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Continuous Culture Studies on Glycogen Synthesis in *Escherichia coli B*

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The correlation between the synthetic rates of nitrogen-containing compounds and of a nitrogen-free substance, glycogen, has been studied in *Escherichia coli B* by the continuous culture technique. The nitrogen source was used as the growth-rate-limiting factor. This kind of limitation did not reduce the synthetic rate of the glycogen in the cells. The net synthetic rate of glycogen was increased by a reduction in the synthetic rate of the nitrogen-containing compounds.

The possibility of maintaining steady-state growth in bacterial cultures was demonstrated in 1950 by Monod [1] and Novick and Szilard [2,3]. Since then a great number of continuous culturing devices have been described (for examples, see Refs. [1–7]), and some experimental work has been reported. Monod and Cohn [8] used the technique in studies on adaptive enzymes, and Novick and Szilard [2,9–11] demonstrated its usefulness in studies of bacterial genetics and physiology. Herbert and collaborators [5], using a 20-litre pilot-plant type apparatus, pointed out the advantages of the technique in industrial fermentation.

A continuous culture system consists in general of a growing bacterial culture, which is fed with a continuous flow of liquid, sterile nutrient medium at a constant rate. The culture volume is kept constant by removal of the bacterial suspension at the same rate as fresh medium enters the culture.

The increase of the bacterial mass in any exponentially growing culture may be expressed by

$$\frac{dx}{dt} = \mu x$$

where x is the dry weight of organisms per unit volume at time t and μ is the specific growth rate. If the flow-rate of fresh medium fed into a continuous culture is called f, the dilution rate, D, will be f/v, where v is the culture volume. In this case the expression for the increase in bacterial mass in the growth vessel is

$$\frac{dx}{dt} = \mu x - Dx$$

Equilibrium will be obtained, if $\mu = D$, *i.e.* if the flow-rate is equal to the specific growth rate. By utilizing the fact that the growth rate in a bacterial culture may be limited by reducing the concentration of one of the essential nutrients in the medium, it is possible to achieve equilibrium over a wide range of growth rates. While the concentration of one of the essential nutrients is low, all others are in excess. The low-concentration nutrient is called the limiting or controlling factor. The concentration of this factor is almost instantaneously reduced when the medium is mixed with the culture, and reaches a level which limits the growth rate. It can be shown that this system tends to attain a self-stabilizing equilibrium, where the growth rate is determined by the flow-rate of the incoming medium, and the bacterial mass is determined by the concentration of the limiting factor.

In equilibrium, the synthetic rate of every component of the cell is equal to the dilution rate, which in turn can be regulated by the experimenter at will to any values that do not exceed the maximum value of μ, *i.e.* the maximum synthetic capacity of the bacterium. Hence it should be clear that the continuous culture method provides definite advantages in the study of synthetic functions of bacteria, since the determinations of the rates of various processes are reduced to measurements of steady-state concentrations.

During continuous culture experiments with *Escherichia coli B* it was noted that the nature of the limiting factor had a profound influence on the chemical composition of the cells. Special attention was paid to the glycogen content of the bacteria. When the nitrogen source was used as the limiting factor, considerable variations in the glycogen content of the cells were noted at different growth rates.

In previous experiments in which conventional cultivation techniques were used Palmstierna [12] found that glycogen accumulated during the lag phase of growth in cultures of *E. coli B*. In collaboration with Palmstierna a number of experiments were carried out, the object of which was to throw light on different aspects of the glycogen synthesis in this bacterium [13–16].

In the experiments described in the present paper, the continuous culture technique has been used in a study of the correlation between the synthetic rates of glycogen and of nitrogen-containing compounds in *E. coli B*.* When the nitrogen source was used as the limiting factor, the synthetic rate of the nitrogen-containing compounds was selectively reduced. The rate of glycogen synthesis was not reduced by this limitation, and it attained its highest values at the lowest synthetic rates of the nitrogen-containing compounds. The main product of synthesis that accumulated inside the cells during nitrogen deficiency appeared to be glycogen.

METHODS

Continuous culture apparatus. In these studies it was possible to use a fairly simple device, which had the advantage of being easily operated. The culture vessel consisted of a 2-litre Erlenmeyer flask which was given a working volume of 1 litre by means of a

* In the present paper "synthesis" always refers to the net synthesis.

tubulature on the wall for removing the emergent bacterial culture. Good mixing and aeration was achieved by placing the flask on a rotary shaker working at 120 r.p.m., and by having the air stream forced through a sintered glass disc and entering the culture fluid as close to the wall of the flask as possible. The neck of the flask was sealed by a rubber stopper which was perforated by glass tubes for the supply of air and fresh medium, and for inoculation. Effluent air left the flask only through the tubulature mentioned, making the transfer of the emergent bacterial suspension to the cooling flask a matter of seconds. The air flow-rate was 1 litre per min, and was indicated by a rotameter. In some instances a small amount of carbon dioxide was added to the air, this also being indicated by a special rotameter. The rotary shaker with the culture vessel operated in a constant temperature-room at 37°C. Fresh medium was stored in 40-litre spherical pyrex flasks and supplied to the culture vessel by means of a hose pressure pump * through latex rubber tubes. The continuously expelled bacterial suspension was cooled on the walls of a cylindrical collecting vessel placed in ice water.

