Stimulation of mitochondrial functions by glucagon treatment

Evidence that effects are not artifacts of mitochondrial isolation

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(Received 25 August 1982/Accepted 25 November 1982)

(1) Activation of rat liver mitochondrial functions following glucagon treatment was demonstrated in mitochondria that had not been isolated by the conventional technique of differential centrifugation and washing in sucrose solutions. Crude liver homogenates in 0.3 M-sucrose or 0.15 M-KCl prepared from rats treated with glucagon showed stimulation of State-3 and uncoupled respiration, carboxylation of pyruvate, and citrulline synthesis comparable with those previously reported in isolated mitochondria. (2) During the isolation procedure of mitochondria the hormonal stimulations of pyruvate carboxylation and citrulline formation were shown not to be enhanced by sequential washing. (3) Mitochondria isolated from glucagon-treated rats by differential centrifugation and washing in 0.3 M-mannitol/1 mM-EGTA, pH 7.0, exhibited a mean rate of citrulline synthesis that was greater than twice that of the control. Liver homogenates prepared in 0.3 M-sucrose or 0.3 M-mannitol showed identical rates of State-3 respiration and percentage stimulations of respiration by glucagon treatment. (4) Addition of glucagon led to a rapid accumulation of malate and aspartate and decreased the amounts of glutamate and citrate in isolated hepatocytes incubated with L-lactate. When gluconeogenesis was inhibited at the phosphoenolpyruvate carboxykinase (EC 4.1.1.32) reaction these phenomena were accentuated, lending support to the interpretation that they are the direct result of stimulation of carboxylation and oxidation reactions in the mitochondria. These results do not support the proposal [Siess, Fahimi & Wieland (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1643–1651] that the mitochondrial effects of glucagon treatment result from a stabilization of mitochondria to detrimental effects of sucrose during their isolation. (5) The mean hormonal stimulation of pyruvate carboxylation in mitochondria isolated in 0.3 M-sucrose was shown to be approx. 2.5-fold when assayed either at 37°C or 25°C. In contrast, on the basis of similar experiments, Siess *et al.* (1981) concluded that the effects of glucagon on hepatic mitochondria are not characteristic of a true hormonal stimulation. Our data indicate this conclusion to be unjustified.

In 1969 we reported that mitochondria isolated from the livers of rats treated with glucagon, adrenaline, or cortisol exhibited accelerated rates of uptake, carboxylation, and decarboxylation of pyruvate (Adam & Haynes, 1969). Subsequently we demonstrated that the mitochondrial responses to these hormones are sufficiently sensitive and rapid to be compatible with a significant role in the control of gluconeogenesis and ureogenesis (Garrison & Haynes, 1975; Titheradge & Haynes, 1980a). In addition to enhanced rates of uptake, carboxylation, and decarboxylation of pyruvate, a number of other functions have been shown to be stimulated in hepatic mitochondria isolated after glucagon ad-

ministration. These include the rates of State-3 and uncoupled respiration (Yamazaki, 1975; Titheradge & Coore, 1976; Halestrap, 1978), uncoupler-dependent ATPase (Yamazaki *et al.*, 1977; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980b), citrulline formation (Yamazaki & Graetz, 1977; Bryla *et al.*, 1977; Triebwasser & Freedland, 1977), succinate dehydrogenase activity (Siess & Wieland, 1978, 1979; Titheradge & Haynes, 1979), and phosphate-dependent glutaminase (Lacey *et al.*, 1981). It has also been found that glucagon treatment produces a net increase of adenine nucleotides within the hepatic mitochondria (Prpic *et al.*, 1978; Titheradge & Haynes, 1980a).

