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Further solutions to an isotope dilution model for partitioning phenylalanine and tyrosine between milk protein synthesis and other metabolic fates by the mammary gland of lactating dairy cows

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Abstract

Phenylalanine (PHE) and to a lesser extent TYR are two commonly used amino acid tracers for measuring protein metabolism in a variety of species and tissues. The model examined in this paper was developed to resolve trans-organ and stable isotope dilution data collected from experiments with lactating dairy cows using these tracers. Two methods of solving the model, i.e., as two four-pool submodels, one representing PHE and the other TYR, or as an integrated eight-pool model, are investigated and the alternative solutions are contrasted. Solving the model as the two four-pool submodels rather than the integrated 8-pool model is preferred as the equations are slightly simpler and their application less susceptible to any compounding of measurement errors. The data used to illustrate the model were taken from experiments conducted to investigate the effects of high and low protein diets on the partitioning of PHE and TYR between milk protein synthesis and other metabolic fates by the mammary gland.

Introduction

The aim of dairy researchers and producers alike is to increase the conversion efficiency of dietary nutrients into milk by dairy animals. The efficiency of converting dietary nitrogen into milk protein output is poor at 25-30% (Lobley, 2003) and in recent years has become a focus of attention, due to the increasing problem of environmental pollution related to emissions of nitrogen (N) to the environment, principally ammonia and nitrous oxide to air, nitrate to groundwater and particulate N to surface waters from dairy production systems (Dijkstra *et al.*, 2013). Milk protein content and yield can be increased by dietary protein supplementation or by gastrointestinal infusion of protein or amino acids. However, the responses attained cannot be predicted accurately using current requirement-based feeding schemes for dairy cows (e.g., Thomas, 2004), or by supply-driven response models (e.g., NASEM, 2021) in view of the large metabolic flexibility of lactating cattle to handle variation in supply of nutrients, in particular supply of amino acids (e.g., Nichols *et al*. 2019b). The synthesis of milk protein in the mammary gland requires high amounts of amino acids, either extracted from the circulation or synthesized *de novo* in the gland. To address such issues, research programmes have focused on identifying and understanding the factors and mechanisms regulating the partitioning of amino acids between milk and constitutive proteins in the mammary gland.

Much of the earlier knowledge on amino acid and protein metabolism in the lactating mammary gland was derived from the perfused mammary gland (e.g., Roets *et al.*, 1983) and from *in vivo* measurements of tissue protein synthesis (e.g., Baracos *et al.*, 1991) using dairy goats. Such studies provided information on the metabolic pathways of milk synthesis but were generally limited by single measurements. Studies of tissue protein metabolism require estimates of the rate of both protein synthesis and degradation, which are routinely estimated in different animals over dissimilar periods. To overcome these difficulties, several laboratories have developed an alternative indirect approach to repeatedly measure the partition and

63 metabolism of amino acids across tissue beds *in vivo* and estimate the rates of constitutive protein
64 turnover. The technique involves sampling the blood supplying and draining a tissue bed and
65 measuring the rate of blood flow across the tissue in combination with the unidirectional uptake
66 and release of a tracer and tracee amino acid of choice. The procedures have been applied
67 across the mammary gland of lactating goats (Oddy *et al.*, 1988; Bequette *et al.*, 2002) and dairy
68 cows (France *et al.*, 1995; Raggio *et al.*, 2006; Huang *et al.*, 2021), generally using leucine as a
69 single tracer amino acid.

70 Phenylalanine (PHE) and to a lesser extent tyrosine (TYR) have been widely used as
71 tracer amino acids, for the study of muscle protein metabolism in particular, in several species,
72 because they have minimal enrichment gradients between extracellular and intracellular
73 compartments, low endogenous turnovers and neither amino acid is synthesised or oxidised in
74 muscle. The major site of catabolism for PHE and TYR is the liver, where PHE can be
75 converted to TYR by hydroxylation and both PHE and TYR are catabolised. For the mammary
76 gland, early *in vitro* studies demonstrated significant conversion of PHE to TYR in bovine
77 secretory tissue (Jorgensen and Larson, 1968). Later *in vivo* evidence suggests hydroxylation
78 of PHE across the mammary gland in ruminants is negligible, even under conditions of
79 increased PHE supply (e.g., Bequette *et al.*, 1999); other recent *in vivo* data with dairy cattle
80 indicate the mammary gland uptake to output ratio of PHE and TYR does not differ from unity
81 at low levels of metabolizable protein supply, but is significantly higher and lower than unity,
82 respectively, at high levels of metabolizable protein supply, indicating PHE hydroxylation may
83 depend on protein supply (e.g., Nichols *et al.*, 2019a). Therefore, in the mammary gland during
84 established lactation, where the amount of secretory tissue is in equilibrium, the uptake of the
85 sum of PHE and TYR reflects their output in milk protein. That PHE and TYR appear to be
86 taken up by the mammary gland in sufficient quantities to match their requirement for milk

protein synthesis has led to the two amino acids being extensively used to estimate blood flow rate across the mammary gland by application of the Fick principle (e.g., Cant *et al.*, 1993).

The model examined herein was initially developed by Crompton *et al.* (2014) to resolve trans-organ and isotope dilution data collected from experiments with lactating dairy cows. The experiments were undertaken to investigate the effects of high and low protein diets on the partitioning of PHE and TYR between milk protein synthesis and constitutive protein synthesis. Stable isotopes were used as the tracer in these studies. The present paper describes an evolution of the work reported by Crompton *et al.* (2014) in which a second method of solving the model is proposed and the alternative solutions contrasted, and a larger data set is investigated. The model provides an effective means of generating information about the fates of phenylalanine and tyrosine in the mammary gland and could be used as part of a more complex system describing amino acid metabolism in the whole ruminant or other species.

