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Role of Pyridoxine in Glutathione Metabolism

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Binkley, Christensen & Jensen (1952) demonstrated that pyridoxal phosphate is a cofactor in the biosynthesis of cysteine from methionine. Blaschko, Datta & Harris (1953) reported that the decrease in urinary excretion of taurine in vitamin B_6 deficiency is in part due to the decrease in the concentration of the pyridoxal phosphate-containing enzyme, cysteinesulphinate decarboxylase, which catalyses the formation of hypotaurine. On the basis of the decrease of pantothenic acid content in the liver of pyridoxine-deficient rats, Williams & Hata (1959) observed that its CoA activity is also decreased. As a result of these findings which point to the existence of metabolic interrelationships between vitamin B_6 and sulphur-containing amino acids, we studied the role of pyridoxine on the metabolism of another physiologically important thiol compound, namely reduced glutathione (GSH). The present paper gives results to show that pyridoxine deficiency induced under various dietary conditions brings about a consistent increase in the concentration of GSH in liver and, to a less extent, in erythrocytes, and accelerates the incorporation of [2-^{14}C]glycine into liver GSH.

EXPERIMENTAL

Preparation of experimental animals and diet. Weanling or 6–8 week-old rats of both sexes of the McCollum strain were used. They were placed in an air-conditioned room maintained at 22–24° and housed in individual screen-bottom cages for 5–6 weeks. A high-fat (Hsu & Chow, 1957) or a high-protein (Hsu & Kawin, 1962) basal diet deficient in pyridoxine was used to induce vitamin B_6 deficiencies. For control, another group of animals was given the same pyridoxine-deficient diet supplemented with pyridoxine hydrochloride (20 mg./kg.). In some studies deoxypyridoxine, an antagonist of pyridoxine, was also administered to groups of rats on the deficient diet to accelerate pyridoxine deficiency.

A complete synthetic diet was also used for certain experiments, and its percentage composition was as follows: essential amino acids, 10·9; non-essential amino acids, 8·0; corn oil, 5·0; sucrose, 72·0; salt mixture, 4·0. All known vitamins except pyridoxine were added to the synthetic diet in a manner similar to the high-fat diet described above. The amino acid composition of the diet, expressed as g./kg., is shown in Table 1. In the preparation of cysteine-free or

Table 1. *Amino acid composition of the diet*

The amino acid contents are expressed as g./kg. of diet.

L-Arginine hydrochloride	8·55
L-Histidine hydrochloride	6·27
DL-Isoleucine	15·39
DL-Leucine	18·24
DL-Methionine	6·84
Cysteine*	3·42
DL-Phenylalanine	10·26
DL-Tryptophan	2·28
DL-Threonine	11·40
DL-Valine	15·96
DL-Alanine	13·68
DL-Aspartic acid	13·68
DL-Glutamic acid†	20·00
DL-Tyrosine	15·96
Glycine	7·98
DL-Lysine hydrochloride	14·25
DL-Serine	5·70
Total	189·86

* Cysteine was omitted in the preparation of the cysteine-free diet, and the DL-methionine content was increased to 9·0 g./kg.

† Glutamic acid was omitted in the preparation of the glutamic acid-free diet.

glutamic acid-free diets, the specific amino acid was quantitatively replaced by sucrose.

Measurements of non-protein thiol compounds and reduced glutathione. The amounts of non-protein SH compounds in erythrocytes and liver were determined by precipitating the protein with metaphosphoric acid at 4°. The nitroprusside solution was added to the filtrate according to the method of Grunert & Phillips (1951). The intensity of the resulting colour was measured quantitatively in the Klett colorimeter with a no. 52 filter. The concentrations of GSH in erythrocytes and tissues were determined in metaphosphoric acid filtrates according to the method of Patterson & Lazarow (1955). The compound resulting from the reaction of alloxan with GSH gives a maximal absorption at 305 mμ. Extinctions were measured in a Beckman model DU spectrophotometer. The haematocrit value was estimated for the blood specimens in Wintrobe tubes by centrifuging at 2000 rev./min. for 15 min. The concentration of GSH in erythrocytes was expressed as μmoles of GSH/100 ml. of packed erythrocytes. The GSH concentrations in liver and other tissues were expressed as μmoles/100 g. wet wt. of tissue.

