The Effect of Lysine on Gluconeogenesis from Lactate in Rat Hepatocytes

By NEAL W. CORNELL, PATRICIA LUND and HANS A. KREBS
*Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.*

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1. In freshly prepared isolated rat liver cells there is a lag in gluconeogenesis from lactate. The magnitude of the lag increases with increasing lactate concentration. 2. The lag is virtually abolished by lysine. 3. A few other amino acids (tyrosine, arginine, asparagine, ornithine) and NH_4Cl had effects similar to, but less pronounced than, lysine during the early stage of incubation. Lysine was unique in accelerating gluconeogenesis beyond the lag period. 4. The effects of the accelerators are not additive. 5. Glycine, serine, threonine, cysteine, tryptophan and histidine at 2 mM markedly inhibit (>20%) gluconeogenesis from lactate. 6. Oleate, which promotes gluconeogenesis from lactate by supplying acetyl-CoA required for the pyruvate carboxylase reaction, had no effect on the lag, yet oleate oxidation showed no lag. 7. Preincubation of cells decreased the lag and decreased the magnitude of the lysine effect. 8. Pyruvate (added at 1 mM to give an initial [lactate]/[pyruvate] ratio of 10) also abolished the lag and decreased the lysine effect by about 50%. 9. Lysine reversed the inhibition by ethanol of gluconeogenesis from lactate. 10. All accelerators increased the rate of re-oxidation of cytosolic NADH as shown by a rapid re-adjustment of the [lactate]/[pyruvate] ratio on addition of 10 mM-lactate. 11. The accelerated rates of gluconeogenesis are associated with an increased formation of aspartate and glutamate and especially alanine. 12. The existence of the lag period can be explained on the basis of the fact that the accumulation of pyruvate during the lag diverts oxaloacetate from gluconeogenesis to malate formation, i.e. that the re-oxidation of cytosolic NADH takes precedence over gluconeogenesis. This means that much oxaloacetate formed by the pyruvate carboxylase reaction has to be transferred twice from the mitochondria to the cytosol by the aspartate shuttle. Under these conditions the operation of the shuttle limits the rate of gluconeogenesis from lactate. Lysine and other accelerators may increase the effectiveness of the shuttle by providing components of the aspartate aminotransferases involved. The question of why lysine specifically accelerates gluconeogenesis beyond the lag period is discussed.

We recently reported (Cornell *et al.*, 1973) that lysine accelerates glucose formation from lactate in isolated rat liver cells. The effect of lysine proved to be of a catalytic nature inasmuch as the amount of extra glucose formed and extra lactate used was much greater than the amount of lysine metabolized. The present paper is concerned with an analysis of the effects of lysine and of other nitrogenous substances on glucose formation from lactate.

Materials and Methods

Rats

Female Wistar rats (180–220 g) were obtained from Charles River U.K. Ltd., Manston Road, Margate, Kent, U.K., and were fed on Oxoid pasteurized breeding diet for rats and mice (Oxoid Ltd., London

S.E.1, U.K.). Animals were starved for 48 h before use.

Preparation of isolated liver cells

Cells were prepared essentially as described by Berry & Friend (1969) with the modifications described by Cornell *et al.* (1973) and Krebs *et al.* (1974). In the experiments involving preincubation, glucose synthesis was initiated by the addition of substrates at 40 min and the flasks were re-gassed with $\text{O}_2 + \text{CO}_2$ (95:5).

The number of observations given in the Tables for any given experimental condition refers to results obtained from separate batches of cells.

Reagents

Urease tablets (type 111-20) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6,

U.K. L-Lysine decarboxylase was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Other purified enzymes and coenzymes were products of Boehringer Corp. (London) Ltd., London W.5, U.K. Other chemicals were reagent grade.

Determination of metabolites

Glucose was determined by the method of Slein (1963), lactate and pyruvate by those of Hohorst *et al.* (1959), 3-hydroxybutyrate and acetoacetate by those of Williamson *et al.* (1962), aspartate by that of Pfeleiderer (1963), glutamate and alanine successively in the same cuvette by a modification of the method of Bernt & Bergmeyer (1963). The cuvettes contained, in a final volume of 2ml, 1ml of Tris-hydrazine buffer, pH9 [100mm-Tris-5% (w/v) hydrazine hydrate-2mm-EDTA], 0.1ml of 2% NAD⁺, 1 μ mol of ADP, sample and water. The glutamate assay was started by addition of 20 μ l of glutamate dehydrogenase in glycerol. On completion, 0.01ml of alanine dehydrogenase was added. The advantage of using pH9 for the determination of alanine rather than pH10 as previously published (Williamson *et al.*, 1967b) is that the extinction change of the 'reagent blank' is much smaller.

NH₃ and urea were determined successively in the same cuvette in the assay system described by Bernt & Bergmeyer (1970) for urea. Asparagine was also determined as NH₃ by addition of asparaginase to the cuvette after prior removal of free NH₃ by reaction with glutamate dehydrogenase in glycerol. L-Lysine was determined manometrically as CO₂ with lysine decarboxylase (Gale, 1970). Saccharopine and α -amino adipate were determined by using a Jeol amino acid analyser. The saccharopine used as a marker was a gift from Dr. J. Hutzler, New York University School of Medicine.

