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Glycogen-like Polyglucose * in *Escherichia coli B* during the First Hours of Growth

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A method is described by which it is possible to estimate the mean dry weight of bacterial cells.

A polysaccharide occurring in *Escherichia coli B* is shown to be of a glycogen-like nature. This glycogen accumulated before the cells began to divide, and the glycogen content of the culture diminished when the nitrogen-containing cell constituents per litre of culture increased at the highest rate the culture conditions allowed.

It is shown that during the period of growth observed, the incorporation of ^{14}C from β-labeled lactate into the glycogen was smaller than could have been expected if all the glycogen had been synthesized from lactate in the medium only.

It is shown that the glycogen incorporated some carbon from carbon dioxide.

If is shown that glycogen could be synthesized from intracellular carbon sources as well as from carbon sources supplied with the medium.

The glycogen increased in the cells during the lag phase and decreased during the log phase in both glucose medium and broth, in the same way as in lactate medium. An increase of the same magnitude was noted in all culture volumes and at all cell concentrations used.

Escherichia coli B contains an alkalistable polyglucose as previously reported [1]. An alkalistable polysaccharide has also been observed in enteric bacteria by Levine *et al.*, detected by means of infra-red spectrophotometry [2]. Isolations of glycogen-like polysaccharides from *Mycobacterium tuberculosis* [3], *Neisseria perflava* [4] and *Bacillus megaterium* [5] have also been reported.

In the present paper the glycogen content of the cells was studied at different phases of growth. A very rapid increase in the glycogen content of the cells was noted to occur before the first cell divisions, followed by a decrease when the cells began to divide.

* For the sake of brevity called glycogen in the present paper.

BACTERIOLOGICAL METHODS

Media. The salt medium with sodium lactate as the sole source of carbon was that originally described by Friedlein [9]. The salt medium with glucose as the sole source of carbon was the one used by Hook and co-workers [10].

The broth was prepared in the usual way and buffered to pH 7.0 with 0.1 M phosphate buffer and sodium chloride was added up to a concentration of 0.5 g per litre. The medium was stored for 24 hours at 0 °C. The precipitate was filtered off and the medium sterilized in steam under pressure (1.5 kg/cm^2). This procedure was repeated twice. In this way it was possible to get a clear broth in which hardly any precipitate was formed during growth and during the cooling down of the culture.

The nitrogen-free medium was that used by Friedlein, from which the ammonium chloride was omitted. In the lactate- and nitrogen-free medium both ammonium chloride and lactate were left out. A completely nitrogen- and carbon-free medium was prepared by using this last-mentioned medium and filtering the air through an "Ascarite" filter, two flasks containing 50 % KOH and one flask containing saturated barium hydroxide. The air was dispersed by means of sintered glass discs.

Cultivation technique. The cultivation techniques are modifications of the techniques used by Cavanna *et al*[6]. Lyophilized cells, grown from one and the same culture of *E. coli B* were used in all the experiments. The cells were spread onto two agar slants and incubated for 24 hours at 37° C. Then the cells were suspended in 5 ml of culture medium. The suspension was inoculated into a 6 litre Erlenmeyer flask containing 4 or 5 litres of medium. The medium in this praeculture was of the same composition as that of the medium in the final culture. The praeculture was grown for 18 hours at 37° C and the cells were spun down at 0 °C in an International refrigerated centrifuge at 2 000 × g for 90 min. The cups were thoroughly rinsed, but not sterilized, before centrifugation. The loss of cells into the supernatant was too small to be estimated in a Beckman spectrophotometer, equipped with turbidimetric facilities. The cells were washed in fresh medium and suspended again in 200 ml of medium. The cell concentration was determined by counting the cells in the Bürker chamber under the phase contrast microscope. The final culture was inoculated with an appropriate amount of the suspension. The cultures were kept at 36.5–36.7 °C by means of a water-bath (volume 1 m^3) and shaken vigourously. The aeration current was filtered through tightly packed, sterilized cotton-wool and dispersed by sintered glass filters. The rate of aeration was 1 litre per minute and litre of medium as estimated by means of rotameters. The media were aerated for at least five minutes prior to inoculation. The air entering the praeculture was moistened by passing it through sterile, distilled water at 37 °C. From a culture of 5 litres, the loss of fluid from the praeculture did not exceed 0.2 litres in 18 hours.

