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Changes in Glycogen and Nitrogen-containing Compounds in *Escherichia coli B* during Growth in Deficient Media

I. Nitrogen and Carbon Starvation

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When *Escherichia coli B* was cultivated in media deficient either in the nitrogen or in the carbon source, there was no increase in the nitrogen-containing compounds in the cells during the phase of constant cell concentration, which was caused by exhaustion of the limiting factor.

During this starvation phase an increase of glycogen in the cells was observed.

During the phase of rapid cell division following the addition of the limiting factor in the nitrogen-deficient culture with sodium lactate as the carbon source, the glycogen per litre of culture decreased rapidly.

When the cells were cultivated in nitrogen-deficient media, substances causing an absorption in the ultra-violet region poured into the medium. During the period of growth following the addition of the nitrogen source, the ultra-violet absorption of the culture fluid decreased rapidly.

In earlier experiments it was shown that the total glycogen in *Escherichia coli B* cultures increased considerably during the lag phase of growth, and decreased abruptly when the cells began to divide[1]. By the use of a synthetic medium deficient in one essential nutrient it is possible to arrest cell division after a certain number of generations in the logarithmic phase of growth[2]. In this paper experiments are described in which this technique is used in order to obtain additional information regarding glycogen synthesis in non-dividing cells. The experiments also include the addition of the limiting factor after a certain period of starvation, as well as observations concerning the phase of cell division that followed.

METHODS

Media. The medium used in all experiments but one was Friedlein's synthetic sodium lactate medium [3]. In one experiment a medium with glucose as the carbon source was used [4]. The nitrogen source was ammonium chloride in all experiments. Praecultures were grown in these media. In the final cultures, media of the same composition as in the praecultures were used, except that they were deficient either in the carbon or in the nitrogen source. In the experiments in which the nitrogen source was the limiting factor, the initial concentration of ammonium chloride was 0.75×10^{-3} M. In the experiment with the carbon source as the limiting factor, the initial sodium lactate concentration was 1.67×10^{-2} M.

Cultivation technique. Praecultures were grown as previously described [1]. The final cultures were inoculated with an amount of cells sufficient to give a concentration of about 10^{8} cells per ml. Prior to inoculation the cells were washed once with the deficient medium. One and a half hour after the cells had ceased dividing in the nitrogen-deficient cultures, and four hours later in the carbon-deficient one, a quantity of the limiting factor was added in order to give approximately the same concentration of those factors as in the complete media, *e. g.* 0.02 M in respect of ammonium chloride and 0.1 M in respect of sodium lactate. In all other respects the techniques were the same as previously described.

Chemical methods. The isolation of glycogen and the estimation of the glycogen-glucose were performed as described in a preceding article [1]. The trichloro-acetic acid (TCA) extractions were performed according to Schneider [5]. The "hot TCA"-extraction was always performed on the material already TCA-extracted in the cold. The micro-Kjeldahl method was used for the nitrogen determinations.

Table 1. Experiment A. Nitrogen starvation. Carbon source: sodium lactate.

