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Formation of hexose 6-phosphates from lactate+pyruvate+glutamate by a cell-free system from rat liver

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A cell-free system prepared from rat liver containing cytosol and mitochondria as well as a number of cofactors and gluconeogenic intermediates at near-physiological concentrations was shown to form hexose 6-phosphates linearly from lactate+pyruvate+glutamate at a rate of 0.82 ± 0.05 μmol/min per g of liver (mean ± S.E.M., $n = 8$, 37 °C). The indicated rates were measured between 20 min and 60 min incubation time, when the system was near steady state. Experiments with either [1-^{14}C]lactate or [U-^{14}C]glutamate revealed that the incorporation of radioactive label into hexose 6-phosphates was proportional to the utilization of lactate+pyruvate and of glutamate during incubation and that both served as gluconeogenic substrates at a ratio of about 2:1. When the [ATP]/[ADP] ratio was lowered from 60 to 19 by addition of ATPase, the rate of hexose 6-phosphate formation fell to one-third. This decrease in gluconeogenic flux was mainly due to a decreased flow through the phosphoglycerate kinase step. Hexose 6-phosphate formation could also be decreased by increasing the ratio [NADH]/[NAD$^+$], either by addition of ethanol or by increasing the initial concentration of lactate+pyruvate at a fixed ratio of 10:1. The observed inhibition was linked to a limitation in the availability of oxaloacetate for the phosphoenolpyruvate carboxykinase reaction and to an increased formation of *sn*-glycerol 3-phosphate. Finally, the rates of hexose 6-phosphate formation in incubations with cytosols from fed rats were only 50% of those observed with cytosols from animals starved for 48 h. One of the limiting steps was found to be the flow through the phosphoenolpyruvate carboxykinase step.

INTRODUCTION

Most of the information on the regulation of gluconeogenesis has been obtained by studies of single purified gluconeogenic enzymes or with experiments using whole cellular systems such as perfused livers or hepatocytes. However, to obtain more detailed information on the regulatory properties of low-M_r compounds such as adenine nucleotides, nicotinamide nucleotides, various ions and intermediary metabolites, it would be preferable to use a cell-free system. Such a system, involving the mitochondrial as well as the cytosolic reactions of gluconeogenesis, has so far only been set up for avian cell preparations. On the one hand, Krebs *et al.* (1964) showed that glucose is formed from lactate at physiological rates in homogenates of pigeon liver, and on the other hand Mendicino & Utter (1962) reported that glucose 6-phosphate is formed from either 3-phosphoglycerate or fumarate by combination of purified gluconeogenic enzymes with chicken liver mitochondria. However, in mitochondria from pigeon as well as from chicken liver, phosphoenolpyruvate carboxykinase is localized within the mitochondria, whereas in rat liver this enzyme is mainly cytosolic. Since most results on hormonal regulation of gluconeogenesis have been obtained with rat liver, and furthermore because pyruvate/phosphoenolpyruvate metabolism is one of the regulatory sites, the avian models are only of limited use.

During the last few years we have succeeded in setting up various cell-free systems for the cytosolic part of gluconeogenesis. Physiological rates for the formation of glucose 6-phosphate could be obtained with glyceraldehyde 3-phosphate (Mörikofer-Zwez & Walter, 1979), 3-phosphoglycerate (Mörikofer-Zwez *et al.*, 1981) or malate (Mörikofer-Zwez *et al.*, 1982) as substrates. The present study describes for the first time a cell-free gluconeogenic system from rat liver comprising the mitochondrial as well as the cytosolic steps of gluconeogenesis. The observed rates of hexose 6-phosphate formation with lactate+pyruvate+glutamate as substrates are comparable with those observed with rat hepatocytes or perfused livers. Furthermore, the dependence of the new system on changes of the redox state, the nucleotide ratio and the nutritional state of the rats is reported. Some results have been presented previously in abstract form (Stoecklin *et al.*, 1983).

EXPERIMENTAL

Materials

Enzymes, coenzymes, adenine nucleotides, 2-oxoglutarate and GSH were purchased from Sigma (St. Louis, MO, U.S.A.). 3-Phosphoglycerate, pyruvate (sodium salt) and dithiothreitol were obtained from Boehringer (Mannheim, Germany), L-(−)-malic acid and L-alanine from Fluka (Buchs, Switzerland), L-[U-^{14}C]glutamic acid was from The Radiochemical Centre (Amersham, Bucks, U.K.), DL-[1-^{14}C]lactate was from New England Nuclear (Boston, MA, U.S.A.) and ITP (sodium salt), L-lactate (sodium salt) and sucrose were from Serva (Heidelberg, Germany). All other reagents were of the highest purity commercially obtainable. Solutions were prepared with double-quartz-distilled water.

Bovine heart mitochondrial ATPase was prepared as described previously (Brawand *et al.*, 1980). To remove $(NH_4)_2SO_4$, the enzyme suspension was centrifuged

before use and the pellet was dissolved in 150 mM-Tris/HCl buffer, pH 7.4.

Male albino Wistar rats from the Swiss Vitamin Institute, Basel, weighing 180–230 g, were used.

Preparation of cystols and mitochondria

Liver cytosols from rats starved for 48 h or from fed rats were prepared as described previously (Mörikofer-Zwez & Walter, 1979). In order to minimize endogenous reactions during the incubations, the resulting cytosols were freed of low-M_r compounds by passage through a column (1.0 cm × 20 cm) of Sephadex G-25 saturated with an elution buffer consisting of 0.14 M-sucrose in 0.05 M-potassium phosphate, pH 7.2. The protein fraction of the cytosol was eluted with the same buffer and was used in the subsequent experiments. Liver mitochondria from fed rats were isolated by the method of Johnson & Lardy (1967) in 0.25 M-mannitol/0.07 M-sucrose/2 mM-Hepes, pH 7.4. For the isolation of mitochondria from rats starved for 48 h, the livers were homogenized with a glass homogenizer with a loosely fitting spherical pestle, thereby causing less mitochondrial damage than by the standard procedure (S. Mörikofer-Zwez, unpublished work). Unless stated otherwise, cytosols from starved rats and mitochondria from fed rats were used.

