Chapter 6

MODELING VITAMIN B6 METABOLISM

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I. METABOLISM

A. METABOLIC PATHWAYS

Vitamin B6 is one of the most versatile enzyme cofactors. Pyridoxal phosphate-containing proteins are found in each IUB enzyme category except ligases (category 6). [Tong and Davis (1995) reported that 2-amino-3-ketobutyrate-CoA ligase is a pyridoxal phosphate enzyme. However, the

EC number for the enzyme is 2.3.1.29, which is in category 2, transferases.] The 1992 edition of Enzyme Nomenclature (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, 1992) lists almost 120 pyridoxal phosphate-containing enzymes. Zubay (1988) has suggested that vitamin B6 catalyzes the rupture of a wider variety of chemical bonds than most other cofactors. The metabolic interconversions of vitamin B6 (Fig. 1) complicate the assessment of requirements and status as well as the development of models of vitamin B6 metabolism. Therefore, we will briefly review our current understanding of the biochemical and physiological pathways before discussing the models. In studies of B6 vitamer concentrations it is essential that the tissue be homogenized in a protein denaturing solution. Otherwise, there can be rapid shifts in the distribution of vitamers, particularly in liver (Coburn *et al.*, 1988b). The metabolic sequence in liver (Colombini and McCoy, 1970; Johansson *et al.*, 1974) appears to be that pyridoxine is phosphorylated to pyridoxine 5′-phosphate (k_1) and then oxidized to pyridoxal 5′-phosphate (k_2). It can then interchange with pyridoxamine 5′-phosphate (k_3,k_{-3}). Similarly, pyridoxamine can be phosphorylated (k_4) and then equilibrated with pyridoxal 5′-phosphate (Johansson *et al.*, 1974). The rate constants (fraction/minute) proposed for these reactions in mouse liver are (Johansson *et al.*, 1974):

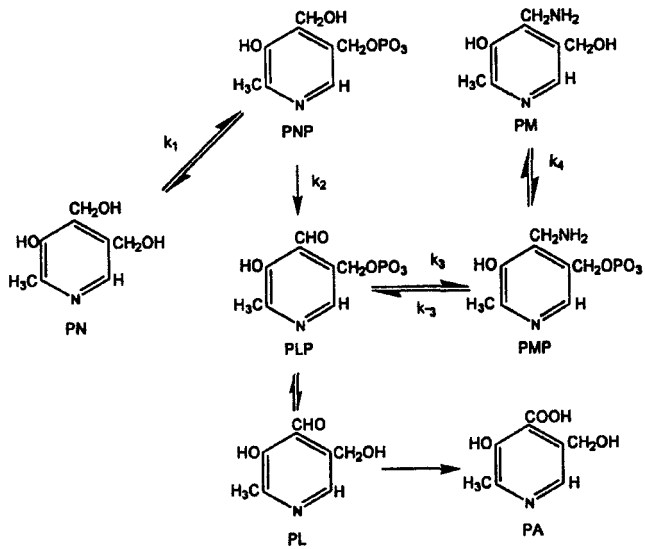

FIG. 1. Proposed major pathway of vitamin B6 metabolism. PN, pyridoxine; PNP, pyridoxine 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PA, 4-pyridoxic acid.

$k_1 = 0.07$, $k_2 = 0.11$, $k_3 = 0.03$, $k_{-3} = 0.07$ and $k_4 = 0.04$. This study did not examine the hydrolysis of pyridoxal 5′-phosphate to pyridoxal and the oxidation of pyridoxal to 4-pyridoxic acid. There was limited conversion of pyridoxine or pyridoxine 5′-phosphate to other compounds in perfused rat muscle (Buss *et al.*, 1980), raising the possibility that earlier data suggesting significant direct conversion of pyridoxine to pyridoxal in the muscle of intact mice (Colombini and McCoy, 1970) might be an artifact.

Hydrolysis of pyridoxal phosphate to pyridoxal followed by oxidation to 4-pyridoxic acid is the major catabolic pathway for vitamin B6 in most mammalian species. In cats, however, the major urinary metabolites are pyridoxine 3-sulfate and *N*-methylpyridoxine (Coburn and Mahuren, 1987). Also, in humans receiving very large vitamin B6 intakes excretion of 5-pyridoxic acid may become significant (Mahuren *et al.*, 1991).

Modeling vitamin B6 metabolism is further complicated by the fact that the activity of the kinase, oxidase, and phosphatase enzymes varies between organs and species. A very simplified diagram of vitamin B6 metabolism is shown in Fig. 2. In the intestine any phosphorylated forms are hydrolyzed. The free vitamers are readily taken up by diffusion into the intestinal wall where significant phosphorylation (Middleton, 1979) and other metabolism (Middleton, 1985) occurs. In mice small doses (up to 14 nmol) of pyridoxine (Sakurai *et al.*, 1988) and pyridoxamine (Sakurai *et al.*, 1992) were converted almost completely to pyridoxal before being released into the portal circulation. While it is clear that the intestinal microflora produce vitamin B6,

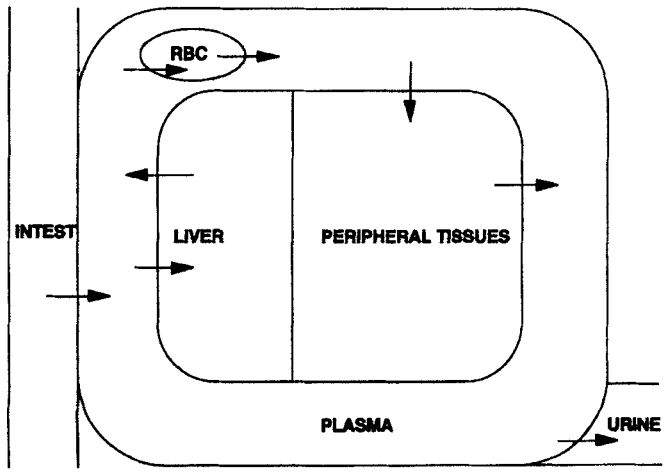

FIG. 2. Schematic illustration of whole-body metabolism of vitamin B6. For clarity, the reaction sequences within each tissue are not shown.

tracer studies found little difference in isotope dilution between conventional and germ-free rats, suggesting that animals receiving adequate vitamin B6 intake do not utilize significant amounts of microbially produced vitamin B6 (Coburn *et al.*, 1989a).

