

The ‘anomalous’ absorption of labelled and unlabelled vitamin C in man

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Previous studies of vitamin C absorption in man using stable isotope probes have given results which cannot easily be reconciled with those obtained using non-isotope measurement. In order to investigate some of the apparent paradoxes we have conducted a study using two consecutive doses of vitamin C, one labelled and one unlabelled, given 90 min apart. Compatibility of the experimental results with two feasible models was investigated. In Model 1, ingested vitamin C enters a pre-existing pool before absorption, which occurs only when a threshold is exceeded; in Model 2, ingested vitamin C is exchanged with a pre-existing flux before absorption. The key difference between these two models lies in the predicted profile of labelled material in plasma. Model 1 predicts that the second unlabelled dose will produce a secondary release of labelled vitamin C which will not be observed on the basis of Model 2. In all subjects Model 1 failed to predict the observed plasma concentration profiles for labelled and unlabelled vitamin C, but Model 2 fitted the experimental observations. We speculate on possible physiological explanations for this behaviour, but from the limited information available cannot unequivocally confirm the model structure by identifying the source of the supposed flux.

Vitamin C: Ascorbate: Absorption: Human subjects

Vitamin C requirements vary between individuals, and are known to increase in disease (Som *et al.* 1981; Basu & Schorah, 1982) particularly when there is risk of tissue damage (Kallner *et al.* 1981). Furthermore, it has been suggested that the efficiency of vitamin C absorption is impaired with ageing (Davies *et al.* 1984), although these findings are controversial (Blanchard *et al.* 1990*a,b*), and there is currently no international consensus on daily requirements.

There is surprisingly little detailed information about the efficiency and kinetics of vitamin C absorption or the sizes of and rates of transfer between the pools in which it is distributed. There have been some attempts to investigate absorption kinetics by investigating the change in plasma concentration following ingestion of vitamin C (Piotrovskij *et al.* 1993; Levine *et al.* 1996); others have used radioactive labelling to follow the distribution of an oral dose (Baker *et al.* 1969, 1971; Kallner *et al.* 1979, 1981). Neither methodology is ideally suited to its purpose, the first is insensitive for intakes representative of normal foods in well-nourished people, and use of the second may be restricted by ethical considerations.

We have recently demonstrated that stable isotope-labelled vitamin C is a potentially useful probe for measurement of the absorption and distribution of vitamin C in man at the non-pharmacological level of doses of a few tens of milligrams (Bluck *et al.* 1996, 2002; Izzard *et al.* 1996; Jones *et al.* 2002; Bates *et al.* 2004). However, when comparing the results of these experiments with those using other methods there have been some elements which up to now remain unexplained. Using a three-compartment

model the apparent size of the accessible pool found using unlabelled material has been reported to be 711 (SE 119) μmol (Piotrovskij *et al.* 1993), and which is identified with the extracellular fluid. The stable isotope technique, on the other hand, gave much larger estimates of the quantity of the vitamin in the accessible pool, 3210 (SE 390) μmol (Bluck *et al.* 1996). This paradox was also apparent in later studies made in this laboratory with greater subject numbers, when, in response to a 30 mg dose of pure labelled material, the average rise in plasma vitamin C concentration measured by non-isotopic methods was found to be about 6 %, compared with only 3 % when determined from the tracer methodology (Bates *et al.* 2004). The conclusion drawn in the most recent work was that in addition to the increment in plasma vitamin C provided by the ingested tracer there was an approximately equal amount appearing simultaneously from another (presumably endogenous) source. (The use of ‘endogenous’ and ‘exogenous’ to describe vitamin C sources in man is questionable practice, since there is no synthetic pathway, and all vitamin C in the human body is, by definition, exogenous. Nevertheless the use of ‘endogenous’ to describe vitamin C existing within the body prior to the period of investigation, and ‘exogenous’ to indicate vitamin C taken up during the course of the study is a convenient shorthand.)

A second feature of the data, not previously reported, was that the tracer appeared to reach a maximum plasma concentration after about 90 min, before exhibiting a slight decline, whereas the total concentration appeared to reach a plateau at a slightly later time.

If first-order kinetics (i.e. exit flowrates proportional to quantity of material in the compartment) are applied universally then, by

Abbreviation: RMS, root mean square.

