The Metabolic Fate of Lactate in Renal Cortical Tubules

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1. Isolated kidney cortex tubules prepared from fed rats and incubated with near-physiological concentrations of [^{14}C]lactate decrease the specific radioactivity of the added lactate. This effect may be attributable to at least two mechanisms; formation of lactate from endogenous precursors, or entry of unlabelled carbon into the lactate pool as a result of substrate cycling, via phosphoenolpyruvate, pyruvate and oxaloacetate, together with equilibration of the oxaloacetate pool with malate and fumarate. Such substrate cycling could occur within a single cell, or between two populations of different cells, one glycolytic and the other gluconeogenic. These possibilities have been investigated by using metabolic inhibitors or alternative metabolic substrates. 2. Tubules from fed rats produced a fall in specific radioactivity of 14.4% when incubated for 40 min with 2 mM-lactate alone. A mathematical treatment of this result is presented, which allows the rate of fall in specific radioactivity to be expressed as the addition of unlabelled lactate to the pool. This corresponds to a rate of formation of unlabelled lactate of $121 \pm 22 \mu\text{mol/h per g dry wt.}$, a rate close to that of gluconeogenesis. In tubules from fasted rats, there was no reduction of the specific radioactivity of lactate, indicating that fasting for 24 h suppresses production of unlabelled-lactate carbon. 3. Addition of 2 mM-fumarate resulted in a significantly greater decrease in the specific radioactivity of lactate, but aspartate (2 mM), malate (2 mM) and glucose (5 mM) were without effect. Total inhibition of gluconeogenesis with 3-mercaptopicolinate did not prevent the fall in specific radioactivity of lactate observed in tubules from fed-rat kidney, thereby excluding significant activity of the substrate cycle pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate. 4. The capacity of pyruvate kinase under the test conditions in tubules prepared from kidneys of fed or starved rats was at least ten times higher than the observed rate of production of lactate, so that failure to observe recycling of lactate in starved-rat tubules indicates suppression of pyruvate kinase activity. 5. The endogenous glycogen and glucose content of isolated renal cortex tubules is too low to account for the dilution of label of lactate. Endogenous concentrations of glycerol and amino acids were also very low. As for glycogen, the possibility that very rapid turnover of these metabolites, in fed rats but not in starved rats, may account for formation of unlabelled lactate cannot be excluded. 6. It is concluded that substrate cycling via phosphoenolpyruvate does not occur to any significant extent in either fed or starved-rat kidney. In fed rats recycling of lactate carbon does occur and the rate of this reaction is similar to the rate of gluconeogenesis at physiological concentrations of lactate. The present results favour participation of oxaloacetate decarboxylase rather than 'malic' enzyme in this cycle.

The main fates of added lactate in renal cortex are conversion to glucose or oxidation to CO_2 and water. It should be emphasized that lactate itself has no other fate than to form pyruvate. The presumption is that lactate and pyruvate are always in equilibrium

via lactic dehydrogenase. Quantitative determination of the rates of both gluconeogenesis and lactate oxidation conventionally depends upon the measurement of differences between initial and final concentrations of metabolites in an incubation mixture, to which lactate and a respiring tissue preparation of kidney cortex (slices or tubule fragments) have been added. This simple approach takes no account of the

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possible existence of glycolysis in the tissue under investigation; glycolysis may continuously remove glucose that is formed and lead to an underestimate of the rate of glucose synthesis. This possibility must be considered in the kidney cortex in particular because of the heterogeneity of cell types, some of which contain predominantly glycolytic enzymes (Guder & Schmidt, 1975).

In addition, substrate cycling may occur in kidney cortex. Substrate cycling is the process whereby the initial substrate of a pathway is reformed by the action of two or more enzymes acting at the same time. In the present case, cycling of pyruvate may occur:

Pyruvate converted to phosphoenolpyruvate via pyruvate carboxylase and phosphoenolpyruvate carboxykinase could then be reformed by the action of pyruvate kinase. This process constitutes a 'substrate cycle' which, according to Newsholme (1976), may be of significance in metabolic regulation, although it is wasteful of ATP.

A high rate of substrate cycling would result in an underestimate of the rate of lactate utilization in kidney cortex.

The existence of glycolytic and gluconeogenic enzymes in kidney cortex is well established. The micro-dissection studies by Guder & Schmidt (1975) indicate that the two pathways may be separated in space, since hexokinase and pyruvate kinase, enzymes of glycolysis, occur in different cell types from those which contain the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase. The measured rate of lactate production from glucose in kidney cortex is only 10% of the rate of gluconeogenesis from lactate (Krebs *et al.*, 1963; Newsholme & Underwood, 1967). Nevertheless, pyruvate kinase is about five times as active in proximal-tubular cells of the kidney as is phosphoenolpyruvate carboxykinase (Guder & Schmidt, 1975). Hence the possibility of substrate cycling at the level of pyruvate, oxaloacetate and phosphoenolpyruvate exists. Rognstad & Katz (1972) suggested that, at initial pyruvate concentrations of 10 mM or more, up to 70% of the carbon derived from pyruvate might be recycled in this way. These studies were performed over the course of 2 h, with high initial concentrations of pyruvate, and it is probable that all of the pyruvate was consumed during this prolonged incubation. Rognstad & Katz (1972) also suggested that the rate of recycling might be considerably less when lactate was the substrate. Saggerson (1978) using similarly high concentrations of pyruvate concluded that about 25% of the pyruvate carbon was recycled in renal tubules.

