Measurement of the turnover of glycogen phosphorylase by GC/MS using stable isotope derivatives of pyridoxine (vitamin B_6)

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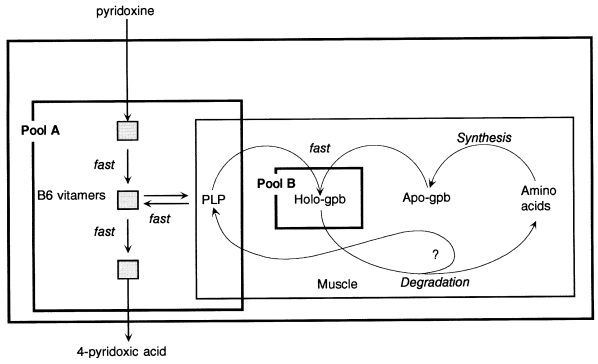
The majority of vitamin B_6 in the body is in skeletal muscle, bound as the cofactor pyridoxal 5′-phosphate to one abundant protein, glycogen phosphorylase. Previous work has established that radiolabelled vitamin B_6 can be used as a turnover label for glycogen phosphorylase. In this study, a stable isotope derivative of pyridoxine {dideuterated pyridoxine; 3-hydroxy-4-(hydroxymethyl)-5-[*hydroxymethyl*-$^2H_2$]-2-methylpyridine} ([$^2H_2$]PN) has been used as a metabolic tracer to study the kinetics of labelling of the body pools of vitamin B_6 in mice. A non-invasive method was developed in which the isotope abundance of the urinary excretory product of vitamin B_6 metabolism, 4-pyridoxic acid, was analysed by GC/MS. The change in isotope abundance of urinary 4-pyridoxic acid following administration of [$^2H_2$]PN reflects the kinetics of labelling of the body pools of vitamin B_6, and yields, non-invasively, the rate of degradation of glycogen phosphorylase.

INTRODUCTION

The vitamin B_6 complex comprises pyridoxine (PN), pyridoxal, and pyridoxamine as well as the 5′-phosphate esters of pyridoxal and pyridoxamine; the last two being the biologically active coenzyme derivatives. PN or pyridoxal 5′-phosphate (PLP) are the vitamers normally obtained in the diet and the principal excretion product of vitamin B_6 metabolism is 4-pyridoxic acid (4-PA), a metabolically irrecoverable form of vitamin B_6 excreted in the urine. Between 70 and 80 % of the vitamin B_6 in man is located in skeletal muscle [1,2], the majority of which is bound as the cofactor, PLP, to glycogen phosphorylase [3]. Since phosphorylase comprises nearly 5 % of the soluble protein in muscle [4] and muscle accounts for about 40 % of the body mass, glycogen phosphorylase is a singularly large pool of vitamin B_6 in the body [3]. Smaller pools comprise vitamers that are not complexed to protein, and the protein-bound cofactor pools (such as aminotransferases) that can exchange rapidly with the free vitamer pool.

Although vitamin B_6 metabolism is complex [5,6], the kinetics of vitamin B_6 assimilation, determined by both a single pulse dose or by long-term administration of PN, can be reconciled most simply as a two-pool model; a 'fast' pool consisting of all free forms of the vitamin and a 'slow' pool that is very resistant to exchange with exogenously added label [2,7–9]. The large, low-turnover pool (of the order of 80–90 % of the total) is in slow equilibrium with a much smaller rapid-turnover pool, such that in the short term, additions and losses only occur from the small pool. The size of the large, slowly exchanging pool suggests that it is associated with muscle glycogen phosphorylase [10]. Because muscle can neither synthesize PN nor 4-PA, this model is also the simplest that is consistent with known metabolic interconversions of the vitamin and the roles of different tissues in that process [5].

We have previously employed specific cofactor labelling techniques to measure the degradation of phosphorylase in mouse skeletal muscle. Labelling of the cofactor pool with [^{3}H]PN, a radiolabelled precursor of PLP, leads to incorporation of label in phosphorylase. PLP is buried in a hydrophobic pocket in close proximity to the phosphorylase active site [11] and resolution of the enzyme requires denaturing treatments. We have proposed that PLP is only released when the enzyme is degraded. The other, much smaller, pools of PLP are either free or bound to other enzymes weakly and reversibly. Thus, all non-phosphorylase pools are very labile, exchange rapidly with circulating vitamer and are rapidly excreted as inactive 4-PA (Scheme 1). We have demonstrated that labile pools of the label decline quickly, but the phosphorylase pool declines more slowly, at the same rate as the turnover rate of the enzyme derived from measurement



Scheme 1 A simple model of vitamin B_6 metabolism

Oral PN is rapidly converted into pyridoxal and pyridoxal phosphate. These forms of vitamin B_6 circulate in the bloodstream and equilibrate with the tightly bound muscle glycogen phosphorylase pool (B) of vitamin B_6. The exchange into and out of this 'slow' pool is dictated by the rate of turnover of glycogen phosphorylase. The rapidly exchanging pools (grouped here as pool A) comprise all forms of vitamin B_6, both free and weakly bound to enzymes such as aminotransferases. This pool provides the input to the metabolic dead-end product, 4-PA, that is excreted in the urine. The slow turnover and large size of the glycogen phosphorylase-bound pool of vitamin B_6 means that the kinetics of the slow process can be studied in isolation.