B. PLASMA

Because the polar nature of pyridoxal phosphate prevents it from readily crossing membranes, the primary source of pyridoxal phosphate in plasma appears to be secretion by the liver, presumably as a protein complex (Lumeng *et al.*, 1974). After a small oral dose the vitamer concentration in human plasma usually peaks in 1–2 hr (Benson *et al.*, 1994; Contractor and Shane, 1970). With large doses the pyridoxal phosphate concentration may not peak until much later (Ubbink *et al.*, 1987). In normal individuals plasma pyridoxal phosphate concentrations do not usually exceed 1000 nmol/liter even at high vitamin B6 intakes (Coburn *et al.*, 1983; Bhagavan *et al.*, 1975). However, they can go higher under conditions such as Down's syndrome (Coburn *et al.*, 1983; Bhagavan *et al.*, 1975) or hypophosphatasia (Whyte *et al.*, 1985) in which the regulatory processes are modified. There appears to be no limit to the concentrations of pyridoxal and pyridoxic acid in plasma (Coburn *et al.*, 1983).

The distribution volume of intravenously administered pyridoxal phosphate is about twice the plasma volume (Lumeng *et al.*, 1974). This could reflect binding to the walls of the vascular system or equilibration of protein bound pyridoxal phosphate with an interstitial pool.

Clearance of pyridoxal phosphate from plasma has been examined in the rat (Bode and van den Berg, 1991), dog (Lumeng *et al.*, 1984), pig (Coburn *et al.*, 1992a), goat (Coburn *et al.*, 1992a), and human (Lui *et al.*, 1985). Bode *et al.* (1987) found that although plasma pyridoxal phosphate kinetics in the rat followed a biexponential curve, the kinetics could be described better by a three-compartment system with a saturable reentry process than by a two-compartment open model. Perhaps more relevant than the clearance values is the amount of pyridoxal phosphate removed per unit time. Based on data from our laboratory plus other sources (Coburn *et al.*, 1984b; Coburn and Mahuren, 1983) the values were estimated to be 3.6 (human), 3.8 (pig), 5 (goat), 25 (dog), and 123 (rat) nmol/kg body wt/hr. The values for the dog are subject to considerable error because the estimated plasma pyridoxal phosphate value (Coburn *et al.*, 1984b) had a very large standard deviation, probably due to variations in vitamin B-6 intake. Therefore, the value might well be comparable to that for humans, pigs, and goats. The reason for the higher value for rats probably reflects a higher metabolic rate. Veitch *et al.* (1975) reported that perfused rat liver

released pyridoxal phosphate at a rate of 2.4 nmol/hr/g wet wt. Assuming that liver is 3% of the body wt (Coburn *et al.,* 1988b), that would amount to 72 nmol/hr/kg body wt, which is reasonably comparable to the clearance reported above. Sorrell *et al.* (1974) reported that vitamin B6 was released from perfused rat liver at a rate of only 0.1–0.2 nmol/hr/g. The lower value may be due in part to the fact that Sorrell *et al.* (1974) deliberately avoided including albumin in the perfusing medium because of its ability to complex vitamins while Veitch *et al.* (1975) did use albumin. Lack of albumin may have allowed some of the secreted pyridoxal phosphate to be removed by the liver since both groups recirculated the perfusing medium during the experiments. The role of protein binding in protecting pyridoxal phosphate from hydrolysis is well recognized (Li *et al.,* 1974; Lumeng *et al.,* 1974). The higher release value is more in line with the observed clearance. In addition, assuming that formation of new tissue requires 15 nmol vitamin B6/g (Coburn, 1990, 1994) and that rats grow about 14%/day (Lumeng *et al.,* 1978), the new tissue in a 50-g rat would require 105 nmol/day. If pyridoxal phosphate were the only source of vitamin B-6 and liver were about 3% of the body wt, it would have to release about 3 nmol/hr/g to meet the growth requirement. Therefore, the high release and clearance values observed in the rat appear compatible with other metabolic measures. This is another example of the marked interspecies differences in vitamin B-6 metabolism.

The importance of alkaline phosphatase in regulating pyridoxal phosphate concentrations in plasma is indicated by the decreased pyridoxal phosphate observed in liver disease characterized by increased alkaline phosphatase (Labadarios *et al.,* 1977) and the high concentrations of pyridoxal phosphate found in hypophosphatasia (low alkaline phosphatase) (Whyte *et al.,* 1985). The relative importance of pyridoxal and pyridoxal phosphate in vitamin B6 transport to peripheral tissues remains uncertain. Limited evidence in pigs and goats suggests that tissues take up approximately equal amounts of pyridoxal and pyridoxal phosphate (Coburn *et al.,* 1992a).

C. ERYTHROCYTES

The role of erythrocytes in vitamin B6 metabolism remains uncertain. Mouse and human erythrocytes have higher oxidase activity and, therefore, convert pyridoxine to pyridoxal phosphate appreciably faster than erythrocytes from rat, hamster, and rabbit (Fonda, 1988). Anemic rats showed increased urinary loss of label administered as pyridoxal, suggesting that uptake by erythrocytes may conserve pyridoxal (Ink and Henderson, 1984).

D. TISSUE DISTRIBUTION

Seven days or more after administration of labeled pyridoxine to mice (Colombini and McCoy, 1970; Dahlkvist *et al.*, 1969), rats (Coburn *et al.*, 1988b), and miniature pigs (Coburn *et al.*, 1985) 70–80% of the total label in the body was located in muscle, 10–20% in liver, and the remainder in other tissues. The percentage in the liver was highest in mice. The uptake and turnover of label were quite high in liver and low in brain (Colombini and McCoy, 1970; Dahlkvist *et al.*, 1969).

