Some Effects of Folic Acid and Vitamin B₁₂ on Nucleic Acid Metabolism in Lactobacillus leichmannii*

JAMES S. DINNING AND RUTH S. YOUNG

From the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas

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Several lines of evidence implicate folic acid and vitamin B₁₂ in nucleic acid metabolism in various microorganisms. In some cases the exact effect has been carefully delineated. It is now well established that folic acid derivatives function in the insertion of 1-carbon units into purines and into the 5-methyl group of thymine (1). It was shown by Shive et al. (2) that thymine would replace folic acid for the organism Lactobacillus leichmannii.

Vitamin B₁₂ has been shown to influence two aspects of nucleic acid metabolism in L. leichmannii. It was observed by Snell et al. (3) that thymine would replace vitamin B₁₂ for L. leichmannii, and subsequent work (4) indicated that any deoxyribonucleoside would replace the vitamin for this microorganism. Recent work (5) has indicated that vitamin B₁₂ is involved in the conversion of ribose to deoxyribose by this organism. In addition to this effect of B₁₂ on deoxyribonucleic acid biosynthesis in L. leichmannii, it has also been shown that vitamin B₁₂ catalyzes the reduction of formate to the thymine 5-methyl group (6), a reaction similarly catalyzed by B₁₂ in chick bone marrow cells (7).

The present experiments were designed to obtain additional information on the influence of these two vitamins on nucleic acid metabolism in L. leichmannii.

**EXPERIMENTAL PROCEDURE**

A culture of L. leichmannii (American Type Culture Collection No. 7830) was obtained from Georgetown University. The general handling of the organism was as is described in the official vitamin B₁₂ procedure (8). The basal medium was the same as is described in the official assay except that folic acid was omitted from the medium. Advantage was taken of the fact that the B₁₂ requirement of the organism can be replaced with deoxyxystidine, and that the folic acid requirement can be replaced with thymine. Thus, it is possible to produce cells deficient in both vitamins and to measure the effects of the vitamins individually and in combination. The organism was grown for 16 hours in this basal medium with supplements as indicated in the various tables of results. The levels of additions to the basal medium were thymine, 5 µg per ml; deoxyxystidine, 2 µg per ml; folic acid, 1 µg per ml; and vitamin B₁₂, 0.2 mg per ml. In certain experiments various C⁴ substrates were included in the growth medium. The C⁴ compounds used, the quantities employed, and the specific activities were as follows: formate-C⁴, 0.8 µc per ml, 1 µc per µmole; thymine-2-C⁴, 0.04 µc per ml, 1 µc per µmole; orotic acid-2-C⁴, 0.1 µc per ml, 1 µc per µmole; and uracil-2-C⁴, 0.04 µc per ml, 0.4 µc per µmole. The total volume of culture medium was 10 ml in all experiments. After incubation for 16 hours at 37°, optical density was measured at 600 mµ with a Coleman spectrophotometer, and the cells were centrifuged and washed three times with 0.9% NaCl solution. The cells were subjected to the combined Schneider (9), Schmidt-Thannhauser (10) fractionation, according to the procedure previously described (6). Aliquots of the nucleic acid fractions were evaporated for C⁴ assay, in an end window Geiger tube. The concentrations of RNA and DNA were determined by the orcinol and diphenylamine procedures, respectively, as outlined in the Schneider fractionation procedure (9).

In the experiments in which the turnover of DNA thymine was measured, the cells were grown with thymine, deoxyxystidine, and with the additions of folic acid and vitamin B₁₂ as indicated in Table III. For the initial growth period, thymine-2-C⁴ was added to the basal medium (0.04 µc per ml). After incubation for 10 hours, the cells were centrifuged, washed four times with 0.9% NaCl solution, and extracted with cold 10% trichloroacetic acid to remove acid-soluble materials. The residue from this extraction was washed one time with cold 10% trichloroacetic acid and the washing discarded. The residue was then suspended in 5 ml of 5% trichloroacetic acid and heated for 30 minutes at 90° to remove nucleic acids. An aliquot of the acid soluble material and of the nucleic acid fraction was evaporated for counting. Another series of tubes treated in the same way during the initial 16-hour growth period was, after washing with 0.9% NaCl solution, resuspended in the identical medium previously employed, except that the C⁴ thymine was replaced with unlabeled thymine. After an additional 5 hours of growth, cells of this series were again harvested. The supernatant solution (incubation medium) was assayed for C⁴ activity and the cells were fractionated as were the cells in the first series. The third series of cells was treated the same as the second, except that they were allowed to grow an additional 20 hours after resuspension in the unlabeled medium. All counts on supernatant solutions, acid-soluble material, and nucleic acid fractions were corrected to infinite thinness.