Abbreviations used: TBDMS, tert-butyldimethylsilyl; 4-PA, 4-pyridoxic acid; PN, pyridoxine; PLP, pyridoxal 5′-phosphate; SIM, selected ion monitoring.

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of the rate of synthesis by amino acid incorporation [12]. The tight association between PLP and phosphorylase allows the cofactor to be released only by proteolysis of the protein, and thus the rate of loss of phosphorylase-bound label is an index of the rate of catabolism of the enzyme. The technique has been used to measure phosphorylase degradation in both normal and atrophic mouse skeletal muscle, including C57BL/6J$^{dy/dy}$ dystrophic mice and denervation-induced muscle atrophy [13–16]. The technique has also been applied to the study of phosphorylase degradation in the pectoralis muscle of growing broiler and layer chickens [17].

Determination of phosphorylase turnover using radiolabelled precursors of PLP requires the killing of two or more animals for direct analysis of phosphorylase-bound PLP in order to obtain a single time point on the labelling curve. The use of radioisotopes, and the need for repeated muscle biopsies, have precluded direct transfer of our methodology to man. We have now extended our methodology to encompass a non-invasive method for the study of vitamin B_6 kinetics and skeletal muscle glycogen phosphorylase turnover, using a stable isotope derivative of vitamin B_6, dideuterated PN ([$^2H_2$]PN). The methodology involves urinalysis of the principal excretion product of vitamin B_6 metabolism, 4-PA, by GC/MS [18]. The use of stable isotope precursors of PLP, coupled with urinalysis, enables repeated sampling of each animal at multiple time points along the labelling curve. From a simple two-pool model of vitamin B_6 kinetics it follows that in the early phase of labelling experiments the isotope abundance of the urinary output of 4-PA will change rapidly, reflecting the isotope abundance of the fast pool. When the fast pool is mostly equilibrated, a gradual exchange into the slow phosphorylase pool will occur at a rate dictated by the degradation and concomitant re-synthesis of the enzyme. By monitoring of the isotope abundance of urinary 4-PA the kinetics of labelling of glycogen phosphorylase, and hence its turnover, can be measured by this method.

EXPERIMENTAL

Preparation of [$^2H_2$]PN

[$^2H_2$]PN was synthesized to an isotopic purity of 96 % as described previously [18,19]. Deuterium is incorporated in the 5′-methylene group of the molecule at a metabolically stable position.

Experimental animals

BALB/C male mice were purchased from Bantin and Kingman, Hull, U.K. At the start of the experiment the animals were 12 weeks old. The animals were maintained on a 12 h light/12 h dark cycle and allowed free access to water and a vitamin B_6-deficient pelleted diet (Dyets Inc., Bethlehem, PA, U.S.A.). [$^2H_2$]PN hydrochloride was dissolved in autoclaved tap water (to prevent microbial destruction of the vitamer) at 34 μM, and changed daily. This dosage (7 mg/l) was chosen to provide the same daily amount of vitamin B_6 to each animal as would have been available from a vitamin B_6-sufficient diet (7 mg/kg) based on food and water consumption measurements conducted prior to the study; it should be stressed that these animals were not vitamin B_6 deficient at any time. In a typical experiment, a group of 20 BALB/C male mice were placed on a vitamin B_6-deficient diet and the drinking water was supplemented with 7 mg/l [$^2H_2$]PN. At regular intervals over the next 50 days, urine samples were collected from some mice by gentle palpation of the bladder of unanaesthetized animals, selected at random. The urine samples were treated with 5 % (w/v) trichloroacetic acid and frozen at −20 °C overnight to precipitate mouse urinary proteins.

Isolation and derivatization of 4-PA from mouse urine

Precipitated material was pelleted by centrifugation and 80 μl of the supernatant fraction was loaded on to a 3-μm Spherisorb ODS-2 column. Fractions containing 4-PA were collected, freeze-dried and used for preparation of the tert-butyldimethylsilyl (TBDMS) ether derivative of 4-PA lactone (4-PA-TBDMS) isolated from HPLC [18].

