The rôle of mitochondrial pyruvate transport in the stimulation by glucagon and phenylephrine of gluconeogenesis from L-lactate in isolated rat hepatocytes

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The sensitivity of glucose production from L-lactate by isolated liver cells from starved rats to inhibition by α -cyano-4-hydroxycinnamate was studied. A small percentage of the maximal rate of gluconeogenesis was insensitive to inhibition by α -cyano-4-hydroxycinnamate, and evidence is presented to show that this is due to pyruvate entry into the mitochondria as alanine. After subtraction of this rate, Dixon plots of the reciprocal of the rate of gluconeogenesis against inhibitor concentration were linear both in the absence and presence of glucagon, phenylephrine or valinomycin, each of which stimulated gluconeogenesis by 30–50%. Pyruvate kinase activity was decreased by glucagon, but not by phenylephrine or valinomycin. Inhibition of gluconeogenesis by quinolinate (inhibitor of phosphoenolpyruvate carboxykinase) or monochloroacetate (probably inhibiting pyruvate carboxylation) caused a significant deviation from linearity of the Dixon plot obtained with α -cyano-4-hydroxycinnamate. Amytal, however, inhibited gluconeogenesis without affecting the linearity of this plot. These data, coupled with a computer simulation study, suggest that pyruvate transport may control gluconeogenesis from L-lactate and that hormones may stimulate this process through an effect on the respiratory chain. An additional role for pyruvate kinase and pyruvate carboxylase is quite compatible with the data presented.

It is well established that glucagon and α -adren-
ergic agents such as phenylephrine stimulate glu-
coneogenesis from L-lactate in isolated rat hepato-
cytes by cyclic AMP dependent- and independent-
mechanisms respectively (Exton & Park, 1969;
Tolbert & Fain, 1974; Cherrington *et al.*, 1976;
Chan & Exton, 1978; Hue *et al.*, 1978). In both
cases the stimulation of the conversion of pyruvate
to phosphoenolpyruvate is known to be an import-
ant site of action of these hormones (Exton & Park,
1969; Ui *et al.*, 1973; Chan & Exton, 1978). A
partial explanation of the action of glucagon may be
the well-documented phosphorylation and inhibition
of pyruvate kinase induced by the hormone (see
Engström, 1980). However, an effect of α -adren-
ergic agents on the activity of this enzyme is less
certain and may well be absent (Hue *et al.*, 1978;
Blair *et al.*, 1979; cf. Chan & Exton, 1978; Foster &
Blair, 1978; Steiner *et al.*, 1980) even though
stimulation of ^{32}P incorporation into the enzyme is
observed (Nagano *et al.*, 1980; Steiner *et al.*, 1980).

An alternative or additional mechanism by which
hormones may stimulate gluconeogenesis at this
point has been suggested from studies on liver

mitochondria isolated from control and hormone-
treated rats. Mitochondria derived from glucagon- or
adrenaline-treated rats show enhanced rates of
pyruvate metabolism when compared with those
from control animals (Adam & Haynes, 1969;
Titheradge & Coore, 1976*a,b*; Halestrap, 1977,
1978*b*). This appears to be a consequence of the
glucagon-treated mitochondria maintaining a more
alkaline matrix and so supporting faster rates of
pyruvate transport into the mitochondria, the rate-
limiting process in mitochondrial pyruvate meta-
bolism (Halestrap, 1978*a,b*).

The intention of the experiments presented in the
present paper is to examine whether pyruvate
transport into the mitochondria is also stimulated *in*
situ in isolated hepatocytes and so may control the
rate of gluconeogenesis from L-lactate *in vivo*. Mitochondria from glucagon-treated animals show a
large number of altered properties when compared
with control mitochondria (see Halestrap *et al.*,
1980; Halestrap, 1981) and it is quite possible that
some of these are artefacts of mitochondrial prep-
aration.

