Biotin has been shown to have no significant influence on the total levels of folates in Escherichia coli. Exogenous biotin caused a redistribution of folate derivatives favouring the intracellular accumulation of unconjugated methylenetetrahydrofolates at the expense of conjugated formyltetrahydrofolates. The decreased levels of the conjugated folates in presence of biotin has been attributed to the observed lowering in the levels of folylconjugate synthetase. The availability of intracellular glutamic acid was not a limiting factor in the presence of biotin. The observed lowering in the concentration of intracellular methionine in the biotin grown cells has been discussed in the light of the decreased levels of conjugated folates in these cells.

Various studies have been reported on the metabolic interrelationship of biotin and folic acid since Luckey et al. (1) first suggested that biotin might influence tissue folate synthesis. In a later report Noronha et al. (2) observed that in folate-deficient rats receiving biotin the fecal excretion of folate exceeded the dietary intake of the vitamin. As it was unlikely that the excreted folates were of tissue origin, it was suggested that biotin favourably influenced the folates synthesized by intestinal microflora and consequently improved tissue folate levels. Marchetti et al. (3–6) indicated that biotin could influence conversion of folic acid to citrovorum factor, as also the interconversion of various tetrahydrofolate one-carbon derivatives. Interrelationship between these two vitamins had also been demonstrated in certain microorganisms (7, 8).

We have now chosen the coliform Escherichia coli B for a detailed study of the influence of exogenous biotin on folate metabolism. The results indicate that biotin is likely to influence the state of conjugation of intracellular folates rather than the total folate levels. These differences may have escaped earlier investigators who had not taken into consideration the levels of conjugated tetrahydrofolate derivatives. We have now employed extraction procedures which minimize degradative and oxidative changes along with digestion of the extracts.
to release conjugated folates as assayable monoglutamyl forms. The folates have been further separated chromatographically and characterized by differential microbiological assays.

EXPERIMENTAL

Maintenance and harvesting of Escherichia coli B. The cells were harvested at late log phase growth at 37°C in a synthetic medium containing 7.0 g of K₂HPO₄, 3.0 g of KH₂PO₄, 0.55 g of sodium citrate, 2.0 g of (NH₄)₂SO₄, 1.0 g of DL-aspartic acid, 5.0 g of glucose and 0.05 g of MgSO₄·7H₂O in one litre at pH 6.8. Biotin, when present, was added in the growth medium at a level of 15 μg/litre. The cells were harvested and washed with isotonic saline by centrifugation (4°C at 5,000 × g for ten minutes). Acetone powders were prepared using chilled acetone and the dried powders were stored up to a week at -20°C before extraction.

Extraction of folates. Weighed aliquots of the acetone powders were extracted in hot 1% ascorbate (stored frozen as a 10% solution adjusted to pH 6.0 with potassium hydroxide) at 75°C for twenty minutes. Under these conditions, oxidative, isomerisation and hydrolytic alterations of the folate compounds present are minimal (11). The suspensions were cooled and the clear extracts obtained by centrifuging were used for the assay of folate activity.

Assay of folate activity. The samples were assayed prior to and after treatment with chicken liver conjugase preparation in order to release simple folates from conjugated forms according to the method of NORONHA and ABOOBAKER (9). Folate activity was measured by the growth responses to Lactobacillus casei and Pediococcus cerevisiae. Calcium leucovorin (Lederle) was used as the reference standard. Corrections were made for the inactive isomer.

Chromatography of E. coli extracts. Up to 1 ml aliquots of the 1% ascorbate extracts of the acetone powder corresponding to a total folate content of about 3 μg were layered on standard analytical DEAE-cellulose column and the folate activity was eluted out by the ascorbate-phosphate gradient as described by SILVERMAN et al. (10). Fifty 5.0 ml fractions were collected. The eluted fractions were subjected to conjugase digestion and assayed for folate activity using L. casei as the test organism. The results are shown in Fig. 1.