One function of the recycling of pyruvate carbon is the so-called 'futile cycle', which may be involved in the regulation of both gluconeogenesis and glycolysis. Another quite different role for such a cycle might be in the complete oxidation of substrates entering the tricarboxylic acid cycle. Thus carbon entering at oxaloacetate would only be oxidized to CO_2 if it was first converted to phosphoenolpyruvate and then to pyruvate. This idea arose from the view that the direct conversion of oxaloacetate to pyruvate, and hence to acetyl-CoA, would not occur at a significant rate under physiological conditions. The present study was undertaken to re-examine the existence of substrate cycling in kidney.

Isolated kidney tubules were incubated with a near-physiological concentration of sodium L-lactate to which was added a quantity of [$\text{U-}^{14}\text{C}$]-lactate. The specific radioactivity of the lactate was determined initially, and again after timed incubations. The specific radioactivity of lactate may fall by a number of mechanisms: lactate may be formed from endogenous precursors, such as glycogen, glycerol or amino acids; two distinct cell populations may be present in kidney tubules, one carrying out glycolysis and the other gluconeogenesis, or recycling of lactate carbon may occur, by some route. In the last case a fall in specific radioactivity may be expected, because any extra lactate formed during continuous removal of labelled lactate will be unlabelled to the extent that carbon atoms may randomize in the oxaloacetate molecule. This randomization is in turn brought about by the formation of fumarate and malate (see Fig. 1).

The primary aim of this study was to establish whether or not at near-physiological concentrations, there is a loss of label from lactate by any route.

Lactate formed by cycling through phosphoenolpyruvate may be expected to have $\frac{1}{2}$ of its initial specific activity after a single pass, if tricarboxylic acid-cycle activity is ignored. Since the tricarboxylic acid cycle turns at about twice the rate of gluconeogenesis in fed rats (Vinay *et al.*, 1978) and since significant oxidation of lactate occurs in kidney tubules, the oxaloacetate pool will be more highly labelled than predicted from pyruvate carboxylase activity alone. This will decrease the rate of entry of unlabelled carbon to the lactate pool, but not its final extent. Any such effect of labelling of tricarboxylic acid-cycle intermediates will be decreased by adding unlabelled oxidizable substrates, such as oleate, in the presence of radioactive lactate (Weidemann & Krebs, 1969). This method of determining substrate cycling is relatively insensitive but has two advantages over the methods applied by Rognstad & Katz (1972) and Saggerson (1978). Cycling can be measured with physiological concentrations of substrates (lactate). Furthermore, formation of un-

[U- 14 C]Lactate is converted via pyruvate to oxaloacetate in which the extra carbon derives from the unlabelled bicarbonate pool. After conversion to the symmetrical molecule, fumarate, randomization of carbon occurs. When oxaloacetate is returned, by whatever route, to pyruvate, $^{14}\text{CO}_2$ is lost, with the formation of 1,2-labelled lactate. The specific activity of the lactate pool would thereby fall to $\frac{1}{2}$ of the starting value.

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Saunders, Smith Kline and French, Philadelphia, PA, U.S.A.) was prepared fresh before each experiment. Oleate was added as the sodium salt from a stock solution of 0.1 M. The final concentration of oleate in the reaction flask was 1 mM, to avoid deleterious effects of higher concentrations on kidney cortex tubules (Guder & Wieland, 1972). All incubations were carried out in duplicate and duplicate determinations of the specific radioactivity of lactate and glucose were made on each sample.

Analytical procedures

Lactate, pyruvate, glucose, malate, fumarate and aspartate were measured on HClO_4 -extracts of the incubation mixtures that had been neutralized with KOH. Standard enzymatic assays were used, as described by Bergmeyer (1974).

To separate lactate and glucose, portions of the neutralized extracts were applied to a column (20 cm $\times$ 1 cm) of Dowex 1 (formate form) and were eluted with a linear gradient of formic acid (Busch *et al.*, 1952). The positions of the radioactive peaks were determined with a continuous-flow scintillation counter and the fractions corresponding to glucose and lactate peaks were collected. After neutralizing the pooled fractions with NaOH, glucose and lactate were determined by using enzymatic methods (Bergmeyer, 1974) using an Aminco DW 2 spectrophotometer. Radioactivity of the samples was determined by counting 0.4-ml portions in 10 ml of scintillation fluid, which consisted of 5.5 g of Permablend (Packard Instruments), 60 g of naphthalene, 400 ml of 2-methoxyethanol and 600 ml of toluene. Specific radioactivity was determined and expressed as c.p.m./ μmol .