Work from this and other laboratories (Hales-

trap, 1978b; Rognstad, 1979; Halestrap *et al.*, 1980) has suggested that an enzyme in a metabolic pathway that is rate-limiting can be recognized by the effect of inhibitors of that enzyme on the rate of the overall pathway. If the inhibition of the whole pathway follows a linear Dixon plot (1/velocity plotted against inhibitor concentration) then the enzyme being inhibited must be rate-limiting. Deviation from linearity at low inhibitor concentrations implies that the enzyme cannot be the rate-limiting step. In this paper we use the specific inhibitor of mitochondrial pyruvate transport, α -cyano-4-hydroxycinnamate, to demonstrate that gluconeogenesis from L-lactate is limited by the rate of pyruvate transport into the mitochondria, and that this transport is stimulated by glucagon and phenylephrine. However, the evidence does not allow us to conclude whether or not the mitochondrial pH gradient is altered by these hormones.

Experimental

Materials

Rats. Male Wistar rats of 300–400 g body weight, starved for 48 h but with free access to water, were used for the preparation of liver cells.

Chemicals. Unless stated below the sources of all chemicals and biochemicals were the same as those given by Halestrap & Denton (1975) and Halestrap (1978a,b). L-Phenylephrine, peroxidase and heparin were obtained from Sigma. Amino-oxyacetate and α -cyano-4-hydroxycinnamate were from Aldrich, and collagenase from Boehringer. Bovine serum albumin was obtained from International Enzymes, Windsor, Berks., U.K. and was defatted by the method of Chen (1967).

Preparation and incubation of liver cells

Rat liver parenchymal cells were prepared by the method of Berry & Friend (1969), but without the use of hyaluronidase and with collagenase present at 0.1 mg/ml. After washing, the cells were suspended at a final protein concentration of approx. 4 mg/ml in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) containing 25 mg of defatted bovine serum albumin/ml, and gassed with O_2/CO_2 (19:1). In the majority of experiments the medium was supplemented with 1 mM-sodium oleate solubilized as described by Ross *et al.* (1967). Samples (0.5 ml) of the cell suspension were preincubated with shaking for 20 min at 37°C in stoppered plastic vials of 8 ml capacity under an atmosphere of O_2/CO_2 (19:1), at which time substrate, hormones and inhibitor were added as required. After a further 20 min and 50 min of incubation (or at variable times for time courses), 0.2 ml aliquots were removed and rapidly added to 20 μ l of $HClO_4$ (20%, w/v).

Determination of free α -cyano-4-hydroxycinnamate concentrations

The use of α -cyano-4-hydroxycinnamate as an inhibitor of gluconeogenesis was complicated by its binding to albumin in the incubation medium (Halestrap, 1975). To correct for this, albumin-free incubation medium (0.25 ml) was dialysed against 2.25 ml of incubation medium containing the required total concentration of inhibitor in gassed, stoppered incubation vials. The dialysis was continued at 37°C with shaking until equilibration was complete (about 7 h). The concentration of free α -cyano-4-hydroxycinnamate inside the dialysis tubing was determined spectrophotometrically at 325 nm (Halestrap, 1976), after 15-fold dilution with Tris/HCl buffer, pH 7.4. The total concentration of α -cyano-4-hydroxycinnamate remaining in the albumin-containing buffer was calculated by subtraction of the amount lost to the medium in the dialysis tubing. Fig. 1 shows the binding curve for defatted albumin (25 mg/ml) in the presence of 1 mM-oleate. The binding of α -cyano-4-hydroxycinnamate was considerably increased in the absence of oleate and new binding curves had to be determined when changes to the incubation conditions were made.

Fig. 1. Binding of α -cyano-4-hydroxycinnamate to defatted bovine serum albumin

Krebs bicarbonate buffer (0.25 ml) was dialysed at 37°C against 2.25 ml of the same buffer containing 25 mg of defatted bovine serum albumin/ml, 1 mM-sodium oleate and α -cyano-4-hydroxycinnamate at the required concentration. Dialysis was carried out under an atmosphere of O_2/CO_2 (19:1) until equilibrium was reached. The concentration of α -cyano-4-hydroxycinnamate inside the dialysis tubing was determined spectrophotometrically as described in the Experimental section.

