Gluconeogenesis in the Kidney Cortex

FLOW OF MALATE BETWEEN COMPARTMENTS

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1. Kidney-cortex slices from starved rats were incubated with L-[U-¹⁴C]lactate or L-[U-¹⁴C]malate plus unlabelled acetate and the specific radioactivity of the glucose formed was determined. In parallel experiments the specific radioactivity of the glucose formed from [1-¹⁴C]acetate plus unlabelled L-lactate and L-malate was determined. 2. By analytical methods the major products formed from the substrates were measured. The glucose formed was purified by paper chromatography for determination of specific radioactivity. 3. The specific radioactivity of the glucose formed from L-[U-¹⁴C]lactate agrees with predictions of a model based on interaction of the gluconeogenic and the oxidative pathways. 4. The specific radioactivity of the glucose formed from L-[U-¹⁴C]malate agrees with the predicted value if rapid malate exchange between the cytosol and mitochondria is assumed. 5. The rate of malate exchange between compartments was estimated to be rapid and at least several times the rate of glucose formation. 6. The specific radioactivity of the glucose formed from [1-¹⁴C]acetate plus unlabelled L-lactate or L-malate agrees with the predictions from the model, again assuming rapid malate exchange between compartments. 7. Malate exchange between compartments together with reversible malate dehydrogenase activity in the mitochondria and cytosol also tends to equilibrate isotopically the NADH pool in these compartments. ³H from compounds such as L-[2-³H]lactate, which form NAD³H in the cytosol, appears in part in water; and ³H from DL-β-hydroxy[3-³H]butyrate, which forms NAD³H in the mitochondria, appears in part in glucose, largely on C-4.

In gluconeogenesis from pyruvate, it has been proposed that malate flows out of the mitochondria to provide the required cytoplasmic reducing equivalents (Lardy, Paetkau & Walter, 1965; Krebs, Gascoyne & Notten, 1967). The preceding paper also supports this hypothesis (Rognstad & Katz, 1970). Experiments by Haslam & Krebs (1968) with isolated mitochondria have suggested inflow of malate to require energy. To investigate the problem of malate flow between the mitochondria and the cytosol in the intact cell, we determined the specific radioactivity of the glucose formed when kidney-cortex slices were incubated with L-[U-¹⁴C]malate plus unlabelled acetate. From the dilution in specific radioactivity of the glucose formed compared with that of the substrate, we have concluded that malate exchange between compartments is rapid, at least under conditions that permit active gluconeogenesis.

EXPERIMENTAL

All enzymes were obtained from either Calbiochem, Los Angeles, Calif., U.S.A., or Sigma Chemical Co., St Louis, Mo., U.S.A. L-[U-¹⁴C]Lactate, [1-¹⁴C]acetate

and [³H]NaBH₄ were from New England Nuclear Corp., Boston, Mass., U.S.A. L-[U-¹⁴C]Malate and [2-¹⁴C]-pyruvate were from Amersham/Searle, Des Plaines, Ill., U.S.A. L-[2-¹⁴C]Lactate was prepared by reduction of [2-¹⁴C]pyruvate with NADH in the presence of lactate dehydrogenase. DL-[2-³H]Malate, DL-[2-³H]lactate and DL-β-hydroxy[3-³H]butyrate were prepared by reduction with [³H]NaBH₄ of, respectively, oxaloacetate, pyruvate and acetoacetate (Lowenstein, 1963). L-[2-³H]Lactate was synthesized as follows: DL-[2-³H]malate (3 μmol), NAD⁺ (15 μmol), acetyl phosphate (10 μmol), CoA (1 μmol), triethanolamine-HCl buffer, pH 8.0 (300 μmol), malate dehydrogenase (5 units), phosphotransacetylase (3 units; EC 2.3.1.8) and citrate synthase (3 units) in a final volume of 3 ml were incubated in a cuvette and NAD³H formation was followed at 340 nm. Pyruvate (20 μmol) and lactate dehydrogenase (10 units) were then added and L-[2-³H]-lactate formation was followed by the decrease in *E*₃₄₀. The reaction mixture was put on a column (1 cm × 11 cm) of Dowex 1 (X8; 100-200 mesh acetate form) (Calbiochem). L-[2-³H]Lactate was eluted with 1 M-formic acid, evaporated to dryness and further purified by paper chromatography with ethanol-aq. NH₃ (sp.gr. 0.88)-water (16:1:3, by vol.). L-[2-³H]Malate was synthesized as follows: DL-[2-³H]lactate (2 μmol), NAD⁺ (10 μmol), L-glutamate (50 μmol), glycine-NaOH buffer, pH 9.5 (200 μmol), lactate dehydrogenase (20 units) and glutamate-pyruvate

transaminase (8 units) were incubated in a 3 ml volume and NAD³H formation was followed at 340 nm. Oxaloacetate (10 μ mol) and malate dehydrogenase were then added. The L-[2-³H]malate formed was purified by a procedure similar to that used for L-[2-³H]lactate.

