Effect of Glucagon on Metabolite Compartmentation in Isolated Rat Liver Cells during Gluconeogenesis from Lactate

By ELMAR A. SIESS, DIETRICH G. BROCKS, HERBERT K. LATTKE
and OTTO H. WIELAND

*Forschergruppe Diabetes und Klinisch-Chemisches Institut, Städtisches Krankenhaus München-Schwabing,
8000 München 40, Kölner Platz 1, Federal Republic of Germany*

(Received 14 February 1977)

1. The subcellular distribution of adenine nucleotides, acetyl-CoA, CoA, glutamate, 2-oxoglutarate, malate, oxaloacetate, pyruvate, phosphoenolpyruvate, 3-phosphoglycerate, glucose 6-phosphate, aspartate and citrate was studied in isolated hepatocytes in the absence and presence of glucagon by using a modified digitonin procedure for cell fractionation. 2. In the absence of glucagon, the cytosol contains about two-thirds of cellular ATP, some 40–50% of ADP, acetyl-CoA, citrate and phosphoenolpyruvate, more than 75% of total 2-oxoglutarate, glutamate, malate, oxaloacetate, pyruvate, 3-phosphoglycerate and aspartate, and all of glucose 6-phosphate. 3. In the presence of glucagon the cytosolic space shows an increase in the content of malate, phosphoenolpyruvate and 3-phosphoglycerate by more than 60%, and those of aspartate and glucose 6-phosphate rise by about 25%. Other metabolites remain unchanged. After glucagon treatment, cytosolic pyruvate is decreased by 37%, whereas glutamate and 2-oxoglutarate decrease by 70%. The $[NAD^+]/[NADH]$ ratios calculated from the cytosolic concentrations of the reactants of lactate dehydrogenase and malate dehydrogenase were the same. Glucagon shifts this ratio and also that of the $[NADP^+]/[NADPH]$ couple towards a more reduced state. 4. In the mitochondrial space glucagon causes an increase in the acetyl-CoA and ATP contents by 25%, and an increase in [phosphoenolpyruvate] by 50%. Other metabolites are not changed by glucagon. Oxaloacetate in the matrix is only slightly decreased after glucagon, yet glutamate and 2-oxoglutarate fall to about 25% of the respective control values. The $[NAD^+]/[NADH]$ ratios as calculated from the [3-hydroxybutyrate]/[acetoacetate] ratio and from the matrix [malate]/[oxaloacetate] couple are lowered by glucagon, yet in the latter case the values are about tenfold higher than in the former. 5. Glucagon and oleate stimulate gluconeogenesis from lactate to nearly the same extent. Oleate, however, does not produce the changes in cellular 2-oxoglutarate and glutamate as observed with glucagon. 6. The changes of the subcellular metabolite distribution after glucagon are compatible with the proposal that the stimulation of gluconeogenesis results from as yet unknown action(s) of the hormone at the mitochondrial level in concert with its established effects on proteolysis and lipolysis.

Intermediary metabolites have now been measured in a number of liver perfusion studies (Schimassek & Mitzkat, 1963; Ross *et al.*, 1967a; Exton & Park, 1969; Williamson *et al.*, 1969c; Veneziale *et al.*, 1970; Veneziale, 1971, 1972; Blair *et al.*, 1973a,b; Ui *et al.*, 1973a,b; Müllhofer *et al.*, 1974; Parrilla *et al.*, 1975, 1976) in order to find out the site(s) of glucagon action in the gluconeogenic pathway. However, as pointed out repeatedly (Exton & Park, 1969; Williamson *et al.*, 1969b; Ui *et al.*, 1973a), the interpretation of the results accumulated has been hampered by the lack of information on metabolite compartmentation in the parenchymal liver cell. At present, evidence for glucagon acting at the levels of mitochondrial pyruvate (Adam & Haynes, 1969; Garrison & Haynes, 1974; Yamazaki & Haynes, 1975; Titheradge

& Coore, 1976a) and/or hydrogen influx (Müllhofer *et al.*, 1974), of pyruvate carboxylase (EC 6.4.1.1) (Williamson *et al.*, 1969c; Ross *et al.*, 1967a), 2-oxoglutarate dehydrogenase (EC 1.2.4.2) (Ui *et al.*, 1973b), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Exton & Park, 1969), fructose 1,6-bisphosphatase (EC 3.1.3.11), phosphofructokinase (EC 2.7.1.11) (Schimassek & Mitzkat, 1963; Williamson *et al.*, 1969c; Clark *et al.*, 1974) and pyruvate kinase (EC 2.7.1.40) (Feliú *et al.*, 1976; Friedrichs, 1976; Pilkis *et al.*, 1976; Blair *et al.*, 1976) is available; however a differentiation between primary and secondary site(s) of action has not been achieved.

Parrilla *et al.* (1975, 1976) attempted to obtain information on the subcellular metabolite distribution by using calculation methods; these, however,

were based on as yet unestablished assumptions, especially with respect to the glucagon-stimulated liver. Therefore we considered the problem suitable for investigation by a different approach. We applied the digitonin fractionation method developed by Zuurendonk & Tager (1974), to investigate the metabolite compartmentation in isolated rat liver cells during gluconeogenesis from lactate in the absence and presence of glucagon in the present study.

