Disequilibrium in the Malate Dehydrogenase Reaction in Rat Liver Mitochondria *in vivo*

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1. When [2-¹⁴C]pyruvate is injected into rats the C3-position of liver glutamate becomes more heavily labelled than the C2-position, thus establishing that oxaloacetate and fumarate are not in equilibrium in rat liver mitochondria *in vivo*. The amount of disequilibrium was shown to be simply related to the value that the C3-label/C2-label ratio would have were no label recycled. This ratio, *z*, was calculated for post-absorptive rats in environmental temperatures of 20° and 30°C from determinations of the distribution of label within glutamate 1, 3 and 10 min after intravenous injection of [2-¹⁴C]pyruvate. The values of *z* (best estimate and range) were 1.65 (1.60–1.69) in rats at 20°C and 2.43 (2.23–2.63) in rats at 30°C. These values of *z* imply the following rates of interconversion in mitochondria of fumarate and oxaloacetate (in terms of the oxaloacetate → citrate flux, *R*) in rats at 20°C:

and in rats at 30°C:

2. The kinetic parameters of malate dehydrogenase and fumarate hydratase and the intra-mitochondrial concentrations of NAD⁺ and NADH under (as far as could be judged) conditions *in vivo* were collated. From them and the best estimates of *R* now available were calculated the rates of interconversion of fumarate, malate and oxaloacetate required to give the found values of *z*. These rates showed that the fumarate hydratase reaction was nearly in equilibrium, but that the malate dehydrogenase reaction was considerably out of equilibrium. The calculations also led to the following conclusions. 3. In livers of rats at 20° and 30°C mitochondrial malate concentrations were respectively about 5 and 1.5 times mean cellular concentrations. 4. Mitochondrial oxaloacetate concentrations were less than 0.2 of the mean cellular concentrations. They were also only 0.65 and 0.55 of the equilibrium concentrations for the malate dehydrogenase reaction in rats at 20° and 30°C respectively. 5. Malate dehydrogenase activity was low because of the very low oxaloacetate concentrations in the mitochondria and the very small fraction of the enzyme complexed with NAD⁺, i.e. in each direction one substrate concentration was very sub-optimal.

Heath and Threlfall (1968) pointed out that the unequal labelling of the C2- and C3-positions* in the glutamate separated from hydrolysates of protein from the livers of rats treated with C2-alanine (Freedman *et al.*, 1960) or C2-pyruvate (Koeppe *et al.*, 1959) showed that oxaloacetate and fumarate were not in equilibrium in rat liver mitochondria *in vivo*. This follows from the way in which label is distributed (Scheme 1). The relevant product from

* C_{*n*}, where *n* is an integer, is used for *n*-¹⁴C-labelled material to conform with Heath (1968) and Heath & Threlfall (1968).

C2-alanine, C2-lactate or C2-pyruvate is C2-oxaloacetate. If this is converted via the citrate cycle directly into glutamate, without prior conversion into fumarate, only the C3-position of glutamate becomes labelled during the first passage of the label round the citrate cycle. If, however, C2-oxaloacetate is converted first into fumarate, which becomes symmetrically C2,3-labelled, glutamate becomes equally labelled in the C2- and C3-positions during the first passage of label round the cycle. During second and subsequent passages of label round the cycle the C2- and C3-positions in glutamate become equally

Scheme 1. Non-symmetrized and symmetrized pathways for the labelling of glutamate from C2-pyruvate

labelled. There are thus two pathways, with nearly all the citrate-cycle intermediates in common; however, one contains pools of fumarate and perhaps malate, but the other does not. Heath (1968) called them the 'sym.' and 'non-sym.' pathways according to whether label was or was not symmetrized by passage through fumarate. A simple representation of the scheme is as follows:

where r_n and r_s are the rates along the non-sym. and sym. pathways respectively, which together add up to the rate of conversion of oxaloacetate into citrate (R), and where r_1 is the rate of conversion of oxaloacetate into fumarate.

We have obtained (in post-absorptive rats in the environmental temperatures 20° and 30°C) much more precise estimates of the relative rates along the two pathways, i.e. of r_n/r_s , than were available from pre-existing information, and have shown that these rates require the malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37) reaction to be out of equilibrium much more than the fumarate hydrat-

ase reaction (L-malate hydro-lyase, EC 4.2.1.2). The reasoning behind the methods adopted is outlined below.

The r_n/r_s ratio is simply related to measurable quantities only in an ideal case, namely that in which the pools of fumarate and malate are negligible, so that label takes the same time to pass from oxaloacetate to glutamate along each pathway, and in

which no label passes more than once round the citrate cycle. In this case Scheme 1 shows that (brackets denote concentrations):

$$[\text{C3-label}] = \text{constant} \times (r_n + 0.5r_s) \quad (1)$$

$$[\text{C2-label}] = \text{constant} \times 0.5r_s \quad (2)$$

Dividing eqn. (1) by eqn. (2):

$$[\text{C3-label}]/[\text{C2-label}] = z = (r_n + 0.5r_s)/0.5r_s$$

or:

$$r_n/r_s = (z-1)/2 \quad (3)$$

In eqn. (3) z is the value of the C3-label/C2-label ratio in the absence of recycling and hold-up in

fumarate and malate. The experimental values of the C3-label/C2-label ratio are affected by both factors. The effects of hold-up in malate and fumarate can be shown to be small and can be allowed for. Recycling can be allowed for if the distribution of label in glutamate is determined after such short times that a negligible amount of label has passed more than twice round the cycle, and if C5- and C1-label are also estimated, to enable the effects of the second passage to be calculated. We therefore determined the total distribution of label within liver glutamate 1, 3 and 10 min after intravenous injection of C2-pyruvate.

The relative rates of flux through the citrate cycle, R , and of conversion of oxaloacetate into fumarate, r_1 , can be related to r_n/r_s , and hence, from the values of R in the literature, r_1 can be calculated. For the simple scheme already shown:

$$R/r_1 = r_n/r_s \quad (4)$$

More complex and realistic schemes are analysed in the Discussion section. The main uncertainty lies in the value of R . The rates of the forward and backward reactions between fumarate and oxaloacetate calculated in this way are those required to explain the inequality in C2- and C3-glutamate labelling.

To decide whether the overall disequilibrium was due to disequilibrium in the malate dehydrogenase reaction or in the fumarate hydratase reaction or in both we have collated the properties of the two enzymes from the literature and have calculated the kinetics of the fumarate-malate-oxaloacetate system from the concentrations of the enzymes and their substrates in liver mitochondria *in vivo*. The results indicated that the malate dehydrogenase reaction was very much further from equilibrium than the fumarate hydratase one.

Experimental

Materials

Chemicals and enzymes. Sodium C2-pyruvate (31.7 mCi/mmol), sodium C1-butyrate, C1-propionate, C1-acetate and C2-acetate, and L-[U- ^{14}C]- and DL-C1-glutamic acid were bought from The Radiochemical Centre, Amersham, Bucks., U.K. DL-C2-Glutamate was bought from Calbiochem, London W.1, U.K. and DL-C3,4-glutamate from Schwarz Bioresearch, New York, U.S.A. Immediately before injection C2-pyruvate was dissolved in 0.9% NaCl, and adjusted to pH 7 with Na_2CO_3 .

Glutamate dehydrogenase [L-glutamate-NAD oxidoreductase (deaminating), EC 1.4.1.2] was bought from Boehringer Corp. (London) Ltd., London W.5, U.K.

Animal techniques. Male albino Wistar rats of the Porton strain (231–254 g) fed on M.R.C. diet 41B (Bruce & Parkes, 1956) were used in the post-

absorptive state (Heath & Threlfall, 1968). Each rat was injected via a tail vein with about 20 μCi of C2-pyruvate in 0.2 ml of 0.9% NaCl. It was stunned by a blow on the head at a set time after injection, and the liver was rapidly removed and dropped into liquid N_2 . The whole process took about 10 s, but after the liver was touched only about 2–4 s elapsed before it was dropped into the liquid N_2 . All concentrations are in terms of wet weight of liver.