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the principle of tracer/tracee indistinguishableness there will be no difference in the behaviour of tracer and incremental tracee (i.e. tracee measured above basal values). However under other circumstances, such as flowrates independent of substrate quantity, this is no longer the case. *A priori*, we devised two simple models which might account for the observed kinetics, in the first of which ingested vitamin C was mixed with a pre-existing pool, and then passed on to the systemic circulation only when a threshold had been exceeded, whilst Model 2 again supposed that the ingested dose entered an existing pool, but that this pool had a continuous flux of endogenous material passing through it.

It is the purpose of the present study to attempt to differentiate between these two putative models as a first step in assigning physiological relevance to the observations made so far.

Methods

Vitamin C (L-ascorbic acid) doses

[1-¹³C]Vitamin C was obtained from Cambridge Isotopes Ltd (Cambridge, MA, USA). The crystalline solid was specified to be >98% isotopically pure, and was stored desiccated for in excess of 3 years under refrigerated conditions at -20°C. GC/MS analysis of the material demonstrated an isotopic purity >97%. Unlabelled vitamin C powder was obtained over the counter from the local pharmacy. Doses of labelled and unlabelled material (30mg) were administered orally dissolved in tap water (200 ml).

Subjects and study day protocol

The protocol was approved by the Cambridge Local Research Ethics Committee. Four healthy non-smoking male volunteers were recruited to the study. Potential recruits were screened by questionnaire; habitual users of vitamin C supplements, and those with clotting or bleeding disorders, were excluded.

Subjects were asked to attend the volunteer suite of Medical Research Council Human Nutrition Research (Cambridge, UK)

on the morning of the study having abstained from food or drink (other than water) since the previous evening. An indwelling forearm cannula was inserted, and two basal blood samples (5 ml) drawn 10 min apart. Five minutes after the second of these the subjects received an oral dose of the [1-¹³C]vitamin C. Blood samples (5 ml) were collected every 15 min thereafter into Sarstedt monovettes (Sarstedt Ltd, Leicester, UK) containing heparin anticoagulant. Immediately after the blood sample taken 90 min post dose a second dose of vitamin C (this time unlabelled) was given. The blood sampling continued as before for a further 90 min. During the whole of the sampling time the cannula was kept patent by flushing with heparin.

Vitamin C assays

The preparation of the samples for assays and the determination both of total vitamin C content and its isotopic composition have been described elsewhere (Bates *et al.* 2004).

Modelling

Vitamin C is taken up by two routes. The reduced form (ascorbic acid) is absorbed in the upper ileum by a saturable energy- and sodium-dependent process (Bates, 1997), whilst the oxidised form, dehydroascorbic acid, is transported by non-specific glucose (GLUT4) transporters (Liang *et al.* 2001). At low (<200 mg) intakes the former mechanism predominates, and this is conveniently described by Michaelis–Menten kinetics. It is well established that bioavailability is complete at doses of the size used (Levine *et al.* 1996), and therefore 100% of the doses were assumed to have been taken up. After absorption the vitamin is supposed to be distributed by exchange with deeper body stores. The two models used are indicated in Fig. 1 and described mathematically in the Appendix.

In practice, a single spreadsheet was used in which either *H* (threshold quantity) or *F* (flux of endogenous material passing through the site of absorption) could be set equal to zero in order to switch between models. The time courses were calculated