Operation. Deficient medium (40 litres in each storage flask) was sterilized separately. The culture vessel was sterilized containing 1 litre of the complete medium. It was inoculated with bacteria from an agar slant, and growth was allowed to proceed until the cell concentration reached a value of about 2×10^9 cells per ml, corresponding to about 500 mg of dry cells per litre. At this stage the continuous addition of deficient medium was started. In the steady-state, the dry weight of cells per litre of culture was usually kept about 120—200 mg. At the beginning of each experiment a dilution rate of about 0.3 h^{-1} was used. During the first 10 h of the continuous run, it was found necessary to add a certain amount of carbon dioxide to the air in order to prevent a complete wash-out of the cells (see Table 4 and Figs. 4 and 5).

When the steady-state was achieved at the selected flow-rate, two or three samples were taken daily. The duration of the test at each flow-rate was in most cases 2 days, but during a continuous run of 5—6 days at the same flow-rate, the variations in the polulation density and the glycogen content of the culture were found to be within the limits of the experimental errors. The flow-rate was changed to lower or higher values stepwise, big changes always being avoided. After a change of the flow-rate had been effected, the culture was run for about 18 h before sampling of the culture at the new steady-state was commenced. Control of the flow-rate was effected by measuring the volume of the bacterial suspension that had collected during a certain interval.

After a continuous run of about 12—14 days, wall-growth occurred in most cases, and the experiment was terminated. Wall-growth was never tolerated during the continuous run, because it affected both the yield of the bacteria and their glycogen content.

When the volume of the bacterial suspension in the collecting vessel was 800 ml, the cells of this sample were quantitatively spun down and dry weight determination was performed according to the method used by Palmstierna [12]. The freeze-dried material was kept at —20°C until it was weighed in for analysis.

The cell concentration was determined in 1 ml-samples taken directly from the outflowing culture. The bacteria were counted in a counting chamber under the phase contrast microscope.

Organism and culture media. Freeze-dried cultures of *Escherichia coli B* were used throughout. Two media were employed: one with sodium lactate ** and one with glucose *** as the carbon source. In both of the media, ammonium chloride was the sole nitrogen source. This nutrilite was used as the limiting factor in most of the experiments, but one experiment was also carried out with lactate as the limiting factor. In the nitrogen-deficient media, the concentration of ammonium chloride was usually about 1.5×10^{-3} M, but in one experiment a concentration of 3.9×10^{-3} M was used. After sterilization the ammonium-N concentration was determined, in every new batch of deficient medium. In the ex-

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* Sigmamotor Inc., Middleport, N.Y., USA.

** Described by Friedlein [17] and having the following composition: sodium lactate 10 g, NH_4Cl 1 g, K_2HPO_4 0.7 g, KH_2PO_4 0.3 g, $Na_2SO_4 \cdot 10H_2O$ 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.02 g, distilled water 1 000 ml.

*** Described by Hook and coworkers [18]: glucose 4 g, NH_4Cl 1 g, $Na_2HPO_4 \cdot 2H_2$ O7.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, distilled water 1 000 ml.

periment with the carbon source as the limiting factor, the sodium lactate concentration was 1.0×10^{-2} M. The pH of the culture medium during the continuous run was 7.0 when the lactate medium was used, and 6.9 with the glucose medium.

Table 1.

