The Redistribution of Carbon Label by the Reactions Involved in Glycolysis, Gluconeogenesis and the Tricarboxylic Acid Cycle in Rat Liver

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A scheme is presented that shows how the reactions involved in gluconeogenesis, glycolysis and the tricarboxylic acid cycle are linked in rat liver. Equations are developed that show how label is redistributed in aspartate, glutamate and phosphopyruvate when it is introduced as specifically labelled pyruvate or glucose either at a constant rate (steady-state theory) or at a variable rate (non-steady-state theory). For steady-state theory the fractions of label introduced as specifically labelled pyruvate that are incorporated into glucose and carbon dioxide are also given, and for both theories the specific radioactivities of aspartate and glutamate relative to the specific radioactivity of the substrate. The theories allow for entry of label into the tricarboxylic acid cycle via both oxaloacetate and acetyl-CoA, for $^{14}\text{CO}_2$ fixation and for loss of label from the tricarboxylic acid cycle in glutamate, but not for losses in citrate. They also allow for incomplete symmetrization of label in oxaloacetate due to incomplete equilibration with fumarate both in the extra-mitochondrial part of the cell and in the mitochondrion on entry of oxaloacetate into the tricarboxylic acid cycle. In the latter case failure both of oxaloacetate to equilibrate with malate and of malate to equilibrate with fumarate are considered.

In the liver the reactions involved in gluconeogenesis, glycolysis and the tricarboxylic acid cycle are interlinked. Krebs, Hems, Weidemann & Speake (1966) pointed out that attempts to calculate the rates of these reactions from experiments with ^{14}C either in perfused organs or *in vivo* were often frustrated because the redistribution of label along the pathways had not been worked out.

With any specifically labelled pyruvate or glucose as substrate experimental methods exist for determining the distribution of label within glucose, glutamate and aspartate, and the relative specific radioactivities of glucose, glucose 6-phosphate, pyruvate, aspartate and glutamate; and with any specifically labelled pyruvate as substrate for determining the fractions of label incorporated into carbon dioxide and glucose, i.e., by chemical analogy, the yields of $^{14}\text{CO}_2$ and [^{14}C]glucose (Ashby, Heath & Stoner, 1965). The aim here is to relate algebraically these distributions of label, relative specific radioactivities and yields to the rates of the reactions involved for the particular case of the rat liver.

Rat liver was chosen because most is known about it. In the kidneys some enzymes may be differently distributed between the mitochondrion and the extramitochondrial part of the cell (cytoplasm),

and in the livers of some other species one enzyme is known to be differently distributed.

For rat liver the basic scheme is taken to be that in Scheme 1, which is based on the work of many authors (e.g. Krebs *et al.* 1966; Walter, Paetkau & Lardy, 1966; Henning, Stumpf, Ohly & Seubert, 1966). Lorber, Lifson, Wood, Sakami & Shreeve (1950) gave a semi-quantitative discussion of the interactions of the tricarboxylic acid cycle and gluconeogenesis that foreshadows the present one in several respects, and Weinman, Strisower & Chaikoff (1957) and Exton & Park (1967) have given preliminary algebraical treatments based on the same principles as the present one.

As the system: oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate in the tricarboxylic acid cycle can be treated in three ways (see under '3. Steady-state theory' in the Theory section) and at least two of these treatments must be combined to correlate experimental results (Heath & Threlfall, 1968) to keep the algebra within reasonable limits the basic scheme is simplified by five omissions: interaction with lipogenesis; the conversion of oxaloacetate into pyruvate via malate; the dilution of pyruvate by unlabelled alanine; the exchange of label between glutamate from the tricarboxylic acid cycle with unlabelled glutamate from exogenous sources;

Scheme 1. Basic scheme for the interaction of glycolysis, gluconeogenesis and the tricarboxylic acid cycle. Most of the variables used in deriving equations are defined by this Scheme. Within dashes: mitochondrial processes; within dots: both intra- and extra-mitochondrial processes; outside dots and dashes: extra-mitochondrial (cytoplasmic) processes.

similar dilution of aspartate label. Inclusion of the first two has been shown to have negligible effects on the treatment (Heath & Threlfall, 1968); inclusion of the third would result in a lowering of all specific radioactivities relative to glucose 6-phosphate, without altering yields or the distribution of label within compounds; inclusion of the last two would result only in a lowering of the specific radioactivities of glutamate and aspartate relative to the other specific radioactivities. Heath & Threlfall (1968) also discuss these dilution processes in the light of experimental findings.

The transfer of radioactivity without net flux of compound (exchange) is considered in the Discussion section and by Heath & Threlfall (1968), and is shown to be allowed for adequately by the scheme.

The substrates considered are specifically labelled pyruvate and glucose. It is assumed either that the substrates are introduced at such low concentrations

that they do not alter any chemical concentrations appreciably, or that they are introduced at a steady rate which maintains the concentrations constant. In the first case labelled lactate and for many purposes alanine are equivalent to pyruvate labelled in the same positions. In the second they may not be, and results refer to the particular balance of pathways set up by the steady substrate concentration.

Both an isotopic steady-state theory and an isotopic non-steady-state theory are developed. For the steady-state theory the specific radioactivity of the substrate is assumed to remain constant throughout an experiment long enough for label to equilibrate in the system and for its distribution to become time-invariant. This corresponds to intravenous infusion and the situation in some perfusions of isolated organs. For the non-steady-state theory the specific radioactivity of the substrate is supposed

to vary with time, as, e.g., after intravenous injection. Non-steady-state theory greatly extends the range of data which can be handled, and makes it possible to show that steady-state theory can often be used with little error when, from the rapid variations in substrate specific radioactivity known to take place, this seems at first sight unlikely. This is important, as the steady-state theory is much more easily programmed. For both theories chemical concentrations are assumed to remain constant.

The next paper (Heath & Threlfall, 1968) demonstrates that the theory developed can correlate many observations made *in vivo*. The present paper is, in effect, a description of methods, and as such contains no conclusions of a biochemical nature.

Throughout positions of labelling are denoted by numerals after 'C', e.g. C1,4-aspartate denotes [1,4-¹⁴C]aspartate.

THEORY

1. Pathways

Glucose
Glycogen

glucose 6-phosphate → PEP* →

glucose. Threlfall & Heath (1968) have shown that the glycolytic and gluconeogenic chains are separated between glucose and the triose phosphates, and may be at the triose phosphate level also. It is irrelevant to the mathematical treatment whether the chains are also separated to PEP or whether they are only separated to the triose phosphates, provided that these are in equilibrium with PEP. [Separation to PEP is in accordance with, e.g., some findings of Fellenberg, Eppenberger, Richterich & Aebi (1962), Krebs (1963), Ashby *et al.* (1965), Exton & Park (1965) and Heath & Threlfall (1968).]

Threlfall & Heath (1968) showed that most of the liver glucose 6-phosphate was in the glycolytic chain, so that in glucose 6-phosphate label from glucose was much diluted by glucose 6-phosphate from glycogen. For this reason when labelled glucose is the primary substrate specific radioactivities are calculated relative to that of glucose 6-phosphate, and an allowance is made for the dilution of glucose label.

PEP → pyruvate. The reaction is mediated by pyruvate kinase (ADP-pyruvate phosphotransferase, EC 2.7.1.40) in the cytoplasm. It is taken as irreversible, in accordance with experiments *in vivo* (Hornbrook, Burch & Lowry, 1965) and *in vitro* (Utter, 1959). Observations earlier explained by its reversal (Hoberman & d'Adamo, 1960) can now be

explained in other ways (Seubert & Huth, 1965; and see below under '2. Redistribution of label').

Pyruvate → acetyl-CoA → tricarboxylic acid cycle. Acetyl-CoA is formed in the mitochondrion by the action of pyruvate oxidase [pyruvate-oxygen oxidoreductase (phosphorylating), EC 1.2.3.3]. There can be no net synthesis of glucose from pyruvate via this pathway.

Pyruvate → oxaloacetate. The reaction is mediated by pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), EC 6.4.1.1]. In recently fed rats this is mostly mitochondrial (Walter *et al.* 1966), but in starved rats up to half may be cytoplasmic (Seubert & Huth, 1965). Oxaloacetate can only enter the tricarboxylic acid cycle to compensate for losses from the cycle: of citrate, 2-oxoglutarate (via glutamate), fumarate, malate or oxaloacetate. Oxaloacetate formed in the cytoplasm probably cannot permeate the mitochondrial membrane, but must be transported via aspartate or malate (Lardy, Paetkau & Walter, 1965; Shrago & Lardy, 1966).

Oxaloacetate + acetyl-CoA → 2-oxoglutarate. The intermediate stages are omitted, as they do not affect the kinetics except when citrate is lost. The primary reaction is mediated by citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7].

Oxaloacetate → PEP. This is mediated by phosphopyruvate carboxylase [GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32], which in the rat, unlike in some other species, is almost wholly cytoplasmic (Nordlie & Lardy, 1963; Nordlie, Varricchio & Holten, 1965). The reaction is reversible, but goes very much faster in the direction shown under physiological conditions (Chang, Maruyama, Miller & Lane, 1966).

2-Oxoglutarate ⇌ glutamate and oxaloacetate ⇌ aspartate. These are linked via aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) and are also catalysed by other transaminases. For steady-state theory the rates do not matter. Non-steady-state theory is based on the finding that the conversion of 2-oxoglutarate into glutamate is faster than the conversion of 2-oxoglutarate into fumarate (Balázs & Haslam, 1965). Aspartate is assumed only to equilibrate with cytoplasmic oxaloacetate (see the Discussion section, and also Heath & Threlfall, 1968).

Glutamate ⇌ protein etc. Only net loss or net gain of glutamate from and to the system is considered, as there are no data with which to solve the equations derived assuming simultaneous loss and gain. The fate or origin of the glutamate involved is irrelevant to the kinetics.

Oxaloacetate ⇌ malate ⇌ fumarate. These reactions mediated by malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37) and

* Abbreviation: PEP, phosphoenolpyruvate.

fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2) are discussed below (under '2. Redistribution of label').

Transport of oxaloacetate, malate and/or fumarate from the mitochondrion to the cytoplasm. Krebs, Gascogne & Notton (1967) pointed out that to provide NADH for the conversion of diphosphoglycerate into glyceraldehyde phosphate a reduced intermediate must leave the mitochondrion, and not oxaloacetate. They suggested malate. Application of the present theory leads independently to the conclusion that the compound transported is not oxaloacetate, but could be malate or fumarate or both.

The two major omissions are:

Citrate $\rightarrow$ *oxaloacetate* + *acetyl-CoA*. This is the lipogenic process (see the introduction) utilizing citrate from the tricarboxylic acid cycle (Sreer, 1965).

Oxaloacetate $\rightarrow$ *malate* $\rightarrow$ *pyruvate*. Malate is probably converted *in vivo* into pyruvate, and not vice versa, by 'malic' enzyme [L-malate-NAD oxidoreductase (decarboxylating), EC 1.1.1.38] (Shrago, Lardy, Nordlie & Foster, 1963; Seubert & Huth, 1965; Shrago & Lardy, 1966). These authors agree that this enzyme is not very active.

