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# Relationships between Biotin and Vitamin $B_{12}$

## EFFECTS OF BIOTIN AND VITAMIN $B_{12}$ ON FOLIC ACID METABOLISM

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1. The effects of dietary biotin compared with vitamin $B_{12}$ on the total content and on the distribution of the various folate derivatives in the liver of rats given a biotin-free diet have been studied. The effect of both vitamins on the conversion *in vitro* of folic acid into citrovorum factor in the same experimental conditions was also examined. 2. In biotin-treated rats as well as in vitamin $B_{12}$-treated rats the total content of folic acid-active substances measured microbiologically by *Pediococcus cerevisiae*, *Streptococcus faecalis* and *Lactobacillus casei* is significantly higher than that in biotin-deficient rats. The liver distribution of various folate derivatives in the three groups of animals is also markedly modified. 3. The amount of citrovorum factor formed in systems with liver homogenate of rats receiving biotin or vitamin $B_{12}$ is higher than that with liver homogenates of deficient rats. 4. The results obtained demonstrate the influence of biotin in the metabolism of folic acid, and the similar actions at this level of both biotin and vitamin $B_{12}$. These results are discussed in relation to the participation of the two vitamins in the metabolism of $C_1$ units, as a biochemical interpretation of the relationships between vitamin $B_{12}$ and biotin.

Interrelationships between biotin and vitamin $B_{12}$ have been observed by Marchetti & Testoni (1964). The administration of vitamin $B_{12}$ to rats receiving a biotin-free diet exerts a favourable effect on the growth rate and a delay of the deficiency symptoms. In addition, vitamin $B_{12}$ seems to have an effect on biotin-dependent enzymic activities. On the other hand, the administration of biotin influences methylation *in vivo* and *in vitro* in the same experimental conditions. This last observation led to the hypothesis of the common participation of both vitamins in the metabolism of $C_1$ units, and this is a biochemical interpretation of the 'sparing action' of vitamin $B_{12}$ in biotin deficiency.

The part played by vitamin $B_{12}$ in the metabolism of $C_1$ units has already been studied. In particular, the role of vitamin $B_{12}$ in methylation has been demonstrated (Oginsky, 1950; Dietrich, Monson & Elvehjem, 1952; Fatterparker, Marfatia & Sreenivasan, 1955; Marchetti, Viviani & Rabbi, 1956; Ranke, Ranke & Chow, 1962; Moruzzi, Marchetti & Viviani, 1963). According to Arnstein (1955), vitamin $B_{12}$ might be involved in the synthesis of labile methyl groups from more highly oxidized precursors such as C-$\alpha$ of glycine or C-$\beta$ of serine.

Kisliuk (1961) showed that an enzyme containing a derivative of vitamin $B_{12}$ is required for the synthesis of the methyl group of methionine. Recent evidence suggests that methylcobalamin may be the actual coenzyme for methionine synthetase (Guest, Friedman, Woods & Smith, 1962; Weissbach, Peterkofsky, Redfield & Dickerman, 1963; Foster, Dilworth & Woods, 1964). With regard to biotin, the only (indirect) evidence for its possible participation in the metabolism of $C_1$ units is that on its influence on folic acid synthesis. Luckey, Pleasants, Gordon & Reyniers (1955) and Noronha, Sreenivasan & Padval (1959) demonstrated that biotin administered to biotin- and folic acid-deficient rats caused an abnormal increase in folic acid excretion and an almost complete restoration of folic acid concentrations in liver.

Therefore the action of biotin on the metabolism of $C_1$ units might be due to its influence on the availability of folic acid or even more of folate cofactors in tissues.

The role of folate coenzymes in the metabolism of $C_1$ units is well known: the hydroxymethyl groups involved in serine–glycine interconversion and in the biosynthesis of methyl groups of thymine, methionine and choline are transferred by tetrahydrofolic acid (O'Brien, 1962). Sakami & Ukstins (1961) and Larrabee, Rosenthal, Cathon & Buchanan (1963) have indicated that a folic acid derivative, $N^5$-methyltetrahydrofolic acid, can be considered as a precursor in methionine synthesis.