E. EXCRETION

While significant amounts of vitamin B6 appear in the feces, label from B6 vitamers is excreted almost exclusively in the urine (Cox *et al.*, 1962; Tillotson *et al.*, 1967). Pyridoxic acid is the major urinary metabolite in many species. Excretion of other vitamin B6 compounds is minimal with normal vitamin B-6 intakes but increases rapidly with larger intakes. Biliary excretion is minimal in both the rat (Lui *et al.*, 1983) and the chicken (Heard and Annison, 1986). Metabolic balance studies in humans also suggested that urinary excretion was almost the sole route of excretion (Lui *et al.*, 1985). Therefore, it appears that urinary excretion of pyridoxic acid may be a good indicator of absorbed vitamin B6. However, with natural diets urinary pyridoxic acid may account for only 50% of the vitamin B-6 intake (Lindberg *et al.*, 1983), suggesting decreased bioavailability in natural foods possibly as a result of the occurrence of glycosidic derivatives (Gregory *et al.*, 1991).

F. PREGNANCY AND LACTATION

Pregnancy and lactation pose some difficult challenges for modeling vitamin B6 metabolism. Pyridoxal phosphate concentrations in plasma decline during pregnancy. Much of this decline appears to be correlated with the increased activity of placental alkaline phosphatase. Barnard *et al.* (1987) found that pyridoxal concentrations in pregnant women increased to compensate for the decline in pyridoxal phosphate.

In the course of testing our chromatographic method for measuring vitamin B6 on a variety of samples, we happened to include goat milk, which contains a very high concentration of pyridoxal phosphate (Coburn and Mahuren, 1983). We then noticed that bovine milk contained about the same total vitamin B-6 content but had a lower concentration of pyridoxal phosphate. Further study revealed a strong inverse relationship between the pyridoxal phosphate content of milk and the alkaline phosphatase

activity in goats, cattle, swine, dogs, and rats (Coburn *et al.,* 1992b). Since it is usually assumed that free pyridoxal phosphate cannot cross membranes because it is charged, the assumption is that in these species significant amounts of pyridoxal phosphate are transported into the milk, probably bound to protein. Surprisingly, human milk fails to show any relationship between alkaline phosphatase and pyridoxal phosphate content. Both compounds are found only in small amounts in human milk. This suggests that human mammary tissue cannot transport pyridoxal phosphate efficiently. Further work is needed to clarify the mechanisms involved in these processes.

While human mammary tissue apparently has limited ability to transport pyridoxal phosphate, about 23% of the label appearing in the fetal compartment was in the form of pyridoxal phosphate when human placenta was perfused with labeled pyridoxal (Schenker *et al.,* 1992). Perfusion with pyridoxal phosphate resulted in limited transport unless the perfusate was recirculated, thus allowing hydrolysis to pyridoxal. The appearance of pyridoxal phosphate on the fetal side during perfusion with pyridoxal suggests that the pyridoxal was phosphorylated and secreted into the fetal circulation presumably bound to protein.

Another interesting aspect of vitamin B6 secretion in ruminant milk is the large quantity of vitamin B6 involved. If we assume that a cow can produce 40 kg milk/day and that the total vitamin B6 content is about 2 μmol/liter, then the total daily output of vitamin B6 would be about 80 μmol or 16 mg. Further work is needed to determine whether this large influx of vitamin B6 is uniformly distributed or selectively processed by the mammary tissue.

II. KINETICS

Our interest in vitamin B6 was stimulated by reports that vitamin B6 metabolism was altered in Down's syndrome (McCoy *et al.,* 1969). Since Down's syndrome is associated with trisomy of chromosome 21, it seemed most likely that we would be dealing with altered rates of metabolism. Therefore, we have been attempting to examine the kinetic aspects of vitamin B6 metabolism.

There have been three basic approaches to examining the kinetics of vitamin B6 metabolism. One has been to examine individual enzymes (Merrill and Henderson, 1990) or tissues (Middleton, 1985; Mehansho and Henderson, 1980; Mehansho *et al.,* 1979, 1980; Hamm *et al.,* 1979, 1980; Buss *et al.,* 1980). A second has been to examine the changes with time after administration of unlabeled (Ubbink *et al.,* 1987; Hamaker *et al.,* 1990;

Chang and Kirksey, 1990; Wozenski *et al.*, 1980; Lui *et al.*, 1985; Spannuth *et al.*, 1977; Contractor and Shane, 1970; Bode and van den Berg, 1991; Ubbink and Serfontein, 1988; Speitling *et al.*, 1990; Kant *et al.*, 1988; Bode *et al.*, 1987) or labeled vitamin B-6 (Butler *et al.*, 1985; Fonda and Eggers, 1980; Fonda *et al.*, 1980; Bode *et al.*, 1992) to intact animals or humans. In some of these cases noncompartmental analysis was used to estimate clearances and distribution volumes. A discussion of the assumptions and calculations for noncompartmental analysis is presented by Wolfe (1992). The third approach has been to gain some insights into the metabolic state of vitamin B6 through the use of compartmental analysis.

A. STUDIES USING RADIOACTIVE TRACERS

The first attempts at compartmental analysis were based on following the excretion of label in the urine after administration of a single dose of labeled pyridoxine (Johansson *et al.*, 1966a,b). The excretion curves were biphasic, suggesting that at least two pools were involved. Johansson *et al.* (1966b) proposed a two-pool model with a small, rapid turnover pool in equilibrium with a large, slow turnover pool (Fig. 3). They assumed that excretion occurred only from the small pool and that the specific activity of the urinary pyridoxic acid was equal to the specific activity of the small pool. These assumptions allowed them to make some estimates of the rate constants and pool sizes. The predicted total pool in 250-g rats after an intraperitoneal dose was about 3–5 mg (Johansson *et al.*, 1966a; Johansson and Tiselius, 1973) or 50–100 nmol/g. This is much higher than the 16 nmol/g found by direct measurement (Coburn *et al.*, 1988b). At least part of the discrepancy probably results from the fact that the line used by Johansson *et al.* to estimate the initial specific activity value appears to decline more slowly than the data. This would lead to an overestimate of the pool size.

