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Formation of Folate Enzymes during the Growth Cycle of Bacteria

III. Changes in Tetrahydrofolate Dehydrogenase Activity during the Active Growth Phases of *Streptococcus thermophilus* and *Lactobacillus arabinosus*

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The formation of tetrahydrofolate dehydrogenase in different growth periods of *Streptococcus thermophilus* and *Lactobacillus arabinosus* has been studied. In both organisms the tetrahydrofolate dehydrogenase activity increased from the very beginning of the lag phase and reached maximum level early in the exponential phase. After the maximum it decreased and was about the same at the beginning of the retardation phase as at the beginning of the lag phase.

In two previous papers of this series we have described the variations occurring in the tetrahydrofolate dehydrogenase activity during the first two growth phases of *Streptococcus faecalis* R,[1] and those occurring in the serine hydroxymethyltransferase activity during the active growth phases of *Escherichia coli* and certain lactic acid bacteria.[2] Both enzymes were typical "lag-phase enzymes" the activity of which increases from the very beginning of the lag phase. As there was no information available on the tetrahydrofolate dehydrogenase activity during the later phases of active growth of lactic acid bacteria, we have now studied the variations in the activity of this enzyme during the entire growth cycle.

EXPERIMENTAL

Reagents. Unless stated otherwise, all the reagents used in this work were analytical grade products from E. Merck AG, or BDH. Dihydrofolic acid was prepared by the method of Futterman.[3] The resulting product was yellowish gray in colour and its absorption maximum was at 282 mμ. The compound was preserved in vacuum at 0° in 0.005 N HCl. For the determinations, the dihydrofolic acid was mixed with a small amount of

0.005 M potassium-phthalate buffer, pH 5.8, containing 1 % 2-mercaptoethanol and dissolved by adding 0.5 N KOH dropwise. The concentration of the solution was determined spectrophotometrically at 282 mμ ($\varepsilon_{282} = 19 \times 10^6$ cm^2 mole^{-1}).

Cultivation of test organisms and the culture media. The *Streptococcus thermophilus* strain KQ was developed from a wild strain of *Str. thermophilus* which had been preserved in sterilized skim milk at 4° with transfers every 4 weeks. The wild strain was transferred to 5 ml of TSHGA medium with a platinum loop. The TSHGA medium contained 2.0 g of Bacto-tryptone (Difco), 0.5 g each of lactose, glucose, sucrose, and yeast extract (Difco), 0.25 g of gelatine, and 0.07 g of ascorbic acid in 100 ml of distilled water. The pH of the medium was 6.7 and sterilization was performed at 115° for 15 min. After incubation at 42° for 20 h, the cells were harvested by centrifugation (Servall RC-2, 15 min, 5400 *g*), washed once with sterilized 0.9 % NaCl solution and suspended in the same solution. The suspension was transferred with a platinum loop to 5 ml of sterilized ScQ medium (Table 1), where the organism grew 20 h at 42°. The cells were harvested by centrifugation (15 min, 4500 *g*), washed once with sterilized 0.9 % NaCl solution and suspended in the same solution. The cultivation in medium ScQ was repeated five times and resulted in a new *Str. thermophilus* strain referred to below as Str KQ. Str KQ was preserved suspended in 5 ml of medium ScQ at +4° with weekly transfers. For each experiment 5 ml of the suspension of Str KQ cells was poured aseptically into 1000 ml of ScQ medium and incubated at 42° for 18 h. The cells were harvested by centrifugation (15 min, 2000 *g*), washed once with 0.9 % NaCl solution and suspended in 2000 ml of ScQ medium at 42°. The growth, which continued at 42°, was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62.

Lactobacillus arabinosus 17—5 (ATCC 8014) was preserved at 4° in a stick culture in TSHGA medium containing 1.5 % Bacto agar (Difco). Transfers were made every 4 weeks. Before each experiment the organism was transferred from the stick culture to 5 ml of sterilized agar-free TSHGA medium, where it grew 5—6 h at 37°. The cells were then poured aseptically into 100 ml of the same medium where the growth continued for 18—20 h at the same temperature. The cells were harvested by centrifugation (15 min, 2000 *g*), washed three times with 0.9 % NaCl solution and suspended in 1500—2000 ml of pre-warmed growth medium at 37°. The Bacto biotin assay medium (Difco) containing added biotin (20 μg/1000 ml) was used as growth medium. The growth took

Table 1. Composition of medium ScQ (pH 6.8.). The medium was autoclaved 15 min at 105° before inoculation.

Component	Quantity
Sucrose	5.0 g
Lactose	5.0 ,,
β-Glycerophosphate (Sigma)	5.4 ,,
Sodium acetate · $3H_2O$	200 mg
Ascorbic acid	200 ,,
NH_4Cl	267 ,,
L-Glutamic acid	147 ,,
L-Cysteine HCl	88 ,,
KCl	748 ,,
$MgSO_4$	304 ,,
Thiamine	1 ,,
Riboflavine	1 ,,
Nicotinic acid	1 ,,
p-Aminobenzoic acid	1 ,,
Pyridoxal · HCl	1 ,,
Ca-pantothenate	0.5 ,,
Biotin	1 μg
H_2O (twice distilled)	ad 1000 ml

place at 37° in a vessel holding 2 litres, and was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62.