Materials and Methods

Animals

Male Sprague-Dawley rats (W. Gassner, Sulzfeld, Germany) weighing about 200 g after starvation for 48 h were used for liver cell preparation. Normal fed rats under Nembutal anaesthesia were bled to obtain the serum added to the incubation medium to improve the glucagon response (Siess & Wieland, 1975).

Chemicals

Coenzymes and enzymes were products of Boehringer (Mannheim, Germany), except for glutaminase (EC 3.5.1.2) which was obtained from Sigma (Munich, Germany), and lactate dehydrogenase (EC 1.1.2.3), which was prepared from baker's yeast by a modification (Wieland & von Jagow-Westermann, 1974b) of the method of Dixon (1955). Glucagon was a gift from Hoechst (Frankfurt-Hoechst, Germany). L-Lysine monohydrochloride was bought from E. Merck (Darmstadt, Germany), and L-lactic acid from Schuchardt (Munich, Germany). Other chemicals were the same as in an earlier study (Siess *et al.*, 1976a).

Preparation and incubation of liver cells

Liver cells were prepared as described (Siess *et al.*, 1976a). In the present study the washing medium also contained 2 mM-lysine to improve the rate of gluconeogenesis (Cornell *et al.*, 1973, 1974). At least 92% of the hepatocytes excluded 0.2% Trypan Blue. The cells were incubated as described (Siess *et al.*, 1976a) in a medium composed of 0.6 ml of Ca²⁺-free Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932), pH 7.4, containing 1.5% gelatin, 1 mM-L(-)-carnitine and 2 mM-lysine, 0.4 ml of rat serum, 0.1 ml of 10% defatted (Chen, 1967) bovine serum albumin in 0.9% NaCl, 0.01 ml of 80 mM-CaCl₂ and 0.05 ml of 325 mM-lactate. Incubation was commenced by the addition of 0.2 ml of the liver cell suspension.

Liver cell fractionation

Separation of the cytosolic and mitochondrial fractions by a modified digitonin method (Zuuren-

donk & Tager, 1974; Siess & Wieland, 1976) and the determination of the total intracellular amounts of metabolites were performed as described by Siess *et al.* (1976a).

Determination of metabolites

Aspartate was determined by the method of Bergmeyer *et al.* (1974), glutamate by the method of Bernt & Bergmeyer (1974), glutamine by the method of Lund (1974), glucose 6-phosphate by the method of Lang & Michal (1974), lactate by the method of Wieland & von Jagow-Westermann (1974a), pyruvate and phosphoenolpyruvate by the method of Czok & Lamprecht (1974), 2-oxoglutarate by the method of Bergmeyer & Bernt (1974), and 3-phosphoglycerate by the method of Czok (1974). Other metabolites were assayed by the methods used by Siess *et al.* (1976a). Oxaloacetate was measured as described previously (Siess *et al.*, 1976b), except that in the present study reaction mixtures with water in place of acetyl-CoA were run in parallel to correct for any background radioactivity.

Calculation of metabolite concentrations

Cytosolic and mitochondrial metabolite concentrations in the absence and presence of glucagon were calculated as described previously (Siess *et al.*, 1976a). As shown by Titheradge & Coore (1976b), glucagon does not change the mitochondrial matrix volume.

Results

Total cellular amounts of metabolites

Total cellular contents of adenine nucleotides, tricarboxylic acid-cycle intermediates, glycolytic and other metabolites determined in isolated hepatocytes incubated for 40 min in the presence of lactate are summarized in Table 1. The values obtained with the isolated liver cells closely resemble those found in liver-perfusion studies performed under comparable conditions, indicating that our cell preparations represent a suitable model for studies on metabolite compartmentation in the liver parenchymal cells. This view is further strengthened by the high capacity for glucose synthesis from lactate, and by the response to glucagon. As shown in Table 2, this hormone stimulated gluconeogenesis, ureogenesis and ketogenesis by 79, 132 and 84% respectively. Concomitantly, glucagon caused an increase in lactate uptake, a decrease of pyruvate oxidation and a rise of the [3-hydroxybutyrate]/[acetoacetate] ratio. In a similar type of experiment the active form of pyruvate dehydrogenase (EC 1.2.4.1) assayed as described by

Table 1. *Contents of metabolites in liver cells from 48h-starved rats after incubation with initially 11.9mm-lactate for 40min in a serum-containing mixture*

Further details are described in the Materials and Methods section. Mean values $\pm$ S.E.M. are given for the numbers of cell preparations in parentheses.

Metabolite	Amount of metabolite (nmol/g dry wt.) present in		
	Perfused liver		
	Isolated hepatocytes	Williamson <i>et al.</i> (1969c)	Parrilla <i>et al.</i> (1975)
ATP	8830 $\pm$ 400 (9)	12380	7740
ADP	2340 $\pm$ 120 (9)	2550	2333
AMP	670 $\pm$ 40 (7)	470	1595
Acetyl-CoA	179 $\pm$ 21 (9)	189	347
CoA	600 $\pm$ 60 (6)	771	412
Aspartate	1470 $\pm$ 110 (10)	3210	1902
Glutamate	13500 $\pm$ 1820 (9)	13090	9481
Citrate	1830 $\pm$ 190 (9)	3700	2118
2-Oxoglutarate	2210 $\pm$ 210 (7)	2840	1763
Malate	1020 $\pm$ 170 (10)	1060	1186
Oxaloacetate	21.9 $\pm$ 1.8 (13)	18.5	18.7
Pyruvate	1060 $\pm$ 60 (6)	1960	995
Phosphoenolpyruvate	770 $\pm$ 80 (9)	980	466
3-Phosphoglycerate	540 $\pm$ 60 (6)	11440	351
Glucose 6-phosphate	190 $\pm$ 10 (5)	239	180

Siess & Wieland (1976) was decreased ($P < 0.0125$) from 17.5 ± 3.4 to $14.7 \pm 3.6\%$ (means $\pm$ S.E.M. for five experiments) by glucagon (E. A. Siess & O. H. Wieland, unpublished work).