The model

Overall scheme

The overall scheme is depicted in Fig. 1. It contains four intracellular and four extracellular pools. The intracellular pools are free PHE (pool 4), PHE in milk protein (pool 3), free TYR (pool 5) and TYR in milk protein (pool 6), while the extracellular ones represent arterial PHE and TYR (pools 1 and 2) and venous PHE and TYR (pools 7 and 8). The flows of PHE and TYR between pools and into and out of the system are shown as arrowed lines. The extracellular arterial PHE pool 1 has a single inflow: entry into the pool, F_{10}, and two outflows: uptake by the mammary gland, F_{41}, and release into the extracellular venous PHE pool, F_{71}. The extracellular arterial TYR pool 2 also has a single inflow: entry into the pool, F_{20}, and two outflows: uptake by the mammary gland, F_{52}, and release into the extracellular venous TYR pool, F_{82}. The milk protein-bound PHE pool 3 has a single inflow: from free PHE, F_{34}, and

two outflows: secretion of protein in milk, F_{03}, and degradation, F_{43}. The intracellular free PHE pool 4 has three inflows: from the degradation of constitutive mammary gland protein, F_{40}, from the extracellular arterial pool, F_{41}, and from degradation of milk protein, F_{43}. The pool has five outflows: secretion in milk, $F_{04}^{(m)}$, synthesis of constitutive mammary gland protein, $F_{04}^{(s)}$, incorporation into milk protein, F_{34}, hydroxylation to the intracellular free TYR pool, F_{54}, and outflow to the extracellular venous PHE pool, F_{74}. The intracellular free TYR pool 5 has four inflows: from the degradation of constitutive mammary gland protein, F_{50}, from the extracellular arterial TYR pool, F_{52}, from the intracellular PHE pool, F_{54}, and from the degradation of milk protein, F_{56}. The pool has five outflows: secretion in milk, $F_{05}^{(m)}$, oxidation and TYR degradation products, $F_{05}^{(o)}$, synthesis of constitutive mammary gland protein, $F_{05}^{(s)}$, incorporation into milk protein, F_{65}, and outflow to the extracellular venous TYR pool, F_{85}. The milk protein-bound TYR pool 6 has one inflow: from the intracellular free TYR pool, F_{65}, and two outflows: secretion of protein in milk, F_{06}, and degradation, F_{56}. The extracellular venous PHE pool 7 has two inflows: bypass from the arterial PHE pool, F_{71}, and release from the intracellular PHE pool, F_{74}, and one outflow out of the system, F_{07}. The extracellular venous TYR pool 8 also has two inflows: bypass from the arterial TYR pool, F_{82}, and release from the intracellular TYR pool, F_{85}, and one outflow from the system, F_{08}.

This scheme can be solved as an eight-pool model (Crompton *et al.*, 2014). Alternatively, it can also be solved by decomposing it into two four-pool schemes (i.e., a PHE sub-model and a TYR sub-model), then linking the two schemes. The PHE and TYR sub-models are both similar structurally to the model of LEU kinetics presented by France *et al.* (1995).

PHE sub-model

The schemes adopted for the movement of total and labelled PHE in the PHE sub-model are shown in Fig. 2a and Fig. 2b respectively. The fundamental equations are (mathematical notation is defined in Table 1):

$$\frac{dQ_1}{dt} = F_{10} - F_{41} - F_{71} \quad (1)$$

$$\frac{dQ_3}{dt} = F_{34} - F_{03} - F_{43} \quad (2)$$

$$\frac{dQ_4}{dt} = F_{40} + F_{41} + F_{43} - F_{04}^{(m)} - F_{04}^{(s)} - F_{34} - F_{54} - F_{74} \quad (3)$$

$$\frac{dQ_7}{dt} = F_{71} + F_{74} - F_{07} \quad (4)$$

and for [^{13}C] labelled PHE:

$$\frac{dq_1}{dt} = I_1 - e_1\left(F_{41} + F_{71}\right) \quad (5)$$

$$\frac{dq_3}{dt} = e_4 F_{34} - e_3\left(F_{03} + F_{43}\right) \quad (6)$$

$$\frac{dq_4}{dt} = e_1 F_{41} + e_3 F_{43} - e_4\left(F_{04}^{(m)} + F_{04}^{(s)} + F_{34} + F_{54} + F_{74}\right) \quad (7)$$

$$\frac{dq_7}{dt} = e_1 F_{71} + e_4 F_{74} - e_7 F_{07} \quad (8)$$

When the system is in steady state with respect to both total and labelled PHE, the derivative terms in these 8 differential equations are zero. For the scheme assumed, the enrichment of the intracellular milk protein-bound pool equalizes with that of the free pool as steady state is approached (i.e., $e_3 = e_4$). After equating intracellular enrichments and eliminating redundant equations, the four differential equations for labelled PHE, Equations (5) to (8), yield the following three identities:

$$I_1 - e_1\left(F_{41} + F_{71}\right) = 0 \quad (9)$$

$$e_1 F_{41} - e_3\left(F_{04}^{(m)} + F_{04}^{(s)} + F_{34} + F_{54} + F_{74} - F_{43}\right) = 0 \quad (10)$$

$$e_1 F_{71} + e_3 F_{74} - e_7 F_{07} = 0 \quad (11)$$

To obtain steady state solutions to this sub-model, it is assumed that free PHE in milk, PHE secreted in milk protein and PHE removal from the venous pool (i.e., $F_{04}^{(m)}$, F_{03} and F_{07}, respectively) can be measured experimentally. Algebraic manipulation of Equations (1) to (4) with the derivatives set to zero, together with Equations (9) to (11) gives:

