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The Design of Experiments using Isotopes for the Determination of the Rates of Disposal of Blood-Borne Substrates *in vivo* with Special Reference to Glucose, Ketone Bodies, Free Fatty Acids and Proteins

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1. The two well-known methods of estimating rates of irreversible disposal (R) of blood-borne substrates *in vivo* by isotope experiments involve estimating the specific radioactivity (S) of the substrate in blood either after single intravenous injection of labelled substrate or during its infusion at a constant rate. The value of R is calculated from the S–time curve, usually by assuming: (i) a metabolic steady state with respect to substrate, (ii) the passage of all substrate through the blood, and (iii) the absence of certain types of recycling via blood. 2. In a theoretical investigation we show how experiments can be performed and R calculated from analyses of blood when one or more of the above assumptions is unjustified, by using glucose, ketone bodies, plasma free fatty acids and proteins as examples. In general the methods require single injection procedures, with estimation of the total quantity of label in the substrate in blood and the substrate concentration instead of only S. Such values give estimates of R with standard errors even when only one blood specimen is taken from each of a group of animals, as is convenient when working with small animals or substrates in low concentration, and when the animals are in a non-steady state in which constant infusion procedures are invalid. 3. Similar methods give the fraction of label injected as one compound which passes through another (the isotopic yield). 4. The methods are not always applicable, and cannot be applied to plasma proteins in some pathological conditions. A questionnaire for assessing their applicability is given.

We and some of our colleagues wished to determine rates of utilization of glucose, ketone bodies and plasma free fatty acids in rats to investigate intermediary metabolism and to define pathological states in metabolic terms. We found that the theory underlying such determinations could not be applied without modification. The necessary modifications, applicable also to the study of other substrates, are described here.

The two standard methods of estimating rates involve respectively single intravenous injection or continuous intravenous infusion, at a constant rate, of tracer quantities of the labelled substrate under investigation. Blood samples are taken at intervals from the start of the experiment and the specific radioactivity (S) of the substrate is determined. During infusion of label at rate D_r an equilibrium value of S, S_E, is eventually attained, and the rate, R, is given by:

$$R = D_r/S_E \qquad (1.1)$$

After a single injection of dose, D, of label, the area, A, under the S–t curve to infinity, i.e.

$$A = \int_0^\infty S\,dt \qquad (1.2)$$

gives R by:

$$R = D/A \qquad (1.3)$$

The rate given by these equations is called the rate of irreversible disposal. The concept of irreversibility in this context is discussed by Baker (1969), Baker & Rostami (1969) and Shipley *et al.* (1967) and also, briefly, later in the present paper.

Eqns. (1.1) and (1.3) hold only when four conditions are satisfied.

Condition 1. There are no isotope effects. This assumption is unavoidable, and we also make it.

Condition 2. All the unlabelled compound replacing that utilized must pass through the blood.

Condition 3. The system must be in a steady state, i.e. all rates between pools and into and out of the system, and all pool sizes, must remain constant.

Condition 4. The substrate is not recycled extensively via a metabolite in the course of the experiment.

Condition 2 is not always satisfied for glucose, condition 3 is not satisfied for glucose or plasma free fatty acids in rats because of the ease with which their metabolism is disturbed by handling (Heath & Stoner, 1968; Heath *et al.*, 1971; Besch & Chou, 1971), and condition 4 is not satisfied for the ketone

bodies, 3-hydroxybutyrate and acetoacetate, which are rapidly interconverted *in vivo*. (Estimation of their combined rate of disposal is relatively simple when they are in isotopic equilibrium, but we do not consider this rare case.) Also the usual experimental procedure is unsatisfactory, because taking successive blood samples from animals as small as rats alters their metabolic state.

It is shown that conditions 2 and 4 do not in fact prohibit determination of the disposal rates of these substrates, and that the steady-state condition, condition 3, can be much relaxed by suitable design of experiments. It is also shown how to combine results from different rats, each providing only one blood sample, to determine mean values of disposal rates with estimates of the random errors on them. The methods are applicable to other substrates, such as plasma proteins, and to the estimation of isotopic yields (see below). Examples of use are given in the three following papers (Heath & Corney, 1973; Barton, 1973; Cunningham, 1973).

In the methods devised the only 'pool' that has to be analysed is blood, which is easily sampled and fairly homogeneous. Such methods are referred to as 'model-free'. They are nearly all single-injection methods, as these, unlike constant-infusion methods, can be used under non-steady-state conditions. The basic equations depend, however, upon steady-state theory, which is therefore considered first.

Definitions

Pool: any quantity of a compound in the body that can be regarded as instantaneously mixed in terms of the time-scale of the experiment.

C_i: concentration of substance in the ith pool.

Q_i: quantity of substance in the ith pool.

V_i: volume of ith pool. $Q_i = C_i V_i$.

q_i: quantity of label in the ith pool.

$S_i = q_i/Q_i$ = specific radioactivity of the compound in the ith pool.

R_{ij}: rate of transfer of compound from ith to jth pool.

k_{ij}: rate coefficient for the above process, i.e. $R_{ij} = k_{ij} Q_i$.

R_i: rate of irreversible disposal from ith pool.

k_i: rate coefficient for the above process.

v_{ij}, K_{ij}, v_i and K_i: corresponding generalized rates and rate coefficients.

R: total rate of irreversible disposal, i.e. $\sum R_i$.

I_i: rate of input of unlabelled compound into ith pool.

I: total rate of input, i.e. $\sum I_i$.

D: dose of label in intravenous injection.

D_r: rate of input of label by infusion.

c: coefficient of error, i.e. S.E.M./mean.

A_i: area under S_i–t curve, i.e. $\int_0^\infty S_i dt$ after intravenous injection.

A_{qi}: area under the q_i–t curve, i.e. $\int_0^\infty q_i dt$ after intravenous injection.

When the pool size, Q_i, is constant:

$$A_{qi} = A_i Q_i \qquad (2.1)$$

S_{Ei}: equilibrium value of S_i during constant infusion.

In most cases the pool into which label is injected or infused will be denoted by subscript 1, and be referred to as pool 1. The exceptions are all given in the section on steady-state systems, 'ketone bodies', in which case two pools may be injected into.

Steady-State Systems

General theory

The theory in this section consists of particular applications of that given by Gurpide *et al.* (1963) and Mann & Gurpide (1966). Tait (1963) and Horton & Tait (1966) developed a similar theory.

There are two crucial theorems.

Theorem 1. Any relationship between rates, dose rates (D_r) and equilibrium specific radioactivities (S_{Ei}) which holds during constant infusion holds also after single injection when doses (D) are substituted for dose rates (D_r) and S–t integrals (A_i) for equilibrium specific radioactivities (S_{Ei}) throughout. In symbolic terms, for any two pools i and j:

$$A_i/A_j = S_{Ei}/S_{Ej} \qquad (3.1)$$

The validity of eqn. (3.1) can be seen intuitively by regarding constant infusion as an infinite set of closely spaced intravenous injections. There is an alternative form of it:

$$S_{Ei} = D_r A_i/D \qquad (3.1A)$$

which states that for any and, therefore, every pool in the system S_E and A are related by the same constant, the ratio of the dose rate during constant infusion, D_r, to the dose by single injection, D. This form is obtained (in our symbolism) from eqn. (9) of Gurpide *et al.* (1963) by continuing integration to $t = \infty$ and dividing both sides by Q_i. The theorem requires only that condition 3 holds.

There are two corollaries: (*a*) equations need only be worked out for the constant infusion case and (*b*) constant infusion has no theoretical superiority over single injection even in the steady state. (In the non-steady state constant infusion methods cannot be used.)

Theorem 2. For any pool:

$$S_{Ei} = (\text{net flow of label}) \div (\text{net flow of unlabelled compound}) \qquad (3.2)$$

This theorem is implicit in eqn. (4) of Mann & Gurpide (1966). In the form of eqn. (3.2) it appears self-evident. It implies that it is not necessary for either label or input of unlabelled compound to enter initially the pool sampled for the equation:

$$S_E = D_r/I \qquad (3.3)$$

to hold. It is only necessary that all of both eventually pass through it.

Theorem 3. If one pool receives all its label and compound from another pool then at equilibrium both pools will contain compound at the same specific radioactivity. This holds providing the specific radioactivity of the donor pool remains constant. The theorem is generally regarded as self-evident.

If a substrate is not synthesized in erythrocytes, erythrocytes constitute a receptor pool with respect to plasma, so that during constant infusion S_E is the same in erythrocytes and plasma and after a single injection so is A. Consequently erythrocytes, plasma or whole blood can be analysed at will to give S_E or S. If S is measured then it may be different in erythrocytes and plasma, as found by Heath & Rose (1969) for glucose, but the S–t integrals, i.e. values of A, will be the same. This is true for all the substrates under consideration, but would not be so for lactate, which is synthesized in erythrocytes.