Time after inoculation in min.	Number of cells per ml $\times 10^{-8}$	Dry weight of cells per litre of culture (mg)	Dry weight per cell (mg $\times 10^{-10}$)	Glycogen-glucose % of dry weight	Glycogen-glucose per litre of culture (mg)	Glycogen-glucose per cell (mg $\times 10^{-12}$)	Cold TCA-N % of dry weight	Cold TCA-N per litre of culture (mg)	Cold TCA-N per cell (mg $\times 10^{-12}$)	Hot TCA-N % of dry weight	Hot TCA-N per litre of culture (mg)	Hot TCA-N per cell (mg $\times 10^{-12}$)	Protein-N % of dry weight	Protein-N per litre of culture (mg)	Protein-N per cell (mg $\times 10^{-12}$)
0	0.75	13.5	1.8	2.1	0.3	3.8	0.6	0.1	1.1	2.6	0.4	4.8	10.1	1.4	18.3
15	0.75	18.2	2.4	5.0	0.9	12.1				2.3	0.4	5.5	7.1	1.3	17.2
30	0.8	18.9	2.4	8.8	1.7	20.8				2.5	0.5	5.9	7.0	1.3	16.5
60	1.1	26.3	2.4	8.8	2.3	21.1				2.8	0.7	6.7	7.2	1.9	17.1
90	1.5	38.6	2.6	8.4	3.2	22.0	0.6	0.2	1.6	3.2	1.2	8.1	7.6	2.9	19.5
140	2.7	79.5	3.0	6.1	4.8	17.9				3.3	2.6	9.7	7.8	6.2	23.1
180	4.5	111.4	2.5	9.3	10.4	23.1				3.1	3.5	7.8	7.3	8.2	18.2
220	4.6	111.1	2.4	12.2	13.6	29.5	0.5	0.6	1.3	3.0	3.3	7.2	7.0	7.8	17.0
240	5.0	119.1	2.4	13.6	16.2	32.4	0.6	0.7	1.4	2.6	3.1	6.1	7.0	8.3	16.6
260	4.9	119.0	2.4	15.3	18.2	37.2				2.5	3.0	6.1	6.9	8.3	16,8
265	addition of ammonium chloride.														
270	5.3	124.8	2.4	13.8	17.2	32.4	0.7	0.9	1.6	2.6	3.3	6.1	6.8	8.4	15.9
280	5.7	132.6	2.3	13.0	17.3	30.4				2.7	3.6	6.3	7.1	9.4	16.5
295	6.8	152.9	2.3	9.9	15.2	22.3	0.6	1.0	1.5	3.0	4.6	6.8	7.3	11.2	16.5
325	8.5	190.0	2.2	7.5	14.2	16.7	0.6	1.2	1.4	3.4	6.4	7.6	7.9	14.9	17.5
355	12.0	295.0	2.5	3.5	10.4	8.7	0.6	1.8	1.5	3.5	10.3	8.6	8.8	25.8	21.5
475	—	833.2	—	0.4	3.5	—	0.6	5.4	—	3.1	25.7	—	9.3	77.2	—
630	—	1 224.0	—	0.6	7.5	—				2.9	35.3	—	9.7	118.4	—

The cells were washed with fresh medium free of the nitrogen source. TCA: trichloro-acetic acid.

RESULTS

Nitrogen starvation. In the nitrogen-deficient cultures (expts. A, C and D), the lag phase was of the same duration as in the complete media. After an initial multiplication period, the cells ceased dividing on account of the deficiency in the medium (Fig. 1, Tables 1, 3 and 4).

In order to obtain a cell concentration of about 5×10^8 cells per ml during the starvation phase, the initial concentration of ammonium chloride in the medium had to be 0.75×10^{-3}M.