Studies made with isolated hepatocytes have provided evidence that the effects of hormone treatment found in isolated mitochondria reflect stimulations of mitochondrial function within the intact liver cell. Siess *et al.* (1977) found that administration of glucagon produced a rise in the cytosolic concentrations of malate and aspartate, strongly suggesting a stimulation of carboxylation of pyruvate. They also observed decreases in the cytosolic concentrations of glutamate and 2-oxoglutarate, a finding consistent with the previously reported stimulation of the rates of oxidation of glutamate in the perfused liver and in isolated mitochondria (Ui *et al.*, 1973; Yamazaki, 1975). There was a rise in mitochondrial ATP in spite of the increased energy requirements of gluconeogenesis. A recent study of transport of pyruvate by mitochondria *in situ* has emphasized the major role of hormonal regulation of this reaction in control of gluconeogenesis (Thomas & Halestrap, 1981).

An alternative hypothesis proposes (Siess *et al.*, 1981) that glucagon affects hepatic mitochondria by stabilizing them during their preparation in sucrose media. It was further proposed that effects observed in mitochondria isolated subsequent to hormone treatment may be artifacts rather than a reflection of events occurring within the intact hepatocyte. The experiments reported here were carried out to address this possibility.

Experimental

Materials

Sodium [^{14}C]carbonate was purchased from New England Nuclear. Enzymes used in metabolite determinations were obtained from Boehringer Mannheim. Glucagon was obtained from Sigma and crystallized bovine serum albumin from Miles Laboratories. 2-Mercaptopicolinate was kindly given by Dr. Harry Saunders, Smith Kline and French Laboratories, Philadelphia, PA, U.S.A.

Methods

Male Wistar rats (150–300 g) fed *ad libitum* except where indicated were used for these studies. Rats were anaesthetized by administration of intraperitoneal pentobarbital (60 mg/kg) and then received either 100 μg of glucagon subcutaneously in 1 ml of 0.9% NaCl/0.05% bovine serum albumin or 1 ml of the suspending vehicle alone. After 20 min the livers were removed and chilled in the medium used for homogenization. They were then homogenized (9 ml of medium/g of liver) using seven strokes of a Teflon/glass homogenizer. In experiments designed to study mitochondrial functions without isolation of the organelles, aliquots of these homogenates were taken directly for assay.

Respiration was measured at 26°C with a Clark oxygen electrode using 0.15 ml of homogenate and 1.8 ml of respiratory assay medium [125 mM-KCl/5 mM- K_2HPO_4 /50 mM-Mops (4-morpholinepropanesulphonic acid)/1 mM-EGTA/0.2 mM- MgCl_2 /0.7 μg of rotenone/ml/5 mM-succinate, pH 7.0]. State-3 respiration was induced with 150 μM -ADP; uncoupled respiration was produced by the addition of 0.5 μM -carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone. Rates of carboxylation of pyruvate were determined as previously described (Adam & Haynes, 1969); formation of citrulline was measured according to Yamazaki & Graetz (1977).

For isolation of mitochondria, either 0.3 M-sucrose or 0.3 M-mannitol/1 mM-EGTA, pH 7.0, was used as the medium for homogenization of livers and washing and suspension of the mitochondria. Nuclei and cellular debris were removed by an initial centrifugation of the crude homogenates at 600 g for 10 min. The resulting supernatant fraction was then centrifuged at 9000 g for 10 min to sediment the mitochondria. In preparations made in 0.3 M-sucrose, the mitochondria were washed twice; when mannitol was used, the mitochondria were washed once.

