Metabolism of [$^3H_8$]Pyridoxine in Mice*

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ABSTRACT: [$^3H_8$]Pyridoxine has been administered by the intravenous route to mice, and perchloric acid extracts of liver and carcass have been fractionated by an ion-exchange chromatographic procedure. The distribution of isotope between pyridoxine 5′-phosphate, pyridoxal, pyridoxal 5′-phosphate, pyridoxamine, and pyridoxamine 5′-phosphate has been calculated at different times after the administration of pyridoxine. Labeled pyridoxine disappeared from the liver within 60 min after the injection. During this period the labeling of pyridoxine 5′-phosphate first increased and reached a maximum after 10–15 min when 40% of the recovered isotope appeared as pyridoxine 5′-phosphate. Concomitantly with the following decrease in labeling of the pyridoxine 5′-phosphate, the labeling of pyridoxal 5′-phosphate increased. The incorporation of isotope into pyridoxamine 5′-phosphate occurred at a slower rate; between days 1 and 7 the ratio between pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate remained constant. In carcass the same processes occurred as in liver but at a slower rate. The specific radioactivity was determined in pyridoxal 5′-phosphate, pyridoxal, pyridoxamine 5′-phosphate, and pyridoxamine between days 1 and 7 after the administration of [$^3H_8$]pyridoxine. It remained approximately constant between days 4 and 7 indicating a slow turnover of the body pool of vitamin B_6.

In animal tissues vitamin B_6 occurs mainly in the form of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate (Lyon *et al.*, 1962). The requirement for vitamin B_6 is covered to a large extent by vitamin B_6 in cereals, in which pyridoxine is a large fraction of the total vitamin B_6 content (Toepfler and Polansky, 1964). Little is known about the interconversion of the different forms of the vitamin *in vivo* in animals and humans (*cf.* Snell, 1964). It has been suggested that a functional vitamin B_6 deficiency may arise through impaired conversion of injected pyridoxine into a metabolically active form and that such a mechanism may, at least partially, be responsible for the antivitamin effect of certain carbonyl reagents, *e.g.*, isoniazid (Snell, 1964). Possibly, such mechanisms may also be involved in aberrations of vitamin B_6 metabolism such as the dependency syndrome (Harris *et al.*, 1956; Harris and Horrigan, 1964) or vitamin B_6 responsive anemia (Hunt *et al.*, 1954; Coursin, 1964).

The purpose of the present study was to obtain information on the conversion of pyridoxine into other forms of the vitamin *in vivo* in a normal animal.

Material and Methods

Compounds. [$^3H_8$]Pyridoxine was obtained from the Radiochemical Centre (Amersham, Bucks, England) with a specific radioactivity of about 1 μCi/μg. According to the manufacturer this preparation was more than 97% radiochemically pure by the following criteria: paper chromatography in (i) 1-butanol–dioxane–ammonia, (ii) 1-butanol–acetic acid–water, and (iii) ethanol–ammonia–water; thin-layer chromatography in acetone–dioxane–ammonia. This was also verified by ion-exchange chromatography on a column of Dowex 50 (Figure 1) and by high-voltage electrophoresis (Clotten and Clotten, 1962). Pyridoxine-HCl, pyridoxamine-HCl, pyridoxal-HCl, pyridoxamine 5′-phosphate, and pyridoxal 5′-phosphate were gifts from Merck Sharp and Dohme, Rahway, N. J. Pyridoxine 5′-phosphate was purchased from Calbiochem, Luzerne, Switzerland.

Animals and Preparation of Tissues. Male mice of the N. M. R. I. strain, weighing about 25 g, which had been kept on a commercial mouse diet (Harld Forss Co., Stockholm, Sweden), were injected in a tail vein with 0.1 ml of a solution of [$^3H_8$]pyridoxine which contained 200 μg/ml. The mice were killed by a blow on the head at different times after the injection.

Tissues were homogenized in an equal weight of water in a VirTis homogenizer (The VirTis Co., Inc., Gardiner, New York, N. Y.). The proteins were precipitated by the addition of an equal volume of 2 N perchloric acid, the mixture was centrifuged, and the proteins were washed twice with 1 N perchloric acid. The combined solutions were titrated to pH 4.2 with 3 M potassium hydroxide and left overnight at 0°. Potassium perchlorate was filtered in the cold and the filtrate was evaporated to dryness *in vacuo* at a bath temperature of 40°.