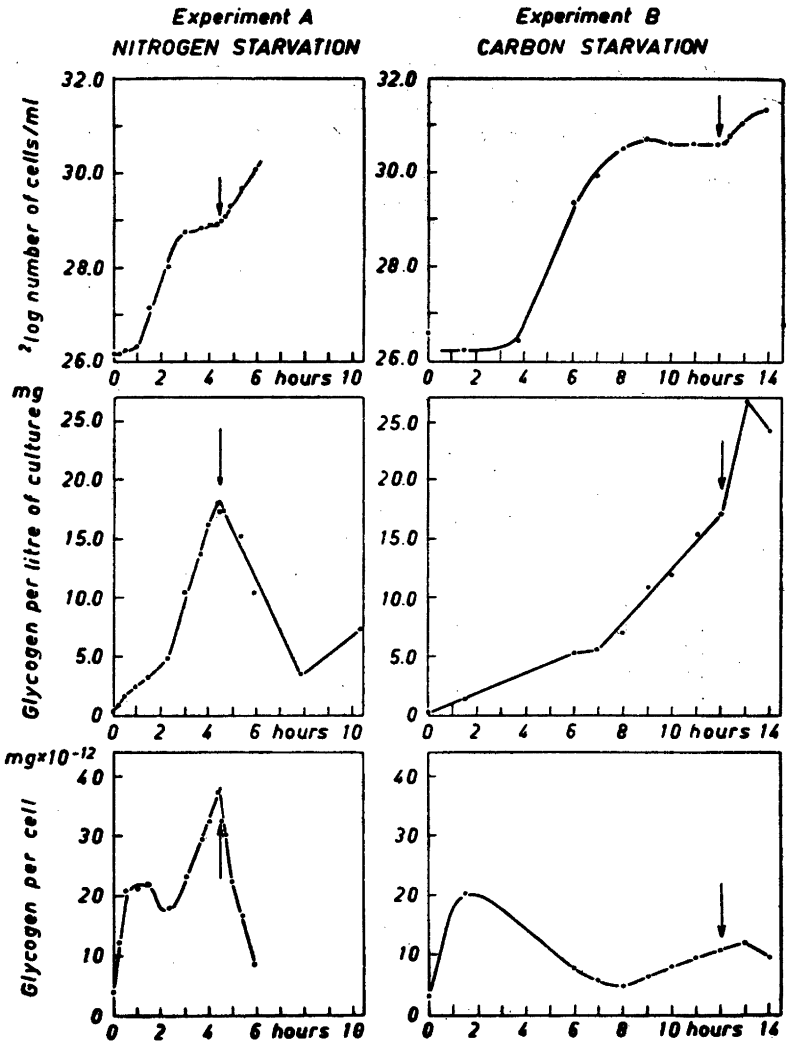

Fig. 1. Experiments A (nitrogen starvation) and B (carbon starvation). The arrows indicate the moment when ammonium chloride and sodium lactate respectively were added.

In one experiment (D, Table 4) it was noted that the glycogen content of the cells had reached a value near maximum in about two hours from the beginning of the starvation phase.* Consequently, when the starvation phase had lasted for that period in expts. A and C, ammonium chloride was added to obtain a concentration of 0.02 M, in order to initiate a second multiplication phase with the cells loaded with glycogen.

After the addition of ammonium chloride, the cells began to divide immediately (Fig. 1).

Glycogen per cell. The glycogen per cell increased during the lag phase and decreased during the first part of the logarithmic phase of growth in the same way as when the cells were cultivated in a complete medium (Fig. 1). However, when the division rate began to decrease, the glycogen per cell increased again. This increase continued linearly during the starvation phase until ammonium chloride was added. In experiment A, where lactate was the carbon source, the glycogen per cell increased from 17.9 to 37.2 $\times 10^{-12}$ mg (Table 1). The same course of development was noted in expt. C, with glucose as the carbon source, and the corresponding figures for the glycogen increase per cell were 26.0 and 75.7 $\times 10^{-12}$ mg, respectively, showing as in earlier experiments that the amount of glycogen was higher in cultures with glucose as the carbon source (Table 3). In both experiments (A and C) the addition

Table 2. Experiment B. Carbon starvation. Carbon source: sodium lactate.