Synthesis of conjugated folates by E. coli extracts. Freshly harvested cells were lysed by sonication in 0.05 M phosphate buffer, pH 7.5 and the extract spun at 10,000 rpm for thirty minutes. The supernatant was treated with Dowex-1-chloride to remove the endogenous folates. The Dowex-treated cell lysate was subjected to 0–63% ammonium sulphate fractionation and the precipitate dissolved in 0.05 M phosphate buffer pH 7.5. This preparation, shown to be active in the synthesis of conjugated tetrahydrofolates from tetrahydrofolic acid by GRIF-FINS and BROWN (12), was assayed accordingly. After three hours of reaction the 2 ml reaction mixture was made up to 1% with ascorbate and boiled for five
Fig. 1. Distribution of folate derivatives in biotin-grown and control cells. Aliquots of the extracts of acetone powder of control cells (O—O) and biotin grown cells (×—×) corresponding to about 3 μg folate activity were chromatographed on standard DEAE-cellulose columns, eluted and assayed microbiologically with L. casei as described in the text.

RESULTS

Inclusion of biotin in the growth medium was without effect on the growth of the organism. The presence of biotin did not influence the total folates biosynthesized by the cells as both the groups were found to be almost equally rich in their total folate contents (L. casei activity after conjugase digestion—Table 1). However in the biotin grown cells the unconjugated folates (values before conjugase digestion) responding to L. casei and P. cerevisiae were significantly higher and accordingly the ratio between the conjugated and unconjugated folates was found
Fig. 2. Decrease in the folyl conjugate synthetase activity of biotin grown cell extracts. The reaction mixture contained in a total volume of 2.0 ml L-glutamic acid 5.0 mM, tetrahydrofolic acid 0.1 mM, ATP 5.0 mM, MgCl₂ 10.0 mM, phosphate buffer pH 7.5, 200 mM and enzyme preparation corresponding to 3 mg protein. ● Control, ×→× biotin grown. After three hours of reaction at 37°C, the altered folates were chromatographically separated and assayed using L. casei. The folate profiles presented have been corrected for endogenous conjugated folates (see text).

Table 1. Folic acid content of Escherichia coli cells grown in presence and absence of exogenous biotin. The values represent an average of five separate experiments.

| Biotin status of medium | L. casei activity | P. cerevisiae activity | Methyl-THFA* |
|-------------------------|-------------------|------------------------|--------------|
|                         | Conjugase digestion | Conjugated | Unconjugated | Conjugase digestion | Conjugated | Unconjugated | Non-methyl-THFA |
|                         | before | after | (µg/g of acetone powder) | before | after | (µg/g of acetone powder) | before | after | (µg/g of acetone powder) |
| +Biotin                 | 9.709 ± 0.289 | 22.390 ± 1.627 | 1.283 ± 0.061 | 3.787 ± 0.216 | 7.965 ± 0.487 | 1.104 ± 0.062 | 1.810 ± 0.083 |
| −Biotin (control)       | 6.024 ± 0.187 | 20.840 ± 1.243 | 2.875 ± 0.074 | 2.453 ± 0.178 | 11.235 ± 0.793 | 3.684 ± 0.287 | 0.855 ± 0.057 |

*Methyltetrahydrofolates correspond to the difference between L. casei activity and P. cerevisiae activity (non-methyltetrahydrofolates). Increase in assayable folate activity after conjugase digestion was taken as a measure of conjugated folate levels.

...to be significantly lowered (p<0.001). Biotin was also found to favour the presence of more methyltetrahydrofolate derivatives (difference between L. casei activity and P. cerevisiae activity) and consequently the ratio of methyltetrahydr-
rofolates to non-methyltetrahydrofolates was increased \( (p<0.01) \) in the presence of biotin.

The distribution of cellular folates as monoglutamates, di- and triglutamates and polyglutamates can be clearly seen from Fig. 1. NORONHA and SILVERMAN (11) had shown earlier that (under identical eluting conditions described for these standard analytical DEAE-cellulose columns) folate activity peak fractions eluting up to fractions 13 (5.0 ml each) are monoglutamyl tetrahydropterate derivatives; fraction 13–24 contain di- and triglutamates while higher polyglutamyltetrahydropterate derivatives elute out beyond fraction 25. The proportion of monoglutamates present in the biotin supplemented cells was higher (9.22%) compared with control cells (2.26%); di- and triglutamyl pteroates were also higher in the presence of exogenous biotin (50.31% as compared to 20.51% in the absence of biotin). However the polyglutamylfolate levels were 37% lower in the presence of exogenous biotin.

In unreported observations the intracellular concentrations of free amino acids were determined by analysis on the 'Unichrom amino acid analyser' (Beckman) after suitable extraction and precipitation of peptides and proteins. It was found that the methionine concentration (816 μmoles/1×10^8 cells) in the cells grown in biotin supplemented medium was less than half that in control cells (2,064 μmoles/1×10^8 cells). However the glutamic acid levels were found to be increased in the presence of biotin (469 μmoles/1×10^8 cells and 304 μmoles/1×10^8 cells in the absence of added biotin). Other amino acids were not significantly affected.