Kidney-cortex slices were obtained from starved rats except where noted. The incubation procedure is given in the preceding paper (Rognstad & Katz, 1970). The incubation medium was that of Krebs, Hems & Gascoyne (1963), which contained no bicarbonate. At the end of the incubation, CO₂ was collected by injecting 0.3 ml of 4 M-NaOH (CO₂-free) into a hanging plastic well and acidifying the medium. After at least 2½ h the well was removed and the contents were added to 4.7 ml of CO₂-free water. A sample of this was taken for manometric CO₂ determination in a Warburg apparatus. The amount of glucose initially present in the slices was found never to exceed 0.1 μ mol. The initial L-lactate content also was less than 0.1 μ mol. Control flasks were also run containing buffer and tissue that were acidified at zero time and a 'CO₂ blank' was determined. This value never exceeded 1 μ mol, and the blank value was subtracted to give the CO₂ values in Table 1.

A sample (8 ml) of the acidified medium (total vol. 10 ml) was put through a column (1 cm $\times$ 10 cm) of Dowex 50 (X8; 50–100 mesh; H⁺ form) and a column (1 cm $\times$ 11 cm) of Dowex 1 (X8; 100–200 mesh; acetate form), arranged in tandem, both of which were washed with water until 25 ml of effluent was collected. This was evaporated to dryness and chromatographed on Whatman 3 MM paper with butan-1-ol-acetic acid-water (4:1:2, by vol.). Glucose spots were located with X-ray film. The specific radioactivity of the purified glucose was measured, with glucose determination by the method of Slein (1965). All radioactivity counting was done with a Packard model 3375 liquid-scintillation counter.

Yields of ³H in water were determined either by freeze-drying of the neutralized medium or, when yields were high, by the difference in ³H radioactivity between identical samples of the neutralized medium with or without prior evaporation.

The location of ³H in glucose was determined by an enzymic procedure. It is assumed that, as found by Hoberman & D'Adamo (1960), all ³H was on either C-4 or C-6. Glucose was converted into 3-phosphoglycerate with purified glycolytic enzymes and the required cofactors in the presence of arsenate plus excess of acetaldehyde and alcohol dehydrogenase to oxidize the NADH formed (Schmidt, Genovese & Katz, 1970). The amount of NAD⁺ added was less than 5% of the original glucose. Unlabelled glucose was later added to ensure turnover of the NADH. The radioactivity of samples of the medium was counted with and without prior evaporation. The difference in ³H radioactivity measured gives the ³H originally located on C-4 of glucose. Trials were run with authentic [4-³H]glucose and [6-³H]glucose to determine the validity of the procedure.

RESULTS AND DISCUSSION

When kidney-cortex slices from starved rats are incubated without any added substrate, a small amount of glucose is formed from endogenous substrates, generally less than 0.5 μ mol/2 h per 125 mg wet wt. However, a considerable amount of carbon dioxide is formed, corresponding to the oxidation of nearly 10 μ mol of acetyl-CoA/2 h per 125 mg. The main source of the acetyl-CoA is presumably lipid. If acetate is added, no additional glucose is formed, but there is usually some increase in carbon dioxide production. Acetate was used in nearly all the experiments described to maintain a relatively constant flow of acetyl-CoA to the tricarboxylic acid cycle, rather than depending on endogenous sources, which may vary between experiments. Addition of acetate also tends to diminish oxidation of pyruvate to acetyl-CoA. This is desirable because measurement of this flow is only approximate.

When lactate and acetate are used as substrates

Table 1. *Metabolism of L-lactate and L-malate by kidney-cortex slices*

Kidney-cortex slices from starved rats were incubated for 2 h at 37°C with substrates as shown plus 20 μ mol of acetate. Glucose formation without substrate was 0.4 ± 0.1 μ mol/2 h per 125 mg wet wt. and has been subtracted to give the values in the table. Substrate oxidation is calculated from the amount of substrate used minus that converted into glucose, lactate and pyruvate. Results are expressed as μ moles/2 h per 125 mg wet wt. n.d., Not determined.

Expt. no.	Substrate	Amount added (μ mol)	Substrate used	Substrate converted into glucose	Lactate formed	Pyruvate formed	Total CO ₂ produced	Substrate oxidized
2	L-Lactate	16	8.7	7.6	—	0.5	37.0	0.6
	L-Malate	16	14.5	11.2	0.8	n.d.	50.4	2.5
3	L-Lactate	16	9.0	6.6	—	0.6	30.9	1.8
	L-Malate	16	13.5	11.6	0.8	n.d.	52.3	1.1
4	L-Lactate	8	7.2	6.6	—	0.1	36.3	0.5
	L-Malate	8	7.0	6.9	0.4	n.d.	40.5	0
6	L-Lactate	16	8.7	5.7	—	0.6	37.9	2.4
	L-Malate	16	11.1	8.5	0.6	0.1	43.7	1.9

most of the lactate consumed is converted into glucose, and only a small amount is oxidized to acetyl-CoA (Table 1). The flow of acetyl-CoA is estimated from the amount of lactate that disappears minus that converted into glucose and pyruvate. This neglects formation of other minor products. An independent method (R. Rognstad & J. Katz, unpublished work) using the pattern of ^{14}C labelling in C-1 and C-5 of glutamate isolated from experiments with L-[U- ^{14}C]lactate or L-[2- ^{14}C]lactate has shown agreement with the method based on analysis.