At certain points some processes are described as irrelevant. This does not mean that they do not take place, or that they are metabolically unimportant. It does mean that the theory does not depend on them.

2. Redistribution of label

The redistributions of label brought about by the reactions already discussed are shown in Schemes 2-5. Key statements are italicized and numbered for later reference.

Scheme 2 shows that C1-PEP gives only C3,4-glucose, C2-PEP only C2,5-glucose, C3-PEP only C1,6-glucose and vice versa. As equilibration at the triose phosphate stage is not quite complete

(Hers, 1957; Rauschenbach & Lamprecht, 1964), C1,2,3- and C4,5,6-labelling in glucose may be unequal. The pentose shunt causes erosion and randomization of glucose label that first affects the C1-position (see, e.g., Wood, Katz & Landau, 1963). Both effects are fairly small, and are neglected in the present treatment. The pentose shunt is unlikely to affect C4,5,6-labelling in glucose, as, to affect these positions, the shunt has to be very fast with much recycling. Therefore it is assumed that:

(2.1) *The relative labelling in the C1-, C2- and C3-positions in PEP is reflected precisely in the relative labelling in the C4-, C5- and C6-positions in the glucose formed from PEP.*

(2.2) *On glycolysis C4-glucose will give C1-PEP, C5-glucose will give C2-PEP and C6-glucose will give C3-PEP.*

(2.3) *C(U)-glucose (uniformly labelled) gives uniformly labelled PEP on glycolysis.*

Scheme 3 shows the conversion of pyruvate into PEP via oxaloacetate.

(2.4) *In forming oxaloacetate any label in carbon dioxide will label oxaloacetate in the C4-position, and in forming PEP all C4-label in oxaloacetate is lost as $^{14}\text{CO}_2$.*

Scheme 4 shows that the interconversion of oxaloacetate and fumarate via malate leads to symmetrization of the label in oxaloacetate. The label can only take up positions symmetrical about the centre of the carbon skeleton, e.g. C2- and C3-oxaloacetate both become C2,3-oxaloacetate,

Scheme 3. Formation of PEP from pyruvate.

Scheme 2. Conversion of PEP into glucose.

Scheme 4. Symmetrization of label in oxaloacetate via malate and fumarate.

but cannot produce C1- or C4-oxaloacetate. Symmetrization may be incomplete, depending on the relative rates of the reactions involved. Thus:

(2.5) *Label in oxaloacetate may be symmetrized via fumarate.*

(2.6) *Symmetrization may be incomplete.*

Also, from statement (2.1) and Scheme 3:

(2.7) *Neither C2- nor C3-pyruvate can label the C4-position in glucose by pathways that do not involve the tricarboxylic acid cycle [extra-(tricarboxylic acid-cycle) pathways].*

Scheme 5 shows the redistribution of label in the tricarboxylic acid cycle when pyruvate moieties enter it either via acetyl-CoA or via oxaloacetate. The possible symmetrization of label in oxaloacetate via fumarate before condensation with acetyl-CoA is ignored in Scheme 5.

All label entering as C1-oxaloacetate is lost as ¹⁴CO₂ before 2-oxoglutarate, and all label that enters as C4-oxaloacetate is lost before fumarate. No label from C1-pyruvate can enter the tricarboxylic acid cycle via acetyl-CoA. Hence from statements (2.1), (2.4) and (2.7):

(2.8) *No tricarboxylic acid-cycle pathway can confer on to glucose any label introduced as C1-pyruvate or ¹⁴CO₂. [Labelling via ¹⁴CO₂ fixation in the cytoplasm (statements 2.4 and 2.6) is, of course, possible.]*

Label entering as C2- or C3-oxaloacetate is randomized. After two turns of the cycle C2-oxaloacetate gives uniformly labelled malate, and after one turn C3-oxaloacetate gives C1,4-malate. Thus both can give C1-oxaloacetate, and hence:

(2.9) *Both C2- and C3-pyruvate can give C3,4-glucose via the tricarboxylic acid cycle.*

Extensive C3,4-labelling was found by Lorber *et al.* (1950) which, from statements (2.7) and (2.9), shows that tricarboxylic acid-cycle pathways can contribute to gluconeogenesis.

Only those moieties which come from C2-pyruvate and which also enter the tricarboxylic acid cycle via acetyl-CoA can label the C5-position in 2-oxoglutarate. Label in liver glutamate reflects labelling in 2-oxoglutarate. Hence:

(2.10) *The (C5-label in glutamate)/(total label in glutamate) ratio, abbreviated to (C5-Glu)/(T-Glu),*

found after C2-pyruvate injection indicates the relative contributions made by the two pathways, entry via acetyl-CoA and entry via oxaloacetate (Freedman & Graff, 1958; Koeppe, Mourkides & Hill, 1959).

(2.11) *Label from C3-pyruvate enters as C2-acetyl-CoA, and is randomized like label entering as C2-oxaloacetate.*

3. Steady-state theory

Three variables in addition to those shown in Scheme 1 are required.

(1) In the cytoplasm passage of label through oxaloacetate may not be accompanied by complete symmetrization (statements 2.5 and 2.6). The fraction of label that retains its position from pyruvate through oxaloacetate to PEP is denoted by α . Scheme 6 shows in terms of α how label is redistributed.

(2) When pyruvate moieties enter the tricarboxylic acid cycle as oxaloacetate the label in oxaloacetate may not be completely symmetrized on entry before it reacts with citrate synthase. Existing data can only be correlated if symmetrization on entry is incomplete (Heath & Threlfall, 1968). The fraction that is not symmetrized is denoted by z . It should be noted that z is not the algebraical equivalent of α .

(3) Label can fail to be symmetrized on entry into the cycle in two ways: either because malate fails to equilibrate with fumarate (Scheme 7a) or because it fails to equilibrate with oxaloacetate (Scheme 7b). The two cases give different equations. Therefore three cases are worked out: the case with complete symmetrization (sym. case); the case with no symmetrization because fumarate does not equilibrate with malate (non-sym. case A); and the case with no symmetrization because oxaloacetate does not equilibrate with malate (non-sym. case B). The fractions of input of label obeying non-sym. cases A and B are denoted by z_f and z_o respectively. To correlate data all three cases are worked out for the chosen values of the variables, and added in the required proportions: sym. case:non-sym. case A:non-sym. case B = $(1 - z_f - z_o):z_f:z_o$. In practice

Scheme 5. Labelling from pyruvate in the tricarboxylic acid cycle. Numbering of carbon atoms is shown to the left of key compounds.

Scheme 6. Partial symmetrization in the conversion of pyruvate into glucose. The fraction α of label retains its position. C4,5,6 of glucose are shown.

Scheme 7. Non-symmetrization of oxaloacetate on entry into the tricarboxylic acid cycle. The symbols are as in Scheme 1. In (a) malate and fumarate do not equilibrate; in (b) oxaloacetate and malate do not equilibrate.

the available data only enable the sym. case to be combined unambiguously with either of the non-sym. cases, and not with both together.

Reference to Scheme 1 shows the variables are: V_1 , V_2 , V_3 , V_5 , V_6 , Δ , R and A . Other variables are α , z_f and z_o . Of these variables not all are independent, as the flow into a compound pool must equal the flow out of it. Thus:

$$V_1 = (1+A)V_2 + V_3 \quad (3.1)$$

As a flow of $\frac{1}{2}V_5$ of glucose 6-phosphate corresponds to a rate of formation of PEP of V_5 :

$$V_5 = AV_2 + \Delta + V_6 \quad (3.2)$$

To develop equations label is supposed to enter the system at a constant rate in a specific position either in pyruvate or in PEP from glucose (statement 2.2) via glucose 6-phosphate. As:

$$\text{Specific radioactivity} = \frac{\text{flow of label}}{\text{flow of compound}} \quad (3.3)$$

the specific radioactivity of each carbon in the system can be directly calculated as far as 2-oxoglutarate in terms of the rate of entry of label in pyruvate or PEP. It is mathematically convenient to divide the tricarboxylic acid cycle between 2-oxoglutarate and succinate, and to denote this

calculation as referring to the zeroth turn of the (tricarboxylic acid) cycle. Label now flows to malate redistributed as in Scheme 5. This flow represents the input for the first turn of the cycle. Specific radioactivities are now calculated relative to this new input, which is itself related to the original input in pyruvate or PEP. And so on for successive turns of the cycle to infinity. The treatment therefore gives the specific radioactivity of each position in each compound for each turn of the cycle, and the results are tabulated in this form in the Appendix. The total specific radioactivity of any position of labelling is obtained by summing the specific radioactivities calculated for every turn of the cycle to infinite turns.

One example is worked out in detail, namely labelling from C2-pyruvate on non-sym. case B, and another in fair detail, namely C2-pyruvate on sym. case, to show the approach. There are in all nine sets of equations: three cases, and for each case the labelling from C1-, C2- and C3-pyruvate.

C2-pyruvate, non-sym. case B. The parameters are as in Schemes 1 and 7(b). There is no equilibration between oxaloacetate and fumarate in the tricarboxylic acid cycle. This implies very low oxaloacetate concentrations, so that losses must be as malate or fumarate or both, not as oxaloacetate, and these receive no label except from 2-oxoglutarate.

Denote the specific radioactivities of pyruvate, PEP, 2-oxoglutarate and malate ($\equiv$ fumarate) by S_p , S_{pp} , S_k and S_m , and those of oxaloacetate in the tricarboxylic acid cycle and in the cytoplasm by S_{o1} and S_{o2} respectively. Numerals in ordinary type in parentheses after 'C' denote positions labelled. *Italicized* numerals merely enumerate derived parameters. They are chosen here to correspond to those in the full programme of nine cases, so they are not always in the order expected in the small part presented here.