Determination of plasma glutamate–oxalate transaminase. This was determined colorimetrically by the method of Reitman & Frankel (1957). The measurement was mainly used as a criterion for assessing the state of pyridoxine deficiency.

Formation of reduced glutathione from [2-^{14}C]glycine and DL-[1-^{14}C]*glutamic acid.* The radioactive amino acids used were [2-^{14}C]glycine and DL-[1-^{14}C]glutamic acid (New England Nuclear Corp., Boston, Mass., U.S.A.). After being kept overnight without food each rat was injected intraperitoneally with a single dose of 10 μc of [2-^{14}C]-glycine (specific activity 3·7 μc/mg.) or 10 μc of DL-[1-^{14}C]-glutamic acid (specific activity 29·4 μc/mg.) in an isoosmotic saline solution for each 200 g. of body weight. Then 4 hr. later the animals were decapitated and the liver specimens were immediately removed for analysis. The total GSH was precipitated as the cadmium mercaptide (Goldzieher, Besch & Velez, 1958) for the determination of its specific activity. The ^{14}C activity in the cadmium mercaptide precipitate was measured in a Nuclear–Chicago model D47 gas-flow counter equipped with a Micromil window and an Ultrascaler. A representative piece of fresh liver weighing approx. 0·5 g. was simultaneously taken for the measurement of total GSH content according to the procedures described above.

RESULTS

The results of the first three experiments are given in Table 2. The concentration of liver non-protein SH compounds of pyridoxine-deficient rats of both sexes was significantly higher than that of their respective pyridoxine-fed controls, whether these controls were fed *ad libitum* or pair-fed. Thus this increase in the concentration of non-protein SH compounds in the livers of pyridoxine-deficient rats was not due to the decrease of dietary intake. The concentration of non-protein SH compounds in erythrocytes in pyridoxine-deficient rats was also higher than that in pyridoxine-treated animals (Expts. 1 and 3, but not Expt. 2), but the difference was insignificant. The administration of deoxypyridoxine to pyridoxine-deficient male and female rats caused a further increase in the concentration of non-protein SH compounds in the liver, except in Expt. 2. An insignificant difference was observed in the concentrations of non-protein SH compounds in erythrocytes as a result of the deoxypyridoxine treatment.

Since nearly all the non-protein SH material in tissues is GSH, it might be expected that the increase in the concentration of non-protein SH compounds in pyridoxine deficiency is primarily due to an increase of this compound. This belief was confirmed when a specific test for GSH was used. The results in Table 3 demonstrate that there was an increase in erythrocyte GSH concentrations in pyridoxine-deficient rats placed on either a high-fat or a high-protein diet. Liver GSH concentrations were determined only on those rats receiving a high-

Table 2. *Effects of pyridoxine deficiency and of deoxypyridoxine administration on the concentrations of non-protein thiol compounds in erythrocytes and liver of rats*

Experimental details are given in the text. The deoxypyridoxine-treated rats were given deoxypyridoxine hydrochloride (0·75 mg. in 0·5 ml. of water) three times weekly. Male rats were used for Expts. 1 and 2, and female rats were used for Expt. 3. The results are given as means ± S.E.M., with the numbers of rats used in parentheses.