The metabolic changes caused by glucagon in isolated liver cells agree with the effects of this hormone observed in liver perfusion (Struck *et al.*, 1965, 1966; Ross *et al.*, 1967a; Schimassek, 1967; Williamson *et al.*, 1969c; Blair *et al.*, 1973b; Ui *et al.*, 1973b). The results of our studies on cytosolic and mitochondrial metabolite distribution in the absence

and presence of glucagon are summarized in Tables 3 and 4.

Cytosolic space

In the cytosolic space statistically significant changes were observed in the amounts of ATP, malate, aspartate, phosphoenolpyruvate, pyruvate, glucose 6-phosphate, 2-oxoglutarate, glutamate and 3-phosphoglycerate, whereas all other metabolites studied remained unchanged (Table 3). The cytosolic ATP concentration was moderately decreased by glucagon, resulting in a fall of the [ATP]/[ADP] ratio from 5.6 to 4.5. The most marked changes occurred in the concentrations of 2-oxoglutarate and glutamate, which fell to about 30% of their control value. This is not due to increased glutamine formation, which was instead decreased (results not shown). The decrease in the cytosolic [pyruvate] by 37% at an unchanged cytosolic [lactate] of 8.2mm caused the [lactate]/[pyruvate] ratio to increase from 13 to 21. The [malate]/[oxaloacetate] couple also displayed a higher state of reduction in the presence of glucagon, as indicated by [malate]/[oxaloacetate] ratios of 47 and 63 respectively, owing to an increase in [malate] by 64% at nearly unchanged oxaloacetate concentrations. Among the glycolytic intermediates measured, glucagon led to an increase in cytosolic phosphoenolpyruvate and 3-phosphoglycerate concentrations by more than 70%, and that of glucose 6-phosphate was 25% higher than in the controls. No significant change of total intracellular glucose was measurable, values being 14.2 ± 0.58 and $15.33 \pm 0.58 \mu\text{mol/g dry wt.}$ (mean $\pm$ S.E.M. for seven different cell preparations) in the absence and presence of glucagon respectively.

Pellet fraction

Table 4 summarizes the metabolite contents found in the pellet fraction after digitonin fractionation of liver cells incubated in the absence or presence of glucagon. As in the cytosolic space, glucagon also affected the ATP concentration in the mitochondrial fraction, but in the opposite direction, as it increased by 25%. Concomitantly the matrix [ATP]/[ADP] ratio rose from 2.2 to 2.5, [ADP] again remaining unchanged. The decreasing effect of glucagon on the amounts of 2-oxoglutarate and glutamate in the mitochondrial fraction was even more pronounced than in the cytosol (amounting to 75 and 80% respectively). Table 4 further shows that glucagon slightly increased the amount of matrix acetyl-CoA without changes in CoA, and the amount of oxaloacetate was somewhat decreased, without apparent changes in citrate, malate or aspartate. Unexpectedly, some 40% of total intracellular phosphoenolpyruvate and about 20% of 3-phosphoglycerate were found in the mitochondrial fraction in both the absence and the presence of glucagon (Table 3).

Table 2. *Effect of glucagon on gluconeogenesis, ureogenesis and ketogenesis in isolated liver cells from 48 h-starved rats in the presence of lactate*

The cells were incubated for 30 min before glucagon (7 µg/ml) was added and incubation continued for 10 min. Initial lactate concentration was 11.9 mM, and 8.5 mM at the beginning of the glucagon incubation. Mean values ± S.E.M. are given for the numbers (*n*) of different cell preparations. Rate measurements are given for the 10 min period between 30 and 40 min of incubation. Statistical significance (*P*) was calculated by Student's *t*-test for paired data.

	<i>n</i>	Metabolite change (µmol/10 min per g dry wt.)		
		Control	Glucagon	<i>P</i>
Glucose	18	40.8 ± 4.0	72.9 ± 4.6	<0.001
Lactate used	8	71 ± 4	99 ± 12	<0.05
Urea	6	10.1 ± 1.0	23.4 ± 4.4	<0.01
Glucose formed from protein as calculated from urea*		10.1	23.4	
Glucose formed from lactate		30.7	49.5	
Lactate used for glucose formation		61.4	99	
Percentage of lactate used for glucose formation		86.5	100	
3-Hydroxybutyrate	12	1.3 ± 0.3	6.9 ± 0.7	<0.001
Acetoacetate	13	4.3 ± 0.5	3.4 ± 1.1	
[3-Hydroxybutyrate]/[acetoacetate]		0.3	2.0	
[Lactate]/[pyruvate]	13		21	

* According to Menahan & Wieland (1969).