Time after inoculation in min.	Number of cells per ml $\times 10^{-8}$	Dry weight of cells per litre of culture (mg)	Dry weight per cell (mg $\times 10^{-10}$)	Glycogen-glucose % of dry weight	Glycogen-glucose per litre of culture (mg)	Glycogen-glucose per cell (mg $\times 10^{-12}$)	Cold TCA-N % of dry weight	Cold TCA-N per litre of culture(mg)	Cold TCA-N per cell (mg $\times 10^{-12}$)	Hot TCA-N % of dry weight	Hot TCA-N per litre of culture(mg)	Hot TCA-N per cell (mg $\times 10^{-12}$)	Protein-N % of dry weight	Protein-N per litre of culture (mg)	Protein-N per cell (mg $\times 10^{-12}$)
0	1.0	12.0	1.2	2.1	0.3	2.5									
90	0.7	19.1	2.6	7.8	1.5	20.3	5.1	1.0	13.2	1.8	0.3	4.6	8.0	1.5	20.7
225	0.9														
360	7.0	133.5	1.9	4.1	5.4	7.7	2.6	3.5	5.0	1.8	2.3	3.4	8.6	11.4	16.3
420	10.0	212.4	2.1	2.7	5.7	5.7	2.2	4.7	4.7	2.0	4.1	4.1	8.9	18.9	18.9
480	15	250.6	1.7	2.8	7.0	4.7	2.4	6.1	4.1	1.6	3.9	2.6	9.0	22.5	15.0
540	17	283.7	1.7	3.9	10.9	6.4	2.5	7.0	4.1	1.5	4.3	2.6	9.1	25.7	15.1
600	16	276.2	1.7	4.6	12.8	8.0	2.3	6.4	4.0	1.7	4.6	2.9	9.5	26.4	16.5
660	16	272.2	1.7	5.7	15.5	9.7	2.1	5.7	3.6	1.6	4.3	2.7	8.9	24.3	15.2
720	16	267.9	1.7	6.4	17.2	10.7	2.2	5.8	3.6	1.4	3.8	2.4	8.8	23.6	14.8
721	addition of sodium lactate														
735	16														
745	18														
780	22	388.4	1.8	6.9	26.8	12.2	1.0	3.7	1.7	2.4	9.4	4.3	8.4	32.8	14.9
840	26	466.9	1.8	5.4	25.2	9.7	0.9	4.0	1.5	2.5	14.8	5.7	8.8	41.2	15.9

The cells were washed with fresh medium free of the carbon source.

The cold TCA-N values are corrected for the amount of nitrogen originating from the washing fluid.

* The starvation phase is considered to have begun when the cell division rate decreases owing to a deficiency in the medium.

Table 3. Experiment C. Nitrogen starvation. Carbon source: glucose.

Time after inoculation in minutes	Number of cells per ml × 10^{-6}	Dry weight of cells per litre of culture (mg)	Dry weight per cell (mg × 10^{-10})	Glycogen-glucose % of dry weight	Glycogen-glucose per litre of culture (mg)	Glycogen-glucose per cell (mg × 10^{-12})
0	1.2	19.0	1.6	6.6	1.3	10.4
60	1.2	23.9	2.0	13.4	3.2	26.7
150 *	1.7	51.1	3.0	11.0	5.6	33.0
300	2.8	101.4	3.6	21.0	21.2	75.8
305	addition of ammonium chloride					
375	5.9	207.1	3.5	11.9	24.6	41.6

* After 200 minutes the number of cells remained constant at 2.8 × 10^6 cells per ml.

of ammonium chloride caused an immediate and rapid decrease in glycogen per cell.

T o t a l g l y c o g e n. In experiments performed in complete media, glycogen per dry weight and litre of culture (total glycogen) began to decrease two hours after inoculation. In the deficient cultures the starvation phase had already begun at the time when this decrease was expected and the total glycogen began instead to increase at this point. This increase was linear and continued during the starvation phase.

After the addition of ammonium chloride the total glycogen decreased rapidly in the culture with lactate as its carbon source, showing that the glycogen synthesis was too small to compensate for the utilization. The lowest figure was noted at the beginning of the stationary phase (3.5 mg per litre o culture). The slight increase in total glycogen that followed in this experiment was primarily due to the increase in the cell mass (Table 1). The culture with glucose as its carbon source (expt. C) did not show the same immediate decrease in total glycogen after the addition of ammonium chloride, but the rate of increase slowed down (Table 3).