Hepatocytes were prepared as previously described (Garrison & Haynes, 1975) from rats starved for 18–24 h. The isolated cells were suspended to a concentration of approx. 30 mg of protein/ml in an oxygenated solution consisting of 119 mM-NaCl/4.8 mM-KCl/2.5 mM- CaCl_2 /1.2 mM- MgSO_4 /1.2 mM- KH_2PO_4 /2.1 mM- NaHCO_3 /22.7 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid], pH 7.4, and kept at 0°C until used. The cells were incubated at a concentration of 12 mg of protein/ml in the same medium at 30°C. The gas phase was air; L-lactate was added at an initial concentration of 20 mM, and the suspensions were agitated at 150 rev./min in a rotary shaker bath. Under these incubation conditions, rates of production of glucose did not differ significantly from those obtained using Krebs–Ringer bicarbonate-buffered medium with O_2/CO_2 (19:1) as the gas phase. Where indicated, 120 μM -3-mercaptopicolinate was present from the beginning of the incubation. After 45 min of incubation, glucagon in 0.007 M-HCl or 0.007 M-HCl alone was added in a volume of 10 μl /ml of incubation medium. The final concentration of glucagon was 2.9 μM . At designated times, aliquots of the suspensions were removed and extracted with HClO_4 , neutralized with KHCO_3 , and analysed for glucose (Werner *et al.*, 1970). The reported glucose values are corrected for the small amounts of glucose produced by hepatocytes incubated without added lactate. This lactate-independent rate of glucose formation was less than 10% of the lactate-dependent rate. Aspartate, malate, and glutamate in

the extracts were measured fluorimetrically by the techniques of Lowry & Passonneau (1972). Citrate was assayed by the method of Matschinsky *et al.* (1968).

Glutamate dehydrogenase (EC 1.4.1.2) was assayed by the method of Martin & Denton (1970). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

To normalize metabolic rates of homogenates to mitochondrial protein content, rates per mg of homogenate protein were multiplied by 3.22. This factor was the ratio of the mean glutamate dehydrogenase activity found in isolated mitochondria (3766 ± 255 nmol/min per mg of protein) to the glutamate dehydrogenase activity of homogenate protein (1171 ± 59 nmol/min per mg).

Statistical analyses were by paired comparisons using Student's one-tailed *t* test.

Results

Hormonal responses in crude homogenates of liver

Because the possibility has been raised that the effects of hormone treatment observed in isolated mitochondria are largely the result of exposure to the nonphysiological conditions of mitochondrial preparation (Siess *et al.*, 1981), we carried out experiments to assess mitochondrial respiration, carboxylation of pyruvate, and citrulline formation in crude homogenates of liver. We felt homogenates should provide substrates with free access to the

mitochondria and allow mitochondrial functions to be evaluated without the necessity of subjecting the organelles to the multiple procedures ordinarily involved in their isolation. Inasmuch as sucrose has been implicated as possibly playing a factitious role in the reported mitochondrial effects, we compared homogenates prepared in 0.15 M-KCl with those prepared in 0.3 M-sucrose as described in the Experimental section.

Table 1 presents data on respiration, pyruvate carboxylation and citrulline synthesis of homogenates from a group of nine experiments. It can be seen from the data of the table that homogenates prepared from glucagon-treated rats showed stimulations of the rates of State-3 and uncoupled respiration comparable with those reported for isolated mitochondria (Yamazaki, 1975). The homogenates displayed oxygraphic traces typical of mitochondrial preparations. Furthermore, the behaviour of homogenates prepared in 0.15 M-KCl was not significantly different from those prepared in 0.3 M-sucrose. To estimate the mitochondrial content of the homogenates, the activity of glutamate dehydrogenase was determined in sucrose homogenates and found to be 1240 ± 104 nmol/min per mg of protein for homogenates from rats serving as controls and 1240 ± 120 nmol/min per mg of protein for homogenates from glucagon-treated rats. For KCl homogenates the corresponding values were 1130 ± 155 and 1070 ± 92 nmol/min per mg of protein. Thus hormone treatment did not produce

Table 1. *Effects of glucagon treatment on respiration, pyruvate carboxylation and citrulline synthesis in liver homogenates*

