Effects of Ammonia and Norvaline on Lactate Metabolism by Hepatocytes from Starved Rats

THE USE OF ^{14}C -LABELLED LACTATE IN STUDIES OF HEPATIC GLUCONEOGENESIS

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1. Hepatocytes from starved rats were incubated with *L*-lactate and NH_4Cl or norvaline, and the rates of the tricarboxylic acid cycle and of gluconeogenesis were calculated from changes in metabolite concentrations or from radioisotopic data from incubations with labelled lactate or propionate. 2. Gluconeogenesis was stimulated by the addition of 10 mM- NH_4Cl , 5 mM-norvaline or 1 mM-oleate by 27, 45 and 59% respectively. NH_4Cl or norvaline also increased lactate uptake. Norvaline inhibited urea synthesis from NH_4Cl by 85%. 3. The effects of NH_4Cl and norvaline were not additive. However, NH_4Cl inhibited and norvaline was without effect on gluconeogenesis from pyruvate, indicating that the two compounds act by different mechanisms. 4. The tricarboxylic acid-cycle flux was increased 80% by lactate, and NH_4Cl caused a further 25% stimulation. Norvaline had no effect on the tricarboxylic acid-cycle flux. NH_4Cl and norvaline tripled and doubled, respectively, flux through pyruvate dehydrogenase. 5. Total ATP formation was calculated to range from 470 to 830 $\mu\text{mol/h}$ per 100 mg of protein, of which the basic metabolic activity accounted for 400–450 $\mu\text{mol/h}$ per 100 mg of protein. ATP formation does not seem to be rate-limiting for gluconeogenesis. 6. Pyruvate recycling was estimated from the ^{14}C yield from $[1-^{14}\text{C}]$ propionate in lactate and glucose to be 10–30% of the flux of phosphoenolpyruvate to glucose. The further addition of NH_4Cl more than doubled the recycling of pyruvate. 7. $[1,4-^{14}\text{C}]$ Succinate was rapidly metabolized by hepatocytes. About 20% of the radioactivity was recovered in glucose, indicating that succinate is also metabolized by intact (non-damaged) hepatocytes. 8. It is concluded that the metabolism of lactate by the liver is too complex to allow simple rate measurements with labelled compounds.

NH_4Cl has been reported to stimulate gluconeogenesis from lactate in hepatocytes, although there is some disagreement about this effect (Cornell *et al.*, 1974; Zahlten *et al.*, 1974; Krebs *et al.*, 1976b; Van der Meer *et al.*, 1976). NH_4Cl also has been found to increase markedly glycogen synthesis from lactate (Katz *et al.*, 1976b). However, there have been few studies on the effect of NH_4Cl on other aspects of lactate metabolism. Norvaline inhibits urea synthesis (Rognstad, 1977), and in the present study we report our findings on the effects of NH_4Cl and norvaline on lactate metabolism.

Radioactive lactate and pyruvate have been used for many years in the study of hepatic gluconeogenesis. The randomization of label in the tricarboxylic acid cycle and pyruvate recycling complicates the interpretation of such experiments. We have examined here the application of various

labelled precursors to the study of lactate metabolism by isolated hepatocytes.

Methods

Hepatocytes were prepared from 140–200 g male Wistar rats, starved for 24 h, by perfusion of the liver with collagenase as described previously (Katz *et al.*, 1975). About 20 mg dry wt. of cells (approx. 80 mg wet wt.) was incubated with shaking at 37°C in 25 ml conical flasks in a total volume of 3 ml. The buffer was Krebs–Henseleit (1932) bicarbonate and the gas phase O_2/CO_2 (19:1). After addition of cells the flasks were gassed for 2 min and incubated for the time indicated. The reaction was stopped by 1 ml of 6% (w/v) HClO_4 and $^{14}\text{CO}_2$ was collected for a period of 1½–2 h in 0.25 ml of phenethylamine/water (1:1, v/v) in a small vial suspended from the stopper. Additions after zero time were performed by injection through the stopper. We found no or only marginal stimulation by albumin of gluconeogenesis and substrate utilization. This is contrary to the obser-

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variations of Nakai *et al.* (1976). The discrepancy may possibly be explained by the different amounts of cells used in the incubations [5–7 mg dry wt./ml; cf. 1.8–2.3 mg/ml in the experiments of Nakai *et al.* (1976)].

Glucose, lactate, pyruvate, β -hydroxybutyrate, acetoacetate, asparagine, aspartate, glutamate, ammonia and urea were determined by standard enzymic analyses (Bergmeyer, 1974). Glutamine was determined as glutamate after incubation of the sample with glutaminase and alanine was measured with alanine dehydrogenase (Bergmeyer, 1974). Allowance was made for initial cell content of metabolites.

Oxygen uptake was determined by Warburg manometry by using the vessels described by Krebs *et al.* (1974) with a carbonate buffer in the centre well. The gas phase was O_2/CO_2 (19:1).