Harvesting procedure. Aliquots of the culture were withdrawn by suction and the samples immediately cooled down to 4 °C by means of a sling of stainless steel tubing through which alcohol of −40 °C circulated. The time taken from the commencement of the suction to the finish of the cooling did not exceed 4.5 minutes, with the samples ranging from 1 to 12 litres. The samples called 0 were taken from the suspension used for inoculation. The samples were stored at 0 °C and centrifuged within 12 hours. It was found, however, that they could be stored for three days at 0 °C without any detectable change in the glycogen content occurring.

Determination of the mean dry weight of the cells. The volume of the cell suspension was determined, and the number of cells per ml estimated in a Bürker chamber under the phase contrast microscope. The cells were spun down at 0 °C and 2 000 × g for two hours in the afore mentioned centrifuge. In order to prevent the cells from whirling up again when the centrifuge slowed down, the bottoms of the centrifuge tubes were covered with a double layer of fine mesh stainless steel netting. The tubes were divided up into four chambers by means of baffles. The losses of cells were checked by estimating the amount of cells in the supernatant with the turbidimeter mentioned. (The supernatant was compared with a sample of the original suspension, diluted 25–50 times with fresh medium, to which formaldehyde was added to give a final concentration of approximately 0.3 %). The losses were lower than 2.4 % when the original suspension contained 10^8 to 5×10^8 cells per ml. When the cell concentration was higher than 5×10^8 cells per ml, the losses were too small to be estimated by the method used. All the data presented have been corrected for the losses of cells during centrifugation.

The cells were washed twice with fresh medium. The centrifugations were performed in an International refrigerated centrifuge typ PR-1 with a multispeed attachment (0 °C, 4 minutes, 24 000 × g at the bottom). The losses amounted to 3 000 cells per ml of washing fluid. This estimate was arrived at by counting the number of colonies produced on endo agar plates. The volume of the washing fluid was less than 60 ml. No corrections were introduced for these small losses.

The cells were washed in medium, since they lost appreciable amounts of ultra-violet absorbing substances into the washing fluid when they were treated with water or 0.9 % sodium chloride. This was especially the case with cells harvested during the last part of the lag phase and the first part of the log phase of growth. No losses of ultraviolet absorbing matter were observed when the cells were washed in fresh medium.

The washed samples were weighed in wet state and lyophilized. All analyses were performed on this freeze-dried material, with the exception of the nitrogen determinations. These determinations were performed on 15 to 25 mg of the material dried to constant dry weight over P_2O_5 at 90 °C *in vacuo*.

The difference between the wet weight and the constant dry weight was taken as medium water. All values presented were corrected for the amount of medium constituents, that is contained in the corresponding amount of medium.

The sum of the corrections amounted to between 8 and 15 % of the freeze dry weight.

The mean dry weight of the cells was calculated by dividing the corrected dry weight of the cell material per unit volume of culture by the number of cells per unit volume of culture.

All cultures were tested for purity by means of blood and endo agar cultures. In no case were any contaminations observed.

CHEMICAL METHODS

Isolation of the glycogen-like polysaccharide. Between 80 and 150 mg of the lyophilized material was treated with 0.1 ml of 30 % KOH per 10 mg of lyophilized material for three hours on a boiling water-bath. Immediately after the hydrolysis, three volumes of water and 8 volumes of 99 % ethanol were added. The precipitate was washed twice with 60 % ethanol and dried with ethanol and ether. When it was expected that the cells would contain small amounts of the polysaccharide only, they were treated with boiling ether prior to the alkaline hydrolysis, in order to remove most of the lipids which go down with the polysaccharide at the first precipitation with alcohol.

The polysaccharide obtained in this way is called here crude glycogen.

When deemed necessary, the crude glycogen was purified in the following way: 100 mg of the crude glycogen was dissolved in 1 ml of water, cooled down to 0 °C and precipitated again by adding glacial acetic acid to a final concentration of 80 %. This procedure was repeated twice. The precipitate was again dissolved and precipitated by adding alcohol to a final concentration of 60 %. The precipitate was dried with ethanol and ether. The glycogen purified by the acetic acid treatment is called here purified glycogen. Analyses of this glycogen are presented in the following.