GC/MS analysis of 4-PA

GC-MS analysis of 4-PA-TBDMS was performed on a Hewlett Packard 5890 series II gas chromatograph interfaced with an HP 5971A mass-selective detector. Derivatized samples were injected on-column using a Hewlett Packard 7673A automatic injector. The analytical column was a 12 m × 0.2 mm (internal diam.) BP-1-coated flexible fused-silica polyimide-clad capillary (immobilized dimethylpolysiloxane, 0.25 μm film thickness; S.G.E., Milton Keynes, U.K.). A 'retention gap' consisting of a length of deactivated flexible fused-silica capillary tubing (0.5 m × 0.53 mm internal diam.; Phase Separations, Deeside, U.K.) was connected between the injection port and the analytical column. Cool on-column injections were performed under the conditions described previously. The GC-MS was operated in selected-ion-monitoring (SIM) mode and ions generated by electron ionization at m/z 222 and 224 were monitored for 4-PA and dideuterated 4-PA respectively [18].

Analysis of data

The ascending and descending urinary isotope ratio data for individual animals were simultaneously fitted as a double-exponential curve using SAAM31 (Simulation, Analysis and Modelling, Laboratory of Mathematical Biology, NIH, Bethesda, MD, U.S.A.). Although vitamin B_6 metabolism is certainly more complex than implied by this simple analysis, a double-exponential equation fits the data well, and yields informative rate constants [20]. The data for the labelling phase were analysed according to the equation:

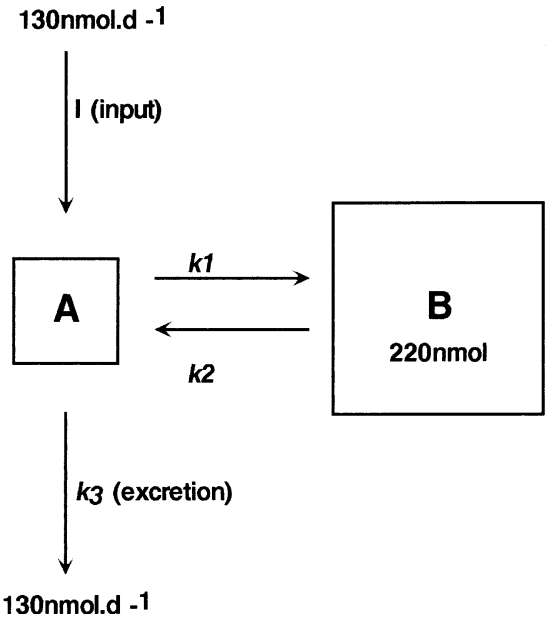
$$A_t = A_\infty \frac{P_f}{P_f + P_s} \cdot (1 - e^{-k_f \cdot t}) + A_\infty \frac{P_s}{P_f + P_s} \cdot (1 - e^{-k_s \cdot t}) \quad (1)$$

where A_t is the isotope abundance of 4-PA at time t, A_∞ is the abundance at infinity, P_f and k_f are the fractional pool size and exponent for the 'fast' component, and P_s and k_s represent the fractional pool size and exponent for the 'slow' component. For data in which labelled animals were placed on an unlabelled diet, the decay of isotope abundance in urinary 4-PA was analysed according to the equation:

$$A_t = A_0 \left(\frac{P_f}{P_f + P_s} \right) \cdot e^{-k_f \cdot t} + A_0 \left(\frac{P_s}{P_f + P_s} \right) \cdot e^{-k_s \cdot t} \quad (2)$$

where A_0 is isotope abundance at zero time; the other parameters are as defined above.

For animals in which there was adequate data (nine mice in total), the ascending and descending urinary isotope ratio data for individual animals were simultaneously fitted as a double-



Scheme 2 The kinetic model of vitamin B_6 metabolism

This scheme reflects the model for which data were analysed, and is analogous to the 'two-pool' model previously proposed. Because skeletal muscle is unable to synthesize pyridoxal phosphate from PN or to synthesize 4-PA from pyridoxal, both of which are liver functions, the model, in which input and output can only occur from the 'fast' pool A, is entirely consistent with the known metabolic capabilities of the tissues.