Analytical procedures

Metabolites were assayed in supernatants after centrifugation of the HClO_4 extracts. Glucose was measured by using glucose oxidase/peroxidase (Huggett & Nixon, 1957), pyruvate by the method of Bücher *et al.* (1963) and ATP by using luciferase (Stanley & Williams, 1969). For determination of the activity of pyruvate kinase, 1 ml of cell suspension was incubated in 20 ml vials under the conditions described above. The cells were separated from the medium 20 min after the addition of hormone by centrifugation for 5 s in an Eppendorf 3200 centrifuge and were immediately frozen in liquid N_2 . The cell pellet was extracted in Tris/acetate (100 mM), pH 6.8, containing KF (100 mM) and EDTA (5 mM), using a Polytron homogenizer (fitted with a PTA 7 micro head) at speed setting 4 for 20 s. The extracts were stored frozen and assayed as described by Denton *et al.* (1979), with phosphoenolpyruvate concentrations in the range 0.2–4 mM. Cell protein concentrations were measured with a modified biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard. Corrections were made for the albumin in the incubation medium.

Results and discussion

Use of α -cyano-4-hydroxycinnamate to inhibit mitochondrial pyruvate transport in the hepatocyte

Previous work from this laboratory has shown that α -cyano-4-hydroxycinnamate may be used to inhibit mitochondrial pyruvate transport in intact tissue and cell preparations without perturbing other aspects of cellular metabolism (Halestrap & Denton, 1975; Mendes-Mourão *et al.*, 1975; Halestrap *et al.*, 1980). We have confirmed that under all conditions used to study gluconeogenesis from L-lactate to be reported in this paper, even the maximal concentration of α -cyano-4-hydroxycinnamate added to hepatocytes ($700 \mu\text{M}$ free concentration) did not change cellular ATP or pyruvate concentrations, nor is gluconeogenesis impaired from substrates not requiring mitochondrial pyruvate transport (Mendes-Mourão *et al.*, 1975). Fig. 2 shows that gluconeogenesis from L-lactate was linear over the period 20 min to 60 min in the presence of α -cyano-4-hydroxycinnamate at all the concentrations tested. Thus the difference in glucose in the medium between 20 and 50 min was used to calculate the rate of gluconeogenesis after the subtraction of the rate of lactate-independent glucose output. It should be noted that a lag period was observed before glucose production became maximal, as has been observed by others (Cornell *et al.*, 1974) and it appears that α -cyano-4-hydroxycinnamate may prolong this. It is also important to stress

Fig. 2. Effect of α -cyano-4-hydroxycinnamate on the time course for gluconeogenesis by isolated rat liver cells. Rat liver cells (3.5 mg of cell protein/ml) were incubated with 1 mM-oleate as described in the Experimental section. After preincubation for 20 min at 37°C , 10 mM-L-lactate and the required amount of α -cyano-4-hydroxycinnamate were added except for one incubation (●) where no L-lactate was added. Incubations were terminated at the times indicated by addition of HClO_4 to a final concentration of 2% (w/v). Each point represents the mean of two separate incubations in the absence (●) or presence of α -cyano-4-hydroxycinnamate at a free concentration of (▲) $40 \mu\text{M}$, (▼) $100 \mu\text{M}$, (○) $400 \mu\text{M}$, (□) $700 \mu\text{M}$.

that all concentrations of α -cyano-4-hydroxycinnamate are expressed in terms of the free concentration, correction having been made for the inhibitor bound to albumin as outlined in the Experimental section.