Chemicals

Enzymes and coenzymes were obtained from the Boehringer Corp. Hyaluronidase was from Miles, and all other chemicals were reagent grade. Radioactive [^{14}C]lactate was obtained from The Radiochemical Centre, Amersham.

Enzyme assays and mitochondrial preparation

Pyruvate kinase was determined in homogenates prepared from isolated tubules. Other enzyme assays were performed on whole kidney-cortex homogenates or on mitochondrial and cytosolic components of the homogenates. The mitochondrial preparation followed the method of Myers & Slater (1957), using two-step centrifugation in 0.25 M-sucrose. Oxaloacetate decarboxylase was assayed according to Wojtczak & Walatys (1974); malic enzyme (Hsu & Lardy, 1969), malic dehydrogenase (Bergmeyer, 1974, p. 485); glutamate dehydrogenase (Schmidt, 1974) and fumarase (Bergmeyer, 1974, p. 452) were assayed in the same extracts.

The determination of pyruvate kinase followed the

method of Carbonell *et al.* (1973). Kidney tubules were prepared as usual from fed or fasted rats and were homogenized in 3 ml of 0.25 M-sucrose. The supernatant obtained after centrifuging for 1 h at 20000 g at 4°C was fractionated using 0–50% and 50–70% saturated $(\text{NH}_4)_2\text{SO}_4$. The pellets were redissolved in 25 mM-Tris/HCl buffer, pH 7.0, containing 0.1 mM-EDTA and 1 mM-dithioerythritol. The 0–50% fraction contained the L-type pyruvate kinase and the 50–75% fraction contained the A-type (Carbonell *et al.*, 1973). The L-type is the allosteric form of pyruvate kinase, which in kidney represents only a small fraction of the total enzyme. Changes in this fraction in particular might be expected to influence the rate of substrate cycling (Rognstad, 1976).

Expression of results and determination of rate of lactate production

Rates of metabolic change are expressed as μmol of metabolite/h per g dry wt. of tubules, determined from initial and final concentrations of lactate or glucose in the incubation mixture.

Most of the results in this study are concerned with the change in specific radioactivity of lactate. Unlabelled lactate enters the pool of labelled lactate, from which lactate is continuously drawn by gluconeogenesis and complete oxidation. This makes a mathematical treatment necessary to evaluate the true rate of entry of unlabelled lactate into that pool. This figure, given as three-carbon units of unlabelled lactate, rather than as single carbon atoms, is the maximum rate of recycling of lactate. The lower limit of detection, based on the measurable fall in specific radioactivity of lactate, is therefore equivalent to 15 μmol of lactate formed/h per g dry wt. of kidney, a recycling rate of 8% of the rate of glucose synthesis.

The rate of entry (β) of unlabelled lactate into the pool can be calculated for each experiment from eqns. (1) and (2) below, knowing the initial (y_0) and final (y_t) amounts of labelled lactate present and the final concentration of unlabelled lactate, z_t :

$$\alpha = \ln \frac{y_0}{y_t} \quad (1)$$

$$z_t = \beta / \alpha \left(1 - \frac{y_t}{y_0} \right) \quad (2)$$

Definitions, and derivation of eqns. (1) and (2)

α = The rate constant for the removal of lactate from the pool, proportional to the concentration of lactate in the range of lactate concentrations (0.5–2 mM) used in this study. β = The rate of formation of unlabelled lactate from all sources, including recycling. t = The duration of incubation;

40 min in this instance. x_0 = Measured lactate at time 0. x_t = Measured lactate at time t . y_0 = Lactate at initial specific radioactivity, at time 0. y_t = Lactate at the same specific radioactivity, at time t . z_t = Amount of unlabelled lactate present at the end of incubation. Thus:

$$x_t = y_t + z_t$$

c_1 and c_2 are constants. dy and dz refer to small decrements in the amount of labelled and unlabelled lactate respectively, in a small unit of time, dt .