Experiment	Dilution rate	Number of cells per ml $\times 10^{-6}$	Dry weight of cells per litre of culture (mg)	Glycogen-glucose, % of dry weight	Total nitrogen, % of dry weight	Total bacterial nitrogen per litre of culture (mg)	Synthetic rate of glycogen
A	0.18	6.5	153.1	21.9	11.2	17.2	35.1
	0.18	6.5	148.4	20.6	11.4	16.9	32.5
	0.18	6.6	153.1	19.3	11.2	17.2	30.9
	0.18	6.5	151.9	17.9	11.3	17.1	28.6
	0.33	6.0	132.0	8.2	13.1	17.3	20.7
	0.36	6.0	135.7	7.8	13.0	17.6	21.7
	0.36	6.0	131.7	7.7	13.1	17.2	21.2
	0.36	6.0	129.5	8.1	13.0	16.8	22 4
	0.59	5.2	130.9	4.1	13.5	17.7	17.9
	0.59	5.2	130.4	3.2	13.3	17.3	14.2
	0.75	5.0	123.7	2.2	14.3	17.7	11.5
	0.94	4.9	100.4	2.3	14.3	14.3	15.1
	0.94	4.9	104.0	2.3	14.1	14.7	15.3
B	0.21	7.7	153.9	16.1	11.8	18.1	28.7
	0.21	7.7	152.0	15.2	11.6	17.7	27.5
	0.47	7.7	133.0	5.7	13.3	17.7	20.2
	0.47	7.7	130.0	6.0	13.6	17.7	20.7
	0.48	6.2	144.0	5.8	13.3	19.2	20.9
	0.48	6.2	138.9	5.5	13.4	18.6	19.7
	0.49	5.4	141.5	5.4	13.4	18.9	19.8
	0.49	5.4	142.1	6.8	13.2	18.7	25.3
	0.49	5.4	138.5	4.8	13.5	18.7	17.4
	0.50	5.9	137.1	5.4	13.4	18.4	20.1
	0.50	5.9	139.4	5.9	13.2	18.4	22.3
	0.50	5.8	142.2	5.4	13.2	18.8	20.4
	0.61	5.4	129.6	2.2	14.0	18.2	9.6
	0.61	5.4	130.8	2.5	14.1	18.3	10.9
	0.66	5.4	141.6	2.3	13.9	19.7	10.9
	0.66	5.4	142.4	2.5	13.8	19.6	12.0
	0.66	5.4	137.3	2.7	13.7	18.8	13.0

Expts. A and B. Carbon source: sodium lactate. Input concentration of ammonium-N: expt. A 20 mg/l; expt. B 22 mg/l. The values given in the last column of the tables represent the amount of glycogen synthesized in one hour per 100 mg bacterial nitrogen. These values were plotted against the dilution rate. The data of the best straight line being fitted by the method of least squares are given below (the values at higher dilutions rates than 0.7 h^{-1} are not included in the calculations). As regards the statistical methods the reader is referred to Fisher [19].

Regression equation: $y = 37.6 - 38.1\,x$
Regression coefficient: -38.1 ± 3.25 ($t = 11.74$; $p < 0.001$)
Correlation coefficient: -0.920 ± 0.030 ($t = 30.7$; $p < 0.001$).

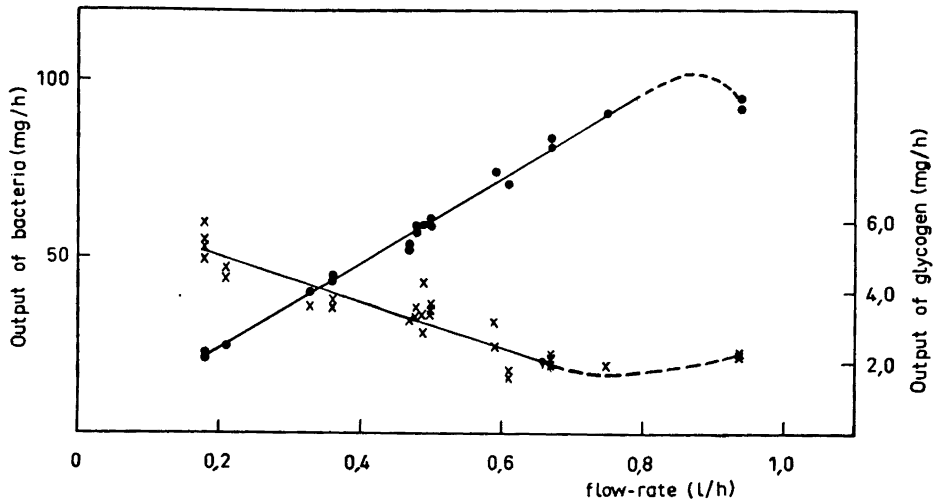

Fig. 1. Output of glycogen and bacteria at different flow-rates. Expts. A and B. Carbon source: sodium lactate. Culture volume: 1 litre. Input concentration of ammonium-N: 20 mg/l (the values of expt. B were corrected to correspond to this input concentration). The dry weight of cells per litre is indicated by dots (the glycogen content has been subtracted from the total dry weight values). The glycogen per litre is indicated by crosses, and the unbroken curve is fitted by the method of least squares.

Chemical methods. The isolation of glycogen and the estimation of the glycogen-glucose were performed as described by Palmstierna [12]. The micro-Kjeldahl method was used for the nitrogen determinations.

