

Biosynthesis and Excretion of Folic Acid during the Growth Cycle of *Lactobacillus arabinosus*

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The folic acid content of *Lactobacillus arabinosus* cells and the excretion of the compound during the active growth phases of the cells have been studied. Marked variations occurred in the folic acid content of the cells as early as during the lag phase (between cell divisions). The folic acid content of the cells increased during the early exponential growth phase, rose to a maximum when one-third of the phase had elapsed, and then decreased rather sharply to the original level. *Lb. arabinosus* did not excrete any folic acid during the lag phase, but did so during the acceleration and retardation phases.

It is a well-known fact that certain microbes are able to synthesize and excrete folic acid. In connection with investigations concerning symbiosis phenomena in lactobacilli it has been found that certain strains, for instance, *Lactobacillus arabinosus* 17-5 (ATCC 8014), both synthesize folic acid and excrete it into the growth medium^{1,2}. Whether variations occur in the biosynthesis and excretion of folic acid during the different growth phases has not been investigated in greater detail and we have therefore undertaken a study of the amounts of folic acid synthesized and excreted by *Lactobacillus arabinosus* 17-5 during the active growth phases. In addition, we especially wanted to find out whether *Lb. arabinosus* synthesizes or excretes folic acid already in the lag phase. Monod's definitions of the bacterial growth phases have been followed in this paper, and according to these definitions we have taken the lag phase to extend over the period when cell division is not taking place before the growth³.

EXPERIMENTAL

Organisms. *Lactobacillus arabinosus* 17-5 (ATCC 8014) and *Streptococcus faecalis* R (ATCC 8043) were used in this study. The bacteriological technique for maintaining stock cultures has been described in an earlier paper.⁴

Preparation of inoculum. In the experiments concerning the exponential growth phase the preparation of the inoculum was conducted as follows. *Lb. arabinosus* cells were transferred from the stock cultures to 7 ml of a glucose-citrate-tryptone-yeast extract medium and incubated at 37° for 16-18 h. The cells were then centrifuged, isola-

ted and washed 4–5 times with 0.9 % sterile saline. After washing the cell mass was dispersed in 100 ml of the saline solution. The turbidity of the suspension was so low that a Klett-Summerson colorimeter did not give any reading. Two drops of this weak suspension was used to inoculate 20 ml of the final growth medium.

The inoculum used in the study of folic acid synthesis and excretion during the lag phase of growth was prepared in the same way except that the bacteria were cultured first in ten tubes each containing 7 ml of the glucose-citrate-tryptone-yeast extract medium and the washed cells were mixed with saline solution to give suspensions with optical density readings of 150–200 (using filter 62), equivalent to 0.4–0.5 mg dry weight of cells per ml of the suspension. One ml of this thick suspension was used to inoculate 20 ml of the final growth medium.

Procedure. The basal medium employed was the one described by Macias⁵ except that the folic acid was left out and thiamine (1 μ g per ml of basal medium) added. The pH was adjusted to 6.8.

In the experiments relating to the lag phase, 10 ml of the basal medium and 9 ml of water were added to large test tubes (20 \times 180 mm). After they had been autoclaved at 112° for 10 min, the tubes were cooled to 37° and 1 ml of the heavy inoculum was added to each. The tubes were then transferred to an incubator at 37°. At intervals of 15 min single tubes were removed from the incubator and cooled in ice-water.

The experiments relating to the exponential phase were conducted in the same way as those relating to the lag phase except that a light inoculum suspension was used and the tubes were removed only after several hours of incubation (Fig. 2). In all experiments the growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter No. 62. The calibration curve plotting bacterial density (mg dry wt. of cells per ml) against turbidity proved to be valid both for the inoculum and for the cultures.

Preparation of samples for folic acid assay. When the turbidities of the suspensions had been measured, the cells were centrifuged down (10 min at 3 500 rpm) and washed four times with 0.9 % saline. For the folic acid assay an enzymatic hydrolysis was performed by the method of Snell⁶ modified as follows. The washed cells were suspended in 5 ml of 0.5 % acetate buffer solution (pH 4.5) to which 1 mg each of papain and diastase and 5 ml of toluene had been added. The solution was then incubated at 37° for 24 h. The enzymes were then destroyed by heating the suspension in an autoclave at 120° for 10 min. The cooled suspension was centrifuged. The clear supernatant was neutralized to pH 6.8 and diluted with distilled water to a volume of 50 ml.

The culture filtrate was also diluted with distilled water to 40 ml and preserved at + 1° until assayed.

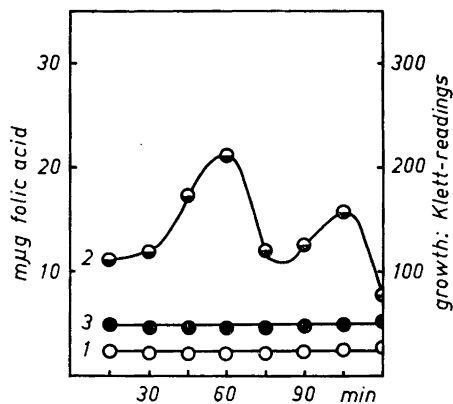


Fig. 1. Formation and excretion of folic acid by *Lb. arabinosus* during the lag phase of growth. 1, Growth curve (right-hand scale); 2, Content of folic acid in the cells (μ g per mg of dry matter); 3, Amount of folic acid excreted by 1 mg (dry wt.) of cells per 20 ml of growth medium.