Results

Time-course of the acceleration by lysine of gluconeogenesis from lactate

When freshly prepared cells were incubated with 10mm-lactate the rate of glucose synthesis was not

constant (Table 1). It was very low initially and reached a constant rate after about 40min. Lysine was most effective during the early stage of the incubation, the mean increase caused by lysine during the 0-20min period being about 150%. Lysine virtually abolished the lag in the onset of gluconeogenesis from 10mm-lactate. Oleate (1mm) which is known to stimulate gluconeogenesis from lactate (Struck *et al.*, 1965) was without effect during the 0-20min period but accelerated it after 20min. Lysine accelerated the rate under all conditions even when it was already increased by oleate.

Effect of lactate concentration on the lag

In the experiments of Table 1 the lactate concentration was 10mm. As shown in Table 2 the extent of lag depends on the lactate concentration; it becomes more pronounced with increasing lactate concentrations. The main effect of lysine at different lactate concentrations was again the abolition of the lag. Hence in the presence of lysine the rate of gluconeogenesis tends to become independent of the initial lactate concentration as long as this is above 2mm.

There was no lag period in the synthesis of glucose over a wide range of pyruvate concentrations, and lysine had no effect on gluconeogenesis from pyruvate.

Stimulation of gluconeogenesis from lactate by amino acids other than lysine

In the experiments of Cornell *et al.* (1973) lysine was the only amino acid that increased to a major extent the rate of gluconeogenesis from lactate. A minor increase was found with ornithine. These results referred to an incubation period of 60min. When it had been established that the main effect of lysine occurred during the early stages of incubation (Table 1) the effect of other amino acids was tested for the 0-20min period. Whereas the calculation of the percentage effect of lysine on gluconeogenesis from lactate was straightforward (see Table 2) because

Table 1. *Time-course of gluconeogenesis from lactate in isolated hepatocytes under various conditions*

Incubation conditions were as described in the Materials and Methods section. The initial concentration of lactate was 10mm, of lysine 2mm and of oleate 1mm. Means $\pm$ S.E.M. are given with the numbers of observations in parentheses.

Period of incubation (min)	Rate of glucose formation (μ mol/min per g wet wt. of cells)				
	No additions	Lactate	Lactate lysine	Lactate oleate	Lactate oleate lysine
0-20	0.104 $\pm$ 0.013 (11)	0.34 $\pm$ 0.05 (9)	1.05 $\pm$ 0.11 (9)	0.33 $\pm$ 0.07 (6)	0.98 $\pm$ 0.10 (6)
20-40	0.076 $\pm$ 0.011 (11)	0.66 $\pm$ 0.08 (9)	1.36 $\pm$ 0.09 (9)	1.35 $\pm$ 0.16 (6)	2.20 $\pm$ 0.11 (6)
40-60	0.105 $\pm$ 0.004 (11)	1.05 $\pm$ 0.09 (9)	1.25 $\pm$ 0.09 (9)	1.64 $\pm$ 0.11 (6)	1.88 $\pm$ 0.13 (6)
0-60	0.094 $\pm$ 0.001 (11)	0.65 $\pm$ 0.06 (9)	1.18 $\pm$ 0.08 (9)	1.11 $\pm$ 0.10 (6)	1.65 $\pm$ 0.12 (6)

lysine alone gave no glucose, the basis of the calculations becomes uncertain when glucogenic amino acids are involved. It is not known to what extent these amino acids inhibit endogenous glucose formation. When lactate is the glucose precursor, lactate removal is almost quantitatively accounted for by pyruvate and glucose formation which indicates

Table 2. *Dependence of the lysine effect on lactate concentration*

The incubation period was 20 min. For the general conditions see the Materials and Methods section.

Lactate concentration (mM)	Rate of glucose formation [$\mu\text{mol/min}$ per g wet wt. of cells, means $\pm$ S.E.M. (3)]		
	No lysine added	2 mM-Lysine	Increase by lysine (%)
0	0.15 $\pm$ 0.02	0.13 $\pm$ 0.01	—
1	0.71 $\pm$ 0.04	0.93 $\pm$ 0.02	32 $\pm$ 5
2	0.69 $\pm$ 0.04	1.17 $\pm$ 0.08	68 $\pm$ 2
5	0.60 $\pm$ 0.05	1.31 $\pm$ 0.12	119 $\pm$ 6
10	0.46 $\pm$ 0.04	1.25 $\pm$ 0.15	169 $\pm$ 11
20	0.34 $\pm$ 0.04	1.07 $\pm$ 0.15	211 $\pm$ 22

that lactate suppresses endogenous glucose formation and that no correction is necessary. Whether added amino acids suppress endogenous glucose formation (which is mainly from amino acids) is uncertain. The effects of amino acids have therefore been calculated in two ways in Table 3. 'Uncorrected' assumes complete suppression of endogenous glucose synthesis, and 'corrected' is calculated on the same basis as the data in Table 2, i.e. the rate of glucose synthesis from amino acid alone is corrected for the endogenous rate. The overall picture is the same whichever method is used; tyrosine, ornithine, arginine and asparagine are powerful accelerators though less effective than lysine. Alanine, phenylalanine and proline fall into a group having small stimulatory effects and a further seven amino acids have no significant effect (citrulline, aspartate, glutamate, glutamine, leucine, isoleucine, valine). At the other end of the range is a group, given in decreasing order of effectiveness, which are inhibitory (histidine, cysteine, threonine, serine, tryptophan, glycine and methionine). It is already known that tryptophan (Mirsky *et al.*, 1957; Veneziale *et al.*, 1967) and histidine (Cornell *et al.*, 1973) can be inhibitory. The mechanism of the inhibition by histidine will be the subject of a subsequent paper.