The preparation and quantitative determination of glucose from the glycogen-like polysaccharide. The crude glycogen was submitted to hydrolysis in either 0.6 N HCl, or N H_2SO_4, for three hours on a boiling water-bath. No difference between the yields of glucose in the two kinds of hydrolysis could be observed. The glucose thus obtained was purified by passing the hydrolysate through a column of "Dowex 50" in the H^+-form and "Dowex 2" in the acetate-form. The columns had a length of 20 cm and an inner diameter of 0.7 cm. The ion exchange resins (40—100 mesh) were thoroughly washed with distilled water prior to use.

The purified glucose solutions were brought to dryness by heating to about 50° C in a strong air current.

Glucose (2, 4, and 10 mg in 10 ml of water) was passed through the columns, and 103 %, 99 % and 97 %, respectively, of the glucose were recovered.

All estimations of glucose were performed according to a modification of Disches' method [7].

Table 1. Isotopic dilution of glycogen in E. coli B.

Recovery of the glycogen (Chemical method) percentage	95.2	97.1	96.4	97.3
Recovery according to the dilution of the isotope, percentage	92.9	96.0	97.5	96.9

^{14}C-glycogen was added to samples of lyophilized cells (ranging between 50 and 100 mg) with a known content of glycogen. The glycogen was isolated and determined as glucose according to a modification of Dische's colorimetric method. The dilution of the isotope ranged between 10 and 60 per cent. Aqueous solutions of the hydrolyzed glycogen was spread directly on aluminium planchets and counted with a windowless gas flow counter, flushed with a helium-*iso*butane mixture. Since the layer of the material on the planchets did not exceed 0.03 mg/cm^2, no correction was made for self-absorption.

Protein isolation and nitrogen determination. Protein was isolated from the cells according to Schneider [8]. The nitrogen determinations were performed according to the micro-Kjeldahl method.

The isolation of glycogen and the determination of glucose checked by isotope dilution. In order to obtain ^{14}C-glycogen of bacterial origin, a culture was performed with sodium lactate, labeled in the β-carbon with ^{14}C, as the sole source of carbon. The isotopic lactate was synthesized by L. Reio and purified by ion exchange chomatography by S. Åqvist [15]. The culture volume was 5 litres, the initial cell concentration 5.5 $\times$ 10^8 cells per ml and the cultivation time 90 min. The concentration of the lactate was one-third of the concentration in the experiments mentioned in the following. The yield of glycogen was 13.1 % of the cells in the lyophilized state. The specific activity of the purified glycogen was 58.7 c.p.m. per microgram of glucose carbon. This ^{14}C-glycogen was used for determinations by the isotope dilution method. (Table 1.)

RESULTS

Analysis of the glycogen. Apart from glucose, no other sugar could be observed on the routine paper chromatograms (butanol saturated with water). The separation of glucose and galactose in this solvent is not satisfactory.

In order to exclude the presence of any sugar other than glucose in the polysaccharide, the purified glycogen was hydrolysed in N sulfuric acid for ten hours and chromatographed according to a modification of the method of Gardell [11], introduced by himself. (Butanol, propanol and water were used in the proportions 3:2:1 instead of in the proportions 4:1:1, and the column was heated to 40° C.) With this kind of hydrolysis, the polysaccharide sugar was destroyed to an extent of between 7.1 and 10.2 %. When hydrolysed and chromatographed in this way, 92.2, 91.7, and 91.3 % of the polysaccharide were recovered as glucose. If a correction is allowed for the amount of sugar destroyed during hydrolysis, this would mean that between 99 and 105 % of the polysaccharide was revocered after chromatography. No traces of mannose, galactose or any other sugar were found. It is possible, however, that there were other sugars besides glucose present in the polysaccharide, and that these sugars were selectively destroyed during hydrolysis, since the linkages in the polysaccharide might influence the effect of the acid on the sugar itself.

Table 2. The content of glycogen-glucose in E. coli B *at different times after inoculation.* (Friedleins sodium lactate medium. culture volume 40 litres.)

