Vitamin B_6 Metabolism. The Utilization of [^{14}C]Pyridoxine by the Normal Mouse*

C. E. Colombini and E. E. McCoy

ABSTRACT: [4,5-*ring*-^{14}C]Pyridoxine at high specific activity was intravenously injected in normal mice which were sacrificed at intervals varying from 1 min to 7 days following injection. The nonphosphorylated and phosphorylated B_6 vitamers synthesized *in vivo* from radioactive pyridoxine were extracted from the livers, brains, and carcasses, separated by high-voltage thin-layer electrophoresis, and quantitated by scintillation counting. The results show that in the liver and brain where pyridoxal 5′-phosphate is known to be engaged in multiple enzymatic activities as coenzyme, the injected pyridoxine was rapidly transformed into pyridoxal and pyridoxine 5′-phosphate which were further converted into pyridoxal 5′-phosphate suggesting that the activities of the pyridoxal phosphokinase and oxidase enzymes were both high.

The carcass is characterized by a slow formation of the phosphorylated vitamers and high levels of pyridoxal indicating, respectively, low pyridoxal phosphokinase and high oxidase activities. The continuous presence of appreciable amounts of pyridoxine, pyridoxal, and pyridoxine 5′-phosphate suggests that vitamin B_6 may be stored in the carcass for future utilization in other organs, while the slow accumulation of equal amounts of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate indicates their availability for coenzyme activity. In all tissues, the synthesis of pyridoxamine 5′-phosphate was slow but steady, and toward the end of the experiment its percentages equaled that of pyridoxal 5′-phosphate. Since the synthesis of pyridoxamine seems to have been poor in all tissues and thus, its contribution to the formation of pyridoxamine 5′-phosphate would be minimal, the most logical source for this vitamer is represented by transamination reactions.

The role attributed to vitamin B_6, in its active forms pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate,[1] as an indispensible cofactor in many fundamental enzymatic transformations and the serious consequences resulting when it becomes unavailable to the organism have been widely recognized by numerous investigators (Harris *et al.*, 1964). In 1964, interest in vitamin B_6 centered on its metabolism, its metabolic functions, as well as on the mechanism involved in normal and impaired enzymatic reactions where PLP is known to act as the coenzyme. In 1969, these problems remain practically unsolved.

Snell (1964) and Williams (1964) indicated that fundamental biochemical problems connected with vitamin B_6 could be approached by employing isotopically labeled B_6 vitamers. The use of [^{3}H]pyridoxine in metabolic studies has been reported in recent years (Booth and Brain, 1962; Brain and Booth, 1964; Cox *et al.*, 1962; Johansson *et al.*, 1966a,b, 1968). None, however, offered a sufficiently detailed picture of the overall *in vivo* transformations of the B_6 vitamers. Moreover, it became evident that tritiated PN was not devoid of serious inconveniences, particularly when applied to metabolic studies (Johansson *et al.*, 1966a; Argoudelis and Kummerov, 1966). For these reasons, the present work reports the *in vivo* synthesis and retention of the B_6 vitamers in mouse liver, brain, and carcass, employing [^{14}C]PN at high specific activity. This represents the initial phase of a broad study directed toward the understanding of the control mechanism of vitamin B_6 dependent normal, as well as abnormal enzymatic reactions and their clinical significance. Since [^{14}C]PN was not commercially available, it was synthesized (Colombini and Celon, 1969).

Materials and Methods

[4,5-^{14}C]Pyridoxine·HCl. This compound was prepared at a specific activity of 7 mCi/mmole by the condensation of [2,3-^{14}C]diethyl fumarate with 4-methyl-5-ethoxyoxazole followed by lithium aluminum hydride reduction of the obtained adduct. Because of the tendency to undergo autoradiolysis, its purity was periodically checked by high-voltage electrophoresis according to the method of Siliprandi *et al.* (1954) and by paper chromatography (Argoudelis and Kummerov, 1966) in *t*-amyl alcohol–acetone–water–concentrated ammonium hydroxide (40:35:20:5, v/v) with the chromatograms analyzed by a radiochromatogram scanner (Baird-Atomic, Model 363). When necessary, purification was accomplished by repeated recrystallizations from a hot ethanol–HCl mixture in the dark. Prior to its injection, an aliquot was dissolved in sterile saline solution (Abbott 4124) at a concentration of 330 μg/ml (10.62 μCi/ml) and was kept frozen in an amber bottle.