Table 3. *Effects of amino acids on gluconeogenesis from lactate in isolated liver cells*

The initial concentrations were for lactate, 10 mM and amino acid 2 mM. Incubation was for 20 min at 37°C. Means $\pm$ S.E.M. are given when there were three or more experiments; means only are given for two experiments. For calculations of the percentage effects of amino acids see the text.

Amino acid	Number of observations	Rate of glucose formation ($\mu\text{mol/min}$ per g wet wt. of cells)				Effect of amino acid on rate (uncorr.) (%)	Effect of amino acid on rate (corr.) (%)
		No substrate	Lactate	Amino acid	Lactate + amino acid		
L-Lys	(8)	0.15 $\pm$ 0.006	0.56 $\pm$ 0.047	0.15 $\pm$ 0.007	1.40 $\pm$ 0.09	+97	+150
L-Tyr	(4)	0.18 $\pm$ 0.01	0.48 $\pm$ 0.06	0.39 $\pm$ 0.05	1.42 $\pm$ 0.05	+63	+106
L-Orn	(3)	0.14 $\pm$ 0.016	0.50 $\pm$ 0.03	0.16 $\pm$ 0.017	1.04 $\pm$ 0.056	+58	+100
L-Arg	(2)	0.15	0.57	0.18	1.16	+55	+93
L-Asn	(4)	0.16 $\pm$ 0.004	0.44 $\pm$ 0.056	0.53 $\pm$ 0.032	1.47 $\pm$ 0.138	+52	+81
L-Ala	(3)	0.18 $\pm$ 0.003	0.61 $\pm$ 0.085	0.56 $\pm$ 0.045	1.35 $\pm$ 0.10	+15	+36
L-Phe	(2)	0.18	0.69	0.21	1.01	+12	+40
L-Pro	(3)	0.18 $\pm$ 0.003	0.61 $\pm$ 0.085	0.30 $\pm$ 0.02	1.02 $\pm$ 0.12	+12	+40
L-Citrulline	(2)	0.15	0.53	0.16	0.61	-12	+13
L-Asp	(2)	0.17	0.51	0.21	0.62	-14	+13
L-Glu	(2)	0.17	0.55	0.20	0.61	-19	+5
L-Gln	(2)	0.18	0.65	0.23	0.70	-20	0
L-Leu	(2)	0.15	0.53	0.14	0.51	-24	-2
L-Ile	(2)	0.15	0.53	0.14	0.49	-27	-6
L-Val	(2)	0.15	0.53	0.15	0.50	-27	-6
L-Met	(2)	0.18	0.69	0.20	0.61	-32	-14
Gly	(2)	0.18	0.69	0.19	0.54	-39	-23
L-Trp	(2)	0.17	0.52	0.19	0.42	-41	-22
L-Ser	(2)	0.18	0.55	0.38	0.44	-43	-41
L-Thr	(3)	0.18 $\pm$ 0.003	0.61 $\pm$ 0.085	0.27 $\pm$ 0.015	0.43 $\pm$ 0.058	-51	-39
L-Cys	(2)	0.18	0.51	0.18	0.33	-52	-35
L-His	(2)	0.17	0.52	0.22	0.35	-53	-39

Time-course of the effect of asparagine on gluconeogenesis from lactate

Although the rate of glucose formation from asparagine was constant during the first hour of incubation (Table 4) the accelerating effect of asparagine on gluconeogenesis from lactate shown in Table 3 disappeared after 20 min. During the second 20 min period the rate with lactate plus asparagine was as expected on the assumption that the rates from the two substrates are additive but during the third 20 min period the rate decreased. The initial acceleration and the later inhibition almost balance so that the rate for the 60 min period was virtually the same as that expected for additive effects. Very similar results were obtained when asparagine was replaced by tyrosine, whereas phenylalanine had a slight effect only (see Table 3). Glutamine, in contrast with asparagine, did not stimulate gluconeogenesis from lactate at any time.

Fate of lysine

The amounts of lysine removed by the isolated liver cells were low in relation to the amounts of glucose formed from lactate, and the increase in lysine removal in the presence of lactate was not significant (Table 5).

Table 4. *Time-course of the effects of asparagine on gluconeogenesis from lactate*

The initial concentration of lactate was 10 mM and of asparagine 2.5 mM. The rates are means $\pm$ S.E.M. for four observations.

Period of incubation (min)	Rate of glucose formation (μ mol/min per g wet wt. of cells) in the presence of			Rate expected for additive effects
	Lactate	Asparagine	Lactate + asparagine	
0-20	0.42 $\pm$ 0.08	0.54 $\pm$ 0.02	1.46 $\pm$ 0.14	0.96
20-40	0.76 $\pm$ 0.20	0.58 $\pm$ 0.06	1.26 $\pm$ 0.13	1.34
40-60	1.04 $\pm$ 0.16	0.54 $\pm$ 0.08	0.99 $\pm$ 0.15	1.58
0-60	0.74 $\pm$ 0.14	0.55 $\pm$ 0.05	1.24 $\pm$ 0.11	1.29

This bears out the earlier conclusion (Cornell *et al.*, 1973) that the effect of lysine on gluconeogenesis from lactate is of a catalytic nature. Addition of lysine caused an increased formation of glutamate, alanine, NH_3 and urea. In the presence of lactate the increase due to addition of lysine after 60 min was 1.45 μ mol of N compared with a decrease of lysine of 1.53 μ mol. Thus only half of the lysine removed was accounted for by the products measured. The deficit was still greater when lysine only was added. There were no increases in the amounts of aspartate and glutamate, and no saccharopine was detectable when the concentration of added lysine was 2 mM. There was a small amount of α -amino adipate (Table 6) but this contributed very little to the nitrogen balance. Saccharopine became detectable when the lysine concentration was raised to 10 mM provided that pyruvate was also added (Table 6). The presence of lactate substantially increased the yield of glutamate, alanine and α -amino adipate whereas the yield of NH_3 was slightly decreased. According to Table 6 addition of oleate increased the yield of α -amino adipate. Presumably lactate, pyruvate and oleate act by contributing to the synthesis of α -oxoglutarate which is required for the initiation of the saccharopine pathway of lysine degradation.

