The Interaction of Glycolysis, Gluconeogenesis and the Tricarboxylic Acid Cycle in Rat Liver *in vivo*

By D. F. HEATH AND C. J. THRELFALL

*Experimental Pathology of Trauma Section, Toxicology Research Unit,
Medical Research Council Laboratories, Carshalton, Surrey*

(Received 4 April 1968)

1. The equations derived by Heath (1968) were applied to data from experiments on rats in four metabolic states: fed, post-absorptive, starved and 2 hr. after an eventually lethal injury. The data used were: (a) The fractions of label injected as C1-, C2- and C3-pyruvate (where the prefix indicates the position of labelling) that are incorporated into carbon dioxide and glucose in post-absorptive and injured rats (yields). Yields could be corrected to yields on label taken up by the liver. (b) The (C5-label in glutamate)/(total label in glutamate) ratio in the liver after C2-pyruvate in rats in all four states. (c) The distribution of label within glutamate after C2-pyruvate or C2-alanine in the livers of fed, post-absorptive and starved rats. (d) The distribution of label within glucose after C2-lactate or C2-pyruvate in starved rats. (e) The relative specific radioactivities of pyruvate, aspartate, glutamate and (in two states only) of glucose 6-phosphate after injection of [U- ^{14}C]glucose into rats in all four states. These data were previously published, except those after (e) and some after (b) above, which are given in this paper. 2. In addition the concentrations of pyruvate, citrate, glutamate and aspartate in the livers of post-absorptive and injured rats were found. Injury decreased glutamate and citrate concentrations and to a smaller extent aspartate and pyruvate concentrations. 3. Non-steady-state theory showed that most of the data could be used without serious error in steady-state theory. Steady-state theory correlated all but one observation (the relative yields of $^{14}\text{CO}_2$ from C2- and C3-pyruvate) listed after (a)–(e) above within the experimental errors, and gave rough estimates of the rates of pyruvate carboxylation, conversion of pyruvate and fat into acetyl-CoA and utilization of glutamate. The main conclusions were: (a) symmetrization of label in oxaloacetate both in the mitochondrion and in the cytoplasm was far from complete, because oxaloacetate did not equilibrate with fumarate in either. From this and other findings it was deduced: (b) that malate or fumarate or both left the mitochondrion, and not oxaloacetate; (c) that there was a loss from the mitochondrion of a fraction of the malate or fumarate or both formed from succinate, and (d) the resulting deficiency of oxaloacetate for the perpetuation of the tricarboxylic acid cycle was made up from pyruvate in fed and post-absorptive rats, but (e) in the starved rat could only be made up by utilization of glutamate. (f) In the fed rat the tricarboxylic acid cycle ran mostly on pyruvate, but in the post-absorptive and starved rat mostly on fat. (g) In the injured rat the tricarboxylic acid cycle was slowed, label in oxaloacetate was completely symmetrized (cf. conclusion a), and the tricarboxylic acid cycle utilized glutamate. (h) The conclusions were not invalidated by isotopic exchange, i.e. flux of label without net flux of compound, nor by interaction with lipogenic processes. (i) In the kidneys interaction between the tricarboxylic acid cycle and gluconeogenesis was different from in the liver, and was much less. The effects on the theory were roughly assessed, and were small. 4. The experiments and optimum experimental conditions required to check the theory are listed, and several predictions, open to experimental confirmation, are made.

In the preceding paper Heath (1968) gave equations for the redistribution of carbon label brought about in rat liver by glycolysis, glucone-

genesis and the tricarboxylic acid cycle when specifically labelled pyruvate or glucose is the primary substrate. In the present paper we show

how to apply the equations to data obtained from experiments *in vivo*.

The equations contain nine independent variables. When equations of this complexity are applied to data obtained from experiments made *in vivo*, the errors of which cannot always be easily assessed, there is a serious risk of drawing incorrect conclusions. To minimize this risk, a wide range of data was used, making allowances for errors, and the equations were applied to data from rats in four metabolic states, which tested the applicability of the theory over a wide range of some of the variables. The finding that not only could data from the rats in the same metabolic state be correlated, but also that data from rats in widely differing metabolic states could be interrelated, is crucial to the credibility of the undertaking.

In view of the length of the exposition the main line of argument has been separated from the details, which are in small type. To simplify presentation further, the experimental data, mostly already published, are brought in as required, and experimental methods are put at the end.

Conventions and symbols are as in Scheme 1 and under '3. Steady-state theory' in the Theory section of Heath (1968), to which frequent reference is made.

METABOLIC STATES

The four states considered were fed, post-absorptive, starved and injured. The fed state was that up to 2.5 hr. after feeding, the post-absorptive state 7–10 hr. after feeding, and the starved state 24–30 hr. after feeding. The post-absorptive state, about which most is known, was studied in detail, and the other states were regarded as perturbations of it.

The states in normal rats were based on observations on male albino Wistar rats of the Porton strain (about 200 g.) from our colony (fed on M.R.C. diet 41 B). They are nocturnal feeders. Food is withdrawn from rats for experiment at 9.00 hr., but they mostly stop eating at about 7.00 hr. and times are taken from 7.00 hr. The fed state is characterized by high blood glucose concentrations (about 1.5 mg. of glucose/ml.), and liver glycogen content rising to a steady value. From 2.5 hr. liver glycogen content falls steadily until about 12 hr. (Stoner, 1958), and the blood glucose concentrations fall initially to steady values (1.1 mg. of glucose/ml.) that are maintained from 7 to 10 hr. after feeding. This period, 7–10 hr. after feeding, we have called 'post-absorptive'. The starved state is presumably established when liver glycogen is depleted, at about 22 hr. (Stoner, 1958). In practice, experiments were carried out 24–30 hr. after withdrawal of food, when blood glucose concentrations were low, varying from rat to rat (0.5–0.9 mg./ml.) and slowly falling. No observations have been made after 30 hr. In assigning rats used by other workers to these states there is an element of guesswork, as the effects of differences in strain and diet are not known.

The injured state was that 1.5–3 hr. after a 4 hr. period of bilateral hind-limb ischaemia with the tourniquets applied at 9.00 hr. The injury is eventually fatal to about 85% of the rats (Stoner, 1961a,b; see also for a fuller description). The essential characteristics are: a fairly steady biochemical state in spite of falling body temperature; a 50% fall in heat production (Stoner & Pullar, 1963); high but steady blood glucose concentrations (> 2 mg./ml.); low liver glutamate and citrate contents; and slightly lowered liver aspartate content (Table 6). These and other results (Ashby, Heath & Stoner, 1965; Heath & Stoner, 1968) imply slowing of the tricarboxylic acid cycle at the citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7) stage. The results obtained here accord with this interpretation.

BASIS OF APPLICATION *IN VIVO*

Basic assumptions. Three assumptions were made that cannot be tested in detail.

(1) In rats from the same colony in the same nutritional state etc. there is no drift in relevant kinetics with time, e.g. over the 4-year period in which the relevant experiments on our colony were carried out.

(2) Rats from different colonies etc. are comparable if their nutritional states are the same.

(3) The relative rates in kidneys are the same as in the liver. On this assumption the data in Table 1, which refer to the liver and kidneys combined, can be correlated with the remaining data that refer to the liver alone. It turned out that this assumption was not justified, but that the errors arising from it were small, because the kidneys are relatively small, and did not invalidate any important conclusions (see under 'Effects of the kidneys' in the General Discussion and Conclusions section).

There are large metabolic differences between the kidneys and the liver (Dzurik, Krajci-Lazary & Niederland, 1963; Kun, Ayling & Baltimore, 1964; Kun & Volfin, 1966). In kidney-cortex slices most of the label introduced as pyruvate appears in CO₂, not in glucose (Krebs, Hems, Weidemann & Speake, 1966), and the kidneys contain less than one-fifth as much pyruvate carboxylase as the liver (calculated from data given by Henning, Stumpf, Ohly & Seubert, 1966), so labelling of glucose from pyruvate is likely to be much less. Kidney O₂ consumption is also much less: about 3.5% of total animal O₂ consumption (Brauer, Leong & Holloway, 1962) against the liver's 12.5% (R. W. Brauer, personal communication).

Preassessment of absolute rates. All the rates given in this paper are in $\mu\text{moles/min./100g. rat body wt.}$ For comparison with data from experiments *in vitro*, which are usually given in the literature in $\mu\text{moles/min./g.}$, the liver weight is

taken as 4g./100g. rat body wt., its value in post-absorptive rats.

Preassessment can be made of R , the rate of rotation of the tricarboxylic acid cycle, and of V_6 , the rate of conversion of triose phosphate into glucose 6-phosphate (Scheme 1 in Heath, 1968), as follows.

R . To assess the rate of rotation of the tricarboxylic acid cycle it was assumed initially that the respiratory quotient of the liver was the same as that of the whole rat, so that the liver would supply the same percentage of the total carbon dioxide excreted from the rat as it consumed of the total oxygen consumption, i.e. 12.5% (see above). On this basis it was calculated from the carbon dioxide excretion rate of the post-absorptive rats used by Ashby *et al.* (1965) that the liver excreted 16 μ moles of carbon dioxide/min. If the whole of this carbon dioxide came from the total oxidation of free fatty acids, i.e. β -oxidation to acetyl-CoA followed by oxidation of the acetyl-CoA in the tricarboxylic acid cycle, then $R = 0.5 \times \text{CO}_2$ excretion rate = 8 μ moles/min. This is the maximum value. In fact, some oxygen is consumed in the formation of ketone bodies, which are only finally oxidized outside the liver, and some oxygen is consumed by microsomal oxidases. The oxygen consumption by either route is not more than a small fraction of the oxygen consumption by the total oxidation to carbon dioxide of carbohydrates and fat via the tricarboxylic acid cycle. In addition, a little glucose was utilized (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section). Consequently the rate of rotation of the tricarboxylic acid cycle should be less than 8 μ moles/min., but not much less. The best estimate that can be made of R is 6 μ moles/min.; but the whole range considered possible, 5–8 μ moles/min., has been investigated.

V_6 . In our post-absorptive rats, R_2 , the rate

of glycogenolysis, is 2.3 ± 0.35 (S.E.M.) μ moles/min. (Stoner, 1958; and later confirmed by him and communicated personally). The (glucose 6-phosphate)/glucose specific-radioactivity ratio 10–20 min. after intravenous injection of [^{14}C]glucose is 0.3–0.45 (Threlfall & Heath, 1968; and under 'Post-absorptive rats: Discussion' in the Results and Discussion section), whence, by eqn. (3.67) and Scheme 1 in Heath (1968): $V_5 = 6.7$ – 7.7 μ moles/min. As:

$$V_5 - V_6 = AV_2 + \Delta \quad (1)$$

and solutions found later show that $AV_2 + \Delta = 1$ – 1.7 μ moles/min., V_6 is 5–6.7 μ moles/min. The best value is taken as 6 μ moles/min., but the value of 5 μ moles/min. is also used.

Errors in data. Statistical errors are allowed for by solving equations for mean values and for means ± 1 S.E.M.

There are other, non-random, errors, especially in the data to which most weight is given (Table 1). Ashby *et al.* (1965) injected C1-, C2- and C3-pyruvate (where the prefix indicates the position of labelling; see Heath, 1968) intravenously into rats and estimated the fractions of injected label incorporated into glucose and carbon dioxide. These are the yields, denoted by $Y_1(\text{glucose})$ etc., where the subscript denotes the position of the label in the injected pyruvate, and the compound in parentheses the compound in which the label was measured. The yields were originally computed on the total label injected. For the present purposes yields were required on the label reaching the liver and kidneys. There are reasons, given below, for expecting the yields to lie within the ranges shown in Table 1. The analysis given in this paper confirms this conclusion. More emphasis, however, is put on the ratios of the yields of [^{14}C]glucose than on their absolute values, as these ratios are not affected by uncertainties in how much label reaches the

Table 1. Yields of $^{14}\text{CO}_2$ and [^{14}C]glucose from C1-, C2-, and C3-pyruvate in post-absorptive and injured rats

The yields are given as $Y_n(\text{compound})$, where n is the position of label in pyruvate and the compound labelled from pyruvate is in parenthesis. The yields were calculated from the results obtained by Ashby *et al.* (1965), omitting on statistical grounds PT1 in Table 7 in their paper. The percentage of C1-pyruvate assumed to be oxidized rapidly outside the liver is given as '% elsewhere'. Errors are S.E.M. values. Lines 4 and 5 assume that $Y_2(\text{glucose})/Y_1(\text{glucose})$ was 1 S.E.M. low. Line numbers are referred to frequently in the text.

Line no.	%	$Y_n(\text{glucose})$			$Y_n(\text{CO}_2)$	
		Y_1	Y_2/Y_1	Y_3/Y_2	Y_1/Y_2	Y_3/Y_2
Controls	0	0.095	3.34 ± 0.50	0.87 ± 0.09	1.54 ± 0.07	1.00 ± 0.05
	10	0.106			1.62	
	20	0.119			1.73	
	10	0.114			1.54	
	20	0.128	2.84	0.936 ± 0.09	1.62	
Injured	0	0.189			1.89	

gluconeogenic organs. The $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ ratio (Table 1) may have a systematic error of up to 5% superposed on its random error.

The yields were computed allowing for the cycling of pyruvate via extracellular spaces and for the $^{14}\text{CO}_2$ from the ^{14}C glucose produced. A continuing slow excretion of $^{14}\text{CO}_2$, attributed to complex recycling, was excluded. Minimum yields can be obtained reliably. Of label injected as C1-pyruvate 76% was accounted for as ^{14}C glucose and $^{14}\text{CO}_2$. (The rest was involved in complex recycling.) More than 95% of C1-pyruvate label reaching the liver is incorporated into glucose and CO_2 according to all the calculations summarized in Table 5. Consequently the minimum yields are obtained by dividing the yields on total label injected by 0.76. They are given in the first line of Table 1. But some label will be incorporated rapidly into CO_2 in other organs. The uncertainty is in how much. No tissue other than liver, kidneys and heart muscle is known to oxidize injected (as opposed to endogenous) pyruvate. Heart muscle oxidizes pyruvate rapidly, but it is fed by a much smaller fraction of the blood circulation than the liver and kidneys (Takacs, Kallay & Skolnik, 1962), and, though it can oxidize pyruvate rapidly, it oxidizes less than a half of the amount taken up when other substrates are available (Garland, Newsholme & Randle, 1964). It is therefore unlikely to oxidize more than about 5% of an injected dose of pyruvate. If, however, the kidneys give much smaller yields of ^{14}C glucose and higher yields of $^{14}\text{CO}_2$ than the liver, perhaps up to 30% of the pyruvate rapidly oxidized could be oxidized in tissues other than the liver. It is therefore necessary to allow for the possibility in the computations, in the way shown below. The computations themselves throw light on this problem (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section). Suppose other tissues oxidized one-tenth of the pyruvate rapidly utilized. Then the yields of ^{14}C glucose given in line 1 in Table 1 should be divided by 0.9. However, $Y_1(\text{CO}_2) - Y_2(\text{CO}_2)$ is only slightly greater than $Y_2(\text{glucose}) - Y_1(\text{glucose})$, i.e. less label is incorporated into CO_2 from C2-pyruvate than from C1-pyruvate mostly because more is incorporated into glucose. Other organs do not produce glucose, so in them $Y_1(\text{CO}_2)$ and $Y_2(\text{CO}_2)$ should be equal, and for the liver the $Y_1(\text{CO}_2)/Y_2(\text{CO}_2)$ ratio should be raised from 1.54 to 1.62. This set of yields (90% in liver, 10% elsewhere) is shown in line 2 in Table 1; and a further set (80% in liver, 20% elsewhere) in line 3 in Table 1. Statistical errors are allowed for in lines 4 and 5 in Table 1, assuming that the errors on $Y_1(\text{glucose})$ and $Y_2(\text{glucose})$ were proportional to their values and such that $Y_2(\text{glucose})/Y_1(\text{glucose})$ was 1 s.e.m. less than its empirical mean.