Radioactive metabolites were separated by ion-exchange chromatography as previously described (Katz *et al.*, 1975). About 2 ml of the neutralized $HClO_4$ extract was applied to a column of Dowex 50W (H^+ form) (1 cm $\times$ 5 cm) on top of Dowex 1 (acetate form) (1 cm $\times$ 8 cm). The columns were eluted with 20–30 ml of water to obtain the neutral fraction. Phosphorylation of this fraction with ATP and hexokinase and application of the reaction mixture to a column of Dowex 1 (acetate form) showed that nearly all the radioactivity in the neutral fraction was in glucose. The Dowex 50 column was eluted with 20 ml of 2M- NH_3 to give the amino acid fraction. Successive elution of the Dowex 1 column with 2M-acetic acid and 2M-ammonium formate, pH 3, gave the lactate and 'organic anions' fractions respectively. In some experiments, ketone bodies and propionate were eluted before the lactate fraction by 0.5M-acetic acid, and succinate and malate after the lactate fraction by 4M-acetic acid and 3M-formic acid respectively.

Glucose degradation was carried out after dismutation to 3-phosphoglycerate and *sn*-glycerol 3-phosphate (Schmidt *et al.*, 1970). The two compounds were separated by ion-exchange chromatography and the specific radioactivity of each was determined (Katz *et al.*, 1976a). The specific radioactivity of the *sn*-glycerol 3-phosphate was equal to that of 3-phosphoglycerate, and it was not degraded. The 3-phosphoglycerate was dephosphorylated by acid phosphatase to glyceralate, which was degraded with periodate as described elsewhere (Genovese *et al.*, 1970).

Two-dimensional paper chromatography was carried out on the neutralized $HClO_4$ extract after electrodialysis as described by Katz & Chaikoff (1954).

Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer 3385: 6 ml of scintillation fluid {750 ml of toluene, 250 ml of Triton X-100, 5 mg of PPO (2,5-diphenyloxazole)

and 60 mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene]} was used to a total of 1 ml of sample plus water.

Results are presented per h per 100 mg dry wt. The dry weight was determined as described elsewhere (Katz *et al.*, 1975) and is essentially protein. Our data may be re-expressed approximately as per g wet wt. of liver by multiplication by 2.5.

Calculations

The flux through the tricarboxylic acid cycle, the pyruvate dehydrogenase and the β -oxidation pathway was calculated from the metabolite changes shown in Table 1. In these calculations, all the oxygen uptake was assumed to be mitochondrial (line 1, Table 2), since no reliable information is available on how much of the oxygen uptake by isolated hepatocytes is mitochondrial [in the perfused liver, 85–90% of the oxygen uptake is cyanide-sensitive (Williamson *et al.*, 1969)].

Oxygen consumption for ketone-body formation (line 2, Table 2) was calculated as $3.5 \times \Delta \text{acetoacetate} + 2.5 \times \Delta \beta\text{-hydroxybutyrate}$ (assuming a fatty acid chain length of 16 carbon atoms, 14 O atoms are used for the formation of 8 molecules of acetyl-CoA, which is equivalent to 4 molecules of acetoacetate; $14/4 = 3.5$). Oxygen consumption for lactate oxidation (line 3, Table 2) was obtained by multiplication by 6 of the lactate not accumulated as glucose, pyruvate or alanine ($\Delta \text{lactate} - 2 \Delta \text{glucose} - \Delta \text{pyruvate} - \Delta \text{alanine}$). The oxygen uptake not accounted for (i.e. line 1–line 2–line 3 of Table 2) was ascribed to fatty acid oxidation (line 4, Table 2). Oxygen consumption due to tricarboxylic acid-cycle activity (line 5, Table 2) was calculated as two-thirds $\times$ oxygen consumption for lactate oxidation (because one-third of the oxygen used for lactate oxidation is used for oxidation of lactate to acetyl-CoA) plus $0.7 \times$ oxygen consumption for fatty acid oxidation [assuming a fatty acid chain length of 16 carbons, β -oxidation uses 14 of the total 46 oxygen atoms per fatty acid; $(46-14)/46 = 0.7$]. The tricarboxylic acid-cycle flux (line 6, Table 2) is this value divided by 4. Flux through pyruvate dehydrogenase (line 7, Table 2) is one-sixth of the oxygen consumption for lactate oxidation. The flux in C_2 units through the β -oxidation pathway (line 8, Table 2) is obtained as $2 \times \Delta \text{ketone bodies}$ plus the oxygen consumption for fatty acid oxidation multiplied by 0.3 (14 out of 46 O atoms are used for β -oxidation of a C_{16} fatty acid; $14/46 = 0.3$).

Chemicals

Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) and other chemicals and enzymes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from Calbiochem (Los Angeles, CA, U.S.A.).

Radiochemicals were from New England Nuclear (Boston, MA, U.S.A.), except for L-[1-¹⁴C]lactate and L-[2-¹⁴C]lactate, which were prepared by reduction of the corresponding labelled pyruvate by NADH and lactate dehydrogenase, and purified by paper chromatography with ethanol/aq. NH₃ (sp.gr. 0.88)/water (16:1:3, by vol.).

Results and Discussion

Metabolite changes

The formation or uptake of various metabolites by isolated hepatocytes in the 60min incubation period is shown in Table 1.