Products of asparagine metabolism

Within the range tested, i.e. up to 5 mM, the rate of removal of asparagine was directly proportional to the concentration of asparagine. The rate was not affected by the presence of lactate but lactate modified the metabolic products of asparagine in that asparagine nitrogen was diverted from the synthesis of urea to the formation of alanine (Table 7). In the experiment of Table 7 asparagine nitrogen was accounted for throughout the incubation by the formation of alanine, glutamate, aspartate, NH_3 and urea. Asparagine carbon was accounted for by the formation of glucose, lactate, pyruvate, alanine, aspartate and glutamate when asparagine only was added. The sum of these products (uncorrected for the amounts formed in the absence of added asparagine)

Table 5. *Products of lysine metabolism in isolated liver cells*

The values are μ mol for 4 ml of suspension containing 74 mg wet wt. of cells, incubated for 40 min (means $\pm$ S.E.M. with the numbers of observations in parentheses). The initial concentration of lactate was 10 mM and of lysine 2 mM.

Substrates added	Metabolite changes						
	Glutamate	Alanine	NH_3	Urea nitrogen	Total nitrogen	Glucose	Lysine
None (7)	0.100 $\pm$ 0.01	0.038 $\pm$ 0.01	0.085 $\pm$ 0.03	1.08 $\pm$ 0.16	1.30 $\pm$ 0.18	0.377 $\pm$ 0.01	
Lactate (5)	0.241 $\pm$ 0.03	0.245 $\pm$ 0.03	0.063 $\pm$ 0.02	0.921 $\pm$ 0.21	1.47 $\pm$ 0.18	2.19 $\pm$ 0.25	
Lactate + lysine (8)	0.477 $\pm$ 0.07	0.812 $\pm$ 0.07	0.132 $\pm$ 0.02	1.50 $\pm$ 0.17	2.92 $\pm$ 0.22	4.54 $\pm$ 0.35	-1.53 $\pm$ 0.42
Lysine (6)	0.125 $\pm$ 0.02	0.052 $\pm$ 0.01	0.286 $\pm$ 0.10	1.65 $\pm$ 0.30	2.11 $\pm$ 0.41	0.297 $\pm$ 0.01	-1.28 $\pm$ 0.23

was 8.89 μmol for the 60min incubation period. The blank gave 1.14 μmol . Thus the corrected yield was 7.75 i.e. equal to the amount of asparagine removed (7.80 μmol). The main metabolic products of asparagine carbon were glucose and lactate.

The fact that lactate removal was almost trebled during the first 20min period whereas the rate of

asparagine removal was constant indicates that the extra glucose formed on addition of asparagine and lactate is due to gluconeogenesis from lactate. The amounts of lactate removed exceeded that of the extra glucose but the excess corresponded to the formation of pyruvate and alanine.

Effects of NH_4Cl

Between 2.6 and 10mM- NH_4Cl accelerated gluconeogenesis from lactate but rather less than lysine, the increase of the rate being about 35% during a 60min period of incubation (Table 8). The higher NH_4Cl concentrations (5–10mM) abolished the lysine effect and 2.5mM decreased it. NH_4Cl also inhibited lysine removal, as may be expected on the basis of Fellows' (1973) finding that NH_4Cl inhibits lysine oxoglutarate reductase, the first step of lysine degradation.

Other factors affecting the lag in gluconeogenesis from lactate

As shown in Table 9 the lag seen with 10mM-lactate (Table 1) can be virtually abolished by preincubating cells in the absence of substrate for 40min. Addition of lysine after preincubation still increased the rate of gluconeogenesis from lactate, but the percentage increase was smaller (59% in the first 20min) than with non-preincubated cells [169% in the first 20min (Table 2)]. Preincubation with 1mM-oleate was even more effective in decreasing the lag and the lysine effect. This could be connected with the fact (Table 1)

Table 6. *Formation of saccharopine and α -amino adipate from lysine in isolated rat liver cells*

The values are μmol for 4ml of suspension containing 70mg wet wt. of cells incubated for 60min. The amino acids were measured by an amino acid analyser. The initial concentrations of lactate were 10mM, of pyruvate 10mM, of oleate 1mM and of lysine 10mM (unless otherwise stated).

Substrates added	Metabolite changes		
	Saccharo- pine	α -Amino- adipate	Lysine
Lysine	<0.01	0.19	-2.1
Lysine; lactate	<0.01	0.96	-3.8
Lysine; pyruvate	0.07	1.80	-6.1
Lysine; lactate; 1 mM-pyruvate	0.02	0.99	
Lysine; lactate; 1 mM- pyruvate; oleate	0.02	1.40	
2mM-Lysine; lactate; 1 mM-pyruvate; oleate	<0.01	0.34	
2mM-Lysine; lactate	<0.01	0.04	
2mM-Lysine; lactate; oleate	<0.01	0.07	

Table 7. *Metabolites formed from lactate and asparagine*

The initial concentration of lactate was 10mM, and of asparagine 5mM. This is a representative experiment. The sum of nitrogenous products of asparagine given in the last vertical column has been corrected for values found in the corresponding controls without asparagine.