Let label enter at rate V_1 , solely in the C2-position in pyruvate. Let the label in PEP be divided between the C2- and C3-positions in the ratio: $A(I)/[1-A(I)]$. Then:

$$S_p(C2) = 1 + A(I)S_{pp}(C2,3) \quad (3.4)$$

$$S_p(C3) = [1-A(I)]S_{pp}(C2,3) \quad (3.5)$$

$$S_p(C2,3) = 1 + S_{pp}(C2,3) \quad (3.6)$$

From eqn. (3.3):

$$S_{o2}(C2,3) = V_3 S_p(C2,3)/(V_2 + V_3 - \Delta) \quad (3.7)$$

$$S_{pp}(C2,3) = (V_2 + V_3 - \Delta)S_{o2}(C2,3)/(V_1 + V_6) \quad (3.8)$$

Substitute eqn. (3.7) in eqn. (3.8), and denote:

$$V_3/(V_1 + V_6) = B(I) \quad (3.9)$$

separate variables and rearrange. Then:

$$S_{pp}(C2,3) = B(I)/[1-B(I)] \quad (3.10)$$

Put:

$$V_3/(V_2 + V_3 - \Delta) = A(2) \quad (3.11)$$

Then eqns. (3.10) and (3.11) in eqn. (3.8) give:

$$S_{o2}(C2,3) = A(2)/[1-B(I)] \quad (3.12)$$

To obtain $A(I)$ explicitly proceed as follows:

$$\begin{aligned} S_{o2}(C2) &= A(2)S_p(C2); \\ S_{o2}(C3) &= A(2)S_p(C3) \end{aligned} \quad (3.13)$$

(Here and throughout the label given for oxaloacetate in the cytoplasm is that before symmetrization.) The fraction of label that retains its position in passing through oxaloacetate is α (see above). Hence:

$$\begin{aligned} S_{pp}(C2) &= A(I)S_{pp}(C2,3) \\ &= \frac{(V_2 + V_3 - \Delta)[\alpha S_{o2}(C2) + (1-\alpha)S_{o2}(C3)]}{(V_1 + V_6)} \end{aligned} \quad (3.14)$$

Substituting eqns. (3.4), (3.5) and (3.10) in eqn. (3.13), and eqn. (3.13) in eqn. (3.14), separating variables and rearranging gives:

$$A(I) = [\alpha - B(I)(2\alpha - 1)]/[1 - B(I)(2\alpha - 1)] \quad (3.15)$$

Put:

$$B(I)/[1-B(I)] = F' \quad (3.16)$$

and substitute in eqns. (3.4), (3.5) and (3.10). Then:

$$\begin{aligned} S_{pp}(C2) &= A(I)F'; \quad S_p(C2) = 1 + A(I)F'; \\ S_{pp}(C3) &= S_p(C3) = [1 - A(I)]F' \end{aligned} \quad (3.17)$$

$$\begin{aligned} S_{o1}(C2) &= V_2 S_p(C2)/R; \\ S_{o1}(C3) &= V_2 S_p(C3)/R \end{aligned} \quad (3.18)$$

The labelling in 2-oxoglutarate can be estimated from the relationships:

$$\begin{aligned} S_k(C5) &= A V_2 S_p(C2)/R; \quad S_k(C4) = A V_2 S_p(C3)/R; \\ S_k(C3) &= S_{o1}(C2); \quad S_k(C2) = S_{o1}(C3) \end{aligned} \quad (3.19)$$

This completes the zeroth turn of the cycle. Label is symmetrized via succinate, so that:

$$S'_m(C1) = S'_m(C4) = 0.5[S_k(C5) + S_k(C2)]$$

and

$$S'_m(C2) = S'_m(C3) = 0.5[S_k(C4) + S_k(C3)] \quad (3.20)$$

The number of primes corresponds to the number of turns of the cycle.

Put:

$$\begin{aligned} (R - V_2)[S'_m(C1) + S'_m(C4)] \\ \equiv (R - V_2)V_2\{A[1 + A(I)F'] + [1 - A(I)]F'\}/R \\ = D(I) \end{aligned} \quad (3.21)$$

and

$$\begin{aligned} (R - V_2)[S'_m(C2) + S'_m(C3)] \\ \equiv (R - V_2)V_2\{A[1 - A(I)]F' + 1 + A(I)F'\}/R \\ = D(2) \end{aligned} \quad (3.22)$$

$D(I)$ and $D(2)$ are the input functions for the first turn of the cycle. Then:

$$\begin{aligned} S'_{o1}(C1) &= 0.5D(I)/R + V_2S'_p(C1)/R; \\ S'_{o1}(C4) &= 0.5D(I)/R \end{aligned} \quad (3.23)$$

and, as $S'_{o1}(C2) = S'_{o1}(C3)$, so that $S'_p(C2) = S'_p(C3)$:

$$S'_{o1}(C2) = S'_{o1}(C3) = 0.5[D(2) + V_2S'_p(C2,3)]/R \quad (3.24)$$

$$S'_{o2}(C1) = \frac{[(V_2 - \Delta)S'_m(C1) + V_3S'_p(C1)]}{(V_2 + V_3 - \Delta)} \quad (3.25)$$

and similarly for $S'_{o2}(C2)$, $S'_{o2}(C3)$ and $S'_{o2}(C4)$.

$$S'_{pp}(C1) = \frac{(V_2 + V_3 - \Delta)[\alpha S'_{o2}(C1) + (1 - \alpha)S'_{o2}(C4)]}{(V_1 + V_6)} \quad (3.26)$$

whence, substituting eqn. (3.21) in eqn. (3.20), simplifying with eqn. (3.9) and putting:

$$(V_2 - \Delta)/(V_1 + V_6)(R - V_2) = D(3)$$

and

$$(V_2 - \Delta)/(V_2 + V_3 - \Delta)(R - V_2) = D(4) \quad (3.27)$$

we obtain:

$$S'_p(C1) = S'_{pp}(C1) = \frac{0.5D(I) \cdot D(3)}{1 - \alpha B(I)} \quad (3.28)$$

$$S'_{o2}(C1,4) = D(I) \left(D(4) + \frac{0.5A(2) \cdot D(3)}{1 - \alpha B(I)} \right) \quad (3.29)$$

Similar reasoning gives:

$$\begin{aligned} S'_p(C2) &= S'_p(C3) = S'_{pp}(C2) = S'_{pp}(C3) \\ &= 0.5D(2) \cdot D(3)/[1 - B(I)] \end{aligned} \quad (3.30)$$

$$S'_{o2}(C2,3) = D(2) \left(D(4) + \frac{A(2) \cdot D(3)}{1 - B(I)} \right) \quad (3.31)$$

Substitution of eqns. (3.28) and (3.30) in eqns. (3.23) and (3.24) gives $S'_{o1}(C1)$ etc., and these and eqn. (3.30) in eqn. (3.19) give:

$$S'_k(C5) = S'_k(C4) = \frac{0.5A V_2 D(2) \cdot D(3)}{R[1 - B(I)]} \quad (3.32)$$

$$S'_k(C3) = S'_k(C2) = \frac{0.5D(2)}{R} \left(1 + \frac{V_2 D(3)}{1 - B(I)} \right) \quad (3.33)$$

$$S'_k(C1) = S'_{o1}(C4) = 0.5D(I)/R \quad (3.34)$$

This ends the first turn of the cycle.

As $S'_k(C5) = S'_k(C4)$ and $S'_k(C3) = S'_k(C2)$, it follows from eqn. (3.20) that:

$$\begin{aligned} S''_m(C1) &= S''_m(C2) = S''_m(C3) = S''_m(C4) \\ &= 0.5[S'_k(C5) + S'_k(C2)] \end{aligned} \quad (3.35)$$

By analogy with eqns. (3.21) and (3.22) we put:

$$\begin{aligned} (R - V_2)[S'_k(C5) + S'_k(C2)] &\equiv \\ \frac{0.5D(2) \cdot (R - V_2)}{R} \left(1 + \frac{(1 + A)V_2 D(3)}{1 - B(I)} \right) &= E(I) \cdot D(2) \end{aligned} \quad (3.36)$$

Then $E(I) \cdot D(2)$ can be substituted for the functions $D(I)$ and $D(2)$ used for the first turn of the tri-carboxylic acid cycle, and hence all equations for this, the second, turn of the tricarboxylic acid cycle can be obtained by substituting $E(I) \cdot D(2)$ for $D(I)$ and $D(2)$ throughout the derivations of equations. Similarly for the third turn of the cycle $E(I)^2 \cdot D(2)$ can be substituted. Hence, as:

$$1 + E(I) + E(I)^2 + \dots$$

$$E(I)^n \text{ to infinity} = 1/[1 - E(I)]$$

the required specific radioactivities can be written down.

Label is also introduced by $^{14}\text{CO}_2$ fixation (statement 2.4). It is assumed that, as the liver releases carbon dioxide rapidly mostly from the tricarboxylic acid cycle, the $^{14}\text{CO}_2$ released is not diluted by less heavily labelled carbon dioxide from other organs. As the contribution made by $^{14}\text{CO}_2$ fixation is fairly small, very little error will be introduced if this assumption is not quite right. Then:

$$\begin{aligned} \text{Rate of release of } \text{CO}_2 \text{ (unlabelled)} \\ = R(\text{citrate} \rightarrow 2\text{-oxoglutarate}) \\ + (R - \Delta)(2\text{-oxoglutarate} \rightarrow \text{succinate}) \\ + (V_2 + V_3 - \Delta)(\text{oxaloacetate} \rightarrow \text{PEP}) \\ + AV_2(\text{pyruvate} \rightarrow \text{acetyl-CoA}) \\ - (V_2 + V_3)(\text{pyruvate} \rightarrow \text{oxaloacetate}) \\ = 2R - 2\Delta + AV_2 \end{aligned} \quad (3.37)$$

Rate of evolution of $^{14}\text{CO}_2 = V_1 Y_2(\text{CO}_2)$, where $Y_2(\text{CO}_2)$ is the fraction of label introduced as C2-pyruvate that is incorporated into carbon dioxide, i.e. the yield of $^{14}\text{CO}_2$ from C2-pyruvate (see the introduction).

Hence, if C_2 is the specific radioactivity of carbon dioxide from C2-pyruvate, from eqn. (3.3):

$$C_2 = V_1 Y_2(\text{CO}_2)/(2R - 2\Delta + AV_2) \quad (3.38)$$

Whence, reasoning as before:

$$S_{o1}(C4) = V_2 C_2/R = S_k(C1) \quad (3.39)$$

$$S_{o2}(C1) = A(2)S_p(C1); \quad S_{o2}(C4) = A(2)C_2 \quad (3.40)$$

$$S_{dp}(C1) = S_p(C1) = (V_2 + V_3 - \Delta)[\alpha S_{o2}(C1) + (1 - \alpha)S_{o2}(C4)]/(V_1 + V_6) \quad (3.41)$$

whence, with eqn. (3.40) in eqn. (3.41), and simplifying with eqns. (3.9) and (3.11):

$$S_{dp}(C1) = S_p(C1) = B(I)(1 - \alpha)C_2/[1 - \alpha B(I)] \quad (3.42)$$

$$S_{o2}(C1,4) = A(2)[1 - B(I)(2\alpha - 1)]C_2/[1 - \alpha B(I)] \quad (3.43)$$

All label is lost as $^{14}\text{CO}_2$ except that accounted for above.

In listing the final formulae the following factors are considered.

(1) Label is measured in aspartate and glutamate, not in oxaloacetate and 2-oxoglutarate. Specific radioactivities are therefore referred to the amino acids. In the steady state these must, of course, equal those in the oxo acids.

(2) In glutamate the position labelled is denoted as n , i.e. $Glu(n)$ is the specific radioactivity of the n th position in glutamate.

(3) For aspartate the distribution of label may be between that of the oxaloacetate from which it is formed and that formed from it which goes to PEP. Therefore detailed labelling is not given, only the specific radioactivities $Asp(1,4)$ and $Asp(2,3)$, as the amounts in these combined positions are unaffected by this uncertainty. (Numerically the uncertainty is small, and the values for individual positions could be bracketed closely from the equations.)

(4) When the primary substrate is pyruvate, the specific radioactivity of glucose rarely in practice approaches that of PEP because $[^{14}\text{C}]$ glucose is diluted by a big pool of unlabelled glucose. Specific radioactivities in PEP are therefore given, denoted by $PP(n)$. The distribution of label is the same in C4,5,6-glucose as in C1,2,3-PEP.

(5) On the scale implied by the treatment the specific radioactivity of intracellular pyruvate is given by $1 + PP(1) + PP(2) + PP(3)$, and the specific radioactivity of the input pyruvate by 1.