Animals and Injection of Labeled PN. Twenty-four male Swiss-Webster mice, averaging 29.3 g and kept on regular Purina Mouse Chow, were used. They received 0.5 ml of labeled PN solution in a tail vein and were sacrificed in pairs

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[1] Abbreviations used are: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, pyridoxine 5′-phosphate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

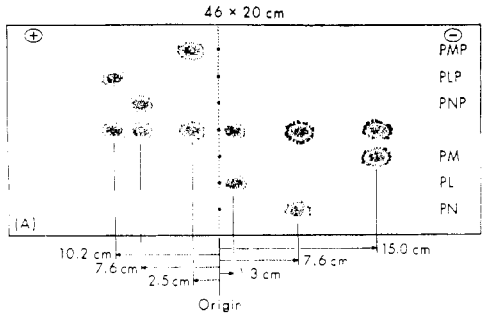

FIGURE 1: Reproduction of a cellulose thin-layer electrophoretic separation of a mixture and individual vitamin B_6 components: PN, PL, PM, PNP, PLP, and PMP.

at intervals varying from a minimum of 1 min to a maximum of 7 days following injection by immersion in liquid N_2.

Tissue Extracts. While still frozen, livers and brains were rapidly removed from the carcasses, weighed, and homogenized with an amount of cold distilled water equal to twice their weight. After the additional removal of the tail, skin, and bladder, the frozen carcasses and an amount of cold distilled water equal to twice their weight were homogenized in a high-speed Waring blender. Cold, 40% trichloroacetic acid was added dropwise to known quantities of the homogenates to obtain a final concentration of 7% in trichloroacetic acid while homogenization continued. Following centrifugation for 15 min at 12,000*g* at 0–4°, the supernatants were stored in brown bottles and the precipitates were reextracted with 7 ml of 7% trichloroacetic acid. After centrifugation the combined supernatants were extracted to remove excess trichloroacetic acid with peroxide-free ethyl ether to a final pH of 5–5.5. All these manipulations were carried out at ice-water temperature. The tissue extracts were then concentrated in the dark under reduced pressure to a volume of exactly 0.5 ml employing a rotating evaporator with a water bath not exceeding 40°.

Electrophoretic Separation and Determination of the B_6 Vitamers. High-voltage thin-layer electrophoretic separation of all B_6 vitamers was employed (Colombini and Celon, 1969). Figure 1 is a reproduction of a plate showing a separated mixture and the position of the individual unlabeled B_6 vitamers at the end of an electrophoretic run, revealed by exposure to ultraviolet light. An aliquot of standard solution containing up to 5 μg each of all six unlabeled vitamers was applied to a layer of prewashed Whatman cellulose powder CF-1 spread onto 46 × 20 cm glass plates alongside 5-μg quantities of individual vitamers. Electrophoresis was run at 750 V for 3 hr or at 600 V for 4 hr, using 0.05 M sodium acetate buffer at pH 5.1, employing a horizontal Savant apparatus. The plates were then dried under a stream of warm air and the separated vitamers were visualized by ultraviolet light exposure.

The same procedure was employed for the separation of the radioactive vitamers contained in the tissue extracts. In this case a maximum of 5–10 μl of tissue extracts was employed for each determination; larger quantities were found to be unsuitable to electrophoresis without further elimination of coextracted materials.

At the same time the quantity of the radioactive vitamers present in 5–10-μl aliquots of tissue extracts was so small as to render their visualization impossible. To overcome this difficulty, 5 μg each of carrier B_6 vitamers was added to the tissue extract samples on the plate before electrophoresis. In this way both labeled and unlabeled vitamers traveled together and were visualized as usual by ultraviolet light exposure. No purity check for the radioactive B_6 vitamers was deemed necessary. Their relative positions had previously been established and they resulted well separated from each other on the plates. In addition it was assumed that each chemically identical labeled and unlabeled vitamer had the same electrophoretic properties.

Radioactivity Measurements. The six spots corresponding to the B_6 vitamers visible under ultraviolet light were marked, carefully scraped off the plates into vials containing 1 ml of methanol, and counted in a liquid scintillation system (Beckman LS 200B). The counting mixture (10 ml) consisted of the following components: 2,5-diphenyloxazole (5 g), 1,4-bis[2-(5-phenyloxazoyl)]benzene (300 mg), Cab-O-Sil (40 g), and toluene (1 l.). The efficiency for ^{14}C varied from 85% for 10^6 dpm to about 92% for 50 dpm.

