RELATIONSHIP OF THE DEPOSITION OF FOLIC AND FOLINIC ACIDS TO CHOLINE OXIDASE OF ISOLATED MITOCHONDRIA*

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In several recent publications (1–4) it has been reported that dietary aminopterin produces a marked decrease in rat liver choline oxidase activity as measured by the whole homogenate technique. A simple folic acid deficiency, however, has been shown to produce only slight changes in the activity of this enzyme (5). It was probable that a simple folic acid deficiency did not deplete the tissues of folic (and folinic) acid to a great enough extent to produce much change in the activity of the whole homogenate. If only minute amounts of a folic acid cofactor were needed by the enzyme, a whole liver homogenate, even from deficient animals, might contain enough of the cofactor to maintain the normal activity of the enzyme. The choline oxidase system of rat liver has been shown to be located in the mitochondrial fraction of liver (6–8). Therefore, the authors felt that, if the mitochondria were isolated, washed, and thus freed of extraneous folic acid, folinic acid, or cofactor containing either of these in bound form, the effects of a simple folic acid deficiency could be demonstrated more clearly.

In the present investigation not only the effect of a simple folic acid deficiency on the choline oxidase of isolated mitochondria has been studied, but also a comparison has been made with the effects on the mitochondrial activity of the enzyme produced by feeding aminopterin to rats. In order to complement the enzyme results, and to study the correlation of the enzyme activity with folic acid and folinic acid[1] concentration in the tissues, all of the samples used for the enzyme determinations were assayed microbiologically for both folic acid and folinic acid. In the course of these

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[1] Or *Leuconostoc citrovorum* factor.

studies, some interesting effects of dietary aminopterin on the concentrations of folic acid and folinic acid in liver fractions have been uncovered. Also some new concepts concerning the assay of enzymes in mitochondria have been derived.

EXPERIMENTAL

Folic Acid Deficiency—Weanling, male rats of the Sprague-Dawley strain were employed as experimental animals. They were divided into two groups, one receiving a complete ration plus sulfasuxidine and the other receiving the same ration with folic acid omitted. The contents of these rations have been published previously (9). The two groups of rats were fed their respective rations for 5 weeks. At the end of this period the average weights of the groups were as follows: normal control, 204 gm.; folic acid-deficient, 105 gm. The rats of both groups were then sacrificed by decapitation, the livers removed, and the mitochondria isolated and washed as previously described (10). The mitochondria from 4 gm. of liver were finally suspended in 25 ml. of ice-cold distilled water and used as such for the enzyme determinations. Aliquots of the whole unfractionated homogenates and of the mitochondrial suspensions were stored at −5° until assayed microbiologically for folic acid and folinic acid.

The basic system used for the enzyme determinations was prepared as follows: Warburg flasks were set up containing 0.6 ml. of Krebs-Ringer phosphate buffer (11), 0.2 ml. of 2×10^{-4} M cytochrome *c* (Sigma Chemical Company), and 0.2 ml. of water in the main compartment. In alternate side arms was added 0.2 ml. of 2 per cent choline chloride or water. Finally 0.5 ml. of the mitochondrial preparation to be assayed for choline oxidase activity was added to the main compartment of the flasks. The final volume in the flasks was brought to 2.2 ml. by adding water to the side arms. The center well of each flask contained 0.2 ml. of 10 per cent potassium hydroxide to absorb carbon dioxide. In certain experiments, as will be pointed out later, extra water was added to the flasks to bring the final volume to 3.2 ml.