Assuming that all newly-formed lactate mixes completely and rapidly with the pool, and that the rate of lactate removal (α) is proportional to its concentration:

$$\frac{dy}{dt} = -\alpha y$$

and:

$$\frac{dz}{dt} = \beta - \alpha z$$

then:

$$y_t = c_1 e^{-\alpha t}$$

$$z_t = \beta/\alpha + c_2 e^{-\alpha t}$$

At time 0, $y_0 = c_1$ and $c_2 = -\beta/\alpha$:

$$y_t = y_0 e^{-\alpha t}$$

$$z_t = \beta/\alpha (1 - e^{-\alpha t})$$

Making, t , the unit of time (40 min) = 1:

$$y = y_0 e^{-\alpha}; \quad z = \beta/\alpha (1 - e^{-\alpha})$$

Substituting:

$$\alpha = \ln \frac{y_0}{y_t} \quad (1)$$

$$z_t = \beta/\alpha \left(1 - \frac{y_t}{y_0} \right) \quad (2)$$

Statistics

Paired samples were compared statistically using the paired t test. This assumes that the samples used

are normally distributed. Significant differences are indicated in Tables 3 and 4 as * $P < 0.05$ and ** $P < 0.005$.

Results

Glucose production from lactate in isolated kidney tubules

The object of preliminary experiments was to establish conditions in which rates of lactate metabolism and glucose synthesis were linear at the lowest concentrations of lactate, and to avoid exhausting all of the added substrate(s).

Glucose formation from lactate by isolated kidney tubules was linear for up to 60 min, at substrate concentrations of 5 mM and above.

At 1 mM-lactate, glucose formation occurred at a lower rate, and ceased after 40 min of incubation when the concentration of lactate had fallen to 0.2 mM or below. With an initial concentration of lactate of 2 mM, the rate of glucose synthesis was somewhat lower than at 10 mM (Table 1) but was approximately linear for 40 min, at which time the lactate concentration had fallen to about half the initial value. Subsequent studies were performed with 2 mM-lactate because the substrate concentrations at the start and finish of the incubation were not very different from those observed in blood under physiological conditions. In both fed and starved rats the rate of lactate removal was about five times the rate of glucose synthesis (see Table 1). The accumulation of pyruvate never exceeded 1% of the lactate removed. From earlier studies it may be assumed that the lactate removed in excess of twice the glucose formed, is completely oxidized (Krebs *et al.*, 1965).

Dilution of radioactive lactate by kidney tubules

During 40 min of incubation with 2 mM-[U- 14 C]-lactate, lactate was removed at the rate of 621 μ mol/h per g dry wt. of kidney (see Table 2). During the same period the specific radioactivity of the remaining lactate pool fell from 80 500 c.p.m./ μ mol to 58 400 c.p.m./ μ mol. Calculations show that the fall in the specific radioactivity is equal to a rate of

Table 1. *Glucose synthesis and lactate utilization in isolated kidney tubules*

Tubules prepared from the cortex of kidneys of fed or 24-h starved rats were incubated for 40 min with 2 mM- or 10 mM-lactate. Results are expressed as μ mol/h per g dry wt.; means $\pm$ S.E.M., with the number of kidneys in parentheses.

Animals	Lactate concn. (mM)	Glucose formed	Lactate consumed	Lactate used glucose formed
Fed	2	111 $\pm$ 5 (15)	-622 $\pm$ 22 (15)	5.60
	10	125 (2)		
Starved 24 h	2	140 $\pm$ 2 (4)	-604 $\pm$ 26 (4)	4.31
	10	171 $\pm$ 6 (4)		

Table 2. *Change in specific radioactivity of lactate in kidney cortex from fed and starved rats*

Isolated renal tubules from fed and 24-h starved rats were incubated for 40 min at 37°C with lactate as substrate. Data are from typical experiments. α , β and the rate of entry of unlabelled lactate to the lactate pool were calculated as described in the Materials and Methods section.

	Rats	
	Fed	24-h starved
Initial lactate concentration (mM)	2.14	1.9
Final lactate concentration (mM)	0.76	1.00
Initial lactate specific radioactivity (c.p.m./ μ mol)	80 500	108 200
Final lactate specific radioactivity (c.p.m./ μ mol)	58 400	108 500
α	1.40	—
β	0.721	—
Rate of entry of unlabelled lactate to the lactate pool (μ mol/h per g dry wt.)	157	0
Rate of glucose synthesis (μ mol/h per g dry wt.)	98	138
Tubules added (mg of dry tubules/flask)	6.9	5.4

Table 3. *Metabolic fate of labelled lactate in kidney tubules from fed and 24-h starved rats: the effect of glucose, 3-mercaptopycolinate and oleate*

Kidney tubules from fed or starved rats were incubated with 2 mM-sodium L-[U- 14 C]lactate for 40 min with the additions indicated. The rates of glucose synthesis and lactate utilization were determined from enzymatic analysis of the incubation mixtures. The specific radioactivity of glucose formed and the lactate remaining was determined by separation on Dowex (formate form) as described in the Materials and Methods section, and the resulting change in specific radioactivity of lactate is expressed as a percentage of the initial specific radioactivity. The fall in lactate specific radioactivity has been calculated in terms of the rate of formation of unlabelled lactate. Results are μ mol/h per g dry wt., with the number of experiments in parentheses. * Significantly different from fed, 2 mM-lactate ($P < 0.05$).

