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The Rate of Gluconeogenesis from Various Precursors in the Perfused Rat Liver

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1. The rates of gluconeogenesis from many precursors have been measured in the perfused rat liver and, for comparison, in rat liver slices. All livers were from rats starved for 48hr. Under optimum conditions the rates in perfused liver were three to five times those found under optimum conditions in slices. 2. Rapid gluconeogenesis (rates of above 0·5μmole/g./min.) were found with lactate, pyruvate, alanine, serine, proline, fructose, dihydroxyacetone, sorbitol, xylitol. Unexpectedly other amino acids, notably glutamate and aspartate, and the intermediates of the tricarboxylic acid cycle (with the exception of oxaloacetate), reacted very slowly and were not readily removed from the perfusion medium, presumably because of permeability barriers which prevent the passage of highly charged negative ions. Glutamine and asparagine formed glucose more readily than the corresponding amino acids. 3. Glucagon increased the rate of gluconeogenesis from lactate and pyruvate but not from any other precursor tested. This occurred when the liver was virtually completely depleted of glycogen. Two sites of action of glucagon must therefore be postulated: one concerned with mobilization of liver glycogen, the other with the promotion of gluconeogenesis. Sliced liver did not respond to glucagon. 4. Pyruvate and oxaloacetate formed substantial quantities of lactate on perfusion, which indicates that the reducing power provided in the cytoplasm was in excess of the needs of gluconeogenesis. 5. Values for the content of intermediary metabolites of gluconeogenesis in the perfused liver are reported. The values for most intermediates rose on addition of lactate. 6. The rates of gluconeogenesis from lactate and pyruvate were not affected by wide variations of the lactate/pyruvate ratio in the perfusion medium.

The rat liver-perfusion technique described by Hems, Ross, Berry & Krebs (1966) has been used in the present investigation to measure the rate of gluconeogenesis from all major physiological precursors. Experiments on the intact perfused organ were prompted by the finding that liver slices failed to form glucose at the expected rates from several precursors that are known to be glucogenic in the intact body (Krebs, Notton & Hems, 1966). Although the rates of gluconeogenesis from some precursors, such as lactate and pyruvate, are more rapid in the perfused liver than in slices, the rate of reaction of those precursors that carry a double or treble negative charge, like the intermediates of the tricarboxylic acid cycle and the dicarboxylic amino acids, were found to be very low in the perfused liver. There are indications that the penetration of the precursors to the cellular site of conversion is a rate-limiting factor in the gluconeogenesis from these substances.

EXPERIMENTAL

The basic method of perfusion was as described by Hems *et al.* (1966). Female Wistar rats weighing 200–250g. and starved for 48hr. were used unless otherwise stated. The substrates were added as 0·2M neutral solutions to a final concentration of 10mM, 38min. after the start of the perfusion. The times of perfusion given in the description of the experiments refer to the time (min.) elapsed since the start of the perfusion, and not to the time of the addition of substrate. Metabolic rates were calculated from the gradient of the plot of total metabolite in the medium versus time and are expressed as μmoles/min./g. of wet liver.

The methods for the determination of glucose, glycogen and lactate were as previously described by Hems *et al.* (1966). α-Oxoglutarate was determined by the method of Bergmeyer & Bernt (1963); oxaloacetate by the method of Löffler & Wieland (1963); malate by the method of Hohorst (1963); succinate by the method of Clark & Porteous (1964); glycerol by the method of Garland & Randle (1962). Fumarate was estimated as malate after the addition of fumarase. Pyruvate, phosphopyruvate, 2- and 3-phospho-

Table 1. *Rates of gluconeogenesis from various precursors in the perfused liver of female rats starved for 48 hr.*

The final substrate concentration was 10 mM. Substrate was added as neutral sodium salts unless stated otherwise. The values are means ± S.E.M. with the number of observations in parentheses. P is the significance of difference of glucose formation with and without substrate and of substrate removed.