Methods

Estimation of glutamate. Glutamate in aqueous solution was estimated enzymically with glutamate dehydrogenase essentially as described by Bergmeyer (1968) by using a Carey recording spectrophotometer.

Radioactivity measurements. For all measurements except of $^{14}\text{CO}_2$ about 1 ml of an aqueous solution was weighed and Cab-O-Sil scintillator fluid (15 ml) was added [700 ml of toluene, 2100 ml of dioxan, 2100 ml of 2-ethoxyethanol, 35 g of 2,5-diphenyloxazole, 280 g of naphthalene and 325 g of Cab-O-Sil (Packard Instrument Co., La Grange, Ill., U.S.A.)]. This suspending fluid was chosen after it was discovered that several 'solvents', such as those described by Bruno & Christian (1961), precipitated glutamate on to the walls of the vessel, even though only μg amounts were present, with a variable (3–15%) decrease in counting efficiency. [Bray's (1960) solution was later found to be more satisfactory than these fluids but was no better than Cab-O-Sil.]

Samples were counted in a Packard Tri-Carb series 4000 liquid scintillation spectrometer. Counts were normalized to constant efficiency by a means of a [^{14}C]toluene standard.

Extraction of glutamate from the liver. Glutamate was extracted and subjected to ion-exchange chromatography as described by Heath *et al.* (1971). After this stage and in all later stages samples were taken for the determination of the specific radioactivity of the glutamate.

As further purification altered the specific radioactivity by a few % all glutamate specimens were chromatographed as 16 cm strips on 3 MM paper (Whatman) for 44 h at 25°C in butan-1-ol-acetic acid-water (4:1:1, by vol.) (Reed, 1950). The ends of the glutamate bands were located with ninhydrin treatment (Gaitonde, 1962), and the glutamate was eluted with water and further purified by passage through Dowex AG-50 and subsequent elution, as described by Heath *et al.* (1971).

To test for purity four glutamate specimens were subjected to two more purification procedures; their specific radioactivities were compared before and after these procedures. (a) Electrophoresis (Biserte *et al.*, 1960). (Specific radioactivity before)/(specific radioactivity after) = 0.985 ± 0.022 (S.E.M.). (b) Chromatography on Whatman no. 1 paper with acetone-water

(2/1, v/v) for 5 h. (Specific radioactivity before)/(specific radioactivity after) = 1.033 ± 0.048 (S.E.M.). As considerable further purification is likely to have taken place during the separations involved in estimating the distribution of label within glutamate, the separation achieved before these tests was judged adequate.

In a few instances glutamine was also separated from liver extracts as described by Heath *et al.* (1971), and its specific radioactivity was determined.

Isotope dilution. To obtain crystalline glutamate each aqueous extract of glutamate, purified as described above, was added to 400 mg of L-glutamic acid in a beaker, then the volume was made up to 15–20 ml with water, and the glutamate was dissolved by boiling. As soon as the material had dissolved completely ethanol (100 ml) was added, and the mixture was left overnight at 4°C for the glutamate to crystallize. The glutamate was filtered on a sintered crucible, then washed first with ethanol–water (4/1, v/v), then ethanol, and finally ethanol–ether (1/3, v/v) before being dried at 100°C for 10 min. The specifically labelled glutamic acids used to test the methods were similarly diluted to 0.25 μ Ci/mmol.

Degradation of glutamic acid. The method used was essentially that described by Phares (1951) and Mosbach *et al.* (1951). The complete degradation was routinely done with between 1.2 and 2.0 mmol of glutamic acid, the mean yield of purified product at each stage being recorded in Table 2. The following modifications of the original method were used.

(1) The pH of the glutamic acid solution was adjusted to between 6.5 and 7.0 before addition of chloramine-T (Koeppe & Hill, 1955), and the reaction was done in air. The recommended N₂ atmosphere was found to make no substantial difference to the yield of the final product, butyric acid.

(2) The crude butyric acid from the steam distillate of the Wolff–Kishner reduction mixture was purified by partition chromatography on acid-treated silicic acid (Sigma, 100 mesh) as described by Mueller *et al.* (1958), scaled up to 50 g of silicic acid–sulphuric acid mixture/column. The fractions containing butyric acid were neutralized with 0.02 M-NaOH, then they were combined, and a portion was examined by g.l.c. Samples were always found to contain less than 0.1% of impurity (propionic acid). The propionic acid obtained from the Schmidt degradation of butyric acid was similarly purified. G.l.c. was performed on a single column Perkin–Elmer model 800 Chromatograph, equipped with a flame-ionization detector. Acids were resolved at 150°C with N₂ carrier gas on a stainless-steel column (2 m $\times$ 3.2 mm outside diameter) packed with 5% FFAP on 80–100 mesh AW–DMCS Chromosorb G.

(3) The ¹⁴CO₂ liberated from the acids by the Schmidt degradation was absorbed in ethanolamine (0.25 ml) in 2-ethoxyethanol (10 ml), and samples

(3 ml) were taken for determination as BaCO₃ and for counting directly in toluene scintillator. Quantitative precipitation of BaCO₃ from the ethanolamine–2-ethoxyethanol solution was achieved by heating the 3 ml sample with 15 ml of 0.4 M-BaCl₂ in a boiling-water bath for 10 min. The precipitate was filtered off (after being left overnight under protection from atmospheric CO₂) on a weighed glass sinter (porosity 4), then it was washed with water and ethanol–diethyl ether (1:1, v/v), and finally dried at 100°C for 15 min.

(4) C1-label was estimated as ¹⁴CO₂ after release by ninhydrin (van Slyke *et al.*, 1941). Various ways of collecting the ¹⁴CO₂ were investigated. Those involving transfer of CO₂ by diffusion either under reduced or at normal pressures never gave complete recoveries in our hands except when non-volatile absorbents were used, e.g. NaOH; and then for reliable counting it was necessary to release the ¹⁴CO₂ by acid treatment and absorb it in ethanolamine. This double transfer was a source of error and was slow. Forced distillation under reduced pressure in the apparatus shown in Fig. 1 gave reliable recoveries quickly. To glutamic acid (about 20 mg weighed precisely) in tube A, ninhydrin (150 mg), a few anti-bumping granules, 1 drop of octanol (anti-foaming agent) and 1 M-orthophosphoric acid (3 ml) were added. Tube B contained 2-ethoxyethanol (3 ml) and ethanolamine (0.15 ml). The apparatus was assembled with the tap (C) open, it was then evacuated by a filter pump. The tap and clip (D) were then closed,

Fig. 1. Apparatus for the determination of C1-label in glutamate

For an explanation of the way this apparatus is used, see the text.

and the apparatus was detached from the vacuum line and both tubes were immersed in a 5-litre beaker of boiling water. After exactly 10 min the apparatus was lifted out of the boiling water and tilted so that the central arm was about 20° to the horizontal upward from tube A, and the central tap was opened. (It was advisable to start with both tubes warm to avoid splash-over.) The heater was removed from the beaker and tube A was immersed in the water and left there for exactly 10 min to allow the CO₂ and water to distil into tube B. The apparatus was removed from the beaker, a little dioxan was placed in the open end of the rubber tube, and the clip was opened to release the vacuum. The liquid in tube B was transferred with washings of scintillator fluid (15 ml of a mixture of toluene, 0.7 litre; dioxan, 2.1 litres; 2-ethoxyethanol, 0.7 litre; 2,5-diphenyloxazole, 35 g; and naphthalene, 280 g) to a scintillation vial. The ¹⁴C radioactivity was counted and the efficiency was determined as described above.

The method was tested by estimations on three types of crystalline specimen: on L-[U-¹⁴C]glutamic acid diluted with L-glutamic acid; on DL-[1-¹⁴C]glutamic acid diluted with L-glutamic acid; and on biological samples prepared and diluted as described above. The 1-¹⁴C-label from [U-¹⁴C]glutamic acid constituted 0.199 ± 0.004 ($\pm$ s.d., $n = 16$) of the total (theory = 0.200); and that from [1-¹⁴C]glutamic acid constituted 0.983 ± 0.019 ($\pm$ s.d., $n = 5$) of the total (theory = 1.000). Duplicated analyses of biological specimens differed on the average by 2.3% ($n = 7$).