Exp.	Time after inoculation in minutes	Number of cells $\times 10^{-8}$, per ml	Mean dry weight of cells, mg $\times 10^{-10}$	Glycogen-glucose			Protein-nitrogen per litre of culture, mg
				per cell mg $\times 10^{-12}$	in per cent of dry weight	per litre of culture, mg	
A	0	1.3	1.5	3.5	2.3	0.5	1.8
	10	1.3	1.7	28.9	16.9	3.8	1.8
	20	1.3	1.9	34.9	18.3	4.6	2.0
	30	1.3	2.2	38.5	17.5	5.1	2.1
	50	1.3	2.3	48.3	21.1	6.4	2.2
	70	1.6	2.5	49.9	19.9	8.0	3.0
	90	1.9	2.5	46.3	18.5	8.8	3.8
	130	3.2	2.7	26.2	9.7	8.4	6.7
	180	6.7	2.3	8.5	3.7	5.7	14.1
	240	22.8	1.6	1.1	0.7	2.5	31.9
	360	52.3	1.4	1.3	0.9	6.6	73.2
	960	— **	—	—	1.3	12.2 *	—
B	0	1.3	2.4	4.3	1.8	0.6	3.0
	30	1.3	2.5	26.9	10.8	3.5	2.6
	60	1.4	3.6	38.2	10.6	5.2	3.6
	90	1.5	3.5	36.7	10.4	5.5	4.4
	120	2.8	3.0	17.1	5.7	4.7	7.3
	240	20.1	1.5	1.0	0.7	2.0	28.1
	360	43.7	1.3	1.1	0.8	4.8	56.8
	960	— **	—	—	0.5	6.2 *	—

* In experiment A no precautions were taken to prevent loss of water from the culture. In experiment B loss of water was prevented by moistening the air entering the culture.

** It was not possible to get a reliable estimate of the cell concentration due to scanty contrast in the cells.

When glucose was added to the hydrolysate, a single symmetrical peak was observed on the chromatogram. When galactose was added, two distinctly separated peaks were obtained.

The polysaccharide was partly degradable with β-amylase and gave the same reddish-brown colour with iodine as that obtained with rabbit and rat liver glycogen.

Due to the difficulty of obtaining a clear solution of the bacterial glycogen, even when purified glycogen was used, the specific optical rotation could not be determined exactly (*cf.* Ref.[2].) It lies somewhere between $+185°$ and $+200°$ (rat liver glycogen $+198°$ to $+200°$).

The polysaccharide had a slight reducing power prior to hydrolysis. After hydrolysis for ten hours in N sulfuric acid it gave a yield of 91 % of the theoretically expected value.

The phosphorus content of three different batches of purified glycogen was 0.8, 0.8, and 0.7 %, the nitrogen content 0.1, 0.15, and 0.3 %, respectively. These values are calculated on the ash-free polysaccharide. The ash content

was 2.4, 1.9, and 2.0 %, respectively. The carbon content of the ash-free polysaccharide was 43.7 ± 0.7 % (four determinations).

The glycogen content of the cells at different intervals after inoculation. The results from the cultures in sodium lactate medium are shown in Figs. 1 and 2, and in the Tables 2, 3 and 4.

The glycogen-glucose content of the cells increased very rapidly during the lag phase of growth. The increase was most pronounced during the first ten minutes after inoculation. The increase in glycogen accounted for most of the increase in dry weight of the cells during the first part of the lag phase.

In the time between ten minutes after inoculation and the time of the first cell divisions (70—90 min. after inoculation) the increase in glycogen-glucose per unit weight of cells was less pronounced. The glycogen-glucose per dryweight and litre of culture, still showed a rapid increase during the same period. This difference is due to the increasing synthesis of other cell constituents during the last part of the lag phase (*cf.* the protein per litre values, Table 2). During the log phase * the glycogen-glucose per cell decreased up to 240 minutes after inoculation, after which it remained constant at a value of the same magnitude as the zero time value. This was also the case with the glycogen-glucose per unit weight value. The glycogen-glycose per dryweight and litre of culture (in the following called total glycogen) also decreased up to 240 minutes after inoculation. The decrease coincided with the time at which the culture attained its highest cell division rate (Fig. 2). The total glycogen-glucose increased again when the cell division rate began to slow down (about 240 min. after the inoculation). This increase in total glycogen-glucose was due to an increase in the cell concentration, without any significant increase in the glycogen-glucose per cell.