The rates of gluconeogenesis, lactate uptake and oxygen consumption observed here are similar to or higher than values reported by others with isolated hepatocytes or perfused liver. Cornell *et al.* (1974) reported a lag period in gluconeogenesis from lactate with isolated hepatocytes of about 40min. This is not observed by us (Fig. 1). This discrepancy may be related to differences in the methods used for the preparation of hepatocytes. In our method the liver is perfused with 5mM-glucose plus 5mM-pyruvate, and when the liver is digested by the collagenase the cells are dispersed and washed immediately. This may tend to diminish the loss of low-molecular-weight metabolites, which occurs when perfusion of the liver is carried out without substrates, and when the liver is

shaken in a large volume of buffer before the cells are washed (Krebs *et al.*, 1976a).

Effects of NH₄Cl. Glucose production with lactate as the only substrate is 30 μ mol/h per 100mg dry wt., corresponding to about 1.2 μ mol/min per g wet wt. of liver, and is stimulated 27% by 10mM-NH₄Cl. Gluconeogenesis from lactate was approximately linear with time in our experiments as long as the lactate was not used up (Fig. 1). The increase in the rate of gluconeogenesis on addition of NH₄Cl is in agreement with the results reported by Zahlten *et al.* (1974) and by Cornell *et al.* (1974). Other reports, however, show no effect (Krebs *et al.*, 1976b) or an inhibition (Krebs *et al.*, 1976b; Van der Meer *et al.*, 1976) of gluconeogenesis by NH₄Cl. The reasons for the discrepancies are not clear, although NH₄Cl seems to inhibit gluconeogenesis at lactate concentrations below 2mM (Van der Meer *et al.*, 1976; Krebs *et al.*, 1976a).

The stimulation of gluconeogenesis by NH₄Cl is paralleled by an increased uptake of lactate (Table 1). Oleate (1mM) stimulates gluconeogenesis, but with little effect on lactate uptake (Table 1). Endogenous ketone-body production is 11.5 μ mol/h per 100mg dry wt. and is lowered 84% by lactate addition (Table 1).

The [lactate]/[pyruvate] ratio of about 9 is not significantly affected by NH₄Cl, whereas the mitochondrial [NADH]/[NAD⁺] ratio, as reflected in the

Table 1. *Changes in metabolites*

Cells were incubated for 1 h with the substrates indicated and added to the following concentration: 10mM-L-lactate, 10mM-NH₄Cl, 5mM-norvaline, 1mM-oleate + 1% albumin. Values are presented as μ mol/h per 100mg dry wt. and are means with the numbers of different cell preparations in parentheses. S.E.M. values are given if $\geq 10\%$ of the mean. The initial content of metabolites per flask (about 20mg dry wt. in 2ml) was about 0.2 μ mol of glucose, 0.01 μ mol of pyruvate, 0.05 μ mol of β -hydroxybutyrate, 0.04 μ mol of acetoacetate, 0.2 μ mol of NH₄⁺, 0.3 μ mol of urea NH₄⁺, 0.3 μ mol of glutamate, 0.4 μ mol of glutamine, 0.1 μ mol of alanine, 0.02 μ mol of aspartate and 0.02 μ mol of asparagine. Abbreviation: n.d., not determined.

Substrates	...	Endogenous	Lactate	Lactate + NH ₄ Cl	Lactate + norvaline	Lactate + NH ₄ Cl + norvaline	Lactate + oleate	Lactate + oleate + NH ₄ Cl
Glucose output		5.0 (11)	29.9 (11)	37.9 (10)	43.3 (4)	40.3 (3)	47.6 (5)	46.5 (3)
Lactate uptake		—	87.6 (13)	137 (12)	124 (3)	150 (3)	107 (5)	130 (3)
Pyruvate formation		0.1 $\pm$ 0.05 (8)	9.0 (8)	2.1 $\pm$ 0.4 (7)	4.7 (3)	1.5 $\pm$ 0.4 (3)	8.5 $\pm$ 1.2 (5)	5.0 $\pm$ 1.6 (3)
β -Hydroxybutyrate formation		0.7 $\pm$ 0.1 (7)	0.2 $\pm$ 0.2 (7)	-0.3 $\pm$ 0.5 (6)	0.2 $\pm$ 0.3 (3)	-0.4 $\pm$ 0.2 (3)	7.6 $\pm$ 1.5 (4)	2.2 (2)
Acetoacetate formation		10.9 (8)	1.6 $\pm$ 0.2 (8)	2.5 $\pm$ 0.5 (7)	1.5 $\pm$ 0.3 (3)	3.2 $\pm$ 0.3 (3)	7.6 $\pm$ 1.5 (5)	10.0 $\pm$ 1.3 (3)
NH ₄ ⁺ uptake		-0.5 $\pm$ 0.9 (4)	-2.1 $\pm$ 0.7 (6)	99.2 $\pm$ 14.1 (6)	-4.1 (1)	7.0 (1)	n.d.	n.d.
Urea NH ₄ ⁺ formation		12.7 $\pm$ 1.6 (6)	11.6 $\pm$ 1.7 (7)	80.5 $\pm$ 11.9 (7)	8.3 $\pm$ 3.7 (3)	13.2 $\pm$ 4.0 (3)	n.d.	n.d.
Oxygen uptake		78.4 $\pm$ 9.3 (6)	109 (6)	138 (7)	115 $\pm$ 14.1 (3)	135 (3)	156 (4)	134 (4)
Glutamate formation		1.5 (1)	1.7 $\pm$ 0.8 (3)	2.4 $\pm$ 0.3 (3)	2.2 (2)	4.8 (2)	n.d.	n.d.
Glutamine formation		1.0 (1)	3.3 $\pm$ 1.3 (3)	5.8 $\pm$ 1.7 (3)	1.5 (2)	2.1 (2)	n.d.	n.d.
Alanine formation		0.6 (2)	2.4 $\pm$ 0.4 (3)	4.8 $\pm$ 1.4 (3)	1.2 (1)	6.4 (2)	n.d.	n.d.
Aspartate formation		0 (1)	0.1 (2)	1.0 (2)	0.1 (1)	0.7 (1)	n.d.	n.d.
Asparagine formation		n.d.	0.2	0.1	0	0.1	n.d.	n.d.
[Lactate]/[pyruvate]		1.4 $\pm$ 0.6 (5)	8.8 (8)	11.4 $\pm$ 1.9 (7)	7.1 $\pm$ 1.1 (3)	8.4 (3)	9.9 $\pm$ 1.3 (5)	13.1 (3)
[β -Hydroxybutyrate]/ [acetoacetate]		0.11 (6)	0.59 $\pm$ 0.10 (8)	0.11 $\pm$ 0.06 (7)	0.55 $\pm$ 0.09 (3)	0.05 $\pm$ 0.04 (3)	1.2 $\pm$ 0.2 (5)	0.27 $\pm$ 0.04 (3)