Results

The method for measuring the distribution of label within glutamate involves conversion of glutamate into butyrate, with the loss of the C1-carbon, and the stepwise degradation of butyrate to propionate, acetate and methylamine, each time again with the loss of C1-carbon, which is measured. As C1-butyrate derives from C5-glutamate, the degradation gives the labelling in glutamate in the order: C1, C5, C4, C3, C2. The method was checked, first by analysing specifically labelled aliphatic acids (Table 1) and secondly by analysing glutamate specifically labelled

in different positions (Table 2), and was precise and specific.

The distribution of label within liver glutamate from rats given C2-pyruvate is shown in Table 3. The sum of the specific radioactivities in the five positions was very close to the overall specific radioactivity, averaging $100.7 \pm 3.1\%$ (s.d.).

At two times after injection of rats in a 20°C environment replicate experiments were done. Although there were considerable differences in C1- and C5-label, discussed below, the C3-label/C2-label ratio was fairly constant at each time, with an overall coefficient of deviation of 0.041 ($n = 6$), i.e. 4.1%. This is also an estimate of the standard error of a single determination.

Specific radioactivities of liver glutamate were also determined, as were some specific radioactivities of glutamine (Table 4).

Discussion

The patterns of labelling were much as expected (Table 3). The C3-label/C2-label ratio decreased with time after injection because of the redistribution of label on recycling (Scheme 1). The relative increase in C1-label was also evidence of recycling. The three ways in which label can reach C1 all involve passage of label at least once round the citrate cycle: via C5-oxoglutarate; via C2-oxoglutarate; or via ¹⁴CO₂ fixation. In the last case ¹⁴CO₂ is only released after label has been round the cycle more than one full turn. Some label appeared in the C1-position very soon (within 1 min) after injection. It has been found (D. F. Heath, unpublished work) that much of the label that reaches 2-oxoglutarate does not enter glutamate but passes directly on round the cycle. This portion of the label can reach 2-oxoglutarate on the second time round very quickly, as the pools in the pathway are all small. Some of this label can then enter glutamate and give the recycled labelling observed.

Table 4 shows that glutamine contained substantial amounts of label. As glutamine and glutamate are in equilibrium, the glutamine label had a buffering effect on glutamate label. This explains in part why the distribution of label within glutamate altered rather little between 3 and 10 min after injection.

Table 1. Recovery of ¹⁴C from specifically labelled carboxylic acids

Results are given as % $\pm$ s.d. with the numbers of samples in parentheses. '0' values are results of analyses

Labelled acid	Carbon 1	Carbon 2	Carbon 3	Carbon 4
CH ₃ -[CH ₂] ₂ - ¹⁴ CO ₂ H	100.6 $\pm$ 2.4 (6)	0	0	0
CH ₃ -CH ₂ - ¹⁴ CO ₂ H	101.2 $\pm$ 1.5 (5)	0	0	—
CH ₃ - ¹⁴ CO ₂ H	99.7 $\pm$ 3.7 (6)	0	—	—
¹⁴ CH ₃ -CO ₂ H	0	99.1 $\pm$ 1.3 (5)	—	—

Table 2. *Distribution of label within specifically labelled glutamic acids*

The percentage found in each position is shown. The 'yield per C atom' is the fraction of the total label in that position in glutamate actually recovered as $^{14}\text{CO}_2$. C1-label was determined separately.

	C2-Glutamic acid	C3,4-Glutamic acid	C(U)-Glutamic acid	Yield per C atom
C1	0.02	0.01	20.0	100%
C2	99.2	0.01	19.5	22%
C3	0.02	50.6	19.6	27%
C4	0.19	49.9	20.6	35%
C5	0.03	0.02	19.2	54%
Total	99.4	100.5	98.9	

Table 3. *Distribution of label within liver glutamate from post-absorptive rats in environmental temperatures of 20° and 30°C*

For details see the text. The fraction of the total label in each carbon position is shown.

Time after injection (min) ...	No. of rat ...	20°C environment									30°C environment		
		1				3	10				1	3	10
		1	2	3	4	5	6	7	8	9	10	11	12
Carbon 1		0.018	0.040	0.103	0.027	0.089	0.217	0.247	0.216	0.224	0.039	0.077	0.192
2		0.128	0.264	0.221	0.266	0.258	0.294	0.308	0.308	0.304	0.102	0.249	0.281
3		0.206	0.444	0.381	0.425	0.338	0.358	0.367	0.402	0.394	0.247	0.426	0.357
4		0.002	0.004	0.006	0.003	0.010	0.006	0.012	0.006	0.006	0.034	0.011	0.010
5		0.652	0.298	0.288	0.244	0.292	0.107	0.133	0.073	0.104	0.603	0.212	0.149
C3-label/C2-label		1.61	1.68	1.72	1.60	1.31	1.22	1.19	1.31	1.30	2.42	1.71	1.27

Relative rates along the sym. and non-sym. pathways

To use eqn. (3) to evaluate r_n/r_s , the experimental values of the C3-label/C2-label ratio in glutamate must be corrected for recycling and for the time-lag in labelling by the sym. pathway caused by passage of label through the fumarate and malate pools.

Correction for the time-lag depends on three factors: the rate of rotation of the citrate cycle; the quantities of malate and fumarate in the liver; and the fractions of these quantities that are in the sym. pathway.

(1) The rate of rotation of the citrate cycle in rats at 20°C will be taken as $1.25 \mu\text{mol/min per g}$, which is a recent estimate by Sauer *et al.* (1970) that is within the range suggested by Heath & Threlfall (1968).

(2) The total malate pool is $0.24 \mu\text{mol/g}$ in rats at 20°C that were like ours in colony, nutritional state etc. (Threlfall, 1970). The equilibrium ratio of [malate]/[fumarate] is about 5 at 37°C and pH 7.5 (Bock & Alberty, 1953). We show below that there is

no reason to suppose that malate and fumarate are significantly out of equilibrium. Consequently, the pools can be regarded kinetically as one pool of $1.2 \times 0.24 = 0.29 \mu\text{mol/g}$.

(3) Anderson *et al.* (1971) concluded that in perfused livers mitochondrial malate only equilibrated slowly with malate in the cytosol, whereas Chappell (1968) argued that equilibration should be rapid. Rognstad (1970) found evidence for rapid equilibration in kidney cortex slices. As these views conflict, we have considered two extreme cases. (a) When all of the liver malate and fumarate is in the symmetric pathway. (b) When only the mitochondrial malate and fumarate pool is in the pathway, and this pool is as small as possible. The mitochondrial malate concentration is variably higher than the concentration in the cytosol (see, e.g., Anderson *et al.*, 1971), so the smallest pool is that with equal concentrations. Then, as the mitochondrial water is about 9% of the cell water (Williamson *et al.*, 1969), about 9% of the

Table 4. *Specific radioactivities of glutamate and glutamine in rat liver*

The no. of each rat is the same as in Table 3.

		$10^{-3} \times$ Specific radioactivity (c.p.m./ μ mol)											
		1					3		10				
		1	2	3	4	10	5	11	6	7	8	9	12
	No. of rat ...	3.0	5.1	1.4	4.8	2.5	6.8	4.2	5.2	6.0	5.6	6.4	5.9
Glutamate		—	—	—	—	—	—	—	—	3.3	4.8	2.3	—
Glutamine		—	—	—	—	—	—	—	—	—	—	—	—

combined pool, only $0.03 \mu\text{mol/g}$, would be in the symmetric pathway. With a citrate-cycle flux of $1.25 \mu\text{mol/min per g}$ such a pool would delay labelling by the negligible amount of 2s. If the whole pool is in the pathway, however, the delay would not be negligible.