The yields of $^{14}\text{CO}_2$ were corrected for the $^{14}\text{CO}_2$ release from the ^{14}C glucose produced assuming that the glucose became uniformly labelled. This was not so (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section), and may have caused a systematic error in $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ (Table 1), which should not have exceeded 5%.

Only the data of Table 1 are entirely suitable for use in steady-state theory, as the yields were computed in a way that makes them time-invariant.

The remaining data consist of analyses carried out at set times, when isotopic equilibration may have been incomplete. By using non-steady-state theory the errors introduced can be allowed for.

Data used in the computations. (1) The yields in Table 1.

(2) The (label in C5-glutamate)/(total label in glutamate) ratios, abbreviated to (C5-Glu)/(T-Glu), estimated 10 min. after intravenous injection of C2-pyruvate (Table 2; cf. Freedman & Graff, 1958; Koeppe, Mourkides & Hill, 1959).

(3) The glutamate/pyruvate and aspartate/pyruvate specific-radioactivity ratios found in liver 10 min. after intravenous injection of $[\text{U-}^{14}\text{C}]$ glucose (Table 3). The 2-oxoglutarate/pyruvate specific-radioactivity ratios found under similar conditions by Bachelard (1965) are also given. They agree with the glutamate/pyruvate specific-radioactivity ratios, as they should on steady-state theory, so both ratios are combined. Pyruvate turns over very rapidly, so the experimental specific radioactivities might be expected to differ from those *in vivo*. It was, however, found in similar experiments that the specific radioactivities of pyruvate were close to those of lactate, which turns over much more slowly. There is therefore no reason to suspect systematic errors in the data in Table 3.

(4) Hoberman & d'Adamo (1960) gave starved rats C2-lactate. In the ^{14}C glucose only 4% of the label was in the C3-position (Table 10), showing that most of the label had not passed through the tricarboxylic acid cycle. They found the ratio of labelling in the C5- and C6-positions to be 0.66/0.34, i.e. that 0.66 of the label retained its position in passing through cytoplasmic oxaloacetate (Scheme 6 in Heath, 1968), i.e. $\alpha = 0.66$. This is the only information that gives a simple estimate of α . However, more symmetrization of label in the cytoplasm is possible, and $\alpha = 0.5$ (complete symmetrization) was used as a variant. Reasons are, however, finally adduced for believing that $\alpha = 0.66$ is about correct, and is more likely to be low than high (see under 'Fed and starved rats: starved rats' in the Results and Discussion section).

(5) The distribution of label in glutamate 1 hr. after giving C2-alanine (Table 4, after Freedman *et al.* 1960). In fed rats the (C5-Glu)/(T-Glu) ratios are higher than in post-absorptive rats (Table 2), and in starved rats are lower. In starved rats this ratio is often nearly zero (Freedman & Graff, 1958; Koeppe *et al.* 1959). The other important feature is the (C3-Glu)/(C2-Glu) ratio, which is greater than 2. Koeppe *et al.* (1959) found more even C2,3-labelling, especially in fed rats, 3 hr. after injection of C2-pyruvate. So long after injection the labelling would take place almost solely via ^{14}C glucose, a pathway that randomizes label. More weight is therefore given to the results obtained

Table 2. *Fraction of glutamate label in the C5-position [(C5-Glu)/(T-Glu)] in rat liver after injection of C2-pyruvate*

Results for post-absorptive and injured rats were obtained as described in the Materials and Methods section. Those for fed rats are taken from Freedman, Rumsey & Graff (1960), and those for starved rats from Koeppe *et al.* (1959) and Freedman & Graff (1958). Freedman and co-workers gave C2-alanine intraperitoneally.

State	(C5-Glu)/(T-Glu)	Mean	S.D.	S.E.M.
Fed	0.35, 0.40	0.375	—	—
Post-absorptive	0.05, 0.06, 0.08, 0.20, 0.22, 0.30	0.15	0.10	0.04
Starved	Several values	0.02	—	—
Injured	0.07, 0.18, 0.18, 0.29, 0.37, 0.42	0.25	0.13	0.05

by Freedman *et al.* (1960) 1 hr. after intraperitoneal injection. Even these, however, will give (C3-Glu)/(C2-Glu) rather lower than it would be in the absence of recycling via glucose.

Computation. Steady-state theory was programmed by Mr B. K. Kelly for the London University Atlas Computer in EXCHLF language.

The equations in the Appendix to the preceding paper (Heath, 1968) give, among other things, $Y_1 - Y_3$ (glucose), $Y_1 - Y_3$ (CO₂) (Table 1), (C5-Glu)/(T-Glu) (Table 2) and (C3-Glu)/(C2-Glu) (Table 4) in the steady state after the injection of specifically labelled pyruvates, and the aspartate/pyruvate and glutamate/pyruvate specific-radioactivity ratios (Table 3) after the injection of [U-¹⁴C]glucose in terms of the following variables:

R , rate of rotation of the tricarboxylic acid cycle;

V_6 , rate of conversion of the triose phosphates into glucose;

α , fraction of label retaining its position during passage through cytoplasmic oxaloacetate; the value of α is related to the fraction of the oxaloacetate that does not equilibrate with fumarate;

V_2 , rate of formation from pyruvate of oxaloacetate utilized by the tricarboxylic acid cycle;

V_3 , rate of formation from pyruvate of oxaloacetate that is not utilized by the tricarboxylic acid cycle, but is utilized in cytoplasm;

Δ , rate of irreversible loss of glutamate from the tricarboxylic acid cycle (presumably to protein);

A , where AV_2 is the rate of formation from pyruvate of acetyl-CoA utilized by the tricarboxylic acid cycle;

z_1 and z_0 , i.e. the fractions of label introduced into the tricarboxylic acid cycle as oxaloacetate that obey the kinetics of non-sym. cases A and B respectively.

When oxaloacetate enters the tricarboxylic acid cycle the label in it will be wholly symmetrized if all the oxaloacetate equilibrates with fumarate before reacting with citrate synthase. These kinetics are given by the sym. case. If, however, a portion of the oxaloacetate reacts with citrate synthase before equilibrating with fumarate, then in this portion label will not be symmetrized.

Equilibration may be incomplete either because malate does not equilibrate with fumarate (non-sym. case A), or because oxaloacetate does not equilibrate with malate (non-sym. case B), or for a combination of both reasons. In principle all three cases can be combined by adding the contributions made by each. In practice existing data did not allow this to be done unambiguously and only one or other of the non-sym. cases could be added to the sym. case. Non-sym. case B was mostly used, because it enabled all the data over the whole experimental range of errors to be correlated, whereas non-sym. case A did not. This did not prove that non-sym. case A was irrelevant, as non-sym. case A could correlate all mean values.

For each computation for post-absorptive rats values of R , V_6 and α , and the non-sym. case to be used, were pre-set, leaving five variables, V_2 , V_3 , Δ , A and z_1 or z_0 (according to the choice of non-sym. case), to be calculated from the data. The data consisted of a line of yields from Table 1 and a value of (C5-Glu)/(T-Glu) from Table 2. As Y_1 (CO₂) had been used to correct data (see above under 'Errors in data') each set contained six pieces of data: three yields of [¹⁴C]glucose and Y_2 (CO₂)/ Y_1 (CO₂) and Y_3 (CO₂)/ Y_2 (CO₂), and a value of (C5-Glu)/(T-Glu). Two methods of computation were used. In one the value of Y_3 (CO₂)/ Y_2 (CO₂), which may be in error (see above under 'Errors in data'), was ignored, so that exact solutions (five unknowns, five knowns) could be obtained. In the other the values of Y_3 (CO₂)/ Y_2 (CO₂) and Y_3 (glucose)/ Y_2 (glucose) were given equal statistical weight, the remaining data being fitted exactly. These solutions are called least-mean-square solutions.

For exact solutions values of V_2 , V_3 , A and Δ were guessed, and the values of the knowns were calculated for the sym. case and for non-sym. case B. The values for the sym. case were multiplied by $(1 - z_0)$ and for non-sym. case B by z_0 , z_0 being chosen to give the best fit. By varying V_2 , V_3 , A , Δ and z_0 in turn and recomputing, a set of difference equations was produced, from which by Newton's method better values of the variables could be obtained. Two iterations gave solutions to three significant figures.

For the least-mean-squares solutions to $Y_3(\text{glucose})/Y_2(\text{glucose})$ and to $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$, z_0 was varied in intervals of 0.05 over the relevant range, and exact values of V_2 , V_3 , Δ and A were found that fitted all but $Y_3(\text{glucose})/Y_2(\text{glucose})$ and $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$. For each set of results so obtained, $Y_3(\text{glucose})/Y_2(\text{glucose})$ and $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ were calculated. Denote the calculated values by r_g and r_c (ratios for glucose and CO_2), the empirical values by $r_g(\text{emp})$ and $r_c(\text{emp})$, and the S.E.M. values by e_g and e_c . Then the error function (e.f.) was calculated:

$$\text{e.f.} = [r_g - r_g(\text{emp})]^2/e_g^2 + [r_c - r_c(\text{emp})]^2/e_c^2 \quad (2)$$

The best set of V_2 , V_3 , Δ , A and z_0 was taken as that which minimized the error function, which is that for a weighted least-mean-squares fit.

From the values of the variables calculated in either of these ways the glutamate/pyruvate and aspartate/pyruvate specific-radioactivity ratios for $[\text{U-}^{14}\text{C}]\text{glucose}$ as substrate and $(\text{C3-Glu})/(\text{C2-Glu})$ for C2-pyruvate as substrate were calculated, and were compared with the data in Tables 3 and 4.

Non-steady-state theory was computed by hand for those sets of variables that seemed most representative of the system.

RESULTS AND DISCUSSION

Post-absorptive rats

Steady-state theory. The fact that when C2-alanine is given to rats C3-labelling in glutamate is greater than C2-labelling (Table 4, quoted from Freedman *et al.* 1960) is sufficient to prove that label in oxaloacetate is not completely symmetrized before reacting with citrate synthase, i.e. in the tricarboxylic acid cycle oxaloacetate and fumarate are not in equilibrium. Therefore the sym. case, which assumes equilibrium, should have failed to correlate the data, as it did. It gave $Y_3(\text{glucose})/Y_2(\text{glucose}) > 1.15$, against 0.87 ± 0.09 found experimentally (Table 1). Mixed sym. and non-sym. cases were therefore used.

The results of numerous computations (about two-thirds of those carried out) are shown in Table 5. The large number was needed to allow for experimental errors and to scan for the effects of varying over their likely ranges the rate of rotation of the tricarboxylic acid cycle (R), the rate of conversion of the triose phosphates into glucose (V_6) and the degree of symmetrization of oxaloacetate in the cytoplasm (α). Only some sets reproduced the relative specific radioactivities after $[\text{U-}^{14}\text{C}]\text{glucose}$ of liver pyruvate, aspartate and glutamate, i.e. the aspartate/pyruvate and glutamate/pyruvate specific-radioactivity ratios in Tables 3 and 5. Attention was confined to these satisfactory sets, which are shown in **bold** type in Table 5. They were chosen as giving the sum of the errors on the aspartate/pyruvate and glutamate/pyruvate

specific-radioactivity ratios less than 0.2, corresponding to probabilities greater than about 0.1.

The satisfactory sets fell into two groups, exact solutions (lines 16–20 and 23) and least-mean-square solutions (lines 4, 8 and 11–13). The exact solutions predicted the experimental value of $(\text{C3-Glu})/(\text{C2-Glu})$ after injection of C2-alanine, but gave $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ 2–2.4 S.E.M. too high. The least-mean-square solutions gave $(\text{C3-Glu})/(\text{C2-Glu})$ too low, but $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ within 1 S.E.M. Both types of solution gave similar values of the variables with one important exception: the exact solutions gave higher z , i.e. they required less symmetrization of oxaloacetate in the tricarboxylic acid cycle. It seemed advisable in the early stages to use both types of solution for further work, to see whether there were any other important differences.

Table 3. *Relative specific radioactivities of pyruvate, glutamate and aspartate, and of pyruvate and 2-oxoglutarate (Bachelard, 1965), after injection of $[\text{U-}^{14}\text{C}]\text{glucose}$*

Relative specific radioactivities were estimated 10 min. after intravenous injection except in Bachelard's (1965) experiments (10 min. after subcutaneous injection). S.E.M. values are given. There were three rats in each group. The aspartate/glutamate specific-radioactivity ratio for injured rats was 1.15 ± 0.05 ($n=9$).

	Specific-radioactivity ratios		
	Aspartate Pyruvate	Glutamate Pyruvate	2-Oxoglutarate Pyruvate
Post-absorptive	0.67 ± 0.10	0.73 ± 0.15	0.67 ± 0.15
		0.70 ± 0.11	
Injured	0.68 ± 0.10	0.66 ± 0.11	

Table 4. *Distribution of label in liver glutamate in rats given C2-alanine (after Freedman *et al.* 1960)*

The experimental results are percentages of total label in each position with recoveries about 95%. The rats were killed 1 hr. after intraperitoneal injection. For calculated results see the text.

Type of rat	Distribution of label (%)					C3/C2 ratio
	C1	C2	C3	C4	C5	
Post-absorptive	15.3	18.1	40.7	1.1	20.3	2.25
Calc.	17.5	19.7	39.2	1.7	20	2.08
Fed	20.5	12.3	19.2	2.0	40.4	1.56
Calc.	20.6	12.1	24.8	2.6	39.9	2.04
Starved*	11.3	26.4	55.3	0.4	3.5	2.09
Calc.	10.1	27.2	62.7	0	0	2.31

*Starved for 40 hr., but given 2.5 g. of glucose by stomach tube shortly before treatment with alanine.