Animals	Substrate	Glucose synthesis	Lactate utilization	Glucose specific radioactivity (c.p.m./ μ mol)	Change in specific activity of lactate (%)	Calculated unlabelled lactate synthesis
Starved	2 mM-Lactate	140 $\pm$ 2 (4)	604 $\pm$ 26 (4)	146 $\pm$ 10 (4)	2.7 $\pm$ 2.2 (4)	15.3 $\pm$ 15 (4)
Fed	2 mM-Lactate	119 $\pm$ 6 (6)	612 $\pm$ 34 (6)	176 $\pm$ 9 (4)	14.4 $\pm$ 3.1 (6)	121 $\pm$ 22 (6)
	2 mM-Lactate + 5 mM-glucose	—	536 $\pm$ 42 (4)	74 $\pm$ 1.1 (4)	17.3 $\pm$ 3.8 (4)	146 $\pm$ 26 (4)
	2 mM-Lactate + 0.5 mM-3-mercaptopycolinate	4.5 $\pm$ 2.0 (5)	477 $\pm$ 38 (5)*	—	9.5 $\pm$ 2.2 (5)	94 $\pm$ 10.6 (5)
	2 mM-Lactate + 1 mM-oleate	119 $\pm$ 12 (4)	480 $\pm$ 18 (4)*	147, 139	13.9 $\pm$ 3.2 (4)	124 $\pm$ 24 (5)
	2 mM-Lactate + 1 mM-oleate + 0.5 mM-3-mercaptopycolinate	0	326 $\pm$ 43 (4)*	—	16.2 $\pm$ 3.4 (4)	177 $\pm$ 48 (4)

formation of unlabelled lactate of 157 μ mol/h per g dry wt. of kidney.

When kidney tubules from 24 h-starved rats were incubated under the same conditions, the rate of glucose formation was 138 μ mol/h per g dry wt., and, in contrast with the results with the fed rat, there was no change in the specific radioactivity of lactate. Thus, in starved-rat kidney, the rate of formation of unlabelled lactate was negligible.

These findings were reproduced in the series of experiments shown in Table 3. Because of the variation between experiments in the starting specific radioactivity of lactate, the results have been expressed as percentage change in specific radio-

activity of lactate in 40 min. In six kidneys from well-fed rats, the mean decrease of specific radioactivity of lactate was 14.4%, equivalent to the formation of 121 $\pm$ 22 μ mol of unlabelled lactate/h per g dry wt. of kidney. Starving the rats for 24 h prevented any significant reduction in the specific activity of lactate.

Endogenous sources of unlabelled lactate in isolated tubules

Two explanations suggest themselves for the decrease in specific radioactivity of lactate in fed rats. Either lactate is formed from an unlabelled endogenous precursor, such as glycogen, glucose or

an amino acid, or unlabelled carbon is incorporated by a cyclic process of the following kind:

The glycogen content of tubules immediately after preparation was only $2.5 \mu\text{mol/g}$ dry wt. of kidney (expressed as glucose, for two kidneys); when tubules were incubated for 40 min without substrate, this decreased to $1 \mu\text{mol/g}$ dry wt., but in the presence of 2 mM-lactate, a small increase in glycogen content (to $6.7 \mu\text{mol/g}$ dry wt.) was observed. Thus glycogen could account for no more than 5% of the unlabelled lactate produced.

Alternative sources, including endogenous lactate, glycerol and glucose, were all present at very low concentrations in tubules after their preparation, and these concentrations did not change after 40 min of incubation in the absence of substrate. It is therefore very unlikely that these precursors contribute significantly to the formation of unlabelled lactate. The possibility that glycerol, for example, is generated by lipolysis and re-oxidized, thereby significantly generating unlabelled lactate is diminished by the finding that there is virtually no formation of unlabelled lactate in the tubules prepared from fasted rats. A similar objection applies to the formation of significant amounts of pyruvate or lactate from endogenous amino acids.

Effect of added glucose, 3-mercaptopycolinic acid or oleate

If the metabolic pathway for cycling exists in kidney tubules, its presence should be more easily demonstrable by addition of an unlabelled precursor of lactate. In particular, glucose should be metabolized to lactate via pyruvate kinase.

Addition of oleate would supply unlabelled acetyl-CoA, which otherwise would arise from the labelled lactate, and thus increase the incorporation of unlabelled carbon into lactate after randomization in the tricarboxylic acid cycle.

Finally, such formation of unlabelled lactate as occurs by substrate cycling should be inhibited if phosphoenolpyruvate carboxykinase were inhibited. To this end, experiments were performed in the presence of 2 mM-lactate with addition of glucose, oleate or 3-mercaptopycolinate.