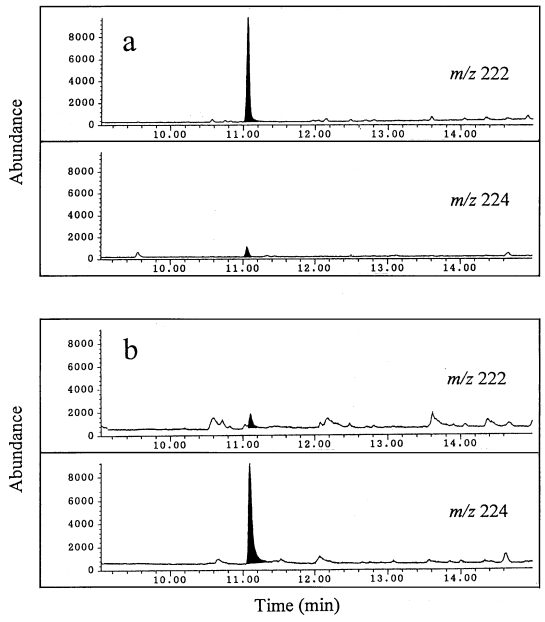


Figure 1 GC/MS analysis of urinary PA

Mice were placed on a vitamin B_6-deficient diet and administered 7 mg/l [$^2H_2$]PN in the drinking water. At time intervals, urine samples were deproteinized and separated on reversed-phase HPLC. The fraction containing PA was evaporated to dryness, derivatized with *N*-methyl-*N*-(tert-butylmethylsilyl)trifluoroacetamide and analysed by GC/MS in SIM mode, acquiring data at *m/z* 222 (TBDMS-PA lactone derivative) and *m/z* 224 (TBDMS-[$^2H_2$]PA lactone derivative). Representative data are shown from single urine samples corresponding to early and late samples with isotope abundances (224/[222 + 224]) of 0.1 (**a**) and 0.87 (**b**). The shaded peak in the GC/MS trace is TBDMS-4-PA lactone.

exponential curve using SAAM31 (Simulation, Analysis and Modelling; Laboratory of Mathematical Biology, NIH, Bethesda, MD, U.S.A.). The results from individual animals were combined using the multiple-studies procedure of Lyne et al. [21]. The equation generated from the multiple-studies analysis was used to generate isotope ratio data for estimating rate constants for a two-pool model with a small, rapid turnover pool in equilibrium with a large slow turnover pool (Scheme 2). Input and output occurred only in the fast pool. Assuming a steady state, the rate constants can be described in terms of input (I), the exponent for the slow phase of the double-exponential equation (k_s) and the pool sizes (A,B). The exponent can be obtained from the data. The input and/or excretion can be measured. The total pool can be estimated from the vitamin B_6 content of skeletal muscle. The rate constants can be described by the following expressions [6].

Excretion:

$$k_3 = I/A \quad (3)$$

Turnover of the slow pool:

$$k_2 = (k_s^2 \cdot A - k_s \cdot I)/[k_s \cdot (A+B) - I] \quad (4)$$

Transfer from small pool (A) to large pool (B):

$$k_1 = k_s \cdot B/A \quad (5)$$

RESULTS AND DISCUSSION

The TBDMS derivative of 4-PA lactone yields a predominant ion at m/z 222 (or 224 for the dideuterated product). Representative GC/MS traces in SIM mode are shown in Figure 1. The peak of material just after 11 min on the GC trace is the PA derivative, confirmed with pure samples of the derivative (results not shown). Thus, the isotope abundance of the urinary 4-PA can be assessed by monitoring at m/z 222 and m/z 224, and calculated as abundance at m/z 224/(total abundance at m/z 222 + m/z 224). Calculation of the isotope abundance ratio takes into account the small amount of unlabelled PN present in the labelled PN preparation and residual PN in the vitamin B_6-deficient diet.

Mice were tagged, placed on the [$^2H_2$]PN regime, and urine samples were obtained at random from individual animals (it was not always possible to obtain sufficient urine from the same individuals). Figure 2 (upper panel) shows the change in isotope abundance measured using the ratio of dideuterated to unlabelled PA present in the individual samples analysed by SIM GC/MS.

During the first days after animals are exposed to [$^2H_2$]PN there is a rapid change in isotope abundance in urinary 4-PA which reflects exchange into a mobile pool of vitamin B_6 that turns over very quickly. Subsequently, exchange into body pools slows as the ingested vitamer equilibrates with the large pool of vitamin B_6 that is bound to muscle glycogen phosphorylase. From the two-pool model the kinetics of vitamin B_6 assimilation and exchange can be analysed as a bi-exponential process (eqn. 1) from which the fractional pool sizes and the rate constants for exchange into both the fast and slow components can be obtained by non-linear curve-fitting (Figure 2, upper panel). The final plateau value predicted by the curve-fitting gave an isotope abundance of 0.93, a value consistent with the experimental data.