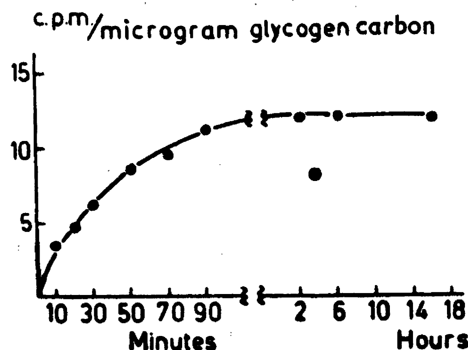

Fig. 1. In experiment A (Table 2) the cells were cultivated in Friedlein's medium in which the lactate was labeled in the β-carbon with ^{14}C. The specific activity was 32.2 c.p.m. per microgram of lactate carbon, as estimated by diluting a sample of the original, highly active sodium lactate. The radioactivity measurements were performed in the same way as described under Table 1. The ^{14}C-lactate used was to a slight extent contaminated with ^{14}C-alanine.

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* The log phase is here defined as follows: beginning when the first cell divisions are found and lasting until the cells have stopped dividing, as checked by counting the cells in the Bürker chamber under the phase contrast microscope.

Table 3. Glycogen-glucose of E. coli B *cells incubated into Friedlein's medium from which nitrogen source has been left out.*

Exp.	Carbon source	Time after inoculation, in minutes	Number of cells × 10^{-8} per ml	Glycogen-glucose in per cent of the dry weight
H	Lactate and CO_2	0 90	1.3 1.4	1.7 7.1
J	CO_2	0 60 90	1.0 1.0 1.0	1.3 3.8 3.5
K	None	0 90	1.6 1.7	2.1 1.4
L	Lactate No CO_2	0 90	1.2 1.2	1.2 9.3

In these experiments, Friedlein's lactate medium was used, but the usual source of nitrogen, ammonium chloride, was omitted. The carbon dioxide is that present in the air entering the culture. In experiments K and L the carbon dioxide was absorbed before the air entered the culture.

Incorporation of labeled lactate into bacterial glycogen. When sodium lactate, labeled in the β-carbon with ^{14}C, was used as the sole source of carbon, the specific activity of the glycogen carbon increased rapidly during the whole lag phase. Thereafter the specific activity remained constant at a value of about one-third of the specific activity of the substrate carbon (Fig. 1).

The glycogen content of the cells when cultivated in other media. When the cells were incubated in the Friedlein medium from which the nitrogen source had been omitted, they were still able to synthesize glycogen for at least 90 min. The glycogen-glucose content increased from 1.7 % to 7.1 % of the dry weight. The increase in glycogen-glucose was considerably lower when both the nitrogen source and the lactate were omitted, but there was still an increase (from 1.3 % at zero time to 3.5 % at 90 min.). If the air entering the culture was freed from carbon dioxide, and both the nitrogen and the lactate were excluded from the medium, no glycogen was formed by the cells. When lactate was present, and the carbon dioxide supply to the culture was prevented, the glycogen-glucose increased from 1.2 to 9.3 % of the dry weight (Table 3).

When the cells were cultivated in a glucose medium, the glycogen-glucose increased during the lag phase and decreased during the log phase in the same way as when the cells were grown in Friedlein's medium (Table 4, expt. G). The major difference consisted in a higher glycogen-glucose content of the cells at the moment of inoculation compared with cells grown in a lactate medium. The glycogen-glucose content of cells grown in broth also behaved in the same way (Table 4, expt. F). The increase in glycogen-glucose was however not so pronounced during the lag phase as in the lactate medium. This may be due to the difficulty of obtaining cells free from medium constituents tending to precipitate at the harvest.