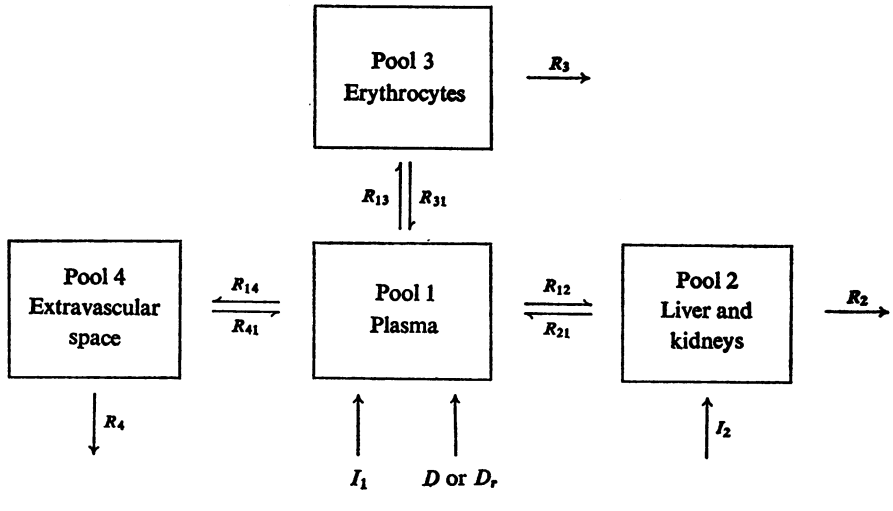
Specific examples

Glucose. Model 1 is a rudimentary model. The pool representing the extrahepatic extravascular space is intended to represent the very large number of pools which must exist; only one is needed for the argument. The representation of the liver and kidneys by one pool is also only an approximation. In the recently fed rat, portal blood should probably be a separate pool containing glucose of lower specific radioactivity than the rest because some unlabelled glucose taken up from the gut is deposited in the liver as glycogen before mixing with the rest of the plasma glucose.

Glucose enters the system in only two ways, via the liver and kidneys by gluconeogenesis and glycogenolysis at rate I_2 and from the gut directly, in effect, into the plasma at rate I_1. Extrahepatic tissues receive label and glucose from the plasma only, so during constant infusion the value of S_E in them will equal the value of S_E in plasma. Therefore, their presence does not affect the calculation of R, which depends only upon the plasma and liver–kidney pools.

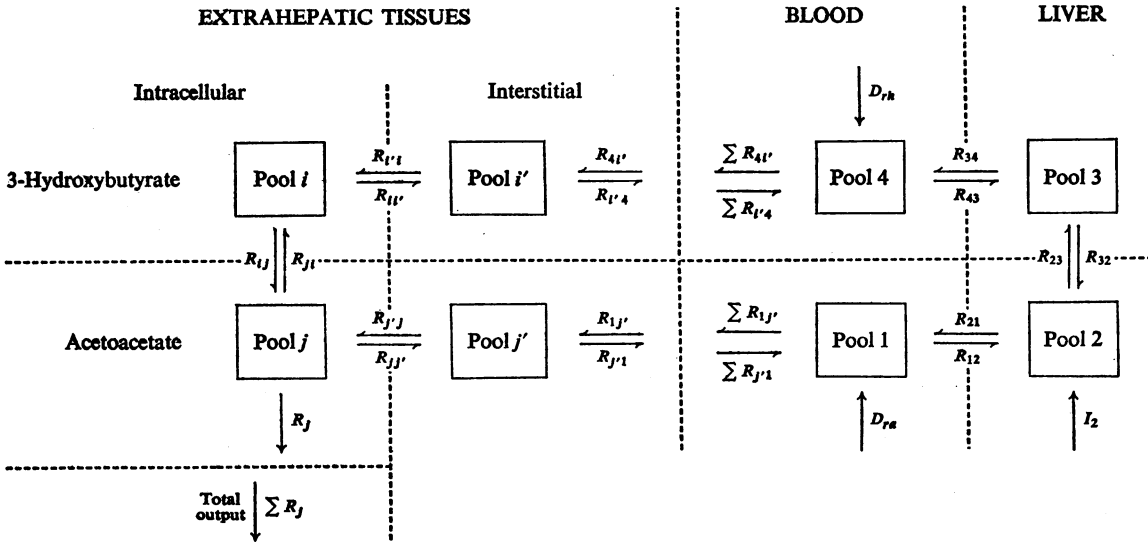
In the recently fed animal gluconeogenesis is nearly zero, so that the liver also receives all its label and glucose from the plasma. (Neglecting the distinction between portal and other blood causes R to be underestimated.) In the starved animal no glucose is absorbed from the gut, but the liver and kidneys secrete glucose and utilize very little, as shown by the near absence of labelling of liver glutamate after [^{14}C]glucose injection (C. J. Threlfall, personal communication), i.e. $I_1 = 0$, $R_2 = 0$. In both cases all the label and all the glucose reach the plasma, and R is given by eqn. (1.3).

In the post-absorptive animal $I_1 = 0$ by definition



Model 1. *Simple pool system for glucose*

For symbolism see the Definitions section. By theorem 3 the erythrocyte pool, pool 3, can be ignored in calculating whole body rates.



Model 2. *Complete model for irreversible disposal of ketone bodies in the steady state*

No separate plasma and erythrocyte pools are required as erythrocytes only receive ketone bodies from plasma.

(no uptake from the gut) but the liver and kidneys utilize glucose (see e.g. Heath & Threlfall, 1968), i.e. $R_2 \neq 0$. Model 1 shows that of the unlabelled input into pool 2, I_2, the fraction $R_2/(R_2+R_{21})$ is lost irreversibly from pool 2, and that the rest, $1-R_2/(R_2+R_{21})$, enters pool 1. Hence the net flow of unlabelled compound into pool 1 is given by:

$$\text{Flow} = I_2[1-R_2/(R_2+R_{21})] = I_2 R_{21}/(R_2+R_{21}) \quad (3.4)$$

As I_2 is the total input, so that $I_2 = R$, eqn. (3.4), with R for I_2, can be substituted into eqn. (3.2) to give:

$$S_{E1} = D_r(R_2+R_{21})/RR_{21} \quad (3.5)$$

On rearranging, and substituting D for D_r and A_1 for S_{E1}:

$$R = D_r(R_2+R_{21})/R_{21}\,S_{E1} = D(1+R_2/R_{21})/A_1 \quad (3.6)$$

Thus R calculated by eqn. (1.3) is too low and must be multiplied by $1+R_2/R_{21}$. For rat liver $R_{21} \approx 400\,\mu$mol/min per 100g body wt. [from the kinetics of glucose transport between plasma and liver (Williams *et al.*, 1968) and the glucose concentration, about 10mM in plasma water]. As R is about $10\,\mu$mol/min per 100g body wt., and only a fraction of this overall rate is accounted for by liver metabolism, R_2/R_{21} is probably less than 0.01. There are not enough data to carry out a similar calculation for the kidney, but as this is a much smaller organ it is not likely to alter the conclusion that eqn. (1.3) would give a result negligibly different from the correct one. Thus in the steady state, R for glucose can be determined from A and eqn. (1.3) with negligible error. How best to measure A is discussed below.

Ketone bodies. These are taken to be acetoacetate and 3-hydroxybutyrate. The complete model is shown as Model 2. Both compounds are synthesized as acetoacetate in the liver at rate I_2, where they are interconverted in the mitochondria. They migrate separately via blood to extrahepatic tissues, where both are utilized as acetoacetate. For references, see Bressler (1963).

The extrahepatic tissues are represented by an indefinite number of pools in groups of four, each group consisting of intra- and extra-cellular pools of acetoacetate and 3-hydroxybutyrate. The total rate of disposal, $\sum R_j$ (Model 2), equals the sum of the net rates of flow of the two ketone bodies from the liver through the blood, i.e.

$$\begin{aligned}\textstyle\sum R_j \equiv R = I_2 &= (R_{21}-R_{12})+(R_{34}-R_{43}) \\ &= \textstyle\sum(R_{1j'}-R_{j'1})+\sum(R_{4i'}-R_{i'4}) \\ &\equiv R_1\,(\text{acetoacetate}) + \\ &\quad R_4\,(3\text{-hydroxybutyrate}) \end{aligned} \quad (3.7)$$

Eqn. (3.7) also defines the rates R, R_1 and R_4.

Mann & Gurpide (1966) show that systems like that in Model 2 can be represented by generalized flow rates. The equivalent model is Model 3(*a*). In their terminology pools 1 and 4 are the 'primary pools', into which label is injected and which are analysed. Each labelled ketone body is infused into a separate group of animals and the quantities shown in Table 1 are determined.

Table 1. *Quantities required and symbolism used in calculating rates of disposal of ketone bodies*

Labelled compound infused	Dose rate	Equilibrium sp. radioactivity	
		Acetoacetate	Hydroxybutyrate
Acetoacetate	D_{ra}	S_{1a}	S_{4a}
3-Hydroxybutyrate	D_{rh}	S_{1h}	S_{4h}

The rate I_1 is the rate of influx into pool 1 of that acetoacetate which has not previously passed (as 3-hydroxybutyrate) through pool 4; I_4 is the corresponding influx of new 3-hydroxybutyrate into pool 4. The sum is the total input of unlabelled material, i.e. I_2 in Model 2, and:

$$I_2 = \sum R_j = R = I_1 + I_4 = v_1 + v_4 \qquad (3.8)$$

As, however, the input into either pool in Model 2 includes material that has already been in the other blood pool neither I_1 nor v_1 equals R_1, nor I_4 nor v_4 equals R_4, although $R_1 + R_4 = v_1 + v_4$.