Thus:

$$PP(1) = \frac{B(I)(1 - \alpha)C_2}{[1 - \alpha B(I)]} + \frac{0.5D(3)}{[1 - \alpha B(I)]} \left(D(I) + \frac{E(I) \cdot D(2)}{[1 - E(I)]} \right) \quad (3.44)$$

$$PP(2) = A(I)F' + \frac{0.5D(2) \cdot D(3)}{[1 - B(I)][1 - E(I)]} \quad (3.45)$$

$$PP(3) = [1 - A(I)]F' + \frac{0.5D(2) \cdot D(3)}{[1 - B(I)][1 - E(I)]} \quad (3.46)$$

$$Asp(1,4) = \frac{A(2)[1 - B(I)(2\alpha - 1)]C_2}{[1 - \alpha B(I)]} + \left(D(4) + \frac{0.5A(2) \cdot D(3)}{[1 - \alpha B(I)]} \right) \left(D(I) + \frac{E(I) \cdot D(2)}{[1 - E(I)]} \right) \quad (3.47)$$

$$Asp(2,3) = \frac{A(2)}{[1 - B(I)]} + \left(D(4) + \frac{A(2) \cdot D(3)}{[1 - B(I)]} \right) \frac{D(2)}{[1 - E(I)]} \quad (3.48)$$

$$Glu(1) = \frac{V_2 C_2}{R} + \frac{0.5}{R} \left(D(I) + \frac{E(I) \cdot D(2)}{[1 - E(I)]} \right) \quad (3.49)$$

$$Glu(2) = \frac{V_2[1 - A(I)]F'}{R} + \frac{0.5[1 - B(I) + V_2 D(3)]D(2)}{R[1 - B(I)][1 - E(I)]} \quad (3.50)$$

$$Glu(3) = \frac{V_2[1 + A(I)]F'}{R} + \frac{0.5[1 - B(I) + V_2 D(3)]D(2)}{R[1 - B(I)][1 - E(I)]} \quad (3.51)$$

$$Glu(4) = \frac{A V_2[1 - A(I)]F'}{R} + \frac{0.5 A V_2 D(2) \cdot D(3)}{R[1 - B(I)][1 - E(I)]} \quad (3.52)$$

$$Glu(5) = \frac{A V_2[1 + A(I)]F'}{R} + \frac{0.5 A V_2 D(2) \cdot D(3)}{R[1 - B(I)][1 - E(I)]} \quad (3.53)$$

Denote the yield of glucose by $Y_2(\text{Glc})$. The rate of labelling of glucose is $V_6 \times$ specific radioactivity of PEP, and the rate of input of label into the system is V_1 . Hence the fraction of the label entering glucose, i.e. the yield, is:

$$Y_2(\text{Glc}) = V_6[PP(1) + PP(2) + PP(3)]/V_1 \quad (3.54)$$

Similarly the fractional loss of label as glutamate probably to protein is given by:

$$Y_2(\text{Prot}) = \Delta[Glu(1) + Glu(2) + Glu(3) + Glu(4) + Glu(5)]/V_1 \quad (3.55)$$

Hence, as label is only lost from the system in glucose, 'protein' (via glutamate) and carbon dioxide:

$$Y_2(\text{CO}_2) = 1 - Y_2(\text{Glc}) - Y_2(\text{Prot}) \quad (3.56)$$

C2-pyruvate, sym. case. For this case oxaloacetate, malate and fumarate behave as a single pool in the tricarboxylic acid cycle, with complete exchange of label. Hence $S_m = S_{o1}$. Otherwise terminology is as before.

Thus:

$$S_{pp}(C2) = \delta S_{pp}(C2,3); \quad S_{pp}(C3) = (1-\delta)S_{pp}(C2,3) \\ = S_p(C3); \quad S_p(C2) = 1 + S_{pp}(C2) \quad (3.57)$$

by analogy with eqns. (3.4), (3.5) and (3.6).

As label is symmetrized on entering the tri-carboxylic acid cycle:

$$S_{o1}(C2) = S_{o1}(C3) = 0.5V_2[1 + S_{pp}(C2,3)]/V \quad (3.58)$$

where:

$$V = R + V_2 - \Delta \quad (3.59)$$

$$S_{o2}(C2) = \frac{[(V_2 - \Delta)S_{o1}(C2) + V_3S_p(C2)]}{(V_2 + V_3 - \Delta)} \quad (3.60)$$

and analogously for $S_{o2}(C3)$.

$$S_{pp}(C2) = \frac{(V_2 + V_3 - \Delta)[\alpha S_{o2}(C2) + (1 - \alpha)S_{o2}(C3)]}{(V_1 + V_6)} \quad (3.61)$$

$$S_{pp}(C3) = \frac{(V_2 + V_3 - \Delta)[(1 - \alpha)S_{o2}(C2) + \alpha S_{o2}(C3)]}{(V_1 + V_6)} \quad (3.62)$$

Defining:

$$B = [V_2(V_2 - \Delta) + VV_3]/V(V_1 + V_6) \quad (3.63)$$

and proceeding as for the zeroth turn of the non-sym. case gives:

$$S_{pp}(C2,3) = B/(1 - B) \quad (3.64)$$

$$\delta = \frac{0.5V_2(V_2 - \Delta) + VV_3[\alpha - B(2\alpha - 1)]}{V_2(V_2 - \Delta) + VV_3[1 - B(2\alpha - 1)]} \quad (3.65)$$

$$S_{o2}(C2,3) = \frac{V_2(V_2 - \Delta) + VV_3}{V(V_2 + V_3 - \Delta)(1 - B)} \quad (3.66)$$

By eqns. (3.64) and (3.65) in eqns. (3.57) and (3.58) equations are obtained for $S_p(C2)$, $S_p(C3)$, $S_{o1}(C2)$ and $S_{o1}(C3)$, and hence for $S_k(C2)$ – $S_k(C5)$. The remaining equations are developed as before.

Glucose as substrate. In the symbolism of Scheme 1 the glucose/(glucose 6-phosphate) specific-radioactivity ratio is given by:

$$R_1/(R_1 + R_2) \quad (3.67)$$

Specific radioactivities of pyruvate, glutamate and aspartate cannot therefore be related to those of glucose except via eqn. (3.67) (if R_1 and R_2 are known) or by the empirical determination of the relative specific radioactivities of the two compounds. Specific radioactivities are therefore given relative to those in specifically labelled glucose 6-phosphate.

Suppose that for any case (sym., non-sym. A or non-sym. B) and any position of labelling, C4-, C5- or C6-, label flows from glucose 6-phosphate to PEP at unit rate. Then, as the total flow of material

through PEP is $V_1 + V_6$, the specific radioactivity of the PEP formed is initially $1/(V_1 + V_6)$, C4-glucose giving C1-PEP and so on. Now, as pyruvate is only formed from PEP, pyruvate has the same specific radioactivity as PEP, i.e. label flows into pyruvate at the rate: $V_1/(V_1 + V_6)$. But when pyruvate was substrate the flow was defined as V_1 . Consequently the specific radioactivities in the system relative to unit flow of label into PEP are those given by the equations for pyruvate as substrate all divided by $(V_1 + V_6)$.

Suppose glucose 6-phosphate is of unit specific radioactivity and singly labelled. Then the rate of flow of label to PEP is $0.5V_5$ (Scheme 1). Hence to obtain specific radioactivities relative to that of glucose 6-phosphate the equations should be multiplied by $0.5V_5/(V_1 + V_6)$. For example, with C5-glucose as primary substrate, on non-sym. case B, the specific radioactivity of C5-glutamate is given by:

$$0.5V_5Glu(5)/(V_1 + V_6) \quad (3.68)$$

where $Glu(5)$ is given by eqn. (3.53).

For pyruvate, similarly, the total relative specific radioactivity is given by the pyruvate/(glucose 6-phosphate) specific-radioactivity ratio:

$$0.5V_5[1 + PP(1) + PP(2) + PP(3)]/(V_1 + V_6) \quad (3.69)$$

where $PP(1)$, $PP(2)$ and $PP(3)$ are given by eqns. (3.44), (3.45) and (3.46).

For uniformly labelled glucose 6-phosphate as substrate the equations for C1-, C2- and C3-pyruvate should be added, and multiplied by $V_5/6(V_1 + V_6)$. The pyruvate/(glucose 6-phosphate) specific-radioactivity ratio is given by:

$$V_5[3 + PP(1) + PP(2) + PP(3)]/6(V_1 + V_6) \quad (3.70)$$

It is therefore only necessary to list the equations for the pyruvates, as those for glucose can be easily obtained in the way described.

General comments. In the sym. case a factor E is obtained, relating the labelling in the i th turn of the tricarboxylic acid cycle to the label in the $(i - 1)$ th turn, for $i > 2$, just as $E(I)$ does in non-sym. case B. E is not the same as $E(I)$. This casts some doubt on the validity of adding the cases as suggested in this section, paragraph (3). The numerical differences between $E(I)$ and E are, however, always very small, so any error can easily be shown to be small also. For non-sym. case A the corresponding factor is identical with that for the sym. case. These two cases can be added with no possibility of error.

4. Non-steady-state theory

Specific radioactivity of substrate. The time-variance of the specific radioactivity of the basic substrate is taken to be found by experiment, and

Scheme 8. Intermediate pools between glucose and pyruvate.

is represented by $F(t)$. In the examples given it is also assumed that $F(t)$ can be expressed as the sum of a number of exponential terms, i.e.

$$F(t) = \sum A_i e^{-\sigma_i t} \quad (4.1)$$

This is nearly always possible with data obtained *in vivo* and greatly eases computation, but it is not strictly necessary. The differential equations set up are correct whatever the form of $F(t)$, and could be integrated numerically.

Usually $F(t)$ is found in plasma, which can be sampled serially, and not in liver cells. Consequently an allowance must be made for diffusion from plasma, and for mixing in any intermediate pools.

For glucose, equilibration of label between liver and plasma is not instantaneous (see, e.g. Hetenyi, Kopstick & Retelsdorf, 1963; Heath & Threlfall, 1968). Isotopic equilibration can be represented as in Scheme 8(a). As equilibration is rapid compared with net utilization:

$$ds_1/dt = k'[F(t) - s_1] \quad (4.2)$$

where s_1 is the specific radioactivity of glucose in the liver and k' is the rate constant for transport from liver to plasma. The equation is identical with that obtained if the liver is treated as an intermediate pool, as in Scheme 8(b), which feeds glucose 6-phosphate at a rate $k'Q_1$, where Q_1 is the size of the liver glucose pool. The glucose 6-phosphate pool is also not negligible ($Q_6 \approx 1.4 \mu\text{moles}/100 \text{ g. rat body wt.}$). Similar reasoning gives:

$$ds_6/dt = k_6[R_1 s_1/(R_1 + R_2) - s_6] \quad (4.3)$$

The factor $R_1/(R_1 + R_2)$ allows for dilution from glycogen (eqn. 3.67 and Scheme 8b).

A simpler treatment may often be adequate.

