ACTA CHEMICA SCANDINAVICA 17 (1963) 947–952

Formation of Folate Enzymes during the Growth Cycle of Bacteria

I. Tetrahydrofolate Dehydrogenase Activity During the Lag and Acceleration Periods of *Streptococcus faecalis* R.

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The variation of tetrahydrofolate dehydrogenase activity in the lag and acceleration phases of growth of *Streptococcus faecalis* R has been studied. The results suggest that the tetrahydrofolate dehydrogenase activity increases during the first two growth phases, and does so from the very beginning of the lag phase. On the basis of these observations tetrahydrofolate dehydrogenase is considered a typical "lag phase enzyme".

The conversion of folic acid (FA)(pteroylglutamic acid) into a tetrahydrofolic acid derivative was first observed by Sauberlich,[1] who isolated 5-formyltetrahydrofolic acid (5-formylFH_4) from the urine of a rat that had been given folic acid with food. The same conversion has been observed to take place in bacterial cells and cell extracts also [2,3]. Greenberg [4] demonstrated that dihydrofolic acid (FH_2) is reduced to tetrahydrofolic acid (FH_4) in the presence of nicotinamide-adenine dinucleotide (NAD) and adenosine-5′-triphosphate (ATP), and he suggested that an active coenzyme, 10-formylFH_4, may be formed from folic acid (FA) by the following reaction series:

$$FA \xrightarrow{2H} FH_2 \xrightarrow{NaDH_2} FH_4 \xrightarrow[ATP]{HCOOH} \text{10-formyl}FH_4$$

The first step in the series is catalyzed by folate reductase, and the second by tetrahydrofolate dehydrogenase. Later Futterman [5] found that the reduction of FA to FH_2 occurs in the presence of $NADPH_2$, whereas either $NADH_2$ or $NADPH_2$ (reduced nicotinamide-adenine dinucleotide phosphate) is required as coenzyme for the further reduction of FH_2. The same system has also been studied by Zakrzewski and Nichol,[6] who employed an enzyme system isolated

from chicken liver, and who held that folate reductase and tetrahydrofolate dehydrogenase are one and the same enzyme.

Investigations concerning the reduction of folic acid in micro-organisms are few in number. Wright [7] isolated from *Clostridium sticklandii* a folate reductase for which coenzyme A (CoA) acts as cofactor and which also requires some electron donor like serine, pyruvic acid, or α-ketoglutaric acid. Blakley *et al.*[8] recently isolated from *Streptococcus faecalis* R a tetrahydrofolate dehydrogenase for which $NADPH_2$ functions as coenzyme, and on which $NADH_2$ has a much weaker effect. Tetrahydrofolate dehydrogenase has been isolated also from chicken liver,[9,10] sheep liver,[11] and calf thymus [12].

The biosynthesis and excretion of folic acid during the growth cycle of *Lactobacillus arabinosus* has been studied in this laboratory [13]. The results obtained show that marked variations occur in the content of folic acid (present as pteroylglutamic acid and *Streptococcus faecalis* R activity) in the cells during the active growth phases. Because of these observations it was of interest to determine whether variations occur also in the formation of folic acid enzymes during cell growth. A study of tetrahydrofolate dehydrogenase is reported here.

EXPERIMENTAL

Cultivation of the test organism. Streptococcus faecalis R (ATCC 8043) was maintained as a stab culture in a glucose-citrate-tryptone-yeast extract(GSHT)-agar medium [18]. The organism was transferred into a fresh agar medium every 2 or 3 weeks.

Before the experiment the bacteria were grown in 300 ml of inoculum medium (GSHT medium),[18] to which they were transferred with a platinum wire, at 37° for 12 – 14 h, after which they were centrifuged (10 min, 3000 rpm) and washed twice with 0.9 % saline. The cell mass was placed in a previously warmed growth medium containing 0.1 μg of folic acid per 100 ml [7]. The growth took place at 30° in a continuously stirred medium in a 1000-ml erlenmeyer flask. Growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62.