The methods of correcting for recycling in both cases are described in Appendix A. The results for rat no. 3 (Table 3) were omitted as the high apparent C1-labelling in this specimen was inconsistent with numerous other experiments (D. F. Heath, unpublished work) as well as the rest of the present series. It may have been caused by contamination with label volatile from acid.

For rats at 20°C the small malate+fumarate pool gave $z = 1.69 \pm 0.05$ (3) and the large pool gave $z = 1.60$. For rats at 30°C the values of z were 2.63 and 2.23 respectively. The mean values 1.65 and 2.43 will be taken for the rats at 20° and 30°C respectively.

It is shown below that the malate+fumarate pool in the mitochondria is probably considerably above 9% of the cellular malate+fumarate, at least in rats at 20°C , so the higher estimate in each case is probably too high. If, as is likely, some label had been recycled more than once by 3min after injection the lower estimate would be too low. Hence the mean values are probably very nearly correct. On substitution of these mean values in eqn. (3) they give $r_n/r_s = 0.33$ for rats at 20°C and $r_n/r_s = 0.72$ for rats at 30°C .

Heath & Threlfall (1968) give an estimate of r_n/r_s (0.43–1.2) from the results given by Freedman *et al.* (1960). Their results were obtained by analysing glutamate from protein, which had, in effect, continuously sampled the $[^{14}\text{C}]$ glutamate over the hour from the time of injection to the time of killing. The computation was very complex, and the estimate only rough, because of the very extensive recycling. The present method involves much simpler computations, and brackets the value of r_n/r_s much more closely. The advantages of taking samples at short times, emphasized by Heath & Threlfall (1968), have been fully borne out in practice.

Rates in the oxaloacetate-malate-fumarate system

To find how disequilibrium was distributed between the malate dehydrogenase and fumarate hydratase reactions and to find values for the rates, the following procedure was adopted. (1) On the assumption that only the malate dehydrogenase reaction was in disequilibrium preliminary estimates of the rates of the forward and backward reactions between malate and oxaloacetate were calculated.

(2) The intramitochondrial malate concentration required to give these rates was calculated from the properties of the enzyme *in vitro* and the conditions *in vivo*. This was the preliminary estimate of the malate concentration.

(3) The preliminary estimate of the malate concentration was used to calculate preliminary estimates of the forward and backward rates of the fumarate hydratase reaction. These rates showed that the fumarate hydratase reaction was slightly out of equilibrium, and consequently that the preliminary estimates of the rates obtained in step (1) were a little low. These were corrected accordingly.

(4) These estimates were used in step (2), and so on by iteration until the malate concentration and the rates for both the malate dehydrogenase and fumarate hydratase reactions were obtained that gave the required overall disequilibrium and which were consistent with the kinetic equations for the enzymes. In practice only two cycles of the procedure were required, as most of the disequilibrium was in the malate dehydrogenase reaction.

To do the computations, it is necessary to give exact numerical values to the parameters. The values used were compiled from the most recent information we were aware of, and are as precise as we could make them. They not only give 'best answers' to the calculations, but provide a list against which other, later and better values can be checked. They do, however, lend a spurious air of precision. There may be quite big errors in the estimates of some parameters. The main guarantee of the validity of the approach is that whenever the possible effects of

errors have been investigated (see, e.g., Appendix B) the general conclusions have remained unaltered.

1. *Preliminary estimates of the rates in the malate dehydrogenase reaction.* As stated above, it is assumed at this stage that only the malate dehydrogenase reaction is in disequilibrium.

The rates depend upon R , the rate of rotation of the citrate cycle. In rats at 20°C R is $1.25\ \mu\text{mol/min per g}$. In rats at 30°C R is probably less. Both in man (Gump *et al.*, 1970) and rat (Brauer *et al.*, 1962) there is evidence that the liver is an important source of heat. Non-shivering thermogenesis is decreased by 40% in rats moved from 20° to 30°C (Stoner, 1969), and it will be assumed that R is decreased proportionally, to $0.75\ \mu\text{mol/min per g}$.

The citrate cycle received the relevant label as oxaloacetate formed from pyruvate. This flux of oxaloacetate into the cycle must be compensated for by an equal outward flux. The inward flux is about 0.3 of the cycle flux, i.e. about $0.4\ \mu\text{mol/min per g}$ in post-absorptive rats at 20°C (Heath & Threlfall, 1968). Two possible Schemes are shown (Scheme 2, *a* and *b*). In both there is a loss, Δ , between citrate and fumarate, but this is a small fraction of R (Heath & Threlfall, 1968) and can be neglected, i.e. Δ can be put equal to zero. In Scheme 2(*a*) the outward flux is as oxaloacetate, and in Scheme 2(*b*) as malate.

We show first that both Schemes 2(*a*) and 2(*b*) give similar rates by estimating r_2 for rats at 20°C in both ways.

Scheme 2. Kinetic schemes for the symmetric and non-symmetric pathways in the livers of rats in an environment at 20°C

Recycling of phosphopyruvate to pyruvate is not shown. In (*a*) the outward flux is as oxaloacetate, and in (*b*) as malate.

Scheme 2(a) conforms to that shown in the Introduction, with $r_2 = r_1$, so that, from eqn. (4):

$$R/r_2 = R/r_1 = r_n/r_s \quad (5)$$

In Scheme 2(b), from each unit of malate formed from oxaloacetate only $(R+r_2-0.4)/(R+r_2)$ units return to oxaloacetate, the rest being lost from the mitochondrion and converted into phosphopyruvate. Then:

$$r_n/r_s = R(R+r_2)/r_2(R+r_2-0.4) \quad (6)$$

On substituting $r_n/r_s = 0.33$ and $R = 1.25 \mu\text{mol/min}$ per g in eqns. (5) and (6) the rates of interconversion of malate, oxaloacetate and citrate become, for Scheme 2(a):

and for Scheme 2(b):

The rates in both directions of the malate dehydrogenase reaction are similar in both Schemes 2(a) and 2(b).

Although the choice of Scheme is not critical, one must be made to save space. Scheme 2(a) has been chosen as the more realistic for the post-absorptive rat. Scheme 2(b) requires net loss of malate from the citrate cycle, and this only takes place in order to provide reducing equivalents when pyruvate or alanine is utilized for gluconeogenesis (Krebs *et al.*, 1967). There is probably little gluconeogenesis from either in the post-absorptive rat; a kinetic analysis (Heath & Threlfall, 1968) favours extensive recycling of phosphopyruvate to pyruvate by pyruvate kinase. This is consistent with Scheme 2(a). It is a feature of Scheme 2(a) that the conclusions presented here are independent of the input rate of oxaloacetate from pyruvate, whereas conclusions based on Scheme 2(b) are dependent upon this flux. It is, of course, unlikely that the system corresponds exactly to Scheme 2(a). Probably it would be better represented by Scheme 2(a) with some superposition of Scheme 2(b); but such a modification would introduce differences in the calculations of a few per cent at the most, which are negligible in comparison with the other uncertainties.

For the rat at 30°C substitution of $r_n/r_s = 0.72$ and $R = 0.75 \mu\text{mol/min}$ per g in eqn. (5) gives:

The flow diagrams (A1) and (A2) are the main experimental findings of this paper.

2. *Preliminary estimate of malate concentration.* The details of the enzymology will be found in

Appendix B. Only the main principles are outlined here.

The information required for the calculation of the malate and oxaloacetate concentrations and their sources are:

(1) The mitochondrial pH. Williams & Woodbury (1971) give the pH of the cytosol as 7.25, and Addanki & Sotos (1969) give the pH in mitochondria in respiration states 3 to 4 as about 0.25 unit higher than the pH of the cytosol. Hence the mitochondrial pH was taken to be 7.5, to which all parameter values were adjusted.

(2) The concentrations of NAD^+ and NADH. The total NAD concentration was calculated from that of the total mitochondrial NAD (Purvis, 1960) corrected for the amount adsorbed (Williamson *et al.*, 1967; Glock & McLean, 1956) divided by the amount of mitochondrial water (Klingenberg & Pfaff, 1966). This, combined with the $[\text{NADH}]/[\text{NAD}^+]$ ratio (Klingenberg & von Häfen, 1963; Williamson *et al.*, 1967; Barton, 1971), gave the concentrations of NADH and NAD^+ . Details are in Appendix B.