The results of these experiments, which are presented in Table I (Group A), have been calculated in terms of per cent of control. In this case the control was taken as the activity of the normal mitochondrial preparation in a 2.2 ml. incubation volume and the activities of the other systems were calculated as per cent of the control. In this way, the effects of variation in absolute activity from animal to animal of any one group could be overcome to a great extent. The results of these experiments indicate that when the total incubation volume was 2.2 ml. there was a marked difference in the mitochondrial choline oxidase activity between the normal and folic acid-deficient rats. However, this difference disappeared when

the volume was increased to 3.2 ml. Therefore, it appears that, when the concentrations of the flask components (including the enzyme) are decreased (by increasing the volume with water), the folic acid deficiency no longer limits the choline oxidase activity of the mitochondrial system. The choice of the 2.2 ml. volume in the first experiments fortuitously gave the expected result, whereas, if the 3.2 ml. volume had been used first, the effect of the folic acid deficiency would have been overlooked. From these results it appears that the cofactor or cofactors for choline oxidase are firmly bound to the mitochondria. If the cofactors had been easily dissociable from the enzyme, dilution of the enzyme would probably have made the cofactor more limiting with a consequent decrease in activity.

TABLE I

Relationship of Simple Folic Acid Deficiency and Dietary Aminopterin to Mitochondrial Choline Oxidase Activity

Group	Treatment of rats	Volume of system	Choline oxidase activity, per cent of control*	
			Normal mitochondria	Deficient mitochondria
		ml.		
A	Normal *vs.* folic acid deficiency	2.2	100	61
		3.2	100	118
B	Normal *vs.* dietary aminopterin	1.5	131	66
		2.2	100	80
		3.2	110	130

* 100 = 54 μl. of O_2 per hour per flask. Each figure is the average of three separate experiments.

In order to study this phenomenon more fully, a series of experiments was carried out in which the substrate concentration was varied while the concentrations of the other flask components were held constant. From earlier work (12) it had been demonstrated that the substrate at high concentrations inhibits the activity of choline oxidase. Therefore, if the choline oxidase activity of the folic acid-deficient mitochondria is less than the normal at a volume of 2.2 ml. but equal to the normal at 3.2 ml., the concomitant decrease in the substrate *concentration* might have been just enough to shift the effective substrate concentration from a point where it would inhibit the less active enzyme to a point where the inhibition no longer appeared. In the following experiments substrate concentration curves were obtained for the normal and for folic acid-deficient mitochondria. The final flask volume was maintained at 2.2 ml., and the concentrations of the flask components other than the substrate were kept the

same as before. The choline chloride was varied from 0 to 7 mg. per flask with several intermediate values as shown in Fig. 1. Here it can be seen that up to a point typical substrate concentration curves were obtained for both the normal and folic acid-deficient mitochondria. Above 3 mg. of choline chloride, however, a marked inhibition of the folic acid-deficient mitochondria occurred, while the normal mitochondria continued to respond positively to increasing amounts of the substrate. Therefore, these results indicate that, when the substrate concentration is high enough to give maximal activity with normal mitochondria, the apparent decrease in

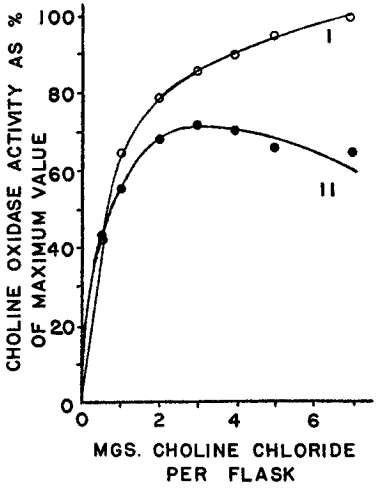

FIG. 1. Relationship of substrate concentration to mitochondrial choline oxidase activity of normal (Curve I) and folic acid-deficient (Curve II) rats. Each curve is the average of five separate experiments. The ordinate is expressed as per cent of the maximal choline oxidase activity obtained. 100 = 70 μl. of O_2 per hour per flask.

activity with folic acid-deficient mitochondria is amplified by the substrate inhibition of the latter. At lower substrate concentrations the difference in the two curves becomes less and they finally approach each other. These experiments indicate, therefore, that enzyme assays in which a normal and an abnormal system are compared may give different results depending upon the substrate concentration, especially if high concentrations of the substrate inhibit the enzyme. The results also indicate that the choline oxidase in folic acid-deficient mitochondria is less active than that in normal mitochondria.

