THE METABOLISM OF SERINE IN PYRIDOXINE DEFICIENCY

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FORMATION of ethanolamine from serine and glycine has been shown in the animal organism (Stetten, 1942; Greenberg and Harris, 1950; Weissbach *et al.*, 1950; Jonsson and Mosher, 1950; Levine and Tarver, 1950). Evidence is also available to show that ethanolamine is an intermediate in the biosynthesis of choline (Arnstein, 1951; Sprinson and Coulon, 1954; Elwyn *et al.*, 1955). Amines are known to be formed as a result of decarboxylation of amino acids (Gale, 1946, Blaschko, 1945). The presence of ethanolamine in the urine of rats (Stein, 1953) and the increase in its excretion with administration of serine (Fishman and Artom, 1945; Luck and Wilcox, 1953) would indicate that serine is the source of ethanolamine. Absence of label in the hydroxymethyl group of ethanolamine isolated after feeding of carboxyl labelled glycine would also suggest that glycine has to be transformed to serine prior to decarboxylation (Greenberg and Harris, 1950). Nord (1919) had shown that an aerobic bacteria produced ethanolamine from serine in putrefying meat. Ethanolamine formation is also reported in intestinal micro-organisms (Melnykowicz and Johansson, 1955).

Although pyridoxine, as pyridoxal phosphate is known to be the coenzyme of amino acid decarboxylases in bacteria (Snell, 1953; Mandeles *et al.*, 1954), its participation in serine decarboxylation in the animal organism is not established. However, from an observed decrease in choline biosynthesis in the vitamin B_6-deficient rat, Stekol *et al.* (1952, 1953) inferred a function for vitamin B_6 in serine decarboxylation.

Serine deamination in *Neurospora* is known to be influenced by pyridoxine (Yanofsky and Reissig, 1953); again, this effect has not been demonstrated in the animal organism. Glycine to serine conversion requires participation of pyridoxal phosphate and has been extensively studied (Deodhar and Sakami, 1952; Blakley, 1956; Alexander and Greenberg, 1955).

The present work relates to a study of serine metabolism in vitamin B_6 deficiency in rats. It is shown that there is no apparent effect of this vitamin on serine decarboxylation *in vitro*, while serine deamination, as well as glycine to serine conversion are influenced by vitamin B_6.

EXPERIMENTAL

Preparation of Tissue

The rat was killed by decapitation and the liver was quickly removed and chilled. It was homogenised to a 20 per cent. suspension in ice-cold distilled water using a Potter-Elvehjem all-glass homogeniser.

Manometric Assay of Serine Decarboxylation

Assays were carried out in Warburg flasks in a nitrogen atmosphere. In preliminary experiments, optimum conditions with respect to pH and substrate concentration, were ascertained. The procedure finally adopted was as follows: The main compartment of the Warburg flask contained 1·0 ml. each of 0·1 M phosphate buffer, pH 6·0 and of the liver homogenate. The side-arm contained 0·5 ml. of 0·1 M DL-serine solution or distilled water in the case of controls. The contents of the side-arm were tipped in after initial equilibration period of 10 minutes at 37 ° C., during which nitrogen gas was passed through the flask. The centre cup contained 0·5 ml. of 1 N H_2SO_4 which was added to the main chamber to stop the reaction. The acid was added when there was no further change in manometric levels, which usually occurred within about 40 minutes.

In some experiments filter-paper strips soaked in 10 per cent. KOH were inserted into the centre cup instead of the 1 N H_2SO_4 and it was always observed that in these cases there was no significant change in pressure, indicating absorption of the carbon dioxide liberated.

Correction for CO_2 retained in the liquid phase was negligible at the prevailing acidic pH.

The ethanolamine formed as a result of serine decarboxylation was identified on circular paper chromatograms in the following manner: The reaction mixture in the Warburg flask was taken in a test-tube and heated on a boiling water-bath for 10 minutes. It was then centrifuged and the supernatant used for chromatography. The technique was similar to that of Giri and Rao (1952). Samples were spotted side by side, with solutions of serine and ethanolamine. A somewhat diffuse band characteristic of ethanolamine was observable. The R_f values for ethanolamine were 0·60 and 0·75 in phenol-water (80 : 20) and butanol-acetic acid-water (40 : 10 : 50) as solvents respectively. The corresponding R_f values for serine were 0·40 and 0·50 respectively.