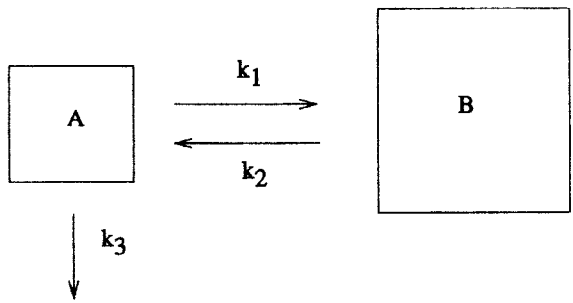

FIG. 3. Two-pool model of vitamin B6 metabolism.

Also, the isotope retention curves and estimates of the rate constants appear to be very sensitive to the dose. It is usually assumed, particularly in radioisotope studies, that the amount of tracer is much smaller than the pool size. Johansson *et al.* (1966a; Johansson and Tiselius, 1973) used doses of 5 to 200 μg of pyridoxine, which they acknowledged would constitute a significant fraction of the pool (Johansson *et al.*, 1966a). This may partially explain why the rate constant(k_3) for excretion from the small pool averages 0.60 when calculated from their data for the 20-μg dose compared with 2.3–4.1 based on the 200-μg dose (Johansson and Tiselius, 1973).

The two-pool model was also applied to humans (Johansson *et al.*, 1966b; Tillotson *et al.*, 1967; Shane, 1970). The predicted total body pools ranged from 107 to 190 μmol following oral administration of label (Johansson *et al.*, 1966b; Tillotson *et al.*, 1966) compared with 345 to 725 μmol following intravenous administration (Johansson *et al.*, 1966b; Shane, 1970). The marked influence of the route of administration results from the difference in the distribution of oral and intravenous doses. One hour after placing pyridoxine in a jejunal loop in rats 81% of the dose was recovered in the liver (Serebro *et al.*, 1966) while only about 3% of an intravenous dose in mice was found in the liver at that time (Colombini and McCoy, 1970). Similarly, in rats the half-lives for the fast and slow portions of the isotope retention curves were about 30 min and 10 hr, respectively, after an intraperitoneal dose compared with 80 min and 9 days after intramuscular administration. The difference between the oral and intravenous results reflects the importance of the intestinal tract and liver in metabolizing vitamin B6. Label administered orally or intraperitoneally enters the portal circulation where it can be taken up and oxidized by the liver. As noted above, oxidation may also occur in the intestinal wall. Only a portion of the label injected into the muscle or peripheral circulation will reach the liver on the first pass. However, the fact that even the highest predicted values for the body pool were less than the 1000 μmol which we had found through muscle biopsies (Coburn *et al.*, 1988a) suggested that the two-pool model was not completely appropriate. The underestimation of the human pool suggested the existence of a pool with a turnover too slow to be detected by the short-term, single dose protocol. Further evidence of such a pool was the inability to achieve uniform labeling of vitamin B6 pools after daily administration of labeled pyridoxine for 73 days in adult rats (Coburn *et al.*, 1989a), 120 days in guinea pigs (Coburn *et al.*, 1984a), and 230 days in miniature swine (Coburn *et al.*, 1985).

This large, slow turnover pool is most likely in muscle, which contains about 70–80% of the vitamin B6 in the body (Coburn *et al.*, 1985, 1988b). The muscle pool is associated primarily with glycogen phosphorylase (Butler *et al.*, 1985). Using pyridoxal phosphate as an indicator of glycogen phospho-

rylase turnover in muscle Butler *et al.* (1985) estimated the half-life at 11.9 days in mice. Turnover in humans consuming normal diets was estimated at 1–4% of the body pool per day (Johansson *et al.*, 1966b; Tillotson *et al.*, 1967). However, when men were restricted to a vitamin B6 intake of only 1.76 μmol/day, pyridoxic acid excretion declined to an amount approximately equal to the intake, thus establishing a new steady state with a net loss of only about 4% of the body pool and no statistically significant decline in vitamin B6 in muscle (Coburn *et al.*, 1991).

B. STUDIES USING STABLE ISOTOPE TRACERS

Although the previous tracer studies in humans utilized radioactive compounds, we felt that stable isotopes would allow us to conduct a wider variety of studies. Deuterium was chosen over carbon-13 because of its lower cost. Methods for incorporating deuterium into the metabolically stable 2-methyl and/or the 5-methylene groups were developed (Coburn *et al.*, 1982). This gave us the capability of labeling vitamin B-6 compounds with two, three, or five deuterium atoms. By selecting routes which utilized inexpensive sources of deuterium, the compounds can be synthesized at a cost for labeled materials of less than $10/g and $250/g for the D2 and D5 forms, respectively. Using these compounds demonstrated that the half-life for labeling of urinary pyridoxic acid in the subjects receiving a restricted vitamin B-6 intake was about 75 days and the half-life of the large pool was estimated at about 1700 days (Pauly *et al.*, 1991). The vitamin B-6 content of muscle in vitamin B-6 deficient rats was also conserved until the deficiency became so severe that the rats started to lose weight (Black *et al.*, 1978). The studies with ^{14}C-pyridoxine mentioned above led Johansson and Tiselius (1973) to conclude that the elimination of vitamin B6 from the body reservoir is slow and is not increased by omitting pyridoxine from the diet. Of particular interest is the observation that retention of isotope was increased to a similar degree whether rats had received a vitamin B6 deficient diet for 24 hr or 6 months prior to administration of tracer (Johansson *et al.*, 1966a). Therefore, in contrast to the common assumption that water-soluble vitamins are readily flushed from the body, vitamin B6 can be conserved during periods of low intake.

C. CALCULATION OF VITAMIN B6 REQUIREMENT FOR GROWTH

If tissues can efficiently conserve vitamin B6, we postulated that the main requirement of vitamin B6 during growth might be to supply the new tissue. As noted above, the overall body vitamin B6 content of several species

averages about 15 nmol/g. We found that multiplying 15 nmol/g times the grams of daily gain yielded a reasonable agreement with experimentally observed requirement data in species ranging from fish to man (Coburn, 1990, 1994). The major exceptions were primates, where data are limited, and prawn, where there may be significant losses due to leaching during feeding.