Preparation of enzyme extracts. During the growth 100-ml specimens were withdrawn and immediately cooled to 0° in ice-water. The cells were centrifuged (10 – 15 min at 3000 rpm) and suspended in acetone at −20° for 1 h. The acetone was then decanted and the cells dried in a vacuum at room temperature for half an hour. The dried cells were suspended in 5 ml of distilled water and incubated at 37° for 15 – 16 h. The suspension was centrifuged until clear (20 min at 15 000 rpm in a Servall RC-2 centrifuge) and the supernatant containing the enzyme was tested for protein by the sulphosalicyclic acid method [19].

Reaction systems. The enzyme activity of each specimen was determined by measuring the changes in extinction at a wave length of 340 mμ with a Beckman DU spectrophotometer fitted with glass cuvettes with a light path of 1 cm. The determinations were made by the method of Osborn and Huennekens [9]. The amount of enzyme solution was 0.5 ml and the total volume 3.3 ml. A 0.05 M phthalate buffer of pH 5.8 was used. Phosphate, acetate, citrate, and other buffers could not be used because the crude enzyme preparation employed reduced these compounds in the presence of $NADH_2$. The reaction time at room temperature (20°) was 10 – 30 min.

Blakley and McDougall [8] gave 6.7 as the optimum pH for tetrahydrofolate dehydrogenase when a phosphate buffer is employed. With a phthalate buffer the optimum pH is 5.8 – 6.0 according to observations made in this laboratory. At pH 6.7 the activity is markedly lower.

Because of the presence of oxidases for $NADH_2$ in the test organism, the enzyme preparation reduced $NADH_2$ also in the absence of FH_2[20]. Another reaction was therefore started without adding FH_2 under otherwise identical experimental conditions. The amount of $NADH_2$ oxidized in this experiment was substracted from the amount oxidized in the first one.

FH_2 was prepared by the method of Futterman [21]. The sample was preserved suspended in 0.005 M HCl in a vacuum at 0°. As needed, some of the suspension was added to a 0.05 M phthalate buffer solution of pH 5.8 and dissolved in 0.01 M KOH containing 10 mM of 2,3-dimercaptopropanol (BAL). The FH_2 content of this solution was measured with a Beckman DU spectrophotometer employing the value 21 000 for the molar absorptivity ε_{283} ($= 19 \times 10^6$ cm^2 mole^{-1}).

RESULTS

In this study Monod's definitions of the bacterial growth phases have been followed. In accordance with these definitions we have taken the lag phase to extend over the period before the beginning of growth when no cell division takes place, and the acceleration phase to extend over the period during which cell division takes place with increasing speed [14].

For the study of tetrahydrofolate dehydrogenase activity during the lag phase of growth of *Str. faecalis* R, the experimental conditions were arranged so that the phase in question lasted about half an hour. A factor that affects the length of the lag and acceleration phases of growth is the period of time during which the test organism is grown in the inoculum medium. The effect of this factor as a regulator of the growth rate is shown in Fig. 2.

Fig. 3 shows the results of an experiment (experiment 1) in which the inoculum growth of the test organism had been so timed that the lag phase lasted about 20 min and the cell division took place comparatively slowly during the acceleration phase. The tetrahydrofolate dehydrogenase activity began to increase immediately at the beginning of the lag phase and continued to do so to the end of the phase. The final activity was one and a half times the activity at the beginning of the phase. During the acceleration phase the tetrahydrofolate dehydrogenase activity continued to increase and reached a maximum about 30 min after growth began. The activity of the enzyme had then increased to about 2 ½ times the level at the beginning of the lag phase. The