Treatment of rats, preparation of liver homogenates, the conditions for measurement of oxygen consumption, and the assay of pyruvate carboxylation and citrulline formation are described in the Experimental section. Succinate served as respiratory substrate. For all groups, $n = 9$; values are means $\pm$ S.E.M. The mean fixation of CO_2 in the pyruvate carboxylation assay was 0.6 ± 0.07 nmol/min per mg of protein for all treatment groups in the absence of added pyruvate with no significant differences among groups. Fixation of CO_2 (nmol/min per mg of protein) in the citrulline assay without added ornithine was 0.81 ± 0.05 and 1.24 ± 0.11 for control and glucagon homogenates, respectively, in sucrose ($P < 0.005$), and 0.84 ± 0.07 and 1.22 ± 0.17 for control and glucagon homogenates, respectively, in KCl ($P < 0.025$). Values in parentheses represent mean rates normalized per mg of mitochondrial protein. The statistical significances of the results calculated by pair comparisons using Student's one-tailed *t* test were: * $P < 0.001$; ** $P < 0.002$; *** $P < 0.01$; **** $P < 0.05$.

Parameter	0.3 M-Sucrose		0.15 M-KCl	
	Control	+ Glucagon	Control	+ Glucagon
State-3 oxygen consumption (nmol of O/min per mg of protein)	42 ± 3 (127)	$54 \pm 2^*$ (163)	38 ± 2 (115)	$51 \pm 3^*$ (154)
State-4 oxygen consumption (nmol of O/min per mg of protein)	8 ± 1	9 ± 1	8 ± 1	9 ± 1
Uncoupled oxygen consumption (nmol of O/min per mg of protein)	43 ± 3	$51 \pm 3^*$	37 ± 3	$49 \pm 4^*$
Respiratory control ratio	5.4 ± 0.4	$6.6 \pm 0.5^{**}$	5.0 ± 0.4	$6.0 \pm 0.3^{****}$
Pyruvate carboxylation (nmol/min per mg of protein)	6.5 ± 1.2 (19.5)	$13.3 \pm 2.0^*$ (42.2)	7.5 ± 1.0 (22)	$13.5 \pm 1.7^*$ (43.5)
Citrulline formation (nmol/min per mg of protein)	1.1 ± 0.2 (3.3)	$4.7 \pm 0.7^*$ (14.1)	1.2 ± 0.2 (3.6)	$4.3 \pm 0.3^{***}$ (13.8)

significant differences in the mitochondrial content of the homogenates.

The data of Table 1 show that the treatment of rats with glucagon led to an increased rate of carboxylation of pyruvate in the crude homogenates as compared with the control preparations. Again, as with the stimulation of respiration, there was no significant difference between KCl- or sucrose-containing homogenates. Table 1 also contains data from assays of the homogenates for ornithine-dependent fixation of CO₂, which is considered to represent citrulline synthesis. A marked stimulation of citrulline synthesis in homogenates from glucagon-treated rats relative to the controls was evident. Just as with respiration and carboxylation of pyruvate, the results were independent of the medium used for preparation of homogenates.

Hormone effects at different stages of mitochondrial isolation

Siess *et al.* (1981) reported that the effect of hormone treatment increased as mitochondrial fractions were washed during the isolation process. This appeared to result from a decrease in activity of the control mitochondria relative to those isolated from glucagon-treated hepatocytes. In Table 2 results are presented from experiments carried out to examine the effects of washing. Assays of pyruvate carboxylation and citrulline formation were made during the course of our routine procedure for isolation of mitochondria in 0.3M-sucrose from intact livers. After the material sedimented by centrifugation at 600g for 10 min was discarded, the mitochondrial pellet was collected at 9000g and washed twice in 0.3M-sucrose. In contrast with the report of Siess *et al.* (1981) we found that two washes in 0.3M-sucrose had minimal effects on the mitochondrial functions examined.