The calculation described above does not include any compensation for protein intake. Protein intake does influence some measures of vitamin B-6 requirement in some circumstances. However, it is interesting to note that the calculation does give good results for carnivores such as cats and fish without any adjustments for their high protein intake. We suspect that in at least some circumstances large increases in protein intake in noncarnivores may force the animal to metabolize an unusually large amount of amino acids for energy and/or other purposes, thus requiring increased activity of aminotransferases and other amino acid processing enzymes with a resulting increase in the vitamin B-6 requirement. The fact that growth in animals receiving high protein intakes is often below maximal levels even with vitamin B-6 supplementation suggests that in some cases the high protein intake significantly alters processes in addition to vitamin B-6 requirement. More study of these interactions is needed.

III. REFINING MODELS OF VITAMIN B6 KINETICS

The conservation of vitamin B6 during low intake has several consequences for modeling vitamin B6 metabolism. First, only 4% of the body pool was lost; and yet, urinary excretion of pyridoxic acid declined to about 10% of baseline values (Coburn *et al.,* 1991). It is usually assumed that pyridoxic acid arises from the oxidation of pyridoxal. While pyridoxal phosphate concentrations in plasma did decline to about 10% of baseline values, pyridoxal concentrations declined only to 50% of baseline values (Coburn *et al.,* 1991). Michaelis–Menten kinetics cannot explain such a large drop in pyridoxic acid excretion without a comparable drop in pyridoxal concentrations. However, a large drop in reaction rate with a relatively small change in total substrate could be achieved if there is binding or compartmentalization of the substrate. The role of protein binding in the metabolism of pyridoxal phosphate has long been recognized (Lumeng *et al.,* 1974). These data suggest that some type of binding may also be involved in pyridoxal metabolism.

A second consequence of these data is that the turnover of the vitamin B6 pool appears to be dependent on vitamin B6 intake. The original turnover estimates of 1%/day were obtained with normal vitamin B6 intakes, which

are about 1% of the body pool/day. A half-life of 1700 days with an intake of 1.76 μmol/day is not unreasonable when one considers that it would take at least 284 days to replace half of the 1000 μmol pool, assuming 100% retention of label.

A third consequence is the question of whether glycogen phosphorylase conserves vitamin B6 because it has a very low rate of degradation or because it recycles the pyridoxal phosphate very efficiently. Based on the rapid decay of the free vitamin B6 pool in muscle, the slow turnover of the protein-bound vitamin B6 pool, the failure to detect apo-phosphorylase, and the agreement between turnover rates based on amino acid or vitamin B6 data, Beynon *et al.* (1986) concluded that recycling would be minimal. We feel that the effect of vitamin B6 intake on turnover rates tends to favor the recycling option under conditions of limited intake. However, more data are needed before a final decision can be made.

The model of Johansson *et al.* (1966b) assumes that excretion occurs only from the small pool and that the specific activity of the urinary pyridoxic acid is equal to the specific activity of the small pool. One assumption of this model which may not be valid is that the input pool is identical to the output pool. The model also requires recycling. It could be written to exclude recycling by allowing excretion directly from the large pool in addition to excretion from the small pool. (The concentration of isotope appearing in the urine is too high to limit excretion solely to the large pool.) The disadvantage of allowing excretion from both pools is that the model becomes nonidentifiable if only urine data are available. Therefore, it is important to recognize that mathematical models are usually imperfect attempts to describe complex metabolic systems. Certain compromises may be made in order to obtain preliminary estimates of selected parameters.

A. IMPROVING THE AGREEMENT OF THE PARAMETER ESTIMATES WITH PHYSIOLOGICAL OBSERVATIONS

Experimental data never fit the model precisely. Often a variety of parameter estimates may give similarly good fits to the model and some of the predicted characteristics may be inconsistent with the physiological observations (Coburn *et al.*, 1985). In the two-pool model used by Johansson *et al.*, the urinary excretion of isotope after a single bolus will follow an equation of the general form

$$Y = Me^{-mT} + Ne^{-nT}.$$

Johansson *et al.* showed that

$$k_1 = m + n - k_2 - k_3 \text{ or}$$
$$k_1 + k_2 = m + n - k_3 \quad (1)$$

$$k_2 = mn/k_3 \text{ or}$$
$$m = k_2k_3/n. \quad (2)$$

Substituting Eq. (2) in Eq. (1) and rearranging yields

$$k_1 + k_2 = (k_2k_3 + n^2 - k_3n)/n. \quad (3)$$

The fractional turnover rate of the total pool equals $k_2k_3/(k_1 + k_2)$ or $k_3A/(A + B)$, where A and B represent the pool sizes. Therefore,

$$k_2k_3/(k_1 + k_2) = k_3A/(A + B) \text{ or}$$
$$k_2/(k_1 + k_2) = A/(A + B). \quad (4)$$

Substituting Eq. (3) for $(k_1 + k_2)$ in Eq. (4) and solving for k_2 yields

$$k_2 = (n^2A - nk_3A)/(n(A + B) - k_3A). \quad (5)$$

At the steady state the excretion, k_3A, must equal the input (I). Therefore, Eq. (5) can be written as

$$k_2 = (n^2A - nI)/(n(A + B) - I). \quad (6)$$

Equation (6) defines the fractional turnover rate(k_2) for the large pool (B) in terms of the exponent (n) for the slow phase of the curve, the small pool (A), the large pool (B), and the intake (I). In the case of normal vitamin B6 intake, 15 nmol/g times g body wt usually yields a reasonable estimate of the total pool. Since muscle usually contains 70–80% of the pool, muscle biopsies provide a means of verifying the pool size. The input and/or excretion can usually be measured. The exponent can be obtained by fitting a curve to the data. The size of pool A must be estimated. However, assuming that A is small relative to B and that n is small, variations in A have relatively little effect on the estimates of k_2. Therefore, Eq. (6) provides a means for making k_2 more consistent with physiological observations.