RESULTS

In continuous cultures of *Escherichia coli B* steady-state growth was obtained at different growth rates. Two media were employed: one with sodium lactate and the other with glucose as the carbon source. In other respects their composition was about the same. Using the nitrogen source as the limiting factor, specific growth rates * of 0.13—0.94 h^{-1} were established. The glycogen content and the total nitrogen content of the cells were determined. The results are presented in Tables 1 and 2. In experiments A and B (Table 1) sodium lactate was the carbon source; in experiments C and D (Table 2), it was glucose.

* The specific growth rate, μ, is defined by

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{d\ (\log_e x)}{dt} = \frac{\log_e 2}{t_d}$$

where t_d is the doubling time, *i.e.* the time required for the concentration of organisms to double.

At equilibrium the specific growth rate (and the specific synthetic rate of every cell constituent) is equal to the dilution rate, D, defined as f/v, where f is the flow-rate in litres per hour and v is the culture volume.

The specific synthetic rate of glycogen increased considerably with decreasing dilution rates. The amount of glycogen synthesized in one hour at an input concentration of 20 mg ammonium-N per litre can be calculated by multiplying the per litre value of glycogen by the dilution rate value. In Figs. 1 and 2 the results are presented in that way. The output of bacterial cells (calculated as dry weight) is also recorded (the glycogen content has been subtracted from the total dry weight of the cells).

The values of the total nitrogen of the cells seemed to form a more accurate basis for the comparison of the synthetic rates of glycogen and nitrogen-

Table 2.

Experiment	Dilution rate	Input conc. of ammonium-N (mg/l)	Number of cells per ml $\times 10^{-6}$	Dry weight of cells per litre of culture (mg)	Glycogen-glucose, % of dry weight	Total nitrogen, % of dry weight	Total bacterial nirogen per litre of culture (mg)	Synthetic rate of glycogen
C	0.34	21	5.3	148.8	12.9	12.0	17.8	36.6
	0.34	21	5.3	146.5	12.5	12.2	17.9	34.8
	0.34	21	5.3	152.4	12.9	12.2	18.6	35.9
	0.35	21	6.0	147.8	11.8	12.2	18.1	33.8
	0.35	21	6.0	148.1	12.8	12.5	18.4	36.0
	0.35	21	6.1	148.4	12.1	12.4	18.4	34.2
	0.49	21	6.5	138.5	6.5	12.9	17.9	24.7
	0.49	21	6.0	139.3	7.2	13.1	18.3	26.9
	0.49	21	6.4	138.4	7.6	12.7	17.6	29.3
	0.66	21	4.1	130.1	5.5	13.6	17.7	26.6
	0.66	21	4.1	133.4	5.4	13.1	17.5	27.1
	0.66	21	4.0	133.5	5.6	14.1	18.8	26.2
	0.66	21	4.0	133.2	6.1	13.7	18.3	29.3
	0.72	21	3.6	135.6	5.4	13.4	18.2	28.9
	0.72	21	3.6	133.9	5.4	13.6	18.1	28.7
	0.72	21	3.7	133.6	4.5	13.8	18.5	23.4
	0.72	21	3.8	135.1	4.7	13.7	18.5	24.8
	0.84	22	6.0	148.4	3.6	13.1	19.4	23.1
	0.84	22	6.0	146.6	3.6	13.3	19.4	22.8
	0.86	22	6.0	146.3	3.4	13.2	19.3	22.2
	0.86	22	6.1	140.4	3.3	13.1	18.4	21.6
D	0.13	26	14	205.5	22.5	9.9	20.3	29.5
	0.17	26	14	205.5	20.0	10.8	22.2	31.5
	0.17	26	14	208.3	21.4	10.6	22.1	34.3
	0.24	26	14	199.2	15.4	11.5	22.9	32.1
	0.35	26	14	176.3	11.0	12.3	21.7	31.3
	0.35	26	14	176.8	11.2	12.3	21.7	31.9

Expts. C and D. Carbon source: glucose. The values of the synthetic rate of glycogen were plotted in the same way as for expts. A and B (the values at lower dilution rates than 0.2 h^{-1} were not included in the calculations).

Regression equation: $y = 40.2 - 20.4\,x$

Regression coefficient: -20.4 ± 2.43 ($t = 8.40$; $p < 0.001$)

Correlation coefficient: -0.872 ± 0.049 ($t = 17.8$; $p < 0.001$).