(3) Kinetic parameters for mitochondrial malate dehydrogenase. These are discussed in Appendix B. They present two problems:

(a) The most complete information is for pig heart malate dehydrogenase (Raval & Wolfe, 1962a,b). As nearly all of the information for the rat liver mitochondrial enzyme agrees with it (Kun *et al.*, 1963), it seemed justifiable to use the pig heart values. This was necessary because the information for rat liver enzyme was not sufficient for our purpose.

(b) Raval & Wolfe's (1962a,b) pig heart information was obtained at 25°C and I 0.05. These constitute the basic set (column A, Table 1B, Appendix B). Williamson *et al.* (1967) redetermined K_{eq} at 38°C and I 0.25, and obtained a very different value. This value is preferable because it was determined under conditions of temperature and ionic strength that are closer to those *in vivo*, but it imposes alterations in the other parameters. The alterations can be made in many ways, but the parameters must obey certain mathematical relationships (Haldane equations) to be self-consistent. We therefore constructed three different self-consistent sets of parameters that incorporated the value of K_{eq} obtained by Williamson *et al.* (1967). There were, therefore, four sets of parameters (Table 1B, Appendix B) from which the intramitochondrial malate and oxaloacetate concentrations could be calculated.

For rats at 20°C all four sets of parameters were used. The calculated concentrations are compared with the mean intracellular concentrations (Table 5). The basic set of parameters (column A, Table 1B, Appendix B) gave a very high malate concentration. The three sets based on a more realistic value of K_{eq} all gave lower estimates similar to each other for the concentrations of both oxaloacetate and malate.

Table 5. Comparison between cellular concentrations of malate and oxaloacetate in post-absorptive rats and those calculated to be in the mitochondria

The values of the parameters were taken from Table 1B, Appendix B, and results calculated from them are coded by the column headings [(A), (B) etc.] in that Table. The cellular concentrations were calculated from Threlfall's (1970) results by using the factor calculated in Appendix C.

	[Malate] (mM)	[Oxaloacetate] (μM)
Rats in 20°C environment		
Cellular concentration	0.47	3.8
Calculated from: (A)	4.70	0.466
(B)	1.54	0.478
(C)	2.28	0.666
(D)	2.05	0.614
(D), best fit	2.25	0.695
Rats in 30°C environment		
Calculated from: (D)	0.68	0.170
(D), best fit	0.72	0.187

As the parameter set (D) (Table 1B, Appendix B) gave about the average results of those obtained with sets (B)–(D) (Table 1B, Appendix B) only this set was used to calculate concentrations in rats at 30°C (Table 5). The calculated concentration is lower than that in rats at 20°C, because the rates to be accounted for are lower.

3. *Preliminary estimate of rates in the fumarate hydratase reaction.* For any given malate concentration and net flux across the enzyme the individual fluxes and the fumarate concentration can be calculated from the values for fumarate hydratase given by Bock & Alberty (1953), Alberty *et al.* (1954), Kuff (1954) and Fahien & Strmecki (1969). The kinetics are described in detail in Appendix B and the

reaction (flow diagrams A1 and A2), i.e. the fumarate hydratase reaction is much closer to equilibrium.

The true rates may be a little lower owing to inhibition of the reaction by ATP (Penner & Cohen, 1969). The inhibition is, however, inversely related to pH and does not take place when the ATP is complexed with magnesium. The concentration of uncomplexed ATP would have to be greater than 1 mM at pH 7.5 before there would be sufficient inhibition to affect the conclusion, so this possibility is ignored.

4. *Overall rates for the system: fumarate–malate–oxaloacetate.* By using parameter set (D) (Table 1B, Appendix B) as the best set for malate dehydrogenase the kinetics of both enzymes were combined to give best estimates of the rates, namely, for rats at 20°C:

and for rats at 30°C:

rates were calculated from parameter set (D) (Table 5) and the net flux (*R*) from fumarate to malate. The preliminary estimates of the rates across the fumarate hydratase reaction were calculated to be, for rats at 20°C:

and for rats at 30°C:

The forward and backward rates are much closer to equality than those in the malate dehydrogenase

The calculation is described in Appendix B. It gives slightly different malate and oxaloacetate concentrations, shown as 'best fit' values in Table 5.

General discussion

The kinetic analysis depended upon combining, and applying to the system *in vivo*, estimates of the kinetic parameters for malate dehydrogenase and fumarate hydratase obtained from several sources and under various conditions *in vitro*. Most of the values were self-consistent, but there were two experimental values of K_{eq} for malate dehydrogenase, as

discussed above. The parameters may have different values *in vivo* from those *in vitro*, and if they were different enough, and in the appropriate directions, the conclusion that disequilibrium in the fumarate-malate-oxaloacetate system lies mostly in the malate dehydrogenase reaction would be incorrect. As such differences would have to involve factors of 10 or more in the values of more than one parameter, we think this is unlikely to be the case. But, although we use the results calculated from parameter set (D) (Table 1B, Appendix B) as the best available, these parameters may be somewhat in error, and can be so without vitiating any conclusions.

The maximum velocities of the malate dehydrogenase reaction in liver mitochondria are much higher than the rates *in vivo*. Why this is so is clear from the state of the enzyme. The results given below were calculated by the methods in Appendix B from parameter set (D) (Table 1B, Appendix B) for rats at 30°C. The terminology of Appendix B is put in parentheses. The rate of the forward reaction is proportional to the fraction of the enzyme complexed with NAD⁺ and to the malate concentration expressed as a fraction of its K_m . Of the total enzyme only 3% was as NAD⁺ complex ($EA/E_t = 0.0306$), so that although the malate concentration was nearly as high as the K_m value (0.72 mM and 0.8 mM respectively) the forward reaction was necessarily slow. For the reverse reaction nearly all the enzyme was complexed with NADH ($EQ/E_t = 0.963$), but the oxaloacetate concentration was only a tiny fraction of the K_m (0.187 μ M and 30 μ M respectively), so that this reaction also was slow. Because one substrate was at a very sub-optimal concentration in both reactions the low effective activity of malate dehydrogenase *in vivo* is understandable.

Our calculated malate and oxaloacetate concentrations are similar to estimates by Williamson *et al.* (1967), Anderson *et al.* (1971) and Anderson & Garfinkel (1971). Like them we calculated the intramitochondrial [NADH]/[NAD⁺] ratio from the intracellular [hydroxybutyrate]/[acetoacetate] ratio, but otherwise the estimates are independent. There is one major difference. Previous estimates have assumed that the malate dehydrogenase reaction was in equilibrium [or, by Anderson & Garfinkel (1971), very nearly so]. We have found that the oxaloacetate concentrations were about 35 and 45% below equilibrium concentration in rats at 20° and 30°C respectively.

The view that the mitochondrial oxaloacetate concentration is mainly determined by the redox potential (Wieland & Löffler, 1963; Löffler *et al.*, 1965) is not inconsistent with disequilibrium in the malate dehydrogenase reaction, as a change in redox state will change the oxaloacetate concentration whether malate dehydrogenase is in equilibrium or not, and, of course, vice versa. The present results

do, however, show that other factors are important. The change in the r_n/r_s ratio with environmental temperature would require a substantial change in oxaloacetate concentration even if the citrate cycle rotated at the same rate under both conditions. If, as seems likely and as we have assumed, the citrate cycle goes slower in rats at 30°C, then for equal redox potentials the oxaloacetate concentration is only one-third as great in rats in an environment at 30°C as in rats in an environment at 20°C (Table 5).

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APPENDIX A

Correction of C3-glutamate/C2-glutamate ratios for recycling of label

The methods given are only approximate but should be adequate as the corrections are small. Four approximations were made:

(1) Some forms of labelling were assumed to have identical time-courses, whereas their time-courses were only similar.

(2) Linear, and not curvilinear, interpolations were made.