Assay of folic acid. The folic acid assay was performed according to the standard procedure ⁷ using *Str. faecalis* R (ATCC 8043). Pteroylglutamic acid was used as standard. No upward or downward drifts were noted. In this connection it should be noted that in addition to pteroylglutamic acid also pteronic acid, 10-formylpteronic acid, 10-formylfolic acid and reduced folic acid derivatives are equally active for this organism (reviewed recently by Rabinowitz ⁸).

RESULTS AND DISCUSSION

In the first stage of the present work the experiments aimed at clarifying whether *Lb. arabinosus* is able to synthesize folic acid and possibly excrete it into the growth medium already in the lag phase. A heavy inoculum was used in these experiments but in the experiments relating to the exponential phase the inoculum was a light one. The advantage of using a heavy suspension lies in the large number of cells available for study. The lag phase lasted 2 h at most.

Two maxima were observed in the curve plotting the folic acid content of the cells against time during the growth of *Lb. arabinosus*. The first maximum occurred when half the lag phase had passed. At this point the cells contained 22 μg of folic acid per mg of dry matter. At the beginning of the lag phase they contained 11 $\mu\text{g}/\text{mg}$, which value was obtained also when two-thirds of the phase (82 min) had passed. The second maximum occurred when 87 % of the lag phase had passed; the concentration of folic acid in the cells was then 14 μg per mg of dry matter. No folic acid was excreted into the medium during the lag phase.

In the experiments relating to the exponential growth phase the folic acid content of the cells increased already from the beginning of the phase (the expo-

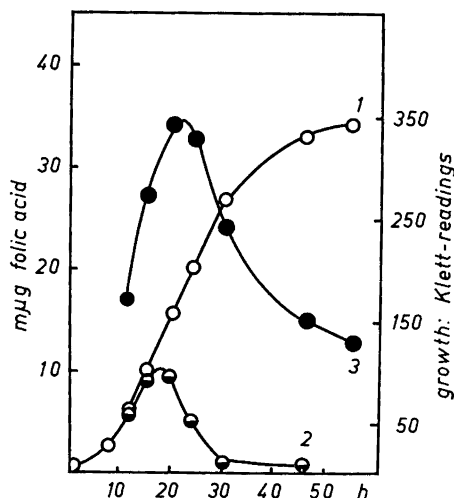


Fig. 2. Formation and excretion of folic acid by *Lb. arabinosus* during the exponential and retardation phases of growth. 1, Growth curve (right-hand scale); 2, Content of folic acid in the cells (μg per mg of dry matter); 3, Amount of folic acid excreted by 1 mg (dry wt.) of cells per 20 ml of growth medium.

ponential growth phase was taken to have begun 11.5 h after the inoculation) and attained a maximum level after 17 h. Thus the maximum occurred at a point where 30 % of the exponential phase had passed. The folic acid content of the cells was at this point 11 $\mu\text{g}/\text{mg}$ (5 $\mu\text{g}/\text{mg}$ at the beginning of the phase).

The folic acid content then decreased relatively rapidly to a point 30 h after the inoculation, when the cells contained 1 μg of folic acid per mg dry weight of cells. The content then remained practically constant. It should be noted that the exponential growth phase ended at this same point, 30 h after the inoculation. The content of folic acid in the cells thus remained constant during the retardation phase.

As Fig. 2 shows, *Lb. arabinosus* excreted folic acid into the growth medium during both the exponential and the retardation growth phases. In the experiment in question the exponential phase began 11.5 h after the inoculation. At this point of growth each mg of cells (dry wt.) had excreted 1.2 μg of folic acid per ml of growth medium. The amount of folic acid excreted then rose sharply, reaching a maximum (2.3 $\mu\text{g}/\text{ml}$ medium excreted by 1 mg cells) at the mid-point of the exponential growth phase. After this the excretion decreased, and at the end of the exponential phase 1 mg of cells excreted 1.6 μg of folic acid per ml of growth medium. During the retardation phase, the folic acid excretion decreased further to a minimum of 0.9 $\mu\text{g}/\text{mg}$. All the experiments were repeated three times; the same results were obtained in all replications.

The most interesting finding of this study is the marked variation in the folic acid content of the cells during the lag phase, but it is very difficult to draw any definite conclusions as to why such variations occur on the basis of the experiments carried out hitherto. As folic acid derivatives participate in the biosynthesis of nucleotides⁸ the increases in the folic acid content may be considered to reflect a very active metabolism of nucleic acid components within the cell.

This work is a part of a more extensive study in progress in this laboratory which is connected with the biosynthesis of folic acid and related compounds and coenzymes during early phases of cell growth.

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