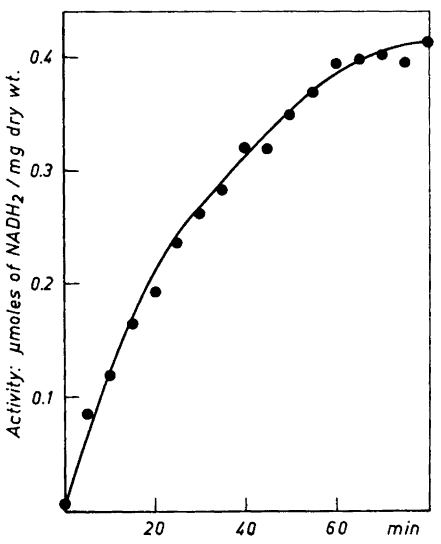

Fig. 1. Dependence of the extent of reaction on time. The reaction mixture contained 0.5 mg of cell protein, ATP 0.2 μmoles, sodium formate 6 μmoles, FH_2 0.45 μmoles, $NADH_2$ 0.25 μmoles, and a 0.05 M phthalate buffer of pH 5.8 to make a volume of 3.3 ml. The reaction was started by the addition of $NADH_2$. Oxidation was followed spectrophotometrically at 340 mμ by reading the reaction mixture against a reference mixture that lacked $NADH_2$.

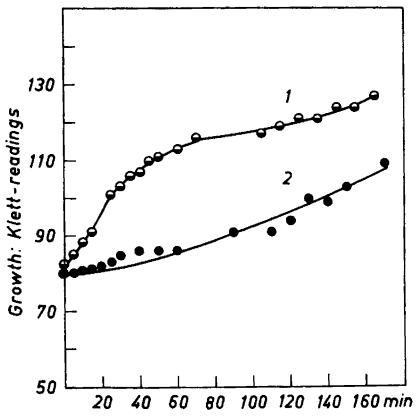

Fig. 2. Effect on the growth rate of *Str. faecalis* R of the period of growth in the inoculum medium. 1, the organism grew 11 h in the inoculum medium before it was transferred to the growth medium, where growth was followed turbidimetrically; 2, the time of inoculum growth was 18 h before the transfer.

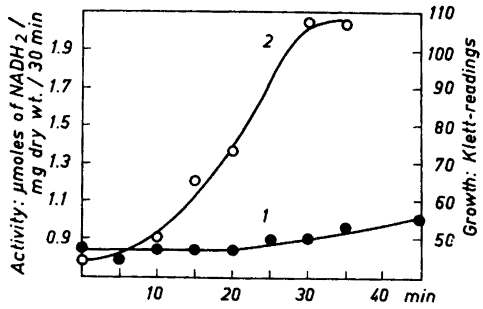

Fig. 3. Tetrahydrofolate dehydrogenase activity during the lag and acceleration phases of growth of *Str. faecalis* R. 1, growth curve (scale on right); 2, activity curve (scale on left). Reaction time 30 min.

reaction time in the determination of enzyme activity was 30 min in this experiment.

In experiment 2 the reaction time in the determination of tetrahydrofolate dehydrogenase was only 15 min. As can be seen from Fig. 4, the enzyme activity increased in a similar way as in experiment 1. The lag phase lasted about 30 min.

In experiment 3 the enzyme activity was followed mainly during the acceleration phase. In fact, under the experimental conditions employed no clear lag phase was observed. The reaction time in the determination of tetrahydrofolate dehydrogenase was 10 min. Fig. 5 shows that a definite increase in enzyme activity occurred from the very beginning of growth, and an abrupt increase during the acceleration phase.

Table 1 summarizes the results of the three experiments. Although the experimental conditions differed somewhat — in particular the length of the lag phase and the reaction time in the determination of tetrahydrofolate dehydrogenase varied — the results of all three experiments conformed completely in showing that the tetrahydrofolate dehydrogenase activity increases during the first two phases of growth of *Str. faecalis* R.

DISCUSSION

The data presented above indicate that the content of active tetrahydrofolate dehydrogenase must reach a certain definite level before cell division can begin. Of course, it is difficult to determine the exact amount of enzyme present in the cell just before the division. If we assume that the level of tetra-

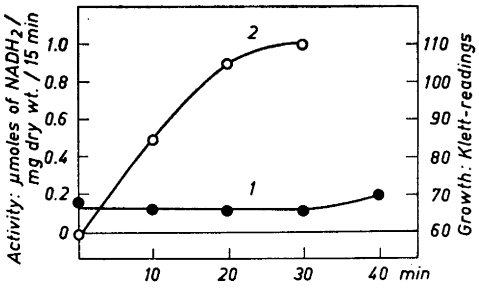

Fig. 4. Tetrahydrofolate dehydrogenase activity during the lag and acceleration phases of growth of *Str. faecalis* R. 1, growth curve (scale on right); 2, activity curve (scale on left). Reaction time 15 min.