Equating in Model 3(*a*) input and output of label from each pool in turn:

$$\left.\begin{array}{l}\text{Acetoacetate infusion}\\ (v_1+v_{14})S_{1a}-v_{41}S_{4a}=D_{ra}\\ v_{14}S_{1a}-(v_4+v_{41})S_{4a}=0\\ \text{Hydroxybutyrate infusion}\\ (v_4+v_{41})S_{4h}-v_{14}S_{1h}=D_{rh}\\ v_{41}S_{4h}-(v_1+v_{14})S_{1h}=0\end{array}\right\} \qquad (3.9)$$

Standard methods give v_1, v_4, v_{14} and v_{41} in terms of S values, and hence, by eqn. (3.8):

$$\begin{aligned}R &= v_1 + v_4\\ &= [D_{ra}(S_{4h}-S_{1h}) + D_{rh}(S_{1a}-S_{4a})]/(S_{1a}S_{4h}-S_{1h}S_{4a})\end{aligned} \qquad (3.10)$$

Bates (1971) also gives an equation which simplifies to eqn. (3.10), but it was derived on the assumptions that the interchange of label between blood acetoacetate and blood 3-hydroxybutyrate only took place in the liver, and that the ketone bodies were in equilibrium in the liver. The present derivation is not limited in these ways.

The form of eqn. (3.10) is not, however, the best to use because it is not practicable to estimate all four specific radioactivities in the same animal. In different animals even in the same nutritional state ketone body concentrations are different (Berry *et al.*, 1965; Young & Renold, 1966) and the rates are functions of the concentrations, so that eqn. (3.10) can only give a mean value, and this would only be precise if very large numbers of animals were used. A simple alternative treatment, which also gives R_1 and R_4 separately, and which is suitable when only one blood sample is taken from each animal, is possible by making two assumptions.

1. Each individual rat is in a steady state. Constancy of the average concentration in blood after injection is evidence for this assumption. When it is not true the theory of non-steady-state systems (see below) must be applied.

2. The values of the rate coefficients are the same in different rats, i.e. as, by definition, $R_{ij} = k_{ij}Q_i$, each rate is proportional to pool size. Also by definition, $S_i = q_i/Q_i$, so that:

$$R_{ij}S_i = k_{ij}Q_iS_i = k_{ij}q_i \qquad (3.11)$$

Eqn. (3.11) implies that any equation such as eqn. (3.9) relating rates (R_{ij}, v_{ij}) and specific radioactivities (S_i) will have an analogue of identical form relating rate coefficients (k_{ij}, K_{ij}) and total label in pools (q_i). Values of q_1 and q_4 are determined experimentally instead of values of S_1 and S_4; and calculation gives k_1 and k_4 instead of R_1 and R_4. As $v_1+v_4 = R_1+R_4$, and $v_1 \equiv K_1Q_1$ and $v_4 \equiv K_4Q_4$, the overall disposal rate, R, can be calculated from either of the identities:

$$R = k_1Q_1 + k_4Q_4 = K_1Q_1 + K_4Q_4 \qquad (3.12)$$

The experimental and theoretical steps are as follows.

(*a*) Values of q_{1a}, q_{4a}, q_{1h} and q_{4h} are determined in whole animals. Eqn. (3.9) is transformed as above and solved for K_1, K_4, K_{14} and K_{41}.

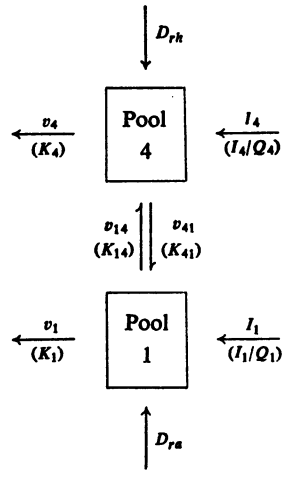
$$\left.\begin{array}{l}K_1 = (D_{ra}q_{4h} - D_{rh}q_{4a})/P\\ K_4 = (D_{rh}q_{1a} - D_{ra}q_{1h})/P\end{array}\right\} \qquad (3.13)$$

$$K_{14} = D_{rh}q_{4a}/P \text{ and } K_{41} = D_{ra}q_{1h}/P \qquad (3.14)$$

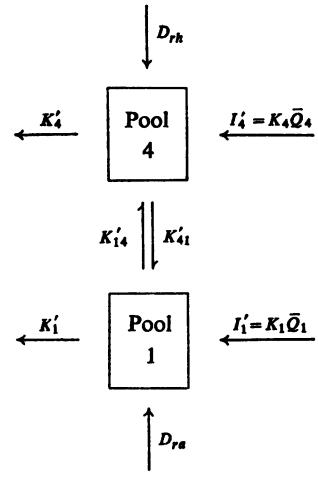
where $P = q_{4h}q_{1a} - q_{1h}q_{4a}$. If only the overall rate, R, is required it can be calculated by substituting eqn. (3.13) into eqn. (3.12).

(*b*) Hepatectomy eliminates transport between pools 1 and 4 via the liver (McGarry *et al.*, 1970). Hepatectomized rats are therefore infused with both carrier ketone bodies at rates I_1' and I_4' (Model 3*b*) to maintain a normal ratio of blood pool sizes, Q_1/Q_4. If extrahepatic rate coefficients are wholly independent of concentration and unaffected by hepatectomy the carrier infusion rates do not matter. In case they are not, it is better to infuse at rates near the mean values of the rates of output from the liver of whole animals.

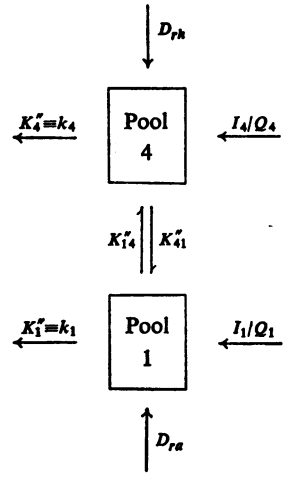
Satisfactory estimates of these rates for this purpose are $K_1\bar{Q}_1$ and $K_4\bar{Q}_4$, where $\bar{Q}$ is the mean value of Q in whole animals. A labelling experiment as in



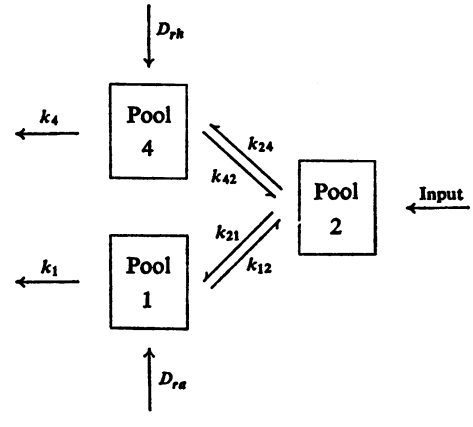
(*a*) Whole body. Rate coefficients are shown in parentheses.



(*b*) After hepatectomy. Carrier infused at rates I_1' and I_4'.



(*c*) Generalized model of liver and blood only.



(*d*) Detailed model of liver and blood, assuming equilibrium in the liver.

Model 3. *Generalized flow models for ketone bodies*

(*a*) above is then carried out to estimate K_{14}' and K_{41}', the generalized rate coefficients for extrahepatic conversion.

(*c*) The transport rates between pools 1 and 4 are the sum of the extrahepatic and hepatic rates. Denoting the coefficients for the latter by K_{14}'' and K_{41}'':

$$K_{14}'' = K_{14} - K_{14}'; \; K_{41}'' = K_{41} - K_{41}' \qquad (3.15)$$

Model 3(*c*) can therefore be set up, representing liver and blood only. With zero extrahepatic exchange K_1'' and K_4'' are identical to k_1 and k_4 respectively. Also, as all unlabelled input must pass through either pool 1 or pool 4 before reaching any extrahepatic pool the unlabelled inputs into these pools must be the same in the liver model, 3(*c*), and the whole animal, model 3(*a*). Hence equating flow of

material into and out of pools 1 and 4 in Models 3(*a*) and 3(*c*) and equating unlabelled inputs in both:

$$\left.\begin{aligned}\text{Pool 1. } &\overbrace{(K_1+K_{14})Q_1-K_{41}Q_4}^{\text{Model 3(a)}}=I_4=\\ &\overbrace{(K_1''+K_{14}'')Q_1-K_{41}''Q_4}^{\text{Model 3(c)}}\\ \text{Pool 4. } &(K_4+K_{41})Q_4-K_{14}Q_1=I_1=\\ &(K_4''+K_{41}'')Q_4-K_{14}''Q_1\end{aligned}\right\} \quad (3.16)$$

Rearranging, equating k_1 with K_1'' and k_4 with K_4'', and substituting from eqn. (3.15) gives:

$$\left.\begin{aligned}k_1&=K_1+K_{14}'-K_{41}'Q_4/Q_1\\ k_4&=K_4+K_{41}'-K_{14}'Q_1/Q_4\end{aligned}\right\} \quad (3.17)$$

whence k_1 and k_4 can be determined, and hence R_1 and R_4 separately by $R_1=k_1Q_1$ and $R_4=k_4Q_4$.