Addition of 5 mM-glucose, which would, by glycolysis to lactate, be expected to enhance the dilution of specific radioactivity of lactate, had no effect (Table 3); the fall in lactate specific radioactivity was 17.3%, and the calculated rate of formation of unlabelled lactate, 146 ± 26 (S.E.M., $n = 4$) $\mu\text{mol/h}$ per g dry wt., was not significantly different from that formed when lactate alone was present. No net utilization of glucose could be

measured in these experiments, because of the high initial glucose concentration. In other experiments, Newsholme & Underwood (1967) have shown a rate of glucose removal by kidney cortex slices of about $50 \mu\text{mol/h}$ per g dry wt. of kidney.

3-Mercaptopycolinic acid, an inhibitor of phosphoenolpyruvate carboxykinase, inhibited glucose synthesis from lactate by 96%, significantly decreased the rate of lactate uptake, but did not significantly limit the observed rate of decrease in specific radioactivity of lactate (Table 3). The calculated rate of formation of unlabelled lactate in the presence of 3-mercaptopycolinate was $94 \pm 10.6 \mu\text{mol/h}$ per g dry wt., not significantly less than the control. It is unlikely therefore that the observed change in specific radioactivity of lactate involved the cycling of carbon through phosphoenolpyruvate. Addition of oleate (1 mM) did not alter the rate of glucose synthesis from lactate, nor did it alter the decrease in specific radioactivity of lactate. The rate of formation of unlabelled lactate in the presence of oleate ($124 \pm 24 \mu\text{mol/h}$ per g dry wt.) was thus not significantly different from that observed with lactate alone. Furthermore the addition of 3-mercaptopycolinate in the presence of oleate suppressed glucose synthesis, but failed to affect the rate of decrease of lactate specific radioactivity. These experiments may be taken to indicate that oleate carbon fails to enter lactate pools under the conditions of the present experiment. Addition of oleate caused only a slight decrease in the specific radioactivity of the glucose synthesized. (Table 3), but the rate of lactate oxidation fell from 314 to $242 \mu\text{mol/h}$ per g dry wt., in tubules from fed rats ($P < 0.05$).

Effect of exogenous substrates on the formation of unlabelled lactate

In an attempt to enhance dilution of the lactate radioactivity, substrates were added that might be expected to form lactate.

In a series of experiments with unlabelled precursors, the rate of lactate accumulation was extremely low: from malate, fumarate, dihydroxyacetone and aspartate (2 mM in each case) the rate was less than $10 \mu\text{mol}$ of lactate/h per g dry wt., and from 5 mM-glucose the rate of lactate accumulation was $16 \pm 4 \mu\text{mol/h}$ per g dry wt. (S.E.M., $n = 3$) in tubules from fed rats, and only 3–6 $\mu\text{mol/h}$ per g dry wt. in tubules from starved rats. But this does not exclude the formation of lactate with its subsequent re-utilization. Thus, under anaerobic conditions, in the presence of 1 mM-KCN and 5 mM-glucose, lactate accumulated at the rate of 217 ± 14 (S.E.M., $n = 4$) $\mu\text{mol/h}$ per g dry wt.

To show this directly, unlabelled substrates were added in the presence of [^{14}C]lactate. When 2 mM-sodium fumarate was added, the expected en-

hancement occurred; glucose synthesis was more than doubled and the specific radioactivity of the glucose formed was decreased to about one-third of the control value (Table 4). The specific radioactivity of the lactate pool fell, so that the calculated rate of addition of unlabelled lactate to the pool was almost doubled (122 increased to 219 $\mu\text{mol/h}$ per g dry wt.). The results from six experiments with lactate alone, with added fumarate (2 mM) or with 3-mercaptopicolinate (0.5 mM) show that the increase in formation of unlabelled lactate was significant when fumarate was added ($P < 0.005$; Table 5). The decrease in specific radioactivity of lactate was not prevented by the further addition of 3-mercaptopicolinate (0.5 mM) sufficient to inhibit glucose synthesis by over 90% (Tables 4 and 5).

In contrast, malate (2 mM) and aspartate (2 mM) produced no change in lactate specific radioactivity when added to tubules incubated with [^{14}C]lactate ($P > 0.1$ in both series). Thus, no extra lactate formation was observed with these two substrates, despite the fact that both were actively metabolized. This was indicated by the extra glucose formed and by the very marked decrease in glucose specific radioactivity (Table 4). Indeed, the rate of lactate

removal was significantly stimulated by addition of 2 mM-aspartate.

The mechanism of this effect is not clear. The increase in gluconeogenesis caused by aspartate may be due to the provision of aspartate for the malate-aspartate shuttle. But this is probably not the only effect. A consequence of the addition of aspartate was that the final concentration of lactate was lower in incubations with lactate + aspartate than with lactate alone. An equivalent rate of addition of unlabelled lactate therefore resulted in a greater decrease (23.3%) in lactate specific radioactivity; this is not indicative of any change in the rate of recycling of lactate in the presence of aspartate. Inhibition of glucose formation from malate or aspartate by adding 3-mercaptopicolinate did not significantly alter the rate of formation of unlabelled lactate (Table 4).