Substrate added	Glucose formed (μmoles/min./g.)	$P<$	Substrate removed (μmoles/min./g.)	$P<$
Nil	0·14 ± 0·03 (5)			
L-Lactate	1·06 ± 0·09 (12)	0·01	1·95 ± 0·23 (12)	0·01
Pyruvate	1·02 ± 0·08 (5)	0·01	4·46 ± 0·70 (3)	0·01
Glycerol	0·48 ± 0·03 (5)	0·01	1·02 ± 0·12 (5)	0·01
Dihydroxyacetone	2·07 ± 0·30 (6)	0·01		
D-Fructose	2·68 ± 0·25 (4)	0·01		
D-Galactose	0·36 ± 0·11 (3)	0·2		
Sorbitol	1·79 ± 0·06 (3)	0·01		
Xylitol	0·67 ± 0·11 (3)	0·01		
L-Serine	0·98 ± 0·04 (3)	0·01		
L-Alanine	0·66 ± 0·12 (6)	0·01		
L-Proline	0·55 ± 0·10 (3)	0·01		
L-Ornithine	0·19 ± 0·05 (5)	0·4		
L-Arginine	0·27 (2)			
L-Valine	0·12 (2)			
L-Isoleucine	0·22 (2)			
L-Threonine	0·40 ± 0·04 (3)	0·1		
β-Aminobutyrate	0·23 ± 0·04 (3)	0·2		
Sodium L-glutamate	0·31 ± 0·04 (6)	0·01	0·15 ± 0·04 (4)	0·1
Ammonium L-glutamate	0·29 ± 0·04 (3)	0·05		
L-Glutamine	0·45 ± 0·08 (4)	0·02	1·31 ± 0·13 (3)	0·01
L-Aspartate	0·23 ± 0·04 (3)	0·2		
L-Asparagine	0·51 ± 0·11 (3)	0·02		
Oxaloacetate	1·22 ± 0·12 (4)	0·01	2·67 ± 0·50 (4)	0·01
Succinate	0·24 ± 0·03 (4)	0·05	0·10 ± 0·07 (3)	0·3
α-Oxoglutarate	0·25 ± 0·05 (4)	0·1	0·19 ± 0·03 (4)	0·01
Fumarate	0·19 ± 0·05 (3)	0·5	0·16 ± 0·02 (3)	0·02
L-Malate	0·23 ± 0·07 (4)	0·3	0·23 ± 0·02 (3)	0·01

glycerate were determined by the combined assay of Czock & Eckert (1963), fructose diphosphate and the two triose phosphates by the method of Bücher & Hohorst (1963).

Slices of rat liver, used for comparison with the perfused organ, were handled as described for mouse liver slices by Krebs, Notton & Hems (1966). The incubation medium was the Krebs & Henseleit (1932) solution. This medium gave higher rates with rat liver, as it did also with rat kidney and mouse liver, than any other medium tested including those of Buchanan, Hastings & Nesbett (1949*a*,*b*) and Hastings, Teng, Nesbett & Sinex (1952).

RESULTS

Glucose synthesis from various precursors. The rate of glucose formation from known glucogenic substances, measured under standard conditions on livers of rats starved for 48 hr., is shown in Table 1. Ready gluconeogenic precursors were lactate and pyruvate. Of the amino acids listed only L-serine, L-alanine and L-proline gave rates of an expected order of magnitude. Surprisingly, glutamate and aspartate reacted very slowly. The increment over the blank was significant with glutamate ($P<0·05$) but doubtful with aspartate ($0·1<P<0·2$). Ornithine gave a slight increment that was not significant ($0·2<P<0·4$). L-Glutamine was somewhat more effective as a glucose former than sodium glutamate or ammonium glutamate. Asparagine reacted more than twice as rapidly as sodium aspartate.

No less surprising was the failure of the intermediates of the tricarboxylic acid cycle to form glucose rapidly. Succinate and α-oxoglutarate showed a significant ($P<0·1$) but very small effect. Malate and fumarate gave no significant increases. The only exception among the intermediates of the tricarboxylic acid cycle was oxaloacetate, which reacted even more rapidly than lactate or pyruvate. The low rates were unexpected because all the enzymes required for the conversion of the precursors into glucose are known to be present in very high activity in rat liver. The failure to form glucose rapidly was matched by the very low rate of removal of the precursors from the perfusion medium.

The highest rate of glucose formation was given by fructose, followed by dihydroxyacetone and sorbitol. Glycerol gave a relatively low rate. Xylitol reacted more rapidly than glycerol.

The rate of substrate removal was approximately twice that of glucose formation with lactate, glycerol and oxaloacetate, and greater than this with pyruvate and glutamine. The amounts of extra glucose formed and of substrate removed with glutamate, succinate and α-oxoglutarate do not show the expected stoicheiometric relation of two to one because of the relatively large endogenous glucose formation. The high rate of glutamine removal was partly due to a conversion into glutamate.

Because slices of female mouse liver gave higher rates of gluconeogenesis than those of male liver (Krebs, Notton & Hems, 1966), a series of experiments was carried out on perfused livers of male rats, with lactate as the substrate, but no differences between the sexes were found.

Modification of the perfusion medium. The essentially negative results obtained with known glucose precursors raise the question of whether the perfusion techniques employed had major shortcomings. The effect of various modifications of the medium was therefore tested. Since Miller, Burke & Haft (1955) had reported relatively high rates of removal of α-oxoglutarate by the perfused rat liver when rat blood was the perfusion medium, the standard medium was replaced by that of Miller *et al.* (1955). Fresh heparinized rat blood was obtained by aortic puncture and diluted with about one-third volume of Ringer solution. A sample of the medium was kept at 37° for the measurement of blood glycolysis. The initial glucose content of this medium was about 3mM. α-Oxoglutarate (20mM) was removed from the medium at the rate of 0·3 ± 0·16 (3) μmole/min./g. wet wt., which is not significantly different from that recorded in Table 1 and much lower than that quoted by Miller *et al.* (1955), who reported a rate of approx. 1 μmole/min./g. Other experiments in which rat blood was used with succinate (10mM) and L-glutamate (10mM) gave results which did not differ from those recorded in Table 1, i.e. no significant amounts of glucose were formed. With L-lactate the rate of removal and conversion into glucose was similar in livers perfused with rat blood and the standard medium.