The mitochondrial samples used for the enzymatic studies above (Table I (Group A) and Fig. 1), as well as aliquots of the whole liver homogenates from which the mitochondria were isolated, were assayed microbiologically

for both folic acid and folinic acid. To release the bound factors 1 ml. aliquots of the samples were incubated for 18 hours at 37° with 0.2 ml. of hog kidney enzyme (13), 7.8 ml. of 0.06 M acetate buffer of pH 4.5, and 1 ml. of 0.4 M L-cysteine hydrochloride. Toluene was added to each incubation mixture. After incubation the samples were autoclaved for 10 minutes, diluted to 25 ml. with water, adjusted to pH 7.0, and filtered. The filtrates were kept frozen until assayed for folic and folinic acids. Folic acid was assayed by the method of Luckey *et al.* (14) using *Streptococcus faecalis* R and folinic acid by the procedure of Sauberlich and Baumann (15) using *Leuconostoc citrovorum* ATCC 8081. Each sample was assayed in quadruplicate and the entire assay, including the incubation with hog

TABLE II

Effects of Simple Folic Acid Deficiency and Dietary Aminopterin on Concentration of Folic and Folinic Acids in Whole Liver and Liver Mitochondria

Group	Treatment of rats	Folic acid concentration,* mμgm. per gm. liver or its mitochondrial equivalent		Folinic acid concentration,* mμgm. per gm. liver or its mitochondrial equivalent	
		Whole liver	Mitochondria	Whole liver	Mitochondria
A	Normal	2464	192	156	11.1
	Folic acid deficiency	331	10.7	44	7.8
B	Normal	2628	82	212	11.0
	Dietary aminopterin	2517	75	83	6.5

* Each figure is the average of three to five samples from different animals run in octuplication.

kidney enzyme, was repeated and the results of both assays averaged together. The results of these analyses, presented in Table II (Group A), indicate that a folic acid deficiency produces a marked decrease in folic acid both in the whole homogenate as well as in the liver mitochondria. About 90 per cent of the folic acid is lost during the 5 week depletion period both from the whole liver and the mitochondria. In the case of folinic acid a marked decrease occurs in the whole liver concentrations, although the relative loss is not as great as that for folic acid. The folic acid deficiency produces a less marked loss in folinic acid from the mitochondria (from 11.1 mμgm. per gm. equivalent of mitochondria to 7.8 mμgm.). Although this decrease is less in the mitochondria, the difference is, nevertheless, highly significant. If one compares the loss in mitochondrial choline oxidase activity caused by a folic acid deficiency (the difference in maxima of Curves I and II in Fig. 1) with the loss in folinic acid from the mitochon-

dria, the relative decreases in enzyme activity and folinic acid are almost identical (100/72 = 1.39; 11.1/7.8 = 1.42). These results lend support to the conclusion that folinic acid rather than folic acid is closely related to mitochondrial choline oxidase activity.

Effects of Dietary Aminopterin—In these experiments adult male rats of the Sprague-Dawley strain were used as experimental animals. They were fed the complete ration fed to the weanling rats in the above experiments except that sulfasuxidine was omitted and 4 mg. of aminopterin[2] per kilo of ration were included. When the marked symptoms caused by the aminopterin were observed (after about 10 days), the animals were used in the enzyme studies. The liver mitochondrial suspensions were prepared from whole liver homogenates as described. The enzyme studies were carried out as above except where noted in Table I (Group B). Aliquots of the whole homogenates and mitochondrial suspensions were stored at −5° for determinations of folic and folinic acids.

In Table I (Group B) are presented the results of the experiments in which the volume of the system used in the enzyme determinations was varied. As in a simple folic acid deficiency (Group A), a significant depression in mitochondrial choline oxidase activity was observed for the rats fed aminopterin when the 2.2 ml. volume was used. This difference, however, disappeared as before when the volume was increased to 3.2 ml. with water. When the volume was decreased to 1.5 ml., the difference again appeared and was considerably greater than that observed for the 2.2 ml. volume.