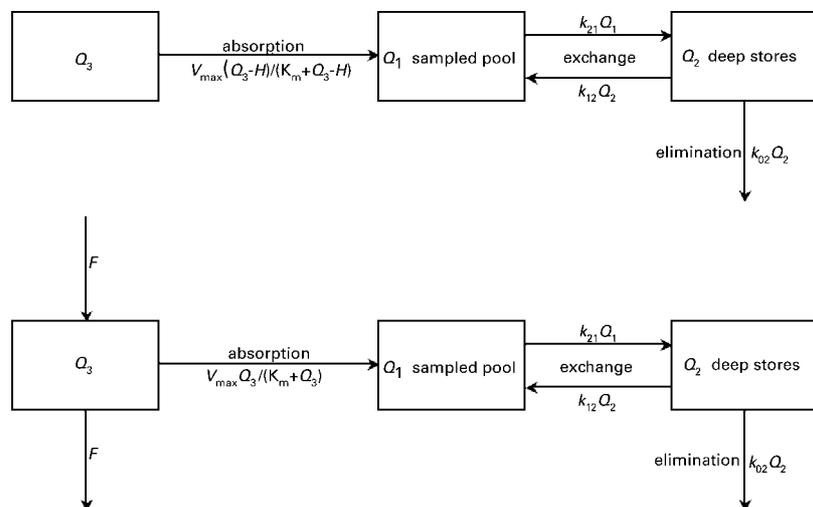


Fig. 1. The two models postulated for vitamin C kinetics in man. In Model 1 (upper) transfer of vitamin C from the site of absorption occurs only when a threshold is exceeded. In Model 2 (lower) a constant flux of endogenous vitamin C is passing through the absorption site.

iteratively using Euler's method and compared with the plasma concentrations of labelled and unlabelled material, C_1 and C_1^* scaled by the apparent distribution volume N

$$\begin{aligned} Q_1 &= C_1 N \\ Q_1^* &= C_1^* N \end{aligned} \quad (1)$$

Two of the kinetic parameters were assigned global values obtained from our previous study: therefore $k_{12} = 0.0044 \text{ min}^{-1}$ and $k_{02} = 0.0008 \text{ min}^{-1}$. These parameters describe irreversible loss and re-appearance in the accessible pool of material from deep stores and are not functions of the absorption process. In practice they are estimated largely from later periods of more prolonged experiments and our adoption of these values allowed us to truncate the blood-sampling protocol and therefore place less burden on the subjects.

Model fitting was achieved by summing the squared residuals between experimental and fitted plasma concentrations. Initially all data points were allocated equal weighting. However, once it became clear which model was better, a weighting scheme was adopted in which the isotope data were weighted more heavily than those from the total measurement by estimates of the relative precisions of the two methods.

Results

The four subjects (mean weight 87.6 kg, BMI 27.3 kg/m²) had baseline plasma vitamin C concentrations in the range 35–70 μM , mean value 52.0 μM , typical of the UK population (Ruston *et al.* 2004). The fluorometric measurements of total vitamin C were insufficiently precise compared with the increments that were being measured to allow plasma time courses for individual subjects to be followed, but as expected the average of the incremental concentrations gave a pattern that clearly exhibited the dosing regime. As a crude estimate of the response to the dose we calculated the increment in plasma concentration for the periods 0–90 min (SE 1.25) μM and 90–180 min (6.67 (SE 1.91) μM). The two values are not significantly different ($P \approx 0.5$), and indicate that absorption of the second dose is unfluenced by the first.

Assuming that the responses to the two doses have equivalent behaviour it is possible to extrapolate to the asymptote to conclude that a 170 μmol (30 mg) oral dose of vitamin C elevated the plasma concentration by 6.2 μM , indicating an approximate maximum volume of distribution of 27 litres, and hence a maximum accessible pool size of 1400 μmol .

As found previously, the increased precision of the isotope methodology allowed time courses to be followed on an individual basis if necessary. All four of the time courses obtained showed a rise in the quantity of label present in plasma that reached a maximum before 90 min had elapsed, and then a small decrease up to the end of sampling at 180 min. The maximum concentration of labelled vitamin C in the plasma was found to be in the range 1.5–3.0 μM , compared with the 6.2 μM expected by total vitamin C measurement. Subjecting the $Q_1^*(0)$, $Q_1^*(90)$ and $Q_1^*(180)$ (taken as the average concentration of plasma label between 0 and 15 min, between 75 and 105 min, and between 165 and 180 min, respectively) to ANOVA indicated a significant effect of the dosing regime ($P < 0.00005$), but this was entirely due to events associated with the first dose (i.e. comparing $Q_1^*(0)$ and $Q_1^*(90)$ and also

$Q_1^*(0)$ and $Q_1^*(180)$ gave $P < 0.005$ by Bonferroni *t*-test, but no significant difference between the later time datasets ($P \approx 0.19$). Therefore there was no detectable washout of the labelled material by the subsequent unlabelled dose. A crude power calculation indicated that this simple measure would detect a rise in plasma concentration of 0.36 μM .