Table 5. *Solutions to steady-state theory for post-absorptive rats*

The data used from Table 1 are referred to by line no. in Table 1. (C5-Glu)/(T-Glu) values are taken from Table 2 (means $\pm$ s.e.m.). The preassigned variables are R (rate of rotation of tricarboxylic acid cycle), α (fraction of label retaining its position during passage through cytoplasmic oxaloacetate), V_6 (rate of conversion of triose phosphates into glucose) and the non-sym. case used, A or B. The values of Δ , A , V_2 , V_3 and z_0 (see the text for definitions) were obtained to fit the data by using the preassigned values of R , α , V_6 and non-sym. case by using either an exact fit (E) or a least-mean-squares fit (LMS) (see the text). For each set the following were calculated: V_1 and V_5 ; and for when $[U-14C]_{\text{glucose}}$ is given the relative specific radioactivities: pyruvate/(glucose 6-phosphate) (Pyr/G6P); glutamate/pyruvate (Glu/Pyr); and aspartate/pyruvate (Asp/Pyr); and for when C2-pyruvate is given the ratio: (C3-glutamate)/(C2-glutamate) (C3-Glu/C2-Glu).

Data			Preassigned values			Solved values				Type of fit			Calculated values					
Table 1 (C5-Glu)			Non-sym. case			Δ	A	V_2	V_3	z	Type of fit	V_1	V_5	Pyr G6P	Glu Pyr	Asp Pyr	C3-Glu C2-Glu	
No.	line no.	R	α	V_6														
1	1	0.15	8	0.66	5	B	0.47	0.39	2.89	0.83	0.50	LMS	4.85	6.60	0.41	0.61	0.72	1.68
2	2	0.15	8	0.66	5	B	0.52	0.34	3.87	1.17	0.40	LMS	6.37	6.84	0.41	0.71	0.82	1.60
3	3	0.15	8	0.66	5	B	0.55	0.28	5.95	1.75	0.30	LMS	9.34	7.20	0.39	0.86	0.93	1.49
4	2	0.15	6	0.66	5	B	0.38	0.36	2.42	0.58	0.40	LMS	3.86	6.24	0.44	0.64	0.74	1.59
5	3	0.15	6	0.66	5	B	0.40	0.31	3.25	0.81	0.35	LMS	5.07	6.41	0.42	0.74	0.83	1.58
6	2	0.15	5	0.66	5	B	0.32	0.36	1.86	0.40	0.40	LMS	2.93	5.99	0.44	0.61	0.71	1.61
7	3	0.15	5	0.66	5	B	0.34	0.32	2.43	0.54	0.35	LMS	3.74	6.11	0.43	0.70	0.79	1.58
8	4	0.15	6	0.66	5	B	0.51	0.38	2.08	0.62	0.40	LMS	3.48	6.29	0.44	0.68	0.74	1.58
9	5	0.15	6	0.66	5	B	0.55	0.34	2.70	0.89	0.35	LMS	4.50	6.46	0.43	0.75	0.82	1.54
10	2	0.19	6	0.66	5	B	0.37	0.53	2.49	0.95	0.60	LMS	4.76	6.69	0.43	0.75	0.83	1.92
11	2	0.11	6	0.66	5	B	0.39	0.24	2.27	0.39	0.30	LMS	3.18	5.92	0.42	0.55	0.67	1.45
12	2	0.15	6	0.66	6	B	0.39	0.36	2.23	0.48	0.40	LMS	3.52	7.19	0.44	0.61	0.71	1.61
13	3	0.15	6	0.66	6	B	0.40	0.32	2.91	0.64	0.35	LMS	4.48	7.33	0.43	0.66	0.79	1.58
14	2	0.15	6	0.50	5	B	0.38	0.40	2.01	0.71	0.45	LMS	3.52	6.17	0.43	0.58	0.74	1.52
15	2	0.15	6	0.66	5	A	0.37	0.31	2.89	0.10	0.55	LMS	3.88	6.26	0.42	0.64	0.74	1.72
16	2	0.15	6	0.66	5	B	0.34	0.43	1.99	0.70	0.72	E	3.55	6.20	0.43	0.60	0.71	2.05
17	3	0.15	6	0.66	5	B	0.36	0.39	2.55	1.04	0.67	E	4.59	6.35	0.42	0.70	0.79	2.04
18	2	0.15	6	0.66	6	B	0.34	0.44	1.85	0.60	0.73	E	3.25	7.15	0.44	0.57	0.68	2.08
19	3	0.15	6	0.66	6	B	0.36	0.40	2.32	0.85	0.68	E	4.09	7.28	0.43	0.66	0.75	2.07
20	2	0.19	6	0.66	6	B	0.34	0.66	1.92	0.90	1.00	E	4.08	7.60	0.44	0.69	0.77	2.50
21	3	0.19	6	0.66	6	B	0.35	0.62	2.35	1.34	1.00	E	5.13	7.80	0.44	0.78	0.85	2.58
22	2	0.11	6	0.66	6	B	0.35	0.27	1.81	0.41	0.52	E	2.72	6.85	0.44	0.49	0.61	1.76
23	3	0.11	6	0.66	6	B	0.36	0.35	2.25	0.57	0.49	E	3.39	6.93	0.43	0.56	0.68	1.76

Fig. 1. Specific radioactivities of pyruvate, aspartate and glutamate after intravenous injection of $[U-^{14}C]$ glucose. Experimental specific radioactivities: $\circ$, aspartate; $\bullet$, glutamate; S.E.M. values are about 10% of means except for aspartate at 1.5 min., where they are about 20%. The number of estimations is given beside each point. The curves are the theoretical specific radioactivities, normalized

Before drawing conclusions it was also necessary to establish that the solutions obtained had not been distorted by neglecting interaction with lipogenesis, by using too simple a model for $^{14}CO_2$ fixation or by using steady-state theory when the relative specific radioactivities given in Tables 2-4 must vary with time after injection, and should therefore be treated by non-steady-state theory.

Interaction with lipogenesis. Citrate leaving the mitochondria is converted by ATP citrate lyase [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] into acetyl-CoA and cytoplasmic oxaloacetate (Srere, 1959, 1965). The pathway could be represented on Heath's (1968) Scheme 1 by a rate, D , leading from before 2-oxoglutarate to cytoplasmic oxaloacetate, and the substitution of $R-\Delta-D$ and $V_2-\Delta-D$ for the rates shown as $R-\Delta$ and $V_2-\Delta$. The effects of this pathway were investigated. The enzyme removes as an acetyl group only the C5,6-carbon atoms in citrate, i.e. those from acetyl-CoA in the citrate synthase reaction (Bhaduri & Srere, 1963). The value of D was set at $0.5 \mu\text{mole/min.}$, about one-quarter of its maximum rate (Shrago & Lardy, 1966) but considerably faster than would be expected from the citrate concentrations present in the liver [$0.27 \mu\text{mole/g.}$; cf. Srere (1959) for dependence of rate on citrate concentration]. The other rates etc. were as in line 19 in Table 5. The additional pathway changed no calculated specific radioactivity by more than 4%, and most by less than 2%. Interaction with lipogenesis can therefore be ignored. Conversely, the sort of data used can give no information on lipogenesis.

$^{14}CO_2$ fixation. The computation of $^{14}CO_2$ fixation involved an approximation that could lead to an overestimate of its importance (see under '3. Steady-state theory: C2-pyruvate, non-sym. case B' in the Theory section of Heath, 1968). In no set was $^{14}CO_2$ fixation calculated to contribute more than 10% of the label in any compound, so that the approximation could not have introduced any appreciable error into the results calculated. This conclusion only applies to these particular calculations. Had, for example, the label in glutamate after injection of C1-pyruvate been calculated the errors might have been considerable.

Non-steady-state theory. Non-steady-state theory enabled us in principle to calculate the variation with time of any of the specific radioactivities discussed from the time-course of the specific radioactivity of glucose or pyruvate in the body (given

so that the curve for glutamate passes through the experimental specific radioactivity at 12 min. The horizontal lines give the specific radioactivities of aspartate (----) and glutamate (—) relative to that of pyruvate at 10 min., calculated from steady-state theory. If steady-state theory is exact, the horizontal lines will intercept the corresponding curves at 10 min. (a) Complete equilibrium between 2-oxoglutarate and glutamate; (b) partial equilibrium, $\beta=0.5$; (c) complete equilibrium, but with an induction period of 1 min. and calculated from a simplified model (see the text).

by Ashby *et al.* 1965) and values of the variables, e.g. any set in Table 5. The two sets that gave the best fit to the data on steady-state theory were chosen: line 19 in Table 5 of the exact solutions, and line 4 in Table 5 of the least-mean-square solutions. In all calculations both sets gave nearly identical results. The general applicability of non-steady-state theory was tested by calculating the time-courses of the specific radioactivities of aspartate, glutamate and pyruvate after intravenous injection of [U-¹⁴C]glucose and comparing them with the experimental values (Fig. 1).

Three approaches were tried.

(1) Complete equilibration between glutamate and 2-oxoglutarate was assumed (Fig. 1a).

(2) Partial equilibration only was assumed. It was supposed that on each turn of the tricarboxylic acid cycle half the 2-oxoglutarate formed by-passed glutamate (Fig. 1b). Both approaches predicted much more rapid initial labelling of aspartate and glutamate than was in fact observed. (Had either approach worked it would have been possible to obtain an independent estimate of the rate of rotation of the tricarboxylic acid cycle, *R*.)

(3) In preliminary experiments it was observed that very little label reached either aspartate or glutamate within 0.5 min. of injection of pyruvate, but that after 1 min. there was a very rapid increase in labelling. This is the expected result if there are several processes between injection of label as pyruvate and its appearance in metabolites (diffusion processes and pools of intermediates) that all take similar times, when their effects simulate an induction period. The first approach, with a further simplification, was therefore combined with an induction period of 1 min. The predicted and calculated specific-radioactivity-time curves agreed well (Fig. 1c).

Although these three approaches differed in how well they reproduced the specific-radioactivity-time curves, they all agreed in predicting that 10 min. after injection of [U-¹⁴C]glucose the relative specific

radioactivities of pyruvate, aspartate and glutamate would be close to their steady-state values. The steady-state values relative to the specific radioactivity of pyruvate are denoted by horizontal lines in Fig. 1. Moreover they predicted that the (C5-Glu)/(T-Glu) ratio after injection of C2-pyruvate would be close to its equilibrium value 10 min. after injection (Fig. 2). The first approach gave (C5-Glu)/(T-Glu) = 0.15, exactly the steady-state value, and the second approach 0.169, i.e. only 12% higher, much less than the biological variation (Table 2). Thus the use of steady-state theory was justified, to some extent because of a fortunate choice of time-interval between injection of C2-pyruvate or [U-¹⁴C]glucose and taking the liver.

In more detail the computations were carried out as follows.

The specific radioactivity of plasma glucose was taken initially to vary as:

$$\text{Specific radioactivity of glucose} = 0.72e^{-2t} + 0.11e^{-0.1t} + 0.17e^{-0.02t} \quad (3)$$

Fig. 2. Calculated (C5-Glu)/(T-Glu) ratios plotted against time after intravenous injection of C2-pyruvate. —, Equilibrium case; ----, partial equilibrium case (see the text for details). For steady-state theory to be applicable without correction the curves should cut (C5-Glu)/(T-Glu) = 0.15 at 10 min.

Table 6. Concentrations of pyruvate, aspartate, citrate and glutamate in the livers of post-absorptive and injured rats

Means $\pm$ s.e.m. are given. For pyruvate the distribution was assumed to be log-normal, as the spread of values was great, so the means are geometric means, and the s.e. range is given (Heath, 1967).

	Concentration (μ moles/g. wet wt.)			
	Pyruvate	Aspartate	Citrate	Glutamate
Post-absorptive	0.123 (12) (0.105–0.145)	0.76 $\pm$ 0.04 (4)	0.270 $\pm$ 0.009 (11)*	4.11 $\pm$ 0.33 (5)
Injured	0.075 (12) (0.063–0.090)	0.46 $\pm$ 0.04 (4)	0.179 $\pm$ 0.016 (12)	1.51 $\pm$ 0.08 (5)

*Start & Newsholme (1968) found 0.226 $\pm$ 0.006 (7), a little lower.

relative to its specific radioactivity at $t=0$ (Ashby *et al.* 1965). Similarly that of pyruvate was taken to be:

Specific radioactivity of pyruvate =

$$0.95e^{-4t} + 0.05e^{-0.065t} \quad (4)$$

derived by Ashby *et al.* (1965). Only the exponent in the second term is critical. Its value, 0.065/min., agrees with 0.072/min. found by Shichiri, Miller & Woodward (1967) for DL-C2-lactate from 5 min. after injection. It seems likely that the second term obtained by Ashby *et al.* (1965) in the equation (4) also represents predominantly the disappearance of label from the combined lactate-pyruvate pool in blood.

The intermediate pool between plasma and the tricarboxylic acid cycle was assigned the turnover constant 1/min. (Heath, 1968). The glutamate and aspartate pool sizes in liver were taken from Table 6. The effective glutamate pool was considered to contain half the free glutamine in the liver (see under '4. Non-steady-state theory: Simplification of the basic scheme' in the Theory section of Heath, 1968), giving $Q_g = 20 \mu\text{moles/4g.}$ For complete equilibrium between 2-oxoglutarate and glutamate (first approach) $k_g = R/Q_g$ and $k_a = (V_2 + V_3 - \Delta)/Q_a$ (eqns. 4.8 and 4.9 in Heath, 1968). For partial equilibrium between glutamate and 2-oxoglutarate (second approach), with $\beta = 0.5$, see eqns. (4.29) and (4.44) in Heath (1968) for calculation of k_g and k_a .

For both approaches and both sets of results (lines 4 and 19 in Table 5) the relative specific radioactivities of glutamate, aspartate and pyruvate after intravenous injection of $[U-^{14}\text{C}]$ glucose were calculated as functions of time (Appendix to the present paper). The relative specific radioactivities were close to their equilibrium values at 10 min. (Figs. 1a and 1b). Similarly the (C5-Glu)/(T-Glu) ratio after injection of C2-pyruvate was calculated (Fig. 2). Thus non-steady-state theory validates the use of the specific-radioactivity ratios in Tables 2 and 3 in steady-state theory to calculate rates.