Substrate added	Incubation time (min)	Substrate removal and product accumulation during incubation (μmol for 4ml of cell suspension containing 62mg wet wt. of cells)								Aspara- gine	Sum of products formed (μmol of N)
		Glucose	Lactate	Pyruvate	Alanine	Aspartate	Glutamate	Urea	NH_3		
None	0				0.01		0.07				
	20	0.26			0.01	<0.05	0.08	0.34	0.01		
	40	0.37			0.02	<0.05	0.09	0.56	0.04		
	60	0.48			0.05	<0.05	0.13	0.76	0.13		
Lactate	20	0.69	-2.55	1.30	0.01	0.19	0.13	0.36	<0.01		
	40	1.81	-5.89	2.20	0.19	0.12	0.21	0.50	0.07		
	60	3.47	-11.06	2.82	0.36	0.15	0.29	0.71	0.08		
Asparagine	20	0.64	+0.23	0.28	0.28	0.55	0.64	1.22	2.13	-2.42	2.60
	40	1.38	+0.81	0.43	0.49	0.58	0.58	4.85	3.43	-5.14	6.73
	60	2.30	+1.77	0.69	0.59	0.81	0.43	6.04	3.57	-7.86	7.80
Lactate	20	2.03	-7.3	3.64	1.77	0.79	0.63	1.15	1.76	-2.64	3.10
Asparagine	40	3.69	-11.70	4.01	3.02	0.07	0.73	2.68	3.12	-5.92	5.67
	60	5.40	-13.19	3.21	3.68	0.83	0.87	4.47	4.20	-8.51	8.11

Table 8. *Effect of NH₄Cl on gluconeogenesis from lactate and on lysine removal*

The initial concentration of lactate was 10mM and of lysine 2mM. Incubation was for 60min. Values are means $\pm$ S.E.M. for three experiments.

NH ₄ Cl added (mM)	Glucose synthesis (μ mol/min per g wet wt. of cells)			Lysine removal (μ mol/min per g wet wt. of cells)
	Lactate	Lactate lysine	Increase by lysine (%)	
None	0.87 $\pm$ 0.01	1.42 $\pm$ 0.13	64 $\pm$ 12	0.39 $\pm$ 0.06
0.5	0.76 $\pm$ 0.05	1.33 $\pm$ 0.15	76 $\pm$ 14	0.41 $\pm$ 0.07
1.0	1.01 $\pm$ 0.08	1.42 $\pm$ 0.11	40 $\pm$ 4	0.38 $\pm$ 0.06
2.5	1.17 $\pm$ 0.09	1.36 $\pm$ 0.06	17 $\pm$ 4	0.28 $\pm$ 0.03
5.0	1.18 $\pm$ 0.07	1.27 $\pm$ 0.06	7 $\pm$ 1	0.08 $\pm$ 0.04
10.0	1.13 $\pm$ 0.08	1.15 $\pm$ 0.07	2 $\pm$ 1	0.10 $\pm$ 0.03

Table 9. *Effect of preincubation of liver cells on rates of gluconeogenesis from lactate*

Preincubation means that cells were incubated either without or with 1mM-oleate for 40min before the addition of 10mM-lactate or 10mM-lactate and 2mM-lysine. Rates are means $\pm$ S.E.M. In calculating the percentage increase by lysine the controls were samples of the same cell suspension.

Period of incubation (min)	Preincubation without substrates (four observations)			Preincubation with 1mM-oleate (three observations)		
	Rate of glucose formation (μ mol/min per g wet wt. of cells)			Rate of glucose formation (μ mol/min per g wet wt. of cells)		
	With lactate	With lactate and lysine	Increase by lysine (%)	With lactate	With lactate and lysine	Increase by lysine (%)
0-20	0.90 $\pm$ 0.07	1.44 $\pm$ 0.13	59 $\pm$ 6	1.33 $\pm$ 0.03	1.51 $\pm$ 0.003	14 $\pm$ 3
20-40	1.09 $\pm$ 0.12	1.43 $\pm$ 0.17	31 $\pm$ 7	1.59 $\pm$ 0.05	1.78 $\pm$ 0.06	13 $\pm$ 7
40-60	0.88 $\pm$ 0.10	1.18 $\pm$ 0.15	33 $\pm$ 5	1.61 $\pm$ 0.16	1.87 $\pm$ 0.04	18 $\pm$ 9
0-60	0.96 $\pm$ 0.09	1.35 $\pm$ 0.14	40 $\pm$ 3	1.51 $\pm$ 0.06	1.73 $\pm$ 0.03	15 $\pm$ 4

that the accelerating effect of oleate has a lag of 10-30min. Thus preincubation of the cells for 40min with 1mM-oleate produces its maximum stimulation of gluconeogenesis when the lactate is added. Under these conditions the stimulatory effect of lysine is decreased to about 15%.