Replacing the Krebs–Henseleit solution by dialysed bovine serum, dialysed human plasma or dialysed rat serum had no effect on the metabolic behaviour of lactate, glutamate, fumarate or malate, nor had prolonged dialysis (48hr.) of the bovine albumin used in the preparation of the standard medium. Substitution of Haemaccel, a plasma expander (Behringwerke, Marburg), for albumin or omission of red cells had no effects. Altering the pH over the range 7·2–7·7 had no effect on the rate of glucose formation from glutamate. Varying the bicarbonate concentration between 5 and 40mM but keeping pH near 7·4 by adjusting the carbon dioxide pressure of the gas mixture was also without effect.

Insulin, which has been reported to accelerate the uptake of amino acids by rat diaphragm (Manchester & Young, 1960), had no effect on the removal of glutamate from the perfusion medium or on the synthesis of glucose when added as a single dose of 0·075unit/ml. at 40min. Hydrocortisone hemisuccinate (0·067mg./ml.) was also without effect on the removal of glutamate.

The non-formation of glucose from expected precursors cannot be interpreted by an immediate utilization of glucose (see Williamson, Garcia, Renold & Cahill, 1966) because the substrates (with the exception of glutamine) were not significantly removed from the medium and the glucose, added to the medium at low concentrations (below 5mM), was not utilized by the liver.

Table 2. *Effect of glucagon on glucose synthesis in perfused liver of rats starved for 48hr.*

Glucagon was added as a single dose (75 μg. total = 0·5 μg./ml.) at 40min. For other details see text and Table 1. n. s., Not significant.

Substrate added (10mM)	Glucose formed		Effect of glucagon	
	Without glucagon (μmoles/min./g.)	With glucagon (μmoles/min./g.)	Absolute (μmoles/min./g.)	Increase (%)
Nil	0·14 ± 0·03 (5)	0·22 ± 0·07 (3)	+0·08	n. s.
L-Lactate	1·06 ± 0·09 (12)	1·86 ± 0·16 (8)	+0·80	75%
Pyruvate	1·02 ± 0·08 (5)	1·44 ± 0·11 (4)	+0·42	41%
Glycerol	0·48 ± 0·03 (5)	0·51 ± 0·04 (3)	+0·03	n. s.
L-Alanine	0·66 ± 0·12 (6)	0·70 ± 0·09 (3)	+0·04	n. s.
L-Proline	0·55 ± 0·10 (3)	0·57 ± 0·07 (5)	+0·02	n. s.
L-Glutamate	0·31 ± 0·04 (6)	0·31 ± 0·02 (4)	0	n. s.
Oxaloacetate	1·22 ± 0·12 (4)	1·35 ± 0·19 (4)	+0·13	n. s.
Fructose	2·68 ± 0·25 (4)	2·94 ± 0·21 (4)	+0·26	n. s.

The following further modifications had no effects on the gluconeogenesis from glutamate: addition to the perfusion medium of butyrate or acetoacetate, i.e. of substances that can promote the conversion of lactate into glucose in slices of kidney cortex (Krebs, Speake & Hems, 1965); replacement of sodium glutamate by ammonium glutamate, prompted by the observation of Chappell & Haarhoff (1966) that NH_4^+ facilitates the penetration of dicarboxylic acids into mitochondria; pretreatment of rats by injection of nicotinamide (500mg./kg.), to increase the NAD and $NADH_2$ content of the liver; perfusion through the hepatic artery or simultaneous perfusion through the artery and portal vein; addition of pyruvate, pyridoxal, pyridoxamine and their phosphates, i.e. agents that facilitate transamination of glutamate. For a full description of these experiments see Ross (1966).

Effect of glucagon on glucose synthesis from various substrates. As shown in Table 2, glucagon (a gift of Dr Otto K. Behrens, Eli Lilly and Co., Indianapolis, Ind., U.S.A.), when added to the perfusion medium at 38min., greatly increased the rate of glucose formation from lactate, as has already been reported by Schimassek & Mitzkat (1963), Struck, Ashmore & Wieland (1965) and Exton & Park (1966). There was also an effect with pyruvate, the absolute increase being 0·80μmole/g./min. with lactate and 0·42μmole/g./min. with pyruvate. By contrast there was no effect of glucagon on the endogenous gluconeogenesis nor with alanine, proline, glutamate, glycerol, oxaloacetate or fructose. The increased rate of glucose synthesis from lactate was paralleled by an increased removal of lactate. The rate of lactate uptake was 3·71 ± 0·15 (4)μmoles/min./g. This accounted for all the glucose formed on addition of glucagon.