$$F_{10} = I_1 / e_1 \quad (12)$$

$$\overline{F_{34} - F_{43}} = F_{03} \quad (13)$$

$$F_{71} = \left(\frac{e_7 - e_3}{e_1 - e_3} \right) F_{07}; \; e_1 \neq e_3 \quad (14)$$

$$F_{41} = F_{10} - F_{71} \quad (15)$$

$$F_{74} = F_{07} - F_{71} \quad (16)$$

$$F_{40} = \left(\frac{e_1 - e_3}{e_3} \right) F_{41} \quad (17)$$

$$\overline{F_{04}^{(s)} + F_{54}} = F_{40} + F_{41} - F_{04}^{(m)} - \overline{F_{34} - F_{43}} - F_{74} \quad (18)$$

where for these equations the italics denote steady state values of flows and enrichments, and the over-lining indicates coupled flows (those which cannot be determined separately by the sub-model). The net flow $\overline{F_{34} - F_{43}}$ may be uncoupled by assuming that a fixed proportion (~0.1) of the nascent milk protein is cleaved during the docking and secretory processes (Razooki Hasan *et al.*, 1982).

TYR sub-model

The schemes adopted for the movement of total and labelled TYR in the TYR sub-model are shown in Fig. 3a and Fig. 3b respectively. The fundamental equations are:

177 $$\frac{\mathrm{d}Q_2}{\mathrm{d}t} = F_{20} - F_{52} - F_{82} \qquad (19)$$

178 $$\frac{\mathrm{d}Q_5}{\mathrm{d}t} = F_{50} + F_{52} + F_{54} + F_{56} - F_{05}^{(m)} - F_{05}^{(o)} - F_{05}^{(s)} - F_{65} - F_{85} \qquad (20)$$

179 $$\frac{\mathrm{d}Q_6}{\mathrm{d}t} = F_{65} - F_{06} - F_{56} \qquad (21)$$

180 $$\frac{\mathrm{d}Q_8}{\mathrm{d}t} = F_{82} + F_{85} - F_{08} \qquad (22)$$

181 and for [^{2}H] labelled TYR:

182 $$\frac{\mathrm{d}\phi_2}{\mathrm{d}t} = \Phi_2 - \varepsilon_2 \left(F_{52} + F_{82} \right) \qquad (23)$$

183 $$\frac{\mathrm{d}\phi_5}{\mathrm{d}t} = \varepsilon_2 F_{52} + \varepsilon_6 F_{56} - \varepsilon_5 \left(F_{05}^{(m)} + F_{05}^{(o)} + F_{05}^{(s)} + F_{65} + F_{85} \right) \qquad (24)$$

184 $$\frac{\mathrm{d}\phi_6}{\mathrm{d}t} = \varepsilon_5 F_{65} - \varepsilon_6 \left(F_{06} + F_{56} \right) \qquad (25)$$

185 $$\frac{\mathrm{d}\phi_8}{\mathrm{d}t} = \varepsilon_2 F_{82} + \varepsilon_5 F_{85} - \varepsilon_8 F_{08} \qquad (26)$$

186 When the system is in steady state with respect to both total and labelled TYR, the derivative
187 terms in these 8 differential equations are zero. For the scheme assumed, the enrichment of the
188 intracellular milk protein-bound pool equalizes with that of the free pool as steady state is
189 approached (i.e., $\varepsilon_6 = \varepsilon_5$). After equating intracellular enrichments and eliminating redundant
190 equations, the four differential equations for labelled TYR, Equations (23) to (26), yield the
191 following three identities:

192 $$\Phi_2 - \varepsilon_2 \left(F_{52} + F_{82} \right) = 0 \qquad (27)$$

193 $$\varepsilon_2 F_{52} - \varepsilon_6 \left(F_{05}^{(m)} + F_{05}^{(o)} + F_{05}^{(s)} + F_{65} + F_{85} - F_{56} \right) = 0 \qquad (28)$$

194 $$\varepsilon_2 F_{82} + \varepsilon_6 F_{85} - \varepsilon_8 F_{08} = 0 \qquad (29)$$

195 To obtain steady state solutions to the sub-model, it is assumed that free TYR in milk, CO_2
196 production, TYR secreted in milk protein and TYR removal from the venous pool (i.e., $F_{05}^{(m)}$,

$F_{05}^{(o)}$, F_{06} and F_{08}, respectively) can be measured experimentally. Algebraic manipulation of Equations (19) to (22) with the derivatives set to zero, together with Equations (27) to (29), gives:

$$F_{20} = \Phi_2 / \varepsilon_2 \qquad (30)$$

$$\overline{F_{65} - F_{56}} = F_{06} \qquad (31)$$

$$F_{82} = \left(\frac{\varepsilon_8 - \varepsilon_6}{\varepsilon_2 - \varepsilon_6} \right) F_{08}; \;\; \varepsilon_2 \neq \varepsilon_6 \qquad (32)$$

$$F_{52} = F_{20} - F_{82} \qquad (33)$$

$$F_{85} = F_{08} - F_{82} \qquad (34)$$

$$\overline{F_{50} + F_{54}} = \left(\frac{\varepsilon_2 - \varepsilon_6}{\varepsilon_6} \right) F_{52} \qquad (35)$$

$$F_{05}^{(s)} = \overline{F_{50} + F_{54}} + F_{52} - F_{05}^{(m)} - F_{05}^{(o)} - \overline{F_{65} - F_{56}} - F_{85} \qquad (36)$$

where the italics denote steady state values of flows and enrichments for these equations, and over-lining indicates coupled flows (which cannot be separately estimated by the sub-model). The net flow $\overline{F_{65} - F_{56}}$ may be uncoupled as described for the uncoupling of $\overline{F_{34} - F_{43}}$.