Mann & Gurpide (1966) give relationships between generalized and true rates of transfer, from which the rates of transfer of both ketone bodies between blood and liver can be calculated if the two ketone bodies are in equilibrium in the liver (evidence for this is given by Williamson *et al.*, 1967). They can then be represented by a single pool, pool 2 (Model 3*d*), and the relationships are:

$$\left.\begin{aligned}v_{14}''&=K_{14}''Q_1=R_{24}R_{12}/(R_{21}+R_{24})\\ v_{41}''&=K_{41}''Q_4=R_{42}R_{21}/(R_{21}+R_{24})\end{aligned}\right\} \quad (3.18)$$

Solving, after substituting $R_{12}+R_1$ for R_{21}, $R_{42}+R_4$ for R_{24}, and $k_{ij}Q_i$ for R_{ij} throughout, gives:

$$\left.\begin{aligned}k_{12}&=\frac{(K_{14}-K_{14}')(K_1Q_1+K_4Q_4)}{(K_4+K_{41})Q_4-K_{14}Q_1}\\ k_{42}&=\frac{(K_{41}-K_{41}')(K_1Q_1+K_4Q_4)}{(K_1+K_{14})Q_1-K_{41}Q_4}\end{aligned}\right\} \quad (3.19)$$

It can be seen by dividing the top and bottom of these equations by either Q_1 or Q_4 that the calculated values of k_{12} and k_{42} are independent of absolute values of Q_1 and Q_4 but do depend upon Q_4/Q_1. It can be shown that if the two ketone bodies are not in equilibrium in the liver the value of k_{12} is unchanged but no unambiguous value for k_{42} can be obtained.

The treatment can be modified in three ways. (1) Equations analogous to eqn. (3.10) can be derived and are listed below, with the same equation numbers as before except for the addition of *S*.

$$\left.\begin{aligned}v_1&=(D_{ra}S_{4h}-D_{rh}S_{4a})/F\\ v_4&=(D_{rh}S_{1a}-D_{ra}S_{1h})/F\end{aligned}\right\} \quad (3.13S)$$

$$v_{14}=D_{rh}S_{4a}/F \quad \text{and} \quad v_{41}=D_{ra}S_{1h}/F \quad (3.14S)$$

where $F=S_{4h}S_{1a}-S_{1h}S_{4a}$.

$$v_{14}''=v_{14}-v_{14}' \quad \text{and} \quad v_{41}''=v_{41}-v_{41}' \quad (3.15S)$$

$$\left.\begin{aligned}R_1&=v_1+v_{14}'-v_{41}'\\ R_4&=v_4+v_{41}'-v_{14}'\end{aligned}\right\} \quad (3.17S)$$

$$\left.\begin{aligned}R_{12}&=[(v_{14}-v_{14}')(v_1+v_4)]/(v_4+v_{41}-v_{14})\\ R_{42}&=[(v_{41}-v_{41}')(v_1+v_4)]/(v_1+v_{14}-v_{41})\end{aligned}\right\} \quad (3.19S)$$

Eqn. (3.18) is unchanged. It should be noticed, however, that in estimating v_{14}' and v_{41}' in hepatectomized rats it is now necessary to infuse carrier at exactly the rates R_1 and R_4, which are not known. A practicable alternative is to estimate v_{14}' and v_{41}' at two or more values of C_1 and C_4 obtained by infusing carrier at different rates, and to calculate by interpolation the values of v_{14}' and v_{41}' in any particular intact rat in which C_1 and C_4 are known, and v_1 and v_4 have been estimated.

(2) The equations can be converted into those for the single injection case by substituting A_t for S_t or A_{qt} for q_t, where:

$$A_{qt}=\int_0^\infty q_t\,dt \quad (3.20)$$

In this equation q_t is the instantaneous time-variable value of q in pool i, not an equilibrium value as in eqns. (3.11), (3.13) and (3.14); D is also substituted for D_r throughout.

(3) If rate coefficients are not independent of pool size the equations relating pool label to pool size, e.g. q_{1a} and q_{4a} to Q_1, must be found empirically, and substituted for q in eqns. (3.13) and (3.14). Solution of the equations and standard statistical methods will then give the rate coefficients as functions of pool size. Heath & Corney (1973) used this procedure to determine how the disposal rate of glucose varied with glucose concentration in injured rats. Alternatively one can relate specific radioactivities to concentrations and use eqns. (3.13S) and (3.14S) simultaneously.

Yields of labelled products. The fraction of the label in an injected substrate that passes through a product has been defined as the isotopic yield, Y (Ashby *et al.*, 1965). For instance, in the terms of Model 3(*a*) the yield of plasma [^{14}C]acetoacetate from hydroxy-[^{14}C]butyrate is $v_{41}/(v_{41}+v_4)$. For most purposes 'back-labelling' can be neglected, e.g. the relabelling of 3-hydroxybutyrate from the [^{14}C]acetoacetate produced. [In this instance the results given by Barton (1973) show that only 1.5–3% of the total label in 3-hydroxybutyrate reaches it in this way.] On this assumption two experiments are needed: one to estimate the generalized disposal rate of the product (v_1+v_{14} in the example) from the area under the *S*–*t* curve (A_{1a}) after injection of labelled product; and another to estimate the area under the product *S*–*t* curve after injection of precursor substrate containing D_s units of label. If x units of label enter the product the yield, Y, as defined is x/D_s. These x units can be regarded as a dose of label injected into

the blood in product, to which eqn. (1.3) applies. Hence denoting the general disposal rate of the product by v_p and the area under the product S–t curve after injection of substrate by A_{ps}:

$$v_p = x/A_{ps} = D_s\, Y/A_{ps}$$

or, on rearrangement:

$$Y = A_{ps}\, v_p/D_s \qquad (3.21)$$

In determining the yield of [^{14}C]glucose from [^{14}C]pyruvate the first experiment has already been shown to give R, i.e. $v_p \equiv R_p$ in this instance; and exchange of glucose between liver cells and plasma is so rapid that A_{ps} in liver and plasma are likely always to have very similar values. Consequently, the yield estimated in blood is also in this case very close to the yield in liver. The method of calculation given here is both easier and more precise than that given by Ashby *et al.* (1965).

Values of Y are obtained as fractions of the substrate label injected. For most purposes one needs the value of Y as a fraction of the substrate at risk, i.e. as the fraction of substrate that reaches the relevant organs and that is metabolized there. For example, in normal rats only 60–70% of pyruvate label reaches the liver and kidneys (Heath & Threlfall, 1968) and only this portion can enter glucose. Corrections for label not reaching relevant organs are usually somewhat speculative, and it is preferable when possible to draw conclusions from ratios of yields. This is possible when the two substrates are chemically the same and differ only in the position of labelling. If the same dose, D_s, of two such substrates is injected, there will be one area for each product (A_{ps1} and A_{ps2}) with two general disposal rates (v_{p1} and v_{p2}) and two yields (Y_1 and Y_2). By eqn. (3.21):

$$Y_1/Y_2 = A_{ps1}\, v_{p1}/A_{ps2}\, v_{p2} \qquad (3.22)$$

In some instances v_{p1} and v_{p2} may be very similar, e.g. for glucose labelled in the two ways given by [2-^{14}C]- and [3-^{14}C]-pyruvate. Then eqn. (3.22) simplifies to:

$$Y_1/Y_2 = A_{ps1}/A_{ps2} \qquad (3.23)$$

and neither v_{p1} nor v_{p2} need be determined.

Non-Steady-State Systems

General principles

The following treatment is relevant to many particular cases, but is not completely general. The pool receiving label and analysed is designated pool 1, and is usually plasma, but may be blood. The instantaneous rate of transport from pool i to pool j is $k_{ij}Q_i$ (see the Definitions section) and k_{ij} may vary.

Rates or rate coefficients may vary during an experiment because R alters, owing to changes in the animal's activity, or because I varies under hormonal influences or with nutritional state etc., or because of changes in the volume of pools or their accessibility. These causes are illustrated by the specific examples given below.

Constancy of specific radioactivity during constant infusion is not evidence that the system is in a steady state. The requirements for constant specific radioactivity are only that D_r and I should be constant (or in a constant ratio), and that all input, I, reaches pool 1 either directly or via pools associated with constant rate coefficients, i.e. if k_{ij} or k_{ji} vary, then $I_i = 0$. Thus suppose that during constant infusion a system has reached a steady state, and that there are two pools, 1 and 2, of which pool 2 receives label and compound only from pool 1. Then $S_{E1} = S_{E2}$ [see text near eqn. (3.3)]. Suppose now that D_r and I are unchanged, but that R is changed, altering the size of pool 2. This cannot affect S_{E2}, which still receives label and compound solely from pool 1. The argument establishes that constant-infusion experiments are unreliable in non-steady-state systems because I and R are not equal.