This present research has been undertaken to verify experimentally the hypothesis of the common participation of vitamin $B_{12}$ and biotin in the metabolic pathway of $C_1$ units, and in particular in the metabolism of folic acid. For this purpose it was decided to study the effect of dietary biotin, compared with that of vitamin $B_{12}$, on the total content and on the distribution of the various folate derivatives in the liver of biotin-deficient rats. The effect of both vitamins on the conversion *in vitro* of folic acid into citrovorum factor was also examined.

## MATERIALS AND METHODS

Weanling male rats of the Wistar strain were used. The animals were divided into three groups each of 12 rats, housed in cages with wire bottoms and fed *ad libitum* on the following diets respectively: biotin-free diet; biotin-supplemented diet; biotin-free diet supplemented with vitamin $B_{12}$. The exact composition of these diets is given in Table 1.

After 60 days, six animals of each group were killed and the livers, removed as rapidly as possible, were used for the determination of the concentrations of folate derivatives and their distribution. The livers, wiped free from blood, were homogenized with 2 vol. of ice-cold water. To the homogenates 2 vol. of ice-cold acetone was added, and the precipitated material was immediately filtered on a Buchner funnel with suction, washed with acetone and ether, and dried under vacuum over $P_2O_5$. Suitable quantities of acetone-dried powders were extracted in 1% (w/v) potassium ascorbate, pH 6·0, by heating the suspensions (20 mg./ml.) for 30 min. in a water bath at 75°. After centrifugation at 0° samples of clear supernatant were put on a column of DEAE-cellulose–Hyflo Super-Cel (20 cm. × 1 cm.) prepared for use by sequentially washing with 25 ml. of 0·5 N-KOH followed by water until the rinse was neutral, and then with 25 ml. of 0·5 M-phosphate buffer, pH 6·0, followed by water until the rinse was free of phosphate. After the extract was absorbed the column was washed with 0·2% potassium ascorbate and then eluted with an ascorbate–phosphate eluent (0·2% potassium ascorbate, pH 6·0, and 0·5 M-potassium phosphate buffer, pH 6·0). The flow rate was 1 ml./min. Thirty 5 ml. fractions were collected in tubes containing 0·1 ml. of 10% (w/v) potassium ascorbate, and, after suitable dilution in 1% potassium ascorbate, were assayed for folic acid activities with *Pediococcus cerevisiae* ATCC 8081, *Lactobacillus casei* ATCC 7469 and *Streptococcus faecalis* R ATCC 8043. Media employed were those generally used and described by Bakerman (1961). For assay, samples were added to basal media, each tube of which (final vol. 10 ml.) contained 10 mg. of potassium ascorbate. The tubes were steamed for 30 min., cooled and inoculated. Bacterial growth was measured turbidimetrically (at 660 mμ) after 20 hr. of incubation. Calcium leucovorin was used as a reference standard and the concentration was adjusted to correct for the presence of the inactive isomer. The identification of various peaks in the elution pattern on DEAE-cellulose of acetone-dried liver powder was obtained by running individual chromatograms with reference samples. [$N^{10}$-Formyltetrahydrofolic acid was prepared by isomerization of $N^5$-formyltetrahydrofolic acid with HCl at 0° and neutralization to pH 6·0; $N^5$-formyltetrahydrofolic acid was the DL-racemate, leucovorin (Lederle); tetrahydrofolic acid was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.] The identity of various folate derivatives was confirmed by their activities in stimulating the growth of the three test organisms and by their properties of stability (Silverman, Law & Kaufman, 1961).

The compound occurring in the region of tubes 8–10 (peak I) was identified as $N^{10}$-formyltetrahydrofolic acid. Further confirmatory evidence that peak I is that of $N^{10}$-formyltetrahydrofolic acid is that it possesses essentially equivalent growth activity for all three test organisms. In addition, when acetone-dried liver powder was extracted at 125° for 30 min. and then chromatographed, peak I disappeared almost completely, whereas peak III increased (isomerization of $N^{10}$-formyltetrahydrofolic acid to $N^5$-formyltetrahydrofolic acid).