If L-[U- ^{14}C]lactate is used as substrate, the molar specific radioactivity of the glucose formed via a pathway that involved synthesis from two L-[U- ^{14}C]lactate molecules without dilution of specific

radioactivity naturally would be twice that of the original lactate. Such a pathway, e.g. via reversal of pyruvate kinase, is no longer considered of any physiological significance. As shown in Table 2, the molar specific radioactivity of the glucose formed in the experiment is even less than that of the lactate. The most likely reason for this dilution in specific radioactivity is found in the 'crossing-over' phenomenon (discussed by Krebs, Hems, Weidemann & Speake, 1966) in which labelled carbon from the gluconeogenic pathway mixes with unlabelled carbon of the oxidative pathway (tricarboxylic acid cycle) because mitochondrial oxaloacetate is an intermediate common to both pathways.

Scheme 1 shows a model of the metabolism of

Scheme 1. Metabolism of L-[U- ^{14}C]lactate and unlabelled acetate in the kidney cortex. The small letters or numbers under each carbon atom represent molar specific radioactivities of these atoms. The rates, V , are molar rates. V_G is the rate of gluconeogenesis from added substrate; V_B is the rate of pyruvate dehydrogenase; V_T is the rate of the tricarboxylic acid cycle; V_A is the rate of formation of acetyl-CoA from added acetate and from endogenous sources; V_N is the rate of gluconeogenesis from endogenous glutamate. M_1 represents the specific radioactivity of the carboxyl carbon of the dicarboxylic acids, oxaloacetate, malate and fumarate, which are assumed to be in isotopic equilibrium. m_2 represents the specific radioactivity of the interior carbon atoms of these dicarboxylic acids. The lower dicarboxylic acid is used to show the specific radioactivity of the inflowing carbon from the forward tricarboxylic acid cycle. When L-[U- ^{14}C]lactate is the substrate, the specific radioactivity of each carbon atom of lactate is set equal to 1. When [1- ^{14}C]acetate is the substrate, the specific radioactivity of C-1 is set equal to 1.

Table 2. *Dilution in specific radioactivity of glucose formed from L-[U-¹⁴C]lactate or L-[U-¹⁴C]malate*

For experimental conditions, see Table 1. V_T is the rate of the tricarboxylic acid cycle; V_B is the rate of the pyruvate dehydrogenase reaction; V_N is the rate of formation of glucose from endogenous substrates; V_E is the rate of exchange of malate between mitochondria and cytosol. All of these are expressed relative to the rate of conversion of glucose, V_G , set equal to 1.0. V_B is estimated from the amount of substrate oxidized (Table 1). V_T is estimated from the amount of CO_2 formed in the experiment, measured manometrically, minus that formed in the pyruvate dehydrogenase reaction (V_B) and also, when malate is substrate, minus that produced from C-4 of malate utilized. R_{exp} is the dilution in specific radioactivity of the glucose formed and is defined by: $R_{\text{exp.}} = (\text{sp. radioactivity of glucose})/2(\text{sp. radioactivity of C-1, C-2 and C-3 of substrate})$. When L-[U-¹⁴C]lactate is the substrate, $R_{\text{theoret.}}$ is calculated by:

$$R = \frac{(2V_G + V_B)(2V_G + 5V_T + 4V_N) + (V_G + V_B)(2V_G + V_T + 2V_N)}{3(2V_G + V_T + 2V_N)(V_G + 2V_T + 2V_N)} \dots \text{(A1)}$$

When L-[U-¹⁴C]malate is the substrate, $R_{\text{theoret.}}$ is calculated by:

$$R = \frac{2(V_G + V_B)}{2V_G + V_B + V_T + 2V_N} \dots \text{(A4)}$$

This equation assumes complete equilibration of intra- and extra-mitochondrial malate ($V_B = \infty$). V_E is calculated from:

$$V_E = \frac{(V_T + 2V_N)(V_G + V_B) - R[(V_G + V_B + V_N)(V_T + 2V_N) - V_N V_B]}{R(2V_G + V_B + 2V_N + V_T) - 2(V_G + V_B)} \dots \text{(A3)}$$

by using the experimental value of R . The equations are derived in the Appendix.