Results

The individual values for each vitamer obtained from the electrophoretic separations of the tissue extracts, followed by quantitation of the radioactivity present in each spot were plotted for each studied tissue as shown in Figures 2–4. These values represent the percentages of labeled B_6 vitamers synthesized and retained at various intervals in each tissue as a function of the injected [^{14}C]PN. From the same values, a second set of curves representing the distribution of the labeled B_6 vitamers could also be plotted but due to limited space is omitted. Nonetheless, these distribution patterns may be visualized from the reported curves.

A first analysis of the data reported in the diagrams clearly indicates that, in all tissues, the first hour immediately following the injections of labeled PN was characterized by a dynamic series of transformations of the B_6 vitamers. Figure 5 represents the currently accepted pathway for the enzymatic conversion of PN into the other forms of the B_6 group.

Liver. Within 1 min after the injection of [^{14}C]PN, the synthesis of the other labeled B_6 vitamers had already begun. The total retained radioactivity was 12.2% and was comprised of 9.63% PN, 1.86% PNP, 0.29% PLP, 0.24% PL, 0.12% PMP, and 0.06% PM, as shown in Figure 2. PN rapidly decreased either because it was excreted unchanged or underwent metabolic transformation, and after 1 hr its percentage was only 0.08. During the same period of time, PL and PNP rose rapidly as long as PN was available reaching maxima of 0.74% at 15 min and 1.94% at 5 min, respectively, then, when the concentration of PN became minimal, their levels declined. The synthesis of PLP occurred at a rapid rate and reached maximum values of 1.57–1.53% between 15 and 60 min from the time of injection; that of PMP occurred at a substantially lower rate and a maximum value of 0.69% was found at 24 hr. At the end of the 7-day experiment, the percentage of the total injected radioactivity still present in the liver was 0.37 and was mainly represented by 0.17 PMP and 0.18 PLP. It may be pointed out that from 60 min after [^{14}C]PN was injected to the end of the experiment, PLP and PMP represented the bulk of the B_6 vitamers remain-

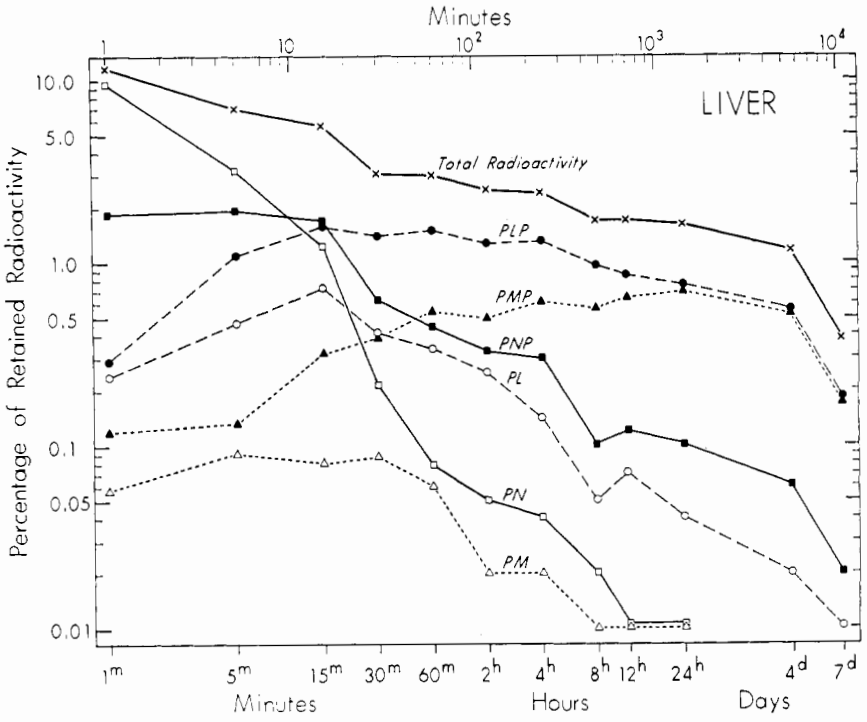

FIGURE 2: Logarithmic curves of the combined and individual radioactive B_6 vitamers synthesized from injected [^{14}C]pyridoxine and retained in the mouse liver for various intervals. Combined B_6 vitamers (x), pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