Preparation of enzyme extracts. During the growth of the test organisms 200-ml samples were withdrawn and immediately cooled in ice water. The cells were centrifuged (15 min, 2000 *g*) and washed twice with 0.9 % NaCl solution. The washed cells were suspended in 2.5 ml of 0.001 M potassium-phthalate buffer of pH 5.8, and cooled to −40° and then thawed at 37°. The freezing and thawing was repeated three times before the suspension was centrifuged (20 min, 20 000 *g*) and the clear supernatant tested for proteins by the sulphosalicylic acid method.[4] In a part of the experiments the cells used in the enzyme extracts were broken up by treating the samples with an MSA ultrasonic apparatus (18−20 kc, 1.5 A) while keeping them in ice water to prevent overheating. After the treatment the suspension was centrifuged (30 min, 32 000 *g*) and the clear supernatant was tested for proteins by the sulphosalicylic acid method.[4] The supernatant was used as such in the determinations.

Determination of enzyme activity. (a) *Chromatographic determination.* A reaction mixture was prepared for the qualitative determination of enzyme activity. This mixture contained 0.2 μmole of FH_4, 0.2 μmole of $NADPH_2$, and 0.1 ml of cell extract in 2 ml of 0.005 M phthalate buffer of pH 5.8. After 60 min 100 μl of the reaction mixture was pipetted onto Whatman No. 1 paper. For comparison a few drops of FH_2 and FH_4 (prepared by the method of Kisliuk [5]) were also pipetted on the same paper. The chromatogram was run in a hydrogen atmosphere by the ascending technique using a 0.05 M phosphate buffer, pH 7.4, containing 1 % of 2-mercaptoethanol as solvent. The paper chromatogram was also developed between two horizontal glass plates in order to prevent oxidation using the same solvent as in the ascending run. Immediately after the run the moist spots were studied in UV light (The Chromatolite, Hanovia). Thin layer chromatography was also employed to study the products of the reactions. A Desaga apparatus was used and Kieselgel G, 0.25 mm thick, from E. Merck AG was employed as adsorbent. Several solvents were tried out before dimethylformamide-water 190:10 was chosen as it gave the best results.[6]

(b) *Spectrophotometric determination.* For the quantitative determination of enzyme activity a mixture of the following components was placed in the quartz cuvette (light path 1 cm) of a Beckman DU spectrophotometer: FH_2, 0.45 μmole, $NADPH_2$, 0.25 μmole, ATP, 0.2 μmole, Na-formate, 6.0 μmoles, cell extract 0.3−0.5 ml, 2-mercaptoethanol, 25 μl, and potassium phthalate buffer of pH 5.8, 150 μl. The total volume was 3.0−3.3 ml. The reaction started when the $NADPH_2$ was added. The rate at which $NADPH_2$ was consumed in the reaction was determined by following the decrease in absorption at 340 mμ ($\varepsilon_{340} = 6.2 \times 10^6$ cm^2 mole^{-1}) and comparing the results with those for a mixture lacking $NADPH_2$. Since the cell extract is believed to contain oxidases which catalyze the oxidation of $NADPH_2$,[7] the decrease in absorption at 340 mμ was determined also without FH_2 present. The decrease in the amount of coenzyme that took place under these conditions was taken into account in the calculation of the enzyme activity.

RESULTS and DISCUSSION

Monod's definitions [8] of bacterial growth phases have been followed in this work.

The existence of tetrahydrofolate dehydrogenase in the cells of *Lb. arabinosus* 17−5 and Str KQ was proved qualitatively by running a paperchromatogram of the reaction mixture after the reaction. The tetrahydrofolic acid formed in the reaction was discerned as a dark spot when the paper was examined in UV light. The R_F value of the spot was 0.64 and it was dyed by a diazostain. When the spot was extracted with a 0.05 M potassium phosphate buffer 0.1 M in 2-mercaptoethanol, and the absorption of the extract was measured, a curve with a peak at 298 mμ was obtained. The dihydrofolic acid spot had the R_F value 0.24 and therefore did not affect the results.

The level of tetrahydrofolic acid was a maximum at the beginning of the exponential phase. This became evident when the enzyme activities of samples withdrawn regularly during the growth were determined by paperchromatography. The activity was markedly lower for the samples taken at the beginning of the lag phase and in the retardation phase. The results were similar also when thin-layer chromatography was employed in the study of the reaction mixtures.

Other spots with a bluish green fluorescence and an R_F value of 0.09 were found just above the starting spots. In size and brightness these spots were directly proportional to the starting spots obtained with the same reaction mixture, but they were not coloured by diazo dying. For this reason they were not considered to contain tetrahydrofolic acid. Evidently these spots are formed by xanthopterine and 2-amino-4-hydroxy-6-methylpterine, the decomposition products of tetrahydrofolic acid. The activity of tetrahydrofolate dehydrogenase was measured quantitatively with a Beckman DU spectrophotometer by determining the absorption at 340 mμ. The amount of $NADPH_2$ oxidized in the reaction was measured. In these experiments the amount of $NADPH_2$ in micromoles per mg of cell protein oxidized during 10 min of reaction was used as a standard in the determinations of tetrahydrofolate dehydrogenase activity.