Chromatographic Procedure. A suitable aliquot of the perchloric acid extract was dissolved in 1–2 ml of 0.05 M ammonium formate buffer at pH 4.25 and put onto a column (diameter 9 mm, height 400 mm) of Dowex 50-X8. The column was eluted first with 100 ml of 0.05 M ammonium formate at pH 4.25. A gradient was then

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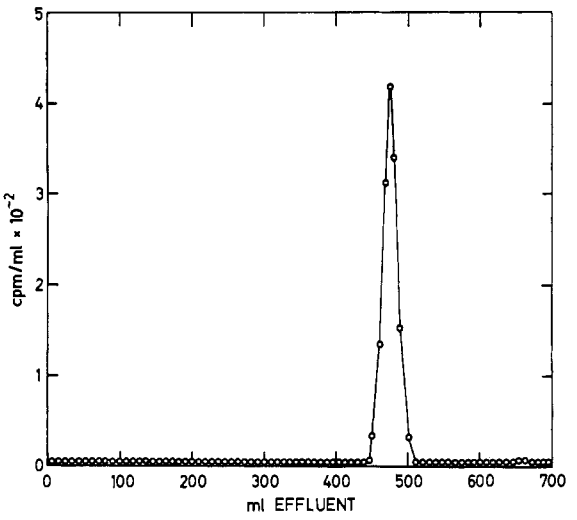

FIGURE 1: Chromatography of [$^3H_8$]pyridoxine on a column (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 (200–400 mesh). The column was equilibrated with 0.05 M ammonium formate at pH 4.25 and eluted with 100 ml of the same buffer, followed by 0.5 M ammonium formate with an increasing pH gradient (4.25–7.5).

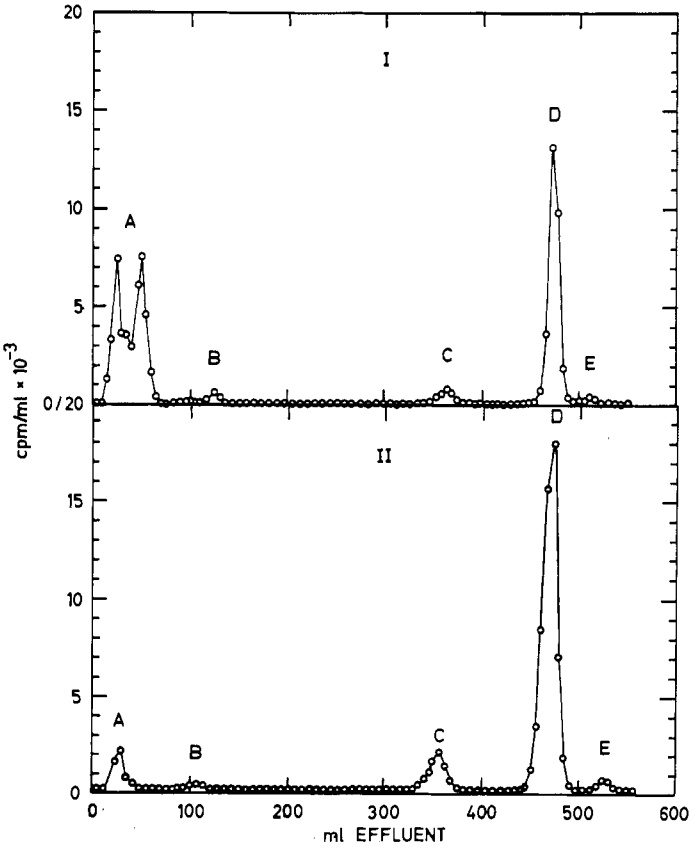

FIGURE 2: Chromatography on 2 columns (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 of a perchloric acid extract of mouse liver 12 min after the injection of [$^3H_8$]pyridoxine. I is the unhydrolyzed extract and II the same extract after hydrolysis. The peaks are: (A) pyridoxine 5′-phosphate and pyridoxal 5′-phosphate, (B) pyridoxamine 5′-phosphate, (C) pyridoxal, (D) pyridoxine, and (E) pyridoxamine.

started with 100 ml of 0.5 M ammonium formate at pH 4.25 in a closed chamber to which was added 0.5 M ammonium formate at pH 7.5. The eluate was collected in fractions of 5-ml volume at a flow rate of about 0.5 ml/min.