The material that emerges at tubes 11 and 12 (peak II) corresponds in microbiological activity (active for *L. casei* and *S. faecalis* but not for *P. cerevisiae*) and stability to $N^{10}$-formyldihydrofolic acid and $N^{10}$-formylfolic acid.

The compound determining peak III (tube 13) was characterized as $N^5$-formyltetrahydrofolic acid by its stability at 125° as well as its activity in stimulating the growth of all three test organisms. The compound determining peak IV (tubes 14 and 15) was identified as $N^5$-methyltetrahydrofolic acid by its property of supporting the growth of *L. casei* but not of *S. faecalis* or *P. cerevisiae*.

The compound eluted in the region of tubes 16 and 17 (peak V) was characterized as tetrahydrofolic acid by its lability to heat in the absence of ascorbic acid and the different activity it showed in stimulating the growth of three test organisms.

Finally, the folates occurring at tubes 19 and 20 (peak VI) and 22–26 (peak VII) could correspond, according to Wittenberg, Noronha & Silverman (1962), to $N^{10}$-formyl and $N^5$-formyl derivatives of pteroylpolyglutamic acid.

For testing the conversion of folic acid into citrovorum factor *in vitro* six rats of each group were killed, and the livers were rapidly removed and homogenized with 4 vol.

Table 1. *Composition of experimental diets*

| Ingredients | Composition (%) Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Casein (vitamin-free) | 20 | 20 | 20 |
| Sucrose | 59 | 59 | 59 |
| Salt mixture no. 4* | 4 | 4 | 4 |
| Groundnut oil | 5 | 5 | 5 |
| Autoclaved egg white | — | 11 | — |
| Raw dried egg white | 11 | — | 11 |
| Vitamin mixture† | 1 | 1 | 1 |
| Biotin | — | 0·00001 | — |
| Vitamin $B_{12}$ | — | — | 0·00001 |

* Hegsted, Mills, Elvehjem & Hart (1941).

† The vitamin mixture contained (mg./g.): thiamine hydrochloride, 0·2; riboflavine, 0·2; pyridoxine, 0·25; calcium pantothenate, 2·0; nicotinic acid, 5·0; inositol, 10·0; *p*-aminobenzoic acid, 25·0; folic acid, 0·02; choline hydrochloride, 100·0; vitamin K, 0·21; diluted in sucrose, 847. Two drops of vitamins A, D and E concentrate were fed orally to each rat once a week.

of 0·08M-phosphate buffer, pH 6·3. Samples (5ml.) of homogenate were pipetted in 50ml. Erlenmeyer flasks containing 5ml. of the above-mentioned buffer, 10mg. of DL-homocysteine, 10mg. of L-serine and 100$\mu$g. of folic acid; the final volume was made up to 11ml. with water and the solutions were layered with toluene. Tests without adding folic acid were also prepared (control tests). The flasks were incubated in a reciprocating shaker at 37° for 2hr. under nitrogen. Then the samples were autoclaved, cooled, diluted and filtered. In the filtrates the citrovorum factor formed was assayed microbiologically with *P. cerevisiae*. Bacto 'CF assay medium Difco' was used as medium and calcium leucovorin as standard. The cultures were incubated for 20hr. at 37° and bacterial growth was measured turbidimetrically.

The quantities of citrovorum factor formed in systems without folic acid were subtracted from those formed in systems with folic acid. All the results were analysed for statistical significance by Fisher's *t* test; a difference between two means was regarded as significant when $P$ was no greater than 0·05.