Linking the PHE and TYR sub-models

The two sub-models can be linked by considering constitutive mammary protein. Assuming a fixed protein composition for constitutive mammary tissue, then the ratios of TYR to PHE in protein synthesised and protein degraded (μmol TYR/μmol PHE) are equal:

$$\frac{F_{50}}{F_{40}} = \frac{F_{05}^{(s)}}{F_{04}^{(s)}} \qquad (37)$$

This assumption allows Equations (18) and (35) to be uncoupled. Differencing these coupled flows:

$$\overline{F_{04}^{(s)} + F_{54}} - \overline{F_{50} + F_{54}} = F_{04}^{(s)} - F_{50} = b \qquad (38)$$

Using Equation (37) to substitute for F_{50} in the above equation:

$$F_{04}^{(s)} - \frac{F_{05}^{(s)} F_{40}}{F_{04}^{(s)}} = b$$

$$\left[F_{04}^{(s)}\right]^2 - bF_{04}^{(s)} - F_{05}^{(s)} F_{40} = 0$$

Solving this quadratic:

$$F_{04}^{(s)} = \frac{b + \sqrt{b^2 + 4F_{05}^{(s)} F_{40}}}{2} \qquad (39)$$

Note that only positive roots of this quadratic are permissible, so any negative roots must be discarded. Therefore:

$$F_{50} = F_{04}^{(s)} - b \qquad (40)$$

$$F_{54} = \overline{F_{04}^{(s)} + F_{54}} - F_{04}^{(s)} \qquad (41)$$

The overall scheme can now be solved by computing Equations (12) to (18), (30) to (36), and (38) to (41) sequentially.

Application

To illustrate application of the model, 4 datasets obtained with 3 cows were taken from an experiment conducted at our laboratories in the United Kingdom with multi-catheterised and mid-lactation cows. The data were taken from a trial using multiparous Holstein-Friesian dairy cows (average body weight 622 kg) 21 weeks into lactation with an average milk yield of 23.2 kg/d. The cows were fed hourly and ad libitum by auto-feeders at two levels of dietary crude protein (CP), based on a diet consisting of chopped Lucerne hay and grass silage [50% of diet dry matter (DM)] with the remaining 50% of diet DM provided as either a low (L, 108 g/kg) or high (H, 206 g/kg) protein concentrate. Dietary CP levels were 117 and 168 g/kg DM for L and H respectively, and so provided different levels of PHE and TYR supply to the small

intestine for absorption. The average daily intakes were 20.1 kg DM. Diets were fed for 6 weeks before the cows were given constant jugular vein infusions of sterile saline for 3 d, followed by a buffered mixture of essential amino acids for a further 3 d. The essential amino acids were administered at a daily rate equivalent to the essential amino acids in 600 g milk protein (316 g essential amino acids/d). On the final day of each 3 d infusion, the animals received a primed, constant jugular vein infusion of [1-^{13}C]PHE (350 mg/h) and [2,3,5,6-^{2}H]TYR (100 mg/h) in sterile saline for 6 h, and 6 hourly blood sample sets were taken simultaneously from catheters in the carotid artery and subcutaneous abdominal vein for the measurement of blood flow rate (by PAH dilution) and nutrient metabolism by the mammary gland. Blood samples were centrifuged at 4 ^{o}C for 20 min at 2000 *g* and the plasma stored at -20 ^{o}C for subsequent analysis.

The relevant experimental measurements are given in Table 2. They are reported for two animals during the saline infusion (1 low protein diet; 1 high protein) and two animals during the amino acid infusion (both low protein diet). Values are based on plasma rather than whole blood values. Phenylalanine and TYR measurements are based on free rather than total (i.e., free plus bound) plasma PHE and TYR. The output of PHE and TYR in milk protein were calculated using the protein content of milk and the amino acid composition of milk proteins (Maas *et al.*, 1997). The effective isotope infusion rates to the mammary gland, I_1 and Φ_2 were calculated from the arterial concentrations and enrichments of PHE and TYR and plasma flow rate across the gland. Flows F_{07} and F_{08} were determined from venous PHE and TYR concentration and plasma flow rate. Flows $F_{04}^{(m)}$ and $F_{05}^{(m)}$ were assigned a value of zero (for further justification see Mehaia and Al-Kanhal, 1992). As the enrichment of the intracellular free pool was not measured directly, e_4 was assumed to equal e_3 and ε_6 was assumed to equal ε_5 as steady state is approached, in line with other reports (e.g., Huang *et al.*, 2021). There was no detectable appearance of labelled $^{13}CO_2$ across the mammary gland,

indicating zero oxidation of TYR ($F_{05}^{(o)}$ = 0); an observation supported by the study of Lemosquet *et al.* (2010). The solutions to the split model described herein are shown in Table 3. Combining input data reported herein and those reported by Crompton *et al.* (2014) enables the solutions from the two 4-pool schemes to be contrasted with corresponding solutions obtained using the integrated 8-pool model (Crompton *et al.*, 2014). The averaged flows from both models are shown in Table 4 and highlight the unanimity of calculated flows between the two schemes. Linking the PHE and TYR sub-models affects flows $F_{04}^{(s)}$, F_{50}, and F_{54} representing constitutive protein synthesis and degradation and PHE hydroxylation, compared to the 8-pool model.