Fig. 1. Time course of glucose formation (a) and lactate uptake (b)

Hepatocytes were incubated as described in the Methods section with 10mM-L-lactate (○), 10mM-L-lactate + 10mM-NH₄Cl (●) or 10mM-L-lactate + 5mM-norvaline (▲).

[β -hydroxybutyrate]/[acetoacetate] ratio, is lowered from 0.6 to 0.1 (Table 1), as reported earlier (Sies *et al.*, 1974).

Urea formation is 13 μ mol/h per 100mg dry wt. and is stimulated 6-fold by NH₄Cl (Table 1).

In the absence of ammonia there is little amino acid accumulation. Addition of NH₄Cl in the presence of lactate causes an increase in amino acids equivalent to 11 μ mol of C₃ units/h per 100mg (Table 1). The major amino acids were glutamine, alanine and glutamate, but very little aspartate accumulated. Several authors have suggested that the stimulation of urea synthesis and gluconeogenesis by ammonia is caused by an increase in the concentration of the amino acids involved in the transport of carbon across the mitochondrial membrane (Zahlten *et al.*, 1974; Cornell *et al.*, 1974; Meijer *et al.*, 1975). In our experiments we found only small accumulation of

aspartate in the presence of NH₄Cl (although the intracellular concentration may have increased significantly), and, as shown by other results (Katz *et al.*, 1976b), NH₄Cl also stimulates the formation of glucose 6-phosphate from dihydroxyacetone. Probably, therefore, the stimulation of gluconeogenesis by NH₄Cl is a more complex phenomenon than indicated by the authors cited above.

Oxygen uptake was increased from about 80 μ mol/h per 100mg by the addition of lactate to 110 μ mol/h per 100mg, or about 4.2 μ mol/min per g wet wt. of liver (Table 1). NH₄Cl causes a further increase by 27% in the oxygen uptake.

Lactate addition caused an 80% increase in the tricarboxylic acid-cycle flux, from 20 to 36 μ mol/h per 100mg dry wt., and decreased the flux through the β -oxidation pathway by 40%, to 24 μ mol of C₂ units/h per 100mg (Table 2). NH₄Cl in the presence of lactate increased flux through the tricarboxylic acid cycle and through pyruvate dehydrogenase by 23% and 335% respectively, and decreased flux through the β -oxidation pathway to 25% of the rate in the absence of NH₄Cl. These effects of NH₄Cl are also seen in the presence of oleate. Oleate in itself causes almost a tripling of the β -oxidation, a 30% increase in tricarboxylic acid-cycle flux and a complete inhibition of pyruvate dehydrogenase. The stimulation of flux through pyruvate dehydrogenase by NH₄Cl is in accordance with the activation of the conversion of pyruvate dehydrogenase into the active form by NH₄Cl (Walajtys-Rode *et al.*, 1977).

A nitrogen balance based on the data of Table 1 showed (per 100mg dry wt.) the incorporation of 15–20 μ g-atoms of nitrogen in urea, glutamate and alanine from endogenous sources, in either the presence or the absence of lactate. Addition of NH₄Cl largely suppressed the catabolism of endogenous protein.

Effects of norvaline. Norvaline increases gluconeogenesis and lactate uptake by 45 and 42% respectively, and did not affect ketone-body production, urea synthesis and amino acid formation from endogenous sources (i.e. in the absence of added NH₄Cl), oxygen uptake or the cytosolic or the mitochondrial [NADH]/[NAD⁺] ratio (Table 1). An increase in gluconeogenesis by norvaline is obtained even at norvaline concentrations of 0.5mM (results not shown).