Table 3. *Effect of glucagon on cytosolic metabolite concentrations in isolated liver cells from 48 h-starved rats incubated in the presence of lactate*

The cells were incubated for 30 min before glucagon (7 µg/ml) was added and incubation continued for 10 min. Initial lactate concentration was 11.9 mM, and 8.5 mM at the beginning of the glucagon incubation. Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses. Significance was calculated by Student's *t*-test for paired data. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0005. The cytosolic portion as a percentage of the total cellular amount is given in brackets.

Metabolite	Amount (nmol/g dry wt.)		Glucagon effect (%)	Concentration (mM)	
	Control	Glucagon		Control	Glucagon
ATP	6020 ± 390 [68.2] (9)	5180 ± 390*** [59.7] (9)	-14	4.36	3.71
ADP	1080 ± 70 [46.2] (9)	1160 ± 130 [45.7] (9)		0.78	0.84
Acetyl-CoA	78 ± 13 [43.6] (9)	75 ± 7 [37.1] (9)		0.057	0.054
CoA	100 ± 30 [16.4] (6)	140 ± 40 [20.9] (6)		0.073	0.102
Aspartate	1360 ± 120 [92.5] (10)	1670 ± 180* [93.6] (10)	+23	0.99	1.22
Glutamate	10570 ± 1770 [80.4] (8)	2990 ± 530**** [85.4] (8)	-72	7.73	2.19
Citrate	790 ± 130 [38.8] (8)	630 ± 120 [37.9] (8)		0.58	0.46
2-Oxoglutarate	1680 ± 250 [76.0] (7)	500 ± 50*** [79.4] (7)	-70	1.23	0.36
Malate	800 ± 140 [78.2] (10)	1310 ± 180**** [84.0] (10)	+64	0.58	0.96
Oxaloacetate	17.1 ± 1.9 [78.7] (11)	20.7 ± 2.1 [83.9] (11)		0.013	0.014
Pyruvate	880 ± 70 [83.0] (6)	550 ± 80**** [75.3] (6)	-37	0.64	0.40
Phosphoenolpyruvate	430 ± 60 [55.8] (9)	740 ± 70*** [59.2] (9)	+72	0.31	0.54
3-Phosphoglycerate	480 ± 50 [80] (6)	860 ± 120** [83.5] (6)	+79	0.35	0.63
Glucose 6-phosphate	200 ± 10 [100] (6)	250 ± 10*** [100] (6)	+25	0.14	0.18

Table 4. *Effect of glucagon on mitochondrial metabolite concentrations in isolated liver cells from 48 h-starved rats incubated in the presence of lactate*

The cells were incubated for 30 min before glucagon (7 μ g/ml) was added and incubation continued for 10 min. Initial lactate concentration was 11.9 mM, and 8.5 mM at the beginning of the glucagon incubation. Mean values $\pm$ S.E.M. are given for the numbers of different cell preparations in parentheses. Significance was calculated by Student's *t*-test for paired data; **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

Metabolite	Amount (nmol/g dry wt.)		Glucagon effect (%)	Concentration (mM)	
	Control	Glucagon		Control	Glucagon
ATP	2810 $\pm$ 280 (9)	3500 $\pm$ 340** (9)	+25	16.98	21.15
ADP	1260 $\pm$ 60 (9)	1380 $\pm$ 90 (9)		7.61	8.34
AMP	480 $\pm$ 90 (7)	460 $\pm$ 70 (7)		2.90	2.78
Acetyl-CoA	101 $\pm$ 11 (9)	127 $\pm$ 10*** (9)	+26	0.61	0.77
CoA	510 $\pm$ 70 (6)	530 $\pm$ 50 (6)		3.08	3.20
Aspartate	110 $\pm$ 14 (11)	115 $\pm$ 20 (11)		0.66	0.69
Glutamate	2570 $\pm$ 370 (8)	510 $\pm$ 180*** (8)	-80	15.52	3.07
Citrate	1040 $\pm$ 130 (10)	1030 $\pm$ 120 (10)		6.28	6.22
2-Oxoglutarate	530 $\pm$ 50 (7)	130 $\pm$ 30** (7)	-75	3.20	0.79
Malate	220 $\pm$ 40 (10)	250 $\pm$ 60 (10)		1.33	1.51
Oxaloacetate	4.65 $\pm$ 0.54 (11)	3.98 $\pm$ 0.40* (11)	-14	0.028	0.024
Pyruvate	180 $\pm$ 20 (6)	180 $\pm$ 30 (6)		1.08	1.08
Phosphoenolpyruvate	340 $\pm$ 60 (9)	510 $\pm$ 70** (9)	+50	2.05	3.08
3-Phosphoglycerate	120 $\pm$ 70 (7)	170 $\pm$ 90 (7)		0.72	1.03
Glucose 6-phosphate	0	0			

On the basis of reports on glucagon stimulation of pyruvate uptake into mitochondria (Adam & Haynes, 1969; Garrison & Haynes, 1974; Yamazaki & Haynes, 1975; Titheradge & Coore, 1976a) the question of intramitochondrial accumulation of pyruvate arose. But as shown in Table 4, no effect of glucagon on the mitochondrial steady-state concentration of pyruvate was detectable. In Table 5 the concentration gradients between the mitochondrial and cytosolic compartment in the absence and presence of glucagon are listed. The values, calculated from the results shown in Tables 3 and 4, reflect the changes in the metabolite distribution between both subcellular compartments produced by glucagon. The gradients of ATP, acetyl-CoA, citrate and pyruvate became steeper, but those of glutamate and malate were flattened by glucagon.