An analysis of measurement errors in experimental enrichments and infusion rates on model solutions was undertaken. Input datasets from the integrated model of Crompton *et al.* (2014) and the split model described herein were averaged to provide the initial unperturbed values for e_1, e_3, e_7, ε_2, ε_6, ε_8, I_1, Φ_2, F_{03}, F_{06}, F_{07}, F_{08}. Inputs to the split model were then perturbed sequentially by 0, ±10% and ±20%. Each calculated flow (y, μmol/min) was plotted against the perturbation (x, %), and a five-point linear regression of y on x was performed to determine the slope of the line produced. The average slope was subsequently scaled by its corresponding unperturbed average flow value, giving the dimensions of the scaled slope of % change in y per % change in x. Results of the error assessment are presented in Table 5. In general, errors in infusion rates and prescribed flows had less impact on the sensitivity of model solutions than errors in the measurement of isotopic enrichment. Perturbing all inputs caused marked changes in the flow representing PHE hydroxylation (F_{54}).

Discussion

Increasing the efficiency of conversion of feed N into milk and meat N in ruminant production is an integral part of the effort to increase global food production while decreasing agriculture's

291 environmental impact. Improving N utilization in the ruminant is dependent on a clear
292 understanding of post-absorptive amino acid metabolism. The present model describes the
293 partitioning of the indispensable amino acid PHE (and TYR) in the bovine mammary gland. It
294 was constructed to interpret isotope dilution data from *in vivo* trans-organ studies with dairy
295 cows undertaken at our laboratories. The model gave estimates of PHE flow across the
296 mammary gland, rates of PHE and TYR incorporation into constitutive and export protein
297 synthesis, and the rate of hydroxylation of PHE to TYR. The four datasets gave biologically
298 feasible solutions i.e., the computed flows were all non-negative, solutions.

299 Hepatic removal of group 1 amino acids (amino acids transferred from the mammary
300 arterial blood supply into milk in a 1:1 ratio, including PHE+TYR) affects the efficiency of
301 conversion of absorbed amino acids into milk amino acids and its associated changes in milk
302 N secretion and urine N excretion (Nichols *et al.*, 2019b). Phenylalanine and TYR net uptake
303 across the mammary gland were highest for the cows receiving the essential amino acid
304 infusion and lowest for the cow receiving saline (range 66 to 96 μmol PHE/min; 62 to 95 μmol
305 TYR/min). The ratio of mammary net uptake to milk output varied from 0.85 to 1.10 for PHE
306 and 0.82 to 0.87 for TYR. The ratio of TYR to PHE in synthesised constitutive protein and
307 degraded constitutive protein was the same in each animal (range 0.70 to 1.72) and averaged
308 1.17. The ratio of constitutive protein synthesis to degradation was 0.84 for both PHE and
309 TYR. However, in mid to late lactation dairy cows, the ratio of PHE and TYR synthesis to
310 degradation should be equal. Model estimates of intracellular PHE and TYR partitioning must
311 be interpreted with caution due to methodological limitations and imposed assumptions. The
312 rate of hydroxylation of PHE to TYR (F_{54}) was small, on average representing 4.2% of PHE
313 inflow. When the integrated model (Crompton *et al.*, 2014) was used to solve the combined
314 data, three out of the 8 datasets gave small negative values (i.e., infeasible solutions) for the

rate of PHE hydroxylation. This indicates that the compounding of measurement errors is likely to be of greater concern when using the integrated scheme as opposed to the split model.

The model is considered simple because it requires relatively few measurements. Due to this simplicity, measurements need to be accurate to avoid errors in the flow calculations. In our model, plasma is the only entity exchanging labelled and unlabelled amino acids with the mammary glands. It might be argued that whole blood would give more reliable measurements regarding amino acid uptake due to the presence of packed cells. However, studies have demonstrated that erythrocytes make only a minor contribution to amino acid uptake by the mammary gland (Mackle *et al.*, 2000), and therefore plasma isotopic enrichment should be a suitable indicator of the labelling of the mammary intracellular pool. There is evidence for ruminants that a proportion of total amino acids in circulating blood and plasma is bound in the form of peptides, and that there appears to be a removal of these by the mammary gland (Hanigan *et al.*, 1991). In the present model, any hydrolysis of peptides at the intracellular level would be masked in the estimate of the constitutive protein degradation (flows F_{40} and F_{50}). The model relies on the assumption that enrichments have reached their steady state, and 6 h constant infusions of isotopically labelled amino acids have been used by others as well (e.g., Huang *et al.*, 2021). However, the 6 h of infusion used in trials reported here might not have been sufficient to enable PHE and TYR present in milk protein to reach their true plateaux. This last hypothesis is supported by Bequette *et al.* (1999) who reported for goats that ~20 h of infusion were required for [1-^{13}C] PHE in casein to effectively reach a true plateau.

Secreted milk contains a heterogeneous mixture of proteins. In the dairy cow, casein proteins comprise approximately 80% of total milk protein whilst the remainder is made up of various whey proteins (Miller *et al.*, 1990). All casein proteins and some 70% of whey proteins are synthesized in the mammary gland. The remaining whey proteins are synthesized in the

liver, transported to the mammary gland and then secreted in milk (Larson, 1979). The present model omits this influx of preformed proteins to the mammary gland and assumes that all protein secreted in milk is synthesized in the mammary gland. Lapierre *et al.* (2012) estimated that some 3.4% of amino acids in milk protein may be contributed by blood-derived proteins. Following the synthesis of most milk proteins, a signal sequence on the newly synthesized protein is recognized by a specific recognition protein. The signal sequence and the specific recognition protein are cleaved during this process and presumably degraded intracellularly since they do not appear in secreted milk. As stated earlier, a value of 0.1 of total protein synthesis is ascribed in the present model calculations to allow for this retention and re-entry process. The process of milk protein synthesis from intracellular amino acids, and the reverse process of milk protein degradation, differ from transport of intracellular amino acids into milk to result in free amino acids in milk. The associated flows were therefore considered separately in constructing the model. However, given that amount of free amino acid in milk is generally low, for model application it is not strictly necessary to determine free PHE and TYR output in milk experimentally.