In the non-steady state, even after a single injection, determination of S_1 is not enough. It is usually sufficient to determine q_1, the total label in the plasma pool of substrate. As stated in the section above the quantity corresponding to A_1 is the q_1–t integral, A_{q1}. By definition:

$$q_1 = S_1\, C_1\, V_1 = S_1\, Q_1 \qquad (4.1)$$

In many experiments V_1, the plasma volume, is constant, and in these q_1 is proportional to $S_1 C_1$.

There are two important cases: that in which the rate coefficients are constant and that in which they vary.

Rate coefficients constant. In general after a single injection the quantity of label in any pool i is related to the quantities in all other pools j ($j \neq i$) by an equation of the form:

$$\mathrm{d}q_i/\mathrm{d}t = -(k_i + \textstyle\sum k_{ij})q_i + \sum k_{ji} q_j \qquad (4.2)$$

When the rate coefficients have constant values such simultaneous equations can be solved to give each q_i value as an explicit function of time which does not contain I, R, or pool sizes. This fact provides one test to decide whether a particular case falls into the class under discussion. At any one time after injection q_1 has a specific value, the same in different animals, so, by eqn. (4.1), S_1 varies inversely as Q_1. If, therefore, in animals with different plasma pool sizes this relationship is found, or, at least, that q_1 at any one time is much less variable than S_1, it is likely that the rate coefficients are constant. Results for ketone bodies in rats meet this criterion (Barton, 1973); and an additional one is required to test whether the animals are in a non-steady state. This is certainly the case when the mean plasma pool sizes change

significantly with time, as then I and R cannot be equal.

The properties of the q–t relationship imply that the q–t integral, A_q, is also independent of changes in I, R or pool size. Tait & Burstein (1964) reached substantially the same conclusion in their discussion of steroid dynamics. Consequently A_q has a particular value, and the problem is to relate A_q to R. Now R is given by:

$$R = k_1 Q_1 + \sum k_i Q_i \quad (4.3)$$

This leads to a simple solution when, and only when:

$$Q_i = \kappa_i Q_1 \quad (4.4)$$

where κ_i is a constant for all i values, i.e. the quantities in all the pools from which there is irreversible loss (at rate R_i from pool i) vary proportionally to Q_1, the plasma pool. This is so when all rates of transfer linking pool i to pool 1 are fast relative to R_i. In specific cases the likelihood of this can be assessed. Substituting eqn. (4.4) in eqn. (4.3) gives:

$$R = Q_1 \sum k_i \kappa_i + k_1 Q_1 = (k_1 + \sum k_i \kappa_i) Q_1 = k Q_1 \quad (4.5)$$

where k is the composite constant: $k_1 + \sum k_i \kappa_i$. The problem reduces to finding k.

For any particular non-steady-state system with time-invariant rate coefficients there is a particular steady-state analogue with the identical values of coefficients, namely the system towards which the non-steady-state system is tending and which it will eventually reach if the rate coefficients remain constant. Consequently, this system will have the same value of A_{q1}. It can be shown that in the steady state for pool 1 there is always a relationship between R and A_1 (not A_{q1}) of the form:

$$R = MD/A_1 \quad (4.6)$$

where M is a constant. When conditions 2 and 4 are satisfied $M = 1$, and eqn. (4.6) simplifies to eqn. (1.3). When it is not, M is some function of the rate coefficients. Eqn. (3.6) and those for ketone body disposal can be expressed in this form. Dividing eqn. (4.6) by Q_1, putting $A_1/Q_1 = A_{q1}$ and $R/Q_1 = k$ from eqn. (4.5):

$$R/Q_1 = k = MD/A_{q1} \quad (4.7)$$

Although the equation relating k and A_{q1} has been derived for the steady state, as A_{q1} and M are unchanged in the non-steady state as long as the rate coefficients remain unchanged, it also applies in this non-steady state, where it gives R by eqn. (4.5) at any time at which Q_1 is measured. The value of R is at all times proportional to Q_1, and the precision of its estimate therefore depends upon the precision with which Q_1 is estimated, unlike in the steady state, in which A_1 can be estimated and R calculated without estimating Q_1. Note, however, that R is not sensitive to systematic errors in Q_1, such as may arise from using average values of V_1 in the calculation. This may be seen by substituting $C_1 V_1$ for Q_1 and $V_1 \int_0^\infty C_1 S_1 \, dt$ for A_{q1} throughout the argument from eqn. (4.4) on, when it will be found that V_1 cancels out.

Rate coefficients vary. Eqn. (4.3) still holds. We only consider cases where eqns. (4.4) and (4.5) also hold, although, as $k_1 + \sum k_i \kappa_i$ may vary it will be represented differently by k_v. When all the dose of label, D, enters pool 1:

$$D = \int_0^\infty k_v q_1 \, dt \quad (4.8)$$

providing all the unlabelled input also passes through this pool. [If not, there is an additional factor M, as in eqn. (4.7), but this also may not remain constant. If it does it can be introduced as a factor on the right-hand side; if it does not the system is too complex for the present treatment.] Suppose a value, denoted by $\bar{k}_v$, is found from an experimental value of A_{q1} by using the equation:

$$\bar{k}_v = D/A_{q1} = \int_0^\infty k_v q_1 \, dt \Big/ \int_0^\infty q_1 \, dt \quad (4.9)$$

The value of $\bar{k}_v$ is within the range of the values that k_v takes up during the experiment, so that if we could find the time the two coincided, t_c, the rate at that time could be calculated exactly from Q_1 at that time by:

$$R = \bar{k}_v Q_1(t_c) \quad (4.10)$$

For applications, see under 'Glucose' (below).

Specific cases

The value of Q_1 must always be determined. In normal animals Q_1 varies as C_1, the plasma concentration. This is not so when plasma volumes change in some pathological states.

Scheme 1 provides a framework which enables one to decide how individual cases should be treated. It is applied below to the substrates under consideration.

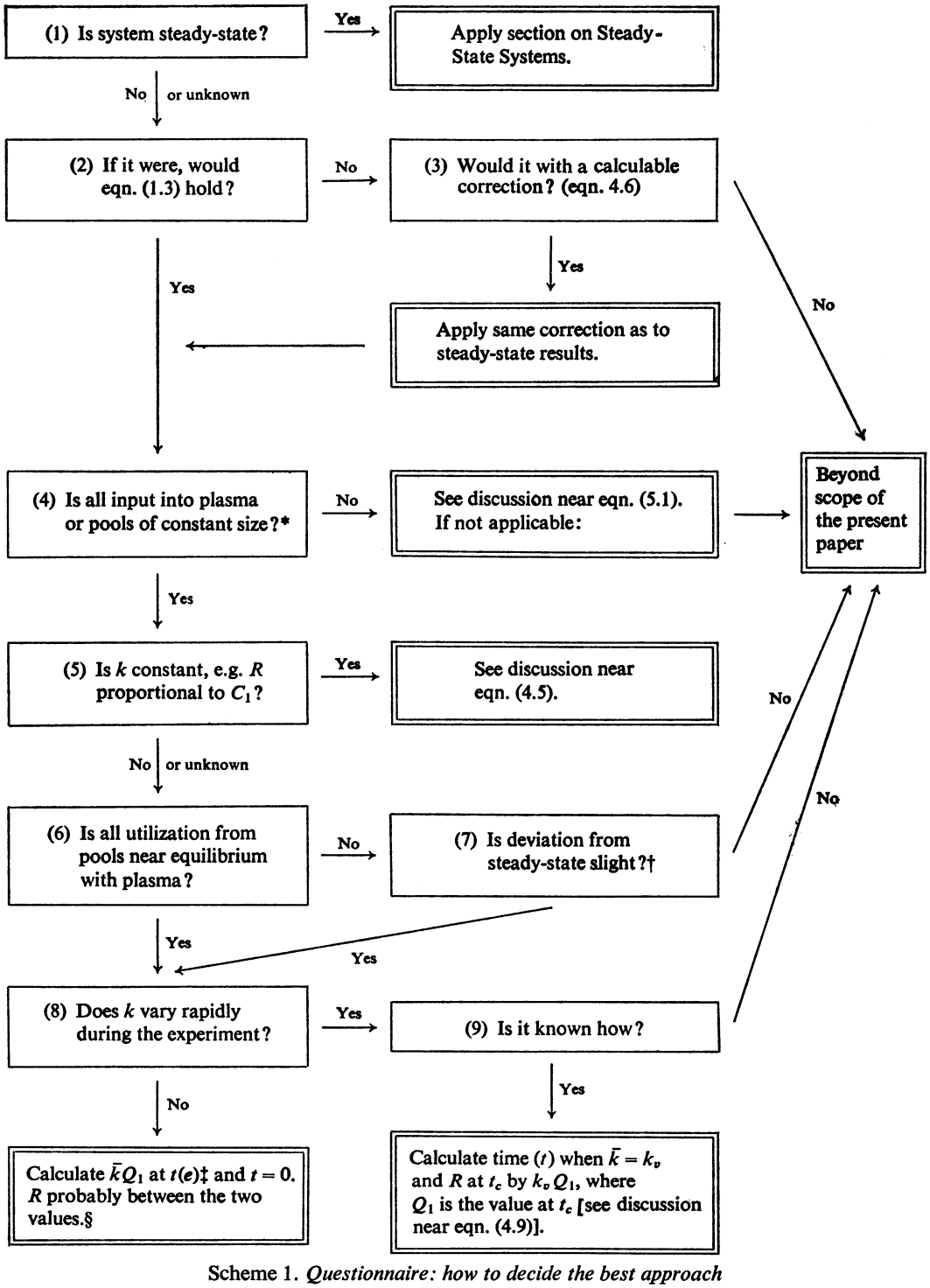
Plasma free fatty acids in rats. The answers to the questionnaire (Scheme 1) are as follows.