It is noteworthy that there is no lag in the ketone-body production from oleate. In a series of experiments the rate of ketone-body production was 89 μ mol/h per g wet wt. during the first 20min and 60 μ mol/h per g wet wt. during the second 20min. This is difficult to reconcile with the view that oleate accelerates gluconeogenesis by producing acetyl-CoA, an allosteric effector of pyruvate carboxylase. Since acetyl-CoA is an intermediate in ketogenesis it must arise promptly on addition of oleate, but there is as yet no information on the steady-state concentration of acetyl-CoA under the conditions of the experiments. As discussed below, oleate does not stimulate during the early stages of incubation because the 'aspartate shuttle' and not pyruvate carboxylase is rate-limiting.

Another factor affecting the lag is the [lactate]/[pyruvate] ratio. When lactate and pyruvate were

Table 10. *Removal of lag period of gluconeogenesis by pyruvate*

The initial concentration of lactate was 10mM, of pyruvate 1mM and of lysine 2mM. Rates given are the averages of two experiments.

Period of incubation (min)	Rate of glucose formation (μ mol/min per g wet wt. of cells)		
	Lactate pyruvate	Lactate pyruvate and lysine	Increase by lysine (%)
0-20	1.01	1.76	74
20-40	1.11	1.57	42

added together at a ratio of 10:1mM there was no lag in gluconeogenesis (Table 10). Under these conditions lysine increased gluconeogenesis by 74% during the first 20min of incubation compared with 169% in the absence of pyruvate (Table 2). During the second 20min period lysine increased the rate by 42%.

Lag in gluconeogenesis from lactate in the perfused liver

Hems *et al.* (1966) in their work on gluconeogenesis from lactate in the perfused rat liver did not notice a lag period. This discrepancy between the performance of the isolated cells and the intact liver is an apparent one. In the perfusion experiments 38 min were allowed to elapse before the addition of lactate, in order to deplete the liver of the residual glycogen still present after 48 h of starvation. Thus the organ was preincubated as a routine. When 10 mM-lactate was present from the start of the perfusion lactate uptake for the three initial successive 15 min periods was near zero, 138 and 251 μmol respectively for 6 g of liver (average of two experiments). During the same intervals when 2 mM-lysine was also present lactate uptake was 173, 275 and 218 μmol respectively (average of two experiments; R. Hems, unpublished work). Glucose release during the first 40 min is generally greater than lactate uptake because of glycogen decomposition. Glucose release therefore is not a reliable measure of gluconeogenesis from lactate during this period in the isolated perfused liver. Isolated liver cells, on the other hand, are depleted of glycogen and glucose during the preparative procedure. The traces remaining are shed during the first 20 min of incubation. Thus the experimental manipulation of the tissue causes disturbances of the

organization of the whole liver and of the isolated cells that can be promptly rectified by lysine; it takes about 40 min incubation in the absence of lysine.

Effect of lysine on gluconeogenesis in the presence of ethanol

In the presence of ethanol lysine trebled the rate of gluconeogenesis from lactate (Table 11) which means that it abolished the well-established inhibition of gluconeogenesis by ethanol (see Krebs *et al.*, 1969). Without lysine the inhibition by ethanol was 45% under the test conditions; in the presence of lysine it was negligible.

Table 11. *Reversal by lysine of the ethanol inhibition of gluconeogenesis from lactate*

Liver cells were incubated for 60 min. The concentration of lactate was 10 mM, of lysine 2 mM, of ethanol 10 mM. The rates are means $\pm$ S.E.M. for five experiments.

Additions	Glucose formation ($\mu\text{mol}/\text{min}$ per g wet wt. of cells)
Lactate	0.78 ± 0.08
Lactate, lysine	1.34 ± 0.11
Lactate, ethanol	0.43 ± 0.08
Lactate, ethanol, lysine	1.25 ± 0.12

Table 12. *Metabolite changes in cell suspensions on incubation with lactate and effectors of gluconeogenesis*

The amounts of glutamate, aspartate and alanine, and the value of the lactate/pyruvate ratio *in vivo* is compared with values found for cell suspensions. The values are μmol of metabolites in 4 ml of cell suspension containing 70 mg of wet cells. The 'expected value' *in vivo* was calculated from data for freeze-clamped liver of 48 h-starved rats (Williamson *et al.*, 1967a,b). The initial concentration of lactate in the incubated perfusions was 10 mM and of amino acids 2 mM. Incubation was for 20 min. The values include the metabolites in the suspension medium and therefore do not represent the concentrations within the cells. In separate experiments the cell to medium gradient was found to be about 100 for glutamate and about 25 for alanine; no reliable value can be given for aspartate because it was undetectable in the incubation medium in some cases. It is probably of the same order as the gradient for glutamate. Values given for the cell suspensions are either means $\pm$ S.E.M. (three observations) or the averages of two experiments.