Both pools are combined to give one intermediate pool with rate constant k_I (k_I is less than k' and k_6). Then the function, $F_g(t)$, defining the specific radioactivity of glucose 6-phosphate, is given by:

$$dF_g(t)/dt = k_I[R_1 F(t)/(R_1 + R_2) - F_g(t)] \quad (4.4)$$

Integration, with $F_g(t) = 0$ at $t = 0$, gives:

$$F_g(t) = \sum A'_i e^{-\sigma_i t} + B e^{-k_I t} \quad (4.5)$$

where:

$$A'_i = k_I A_i R_1/(R_1 + R_2)(k_I - g_i) \quad (4.6)$$

and

$$\sum A'_i + B = 0 \quad (4.7)$$

(A_i and g_i are the empirical constants in eqn. 4.1).

The approximate equation (eqn. 4.5) overestimates the specific radioactivity of glucose 6-phosphate seriously for $t < 0.5/k_I$, but introduces little error for $t > 2/k_I$. As some early work suggested that k_I is in the range 0.5–1.5/min., errors are confined to very short times after starting an experiment.

For pyruvate intracellular concentrations are much less than plasma concentrations (Threlfall & Stoner, 1961), so injected pyruvate may well be utilized before it has penetrated far into the cell. There is, however, an intracellular pool of alanine, with which pyruvate label will to some extent equilibrate. At a guess k_I for pyruvate is about 1–2/min.

The introduction of these intermediate pools may seem superfluous and dubious, especially as there are no accurate estimates of k_I and very little possibility of obtaining them. In fact, any value of $k_I < 2/\text{min.}$ has a considerable smoothing effect on

the estimates made of the specific radioactivity of aspartate at short times after injection, and this agrees with experiment. An intermediate pool is therefore needed. The exact value of k_I over the range 1–2/min. proves, however, of relatively little importance.

Simplification of the basic scheme. The theory depends on certain assumptions.

(1) At least half of the label passing through 2-oxoglutarate equilibrates with glutamate (see above under '1. Pathways'). Two cases are worked out: for complete equilibration and for partial equilibration.

(2) The reactions oxaloacetate $\rightarrow$ aspartate and 2-oxoglutarate $\rightarrow$ glutamate will be assumed to proceed at the same absolute rates, on the grounds that both reactions are linked via L-aspartate aminotransferase. Other transferases also catalyse these reactions, so the approximation will lead to error unless Assumption (1) holds.

(3) The whole free glutamate and aspartate pools are involved, i.e. there is no compartmentation.

(4) As there is partial equilibrium between glutamine and glutamate (Waelsch, Berl, Rossi, Clarke & Purpura, 1964), half the free glutamine

is assumed to be in the glutamate pool for the purposes of calculation. This is the 'best-strategy' assumption to make about an unknown degree of partial equilibrium. Errors in this assumption cannot cause errors greater than 20% on one variable (k_g).

(5) The only pools big enough to include are aspartate and glutamate (including half the glutamine).

(6) Some label leaves the tricarboxylic acid cycle via malate and returns via pyruvate. This process is neglected to simplify the mathematics. A trial computation suggests that this simplification has very little effect on numerical results.

Complete equilibration between glutamate and 2-oxoglutarate. The basic assumptions lead to the scheme shown in Scheme 9. Material passes through successive pools of glutamate, each pool representing one turn of the cycle; and through aspartate, each representing the extra-(tricarboxylic acid cycle) system associated with the corresponding turn of the tricarboxylic acid cycle. To reconcile the treatment with that of the steady state the first glutamate pool is denoted by (0), i.e. zeroth turn and so on.

Scheme 9. Kinetic scheme for non-steady-state theory corresponding to Scheme 1 for steady-state theory. There are some simplifying approximations (see the text).

The same symbols are used as in Scheme 1 with the addition of Q_a , k_a , Q_g and k_g , representing the pool sizes and turnover constants of aspartate and glutamate respectively. As label flows through the tricarboxylic acid cycle at rate R (Scheme 1):

$$k_g = R/Q_g \quad (4.8)$$

and as label flows through cytoplasmic oxaloacetate at rate $V_2 + V_3 - \Delta$:

$$k_a = (V_2 + V_3 - \Delta)/Q_a \quad (4.9)$$

Q_a and Q_g are determined experimentally, so no new unknowns are required.

For computation the equations of the system are best derived in the following way. Define an input function, $F'(t)$, corresponding to $F_g(t)$ or $F(t)$, giving the specific radioactivity of the substrate. Calculate initially as though no label was lost from the system as labelled glucose, carbon dioxide or glutamate. Define $q'_a(i)$ as the quantity of label in the i th aspartate pool and $q'_g(i)$ as the quantity of label in the i th glutamate pool. These quantities will be called preliminary estimates. Let label flow into the first glutamate and aspartate pool at rate $F'(t)$. Then:

$$dq'_g(0)/dt = F'(t) - k_g q'_g(0) \quad (4.10)$$

$$dq'_g(i)/dt = k_g [q'_g(i-1) - q'_g(i)] \quad (4.11)$$

where $i \neq 0$.

$$dq'_a(0)/dt = F'(t) - k_a q'_a(0) \quad (4.12)$$

$$dq'_a(i)/dt = q'_g(i-1) - k_a q'_a(i) \quad (4.13)$$

where $i \neq 0$.

Standard methods enable each q to be calculated explicitly in terms of the constants in the input functions (eqns. 4.1 and 4.7) and k_a and k_g . For example, for pyruvate as substrate:

$$q'_g(i) = \sum A_i e^{-a_i t} + (B + Ct + Dt^2 + \dots + Jt^{i-1})e^{-k_g t} \quad (4.14)$$

where each coefficient, A_i , B , C etc. can be derived from the coefficients in the equation for $q'_g(i-1)$ by substitution of both in eqn. (4.11) and equating coefficients, with $q'_g(i) = q'_g(i-1) = 0$ at $t = 0$.

The correct values are calculated as follows, taking [U-¹⁴C]glucose as an example.

In the Appendix the equations giving the specific radioactivities of each position in pyruvate, aspartate and glutamate with C1-, C2- and C3-pyruvate as substrate are listed for each case and for each turn of the tricarboxylic acid cycle. Suppose that in this instance we want the total specific radioactivities on each turn of aspartate and glutamate, denoted by 'weights', $W_g(i)$ and $W_a(i)$ for the i th turn, and for equal contributions from sym. case and non-sym. case B, i.e. $z_f = 0$

(non-sym. case A), $z_0 = 0.5$ (0.5 non-sym. case B) and $1 - z_0 - z_f = 0.5$ (0.5 sym. case). For $Glu(n)$ add all the equations for $i = 0$ for both the sym. case and non-sym. case B, and multiply by 0.5. Convert for comparison with glucose 6-phosphate by multiplying by $V_5/6(V_1 + V_6)$ (see above under '3. Steady-state theory: Glucose as substrate'). The result is $W_g(0)$. Proceed similarly for aspartate and the other turns of the cycle. [This procedure makes some allowance for cycling of label via cytoplasmic oxaloacetate back to the tricarboxylic acid cycle, as the weights are estimated from steady-state theory, which allows for this. Consequently Assumption (6) introduces only small errors.]

Denote the specific radioactivities of the i th pools of glutamate and aspartate by $S_g(i)$ and $S_a(i)$.

For the zeroth glutamate pool in the steady state:

$$S_g(0) = W_g(0)S \quad (4.15)$$

where S is the (constant) specific radioactivity of glucose 6-phosphate.

The correct form of eqn. (4.10) is:

$$dq_g(0)/dt = kF_g(t) - k_g q_g(0) \quad (4.16)$$

where k is the proportionality constant required to give eqn. (4.15) in the steady state when $F_g(t) = S$.

In the steady state:

$$kF_g(t) = kS = k_g q_g(0) \quad (4.17)$$

whence:

$$S_g(0) = q_g(0)/Q_g = kS/k_g Q_g = W_g(0)S \quad (4.18)$$

i.e.:

$$k = k_g Q_g W_g(0) \quad (4.19)$$

As from eqn. (4.18) $S_g(0) = q_g(0)/Q_g$ it follows that:

$$S_g(0) = k_g W_g(0) q'_g(0) \quad (4.20)$$

Thus $S_g(0)$ is related to the preliminary estimate of label in the zeroth glutamate pool, $q'_g(0)$.

Similarly:

$$S_g(i) = k_g W_g(i) q'_g(i) \quad (4.21)$$

and:

$$S_a(0) = k_a W_a(0) q'_a(0) \quad (4.22)$$

where $q'_a(0)$ is obtained by integrating eqn. (4.12).

In the steady state, eqns. (4.10) and (4.11) give:

$$q'_g(i-1) = S/k_g \quad (4.23)$$

and eqn. (4.13) gives:

$$q'_a(i) = q'_g(i-1)/k_a = S/k_a k_g \quad (4.24)$$

As:

$$S_a(i) = W_a(i)S \quad (4.25)$$

the same reasoning as before shows that:

$$S_a(i) = k_a k_g W(i) q'_a(i) \quad (4.26)$$

when $i \neq 0$.

The total specific radioactivities are given by:

$$S_g = \sum_{i=0}^{\infty} S_g(i); \text{ and } S_a = \sum_{i=0}^{\infty} S_a(i) \quad (4.27)$$

For $i > 1$ about half the label is lost as $^{14}\text{CO}_2$ on each turn of the tricarboxylic acid cycle, and more is lost to form glucose and as glutamate, so $W(i+1)/W(i) < 0.5$. Hence from eqns. (4.21) and (4.26) in eqn. (4.27) it can be seen that the series converge rapidly, and need not be summed beyond $i=7$ or $i=8$ to give S_a and S_g to 1%.

The specific radioactivity of pyruvate is obtained similarly.

For specifically labelled glucose the factor $0.5V_5/(V_1 + V_6)$ is substituted for $V_5/6(V_1 + V_6)$ (see above under '3. Steady-state theory: Glucose as substrate').

For specific positions of labelling, say C5 in glutamate, only the relevant weights are used.

Partial equilibrium between 2-oxoglutarate and glutamate. The derivations of the equations are only sketched in. A full treatment is very long.

Scheme 10 shows the rates of transport of material through 2-oxoglutarate and glutamate, and their specific radioactivities, σ_k and σ_g , when precursor, specific radioactivity = $f(t)$, enters 2-oxoglutarate at rate R .

Assumption (5) requires that $Q_k \ll Q_g$ (Scheme 10) Then:

$$\sigma_k = \frac{\text{flow of label}}{\text{flow of compound}} = \frac{Rf(t) + (k_g Q_g - \Delta)\sigma_g}{R + k_g Q_g - \Delta} \quad (4.28)$$

Define:

$$\beta = (k_g Q_g - \Delta)/(R + k_g Q_g - \Delta) \quad (4.29)$$

Scheme 10. Incomplete equilibration between glutamate and 2-oxoglutarate.

Then:

$$\sigma_k = (1 - \beta)f(t) + \beta\sigma_g \quad (4.30)$$

It should be noted that k_g is now not defined by eqn. (4.8). Its value must be chosen to fit experimental data, so this treatment includes one new variable.

For the glutamate pool:

$$d\sigma_g/dt = k_g(\sigma_k - \sigma_g) = (1 - \beta)k_g[f(t) - \sigma_g] \quad (4.31)$$

(substituting eqn. 4.30 into the first form of the equation).