In order to test which model fitted best, the population data were used for the unlabelled material and the individual data for labelled material. A 10 min delay was allowed to account for gastric emptying. When minimising unweighted residuals the root mean square (RMS) deviation of fitted to experimental values was greater for Model 1 than Model 2 (0.88 μM v. 0.63 μM); almost all of the improvement in using Model 2 was in the isotope data (RMS values 0.62 μM for Model 1 and 0.17 μM for Model 2), rather than in the total data (0.61 in both cases).

When the data were weighted according to estimated measurement precision (see Table 1) the fitted values for Model 2 were, $F = 8.1$ (SE 1.2) $\mu\text{mol/min}$, $V_{\text{max}} = 3.4$ (SE 0.9) $\mu\text{mol/min}$, $K_m = 256$ (SE 130) μmol , $k_{12} = 0.0046$ (SE 0.0017) min^{-1} and $N = 11.7$ (SE 1.5) litres, which when combined with the initial plasma concentrations gave an estimate of accessible pool size of 623 (SE 123) μmol .

Discussion

Although a simple three-compartment model appears suitable for describing the absorption and redistribution of vitamin C in man whether measured directly (Piotrovskij *et al.* 1993) or by isotope techniques (Bluck *et al.* 1996), model shortcomings become apparent when comparing results from the two methodologies. In particular, when the dose given is solely labelled material the pool size of the accessible compartment is apparently much greater when measured using isotope-specific techniques than those which do not distinguish between tracer and tracee. This implies that there is a release of unlabelled material, which is transported to the plasma concurrently with the dose given. Similar observations have recently been reported for folate absorption studies (Wright *et al.* 2003).

The behaviour is confirmed in the present study. The initial dose was of labelled material only. When analysed by an isotope non-specific method an estimate of the rise in plasma vitamin

Table 1. Parameters obtained for the four subjects from fits to Models 1 and 2

Parameter*	Subject			
	1	2	3	4
Model 1				
N (litre)	9.7	28.2	30.2	28.2
H (μmol)	573	963	600	645
V_{max} ($\mu\text{mol/min}$)	8.2	3.4	3.1	3.7
K_m (μmol)	213	12	1	15
k_{21} (min^{-1})	0.0189	0.0004	0.0002	0.0005
Model 2				
N (litre)	14.9	11.7	7.6	12.5
F ($\mu\text{mol/min}$)	5.1	9.9	10.4	7.0
V_{max} ($\mu\text{mol/min}$)	3.3	1.1	5.4	3.8
K_m (μmol)	160	21	625	218
k_{21} (min^{-1})	0.0030	0.0011	0.0091	0.0052

* For details of parameters, see Appendix.

C concentration of about $6 \mu\text{M}$ was obtained, corresponding to a 12% increase. When the same samples were analysed by GC/MS for isotope content, however, the enrichment observed indicated a rise of 3–6%. Given that the dose was isotopically pure (as confirmed by GC/MS analysis performed concurrently with the other measurements described), this can only be explained by postulating that although only labelled substrate is given, what appears in the plasma is a mixture of labelled and (presumably endogenous) unlabelled material. We could envisage two mechanisms whereby this might occur. In Model 1 the dose is mixed with a pre-existing pool, and transfer to the plasma occurs only while the amount of vitamin C in this pool exceeds a threshold. Model 2 supposes that the exogenous labelled vitamin C is continuously diluted by a flux of endogenous material either before or during absorption.

The purpose of the current study was to attempt to determine which of these two scenarios was most in concordance with experimental observations. In the first instance when absorption has been completed the putative pool with which the dose is mixed will retain a considerable fraction of the previous vitamin C intake. Future intake will similarly mix, and the vitamin C released to the plasma will contain a sizeable proportion of material from a previous dose. In Model 2 this will not be the case. The fraction of the dose not transferred to the plasma is swept away from the site of absorption, presumably to some remote store.

This contrasting behaviour was tested by the experimental design in which a double dose of vitamin C was used, the first of which was labelled and the second unlabelled. In Model 1 the appearance of labelled vitamin C in the plasma would be biphasic with the second response due to washout of material retained from the first dose in the intermediate pool by the second dose. Model 2 suggested that the labelled kinetics should represent a single absorption phase due solely to the initial dosing.

The experimental evidence is unequivocally in favour of Model 2. None of the four subjects exhibited any observable second-phase response in their isotope kinetics, and there was a reduction in the residual error between observed and fitted data of 70% when Model 2 was compared with Model 1. The clearest demonstration of the shortcomings of Model 1 is obtained by fitting the isotope data to the first 90 min only. The comparison between the predictions of the two models for one of the subjects is shown in Fig. 2.

