UTILIZATION OF HOMOCYSTINE FOR GROWTH IN PRESENCE OF VITAMIN B₁₂ AND FOLIC ACID*

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It has been shown in this laboratory that rats bred on our preexperimental food will grow on a synthetic diet in which homocystine is the only sulfur-containing amino acid and which contains no known sources of 'labile methyl' groups (1, 2). The ability to utilize homocystine is eventually lost on the synthetic diet, probably due to the exhaustion of stored factors involved in homocystine metabolism. However, this ability to utilize homocystine can be restored by supplementation of the diet with liver extract Lilly (LEL). The liver preparation represents the antianemia fraction formerly called Cohn fraction G (2). In order to find out whether the antianemia principle in the LEL is the active agent in the utilization of homocystine, two Lederle preparations were also fed to rats on a homocystine-synthetic diet containing 2 per cent sulfasuxidine. One preparation, liver extract parenteral (Lederle), is similar to LEL, the other, Lederle concentrated solution of liver extract, is a product derived from the preceding preparation low in folic acid but 4 to 5 times as high in antianemia potency. Although the liver extract parenteral gave a growth response similar to that of LEL when given both orally and by intramuscular injection, the concentrated solution of liver extract gave negative results with both methods of administration (2). It was assumed from these results that the component of the liver preparations active in the utilization of homocystine was not identical with the antianemia principle, although its distribution parallels that of the latter to some degree.

Since this original work was published, crystalline folic acid and vitamin B₁₂ have become available. When the previously cited experiments were carried out, 2 per cent sulfasuxidine was added to the basal diet to inhibit possible bacterial synthesis of factors involved in the utilization of homocystine. At that time, ryzamin-B was our source of folic acid and the intestinal synthesis of additional amounts was inhibited by the sulfasuxidine. Recently some observations were made on the rôle of crystalline

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folic acid in the utilization of homocystine by the rat (3) in the presence and absence of sulfasuxididine, with and without the addition of LEL. It was found that doses of 20 γ of crystalline folic acid enabled some rats, which had lost their ability to utilize homocystine for growth, to reestablish this capacity without the aid of LEL. The fact that only some rats could resume growth seems to indicate that other factors were involved. "These animals retain a latent capacity for the utilization of homocystine which seems to depend for its manifestation on a fairly high level of folic acid and is not destroyed by the sulfa drug." The involvement of other B vitamins, particularly vitamin B_{12}, has been suggested (3) and, therefore, crystalline vitamin B_{12}, cobione (Merck), was added to the synthetic diet.

The present experiments were run to determine the combined effect of crystalline vitamin B_{12} and folic acid on the ability of the rat to utilize homocystine on a "labile methyl"-free diet. In view of our last experiments with folic acid (3), the addition of 2 per cent sulfasuxidine was considered unnecessary. However, for the sake of comparison with former work experiments were carried out in both the presence and the absence of sulfasuxidine. The experiment with the concentrated solution of liver extract (Lederle), which yielded negative results in early experiments (2), was repeated in the presence of crystalline folic acid.

**Diet**

The composition of the basal (17 per cent) amino acid diet was the same as that used in previous experiments and from the same sources (2-4). DL-Homocystine was fed in the concentration of 0.83 per cent and was prepared in this laboratory (100 ± 0.5 per cent by disulfide determination (3)). When sulfasuxidine was fed, it was added to the basal diet which was given *ad libitum*.

The standard daily dose of B vitamins fed was 500 γ each of nicotinic acid, p-aminobenzoic acid, and inositol, 200 γ of calcium pantothenate, 40 γ each of thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, and 2 γ of biotin. 62.5 mg. of unfortified ryzamin-B (Wellcome Research Laboratories) and 20 γ of folic acid (folvite, Lederle) were later added to the vitamin supplement; in one group they were added at the beginning of the experiment. The preparation of vitamin B_{12} used was cobione (Merck); the liver extract was Lederle concentrated solution liver extract (2).

**EXPERIMENTAL**

Three groups of female rats born and bred in this laboratory were used, four animals in each group. The rats were 35 days old at the beginning of the experiment. The preexperimental conditions were the same as
those previously described (2-4). The rats were allowed to lose weight for 14 days on the basal diet, which contained no sulfur-containing amino acids. In Groups A and B, 2 per cent sulfasuxidine was added to the basal diet. After 14 days 0.83 per cent homocystine was also added to the diet and the amount of food given was restricted to 3 gm. per rat per day. Rats receiving sulfasuxidine grew slowly for approximately 2 weeks, when the usual drop in weight occurred. At this point, 62.5 mg. of ryzamin-B and 20 γ of folic acid per day were administered to each rat for 3 to 4 days in order to aid recovery. The rats were then kept on the

![Fig. 1. Average daily growth curves. The basal diet contained 0.83 per cent homocystine and, in Groups A and B, 2 per cent sulfasuxidine. All supplements were given orally. The circles represent a change of period. The initial period in all the curves represents a leveling of the growth in the presence of daily doses of 62.5 mg. of ryzamin-B, 2 γ of biotin, and 20 γ of folic acid added to the vitamin B group supplement. Curve A, second period, represents the growth response of rats to a daily dose of 0.1 cc. (2 γ) of the Lederle concentrated solution of liver extract per rat and the third period, that to 0.2 cc. (4 γ) of the same solution. Curve B, second period, represents the growth response to 2 γ of vitamin B₁₂ (cobione) per day per rat. Curve C, second period, shows growth response to 2 γ of vitamin B₁₂ (cobione) per day per rat, which was increased in the third period to 4 γ of vitamin B₁₂, when it was discontinued (fourth period).]