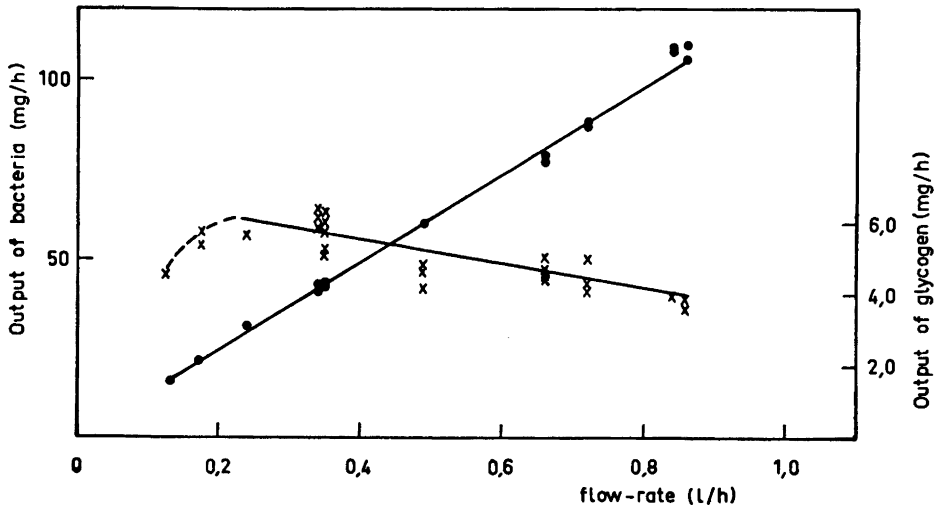

Fig. 2. Output of glycogen and bacteria at different flow-rates. Expts. C and D. Carbon source: glucose. Culture volume: 1 litre. The values are corrected to correspond to an input concentration of 20 mg ammonium-N per litre. For a description of the symbols see Fig. 1.

containing compounds. The amounts of glycogen synthesized in one hour per 100 mg bacterial nitrogen are recorded in the last column of the tables. It is seen that the synthetic rate of glycogen increases considerably when the dilution rate is diminished, *i.e.* when the synthetic rate of the nitrogen-containing compounds is reduced.

In the cultures grown in the lactate medium, the minimum content of glycogen in the continuously cultivated cells appeared to be reached at the highest dilution rates (>0.7 h^{-1}). A slight increase in the synthetic rate of glycogen might consequently have been expected with a further increase in the dilution rate. However, at the highest dilution rate, at which steady-state growth was obtained, the cell concentration was considerably lower than at the lower dilution rates. It is probable that the highest dilution rate, 0.94 h^{-1}, is very close to the maximum value of μ, and thus represents the highest dilution rate that can be attained. The quantitative relationships are best illustrated by the "output" curves in Fig. 1, representing the production of cells and glycogen by the continuous culture.

It appeared that the maximum content of glycogen in the continuously cultivated cells was reached in the cultures grown in glucose medium at the lowest dilution rates (<0.2 h^{-1}). The rate of glycogen synthesis, therefore, decreases at the lowest dilution rates in these cultures (Table 2, Fig. 2).

If the straight lines obtained from the values of the synthetic rate of glycogen in Tables 1 and 2 are drawn to the y axis, both will cut this axis at a value of about 40. This value is comparable with the initial synthetic rate of

glycogen * in batch cultures during nitrogen starvation, *i.e.* during a period when there is no synthesis of nitrogen-containing compounds [13]. This is also the case in the experiment recorded in Table 3, where continuously cultivated cells (dilution rate 0.46 h^{-1}) were used as inoculum in a batch culture. The medium contained no nitrogen source.

It was assumed in the calculations that the small differences in the input concentration of ammonium-N had no influence on the specific synthetic rate of glycogen. It was confirmed by one experiment in which the input concentration was increased to 54 mg ammonium-N per litre that this assumption was justified. The cell concentration was 385 mg dry weight per litre of culture. The synthetic rate of glycogen observed in this experiment was 26.5 ± 0.3 (mg per hour per 100 mg bacterial nitrogen; two determinations) at a dilution rate of 0.51 h^{-1} (carbon source: glucose).

In the continuous cultures where the carbon source was used as the limiting factor, no significant variations in the glycogen content of the cells were observed at different dilution rates. In one experiment in which sodium lactate was used as the limiting factor, glycogen determinations gave the following results: 1.5 % of the dry weight at a dilution rate of 0.33 h^{-1} and 1.1 % at a dilution rate of 0.14 h^{-1}.

Substances causing an absorption in the ultra-violet region poured out into the medium in the cultures with the nitrogen source as the limiting factor. The absorption increased considerably with decreasing dilution rates. The absorption curves for the cell-free culture medium from samples taken at different dilution rates are presented in Fig. 3.

During a certain period after the continuous addition of new medium had been started, it was found necessary to add carbon dioxide to the air

Table 3.