B. COMPARISON OF SINGLE BOLUS VS CONTINUOUS DOSING PROTOCOLS

Since we had been unable to achieve uniform labeling of vitamin B6 pools by daily administration of labeled pyridoxine to adult animals, we

started with weanling rats on the assumption that if they were fed only ^{14}C-labeled pyridoxine, by the time the rats reached adult size the vitamin B6 pools would be over 90% labeled even if none of the vitamin B6 present at weaning were lost. After 130 days the specific activity of urinary pyridoxic acid was essentially equal to the specific activity of the ingested material (Coburn *et al.*, 1988b). At that time half of the animals were continued on the same vitamin B6 intake except that it was labeled with ^{3}H instead of ^{14}C. The other half were given a single 1-μmol dose of [^{3}H]-pyridoxine followed by a daily dose of unlabeled pyridoxine. The animals were sacrificed at intervals over the following 120 days. This allowed us to determine whether the decline in the ^{3}H content of the muscle after a single dose of label accurately reflected the decline of the ^{14}C which had been incorporated over 130 days. The curves for the decline in ^{14}C, decline in ^{3}H in the single dose rats, and the rise in ^{3}H in the daily dose rats could all be fit by the same exponents, indicating that the daily dose and single dose protocols both accurately reflected the turnover of the vitamin B6 pool in muscle (Coburn *et al.*, 1989b). In addition, after the initial washout of the rapid turnover pools the decline in the specific activity of pyridoxic acid in the urine of the single dose rats was parallel to the decline of the ^{3}H content of the muscle of those rats. This suggests that a single bolus of labeled pyridoxine followed by examining urinary pyridoxic acid can be used to evaluate the turnover of vitamin B-6 in muscle, noninvasively.

C. INFLUENCE OF ALTERED METABOLIC STATES DUE TO FASTING AND MICROGRAVITY

As noted above, the body is able to conserve vitamin B-6 quite efficiently when vitamin B6 is the only limiting nutrient. What would happen during fasting? We had the opportunity to analyze plasma and urine samples collected during a 21-day fast during which volunteers consumed only water (Coburn *et al.*, 1995). Plasma pyridoxal phosphate and urinary excretion of pyridoxic acid for the fasting group were similar to the low vitamin B-6 group for about 10 days. Then the values in the fasting group tended to return to normal while those in the low vitamin B-6 group continued to decline. The most likely explanation is that as the fasting group started to lose weight vitamin B-6 was released from the muscle.

The above discussion clearly illustrates the importance of muscle in vitamin B6 metabolism under altered nutrient intake. What would happen to vitamin B6 metabolism if muscle metabolism were altered without altering vitamin B6 intake. Such circumstances exist in a microgravity environment or under conditions of prolonged bed rest. Muscle mass decreases even though nutrient intake is adequate. We had an opportunity to measure

pyridoxic acid excretion in subjects undergoing 17 weeks of bed rest (Coburn *et al.*, 1995). In this case there was lean tissue loss of about 4% but no loss of body weight (LeBlanc *et al.*, 1992). Pyridoxic acid excretion increased about 10% during the bed rest period. The loss of a higher percentage of pyridoxic acid than lean tissue raised the possibility of selective loss of vitamin B-6-containing proteins such as glycogen phosphorylase. In fact, based on fiber length glycogen phosphorylase activity did decline about 50% in fast glycolytic fibers of rats subjected to space flight compared with ground-based controls, leading Manchester *et al.* (1990) to speculate that the fibers might be shifting to a low glycogenolytic type. Although increased pyridoxic acid excretion was detected in the first week of bed rest in the 17-week study, no increase in pyridoxic acid excretion was observed in a second 14-day bed rest study (Coburn *et al.*, 1995). The reason for the discrepancy between the two studies is not known at this time but may be related to differences in vitamin B-6 intake and/or protein metabolism.

D. COMPARISONS BETWEEN METABOLISM OF PYRIDOXINE, PYRIDOXAL, AND PYRIDOXAMINE

There is little information on the relative rates of pyridoxine, pyridoxamine, and pyridoxal metabolism. Wozenski *et al.* (1980) concluded that pyridoxamine might be metabolized slower than pyridoxine. Pyridoxal may be metabolized faster than pyridoxine (Shane, 1970). Having access to three labeled forms of vitamin B6 allowed us to compare the metabolism of pyridoxine, pyridoxal, and pyridoxamine, simultaneously, in humans (Benson *et al.*, 1994). The protocol involved drinking a solution containing 5 μmol each of D2-pyridoxamine, D3-pyridoxal, and D5-pyridoxine. Urine samples were collected at intervals over the following 8 hr and the appearance of the three vitamers in pyridoxic acid was determined. About 44% of the pyridoxal dose was excreted compared with about 14% of the pyridoxine and pyridoxamine. These data have two important implications for modeling vitamin B6 in humans. First, while small doses of pyridoxine and pyridoxamine in mice (Sakurai *et al.*, 1988, 1991, 1992) and pigs (Coburn *et al.*, 1994) appear to be converted completely to pyridoxal (presumably via phosphorylation, oxidation, and hydrolysis) in the intestinal wall before entering the portal circulation, to date we have been unable to develop a model which can explain our human data simply by delaying the appearance of pyridoxine and pyridoxamine in the portal circulation as pyridoxal. If these two compounds were converted completely to pyridoxal in the intestinal wall and if pyridoxic acid is formed primarily in the liver, then pyridoxine and pyridoxamine would have to be lost to the same extent as pyridoxal

although at a slightly later time due to the extra oxidation steps. The fact that they did not follow the pyridoxal excretion curve suggests that in humans pyridoxine and pyridoxamine had an opportunity to enter pools that were not immediately equilibrated with pyridoxal. We interpret these data to suggest that pyridoxal enters the liver where much of it is immediately oxidized to pyridoxic acid while pyridoxine and pyridoxamine are phosphorylated and oxidized to pyridoxal phosphate which can enter a variety of pools and thus is somewhat protected from immediate oxidation to pyridoxic acid.