It did not, however, reproduce the early parts of specific-radioactivity-time curves. The third approach was based on the finding that after pyruvate injection very little label appeared in aspartate within 0.5 min. In view of the rapid uptake of pyruvate by the liver (Busch, 1955; Ashby *et al.* 1965) this implies that there is, not one 'pool' between injection and aspartate, but several in series, none of which is rate-controlling. Under these circumstances there is an effective induction period, in these experiments of about 1 min. Introducing an induction period, however, was not in itself enough to correct for the over-rapid calculated rise in the specific radioactivity of glutamate after injection of $[U-^{14}\text{C}]$ glucose, nor was an additional adjustment in the value of k_r . There may be a physiological explanation. The first term in eqn. (3) is that required if labelled glucose mixes with the whole of the plasma pool of glucose before transport to and from extracellular spaces. Ashby *et al.* (1965) pointed out that transport to part of the extracellular space was so fast that this picture is too simple. If mixing with part of the extracellular space and equilibration in plasma are indistinguishable kinetically (an extreme case), then the specific radioactivity of glucose would be given by the last two terms of eqn. (3) only, i.e.:

Specific radioactivity of glucose =

$$0.11e^{-0.1t} + 0.17e^{-0.02t} \quad (5)$$

As the extracellular space includes liver water, no intermediate pool is required. An induction period is needed, to allow for the time taken for label to reach the liver and to pass through the pools of chemical intermediates. The relative specific radioactivities were calculated on this basis, by using an induction period of 1 min. The results are in good agreement with experiment (Fig. 1c).

These adjustments to the early parts of the specific radioactivity-time curves do not affect the conclusions already reached about relative specific radioactivities 10 min. after injection, but they do make it impossible to use the curves to calculate R or β or both. A possible experimental method is described below (see under 'Further experiments' in the General Discussion and Conclusions section).

Discussion. The results in Table 5 were not distorted appreciably by neglecting lipogenesis, by approximating $^{14}\text{CO}_2$ fixation, or by using steady-state theory. They were therefore a reliable basis for further development. None required more than 20% of the pyruvate rapidly oxidized to be oxidized in tissue other than the liver and kidneys, in accordance with the original supposition (see under 'Errors in data' in the Basis of Application *in vivo* section).

The calculated rate varied with the value assumed for the rate of rotation of the tricarboxylic acid cycle, R . To simplify the treatment this rate is taken to be $6 \mu\text{moles/min./4g.}$ of liver. This was the best estimate that could be made from data obtained *in vivo* (see under 'Preassessment of absolute rates' in the Basis of Application *in vivo* section) and is in the range implied by the work of Hems, Ross, Berry & Krebs (1966) on perfused liver. The rate $6 \mu\text{moles/min./4g.}$ of liver corresponded to an oxygen consumption of $17 \mu\text{moles/min./4g.}$ of liver (calculated from line 19 in Table 5). Hems *et al.* (1966) found an oxygen consumption of $9 \mu\text{moles/min./4g.}$ with no added substrate, and $32 \mu\text{moles/min./4g.}$ with lactate, ornithine and ammonia, of which $17 \mu\text{moles/min./4g.}$ could be accounted for by the basal rate plus the necessary ATP formation. The estimate used of the rate of rotation of the tricarboxylic acid cycle may be wrong. When a better estimate of R is available it will be easy to correct the other rates from the results given in Table 5, unless R lies considerably outside the range $5\text{--}8 \mu\text{moles/min./4g.}$ of liver, which seems improbable.

The calculated rates also varied, though to a smaller extent, with the rate assumed for the conversion of the triose phosphates into glucose, i.e. they varied with V_6 . This rate is taken to be $6 \mu\text{moles/min./4g.}$ of liver, which is exactly midway between the rates calculated from other experiments. Hems *et al.* (1966) found a rate of $8.2 \mu\text{moles/min./4g.}$ of liver in livers perfused with high lactate concentrations. At the physiological lactate concentrations *in vivo* the rate should be less.

Table 7. *Ranges of rates in the post-absorptive rat*

For definition of symbols see the text.

	A	z_0	Δ	V_2 ($\mu\text{moles/min./4g. of liver}$)	V_3
Exact fit (E)	0.25-0.66	0.49-1.00	0.28-0.47	1.8-2.9	0.57-1.04
Least-mean-squares fit (LMS)	0.24-0.59	0.30-0.55	0.30-0.51	2.1-2.9	0.39-0.86

Hornbrook, Burch & Lowry (1965) found a net rate *in vivo* after a big dose of lactate of $4 \mu\text{moles/min./4g. of liver}$. This did not allow for breakdown of the glucose formed, and was therefore probably lower than the true rate *in vivo*.

With R and V_6 both fixed at $6 \mu\text{moles/min./4g. of liver}$ the likely ranges of the other rates were calculated by interpolation and extrapolation from Table 5. The ranges (Table 7) are considerable, and the attempt to estimate absolute rates was only moderately successful. A big range of A was expected, as $A V_2$ represents the rate of conversion of pyruvate into acetyl-CoA utilized by the tricarboxylic acid cycle, which the wide range of experimental values of $(\text{C5-Glu})/(\text{T-Glu})$ (Table 2) showed was very variable in post-absorptive rats. The ranges in other rates are almost entirely due to errors in the data, especially in $Y_2(\text{glucose})/Y_1(\text{glucose})$ (Table 1).

Nearly all the calculations subsequently referred to were carried out by using sets of rates from both exact and least-mean-square solutions (see Tables 5 and 7 and under 'Computation' in the Basis of Application *in vivo* section). In all respects but one they gave almost indistinguishable results. For most purposes, therefore, it is a matter of convenience, to avoid repetition, which type of solution is chosen for illustration. There is, however, one difference with important implications: the exact solutions required more asymmetry (higher z ; Table 7) than the least-mean-square solutions, i.e. they required more oxaloacetate to react with citrate synthase without equilibrating with fumarate. The most direct estimate of asymmetry is given by the $(\text{C3-Glu})/(\text{C2-Glu})$ ratio after injection of C2-alanine or C2-pyruvate; the higher this ratio the greater the asymmetry. Reasons have already been given for thinking that the experimental value of this ratio (Table 4) is lower than its steady-state value (see under 'Data used in the computations' in the Basis of Application *in vivo* section), and only solutions which give ratios at least as high as the experimental values are likely to be correct. This strongly favours the exact solutions. The objection to this argument is that a great deal of weight is placed on a single determination of the ratio, and that the nutritional state of the rat in which it was determined cannot be exactly matched

with the post-absorptive state in our rats. Two considerations diminish the force of this objection. First, the experimental values were substantially independent of the nutritional state of the rat (Table 4), suggesting that the asymmetry is a constant feature, so that matching of nutritional states is not important when estimating asymmetry. Secondly, it is shown below, when the contribution made by the kidneys is considered in detail (see under 'Effects of the kidneys' in the General Discussion and Conclusions section), that there is a general tendency for the present treatment to underestimate asymmetry. For these reasons exact solutions were preferred, even though the experimental value of $Y_3(\text{CO}_2)/Y_2(\text{CO}_2)$ was poorly reproduced (see above under 'Steady-state theory'). The best solution so far is taken as that in line 19 in Table 5, and the network of pathways with the rates in it is shown in Scheme 1.

To check the validity of the approach some implications of the rates in line 19 in Table 5 are now worked out and are compared with experimental data. In all cases at least one other set of rates was used and gave very similar results. Thus the conclusions did not depend on the use of one particular set of rates, but were probably valid for all those sets of rates considered satisfactory, i.e. those in **bold type** in Table 5.

(1) For rats in the same metabolic state it seems likely that most overall metabolic rates will be fairly constant. The transfer of label is, however, very dependent on how much of the acetyl-CoA used by the tricarboxylic acid cycle is formed from pyruvate, which was labelled, and how much from fatty acids, which were not, i.e. the quantities of label in metabolites depend to a considerable extent on the rate of formation of acetyl-CoA from pyruvate, $A V_2$. This rate was very variable (see above), and variations in it might be expected to account for much of the variance in the data in Tables 1 and 3. This was the case. The calculated standard deviations in the data were only a little less than the experimental ones (Table 8).

For the calculation the variables V_2 , V_3 , Δ and z were kept constant, and A was varied to give $(\text{C5-Glu})/(\text{T-Glu}) \pm 1 \text{ s.d. from its mean, i.e. } 0.25 \text{ and } 0.05$. Values of $Y_1(\text{glucose})$ etc. were calculated for these values of A . From the

Scheme 1. Best estimates of rates in the livers of post-absorptive rats. Rates are in $\mu\text{moles/min./4g. of liver, i.e. per 100g. rat body wt.}$ If the maximum allowance is made for interaction with the kidneys certain rates should be different: $V_2=2.0$; $AV_2=0.7$; $V_3\approx 0.5$. Some other rates must then be adjusted to balance.

Table 8. *Experimental s.d. values on various quantities and s.d. values calculated assuming that the only cause of variance was variance in (C5-Glu)/(T-Glu)*

The experimental s.d. values were calculated from Table 3 or from the original results obtained by Ashby *et al.* (1965). For derivation of theoretical s.d. values see the text. s.d. values are expressed as percentages of mean values.

Quantity	Y(glucose)			Y(CO ₂)		Relative specific radioactivities	
	Y ₁	Y ₂	Y ₃	Y ₂ /Y ₁	Y ₃ /Y ₂	Glutamate	Aspartate
						Pyruvate	Pyruvate
Experimental s.d. (%)	35	23	15	25	25	38	19
Theoretical s.d. (%)	25	22	6	14	17	25	13

differences between these values and the values when A was chosen to give the mean value of (C5-Glu)/(T-Glu) (0.15, Table 2; $A=0.4$, line 19 in Table 5) the variances in $Y_1(\text{glucose})$ etc. due to variance in (C5-Glu)/(T-Glu) were calculated. They were converted into s.d. values expressed as percentages of the means (Table 8). They were always less than the empirical errors, and by about the amounts required to allow for the experimental errors in the methods used. The finding implies that V_2 and V_3 were probably nearly constant in post-absorptive rats, but that they could

only be precisely calculated from yields by using very large numbers of rats.

(2) The calculated and empirical values of C1-Glu and C4-Glu after injection of C2-alanine agreed (Table 4). [The (C5-Glu)/(T-Glu) and (C3-Glu)/(C2-Glu) ratios have been used to calculate rates, and so cannot be used to check them.]

(3) The experimental and calculated pyruvate/

(glucose 6-phosphate) specific-radioactivity ratios after injection of [^{14}C]glucose agreed. The experimental pyruvate/glucose and (glucose 6-phosphate)/glucose specific-radioactivity ratios were 0.178 ± 0.033 (3) and 0.38 ± 0.02 (4) respectively, giving a pyruvate/(glucose 6-phosphate) specific-radioactivity ratio 0.47 ± 0.09 . All sets in Table 5 predicted pyruvate/(glucose 6-phosphate) specific-radioactivity ratios in the range 0.41–0.44.

There are no more data known to us for post-absorptive rats with which to check the validity of the approach. Some implications of the scheme are now listed.

(1) The net consumption of glucose by the tricarboxylic acid cycle was fairly small. It is given by:

$$\begin{aligned}\text{Glucose consumption} &= 0.5(AV_2 + \Delta) \\ &= 0.5(V_5 - V_6) \quad (6)\end{aligned}$$

The factor 0.5 appears because the rates are given for C_3 units. The rate depends mainly on A , as net oxidation can only take place when pyruvate moieties enter the tricarboxylic acid cycle via acetyl-CoA, and not when they enter via oxaloacetate. For $(\text{C5-Glu})/(\text{T-Glu}) = 0.05, 0.15$ and 0.25 (mean ± 1 s.d.) glucose consumption by the liver was calculated to be about 0.3, 0.6 and 1.1 $\mu\text{moles/min.}$ respectively.

(2) The tricarboxylic acid cycle in the liver in post-absorptive rats ran mainly on fat. The rate of acetyl-CoA formation from fat is given by:

$$R - AV_2 \quad (7)$$

and, for $R=6$, was 5.7, 5.1 and 4.1 $\mu\text{moles/min.}$ over the same range of $(\text{C5-Glu})/(\text{T-Glu})$. The calculated variation with $(\text{C5-Glu})/(\text{T-Glu})$ in the percentage of mitochondrial acetyl-CoA originating from pyruvate is shown in Fig. 3.

(3) The rate of pyruvate carboxylation by pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), EC 6.4.1.1] was apparently not controlled by the concentration of pyruvate in the liver, i.e. if there was no other controlling factor higher rates of carboxylation would have been expected [$V_2 + V_3 \approx 3 \mu\text{moles/min./4g.}$ of liver from the present calculations; about 9 $\mu\text{moles/min./4g.}$ of liver expected from the pyruvate concentration (Table 6)].

After correction of concentrations of liver pyruvate to molarity in liver water (0.7 ml. of water/g. of liver) the concentration in post-absorptive rats was 0.18 mM. Walter, Paetkau & Lardy (1966) and Henning & Seubert (1964) found the maximum carboxylation capacity, $V_{\text{max.}}$, to be about 20 $\mu\text{moles/min./4g.}$ of liver, and Henning & Seubert (1964) the K_m for pyruvate to be about $2 \times 10^{-4} \text{M}$; whence, by the Michaelis-Menten equation, $(V_2 + V_3)_{\text{max.}} \approx 9 \mu\text{moles/min./4g.}$ of liver. This estimate may well be too high, as there must be a diffusion gradient between the cytoplasm

and the mitochondrion, and no allowance was made for the pyruvate in the blood in the liver, so the concentration of mitochondrial pyruvate may have been appreciably less than 0.18 mM.

(4) The observation by Freedman *et al.* (1960) that $(\text{C3-Glu})/(\text{C2-Glu})$ after intraperitoneal injection of C2-alanine was about 2 (Table 4) implied that more than half of the oxaloacetate reaching the tricarboxylic acid cycle from pyruvate reacted with citrate synthase before it equilibrated with fumarate (Table 5). The present analysis showed that the ratios of the yields of [^{14}C]glucose from specifically labelled pyruvates not only confirmed this, but also required that more of the phosphoenolpyruvate formed from pyruvate was produced via the tricarboxylic acid cycle than was formed by the pathways by-passing the cycle, i.e. $(V_2 - \Delta) > V_3$ (Table 7 and Scheme 1). The two findings combine to throw some light on the interaction of the tricarboxylic acid cycle and gluconeogenesis. The lack of symmetrization in oxaloacetate shows that the concentration of oxaloacetate in the tricarboxylic acid cycle is minute, in agreement with the conclusions reached from studies of the redox potentials and malate concentrations in mitochondria by Krebs (1966). Consequently label from the tricarboxylic acid cycle cannot be transferred to the cytoplasm via aspartate and is unlikely to be transferred as oxaloacetate itself. Transfer as oxaloacetate can probably be ruled out anyway (Walter *et al.* 1966; Shrago & Lardy, 1966). (The argument shows also that aspartate receives label only from cytoplasmic oxaloacetate, in accordance with the basic scheme.) The label must therefore be transferred in malate or fumarate or both. The transfer of label corresponds to a net transfer of compound, i.e. the data cannot be explained by the replacement of labelled malate in the mitochondria by unlabelled malate from the cytoplasm: in the steady state the cytoplasmic malate will be as heavily labelled as the mitochondrial, and there is no cytoplasmic source of the quantities of malate required. Thus the tricarboxylic acid cycle donates gluconeogenic material to the cytoplasm, and this material is malate or fumarate or both, not oxaloacetate. Krebs, Gascoyne & Notton (1967) came to the same conclusion on different grounds. They favoured transfer of malate. The rate of transfer in post-absorptive rats was estimated to be about 2 $\mu\text{moles/min./4g.}$ of liver (Scheme 1).