Expt. no.	Substrate	Amount added (μmol)	Acetate (μmol)	Sp. radioactivity of substrate (c.p.m./ μmol)	Sp. radioactivity of glucose (c.p.m./ μmol)	Sp. radioactivity of CO_2 (c.p.m./ μmol)	$R_{\text{exp.}}$	V_T	V_B	V_N	$R_{\text{theoret.}}$	V_E	m_1 (c.p.m./ μmol)
1	L-[U- ¹⁴ C]Lactate	8	0	152 000	149 000	—	0.49	—	—	—	—	—	—
	L-[U- ¹⁴ C]Malate	8	0	99 500	89 200	—	0.60	—	—	—	—	—	—
2	L-[U- ¹⁴ C]Lactate	16	20	91 700	80 700	10 900	0.44	2.20	0.07	0.12	0.44	7.1	11 500
	L-[U- ¹⁴ C]Malate	16	20	49 400	48 000	—	0.65	1.50	0.21	0.08	0.63	—	—
3	L-[U- ¹⁴ C]Lactate	16	20	152 000	153 000	—	0.50	2.08	0.24	0.12	0.49	—	—
	L-[U- ¹⁴ C]Malate	16	20	101 000	92 700	—	0.61	1.53	0.09	0.07	0.58	5.4	—
4	L-[U- ¹⁴ C]Lactate	8	20	121 000	104 000	13 400	0.43	2.40	0.07	0.12	0.42	—	14 800
	L-[U- ¹⁴ C]Malate	8	20	109 000	79 800	—	0.49	2.11	0	0.12	0.46	9.6	—
5	L-[U- ¹⁴ C]Lactate	8	10	133 000	110 000	—	0.41	—	—	—	—	—	—
	L-[U- ¹⁴ C]Malate	8	10	120 000	84 800	—	0.47	—	—	—	—	—	—
6	L-[U- ¹⁴ C]Lactate	16	20	280 000	252 000	37 600	0.45	2.73	0.37	0.15	0.45	—	37 100
	L-[U- ¹⁴ C]Malate	16	20	351 000	329 000	—	0.62	1.60	0.21	0.10	0.60	10.6	—

L-[U- ^{14}C]acetate and unlabelled acetate in the kidney-cortex. The rates (V values) can all be estimated from the analytical results. There are a number of approximations involved in the model. The specific radioactivity of the carbon dioxide fixed in the pyruvate carboxylase reaction is set equal to the specific radioactivity of the carbon dioxide produced in the tricarboxylic acid cycle. This is strictly true only if V_B , the rate of the pyruvate dehydrogenase reaction, is zero. Mitochondrial oxaloacetate, malate and fumarate are assumed to be in complete isotopic equilibrium. This is a good approximation, as shown by Walter, Paetkau & Lardy (1966). Recycling of phosphoenolpyruvate to pyruvate is neglected. In section

A of the Appendix is calculated, on the basis of the model, the expected dilution in specific radioactivity (denoted by R) of the glucose formed. R is defined as the ratio of the specific radioactivity of the glucose formed to twice the specific radioactivity of C-1, C-2 and C-3 of the substrate. Table 2 shows the comparison of the experimental values of R and the values of R calculated on the basis of Scheme 1. The agreement between experimental and theoretical values is good. In view of the approximations used, Scheme 1 does not necessarily represent the complete physiological situation, but it probably includes the major metabolic pathways involved. Thus the major cause of the dilution in specific radioactivity indeed appears to be the

Scheme 2. Metabolism of L-[U- ^{14}C]malate and unlabelled acetate in the kidney cortex. x represents the specific radioactivity of each carbon atom of extramitochondrial malate, and m represents the specific radioactivity of each carbon atom of intramitochondrial malate. V_E represents the rate of exchange flow of malate between these compartments. When [1- ^{14}C]acetate is the substrate, V_E is set equal to infinity, and $x = m$.

interaction of the gluconeogenic pathway and the tricarboxylic acid cycle.

One of the assumptions used in the model is that the specific radioactivity of the carbon dioxide fixed in the pyruvate carboxylase reaction was the same as that of the carboxyl carbon atoms of the dicarboxylic acids, or C-3 and C-4 of glucose. Table 2 shows that the values of m_1 (representing C-3 and C-4 of glucose), calculated from the equations of the Appendix and the experimental specific radioactivity of glucose, are quite close to the specific radioactivities of the carbon dioxide formed. Use of this assumption hence causes little error.

In gluconeogenesis from malate plus acetate, it is again found that most of the malate that disappears is converted into glucose, with a small amount being oxidized to carbon dioxide via acetyl-CoA (Table 1). Since the conversion of malate into glucose involves strictly a cytoplasmic pathway, it might be expected that the molar specific radioactivity of the glucose formed would be twice that of C-1, C-2 and C-3 of L-[U- 14 C]malate, i.e. values for R of 1.0 might be expected. Actually the dilution in specific radioactivity approaches that found when L-[U- 14 C]-lactate is converted into glucose (Table 2). The simplest explanation for the dilution in specific radioactivity would seem to be that of an exchange flow of malate between the cytosol and the mitochondria, in this way again producing a mixing of labelled carbon atoms from the gluconeogenic pathway with unlabelled carbon atoms from the tricarboxylic acid cycle.