	Expt. 1		Expt. 2		Expt. 3	
Dietary regimen	Concn. in erythrocytes (μmoles/100 ml.)	Concn. in liver (μmoles/100 g.)	Concn. in erythrocytes (μmoles/100 ml.)	Concn. in liver (μmoles/100 g.)	Concn. in erythrocytes (μmoles/100 ml.)	Concn. in liver (μmoles/100 g.)
Pyridoxine-deficient	345 ± 21·5 (6)	663 ± 45·9	290 ± 24·1 (6)	570 ± 25·1	340 ± 12·5 (6)	685 ± 25·8
Deoxypyridoxine-treated	296 ± 20·8 (5)	791 ± 56·1	260 ± 5·1 (5)	564 ± 20·5	323 ± 19·4 (5)	801 ± 48·6
Pyridoxine-treated (pair-fed)	289 ± 13·8 (7)	388 ± 24·6	261 ± 45·4 (4)	389 ± 7·6	263 ± 26·5 (4)	484 ± 23·8
Pyridoxine-treated (*ad libitum*)	—	—	277 ± 35·9 (5)	410 ± 32·9	274 ± 27·0 (5)	527 ± 18·5

Table 3. *Effects of pyridoxine deficiency on the concentration of reduced glutathione in erythrocytes and liver and on the glutamate–oxaloacetate-transaminase activity in plasma of rats*

Experimental details are given in the text. Male rats were used for the high-protein-diet experiment, and female rats were used for the high-fat-diet experiment. The results are given as means±S.E.M., with the numbers of rats used in parentheses.

	Concn. of GSH			Glutamate–oxaloacetate-transaminase activity	
Nature of diet ...	High-protein diet	High-fat diet		High-protein diet	High-fat diet
Dietary regimen	In erythrocytes (μmoles/100 ml.)	In erythrocytes (μmoles/100 ml.)	In liver (μmoles/100 g.)	In plasma (units/ml.)	In plasma (units/ml.)
Pyridoxine-deficient	261±20·4 (5)	263±10·3 (8)	638±14·4 (6)	43± 5·1 (5)	79±10·9 (6)
Pyridoxine-treated	178·9±12·6 (6)	205± 7·5 (5)	505±44·8 (5)	152±13·4 (6)	165±22·7 (6)

Table 4. *Effect of pyridoxine deficiency on the concentrations of reduced glutathione in tissues and erythrocytes of female rats*

Experimental details are given in the text. The results are given as means±S.E.M.; six rats were used in each group.

	Concn. of GSH				
Dietary regimen	In erythrocytes (μmoles/100 ml.)	In liver (μmoles/100 g.)	In kidney (μmoles/100 g.)	In spleen (μmoles/100 g.)	In brain (μmoles/100 g.)
Pyridoxine-deficient	301±11·2	428± 9·2	170±6·4	211±11·9	115± 8·2
Pyridoxine-treated	224± 8·5	286±30·5	160±5·1	205±23·2	111±13·5

fat diet, and were also found to be elevated in pyridoxine-deficient animals. The results in Table 3 also reveal that plasma glutamate–oxaloacetate-transaminase activities were markedly decreased in pyridoxine-deficient animals as compared with their corresponding pyridoxine-supplemented controls.

The effect of pyridoxine deficiency on GSH concentrations in other organs was studied in another experiment (Table 4). The results demonstrate again an increase in the concentration of GSH in erythrocytes and liver as a result of pyridoxine deficiency. However, no significant difference in the concentrations of GSH in kidney, spleen and brain was observed between the pyridoxine-deficient and control groups.

If the elevation of GSH concentrations in liver and erythrocytes in pyridoxine-deficient rats is due only to an accumulation of its precursors, e.g. cysteine, glutamic acid or glycine, it is conceivable that dietary deprivation of any one of the three amino acids may prevent the elevation of GSH concentrations in liver or erythrocytes. Such a study is of particular interest since it has been demonstrated that pyridoxal phosphate, an active form of vitamin B_6, is involved in the synthesis of cysteine from methionine.

The results in Tables 5 and 6 demonstrate that restriction of dietary cysteine or glutamic acid did not abolish the elevation of the concentration of liver GSH resulting from pyridoxine deficiency.