A net loss from the tricarboxylic acid cycle of malate can only be easily visualized as in Scheme 2, some malate as it is formed diffusing from the mitochondrion. The fraction of the total malate produced that diffuses will depend on the topography of the mitochondrion, and on the diffusion gradient for malate/fumarate between the mitochondrion and the cytoplasm. If topography is the

Fig. 3. Calculated percentages of the acetyl-CoA used by the tricarboxylic acid cycle in liver that are produced from pyruvate as a function of $(\text{C5-Glu})/(\text{T-Glu})$.

Scheme 2. Possible form of the system succinate $\rightarrow$ oxaloacetate in the mitochondrion, showing irreversible loss to the cytoplasm of about a third of the malate (and perhaps fumarate), causing a deficiency of oxaloacetate that is made up from pyruvate.

only factor, then a constant fraction of the contents of the tricarboxylic acid cycle will diffuse into the cytoplasm under all normal conditions. This fraction is given by:

$$\text{Fraction diffusing} = (V_2 - \Delta)/(R - \Delta) = 0.3475 \quad (8)$$

The estimated value was obtained by substituting the values of V_2 , Δ and R from line 19 in Table 5 in eqn. (8). In considering other metabolic states this estimate is used, as there are no data on which to correct it for changes in diffusion gradient. The diffusion gradient may depend on redox potentials and on the activities of gluconeogenic enzymes, especially of phosphopyruvate carboxylase [GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32], factors that vary with the nutritional

status of the rat (Shrago, Lardy, Nordlie & Foster, 1963). It turned out that the assumption that the fraction remained constant gave plausible results for fed and starved rats, which suggests that these nutrition-dependent factors may not affect the fraction diffusing very much.

The scheme implies that insufficient oxaloacetate is formed from malate for the perpetuation of the tricarboxylic acid cycle, and that this deficiency must be made good, or the cycle will stop. In the post-absorptive rat it is made good from pyruvate.

It must be emphasized that this picture is for liver *in vivo*. It is unlikely to be correct when *in vitro* or in perfused livers the system is subjected to much higher pyruvate concentrations. Then oxaloacetate formation can greatly exceed the requirements of the tricarboxylic acid cycle, its concentration will not be low, label may well be symmetrized [as found *in vitro* by Walter *et al.* (1966) and by Haynes (1965)] and oxaloacetate may be lost from the mitochondrion via aspartate. As shown below (see under 'Injured rats') this was substantially the situation in injured rats.

Fed and starved rats

The only ways in which the experimental data already presented show fed and starved rats to differ from post-absorptive ones is in the fractions of the acetyl-CoA used by the cycle that are derived from pyruvate. The high values in fed rats of $(\text{C5-Glu})/(\text{T-Glu})$ after intravenous injection of C2-pyruvate show that a much higher fraction enters this way in fed rats, and the very low values of this ratio in starved rats show that in them very much less enters in this way (Table 2). It was found that, when these differences were allowed for, many other differences between fed, starved and post-absorptive rats could be readily explained without varying most of the other rates. This provided a series of tests of the general validity of the theoretical approach.

Fed rats. All rates were assumed to be as in line 19 in Table 5 for post-absorptive rats except the rate of conversion of pyruvate into acetyl-CoA. In fed rats $(\text{C5-Glu})/(\text{T-Glu})$ is found to be 0.35–0.40 (Table 2) 1 hr. after intraperitoneal injection of C2-alanine. These estimates are probably below their steady-state values (Fig. 2), and the best estimate was taken to be 0.40. The rate of conversion of pyruvate into acetyl-CoA (AV_2) was adjusted to give this value ($A=2.00$), and the results in Table 9 were computed. The results predicted several experimental findings.

(a) The tricarboxylic acid cycle runs mainly on pyruvate, not on fat.

(b) Glucose consumption by the liver is high.

(c) After injection of $[\text{U-}^{14}\text{C}]$ glucose the aspartate/glutamate specific-radioactivity ratio should

Table 9. *Some computed rates etc. in recently fed rats*

Rates are in $\mu\text{moles/min./4g. of liver}$. The required value of Δ is 2.0.

Acetyl-CoA formation from pyruvate (ΔV_2)	4.6
Acetyl-CoA formation from free fatty acids (eqn. 7)	1.4
Net utilization of glucose	2.5
$Y_3(\text{glucose})$	0.27
$Y_2(\text{glucose})$	0.19
$Y_1(\text{glucose})$	0.048

be 0.83, 0.30 lower than in post-absorptive rats. The ratio found 12 min. after intravenous injection was 0.87 (mean of two results), i.e. 0.26 lower than in post-absorptive rats in strictly comparable experiments (Fig. 1).

(d) The profile of labelling in glutamate after injection of C2-alanine is reproduced fairly well (Table 4). The experimental value of (C3-Glu)/(C2-Glu) is rather lower than that predicted, as was expected. The contribution made to labelling in glutamate by recycling via ^{14}C glucose increases the greater the utilization of glucose by the tricarboxylic acid cycle; and the randomization in ^{14}C glucose increases the more label enters the tricarboxylic acid cycle via acetyl-CoA. Both recycling via ^{14}C glucose and the entry of label via acetyl-CoA are much more important in fed than in post-absorptive and starved rats, and both should lower experimental values of (C3-Glu)/(C2-Glu) to below those calculated, as here, by a method neglecting recycling via ^{14}C glucose. Some increase in fed rats in symmetrization of label in mitochondrial oxaloacetate cannot, however, be ruled out.

Theory also predicted that all yields of ^{14}C glucose from C1-, C2- and C3-pyruvate should be lowered, and that the $Y_3(\text{glucose})/Y_2(\text{glucose})$ ratio should be raised by an easily measurable amount.

Feeding may well change other rates than the rate of conversion of pyruvate into acetyl-CoA (ΔV_2), but the metabolic consequences of such changes were calculated to be relatively small. In fed rats small variations in (C5-Glu)/(T-Glu) correspond to big variations in the percentage of the acetyl-CoA used by the tricarboxylic acid cycle that comes from pyruvate (Fig. 3). Only when (C5-Glu)/(T-Glu) is fairly small is it a sensitive index of pyruvate metabolism by this pathway, as in starved and post-absorptive rats.

The total rate of formation of pyruvate in the fed rat (V_1) was about $9 \mu\text{moles/min./4g. of liver}$, greater than the rate of formation of glucose from the triose phosphates, $6 \mu\text{moles/min./4g. of liver}$ assumed (V_6). It can be shown to follow from the results obtained by Threlfall & Heath

(1968) that if V_1 was greater than $0.75 V_6$ the gluconeogenic and glycolytic chains would have to be compartmented at the triose phosphate stage to explain some of the results obtained by Ashby *et al.* (1965). The high values of (C5-Glu)/(T-Glu) in fed rats and the higher of the values in post-absorptive rats (Table 2) all imply such high rates of formation of acetyl-CoA from pyruvate that V_1 must have exceeded $0.75 V_6$, and therefore that some compartmentation of the triose phosphates must exist.

Starved rats. The three sets of data considered are in Table 10. The experimental conditions should be noted. As (C5-Glu)/(T-Glu) in starved rats is very low (Table 2) the rate of conversion of pyruvate into acetyl-CoA (ΔV_2) is taken as zero in all computations.

The theory predicts that in the starved rat the liver must oxidize glutamate. Hoberman & d'Adamo (1960) found that of C4 + C5 + C6 label in glucose from C2-lactate only 4% of the label was in the C4-position (Table 10), and pointed out that little of the label in glucose could have passed through the tricarboxylic acid cycle. As the tricarboxylic acid cycle loses a roughly constant fraction of its contents as malate to the cytoplasm (see above under 'Post-absorptive rats: Discussion'), this requires malate in the cycle to be very lightly labelled. In the starved rat the input of label into the cycle is proportional to the rate of entry into the tricarboxylic acid cycle of oxaloacetate from pyruvate, as no label enters via acetyl-CoA. This rate (V_2) must therefore have been small. But if about one-third of the malate is lost (eqn. 8), and the oxaloacetate introduced is inadequate to make up the loss, the tricarboxylic acid cycle will stop unless the deficiency is made up from elsewhere. The only source of sufficient material is glutamate, i.e. in the starved rat glutamate from protein or the circulation enters the tricarboxylic acid cycle, unlike in the post-absorptive and fed rat, where it leaves it. In mathematical terms, Δ is negative.

In the computations that follow there were three independent variables: the rate of utilization of glutamate by the tricarboxylic acid cycle ($-\Delta$); the rate of formation of cytoplasmic oxaloacetate directly from pyruvate; and the fraction of label that retained its position in passing through oxaloacetate (α). Each set of data in Table 10 allowed at least one variable to be fixed within narrow limits, but left at least one uncertain.

For computations V_2 was calculated from eqn. (8). The equations for the steady state (Heath, 1968) were modified for Δ negative (Appendix to the present paper) and were programmed. The calculated values of Δ (and hence V_2) and V_3 were nearly proportional to the values of R used. We assumed that in this early stage of starvation (18–30 hr.) the tricarboxylic acid cycle would go at about the same rate as in the post-absorptive rat, and took $R - \Delta = 6 \mu\text{moles/}$

Table 10. *Data from starved rats*

In interpreting the results obtained by Hoberman & d'Adamo (1960) we assume with them that C3-label=C4-label in glucose; and in interpreting the results obtained by Lorber, Lifson, Wood, Sakami & Shreeve (1950) that the labelling proportions C3/C2/C1 in glucose are the same as the labelling proportions C4/C5/C6. Our results are means of two, s.d. about 20%.

Authors	Compound injected	Dose	Priming dose of glucose	Data		
				Label within glucose (%)		
				C3=C4	C5	C6
				C3,4	C2,5	C1,6
Hoberman & d'Adamo (1960)	C2-lactate	2.4 g./kg. (oral)	None	4	63	33
Lorber <i>et al.</i> (1950)	C2- and/or C3-lactate (¹⁴ C and/or ¹³ C)	1.8 g./kg. (oral)	4 g./kg. at same time	11	52	37
				Relative specific radioactivities 12 min. after injection		
				Aspartate Glucose	Glutamate Glucose	
Present paper	[U- ¹⁴ C]Glucose	Tracer	None	0.060	0.040	

min./4g. of liver, so that, with Δ negative, the 2-oxoglutarate $\rightarrow$ fumarate reactions were supposed to go a little faster and the oxaloacetate $\rightarrow$ 2-oxoglutarate reactions a little slower than in the post-absorptive rat. From the proportionality relationship rough values of Δ , V_2 and V_3 can easily be calculated for other assumptions. Results were nearly independent of V_6 and z , which were taken as 6 and 0.68 as usual (line 19 in Table 5). There remained three variables, Δ , V_3 and α , with which to explain in each case two results (V_2 is a dependent variable), so unambiguous solutions could not be obtained. In fact, however, each set of data fixed one or two variables within narrow limits.

The fraction of label that retained its position during passage through cytoplasmic oxaloacetate (α) was required to be about 0.75 for the results obtained by both Lorber *et al.* (1950) and Hoberman & d'Adamo (1960). The possible ranges were 0.7–0.8 in the first case and 0.70–0.75 in the second. (Our data gave no information on α , as they are independent of its value.) These values are higher than those used in the computations for post-absorptive rats (0.66), but not sufficiently so to warrant recomputation.

The results obtained by Hoberman & d'Adamo (1960) and by Lorber *et al.* (1950) were consistent with wide ranges of rates of glutamate utilization and pyruvate carboxylation (Table 11 shows typical solutions) and therefore gave little information on them. Our data required a high rate of glutamate utilization and fixed the rates of pyruvate carboxylation. To refine the estimates allowance was made for utilization of aspartate and alanine, as well as of glutamate. The best estimates are shown (line 5 in Table 11).

Specific radioactivities after injection of [U-¹⁴C]glucose can only be calculated relative to that of glucose 6-phosphate. In the starved rat there is little glycogen to dilute label in glycolytic glucose 6-phosphate, so the specific radioactivities relative to that of glucose were taken as the same as specific radioactivities relative to that of glucose 6-phosphate. In two starved rats the specific radioactivities of glucose and glucose 6-phosphate were shown to be equal 12 min. after intravenous injection of [U-¹⁴C]glucose. To fit the data Δ had to be negative. It was assumed that the glutamate utilized originated in protein, and that for every mole of glutamate utilized 0.3 mole of alanine and 0.5 mole of aspartate were utilized. The alanine would decrease both relative specific radioactivities by the factor $(V_2 + V_3)/(V_2 + V_3 - \Delta)$. The aspartate would dilute additionally the aspartate pool by $(V_2 + V_3 - \Delta)/(V_2 + V_3 - 1.5\Delta)$, and decrease the specific radioactivity of aspartate correspondingly. [The derivations are obvious from Scheme 1 in Heath (1968).]

The nutritional status of our rats was not in doubt, and they are taken as the base on which to discuss the others. The profile of labelling calculated to be in glutamate after injection of C2-pyruvate or C2-alanine agreed well with experiment (Table 4). In liver of starved rats pyruvate concentrations are low (Ray, Foster & Lardy, 1966; Start & Newsholme, 1968), so the total rate of carboxylation of pyruvate, cytoplasmic + mitochondrial, ($V_2 + V_3$), should be less than in post-absorptive rats. The rates were less: 1.2 against 3 μ moles/min./4g. of liver (Table 11; cf. Scheme 1). The maximum rate of carboxylation consistent with the pyruvate concentrations in liver is in the range 2.1–7.6 μ moles/min./4g. of liver (for calculation see above under 'Post-absorptive rats: Discussion'). The range is

Table 11. *Results of computations in starved rats*

For data see Table 10. In the results of Hoberman & d'Adamo (1960) the distribution of label with glucose from C2-lactate was fitted precisely, with $\alpha=0.73$ pre-set, and V_3 and Δ were varied. In the results of Lorber *et al.* (1950), the same, but Δ varied over a wide range, pre-set, and α and V_3 were varied to fit the distribution for each pre-set Δ . In the present results, the aspartate/glucose and glutamate/glucose specific-radioactivity ratios were fitted precisely, and V_3 and Δ were varied; the value of α was irrelevant. In all computations $V_6=6$, $z=0.68$ (Table 5, line 19). For the specific radioactivities of CO_2 involved in carboxylation see the Appendix to the present paper.