Scheme 2 shows the model of the metabolism of L-[U- 14 C]malate and unlabelled acetate by the kidney cortex. Separate pools of extramitochondrial and intramitochondrial malate are assumed, with the rate of exchange flow of malate between these pools denoted by V_E . In section B of the Appendix is calculated the expected dilution in glucose specific radioactivity ($R_{\text{theoret.}}$) for L-[U- 14 C]-malate as substrate, when V_E is assumed to be infinite (eqn. A4) or when intermediate values of V_E are assumed (eqn. A2). It is seen (Table 2) that the values of $R_{\text{theoret.}}$ calculated on the basis of a single malate pool (i.e. $V_E = \infty$) are close to the experimental values of the dilution ($R_{\text{exp.}}$). If the experimental values of R are used to calculate V_E , from eqn. (A3), rates of 5–10 times the rate of glucose formation are found. Fig. 1 shows a plot of V_E versus R , calculated from eqn. (A2), for an example with typical values of V_T (rate of tricarboxylic acid cycle), V_B (rate of pyruvate dehydrogenase) and V_G (rate of gluconeogenesis from added substrate). At high rates of exchange the curve is rather flat and accurate determination of V_E is difficult in this region. As V_E approaches V_G , R becomes very sensitive to the rate of exchange. The values of V_E calculated in Table 2 can only be

Fig. 1. Effect of the rate of malate exchange between mitochondria and cytosol on the dilution of the specific radioactivity of glucose formed from L-[U- 14 C]malate. An example with typical values of the experimental rates is considered: $V_G = 1$; $V_T = 1.5$; $V_B = 0.1$. V_N is set equal to 0. Various values of V_E are assumed, and R is calculated for each of these values according to eqn. (A3).

Fig. 2. Radioautograph of desalted medium from the incubation of L-[U- 14 C]malate and unlabelled acetate with kidney-cortex slices. The numbers denote the following compounds: 1, malate; 2, glucose; 3, citrate plus isocitrate; 4, glutamate; 5, lactate; 6, succinate; 7, fumarate; 8, α -oxoglutarate (?); 9, unknown; 10, alanine; 11, aspartate. The method is that of Katz & Chaikoff (1954).

considered approximate, since these are in the region where R is insensitive to V_E . However, it should be safe to say that the exchange rate is rapid

and at least several times the rate of glucose synthesis.

In addition to the approximations used in setting up the models there are other considerations that also make an exact determination of the exchange rate difficult. When the medium from an incubation with L-[U-¹⁴C]malate is electrolytically desalted and subjected to two-dimensional chromatography and the radioautographs are examined, there is radioactivity in several other compounds besides glucose and the substrate (Fig. 2). Succinate, glutamate, citrate and other intermediates become labelled. This may mean that exchange of label also occurred with the pre-existing pools of these various intermediates, in addition to the exchange with the large inflow of acetyl-CoA from endogenous sources. However, the amount of radioactivity in these compounds is less than one-tenth that in carbon dioxide and glucose, and neglect of this exchange should not cause a large error in the calculated exchange rate. Another factor to be considered is that a small proportion of cells other than cortex cells may be present, e.g. medulla cells, which oxidize acetate but form no glucose. This would mean that the dilution by unlabelled acetyl-CoA in the kidney-cortex is overestimated by the manometric carbon dioxide results. The two possibilities discussed in this paragraph would give opposite effects. Neglect of exchange with pre-existing pools of intermediates means that V_E is overestimated, whereas neglect of oxidation of acetate by non-cortex cells causes V_E to be underestimated.

In two experiments the specific radioactivity of the residual malate after incubation with L-[U-¹⁴C]malate was determined and found to be unchanged from the original. However, the specific radioactivity of the residual lactate from an incubation with L-[U-¹⁴C]lactate was about 10% less than that of the original substrate. This may reflect some

dilution of the lactate pool due to recycling of phosphoenolpyruvate to pyruvate.

A corollary of the exchange is illustrated by the results of Table 3. Here, labelled [1-¹⁴C]acetate and unlabelled L-lactate or L-malate were incubated with kidney-cortex slices and the specific radioactivity of the glucose was again determined. In this case, labelled carbon from the oxidative pathway mixes with unlabelled carbon from the gluconeogenic pathway either by exchange in the case of malate or probably by a convergence of the two pathways in the case of lactate. Again the specific radioactivity of the glucose formed agrees reasonably well with predictions based on the model shown in Scheme 1 and described in Section C of the Appendix, assuming only a single malate pool.