All experiments were repeated at least two times and the agreement between duplicate experiments was quite good.

**RESULTS AND DISCUSSION**

The data given in Table I represent the average of several experiments. Since the primary objective of these experiments was to elucidate the influence of folic acid and vitamin B₁₂ on...
various parameters of nucleic acid metabolism, it was felt desirable to present the results in a more convenient fashion. For this purpose, a tabulation was made of the magnitude of effect of the variables studied on the various parameters of nucleic acid metabolism. These data are given in Table II. As an example of the calculation, the influence of folic acid on RNA concentration would be determined as follows. The RNA concentration in cells grown without folic acid would be compared to that in comparable cells grown with folic acid. Cells grown with deoxycytidine and thymine would be compared with cells grown with folic acid, deoxycytidine, and thymine; cells grown with B12, deoxycytidine, and thymine would be compared with cells grown with folic acid, B12, deoxycytidine, and thymine; and cells grown with B12 and thymine would be compared with cells grown with folic acid, B12, and thymine. The average percentage change due to addition of folic acid to the medium could then be calculated. These data are given in Table II.

The addition of thymine to the medium significantly reduced the incorporation of formate into DNA, the incorporation of erotic acid into DNA, and the incorporation of uracil into DNA. This must reflect a dilution of the isotope in the DNA and suggests that these substrates were being incorporated into DNA thymine. Deoxycytidine reduced the incorporation of erotic acid into DNA, indicating that erotic acid was being incorporated into DNA thydine.

RNA concentration was not significantly influenced by vitamin B12, but was slightly reduced by folic acid. Folic acid reduced the concentration of DNA, and B12 increased the concentration of DNA. Nucleic acid concentrations are expressed as quantity of nucleic acid per unit of optical density of cells in the original growth tubes. This presumably reflects the concentration of nucleic acid per unit of cellular mass (11). This effect of B12 is in agreement with other reports (12) which indicate that B12 increases the DNA content of several species of microorganisms. The effect obtained with folic acid, however, is not in agreement with other reports (12, 13) which in general suggest that limiting amounts of folic acid in the medium result in reductions in DNA concentrations per cell. The exact explanation for the folic acid effect on DNA concentration is not apparent. It may be related to the fact that the organisms grew more rapidly in the presence of folic acid and may have approached a stationary phase.

Folic acid increased the incorporation of formate into both RNA and DNA, in agreement with the known role of this vitamin in purine and thymine biosynthesis. The incorporation of formate into DNA was increased by B12 when the medium contained folic acid, which is in agreement with our earlier report (6), indicating an effect of B12 on the conversion of formate to the 5-methyl group of thymine. Neither vitamin significantly influenced the incorporation of erotic acid into RNA; however, folic acid increased the incorporation of erotic acid into DNA, and B12 was necessary for incorporation of erotic acid into DNA.

Similar results were obtained with uracil, with neither vitamin influencing the conversion of uracil to RNA and with B12 being essential for the incorporation of uracil-C14 into DNA. These results again point out the important role of vitamin B12 in the methylation of pyrimidine precursors to thymine.

Incorporation of thymine into DNA was decreased in the presence of folic acid and was significantly increased when B12 was added to the medium. In order to evaluate this effect of B12 on the incorporation of thymine into DNA, it seemed necessary to determine whether DNA thymine in this organism was in metabolic equilibrium with thymine in the medium, or whether, if once incorporated into the DNA, it remained as an optical density. One pathway of thymine degradation which occurs in animal tissue (14) would result in the conversion of carbon 2 to carbon dioxide. During the next 15 hours of growth in the unlabeled medium, there was further loss of DNA counts and a considerable increase in supernatant counts, again with optical densities slightly increased. These results demonstrate actual
Summary of individual effects of folic acid, vitamin B₁₂, thymine, and deoxyuridine on nucleic acid metabolism in Lactobacillus leichmannii

The results are expressed as percentage change resulting from addition of the substance in question to the medium. See text for details of calculation.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Effect of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folic acid</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA conc.</td>
<td>-29</td>
</tr>
<tr>
<td>DNA conc.</td>
<td>-41</td>
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<tr>
<td>Formate-C¹⁴ incorporation into RNA</td>
<td>+544</td>
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<tr>
<td>Formate-C¹⁴ incorporation into DNA</td>
<td>+3840</td>
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<tr>
<td>Orotic acid-2-C¹⁴ incorporation into RNA</td>
<td>+21</td>
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<tr>
<td>Orotic acid-2-C¹⁴ incorporation into DNA</td>
<td>+72</td>
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<tr>
<td>Uracil-2-C¹⁴ incorporation into RNA</td>
<td>+4</td>
</tr>
<tr>
<td>Uracil-2-C¹⁴ incorporation into DNA</td>
<td>+33</td>
</tr>
<tr>
<td>Thymine-2-C¹⁴ incorporation into DNA</td>
<td>-47</td>
</tr>
</tbody>
</table>