Incubation procedure

Formation of gluconeogenic and other metabolites was studied in a standard incubation mixture containing 33 mM-Tris/HCl buffer, pH 7.4, 0.66 mM-GSH, 1.0 mM-dithiothreitol, 6 mM-$MgSO_4$, 1.63 mM-NAD^+, 3 mM-ATP, 2 mM-ITP, 90 μM-$MnCl_2$, 80 mM-KCl, 21 mM-$KHCO_3$, 2.0 mM-lactate, 0.2 mM-pyruvate, 1.0 mM-malate, 0.5 mM-3-phosphoglycerate, 3.0 mM-glutamate, 2.0 mM-aspartate, 0.6 mM-alanine and 0.2 mM-2-oxoglutarate. The concentrations of lactate and pyruvate were varied where indicated. The incubation medium was equilibrated with O_2/CO_2 (19:1). Incubations were carried out in stoppered Erlenmeyer flasks (25 ml) in a shaking water bath (frequency 100 strokes/min). After a preincubation period of 3 min at 37 °C, the reaction was started by simultaneous addition of 0.1 ml of mitochondrial suspension containing 0.6 mg of protein and of 0.65 ml of cytosol containing 1.0 mg of protein. The final incubation volume was 1.5 ml, and the final P_i concentration originating from the cytosol was 2.2 mM. Reactions were stopped after 20, 40 or 60 min at 37 °C with ice-cold $HClO_4$ (final concn. 0.4 M). When nucleoside triphosphates and nucleoside diphosphates were measured, the acidic samples were supplemented with 15 mg of pepsin to destroy remaining adenylate kinase activity (Brawand & Walter, 1974). The samples were treated further as described previously (Mörikofer-Zwez *et al.*, 1981).

Analysis of metabolites

In the neutralized extracts, glutamate, alanine, aspartate and 2-oxoglutarate were measured enzymically (Bergmeyer, 1974). All other metabolites were determined as described previously (Mörikofer-Zwez *et al.*, 1982). ITP and ATP on the one hand and IDP and ADP on the other hand were measured together, because the enzymic assays are not specific for adenine nucleotides (Bergmeyer, 1974; Mörikofer-Zwez *et al.*, 1982). Measurement of the adenine nucleotides by h.p.l.c. showed, however, that the ratios for ATP/ADP were essentially the same as for (ATP+ITP)/(ADP+IDP) (Walter *et al.*, 1982, 1983). Formation of [^{14}C]hexose 6-phosphates was studied with L-[1-^{14}C]lactate or [U-^{14}C]glutamate as substrate. Labelled fructose 6-phosphate and glucose 6-phosphate formed during incubation were converted into glucose with phosphoglucoisomerase and microsomal glucose-6-phosphatase (Bergmeyer, 1974). Glucose was then separated from the ionic metabolites as described by Exton & Park (1967), and the incorporation of ^{14}C into glucose was determined as described previously (Mörikofer-Zwez *et al.*, 1982). Protein was determined by the biuret method against an albumin standard.

Calculations

Data on metabolite formation and use of substrates represent net values calculated from the difference between incubated samples and zero-time controls. Metabolite formation between 20 and 60 min incubation time was calculated as the difference of net metabolite formation at 20 and 60 min incubation time. For the calculation of formation rates, 6 ml of cytosol was considered equivalent to 1 g wet wt. of liver. The dilution factor originating from Sephadex filtration of the cytosol was taken into account (for details see Mörikofer-Zwez, 1983).

RESULTS

Characterization of the incubation system

Preliminary experiments confirmed our previous observation (Mörikofer-Zwez *et al.*, 1982) with cell-free systems that a linear formation of glucose 6-phosphate was obtained only after all the gluconeogenic intermediates had reached their steady-state concentration. For example, with malate as gluconeogenic substrate, the concentration of 3-phosphoglycerate proved to be critical and the rate of glucose 6-phosphate formation increased in parallel with that of 3-phosphoglycerate. When 3-phosphoglycerate was added at physiological concentrations at the beginning of the incubation, the time for reaching steady-state conditions could be abbreviated considerably. In the system with lactate+pyruvate+glutamate as gluconeogenic substrates described in the present paper, we found that, besides 3-phosphoglycerate, the concentrations of malate, alanine, aspartate and 2-oxoglutarate were also critical for reaching a linear rate of glucose 6-phosphate formation. These compounds were therefore added at physiological concentrations as reported in the literature (Williamson *et al.*, 1967; Soboll *et al.*, 1976; Brocks *et al.*, 1980). Furthermore, 2 mM-lactate, 0.2 mM-pyruvate and 3.0 mM-glutamate were included as substrates in the incubation mixtures. In order to provide bicarbonate for pyruvate carboxylase, the incubation medium was supplemented with 21 mM-HCO_3^- and equilibrated with CO_2/O_2 (1:19). Since the oxygen partial pressure in the incubation was high, 1 mM-dithiothreitol was also included to avoid inhibition of phosphoenolpyruvate carboxykinase by oxidation (Brinkworth *et al.*, 1981). Other cofactors added were the same as in the system with malate as gluconeogenic precursor (Mörikofer-Zwez *et al.*, 1982).

The time-dependent changes during 60 min for the system described above are shown in Fig. 1. The main products formed were hexose 6-phosphates, citrate, aspartate and alanine. They were rapidly formed between