Despite its limitations, the model described provides a useful vehicle for obtaining information on the uptake and partitioning of PHE and TYR by the bovine mammary gland, indicating aspects of regulation that could be manipulated to direct more of the amino acid towards milk protein synthesis. Solving the model as the two four-pool scheme rather than integrated 8-pool form is preferred as the equations are simpler and their application less susceptible to any compounding of measurement errors.

Concluding remarks

Solving the mammary model as two four-pool submodels rather than the integrated 8-pool scheme is preferred as the equations are slightly simpler and their application less susceptible

to the compounding of measurement errors. The mammary model per se can be applied to other amino acids with similar metabolic fates within the tissue of study, thus used in research employing stable isotope techniques. In terms of practical usage, model solutions permit calculation of PHE and TYR flows for milk protein synthesis and constitutive protein turnover. The efficient use of amino acid N by dairy cows is of major practical importance given that amino acid surpluses are voided in urinary N compounds, in particular urea, which is highly susceptible to volatilization and associated environmental issues such as ammonia deposition and nitrous oxide (a greenhouse gas) formation. Furthermore, linking models describing the dynamics of several amino acids allows quantitative description of inter-organ amino acid metabolism, suggesting aspects of regulation that could be manipulated to direct more amino acids towards milk protein and therefore reduce urinary N excretion.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. All experimental procedures used were licensed, regulated, and inspected by the UK Home Office under the Animals (Scientific Procedures) Act, 1986.

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461

Table 1. Principle symbols used for the kinetic model

F_{ij}	Flow of PHE[1] or TYR[1] to pool i from j; F_{i0} denotes an external flow into pool i and F_{0j} denotes a flow from pool j out of the system	μmol/min
I_i	Effective rate of constant infusion of ^{13}C labelled PHE into primary pool i	μmol/min
Φ_i	Effective rate of constant infusion of ^{2}H labelled TYR into primary pool i	μmol/min
Q_i	Quantity of PHE[1] or TYR[1] in pool i	μmol
q_i	Quantity of ^{13}C labelled PHE in pool i	μmol
ϕ_i	Quantity of ^{2}H labelled TYR in pool i	μmol
e_i	Enrichment of ^{13}C PHE in pool i: ($= q_i/Q_i$)	molar % excess
ε_i	Enrichment of ^{2}H TYR in pool i: ($=\phi_i/Q_i$)	molar % excess
t	Time	min

[1]Total material (i.e., tracee + tracer).

467 **Table 2.** Experimental and other inputs (symbols are defined in the text and Table 1)
468

Cow		1402	6004[1]	6130	6130[1]
Dietary CP (g/kg DM)[2]		168	118	116	116
Milk yield (L/d)[2]		22.2	27.7	20.9	22.0
Plateau	e_1	4.76	3.53	6.11	4.11
Enrichment (molar % excess)	e_3	3.33	2.19	4.19	3.04
	e_7	4.51	3.36	5.98	3.95
	ε_2	1.57	1.56	2.22	2.02
	ε_6	0.96	1.02	1.29	1.32
	ε_8	1.49	1.48	2.06	1.84
Flow (μmol/min)	I_1	12.9	13.7	12.4	15.0
	Φ_2	4.12	3.80	4.09	4.54
	F_{03}	84.5	113	69.5	81.9
	F_{06}	86.6	116	71.2	83.9
	$F_{04}^{(m)}$	0	0	0	0
	$F_{05}^{(m)}$	0	0	0	0
	$F_{05}^{(o)}$	0	0	0	0
	F_{07}	190	293	136	276
	F_{08}	189	149	122	154

469
470 [1]Essential amino acid infusion
471 [2]Average over the 3-day infusion period.
472
473

Table 3. Phenylalanine and tyrosine uptake and partition by the mammary gland of four lactating dairy cows obtained using the two four-pool models (symbols are defined in the text and Table 1)

Cow	1402	6004[1]	6130	6130[1]
Flow (μmol/min)				
F_{10}	270	388	202	366
$\overline{F_{34} - F_{43}}$	84.5	113	69.5	81.9
F_{71}	157	255	127	233
F_{41}	114	133	74.8	133
F_{74}	32.8	37.5	8.88	42.9
F_{40}	48.6	81.1	34.2	46.9
F_{20}	262	244	184	225
$\overline{F_{65} - F_{56}}$	86.6	116	71.2	83.9
F_{82}	162	128	101	114
F_{52}	99.7	116	83.3	110
F_{85}	26.7	20.7	21.3	40.2
$F_{05}^{(s)}$	50.1	40.9	50.8	44.8
$F_{04}^{(s)}$	40.8	58.4	29.5	44.1
F_{50}	59.7	56.8	58.9	47.5
F_{54}	4.03	4.53	1.11	10.9

[1]Essential amino acid infusion

Table 4. Phenylalanine and tyrosine uptake and partition by the mammary gland of lactating dairy cows, obtained using the two four-pool models (symbols are defined in the text and Table 1) and the corresponding solutions obtained using the eight-pool model of Crompton *et al.* (2014). Values are means across datasets (both those reported here and those reported by Crompton *et al.* (2014)). Figures in parentheses are standard errors of the means.