Isotope Determination. The isotope content was determined on each chromatographic fraction in a liquid scintillation spectrometer (Tri-Carb, Packard Co., La Grange, Ill.). To 0.4 ml of each fraction was added 16 ml of a mixture of the following composition: 2,5-diphenyloxazole (10 g), 1,4-bis[2-(4-methyl-5-phenyloxazoly)]benzene (0.3 g), toluene (1000 ml), and methyl Cellosolve (600 ml). The efficiency for tritium in this system was about 15%.

Assay of Vitamin B_6 Activity. Vitamin B_6 activity was determined with a microbiological technique with *Saccharomyces carlsbergiensis* ATCC 9080, strain 4288. An aliquot of each chromatographic fraction (1 ml) was added to 1 ml of 0.4 M sulfuric acid and the solution was kept in an autoclave at 120° for 1 hr. The solution was then adjusted to pH 5.0–5.2 with a potassium citrate buffer at pH 12.3. Part of this solution (1 ml) was then used in the microbiological assay, which was carried out essentially as described by Atkin *et al.* (1943).

Results

Short-Time Experiments. A series of mice were injected intravenously with 20 μg (20 μCi) of [$^3H_8$]pyridoxine and killed at different times within the first hour. Figure 2 shows ion-exchange chromatograms of the perchloric acid extract of liver from the experiment in which homogenization of the liver was started 12 min after the injection.

The main radioactive compound seen in Figure 2 is pyridoxine (peak D) but radioactive material also appears at the position of pyridoxal 5′-phosphate and pyridoxine 5′-phosphate (peak A) and in small amounts at the position of pyridoxamine 5′-phosphate (peak B), pyridoxal (peak C), and pyridoxamine (peak E). Figure 3 illustrates the changes in the percentage distribution of isotope between the different forms of vitamin B_6 in mouse liver during the first hour after injection. Labeled pyridoxine disappeared rapidly and accounted for less than 1% of the total isotope after 60 min. The labeling of pyridoxine 5′-phosphate reached its maximum about 10 min after injection of the [$^3H_8$]pyridoxine and then declined. In contrast, isotope in pyridoxal 5′-phosphate increased and after 60 min this compound accounted for about 70% of the recovered isotope. The amount of isotope in the other forms of the vitamin was small.

Figure 4 shows an ion-exchange chromatogram of a perchloric acid extract of the carcass from a mouse which had been killed 35 min after injection of labeled pyridoxine. The top curve shows the distribution of isotope in the chromatogram and the bottom curve the result of microbiological assay on the individual fractions. The main labeled compound at this time is pyridoxine (peak D), followed by pyridoxine 5′-phosphate and pyridoxal 5′-phosphate (peak A).

The distribution of isotope between the different forms of the vitamin in carcass is illustrated in Figure 5. The labeling of pyridoxine declined immediately after the injection, but after 60 min 60% of the total isotope was still present as pyridoxine. The labeling of pyridoxine 5′-phosphate increased, and this form of the vitamin accounted for 25% of the recovered isotope after 60 min. At this time only small amounts of isotope had been incorporated into other forms of the vitamin.

Long-Time Experiments. Figure 6 shows the distribution of recovered isotope between the different forms of the vitamin in the liver from days 1 to 7 after the intravenous administration of [$^3H_8$]pyridoxine. The initial values in this figure are the 60-min values from Figure 2. Twenty-four hours after the administration of labeled pyridoxine the isotope appeared mainly in pyridoxal 5′-phosphate and in pyridoxamine 5′-phosphate; less then 10% appeared as free pyridoxal or pyridoxamine. From days 1 to 7 there was no significant change in the distribution of isotope between the different forms of the vitamin.