The protein-nitrogen per cell decreased slightly at the beginning of the starvation phase, and then remained constant (Table 1).

Ultra-violet absorption of the culture fluid. In cultures grown in an unmodified Friedlein's medium, the UV absorption of the cell-free culture fluid never exceeded an extinction of 0.15 at 260 mμ (read against fresh medium, 1 cm cuvettes). The case was the same in the carbon-starved culture (expt. B). In nitrogen-starved cultures grown in lactate medium, as described above (expt. A), substances poured from the cells into the medium, causing an absorption in the UV region (Figs. 2 and 3). At 260 mμ the absorption increased from 0.15 at 90 min. after inoculation to 0.6 at 5 hours after inoculation. By that time the starvation phase had lasted for about 2 hours (Fig. 2). When the ammonium chloride was added, the UV absorption of the culture fluid decreased rapidly. The same results were obtained in three different experiments, two in 5 litre cultures, and one in a 200 ml culture.

Carbon starvation. One carbon-deficient culture (expt. B) was also investigated (Fig. 1, Table 2). In lactate-deficient media there was a considerable increase in the duration of the lag phase when the sodium lactate concentration

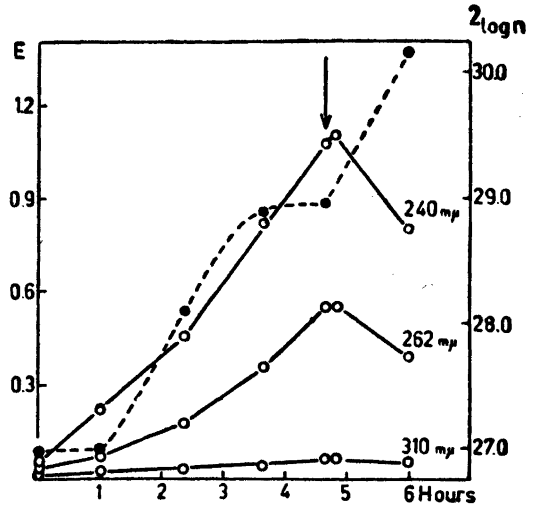

Fig. 2. Ultra-violet absorption of the cell-free medium in a nitrogen-starved culture. Broken curve: ^{2}log number of cells per ml. Unbroken curves: specific absorption (E) of the medium. The arrow indicates the moment when ammonium chloride was added.

was lower than 3.0×10^{-2} M. The initial concentration of lactate in expt. B was 1.67×10^{-2} M, giving a lag phase of about three hours' duration. The cell concentration during the starvation phase reached rather a high level, 1.6×10^9 bacteria per ml, which was about three times as much as the cell concentration during this phase in the nitrogen-starved cultures. The period of constant cell

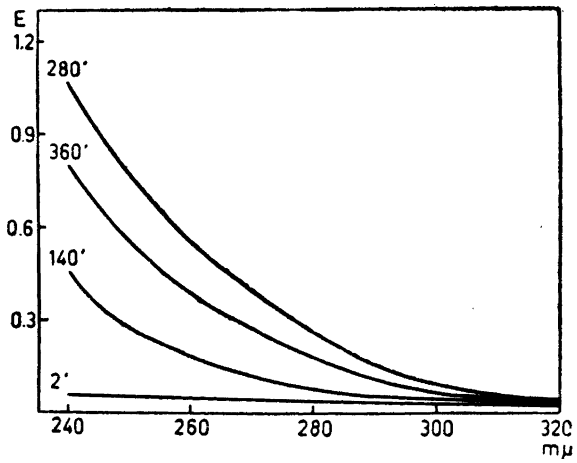

Fig. 3. The same experiment as in Fig. 2. Absorption curves obtained at different times, indicated by the figures at the end of each curve. The extinction was determined at intervals of 5 mμ except in the region 250—270 mμ, where the intervals were 2 mμ.