(3) It was assumed that no label had reached 2-oxoglutarate more than twice. In practice no distribution more than 3 min after injection had to be used for the calculation, so little error should have arisen from this assumption.

(4) The fixation of $^{14}\text{CO}_2$ was assumed to be negligible in the first 3 min after injection. The basic quantity used was the total label in the i th position at each time, p_i , where:

found that label from intravenous C2-pyruvate first reached glutamate at this time.

Besides the empirical values of p_i three derived quantities were needed:

(1 and 2) p_2' and p_3' , the labelling conferred on the C2- and C3-positions in glutamate by the sym. pathway on the first turn of the cycle. By definition, $p_2' = p_3'$.

(3) p_3'' , the labelling conferred on the C3-position by the non-sym. pathway on the first turn. This had a somewhat similar time-course to C5-labelling, p_5 . It was assumed that the time-course was the same for both.

Two cases were considered, in which there was negligible hold-up of label in the sym. pathway by the fumarate+malate pool and in which there was significant hold-up (see the main paper).

$$p_i = \left(\frac{\text{Glutamate}}{\text{concn.}} \right) \times \left(\frac{\text{Specific radioactivity}}{\text{of glutamate}} \right) \times \left(\frac{\text{Fraction of label}}{\text{in } i\text{th position}} \right) \quad (1A)$$

Specific radioactivities are given in Table 4 of the main paper and fractions of label in Table 3 of the main paper. Glutamate concentrations (to which results were rather insensitive) were not determined in these experiments, to save label, and for the present purposes were taken from Heath *et al.* (1971) who determined them in the livers of rats in an environment at 20°C. The same values were used for rats in an environment at 30°C. Values of p_i are listed in Table 1A.

Zero time was taken as 0.5 min after injection, because C. J. Threlfall (personal communication)

1. Negligible hold-up in malate and fumarate

If the mitochondrial pools were small and only these held label up in the sym. pathway then the time-courses of glutamate labelling by the sym. and non-sym. pathways would be very similar and could be assumed to be identical. The best estimate was then that from the glutamate labelling at 0.5 min after zero time, when recycling was minimal. Also at this time practically no label could have passed through glutamate. (Label via recycling came from that which by-passed glutamate during the first time round the cycle; see the main paper.) Consequently p_i measured

Table 1A. Total label in specified positions within glutamate

Quantities of label are defined by eqn. (1A) and are expressed as $10^{-3} \times \text{c.p.m./g}$ of liver wet wt. The number of each rat is the same as in Table 3 of the main text. Times are given in min after zero time, as described in the text of this Appendix.

Time after zero ...	No. of rat ...	20°C environment				30°C environment	
		0.5			2.5	0.5	2.5
		1	2	4	5	10	11
p_1		0.12	0.40	0.28	1.43	0.19	0.76
p_2		0.77	2.62	2.56	4.14	0.50	2.45
p_3		1.24	4.43	4.07	5.41	1.22	4.18
p_5		3.85	3.02	2.28	4.62	2.97	2.08
p_3''		0.47	0.81	1.51	1.27	0.72	1.73

the quantity of label that had entered glutamate and so the quantity that had passed through 2-oxoglutarate. The corrected C3-glutamate/C2-glutamate ratio, z , was given by:

$$z = (p_3'' + p_2')/p_2' \quad (2A)$$

Because each liver yielded a value of z the results were completely independent of the liver glutamate concentration.

The argument given below is illustrated by Scheme 5 of Heath (1968). Because the C5- and C2-labelling in 2-oxoglutarate first time round both have the same time-course (see above) and give C1-labelling the second time round, the C1-label in glutamate was proportional to C5-label and C2-label first time round, i.e.

$$p_1 = \alpha(p_5 + p_2') \quad (3A)$$

where α is a proportionality constant.

Similarly label in the C3-position first time round, $p_2' + p_3''$, gives $\alpha(p_2' + p_3'')$ on recycling in each of the C2- and C3-positions. There are no other redistributions that affect either C2- or C3-labelling. Hence:

$$p_2 = p_2' + \alpha(p_2' + p_3'') \quad (4A)$$

$$p_3 = (p_2' + p_3'')(1 + \alpha) \quad (5A)$$

Subtracting eqn. (4A) from eqn. (5A) gives:

$$p_3'' = p_3 - p_2 \quad (6A)$$

Eqn. (6A) in eqn. (4A), followed by re-arrangement, gives:

$$p_2' = [p_2 - \alpha(p_3 - p_2)]/(1 + \alpha) \quad (7A)$$

Solutions for p_2' and α are obtained most quickly by noting that eqn. (3A) re-arranges as:

$$\alpha = p_1/(p_5 + p_2') \quad (8A)$$

and substitution of the experimental value p_2 for p_2' in eqn. (8A) gives a preliminary value of α . This, in eqn. (7A), gives a preliminary value of p_2' , which is in turn substituted in eqn. (8A) and so on. At most

two iterations are needed. This procedure was used on the results of rats nos. 1, 2, 4 and 10, (Table 1A) to give, by eqn. (2A), the values of z quoted in the text.

2. The whole cellular malate+fumarate pool in the symmetrized pathway

The interposition of the combined pool affects the time-course of labelling as shown in Scheme A1. C2-Pyruvate gave labelling in the i th position by the non-sym. pathway which was some function of time, $f_i(t)$, and by the sym. pathway which was, because of the interposition of the malate+fumarate pool, some other function, $F_i(t)$. As passage of label through this pool is a first-order process it does not matter where in a scheme it is put, so that the equivalent sym. pathway shown in Scheme A1 is kinetically identical. For this:

$$dF_i(t)/dt + kF_i(t) = \text{constant} \times f_i(t) \quad (9A)$$

(The constant allows for different amounts differently distributed passing down each pathway.) The value of k is given by: net flow rate/pool size, i.e. for rats in an environment at 20°C by $1.25/0.29 = 4.3/\text{min}$. Experimentally p_3'' and p_5 measure label that goes via the non-sym. pathway, and were fairly constant by 1 min after injection (Table 1A). Consequently from that time $f_i(t)$ can be regarded as a constant, and, on integration, eqn. (9A) becomes:

$$F_i(t) = \text{constant} \times [1 - \exp(-4.3t)] \quad (10A)$$

By 0.75 min $F_i(t)$ will be within 4% of its final value and by 1.0 min within 1.4% of it. The conclusion is barely affected by such relatively slow rates of change of $f_i(t)$ (i.e. of p_3'' and p_5) with time as were consistent with the results from 0.5 min after zero time. Eqn. (10A) is not applicable before this, because $f_i(t)$ is changing rapidly. Eqn. (10A) implies that by 1.5 min after zero time, in the absence of recycling, C3-glutamate/C2-glutamate would be close to z .

Non-symmetrized pathway *Symmetrized pathway* *Equivalent symmetrized pathway*

Scheme A1. Kinetic schemes for the symmetrized and non-symmetrized pathways

Table 2A. Comparison of experimental values of the C3-label/C2-label ratio with calculated values of z

For details see the text of this Appendix.

	20°C environment		30°C environment	
Time after zero (min)	0.5	1.5	0.5	1.5
Experimental ratio	1.63	1.47	2.42	2.07
Corrected ratio (z)	1.69	1.60	2.63	2.23

To correct for recycling, computations must be done in terms of the label that has passed into and through glutamate, i.e. q_t , and not p_t , where:

$$q_t = \int_0^t p_t dt \quad (11A)$$

because some label will have both entered and left glutamate by 1.5 min after zero time. Values of q_t , which are somewhat dependent upon glutamate concentrations, were calculated by assuming that the value of p_t increased linearly from 0 at zero time to its experimental value at zero time + 0.5 min, and then linearly from this value to its value at zero time + 2.5 min. Hence by 1.5 min after zero time for rats in an environment at 20°C: $q_1 = 0.624$, $q_2 = 3.02$, $q_3 = 4.60$ and $q_5 = 4.21$. The calculation is then con-

tinued as before, substituting q_t for p_t in eqns. (7A) and (8A). For rats in an environment at 20°C the best estimate of the value of z in this case was 1.60. For rats in an environment at 30°C it was 2.23.