Serine Deamination

1·0 ml. of the liver homogenate was incubated with 1·0 ml. of 0·1 M phosphate buffer pH 7·4 and 1·0 ml. of 0·05 M serine solution at 37° C.

for an hour. At the end of the incubation period, the reaction was stopped and the mixture was deproteinised by adding 1·0 ml. of 20 per cent. trichloroacetic acid solution. An aliquot of the supernatant after centrifugation was used for determinations of ammonia and pyruvic acid formed. A control experiment without serine was performed side by side. *Ammonia* was determined by direct Nesslerization (Johnson, 1941) and *Puyrvic acid* by the method of Friedemann and Haughen (1943).

Glycine to Serine Conversion

1·0 ml. of the liver homogenate was incubated under toluene with 0·1 M phosphate buffer pH 7·0, 0·5 ml. of 0·2 M glycine, 0·5 ml. of 0·2 M sodium formate, 0·4 ml. of 0·05 M sodium citrate and 0·1 ml. of 0·05 M sodium fumarate at 37° C. for 5 hours. In a number of preliminary experiments it was noted that this system was the best for following serine synthesis from glycine. The citrate-fumarate mixture served as a sparking system (Fatterpaker, Marfatia and Sreenivasan, 1951). A control experiment with 0·5 ml. of distilled water substituting the glycine solution was run alongside. At the end of the incubation period 0·5 ml. of 1 N H_2SO_4 was added and the mixture was deproteinised by heating on a boiling water-bath for 5 minutes. An aliquot of the supernatant after centrifugation was used for the estimation of serine. The difference in serine values with and without added glycine represented net synthesis of serine.

Serine was assayed microbiologically with *Leuconostoc mesenteroides* P-60 (Steele *et al.*, 1949) and with *Lactobacillus casei* (Alexander, Beckner and Elvehjem, 1953). The assays were conducted in a final volume of 2·0 ml. per tube. The standard solution of *dl*-serine contained 20 μg. per ml. and the standard curve was obtained between 0–20 μg. of serine for every assay. The results are expressed as *l*-serine with appropriate reference to the standard curve. The latter organism was found to be more suitable and was therefore employed generally.

Assay of Pyridoxine

1·0 g. of homogenised liver was hydrolysed in 1 N NaOH by autoclaving for an hour at 15 lb. pressure, in a volume of 10 ml. The alkaline extract was cooled, neutralised with hydrochloric acid and made up to 50 ml. volume. Aliquots were used for the assay of pyridoxine with *Neurospora sitcphila* (Stokes *et al.*, 1943). The range of the assay was 0·1–1·0 μg.

Total Liver Nitrogen

1·0 ml. of the liver homogenate was taken in a Kjeldahl flask to which was added 1·0 g. of a salt mixture containing potassium sulphate and copper

sulphate (10 : 1) and 2·0 ml. of sulphuric acid (A.R.). The digestion was carried out over a micro-burner for 5–6 hours till a clear solution was obtained. The solution was diluted with water, neutralised slowly in the cold with NaOH and made up to volume. Colour was then developed by direct Nesslerization. To 1·0 ml. of the aliquot was added 1·0 ml. water, 2·0 ml. 2 N NaOH and 1·0 ml. of Nessler's reagent. Colour measurements were made on a Klett-Summerson photoelectric colorimeter with 420 mμ. filter in position. A standard curve was obtained with a solution of NH_4Cl in the range 0–15 μg. of nitrogen for reading off the values for total nitrogen.

Liver Choline

Free Choline.—2–3 g. of homogenised liver in 50 ml. of distilled water were heated at 100° C. for one hour (Leucke and Pearson, 1944). The mixture was filtered and aliquot used for determination of free choline.

Total Choline.—A suspension of the homogenised liver was hydrolised with 3 per cent. H_2SO_4 by autoclaving it for 2 hours at 15 lb. pressure (Horowitz and Beadle, 1943). It was cooled, neutralised with barium carbonate and made to volume. It was then filtered and the filtrate used for determination of total choline.

5–10 ml. aliquots prepared as above were passed through previously activated DeCalso columns which were then washed with distilled water. Choline adsorbed was next eluted with 10 ml. of 5 per cent. NaCl solution.

An aliquot of the eluate containing about 10–20 μg. of choline was used for precipitation as periodide by the method of Appleton *et al.* (1953). The periodide was centrifuged in specially tapered tubes and dissolved in redistilled ethylene dichloride. The extent of absorption was measured on a Beckman DU spectrophotometer at 365 mμ and results read off from a standard curve for choline in the range 0–15 μg.