Table 4. Experiment D. Nitrogen starvation. Carbon source: sodium lactate. No addition of the limiting factor.

Time after inoculation in minutes	Number of cells per ml $\times 10^{-8}$	Dry weight of cells per litre of culture (mg)	Dry weight per cell (mg $\times 10^{-10}$)	Glycogen-glucose % of dry weight	Glycogen-glucose per litre of culture (mg)	Glycogen-glucose per cell (mg $\times 10^{-12}$)
0	1.1	14.3	1.3	1.9	0.3	2.5
90	1.1					
140	2.1	69.3	3.3	5.4	3.7	17.7
240	6.0					
300	6.3	96.5	1.5	18.2	17.5	27.8
540	5.7 *	117.5	2.1	21.4	25.2	44.1

* Difficult to count in the Buerker chamber because of the scanty contrast.

concentration due to starvation in experiment B was not interrupted as early as in the nitrogen-starved cultures. The time chosen in each case corresponded roughly to that of the lag phase.

After the addition of sodium lactate (12 hours after inoculation) there was a short lag before the cells began to divide (Fig. 1). The increase in cell numbers was small, and the division rate low, during the period of multiplication that followed, probably because the cell concentration was near its maximum value in the medium used. Two hours after the addition, the cell concentration was 2.6×10^9 bacteria per ml.

Glycogen per cell. The glycogen per cell reached a value of about 20×10^{-12} mg during the lag phase (Table 2). During the logarithmic phase it decreased to a minimum value of 4.7×10^{-12} mg eight hours after inoculation. At this point the division rate slowed down, due to the deficiency in the medium, and the glycogen began to increase again. Compared with the nitrogen-starved cultures, this increase proceeded at a very slow rate, the glycogen per cell amounting twelve hours after inoculation to 10.7×10^{-12} mg. Since the dry weight per cell was constant during the whole starvation phase, the same rate of increase was found in glycogen per mg of dry cells. During the first hour after the addition of sodium lactate, the glycogen per cell showed a slight increase, followed by a decrease.

The "per cell" values do not of course account for different types of cells that may be present in a culture. No attempt has been made as yet to study more closely the differences between cells belonging to the same population.

Total glycogen. Glycogen per dry weight and litre of culture increased throughout the lag, logarithmic, and starvation phases in approximately the same way as in the nitrogen-starved cultures. The rate of increase, however, was much slower (Fig. 1).

In spite of the low content of glycogen in the carbon-starved cells, the values of total glycogen are similar to those in the nitrogen-starved cultures. This is due to the fact that the dry weight of cells per unit volume during the starvation phase was about 2.5 times greater in the carbon-starved culture (B) than in the nitrogen-starved cultures.

After the addition of lactate in expt. B, the total glycogen increased considerably within the first hour, followed by a decrease during the second hour.

Influence of carbon dioxide. Carbon deficient cultures (lactate starvation) were aerated with an air-CO_2-mixture. One per cent CO_2 increased the rate of glycogen synthesis 1.8 times and 4 per cent CO_2 increased the rate 2.5 times that in experiment B (aerated with common air).

DISCUSSION

Several authors have reported experiments dealing with the chemical composition of bacteria cultivated in deficient media[6-8]. When cultivated in media with high carbohydrate and low nitrogen content, *Escherichia coli* and *Aerobacter aerogenes* showed a high polysaccharide content in the stationary phase[7].

Dagley and Dawes[8] determined the total polysaccharides of *E. coli* cells cultivated in deficient media. The interpretation of their results is rendered difficult because of the inhomogeneity of the substances determined. Evidently the polysaccharide material that accumulates during prolonged nitrogen starvation represents different kinds of carbohydrates which may be more or less actively participating in the metabolism.

In a later article, Dagley, Dawes and Morrison[9] describe experiments with *Aerobacter aerogenes*, in which pyruvate was determined in the medium under different conditions of growth.