Table 4. The glycogen-glucose content of E. coli B, *varying the size of the culture, the initial cell concentration and the composition of the medium.*

Exp.	Time after inoculation in minutes	Number of cells × 10^{-8} per ml	Glycogen-glucose in per cent of dry weight
C	0	1.0	2.1
	60	1.1	14.2
	360	—	2.2
D	0	10.5	2.1
	90	12.1	9.2
E	70	5.1 *	11.3
F	0	0.9	2.4
	60	0.9	8.0
	360	66.7	1.6
G	0	1.4	6.2
	60	1.8	12.1
	360	12.0	4.7

The cultures C, D, and E were performed in Friedlein's lactate medium. The size of the culture was 5 litres in exp. C and D. In exp. E the culture volume was 70 litres. This culture was performed in a stainless steel tank. Culture F was performed in a broth medium, culture G in a glucose medium. The culture volumes in both F and G was 5 litres.

The influence of cell concentration and culture volume on the increase in glycogen during the lag phase. The culture volume did not affect the increase in glycogen during the lag phase. Cultures performed in 5, 40, and 70 litres (Expts. A—E) all showed a glycogen increase of the same magnitude during the lag phase.

The initial cell concentration did not affect the increase in glycogen during the first 60 to 90 minutes after inoculation (Table 4, expts. C, D and E).

DISCUSSION

The observation that glycogen had accumulated in lactate, glucose as well as in broth cultures during the lag phase of growth, and that in all these media the glycogen decreased during the log phase, shows that the changes in glycogen were not due to specific actions of the various carbon sources. As shown in Table 2 and Fig. 2 there was a decrease in total glycogen during the first part of the log phase, coinciding with high rates of synthesis of the nitrogen-containing cell constituents. The lag phase may in respect of the glycogen be pictured as an endergonic phase of growth and the first part of the log phase as an exergonic phase.

During the last part of the log phase there was an increase in total glycogen, but there was no significant increase in the low amount of glycogen per cell.

* The initial cell concentration was 5.3 × 10^{8} cells per ml as estimated from the cell concentration in the praeculture.

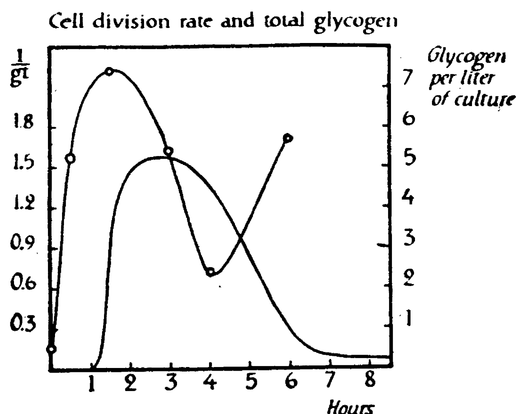

Fig. 2. The cell division rate was estimated by taking the reciprocal value of the mean generation time in hours (gt). Samples for counting were taken every 15 minutes until 6 hours after inoculation. After that the intervals were 40 minutes. The glycogen-glucose (in mg) per litre of culture is shown by the open circles. The rate of formation of nitrogen-containing compounds showed its maximum at the same time as the cell division rate was at its maximum.

It might be suggested that the small residual amount of glycogen left in the cells be considered as a primer for glycogen synthesis.

Hershey and Bronfenbrenner [12], observing the uptake of oxygen in relation to the phase of growth in *E. coli*, found that the uptake of oxygen per hour and gram of bacterial nitrogen was constant during all phases of growth. No observations were made during the first 150 minutes of growth. It is, however, evident that the oxygen uptake per unit weight of bacterial nitrogen and hour was the same at the beginning of the log phase as at the end of this phase. This means that the oxygen uptake was the same whether the synthesis of nitrogen-containing compounds proceeded at a high rate or at a low rate. It seems justifiable to conclude that the aerobic mechanisms functioned at the same rate throughout all phases of growth. Since the high rate of synthesis of nitrogen-containing compounds during the first part of the log phase was not accompanied by a high rate of oxygen consumption, the energy, necessary for these syntheses, may partly have been made available by anaerobic breakdown of carbohydrate. An auxiliary source of this carbohydrate was the glycogen accumulated during the lag phase.