The magnitude of the effect of glucagon on the gluconeogenesis from lactate depended on the time of addition of the hormone. It was much more effective when it was added at 40min. than at 85 min. (Table 3). Lactate alone was equally effective when added at 40 and 85min. The response of glucagon on prolonged perfusion was lost irrespective of the addition of lactate at 40min. No explanation can as yet be offered for the diminished response to glucagon at 85min. The metabolic processes measured (oxygen consumption, urea synthesis, glucose synthesis from lactate) and the concentration of intermediary metabolites, in particular of the adenine nucleotides, appear to be the same at 40min. and at 85min. There was no indication that addition of glucagon at 0min. was more effective than at 40min.

Concentrations of intermediary metabolites in the perfused liver. The steady-state concentrations of intermediates of glycolysis and gluconeogenesis showed no clear-cut changes in the first 40min., when the liver was perfused with the standard medium without addition of substrate, except for a drop in the concentration of lactate (Table 4). This drop may be expected on account of gluconeogenesis from the small amounts of lactate present. At 85min. the lactate concentration showed a further fall and that of malate also decreased, and as the pyruvate concentration did not fall the lactate/pyruvate ratio was substantially lower than that of the liver *in vivo* (given in Table 4 under the heading 'liver not perfused'). The main change occurring during the early stages of perfusion (at 5 and 15min.) was an approximate doubling of the concentration of α-glycerophosphate, an intermediate not on the main pathway of carbohydrate degradation or synthesis. Later the concentration of α-glycerophosphate gradually fell and at 85min. it was one-ninth of the maximal value. Similar observations have been recorded by Schimassek (1963). The causes of the initial rise in the concentration of α-glycerophosphate remain to be explored; it could not be correlated to the lactate/pyruvate ratio.

On addition of 10mM-lactate the steady-state

Table 3. *Effect of time of addition of glucagon and lactate on the rate of gluconeogenesis from lactate in the perfused rat liver*

The general conditions were as described for Table 1. The concentration of lactate was 10mM. Times stated are minutes after the start of perfusion.

Time of addition of lactate (min.)	Glucagon added		Glucose formed	
	Time (min.)	Final concn. (μg./ml.)	Time (min.)	Rate (μmoles/min./g.)
40		0	40–130	1·06 ± 0·09 (12)
40	40	0·5	40–130	1·86 ± 0·16 (4)
85		0	85–130	1·03 ± 0·09 (3)
85	85	0·5	85–130	1·20 ± 0·13 (3)
40	85	0·5	40–85	1·12 ± 0·10 (3)
			85–130	1·14 ± 0·12 (3)

Table 4. *Contents of intermediary metabolites of gluconeogenesis in the perfused rat liver*

Livers of rats starved for 48hr. were perfused for various times and a sample of liver was taken by the rapid-freezing technique [see Hems *et al.* (1966) and text]. Lactate (10mM) was added 40min. after the start of perfusion. The figures are means ± S.E.M. Data on the concentration of the adenine nucleotides for the same conditions were given by Hems *et al.* (1966).

	Liver not perfused (48hr. starved)	Liver perfused without added substrate				Liver perfused with lactate	
Time of perfusion (min.) ...		5	15	40	85	85	130
No. of observations	7	5	3	4	4	6	6
Content of metabolite (μmoles/g. wet wt.)							
Lactate	0·92±0·20	0·47±0·04	0·45±0·04	0·66±0·06	0·32±0·08	3·26±0·26	1·53±0·50
Pyruvate	0·07±0·01	0·12±0·01	0·07±0·01	0·06±0·01	0·07±0·01	0·23±0·03	0·12±0·02
Malate	0·52±0·07	0·36±0·05		0·46±0·05	0·18±0·05	0·62±0·12	0·26±0·04
Oxaloacetate					0·0035 (2)	0·0039 (2)	
Phosphoenolpyruvate	0·03±0·01	0·05±0·01	0·02±0·01	0·02±0·002	0·05±0·01	0·21±0·02	0·23±0·03
2-Phosphoglycerate	0·03±0·01	0·03±0·01	0·02±0·003	0·01±0·003	0·01±0·004	0·05±0·003	0·08±0·01
3-Phosphoglycerate	0·08±0·01	0·09±0·03	0·04±0·01	0·03±0·004	0·10±0·02	0·23±0·09	0·45±0·03
Triose phosphates	0·05±0·03			0·04±0·004	0·04±0·003 (2)	0·05±0·01 (2)	
α-Glycerophosphate	0·99±0·08	1·90±0·35	1·83±0·20	0·96±0·08	0·22±0·05	0·38±0·10	0·18±0·04
Fructose diphosphate					0·032 (2)	0·033 (2)	
Glucose 6-phosphate	0·05±0·01	0·01±0·004	0·01±0·003	0·01±0·003	0·03±0·01	0·08±0·02	0·09±0·03
Inorganic phosphate	4·1 ±0·8	6·0 ±1·4 (2)	4·3 ±0·7 (3)	4·7 ±1·7 (2)	3·9 ±0·01 (2)	4·55±0·4	2·9 ±0·8 (2)
Ratio lactate/pyruvate	13·7±3·6	3·9 ±0·6	6·5 ±0·8	11·0 ±1·9	4·6 ±1·2	14·2 ±2·5	12·6 ±2·0
Ratio malate/oxaloacetate					51	158	