To proceed further σ_g must be split into its constituent parts. (The following verbal definition is imperfect, and must be interpreted in the light of the subsequent equations.) Define $\sigma_g(n, i)$ as the contribution made to the specific radioactivity of glutamate by label passing through glutamate for the $(n+1)$ th time and round the tricarboxylic acid cycle for the i th time. It follows that i cannot be less than n . Then the specific radioactivity of glutamate is given by:

$$S_g = \sum_{n,i=0}^{\infty} \sigma_g(n, i) \quad (4.32)$$

Suppose [$\text{U-}^{14}\text{C}$]glucose is the substrate, as in the preceding section. The input function for the zeroth turn of the tricarboxylic acid cycle is $W_g(0)F_g(t)$, whence from eqn. (4.31):

$$d\sigma_g(0, 0)/dt = (1 - \beta)k_g[W_g(0)F_g(t) - \sigma_g(0, 0)] \quad (4.33)$$

Eqn. (4.30) implies that of the label passing through 2-oxoglutarate the fraction $(1 - \beta)$ emerges with the time-dependence of the input, $f(t)$, and passes round the tricarboxylic acid cycle as though it had never entered glutamate. It therefore provides an input for the first turn of the cycle of $(1 - \beta)W_g(1)F_g(t)$, i.e.:

$$d\sigma_g(0, 1)/dt = (1 - \beta)k_g[(1 - \beta)W_g(1)F_g(t) - \sigma_g(0, 1)] \quad (4.34)$$

and so on for $\sigma_g(0, i)$. Hence:

$$\begin{aligned} d\sigma_g(0)/dt &= \sum_0^{\infty} d\sigma_g(0, i)/dt \\ &= (1 - \beta)k_g[F_g(t) \sum_0^{\infty} (1 - \beta)^i W_g(i) - \sigma_g(0)] \end{aligned} \quad (4.35)$$

[[$(1 - \beta)^0 = 1$ by definition.] This can be solved by standard methods.

To calculate the contribution made by label entering glutamate for the second time, i.e. $n=1$, note from eqn. (4.30) that in the steady state, when $\sigma_g = f(t)$, β of the label passes round the tricarboxylic

acid cycle with the time-dependence of σ_g . Hence:

$$d\sigma_g(1,1)/dt = (1-\beta)k_g[\beta W_g(1)\sigma_g(0,0)/W_g(0) - \sigma_g(1,1)] \quad (4.36)$$

Now $\sigma_g(1,2)$ has two constituents.

(A) Of the input in eqn. (4.36), $(1-\beta)$ passes through 2-oxoglutarate with the time-dependence of $\sigma_g(0,0)$, giving:

$$d\sigma_g(1,2)/dt = (1-\beta)k_g[\beta(1-\beta)W_g(2)\sigma_g(0,0)/W_g(0) - \sigma_g(1,2)] \quad (4.37)$$

(B) The output from $\sigma_g(0,1)$, i.e. $\beta\sigma_g(0,1)$, also contributes:

$$d\sigma_g(1,2)/dt = (1-\beta)k_g[\beta W_g(2)\sigma_g(0,1)/W_g(1) - \sigma_g(1,2)] \quad (4.38)$$

As $\sigma_g(0,0)$ and $\sigma_g(0,1)$ have the same time-dependence, their values are always proportional to the constants in their input functions, i.e. comparing eqns. (4.33) and (4.34):

$$\sigma_g(0,1) = (1-\beta)W_g(1)\sigma_g(0,0)/W_g(0) \quad (4.39)$$

Substituting eqn. (4.39) in eqn. (4.38) and adding eqn. (4.38) to eqn. (4.37) gives:

$$d\sigma_g(1,2)/dt = (1-\beta)k_g[2\beta(1-\beta)W_g(2)\sigma_g(0,0)/W_g(0) - \sigma_g(1,2)] \quad (4.40)$$

Proceeding stepwise it can be shown that:

$$d\sigma_g(1)/dt = \sum_1^{\infty} d\sigma(1,i)/dt = (1-\beta)k_g[\beta\sigma_g(0,0) \sum_1^{\infty} i(1-\beta)^{i-1}W_g(i)/W_g(0) - \sigma_g(1)] \quad (4.41)$$

To extend the treatment to higher values of n proceed as follows. Calculate from $\sigma_g(\overline{p-1}, \overline{p-1})$, i.e. $\sigma_g(n,i)$ where $n=i=p-1$, $\sigma_g(p,p)$ by:

$$d\sigma_g(p,p)/dt = (1-\beta)k_g[\beta W_g(p)\sigma_g(\overline{p-1}, \overline{p-1})/W_g(p-1) - \sigma_g(p,p)] \quad (4.42)$$

Then calculate $\sigma_g(p+1)$, i.e.:

$$\sum_{i=(p+1)}^{\infty} \sigma_g(\overline{p+1}, i)$$

from:

$$d\sigma_g(p+1)/dt = (1-\beta)k_g\{f[\beta, W_g(i)]\sigma_g(p,p)/W_g(p) - \sigma_g(p+1)\} \quad (4.43)$$

The function $f[\beta, W_g(i)]$ can be calculated algebraically, but the functions are listed in Table 1 for n up to 8.

Table 1. Values of the coefficients for use in the equations of the non-equilibrium case

In eqn. (4.43) the function $f[\beta, W_g(i)]$ is always of the form:

$$\sum_{i=p+1}^{\infty} N\beta(1-\beta)^{i-p-1}W_g(p+1)$$

where N is a whole number, which is listed below. For example, to relate $\sigma_g(1)$ to $\sigma_g(0,0)$, $p+1=1$, so:

$$f[\beta, W_g(i)] = \beta W_g(1) + 2\beta(1-\beta)W_g(2) + 3\beta(1-\beta)^2W_g(3) + \dots + i\beta(1-\beta)^{i-1}W_g(i)$$

where the numerical coefficients are taken from the first column.

In eqn. (4.50) the function $f[\beta, W_a(i)]$ is always of the form:

$$\sum_{i=p+1}^{\infty} N\beta(1-\beta)^{i-p-1}W_a(i)$$

where N is a whole number. Values of N are listed to $p+1=8$ and $\dot{p}+1=9$. Trial computations indicate that values may be required to $\dot{p}>7$ or $p>8$. The development of further numbers is, however, obvious. The rows are binomial coefficients with the first member missing.

		Values of N							
$p+1=$		1	2	3	4	5	6	7	8
$\dot{p}+1=$		2	3	4	5	6	7	8	9
$W_g(1)$	$W_a(2)$	1							
$W_g(2)$	$W_a(3)$	2	1						
$W_g(3)$	$W_a(4)$	3	3	1					
$W_g(4)$	$W_a(5)$	4	6	4	1				
$W_g(5)$	$W_a(6)$	5	10	10	5	1			
$W_g(6)$	$W_a(7)$	6	15	20	15	6	1		
$W_g(7)$	$W_a(8)$	7	21	35	35	21	7	1	
$W_g(8)$	$W_a(9)$	8	28	56	70	56	28	8	1

As the series all converge fairly rapidly, there is often no need to sum beyond $\sigma_g(8,8)$, or for $t < 30$ min. beyond $\sigma_g(7,7)$.

This treatment imposes no limitations on the degree of equilibration. If, however, Assumption (2) (see above under 'Simplification of the basic scheme') is to be a reasonable approximation β should probably be at least 0.5.

On Assumption (2):

$$k_a Q_a = k_g Q_g \quad (4.44)$$

With o and a as subscripts denoting oxaloacetate and aspartate, analogy with eqns. (4.28), (4.29), (4.30) and (4.31) gives:

$$\sigma_o = \frac{(V_2 + V_3 - \Delta)f(t) + k_a Q_a \sigma_a}{V_2 + V_3 - \Delta + k_a Q_a} = (1 - \beta_a)f(t) + \beta_a \sigma_a \quad (4.45)$$

where:

$$\beta_a = k_a Q_a / (V_2 + V_3 - \Delta + k_a Q_a) \quad (4.46)$$

and

$$d\sigma_a/dt = (1 - \beta_a)k_a[f(t) - \sigma_a] \quad (4.47)$$

From eqn. (4.44) in eqn. (4.46) β_a and k_a can be calculated from k_g , so there is no new constant involved. [Plainly one can assume eqn. (4.44) does not hold, but then a new constant is required, and empirical verification becomes exceedingly difficult.]

Deriving as eqn. (4.35):

$$d\sigma_a(0)/dt = (1 - \beta_a)k_a[F_g(t) \sum_0^\infty (1 - \beta)^i W_a(i) - \sigma_a(0)] \quad (4.48)$$

(It should be noted that within the square brackets is β , not β_a).

Similarly:

$$d\sigma_a(1)/dt = (1 - \beta_a)k_a[\beta\sigma_g(0,0) \sum_1^\infty (1 - \beta)^{i-1} W_a(i)/W_g(0) - \sigma_a(1)] \quad (4.49)$$

To calculate $\sigma_a(i)$ when $i > 1$, denoting specific values of n and i by $\hat{p}$, it can be shown that:

$$d\sigma_a(\hat{p} + 1)/dt = (1 - \beta_a)k_a\{f[\beta, W_a(i)]\sigma_g(\hat{p}, \hat{p})/W_g(\hat{p}) - \sigma_a(\hat{p} + 1)\} \quad (4.50)$$

where $f[\beta, W_a(i)]$ is given in Table 1.

The same approach can be used to calculate the rate of $^{14}\text{CO}_2$ excretion. This is not discussed here.

DISCUSSION

Label can move without net movement of compound (exchange). The scheme was chosen to allow for this to the extent that seemed plausible under physiological conditions. It was not intended that the scheme should be used unmodified for systems in which the substrate concentrations were grossly unphysiological. Was the allowance adequate?

'Exchange' can refer to such things as the transfer of label from acetyl-CoA to glucose, although there can be no net synthesis of glucose from acetyl-CoA. This type of exchange was fully covered by the randomization and symmetrization processes introduced (Schemes 4-6).

Exchange also refers to transfer of label by the reversal of reactions, so that, e.g., in the closed system malate $\rightleftharpoons$ oxaloacetate at equilibrium label introduced as malate will eventually confer equal specific radioactivities on malate and oxaloacetate without net conversion of malate into oxaloacetate. If the whole tricarboxylic acid cycle consisted of such reversible processes then label could move

round it both ways, although the net flow is only in one. In the tricarboxylic acid cycle, however, exchange has been fully allowed for if two reactions, 2-oxoglutarate $\rightarrow$ succinate and succinate $\rightarrow$ fumarate, are irreversible; and some reversibility in the first is acceptable. All tricarboxylic acid-cycle reactions can be made to reverse, but labelling experiments suggest that under physiological conditions succinate $\rightarrow$ fumarate does not, as even when mitochondria are treated with unphysiologically high concentrations of labelled malate label only appears slowly in succinate (Walter *et al.* 1966). Consider the cycle in the forward direction. The system fumarate, malate and oxaloacetate was treated as an exchanging system, all possible cases being covered by combinations of the sym. case and non-sym. cases A and B (all three compounds in equilibrium, or either adjacent pair with the third not). In the system oxaloacetate + acetyl-CoA $\rightleftharpoons \dots \rightleftharpoons$ 2-oxoglutarate no label changes its position in either direction. As label only originates in acetyl-CoA and oxaloacetate, if label returns from 2-oxoglutarate to these compounds it is as though it had never left them. The kinetics are controlled entirely by the net output rates, $R - \Delta$ from 2-oxoglutarate and Δ from glutamate. Only if the reaction 2-oxoglutarate $\rightarrow$ succinate is reversible is labelling affected, because succinate formation is accompanied by symmetrization. This will lead to more equality between C3- and C2- labelling in glutamate when C2-pyruvate is the substrate than would be the case if the reaction was irreversible. Heath & Threlfall (1968) show that this effect must be small. In glutamate only the distribution of label is affected: the total specific radioactivity of glutamate is not. It has been assumed that retrograde transfer of label from succinate to oxaloacetate is negligible, as the chain is very long.