Mannitol as suspending material

Siess *et al.* (1981) have reported data showing that the use of solutions of mannitol for preparation and suspension of mitochondria increased the rates of carboxylation of pyruvate in both control and glucagon-treated mitochondria. Although this phenomenon resulted in a decreased percentage effect of glucagon treatment, the data of these investigators appear to indicate that mannitol did not significantly decrease the hormone effect as defined by the absolute difference between control and treated groups. Our own experience involving several unpublished experiments of this type is in agreement with this conclusion. In contrast, in the case of citrulline formation we found a doubling in the rate from 13.0 ± 3.7 nmol/min per mg of protein for control mitochondria to 27.2 ± 5.5 nmol/min per

Table 3. *Effect of temperature of assay on carboxylation of pyruvate*

Treatment of rats, preparation of mitochondria, and assay procedure are described in the Experimental section. For both groups $n = 6$; values are means $\pm$ S.E.M. The statistical significance of the results calculated by pair comparisons using Student's one-tailed t test was $P < 0.001$ at both temperatures. The 95% confidence limits of the ratios are indicated by values in parentheses.

Mitochondria	Pyruvate carboxylation (nmol/min per mg) at:	
	37°C	25°C
Control	13.6 ± 1.0	3.2 ± 0.16
Glucagon-treated	37.5 ± 2.2	8.2 ± 0.31
Mean glucagon/ control	2.76 (2.13–3.59)	2.59 (2.21–3.06)

Table 2. *Pyruvate carboxylation and citrulline synthesis at three stages of mitochondrial isolation*

Treatment of rats, isolation of mitochondria, and assays of pyruvate carboxylation and citrulline formation are described in the Experimental section. For both control and glucagon-treated groups, $n = 6$; values are means $\pm$ S.E.M. The statistical significances of the results calculated by pair comparisons using Student's one-tailed t test were: * $P < 0.001$, ** $P < 0.005$, *** $P < 0.025$. These are probability estimates that the difference between pairs in control and glucagon-treated groups are chance variations. In addition, the activity of the glucagon-treated mitochondria showed a significant decline in pyruvate carboxylation between the first and second washes ($P < 0.005$).

Stage	Pyruvate carboxylation (nmol/min per mg of protein)		Citrulline formation (nmol/min per mg of protein)	
	Control	Glucagon-treated	Control	Glucagon-treated
9000 g pellet	24.2 ± 1.5	$46.2 \pm 3.1^*$	8.9 ± 2.0	$23.6 \pm 4.7^{**}$
First wash	23.1 ± 1.1	$49.9 \pm 2.3^*$	10.1 ± 2.2	$25.2 \pm 4.5^{**}$
Second wash	20.4 ± 2.7	$44.7 \pm 1.7^*$	9.2 ± 2.0	$23.5 \pm 5.0^{***}$

mg of protein for mitochondria isolated from glucagon-treated rats (mean $\pm$ S.E.M.; $n = 5$; $P < 0.05$) when mitochondria were isolated in a mannitol/EGTA medium. Recently, Verhoeven *et*

al. (1982) have reported similar data and have concluded that the stimulation of citrulline synthesis by glucagon is due to an activation of carbamoyl phosphate synthetase (EC 2.7.2.5). In a brief series

Fig. 1. Effects of glucagon and 3-mercaptopycolinate on malate and aspartate levels

Preparation and incubation of hepatocytes and measurement of malate and aspartate are described in the Experimental section. For values at 46 min $n = 4$; for all other points $n = 7$; values are means $\pm$ S.E.M. expressed as a percentage of the value at 45 min. Hepatocytes, approximately 12 mg of protein/ml, were incubated at 30°C under air with 20 mM L-lactate and, where indicated, 120 μ M 3-mercaptopycolinate. Glucagon (O---O) or vehicle (●—●) was added at 45 min as indicated by the arrow. (a) Malate levels in the absence of inhibitor. The mean value at 45 min was 1.20 ± 0.07 nmol/mg of cell protein. (b) Malate levels in the presence of 3-mercaptopycolinate. The mean value at 45 min was 11.24 ± 0.06 nmol/mg of cell protein. (c) Aspartate levels in the absence of inhibitor. The mean value at 45 min was 1.11 ± 0.06 nmol/mg of cell protein. (d) Aspartate levels in the presence of 3-mercaptopycolinate. The mean value at 45 min was 2.96 ± 0.22 nmol/mg of cell protein.