Sensitivity of gluconeogenesis from L-lactate to inhibition by α -cyano-4-hydroxycinnamate

Fig. 3 demonstrates the sensitivity of gluconeogenesis from L-lactate to inhibition by α -cyano-4-hydroxycinnamate in the presence and absence of glucagon ($0.2 \mu\text{M}$), phenylephrine ($20 \mu\text{M}$) or valinomycin (5 nM). The stimulation of gluconeogenesis by valinomycin at very low concentrations is well established (Tolbert & Fain, 1974; Haynes *et al.*, 1974; Friedmann & Dambach, 1980) and the concentration of this effector used in the present experiments was determined as that which gave maximal stimulation under the incubation conditions used. Higher concentrations of valinomycin progressively inhibited gluconeogenesis (Haynes

Fig. 3. Effects of glucagon, phenylephrine and valinomycin on the inhibition of gluconeogenesis from L-lactate by α -cyano-4-hydroxycinnamate

Rates of hepatocyte gluconeogenesis from L-lactate were determined exactly as described in the Experimental section. Hepatocytes (4 mg of cell protein/ml) were incubated in the presence of 1 mM-oleate and 25 mg of albumin/ml at 37°C. After 20 min preincubation, 10 mM-L-lactate was added with the required amount of α -cyano-4-hydroxycinnamate and hormones at the concentrations indicated below. The rate of glucose output was determined over the period from 20 to 50 min after the addition of L-lactate. Each point represents the mean of two separate incubations. In (a) the inhibitor titration curve in the presence (○) and absence (●) of 0.2 μ M-glucagon has been fitted by non-linear regression to a modified equation for non-competitive inhibition as described in the text. The data is replotted in (b) as a Dixon plot with the calculated V' parameter subtracted from all of the rates. In addition the effects of (□) 20 μ M-phenylephrine and (Δ) 5 nM-valinomycin are shown.

Table 1. Combined parameter values from non-linear regression of α -cyano-4-hydroxycinnamate titrations of gluconeogenesis

This table summarizes the data from several experiments similar to that shown in Fig. 3. L-Lactate (10 mM) was used as substrate throughout. For each experiment the data were fitted by non-linear regression to the equation $V_i = [V_0/(1 + [I]/K_i)] + V'$ as explained in the text. Each value is the mean $\pm$ S.E.M. of parameters calculated from at least four separate experiments with statistical significance of differences from the control means calculated using a paired t test: * $P < 0.001$, † $P < 0.01$, ‡ $P < 0.05$.

Condition	Rate of gluconeogenesis in the absence of inhibitor (nmol/min per mg of protein)	Parameter values			Number of experiments
		V_0 (nmol/min per mg of protein)	K_i (μ M)	V' (nmol/min per mg of protein)	
Control	5.24 ± 0.41	4.01 ± 0.22	71.3 ± 6.3	1.23 ± 0.25	7
Glucagon (0.2 μ M)	$8.23 \pm 0.53^*$	$6.55 \pm 0.53^\dagger$	73.5 ± 20.4	1.68 ± 0.30	4
Phenylephrine (20 μ M)	$7.34 \pm 0.74^\dagger$	$6.36 \pm 0.48^*$	66.3 ± 11.3	0.82 ± 0.35	5
Valinomycin (5 nM)	$7.23 \pm 1.18^\dagger$	$6.15 \pm 0.79^\ddagger$	71.8 ± 17.9	1.08 ± 0.39	4

et al., 1974; Tolbert & Fain, 1974). The concentrations of glucagon and phenylephrine used were also those giving maximal stimulation ($37.2 \pm 5.0\%$ with glucagon and $30.2 \pm 5.1\%$ with phenylephrine). This stimulation is similar to that observed by others (Garrison & Haynes, 1975; Siess *et al.*, 1977; Titheradge *et al.*, 1979; Garrison & Borland, 1979). Under all these conditions inhibition by α -cyano-4-hydroxycinnamate was apparent at even the lowest inhibitor concentration used (10 μ M). The data gave linear Dixon plots provided that a constant inhibitor-insensitive rate of gluconeogenesis was subtracted (Fig. 3b). This inhibi-

tor-insensitive rate can be determined by performing non-linear regression on the data to the modified equation for non-competitive inhibition:

$$V_i = \frac{V_0}{(1 + [I]/K_i)} + V' \quad (1)$$

where V_i is the inhibited rate of gluconeogenesis, V_0 the control rate in the absence of inhibitor, K_i the dissociation constant for the inhibitor-carrier complex, $[I]$ the concentration of inhibitor and V' the rate of inhibitor-insensitive gluconeogenesis. As indicated in Fig. 3(a) the fit of the data to this

equation is extremely good and the combined data from several such experiments are given in Table 1. However, the fit of the data to eqn. (1) begs the question as to the meaning of the V' term.