In carcass (Figure 7) about 7% of the total isotope remained as pyridoxine 1 day after injection of [$^3H_8$]pyridoxine. At this time about equal amount of isotope (~25%) appeared in pyridoxal 5′-phosphate and in pyridoxamine 5′-phosphate and about 20% of the isotope appeared in each of pyridoxal and pyridoxamine. The distribution of isotope between the different forms of vitamin B_6 was essentially unchanged from days 1 to 7. Figure 8 shows a plot of the specific radioactivity of pyridoxamine 5′-phosphate, pyridoxamine, pyridoxal 5′-phosphate, and pyridoxal in the liver from days 1 to 7 after the injection of [$^3H_8$]pyridoxine. Figure 9 shows the specific radioactivity of the same compounds in carcass. It is seen from these figures that the specific radioactivity was approximately the same in the different forms of the vitamin, and also that there was no marked change in the specific radioactivity from days 4 to 7 after the administration of the labeled pyridoxine.

Discussion

Tritium-labeled pyridoxine has been used to study the excretion of vitamin B_6 in the rat (Cox *et al.*, 1962), the absorption of pyridoxine from the gut in rats (Booth and Brain, 1962) and humans (Brain and Booth, 1964), and the turnover and body pool of the vitamin (Johansson *et al.*, 1966a,b). It has been pointed out by Argoudelis and Kummerow (1966) that commercially available pyridoxine labeled by the Wilzbach (1957) technique may contain appreciable amounts of labeled impurities and that results obtained with such preparations may be invalid. Our preparation was obtained from another source than that referred to by Argoudelis and Kummerow and was radiochemically pure as evidenced by paper and thin-layer chromatography, ion-exchange chromatography, and high-voltage electrophoresis. Only after prolonged storage and repeated thawings of frozen solutions have we occasionally observed labeled degradation products of pyridoxine. These are however easily removed by ion-exchange chromatography. In a study of pyridoxine metabolism in animals it would be desirable to administer pyridoxine in "trace" amounts in order to conserve a physiological steady state. It has for instance been demonstrated that a proportionally larger amount of isotope is excreted in the urine when the

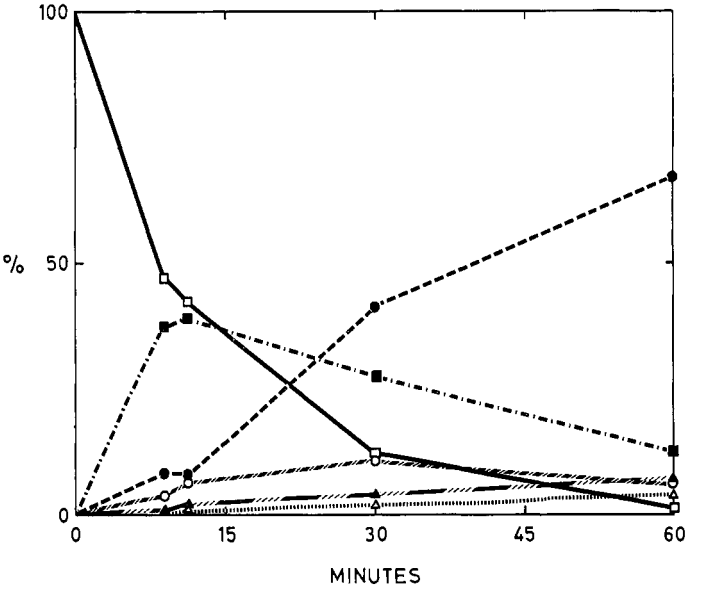

FIGURE 3: Distribution of radioactivity in mouse liver between different forms of vitamin B_6 during the first hour after intravenous administration of [$^3H_8$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