Fig. 2. Effects of glucagon and 3-mercaptopycolinate on glutamate and citrate levels in hepatocytes

Preparation and incubation of hepatocytes and measurement of glutamate and citrate are described in the Experimental section. For values at 46 min $n = 4$; for all other points $n = 7$; values are means $\pm$ S.E.M. expressed as a percentage of the value at 45 min. Hepatocytes, approx. 12 mg of protein/ml, were incubated at 30°C with 20 mM-L-lactate and, where indicated, 120 μ M-3-mercaptopycolinate. Glucagon (○---○) or vehicle (●—●) was added at 45 min as indicated by the arrow. (a) Glutamate levels in the absence of inhibitor. The mean value at 45 min was 9.56 ± 0.31 nmol/mg of cell protein. (b) Glutamate levels in the presence of 3-mercaptopycolinate. The mean value at 45 min was 10.54 ± 0.34 nmol/mg of cell protein. (c) Citrate levels in the absence of inhibitor. The mean value at 45 min was 3.44 ± 0.28 nmol/mg of cell protein. (d) Citrate levels in the presence of 3-mercaptopycolinate. The mean value at 45 min was 6.06 ± 0.70 nmol/mg of cell protein.

of experiments (three replicates) rates of State-3 respiration were determined in homogenates prepared in 0.3 M-sucrose or 0.3 M-mannitol to assess the influence of homogenization medium on the hormone effect. In homogenates prepared in 0.3 M-sucrose, oxygen consumption rates under State-3 conditions for control and glucagon-treated groups were 43 ± 3 and 65 ± 2 nmol/min per mg of protein (mean $\pm$ S.E.M.; $P < 0.001$) and in homogenates prepared in 0.3 M-mannitol, rates were 43 ± 3 and 66 ± 1 nmol/min per mg of protein for the corresponding groups (mean $\pm$ S.E.M.; $P < 0.001$). Thus, there appeared to be no significant difference in the control rates of respiration or the stimulation produced by glucagon between homogenates prepared in sucrose or mannitol, contrary to the data reported by Siess *et al.* (1981) for isolated mitochondria.

Effect of temperature on hormone effect

The data of Siess *et al.* (1981) indicated that assay of the mitochondrial pyruvate carboxylation reaction at 25°C rather than 37°C decreased the hormone effect due to a disproportionate decrease in activity of the glucagon-treated mitochondria relative to the control group. We could not reproduce these results. In our hands, as can be seen from the data of Table 3, the ratio of activities of the glucagon-treated mitochondria to the control mitochondria did not differ significantly at the two temperatures.

Evidence of mitochondrial stimulation in intact cells

Measurements of intermediates of gluconeogenesis have suggested strongly that mitochondrial functions are stimulated by hormones. Thus, as mentioned in the Introduction, the cellular content of both malate and aspartate rise after glucagon administration (Siess *et al.*, 1977) indicating the presence of a metabolic 'push' from the mitochondria rather than only a 'pull' at the phosphoenolpyruvate carboxykinase reaction or beyond, in which case a decline in these intermediates would be anticipated. To confirm and refine these observations, we followed the time course of the hormone-produced changes in metabolic intermediates while phosphoenolpyruvate carboxykinase (EC 4.1.1.32) was inhibited by 3-mercaptopicolinate. The inhibitor was used to assess the possibility that hormonal control of the cytoplasmic phase of the gluconeogenic pathway is responsible for the observed hormone effects on malate and aspartate levels in hepatocytes.