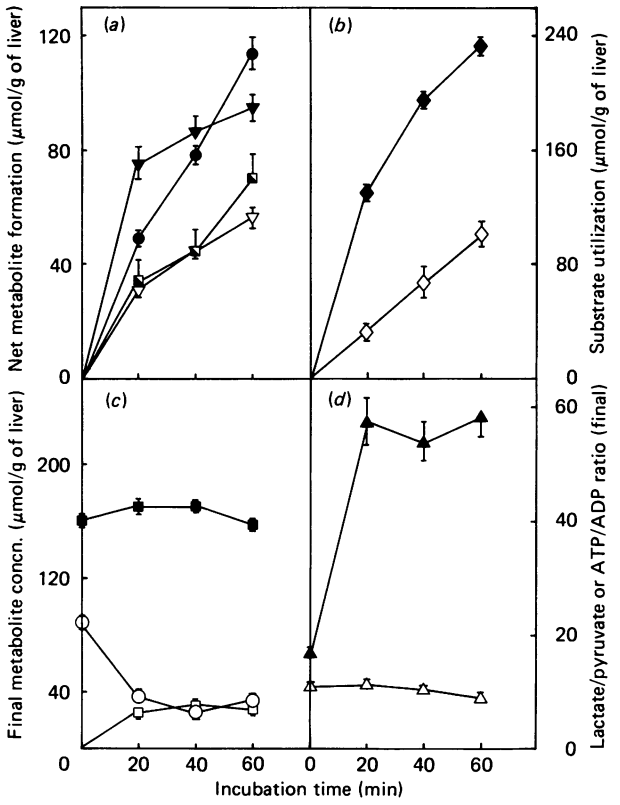

Fig. 1. Time-dependence of metabolite-changes, redox state and nucleotide ratio in the presence of lactate + glutamate

(*a*) Formation of hexose 6-phosphates (●, as C_3 units), citrate (▽), alanine (▼) and aspartate (◪), (*b*) utilization of lactate + pyruvate (◆) and glutamate (◇), (*c*) the sum of the final concentrations of 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate (○), of malate + fumarate (■) and of *sn*-glycerol 3-phosphate (□), and (*d*) the ratio of ATP/ADP (▲) and the ratio of lactate/pyruvate (△) were determined in the standard incubation medium as described in the Experimental section. Results are expressed as means ± S.E.M. from eight independent experiments.

0 and 20 min, and subsequently increased at a lower, but linear, rate for the next 40 min. Of the main substrates used, glutamate disappeared linearly with time from 0 to 60 min, whereas the utilization of lactate + pyruvate decreased from 6.5 μmol/min per g of liver between 0 and 20 min to 2.6 μmol/min per g between 20 and 60 min incubation time. A net formation of *sn*-glycerol 3-phosphate only occurred during the first 20 min, whereas afterwards its concentration remained constant. A similar phenomenon was observed for 3-phosphoglycerate, added initially at a concentration of 0.5 mM. Its concentration decreased during the first 20 min and then remained constant for the rest of the incubation at 0.11 mM. Between 20 and 60 min the total pool of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate was in a steady state. Added malate was rapidly converted into fumarate through the action of fumarase until equilibrium was attained. However, the concentration of these two metabolites together remained stable at 1 mM over the whole incubation period. Furthermore, the concentration of 2-oxoglutarate was also constant at 0.2 mM throughout the incubation (results not shown). Finally, the lactate/pyruvate ratio remained relatively stable over the whole incubation period, whereas the ATP/ADP ratio increased markedly during the first 20 min to 57 and was stable afterwards. The experiments of Fig. 1 clearly show that, between 20 and 60 min, the gluconeogenic incubation system is in a steady state with respect to the concentrations of gluconeogenic intermediates, to the redox state and to the nucleotide ratio. During this incubation period the formation rate of hexose 6-phosphates amounted to 0.82 ± 0.05 μmol/min per g of liver (mean ± S.E.M., $n = 8$, 37 °C).

When only lactate + pyruvate were added as substrates (system without added amino acids, Table 1), the formation of hexose 6-phosphates during the 20–60 min incubation period was decreased by more than 80%. On the other hand, *sn*-glycerol 3-phosphate accumulated as a result of the more reduced redox state favouring *sn*-glycerol 3-phosphate formation from dihydroxyacetone phosphate at the expense of the gluconeogenic

Table 1. Effect of changes in the incubation mixture on metabolite formation

The formation and utilization of intermediary metabolites between 20 min and 60 min incubation time were determined as described in the Experimental section. Results are expressed as means ± S.E.M. from a single experiment with four determinations.

Metabolite(s)	Metabolite changes (μmol/min per g of liver)		
	Complete system	Without added amino acids	Without added lactate + pyruvate
Hexose 6-phosphate	0.67 ± 0.03	0.11 ± 0.02	0.54 ± 0.04
sn-Glycerol 3-phosphate	0.03 ± 0.02	0.39 ± 0.05	−0.08 ± 0.04
Sum of 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate	0.01 ± 0.10	−0.26 ± 0.02	0.15 ± 0.06
Sum of malate + fumarate	−0.05 ± 0.10	−0.33 ± 0.04	−0.77 ± 0.04
Aspartate	0.03 ± 0.12	−0.10 ± 0.05	0.33 ± 0.09
Alanine	1.07 ± 0.18	−0.08 ± 0.03	−0.15 ± 0.09
Glutamate	−1.50 ± 0.17	0.08 ± 0.08	−1.53 ± 0.08
Sum of lactate + pyruvate	−2.01 ± 0.10	−1.07 ± 0.10	−0.10 ± 0.05
Ratio of lactate/pyruvate (final) . . .	8.2 ± 0.3	29.3 ± 2.6	1.1 ± 0.3

Table 2. Net formation and utilization of metabolites under standard conditions

The formation and utilization of intermediary metabolites between 20 and 60 min incubation time was determined as described in the Experimental section. Results are expressed as means ± S.E.M. of eight independent experiments.

Metabolites	Metabolite changes (μmol/g of liver)
Hexose 6-phosphates	32.8 ± 2.0
sn-Glycerol 3-phosphate	3.2 ± 1.2
Sum of 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate	−2.4 ± 1.6
Sum of malate + fumarate	−11.6 ± 4.8
Citrate	26.0 ± 1.6
2-Oxoglutarate	−7.6 ± 0.8
Pyruvate	−5.6 ± 0.4
Lactate	−98.4 ± 3.6
Glutamate	−68.0 ± 6.8
Aspartate	23.6 ± 8.0
Alanine	22.8 ± 4.8
Substrates used . . .	−199.9 ± 17.6
Sum of products formed* . . .	135.2 ± 12.8
Recovery (%) . . .	72.5 ± 14.3

* For calculation of recovery, hexose 6-phosphates are calculated in C_3 units.

reaction sequence. When the incubations were carried out in presence of the amino acids but in absence of lactate + pyruvate, the system was more oxidized, as indicated by the decreased ratio of lactate/pyruvate. As a result, the utilization of malate by oxidation to oxaloacetate was strongly enhanced, thus activating phosphoenolpyruvate carboxykinase. Consequently 3-phosphoglycerate accumulated and the inhibition of glyceraldehyde 3-phosphate dehydrogenase by low concentrations of NADH was partially overcome. Since the NADH-dependent conversion of dihydroxyacetone phosphate into *sn*-glycerol 3-phosphate was also decreased, the formation rate of hexose 6-phosphates was inhibited by only 20% as compared with the complete incubation system.