Time after inoculation (min)	Number of cells per ml $\times 10^{-8}$	Dry weight of cells per litre of culture (mg)	Glycogen-glucose, % of dry weight	Total nitrogen, % of dry weight	Total bacterial nitrogen per litre of culture (mg)
0	5.7	124.5	6.6	13.1	16.3
30	6.5	129.3	10.6	12.9	16.7
60	7.5	138.2	15.1	12.2	16.8
90	7.9	141.5	17.8	12.1	17.2

Cells from a continuous culture incubated in nitrogen-free medium. For further details, see the text.

* During the starvation phase in a nitrogen-starved culture the bacterial nitrogen per litre of culture is constant. The value of glycogen increase (calculated as mg glycogen per 100 mg bacterial nitrogen per h) was multiplied with $\log_e 2$ for the comparison with the values obtained in the continuous culture.

Calculated in this way the mean synthetic rate of glycogen during the first hour of the experiment recorded in Table 3 was 45.8.

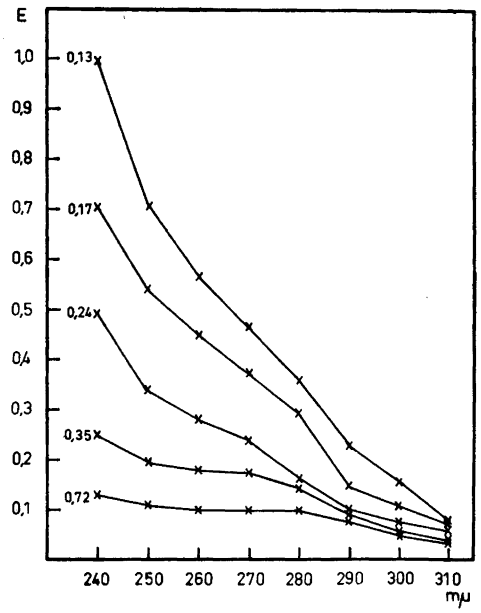

Fig. 3. Specific absorption (E) of the cell-free culture medium at different dilution rates, indicated by the figures at the end of each curve. Measurements were made on samples from expt. D, except those recorded at the dilution rate 0.72 h^{-1}, which were made on a sample from expt. C.

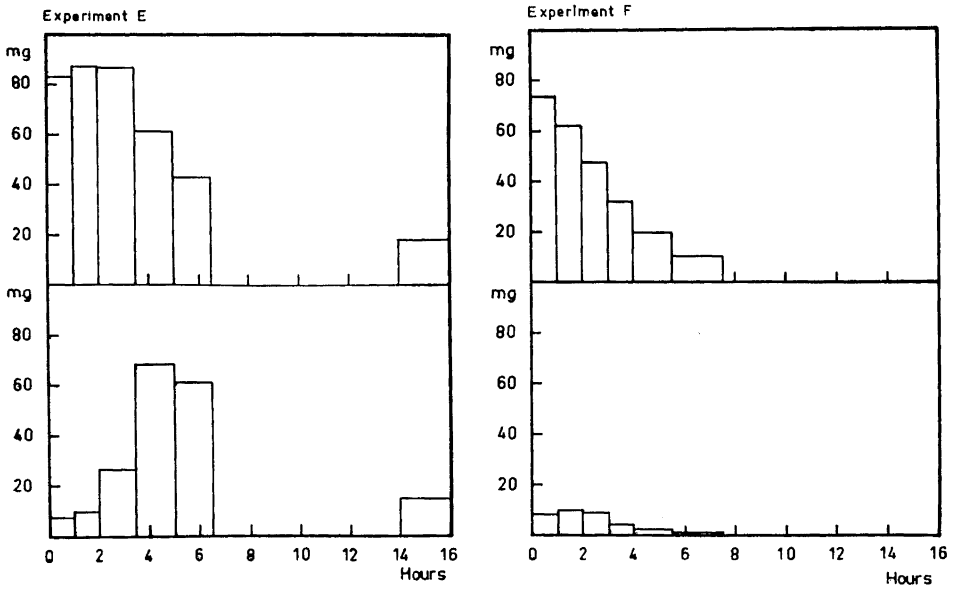

Fig. 4. Expts. E and F. The steeples in the upper parts of the figure indicate the total bacterial nitrogen per litre of culture. In the lower parts of the figure the glycogen per litre of culture is represented. At zero time the continuous addition of fresh medium was started. Dilution rate 0.29 h^{-1}.
Aeration: expt. E: Air+carbon dioxide (5 %); expt. F: Air only.

entering the culture, since otherwise the bacteria were completely washed-out. In order to investigate the role of the carbon dioxide during this period, the following experiment was carried out. Two cultures were allowed to grow in the lactate medium until they reached a cell concentration of about 2×10^9 whereupon the continuous addition of deficient medium (20 mg ammonium-N per litre) was begun. The dilution rate was 0.29 h^{-1} in both cultures. About 5 % carbon dioxide was added to the air entering one of the cultures (expt. E); the other was aerated with air alone (expt. F). The emergent bacterial suspensions were collected and the cells from each sample analyzed (Table 4, Fig. 4). The time required for collecting each sample was 1—2 h (see Table 4).