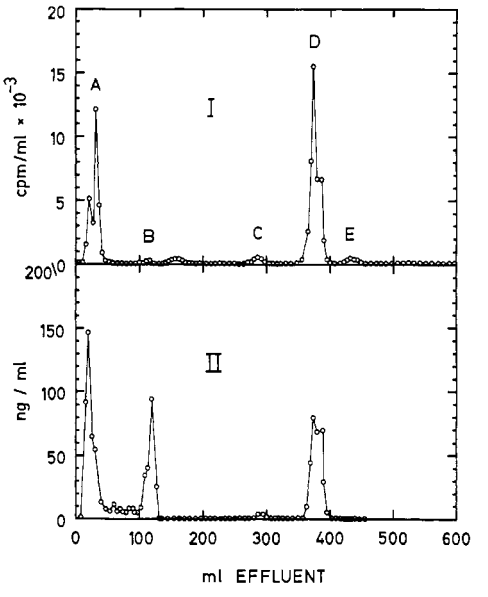

FIGURE 4: Chromatography on a column (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 (200–400 mesh) of a perchloric acid extract of mouse carcass 35 min after the injection of [$^3H_8$]pyridoxine. The column had been equilibrated with 0.05 M ammonium formate at pH 4.25. It was eluted first with 100 ml of the same buffer, followed by 0.5 M ammonium formate with an increasing pH gradient (4.25–7.5). The *top curve* shows isotope determination and the *bottom curve* the microbiological assay with *S. carlsbergiensis*. The peaks are (A) pyridoxine 5′-phosphate and pyridoxal 5′-phosphate, (B) pyridoxamine 5′-phosphate, (C) pyridoxal, (D) pyridoxine, and (E) pyridoxamine.

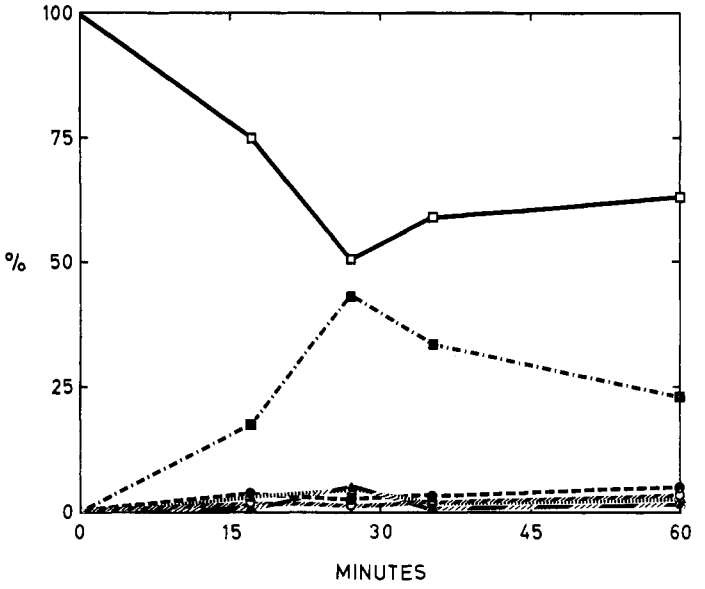

FIGURE 5: Distribution of isotope between the different forms of vitamin B_6 in mouse carcass during the first hour after the intravenous administration of [$^3H_8$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

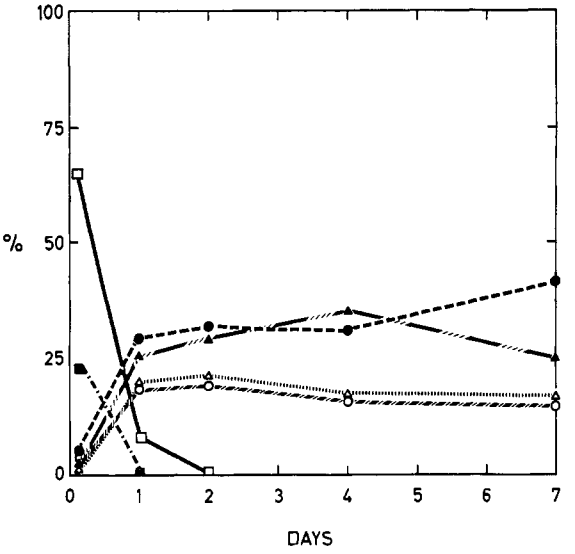

FIGURE 7: Distribution of isotope between different forms of vitamin B_6 in mouse carcass from days 1 to 7 after the intravenous administration of [$^3H_8$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

amount of administered pyridoxine is increased (Johansson *et al.*, 1966a). In this study 20 μg of pyridoxine was injected as a compromise between the desire to keep the injected amount as low as possible and the need for sufficient amount of isotope in the chromatographic separations.