	Metabolite changes ($\mu\text{mol}/4\text{ml}$ of suspension containing 70 mg of cells)						Lactate/ pyruvate
	Glucose	Glutamate	Aspartate	Alanine	Lactate	Pyruvate	
'Expected value' <i>in vivo</i>		0.17	0.052	0.032			16.6
Un-incubated specimen	0.12 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.015 ± 0.001			
Suspension incubated with:							
No substrate	0.24 ± 0.01	0.12 ± 0.01	0.05 ± 0.02	0.03 ± 0.01			
Lactate	0.63 ± 0.06	0.18 ± 0.01	0.08 ± 0.03	0.09 ± 0.01	37.9 ± 0.8	1.27 ± 0.12	32.9 ± 4.3
Lactate, Asn	1.93 ± 0.15	0.53 ± 0.06	0.41 ± 0.08	1.13 ± 0.12	34.7 ± 0.39	3.44 ± 0.18	10.2 ± 0.62
Lactate, Tyr	1.86 ± 0.13	0.39 ± 0.05	0.10 ± 0.04	0.53 ± 0.11	33.8	2.91	11.6
Lactate, Lys	1.73 ± 0.19	0.48 ± 0.04	0.11 ± 0.01	0.45 ± 0.04	33.4 ± 1.0	2.19 ± 0.14	15.6 ± 1.0
Lactate, Orn	1.41 ± 0.09	0.48 ± 0.09	0.11 ± 0.01	0.33 ± 0.17	34.6	2.50	13.9
Lactate, 0.5 mM- NH_4Cl	0.52 ± 0.03	0.10 ± 0.02	0.09 ± 0.02	0.07 ± 0.01	34.8	1.25	27.8
Lactate, 5.0 mM- NH_4Cl	1.15 ± 0.29	0.35 ± 0.04	0.16 ± 0.01	1.11 ± 0.25	32.7 ± 1.2	2.86 ± 0.21	11.5 ± 1.4
Lactate, Gln	0.84 ± 0.07	0.26 ± 0.06	0.07 ± 0.01	0.14 ± 0.21	36.7	1.56	23.6
Lactate, Pro	1.24 ± 0.11	0.28 ± 0.03	0.11 ± 0.01	0.21 ± 0.03	36.6 ± 1.5	2.07 ± 0.15	17.9 ± 1.7
Lactate, His	0.45 ± 0.03	0.73 ± 0.14	0.10 ± 0.03	0.09 ± 0.03	39.1	0.90	44.5

Metabolite changes during the lag

As a lag is often indicative of the need for a build-up of optimal concentrations of reactants, a comparison was made between freshly prepared liver cells, incubated cells and freeze-clamped liver of the concentrations of cell constituents involved in the 'aspartate shuttle' (see the Discussion section). This shows (Table 12) that 50% of glutamate and alanine were lost during the cell preparation; such losses would be caused either by metabolic degradation or by diffusion into the suspension medium. On incubation the concentrations *in vivo* were restored or even exceeded. This process is much accelerated by lysine and other nitrogenous substances, but since it also occurs without addition of nitrogenous substances, the amino acids must partly arise from proteolysis.

A striking effect of all accelerators was to increase the accumulation of alanine and pyruvate, and to bring about a rapid lowering of the [lactate]/[pyruvate] ratio. Within 20 min this reached the physiological range of 10–20 (Table 12), but without the accelerators 40–60 min were required to attain this value.

Discussion

Common features of accelerators of gluconeogenesis from lactate

The various substances which promote gluconeogenesis from lactate have certain metabolic characteristics in common which provide clues to their mechanism of action. First, all cause an increase in the concentration of pyruvate, and thereby lower the [lactate]/[pyruvate] ratio. Secondly, only those substances which react with 2-oxoglutarate to form glutamate are accelerators; glutamine and histidine which form glutamate via other reactions are not

accelerators. Thirdly, all bring about an accumulation of alanine roughly parallel to the formation of glucose (Table 12). Fourthly, their effects are not additive, possibly because of competition for 2-oxoglutarate.

The parallelism between glucose and alanine formation may be connected with the rise of the pyruvate concentration and the fact that pyruvate is a precursor of both alanine and glucose. The exceptionally large increases of alanine (up to 70-fold compared with about 10-fold for glutamate and 2-fold for pyruvate) can be accounted for by the fact that the concentrations of glutamate, 2-oxoglutarate and pyruvate in the cell favour the formation of alanine by the alanine aminotransferase. After 20 min incubation the mass action ratio in the alanine aminotransferase system

$$\frac{[\text{2-oxoglutarate}][\text{alanine}]}{[\text{glutamate}][\text{pyruvate}]}$$

calculated from the separate experiments mentioned in the legend of Table 12 was found to be 0.44 in the presence of lactate, 0.35 in the presence of lactate and asparagine and 0.32 in the presence of lactate and lysine, the equilibrium constant at 38°C being 1.52 (Krebs & Veech, 1970). Thus at the measured concentrations of glutamate, 2-oxoglutarate and pyruvate the concentrations of alanine, though relatively high, were still three to four times less than those expected for equilibrium.

Nature of the action of lysine

Lysine (and other substances which promote gluconeogenesis from lactate) must affect one or several of the following intermediary stages:

When pyruvate accumulates in the cell another set of reactions must take place because for each molecule of pyruvate that accumulates one molecule of NADH must be disposed of by transfer to the mitochondria and oxidation by the respiratory chain. This disposal is taken to involve the steps (Borst, 1963):

followed by reactions (4), (5), (6) and (7). Thus when the conversion of lactate into glucose is stoichiometric oxaloacetate coming out of the mitochondria is quantitatively converted into phosphoenolpyruvate but when pyruvate is an end-product of lactate metabolism an equivalent amount of oxaloacetate carbon, in the form of malate and aspartate, shuttles between cytosol and mitochondria, transferring NADH to the mitochondria. This also occurs when lactate supplies the carbon skeleton of alanine. The data in Table 12 show that under many conditions the amounts of pyruvate plus alanine formed from lactate can be comparable with the amounts of lactate converted into glucose. Hence the sequence of reactions (11)–(13) and (4)–(7) may substantially contribute to the metabolism of liver cells in the presence of lactate.