The experiments C and D (Table 4) were performed in order to rule out the possibility that the decrease in glycogen during the first part of the log phase was caused by anaerobic conditions in the medium. Anaerobic or semi-anaerobic conditions might have been induced by high cell concentrations. If a high cell mass per unit volume of culture were to produce oxygen deficiency in the medium, this would cause an inhibition of the glycogen synthesis from lactate. No significant lowering of glycogen per unit weight of bacteria was observed, even though the initial cell concentration was increased ten times. Furthermore, preliminary experiments have shown that it was possible to diminish the aeration to 100 ml per minute and litre of culture, without any changes in

the length of the lag phase and the mean generation time occurring. These results seem to exclude the possibility that the decrease in glycogen during the first part of the log phase was caused by oxygen deficiency in the medium.

Roberts *et al.*[13], observing the incorporation of ^{14}C from various sources, concluded that in the case of proliferating *E. coli* cultures, the tricarboxylic acid cycle furnishes 50 % of the carbon required for protein synthesis, but is relatively unimportant as an oxidative mechanism. They express the view that the function of the tricarboxylic acid cycle is mainly a synthetic one [14] in this case. It might be suggested that the energy derived from the oxidative breakdown is sufficient for the syntheses during the lag phase, but that the capacity of the cycle is insufficient to furnish the carbon for the syntheses and the energy required during the first part of the log phase. The energy output from the cycle may be less at this time, due to the disappearance of considerable amounts of tricarboxylic acid components in the form of nitrogen containing compounds. This would imply that less energy-rich bonds were produced by the cycle, and consequently the situation would be favourable for the formation of these compounds by means of glycolysis. It should however not be overlooked that there may be other ways of oxidative breakdown in *E. coli* than that of the tricarboxylic acid cycle.

An attempt was made to get some more information about the oxygen consumption of cells in the lag phase by the Warburg "indirect" manometric method. No reproducible results could be obtained.

In the experiment performed in ^{14}C-lactate medium, the specific activity of the glycogen carbon showed a very sharp increase during the lag phase and thereafter remained at a level of about one-third of the specific activity of the lactate carbon in the medium. If every glucose molecule in the glycogen had been synthesized from two lactate molecules only, the specific activity of the glycogen carbon would have shown a still sharper increase during the lag phase, and would have reached approximately the same specific activity during the log phase as that of the medium lactate. The dilution of the glycogen carbon during the lag phase may at least in part be ascribed to dilution from cellular carbon already present at the moment of inoculation. That such was the case is indicated by the results presented in Table 3, which show that glycogen was synthesized to some extent, even when there was no carbon source except CO_2 in the medium. Hammarsten, Palmstierna, Reio and Åqvist [15] have shown that the glycogen in cells praecultured for 18 hours in β-labeled ^{13}C-lactate medium contained increasing amounts of ^{13}C during the lag phase when cultivated in a medium free from ^{13}C. This proves that the glycogen carbon was diluted by carbon of cellular origin during this phase.

A synthesis of glycogen from internal carbon sources was made probable also by the experiments described in Table 3. The role of carbon dioxide in this synthesis was also made evident. If carbon dioxide was available a synthesis of glycogen from cellular carbon sources occurred (expt. J), in this case in the absence of the organic carbon source. No such synthesis occurred if both carbon dioxide and the organic carbon source were excluded, indicating that the mechanism of the synthesis of glycogen from cellular carbon was sensitive to carbon dioxide deficiency. The result that the glycogen synthesis

was uninhibited if lactate but no carbon dioxide was provided, may be explained by a production of carbon dioxide from lactate-carbon (expt. L).

Ehrensvärd and Palmstierna [16] have shown that certain amounts of ^{14}C were incorporated into bacterial glycogen when the culture was aerated with $^{14}CO_2$-containing air. This finding agrees with the results obtained by Hastings *et al.*[17] when working with rats. The incorporation of CO_2-carbon does not explain the low specific activity of this substance when grown on β-labeled lactate, since the CO_2 was incorporated into C_3 and/or C_4. The β-carbon from lactate may be expected in the positions C_1 and C_6.

The observation that glycogen had accumulated to an approximately equal extent in lactate, glucose or broth cultures during the lag phase, and that in all of these media the glycogen decreased during the first part of the log phase, shows that the changes in glycogen were not due to a specific action of the lactate.

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