As reported previously[1], the glycogen accumulation and utilization seems to be correlated to the synthesis of the nitrogen-containing compounds in the

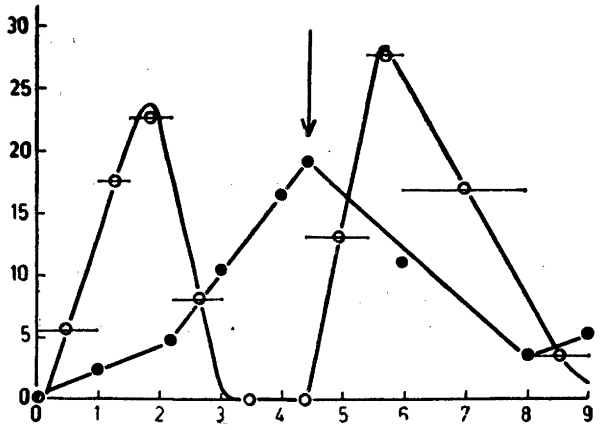

Fig. 4. Total glycogen and rate of protein synthesis in a nitrogen-starved culture. Abscissa: Time in hours. Ordinate: Glycogen per litre of culture in mg and *per cent increase in protein-nitrogen per litre of culture in 10 minutes. The arrow indicates the moment when ammonium chloride was added. The net synthesis of protein was arrived at by estimating the increase in protein nitrogen per litre of culture in per cent of the preceding value. The curve* O——O *was drawn through the midpoint of these time intervals. The curve* ●——● *represents the glycogen per litre values in mg. The curves show that the glycogen increased relatively slowly during the time when the net synthesis of protein showed its first maximum, and that the net synthesis of glycogen increased at a higher rate when there was no protein synthesis due to nitrogen starvation. After the addition of ammonium chloride, the net synthesis of protein increased very rapidly. The glycogen decreased sharply at the same time.*

cells. This view is supported by our present results. In the experiments presented in this paper, as well as in those previously reported a low net synthesis of nitrogen-containing compounds was accompanied by a high net synthesis of glycogen. During a phase of growth in which the synthesis of nitrogen-containing compounds was limited by nitrogen starvation (the starvation phase), glycogen accumulated rapidly.

The high rate of synthesis of nitrogen-containing compounds observed immediately after the addition of ammonium chloride was accompanied by a sharp decrease in the total glycogen in the culture, which also lends support to the view expressed. (Fig. 4.) In one experiment described by Dagley *et al.*[9], the addition of the nitrogen source to a nitrogen-starved culture caused an arrest in the pyruvate production from the cells. This may be explained by a high rate of utilization of the pyruvate, presumably for the synthesis of protein.

The fact that there was no lag before the cell concentration began to increase logarithmically after the addition of ammonium chloride shows that the cells were well prepared for cell division.

The material that poured into the medium during nitrogen starvation causing an absorption in the UV region (Fig. 2), was probably not of protein or nucleic acid origin. The fact that these compounds were dialysable to at least 90 % as measured by the absorption, showed that the UV absorption in the medium was not caused by non-dialysable protein or polynucleotides from autolysed cells. Furthermore, no maximum in the absorption at 260 mμ was recorded (Fig. 3), which would have been the case if cellular material of nucleic acid origin had been released to the medium.

The immediate decrease in the UV absorption in the medium after the addition of ammonium chloride shows that as soon as a nitrogen source was available, the cells were able to take up, or possibly, destroy the substances causing the absorption.

During the starvation phase in the carbon-deficient culture (expt. B) there was no increase in nitrogen-containing compounds (cold and hot TCA fractions, and protein). There was in fact a slight decrease (Table 2). In spite of the low concentration of the carbon source in the medium, there was still an accumulation of glycogen. The reactions involved in cell division and in the synthesis of the nitrogen-containing compounds are apparently more sensitive to a lowering of the concentration of the organic carbon source than is the synthesis of glycogen.

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