concentrations of most intermediates rose. Exceptions were triose phosphate, oxaloacetate and fructose diphosphate, which did not measurably change, and ATP and AMP, which fell. A decrease of ATP has been reported before (Hems *et al.* 1966) and may be connected with the increased consumption of ATP during gluconeogenesis. The findings give no clear-cut indication about the rate-controlling steps of gluconeogenesis but are compatible with the generally accepted view that the carboxylation of pyruvate and the activity of fructose diphosphatase are rate-limiting steps. Under the conditions of the perfusion the factor limiting gluconeogenesis was evidently the availability of substrate. This holds for the starved liver not supplied with substrate, where the absence of gluconeogenesis is not necessarily due to feedback inhibitions or insufficiency of enzyme activities. No striking effects of the addition of substrate on the concentration of specific intermediates are therefore to be postulated.

As may be expected the addition of lactate shifts the ratios of the three redox couples (lactate/pyruvate, malate/oxaloacetate, α-glycerophosphate/triose phosphate) in favour of the reduced component, with the lactate and malate systems by a factor of about 3. It is remarkable how rapidly the lactate/pyruvate ratio was adjusted when it was upset by addition of large amounts of lactate. It was calculated to be 160 immediately on addition of lactate and 14·5 after 45min.

Time-course of glucose formation from pyruvate. Pyruvate was removed from the medium with great speed, so that most of the added 1500μmoles had disappeared within 40min. (Table 5). The rate was approximately linear (5·60μmoles/g./min.) Apart from glucose, large amounts of lactate were formed initially. The rate of lactate formation fell later and eventually lactate formed earlier disappeared. Glucose formation was approximately linear until near the end of the perfusion period, with the lactate formed intitially from pyruvate as the main precursor of glucose towards the end of the perfusion. The lactate/pyruvate ratio, very low after addition of pyruvate, rose within 1hr. to the pre-addition value (20 in this experiment).

Time-course of glucose formation from oxaloacetate. Glucose formation from oxaloacetate, like that from pyruvate, was linear with time (Table 6). Initially there was also a formation of pyruvate and lactate but the concentration of both these products fell later.

Balance of metabolites in the presence of pyruvate and oxaloacetate. In a series of four experiments almost all the added oxaloacetate (98%) could be accounted for as glucose, lactate or pyruvate (Table 7), glucose being the main product. With pyruvate, glucose and lactate accounted for 67% of the substrate removed. Other products (ketone bodies, malate, fumarate) accounted for only minor quantities and most of the missing 33% of the pyruvate used must have served as the substrate of respiration.

Comparison of gluconeogenesis in perfused liver and liver slices. The rates of gluconeogenesis obtained with slices of rat liver after 48hr. starvation and phlorrhizin treatment, expressed in the same units as those obtained with the perfused livers (Table 1), are shown in Table 8. The carbohydrate formed from endogenous sources in controls incubated without substrate, as given in the heading of the Table, has been deducted. In general the rates observed were much lower in the slices than in the perfused organ. Exceptions are the endogenous gluconeogenesis and gluconeogenesis from glycerol and xylitol, which in slices were somewhat higher than in the perfused organ. The rates with lactate and pyruvate were about 50% and with most substrates (alanine, serine, proline, fructose, dihydroxyacetone, oxaloacetate, sorbitol, glutamine, asparagine) between 20 and 30% of those observed in the perfused liver. Substrates that gave little or

Table 5. *Time-course of glucose formation from pyruvate in the perfused rat liver*

Pyruvate (1500μmoles) was added at 38min. and samples were taken at intervals. The figures are μmoles of metabolite in total perfusion medium. The rat was starved for 48hr. The liver weighed 5·62g. wet (1·61g. dry). For further details see text.

Time (min.) ...	10	40	55	70	85	100	115	130
Pyruvate								
Found (μmoles)	6·0	1380	920	435	87	29	17	33
Change/15min. period (μmoles)			−460	−485	−348	−58	−12	+16
Lactate								
Found (μmoles)	134	222	548	775	860	698	445	365
Change/15min. period (μmoles)		(+88)	+326	+227	+85	−162	−253	−80
Glucose								
Found (μmoles)	103	103	174	275	356	432	532	580
Change/15min. period (μmoles)			+71	+101	+81	+76	+100	+48
Ratio lactate/pyruvate	20	0·16	0·60	1·8	9·9	23	26	11

Table 6. *Time-course of glucose formation from oxaloacetate in the perfused rat liver*

Oxaloacetate (1450 μmoles) was added at 38 min. and samples were taken at intervals. The figures are μmoles of metabolite in total perfusion medium. The rat was starved for 48 hr. and the liver weighed 5·02 g. wet (1·52 g. dry). For further details see text.