Model	Two 4-pool models solution	8-pool model solution
Flow (μmol/min)		
F_{10}	276 (23.4)	276 (23.4)
$\overline{F_{34} - F_{43}}$	91.7 (4.82)	91.7 (4.82)
F_{71}	168 (17.3)	168 (17.3)
F_{41}	108 (7.12)	108 (7.12)
F_{74}	25.2 (4.11)	25.2 (4.11)
F_{40}	59.0 (6.14)	59.0 (6.14)
F_{20}	231 (11.1)	231 (11.1)
$\overline{F_{65} - F_{56}}$	93.9 (4.93)	93.9 (4.93)
F_{82}	132 (10.1)	132 (10.1)
F_{52}	98.6 (4.91)	98.6 (4.91)
F_{85}	22.1 (3.91)	22.1 (3.91)
$F_{05}^{(s)}$	53.5 (5.10)	53.5 (5.10)
$F_{04}^{(s)}$	46.3 (4.72)	46.1 (4.77)
F_{50}	67.2 (5.15)	67.1 (4.93)
F_{54}	3.79 (1.24)	3.96 (1.29)

Table 5. Average slope (%) for each of the flows calculated using the model described herein, obtained by perturbing each input in turn (symbols are defined in the text and Table 1)[1]

Flow	Unperturbed	Input perturbed[3]											
	(μmol/min)[2]	e_1	e_3	e_7	ε_2	ε_6	ε_8	I_1	Φ_2	F_{03}	F_{06}	F_{07}	F_{08}
F_{10}	280							1.0					
$\overline{F_{34}-F_{43}}$	91.7	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	1.0	0.02	0.02	0.02
F_{71}	172	-4.0	-0.29	3.1								1.0	
F_{41}	107	6.4	0.47	-5.0				2.6				-1.6	
F_{74}	24.2	28	2.1	-22								1.0	
F_{40}	58.2	7.4	-2.5	-5.0				2.6				-1.6	
F_{20}	232				0.00				1.0				
$\overline{F_{65}-F_{56}}$	93.9	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	1.0	0.02	0.02
F_{82}	133				-3.0	-0.25	2.6						1.0
F_{52}	99.1				4.0	0.33	-3.5		2.3				-1.3
F_{85}	22.4				18	1.5	-16						1.0
$F_{05}^{(s)}$	54.4	-0.04	-0.04	-0.04	7.3	-2.9	-4.7	-0.04	7.3	-0.04	-1.7	-0.04	-4.7
$F_{04}^{(s)}$	46.4	7.5	-2.8	-5.2	1.1	-0.44	-1.0	5.0	2.9	-0.82	-1.1	-3.4	-2.7
F_{50}	68.3	-1.2	0.30	0.71	6.6	-2.5	-4.4	-2.9	4.4	0.79	-0.69	2.0	-3.2
F_{54}	3.40	23	-6.0	-14	-15	5.4	14	57	-40	-16	14	-39	36

[1]The slope for each flow is expressed relative to the value of the flow obtained when no perturbation is made. Only slopes which differ from zero are shown
[2]Values calculated from the mean of inputs reported in Table 2 and inputs reported by Crompton *et al.* (2014)
[3]Model solved by perturbing each input in turn by 0%, ±10% and ±20%

Fig. 1. Scheme for the uptake and utilisation of PHE and TYR by the mammary gland of lactating dairy cows as described by Crompton *et al.* (2014). The small circles indicate flows out of the system that need to be measured experimentally.