(1) No. There are rapid variations, often caused by the experimental procedure.

(2) Yes, as only plasma free fatty acids are being considered.

(3) Not applicable.

(4) No. Free fatty acids are released in the cells of adipose tissue by lipolysis, and the free fatty acid pools in these cells presumably vary with its rate. If the usual assumption that there is little exchange of label with these pools is correct then the input is in effect directly into plasma. Otherwise the system is outside the scope of the present paper.



Scheme 1. *Questionnaire: how to decide the best approach*

For further details see the text.

* k_{1j} and k_{j1} constant if $I_1 \neq 0$ implies pools of constant size.

† If so, then k and Q_1 both change slowly, and the range of values given in the block after question (8) will be small. In effect, eqn. (1.3) is nearly correct. 'Yes' to this question implies a 'no' to question (8).

‡ $t(e)$ is t at end of experiment.

§ Fig. 1 suggests that the correct value of Q_1 will usually be close to that at the time at the centroid of the q–t curve.

(5) Yes (Baker & Rostami, 1969; Boberg, 1969).

The system is in a non-steady state with constant rate coefficients. As all input is directly into the plasma, M in eqn. (4.7) is unity, and:

$$k = D/A_{q1} \qquad (5.1)$$

and R at any time is given by substituting eqn. (5.1) in eqn. (4.5).

Plasma proteins in normal animals and man. The answers to the questionnaire (Scheme 1) are as follows.

(1) Presumably no, as an experiment takes several days for fibrinogen and several weeks for albumin and globulins. But for albumin and globulin the experiments are so long that circadian variations can probably be ignored, a mean value of R being obtained.

(2) Yes. See (4) and (6) below.

(3) Not applicable.

(4) Yes, input directly into plasma from liver except for γ-globulin (see e.g. McFarlane, 1964).

(5) Not known.

(6) Losses direct from plasma (Rossing, 1967; Regoeczi, 1970).

(7) Not applicable.

(8) No. This follows from the finding that R by ^{131}I-labelled protein disappearance agrees well with I estimated over very much shorter time-periods by experiments with $H^{14}CO_3^-$ (McFarlane *et al.*, 1965).

The procedure is as for glucose below with even less likelihood of errors.

Plasma proteins in injured animals and man. The answers to the questionnaire (Scheme 1) are as follows.

(1) No. Very rapid changes in pool sizes have been reported, and q_1 may vary periodically (Heath, 1970, quoting J. W. L. Davies, personal communication.)

(2) For burn injury no, as there are losses from the burnt surface (Davies *et al.*, 1969). For other injuries, probably yes.

(3) For burns, no. No systematic correction can be applied.

(4) Yes, into plasma (except some globulins).

(5) Unknown.

(6) No. Protein may be sequestered for long periods in an oedematous region near a fracture and then return (Mouridsen, 1969). A short experiment would give a higher $\bar{k}_v$, as loss to such a region would appear irreversible; a longer one a lower $\bar{k}_v$.

(7) No.

It is impossible to analyse this system from plasma results only. Values of R can be calculated by combining estimation of label in plasma proteins with whole body counting and/or counts of label excreted in urine and in exudates (Davies *et al.*, 1969). The protein synthetic rate, I, cannot be found by these means, as R and I cannot be equated in the non-steady state, and may be very different if at the time of injection there is a big extravascular pool that later contracts to normal size.

Ketone bodies in rats. The answers to the questionnaire (Scheme 1) are as follows.

(1) Yes. Plasma pool sizes do not alter significantly with time during the short time of an experiment (Barton, 1973). Hence steady-state theory could be applied, as already shown.

Glucose in rats. Strictly the system: glucose $\rightleftharpoons$ gluconeogenic substrates should be treated as a whole like the ketone body system, but because of the number of substrates it appears to be impracticable to carry out the requisite experiments. Consequently our treatment, like that of most workers, ignores recycling. This is equivalent to regarding some part of the conversion of glucose into, e.g., lactate as reversible, so that the disposal rate obtained is lower than if recycling was allowed for. The answers to the questionnaire (Scheme 1) are as follows.

(1) No. The value of R varies 3-fold during 24h of starvation (Heath & Corney, 1973) and is affected by handling (see below).

(2) Yes (for starved and fed rats) or no (for post-absorptive rats).

(3) Yes for post-absorptive rats; see eqn. (3.6).

(4) Effectively yes, as input is either directly into plasma or into the liver and kidney pools; although the latter may change, they are too small to affect the results.

(5) No. The value of R varies more than C_1 (Heath & Corney, 1973), consequently $\bar{k}_v$ must vary.

(6) Dependent upon nutritional state. See answer to (8) below.

(7) Yes, usually.

(8) It may during feeding, when deposition of glycogen is rapid. Then, as up to 30% of the extravascular space equilibrates fairly slowly with plasma glucose (Ashby *et al.*, 1965), the answer to question (9) becomes 'no', and a value for R cannot be found reliably. In other nutritional states rapid changes in C_1 only occur as the result of handling. Handling raises the value of C_1, but probably mostly before injection (Besch & Chou, 1971; Heath & Corney, 1973). Shortly after injection the value of C_1 falls, but too slowly to introduce errors.

Allowing for the variation of C_1 with time in calculating R involves expressing k_v as a function of time, and substituting this function in eqn. (4.9). Three lines of reasoning suggest putting k_v proportional to C_1^n (where the value of n depends upon the causes of the change in C_1) and calculating the time variation of k_v from that of C_1^n, which is found experimentally.

(*a*) The variations of k_v with nutritional state and severity of injury approximate to simple functions of C_1 (Heath & Corney, 1973), which can be represented by C_1^n.

(*b*) Depocas (1964) infused rats with glucose and [^{14}C]glucose and estimated values of R at various values of C_1. Excluding the three highest, very unphysiological values of C_1 and the unexpectedly high value of C_1 in starved, anaesthetized rats, one finds k_v, i.e. R/C_1, to be proportional to C_1^n where $n = 1.21 \pm 0.23$ (17 degrees of freedom; S.E.M.). de Bodo *et al.* (1959) obtained results in dogs given short massive infusions of glucose consistent with $n = 0.5$ to 1.5. (Prolonged infusions in man and dog tend to give values of n of about zero, as found e.g. by Reichard *et al.*, 1958.)

(*c*) The C_1–t curve after mild stress is often a much less extreme version of that found after a glucose-tolerance test (Heath & Corney, 1973), i.e. the initial rise of C_1 is followed by a decrease through a minimum and a return to normal values. Atkins (1971*a*) has analysed such curves theoretically, and gives a brief review. In outline, his analysis is consistent with the view that k_v is proportional to plasma insulin concentration, which rises rapidly very shortly after C_1 rises, and then decreases rather more slowly than C_1. It varies roughly with C_1^n ($n>1$) until the minimum in C_1, but then continues to decrease towards its resting value. This suggests that when C_1–t curves are of this type the k_v–C_1^n relationship should not be calculated from values of C_1 after the minimum.

From the suggested relationship two values of R can be calculated, $R(t_c)$ and $R(e)$. The first is given by eqn. (4.10). Its calculation depends upon calculating $Q_1(t_c)$, and hence upon t_c. As methods for calculating t_c are closely related to those for A_{q1}, the subject of the next section, they are given at its end. $R(e)$ is the value of R to which R tends by the end of the experiment. Variation of k_v as C_1^n implies that:

$$k_v(t) = k_v(\infty)[C_1(t)]^n/[C_1(\infty)]^n \qquad (5.2)$$

which gives in eqns. (4.8) and (4.9), after rearrangement:

$$k_v(\infty) = D[C_1(\infty)]^n/\int_0^\infty [C_1(t)]^n q_1 \,dt \qquad (5.3)$$

By the end of the experiment the animal should have reached a resting state, so that the 'end' experimental value of C_1, $C_1(e)$, can be substituted for $C_1(\infty)$. The value of $k_v(\infty)$ is also better regarded as a value to which k_v is tending by the end of the experiment than as an 'infinite-time' value. There is also evidence, summarized above, that $n \simeq 1$. Modifying eqn. (5.3) to incorporate these changes gives:

$$k_v(e) = D[C_1(e)]/\int_0^\infty C_1(t) q_1 \,dt \qquad (5.4)$$

and:

$$R(e) = k_v(e) \cdot Q_1(e) = k_v(e) \cdot C_1(e) \cdot V_1(e) \qquad (5.5)$$

Experimental Design and Statistics

Choice of method

Although special circumstances may favour constant-infusion methods, single-injection experiments are usually better for several reasons. Some are mentioned by Searle & Cavalieri (1972), who demonstrated experimentally the equivalence of the two procedures for determining R for lactate in man.