Outside the tricarboxylic acid cycle it is a reasonable assumption that the overall reaction pyruvate $\rightarrow$ acetyl-CoA is irreversible, and Heath & Threlfall (1968) adduce confirmatory evidence. 'PEP' was stated to include any triose phosphates in the glycolytic and gluconeogenic chains with which it was in equilibrium (see under '1. Pathways' in the Theory section), so the requirements for V_5 and V_6 are that each should represent any one irreversible step in the glycolytic and gluconeogenic chains respectively. Threlfall & Heath (1968) adduced evidence that there are such steps. The reasoning which suggests that the transfer of malate to the cytoplasm is substantially irreversible is given by Heath & Threlfall (1968), along with a way of modifying the scheme if it is not. Cytoplasmic oxaloacetate is part of a partially equilibrating system, oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate, within which isotopic exchange was allowed for by

introducing the variable α . It is not necessary to suppose that the reaction pyruvate $\rightarrow$ oxaloacetate is irreversible. If it is reversible, e.g. because of rapid exchange with carbon dioxide, there is still no redistribution of label involved. Redistribution only takes place insofar as label in oxaloacetate is symmetrized via fumarate or randomized in the tricarboxylic acid cycle. If the reaction is reversible, however, the interpretation of the rates V_2 and V_3 is different. These rates represent the net rates of utilization of oxaloacetate formed directly from pyruvate by the tricarboxylic acid cycle and by the cytoplasmic system respectively. These are necessarily equal to the net rates of oxaloacetate formation by the two pathways, but these are less than the forward rates.

There remain two more doubtful cases, namely the oxaloacetate $\rightarrow$ PEP and PEP $\rightarrow$ pyruvate reactions. Both are known to be reversible. The first is known to equilibrate heavily in favour of PEP (see under '1. Pathways' in the Theory section). The second is generally believed to equilibrate heavily in favour of pyruvate (see under '1. Pathways' in the Theory section and the study by Kerson, Garfinkel & Mildvan, 1967), but this view has been contested by Krinsky (1959) and Hoberman & d'Adamo (1960). Hoberman & d'Adamo (1960) attributed the preponderance of C5- over C6-labelling in glucose in starved rats given C2-lactate (statement 2.6) to the reversal of the pyruvate kinase reaction, and assumed that label passing through oxaloacetate was necessarily completely symmetrized. I have carried out detailed computations to see what would happen if both the PEP kinase and pyruvate kinase reactions were reversible. They showed that, to affect anything by more than a few per cent, the back reactions would have to be comparable in rate with the net forward reactions, which is, of course, inconsistent with equilibria strongly favouring the forward reactions; and to explain the results obtained by Hoberman & d'Adamo (1960) the back reaction (pyruvate $\rightarrow$ PEP) would have to be three times the net forward reaction. This seemed unlikely, in view of the evidence to the contrary already referred to. The matter is open to experiment. If Hoberman & d'Adamo (1960) were right, then in the starved rat given C2-lactate the C2- and C3-positions in aspartate should be equally labelled. If Scheme 1 is right then the C2/C3 ratio in aspartate should be at least as great as the C5/C6 ratio in glucose (see under '2. Redistribution of label', from Schemes 2 and 3, in the Theory section). Intermediate values would imply some reversal of the pyruvate kinase reaction but some lack of symmetrization in label passing through cytoplasmic oxaloacetate, i.e. $\alpha > 0.5$ (see under '3. Steady-state theory' paragraph (1) and Scheme 6 in the

Theory section). The scheme is easily modified to allow for such cases.

I have assumed that aspartate is only in equilibrium with cytoplasmic oxaloacetate, and not with mitochondrial oxaloacetate. This is a necessary assumption if the possibility is allowed that label in oxaloacetate may be symmetrized to different extents in the cytoplasm and the mitochondria; otherwise both oxaloacetate pools are in equilibrium with each other via aspartate. Computations show that symmetrization in oxaloacetate entering the tricarboxylic acid cycle is incomplete (Heath & Threlfall, 1968), and this implies that the oxaloacetate concentrations in the mitochondria are very low. The assumption is not, therefore, biochemically implausible.

The variable V_3 is shown as representing conversion of pyruvate into oxaloacetate in the cytoplasm. The same mathematics would apply if in fact all conversion of pyruvate into oxaloacetate takes place in the mitochondrion, but some is converted into aspartate, and this is transported to the cytoplasm. This does not, of course, imply equilibrium between oxaloacetate in the tricarboxylic acid cycle and aspartate.

Steady-state theory by itself cannot give absolute rates, only relative rates. To get absolute rates at least one absolute rate must be estimated independently. There are two possibilities.

(1) R , the rate of rotation of the tricarboxylic acid cycle. By assuming that only the tricarboxylic acid cycle utilizes oxygen in the liver, a maximum value of R can be calculated from the rate of oxygen consumption. This estimate is not likely to be far out *in vivo*, and the main error in R will come from the error in the estimate of oxygen consumption.

(2) V_5 . For livers in which glycogenolysis is rapid R_2 (Scheme 1) can be estimated. It is equal to the rate of glycogen disappearance. The relative specific radioactivities of glucose and glucose 6-phosphate can also be estimated some time after an injection of [^{14}C]glucose. The ratio will probably be constant from 4 min. after intravenous injection (see under '4. Non-steady-state theory: specific radioactivity of substrate' in the Theory section). Hence $R_1 + R_2 (= \frac{1}{2}V_5)$ can be estimated by eqn. (3.67). The main error is in the estimation of the rate of glycogenolysis, R_2 .

Non-steady-state theory can give absolute rates readily provided that 2-oxoglutarate and glutamate are in complete isotopic equilibrium. Then, as k_g depends on R (eqn. 4.8), the shape of the glutamate specific-radioactivity-time curve for any known substrate specific-radioactivity-time curve, $F'(t)$, is fixed within narrow limits. The variations in other variables mainly affect the peak value of the glutamate specific radioactivity. If isotopic equilibrium between glutamate and 2-oxoglutarate is

incomplete, i.e. $\beta \neq 1$, absolute rates may still be estimated by bringing in the variation with time of the specific radioactivity of aspartate. The procedure is, however, both experimentally and mathematically more difficult.

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APPENDIX

Tables for redistribution of label

For basic symbolism, see Scheme 1 and under '3. Steady-state theory' in the Theory section of the main paper.

There are Tables for the three cases: sym., non-sym. A and non-sym. B, with two substrates, C1- and C2-pyruvate, in each. Equations for other substrates can be derived from these by the transformations given below.

Each Table contains a list of subsidiary symbols, followed by the number of turns of the cycle, i , to $i=2$. For $i>2$, each equation in the Table under ($i-1$) can be multiplied by the constant factor, E or $E(I)$, to give the corresponding equation under i . To the left are the compounds in which labelling is given with the carbon numbers to which the equations apply, e.g. the top left-hand equation in Table A.1 under $i=0$ gives the labelling from

C1-pyruvate in C1,4-aspartate on the sym. case for the zeroth turn of the tricarboxylic acid cycle. At the end of each Table the way is given of converting equations for C2-pyruvate as substrate into those for C3-pyruvate as substrate.

When there is no entry in a Table, this is because there is no labelling, e.g. there is no C2,3-labelling in aspartate from C1-pyruvate, so there is no entry for C2,3-aspartate.

The specific radioactivities of aspartate, glutamate and phosphoenolpyruvate are denoted by the abbreviations used in eqns. (3.44)–(3.53) of the main paper. The specific radioactivity of carbon dioxide from C(n -labelled)-pyruvate is denoted by C_n (see under '3. Steady-state theory: C2-pyruvate, non-sym. case B' in the Theory section of the main paper).

After the Tables various additional equations are given that enable relative specific radioactivities and yields (see under '3. Steady-state theory: C2-pyruvate, non-sym. case B' in the Theory section

of the main paper) to be calculated, and the equations for labelled glucose 6-phosphate as substrate to be developed.

Table A.1. *Sym. case (label in oxaloacetate is completely symmetrized on entering the tricarboxylic acid cycle)*

Secondary symbols:

$$V = R + V_2 - \Delta; \quad M = 0.5V_2(V_2 - \Delta)/V(V_1 + V_6); \quad N = VV_3/V(V_1 + V_6); \quad B = 2M + N; \quad J = M + \alpha N; \\ L = M + (1 - \alpha)N; \quad D = (V_2 - \Delta)/V(V_1 + V_6); \quad F = [VV_3 + V_2(V_2 - \Delta)]/V(V_2 + V_3 - \Delta); \\ H = (V_2 - \Delta)/V(V_2 + V_3 - \Delta); \quad E = \frac{0.5(R - \Delta)}{1 - B} \left(\frac{AV_2D}{R} + \frac{1 - B + V_2D}{V} \right); \quad \delta = \frac{M + N[\alpha - B(2\alpha - 1)]}{2M + N[1 - B(2\alpha - 1)]}; \\ I'(1) = \frac{V_2(R - \Delta)}{1 - B} \left(\frac{A(1 - B + \delta B)}{R} + \frac{0.5}{V} \right); \quad I'(2) = \frac{V_2(R - \Delta)}{1 - B} \left(\frac{A(1 - \delta)B}{R} + \frac{0.5}{V} \right)$$

Turns of cycle $i =$	0	1	2
C1-pyruvate			
<i>Asp</i> (1,4)	$F[1 + PP(1) + C_1] = X_1$	0	0
<i>Glu</i> (1)	$\frac{0.5V_2X_1}{FV}$	0	0
<i>PP</i> (1)	$\frac{J + LC_1}{1 - J}$	0	0
C2-pyruvate			
<i>Asp</i> (1,4)	$\frac{F(1 - J + L)C_2}{1 - J}$	$\left(H + \frac{0.5DF}{1 - J} \right) I'(1) = X_2.I'(1)$	$E.X_2.I'(2)$
<i>Asp</i> (2,3)	$\frac{F}{1 - B}$	$\left(H + \frac{DF}{1 - B} \right) I'(2) = X_3.I'(2)$	$E.X_3.I'(2)$
<i>Glu</i> (1)	$\frac{0.5V_2(1 - J + L)C_2}{V(1 - J)}$	$\frac{0.5(1 - J + 0.5V_2D)I'(1)}{V(1 - J)} = X_4.I'(1)$	$E.X_4.I'(2)$
<i>Glu</i> (2)	$\frac{0.5V_2}{V(1 - B)}$	$\frac{0.5(1 - B + V_2D)I'(2)}{V(1 - B)} = X_5.I'(2)$	$E.X_5.I'(2)$
<i>Glu</i> (3)	As for <i>Glu</i> (2)		
<i>Glu</i> (4)	$\frac{AV_2(1 - \delta)B}{R(1 - B)}$	$\frac{0.5AV_2D.I'(2)}{R(1 - B)} = X_6.I'(2)$	$E.X_6.I'(2)$
<i>Glu</i> (5)	$\frac{AV_2(1 - B + \delta B)}{R(1 - B)}$	$X_6.I'(2)$	$E.X_6.I'(2)$
<i>PP</i> (1)	$\frac{LC_2}{1 - J}$	$\frac{0.5D.I'(1)}{1 - J} = X_7.I'(1)$	$E.X_7.I'(2)$
<i>PP</i> (2)	$\frac{\delta B}{1 - B}$	$\frac{0.5D.I'(2)}{1 - B} = X_8.I'(2)$	$E.X_8.I'(2)$
<i>PP</i> (3)	$\frac{(1 - \delta)B}{1 - B}$	$X_8.I'(2)$	$E.X_8.I'(2)$

C3-pyruvate

In C2-pyruvate substitute C_3 for C_2 , and interchange $I'(1)$ and $I'(2)$; $(1 - \delta B)$ and $1 - B + \delta B$ in *Glu*(4) and *Glu*(5); and δB and $(1 - \delta)B$ in *PP*(2) and *PP*(3).