Authors	Δ	α	V_3	V_2	Computed specific radioactivities and yields				
					Aspartate	Glutamate	Aspartate	$Y_1(\text{glucose})$	$Y_2(\text{glucose})$
					Glucose	Glucose	Glutamate		
Hoberman & d'Adamo (1960)	-1.4	0.73	2.0	0.69	0.157	0.052	3.0	0.501	0.860
Lorber <i>et al.</i> (1950)	0	0.71	0.90	2.09	0.244	0.169	1.44	0.193	0.581
	-0.5	0.74	0.51	1.59	0.148	0.114	1.30	0.178	0.587
	-1.0	0.77	0.28	1.09	0.077	0.067	1.15	0.170	0.603
Present paper	-1.36	0.77	0.42	0.74	Experimental values			0.280	0.690
					0.060	0.040	1.5		

that given by the low pyruvate concentration reported by Ray *et al.* (1966) and by the high one reported by Start & Newsholme (1968). Like in the post-absorptive rat (see above under 'Post-absorptive rats: Discussion') the rate calculated in this way is higher than that required by the other data, i.e. $1.2 \mu\text{moles/min./4g.}$ of liver.

Two types of comparison with the other data are possible.

(1) The ratios of the rates of carboxylation of pyruvate in the cytoplasm and in the mitochondria (V_3/V_2) can be compared. This ratio in post-absorptive rats was about 0.3. Each set of data in Table 11 gave estimates of this ratio within narrow limits. Our data gave a ratio about 0.6. The results obtained by Lorber *et al.* (1950) gave a ratio about 0.3. The rats in their experiments had been starved and then primed with glucose (Table 11), and may well therefore have been in a less starved state than ours. The results obtained by Hoberman & d'Adamo (1960) gave a ratio about 3, i.e. the big dose of lactate they had been given enormously increased the relative rate of cytoplasmic carboxylation. Their results imply that $Y_2(\text{glucose})/Y_1(\text{glucose})$ should be about 1.7 (Table 11), against 2.5 calculated for our rats. This difference should be easily demonstrable by experiment.

(2) The aspartate/glutamate specific-radioactivity ratios after intravenous injection of $[\text{U-}^{14}\text{C}]$ -glucose can be calculated from the rates and compared. The ratio was not very sensitive to the absolute rates of carboxylation (V_2 and V_3 ; lines 2-4 in Table 11), so the uncertainties in these rates did not affect the conclusions. For our starved rats the experimental ratio was 1.5, and for our

post-absorptive rats was about 1.15 (Table 3). The calculated values of the ratio for the rats used by Lorber *et al.* (1950) lay between these values (Table 11), i.e. their rats were in an intermediate state, as suggested above. For the rats used by Hoberman & d'Adamo (1960) the calculated value was about 3.0, i.e. very high, again confirming that the big dose of lactate these rats had received had altered their metabolic state.

The abnormality of the rats used by Hoberman & d'Adamo (1960) in comparison with ours is indirectly confirmed by some experiments of Friedmann, Goodman & Weinhouse (1965). They gave fed and starved rats tracer doses of C3-pyruvate and determined among other things the fractions of total glucose label in the C6-position to be 0.25-0.26 in both cases, i.e. starvation made little difference. The calculated fractions expected to be in this position for our post-absorptive and starved rats (from line 19 in Table 5 and line 5 in Table 11, $\alpha=0.66$) are 0.255 and 0.224, not much different from each other, and very like the fractions found by Friedmann *et al.* (1965). In the work by Hoberman & d'Adamo (1960), when the rats were given C2-lactate, 0.32 was found in the C5-position, i.e. after C3-lactate 0.32 should have been in the C6-position, a considerably greater fraction.

To conclude: starved rats utilized glutamate at about $1.4 \mu\text{moles/min./4g.}$ of liver, about half the maximum rate of gluconeogenesis from glutamate found by Ross, Hems & Krebs (1967) in perfused livers from rats starved for 48 hr. They carboxylated pyruvate more slowly than did fed and post-absorptive rats. The rate of carboxylation to cytoplasmic oxaloacetate was somewhat less decreased than the rate of carboxylation to oxaloacetate used by the

tricarboxylic acid cycle. About 0.75 of the label in cytoplasmic oxaloacetate retained its position in rats differing metabolically in other ways, i.e. equilibration with fumarate was far from complete. Rats given only big doses of lactate differed markedly in that apparently pyruvate carboxylation to cytoplasmic oxaloacetate was much faster than carboxylation to mitochondrial oxaloacetate.

Injured rats

For injured rats the yields of $[^{14}\text{C}]$ glucose and $^{14}\text{CO}_2$ are given in Table 1, and values of (C5-Glu)/(T-Glu) in Table 2. To begin with only mean values were used, i.e. with no correction of yields for rapid oxidation of pyruvate in organs other than the liver and kidneys, and with no allowance for errors in any data.

As:

$Y_2(\text{glucose}) - Y_1(\text{glucose}) = Y_1(\text{CO}_2) - Y_2(\text{CO}_2)$
there was no loss of label via glutamate. Glutamate could have been utilized, i.e. Δ was either zero or negative (see Appendix to the present paper).

$Y_3(\text{glucose})$ exceeds $Y_2(\text{glucose})$, which implies that there was much more symmetrization of oxaloacetate in the tricarboxylic acid cycle in injured rats than in post-absorptive rats. All calculations confirmed that the best fit to data was obtained by using the sym. case only, i.e. with complete symmetrization of label. This requires a much higher concentration of oxaloacetate in the tricarboxylic acid cycle than in the post-absorptive rat, and opens up the possibility that both cyto-

plasmic and mitochondrial oxaloacetate might be in equilibrium with aspartate. In the sym. case this situation can be represented by putting the rate of carboxylation of pyruvate to cytoplasmic oxaloacetate (V_3) equal to zero. Both cytoplasmic and mitochondrial oxaloacetate should then have the same specific radioactivities in the steady state. The best fit was, in fact, always obtained with $V_3 = 0$ (or V_3 slightly negative, but insignificantly so).

Computed rates must be consistent with a decreased rate of gluconeogenesis in the injured rats (30% less than in post-absorptive rats; Ashby *et al.* 1965), and with the decreased concentrations of citrate, glutamate and aspartate in the liver at the time studied (Table 6). Computation showed that it was impossible on steady-state theory to account for both the yields of $[^{14}\text{C}]$ glucose from specifically labelled pyruvates and the relative specific radioactivities of glutamate, aspartate and pyruvate after injection of $[\text{U-}^{14}\text{C}]$ glucose unless glutamate was utilized by the tricarboxylic acid cycle. The same conclusion was reached by using non-steady-state theory. If glutamate was utilized (negative Δ) a good fit to the data could be obtained whatever the rate of rotation of the tricarboxylic acid cycle (R) over a wide range (Table 12).

When $V_3 = 0$ the value of α is indifferent, as all label reaching cytoplasmic oxaloacetate is symmetrized on arrival. To use the computer programme a non-zero value must be put in for technical reasons, but any will do. Initially values

Table 12. *Comparison between experimental and computed yields of $[^{14}\text{C}]$ glucose and relative specific radioactivities for injured rats*

For various R values either values of $Y_2(\text{glucose})$, 0.566 (Table 1), and (C5-Glu)/(T-Glu), 0.25 (Table 2), were fitted with A and V_2 as variables; or these values and $0.5 \times \text{aspartate/pyruvate specific-radioactivity ratio} + 0.5 \times \text{glutamate/pyruvate specific-radioactivity ratio} = 0.67$ (Table 3) with A , V_2 and Δ as variables. $V_3 = 0$ and $V_6 = 6$ throughout. Other experimentally determined quantities were calculated. The experimental values are listed at the top.

R	V_2	A	Δ	$Y_n(\text{glucose})$		Relative specific radioactivities after $[\text{U-}^{14}\text{C}]$ glucose		
				Y_1	Y_3	Aspartate Pyruvate	Glutamate Pyruvate	Aspartate Glutamate
				Experi- mental 0.189	0.685	0.68	0.66	1.15
4	7.88	0.258	0	0.214	0.662	1.18	1.23	0.96
3	4.08	0.303	0	0.209	0.673	1.10	1.11	0.99
2	2.06	0.334	0	0.205	0.682	1.02	1.00	1.02
1.5	1.38	0.347	0	0.203	0.685	0.98	0.96	1.02
1.0	0.83	0.358	0	0.201	0.687	0.94	0.92	1.02
0.5	0.37	0.371	0	0.199	0.688	0.91	0.88	1.03
4	4.33	0.315	-2.09	0.182	0.680	0.73	0.61	1.19
3	2.66	0.340	-1.10	0.186	0.687	0.71	0.62	1.16
2	1.48	0.359	-0.54	0.187	0.690	0.71	0.63	1.14
1.5	1.04	0.367	-0.34	0.188	0.692	0.72	0.63	1.13

of V_2 and A were calculated for various values of R to fit exactly the experimental values of $Y_2(\text{glucose})$ (Table 1) and $(\text{C5-Glu})/(\text{T-Glu})$ (Table 2) by using steady-state theory. Throughout $V_3 = \Delta = 0$. From the calculated values of V_2 and A values of $Y_1(\text{glucose})$ and $Y_3(\text{glucose})$ and the aspartate/pyruvate, glutamate/pyruvate and aspartate/glutamate specific-radioactivity ratios after intravenous injection of $[\text{U-}^{14}\text{C}]\text{glucose}$ were calculated. The calculated yields agreed with the experimental ones (Table 12), but the aspartate/pyruvate and glutamate/pyruvate specific-radioactivity ratios were too high, and the aspartate/glutamate specific-radioactivity ratio was too low (Table 12).

The discrepancies between the experimental and calculated relative specific radioactivities might be explained if the experimental values were higher than the steady-state values. To see if this was so, non-steady-state theory was applied (procedure associated with Fig. 1c) by using the specific-radioactivity-time function for $[\text{U-}^{14}\text{C}]\text{glucose}$ calculated from the results obtained by Ashby *et al.* (1965) with injured rats:

Specific radioactivity of glucose =

$$0.08e^{-0.09t} + 0.15e^{-0.006t}. \quad (9)$$

For Δ negative or zero:

$$k_g = (R - \Delta)/Q_g \quad (10)$$

where $Q_g = 7.5 \mu\text{moles/4g. of liver}$ [1.25 times the glutamate concentration (Table 6) to allow for glutamine]. For $R > 2$ the non-steady-state aspartate/pyruvate and glutamate/pyruvate specific-radioactivity ratios were calculated to be slightly higher 10 min. after injection of $[\text{U-}^{14}\text{C}]\text{glucose}$ than the steady-state ratios, i.e. the discrepancies between theory and experiment were increased. For $R = 1$ the non-steady-state values were calculated to be 0.61 and 0.81 respectively, in fair agreement with experiment, but the aspartate/glutamate specific-radioactivity ratio was 1.33, greater than that found (Table 3, $P < 0.01$). It was, in fact, impossible on either steady-state or non-steady-state theory to account satisfactorily for all three ratios with $\Delta = 0$.

Negative values of Δ were therefore used for $R > 1.5$ to fit exactly $Y_2(\text{glucose})$, $(\text{C5-Glu})/(\text{T-Glu})$ and the mean value (0.67) of the aspartate/pyruvate and glutamate/pyruvate specific-radioactivity ratios. For $R > 1.5$, $R - \Delta > 1.8$, which by eqn. (10) gives values of k_g too high to require any corrections to steady-state theory. Table 12 shows that all three relative specific radioactivities and all the other experimental data could be reproduced satisfactorily for $R = 1.5\text{--}4.0$ with Δ negative.

Although the computations did not directly limit the possible rate of rotation of the tricarboxylic acid cycle (R), indirectly they did so. The rates of carboxylation (V_2) and of glutamate utilization ($-\Delta$) rose steeply with the rate of rotation of the tricarboxylic acid cycle assumed. The computations were carried out assuming that all the rapid oxidation of ^{14}C pyruvate to $^{14}\text{CO}_2$ took place in the liver. This is improbable. If only a few per cent had been oxidized elsewhere, calculation showed that the rates of carboxylation and glutamate utilization would have had to be considerably higher than those in Table 12 for any given rate of

rotation of the tricarboxylic acid cycle. As the pyruvate concentrations in the liver of injured rats were lower than in those of post-absorptive rats (Table 6), the rate of carboxylation of pyruvate was unlikely to be increased, i.e. V_2 was likely to be less than $3 \mu\text{moles/min./4g. of liver}$. It is therefore unlikely that the rate of rotation of the tricarboxylic acid cycle (R) could have exceeded $2 \mu\text{moles/min./4g. of liver}$ in the injured rat, i.e. the tricarboxylic acid cycle was inhibited at least 70% by injury. The present analysis therefore confirms the conclusions already reached (see the Metabolic States section), and shows in addition that there was a net utilization of glutamate after injury analogous to that found in the starved state. The symmetrization of label in oxaloacetate shows that in the injured rat the concentration of mitochondrial oxaloacetate was very much higher than in the post-absorptive rat. The inhibition of the tricarboxylic acid cycle must therefore lie between oxaloacetate, whose concentration was increased, and citrate, whose concentration was decreased (Table 6). Inhibition is therefore at the citrate synthase stage, thus confirming the conclusions reached by Ashby *et al.* (1965) and by Heath & Stoner (1968). The most plausible explanation of the inhibition is a decrease in the rate of acetyl-CoA formation. If this is so, then a decrease in the acetyl-CoA concentration is to be expected.

The finding that glutamate was utilized provides a partial explanation of why liver glutamate is low 2 hr. after the removal of the tourniquets (Table 6) although from shortly after their removal there is a continuing release of amino acid nitrogen into the blood (Hoar & Haist, 1944; Stoner & Green, 1948). It also implies that there is a decrease of glucose consumption by the liver, and probably some net gluconeogenesis that helps to maintain the high plasma glucose concentrations observed. These glucose concentrations can be explained within the variance of the data by glycogenolysis (Stoner, 1958), but some contribution by amino acids would be consistent with the data.

GENERAL DISCUSSION AND CONCLUSIONS

The theoretical treatment developed by Heath (1968) has been shown to correlate the data in Tables 1–4, including the errors on those data, where they are known, and a number of other observations referred to at various places in the text. It also predicted that glutamate should be utilized by the tricarboxylic acid cycle in the starved rat (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section) and that the tricarboxylic acid cycle in the injured rat was inhibited at the citrate synthase stage, as previously concluded (see under 'Injured rats' in the Results

and Discussion section). We think this is enough to show that the approach is basically sound, and we are concerned here with factors that may have distorted the conclusions, with putting the conclusions in a broader context and with devising further experiments. The main aim of further experiments will be to refine the estimates of the rates. The present estimates are only rough ones (Table 7), partly because of experimental errors, but more because the type of experiment was not ideal for the purpose.

The two factors that may have introduced distortion were the assumption that kidneys behaved like liver in this context, and the neglect of isotopic exchange. It is shown that the assumption about the kidneys probably did cause some distortion, but that neglect of exchange probably caused little or none.