It is unsurprising that either model fits the non-isotope data equally well, since substitution of $\tilde{Q} = Q_3 - H$ in the first three equations of model 1 (Appendix) gives equations equivalent to

those of model 2 (Appendix) and identical in form to those of the simple three-compartment model.

The size of the accessible pool obtained from Model 2, 615 (SE 123) μmol , is comparable with that obtained from the isotope non-specific data of Piotrovskij *et al.* (1993), who found approximately 700 μmol , but smaller than the average value obtained from isotope data alone of about 3200 μmol (Bluck *et al.* 1996). The accessible pool is now identified with the extracellular fluid with a mean volume of 11.7 litres and a vitamin C concentration of 52.6 μM .

The absorption kinetics found are also in broad agreement with previous investigations (Piotrovskij *et al.* 1993; Graumlich *et al.* 1997). Our data indicate a pathway with the same maximum transfer rate, but which saturates at levels of ingested vitamin C midway between the results from the other two studies. In fact the value of K_m found in the current work is almost of the same magnitude as the dose given. Under these conditions there is relatively little deviation from linear behaviour in the transport kinetics, and abandonment of the complexities of Michaelis–Menten kinetics in favour of a simple first-order process, as was done by us previously (Bluck *et al.* 1996; Izzard *et al.* 1996; Bates *et al.* 2004) is justified.

From the current data the average size of the accessible pool is estimated at 615 (SE 123) μmol , with a slowly exchanging pool of about the same size. Combined these account for 10–20% of the total vitamin C stores of the average adult (Naidu, 2003). Even allowing for underestimation due to the use of previously obtained coefficients, it is unlikely that all of the large pools of the vitamin are identifiable from the plasma kinetics alone at their current resolution. The existence of sizeable ‘hidden’ pools allows speculation on the source of the vitamin C flux envisaged to account for the observed absorption behaviour. The required flux, about 8 $\mu\text{mol}/\text{min}$, represents complete turnover of the total body stores in 14–24 h (cf. the known turnover of $< 400 \mu\text{mol}/\text{d}$; Naidu, 2003). Clearly recycling must play an important role. It is a relatively straightforward matter to include some recycling into the model. Referring to Fig. 1 (lower) the flux F leaving pool Q_3 can be fed back into that pool via another pool (not shown). The minimum size of this source pool can be estimated from the minimum detectable rise in plasma vitamin C during the second phase of the present study as approximately 1100 μmol , equivalent to 200 mg. This is somewhat greater than the size of pool associated with the extracellular fluid.

The usual approach to modelling kinetic data is, through an understanding of the physiology, to develop a model to be used

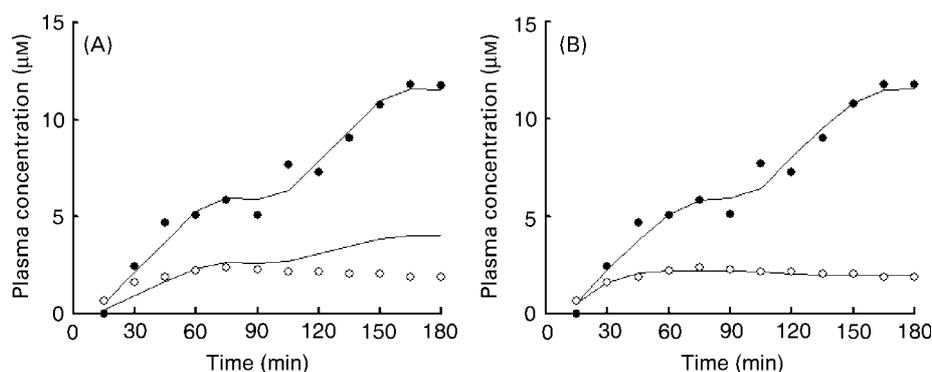


Fig. 2. Comparison of predictions from model 1(A) and model 2(B) for a typical subject. Total plasma vitamin C data (●); isotope data (○). The solid lines represent the best fit to the combined data with fitting to the isotope data restricted to the first 90 min.