The consequences of malate exchange between compartments include effects on the specific radioactivity of the NAD³H produced from tritiated substrates that form NAD³H in either the cytosol or the mitochondria. Hoberman & D'Adamo (1960) were the first to use L-[2-³H]- and L-[2-³H] lactate as tracers for reductive hydrogen in gluconeogenesis. In gluconeogenesis from L-[2-³H]lactate, the molar ³H specific radioactivity of the glucose formed, in the absence of isotope discrimination or exchange reactions with water, should be about 1.0. The NAD³H formed in the lactate dehydrogenase reaction reduces 1,3-diphosphoglycerate to [1-³H]-glyceraldehyde phosphate, which isomerizes to dihydroxy[1-³H]acetone phosphate. When the triose phosphates react via aldolase, the ³H on dihydroxyacetone phosphate is lost to water (Rose & Rieder, 1955), and only one ³H atom (from [1-³H]glyceraldehyde phosphate) is incorporated into glucose, on C-4. However, if malate moves rapidly into and out of the mitochondria, and if the malate dehydrogenases in the cytosol and mitochondria both operate reversibly, isotopic mixing of the cytoplasmic and mitochondrial NADH

Table 3. *Molar specific radioactivity of glucose formed from [1-¹⁴C]acetate plus unlabelled L-lactate or L-malate*

For experimental conditions, see Table 1. The incubation medium contained 16 μ mol of unlabelled substrate plus 20 μ mol of [1-¹⁴C]acetate. $G_{exp.}$ is the ratio of the specific radioactivity of the glucose formed to that of the substrate [1-¹⁴C]acetate; V_A is the rate of acetate oxidation; V_T is the rate of the tricarboxylic acid cycle. V_G is the rate of glucose formation, is set equal to 1. $G_{theoret.}$ is calculated from: $G = 2V_A/(V_G + 2V_T + 2V_N)$ (eqn. A5) when lactate is the substrate, and from $G = V_A/(V_G + V_T + V_N)$ (eqn. A6) when lactate is the substrate.

Expt. no.	Unlabelled substrate	Sp. radioactivity of acetate (c.p.m./ μ mol)	Sp. radioactivity of glucose (c.p.m./ μ mol)	$G_{exp.}$	V_T	V_A	V_N	$G_{theoret.}$
3	L-Lactate	670 000	353 000	0.53	2.08	1.39	0.12	0.52
	L-Malate	670 000	246 000	0.37	1.53	0.98	0.07	0.38
6	L-Lactate	1 600 000	770 000	0.48	2.73	1.64	0.15	0.49
	L-Malate	1 650 000	630 000	0.38	1.60	1.12	0.10	0.41

pools will occur. This will cause production of ^3H in water from L-[2- ^3H]lactate or L-[2- ^3H]malate and further dilution of the specific radioactivity of the glucose formed. Although such effects do appear (Table 4), it is impossible to know how much is due to malate exchange or how much to alternative exchange mechanisms (e.g. aldolase, triose phosphate isomerase exchange; Katz & Rognstad, 1966). If the rates of both malate dehydrogenase reactions and of malate exchange flow were infinite, complete isotopic equilibration of cytoplasmic and mitochondrial NAD ^3H pools would occur. When lactate is the substrate, the rate of mitochondrial NADH turnover is roughly seven times the rate of cytoplasmic NADH turnover, since the rate of the tricarboxylic acid cycle is about $2\frac{1}{2}$ times the rate of glucose formation and three NADH molecules are formed in each turn of the tricarboxylic acid cycle. Complete isotopic equilibration of these pools would result in a sevenfold dilution in the specific radioactivity of the glucose formed from L-[2- ^3H]lactate. Since about a threefold dilution is found, complete isotopic mixing does not occur. One reason for this may be that, although the malate dehydrogenase exchange reactions are active, they are not infinite.

Table 4. *Gluconeogenesis from L-[2- ^3H]lactate and L-[2- ^3H]malate*

For experimental conditions, see Table 1. The specific radioactivity of substrate was set equal to 1.0.

Expt. no.	Substrate	Yield of ^3H in water (% of injected dose)	^3H sp. radioactivity of glucose
1	L-[2- ^3H]Lactate	80	0.28
	L-[2- ^3H]Malate	86	0.39
4	L-[2- ^3H]Lactate	68	0.32
	L-[2- ^3H]Malate	58	0.31

Again, a corollary of this exchange should be appearance of ^3H in glucose from substrates that produce NAD ^3H in the mitochondria. Thus, when DL- β -hydroxy[3- ^3H]butyrate is used as substrate together with lactate or pyruvate, a small amount of ^3H is found in the glucose formed (Table 5). D- β -Hydroxy[3- ^3H]butyrate forms NAD ^3H by an enzyme that is thought to be located exclusively in mitochondria (Lehninger, Sudduth & Wise, 1960), whereas L- β -hydroxy[3- ^3H]butyryl-CoA would form NAD ^3H via the mitochondrial enzymes that oxidize fatty acids. It is noteworthy that most of the ^3H from DL- β -hydroxy[3- ^3H]butyrate found in glucose is on C-4 when either lactate or pyruvate is the substrate. This again would be consistent with rapid malate exchange masking any isotopic difference in the net pathways from lactate and pyruvate. However, the interpretation of the results with DL- β -hydroxy[3- ^3H]butyrate must be considered as very tentative. Other pathways may operate that result in an exchange of reductive hydrogen atoms between the mitochondria and the cytosol, or perhaps some cytoplasmic enzyme reacts with the D- or L-isomer.