TABLE II
Summary of individual effects of folic acid, vitamin B₁₂, thymine, and deoxyuridine on nucleic acid metabolism in Lactobacillus leichmannii

In three series of tubes (12 tubes), the organism was grown for 16 hours in the presence of thymine-2-C¹⁴ and one series taken for assay of acid-soluble material and nucleic acid-C¹⁴. In the two additional series, the cells were exhaustively washed with 0.9% NaCl solution and resuspended in their former media, except the C¹⁴-thymine was replaced with unlabeled thymine. After 5 and 20 hours respectively, cells from the two series were harvested and the C¹⁴ activity of the supernatant solution (incubation medium), cellular acid-soluble material, and nucleic acids determined. Deoxyuridine was present in all tubes. The incubation volume was 10 ml and the c.p.m. refers to the entire incubation volume.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>After 16 hours in labeled medium</th>
<th>5 hours after resuspension in unlabeled medium</th>
<th>20 hours after resuspension in unlabeled medium</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>O.D. Acid-soluble material</td>
<td>O.D. Acid-soluble material</td>
<td>O.D. Acid-soluble material</td>
</tr>
<tr>
<td></td>
<td>c.p.m. Nucleic acid</td>
<td>c.p.m. Nucleic acid</td>
<td>c.p.m. Nucleic acid</td>
</tr>
<tr>
<td>None</td>
<td>0.34 278.16350.52</td>
<td>29.1892 161.058 15</td>
<td>1100 460</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.52 406.12800.64</td>
<td>72.1448 322.065 44</td>
<td>855 437</td>
</tr>
<tr>
<td>B₁₂</td>
<td>0.42 783.95000.61</td>
<td>319.7101 736.068 58</td>
<td>4420 1450</td>
</tr>
<tr>
<td>Folic acid + B₁₂</td>
<td>0.62 478.54000.63</td>
<td>101.6804 270.856 29</td>
<td>4100 500</td>
</tr>
</tbody>
</table>

turnover of DNA thymine during growth of the organism. There was evidence that in cells grown without B₁₂ there was some turnover of DNA thymine after incubation for 20 hours in the unlabeled medium; however, the magnitude of this effect was much less than when the cells were grown in the presence of B₁₂. Folic acid appeared to inhibit this turnover and thus, in this experiment the results obtained by measuring the disappearance of thymine from DNA are in agreement with those obtained in the experiments in which the incorporation of thymine into DNA was measured. These results suggest that B₁₂ somehow catalyzes the reaction involved in the entry and release of thymine from the DNA of L. leichmannii. The exact mechanism of this effect remains to be elucidated. It cannot be stated with certainty from these experiments whether the observed turnover of DNA thymine represents turnover in the intact cell or whether it reflects cell lysis with degradation of the DNA. Such lysis would be counterbalanced by division of other cells to account for the increase in optical density.

These experiments again demonstrate the important role of vitamin B₁₂ in the methylation of thymine precursors in the formation of DNA thymine. The experiments with labeled formate, labeled erotic acid, and labeled uracil all point to the involvement of B₁₂ in this methylation process. The exact explanation for the effect of B₁₂ in catalyzing the turnover of DNA thymine in L. leichmannii is not apparent, if based on present knowledge of the mode of action of the vitamin. The elucidation of the nature of this effect may prove to be of considerable fundamental interest.

SUMMARY
Folic acid and vitamin B₁₂ were both found to exert very significant effects on nucleic acid metabolism of Lactobacillus leichmannii. Folic acid increased the incorporation of formate into deoxyribonucleic acid and ribonucleic acid, and reduced the concentration of deoxyribonucleic acid per unit of cell mass. B₁₂ increased the deoxyribonucleic acid concentration per unit of cell mass and was found to be necessary for the incorporation of erotic acid or uracil into deoxyribonucleic acid. B₁₂ also increased incorporation of formate into deoxyribonucleic acid and the incorporation of thymine into deoxyribonucleic acid. It was found that deoxyribonucleic acid thymine of L. leichmannii is in metabolic equilibrium with thymine in the medium and that this exchange is catalyzed by vitamin B₁₂.

REFERENCES
Some Effects of Folic Acid and Vitamin B₁₂ on Nucleic Acid Metabolism in *Lactobacillus leichmannii*

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