to derive useful parameters by fitting data to it. The fit of data to a model is not normally regarded as proof of a model's legitimacy. In this instance, unexpected kinetic observations have forced us to invent models for their explanation and we now need to enquire if corresponding physiological mechanisms can be found. Identifying specific physiological entities capable of supplying the required flux, and compartments for storing sufficient material, is problematic. Gastric juice is known to contain relatively high concentrations of vitamin C, of the order of 250 μM (Schorah, 1992), but in order to achieve the required flux a secretion of 45 ml/min is required, a factor of 30 times higher than realistic rates. Alternatively, one possible reservoir of vitamin C with which the pool might be identified is the liver. The normal adult liver weighing 1.2–1.3 kg contains about 1350–1700 μmol vitamin C (Hornig, 1975). However, it is difficult to understand the origin of the flux, or why the system should behave more like Model 2 than Model 1.

Conclusions

We have investigated two models of the absorption of vitamin C in man in an attempt to rationalise the discrepancies found between the kinetics obtained from non-labelled and tracer techniques. The model that best agrees with experimental observation requires that a high flux of endogenous material flows through the pool from which absorption takes place. The origin of this flux has not been identified physiologically.

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Appendix

Reference to Fig. 1 which depicts the two models considered enables the equations governing the kinetics to be written.

Model 1

$$\begin{aligned} \frac{dQ_3}{dt} &= -\frac{V_{\max}}{(K_m + Q_3 - H)}(Q_3 - H) & Q_3(0) &= H + D \\ & & Q_3(90 + \delta t) &= Q_3(90) + D \\ \frac{dQ_1}{dt} &= \frac{V_{\max}}{(K_m + Q_3 - H)}(Q_3 - H) - k_{21}Q_1 + k_{12}Q_2 \\ \frac{dQ_2}{dt} &= k_{21}Q_1 - (k_{12} + k_{02})Q_2 \\ \frac{dQ_3^*}{dt} &= -\frac{V_{\max}}{(K_m + Q_3 - H)}\left(1 - \frac{H}{Q_3}\right)Q_3^* & Q_3^*(0) &= D \\ \frac{dQ_1^*}{dt} &= \frac{V_{\max}}{(K_m + Q_3 - H)}\left(1 - \frac{H}{Q_3}\right)Q_3^* - k_{21}Q_1^* + k_{12}Q_2^* \\ \frac{dQ_2^*}{dt} &= k_{21}Q_1^* - (k_{12} + k_{02})Q_2^* \end{aligned} \quad (\text{A1})$$

Model 2

$$\begin{aligned}
 \frac{dQ_3}{dt} &= -\frac{V_{\max}}{(K_m + Q_3)} Q_3 & Q_3(0) &= D \\
 & & Q_3(90 + \delta t) &= Q_3(90) + D \\
 \frac{dQ_1}{dt} &= \frac{V_{\max}}{(K_m + Q_3)} Q_3 - k_{21}Q_1 + k_{12}Q_2 \\
 \frac{dQ_2}{dt} &= k_{21}Q_1 - (k_{12} + k_{02})Q_2 \\
 \frac{dQ_3^*}{dt} &= -F\frac{Q_3^*}{Q_3} - \frac{V_{\max}}{(K_m + Q_3)} Q_3^* & Q_3^*(0) &= D \\
 \frac{dQ_1^*}{dt} &= \frac{V_{\max}}{(K_m + Q_3)} Q_3^* - k_{21}Q_1^* + k_{12}Q_2^* \\
 \frac{dQ_2^*}{dt} &= k_{21}Q_1^* - (k_{12} + k_{02})Q_2^*
 \end{aligned} \tag{A2}$$

In these equations D represents the dose given at both $t = 0$ and $t = 90$ min. Q denotes the total amount of vitamin C and Q^* the amount of labelled vitamin C in pools identified by the subscripts 1, 2, 3. k denotes a fractional rate constant for transfer of material between pools thus k_{12} indicates transfer to pool 1 from pool 2. H is a threshold quantity and F is a flux of endogenous material passing through the site of absorption. V_{\max} and K_m are the usual Michaelis–Menten parameters. The numbering of the pools has been chosen to remain consistent with previous studies (Piotrovskij *et al.* 1993; Bluck *et al.* 1996), although it should be noted that in the former paper these did not follow the conventional ordering of subscripts on rate constants.