Norvaline is an inhibitor of ornithine transcarbamoylase (Marshall & Cohen, 1972) and apparently is metabolized very slowly, as shown by the lack of accumulation of NH₄⁺ and amino acids in incubations with lactate plus norvaline (Table 1). Norvaline in the presence of lactate has no effect on the tricarboxylic acid-cycle flux, decreases flux through the β -oxidation pathway by 58%, and more than doubles the flux through pyruvate dehydrogenase, from 13 to 28 μ mol/h per 100mg dry wt.

Table 2. *Flux through the tricarboxylic acid cycle, pyruvate dehydrogenase and the β -oxidation*

Fluxes were calculated from the average values of Table 1 and as described in the text. Fluxes are $\mu\text{mol/h}$ per 100mg dry wt. and oxygen values are $\mu\text{g-atoms/h}$ per 100mg dry wt.

	Endo- genous	Lactate	Lactate + NH_4Cl	Lactate+ norvaline	Lactate+ NH_4Cl + norvaline	Lactate+ oleate	Lactate+ NH_4Cl + oleate
1. Observed O uptake	157	218	277	229	269	312	268
2. O for ketone bodies	42	6	8	5	10	45	40
3. O for lactate to CO_2	—	76	256	166	310	-2	88
4. O for fatty acids to CO_2	114	136	13	58	<0	268	140
5. O for tricarboxylic acid cycle	80	146	180	151	155	186	156
6. Tricarboxylic acid-cycle flux	20	36	45	38	39	47	39
7. Pyruvate dehydrogenase flux	0	13	43	28	52	0	15
8. β -Oxidation flux, C_2 units	40	24	6	10	7	71	45

Table 3. *ATP balance of isolated hepatocytes*

The calculations were carried out as described in the text and by using the values in Table 1. Results are μmol of ATP/h per 100mg dry wt.

Substrates	...	Endogenous	Lactate	Lactate+ norvaline	Lactate+ NH_4Cl	Lactate+ NH_4Cl + norvaline
1. Total ATP formation		470	653	688	830	807
2. ATP for glucose		30	180	260	227	242
3. ATP for urea		25	23	17	161	26
4. ATP not accounted for		415	450	411	442	539
5. % of ATP used for gluconeogenesis and urea formation		12	31	40	47	33

Effects of NH_4Cl plus norvaline. Norvaline and NH_4Cl have qualitatively similar effects on gluconeogenesis and lactate uptake, and no additive effects of the two compounds occur (Table 1). Norvaline suppressed urea formation from added NH_4Cl by about 85%, and the combination of NH_4Cl and norvaline causes a 24% rise in oxygen uptake in spite of the suppression of urea synthesis (Table 1). It appears, however, that the two compounds act differently, as norvaline has no effect on gluconeogenesis from pyruvate, whereas NH_4Cl inhibits gluconeogenesis from pyruvate by 35–40% (N. Grunnet & J. Katz, unpublished work; Zahlten *et al.*, 1974). Norvaline has no effect on the cytosolic or the mitochondrial $[\text{NADH}]/[\text{NAD}^+]$ ratio (Table 1), and whether norvaline or a metabolite of norvaline is the effector remains an open question. Interestingly, lysine, which is structurally similar to norvaline, also increases gluconeogenesis from lactate (Cornell *et al.*, 1974).

ATP utilization. The ATP consumption for gluconeogenesis and urea formation was calculated by assuming the expenditure of 6 and 4 mol of ATP/mol of glucose or urea formed, respectively, and the ATP consumption for these pathways was compared with the total ATP formation as calculated from the oxygen uptake and a P/O ratio of 3 (Table 3). From 12 to nearly 50% of the ATP production is used for formation of glucose and urea. The energy utilization

for basal metabolism appears constant at 400–450 μmol of ATP/h per 100mg dry wt., except when urea synthesis from lactate+ NH_4Cl is blocked by norvaline (Table 3). Thus when urea synthesis occurs, NH_4Cl has no uncoupling effect, in accordance with the observations of Häussinger *et al.* (1975), but contrary to the results obtained by Hems *et al.* (1966). In the presence of NH_4Cl there is an increased formation of ATP required for urea synthesis (Table 3). When urea synthesis is blocked by norvaline, dissipation of the excess ATP apparently takes place. Thus the ATP formation does not seem to be rate-limiting for gluconeogenesis, in accordance with the conclusion of Krebs *et al.* (1976b).

Isotopic data

The distribution of radioactivity between metabolites after 1 h of incubation with L-lactate, labelled uniformly or in position 1 or 2, is shown in Table 4. Recovery was 96–100%. The residual radioactivity in lactate at the end of incubation agreed within a few per cent with the residual lactate as determined by enzymic analysis. The specific radioactivity of lactate at the end of incubation was 80–95% of the initial value.

NH_4Cl and norvaline caused a marked increase in the lactate uptake and a 50–100% increase in ^{14}C yield in glucose and CO_2 (Table 4). NH_4Cl , as

Table 4. *Distribution of radioactivity from labelled lactate*

The distribution of radioactivity in various metabolite fractions is shown as percentage of the added radioactivity. The metabolite fractions were obtained by ion-exchange chromatography as described in the Methods section. Lactate, NH_4Cl , norvaline and oleate were added to concentrations of 10, 10, 5 and 1 mM + 1% albumin respectively. For the same substrate supplement the number of experiments varies with the position of the label, which accounts for the apparent discrepancy in remaining lactate between the three positions of label. Results are average values of at least three experiments, except when otherwise indicated. Incubation time was 1 h.