Time (min.) ...	0	10	40	55	70	85	100	115	130
Oxaloacetate									
Found (μmoles)	1·5	2·0	1260	1000	815	570	335	300	174
Change/15 min. period (μmoles)				−260	−185	−245	−235	−35	−126
Pyruvate									
Found (μmoles)	1·4	11·7	85	225	159	162	114	73	49
Change/15 min. period (μmoles)				+140	−66	+3	−48	−41	−24
Lactate									
Found (μmoles)	137	153	150	229	346	384	426	394	341
Change/15 min. period (μmoles)				+79	+117	+38	+42	−32	−53
Glucose									
Found (μmoles)	44	74	103	200	294	390	480	575	630
Change/15 min. period (μmoles)		+30	+29	+97	+94	+96	+90	+95	+55
Ratio lactate/pyruvate		13·1	1·8	1·0	2·2	2·4	4·2	7·9	14·0

Table 7. *Balance of products of pyruvate and oxaloacetate metabolism in the perfused rat liver*

Substrates were added at 38 min. and the metabolites were determined at 40 min. (initial) and 130 min. (final). The figures given are the difference between the initial and the final amounts of metabolites in the perfusion medium (mean ± S.E.M., 4 experiments in each group). Disappearance is indicated by the − sign and formation by the + sign. For the calculation of the balance it was assumed that the formation of glucose required two substrate molecules. The rats were starved for 48 hr.

Substrate added	None	Pyruvate (1500 μmoles)	Oxaloacetate (1500 μmoles)
Changes (μmoles)			
Glucose, observed	+62 ± 10	+ 466 ± 44	+ 556 ± 42
Glucose, corrected for control		+ 404	+ 494
Lactate, observed	−29	+ 144 ± 43	+ 189 ± 46
Lactate, corrected for control		+ 173	+ 218
Pyruvate, observed		−1460 ± 8	+ 52 ± 30
Oxaloacetate, observed			−1276 ± 30
Sum of products in substrate equivalents, corrected for control (μmoles)		981	1258
Substrate recovered as product (%)		67	98

no increment on perfusion were also inactive in slices. Thus qualitatively the glucogenic activity of the slices was similar to that of the perfused organ, but with the exception of gluconeogenesis from endogenous precursors, glycerol and xylitol, the absolute rates were two- to five-fold as high in the perfused organ.

Unlike the perfused livers the slices did not respond to glucagon in the presence of lactate and pyruvate. Since in the perfused liver, glucagon raised the rates of gluconeogenesis from lactate and pyruvate by 75 and 40% respectively (Table 2), the absence of response to glucagon of the slices implies that the maximal rates observed in the perfused liver, i.e. those in the presence of saturating amounts of glucagon, were three- to five-fold as high in the perfused organ as in slices for all substrates except glycerol, xylitol and endogenous precursors.

Gluconeogenesis from substrate combinations. It was thought that transamination may promote gluconeogenesis from amino acids but simultaneous addition of glutamate plus pyruvate or glutamate plus oxaloacetate (all substrates 10 mM) gave no higher rates than pyruvate or oxaloacetate alone, and the rate of glutamate removal was not increased. Combinations of amino acids in the form of an enzymic hydrolysate of casein (Tryptone, Oxoid Ltd.) gave no higher rates than the single component amino acids such as alanine, serine or proline. Lactate plus pyruvate gave no higher rates than lactate alone. Aspartate (10 mM) plus α-oxoglutarate (10 mM), which would be expected to generate

Table 8. *Gluconeogenesis from various precursors in rat liver slices*

The rats were starved for 48hr. and treated with phlorrhizin (1g./kg. of rat) 4hr. before killing. The substrate concentrations were 10mM. All values are corrected for the carbohydrate formation without added substrate, which was 0·19±0·01 μmole/g./min. (19). The values are means ±S.E.M., together with the number of observations in parentheses.

Substrate added	Glucose plus glycogen formed (μmole/g. wet wt./min.)
L-Lactate	0·55±0·05 (6)
Pyruvate	0·40±0·05 (4)
Fumarate	0·03±0·01 (4)
Succinate	0·03±0·01 (4)
Oxaloacetate	0·22±0·02 (4)
L-Malate	0·07±0·02 (3)
α-Oxoglutarate	0·03±0·01 (4)
Propionate	0·15±0·05 (4)
L-Aspartate	0·03±0·03 (5)
L-Ornithine	0·07±0·04 (4)
L-Proline	0·17±0·07 (4)
L-Glutamate	0·02±0·01 (6)
L-Alanine	0·23±0·08 (5)
L-Serine	0·20±0·03 (4)
L-Threonine	0·10±0·03 (4)
L-Glutamine	0·08±0·03 (4)
L-Asparagine	0·08±0·03 (4)
Xylitol	0·49±0·04 (4)
Sorbitol	0·77±0·19 (4)
Glycerol	0·62±0·05 (4)
D-Galactose	0·12±0·01 (4)
Dihydroxyacetone	0·38±0·12 (4)
D-Fructose	0·81±0·12 (4)

oxaloacetate in both cytoplasm and mitochondria (if the substrates penetrated these areas), gave no higher rates than these substrates alone.