Table 5. *Effect of pyridoxine deficiency on the concentrations of reduced glutathione in erythrocytes and liver of male rats on a cysteine free diet*

Experimental details are given in the text. The results are given as means±S.E.M.; six rats were used in each group.

	Concn. of GSH	
Dietary regimen	In erythrocytes (μmoles/100 ml.)	In liver (μmoles/100 g.)
Pyridoxine-deficient	234±13·6	371±10·9
Pyridoxine-treated	212± 9·2	298±20·7

Table 6. *Effect of pyridoxine deficiency on the concentrations of reduced glutathione in erythrocytes and liver of female rats on a glutamic acid-free diet*

Experimental details are given in the text. The results are given as means±S.E.M.; five rats were used in each group.

	Concn. of GSH	
Dietary regimen	In erythrocytes (μmoles/100 ml.)	In liver (μmoles/100 g.)
Pyridoxine-deficient	201±19·3	398±20·5
Pyridoxine-treated	174±16·5	286±14·6

Even when the precursor, cysteine or glutamic acid, was absent from the diet, the magnitude of the increase in the concentration of liver GSH caused by pyridoxine deficiency remained about the same as that found in pyridoxine-deficient animals supplied

Table 7. *Incorporation of* ^{14}C *of* [2-^{14}C]*glycine into liver glutathione in pyridoxine-deficient and pyridoxine-treated male rats*

Experimental details are given in the text. The results are given as means ± S.E.M.; four rats were used in each group.

Dietary regimen	Concn. of GSH in liver (μmoles/100 g.)	Radioactivity of GSH in liver (counts per min./ mg. of GSH)	Radioactivity of GSH in liver (counts per min. in GSH/g. of liver)	Percentage of injected radioactivity found in liver as GSH
Pyridoxine-deficient	414 ± 38·5	14300 ± 790	18110 ± 880	1·91 ± 0·20
Pyridoxine-treated	210 ± 21·2	11770 ± 1220	7660 ± 1100	0·77 ± 0·12

Table 8. *Incorporation of* ^{14}C *of* [1-^{14}C]*glutamic acid into liver glutathione in pyridoxine-deficient and pyridoxine-treated male rats*

Experimental details are given in the text. The results are given as means ± S.E.M.; four rats were used in each group.

Dietary regimen	Concn. of GSH in liver (μmoles/100 g.)	Radioactivity of GSH in liver (counts per min./ mg. of GSH)	Radioactivity of GSH in liver (counts per min. in GSH/g. of liver)	Percentage of injected radioactivity found in liver as GSH
Pyridoxine-deficient	325 ± 20	649 ± 57	697 ± 54	0·066 ± 0·003
Pyridoxine-treated	195 ± 16	221 ± 30	450 ± 14	0·046 ± 0·001

with ample precursors: cysteine and glutamic acid. In neither case did the pyridoxine deficiency have a significant effect on erythrocyte GSH concentrations.

Table 7 illustrates the incorporation of [2-^{14}C]-glycine into liver GSH in pyridoxine-deficient and pyridoxine-fed rats. The mean value of total GSH in liver of pyridoxine-deficient rats was almost twice that of pyridoxine-treated ones. Its specific activity, expressed as counts per min./g. of liver, was only slightly and insignificantly higher in pyridoxine-deficient rats. However, the pyridoxine-deficient rats utilized 1·9 % of the injected radioactivity for the synthesis of liver GSH as compared with 0·77 % for rats receiving pyridoxine.

The results in Table 8 indicate that pyridoxine-deficient rats incorporated more of [1-^{14}C]glutamic acid into liver GSH than did the pyridoxine-supplemented rats when the comparison was made as percentage of the administered dose found as liver GSH. Radioactivity from [1-^{14}C]glutamic acid, whether in normal or in pyridoxine-deficient rats, is incorporated into liver GSH to a much less extent than that from [2-^{14}C]glycine.