Substrates	Position of lactate label	Percentage of label in:					
		Glucose	CO_2	Amino acids	Organic anions	Lactate	Total
Lactate (10mM)	U-	23	14	3	6	54	100
	1-	11	34	2	5	46	98
	2-	22	9	5	6	55	96
Lactate (10mM) + NH_4Cl (10mM)	U-	36	27	10	4	20	97
	1-	16	62	7	2	11	98
	2-	44	22	12	4	17	98
Lactate (10mM) + norvaline (5mM)	1-	16	48	2	3	32	99
	2-	44	12	5	5	35	100
Lactate (10mM) + NH_4Cl (10mM) + norvaline (5mM)	1-*	19	61	4	3	14	99
	2-*	48	19	12	5	16	100
Lactate (10mM) + oleate (1mM)	1-	15	32	2	5	43	97
	2-†	34	4	4	7	51	100

* Two experiments.

† One experiment.

Table 5. *Radioisotope distribution in C-4, C-5 and C-6 of glucose from $[2\text{-}^{14}\text{C}]\text{lactate}$ or $[2\text{-}^{14}\text{C}]\text{propionate}$*

The neutral fraction from the ion-exchange chromatography was degraded as described in the Methods section. The specific radioactivity of glucose carbon atoms is expressed relative to that of C-5. In all experiments 10mM-lactate was present, and 1–2 μCi (<30 nmol) of the radioisotopes was added at zero time. Results are averages and ranges of one to five experiments.

Label	Additions	Relative specific radioactivity of glucose		
		C-4	C-5	C-6
$[2\text{-}^{14}\text{C}]\text{Lactate}$	None	20 (17–20)	100	75 (72–80)
	NH_4Cl (3–13mM)	26 (23–30)	100	80 (79–81)
	Norvaline (5mM)	16	100	81
	NH_4Cl (3–13mM) + norvaline (5mM)	22 (20–24)	100	80 (77–84)
$[2\text{-}^{14}\text{C}]\text{Propionate}$	None	17	100	97
	NH_4Cl (10mM)	13	100	102
	Oleate (1mM)	12	100	97
	NH_4Cl (10mM) + oleate (1mM)	10	100	98

expected, more than doubled the radioactivity incorporated into amino acids. The only amino acids that were labelled to any appreciable extent were alanine, glutamate and glutamine, as revealed by two-dimensional paper chromatography of the HClO_4 extract (results not shown). The specific radioactivity of C-4 and C-6 of glucose relative to that of C-5 was about 0.2 and 0.75 respectively, after incubation with L- $[2\text{-}^{14}\text{C}]\text{lactate}$, and NH_4Cl in-

creased the relative radioactivity of both carbon atoms (Table 5).

In some experiments cells were incubated with unlabelled L-lactate and either a tracer dose (<30 nmol/3 ml) or substrate concentrations (1–10mM) of $[1\text{-}^{14}\text{C}]\text{propionate}$ or $[1,4\text{-}^{14}\text{C}]\text{succinate}$. When a tracer dose of these labelled compounds was used, less than 2% of the initial radioactivity remained in the labelled substrate after 15 min of incubation. This

was true also after 1 h of incubation when propionate was added to a concentration of 1 mM. The distribution pattern of ^{14}C after 60 min of incubation with a tracer dose of radioactive substrate did not differ much from that observed after 15 min of incubation. The radioactivity in the glucose, lactate and CO_2 in experiments with labelled propionate or succinate and in the presence of various substrates is shown in Table 6. The results of glucose degradations with $[2\text{-}^{14}\text{C}]\text{propionate}$ as the labelled substrate are shown in Table 5.

$[1,4\text{-}^{14}\text{C}]\text{Succinate}$ gave essentially the same results as $[1\text{-}^{14}\text{C}]\text{propionate}$ (Table 6). Mapes & Harris (1975) proposed that succinate was metabolized exclusively by damaged cells. We have confirmed their results with respect to increased oxygen uptake and malate accumulation after succinate addition, but the incorporation into glucose of 20% of the radioactivity from $[1,4\text{-}^{14}\text{C}]\text{succinate}$ indicates that a considerable fraction of the succinate could be metabolized by intact cells, in keeping with recent results on Ehrlich ascites-tumour cells (Spencer, 1976).

Glucose specific radioactivity. Labelled lactate, pyruvate or alanine are commonly used to estimate the rate of gluconeogenesis. The glucose formation is calculated from the ^{14}C yield in glucose and the specific radioactivity of the substrate, sometimes taking into account the loss of radioactivity in the decarboxylation of oxaloacetate. As shown below, such calculations may underestimate gluconeogenesis by up to 40%.