Pyridoxine Deficiency in Rats

Young rats (Wistar) weighing 80–100 g. were fed on a purified diet of the following percentage composition (based on Meister *et al.* 1953; Beaton *et al.*, 1953); alcohol-extracted casein 18, starch 60, sucrose (vitaminised) 10, salt mixture (U.S.P. IV) 4, sesame oil 6, and shark liver oil 2. Vitamins included in the diet were (as mg./kg.): Thiamine HCl 6, riboflavin 10, niacin 30, calcium pantothenate 30, *p*-aminobenzoic acid 100, biotin 1, folic acid 5, vitamin K 3 and vitamin B_{12} 0·5.

One group of animals was reared on this vitamin B_6-deficient diet while a second group had the same diet with 10 mg./kg. pyridoxine HCl added.

To the deficient animals desoxypyridoxine was given intraperitoneally to hasten deficiency. This was at 0·5 mg. per rat on alternate days. Control rats were given mock injections of saline. There was no weight increase after the second week in the deficient group. Typical signs of deficiency such as dermatitis on the forepaws and the snout were noticed in 3–4 weeks' time. Animals were sacrificed for the various determinations after the 5th week. All results reported are averages from at least four different animals for each group.

RESULTS

Serine decarboxylation was rapid and most of the CO_2 was produced within the first 20–30 minutes of incubation and there was no further change in manometric levels after 40 minutes. The final concentration of the *dl*-serine added to the incubation mixture was 0·016 M (0·5 ml. of 0·1 M *dl*-serine in 3·0 ml.) and as may be seen from Table I, maximal activity was obtained at this concentration.

TABLE I

Effect of substrate concentration on serine decarboxylation

Final concentration of serine	Decarboxylation of serine QCO_2 (μl. CO_2 per mg. N/30 min.)
0·003 M	0·84
0·008 M	1·99
0·016 M	3·53
0·025 M	3·80
0·033 M	3·83

The optimal pH for reaction was found to be 6·0 (Table II). Buffers with pH ranging from 4·0–7·4 were used.

TABLE II

Effect of pH on serine decarboxylation

pH	Buffer	Decarboxylation of serine QCO_2 (μl. CO_2 per mg. N/30 min.)
4·0	0·2 M Acetate	2·42
5·0	,,	2·66
5·6	,,	3·35
6·0	0·1 M Phosphate	3·64
7·0	,,	1·97
7·4	,,	1·35

In view of the known activation of certain amino acid decarboxylases by pyridoxal phosphate (Gale, 1946; Roberts and Frankel, 1951; Mandeles *et al.*, 1954), its effect was studied on the present system. A pyridoxal phosphate preparation was obtained by a method similar to that of Umbreit and Waddel (1949) and Hurwitz (1953). 50 μg. of pyridoxal hydrochloride in 1·0 ml. of 0·1 M phosphate buffer pH 7·4, were incubated with 1·0 ml. of liver homogenate, 0·4 ml. of 0·05 M sodium citrate and 0·1 ml. of 0·05 M sodium fumarate, for three hours at 37° C. The mixture was deproteinised by heating on a boiling water-bath for 5 minutes, centrifuged and the supernatant used. Addition of this pyridoxal phosphate preparation at a concentration equivalent to 20 μg. of pyridoxal did not make any difference to serine decarboxylation but had a slight (9 per cent.) activating effect on synthesis of serine from glycine.

The inhibitory action, if any, of added hydroxylamine HCl on these enzyme systems was also studied.

TABLE III

Hydroxylamine inhibition studies

Addition to normal system	Serine decarboxylation QCO_2 (μl. CO_2 per mg. N/30 min.)	Glycine to serine synthesis (μ moles of serine per mg. N)	Serine deamination (μ moles of NH_3 per mg. N)
None	3·86	0·96	3·06
Hydroxylamine (10^{-3} M)	3·80	0·77	0·49

Hydroxylamine addition inhibited glycine to serine conversion by 20 per cent. and serine deamination by 85 per cent. However, it had no effect on serine decarboxylation. The insensitivity of serine decarboxylase to hydroxylamine could not be attributed to a pyridoxal phosphate coenzyme being firmly bound to the enzyme since the activity of this enzyme is not influenced in dietary vitamin B_6 deficiency (Table IV). On the other hand, there was marked reduction of activity in glycine to serine synthesis and in serine deamination as a result of vitamin B_6 deficiency.