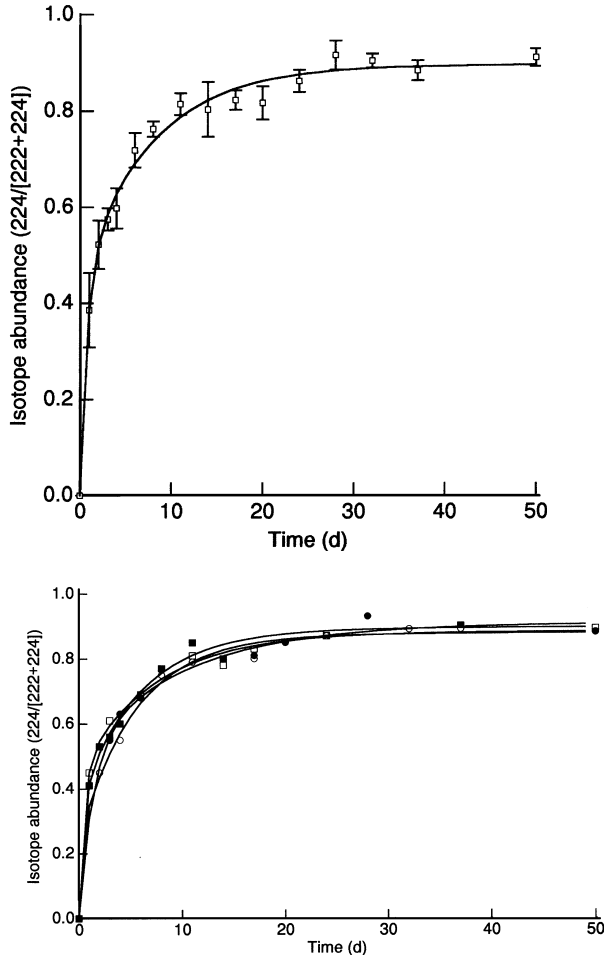


Figure 2 Exchange of [$^2H_2$]PN with body vitamin B_6 pools

Mice were placed on a vitamin B_6-deficient diet and administered 7 mg/l [$^2H_2$]PN in the drinking water. At time intervals, urine samples were analysed for the isotope abundance of urinary PA. In the upper panel, each point is the mean of between 6 and 11 individual animals (results are shown as mean ± S.D.; error bars would have been very difficult to see if presented as ± S.E.M.). The curve represents the best fit line for a bi-exponential curve fitted by non-linear curve fitting (see the text). In the lower panel, data from four representative individuals are shown, together with the best fit bi-exponential lines.

Table 1 Individual variation in vitamin B_6 exchange kinetics

Individual mice were placed on a vitamin B_6-deficient diet and administered 7 mg/l [$^2H_2$]PN in the drinking water. At different times, urine samples were analysed for the isotope abundance of urinary PA. The data were analysed by non-linear curve-fitting of a bi-exponential function (for a description of the function and definition of parameters, see the text); data are only presented for the rate of exchange (k_s) into the slow phosphorylase pool (P_s). Each analysis is presented as mean ± S.E.M. The number of data points obtained from each individual animal is given by *n*.

Animal	*n*	k_s (day^{-1})	P_s
1	11	0.14 ± 0.03	0.43 ± 0.05
2	9	0.17 ± 0.04	0.54 ± 0.06
3	8	0.10 ± 0.03	0.38 ± 0.11
4	10	0.17 ± 0.04	0.62 ± 0.12
Pooled data	135*	0.13 ± 0.03	0.45 ± 0.05

* For the whole data set, between 5 and 11 animals were sampled on each of 16 days.

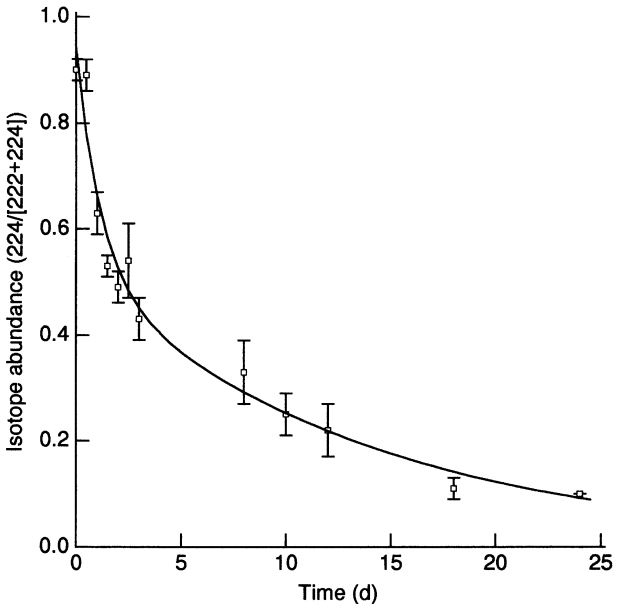


Figure 3 Decay of [$^2H_2$]PN-labelled vitamin B_6 pools

Mice were placed on a vitamin B_6-deficient diet and administered 7 mg/l [$^2H_2$]PN in the drinking water for 50 days. At the end of this period, the [$^2H_2$]PN was replaced by unlabelled PN at the same concentration. At subsequent time intervals, urine samples were analysed for the isotope abundance of urinary PA. Each point is the mean of between 3 and 7 individual animals (results are shown as mean ± S.D). The curve represented the best fit line for a bi-exponential decay curve fitted by non-linear curve-fitting.