The glucose specific radioactivity at the end of the 60 min incubation period is shown in Table 7. If half of the radioactivity in the carboxyl group of lactate is lost in the phosphoenolpyruvate carboxykinase

reaction, and if oxaloacetate is not diluted in the tricarboxylic acid cycle, the glucose specific radioactivity should be equal to that of lactate with $[1\text{-}^{14}\text{C}]\text{lactate}$, twice that of lactate with $[2\text{-}^{14}\text{C}]\text{lactate}$ and 1.67 times that of lactate with $[\text{U-}^{14}\text{C}]\text{lactate}$. With lactate as the only substrate, the measured glucose specific radioactivity is well below these values (Table 7), and the rate of gluconeogenesis may be underestimated by as much as 40%, if dilution of oxaloacetate in the tricarboxylic acid cycle is neglected. Thus the error in the rate of gluconeogenesis as measured by incorporation of label into glucose can be considerable and depends on the experimental conditions and also on the position of the label in the substrate.

The glucose/lactate specific-radioactivity ratio should be twice as great for $2\text{-}^{14}\text{C}$ -labelling as for $1\text{-}^{14}\text{C}$ -labelling and 1.67 times as great for $\text{U-}^{14}\text{C}$ -labelling as for $1\text{-}^{14}\text{C}$ -labelling (cf. above). The observed ratios are 2.3 and 1.9 respectively (Table 7). This deviation can be explained by a less than complete isotopic equilibration via fumarate of C-1 and C-4 of oxaloacetate, as more than 50% of the ^{14}C incorporated into oxaloacetate from $[1\text{-}^{14}\text{C}]\text{lactate}$ will be lost in the reaction catalysed by phosphoenolpyruvate carboxykinase, whereas the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{-}$ or $[3\text{-}^{14}\text{C}]\text{-lactate}$ into glucose will be less dependent on the degree of randomization via fumarate of oxaloacetate. The ratios of 2.3 and 1.9 are compatible with a degree of equilibration of the oxaloacetate carbons of about 0.85, in accordance with the value of about 0.8 found for degradation of glucose (Table 5). The incomplete equilibration of oxaloacetate is in accordance with results of Tischler *et al.* (1977), which show that both cytosolic and mitochondrial malate dehydrogenase

Table 6. *Radioactivity in glucose, lactate and CO_2 from labelled propionate or succinate*

Cells were incubated for 1 h with 10 mM L-lactate and labelled propionate or succinate: $1\text{--}2\text{ }\mu\text{Ci}$ ($<30\text{ nmol}$) of the labelled compounds was added after 10 min incubation with the substrates. The ^{14}C yield is the percentage of added ^{14}C . Results are mean and range and n is the number of cell preparations.

Label	Additions (mM)	^{14}C Yield in			n
		Glucose	Lactate	CO_2	
$[1\text{-}^{14}\text{C}]\text{Propionate}$	None	22 (15–26)	2.2 (1.7–3.3)	67 (60–71)	8
	NH_4Cl (10)	20 (11–26)	4.0 (2.5–5.2)	63 (55–71)	8
	Norvaline (5)	27	2.1	66	1
	Oleate (1)	22	2.2	63	1
	Propionate (0.1)	26	—	70	1
	Propionate (0.5)	28	—	70	1
	Propionate (1)	29 (28–29)	—	66 (65–68)	2
$[2\text{-}^{14}\text{C}]\text{Propionate}$	None	71 (69–72)	4.6 (4.3–4.9)	14 (12–17)	2
	NH_4Cl (10)	62 (61–62)	9.8 (7.5–12.0)	13 (12–15)	2
	Oleate (1)	79 (77–81)	4.2 (4.0–4.4)	8.8 (8.0–9.5)	2
	Oleate (1) + NH_4Cl (10)	56	17	8.4	1
$[1,4\text{-}^{14}\text{C}]\text{Succinate}$	None	19 (16–22)	—	67 (64–71)	2

Table 7. *Glucose specific radioactivity*

The specific radioactivity of glucose was calculated from radioactivity (c.p.m.) in the neutral fraction from ion-exchange chromatography (see the Methods section) and from the glucose formation, and is shown relative to the mean (average of initial and final) lactate specific radioactivity. Lactate, NH_4Cl , norvaline and oleate were added to concentrations of 10 mM, 10 mM, 5 mM and 1 mM respectively. Results are mean and range: *n* indicates the number of cell preparations. Incubation time was 1 h.

Substrates	Lactate label	Glucose specific radioactivity	
		Lactate specific radioactivity	<i>n</i>
Lactate	U-	1.25 (1.19–1.37)	5
	1-	0.63 (0.54–0.77)	8
	2-	1.52 (1.50–1.59)	5
Lactate + NH_4Cl	U-	1.54 (1.47–1.62)	5
	1-	0.85 (0.63–0.99)	8
	2-	1.90 (1.68–2.24)	6
Lactate + norvaline	1-	0.71 (0.63–0.77)	3
	2-	1.66 (1.52–1.80)	2
Lactate + NH_4Cl + norvaline	U-	1.57	1
	1-	0.92 (0.88–0.96)	2
	2-	2.04 (1.84–2.34)	3
Lactate + oleate	1-	0.67 (0.66–0.68)	2
Lactate + NH_4Cl + oleate	1-	0.80 (0.75–0.85)	2

are displaced from equilibrium in isolated hepatocytes. Müllhofer *et al.* (1977a,b) found complete equilibration of oxaloacetate in experiments with both perfused rat liver and isolated rat hepatocytes. The reason for this discrepancy is not clear.