Effects of the kidneys. Busch (1955) showed that of injected [^{14}C]pyruvate 60–70% went initially to organs other than the liver. The results obtained by Ashby *et al.* (1965) combined with the treatment of the present paper showed that 60–70% was eventually metabolized by the kinetics appropriate to the liver. These observations are easily reconciled if the major primary metabolites in most organs are lactate and alanine [this is consistent with Busch's (1955) observations], much of which returns to the blood stream and is utilized by the liver. The labelling of alanine and lactate represents isotopic exchange and not net formation. Of the label in that portion of the labelled pyruvates rapidly metabolized about 80% could be accounted for by 'liver' kinetics (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section), leaving 20%, excreted as $^{14}\text{CO}_2$, to be accounted for in other ways. This residual 20% was rather more than expected (see under 'Errors in data' in the Basis of Application *in vivo* section), and led us to look for more positive evidence that liver kinetics were not wholly adequate. This evidence was found in the labelling of C5-glutamate in the kidney.

Freedman *et al.* (1960) found that after intraperitoneal injection of C2-alanine the (C5-Glu)/(T-Glu) ratio in the kidneys was 0.6 in the fed rat and 0.5 in the starved rat. These are impossibly high values on 'liver' kinetics (Fig. 3). But the whole distribution of label in glutamate closely resembled the distribution that Koeppe & Hahn (1962) found in rat brain after intraperitoneal injection of C2-glucose (equivalent in this context to C2-pyruvate). Koeppe & Hahn (1962) pointed out that this distribution requires nearly all the label entering the tricarboxylic acid cycle to do so via acetyl-CoA, and that very little can be supposed to enter via oxaloacetate. In terms of Heath's (1968) Scheme 1 the kidney results require a very

small V_2 , and $A > 5$, unlike the liver results. [The high proportion of acetyl-CoA formed from pyruvate in the kidney suggests that the sparing effects of fatty acids etc. on pyruvate utilization (Struck, Ashmore & Wieland, 1965; Teufel, Menahan, Shipp, Böning & Wieland, 1967; Jagow, Westermann & Wieland, 1968) should be greater in the kidney than in the liver.] As very little label enters the kidney tricarboxylic acid cycle via oxaloacetate very little can leave it as oxaloacetate, malate or fumarate, and so very little can become incorporated into glucose via the tricarboxylic acid cycle. Thus the gluconeogenic chain from pyruvate interacts very much less with the tricarboxylic acid cycle than in the liver, and consequently gluconeogenesis takes place mostly via the dicarboxylic acid shuttle, i.e. $V_3 > V_2$. As there is net glycolysis in the kidneys at considerably lower plasma glucose concentrations than those at which there is net glycolysis in the liver (Dzurik *et al.* 1963), probably in the post-absorptive rat the kidneys do not transfer much pyruvate label to glucose. Most of the label in pyruvate entering the kidney will enter the tricarboxylic acid cycle, and be excreted as $^{14}\text{CO}_2$. This could account for much of the $^{14}\text{CO}_2$ excretion that could not be accounted for by liver kinetics.

Assuming that the kinetics in the kidney have been deduced correctly, one can now see how the contributions made by the kidney to the yields of [^{14}C]glucose from labelled pyruvate could alter the conclusions drawn about the kinetics in the liver. The results obtained by Freedman *et al.* (1960) require gluconeogenesis in the kidney to take place substantially according to the kinetics of the dicarboxylic acid shuttle. For the shuttle $Y_3(\text{glucose})/Y_2(\text{glucose}) = 1.0$, and $Y_2(\text{glucose})/Y_1(\text{glucose})$ cannot exceed 2.0. As there is evidence that much label retains its position in passing through cytoplasmic oxaloacetate (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section and Table 11), the expected value of $Y_2(\text{glucose})/Y_1(\text{glucose})$ is about 1.5 ($\alpha = 0.66$). The overall values in the whole rat were: $Y_3(\text{glucose})/Y_2(\text{glucose}) = 0.87$; $Y_2(\text{glucose})/Y_1(\text{glucose}) = 3.3$ (Table 1). There is no evidence on which to assess how much labelling in glucose from labelled pyruvate takes place in the kidneys, but, however little it is, it will cause estimates of $Y_2(\text{glucose})/Y_1(\text{glucose})$ on the whole rat to be lower than in the liver, and of $Y_3(\text{glucose})/Y_2(\text{glucose})$ to be higher. Thus in the liver in the post-absorptive rat $Y_2(\text{glucose})/Y_1(\text{glucose})$ was probably greater than 3.3, and $Y_3(\text{glucose})/Y_2(\text{glucose})$ was probably less than 0.87. By interpolating and extrapolating from the results in Table 5 the effects on the rates of increasing $Y_2(\text{glucose})/Y_1(\text{glucose})$ and of decreasing $Y_3(\text{glucose})/Y_2(\text{glucose})$ were estimated.

The results suggested that in the liver mitochondrion oxaloacetate was not in equilibrium with malate. That oxaloacetate is not in equilibrium with fumarate has already been established (see under 'Post-absorptive rats: Steady-state theory' in the Results and Discussion section).

When $Y_3(\text{glucose})/Y_2(\text{glucose})$ was decreased the calculated value of $(\text{C3-Glu})/(\text{C2-Glu})$ in the liver after intraperitoneal injection of C2-alanine was higher than that found experimentally (Table 4). But the experimental value was probably low (see under 'Data used in the computations' in the Basis of Application *in vivo* section), so a decreased value of $Y_3(\text{glucose})/Y_2(\text{glucose})$ is plausible. The value of $(\text{C3-Glu})/(\text{C2-Glu})$ gave the amount of asymmetry in mitochondrial oxaloacetate, and was a very sensitive index of it (Table 5). It therefore seems that there must have been more asymmetry than was calculated by the best exact solution (line 19 in Table 5). The asymmetry was calculated as the contribution to the total kinetics made by non-sym. case B, i.e. by the case that assumes no equilibration between oxaloacetate and malate, and this case contributed therefore more than 0.68 of the total. In general, non-sym. case A can be substituted for non-sym. case B in the calculations, i.e. it can be assumed that malate and oxaloacetate are in equilibrium, but that fumarate and malate are not (see under 'Computation' in the Basis of Application *in vivo* section). But when non-sym. case A was substituted for non-sym. case B the contribution required of it was always much greater, and computation showed that to explain the experimental value of $(\text{C3-Glu})/(\text{C2-Glu})$ the whole kinetics would have to be accounted for by non-sym. case A ($z_1=1.00$), and that it was impossible to explain higher values of $(\text{C3-Glu})/(\text{C2-Glu})$. Thus there was probably some lack of equilibration between oxaloacetate and malate. As it is intrinsically unlikely that there was no equilibration at all between malate and fumarate, it is most likely that both the malate $\rightleftharpoons$ fumarate and oxaloacetate $\rightleftharpoons$ malate systems were some way from equilibrium.

This conclusion cannot be regarded as firmly established, because it depended on a detailed interpretation of the $(\text{C3-Glu})/(\text{C2-Glu})$ ratios in Table 4; but disequilibrium between both oxaloacetate and malate and malate and fumarate require consideration in any detailed study of the kinetics of the liver mitochondrion.

The other effects of allowing for the contributions to yields made by the kidneys were trivial. An increase in $Y_2(\text{glucose})/Y_1(\text{glucose})$ lowered the estimate of the rate of carboxylation of pyruvate to cytoplasmic oxaloacetate (V_3). However, this and other effects of increasing $Y_2(\text{glucose})/Y_1(\text{glucose})$ were to some extent compensated for by the effects of decreasing $Y_3(\text{glucose})/Y_2(\text{glucose})$. Allowing for the different kinetics in the kidney also decreased slightly the calculated rates of mitochondrial carboxylation of pyruvate (V_2) and of glutamate loss (Δ) (see also Scheme 1).

In starved rats the intervention of the kidneys may explain the apparently anomalous nature of

the results obtained by Hoberman & d'Adamo (1960) (Table 10). The other data in Table 10 and the observations made by Friedmann *et al.* (1965) all required most of an injected dose of labelled pyruvate or lactate to enter the tricarboxylic acid cycle en route to incorporation into glucose (Table 11, $V_2 > V_3$, and see under 'Fed and starved rats: Starved rats' in the Results and Discussion section). After a big dose of C2-lactate, however, the labelling in glucose found by Hoberman & d'Adamo (1960) was that expected if a high proportion of the lactate was metabolized by the dicarboxylic acid shuttle, i.e. directly via cytoplasmic oxaloacetate ($V_2 < V_3$; Table 11). The other data showed that the liver was adapted or controlled to utilize little glucose and pyruvate. It is possible that in the starved rat the kidney can utilize pyruvate or lactate readily, converting it into glucose, and that after a big dose some of this was then laid down in liver glycogen, in which Hoberman & d'Adamo (1960) analysed it.

Exchange of label without net flux of compound. Heath (1968) pointed out that a decision as to how far 'isotopic exchange' could invalidate the treatment was much easier to reach when at least approximate rates had been estimated, as has been done in the present paper. The present results lead straightforwardly to the conclusion that exchange was properly neglected.

In what follows, the first four points refer to Heath's (1968) introduction, the rest to his Discussion section.

(1) Interaction with lipogenesis, which involves a complex cyclic exchange of label via oxaloacetate + acetyl-CoA $\rightleftharpoons$ citrate, was shown to alter no estimates of rates, yields etc. by detectable amounts (see under 'Post-absorptive rats: Interaction with lipogenesis' in the Results and Discussion section).

(2) Back conversion of oxaloacetate into pyruvate via malate ['malic' enzyme; L-malate-NAD oxidoreductase (decarboxylating), EC 1.1.1.38] is stated by Seubert & Huth (1965) only to proceed at about $0.5 \mu\text{mole/min./4g. of liver}$. This rate is so much lower than the net rate of carboxylation of pyruvate ($V_2 + V_3$) in post-absorptive rats that its effect in them is plainly negligible. In starved rats this is not necessarily so, as the carboxylation rate was smaller (Table 11). Its main effect would be to increase the symmetrization of labelling in both cytoplasmic and mitochondrial oxaloacetate. The fact that in both symmetrization was far from complete, and seemed to be independent of nutritional state (see under 'Post-absorptive rats: Discussion' and under 'Fed and starved rats: Starved rats' in the Results and Discussion section), suggests that in practice neglect of this reaction makes very little difference.

(3) In the post-absorptive rat a loss of label as glutamate was required to correlate the relative yields of $^{14}\text{CO}_2$ and ^{14}C glucose (Table 7, Δ about 0.4). Hypothetically this loss of label could be accompanied by a net gain of glutamate if there were exchange of label with exogenous glutamate. A small exchange cannot be excluded. A big exchange can probably be ruled out, as it would appreciably lower the

specific radioactivity of glutamate relative to that of aspartate, and these relative specific radioactivities were adequately correlated in both post-absorptive rats (Fig. 1 and Table 5) and fed rats (see under 'Fed and starved rats: Fed rats' in the Results and Discussion section). Should exchange of aspartate label have been very similar to the exchange of glutamate label, then this argument does not hold. However, a big net consumption of glutamate or aspartate or both in fed and post-absorptive rats is unlikely, this being associated with starvation.

(4) Dilution of the system with unlabelled glutamate, aspartate and alanine in the starved state is covered by the Appendix to the present paper (negative Δ) and the treatment given under 'Fed and starved rats: Starved rats' in the Results and Discussion section. Exton & Park (1967) suggest that alanine is taken up by liver preferentially to glutamate. If so, the dilution by alanine was somewhat underestimated in the treatment given under 'Fed and starved rats: Starved rats' in the Results and Discussion section. Numerical calculations show that doubling the uptake of alanine would decrease the calculated specific radioactivities given in Table 11 by only 10–15%.

(5) If the reaction 2-oxoglutarate $\rightarrow$ succinate is reversible, then label in 2-oxoglutarate will become symmetrized. As there was little symmetrization ($z_0 > 0.68$; see above under 'Effects of the kidneys'), exchange in this reaction cannot be rapid. It seems more plausible to associate such symmetrization as there was with the oxaloacetate $\rightleftharpoons$ fumarate system, as was done, but some exchange in 2-oxoglutarate $\rightleftharpoons$ succinate cannot be ruled out. Trial computations show that no estimate of rate would be affected by more than a few per cent, except of V_3 , which could be increased by up to 30%, if all symmetrization was due to equilibration between 2-oxoglutarate and succinate.

(6) If the reaction pyruvate $\rightarrow$ acetyl-CoA is reversible, or if pyruvate is diluted by unlabelled alanine, then the pyruvate/(glucose 6-phosphate) specific-radioactivity ratio will be lower than calculated. This was not so. The found ratio was similar to that calculated (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section).

(7) The finding that α was the same for rats in two states (see Table 11 and under 'Fed and starved rats: Starved rats' in the Results and Discussion section) suggests that the pyruvate kinase reaction is irreversible.

Heath (1968) showed that the present treatment would not be invalidated if the carboxylation of pyruvate to oxaloacetate was reversible. Calculations in the present paper suggest that it may be: the rates of carboxylation, i.e. $V_2 + V_3$, were less than would be expected from the pyruvate concentrations in the liver in both post-absorptive rats (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section) and starved rats (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section). As estimates of pyruvate concentrations vary considerably with the way in which liver samples are taken (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section) and no allowance can be made for diffusion gradients (see under 'Post-absorptive rats: Discussion' in the Results and

Discussion section), the amount of the discrepancy is doubtful, but it seems likely that there is a real one. This could be explained by reversibility. It seems, however, very unlikely that in the mitochondrion the carboxylase reaction could be reversible, because the oxaloacetate concentrations are very low (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section). More likely the rates of carboxylation are lower than expected from the pyruvate concentrations, perhaps because the concentrations of cofactors (ATP, acetyl-CoA and Mg^{2+}) are not optimum (Keech & Utter, 1963; Mehlmán, Walter & Lardy, 1967). Although, therefore, the pyruvate concentrations found in excised livers could give higher rates of pyruvate carboxylation than are required by the present scheme, this is very weak evidence against the general view that the rate of oxaloacetate formation is rate-controlling for gluconeogenesis (Krebs, 1964; Henning, Huth & Seubert, 1964; Seubert & Huth, 1965; Krebs, Speake & Hems, 1965).

Williamson, Herczeg, Coles & Danish (1966) and Williamson, Olson, Herczeg & Coles (1967) have provided evidence that carboxylation of pyruvate to oxaloacetate also controls the rate of the citrate synthase reaction in the mitochondrion in the absence of an exogenous source of glutamate. This is entirely in line with the conclusions drawn in the present paper, i.e. that when insufficient oxaloacetate is formed, as in the liver in the starved rat, the deficiency is made up by glutamate. It follows that citrate concentrations should be lowered by starvation, as found by Angielski & Szutowicz (1967) in unanaesthetized rats and by Start & Newsholme (1968) in anaesthetized rats. The decreases in citrate concentration were fairly small, as was to be expected as citrate can be formed from 2-oxoglutarate.