Determination of pyruvate kinase activity in renal tubules

The failure to form lactate under conditions in which phosphoenolpyruvate is so readily formed could be due to inactivity of pyruvate kinase under the test conditions. Pyruvate kinase was determined

Table 4. *Effect of added substrates upon the metabolic fate of lactate in kidney tubules from fed rats*

Conditions of incubations were as described in the legend to Table 3. n = number of experiments. Values are given as $\mu\text{mol/min}$ per g dry wt. of tubules. Significantly different from incubation with 2 mM-lactate alone (* $P < 0.05$; ** $P < 0.005$)

Substrate	n	Glucose synthesis	Lactate utilization	Glucose specific activity (c.p.m./ μmol)	Change in specific activity of lactate (%)	Calculated unlabelled lactate synthesis
2 mM-Lactate	9	113 $\pm$ 6	635 $\pm$ 26	156 $\pm$ 5	13.7 $\pm$ 2.9	112 $\pm$ 17
2 mM-Lactate + 2 mM-fumarate	6	287 $\pm$ 18**	670 $\pm$ 27	45 $\pm$ 5.3**	27.0 $\pm$ 5.7	219 $\pm$ 49**
2 mM-Lactate + 2 mM-fumarate + 0.5 mM-3-mercaptopicolinate	3	20.3 $\pm$ 4.6*	503 $\pm$ 23*	—	14.7 $\pm$ 2.8	144 $\pm$ 40
2 mM-Lactate + 2 mM-malate	5	286 $\pm$ 33**	685 $\pm$ 21	52 $\pm$ 5.4**	18.9 $\pm$ 4.5	145 $\pm$ 22
2 mM-Lactate + 2 mM-malate + 0.5 mM-3-mercaptopicolinate	2	14	492	—	14.6	106
2 mM-Lactate + 2 mM-aspartate	5	191 $\pm$ 9.5**	879 $\pm$ 63**	100 $\pm$ 2.7**	23.3 $\pm$ 7.2	125 $\pm$ 37
2 mM-Lactate + 2 mM-aspartate + 0.5 mM-3-mercaptopicolinate	3	2.1 $\pm$ 2.1*	670 $\pm$ 21	—	15.8 $\pm$ 4.3	153 $\pm$ 22

Table 5. *Effect of added fumarate upon the metabolic fate of lactate in kidney tubules from fed rats*

The rates of formation of unlabelled lactate in the six experiments shown in Table 4, in which tubules from fed rats were incubated with 2 mM-[^{14}C]lactate alone, with 2 mM-fumarate or with 2 mM-fumarate + 0.5 mM-3-mercaptopicolinate, are given individually.

Substrate	Experiment no.	Lactate formed ($\mu\text{mol/h}$ per g dry wt.)						P
		53	54	55	58	62	63	Mean $\pm$ S.E.M.
2 mM-Lactate	...	194	200	119	87	70	60	122 $\pm$ 25 (6)
2 mM-Lactate + 2 mM-fumarate		395	308	229	179	63	137	219 $\pm$ 49 (6)
					179	63	137	126 $\pm$ 42 (3)
2 mM-Lactate + 2 mM-fumarate + 0.5 mM-3-mercaptopicolinate		—	—	—	189	65	178	144 $\pm$ 40 (3)

directly in isolated tubules prepared from kidneys of fed and starved rats (Table 6) and found to be present in both fed and starved animals; the total activity of 2500 and 3200 $\mu\text{mol/h}$ per g dry wt. respectively was more than 20 times higher than the calculated rate of lactate formation. The differences in the rate of lactate formation between tubules from fed and starved rats could not be attributed to any change in the proportion of the enzymes present in the adaptive form; in both dietary states, 19–25% of the enzyme was found in the L form (Table 6).

Distribution of some enzymes relevant to substrate cycling

The persistence of recycling of lactate carbon in the presence of the inhibitor of phosphoenolpyruvate carboxykinase, and the formation of lactate from fumarate but not from malate or aspartate, could be explained if an alternative pathway for conversion of oxaloacetate to pyruvate was present in kidney. One such pathway, via oxaloacetate

decarboxylase, has been described in kidney (Bennett & Alleyne, 1978). Kidneys of fed rats were homogenized in 0.25 M-sucrose and mitochondrial and supernatant fractions were prepared by centrifugation. Both malic enzyme and oxaloacetate decarboxylase activities were present; ‘malic’ enzyme was predominantly in the supernatant fraction, while oxaloacetate decarboxylase predominated in the mitochondrial fraction. Of significance for the special role of fumarate, fumarase was 3-fold more active in the mitochondrial fraction than in the cytosol (Table 7).