The reasons for preferring single-injection methods are as follows.

(i) Constant-infusion procedures are only viable if the system is in a steady state (see the section on Non-steady-state systems, 'General principles').

(ii) Although R is defined as the rate of irreversible disposal, there is not always a clear distinction between reversible and irreversible losses. For example, after injection into recently fed animals [^{14}C]glucose undergoes slow recycling via glycogen: [^{14}C]glucose → [^{14}C]glycogen → [^{14}C]glucose.

In short experiments most [^{14}C]glucose is 'irreversibly' removed, but in long experiments most will eventually return. In a single-injection experiment slow recycling shows up as a 'tail' of low specific radioactivity, and can usually be eliminated by extrapolating the previous part of the exponential curve to zero [see eqn. (6.4) and nearby discussion]. During constant infusion, however, a slow upward drift in specific radioactivity could imply either recycling or a change in metabolic state. Even if it is known to be due to recycling, extrapolation of S_1 to the appropriate value of S_{E1} is less certain than extrapolating S_1 or q_1 to zero.

(iii) Single-injection experiments are shorter. Not only does this clarify the meaning of R, but it means that, if the system is such that values of R can only be calculated by using steady-state theory, an approximation to a steady state is more likely to be achieved than during a longer constant-infusion experiment.

(iv) Single-injection experiments are simpler, require fewer samples, and do not need an infusion cannula, which may disturb the animal.

Estimation of A_1 or A_{q1}

There are two types of curve: the disappearance curve, when the compound analysed is that injected (Fig. 1); and the product curve, when it is different (Fig. 2). There are also two types of experiment: in one the blood is sampled at intervals to give A_{q1} for an individual animal; in the other an animal is killed for each specimen of blood. The statistics differ. In the first type the coefficient of deviation of q_1 is often time-invariant, i.e. there is a constant fractional error on q_1 over all the q_1–t curve, this depending on the precision of the analytical procedures. In the second at any one time the values of q_1 will show biological variance in addition, and the

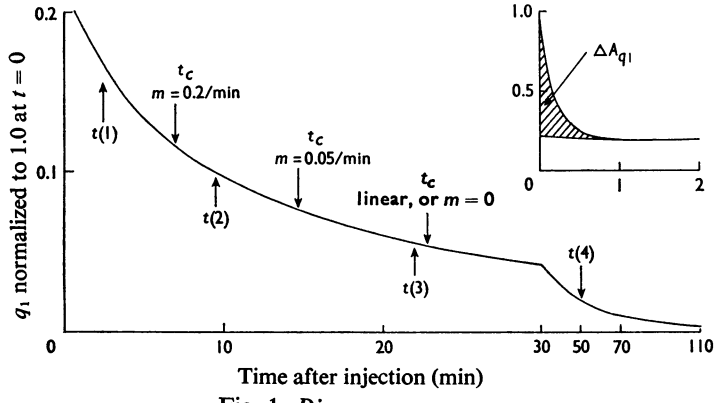


Fig. 1. *Disappearance curve*

The curve shown is the exponential representation (eqn. 6.3) of the label in blood after intravenous injection of [^{14}C]glucose into recently fed rats. The early part of the curve is shown inset, with the additional area, ΔA_{q1}, shaded. In the main figure the optimal sampling times, $t(1) - t(4)$, are shown, and the coincidence times, t_c, for plausible values of m in eqn. (6.11) and for a linear variation of C_1 with t (eqn. 6.12). As $m \rightarrow 0, t_c$ tends to the 'linear' value.

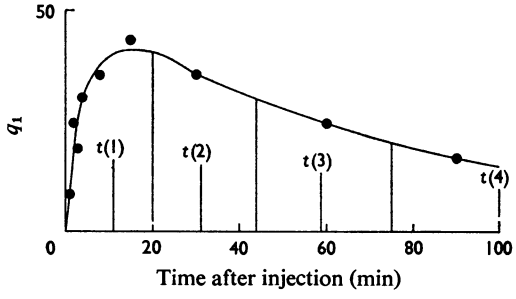


Fig. 2. *Product curve,* [^{14}C]*glucose from* [^{14}C]*acetate*

The area under the curve was divided into five parts, the last four (only 2½ shown) being equal, with centroids at $t(2)$–$t(5)$. The first sub-area is not equal to the rest. See text for the choice of its sampling time, $t(1)$.

coefficient of error of q_1 (S.E.M. of $q_1 \div$ mean q_1) must be found experimentally.

Several authors have chosen to estimate A_1 by finding the best multi-exponential fit to their results, and integrating it analytically (see, e.g., Matthews, 1957; Nosslin, 1964; Baker *et al.*, 1959). The procedure is equally applicable to determining A_{q1}, but has several disadvantages. It requires values of q_1 at many times to evaluate the parameters; the best times for estimating the parameters are not the best for estimating A_{q1}; the values of the parameters are often very sensitive to small experimental errors (see, e.g., Atkins, 1971*b*); and the errors in A_{q1} are not readily calculable from the errors in the parameters because the latter are highly correlated. The approach given below has already been shown to overcome these difficulties in some cases (Corney & Heath, 1970).

The basic approach consists of dividing the area into n contiguous areas, $A(1)$ to $A(n)$, within each of which a value of q_1 is determined at some particular time, t. Thus n values of q_1, $q_1(1)$ to $q_1(n)$, are determined at times $t(1)$ to $t(n)$. Suppose that the shape of the q_1–t curve has been sketched fairly precisely in preliminary experiments. From this sketch a set of factors can be calculated such that $A(i) = f(i)q(i)$. Consequently the total area in a new experiment in which $q(1) \ldots q(n)$ are determined is given by:

$$A_{q1} = \sum_1^n f(i)q(i) \qquad (6.1)$$

If the error on $q(i)$ is $c(i)q(i)$, then the random error on A_{q1} is given by:

$$e_A^2 = \sum_1^n [c(i)f(i)q(i)]^2 = \sum_1^n [c(i)A(i)]^2 \qquad (6.2)$$

Two errors have been neglected: that from systematic errors in the sketched curve, which was only drawn from a small sample of points, and that from differences in the shape of the curve from animal to animal. Consideration of actual curves shows how far such errors can be assessed or neglected.

Disappearance curves. A specific example, [^{14}C]-glucose disappearance from the blood of recently fed rats as determined in these laboratories (Fig. 1), is used to illustrate principles. It is convenient for this purpose to represent the curve in the multiexponential form:

$$q_1 = 0.782e^{-6.6t} + 0.087e^{-0.24t} + 0.129e^{-0.04t} + 0.002e^{-0.002t} \qquad (6.3)$$

The contributions to A_{q1} made by the successive terms are given by:

$$A_{q1} = \int_0^\infty q_1\,dt = 0.782/6.6 + 0.087/0.24 + 0.129/0.04 + 0.002/0.002 \equiv 0.118 + 0.363 + 3.225 + 1.000 = 4.706 \quad (6.4)$$

The aim is to determine A_{q1} without including the 'tail' caused by recycling, as this part of the area [the last term in eqn. (6.4)] cannot be accurately assessed. It is best eliminated by truncating the experiment, in this instance at 50–60min after injection. For [^{14}C]glucose and labelled albumin and fibrinogen the remainder of the curve falls into two parts, a relatively very rapid fall in q_1, representing reversible loss to extravascular space [the first term in eqn. (6.3)], and a much slower subsequent fall [the two central terms in eqn. (6.3)]. The main area is associated with the latter, and will be denoted by A'_{q1}. There is an additional small area under the early part of the curve, ΔA_{q1} (Fig. 1, inset), which with A'_{q1} makes up A_{q1}. The value of ΔA_{q1} can be determined on a few rats and added as a correction to A'_{q1}. For labelled free fatty acids and ketone bodies the loss to extravascular space is mostly irreversible and the early part of the area is too large to add as a correction. In these cases the whole area, A_{q1}, must be determined as described below for A'_{q1}.