From the fact that oleate stimulates gluconeogenesis from lactate and pyruvate in rat liver (Struck *et al.*, 1965, 1966; Ross *et al.*, 1967a; Teufel *et al.*, 1967; Söling *et al.*, 1968; Williamson *et al.*, 1969a; Johnson *et al.*, 1972; Krebs *et al.*, 1974; Siess & Wieland, 1975), it was originally suggested (Struck *et al.*, 1966; Williamson *et al.*, 1966a,b; Ross *et al.*, 1967b; Söling *et al.*, 1968) that glucagon might act by increasing fatty acid supply. However, this view seemed to be incompatible with later observations, which indicated that glucagon can stimulate gluconeogenesis even in the presence of maximally effective concentrations of fatty acids (Ross *et al.*, 1967b; Exton *et al.*, 1969). Moreover, Fröhlich & Wieland (1971) have shown that glucagon still promoted gluconeogenesis when liver lipolysis was inhibited by glycodiazine, indicating that glucagon

and fatty acids operate by different mechanisms. This view is further substantiated by the results presented in Table 6. Oleate did not cause the marked decrease in cellular glutamate and 2-oxoglutarate concentrations as seen in the presence of glucagon, although it stimulated glucose formation by 82% (results not shown), in agreement with previous results (Siess & Wieland, 1975). Moreover, oleate did not change the amount of phosphoenolpyruvate, in contrast with glucagon (Table 6).

Discussion

Validity of the digitonin method

The subcellular distribution of malate, citrate, glutamate and aspartate in rat liver without added glucagon was studied by Soboll *et al.* (1976) using a completely different approach. Accordingly, 70, 35, 76 and 89% of total cellular malate, citrate, glutamate

and aspartate, respectively were located in the cytosol when the liver from a starved animal was perfused with lactate. As shown in Table 3 nearly identical values were found by fractionation of isolated liver cells with the digitonin method. Also, both methods yielded very similar results for the cytosolic and mitochondrial metabolite concentrations (Table 7). This suggests that the information on metabolite compartmentation as revealed by the digitonin procedure is valid also for glucagon-stimulated liver cells.

Effect of glucagon on subcellular metabolite distribution

To our knowledge the data of the present study disclose for the first time in which compartment and to what extent changes in metabolite concentrations may occur during glucagon action. Our experiments revealed that cytosolic and matrix ATP contents change in opposite directions. Further, it is clear that the rise of total acetyl-CoA in the presence of glucagon is due to the accumulation of acetyl-CoA in the mitochondrial compartment only. Another noteworthy result is that in rat liver some 40% of the cellular phosphoenolpyruvate is apparently located within the mitochondria. This observation raises the question to what extent the mitochondrial pellet fraction might be contaminated by cytosolic and nuclear metabolites during the fractionation procedure. From the fact that glucose 6-phosphate, a metabolite present in both the cytosol and the nucleus (Siebert, 1961) was not detectable in the pellet, one can estimate that the carry-over of cytosolic and nuclear constituents contributes less than 5% of the amount of metabolite in the pellet. The possibility that the lack of glucose 6-phosphate in the pellet was due to splitting by glucose 6-phosphatase (EC 3.1.3.9) present in the endoplasmic-reticulum fragments of this fraction (Siess & Wieland, 1976) seems to be ruled out, as no liberation of glucose from exogenously added glucose 6-phosphate to the digitonin medium was detectable. Moreover,

Table 5. Effect of glucagon on the ratio between the mitochondrial and cytosolic metabolite concentration
For further details see legend to Table 2.

Metabolite	Mitochondrial/cytosolic concentration ratio	
	Control	Glucagon
ATP	3.89	5.7
ADP	9.76	9.93
Acetyl-CoA	10.70	14.26
CoA	50.41	31.37
Aspartate	0.66	0.57
Glutamate	2.00	1.40
Citrate	10.88	13.52
2-Oxoglutarate	2.60	2.19
Malate	2.27	1.58
Oxaloacetate	2.15	1.71
Pyruvate	1.68	2.69
Phosphoenolpyruvate	6.53	5.69
3-Phosphoglycerate	2.06	1.62

Table 6. Different effects of glucagon and oleate on total cellular amounts of glutamate, 2-oxoglutarate and phosphoenolpyruvate in isolated liver cells from 48 h-starved rats

Mean values $\pm$ S.E.M. for five experiments are given. The cells were incubated as described in the Materials and Methods section for 30 min before glucagon (7 μ g/ml) or/and albumin-bound oleate (0.7 mM) (Siess & Wieland, 1976) was added and incubation continued for 10 min. The controls received the respective solute(s). * $P < 0.05$ against control without oleate. † $P < 0.005$ against control without glucagon.