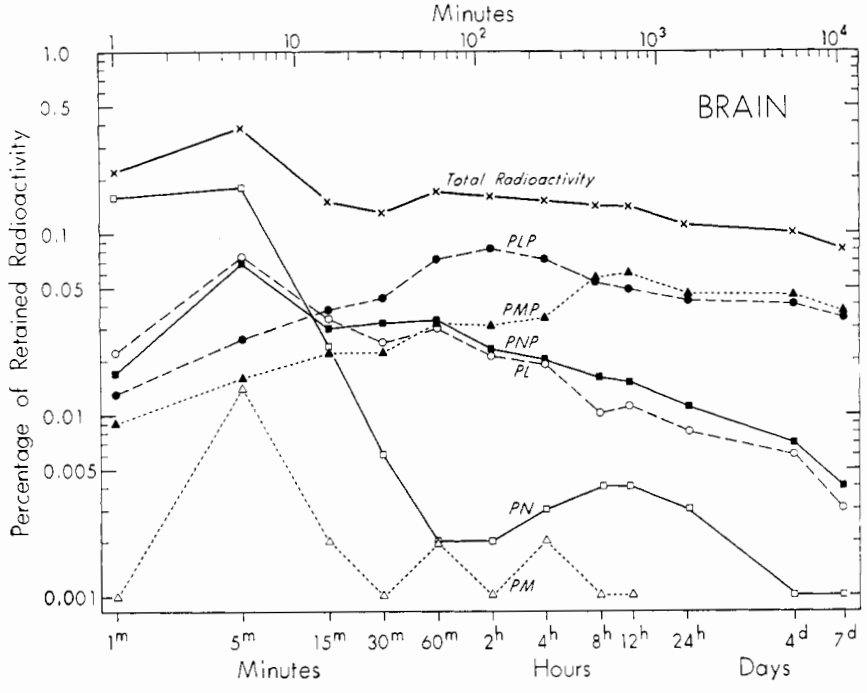

FIGURE 3: Logarithmic curves of the combined and individual radioactive B_6 vitamers synthesized from injected [^{14}C]pyridoxine and retained in the mouse brain for various intervals. Combined B_6 vitamers (x), pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

ing at practically constant levels; PNP and PL progressively declined while PN and PM decreased to minimal values.

The upper curve in Figure 2 represents the changes in radioactivity levels of the combined B_6 vitamers as percentages of the injected radioactivity. These levels declined rapidly from 12.2 to 3.15% during the first 30 min and then at a slow rate during the remainder of the 7-day period.

Brain. In this organ, the accumulation and disappearance

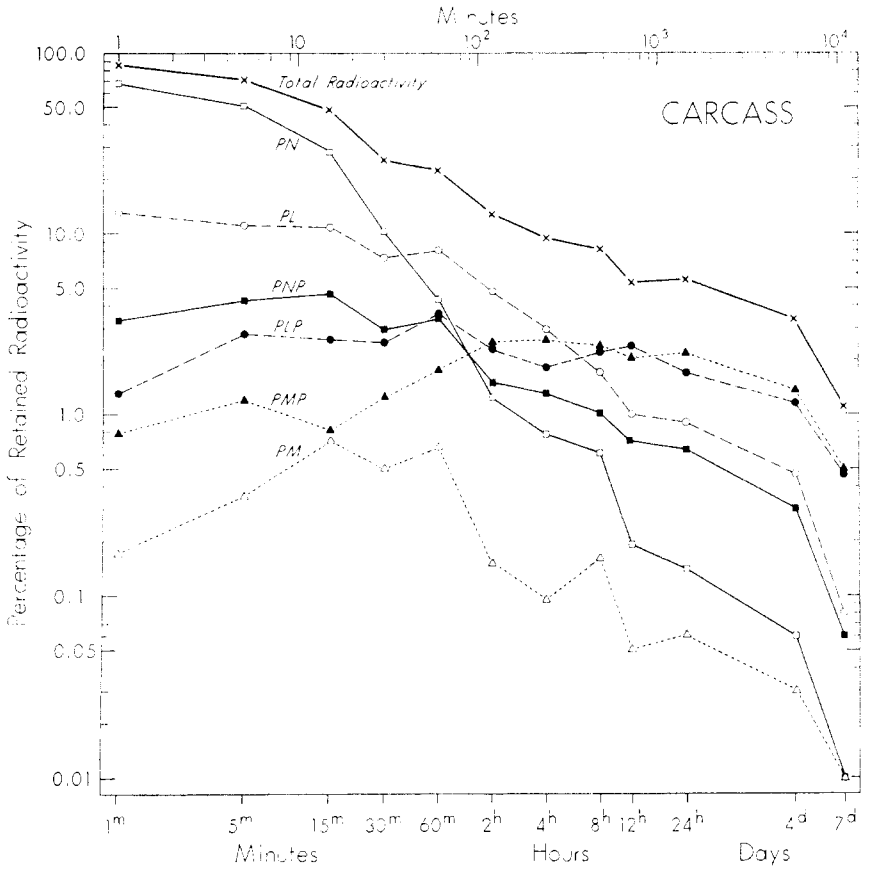

FIGURE 4: Logarithmic curves of the combined and individual radioactive B_6 vitamers synthesized from injected [^{14}C]pyridoxine and retained in the mouse carcass for various intervals. Combined B_6 vitamers (x), pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