A preliminary experiment on a very few animals reveals, in our experience, the outline of the main area, and at what time after injection the curve should be truncated to eliminate the 'tail'. Determination of A'_{q1} then proceeds as follows. The preliminary estimate of the main area is used to find optimal sampling times. The area is divided perpendicularly to the time axis into n equal areas, $A(1)$ to $A(n)$, by any convenient means such as calculation from a smoothed equation or plotting the curve, cutting out and weighing. The centroid of each is found, and the times at the centroids are the best sampling times. An experiment consists of estimating q_1 at those times. The values, $q(1)$ to $q(n)$ (omitting subscript '1') are then related by joining each two adjacent values by a single exponential function:

$$q(i+1) = q(i)\cdot e^{-g(i,i+1)[t(i+1)-t(i)]} \quad (6.5)$$

(In this and the following equations symbols in parentheses indicate sample numbers in sequence and are not multipliers. Those in square brackets are multipliers.) The values of $g(i, i+1)$ are given by:

$$g(i,i+1)[t(i+1)-t(i)] = \ln[q(i)/q(i+1)] \quad (6.6)$$

The first exponential coefficient is used to extrapolate q to zero time, when its value, $q(0)$, is given by:

$$q(0) = q(1)\cdot \exp[g(1,2)\cdot t(1)] \quad (6.7)$$

For [^{14}C]glucose and labelled plasma proteins four sampling times only are required to decrease systematic errors in A'_{q1} to below 2% (Corney & Heath, 1970). Those for fed rats are shown in Fig. 1. The value of A'_{q1} is given by:

$$A'_{q1} = [q(0)-q(2)]/g(1,2) + [q(2)-q(3)]/g(2,3) + q(3)/g(3,4) \quad (6.8)$$

When, also, the coefficient of error of q_1, namely c_1, is the same at all four sampling times, that of A'_{q1}, namely c'_{q1}, is given by:

$$c'_{q1} = c_1/2 \quad (6.9)$$

Errors of up to 20% in choosing sampling times have little effect. Such errors can be estimated from the individual terms in eqn. (6.8), which for perfectly chosen times should be in the proportion 3:2:3.

When c_1 varies with time eqn. (6.2) is applied, by using the experimental values of c_1 at each time, i.e. $c_1(i)$ is the coefficient of error of $q(i)$ at $t(i)$. The area $A(i)$ is taken to consist of half of that between $t(i-1)$ and $t(i)$ plus half of that between $t(i)$ and $t(i+1)$, except at the ends, where $A(1)$ begins at zero time and A(last) goes to infinity. In practice this procedure slightly underestimates the error on A_{q1} if the values of $c(i)$ are very variable.

Cunningham (1973) shows that for labelled free fatty acids five or six points are required. In general, for n points:

$$A'_{q1} = \frac{q(0)-q(2)}{g(1,2)} + \sum_{2}^{(n-2)} \frac{q(i)-q(i+1)}{g(i,i+1)} + \frac{q(n-1)}{g(n-1,n)} \quad (6.10)$$

Product curves. A typical product curve such as Fig. 2 showing [^{14}C]glucose from [1-^{14}C]acetate in the starved rat [there is, of course, no net synthesis of glucose from acetate, although label is transferred (Krebs *et al.*, 1966)] falls into two parts: a multi-exponential 'tail' to which the above procedures apply, and an early, non-exponential part. In the preliminary results of Fig. 2 the q_1–t curve was exponential from 20min after injection, and the area under it could be estimated without systematic error in various ways in later experiments. The method shown involves subdivision into four equal areas, with sampling at their centroids, at 31, 59, 100 and 185min after injection. The last point involves a big extrapolation from the results of the preliminary experiment, and may need revision later. The best that can be done with the early part of the curve is to sketch it in, and to determine the area $A(1)$ under the sketched curve. An arbitrary time $t(1)$ is then chosen during this period, and $A(1)$ is related to the value of $q(1)$ at this time by: $A(1) = q(1)f(1)$. For example, for $t(1) = 11$min, $f(1) = 17.3$. (This part of the curve could be subdivided, but there is no need in this instance.) In later experiments $q(1)$ would always be

determined at 11 min, and multiplied by 17.3 to give $A(1)$. The value of $A(1)$ contains, besides the error proportional to that in $q(1)$, a random error from differences in $f(1)$ from rat to rat and a systematic error from incorrect sketching of the curve. These errors are unknown but they are limited by plausible ways of joining the experimental points. In this instance it seems unlikely that $f(1)$ could be more than 5% in error. As $A(1)$ is under a fifth of A_{q1} the error introduced into A_{q1} is very small, 2% or less.

The subjective element in estimating A_{q1} for product curves does not affect ratios of areas in those cases when the product curves are similar in shape, as, e.g., of [^{14}C]glucose from [2-^{14}C]- and [3-^{14}C]-pyruvate or from [1-^{14}C]acetate and [1-^{14}C]octanoate, as the systematic errors alter both areas by equal fractions.

Coincidence times, t_c. As already stated [just above eqn. (5.2)] it is assumed that k_v varies as C_1^n. Two important cases assume that C_1 decreases exponentially with time to a constant value, $C_1(\infty)$, that is:

$$C_1(t) = C_1(\infty) + \beta e^{-mt} \tag{6.11}$$

or varies linearly with time:

$$C_1(t) = C_1(0) + \beta' t \tag{6.12}$$

where β and β' are constants. Substitution of eqn. (6.11) or (6.12) in eqn. (5.2) followed by binomial expansion and rearrangement gives, when β and β' are small compared with $C_1(\infty)$ or $C_1(0)$:

$$k_v(t) = k_v(\infty) + \alpha e^{-mt} \tag{6.13}$$

$$k_v(t) = k_v(0) + \alpha' t \tag{6.14}$$

Substituting eqn. (6.13) into eqn. (4.9) gives:

$$\bar{k}_v = \int_0^\infty [k_v(\infty) + \alpha e^{-mt}] q_1 \, dt \Big/ \int_0^\infty q_1 \, dt$$

$$= k_v(\infty) + \alpha \int_0^\infty e^{-mt} q_1 \, dt \Big/ \int_0^\infty q_1 \, dt$$

$$\equiv k_v(\infty) + \alpha e^{-mt_c} \tag{6.15}$$

Comparing coefficients of α in eqn. (6.15), rearranging, and writing A_{q1} for $\int_0^\infty q_1 \, dt$ gives:

$$t_c = \frac{1}{m} \ln \frac{A_{q1}}{\int_0^\infty e^{-mt} q_1 \, dt} \tag{6.16}$$

Similar reasoning for the linear case gives:

$$t_c = \int_0^\infty t q_1 \, dt / A_{q1} \tag{6.17}$$

If q_1 is expressed as a multi-exponential function:

$$q_1 = \sum B_j e^{-g_j t} \tag{6.18}$$

then the integrals in eqns. (6.16) and (6.17) are given by $\sum B_j/(g_j+m)$ and $\sum B_j/g_j^2$ respectively. These forms are convenient for theoretical purposes. Their use shows that the values of t_c do not vary much over likely ranges of m (Fig. 1); and that for linear variations in C_1 the value of t_c is independent of the slope of the C_1–t curve. For practical purposes it is better to avoid the explicit use of multi-exponential functions for the reasons already given, and to calculate directly from the results. A treatment similar to that given in eqns. (6.5)–(6.8) gives values of the integrals for n successive values of q_1 by:

$$\int_0^\infty e^{-mt} q_1 \, dt = \frac{q(0) - q(2) \cdot e^{-mt(2)}}{g(1,2) + m} + \sum_2^{(n-2)} \frac{q(i) \cdot e^{-mt(i)} - q(i+1) \cdot e^{-mt(i+1)}}{g(i, i+1) + m} + \frac{q(n-1) \cdot e^{-mt(n-1)}}{g(n-1, n) + m} \tag{6.19}$$

$$\int_0^\infty t q_1 \, dt = \frac{q(0) - q(2)[1 + g(1,2) \cdot t(2)]}{[g(1,2)]^2} + \sum_2^{(n-2)} \frac{q(i)[1 + g(i,i+1) \cdot t(i)] - q(i+1)[1 + g(i,i+1) \cdot t(i+1)]}{[g(i,i+1)]^2} + \frac{q(n-1)[1 + g(n-1,n) \cdot t(n-1)]}{[g(n-1,n)]^2} \tag{6.20}$$

These functions are readily evaluated by a programmable desk-top computer.

Most C_1–t curves can be represented by eqn. (6.11) or (6.12), but some are irregular. In such a case there is no way of estimating t_c, and the best that can be done is to estimate the value of R with a weighted mean value of Q_1. A suitable weighting is provided by:

$$Q_1(t_c) = \sum Q_1(i) A(i) / A_{q1} \tag{6.21}$$

where $Q_1(i)$ is the value of Q_1 at the ith sampling time associated with the area $A(i)$.

Precision

Certain of the procedures involve approximations. The likely magnitudes of the systematic errors in values of R caused by them are as follows.

(1) Eqn. (4.5) is an approximation in the non-steady state. Errors from this source cannot be investigated experimentally, but mathematical simulation showed that in the case of glucose, probably the most sensitive of those for which the

methods of this paper are recommended, they would rarely exceed 2% and never 4%.

(2) The interpolation technique for estimating areas is reliable to within about 2% (Corney & Heath, 1970).

(3) As the concentration-dependence of k_v is usually not known exactly, estimates of t_c (immediately preceding section) may be in error. The effects are hard to assess. For glucose errors in values of R from this cause appear to be $\leqslant 2\%$ (Heath & Corney, 1973).

(4) The optimal times of sampling are difficult to assess when there is extensive recycling of label. This is probably the major source of error. It is in most instances open to experimental investigation by, e.g., double-labelling experiments or experiments in which the redistribution of label is studied.

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