In the absence of inhibitor, glucose production increased from a control rate of 23.1 ± 1.8 μ g of glucose/mg of cell protein per h ($n = 7$) to 32.7 ± 2.3 μ g of glucose/mg of cell protein per h ($n = 7$) following glucagon addition. Incubation with

120 μ M-3-mercaptopicolinate inhibited control rates of gluconeogenesis to 6.1 ± 0.9 μ g of glucose/mg of cell protein per h ($n = 7$) and reduced glucagon-stimulated gluconeogenesis to a rate of 6.4 ± 1.0 μ g of glucose/mg of cell protein per h ($n = 7$), thus abolishing the hormonal stimulation of glucose production. The cellular content of malate and aspartate rose approx. 9- and 3-fold, respectively, when glucose synthesis from lactate was inhibited by 3-mercaptopicolinate (legend to Fig. 1). Treatment with glucagon caused a further increase in the concentrations of these compounds, as would be anticipated if there were a stimulation of carboxylation of pyruvate (Figs. 1a and 1b).

In agreement with Siess & Wieland (1980), who reported a rapid decline in glutamate content in hepatocytes treated with glucagon, we found a decline in glutamate content within 1 min after glucagon addition (Fig. 2a). A similar reduction, although somewhat delayed, was observed in the presence of the inhibitor (Fig. 2b). In contrast to the report that the citrate content of hepatocytes did not change after glucagon addition (Siess *et al.*, 1977), we found (Figs. 2c and 2d) that glucagon caused a rapid drop in the citrate content of hepatocytes. In hepatocytes incubated with 3-mercaptopicolinate the content of citrate subsequently increased to levels greater than controls (Fig. 2d). In the absence of inhibitor, after glucagon addition the levels of citrate remained below those of controls (Fig. 2c).

Discussion

Hormonal stimulation of hepatic gluconeogenesis from pyruvate requires that there be an increased rate of flux through the pyruvate carboxylase (EC 6.4.1.1) reaction within the mitochondria. We have proposed that this increased flux is the consequence of hormonal control at the mitochondrial level (Adam & Haynes, 1969; Garrison & Haynes, 1975). Suggested mechanisms for such control include enhanced transport of pyruvate into the organelles (Adam & Haynes, 1969; Titheradge & Coore, 1976; Halestrap, 1978), an increased ATP/ADP ratio (Yamazaki, 1975; Titheradge *et al.*, 1979) and a rise in matrix pH and fall in matrix glutamate (Siess *et al.*, 1977). Alternatively, the increased flux through the pyruvate carboxylase reaction could be a secondary result of hormonal controls later in the pathway of gluconeogenesis. In this case changes in steady-state concentrations of metabolic intermediates could be involved in positive or negative feedback control of the pyruvate carboxylase reaction. For example a stimulation of phosphoenolpyruvate carboxykinase would lower the cytosolic concentrations of oxalacetate and malate, which is in equilibrium with it. If either of these acids, at cytosolic concentrations, inhibited the

carboxylase reaction, their decline would allow a greater enzymic activity. Glucagon increases the cellular concentrations of malate and aspartate (Siess *et al.*, 1977). The present experiments indicate this is true even when the activity of phosphoenolpyruvate carboxykinase is inhibited. These findings support the concept that the carboxylation of pyruvate is not stimulated through release of a negative feedback control by the C_4 acids. In addition, we are not aware of studies of purified pyruvate carboxylase that have revealed feedback inhibition by these compounds.

Furthermore, our experiments indicate mitochondrial pyruvate carboxylation is not stimulated by positive feedback control mediated by metabolic components of later stages of gluconeogenesis beyond the phosphoenolpyruvate carboxykinase reaction. In our experiments (Fig. 1) in which gluconeogenesis has been largely blocked by 3-mercaptopycolinate, there were elevated concentrations of malate and aspartate that were increased further by glucagon. These responses are not characteristic of a positive effector generated beyond the C_4 acid stage of gluconeogenesis; if such a mechanism were to mediate the effects of glucagon on the mitochondria, inhibition of formation of the putative positive effector by 3-mercaptopycolinate should prevent glucagon from increasing malate and aspartate levels.