Discussion

Substrate or ‘futile’ cycling in kidney cortex

The present findings make it unlikely that there is a significant flow of carbon through the ‘substrate’ cycle:

in rat kidney cortex. In starved animals, in which the rate of conversion of lactate to phosphoenolpyruvate and hence to glucose is significantly increased, there is no measurable return of carbon to lactate. In fed rats, lactate is reformed at the same rate as glucose synthesis under these conditions but not by the above cycle. This is inferred from (i) the failure of 3-mercaptopicolinate to inhibit lactate formation; (ii) the inability of substrates such as aspartate and malate to decrease the specific radioactivity of lactate even though carbon from either substrate enters glucose; (iii) the ability of fumarate to generate lactate even when phosphoenolpyruvate carboxykinase and gluconeogenesis are substantially inhibited.

If ‘substrate-cycling’ is a metabolic regulatory process (Newsholme & Start, 1973) it would be expected to occur at a substantial rate; Rognstad &

Table 6. *Pyruvate kinase activity in isolated tubules of fed and 24-h starved rats*

Kidney tubules were prepared as usual from fed or starved rats. Pyruvate kinase activity was determined in $(\text{NH}_4)_2\text{SO}_4$ fractions of a homogenate of tubules (see the Materials and Methods section). Results are expressed for the A and L forms of the enzyme as defined by Carbonell *et al.* (1973).

	Pyruvate kinase activity (μmol of pyruvate formed/h per g dry wt. of tubules)	
	Fed	Starved
Total activity	2469	3240
A Fraction	1488	1545
L Fraction	285	390

Table 7. *Subcellular distribution of enzymes in rat kidney cortex*

Cortex from two kidneys was homogenized in 0.25 M-sucrose and mitochondria were separated by a two-step centrifugation. Enzymes were assayed as described in the text. Protein assay was by the method of Lowry *et al.*, 1951.

Enzyme	Experiment	Enzyme activity (units/mg of protein) in	
		Soluble fraction	Mitochondria
Oxaloacetate decarboxylase	1	0.0026	0.0063
	2	0.0016	0.0063
‘Malic’ enzyme	1	0.034	0.0079
	2	0.023	0.0035
Fumarase	1	0.071	0.223
	2	0.090	0.249
Malic dehydrogenase	1	8.96	4.26
	2	5.73	1.26
Glutamic dehydrogenase	1	0.0036	0.785

Fig. 2. Pathway of lactate carbon atoms in kidney cortex tubules

Lactate-carbon is partially recycled via oxaloacetate and not via phosphoenolpyruvate. Added substrates enter the cell and, in the case of aspartate and malate, form oxaloacetate, predominantly in the cytosol. Fumarate, metabolized predominantly in the mitochondrion, generates oxaloacetate within the mitochondrion as well as in the cytosol.

Katz (1972) suggested 25% of the rate of gluconeogenesis. The present experiments indicate that the rate of substrate cycling is much lower. The sensitivity of the method is such that recycling of lactate cannot exceed 8% of the rate of conversion of phosphoenolpyruvate to glucose. The true figure may well be less than this.

Alternative sources of lactate in well-fed-rat kidney cortex

The finding that 3-mercaptopycolinate fails to prevent lactate formation in well-fed animals means that an alternative pathway, probably from oxaloacetate to lactate, exists in kidney cortex. The possibilities are either oxaloacetate decarboxylase or 'malic' enzyme. The former is the more likely one since it is largely mitochondrial (Wojtczak & Walajtys, 1974, and the present paper) and requires no NADP. However, this enzyme has a high K_m for oxaloacetate (0.1–0.5 mM, according to Dean & Bartley, 1973). The one substrate that enhanced lactate production in kidney cortex, fumarate, may be expected to generate high concentrations of oxaloacetate in the mitochondria. Fumarase is present at a considerably higher concentration in the mitochondria than in the cytosol (see Table 7).

The failure of malate and aspartate to form lactate may at first sight appear surprising, since both substrates formed substantial amounts of glucose and hence may be expected to dilute both oxaloacetate and phosphoenolpyruvate pools. However, from the above considerations, it will be seen that recycling of lactate occurs in the kidney of fed rats by a route that does not involve phosphoenolpyruvate carboxykinase. For cycling to occur it

seems probable that a high concentration of oxaloacetate must arise in the mitochondrion. Malate, because of the high activity of malate dehydrogenase in the cytosol, forms oxaloacetate outside the mitochondrion with the subsequent formation of glucose. Aspartate also forms predominantly extra-mitochondrial oxaloacetate with glucose synthesis, with the result that no dilution of the lactate specific radioactivity occurs (Fig. 2).

While this work was in progress, Rognstad (1979) suggested that the pathway of recycling of carbon via oxaloacetate decarboxylase may also be active in rat hepatocytes metabolizing high concentrations of pyruvate.

Further work is required to clarify the properties of an intramitochondrial oxaloacetate decarboxylase in kidney and to explain why it is inactive in the kidney of starved rats.

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