A balance study (Table 2) revealed that, during steady state, hexose 6-phosphates (calculated in C_3 units) accounted for almost 50% of product formation. Substrates used were mainly lactate + pyruvate (54%) and glutamate (35%), whereas utilization of gluconeogenic intermediates accounted for only 11% of total substrate use. The products listed represent 73% of the substrates used.

The fraction of hexose 6-phosphates derived from either lactate or glutamate was determined with L-[1-^{14}C]lactate or L-[U-^{14}C]glutamate as substrate (Table 3). Since the isotopic dilution of the label in the pools of added intermediates leads to an underestimation of the incorporation of ^{14}C from lactate or glutamate into hexose 6-phosphates, two approximations were made for the calculation. In the first approximation, leading to a minimum value, the assumption was made that the label of the respective substrate is not diluted in the pools of added intermediates. The amount of hexose 6-phosphates formed from either labelled lactate or glutamate was then calculated on the basis of the initial specific radioactivity of the respective substrate, taking into account the loss of $^{14}CO_2$ during the conversion of lactate or glutamate into phosphoenolpyruvate. In the second approximation, leading to a maximum value, it was assumed that the label of L-[1-^{14}C]lactate is evenly distributed in the pools of lactate, pyruvate, alanine, malate, fumarate, aspartate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate and *sn*-glycerol 3-phosphate at 20 and 60 min incubation time. Since, under incubation conditions, pyruvate kinase is active (results not shown), it had to be assumed that the label of L-[U-^{14}C]glutamate is evenly distributed not only in the pools of glutamate, 2-oxoglutarate, aspartate, malate, fumarate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate and *sn*-glycerol 3-phosphate but also in lactate, pyruvate and alanine. The specific radioactivity of lactate or glutamate at 20 min or 60 min incubation time was then calculated as:

$$\text{Sp. radioactivity of lactate (c.p.m./}\mu\text{mol)} = \frac{\text{c.p.m. (total)} - \text{c.p.m. (glucose)}}{\mu\text{mol of } ^{14}\text{C (total)}}$$

$$\text{Sp. radioactivity of glutamate (c.p.m./}\mu\text{mol)} = \frac{\text{c.p.m. (total)} - \text{c.p.m. (glucose)}}{\mu\text{mol of } ^{14}\text{C (total)}} \times 5$$

The total amount of ^{14}C was calculated for either substrate as the sum of the intermediates in the labelled pool, each multiplied by its number of labelled C atoms. The amount of hexose 6-phosphates formed was then calculated by using the specific radioactivities at 20 or 60 min of incubation. The data of Table 3 show that, between 20 and 60 min incubation time, label is incorporated into hexose 6-phosphates from both substrates. During this incubation period maximally 35.9% of the hexose 6-phosphates were formed from lactate, whereas 18.5% were derived from glutamate. This incorporation of label from lactate and glutamate in the ratio 2:1 corresponds to the utilization of these substrates in the balance study.

Table 3. Formation of hexose 6-phosphates from [1-^{14}C]lactate or [U-^{14}C]glutamate

Formation of hexose 6-phosphates from [1-^{14}C]lactate or [U-^{14}C]glutamate was determined as described in the Experimental section in the standard incubation mixture containing 1 μCi of L-[1-^{14}C]lactate or 2 μCi of L-[U-^{14}C]glutamate. Results (means ± S.E.M. for the numbers of experiments given in parentheses) are expressed as percentages of net hexose 6-phosphates formed. For the calculation of minimum values and maximum values see the text.

	Incubation period (min)	Formation of [^{14}C]hexose 6-phosphates (%) from:	
		L-[1-^{14}C]Lactate (4)	L-[U-^{14}C]Glutamate (3)
Minimim value	0–20	2.1 ± 0.5	1.0 ± 0.1
	0–60	6.3 ± 1.1	4.3 ± 0.4
	20–60	9.5 ± 1.5	7.4 ± 1.2
Maximum value	0–20	6.4 ± 1.1	2.5 ± 0.2
	0–60	22.9 ± 3.6	10.9 ± 0.5
	20–60	35.9 ± 5.7	18.5 ± 1.8

Table 4. Effect of the nutritional state of the rats used for the preparation of cytosol and mitochondria on metabolite formation

The formation and utilization of intermediary metabolites between 20 and 60 min incubation time was determined as described in the Experimental section. The sum of all phosphorylated metabolites is the sum of hexose 6-phosphates + *sn*-glycerol 3-phosphate + dihydroxyacetone phosphate + 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate. Results are expressed as means ± S.E.M. for the numbers of experiments shown in parentheses.

		Metabolite changes (μmol/min per g of liver)		
State of rats { cytosol . . . / mitochondria . . .		Starved 48 h / Fed	Starved 48 h / Starved 48 h	Fed / Fed
Metabolite(s)		(8)	(4)	(4)
Hexose 6-phosphates		0.82 ± 0.05	0.83 ± 0.10	0.43 ± 0.06
Sum of malate + fumarate		−0.29 ± 0.12	−0.27 ± 0.09	0.57 ± 0.25
Sum of lactate + pyruvate		−2.59 ± 0.10	−2.71 ± 0.09	−2.11 ± 0.10
Glutamate		−1.70 ± 0.17	−1.61 ± 0.49	−1.58 ± 0.27
Sum of all phosphorylated metabolites* . . .		1.66 ± 0.10	1.81 ± 0.06	0.57 ± 0.23

* Hexose 6-phosphates are calculated as C_3 units.