Since a high specific radioactivity of the pyridoxine was required, tritium-labeled pyridoxine had to be used. We have recently found that about 20% of the tritium atoms is located at the carbon in the 4-hydroxymethyl group (S. Lindstedt, S. Johansson, and H.-G. Tiselius, unpublished data). This tritium is metabolically labile and is, *e.g.*, lost in the conversion into 4-pyridoxic acid; the remaining tritium is apparently metabolically stable in animals in the reactions discussed in this paper.

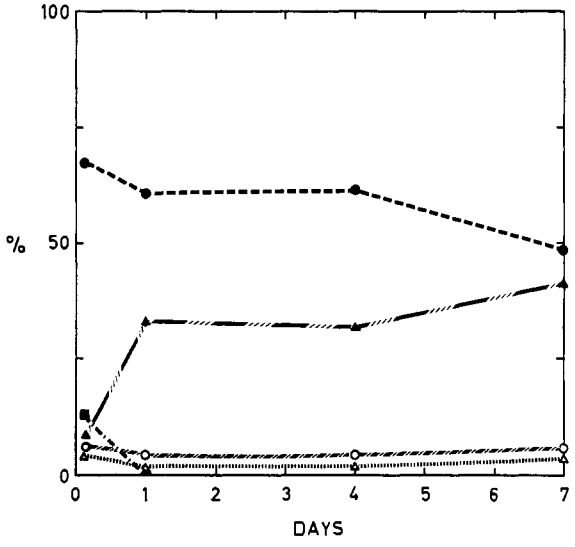

FIGURE 6: Distribution of isotope between different forms of vitamin B_6 in the liver from days 1 to 7 after the intravenous administration of [$^3H_8$]pyridoxine. Pyridoxine (□), pyridoxine 5-phosphate, (■) pyridoxal (○), pyridoxal 5-phosphate, (●) pyridoxamine (△), and pyridoxamine 5-phosphate (▲).

Problems involved in the extraction of vitamin B_6 from biological material have been discussed by Storvick and Peters (1964). The method used in this study is essentially that which has been used by Bain and Williams in their chromatographic studies of the concentration of different forms of vitamin B_6 in animal tissues (Bain and Williams, 1960; Lyon *et al.*, 1962). In our experience at least 85% of the total amount of labeled compounds is extracted by this procedure.

A chromatographic system for the fractionation of the different forms of vitamin B_6, described by Bain and Williams (1960), utilizes a two-layered column of the anion exchanger Dowex 1 and the cation exchanger Dowex 50 and a series of different buffers for elution. In our hands, with large volumes of tissue extract, this system did not give consistently reproducible results. A simpler chromatographic system with the cation exchanger Dowex 50 and volatile buffers was developed, but this system did not completely separate pyridoxine 5′-phosphate and pyridoxal 5′-phosphate. When both these compounds were present their relative amounts could be calculated either from a comparison of chromatograms of hydrolyzed and unhydrolyzed tissue extracts (*cf.* Figure 2) or by rechromatography of pyridoxine and pyridoxal obtained by hydrolysis of the unresolved mixture.

Present-day concept of the interconversion of the different forms of vitamin B_6 is mainly based on *in vitro* experiments and on the isolation of different enzymes responsible for the interconversions (Snell, 1964). However, Rabinowitz and Snell (1949) administered pyri-

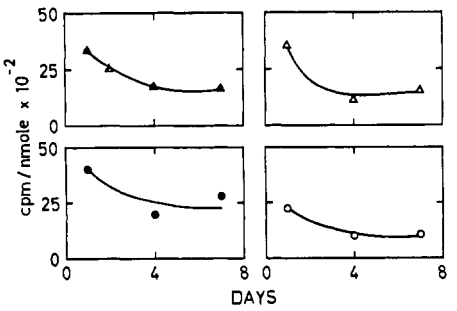

FIGURE 8: Specific radioactivity of pyridoxal (○), pyridoxal 5-phosphate (●), pyridoxamine (△), and pyridoxamine 5-phosphate (▲) in mouse liver after the intravenous administration of [$^3H_8$]pyridoxine.

