introduction of this modification, the molecular weight determinations were reproducible (see Table 1). The glycogen could be stored in neutral water solutions for several days at 0°C without undergoing any change in the molecular weight. The molecular weights of the bacterial

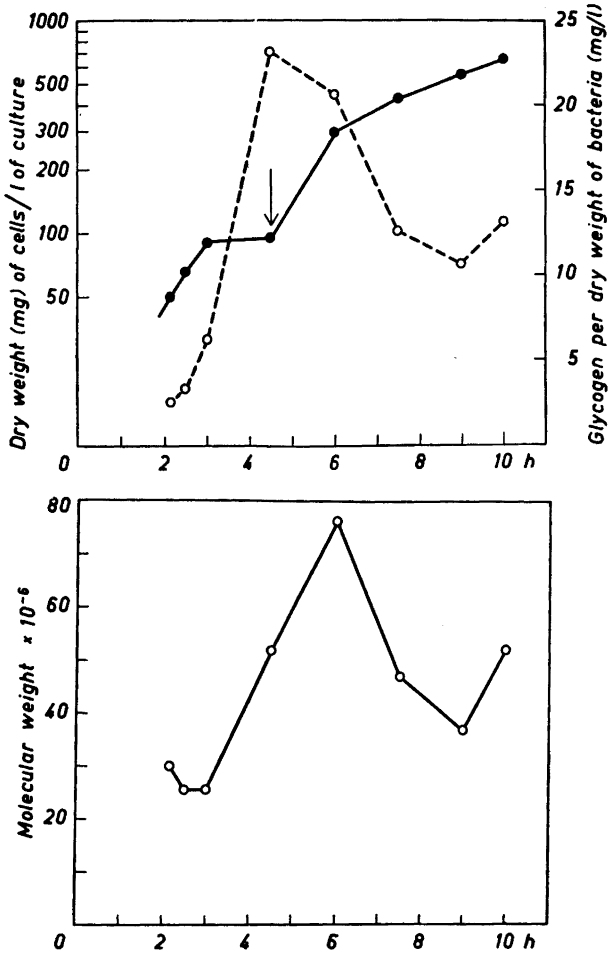

Fig. 1. Experiment D. Nitrogen-starved culture. The curve ● ——— ● represents the dry weight of cells per litre of culture (the glycogen content has been subtracted from the total weight). The arrow indicates the moment when ammonium chloride was added (see the text for further details regarding this experiment). The curve O — — — O represents the glycogen per litre of culture. In the lower part of the figure, the variation in molecular weight is shown.

glycogen isolated by the trichloro-acetic acid method used by Stetten *et al.*[9] were found to be considerably lower than those obtained by the alkaline isolation procedure.

In order to test the reproducibility of the methods used, the following experiment was carried out (expt. A). Cells from a 5-litre nitrogen-starved culture were harvested at 270 min after the inoculation, spun down and freeze-dried. The material was divided into eight equal parts. The glycogen was isolated separately from these samples by the methods described above. One part was taken for determination of the glycogen content of the cells; the molecular weights of the remaining seven samples of glycogen were then determined. The results are recorded in Table 1.

RESULTS

In determining the molecular weight, alkaline hydrolysis was found to be the most reliable method for the preparation of glycogen from *E. coli* cells (Table 1, expt. A).

During the accumulation of glycogen in the bacterial cells, the molecular weight of the glycogen increased considerably. When the glycogen content of the culture (calculated per dry weight of cells in one litre of culture) decreased during a subsequent utilization, the molecular weight decreased again (Table 2, Fig 1). This decrease was demonstrated most clearly in expt. D, in which the glycogen content of the culture reached lower values than in expts. B and C. The results are summarized in Table 2.

DISCUSSION

It is not possible to decide whether the glycogen isolated by one method is more "native" than that isolated by another. If a continuous change in the molecular weights is found during bacterial growth, however, extraction artefacts seem less likely.

On the basis of data obtained from isotope experiments (enzymatic degradation of ^{14}C-labelled rat liver glycogen), Stetten and Stetten [16,17] put forward the hypothesis that the glucose residues incorporated during glycogen synthesis may be added to pre-existing molecules, and preferentially attached to the end groups of these. This view was supported by the experiments reported earlier by Holme and Palmstierna [1,2], who investigated the glycogen metabolism of *E. coli B*. However, another explanation may be that the glycogen is inhomogeneous as regards its metabolic activity.

The possibility of obtaining accurate quantitative data on glycogen synthesis afforded by our system made it probable that molecular weight determinations on the glycogen would give valuable information. Our present results indicate that a correlation exists between the glycogen content of the bacteria and the molecular weight of this substance. However, the fact that the light-scattering method gives weight-average values, made a further analysis of the quantitative data impossible. It will probably be possible to obtain further information by fractionation of the glycogen.

In most cases there is no marked immediate decrease in glycogen per litre of culture after the addition of ammonium chloride to the nitrogen-starved cultures. A possible explanation may be that there has been a considerable overflow metabolism during the starvation period, *i.e.* low molecular intermediates have poured out into the medium [15,18]. These intermediates are possibly more easily utilized by the cells than the glycogen is. During this delay in glycogen utilization an increase in the molecular weight was observed. The explanation of this increase need not necessarily be that there has been a further synthesis of glycogen; it can just as well be due to the formation of larger molecules through the unification of smaller ones.

While this investigation was proceeding. Stetten *et al.*[9] published a work in which the light-scattering method was used for molecular weight determinations of liver and muscle glycogen from rat and rabbit. It was noted that the glycogens were metabolically inhomogeneous with regard to molecular weight.

The reproducibility of our results indicates that the nitrogen starvation technique offers definite advantages in the study of glycogen synthesis, and appears to be well suited for further investigation.

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