In expt. E, where carbon dioxide was added to the air, the glycogen content of the cells increased considerably during the first part of the contiunous run. The cell concentration decreased after an initial increase. Fourteen hours after the continuous addition of deficient medium had been started, steady-state concentrations of cells and glycogen were noted. Addition of carbon dioxide to the air in a culture in the steady-state had no measurable influence on the glycogen content of the cells. In expt. F, in which no carbon dioxide was added, the cell concentration decreased immediately. The glycogen content of the cells was low. Forteen hours after the start of the continuous run, no cells could be detected in the counting chamber.

Table 4.

Experiment	Sample (h)	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	Total nitrogen, % of dry weight	Glycogen-glucose per litre of culture (mg)	Total bacterial nitrogen per litre of culture (mg)
E	0—1	25	571.7	2.3	1.3	14.6	7.4	83.3
	1—2	39	609.7	1.6	1.6	14.3	9.8	87.0
	2—3.5	23	606.8	2.6	4.4	14.3	26.7	86.5
	3.5—5	21	511.8	2.4	13.4	11.9	68.6	60.8
	5—6.5	19	384.0	2.0	16.0	11.2	61.4	43.1
	14—16	5.4	149.6	2.8	9.9	12.1	14.8	18.1
F	0—1	27	509.3	1.9	1.6	14.4	8.1	73.5
	1—2	22	438.0	2.0	2.3	14.2	10.1	62.2
	2—3	18	335.4	1.9	2.6	14.1	8.7	47.4
	3—4	13	223.7	1.7	1.8	14.3	4.0	31.9
	4—5.5	9.0	136.7	1.5	1.4	14.3	1.9	19.5
	5.5—7.5	4.5	71.9	1.6	0.9	14.2	0.6	10.2
	14—16	<0.1	—					

Expts. E and F. Carbon source: sodium lactate. At zero time the continuous addition of fresh medium was started. Dilution rate 0.29 h^{-1}.
Aeration: expt. E: Air + carbon dioxide (5 %)
expt. F: Air only.

DISCUSSION

The application of the continuous culture technique to metabolic studies has given valuable information concerning the regulation of the rates of various synthetic processes [8,11]. A substance of general interest, namely glycogen, shown by Palmstierna [12] to be present in *E. coli* cells was the subject of continuous culture studies in the present investigation. Due to its function as a pool of energy and carbon in the cell, the regulation of its synthesis seemed to be of special interest.

Since the glycogen contains no nitrogen, its synthesis is not prevented by nitrogen deficiency in *E. coli* cells. On the other hand, energy-consuming processes like the syntheses of nucleic acids and protein are readily arrested by nitrogen starvation. When the nitrogen source was used as a growth-limiting substance in the continuous culture, the glycogen synthesis proceeded without being reduced by this limitation, and the synthetic rate could be shown to depend on the synthetic rate of the nitrogen-containing compounds.

This system also offers the possibility of studying the influence of various environmental factors on the synthetic rate of glycogen in the bacterial cells, *e.g.* temperature, pH, oxygen tension, concentration of different medium constituents and other substances. In the present investigation it was observed that the rate of glycogen synthesis depended on the nature of the carbon source in the culture medium. At a high synthetic rate of the nitrogen-containing compounds there was a lower synthetic rate of glycogen when lactate was the carbon source than when glucose was used. A possible explanation of this could be that the lactate must undergo a long series of transformations before it can be incorporated into the glycogen. The glucose on the other hand, is more easily incorporated in spite of the rapid breakdown. The difference in the synthetic rates appears to diminish when the synthetic rate of the nitrogen-containing compounds is reduced, *i.e.* when the carbon source is not utilized to any great extent for the synthesis of these compounds and consequently a greater part is available for glycogen synthesis.

It should be pointed out that glycogen appears to be the main substance that accumulates in the cells of *E. coli B* during nitrogen deficiency. This is made probable by the constant values obtained when the glycogen content of the cells is subtracted from the total dry weight. This has also been confirmed by using the nitrogen starvation technique in batch cultures.

As a rough method of estimating the "overflow metabolism" to the medium the ultra-violet absorption of the cell-free culture medium has been determined. The nature of these substances has not been investigated. It has been shown by Dagley *et al.* [20] that pyruvate is poured out at high rates during nitrogen starvation in batch cultures of *Aerobacter aerogenes*.