Substrate(s) added	Metabolite (μ mol/g dry wt.)					
	Glutamate		2-Oxoglutarate		Phosphoenolpyruvate	
	-Glucagon	+Glucagon	-Glucagon	+Glucagon	-Glucagon	+Glucagon
Lactate	15.8 $\pm$ 1.1	6.1 $\pm$ 0.8†	2.21 $\pm$ 0.13	0.62 $\pm$ 0.21†	0.79 $\pm$ 0.07	1.40 $\pm$ 0.12†
Lactate plus oleate	20.9 $\pm$ 1.7*	12.9 $\pm$ 3.2†	1.76 $\pm$ 0.21*	0.49 $\pm$ 0.09†	0.71 $\pm$ 0.04	1.44 $\pm$ 0.20†

Table 7. Subcellular distribution of metabolites in perfused liver and isolated liver cells from starved rats in the presence of lactate as revealed by different methods

Metabolite	Concentration (mM)											
	Williamson (1969)			Parrilla <i>et al.</i> (1975)			Soboll <i>et al.</i> (1976)			Present study		
	Cyto-solic	Mito-chondrial	m/c	Cyto-solic	Mito-chondrial	m/c	Cyto-solic	Mito-chondrial	m/c	Cyto-solic	Mito-chondrial	m/c
Aspartate	0.80	0.80	1.0*	0.80	0.14	0.18	1.70	0.88	0.52	0.99	0.66	0.67
Glutamate	6.11	6.11	1.0*	3.98	0.90	0.23	5.50	8.00	1.45	7.73	15.52	2.01
Citrate	0.14	12.89	92.07	0.74	1.60	2.16	0.55	4.80	8.73	0.58	6.28	10.83
Malate	0.26	0.38	1.46	0.47	0.37	0.79	0.71	1.38	1.94	0.59	1.33	2.25
Model	Isolated perfused liver									Isolated hepatocytes		
Method of determination	Calculation from mass-action equilibria						Measurement after tissue fractionation in non-aqueous media			Measurement after cell fractionation with digitonin		
* Equal distribution was assumed.												

Table 8. Carry-over of various compounds from the digitonin medium into the pellet during fractionation of hepatocytes (about 5 mg dry wt.) by the standard digitonin procedure

Substance added to the digitonin medium ...	[¹⁴ C]Sucrose	[¹⁴ C]Citrate	[¹⁴ C]Oxaloacetate	[³ H]Acetyl-CoA	Glucose 6-phosphate
Amount added (nmol)	260	11.9	1.4	1.6	5000
(c.p.m.)	82000	75000	13000	54000	—
Percentage recovered in the pellet	0.3	0.8	0.6	1.1	0.23
Carry-over from cytosol (% of amount in pellet; calculated from Tables 3 and 4)	—	0.6	2.2	0.9	0

¹⁴C- or ³H-labelled compounds such as citrate, aspartate, oxaloacetate, acetyl-CoA or sucrose when present during the digitonin fractionation procedure were recovered in the mitochondrial pellet fractions in amounts ranging from 0.2 to 1% (Table 8). As to the compartmentation of phosphoenolpyruvate, we showed that pyruvate kinase, ADP and Mg²⁺ added to the digitonin medium in amounts that caused complete disappearance of exogenous phosphoenolpyruvate failed to lower the phosphoenolpyruvate content of the pellet fraction, indicating that this portion of cellular phosphoenolpyruvate was confined within the mitochondrial membrane(s), and therefore was not accessible for pyruvate kinase. Thus it appears that the amounts of metabolites measured in the pellet fraction are representative of the mitochondrial contents in the incubated cells.

Conflicting results on subcellular metabolite distribution obtained by calculation procedures and by the digitonin method

Parrilla *et al.* (1975) have computed the cytosolic and mitochondrial amounts of metabolites in perfused livers from starved rats during glucagon-

stimulated gluconeogenesis from lactate on the basis of mass-action equilibria (Krebs & Veech, 1969; Greenbaum *et al.*, 1971). In contrast with the marked increases that they found in mitochondrial [oxaloacetate], [aspartate], [malate], [2-oxoglutarate] and [glutamate] after glucagon treatment, our measurements presented in Table 4 indicate a small decrease in [oxaloacetate], no change in [malate] and [aspartate] and a marked decrease in [2-oxoglutarate] and [glutamate]. Other striking differences concern cytosolic [malate] and [aspartate]; according to Parrilla *et al.* (1975) these were decreased by glucagon, but the digitonin procedure reveals an increase in both metabolites in the cytosol. As there is little reason to question the validity of our data obtained with the digitonin method, it follows that the suppositions on which the calculations are based may not be fulfilled, especially under glucagon stimulation. For instance, it is assumed that the reactants of malate dehydrogenase (EC 1.1.1.37) are in equilibrium in mitochondria. Stucki & Walter (1972), however, concluded from experiments with isolated rat liver mitochondria that the malate and oxaloacetate pools are not in equilibrium, and thus by calculation (Veech *et al.*, 1969) falsely low oxaloacetate concentrations

Table 9. *Nicotinamide dinucleotide redox state in isolated hepatocytes incubated with initially 11.9 mM-lactate*
Further details are given in the legend to Table 2.

Compartment	Redox state of free nicotinamide dinucleotides	Redox ratio calculated from:	Redox ratio	
			Control	Glucagon
Cytosol	NAD ⁺ /NADH	Lactate dehydrogenase	806	530
	NAD ⁺ /NADH	Malate dehydrogenase	769	568
	NADP ⁺ /NADPH	'Malic' enzyme	0.038	0.014
Mitochondria	NAD ⁺ /NADH	Malate dehydrogenase	757	572
	NAD ⁺ /NADH	3-Hydroxybutyrate dehydrogenase	67	10
	NAD ⁺ /NADH	Glutamate dehydrogenase	52	—

would result. If it is accepted, however, that the malate dehydrogenase reaction is in equilibrium, it follows (Table 9) that malate dehydrogenase and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) do not share a common NAD⁺ pool. As shown in Table 9 there is a striking similarity between the cytosolic and the mitochondrial [NAD⁺]/[NADH] ratios derived from the malate dehydrogenase equilibrium in the absence and presence of glucagon. Thus it is tempting to speculate that there exist at least two NAD⁺ pools in the mitochondrion, one of which shows the same redox level as that in the cytosol. Thus it appears that the mitochondrial malate and oxaloacetate concentrations cannot be derived from [3-hydroxybutyrate]/[acetoacetate] ratios.