## RESULTS

From the results of Table 2 it appears that the total liver content of folic acid-active substances, measured microbiologically by *P. cerevisiae*, *S. faecalis* and *L. casei*, is significantly higher in the biotin-treated rats ($P < 0·001$, $P < 0·02$ and $P < 0·02$ respectively) and in the vitamin $B_{12}$-treated rats ($P < 0·01$, $P < 0·01$ and $P < 0·01$ respectively) than in biotin-deficient rats. Also, the distribution of various folate derivatives in liver is markedly modified in the three groups of tested animals. The results of Table 3 and Fig. 1 show significant increases in the amounts of various folate derivatives in the liver of rats receiving biotin compared with the deficient animals. Particularly increased are: $N^{10}$-formyldihydrofolic acid and $N^{10}$-formylfolic acid ($P < 0·001$), $N^5$-formyltetrahydrofolic acid ($P < 0·001$), $N^5$-methyltetrahydrofolic acid ($P < 0·01$), tetrahydrofolic acid ($P < 0·001$) and $N^5$-formyl derivatives of tetrahydropteroylpolyglutamic acid ($P < 0·001$).

Larger amounts of folic acid-active substances were also noted in the liver of rats receiving vitamin $B_{12}$ than in those of deficient animals ($P < 0·001$). In the liver of vitamin $B_{12}$-treated rats $N^{10}$-formyltetrahydrofolic acid ($P < 0·02$) and $N^{10}$-formyl

Table 2. *Effects of dietary biotin and vitamin $B_{12}$ on folic acid activities of rat liver*

Each value is given as the mean ± S.E.M. of six determinations on different animals. Experimental details are given in the text.

| Group | Rat nutritional status | Folic acid activity (m$\mu$g./mg. of acetone-dried liver powder) By *P. cerevisiae* | By *L. casei* | By *S. faecalis* R |
|---|---|---|---|---|
| 1 | Biotin-deficient | 4·48 ± 0·36 | 6·17 ± 0·59 | 5·83 ± 0·51 |
| 2 | Biotin-treated (100$\mu$g./kg. of diet) | 6·07 ± 0·43 | 8·72 ± 0·73 | 8·10 ± 0·57 |
| 3 | Biotin-deficient vitamin $B_{12}$-treated (100$\mu$g./kg. of diet) | 6·44 ± 0·51 | 9·55 ± 0·77 | 9·27 ± 0·59 |

Table 3. *Effects of dietary biotin and vitamin $B_{12}$ on the distribution of various folate derivatives*

Each value is given as mean ± S.E.M. of the six determinations on different animals. Experimental details are given in the text.

| Compound | Concn. of folic acid derivatives (m$\mu$g./mg. of acetone-dried liver powder) Biotin-deficient rat | Biotin-treated rat | Biotin-deficient vitamin $B_{12}$-treated rat |
|---|---|---|---|
| $N^{10}$-Formyltetrahydrofolic acid | 4·09 ± 0·30 | 4·42 ± 0·43 | 5·52 ± 0·39 |
| $N^{10}$-Formyldihydrofolic acid and $N^{10}$-formylfolic acid | 0·60 ± 0·04 | 0·92 ± 0·05 | 1·97 ± 0·02 |
| $N^5$-Formyltetrahydrofolic acid | 0·19 ± 0·01 | 0·45 ± 0·03 | 0·56 ± 0·03 |
| $N^5$-Methyltetrahydrofolic acid (prefolic A) | 0·43 ± 0·04 | 0·67 ± 0·05 | 0·85 ± 0·09 |
| Tetrahydrofolic acid | 0·30 ± 0·01 | 0·90 ± 0·03 | 0·61 ± 0·02 |
| $N^{10}$-Formyltetrahydropteroylpolyglutamic acid | 0·65 ± 0·09 | 0·80 ± 0·13 | 1·20 ± 0·14 |
| $N^5$-Formyltetrahydropteroylpolyglutamic acid | 0·95 ± 0·12 | 2·30 ± 0·28 | 1·85 ± 0·21 |

derivatives of tetrahydropteroylpolyglutamic acid ($P < 0.001$) also showed a significant increase.

From the results of Table 4 it appears that the quantity of citrovorum factor formed is higher in systems with the liver homogenate of biotin-treated rats ($P < 0.01$) and of vitamin $B_{12}$-treated rats ($P < 0.02$) than that with liver homogenate of biotin-deficient rats.

## DISCUSSION

The present results confirm the participation of biotin in the metabolism of folic acid. Moreover, the same effect of biotin and vitamin $B_{12}$ on folic acid metabolism is presumably a consequence of the common participation of the two vitamins in the metabolism of $C_1$ compounds.