When glycerol was one of the components of a substrate mixture the yields of carbohydrate were additive, as is already known for kidney-cortex slices (Krebs, Hems & Gascoyne, 1963). A special case of substrate combination is that of mixtures consisting of a fatty acid (which alone is not glucogenic) with lactate. Butyrate (2mM) or oleate (0·5mM) increased the rate of gluconeogenesis from lactate in the perfused liver under some conditions, as they do in rat kidney-cortex slices. This effect is attributable to a sparing action or an activation of pyruvate carboxylase by acetyl-coenzyme A derived from the fatty acids (see Krebs *et al.* 1965) or to both.

DISCUSSION

Comparison of the rates of gluconeogenesis in sliced and perfused rat liver. The results show that there are major differences between sliced and perfused liver with respect to the rates of gluconeogenesis from all substrates tested except glycerol. For most precursors the maximal rates were three- to five-fold as high in the perfused organ. Since in the presence of excess of substrates the rates are limited by the activity of 'pace-maker' enzymes, some of these must be impaired in the slices. Further investigation is required to discover which of the 'pace-makers' are affected. The impairment may be due to instability of the enzyme proteins or to changes in the concentrations of cofactors. The impairment of the gluconeogenic enzymes is in contrast with the urea-synthesizing system. The rates of urea synthesis from ammonia and the accelerating effects of ornithine on this synthesis are similar in sliced and perfused liver (Hems *et al.* 1966).

If, then, there are major quantitative differences between slices and perfused rat liver, qualitatively the behaviour of the two preparations is very similar, i.e. substances that fail to react readily in slices are also ineffective in the perfused organ. The low rates of disappearance of the substrates from the perfusion medium (see Table 1) indicate that it is not only gluconeogenesis but the metabolic utilization generally, including the uptake and storage of amino acids, which is restricted in liver tissue. This fact, together with the ready utilization of glutamate by liver mitochondria in homogenates (see Krebs & Bellamy, 1960) suggests that the rate of entry of substrates like glutamate to the site of enzymes governs the rate of their metabolism. There appears to be a barrier in the intact cell that is no longer effective in isolated mitochondria or in homogenates. In the experiments of Krebs & Bellamy (1960, Table 1) rat liver homogenate oxidized glutamate at a rate of 1·4μmoles/g./min. at 37°. In the perfused liver (Table 1) the rate was 0·15μmole/g./min.

Gluconeogenic precursors in rat liver. Of the precursors which because of their behaviour in the intact body or in kidney slices are expected to be gluconeogenic, only those reacted readily in the liver that have no double or treble negative charge (glycerol, polyols, fructose, lactate, pyruvate, propionate, serine, alanine, proline). The intermediates of the tricarboxylic acid cycle did not react, with the exception of oxaloacetate. A major difference between oxaloacetate and the other intermediates is that the intermediary stages between oxaloacetate and carbohydrate all occur in the cytoplasm, whereas the other di- and tricarboxylic acids must penetrate the mitochondria to be converted into oxaloacetate before they enter the gluconeogenic pathway. Since malate dehydrogenase (EC 1.1.1.37) is present in the cytoplasm and since the required NAD can be regenerated from $NADH_2$ through the $NADH_2$-linked conversion of

1,3-diphosphoglycerate into glyceraldehyde 3-phosphate, malate (and fumarate) would be expected to form carbohydrate, if the mitochondrial membranes rather than access to the cytoplasm were the permeability barrier. Why this expectation is not fulfilled requires further investigation.

Exceptional behaviour of glycerol. The fact that the rate of conversion of glycerol into carbohydrate was not impaired on slicing may be explained on the basis of two special features of glycerol metabolism. First, the pathway leading from glycerol to carbohydrate differs in a major way from those of most other substrates in that it does not involve the stages between oxaloacetate and glyceraldehyde 3-phosphate, and the total number of stages is smaller; hence the risk of impairment is smaller. Secondly, the rate of gluconeogenesis from glycerol is relatively low and is limited by the activity of glycerol kinase (EC 2.7.1.30). The capacity of the steps catalysing the subsequent stages is greatly in excess of that of glycerokinase, as the rapid gluconeogenesis from dihydroxyacetone and fructose indicates. Thus, if the activity of glycerokinase remained normal, a partial loss of activity of these enzymes would not affect the rate of gluconeogenesis from glycerol.