Endogenous gluconeogenesis. The present results indicate a partial inhibition of gluconeogenesis from endogenous sources by added lactate. This is most easily seen by comparing the glucose specific radioactivity as measured in incubations with $[1-^{14}\text{C}]$ -lactate (Table 7) with the glucose specific radioactivity calculated by assuming that endogenous gluconeogenesis is either fully inhibited or unaffected by lactate addition: with endogenous gluconeogenesis fully inhibited and $[1-^{14}\text{C}]$ lactate as the label, the specific radioactivity of glucose should equal that of lactate (see above) multiplied by the dilution of oxaloacetate in the tricarboxylic acid cycle. This dilution is (flux to phosphoenolpyruvate)/(flux to phosphoenolpyruvate + tricarboxylic acid-cycle flux). With a tricarboxylic acid cycle and pyruvate kinase flux of about 0.5 (Table 1) and 0.1 (see below) of the flux to glucose respectively, the calculated glucose specific radioactivity is 0.69 of that of lactate. If endogenous gluconeogenesis was unaffected by lactate addition, the calculated relative specific radioactivity of glucose becomes 0.58, as endogenous glucose production is about 15% of that with lactate (Table 1). The measured glucose specific radioactivity of 0.63 (Table 7) is intermediate to these calculated values, thus indicating a partial inhibition of endogenous gluconeogenesis by lactate.

When NH_4Cl is added in the presence of lactate, the specific radioactivity of glucose is 0.85 of that of lactate with $[1-^{14}\text{C}]$ lactate as the label (Table 7). The

glucose specific radioactivity, calculated as above and assuming that half of the phosphoenolpyruvate is recycled to pyruvate (see below), is 0.75 if the endogenous gluconeogenesis was fully inhibited, and even lower if endogenous gluconeogenesis took place. These results thus suggest a complete inhibition of endogenous gluconeogenesis in the presence of NH_4Cl . This conclusion is supported by the nitrogen-balance data discussed above.

Our results thus provide evidence for a partial inhibition of endogenous gluconeogenesis after lactate addition, in accordance with the view of Cornell *et al.* (1974), but contrary to the suggestions of Exton *et al.* (1972).

Pyruvate recycling. Recycling of pyruvate through the reactions of the futile cycle pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate will give rise to formation of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ lactate without any change in net metabolism, and therefore to a larger production of $^{14}\text{CO}_2$ than expected from the metabolism of lactate. Pyruvate recycling has been observed in the perfused rat liver and in isolated hepatocytes (Friedman *et al.*, 1971; Rognstad, 1975; Rognstad & Katz, 1977), whereas Müllhofer *et al.* (1977a,b) found no evidence for this pathway.

In the present experiments (Table 6) pyruvate recycling was estimated from the incorporation of radioactivity from $[1-^{14}\text{C}]$ propionate into lactate, similar to the use of $^{14}\text{CO}_2$ by Rognstad (1975). The specific radioactivity of phosphoenolpyruvate is taken to be half that of glucose, and the radioactivity entering pyruvate from phosphoenolpyruvate is assumed to be trapped in the large lactate pool present. The flux through pyruvate kinase is multiplied by 1.5 to correct for incomplete trapping of

radioactivity in the lactate pool (Rognstad, 1975). Pyruvate kinase flux may be overestimated by this method owing to incorporation of ^{14}C from $[1-^{14}\text{C}]$ -propionate into lactate via the reaction catalysed by 'malic' enzyme or pyruvate carboxylase. In all probability, the error in the estimation of the pyruvate kinase flux in hepatocytes from starved rats is, however, small, as long as the rate of gluconeogenesis is much larger than the rate of endogenous glucose formation (Rognstad, 1976). The pyruvate kinase flux was calculated to be 0.16 (range 0.09–0.36, $n = 10$) and 0.37 (range 0.18–0.75, $n = 10$) of the phosphoenolpyruvate flux to glucose with lactate and lactate + NH_4Cl respectively as the substrates. Norvaline or oleate did not affect pyruvate recycling. The values with lactate as the substrate for pyruvate recycling are similar to those reported by Rognstad & Katz (1977). Thus 10–30% of the phosphoenolpyruvate is recycled to pyruvate with lactate as the sole substrate and NH_4Cl more than doubles the rate of pyruvate recycling also in the presence of oleate.

Stimulation of gluconeogenesis by, e.g., cyclic AMP may be caused partially by inhibition of the recycling of pyruvate (Rognstad & Katz, 1977). On the contrary, NH_4Cl stimulates gluconeogenesis, but nevertheless also stimulates the recycling of pyruvate.

Conclusion

The radioisotope data obtained in this work show that the description of the metabolism of lactate (or pyruvate or alanine) by the liver is complicated by the existence of pyruvate recycling, various degrees of inhibition of endogenous gluconeogenesis and lack of equilibration of oxaloacetate via fumarate. Thus the importance of using more than one type of label for a given substrate and different types of labelled compounds in the study of metabolic pathways is evident, and in practice the use of isotopes may often be in checking metabolic models and not in measuring rates.

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