Cytosolic free [NAD(P)⁺]/[NAD(P)H] ratios provide evidence for lactate dehydrogenase, malate dehydrogenase and 'malic' enzyme catalysing equilibrium reactions

The general assumption of lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase reactions being in equilibrium in the cytosol has not been checked so far by direct experimental proof. This is now provided by our findings, which indicate that almost identical free [NAD⁺]/[NADH] ratios (Table 9) can be derived from the measured cytosolic ratios of the [lactate]/[pyruvate] and [malate]/[oxaloacetate] couples (Table 3). The same is true under glucagon stimulation, although in this situation the free [NAD⁺]/[NADH] ratio was considerably lower. It seems noteworthy that the free [NAD⁺]/[NADH] ratios derived from our cytosolic metabolite concentrations in the absence of glucagon agree well with those reported by Williamson (1969) for lactate-perfused isolated rat liver. Also with respect to the cytosolic free [NADP⁺]/[NADPH] ratio the value derived (Table 9) from our cytosolic pyruvate and malate concentrations by setting [CO₂] equal to 1.16 mM (Krebs & Veech, 1969) agrees with that calculated (Williamson, 1969), suggesting that the

'malic' enzyme (EC 1.1.1.40) is maintained close to equilibrium as proposed by Krebs & Veech (1969).

Site(s) of glucagon action

The data summarized in Table 6 strongly support the earlier view (Ross *et al.*, 1967b; Exton *et al.*, 1969; Fröhlich & Wieland, 1971) that glucagon does not act by merely increasing fatty acid supply. Although glucagon, like oleate, increases the redox state, the phosphate potential (Siess & Wieland, 1976) in the mitochondrial space, and to a lesser degree ketogenesis also, it differs markedly in its effect on cellular glutamate, 2-oxoglutarate, and phosphoenolpyruvate concentrations (Table 6). Further, oleate in the presence of lactate stimulated citrate formation (Siess *et al.*, 1976a), in contrast with glucagon (Tables 3 and 4).

The matrix pyruvate concentration remains unchanged during glucagon action (Table 4) and is well above the *K_m* of pyruvate carboxylase (Söling *et al.*, 1970; McClure & Lardy, 1971; Seufert *et al.*, 1971). Thus activation of this enzyme would imply an increase in *V_{max}*. This could be achieved by [acetyl-CoA] (Keech & Utter, 1963; Utter *et al.*, 1964; Seufert *et al.*, 1971; von Glutz & Walter, 1976) and the [ATP]/[ADP] ratio (Stucki *et al.*, 1972; von Glutz & Walter, 1976), which are elevated by glucagon in the matrix, the effect of [acetyl-CoA] being potentiated (Seufert *et al.*, 1971) by the increase in matrix pH (Titheradge & Coore, 1976b). Pyruvate carboxylase would become more active also if the great restraint under which the enzyme operates in the intact mitochondria (Haynes, 1972) is lessened. According to Scrutton & White (1974) glutamate is a potent inhibitor of rat liver pyruvate carboxylase, giving about 50% inhibition at 5 mM. As the matrix glutamate concentration normally is about 15 mM (Table 4) this finding could explain why pyruvate carboxylase is considerably more active in the mitochondrial extract than in the intact structure (Haynes, 1972). Moreover, it is reasonable to assume that the fall in matrix glutamate concentration from 15 to 3 mM (Table 4)

after glucagon will be accompanied by pyruvate carboxylase activation. There was no increase in oxaloacetate in the mitochondria, most likely owing to its conversion into malate and aspartate, which leave the mitochondrion and accumulate in the cytosol according to the current concept originally proposed by Lardy *et al.* (1965). Matrix contents of aspartate and malate also remained unchanged under glucagon stimulation. Thus one might conclude that neither the conversion of oxaloacetate into malate and aspartate nor the efflux of both metabolites into the cytosol is rate-limiting under conditions of glucagon-stimulated gluconeogenesis. The most striking event observable after glucagon is the large fall in 2-oxoglutarate and glutamate. Obviously, these two intermediates are utilized for formation of malate and aspartate and are released from the mitochondria at rates faster than they can be replenished. The crossover between citrate and 2-oxoglutarate suggests that the 2-oxoglutarate dehydrogenase step must be operating at much higher speed than the citrate synthase (EC 4.1.3.7) under these conditions. Therefore the mitochondrial accumulation of phosphoenolpyruvate (Table 4) probably results from an increased supply of GTP and not from increased availability of matrix oxaloacetate, which in fact was decreased (Table 4). Although little attention has been paid so far to the mitochondrial phosphoenolpyruvate carboxykinase in rat liver, our observations on the increased matrix content of phosphoenolpyruvate after glucagon would suggest that this enzyme plays a significant role in gluconeogenesis also in the rat. According to Usatenko (1970) and Saggerson & Evans (1975), phosphoenolpyruvate carboxykinase activity in rat liver mitochondria amounts to 0.19 and 0.10 $\mu\text{mol}/\text{min}$ per g wet wt. at 30°C respectively.