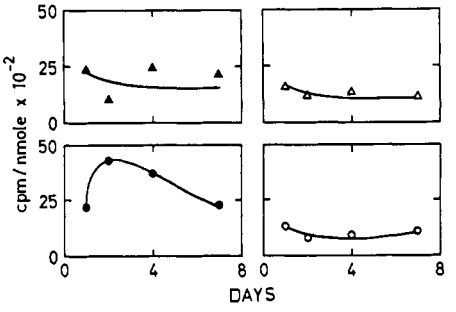

FIGURE 9: Specific radioactivity of pyridoxal (○), pyridoxal 5-phosphate (●), pyridoxamine (△), and pyridoxamine 5-phosphate (▲) in mouse carcass after the intravenous administration of [$^3H_8$]pyridoxine.

doxine, pyridoxamine, and pyridoxal to humans and determined these compounds in urine with a differential microbiological assay. The excretion of 4-pyridoxic acid was increased when either of these compounds was administered. Injection of pyridoxine resulted in a greatly increased excretion of pyridoxal and pyridoxamine, but pyridoxine excretion was not increased after the feeding of pyridoxal or pyridoxamine. Since the microbiological assay was carried out on hydrolyzed samples it could not be decided if these compounds were excreted in free or phosphorylated forms.

The present study has demonstrated a rapid phosphorylation of pyridoxine in the liver; practically no free pyridoxine could be detected 60 min after the intravenous injection of [$^3H_8$]pyridoxine. Ten minutes after the injection of [$^3H_8$]pyridoxine about 40% of the recovered isotope appeared as pyridoxine 5′-phosphate and the relation between pyridoxine and pyridoxine 5′-phosphate resembled a classic precursor–product relationship. ATP:pyridoxal 5′-phosphotransferase (EC 2.7.1.35) has been partially purified from rat liver and brain, from beef and human brain, and also from several microorganisms (McCormick and Snell, 1959, 1961; McCormick *et al.*, 1961). Pyridoxal was the preferred substrate, but pyridoxine and pyridoxamine were also phosphorylated; the K_s values for these substrates were reported as 0.15, 0.25, and 1.5×10^{-4} M for the rat liver enzyme. The same authors also studied the distribution of the enzyme in rat tissue and found high activity in liver, kidney, spleen, and brain. The activity in skeletal muscle was low, which agrees with our finding that labeled pyridoxine disappeared considerably slower from carcass than from liver. It is plausible that some of the pyridoxine 5′-phosphate which is found in carcass may have originated in liver or other parenchymatous organs, but present data do not allow any estimate of such transfer.

The isotope content of the pyridoxal 5′-phosphate in liver started to rise immediately after the injection, but very little pyridoxal 5′-phosphate had appeared in carcass within the first 60 min after injection of labeled pyridoxine although a fairly high concentration of labeled pyridoxine 5′-phosphate had been attained at this time. An enzyme catalyzing the oxidation of pyridoxine 5′-phosphate and of pyridoxamine 5′-phosphate to pyridoxal 5′-phosphate was first demonstrated in rabbit liver (Pogell, 1958). This enzyme–pyridoxamine 5′-phosphate:O_2 oxidoreductase (deaminating), (EC 1.4.3.5) has been purified about 65-fold (Wada and Snell, 1961). Its distribution in different tissues has not been studied, but its concentration in muscle is apparently low.

In carcass, and to a smaller extent in liver, isotope was recovered in pyridoxal and pyridoxamine. A direct conversion of pyridoxine into pyridoxal has been demonstrated (Braunstein and Bukin, 1956), but these compounds may also originate from the action of phosphatases on the respective phosphorylated forms.

In liver the appearance of isotope in pyridoxamine 5′-phosphate was slower than in pyridoxal 5′-phosphate, whereas in carcass these two forms of the vitamin increased almost in parallel in isotope content. The enzymatic mechanisms behind the formation of pyridoxamine and pyridoxamine 5′-phosphate are not as obvious as those involved in the interconversions discussed above. In the course of enzymatic transamination enzyme-bound pyridoxal 5′-phosphate would be transiently converted into pyridoxamine 5′-phosphate. An enzyme catalyzing the reversible transamination between pyridoxamine and oxaloacetate has been purified from rabbit liver (Wada and Snell, 1962). Since the results strongly support the idea that phosphorylation is the primary step in the metabolic transformation of pyridoxine, this reaction is probably of minor significance *in vivo*.