This final value implies that the mice were receiving a small amount of unlabelled PN, either in the diet (nominally PN-deficient) or in the [$^2H_2$]PN samples (initial isotopic purity = 0.96). The labelling period, of 50 days, encompasses about 10 half-lives of the slow pool, and it is reasonable to assume that the animals were in steady state, and that the total body pools of vitamin B_6 were at the same isotope abundance as the ingested material. Even at this level of labelling, in which over 90% of the body pool of vitamin B_6 is dideuterated, we have noticed no ill effects on the animals.

Unlike our radiolabelling methods, which require killing of several animals at each time point [13–17], the new protocol permitted repeated urinalyses from the same animal. Although urine sampling was random, several individual animals generated sufficient data points to describe both fast and slow phases of exchange, and the data could be analysed from a single animal. Figure 2(lower panel) shows the data and the corresponding fitted curves from four such animals. The rapidity of exchange into the fast pool, and the difficulty associated with rapid urine sampling, precludes acquisition of large numbers of data points to define this early phase. However, each animal yielded enough data to define k_s with some accuracy (Table 1). It is interesting to note the range of values for k_s that are derived, from 0.10 to 0.17 day^{-1}. It is unclear whether this reflects the extent of biological 'between animal' variation in the rate of turnover of a single protein but this methodology should, in future, permit us to address this question. The pooled data yielded a value of k_s of 0.13 ± 0.03 day^{-1}.

A second use of the labelling method is provided when the [$^2H_2$]PN is withdrawn and replaced with unlabelled PN at the same dosage. After 50 days on [$^2H_2$]PN, when the isotope