Effect of the nutritional state of the rats on gluconeogenesis

In the preceding experiments, the incubations were carried out with cytosols from rats starved for 48 h, because here the activity of a key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, is high, whereas the activity of the key glycolytic enzymes pyruvate kinase and phosphofructokinase is low. Mitochondria, however, were isolated from livers of fed rats, because those from starved animals are less resistant to mechanical stress. When mitochondria from starved rats were used, prepared by a gentle homogenization procedure (see the Experimental section), no changes in the metabolite pattern were observed (Table 4). However, when the incubations were carried out with cytosols from fed rats instead of from rats starved for 48 h, the formation of hexose 6-phosphates was lowered from 0.82 to 0.43 μmol/min per g of liver. Furthermore, the overall formation rate of phosphorylated metabolites was lowered from 1.66 to 0.57 μmol/min per g of liver, and malate + fumarate accumulated. The metabolite changes caused by the nutritional state of the rats used for the preparation of cytosol are summarized in a cross-over plot (Fig. 2), showing only one cross-over, between malate and the sum of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate.

Effect of the ratio of ATP/ADP

By analogy with our previous studies with 3-phosphoglycerate and malate as gluconeogenic substrates (Mörikofer-Zwez *et al.*, 1981, 1982), the conversion of glutamate + lactate was also found to be strongly dependent on the ATP/ADP ratio. Fig. 3 shows that the net rate of hexose 6-phosphate formation, as measured between 0 and 60 min incubation time, linearly decreased from 1.10 to 0.38 μmol/min per g of liver, in correlation with a decrease in the final ATP/ADP ratio from 60 to 19. The changes in the nucleotide ratio were achieved by addition of purified mitochondrial ATPase. From the metabolite pattern (not shown) it can be calculated that the gluconeogenic flux was inhibited at the phosphoglycerate kinase step by 25% (ATP/ADP = 41.3) or by 66% (ATP/ADP = 18.8) as compared with the control (ATP/ADP = 59.8). Furthermore the final concentrations of malate + fumarate fell from 0.93 mM to 0.57 mM, indicating that mitochondrial reactions also

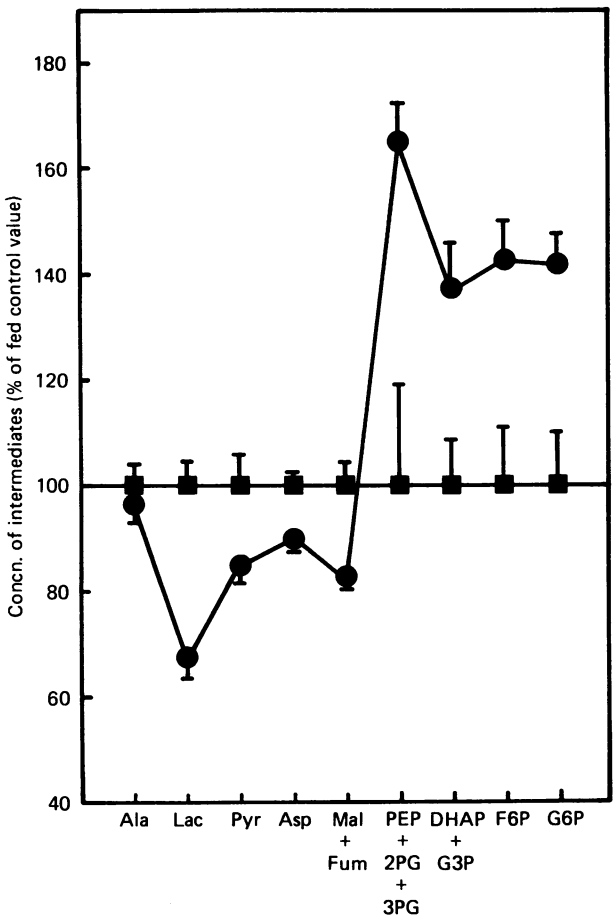

Fig. 2. Effect of the nutritional state of the rats used for the preparation of cytosol on the final concentrations of intermediates

Concentrations of intermediates obtained by incubating cytosols from rats starved for 48 h were plotted as percentages of those obtained by incubating cytosols from fed rats. The concentrations of intermediates were determined at 60 min incubation time in the standard incubation medium as described in the Experimental section. Results are expressed as means ± S.E.M. from eight (rats starved 48 h) and four (fed rats) independent experiments. Control values (fed rat), in μmol/incubation are: alanine (Ala), 1.86; lactate (Lac), 1.46; pyruvate (Pyr), 0.13; aspartate (Asp), 3.93; malate + fumarate (Mal + Fum), 1.72; phosphoenolpyruvate + 2-phosphoglycerate + 3-phosphoglycerate (PEP + 2PG + 3PG), 0.20; dihydroxyacetone phosphate + *sn*-glycerol 3-phosphate (DHAP + G3P), 0.24; fructose 6-phosphate (F6P), 0.09; glucose 6-phosphate (G6P), 0.27.

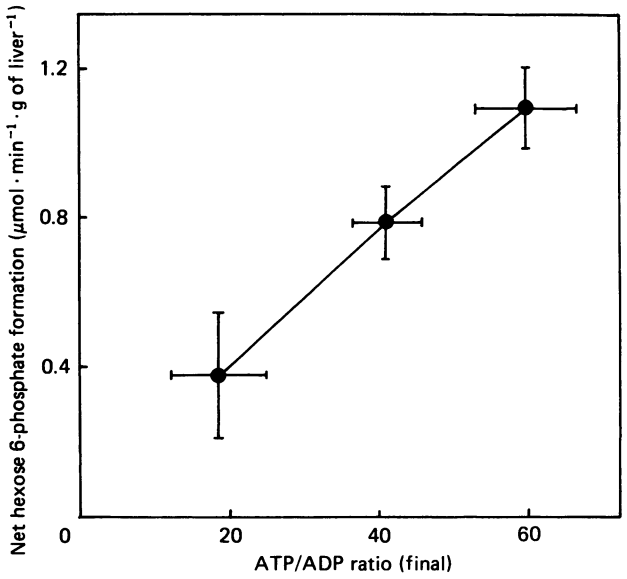

Fig. 3. Effect of changes in the ratio of ATP/ADP on the formation of hexose 6-phosphates from lactate + glutamate

The net hexose 6-phosphate formation rate and the final ratio of ATP/ADP were determined in the standard incubation medium as described in the Experimental section. Incubation time was 60 min. Between 0 and 0.2 unit (μmol/min) of purified mitochondrial ATPase was added to lower the ATP/ADP ratio. Results are expressed as means ± S.E.M. from four independent experiments.

were influenced by lowering the nucleotide ratio from 60 to 19. The lactate/pyruvate ratio remained constant at about 10. However, since the malate concentration decreased, the oxaloacetate concentration can be calculated to fall from 17 μM to 9 μM. As a consequence the activity of phosphoenolpyruvate carboxykinase was decreased from 1.94 to 1.15 μmol/min per g of liver.