Rabinowitz and Snell (1949) in their study on the metabolism of vitamin B_6 in humans failed to account for more than about 45% of the administered amount in the urine. Later studies with labeled pyridoxine have demonstrated that a variable fraction of administered pyridoxine is retained in the body and excreted at a slow rate (Johansson *et al.*, 1966a,b). Based on the shape of the isotope retention curve a metabolic model for the *in vivo* metabolism of pyridoxine has been proposed, and it has been suggested that the total amount of vitamin B_6 in the organism may be estimated by the principle of isotope dilution from knowledge of the total administered isotope and the specific radioactivity of the urinary 4-pyridoxic acid. This procedure requires that the different forms of the vitamin reach the same specific radioactivity after an initial period of equilibration. To test this hypothesis the specific radioactivity of pyridoxal 5′-phosphate, pyridoxal, pyridoxamine 5′-phosphate,

and pyridoxamine was determined at different times after the administration of [$^3H_8$]pyridoxine. They were found to reach approximately the same specific radioactivity. However, the values are probably not very accurate since the microbiological assay has a fairly large methodological error. For this reason, no corrections were used for loss of tritium atoms in the conversion of pyridoxine into pyridoxal and pyridoxamine or for the slightly different growth response of *S. carlsbergiensis* to the three forms of the vitamin. The slow decline in specific radioactivity of the different forms of the vitamin between days 4 and 7 confirms previous results, demonstrating a slow turnover of the body pool of vitamin B_6 (Johansson *et al.*, 1966a,b).

References

Argoudelis, C. J., and Kummerow, F. A. (1966), *Biochemistry 5*, 1.

Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N. (1943), *Ind. Eng. Chem., Anal. Ed. 15*, 141.

Bain, J. A., and Williams, H. L. (1960), *in* Inhibition in the Nervous System and γ-Aminobutyric Acid, New York, N. Y., Pergamon, p 275.

Booth, C. C., and Brain, M. C. (1962), *J. Physiol. (London) 164*, 282.

Brain, M. C., and Booth, C. C. (1964), *Brit. J. Gastroenterol. 5*, 241.

Braunstein, A. E., and Bukin, Y. V. (1956), *Dokl. Acad. Nauk Arm. SSR 106*, 96.

Clotten, R., and Clotten, A. (1962), *in* Hochspannungselektrophorese, Stuttgart, George Thieme, p 63.

Coursin, D. B. (1964), *Vitamins Hormones 22*, 756.

Cox, S. H., Murray, A., and Boone, I. U. (1962), *Proc. Soc. Exptl. Biol. Med. 109*, 242.

Harris, J. W., and Horrigan, D. L. (1964), *Vitamins Hormones 22*, 750.

Harris, J. W., Whittington, R. M., Weisman, R., Jr., and Horrigan, D. L. (1956), *Proc. Soc. Exptl. Biol. Med. 91*, 427.

Hunt, A. D., Stokes, J., McCrory, W. W., and Stroud, H. H. (1954), *Pediatrics 13*, 140.

Johansson, S., Lindstedt, S., and Register, U. (1966a), *Am. J. Physiol. 210*, 1086.

Johansson, S., Lindstedt, S., Register, U., and Wadström, L. (1966b), *Am. J. Clin. Nutr. 18*, 185.

Lyon, J. B., Jr., Bain, J. A., and Williams, H. L. (1962), *J. Biol. Chem. 237*, 1989.

McCormick, D. B., Gregory, M. E., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2076.

McCormick, D. B., and Snell, E. E. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 1371.

McCormick, D. B., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2085.

Pogell, B. M. (1958), *J. Biol. Chem. 232*, 761.

Rabinowitz, J. C., and Snell, E. E. (1949), *Proc. Soc. Exptl. Biol. Med. 70*, 235.

Snell, E. E. (1964), *Vitamins Hormones 22*, 485.

Storvick, C. A., and Peters, J. M. (1964), *Vitamins Hormones 22*, 833.

Toepfler, E. W., and Polansky, M. M. (1964), *Vitamins Hormones 22*, 865.

Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.

Wada, H., and Snell, E. E. (1962), *J. Biol. Chem. 237*, 127.

Wilzbach, K. E. (1957), *J. Am. Chem. Soc. 79*, 1013.