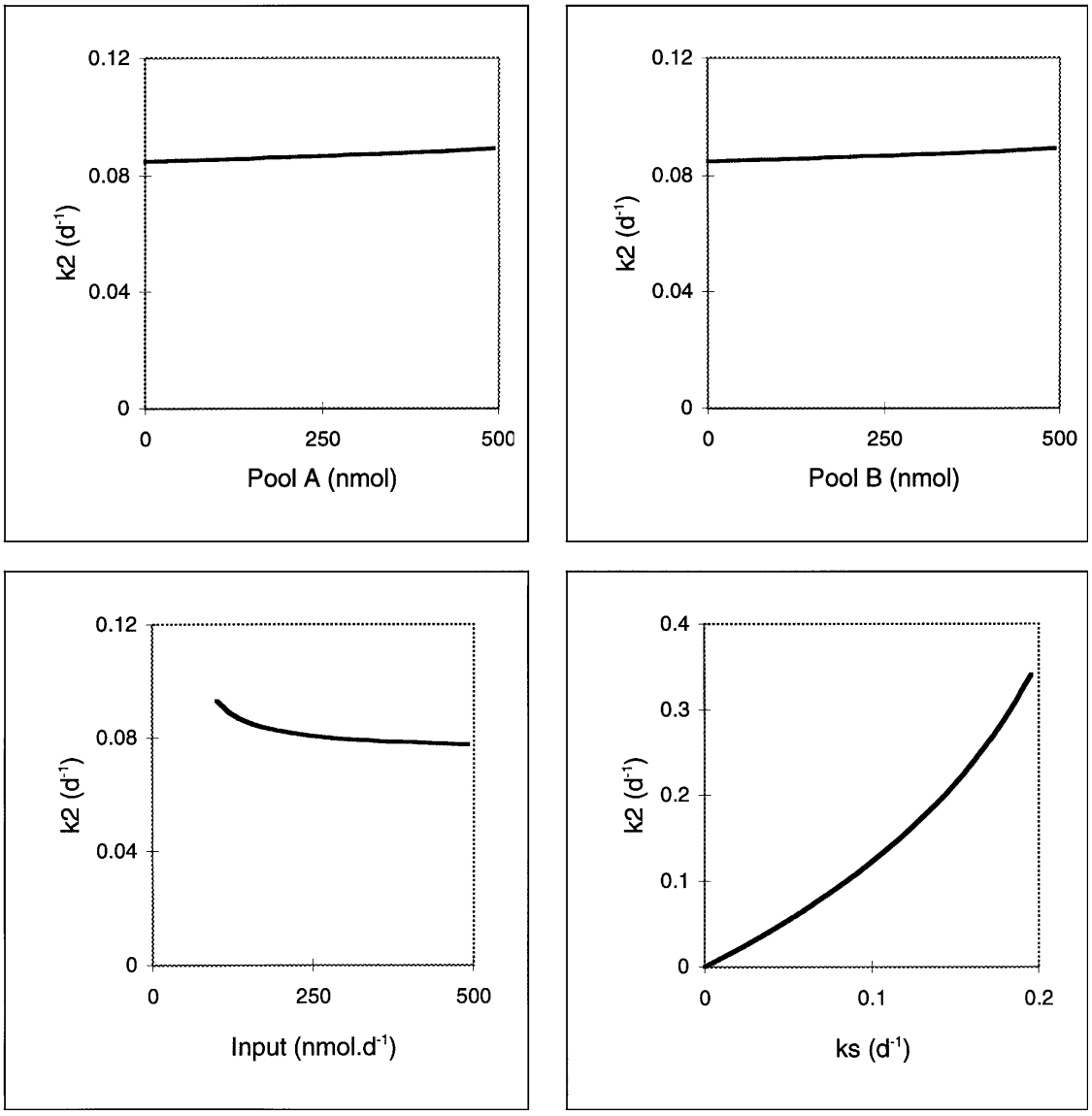


Figure 4 Parameter sensitivity of the analysis of the two-pool model

The derivation of k_2 from eqn. (4) requires knowledge of the input (I) and rapid and slow pool sizes (A and B) in addition to k_s, the exponent for the slow component of the bi-exponential analysis. To assess the sensitivity of the estimate of k_2 to the different parameters, values of k_2 were calculated for a range of values of each parameter in turn, while the other parameters were kept constant at $I = 120$ nmol·day^{-1}, $A = 200$ nmol, $B = 220$ nmol and $k_s = 0.075$ day^{-1}.

abundance of urinary PA from the animals had reached a plateau value of approx. 0.93, the drinking water was dosed with 7 mg/l unlabelled PN in place of [$^2H_2$]PN. Figure 3 shows the decline in isotope abundance over the next 25 days following withdrawal of [$^2H_2$]PN, as exchange into the body pools of vitamin B_6 occurs and the labelled vitamers are displaced. Each datum represents the mean ± S.D. of an average of between 3 and 7 animals. The data obtained for the decline in isotope abundance (A_t) after withdrawal of [$^2H_2$]PN was analysed by non-linear curve-fitting according to eqn. (2). These results indicated that the exponent for the slow component in mice had a rate constant of 0.08 day^{-1}. This value is lower than that obtained during the labelling phase, which may reflect the different time window (24 days rather than 50 days); a longer analysis period would give a more detailed definition of k_s. However, the data confirm a model in which a significant proportion of the body vitamin B_6 is inaccessible over short periods. The size of the slow compartment (55% of the total) was rather lower than expected from previous observations on the distribution of vitamin B_6 in the body in other species. This might imply that a proportion of the glycogen phosphorylase in mouse skeletal muscle is relatively labile. Of course, this labile pool would not be observed in the [G-^{3}H]PN labelling protocol that we have used previously, because after administration of isotope, animals are left for a period of 10 days to deplete all labile pools. Subsequently, measurement of the loss of label from the phosphorylase pool would only yield the rate of degradation of the most stable component. This relatively labile phosphorylase pool may, for example, represent a nascent or glycogen-free form of the protein, and deserves further investigation.

Compartmental analysis of the isotope abundance data using the SAAM31 package permits the data from the labelling and

'chase' phases to be combined. Rather than use the ascending and descending data separately, we employed the multiple-studies analysis of Lyne et al. [21] to obtain the most accurate estimate of the exponents from the animals for whom adequate data existed for both phases. Nine animals had adequate data to provide good estimates of both the ascending and descending curves. The exponent of the slow component of the curve estimated from these animals was 0.075 ± 0.01 day^{-1} (mean $\pm$ S.D.). To calculate a rate constant for turnover of the large pool using eqn. (4), estimates of input (I), and the two pool sizes (A and B) were required. Water intake by our mice was between 3.5 and 4.0 ml/day, giving a mean input of 130 nmol/day. Our own analysis of muscle PLP levels yields a value of 18 nmol/g wet weight (R. J. Beynon and D. M. Leyland, unpublished work) which compares well with other values published in the literature [22,23]. Skeletal muscle occupies approx. 40 % of body mass [24], which yields a pool size for B of 220 nmol. Finally, the pool size of A is harder to assess, but a total body pool of 15 nmol/g would yield, by calculation, an estimate of the pool size for A of about 200 nmol. It is interesting to note that in the mouse, the phosphorylase pool is not as large a fraction of the total vitamin B_6 pool as in other species, comprising approx. 50 % of the body total. In fact, this figure is totally consistent with that obtained from bi-exponential fitting of the decline in isotope abundance in the second phase of the study (Figure 3), which yielded a phosphorylase pool size of 55 % of the total body pool.