original supplement of the vitamin B group and given the basal food ad libitum. When the growth curve had leveled (in approximately 50 days), it was assumed that the sulfasuxidine had become effective and, therefore, at this point, ryzamin-B and additional biotin were added, followed in a short time by folic acid (2-4). If the growth curve of the rats remained level upon the addition of these extra vitamins, the animals were considered ready for vitamin B₁₂ assay. Those rats which did not receive the sulfa drug were fed the full supplement of B vitamins, including ryzamin-B, folic acid, and biotin, from the beginning of the experimental period. After the rats had been kept for 14 days on the basal diet free
of sulfur-containing amino acids, 0.83 per cent homocystine was added. The growth curves of these rats are usually level in about 40 to 50 days. When the homocystine was first fed, the basal food was limited to 5 gm. per rat per day; after approximately 20 days the animals were fed ad libitum. The food consumption averaged about 6.0 gm. per day for each rat during this period.

The four rats represented by Curve A, Fig. 1, were conditioned on the 2 per cent sulfasuxidine diet (initial period) and then given individual daily oral doses of 0.1 cc. of Lederle concentrated solution of liver extract containing 2 γ of vitamin B₁₂ according to biological assay (second period). This amount, which was added to the vitamin supplement, was increased to 0.2 cc. (4 γ of vitamin B₁₂) (third period). The rats weighed 120, 130, 110, and 132 gm. when conditioned and showed a daily gain of 0.8, 1.0, 0.9, and 0.8 gm. when supplemented with 0.1 cc. of Lederle solution. A daily gain of 1.5, 1.1, 0.9, and 0.8 gm. was recorded with 0.2 cc. of Lederle solution. The average gain for 2 γ of vitamin B₁₂ (0.1 cc.) was 0.9 and that for 4 γ of vitamin B₁₂ (0.2 cc.) 1.1 gm.

Four rats were used in Group B. Curve B, Fig. 1, represents two of these rats. The animals weighed 122 and 120 gm. after having been conditioned on the 2 per cent sulfasuxidine diet (initial period). They were given daily doses of 2 γ of vitamin B₁₂ for 30 additional days (second period); the gain per day averaged 0.86 and 0.80 gm. The other two rats fed a shorter period showed a similar growth response to 2 γ of vitamin B₁₂.

The four rats, the growth of which is represented by Curve C, Fig. 1, were conditioned as described without sulfasuxidine (initial period). When they had leveled, 2 γ of vitamin B₁₂ were added to the daily vitamin supplement of each rat for a period of 15 days (second period), after which the amount was increased to 4 γ of vitamin B₁₂ for 16 additional days (third period). The initial weight at the beginning of the vitamin B₁₂ assay was 158, 158, 124, and 131 gm. respectively; the average gain per day with 2 γ of vitamin B₁₂ was 0.7, 0.8, 0.4, and 0.5 gm. When 4 γ of vitamin B₁₂ were administered, the average daily gain was 0.8, 0.8, 0.6, and 1.1 gm. The average gain for the two periods was 0.6 and 0.8 gm. respectively.

DISCUSSION

Rats fed a "labile methyl"-free diet containing 0.83 per cent homocystine as the sole source of sulfur amino acids and the eight B vitamins gradually stopped growing if their intestinal synthesis of folic acid was suppressed by sulfasuxidine and none was given in the diet. When folic acid was added to the diet, some of the rats resumed growth while others did not (3). However, all the animals eventually ceased growing. This
seemed to indicate that an additional factor or factors were involved. Addition of crystalline vitamin B₁₂ to the vitamin supplement, at this point, caused a resumption of growth in all the animals at the rate of approximately 0.8 to 1.0 gm. daily (Curves B and C, Fig. 1). Removal of vitamin B₁₂ resulted in a continued growth for a short period, followed by a slow leveling of the curves. If an equimolar amount of methionine was fed with the basal diet instead of the homocystine at the point mentioned above, there was a resumption of growth of approximately 1 gm. a day without the addition of vitamin B₁₂.

Since vitamin B₁₂ has been shown to be the antianemia principle in liver preparations (5), it seems probable that the component of our liver extracts active in the utilization of homocystine was vitamin B₁₂. It has been shown that liver extracts rich in the pernicious anemia factor are only partially effective in anemia when given alone, but in combination with folic acid give complete recovery (6). In our former experiments, when the Lederle concentrated solution liver extract was used, as stated before, our source of folic acid was obviously inadequate. In the present experiment (Curve A, Fig. 1), growth was obtained with the Lederle liver extract proportional to its vitamin B₁₂ content, on addition of folic acid. Our data seem to indicate that, as in the case of pernicious anemia, the growth response of rats to vitamin B₁₂ on the homocystine diet seems to depend on the folic acid supply whether the source is from intestinal synthesis or from the diet. Our earlier negative experiments with the Lederle extract can now be explained in the light of our present experience with folic acid.

Since our diet was devoid of methionine and all known "labile methyl" donors, including choline, it appears that the growth response obtained in our animals is indicative of the synthesis of methionine in the rat. Since slow growth was obtained, it is obvious that this synthesis is limited but nevertheless real. Further studies on this problem are at present in progress.

SUMMARY

When growth had leveled on a "labile methyl"-free diet containing folic acid, addition of crystalline vitamin B₁₂ promoted growth.

The Lederle concentrated solution of liver extract given orally in place of vitamin B₁₂, under similar conditions, also promoted growth which was apparently proportional to its vitamin B₁₂ content.

It appears that the previous negative results obtained with the Lederle concentrated solution of liver extract (2) were due to folic acid deficiency.

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