Significance of the inhibitor-insensitive rate of gluconeogenesis

Experiments with isolated mitochondria suggest that a small amount of pyruvate may enter the mitochondria independently of the pyruvate carrier by free diffusion. Such entry is only of appreciable significance at high pyruvate concentrations (>1 mM) and low pH (<7.0) (Halestrap, 1978a,b; Pande & Parvin, 1978). Our estimate of the cytoplasmic pH under the incubation conditions used was determined, using 5,5'-dimethylloxazolidine-2,4-dione as described by McGivan (1979), to be 7.2. Based on the cell membrane pH gradient of 0.2 and the data of Fig. 4 the intracellular pyruvate concentration can be calculated to be about 0.3 mM. Thus free diffusion of pyruvate into the mitochondria would be expected to be slow. However work from this and other laboratories (Mendes-Mourão *et al.*, 1975; De Rosa & Swick, 1975; Dieterle *et al.*, 1978) has demonstrated that alanine

may be transaminated to pyruvate in both the cytoplasm and the mitochondria and so allow pyruvate to enter the mitochondria as alanine independently of the pyruvate transporter. The extent to which this might occur in the intact cell was tested using amino-oxyacetate to block glutamate-oxaloacetate transaminase (Rognstad & Katz, 1970) in liver cells synthesizing glucose from pyruvate (Table 2). Lactate cannot be used in these experiments since transaminases are also involved in transporting oxaloacetate out of the mitochondria as aspartate when lactate (but not pyruvate) is the gluconeogenic substrate (Longshaw *et al.*, 1972; Anderson *et al.*, 1971). The data of Table 2 clearly demonstrate that at 10 mM-pyruvate neither amino-oxyacetate (0.5 mM) nor α -cyano-4-hydroxycinnamate (0.4 mM) are effective inhibitors of gluconeogenesis. In the presence of both, however, 80% inhibition was observed without any effect on cellular ATP content, suggesting strongly that pyruvate may enter the mitochondria as alanine in the absence of amino-oxyacetate. The mechanism by which alanine enters the mitochondria is not known, but a concentration dependence at physiological concentrations of alanine might be expected. If it is assumed that glutamate-pyruvate transaminase is near to equilibrium in the cell (Rognstad, 1980), then a correlation between the intracellular concentration of pyruvate and the rate of inhibitor-insensitive

Fig. 4. Correlation between the rate of α -cyano-4-hydroxycinnamate-insensitive gluconeogenesis and the pyruvate concentration in the medium

Experiments were performed as described in the Experimental section and the legend to Fig. 3 and the data were fitted to the equation explained in the text. Pyruvate assays were performed on the same HClO_4 extracts that were used for glucose determinations. To obtain a range of pyruvate concentrations the substrates added to the incubation medium were varied as follows: (●) 10 mM-L-lactate, (■) 10 mM-L-lactate + 1 mM-oleate, (▲) 10 mM-L-lactate + 15 mM-ethanol, (▼) 5 mM-L-lactate + 1 mM-oleate. Linear regression of the data gave a significant correlation coefficient of 0.904.

Table 2. Effects of α -cyano-4-hydroxycinnamate and amino-oxyacetate on gluconeogenesis from pyruvate. Isolated liver cells (4 mg of cell protein/ml) were incubated in the presence of 1 mM-oleate and 25 mg of albumin/ml at 37°C as described in the Experimental section. After preincubation for 20 min, 10 mM-pyruvate was added with inhibitors as indicated and the rate of glucose output was determined over the period from 20 to 40 min after this. Each value is the mean $\pm$ S.E.M. of rates determined from three separate incubations, with the pyruvate-independent rate of glucose output subtracted. The statistical significance of differences from the control mean were calculated using Student's *t* test: **P* < 0.001.