As gluconeogenesis from pyruvate is not affected by lysine, the reactions promoted by lysine must either be specific for gluconeogenesis from lactate or depend on the pyruvate concentration. This rules out the majority of steps as sites of action of lysine. Gluconeogenesis from pyruvate shares reactions (2), (3), (8), (9) and (10) with those of gluconeogenesis from lactate, but (4), (5), (6) and (7) are replaced by other reactions because gluconeogenesis from pyruvate necessitates the supply of one molecule of NADH in the cytoplasm for each molecule of pyruvate used, whereas with lactate as the precursor NADH is formed by reaction (1). The reactions which replace (4), (5), (6) and (7) when pyruvate is the precursor are:

These are the same reactions as (11), (12) and (13), except that (11) and (13) occur in different compartments and that the transfer in (12) is in the opposite direction.

Vol. 142

Thus, when lactate is the precursor, oxaloacetate is transferred from mitochondria to cytosol by the aspartate shuttle, but when pyruvate is the precursor oxaloacetate is transferred, together with reducing equivalents, in the form of malate (Borst, 1963; Lardy *et al.*, 1965; Haynes, 1965; Walter *et al.*, 1966;

Krebs *et al.*, 1967). In this situation malate is not 'shuttled' to and fro because it arises afresh through reaction (11a). As this is one of the major differences between gluconeogenesis from lactate and from pyruvate it follows that the site of action of lysine and related substances is likely to concern the effectiveness of the aspartate shuttle. In accordance with this conclusion is the finding of Rognstad & Katz (1970) that in kidney-cortex slices gluconeogenesis from lactate is inhibited by amino-oxyacetate, whereas gluconeogenesis from pyruvate is not. In other experiments this was found to apply also to isolated hepatocytes. Amino-oxyacetate, first used by Wallach (1960), somewhat specifically inhibits pyridoxal enzymes such as aminotransferases, especially alanine aminotransferase and aspartate aminotransferase (Hopper & Segal, 1964).

Another difference between lactate and pyruvate as precursors concerns the concentrations of lactate and pyruvate, and therefore the initial [lactate]/[pyruvate] ratio, as shown in Table 12. When lactate is the added substrate this ratio changes during the lag, mainly owing to the build-up of the pyruvate concentration through reactions (11)–(14) and this change is accelerated by lysine and other substances. The common feature, then, of the two effects of lysine, a rapid build-up of pyruvate concentrations and the shortening of the lag on the one hand, and the continued raised ultimate rate of gluconeogenesis, may both be attributed to effects of lysine on the aspartate shuttle.

As for the question of how the promotion of the

rate of the shuttle, i.e. reactions (4)–(7), may be effected by lysine, the rate of the aspartate shuttle is expected to depend on the mitochondrial concentration of glutamate. As shown in Table 12 freshly

prepared cells contain much less glutamate than freeze-clamped liver and on incubation the normal glutamate concentration is restored, a recovery much accelerated by lysine and other amino-group donors. This justifies the assumption that part of the lysine effect is due to the promotion of glutamate formation. This effect could account for the removal, or shortening, of the lag, but it does not account for the raised ultimate rate of gluconeogenesis, an effect specific for lysine. The raised ultimate rate may be caused either by lysine directly or by a metabolic product, for instance aminoadipate. The latter may play a role in the transport of aspartate across the mitochondrial membrane (see Azzi *et al.*, 1967). The failure of added glutamate or aspartate to stimulate gluconeogenesis from lactate (see Table 3) is explained by the relative impermeability of the liver cell to dicarboxylic acids (Hems *et al.*, 1968).

Effect of the lactate concentration on the lag period

As shown in Table 2 the lag in onset of gluconeogenesis increases as the initial concentration is increased above 2mM. This can be explained on the assumption that at higher concentrations of lactate the operation of the aspartate-glutamate shuttle is a factor limiting the rate of gluconeogenesis from lactate (see Berry & Kun, 1972) because the shuttle also serves to build up the physiological [lactate]/[pyruvate] ratio [reactions (11)–(14) and (5)–(7)]. As the pyruvate formed by this reaction is discharged into the medium the volume of the shuttle is very substantial and may be of the same order as that required for the conversion of lactate into glucose (see Table 12).

Another way of expressing the situation is to say that, when lactate forms glucose as well as pyruvate, malate dehydrogenase and phosphoenolpyruvate carboxylase compete for oxaloacetate in the cytosol, and at high lactate concentrations the malate dehydrogenase tends to win. Since the malate dehydrogenase reaction in this context is a step towards establishing the normal redox state of the NAD couple (upset by the addition of much lactate) the facts may also be expressed by the statement that optimal rates of gluconeogenesis require the redox state of the NAD couple to be within a 'normal' range.

It is also noteworthy that in spite of the high K_m value of lactate dehydrogenase for lactate, about 20mM, after the lag period the rate of gluconeogenesis between 2 and 10mM is independent of the lactate concentration. This confirms that the lactate dehydrogenase reaction is not rate-limiting, a conclusion implied in the recognition that lactate dehydrogenase is an 'equilibrium' enzyme, the activity of which is high enough to establish equilibrium readily.

Occurrence of a lysine effect in vivo

Whether lysine has an effect *in vivo* under normal conditions is an open question. As lysine was effective at concentrations occurring *in vivo* (Cornell *et al.*, 1973) it is likely to function *in vivo* in situations analogous to the loss of cell constituents during the preparation of the cells. This could happen during hypoxia or during the manipulations involved in organ transplantation; an organ to be transplanted is exposed to the same kinds of stresses as is the tissue handled experimentally.

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