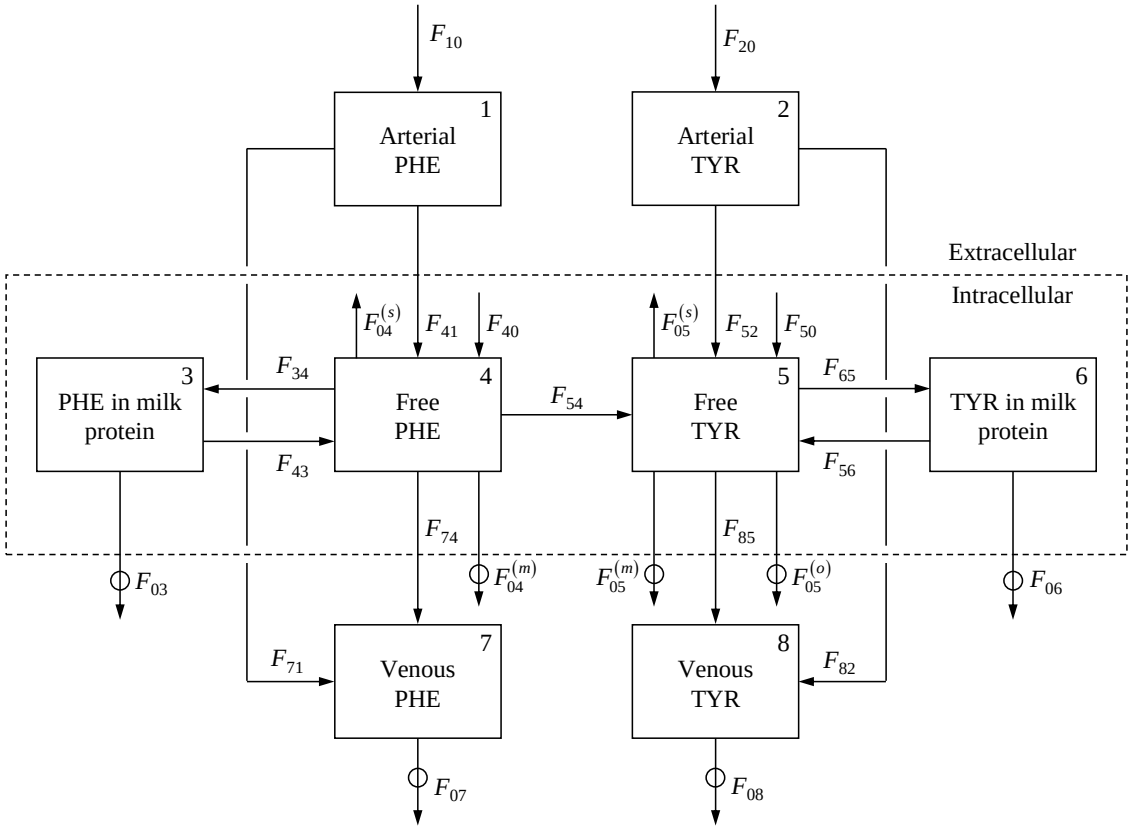
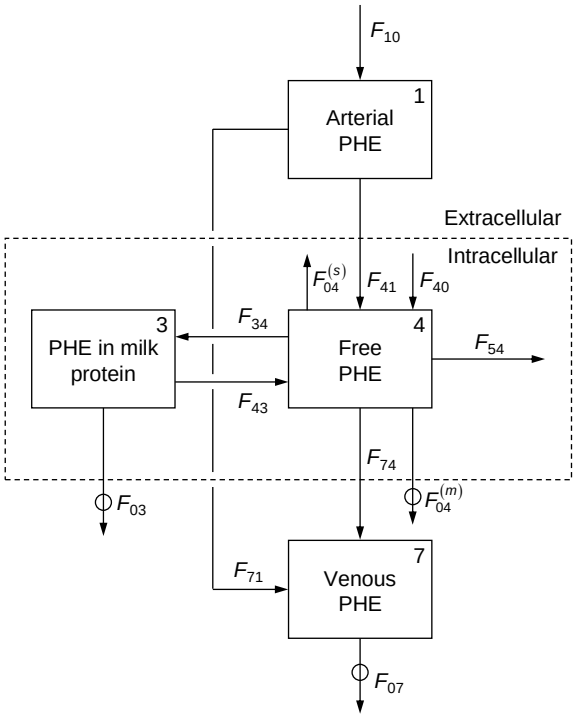


Fig. 2. Scheme for the uptake and utilisation of PHE by the mammary gland of lactating dairy cows: (a) total PHE and (b) [^{13}C] labelled PHE. The small circles in Fig. 2a indicate flows out of the system which need to be measured experimentally.

(a)



(b)

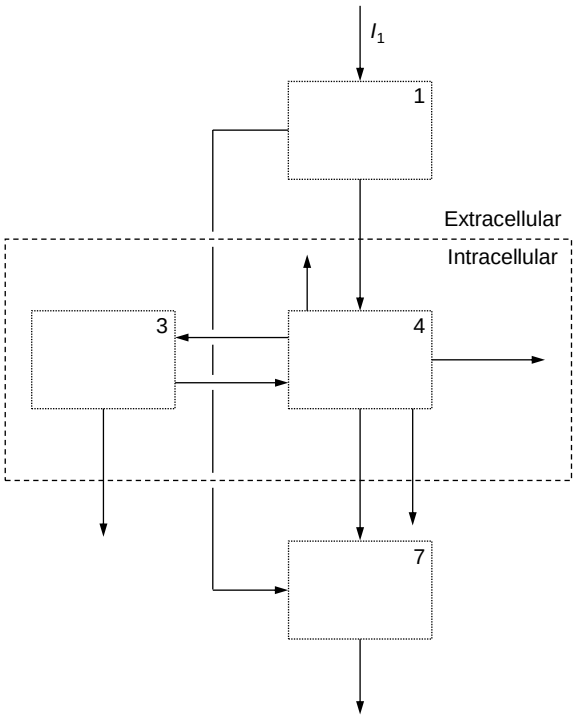
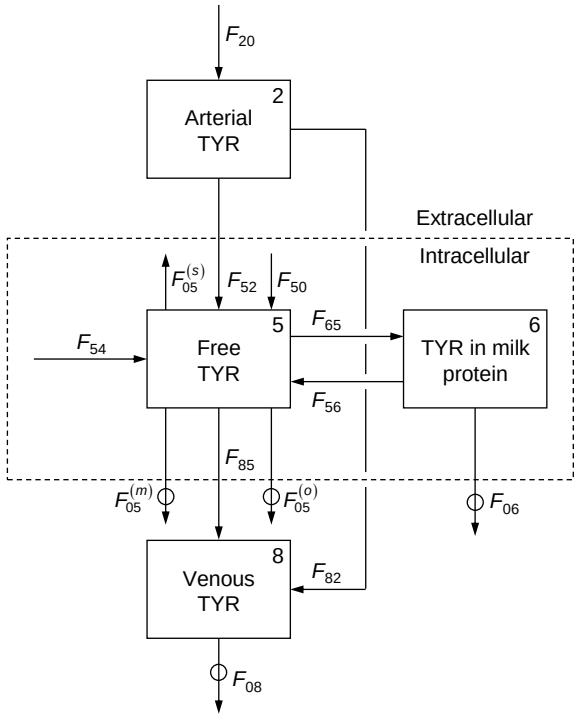


Fig. 3. Scheme for the uptake and utilisation of TYR by the mammary gland of lactating dairy cows: (a) total TYR and (b) [^{2}H] labelled TYR. The small circles in Fig. 3a indicate flows out of the system which need to be measured experimentally.

(a)



(b)