of the total radioactivity followed a different course from that in the liver. As the upper curve in Figure 3 shows, the total radioactivity reached a maximum percentage only after 5 min, perhaps indicating the existence of a moderate blood brain barrier, then diminished very rapidly but leveled off after only 15 min from the initial injection of labeled PN. The total radioactivity remained at an almost constant level for about 12 hr at which time it was mainly represented by PLP and PMP. The maximal percentage for PLP was 0.08 after 2 hr and for PMP 0.06 after 12 hr. The synthesis and disappearance of PL and PNP were typical of the classical precursor–product relationship. They attained practically the same percentages, 0.075 and 0.07, respectively, after 5 min as long as high quantities of PN were available, then were transformed, not as rapidly as in liver, into PLP and PMP. Between 8 and 12 hr after injection of [^{14}C]PN, PLP, and PMP reached an equilibrium that was maintained until the end of the experiment. On day 7 the total radioactivity in brain was 0.08% of that injected or 21% of its value at 5 min. In contrast, at the end of the same period, the radioactivity in liver, in reference to its maximum at 1 min, was only 3%.

Carcass. The carcass appeared to be the storage site for the radioactive B_6 vitamers. As Figure 4 indicates, at 1 min the total radioactivity was 86.75% of that injected; it then diminished rapidly during the first 30 min, and at the end of the 7-day experiment it was still 1.10%. As expected, the percentage of PN was highest after 1 min (68.27) then decreased throughout the experiment, but not as rapidly as in the liver and brain, and was still 0.012 on day 7. Synthesis of PLP and PMP did not seem to be favored in the carcass and their maxima, 3.63% for PLP after 1 hr and 2.60% for PMP after 4 hr, certainly did not compare with those found in the liver and brain. In contrast, PL had a maximum of 12.93% after 1 min while PNP was 3.29%. When PNP attained a maximum percentage of 4.67 after 15 min, PL was still 10.83%; their respective levels remained substantially high for most of the experiment. In all tissues, the levels of PM were found only in trace amounts compared with the other vitamers.

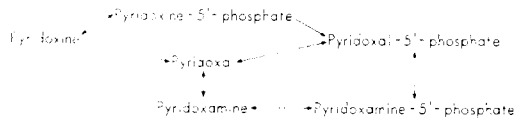

FIGURE 5: Pathway of the conversion of pyridoxine into the other forms of vitamin B_6.

Discussion

Some authors (Brain and Booth, 1964; Johansson *et al.*, 1966b) who had previously been engaged in studies with

generally tritium-labeled[2] PN reported various inconveniences that rendered this compound unsuitable for the present study. The very high specific activity of this compound may have been the cause for autoradiolysis and the consequent formation of undesired labeled impurities. In addition, two of the eight tritium atoms contained in labeled PN may be lost in the course of the metabolic conversion of this vitamer into 4-pyridoxic acid. Since the radioactivity is not equally distributed among the eight tritium atoms, errors of interpretation would certainly arise should no quantitative estimation of tritium distribution in the molecule be carried out. Finally, tritium-labeled PN as well as PL and PLP were found to readily undergo isotope exchange when subjected to high-voltage electrophoresis.[3] In view of these shortcomings, [^{14}C]PN was chosen for this study.

Labeled PN was intravenously injected in the tail of the mice in order to ensure its prompt transport and utilization in all parts of the body and also a rapid elimination of any excess, since doses of 165 μg of radioactive PN may be regarded as high. It appears, however, as if the elimination time of excess PN may be independent of or would cover broad ranges of administered doses, if one considers that in the mice receiving only 20 μg of [^{3}H]PN (Johansson *et al.*, 1968) this compound was cleared from the liver in 1 hr. This is the same time observed for the liver and brain of the present work.