Haslam & Krebs (1968) have found that ATP and other high-energy compounds stimulated the entry of malate into isolated liver mitochondria. Our results in the present paper suggest rapid flow of malate through the mitochondrial membrane. Since a high ATP concentration is required for active gluconeogenesis, our results with intact cells do not conflict with their studies on isolated mitochondria. Preliminary experiments with low concentrations of dinitrophenol, which depress but do not abolish gluconeogenesis, indicate no apparent decrease in the rate of intercompartmental exchange.

If one accepts the hypothesis (Lardy *et al.* 1965; Krebs *et al.* 1967) that glucose formation from either pyruvate or L-lactate involves the mito-

Table 5. *Tritium incorporation into glucose from DL- β -hydroxy[3- ^3H]butyrate*

For experimental conditions, see Table 1. About 1×10^6 c.p.m. of ^{14}C and 30×10^6 c.p.m. of ^3H were used in each incubation. The $^3\text{H}/^{14}\text{C}$ ratio of the substrate mixture was set equal to 1.0.

Expt. no.	Substrates (μmol added)	Yield of ^3H in water (% of injected dose)	$^3\text{H}/^{14}\text{C}$ ratio of glucose	Distribution of ^3H in glucose (%)	
				C-4	C-6
8	L-[2- ^{14}C]Lactate (20) + DL- β -Hydroxy[3- ^3H]butyrate (20)	n.d.	0.030	72	28
	[2- ^{14}C]Pyruvate (20) + DL- β -hydroxy[3- ^3H]butyrate (20)	n.d.	0.024	72	25
2	L-[U- ^{14}C]Lactate (20) + DL- β -hydroxy[3- ^3H]butyrate (20)	28	0.060		
	[U- ^{14}C]Pyruvate (20) + DL- β -hydroxy[3- ^3H]butyrate (20)	29	0.047		
	L-[U- ^{14}C]Malate (20) + DL- β -hydroxy[3- ^3H]butyrate (20)	31	0.041		

chondrial pyruvate carboxylase reaction, it seems likely that malate should flow out of the mitochondria when pyruvate is the substrate, whereas oxaloacetate, or a compound on this oxidation level, should be transferred when lactate is the substrate.

From the difference in the NAD^+/NADH concentration ratios in the cytosol and mitochondria of liver, Krebs (1967) has shown that equilibration of both malate and oxaloacetate between the cytosol and the mitochondria (or at least the mitochondrial space containing malate dehydrogenase) does not occur. Our results showing rapid malate exchange suggest that no control mechanism on malate outflow operates and that malate equilibration between compartments is rapidly established.

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APPENDIX

(A) Specific radioactivity of glucose formed from $\text{L-[U-}^{14}\text{C]lactate}$

All models assume metabolic and isotopic steady state. (In some experiments there is a considerable decrease in the rate of gluconeogenesis as the substrate becomes depleted. The approach used is still valid, but in these cases the rates must be interpreted as average flow of carbon during the incubation period.) Scheme 1 in the main paper shows the model used to represent the metabolism of $\text{L-[U-}^{14}\text{C]lactate}$ by the kidney cortex. Some simplifying approximations have been used: (1) Mitochondrial oxaloacetate, malate and fumarate are assumed to be in complete isotopic equilibrium. (2) The carbon dioxide fixed in the pyruvate carboxylase reaction is assumed to have the same specific radioactivity as the carboxyl carbon of the mitochondrial dicarboxylic acids; this is a good approximation if V_B is small. (3) Recycling of phosphoenolpyruvate back to pyruvate is neglected. (4) The small amount of glucose formed from endogenous sources, V_N , is arbitrarily assumed to be from endogenous glutamate; this is a small inflow (about 5% of V_T).

The specific radioactivity of each carbon atom of $\text{L-[U-}^{14}\text{C]lactate}$ is set equal to 1. Equations are written below that set inflow of radioactivity into any carbon atom equal to outflow of radioactivity

from that carbon atom, assuming an isotopic steady state.