Data on liver analyses of normal and pyridoxine-deficient animals are shown in Table V. Of significance are the values for free and total choline. Any effect on the decarboxylation of serine should be reflected in the values

TABLE IV

Effect of pyridoxine deficiency

Group	Serine decarboxylation QCO_2 (μl. CO_2 per mg. N/30 min.)	Serine deamination		Glycine to serine synthesis (μ moles of serine per mg. N)
		Pyruvic acid	Ammonia	
		μ moles per mg. N		
$-B_6$	3·87±0·14	3·08±0·04	3·03±0·05	0·63±0·02
$+B_6$	3·83±0·11	4·35±0·02	4·38±0·04	1·14±0·03

of choline. The values for total choline are reduced in the deficient rats but the levels of free choline seem to be almost unaltered.

DISCUSSION

Mammalian decarboxylases are of significance in the elaboration of specialised substances such as histamine, taurine, 5-hydroxytryptamine, ethanolamine, etc. The formation of ethanolamine provides the ethanolamine moiety of choline. The production of acetate from ethanolamine helps in the biosynthesis of long chain complex substances like sphingosine, fatty acids and cholesterol (Sprinson and Coulon, 1954). Ethanolamine also serves as an intermediate in the alternate serine to glycine pathway (Weissbach and Sprinson, 1953). Since serine-β-carbon can provide the methyl group of choline, serine becomes concerned with the biosynthesis of both the methyl and ethanolamine moieties of choline.

The present studies indicate that serine decarboxylase of rat liver is unique in that it is apparently not influenced by pyridoxine deficiency. The role of pyridoxal phosphate has been established in almost all bacterial amino acid decarboxylases and in some mammalian decarboxylases. The mammalian decarboxylases studied do not all behave similar to bacterial decarboxylases. Thus, while normally decarboxylases function at acidic pH values, 5-hydroxytryptophan decarboxylase shows an optimum pH of 8·1 (Clark *et al.*, 1954). Again, inhibition of glutamic acid decarboxylase (Roberts and Frankel, 1951) by hydroxylamine could be reversed by pyridoxal phosphate, but such a reversal could not be shown with 5-hydroxytryptophan decarboxylase (Clark *et al.*, 1954; Beiler and Martin, 1954). However, it has been shown recently that pyridoxal phosphate does have a function in 5-hydroxytryptophan decarboxylase (Buxton and Sinclair, 1956). Bacterial histidine decarboxylase (Rodwell, 1953) was also not considered as requiring pyridoxal phosphate,

TABLE V

Liver analyses

Group	Liver weight per 100 g. body weight	Pyridoxine in liver μg./g. fresh weight	Liver nitrogen		Liver choline	
			Per cent. fresh weight	Per cent. dry weight	Free mg./g. fresh weight	Total mg./g. fresh weight
$-B_6$	4·58±0·42	4·10±0·02	3·43±0·14	11·7±1·4	0·21±0·01	5·50±0·05
$+B_6$	3·37±0·40	6·91±0·01	3·91±0·24	13·6±0·9	0·23±0·01	5·83±0·03

but this requirement has since been shown (Guirard and Snell, 1954). Although the possibility exists that a function for pyridoxal phosphate in serine decarboxylation by the rat liver enzyme could not be demonstrated because of firmly bound coenzyme further work is needed to ascertain this possibility.

Serine when administered is excreted as ethanolamine in urine (Fishman and Artom, 1945; Luck and Wilcox, 1953). Similarly, administration of cysteine (Awapara and Wingo, 1953; Blaschko *et al.*, 1953) results in excretion of taurine. It is reported that in vitamin B_6 deficiency, the excretion of taurine is considerably reduced, though such an effect is not evident on ethanolamine excretion (Blaschko *et al.*, 1953).

The results on liver levels of choline (Table V) show a definite influence of vitamin B_6 on total choline content but not on the free choline to the same extent.

Although a function for vitamin B_6 in serine decarboxylation seems doubtful the present experiments have clearly brought out a relationship of this vitamin to glycine-serine conversion and in serine deamination. The participation of vitamin B_6 in glycine to serine transformation has been reported (Lascelles and Woods, 1950; Deodhar and Sakami, 1952; Blakley, 1955), and is explained on the basis of a Schiff base formation (Alexander and Greenberg, 1955). Involvement of vitamin B_6 in the deamination of serine has only been shown hitherto in micro-organisms.

SUMMARY

Deficiency of vitamin B_6 in rats had no effect on serine decarboxylation, though, serine deamination and glycine to serine synthesis were reduced.

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