The quantitation of the labeled B_6 vitamers extracted from the studied tissues posed a particularly difficult problem. In spite of the efforts of many investigators in the field of vitamin B_6, a rapid method for the extraction, simultaneous separation, and determination of all of the vitamers of the B_6 group had not been devised. Interference from coextracted biological materials and prolonged and elaborate manipulations of the tissue extracts have been common drawbacks in the analytical procedures so far developed for the determination of these compounds. This would have been particularly true in the present study which is concerned with small animals and consequently with extremely small quantities of radioactive metabolites. A convenient method was thus developed which permits the simultaneous determinations of the percentages of all radioactive B_6 vitamers extracted from the studied tissues.[4]

The results show that in the liver and brain during the first hour, radioactive PN undergoes a rapid and complete transformation into PL and PNP and then on to PLP, resembling a precursor–product relationship and indicating that the enzymes involved in these transformations have very high activities. Wada and Snell (1961) indicated that the main pathway of PLP formation from PN in animal tissue might be *via* PNP. In addition, McCormick *et al.* (1961) found that in liver, brain, kidney, and spleen, the pyridoxal phosphokinase activity was very high and was capable of phosphorylating PN, PL, and PM. On the other hand, the enzymatic oxidation of PNP to PLP was found to occur rapidly in the liver and, as Wada and Snell (1961) pointed out, this oxidative enzyme is capable of synthesizing PL from PN, although at a slow rate. Our results suggest that in the liver and brain the kinase activity responsible for the transformation of PN to PNP and of PL to PLP is very high and that the activity responsible for the oxidation of PN to PL and of PNP to PLP is likewise high, thus indicating that the synthesis of PLP from PN occurs *via* PNP as well as *via* PL.

It is perhaps significant to note that after the first hour, when PN was no longer available in these two organs and PLP seemed to have reached a saturation point maintaining its high levels until the end of the experiment, the percentages of PL and PNP decreased at a slow rate, yet remained at appreciable levels throughout most of the experiment. Because of the high rates of conversion of PL and PNP into PLP previously observed, one would expect that, once PN had been utilized, PL and PNP would undergo rapid and complete transformation into PLP. That this is not the case might be explained by considering the carcass as the storage site for vitamin B_6 which constantly supplies those organs, areas of intense enzymatic activity requiring PLP as coenzyme, with the required amounts of vitamers necessary for maintaining the proper equilibrium between PLP and its precursors. This equilibrium is controlled by PLP which is known to inhibit the enzymatic oxidation of PNP (Wada and Snell, 1961).

The synthesis of PMP in brain and liver was found to occur at a steadily increasing rate reaching high levels similar to those of PLP between 8 and 24 hr after the injection of labeled PN. Since the synthesis of PM seemed to have been poor throughout the whole experiment, its contribution toward the formation of PMP may be considered minimal. Thus, the most logical source for this vitamer could be represented by transamination reactions, the synthesis of PMP therefore being secondary and conditioned to that of PLP.

In the carcass, the slow and steady synthesis of PLP throughout the experiment is in accord with the findings of McCormick *et al.* (1961) who reported minimal pyridoxal phosphokinase activity in muscular tissue. On the contrary, the high rate of synthesis of PL is indicative of the presence of high oxidase activity. Furthermore, the constant presence of relatively high quantities of PL and moderate percentages of PNP and the slow disappearance of PN during the 7-day period suggest that the carcass may operate as a storage site for B_6 vitamers. These would be supplied as substrate for the synthesis of PLP to organs such as the brain, liver, spleen, and kidney when repletion of this coenzyme is required.

The presence of practically equivalent quantities of PLP and PMP found 2 hr following injection of radioactive PN and thereafter, may be explained as the result of their participation in transamination reactions as coenzymes.

From the manner in which radioactive PN is transformed into the other vitamers and from their retention patterns, it appears that the formation of PLP finds the ideal milieu when both pyridoxal phosphokinase and oxidase activities are high such as in the liver and brain. On the contrary, when one of the two is low, such as in muscular tissue, the synthesis of PLP is not favored.

[2] This designation is reserved for compounds possessing a random distribution of radioactivity. Under the conditions which lead to random labeling, many potential sites for labeling usually contain little or no radioactivity.

[3] This was observed for each of the three-mentioned labeled compounds which had previously been freed from labiles and purified by ion-exchange resin. Very likely the exchange was catalyzed by the high-current flux which caused scattering of radioactivity over the entire electrophoretic plate.

[4] Complete details for the extraction, separation, and quantitation of all B_6 vitamers and other biological material by this method will appear in *Analytical Biochemistry*.

Acknowledgment

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