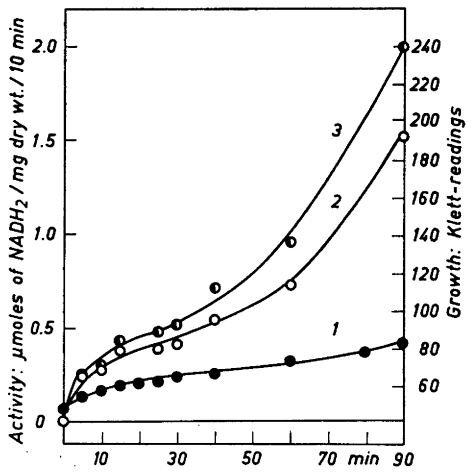

Fig. 5. Tetrahydrofolate dehydrogenase activity during the lag and acceleration phases of growth of *Str. faecalis* R. 1, growth curve (scale on right); 2, activity per mg dry weight; 3, total activity (scale on left). Reaction time 10 min.

hydrofolate dehydrogenase activity to be reached approximately corresponds to the levels at the end of the lag phase in our experiments, the tetrahydrofolate dehydrogenase content of the cell before division begins would be, when expressed as $NADH_2$, 1.7 μmole/mg dry weight of cells. The amount of active enzyme in the cells then rises sharply at an increasing rate during the cell division as shown by our experiments.

Table 1. Comparison of the results of three experiments. The numbers give the activities at different points in the lag and acceleration phases (expressed as μmoles of $NADH_2$/mg dry weight of cells). The reaction time in the determination of tetrahydrofolate dehydrogenase was 15 min in experiment 1 and 10 min in experiment 3. To make possible an internal comparison, the activity values obtained in these two experiments have been recalculated to correspond to the values obtained in experiment 2 where the reaction time was 30 min.

	Experiment 2 (Fig. 4)		Experiment 1 (Fig. 3)	Experiment 3 (Fig. 5)	
Growth time, min	Activity after 15 min	Calculated activity after 30 min	Activity after 30 min	Activity after 10 min	Calculated activity after 30 min
5	0.25	0.5	0.8	0.25	0.75
10	0.5	1.0	0.9	0.3	0.9
15	0.7	1.5	1.2	0.4	1.2
20	0.9	1.8	1.4	0.43	1.29
25	0.98	1.96	1.8	0.47	1.41
30	1.0	2.0	1.95	0.52	1.56

The results show also that tetrahydrofolate dehydrogenase must be included among those enzymes the activity of which increases in the cells from the very beginning of the lag phase and which we call "lag phase enzymes". According to experiments made in this laboratory the group includes the following *E. coli* enzymes: the isoleucine-glutamic, valine-glutamic, and leucine-glutamic transaminases,[15] tryptophan synthetase,[16] and serine-hydroxymethyltransferase [17].

In this connection a comparison of the results obtained in the present study with one of our previous observations is of interest. We found that marked variations occur in the folic acid content (microbiological assay, *Streptococcus faecalis* R ATCC 8043 as test organism) of *Lactobacillus arabinosus* 17—5 (ATCC 8014) cells during the lag phase [13]. The cause of these variations might be an early formation of folate enzymes at the very beginning of the lag phase. Tetrahydrofolate dehydrogenase provides an example. One might suppose that free folic acid (and related cofactors with *Streptococcus faecalis* R activity) is continuously consumed by tetrahydrofolate dehydrogenase, and also possibly in the formation of other folate coenzymes. Since marked variations occur in the folic acid content of the cells, the different folate enzymes may not all be formed at the same rate during the lag phase (and possibly not at the same periods within the lag phase either). It is also possible that polyglutamyl conjugates, to which the test organism does not react, are formed from folic acid during the lag phase.

As a typical lag phase enzyme, tetrahydrofolate dehydrogenase very likely is important for cell division. As a folate enzyme it takes part in the metabolism one carbon (C_1) units and, accordingly, the increase in its activity during the first phases of growth of the organism also points to its importance in the metabolic processes.

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Received November 19, 1962.