Rapid conversion of xylitol into glucose. The relatively high rate of glucose formation from xylitol (as fast as that from glycerol) is unexpected because xylitol and the substances from which it can be formed (*myo*-inositol, D-glucuronate, L-xylulose) occur normally in relatively small quantities. Gluconeogenesis from xylitol involves a conversion of xylitol into D-xylulose phosphate, and the action of transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2). A high activity of these enzymes must therefore be postulated. Transketolase and transaldolase are difficult to assay; no precise information on their relative activities is available.

Primary sites of action of glucagon. Recent work on the action of glucagon in the perfused liver (Schimassek & Mitzkat, 1963; Exton & Park, 1966; Struck, Ashmore & Wieland, 1965; Sokal, 1966) indicates that glucagon may affect carbohydrate metabolism at two different sites of the metabolic chain. One effect is concerned with glycogenolysis; it causes a conversion of liver glycogen into glucose and a depletion of the glycogen store of the liver (see Foa, 1964). The other, first observed by Schimassek & Mitzkat (1963), is concerned with the promotion of gluconeogenesis from lactate and pyruvate. The question arises whether these two effects have a common denominator. The earlier observations on multiple effects of glucagon, including increased proteolysis and urea synthesis, could be explained on the assumption that there is one primary effect of glucagon, namely the promotion of glycogenolysis, and that all other effects are the consequence of the depletion of liver glycogen. Any glycogen depletion, be it by starvation or by a low carbohydrate diet, promotes gluconeogenesis and the assumption that the primary action of glucagon is the mobilization of glycogen stores was the simplest hypothesis to account for all the earlier observations. A further simple assumption is the view that the physiological depletion of liver glycogen irrespective of the ultimate cause is always mediated by glucagon. Although adrenaline can also mobilize liver glycogen, it is unlikely to do this under physiological conditions as the concentrations necessary to promote glycogenolysis are too high (Sokal & Sarcione, 1964). Once the glycogen stores of the liver have reached low concentrations various regulatory mechanisms cause modifications in the activities of enzymes including those concerned with proteolysis, degradation of amino acids, urea synthesis and gluconeogenesis.

However, the glycogenolytic effect of glucagon cannot account for the observation that glucagon stimulates the conversion of lactate and pyruvate into carbohydrate in a fully depleted liver. Thus at least two sites of action of glucagon on the intermediary metabolism of carbohydrate have to be postulated. This still leaves it open, whether a common agent such as AMP or 3′,5′-cyclic-AMP mediates both effects, the glycogenolytic action by the activation of phosphorylase and the gluconeogenic effect by the activation of pyruvate carboxylase, either directly (Exton, Jefferson, Butcher & Park, 1966) or through the mediation of fatty acids (Struck *et al.* 1965). The fact that the 'direct' gluconeogenic effect of glucagon is found with lactate and pyruvate only, but not with oxaloacetate, proline, glycerol or fructose, suggests that the stage between pyruvate and oxaloacetate is the site of action of this glucagon effect. This has also been suspected by Exton *et al.* (1966) on the basis of measurements of the changes in the steady-state concentrations of the intermediates. Schimassek & Mitzkat (1963) observed that glucagon caused a great rise of the concentrations of glucose 6-phosphate and fructose 6-phosphate and a fall in that of fructose diphosphate in the perfused liver and concluded that the site of action of glucagon is at the level of these phosphates. These observations are probably related to the glycogenolytic action of glucagon; if this were the site of action of the gluconeogenic effect glucagon should also promote gluconeogenesis from oxaloacetate, which is not the case.

Importance of the lactate/pyruvate ratio. A noteworthy observation is the independence of the rate of gluconeogenesis from pyruvate on the lactate/pyruvate ratio in the perfusion medium. On

addition of pyruvate this ratio is very low (less than 0·2; see Table 5). It steadily rises and approaches the physiological range of 10–20. It was also low (1·0) on addition of oxaloacetate, which is readily decarboxylated to pyruvate (Table 6). When the physiological conditions are not disturbed by the addition of large quantities of pyruvate, a precursor of pyruvate, or lactate, the lactate/pyruvate ratio in the medium is similar to that of the cytoplasm (Schimassek, 1963) and reflects the $NADH_2/NAD$ ratio in the cytoplasm. The occurrence of gluconeogenesis is taken to be dependent on this ratio not being too low; otherwise the conditions are unfavourable for the reversal of the triose phosphate dehydrogenase. The fact that gluconeogenesis occurred at low lactate/pyruvate ratios in the medium may mean that under the conditions of the experiments the ratio in the perfusion medium no longer reflects the redox state of the NAD couple in the liver cell cytoplasm.

Formation of lactate from pyruvate and oxaloacetate. The perfused liver formed relatively large amounts of lactate from pyruvate and oxaloacetate. Krebs, Notton & Hems (1966) made analogous observations with rat kidney-cortex slices and mouse liver slices and discussed the origin of the required $NADH_2$.

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