E. INCORPORATION OF PROTEIN-BINDING CALCULATIONS

The second important feature of the triple label studies is the increase in endogenous pyridoxic acid excretion from 3 to 6 hr compared with 2 to 3 hr (Fig. 4). (The larger excretions after 6 hr reflect longer collection periods.) If there is no limitation on pool sizes, increasing the pool size by adding isotope will increase total loss from the pools because labeled as well as unlabeled material is being lost. However, loss of unlabeled material will increase only if there is a limit on pool sizes. This flushing effect has been recognized since the earliest tracer studies of vitamin B6 metabolism (Brain and Booth, 1964; Johansson *et al.*, 1966b). One way to cause such

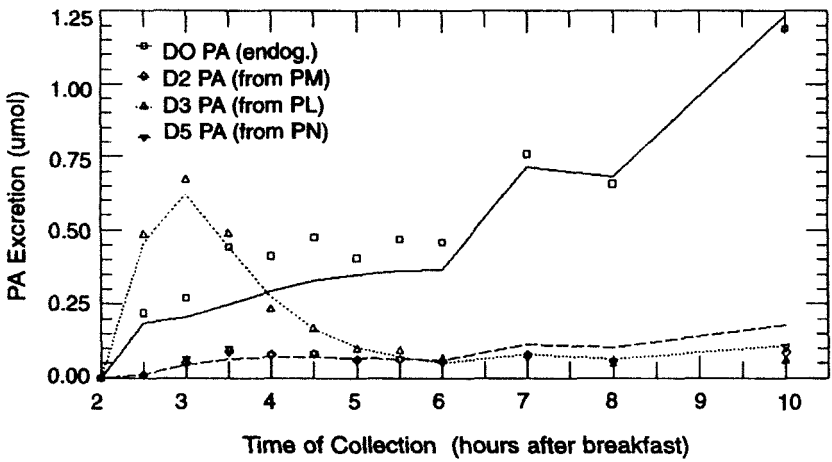

FIG. 4. Appearance of label from pyridoxine, pyridoxamine, and pyridoxal in urinary pyridoxic acid after simultaneous oral administration of 5 μmol of a deuterated form of each vitamer to a healthy man. Lines are predictions of the model described in Fig. 5 and Table I. (Analysis and figure were produced by SAAM31 from the Laboratory of Mathematical Biology, National Cancer Institute, Bethesda, MD 20892.)

an effect is through the use of binding sites. If the binding sites are normally almost filled and if losses occur only from the unbound material, then a small increase in the total pool can produce a large increase in the free pool with a consequent increase in excretion. For example, the intrinsic dissociation constant, K_i, for binding of a ligand to a protein assuming no interaction between sites is (Stenesh, 1993):

$$K_i = [\text{vacant sites}][\text{free ligand}]/[\text{bound sites}].$$

Rearranging,

$$[\text{free ligand}] = K_i\,[\text{bound sites}]/[\text{vacant sites}],$$

since

$$[\text{vacant sites}] = [\text{total sites}] - [\text{bound sites}],$$

then

$$\begin{aligned}[\text{free ligand}] &= K_i\,[\text{bound sites}]/([\text{total sites}] - [\text{bound sites}])\\ [\text{bound sites}] &= [\text{bound ligand}]\\ [\text{total ligand}] &= [\text{free ligand}] + [\text{bound ligand}].\end{aligned} \tag{7}$$

Therefore,

$$[\text{bound ligand}] = [\text{total ligand}] - [\text{free ligand}] \tag{8}$$

Substituting Eq. (8) into Eq. (7) and solving for [free ligand] yields a quadratic equation in terms of K_i, [total binding sites], and [total ligand]; all of which it may be possible to measure or estimate. The solution to the equation is

$$\begin{aligned}[\text{free ligand}] = ([\text{total ligand}] - K_i - [\text{total binding sites}]\\ \pm ((([\text{total binding sites}] + K_i - [\text{total ligand}])^{**}2)\\ + 4^{*}K_i^{*}[\text{total ligand}])^{**}0.5)/2.\end{aligned}$$

One of the simplest models which gives a reasonable approximation of our data is shown in Fig. 5. As noted above, pyridoxamine and pyridoxine must enter at a location different from pyridoxal. For simplicity, the path from pool 3 to 4 to 2 is shown as unidirectional. While the model is not identifiable from urine data alone, there are some constraints. The size of

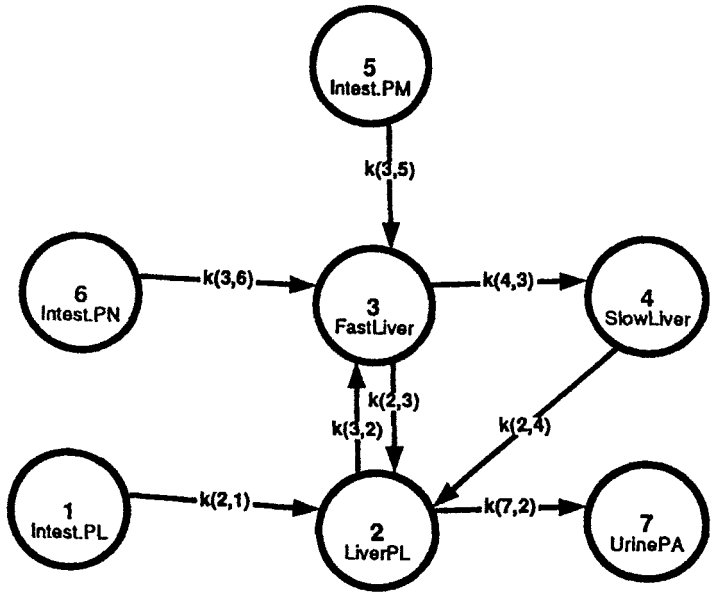

FIG. 5. Preliminary model to fit appearance of label from pyridoxal, pyridoxine, and pyridoxamine in urine as pyridoxic acid (Figure produced by SAAM II from the SAAM Institute, University of Washington, Seattle, WA 98195.)

pool 2, which is controlled by $k(7,2)$, is restricted by the highest D2/D0 ratio observed. The turnover rates and steady-state pool sizes are shown in Table I. $k(2,4)$ incorporates the binding equations described above. This requires estimates of the binding constant and the number of binding sites. A wide variety of combinations of these values will fit the model as long as the resulting flux through pool 4 is about 0.4–0.7 μmol/hr. We have arbitrarily utilized the smallest number of binding sites (30 μmol) which gave a reasonably good fit to the data (Fig. 4). The dissociation constant was 0.15. These values yield a steady-state size of 32.6 μmol for pool 4. The fact that a slow pool of 32.6 μmol is adequate to model this short-term experiment does not limit the total body pool to that size. It has proven difficult to get an optimal fit to both the exogenous and endogenous data simultaneously, probably as a result of the complexities of vitamin B6 metabolism. Our next goal is to add plasma and the large, slow turnover pool so that the model will be able to simulate both short-term and long-term metabolism as well as distinguish between oral and intravenous dosing.