Condition	Rate of gluconeogenesis (nmol/min per mg of protein)	Inhibition (%)
Control	2.22 ± 0.12	—
Amino-oxyacetate (0.5 mM)	2.23 ± 0.05	0
α -Cyano-4-hydroxycinnamate (0.4 mM)	1.84 ± 0.11	17
Amino-oxyacetate (0.5 mM) + α -cyano-4-hydroxycinnamate (0.4 mM)	$0.46 \pm 0.10^*$	79

gluconeogenesis (V') might be expected. The data of Fig. 4 suggest that this is so, if the further assumption is made that the pyruvate concentration of the medium bears a constant relationship to that in the cytoplasm. The presence of a rapid transport mechanism for pyruvate and lactate in the plasma membrane of the hepatocyte (Leeks & Halestrap, 1979) supports this assumption.

In Fig. 4 some data is included demonstrating a negative V' term. This apparently strange observation is explicable in that some pyruvate entering the mitochondria is oxidized through pyruvate dehydrogenase rather than directed through pyruvate carboxylase towards glucose production. Normally pyruvate dehydrogenase activity was low in our cells (<2 munits/mg of cell protein at 37°C) in comparison with the alanine pathway for pyruvate entry into the mitochondria. However, at low pyruvate concentrations the alanine pathway was diminished, becoming less than the flux through pyruvate dehydrogenase. Under these circumstances pyruvate entering the mitochondria may be preferentially oxidized through pyruvate dehydrogenase (K_m for pyruvate about 30 μM) rather than pyruvate carboxylase (K_m for pyruvate about 100 μM) (Denton & Halestrap, 1979). Thus the rate of pyruvate transport is greater than the rate of gluconeogenesis by an amount included in the V' term. A more mathematical consideration of this is given in the Appendix.

Interpretation of the inhibitor titrations of gluconeogenesis

The data presented so far suggest that pyruvate

transport into the mitochondria may control the rate of gluconeogenesis. However, in the Appendix we include a computer simulation of the effects of a pyruvate transport inhibitor on the rate of gluconeogenesis that indicates that this conclusion must be treated with caution. These simulation studies imply that the observed linear Dixon plots are compatible with situations where gluconeogenesis may also be limited by pyruvate carboxylase and pyruvate kinase activities. Unfortunately phosphoenolpyruvate carboxykinase activity is not included as a separate entity in the simulation studies and conclusions cannot be drawn as to the effects of its activity on the Dixon plots. However, no suitable regulatory mechanisms are known for the acute control of this enzyme (see Denton & Halestrap, 1979). Furthermore, in Fig. 5(a) we show data on the effects of 4 mM-quinolinate, a well established inhibitor of phosphoenolpyruvate carboxykinase (Elliott *et al.*, 1977; McDaniel *et al.*, 1972), on the inhibition of gluconeogenesis by α -cyano-4-hydroxycinnamate. This concentration of quinolinate inhibits gluconeogenesis by about 50%, implying that phosphoenolpyruvate carboxykinase is now the rate-limiting process, and this is associated with Dixon plots for α -cyano-4-hydroxycinnamate that are no longer linear. This suggests that phosphoenolpyruvate carboxykinase is unlikely to be the rate-limiting process in the absence of quinolinate when Dixon plots are linear. In opposition to this conclusion are the data of Rognstad (1979) who showed that inhibition of phosphoenolpyruvate carboxykinase activity with mercaptopicolinate gave inhibition of gluconeogenesis from L-lactate which

Fig. 5. Effects of quinolinate, monochloroacetate and amytal on α -cyano-4-hydroxycinnamate inhibition of gluconeogenesis from L-lactate

Rates of gluconeogenesis from 10 mM-L-lactate in the presence of 1 mM-oleate were measured as detailed in the Experimental section and the legend to Fig. 3. Inhibitors were added together with the lactate and the rate of glucose output was determined over the period 20–50 min after this. The data are plotted as Dixon plots with the calculated control V' parameter subtracted from all rates. In (a) the plots represent (●) control, (■) 0.5 mM-monochloroacetate, (▲) 4 mM-quinolinate and in (b) (●) control, (■) 0.6 mM-amytal.

obeyed a linear Dixon plot. The simplest way to reconcile these two observations is to suggest that the activities of the enzyme and the pyruvate carrier are very closely matched, inhibition of either giving inhibition of gluconeogenesis.