The effect of vitamin $B_{12}$ on folic acid metabolism and in particular on the formation of citrovorum factor has been much studied (Drysdale, Betheil, Lardy & Bauman, 1951; Dietrich, Monson & Elvehjem, 1951; Doctor *et al.* 1953; Doctor, Elam, Sparks, Lyman & Couch, 1954; Dawbarn, Hine & Smith, 1958; Gardiner & Silverman, 1960; Moruzzi *et al.* 1963). However, the results obtained do not as yet concur and this is probably due to different experimental conditions.

Besides, the exact nature of the effect is not yet known. Vitamin $B_{12}$ might act directly or indirectly in one or more of the reactions of the enzymic process mentioned.

With respect to the action of biotin our results do not correspond to those of Halevy & Guggenheim (1958), who did not observe any differences either on the hepatic concentrations of citrovorum factor or in the conversion *in vivo* of folic acid into citrovorum factor. On the other hand, these authors noted no effect of biotin on the hepatic concentrations of folic acid.

Also, with regard to biotin the exact mechanism of action is not yet known: it might have a direct coenzymic effect or an indirect effect on a reaction of the enzyme system. It seems unlikely that biotin acts by increasing the tissue storage of cofactors such as ATP, NADPH, pyridoxal 5-phosphate and $Mg^{2+}$ involved in this process. The addition of suitable amounts of these substances to the reaction mixtures did not modify significantly the amount of citrovorum factor formed in the systems with liver homogenates of biotin-deficient animals (Marchetti, Landi & Pasquali, 1964).

In conclusion, the parallel effects of biotin and vitamin $B_{12}$ on folic acid metabolism and therefore on numerous metabolic processes that involve the transfer and the utilization of $C_1$ units at the various oxidation levels could explain the nutritional and metabolic relationships between the two vitamins already observed by Marchetti & Testoni (1964).

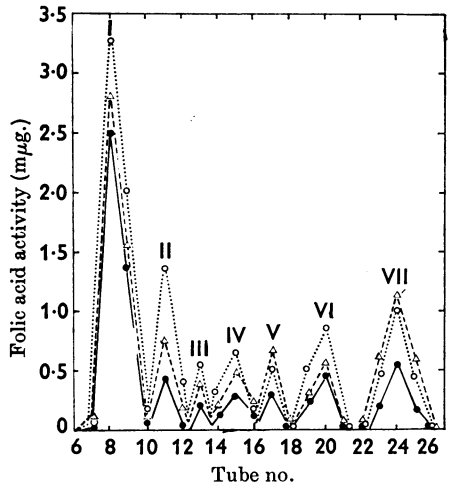


Fig. 1. Chromatograms of the liver folate derivatives of biotin-deficient rats (●——●), biotin-treated rats (△----△) and biotin-deficient vitamin $B_{12}$-treated rats (○····○). Peak I, $N^{10}$-formyltetrahydrofolic acid; peak II, $N^{10}$-formyldihydrofolic acid and $N^{10}$-formylfolic acid; peak III, $N^5$-formyltetrahydrofolic acid; peak IV, $N^5$-methyltetrahydrofolic acid; peak V, tetrahydrofolic acid; peak VI, $N^{10}$-formyltetrahydropteroylpolyglutamic acid; peak VII, $N^5$-formyltetrahydropteroylpolyglutamic acid.

Table 4. *Effects of dietary biotin and vitamin $B_{12}$ on the conversion of folic acid into citrovorum factor by rat-liver homogenate*

Each value is given as the mean ± S.E.M. of six determinations on different animals. Experimental details are given in the text.

| Group | Rat nutritional status | Citrovorum factor formed (μg./g. wet wt. of liver) |
|---|---|---|
| 1 | Biotin-deficient | 2·28 ± 0·47 |
| 2 | Biotin-treated (100 μg./kg. of diet) | 4·86 ± 0·56 |
| 3 | Biotin-deficient vitamin $B_{12}$-treated (100 μg./kg. of diet) | 3·91 ± 0·35 |

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