As to the accumulation of phosphoenolpyruvate in the cytosol, the question arises whether glucagon acts by activation of phosphoenolpyruvate carboxykinase or by inhibition of pyruvate kinase (or both). In isolated rat liver cells glucagon, presumably by enzyme phosphorylation (Ljungström *et al.*, 1974), increases the K_m of pyruvate kinase for phosphoenolpyruvate by a factor of about 2 (Feliu *et al.*, 1976; Friedrichs, 1976) with no change of V_{max} . From this the pyruvate kinase reaction was proposed to be under glucagon control (Friedrichs, 1976; Pilgis *et al.*, 1976; Blair *et al.*, 1976). However, the twofold increase in cytosolic phosphoenolpyruvate after glucagon treatment (Table 3), by compensating for the lowering of substrate affinity, may keep the enzyme activity unchanged.

Concerning the fructose 1,6-bisphosphatase-phosphofructokinase substrate cycle as a possible site of glucagon action (Schimassek & Mitzkat, 1963; Clark *et al.*, 1974), the concept of enzyme regulation by changes in the cytosolic concentrations of

effectors such as citrate, ATP and AMP [for review see Scrutton & Utter (1968)] is not supported by our measurements. In fact, cytosolic citrate and ATP tended to decrease rather than to increase and AMP remained unchanged (results not shown). Other possibilities to explain the prevalence of fructose 1,6-bisphosphatase over phosphofructokinase under glucagon stimulation emerge from the increase in cytosolic 3-phosphoglycerate. First, 3-phosphoglycerate or oleate protect fructose 1,6-bisphosphatase against inactivation by ATP and ADP, as has been demonstrated for the enzyme from rabbit liver (Pogell *et al.*, 1971; Carlson *et al.*, 1973). Secondly, inactivation of liver phosphofructokinase has been shown to occur by liver fructose 1,6-bisphosphatase depending on the presence of oleate and 3-phosphoglycerate (Proffitt *et al.*, 1976). Thus it is tempting to speculate that glucagon, by stimulation of both lipolysis (Struck *et al.*, 1965; Williamson *et al.*, 1966*b,c,d*; Bewsher & Ashmore, 1966; Menahan & Wieland, 1969; Claycomb & Kilsheimer, 1969) and 3-phosphoglycerate formation (Williamson *et al.*, 1969*c*; Parrilla *et al.*, 1975; the present paper) could cause phosphofructokinase inactivation in isolated liver cells. Although we have not measured free fatty acids, it seems noteworthy in this connexion that the content of long-chain acyl-CoA in isolated hepatocytes rose from 49.7 ± 3.1 to 55.7 ± 4.3 nmol/ 10^8 cells (mean $\pm$ S.E.M. for five different cell preparations, $P < 0.025$) after glucagon (E. A. Siess & O. H. Wieland, unpublished work). This would explain the decrease by 50% in fructose 1,6-bisphosphate in the perfused liver (Schimassek & Mitzkat, 1963; Williamson *et al.*, 1969*c*; Blair *et al.*, 1973*a*), which in turn could lead to marked inhibition of pyruvate kinase, since at the cytosolic ATP and phosphoenolpyruvate concentrations of about 4 mM and 0.5 mM respectively, pyruvate kinase activity will largely depend on allosteric activation by fructose 1,6-bisphosphate (Rozenfurt *et al.*, 1969; Llorente *et al.*, 1970; Ljungström *et al.*, 1976).

In conclusion, the changes in the metabolite concentrations that we have observed in the liver cell compartments after glucagon treatment seem to support the presently available evidence suggesting that the stimulation of gluconeogenesis results initially from some as yet unknown action(s) of the hormone at the mitochondrial level synchronous to the established effects on proteolysis (Miller, 1965; Penhos *et al.*, 1966; Struck *et al.*, 1966; Williamson *et al.*, 1966*a*; Schimassek, 1967) and lipolysis (Struck *et al.*, 1965; Bewsher & Ashmore, 1966; Williamson *et al.*, 1966*b,c,d*; Menahan & Wieland, 1969; Claycomb & Kilsheimer, 1969). The stimulation of ATP generation found in isolated mitochondria from glucagon-treated rats (Yamazaki, 1975) would explain our observation of an elevated ATP content in the mitochondria of the glucagon-

stimulated liver cells. The accumulation of mitochondrial phosphoenolpyruvate may be an important consequence of an increased supply of GTP, which should become quite significant if one considers the large depletion of the substrates for this phosphorylation step. Whether this is due to the increased mitochondrial efflux of C_4 dicarboxylates or to an activation of 2-oxoglutarate dehydrogenase as proposed by Ui *et al.* (1973b) remains to be clarified. It is conceivable that the mitochondrially formed phosphoenolpyruvate leads, by enlargement of the cytosolic phosphoenolpyruvate pool, to an increase of 3-phosphoglycerate. The latter would then promote the flow forward to glucose by activation of fructose 1,6-bisphosphatase and/or inactivation of phosphofructokinase as discussed above. Concomitantly, the resulting decrease in fructose 1,6-bisphosphate would curtail recycling of phosphoenolpyruvate through pyruvate kinase.

The expert technical assistance of Mrs. E. Gundel and Miss E. Daller is greatly acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

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