A similar conclusion can be reached with respect to pyruvate carboxylase. In Fig. 5(a) the effects of 0.5 mM-monochloroacetate on gluconeogenesis are investigated. This compound probably inhibits gluconeogenesis at the level of pyruvate carboxylase if it behaves like dichloroacetate (Demaugre *et al.*, 1978*a,b*) and, as with quinolinate, Dixon plots using α -cyano-4-hydroxycinnamate cease to be linear. However, simulation studies indicate that the best data fit is obtained if it is assumed that pyruvate transport and pyruvate carboxylase activities are closely matched and both are activated by glucagon and phenylephrine. Mechanisms exist for such concerted activation. An increase in mitochondrial transmembrane pH gradient would activate pyruvate transport and increase ATP concentrations, so stimulating pyruvate carboxylase (see Halestrap, 1978*b,c*; Halestrap *et al.*, 1980). Similarly, an increase in mitochondrial ketone body output would stimulate pyruvate transport (Halestrap, 1978; Halestrap *et al.*, 1980) and would be associated with elevated mitochondrial acetyl-CoA concentrations which stimulate pyruvate carboxylase (see Denton & Halestrap, 1979). It has been demonstrated that glucagon increases ketone body production by liver cells (Siess *et al.*, 1977; McGarry & Foster, 1980).

Liver pyruvate kinase is known to be inhibited when phosphorylated by cyclic AMP-dependent protein kinase through a rise in the K_m for phosphoenolpyruvate, and inhibition of its activity following glucagon treatment of liver cells is well established (see Engström, 1980). In Table 3 we

present data confirming this observation but demonstrating that phenylephrine and valinomycin had no effect on enzyme activity under conditions in which stimulation of gluconeogenesis was apparent. This agrees with data obtained by others (Hue *et al.*, 1978; Blair *et al.*, 1979) although small changes in enzyme activity and phosphorylation following phenylephrine treatment have been reported by some workers (Foster & Blair, 1978; Chan & Exton, 1978; Nagano *et al.*, 1980; Steiner *et al.*, 1980). Covalent modification of pyruvate kinase cannot account for the stimulation of gluconeogenesis by valinomycin and phenylephrine in our experiments, but a change in the activity of the enzyme *in situ* through alterations in the concentrations of inhibitors or activators such as alanine and fructose-1,6-bisphosphate cannot be eliminated. However, there are data to suggest that, whereas glucagon does decrease recycling of phosphoenolpyruvate through pyruvate kinase, phenylephrine does not (Rognstad, 1975, 1976). The computer simulation included in the Appendix shows that hormonal inhibition of pyruvate kinase could cause suitable activation of gluconeogenesis whilst maintaining linear Dixon plots, provided that two conditions are met. Firstly, that pyruvate transport is not significantly faster than pyruvate carboxylase and secondly, that recycling through pyruvate kinase in the control cells is at least as fast as the net rate of gluconeogenesis.

Interpretation of the K_i value for α -cyano-4-hydroxycinnamate

In studies of pyruvate metabolism by isolated mitochondria, the K_i for inhibition by α -cyano-4-hydroxycinnamate gave valuable information about the mitochondrial pH gradient and changes

Table 3. *Hormonal effects on pyruvate kinase activity in hepatocytes from starved rats*

Isolated liver cells were incubated under identical conditions to those used for the determination of rates of gluconeogenesis from L-lactate, as described in the Experimental section and the legend to Fig. 3. After 40 min incubation with L-lactate and other additions as indicated, the cells were separated from the medium by centrifugation at 5000 g_{av} for 5 s. The supernatant was removed and the pellet rapidly frozen in liquid N_2 . Pyruvate kinase was extracted and assayed using phosphoenolpyruvate in the range 0.2–4 mM as described in the Experimental section. The data were fitted by non-linear regression to the equation for co-operative kinetics:

$$v = V_{\max.}/1 + \left[\frac{K_{0.5}}{[S]} \right]^h.$$

Each value is the mean $\pm$ S.E.M. of parameters calculated from at least three separate experiments, with statistical significance of differences from the control means calculated using Student's *t* test: * $P < 0.001$, † $P < 0.01$.