It can be shown that the numerical effects of correcting for recycling are fairly small by comparing the experimentally determined values of the C3-label/C2-label ratio at 0.5 and 1.5 min after zero time (the latter by linear interpolation) with the values of z calculated above for the two cases (Table 2A).

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APPENDIX B*The kinetics of mitochondrial malate dehydrogenase and fumarate hydratase in vivo and in vitro**Malate dehydrogenase*

Cleland's (1963) symbolism is used throughout, as only he gives all the relevant equations explicitly.

This symbolism is defined in Table 1B, where it is compared with that used by the two groups of authors who provided most of the values: Raval &

Wolfe (1962*a,b*) on pig heart malate dehydrogenase at 25°C in *I* 0.05, and Kun *et al.* (1963) on rat liver mitochondrial malate dehydrogenase at 22°C in *I* 0.1.

The overall kinetic equation for the reaction, which Raval & Wolfe (1962*a*) showed was Ordered Bi Bi, is:

$$v = [V_1 V_2 (AB + PQ/K_{eq.})] \div \{V_2 [K_{ia} K_b + K_b A + K_a B + AB + B(K_a Q/K_{ia} + AP/K_{ip})] + V_1 (K_q P + K_p Q + PQ + K_q AP/K_{ia} + BPQ/K_{ib})/K_{eq.}\} \quad (1B)$$

The rate of transfer of label in oxaloacetate to malate, v_{PB} , is given by:

$$v_{PB} = V_2 P(EQ/E_t)/K_p \quad (2B)$$

where EQ/E_t is the fraction of enzyme in the EQ form, i.e. $E \cdot NADH/E_t$. The equation for EQ/E_t is given by Cleland (1963).

The basic values of the parameters, listed in column (A), Table 1B, were chosen as follows:

(1) V_2 . Berkeš-Tomašević & Holzer (1967) determined V_2 for mitochondrial malate dehydrogenase from rat liver, and give 0.124 mmol/min per g of liver wet wt. at 25°C and pH 7.4. As Anderson & Garfinkel (1971) suggested, we have doubled this estimate to allow for the higher temperature *in vivo*.

(2) Michaelis and inhibition constants. Only Raval & Wolfe (1962*a,b*) give enough information to calculate all these, and theirs are the only experiments done at pH 7.5, so their values are used although they were obtained on pig heart malate dehydrogenase at 25°C in *I* 0.05. Kun *et al.* (1963) give values for K_a , K_b , K_p and K_q at other pH values for rat liver mitochondrial malate dehydrogenase, which agree with the values given by Raval & Wolfe (1962*b*) at matching pH values. [Kun *et al.* (1963) give two values for K_b , the higher of which agrees with Raval & Wolfe's (1962*b*). This is also the relevant value for malate concentrations in the range under consideration.] Raval & Wolfe (1962*a,b*) showed that though the mechanism was Ordered Bi Bi it approximated under most conditions to a Theorell-Chance one (Theorell & Chance, 1951), in which terms in K_{ip} and K_{ib} should be negligible except when substrate concentrations are very high. We checked this on their very precise results at pH 8.0 (Raval & Wolfe, 1962*a*). Terms in K_{ip} and K_{ib} contributed only about 0.1% to the denominator in eqn. (1B) over likely ranges of substrate concentration, and were therefore ignored in all subsequent calculations.

(3) $K_{eq.}$. The value is taken from Raval & Wolfe (1962*b*).

(4) V_1 . As Raval & Wolfe (1962*b*) only give V_1 to one significant figure, it was calculated from V_2 and the Haldane relationship (Alberty, 1953):

$$K_{eq.} = V_1 K_p K_{ia} / V_2 K_{ia} K_b \quad (3B)$$

(5) A and Q ([NAD⁺] and [NADH]). Klingenberg

& Pfaff (1966) showed that of the total water in mitochondria about 60%, 1.56 μl/mg of protein, was inaccessible to externally applied NAD. Purvis (1960) found 3 nmol of NAD/mg of protein in mitochondria not treated with nicotinamide. These mitochondria had presumably retained only the NAD in their

inaccessible space, in which the NAD concentration was therefore 3/1.56 = 1.9 mm. Of this about 15% was probably bound as NADH (Williamson *et al.*, 1967; Glock & McLean, 1956), leaving as free NAD 0.85 × 1.9 = 1.6 mm. The ratio (free [NADH])/(free [NAD⁺]) in the mitochondrion was 0.187, calculated from the value of 1.20 found for the [3-hydroxybutyrate]/[acetoacetate] ratio in the livers of post-absorptive rats at 20°C by Barton (1971) and the $K_{eq.}$ (adjusted to pH 7.5) of β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) given by Williamson *et al.* (1967). These authors discuss and justify the method. The NAD⁺ and NADH concentrations given in Table 1B were calculated from their ratio and the overall NAD concentration.

The basic set of parameters in column (A), Table 1B, all refer to 25°C and *I* 0.05. Williamson *et al.* (1967) found $K_{eq.} = 8.78 \times 10^{-5}$ (corrected to pH 7.5) at 38°C and *I* 0.25 (pig heart malate dehydrogenase). This value is probably to be preferred to the lower one in column (A), as the ionic strength and temperature are the same as *in vivo*. The difference could be due to changes in V_1 , V_2 or the Michaelis and inhibitor constants. Possible variations are explored in columns (B–D), in which groups of parameters are changed in turn, self-consistency being achieved by applying eqn. (3B) or the relationship:

$$K_{eq.} = V_1^3 K_p K_q / V_2^3 K_a K_b \quad (4B)$$

[This equation, due to Alberty (1953), was shown by Raval & Wolfe (1962*a,b*) to hold fairly well for their values.] How eqns. (3B) and (4B) were applied is shown in the legend to Table 1B.

The concentrations of malate and oxaloacetate required to give the net forward rate R and the backward rates (oxaloacetate → malate) shown in the flow diagrams (A1) and (A2) (main paper) were calculated by substituting values from a self-consistent set of parameters in eqns. (1B) and (2B) and solving by trial and error.

Fumarate hydratase

It is again convenient to use Cleland's (1963) symbolism, when $V_{max.}$, fumarate → malate, becomes V_1 ; $V_{max.}$, malate → fumarate, V_2 ; $K_m(\text{fumarate})$, K_a ; $K_m(\text{malate})$, K_p . Neither V_1 nor V_2 has been measured

Table 1B. *Kinetic parameters for malate dehydrogenase*

The symbolism is as given by (1) Cleland (1963), (2) Raval & Wolfe (1962a) and (3) Kun *et al.* (1963). The values obtained as described in the text for mitochondrial malate dehydrogenase are in column (A). Columns (B), (C) and (D) are possible values for mitochondrial malate dehydrogenase for the change in K_{eq} . (see the text). In (B) the whole change was attributed to an increase in V_1 , in (C) to a decrease in V_2 . The values were calculated by eqn. (4B). In (D) V_1 and V_2 were kept constant and K_a , K_b , K_p and K_q were altered by equal factors, i.e. from eqn. (4B) K_a and K_b were divided by $4\sqrt{878/320}$, and K_p and K_q were multiplied by the same factor. In all sets the ratio: K_{iq}/K_{ia} was then calculated from eqn. (3B), and K_{ia} and K_{iq} were then respectively each divided and multiplied by the same factor to accord with the calculated ratio. All values shown are $10^4 \times$ true values, i.e. all K values are in units of $10^4 \times M$, all V values in units of $10^4 \times \text{mol/min per g of liver wet wt.}$, and all K_{eq} values in units of $10^4 \times$ dimensionless quantity.