The estimate of k_2 is quite insensitive to the values of I, A and B (Figure 4), and our value of k_2 is likely to be reasonably accurate and unaffected by small errors in the calculations of pool sizes. Moreover, our analysis differs from many studies in compartmental analysis because our primary interest is in the rate constants, rather than the pool sizes. Previous studies with oral doses of radiolabelled PN severely underestimated the size of the total body pool, because the phosphorylase pool is refractory to exchange and incorporation of the label. By using more realistic pool sizes, we have derived reliable values of the turnover rates of the different pools. Using the values cited, the fractional turnover of the large pool was calculated to be 0.09 day^{-1}. This compares with rate constants for phosphorylase degradation, measured as the loss of [^{3}H]PN pulse-radiolabelled phosphorylase in intact animals, of 0.12 day^{-1} for gastrocnemius muscle [14] and 0.13 day^{-1} for total hind-leg and back muscle [15] obtained previously. However, the two-pool model requires recycling, both mathematically and physiologically. The isotope content of the phosphorylase pool would fall more slowly, yielding an observed half-life of about 9.5 days which is reasonably close to the 11.9 days obtained earlier by direct measurement in muscle.

Although vitamin B_6 metabolism is complex, and involves multiple metabolites in many different pools (see [5]), the 'two-pool' model is particularly relevant to an analysis of the behaviour of the muscle glycogen phosphorylase slow pool. The existence of the large, slowly exchanging pool of vitamin B_6 bound to glycogen phosphorylase may have important consequences for metabolism of this vitamin, particularly when addressed to man. The human daily requirement for vitamin B_6 is generally considered to be about 10 μmol, which must be seen in the context of the human muscle pool of vitamin B_6 which is of the order of 900 μmol (200 mg). However, in the adult the vitamin B_6 maintenance needs may be as low as 20 % of the 10 μmol cited above [25,26]. The most plausible explanation for this finding is that the daily requirement of vitamin B_6 has to function to replenish the 'fast' pools, but that short-term input to, or removal from, the slow phosphorylase-bound pool is not possible. During growth, however, additional PN has to be supplied to match the expansion in muscle glycogen phosphorylase.

Vitamin B_6 depletion or supplementation is unable to exert any dramatic effect on muscle glycogen phosphorylase in man [27] and it is not appropriate to consider the phosphorylase pool as a store of vitamin B_6. In growing rats, vitamin B_6 deficiency results in a decrease in muscle glycogen phosphorylase activity [28], and this effect was much less marked in adult animals. This is consistent with the expected age-dependent decline in the rate of turnover of glycogen phosphorylase and the need for incorporation of PLP into phosphorylase during muscle growth.

Skeletal muscle is not able to oxidize PN or pyridoxal to PA, and thus vitamin B_6 released from muscle phosphorylase, and subsequently from the muscle itself, should be metabolically available. Delivery of vitamin B_6 to other tissues from muscle would be dependent on the rate of turnover of the enzyme, and the efficiency of glycogen phosphorylase as a PLP 'trap'. It has been noted that, in man, at a restricted vitamin B_6 intake, the rate of exchange of the 'slow' pool with the fast and dietary/excretory pools may be lower than 0.01 day^{-1} [29]. In any 24 h period, the release of available vitamin B_6 from skeletal muscle could approximate to or exceed the lower value for a dietary need of about 2 μmol/day, although, obviously, muscle phosphorylase could not be depleted for any length of time to sustain the body requirements. However, it is possible that the muscle pool might function as a 'buffer' to suppress the effects of day-to-day variation in dietary vitamin B_6 intake.

Such a buffer role for PLP bound to glycogen phosphorylase might be of particular significance to those patients suffering from McArdle's disease, which is a lack of functional muscle glycogen phosphorylase ([30–32], for a recent review see [33]). The most common biochemical phenotype is the complete failure to express the protein in muscle [34] caused by a nonsense or frameshift mutation in exon 1 of the myophosphorylase gene [35–37]. Thus, the majority of McArdle's patients lack a putative vitamin B_6 'buffer', and may be more susceptible to variation in vitamin B_6 intake. Indeed, preliminary observations [38] have shown that a number of McArdle's patients exhibit signs of subclinical vitamin B_6 deficiency (assessed by erythrocyte aspartate aminotransferase stimulation). In the absence of glycogenolysis, McArdle's patients might be more dependent on other sources of energy for muscle contraction, including amino acids, and as a consequence, their vitamin B_6 status might be of greater significance for optimal muscle performance. Stable isotope labelling, coupled with the non-invasive methodology that we have developed, will allow us to assess vitamin B_6 kinetics in normal and McArdle's patients, and test this hypothesis directly. These experiments would also yield the rate of degradation of human muscle phosphorylase. The ability to measure the rate of degradation of a major sarcoplasmic protein, non-invasively, can also be applied to other clinical conditions, and may find application in a number of investigations into human muscle protein turnover.

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