As noted earlier, our data suggested that a 50% decrease in pyridoxal concentration might result in a 90% decrease in pyridoxic acid excretion (Coburn *et al.*, 1991). The pyridoxal data suggested that the maximum pool

TABLE I

PARAMETERS AND STEADY-STATE POOL SIZES FOR THE MODEL SHOWN IN FIG. 5 AS APPLIED TO THE DATA IN FIG. 4

Path	Fractional turnover rates (μmol/hr)
$k(2,1)$	1.6
$k(2,3)$	0.23
$k(2,4)$	0.21
$k(3,2)$	1.3
$k(3,6)$	0.98
$k(3.5)$	0.98
$k(4,3)$	1.3
$k(7,2)$	0.84

Steady-state pool sizes with a vitamin B6 intake of 0.357 μmol/hr

Pool	μmol
2	0.4
3	0.6
4	32.6

that ingested pyridoxal encounters before some of it is oxidized is about 2.5 μmol. These observations allow the following series of equations to be developed. Let

F_1 = [free ligand] under normal vitamin B6 intake,
B_1 = [bound sites] = [bound ligand] under normal vitamin B-6 intake,
T = [total binding sites],
K_d = dissociation constant,
F_2 = [free ligand] under low vitamin B-6 intake, and
B_2 = [bound sites] = [bound ligand] under low vitamin B6 intake.

Then,

$$F_1 = K_d B_1/(T\text{-}B_1)$$

and

$$F_2 = K_d B_2/(T\text{-}B_2).$$

Since we are assuming that low vitamin B-6 intake reduces the pyridoxal pool by half

$$F_2 + B_2 = 0.5(F_1 + B_1).$$

Since we are assuming that low B6 intake reduces free pyridoxal by 90%

$$F_2 = 0.1F_1$$

Using these equations we can solve for F_1 in terms of K_d and T yielding a quadratic equation with the solution

$$F_1 = (-0.84K_d + 0.15T \pm (((0.84K_d - 0.15T)**2) - (4*0.04*0.4*K_d*T))**0.5)/(2*0.04).$$

Assuming that K_d is 0.1 and T is 2.5, one set of solutions is

$$F_1 = 0.361 \quad B_1 = 2.215 \qquad F_2 = 0.0361 \quad B_2 = 1.252$$

These results meet the stipulation that a 50% decline in the total pool causes a 90% decline in the free ligand. These equations can easily be incorporated into the model and updated as more accurate data on binding constants and pool sizes become available.

One key question which must be considered when incorporating such equations into models is the rate of equilibration. The above equations assume instantaneous equilibration of bound and free ligand. One alternative is that equilibration requires a significant amount of time. Another possibility is that sites become available only with the turnover of protein. The latter situation would be more likely with pyridoxal phosphate than pyridoxal. If equilibration is not instantaneous, the model may have to include separate compartments for free and bound material with rate constants governing the exchange. In the case of slow equilibration, the binding rate might be set equal to X and the release rate set equal to $X*F/B$, where F is [free ligand] and B is [bound ligand] calculated as above. This assures that the relative pool sizes are compatible with the binding equation. Modeling programs such as SAAM (available from the Laboratory of Mathematical Biology, National Cancer Institute, Bethesda, MD 20892) can be written so that these values are recalculated continuously throughout the experiment to adjust for changing conditions. This allows simulation of a variety of circumstances in addition to steady-state conditions. If it is desired to limit turnover of ligand to turnover of protein, input into the bound pool can be limited to a specified number of binding sites. If it is assumed that the binding sites have first priority on the supply pool, then the rate to the bound pool could be expressed as S/P, where S is new binding sites available/unit time and P equals the size of the supply pool. If binding were less

tight, the amount bound could be calculated as described above. Again, SAAM can recalculate the rates, continuously. Therefore, a variety of techniques can be used to incorporate the effects of protein binding into models of vitamin B-6 metabolism.

A key point, particularly for modeling short time periods, is that the initial pool sizes should normally be set to steady-state conditions. If other values are chosen, upon starting the model the pools will move toward steady-state sizes, and this may complicate the interpretation of the performance of the model. Iterating to improve the fit of the model may alter the steady-state pool sizes and therefore require resetting the initial conditions. One way to establish new steady-state conditions automatically within the SAAM program is to run the model for a sufficient time to reach steady state before starting the experiment. In the case of vitamin B6 in humans, we routinely use 10,000 hr to establish a steady state. The experiment can then be started using the *QO* or *T*-interrupt procedures in the SAAM program.

IV. CONCLUSIONS

1. Models of vitamin B6 metabolism should allow for differences between oral and intravenous administration of label.
2. At least some tissues have the ability to conserve vitamin B6 during periods of low intake, making turnover dependent on intake.
3. The conservation and flushing effects observed in vitamin B6 metabolism can be simulated by binding relationships.
4. Monitoring isotope excretion in pyridoxic acid after simultaneous administration of D2-pyridoxamine, D3-pyridoxal, and D5-pyridoxine provides a practical way to examine short-term metabolism of these compounds.
5. Monitoring isotope excretion starting 2 weeks after a single, large, oral dose of label may provide information about the turnover of vitamin B-6 in slow turnover pools such as muscle.

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