During the lag phase of the *Str. thermophilus* strain KQ the activity increased from 0.026 to 0.046 μmole of $NADPH_2$/mg/10 min. The increase is thus 1.76-fold in the lag phase (Fig. 1). When 17 % of the exponential phase was over the activity was maximal, 0.39 μmole of $NADPH_2$/mg/10 min, which means a further 8.5-fold increase. The activity sharply decreased after the maximum and at the beginning of the retardation phase it was almost equal to the activity at the beginning of the exponential phase (Fig. 2).

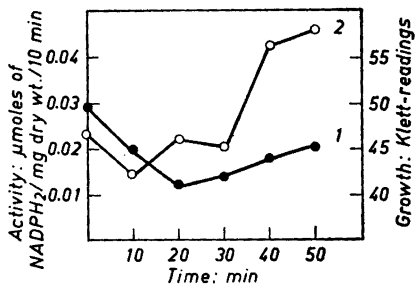

Fig. 1. Tetrahydrofolate dehydrogenase activity during the lag phase of growth of the *Str. thermophilus* strain KQ. 1, Growth; 2, enzyme activity.

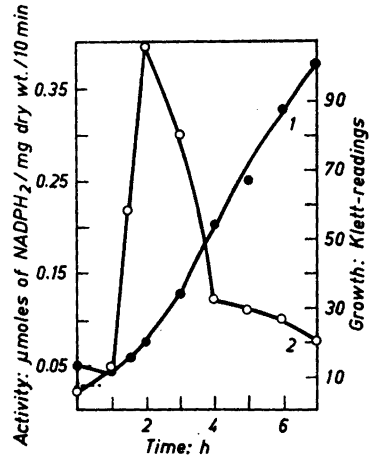

Fig. 2. Tetrahydrofolate dehydrogenase activity during the growth of *Str. thermophilus* strain KQ. 1, Growth; 2, enzyme activity.

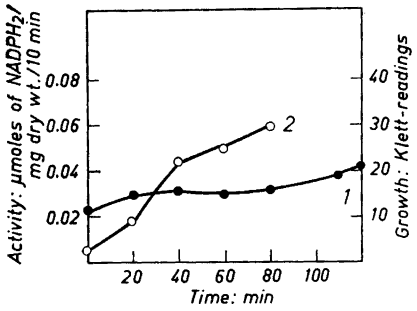

Fig. 3. Tetrahydrofolate dehydrogenase activity during the lag phase of growth of *Lb. arabinosus* 17—5. 1, Growth; 2, enzyme activity.

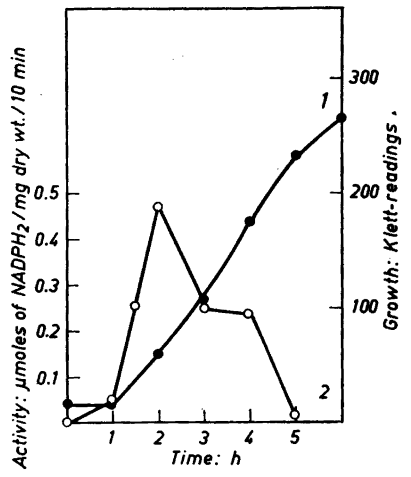

Fig. 4. Tetrahydrofolate dehydrogenase activity during the growth of *Lb. arabinosus* 17—5. 1, Growth; 2, enzyme activity.

During the lag phase of *Lb. arabinosus* 17—5 the tetrahydrofolate dehydrogenase activity increases 12-fold from 0.005 to 0.06 µmole of $NADPH_2$/mg/10 min (Fig. 3). The maximal value during the exponential phase, 0.475 µmole of $NADPH_2$/mg/10 min, was reached when 20 % of the phase was over. After that the activity decreased and at the beginning of the retardation phase it was almost equal to the activity at the beginning of the lag phase (Fig. 4). Similar variations were observed in enzyme activity also in repeated experiments where this activity was determined spectrophotometrically. Similarly, the chromatographic method used in the qualitative determinations showed that tetrahydrofolate dehydrogenase is most active at the beginning of the exponential phase of growth.

When the results obtained in this work are compared with those we have obtained earlier, it becomes evident that the tetrahydrofolate dehydrogenase activity in *Str. thermophilus* and *Lb. arabinosus* increased during the first growth phases as it did in the *Str. faecalis* R cells also.[1] It has thus been possible to prove that tetrahydrofolate dehydrogenase appears as a so-called lag phase enzyme in three different strains of lactic acid bacteria. In addition, it is interesting to note that the activity of tetrahydrofolate dehydrogenase was maximal already at the beginning of the exponential phase of growth.

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