Effect of redox state

The results in Fig. 4 show that a change in the initial concentration of lactate + pyruvate at a constant ratio of 10:1 led to a more reduced redox ratio and to an inhibition of hexose 6-phosphate formation, whereas *sn*-glycerol 3-phosphate accumulated. Furthermore, a decreased formation of the sum of 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate was observed, with increasing final ratios of lactate + pyruvate and a concomitant accumulation of malate + fumarate. Similar effects were observed when the final ratio of lactate/pyruvate was increased by addition of ethanol to the incubation medium. Fig. 5 shows that addition of 1 mM- and 5 mM-ethanol resulted in an increase of the lactate/pyruvate ratio from 10.3 to 17.1 and 28.9 respectively. At the same time the synthesis of hexose 6-phosphate was decreased, whereas the formation of *sn*-glycerol 3-phosphate and malate was increased.

DISCUSSION

The present study describes for the first time the conversion of lactate + pyruvate + glutamate into hexose 6-phosphates in a broken-cell preparation of rat liver, thus combining the mitochondrial and the cytosolic parts of the gluconeogenic pathway. The formation rate of hexose 6-phosphates amounts to 0.82 ± 0.05 μmol/min per g of liver under standard conditions. This rate is within the physiological range, since glucose formation from C_3 precursors in hepatocytes (Whitton *et al.*, 1978) or in perfused livers from starved rats (Ross *et al.*, 1967; Williamson *et al.*, 1969) has been reported to occur at rates between 0.5 and 1.3 μmol/min per g of liver. Microsomal fractions for the conversion of glucose 6-phosphate into glucose were not included in the present incubation system, because their membranes contain a

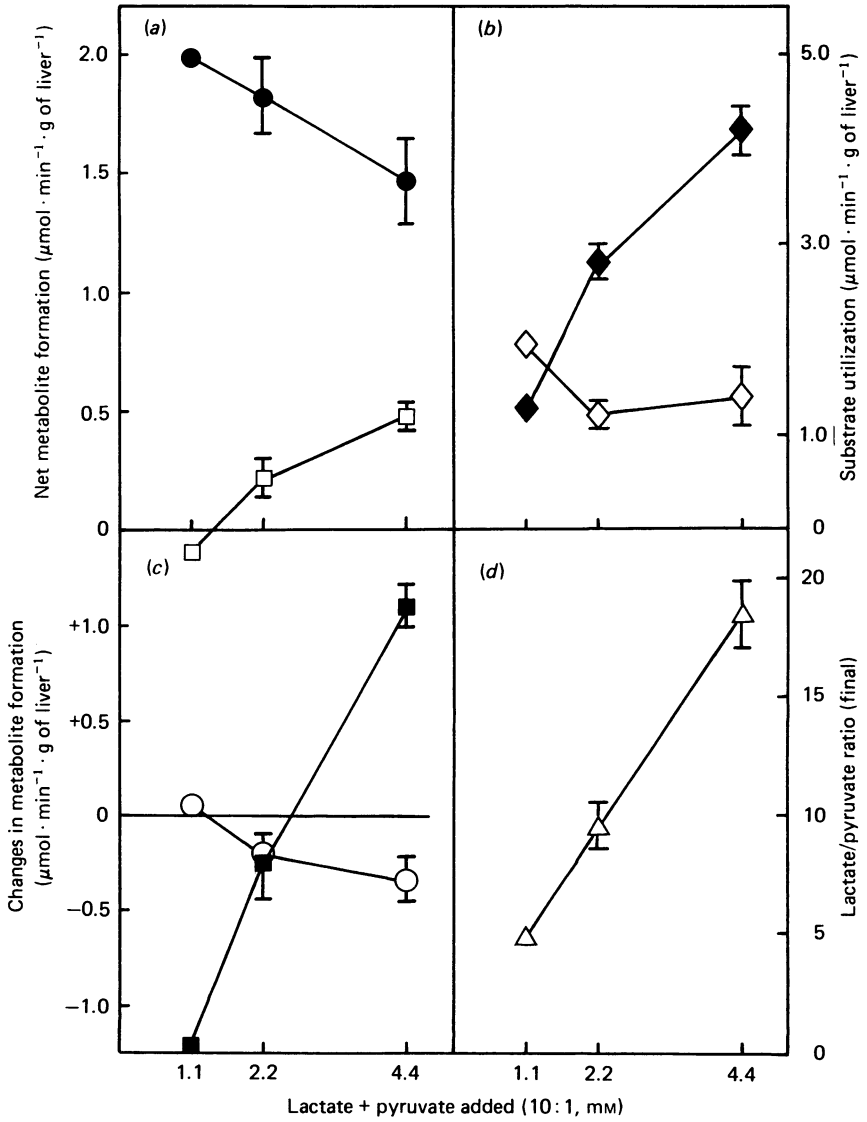

Fig. 4. Effect of increased concentration of lactate + pyruvate on metabolite changes and redox state

(*a*) Formation of hexose 6-phosphates (●, as C_3 units) and *sn*-glycerol 3-phosphate (□), (*b*) utilization of lactate + pyruvate (◆) and glutamate (◇), (*c*) formation or utilization of the sum of 3-phosphoglycerate + 2-phosphoglycerate + phosphoenolpyruvate (○) and malate + fumarate (■), and (*d*) the final ratio of lactate/pyruvate (△) were determined under standard incubation conditions. The initial concentration of added lactate + pyruvate (10:1) was varied from 1.1 to 4.4 mM. Incubation period was between 20 and 60 min. Results are expressed as means of two independent experiments (1.1 mM lactate + pyruvate added) or as means ± S.E.M. from six independent experiments (other concentrations of lactate + pyruvate added).

protein that inactivates phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase (Francis & Ballard, 1980).