Differences between metabolic states. The differences between fed and post-absorptive rats could all be explained by the known increase in the rate of formation of acetyl-CoA from pyruvate in the former (see under 'Fed and starved rats: Fed rats' in the Results and Discussion section). The differences between starved and post-absorptive rats could all be explained by the known decrease in this rate in the starved rats, and by their utilization of glutamate. This did not rule out the possibility of other rates varying, and it seems likely that other rates do vary. Freedman *et al.* (1960), however, found values of (C3-Glu)/(C2-Glu) after intraperitoneal injection of C2-alanine that were similar in all three metabolic states, fed, starved and post-absorptive, and a consideration of the errors in these estimates suggested that the asymmetry in mitochondrial oxaloacetate may be even more similar than at first appears (see under 'Fed and starved

rats: Fed rats' in the Results and Discussion section). This implies that the system oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate in the mitochondrion is in disequilibrium to a very similar extent in all three states, which imposes a considerable restriction on any detailed treatment of this system. For the treatment given in this paper it was assumed that a constant fraction of the malate/fumarate formed in the tricarboxylic acid cycle diffused to the cytoplasm in all three states. This was admitted to be a doubtful approximation (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section). It is, however, a feature of the methods used here that, given suitable data, they enable one to find out what fraction is in fact lost. This still has to be done for the fed and starved rat. If it is done, the results could hardly fail to throw light on the control of gluconeogenesis and the tricarboxylic acid cycle.

In the injured rat previous evidence had shown that the tricarboxylic acid cycle was inhibited at the citrate synthase stage (see the Metabolic States section). The results in the present paper confirmed and extended this conclusion. Label in oxaloacetate in the tricarboxylic acid cycle was found to be symmetrized (see under 'Injured rats' in the Results and Discussion section), and the kinetic distinction between mitochondrial and cytoplasmic oxaloacetate was lost ($V_3 = 0$; see under 'Injured rats' in the Results and Discussion section). These findings can only be explained by much higher mitochondrial concentrations of oxaloacetate than in normal rats, so that there was complete equilibration between oxaloacetate, malate and fumarate in both the mitochondrion and the cytoplasm, and exchange between both parts of the cell. High oxaloacetate and low citrate concentrations show that the reaction slowed by injury must lie between the two in the tricarboxylic acid cycle, i.e. must be the citrate synthase reaction. It is noteworthy that the tricarboxylic acid cycle utilized glutamate like the starved rat, as though a decrease in citrate formation by citrate synthase automatically results in utilization of glutamate, whether the decrease is brought about by inhibition of citrate synthase or by shortage of oxaloacetate.

Further experiments. The main weakness in the application of Heath's (1968) theory was the need to choose a rate of rotation of the tricarboxylic acid cycle (R) on imperfect evidence. The errors on this rate are probably not very great; even large errors ($\pm 30\%$) would not much affect the estimates of relative rates, but estimates of absolute rates are affected rather more than proportionately to the value of R used in the computations. A possible kinetic approach would involve the estimation of the specific radioactivities of liver glucose, glucose 6-phosphate, aspartate, glutamate and C1- and

C5-glutamate at closely spaced time-intervals after intravenous injection of C2-glucose. Whether this could provide information adequate to calculate the rate of rotation of the tricarboxylic acid cycle, allowing for incomplete equilibration between 2-oxoglutarate and glutamate, would be best investigated by an initial computer simulation. Even if the rate of rotation of the cycle could not be calculated reliably from the suggested experiments they could well put non-steady-state theory on a firmer basis. At present non-steady-state theory can only be used to correct data for steady-state theory, a use that requires little precision.

Most of the remaining experiments that need to be done are in principle repetitions of experiments already carried out by other workers on their colonies, but non-steady-state theory suggests that sampling is best done at different times.

(1) To find the asymmetry in label in mitochondrial oxaloacetate (z), the value of (C3-Glu)/(C2-Glu) in the liver after administration of C2-pyruvate should be estimated. The optimum time of sampling is 10–15 min. after intravenous injection.

(2) To estimate the asymmetry in label in cytoplasmic oxaloacetate (α) and the contribution of the tricarboxylic acid cycle to label in glucose the distribution of label between C4-, C5-, and C6-glucose should be estimated after intravenous injection of C2-pyruvate. Blood glucose should be taken about 15 min. after injection. By this method there is no need to disturb the metabolic state of the rats by priming with glucose, which is necessary when glycogen labelling is studied.

(3) To see whether the pyruvate kinase reaction is reversible the C2- and C3-label in aspartate should be compared with the C5- and C6-label in glucose after intravenous injection of C2-pyruvate in the starved rat, as suggested by Heath (1968).

(4) To estimate more closely the fraction of injected pyruvate oxidized rapidly in organs other than the liver, $Y_2(\text{glucose})$ and $Y_2(\text{CO}_2)$ should be estimated in starved rats, where $Y_2(\text{glucose})$ is expected to be much greater and $Y_2(\text{CO}_2)$ much less than in post-absorptive rats (Table 11).

The following predictions are open to experimental check.

(1) In the post-absorptive rat $Y_2(\text{glucose})$ should be positively correlated with (C5-Glu)/(T-Glu) (see under 'Post-absorptive rats: Discussion' in the Results and Discussion section).

(2) In the starved rat given tracer doses of [^{14}C]pyruvate $Y_2(\text{glucose})/Y_1(\text{glucose})$ should be 2.5, against 1.7 in rats given large doses of [^{14}C]lactate (see under 'Fed and starved rats: Starved rats' in the Results and Discussion section).

(3) In the injured rat, where the label in oxaloacetate should be wholly symmetrized (see under

'Injured rats' in the Results and Discussion section), the C2-label should equal the C3-label in aspartate and glutamate and the C5-label should equal the C6-label in glucose after intravenous injection of C2-pyruvate.

On the present evidence it is clear that the scheme proposed by Heath (1968) works sufficiently well to provide a useful method for analysing the processes involved in both normal and pathological states. It should be applicable to the study of the alterations in metabolism associated with diabetes, with the action of adrenocortical hormones and with thermoregulation.

MATERIALS AND METHODS

Rats. Male albino Wistar rats of the Porton strain (180–240 g.) were fed on M.R.C. diet 41B. Injections were via the tail vein.

Compounds. Specifically labelled [^{14}C]pyruvates, C1-glutamate and [^{14}C]glucose were bought from The Radiochemical Centre, Amersham, Bucks., and were dissolved in 0.9% NaCl immediately before injection. Rats received 5–10 $\mu\text{C}/100\text{g. body wt.}$

Liver samples. Livers were taken from stunned rats and extracted as described by Threlfall & Heath (1968).

^{14}C radioactivity measurements. These were carried out with a Packard automatic scintillation counter (series 4000), with [^{14}C]toluene as an internal standard, except for $\text{Ba}^{14}\text{CO}_3$ estimations, which were carried out as described by Heath & Stoner (1968).

Determination of citrate in liver. This was carried out as described by Beutler & Yeh (1959).

Determination of glucose 6-phosphate. The method was as described by Threlfall & Heath (1968).

Determination of concentrations and specific radioactivities of glutamate and aspartate. The first part of the procedure was as described by Threlfall & Heath (1968), i.e. these amino acids were eluted from the Dowex 1 column with the NAD^+ and were located by its u.v. absorption on the LKB monitoring system. The combined fractions were evaporated to dryness at room temperature in a stream of air, dissolved in water (0.5 ml.) and chromatographed as a 10 cm. band on Whatman no. 1 paper for 36 hr. at 25° in butan-1-ol-acetic acid-water (4:1:1, by vol.) (Reed, 1950). The ends of the glutamic acid and aspartic acid bands were located by ninhydrin treatment (Gaitonde, 1962) and the remainder was eluted with water by downward elution into a deposit vessel. The dried samples were dissolved in water (200–400 $\mu\text{l.}$) and an estimate of the amino acid content was made with ninhydrin reagent (Gaitonde, 1962). Whatman no. 1 chromatography papers were spotted with the individual amino acids at two loads (10 and 20 $\mu\text{g.}$) in duplicate on each sheet and also with a standard mixture, 20 $\mu\text{g.}$ of glutamic acid + 20 $\mu\text{g.}$ of aspartic acid. The papers were run for 36 hr. in the above solvent and, after drying, one sample at each level and the standard mixture were treated with the ninhydrin reagent at 60° for 30 min. The colour was eluted with ethanol-water (3:2, v/v) (4 ml.) for 2 hr. with occasional shaking and read at 565 $\text{m}\mu$. The duplicate unsprayed samples were located by reference to the standard and sprayed samples and eluted directly into scintillation-counting vials with water (about 3 ml.). After drying,

water (1.0 ml.) and scintillation fluid (10 ml.) were added and the samples were counted for 50 min. Each paper was run in duplicate and results were averaged.

Determination of (C5-Glu)/(T-Glu). Glutamic acid, separated as above, was isotopically diluted with carrier glutamic acid (400 mg.) and aspartic acid (40 mg.) and recrystallized from ethanol-water (2:1, v/v). This process was shown to separate glutamate from any labelled aspartic acid, which was not the case if carrier aspartic acid was omitted. Total glutamate label was measured by oxidizing portions to CO_2 as described by Heath & Dutton (1958). C5-glutamate was measured by Phares's (1951) method, scaled up threefold and with the temperature raised to 65° in 15 min. and held at 65 – 70° for 15 min. The yields of CO_2 were 75–90%. It was shown that C1-glutamate yielded only about 1% of its label as $^{14}\text{CO}_2$ in the reaction. The $^{14}\text{CO}_2$ was measured as $\text{Ba}^{14}\text{CO}_3$ as described above.

Determination of pyruvate. Frozen liver was extracted with tungstic acid- H_2SO_4 mixture (Cavallini & Frontali, 1954), and the 2,4-dinitrophenylhydrazone of pyruvic acid was made and extracted by the method of Neish (1953a,b). Separation of the acidic hydrazones was achieved in 2 hr. by thin-layer chromatography on silica gel with butan-1-ol-aq. NH_3 (sp. gr. 0.88)-ethanol (7:2:1, by vol.) (Hawary & Thompson, 1953). The yellow spots of 2,4-dinitrophenylhydrazone of pyruvic acid were eluted from the silica gel with ammoniacal ethanol, dried down and dissolved in 0.1 N-HCl.

The pyruvic acid content of the samples was determined by the polarographic method of Neish (1953a,b) and the radioactivity was determined on the same sample after removing the HCl.

Our special thanks are due to Mr B. K. Kelly, of the Medical Research Council Computer Services Centre, London, W.C. 1, who not only programmed the steady-state theory for us, but also saw the many computations through the Atlas (London University) Computer. He is prepared to provide a print-out of the programme in EXCHLF language to bona-fide enquirers. We also thank Mr K. Wilford and Miss M. O'Donnell for technical assistance, and Mrs N. P. Brewster for typing the paper.

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APPENDIX

Specific radioactivity of pyruvate in the non-steady state

For [U-¹⁴C]glucose as substrate in the steady state:

Pyruvate/(glucose 6-phosphate) specific-radioactivity ratio =

$$V_5[3 + PP(1) + PP(2) + PP(3)]/6(V_1 + V_6) \quad (\text{A.1})$$

[Heath's (1968) eqn. (3.70)].

In the non-steady state this splits into two portions.

(a) $0.5 V_5/(V_1 + V_6)$, which varies as the input function, $F'(t)$ (Scheme 9 in Heath, 1968), i.e. as the

specific radioactivity either of the intermediate pool or, if there is none, of the substrate.

(b) $V_5[PP(1) + PP(2) + PP(3)]/6(V_1 + V_6)$, which is the labelling due to recycling. This varies as the specific radioactivity of aspartate, S_a or σ_a . To calculate, sum the weighting coefficients after 'PP' in the relevant Tables in the Appendix to Heath (1968), and weight for the degree and type of asymmetry, i.e. $(1-z)$ of sym. case + z of non-sym. case A or B. Denote the weighting coefficient for the i th turn of the tricarboxylic acid cycle by $F(W_p)_i$ for pyruvate and by $F(W_a)_i$ for aspartate.

Then:

Pyruvate/(glucose 6-phosphate) specific-radioactivity ratio =

$$\frac{V_5}{V_1 + V_6} \left(0.5 F'(t) + \frac{1}{6} \sum_0^{\infty} \frac{F(W_p)_t}{F(W_a)_t} S_a(i) \right) \quad (\text{A.2})$$

In practice $F(W_p)_t/F(W_a)_t$ is very nearly independent of (i) , so that the last term becomes $\gamma S_a/6$, where γ is $F(W_p)_t/F(W_a)_t$, and the specific radioactivity of pyruvate can be calculated directly from the specific radioactivities of the intermediate pool and aspartate.

Steady-state theory when Δ is negative

Scheme 1 in Heath (1968) shows that, if Δ is negative, the glutamate and 2-oxoglutarate pools are diluted by unlabelled glutamate and 2-oxoglutarate, so that their specific radioactivities are $R/(R-\Delta)$ of what they would have been with $\Delta \geq 0$; and there is no loss of label from the system via glutamate. The Tables in Heath's (1968) Appendix give the correct equations if they are modified as follows.

Calculate:

$$R/(R-\Delta) = Z$$

Under $i=0$ multiply all glutamate equations, i.e. Glu(C1) etc., by Z .

For $i \neq 0$, substitute throughout $Z.I'(1)$, $Z.I'(2)$, $Z.I(1)$, $Z.I(2)$, $Z.D(1)$, $Z.D(2)$, $Z.E$ and $Z.E(1)$ for $I'(1)$, $I'(2)$, $I(1)$, $I(2)$, $D(1)$, $D(2)$, E and $E(1)$.

As $Y_n(\text{Prot}) = \text{loss of label via glutamate} = 0$, eqn. (A.7) in Heath (1968) is no longer applicable, and:

$$Y_n(\text{CO}_2) = 1 - Y_n(\text{Glc}) \quad (\text{A.3})$$

substitutes for Heath's (1968) eqn. (A.8).

Terms in C_n are ideally calculated as before. These involved experimental yields, $Y_n(\text{CO}_2)$ (see under '3. Steady-state theory' in the Theory section in Heath, 1968). For the trial calculations quoted in Table 11 of the main paper these yields had to be guessed. They were assigned the values: $Y_1(\text{CO}_2) = 0.6$, $Y_2(\text{CO}_2) = 0.2$, corresponding to $Y_1(\text{glucose}) = 0.4$ and $Y_2(\text{glucose}) = Y_3(\text{glucose}) = 0.8$. These were close enough to the calculated yields for no iteration to be necessary, as the contributions of terms in C_n were always small.

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