An expansion of these results was carried out as before with the substrate concentration curves, which are presented in Fig. 2. Results very similar to those for a simple folic acid deficiency (Fig. 1) were observed. Therefore, it appears that dietary aminopterin has the same effect on mitochondrial choline oxidase as a folic acid deficiency. In fact, the effects are practically indistinguishable.

In Table II (Group B) are presented the results of assays for folic and folinic acids of the enzyme samples used in the above experiments. These data indicate that aminopterin has no effect either on whole liver folic acid or on mitochondrial folic acid. However, the effect on whole liver and mitochondrial folinic acid is quite marked in that dietary aminopterin depresses the folinic acid concentration to almost the same extent as a simple folic acid deficiency. The fact that the control values for folic and folinic acids in Group B (Table II) are different in some cases from those in Group A can be explained on the basis that *adult* animals fed a ration

[2] The authors wish to thank Dr. W. L. Williams and Dr. J. J. Oleson of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for generous samples of folinic acid (leucovorin, Lederle) and aminopterin, respectively.

lacking in sulfasuxidine were used in the present experiments (Group B), whereas *weanling* rats fed a ration containing 2 per cent sulfasuxidine were used in the preceding experiments (Group A). The relative loss in folinic acid, however, was the same in both cases.

The decrease in mitochondrial folinic acid caused by dietary aminopterin again very closely parallels the loss in mitochondrial choline oxidase activity, just as with a simple folic acid deficiency. These results again indicate that folinic acid, rather than folic acid, in liver tissue is involved in maintenance of mitochondrial choline oxidase activity.

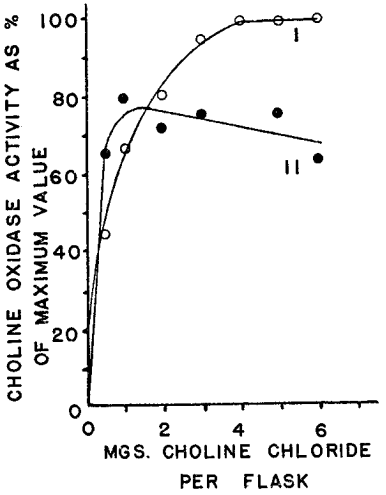

FIG. 2. Relationship of substrate concentration to mitochondrial choline oxidase activity of normal (Curve I) and aminopterin-fed (Curve II) rats. Each curve is the average of three separate experiments. The ordinate is expressed as per cent of the maximal choline oxidase activity obtained.

SUMMARY

1. A folic acid deficiency significantly depresses the activity of choline oxidase in isolated rat liver mitochondria. The relation of the substrate concentration to this effect in the mitochondrial system has been found to be very important in interpreting enzyme data in which the normal and the abnormal systems are compared. Almost exactly similar results were obtained in studies with the liver mitochondria of rats fed aminopterin.

2. Analyses of the whole liver and liver mitochondria of normal and folic acid-deficient rats for folic and folinic acids indicate that these substances are markedly decreased in a folic acid deficiency. Both whole liver and liver mitochondria lose about 90 per cent of their folic acid content during a 5 week depletion period. About 70 per cent of the whole

liver folinic acid and about 30 per cent of the mitochondrial folinic acid are lost during the depletion period. The loss in mitochondrial folinic acid almost exactly parallels the loss in choline oxidase activity.

3. Dietary aminopterin has no effect on either whole liver or mitochondrial folic acid. However, it induces a marked loss in folinic acid both from whole liver and mitochondria. This loss is approximately the same as that produced by a simple folic acid deficiency. Correlation of these data with the mitochondrial enzyme results for aminopterin-fed rats leads to exactly the same conclusions as indicated for a simple folic acid deficiency.

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