In the standard incubation system, glutamate and lactate + pyruvate were the main substrates during the incubation period from 20 to 60 min, whereas all the other added compounds, such as malate, 3-phosphoglycerate and 2-oxoglutarate, reached steady-state concentrations after 20 min incubation time. Besides hexose 6-phosphates, the major products formed were citrate, aspartate and alanine. These products corresponded to 73% of the substrate used. On the basis of earlier work with a similar incubation system (Mörikofer-Zwez *et al.*, 1982), it is likely that a large part of the 27% of substrate unaccounted for in the balance study was oxidized by the mitochondria. Because the urea cycle in the present system was only functioning at a very limited rate, owing to the lack of ornithine (F. Brawand, unpublished work), two-thirds of the amino nitrogen lost by glutamate was recovered in alanine and aspartate. These two amino acids presumably accumulated because 2-oxoglutarate formed by transamination from glutamate was constantly removed in the citric acid cycle, thereby shifting the two transaminase reactions towards aspartate and alanine respectively. The continuous accumulation of citrate was unexpected, since it would be thought to be metabolized in the citric acid cycle. This observation, as well as the influence of an operative urea cycle on gluconeogenesis in our standard system, require further investigation.

Somewhat surprising was the finding that an increase

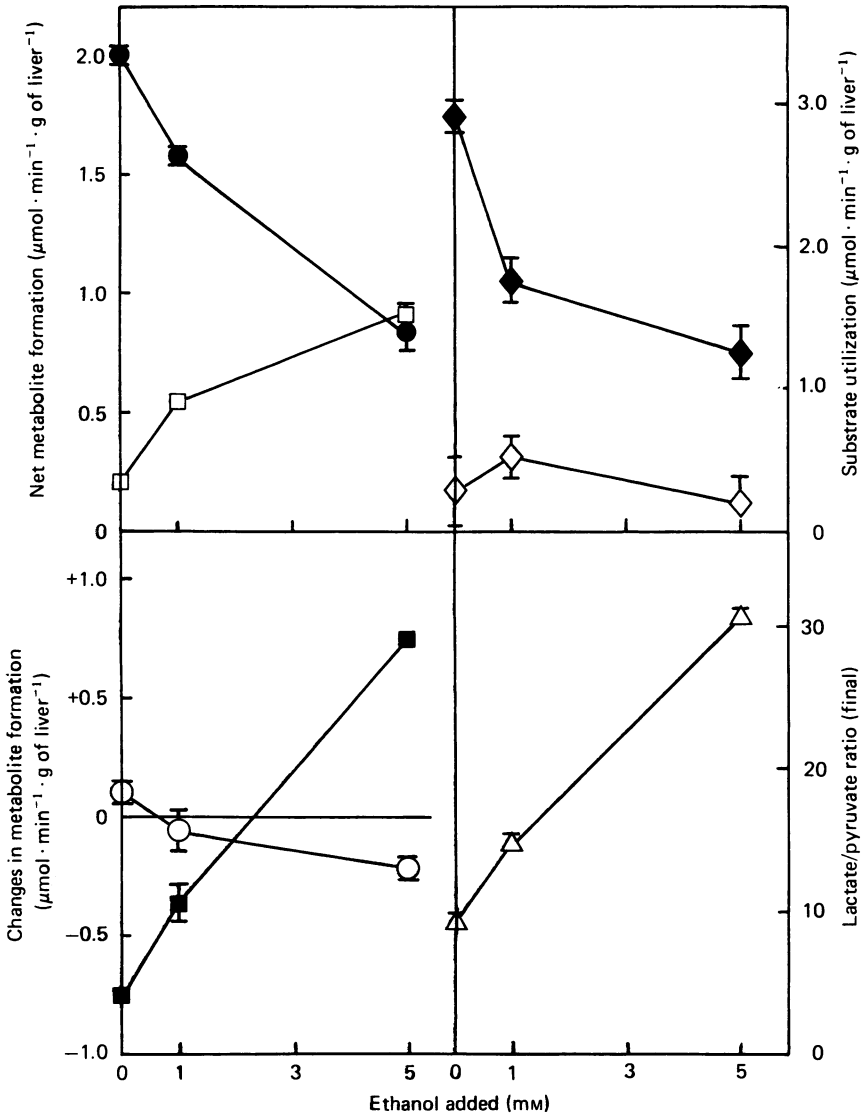

Fig. 5. Effect of ethanol addition on metabolite changes and redox state

Parameters determined and symbols are the same as in Fig. 4. Incubations were carried out in the standard incubation medium containing 0–5.0 mM-ethanol as described in the Experimental section. Incubation period was between 20 and 60 min. Results are expressed as means ± S.E.M. from a single experiment with four determinations.

in the initial concentrations of lactate + pyruvate at a fixed ratio of 10:1 in the standard incubation medium did not lead to an increase in hexose 6-phosphate formation, but resulted in a more reduced status of the system and consequently in an inhibition of gluconeogenesis. The reason for the increasing lactate/pyruvate ratios is probably connected to the fact that a larger portion of the pyruvate is metabolized in the citric acid cycle as a result of the higher pyruvate concentrations. This extra NADH, produced by the transformation of lactate to pyruvate, cannot be fully reoxidized by glyceraldehyde-3-phosphate dehydrogenase and *sn*-glycerol-3-phosphate dehydrogenase. As a result, NADH accumulates, explaining the higher lactate/pyruvate ratio.