Different methods for starting the continuous run of the cultures have been applied. In experiment E the method used in the present investigation is demonstrated. Carbon dioxide was added to the air during the dilution period. In experiment F, where no carbon dioxide was added, there was a more rapid decrease than in experiment E during the first two hours, when the concentration of ammonium chloride could not be limiting. During the following period of rapid dilution, the decrease in bacterial nitrogen per litre of culture

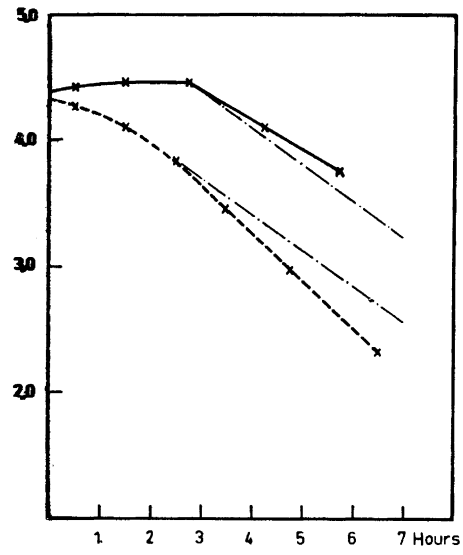

Fig. 5. The first part of the continuous run in expts. E (unbroken curve) and F (broken curve). The curves represent the $\log_e$ for the total bacterial nitrogen per litre of culture (the points are placed at the midpoints of the time intervals represented by the steeples in Fig. 4). The thin broken curves represent the "wash-out rate", *i.e.* the rate at which the organisms initially present in the vessel would be washed out if no growth occurred.

seemed to be more rapid than the wash-out rate (Fig. 5). This may be explained by a lysis of the cells. Fourteen hours after the start of the continuous addition, the number of cells in the culture vessel was less than 10^7 per ml and no increase was noted upon further incubation. In experiment E, where carbon dioxide was added to the air entering the culture the decrease in bacterial nitrogen per litre of culture seemed to be somewhat less rapid than the wash-out rate, but the difference is too small to be considered as significant (Fig. 5). It is however evident that during some part of the time required for stabilizing the culture, there must have been a stimulation of the synthesis of the nitrogen-containing compounds by the carbon dioxide since steady-state concentration of the cells was obtained at fourteen hours after the start of the continuous run.

Obviously the method of nitrogen limitation offers excellent possibilities for the control of glycogen yields. The relationships described might well serve as a model for the commercial production of nitrogen-free substances other than glycogen, cellular or extracellular.

REFERENCES

1. Monod, J. *Ann. Inst. Pasteur*, **79** (1950) 390.
2. Novick, A. and Szilard, L. *Proc. Natl. Acad. Sci. N.Y.* **36** (1950) 708.
3. Novick, A. and Szilard, L. *Science* **112** (1950) 715.
4. Hedén, C.-G., Holme, T. and Malmgren, B. *Acta Pathol. Microbiol. Scand.* **37** (1955) 42.
5. Herbert, D., Elsworth, R. and Telling, R. C. *J. Gen. Microbiol.* **14** (1956) 601.
6. Karush, F., Iacocca, V. F. and Harris, T. N. *J. Bacteriol.* **72** (1956) 283.
7. Perret, C. J. *J. Gen. Microbiol.* **16** (1957) 250.
8. Monod, J. and Cohn, M. *Advances in Enzymol.* **14** (1952) 67.
9. Novick, A. and Szilard, L. *Cold Spring Harbour Symposia Quant. Biol.* **16** (1951) 337.
10. Novick, A. and Szilard, L. *Nature* **170** (1952) 926.

11. Novick, A. and Szilard, L. *Dynamics of Growth Processes*, Princeton Univ. Press, Princeton, New Jersey 1954.
12. Palmstierna, H. *Acta Chem. Scand.* **10** (1956) 567.
13. Holme, T. and Palmstierna, H. *Acta Chem. Scand.* **10** (1956) 578.
14. Holme, T. and Palmstierna, H. *Acta Chem. Scand.* **10** (1956) 1553.
15. Holme, T. and Palmstierna, H. *Acta Chem. Scand.* **10** (1956) 1557.
16. Holme, T., Laurent, T. and Palmstierna, H. *Acta Chem. Scand.* **11** (1957) 757.
17. Friedlein, F. *Biochem. Z.* **164** (1928) 273.
18. Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G. and Beard, J. W. *J. Biol. Chem.* **165** (1946) 241.
19. Fisher, R.A. *Statistical Methods for Research Workers*, Oliver & Boyd, Edinburgh 1950.
20. Dagley, S., Dawes, E.E. and Morrison, G.A. *J. Bacteriol.* **61** (1951) 433.

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