The extent of in vitro synthesis of polyglutamyl tetrahydrofolates by the cell extracts during the conjugate synthetase reaction is evident from Fig. 2 which shows a typical chromatogram of the reaction products. The tetrahydrofolate-glutamate conjugating system of the partially purified extracts from control cells (934±87 μg polyglutamyl folates synthesized per mg protein) was significantly more effective \( (p<0.05) \) than extracts from biotin grown cells (573±63 μg polyglutamyl folates synthesized/mg protein). The conjugated folates formed in vitro from added tetrahydrofolate were mainly triglutamyl tetrahydropterate derivatives (peak fractions in tubes 24 and 27, Fig. 2).

In other experiments the enzymatic breakdown of conjugated folates (conjugase activity) by sonicated extracts of E.coli cells was studied according to NORONHA and ABOOBAKER (9) and found to be negligible irrespective of the biotin status of the growth medium.

**DISCUSSION**

Our results indicate that the presence of exogenous biotin in the growth
medium favoured the accumulation of simpler folates in *E. coli* cells but had no marked influence on the total folates synthesized. This was conclusively evident from the effect of biotin on the distribution pattern of various intracellular folate derivatives presented in Fig. 1. These observations on the role of exogenous biotin in favouring the accumulation of conjugated tetrahydrofolate derivatives in *E. coli* at the expense of conjugated forms, corroborate our findings pertaining to a similar influence of biotin in enhancing rat liver and blood unconjugated folate levels rather than the total folate content (in preparation).

Presumably the factors that determine the state of conjugation of folates are (i) the availability of glutamate for condensing with tetrahydrofolates in step-wise $\gamma$-glutamyl linkages (ii) level of folylconjugate synthetase activity which is responsible for the synthesis of conjugated tetrahydrofolates (12) and (iii) the folylconjugase activity which could break down the polyglutamates to lower levels of conjugation. The observed intracellular levels of glutamate (see RESULTS) rule out the availability of this amino acid as a limiting factor contributing to the decreased levels of conjugated tetrahydrofolates in the presence of exogenous biotin. We have been unable to observe any substantial levels of folyl conjugase activity in sonicated extracts of *E. coli*. Biotin supplementation however, was observed to decrease the folylconjugate synthetase activity of the organism, possibly explaining the lowered levels of intracellular polyglutamyltetrahydrofolates in this group. Relevent here is the observation by GUEST et al. (15) that conjugated forms rather than simple methyltetrahydrofolate are the preferred cofactors for methionine synthesis in *E. coli*. Thus despite the higher proportion of simple methyltetrahydrofolates (Table 1), biotin supplementation resulted in decreased intracellular levels of methionine (see RESULTS). NORONHA and SILVERMAN (13) have suggested that methionine provides a suitable methyl acceptor in the form of S-adenosylhomocysteine which could serve to release ‘trapped’ methyltetrahydrofolates. S-adenosylmethionine is also known to inhibit the 5,10-methylenetetrahydrofolate reductase activity (21). Thus the lowering in intracellular methionine levels would in turn explain the higher proportion of methyltetrahydrofolates associated with biotin supplemented cells (Table 1). GRIFFIN and BROWN (12) and BUEHRING et al. (20) have evidence to suggest that in *E. coli* and rat liver methyltetrahydrofolates could not be converted to higher conjugated forms, further explaining our present observations on the reduced levels of conjugated folate derivatives, as unconjugated methyltetrahydrofolates accumulate when biotin is supplemented.

During avian embryo development NADKARNI and NORONHA (16) showed that unconjugated methyltetrahydrofolates were initially present in the yolk and in rat serum (23) and suggested that this form preferably served as the storage and transport form of the vitamin. Conjugated formyltetrahydrofolates are present in the developing tissue sites (16) and are reported to be more active in the coenzymatic functions of this vitamin (14, 17–19). The latter are not easily
absorbed and transported (22). While biotin may have a role in mobilizing scarce folates stores in folate deficiency (1, 2) by favouring the accumulation of unconjugated methyltetrahydrofolates at the expense of other conjugated tetrahydrofolate derivatives, we have no evidence for a role of biotin in enhancing the total cellular folate levels as erroneously suggested by earlier workers.

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