Meaning	Cleland (1)	Raval & Wolfe (2)	Kun <i>et al.</i> (3)	(A)	(B)	(C)	(D)
Net velocity	v	—	—	—	—	—	—
Maximum velocity							
Malate $\rightarrow$ oxaloacetate	V_1	V_f	$V_{max.}$	0.53	0.74	0.53	0.53
Oxaloacetate $\rightarrow$ malate	V_2	V_r	$V_{max.}$	2.48	2.48	1.77	2.48
[NAD ⁺]	A	(DPN)	NAD ⁺	13.5	13.5	13.5	13.5
[Malate]	B	(M)	Malate	—	—	—	—
[Oxaloacetate]	P	(OAA)	OAA	—	—	—	—
[NADH]	Q	(DPNH)	NADH	2.5	2.5	2.5	2.5
Michaelis constants							
NAD ⁺	K_a	K_{DPN}	$K_{m(NAD^+)}$	1.4	1.4	1.4	1.09
Malate	K_b	K_M	$K_{m(malate)}$	8.0	8.0	8.0	6.2
Oxaloacetate	K_p	K_{OAA}	$K_{m(OAA)}$	0.30	0.30	0.30	0.39
NADH	K_q	K_{DPNH}	$K_{m(NADH)}$	0.18	0.18	0.18	0.23
Inhibitor constants							
NAD ⁺	K_{ia}	$K_{DPN.M}/K_M$	—	7.5	5.4	5.4	5.9
Malate	K_{ib}	*	—	—	—	—	—
Oxaloacetate	K_{ip}	*	—	—	—	—	—
NADH	K_{iq}	$K_{DPNH.OAA}/K_{OAA}$	—	0.030	0.042	0.042	0.038
Equilibrium constant	$K_{eq.}$	$K_{eq.}$	—	0.32	0.878	0.878	0.878

* Not given, but calculable from the other values.

for rat liver mitochondria, but both Kuff (1954) in mouse liver mitochondria and Fahien & Strmecki (1969) in bovine liver mitochondria found $V_1 \approx 1 \mu\text{mol/min per mg of protein}$, i.e. about $60 \mu\text{mol/min per g of liver wet wt.}$ We have used twice this value for 38°C . K_a , K_q and $K_{eq.}$ are pH-dependent and also dependent upon ionic strength. From Alberty *et al.* (1954) and Bock & Alberty (1953) the values at pH 7.5 and $I 0.25$ for pig heart fumarase are about: $K_a = 1.5 \text{ mM}$, $K_p = 7.0 \text{ mM}$, $K_{eq.} = 5.0$ and $V_1/V_2 = 1.07$. Alberty *et al.* (1954) described a very complex kinetic scheme involving substrate activation. Lin *et al.* (1971) have established that the enzyme is heterogeneous, which may explain the complexity. Whatever the explanation, at the fairly low substrate concentrations likely to be present

in mitochondria *in vivo* the simple kinetics described by Bock & Alberty (1953) should be applicable. Then for the net forward reaction, rate R :

$$R = (V_1 A/K_a - V_2 P/K_p)/(1 + A/K_a + P/K_p) \quad (5B)$$

and the rate of labelling of fumarate by malate is:

$$v_{PA} = V_2(A/K_a + P/K_p)/(1 + A/K_a + P/K_p) \quad (6B)$$

For rats in an environment at 20°C , $R = 1.25 \mu\text{mol/min per g}$ and P ([malate]) = 2.05 mM (Table 5 of main paper), whence $A = 0.435 \text{ mM}$, and $v_{PA} = 41.2 \mu\text{mol/min per g}$. Similarly for rats in an environment at 30°C , with $R = 0.75 \mu\text{mol/min per g}$ and $P = 0.68 \text{ mM}$, $A = 0.147 \text{ mM}$, and $v_{PA} = 18.3 \mu\text{mol/min per g}$.

Malate dehydrogenase and fumarate hydratase

In Scheme 2(a) of the main paper:

whence the fraction of oxaloacetate converted into fumarate is: $r_3 r_2 / (R + r_3)(R + r_2)$. In calculating rates for the malate dehydrogenase reaction we assumed that r_3 was infinite, so that $r_2 = r_1$ (main paper). Hence:

$$r_1 / (R + r_1) = r_3 r_2 / (R + r_3)(R + r_2) \quad (7B)$$

where for rats in an environment at 20°C, $r_1 / (R + r_1)$ has the value given in flow diagram (A1, main paper), i.e. $3.8/5.05 = 0.7525$. The flow rates calculated for fumarate hydratase, and shown in flow diagram (A3, main paper) are preliminary estimates of r_3 and $R + r_3$, i.e. $r_3 / (R + r_3) = 41.2/42.45 = 0.970554$. Substitution of these values of $r_3 / (R + r_3)$ and $r_1 / (R + r_1)$ in eqn. (7B) gives preliminary estimates of $r_2 / (R + r_2)$ and hence of r_2 , where r_2 refers specifically to the flux of oxaloacetate to malate in the scheme involving both enzymes. From this value of r_2 a new malate concentration could be calculated as described in the section on malate dehydrogenase, and hence a new value of $r_3 / (R + r_3)$ by the methods described in the section on fumarate hydratase. Iteration of these procedures gave the values in flow diagrams (A5) and (A6) of the main text. The values converge very rapidly to their best estimates.

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APPENDIX C*Calculation of concentrations in cell water in rat liver*

The treatment is in some respects like that given by Hohorst *et al.* (1959).

When livers are excised or whole lobes are clamped in tongs cooled in liquid N₂ the specimen contains 0.08–0.09 g of blood/g of tissue wet wt. Numerical examples are based on this result. For half lobes, or in decapitated rats, the blood content is 0.05–0.06 g/g of tissue (C. J. Threlfall, personal communication). Rat blood has a density of 1.054 g/ml, and contains 0.86 ml of water/ml (Spector, 1956), whence the solid content is 0.016 g/g of liver. The total solid content of liver is 0.288 g/g of liver (Threlfall & Stoner, 1961), i.e. 0.272 g/g of solids other than blood solids. The extravascular, extracellular space, V_E , is best estimated as 0.1 ml/g of exsanguinated liver (Williams

& Woodbury, 1971), i.e. $V_E = 0.092$ ml/g of liver sample. The cellular water is therefore: $(1 - 0.085 - 0.272 - 0.092) = 0.55$ ml/g. This gives the following equations. With no solute in blood:

$$\text{Concn. in cell water} \equiv C = 1.8 C_L \quad (1C)$$

$$= 0.5 C_D \quad (2C)$$

where C_L is the concentration in whole liver and C_D is the concentration per unit of dry weight. The two factors are 1/0.55 and 0.272/0.55 respectively, the latter assuming that the dry weight of the exsanguinated liver is found.

The following interrelationships are helpful when the concentrations are not the same in erythrocyte

water, plasma water and extracellular water. The water content of erythrocytes is 67% (v/v) (Spector, 1956), whence the water volume, V_{RW} , in erythrocytes is:

$$V_{RW} = 0.67 H_C V_B \quad (3C)$$

where H_C is the fractional haematocrit value corrected for entrapped plasma (Constable, 1963), and V_B is the volume of blood entrapped in the tissue. The volume of plasma water, V_{PW} , is:

$$V_{PW} = 0.914 (1 - H_C) V_B \quad (4C)$$

as the water content of plasma is 0.914 g/ml (Dicker, 1948). Hence if C_{RW} , C_{PW} and C_{EW} are the concentrations in the erythrocyte water, plasma water and extracellular water respectively:

$$C = (C_L - C_{RW} V_{RW} - C_{PW} V_{PW} - C_{EW} V_{EW}) / 0.55 \quad (5C)$$

It is usually assumed that $C_{EW} = C_{PW}$ (and that V_{EW} is the extravascular, extracellular space), but this may be wrong if the plasma concentration is much

higher than the cellular concentration, as, for example, of pyruvate in the starved rat (Threlfall, 1970). In such cases the best estimate is to take:

$$C_{EW} = (C_{PW} + C) / 2 \quad (6C)$$

i.e. that C_{EW} is the mean of the plasma and cellular concentrations. C_{RW} and C_{PW} may well be different. In control post-absorptive rats, for glucose $C_{RW} = 0.51 C_{PW}$ (Heath & Rose, 1969).

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