To test the validity of the broken-cell preparation as a model for whole-cell systems, the effects of changes in the nutritional state were investigated. With cytosols from starved rats, the rate of hexose 6-phosphate formation was twice that obtained with cytosols from fed rats. This finding is in full agreement with the well-known changes in whole-cell systems (Ross *et al.*, 1967). In addition, the cross-over plot of the intermediates in Fig. 2 was very similar to that obtained by Exton & Park (1969), using isolated perfused livers, and point to a possible regulatory role of phosphoenolpyruvate carboxykinase and/or of pyruvate kinase. The results in Table 4 show that an accumulation of malate occurred in the experiments with cytosols from fed rats, whereas in those from starved rats a net utilization of malate was observed. At the same time, formation of metabolites beyond the phosphoenolpyruvate carboxykinase step (sum of phosphorylated metabolites in Table 4) was 3 times higher in the cytosols from starved rats than in those from fed animals. These results are in agreement with the fact that phosphoenolpyruvate carboxykinase is induced under starvation (Tilghman *et al.*, 1976) and indicate an

important role for this enzyme in the stimulation of gluconeogenesis observed in the starved state. It cannot, however, be decided to what extent also a decrease in the activity of pyruvate kinase, known to occur under starvation (van Berkel *et al.*, 1977), contributes to the observed effect.

In agreement with results obtained from experiments with perfused livers, infusion of ethanol caused a marked increase in the cytosolic NADH/NAD^+ ratio and an inhibition of gluconeogenesis (Krebs *et al.*, 1969). From the results of the experiment in Fig. 5 it can be concluded that the increased ratio of NADH/NAD^+ has three consequences, which together are responsible for the observed decrease in hexose 6-phosphate formation. (*a*) The utilization of lactate + pyruvate decreases considerably, because the pyruvate concentration on addition of ethanol decreased from 0.08 to 0.04 mM, thereby decreasing the flow through pyruvate carboxylase. (*b*) Even though less pyruvate is metabolized, more malate is found. Owing to the highly reduced state, the oxaloacetate concentration can be calculated to fall from 18 μM to about 7 μM on addition of ethanol, thereby limiting the substrate for phosphoenolpyruvate carboxykinase and resulting in the accumulation of malate. (*c*) It would be expected that the flow through glyceraldehyde-3-phosphate dehydrogenase would be higher in the presence of ethanol because the NADH/NAD^+ ratio is increased. It can, however, be calculated from the sum of trioses + hexose 6-phosphates that the flow through glyceraldehyde-3-phosphate dehydrogenase remains unchanged on addition of ethanol. It appears therefore that the lower concentration of substrate compensates for the expected effect of the higher NADH/NAD^+ ratio, resulting in an unchanged flow through this enzyme. On the other hand, owing to the more reduced state, a larger proportion of the glyceraldehyde 3-phosphate is metabolized to *sn*-glycerol 3-phosphate in the presence of ethanol than in the controls, thereby inhibiting the formation of hexose 6-phosphates.

As outlined in the Results section, omission of either lactate + pyruvate or of the amino acids led to changes in the redox state of the system and as a consequence to inhibition of hexose 6-phosphate formation. On one hand, without lactate + pyruvate but with the amino acids, the availability of NADH for the glyceraldehyde 3-phosphate dehydrogenase became limiting, owing to the lack of lactate. Even though malate oxidation provided some of the missing NADH, the system became more oxidized. On the other hand, when the amino acids were not added but pyruvate + lactate was present, oxaloacetate formed by pyruvate carboxylation could leave the mitochondria only via malate, because intramitochondrial transamination of oxaloacetate to aspartate was not possible. Together with the added lactate, this resulted in an excess of reducing equivalents in the cytosol, leading to a more reduced state of the system. The present cell-free system therefore demonstrates very clearly that the oxidoreduction status of the liver favourable for gluconeogenesis can only be preserved if lactate + pyruvate as well as the amino acids are present.

When the ratio of ATP/ADP was decreased by addition of ATPase, the flow through phosphoglycerate kinase and phosphoenolpyruvate carboxykinase was decreased; however, at a nucleotide ratio of 19 the rate of hexose 6-phosphate formation was still one-third of the maximal rate observed at a ratio of 60. The nucleotide ratios in this study represent extramitochondrial values, because of the low concentration of mitochondrial protein added. The measured cytosolic ratio of total ATP/ADP in intact liver has been reported to lie between 5 and 10 (Akerboom *et al.*, 1978; Soboll *et al.*, 1980; Schwenke *et al.*, 1981). However, as suggested by Veech *et al.* (1979) and Iles *et al.* (1985), the ratio of free ATP/free ADP may be higher than the measured cytosolic ratios, owing to binding of ADP to proteins. Since the protein concentration is very low in the present cell-free system, much less binding of ADP is to be expected. It is therefore quite likely that the ATP/ADP ratios used in these experiments are quite close to the actual physiological ratios.

The cell-free gluconeogenic model system described here for the first time closely reflects many features of cellular hepatic systems used so far in the study of the gluconeogenic pathway: the gluconeogenic rate is within the physiological range, starvation activates and ethanol inhibits gluconeogenic flux. There is, however, one principal difference between this broken-cell system and all cellular systems, including those where the plasma membrane is perforated by treatment with filipin (Gankema *et al.*, 1981) or digitonin (Aragon *et al.*, 1980). In cellular systems, the biochemically important concentration of free effectors in most cases cannot be measured, but can only be calculated on the basis of constants which may or may not apply in a particular case. The present system, however, operates at very high dilution, where binding of low-M_r compounds to proteins is negligible. Therefore, effects observed in response to measurable concentrations of low-M_r compounds can be taken to represent the action of the free effector. This special property of the system has allowed us to study the relationship between gluconeogenesis and the ratio of free ATP/free ADP. Furthermore, the effect of the redox state could be assessed in relation to changing concentrations of free oxaloacetate and free pyruvate. The possibility of studying the influence of free effectors could be extended to investigations on the effect of fructose 2,6-bisphosphate, Mn^{2+} and P_i on gluconeogenesis.

The gluconeogenic system described is also one of the few extended multienzyme systems operative outside the cellular environment. Since the steady-state concentrations of the intermediary metabolites can be manipulated, it provides the means for the determination of kinetic parameters under near-physiological conditions. On a more restricted basis, this property of cell-free gluconeogenic systems has been used for the study of fructose-1,6-bisphosphatase (Mörikofer-Zwez, 1983).

Furthermore, the system presented allows one to study the gluconeogenic pathway either alone or in combination with other metabolic pathways. Addition of ornithine and ammonia opens the possibility of studying the interrelationship between urea synthesis and gluconeogenesis, whereas addition of NADP(H) or UTP will permit investigations on the interdepenence of different glucose-utilizing pathways.

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