Acetyl-CoA:

$$V_B(1) = aV_T$$

C-4 and C-5 of α -oxoglutarate:

$$V_T a = b(V_T + V_N)$$

C-2 and C-3 of α -oxoglutarate:

$$V_T m_2 = m(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$V_G \left(\frac{1+m_1}{2} \right) + (V_T + V_N) \left(\frac{b+n}{2} \right) \\ = m_1(V_G + V_T + V_N)$$

C-2 and C-3 of dicarboxylic acids

$$V_G(1) + (V_T + V_N) \left(\frac{b+n}{2} \right) = m_2(V_G + V_T + V_N)$$

Solving these equations yields:

$$m_2 = \frac{2V_G + V_B}{2V_G + V_T + 2V_N}$$

and

$$m_1 = \frac{V_G + V_B + V_T m_2}{V_G + 2V_T + 2V_N}$$

The molar specific radioactivity of the glucose formed will be $4m_2 + 2m_1$. Dividing this by 6 gives the ratio (R) of the expected molar glucose specific

activity to that of glucose formed from two C₃ molecules of undiluted specific radioactivity:

$$R = (4m_2 + 2m_1)/6 = (2m_2 + m_1)/3$$

From the values of m_2 and m_1 above:

$$R = \frac{(2V_G + V_B)(2V_G + 5V_T + 4V_N) + (V_G + V_B)(2V_G + V_T + 2V_N)}{3(2V_G + V_T + 2V_N)(V_G + 2V_T + 2V_N)} \quad (A1)$$

(B) *Specific radioactivity of glucose formed from L-[U-¹⁴C]malate*

The model used is shown in Scheme 2. The specific radioactivity of each carbon atom of extramitochondrial malate is denoted by x , and of that of intramitochondrial malate by m . The molar specific radioactivity of the glucose formed will be $6x$. The predicted dilution in specific radioactivity, R , will be $6x/6$ or x . The specific radioactivity of each carbon atom of L-[U-¹⁴C]malate is set equal to 1. As above, inflow of radioactivity is equated to outflow, in the isotopic steady state.

Acetyl-CoA:

$$V_B x = a V_T$$

C-4 and C-5 of α -oxoglutarate:

$$a V_T = b(V_T + V_N)$$

C-2 and C-3 of α -oxoglutarate:

$$m V_T = n(V_T + V_N)$$

Intramitochondrial malate:

$$V_E x + (V_T + V_N) \left(\frac{b+n}{2} \right) = m(V_T + V_E + V_N)$$

Extramitochondrial malate:

$$(V_G + V_B)(1) + (V_E + V_N)m = x(V_G + V_B + V_E + V_N)$$

Solving these equations yields:

$$x = R = \frac{(V_G + V_B)(V_T + 2V_E + 2V_N)}{(V_G + V_B + V_E + V_N)(V_T + 2V_E + 2V_N) - (V_E + V_N)(2V_E + V_B)} \quad (A2)$$

Rearranging to solve for V_E gives:

$$V_E = \frac{(V_T + 2V_N)(V_G + V_B) - R[(V_G + V_B + V_N)(V_T + 2V_N) - V_N V_B]}{R(2V_G + V_B + 2V_N + V_T) - 2(V_G + V_B)} \quad (A3)$$

If $V_E = \infty$, eqn. (A2) above reduces to:

$$R = \frac{2(V_G + V_B)}{2V_G + V_B + V_T + 2V_N} \quad (A4)$$

This gives the value of the dilution in glucose specific radioactivity expected if extramitochondrial and intramitochondrial malate are in complete isotopic equilibrium.

(C) *Specific radioactivity of glucose formed from [1-¹⁴C]acetate plus either L-lactate or L-malate*

Schemes 1 and 2 show the models used when L-lactate or L-malate respectively is the unlabelled

substrate [however, in this case the specific radioactivities (m_2) of the interior carbon atoms of the dicarboxylic acids and that of C-2 of acetyl-CoA will be zero]. The same approximations as in the previous sections are used. The molar specific

radioactivity, G , of glucose formed will be $2m_1$. This is also the ratio of the glucose specific radioactivity to that of [1-¹⁴C]acetate, since the latter is set equal to 1. Equations are written for isotopic steady state when lactate is the unlabelled substrate (Scheme 1).

Acetyl-CoA:

$$V_A(1) = a V_T$$

C-5 of α -oxoglutarate:

$$a V_T = b(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$(V_G m_1)/2 + (V_T + V_N)b/2 = m_1(V_G + V_T + V_N)$$

Solving these equations yields:

$$m_1 = \frac{V_A}{V_G + 2V_T + 2V_N}$$

$$G = 2m_1 = \frac{2V_A}{V_G + 2V_T + 2V_N} \quad (A5)$$

When malate is the unlabelled substrate (Scheme 2), we consider only the case where extramitochondrial and intramitochondrial malate are assumed in isotopic equilibrium ($x = m$).

Acetyl-CoA:

$$V_A(1) = a V_T$$

C-5 of α -oxoglutarate:

$$a V_T = b(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$(V_T + V_N)b/2 = m(V_G + V_T + V_N)$$

Solving these equations yields:

$$m = \frac{V_A}{2(V_G + V_T + V_N)}$$

$$G = 2m = \frac{V_A}{V_G + V_T + V_N} \quad (A6)$$