Table A.2. *Non-sym. case A (label in oxaloacetate is not symmetrized on entering the tricarboxylic acid cycle, because malate does not equilibrate with fumarate)*

Secondary symbols:

$$\gamma = \frac{\alpha - B(2\alpha - 1)}{1 - B(2\alpha - 1)}; \quad I(1) = \frac{V_2(R - \Delta)}{1 - B} \left(\frac{A[1 - B(1 - \gamma)]}{R} + \frac{B(1 - \gamma)}{V} \right); \quad I(2) = \frac{V_2(R - \Delta)}{1 - B} \left(\frac{AB(1 - \gamma)}{R} + \frac{1 - B(1 - \gamma)}{V} \right)$$

All other symbols as in Table A.1.

Turns of cycle $i =$	0	1	2
C1-pyruvate			
<i>Asp</i> (1,4)	$F[1 + PP(1) + C_1]$	0	0
<i>Glu</i> (1)	$\frac{V_2 C_1}{V}$	0	0
<i>PP</i> (1)	$\frac{B[\alpha + (1 - \alpha)C_1]}{1 - \alpha B}$	0	0
C2-pyruvate			
<i>Asp</i> (1,4)	$\frac{F[1 - B(2\alpha - 1)]C_2}{1 - \alpha B}$	$\left(H + \frac{0.5DF}{1 - \alpha B} \right) I(1) = X_9.I(1)$	$E.X_9.I(2)$
<i>Asp</i> (2,3)	$\frac{F}{1 - B}$	$X_3.I(2)$	$E.X_3.I(2)$
<i>Glu</i> (1)	$\frac{V_2 C_2}{V}$	$\frac{0.5I(1)}{V}$	$\frac{0.5E.I(2)}{V}$
<i>Glu</i> (2)	$\frac{BV_2(1 - \gamma)}{V(1 - B)}$	$X_5.I(2)$	$E.X_5.I(2)$
<i>Glu</i> (3)	$\frac{V_2[1 - B(1 - \gamma)]}{V(1 - B)}$	$X_5.I(2)$	$E.X_5.I(2)$
<i>Glu</i> (4)	$\frac{AV_2B(1 - \gamma)}{R(1 - B)}$	$X_6.I(2)$	$E.X_6.I(2)$
<i>Glu</i> (5)	$\frac{AV_2[1 - B(1 - \gamma)]}{R(1 - B)}$	$X_6.I(2)$	$E.X_6.I(2)$
<i>PP</i> (1)	$\frac{(1 - \alpha)BC_2}{1 - \alpha B}$	$\frac{0.5D.I(1)}{1 - \alpha B} = X_{10}.I(1)$	$E.X_{10}.I(2)$
<i>PP</i> (2)	$\frac{\gamma B}{1 - B}$	$X_8.I(2)$	$E.X_8.I(2)$
<i>PP</i> (3)	$\frac{(1 - \gamma)B}{1 - B}$	$X_8.I(2)$	$E.X_8.I(2)$

C3-pyruvate

In C2-pyruvate substitute C_3 for C_2 , and interchange $I(1)$ and $I(2)$; $B(1 - \gamma)$ and $1 - B(1 - \gamma)$; and γB and $(1 - \gamma)B$.

Table A.3. *Non-sym. case B (label in oxaloacetate does not symmetrize on entering the tricarboxylic acid cycle, because malate does not equilibrate with oxaloacetate)*

Secondary symbols:

$$\begin{aligned} B(1) &= V_3/(V_1 + V_6); \quad A(2) = V_3/(V_2 + V_3 - \Delta); \quad D(3) = (V_2 - \Delta)/(V_1 + V_6)(R - V_2); \\ F(10) &= 1 - \alpha B(1); \quad D(4) = (V_2 - \Delta)/(V_2 + V_3 - \Delta)(R - V_2); \quad F(13) = 0.5 D(3)/F(10); \\ F(17) &= D(4) + A(2).F(13); \quad F(6) = V_2/R; \quad F(7) = (R - V_2)F(6); \quad F(2) = B(1)(2\alpha - 1); \\ A(1) &= [\alpha - F(2)]/[1 - F(2)]; \quad F(4) = B(1)/[1 - B(1)]; \quad F(3) = [1 + A(1)F(4)]; \quad F(5) = [1 - A(1)]F(4); \\ D(1) &= F(7)[AF(3) + F(5)]; \quad D(2) = F(7)[AF(5) + F(3)]; \quad F(9) = 0.5(R - V_2)/R; \\ F(8) &= V_2 D(3)/[1 - B(1)]; \quad E(1) = F(9)[(A + 1)F(8) + 1]; \quad F(16) = 0.5 D(3)/[1 - B(1)]; \\ F(19) &= D(4) + 2A(2).F(16); \quad F(18) = 0.5/R \end{aligned}$$

The equations appear different at first sight, as they are presented in a form close to that from which a computer programme has been prepared.

Turns of cycle $i=0$	1	2
C1-pyruvate		
$Asp(1,4)$	$A(2)[1 + PP(1) + C_1]$	0
$Glu(1)$	$\frac{V_2 C_1}{R}$	0
$PP(1)$	$\frac{B(1)[\alpha + (1 - \alpha)C_1]}{1 - \alpha B(1)}$	0
C2-pyruvate		
$Asp(1,4)$	$\frac{A(2)[1 - B(1)(2\alpha - 1)]C_2}{1 - \alpha B(1)}$	$F(17).D(1)$
$Asp(2,3)$	$\frac{A(2)}{1 - B(1)}$	$F(19).D(2)$
$Glu(1)$	$\frac{V_2 C_2}{R}$	$F(18).D(1)$
$Glu(2)$	$\frac{V_2[1 - A(1)]F(4)}{R}$	$F(18)[1 + F(8)]D(2) = X_{11}.D(2)$
$Glu(3)$	$\frac{V_2[1 + A(1)F(4)]}{R}$	$X_{11}.D(2)$
$Glu(4)$	$\frac{A V_2[1 - A(1)]F(4)}{R}$	$\frac{A V_2.F(16).D(2)}{R} = X_{12}.D(2)$
$Glu(5)$	$\frac{A V_2[1 + A(1).F(4)]}{R}$	$X_{12}.D(2)$
$PP(1)$	$\frac{B(1)(1 - \alpha)C_2}{1 - \alpha B(1)}$	$F(13).D(1)$
$PP(2)$	$A(1).F(4)$	$F(16).D(2)$
$PP(3)$	$[1 - A(1)]F(4)$	$F(16).D(2)$

C3-pyruvate

In C2-pyruvate substitute C_3 for C_2 , and interchange $D(1)$ and $D(2)$; $[1 - A(1)]F(4)$ and $1 + A(1)F(4)$; and $A(1)F(4)$ and $[1 - A(1)]F(4)$.

Relative specific radioactivities in the steady state.
For each compound and position of label divide the entry under $i = 2$ by $(1 - E)$ for sym. case or non-sym.

case A, or by $[1 - E(1)]$ for non-sym. case B, and add the result to the entries under $i = 0$ and $i = 1$. Denote by $f(S)$.

For pyruvate as substrate, the specific radioactivity of pyruvate, P , is:

$$P = 1 + PP(1) + PP(2) + PP(3) \quad (\text{A.1})$$

Specific radioactivities relative to pyruvate are given by $f(S)/P$.

For glucose as substrate specific radioactivities can be calculated relative to that of glucose 6-phosphate. For C4-, C5- and C6-glucose as substrate use the $f(S)$ values for C1-, C2- and C3-pyruvate. For the specific radioactivity of pyruvate relative to that of glucose 6-phosphate, if glucose is C(n)-labelled then, for $P(n)$:

$$f(S) = 1 + PP(n) \quad (\text{A.2})$$

For labelling in other positions of pyruvate:

$$f(S) = PP(\text{not } n) \quad (\text{A.3})$$

Then for specifically labelled glucose, specific radioactivities are given by:

$$0.5 V_5 \cdot f(S) / (V_1 + V_6) \quad (\text{A.4})$$

Assuming that C3-, C2- and C1-labelling is the same as C4-, C5- and C6-labelling when $[U-^{14}\text{C}]$ glucose is substrate (see under '2. Redistribution of label' in

the Theory section of the main paper), specific radioactivities are given by:

$$V_5[f_1(S) + f_2(S) + f_3(S)] / 6(V_1 + V_6) \quad (\text{A.5})$$

where f_1 , f_2 and f_3 are the functions from C1-, C2- and C3-pyruvate respectively.

For mixed cases, i.e. partial symmetrization, see under '3. Steady-state theory' in the Theory section of the main paper.

Relative labelling in C4, C5 and C6 of glucose formed from pyruvate is given by the relative values of $PP(1)$, $PP(2)$ and $PP(3)$ (statement 2.1 in the main paper).

Yields of $[^{14}\text{C}]$ glucose, $^{14}\text{CO}_2$ and ^{14}C -labelled 'protein'. The yields are the fractions of the label introduced as pyruvate and utilized in the system that are incorporated into glucose and carbon dioxide and lost as glutamate. They are denoted by $Y_n(\text{Glc})$, $Y_n(\text{CO}_2)$ and $Y_n(\text{Prot})$, where the subscript denotes the position labelled in the original substrate.

$$Y_n(\text{Glc}) = V_6[PP(1) + PP(2) + PP(3)] / V_1 \quad (\text{A.6})$$

$$Y_n(\text{Prot}) = \Delta[Glu(1) + Glu(2) + Glu(3) + Glu(4) + Glu(5)] / V_1 \quad (\text{A.7})$$

$$Y_n(\text{CO}_2) = 1 - Y_n(\text{Glc}) - Y_n(\text{Prot}) \quad (\text{A.8})$$