The findings that the oxidation of glutamate is stimulated by glucagon within seconds in the perfused liver (Ui *et al.*, 1973) and that glucagon causes a rapid fall in glutamate (Siess *et al.*, 1977; the present paper) and 2-oxoglutarate (Siess *et al.*, 1977) in hepatocytes support the concept of activation of mitochondrial oxidative metabolism. Similarly the increase in mitochondrial ATP found after glucagon treatment is difficult to explain as a passive response to the increased cytosolic demands of gluconeogenesis, in which case a decreased or unchanged mitochondrial content of ATP would be anticipated. Taken together, this evidence places the burden of proof on any hypothesis proposing that hormonal activation of mitochondrial functions is by feedback control.

Mitochondria prepared from livers of glucagon-treated rats or hepatocytes show enhanced rates of pyruvate carboxylation and oxidative phosphorylation (Adam & Haynes, 1969; Yamazaki, 1975) as would be predicted from what is known of the changes in metabolites and adenine nucleotides discussed above. Our studies presented here show that these effects are not the consequence of exposure to sucrose during isolation of mitochondria, as the hormone effects are present in crude homogenates prepared in 0.15 M-KCl.

The data of Siess *et al.* (1981) indicate that the use of mannitol for isolating mitochondria tends

to diminish the effect of hormone treatment in terms of percentage change. One possible explanation for these results is that mannitol may be less completely excluded from the mitochondrial matrix than is sucrose. The use of mannitol then would yield mitochondria with somewhat expanded matrix volumes relative to mitochondria prepared in sucrose media. We have shown that slightly hypo-osmotic media (Adam & Haynes, 1969) or the presence of valinomycin and K^+ ions (Haynes *et al.*, 1974) stimulate carboxylation and decarboxylation of pyruvate. It has been known for many years that respiration of isolated mitochondria is stimulated in hypo-osmotic media (Johnson & Lardy, 1958). Nicholls & Lindberg (1972) have described a similar effect of matrix density on respiration and activity of the uncoupler-dependent ATPase. The experiments reported here and those of Verhoeven *et al.* (1982) indicate that hormonal stimulation of citrulline formation is strong in mitochondria isolated in a mannitol medium. In relation to the hypothesis that mannitol expands the mitochondrial matrix, it is of interest that we have found that, in contrast with several other functions, citrulline formation by isolated mitochondria is not stimulated by hypo-osmoticity (H. Hamman & R. C. Haynes, unpublished work).

This defence of isolated mitochondria as a preparation useful in the study of hormonal control of gluconeogenesis does not imply that all of the responses of isolated mitochondria reflect qualitatively and quantitatively their activity in the intact cell. Several years ago we called attention to the discrepancy between activation of pyruvate decarboxylation in isolated mitochondria from hormone-treated rats and the inhibition of pyruvate decarboxylation noted in perfused liver after hormone treatment (Garrison & Haynes, 1975). It is evident, nevertheless, that mitochondria do demonstrate persistent alterations in several functions after hormonal treatment that are consistent with their activities *in vivo*. Continued study of these effects should prove useful in elucidating the mechanisms by which gluconeogenesis and ureogenesis are controlled.

Elegant experiments by Rognstad & Katz (1977) have clearly shown that hormones can effectively stimulate gluconeogenesis without decreasing the activity of pyruvate kinase (EC 2.7.1.40). This, together with a lack of definitive evidence for an acute effect on the phosphoenolpyruvate carboxykinase reaction, emphasizes the importance of hormonal control of gluconeogenesis at the mitochondrial level.

This research was supported by N.I.H. grant AM 14347. The authors express their appreciation for the technical assistance of Mr. Ralph Picking and Miss Christina Holstrom.

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