DISCUSSION

The present study shows that dietary deficiency of pyridoxine results in a derangement of GSH metabolism. These findings agree with the observation of Beaton (1955), who reported that rats with a vitamin B_6 deficiency induced by giving a high-fat diet had a greater amount of GSH in erythrocytes and livers than did controls supplemented with vitamin B_6. The present results further reveal that the abnormal increase in the concentration of hepatic GSH also occurred in pyridoxine-deficient rats when they received a high-protein diet or a synthetic diet free of either cysteine or glutamic acid. This suggests that a limited dietary supply of these two amino acids will not decrease the biosynthesis of GSH in pyridoxine-depleted rats.

Deficiency of pyridoxine has been reported to result in a decrease in serum protein synthesis (Pike & Brown, 1959) and in the formation of antibodies (Stoerk, Eisen & John, 1947). On the other hand, the presence of relatively large quantities of GSH in most tissues suggested that this compound might play an important role in the biosynthesis of proteins (Hanes, Hird & Isherwood, 1950). It is not clear from the present study whether the elevation in the concentration of liver GSH in pyridoxine-deficient animals is the consequence of metabolic blocks at certain reaction sites leading to the biosynthesis of the peptide bonds of proteins. From our tracer measurements it appears that, in the pyridoxine-deficient rats, the formation of liver GSH from [2-^{14}C]glycine, and possibly [1-^{14}C]glutamic acid, was increased; this may in part be responsible for the increase of hepatic GSH concentrations.

An accumulation of L-cystathionine, an intermediate in transulphuration from methionine to cysteine, has been reported in the urine, the liver and the brain of pyridoxine-deficient rats (Hope, 1958). Ashwood-Smith & Smith (1959) demonstrated that the concentration of GSH in all three

parts of brain (cortex, cerebellum and medulla) fell significantly after 6–24 weeks of pyridoxine deficiency. This observation, unfortunately, was based on only one rat in each group at different intervals and needs confirmation. Their results were not in agreement with our present findings showing that the mean values of total brain GSH of pyridoxine-deficient rats were almost identical with those of pyridoxine-treated rats (see Table 4). In addition, the rats receiving a cysteine-free diet had a high concentration of liver GSH when pyridoxine became a limiting factor.

SUMMARY

1. The effect of pyridoxine deficiency induced under various dietary regimens, on the concentration of non-protein SH compounds (essentially GSH) in erythrocytes and tissues was studied in rats.

2. Deficiency of pyridoxine resulted in an elevation of GSH concentrations in erythrocytes and liver, but not in kidneys, spleen and brain.

3. An increase of GSH concentration in liver was also observed when pyridoxine-deficient rats received a diet free of cysteine or glutamic acid.

4. The incorporation of [2-^{14}C]glycine and [1-^{14}C]glutamic acid into the GSH moiety was enhanced by pyridoxine deficiency.

5. It is suggested that pyridoxine is an important regulator in glutathione metabolism.

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A Note on the Construction of a Modified Tissue Homogenizer

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Aldridge, Emery & Street (1960) described a tissue homogenizer, based on that of Potter & Elvehjem (1936), which incorporated modifications specifically designed so that homogenization was both efficient and reproducible and also so that if the homogenizer was broken it could be replaced by one with an identical performance.

The present note concerns further modification in the design of this instrument, which, while retaining all the functional advantages of that described by Aldridge *et al.* (1960), greatly simplifies the construction and, in our experience, has several other advantages, which are discussed below.

The instrument described by Aldridge *et al.* (1960) consists of a rotating Perspex pestle with a cylindrical body and a cone-shaped end (90° angle). The body of the pestle is machined so as to fit into a precision-bore glass tube leaving a precisely defined clearance between the two surfaces; the cone-shaped end fits accurately into a corresponding conical depression in a Perspex plug which closes the end of the tube and forms the base of the homogenizer. The Perspex base-plug is machined so as to be a sliding fit into the tube and is cemented in place with Bostik D.

In our experience it is not easy to ensure that the annular space between the Perspex base-plug and