Condition	$V_{\max.}$ (mol/min per mg cell protein)	$K_{0.5}$ for phosphoenolpyruvate (mM)	Hill coefficient (<i>h</i>)	Number of experiments
Control	0.131 $\pm$ 0.019	0.87 $\pm$ 0.03	2.27 $\pm$ 0.06	6
Glucagon (0.2 μ M)	0.145 $\pm$ 0.022	1.42 $\pm$ 0.05*	2.83 $\pm$ 0.16†	6
Phenylephrine (20 μ M)	0.130 $\pm$ 0.025	0.90 $\pm$ 0.05	2.39 $\pm$ 0.05	4
Valinomycin (5 nM)	0.129 $\pm$ 0.37	0.83 $\pm$ 0.03	2.44 $\pm$ 0.13	3

induced in this parameter by hormones (Halestrap, 1978b). However, in the experiments on intact hepatocytes reported in Table 1, interpretation of the K_i value in terms of the mitochondrial ΔpH is not possible for two reasons. Firstly, the dependence of the K_i on ΔpH is a result of the behaviour of the inhibitor as a weak acid, causing it to be concentrated within the mitochondria by the pH gradient (Halestrap, 1975; 1978b). In the intact cell the concentration of the inhibitor within the mitochondria will be given by:

$$[\text{Inhibitor}]_{\text{mito.}} = [\text{Inhibitor}]_{\text{medium}} \times \frac{[\text{H}^+]_{\text{medium}}}{[\text{H}^+]_{\text{mito.}}}$$

The cytoplasmic pH is not involved since both the medium/cytoplasmic and cytoplasmic/mitochondrial gradients include this term, which cancels out in the determination of the medium/mitochondrial gradient. Thus a decrease in cytoplasmic pH without a change in mitochondrial pH would not affect the K_i value but would increase mitochondrial ΔpH and pyruvate transport. The effects of glucagon on cytoplasmic pH have not been reported, but we have not observed any significant changes in the uptake of the pH marker 5,5'-dimethylloxazolidine-2,4-dione (results not shown). Thus changes in cytoplasmic pH are unlikely to be induced by glucagon and so influence the relationship between K_i and the mitochondrial ΔpH . The second difficulty associated with interpreting the K_i value is that it may be influenced by ketone bodies displacing the inhibitor from the pyruvate carrier on the inside of the mitochondrial membrane (Halestrap, 1978a,b; Halestrap *et al.*, 1980). Since the output of ketone bodies is increased by glucagon (Siess *et al.*, 1977; McGarry & Foster, 1980) a rise in K_i might be expected if ΔpH remained constant.

The data of Table 1 show no significant change in K_i values with glucagon, phenylephrine or valinomycin, although the errors on the means are such that small changes would be hard to see. The errors within a single experiment were relatively low, as indicated in Fig. 3, but there was considerable variation in the K_i values between experiments and the effects of hormones on this parameter. This variation may be explained, in part, by differences in ketone body output between experiments. Thus measurement of K_i values in the presence of fatty-acid-free albumin (low ketone body output) gave a mean value $\pm$ S.E.M. for the K_i of $48 \pm 4.6 \mu\text{M}$ ($n=4$) compared with $71 \pm 6.3 \mu\text{M}$ ($n=7$) in the presence of added oleate (high ketone body output). Although some experiments were carried out in the absence of oleate, this condition was not used routinely since rates of gluconeogenesis were considerably lower (about 40%) and the inhibitor-independent rate much higher (see Fig. 4) than in the

presence of oleate. However, pyruvate transport still appeared to limit the rate of gluconeogenesis and be stimulated by glucagon (results not shown). Again, no significant change in K_i value was observed, but variation between experiments was too large to allow any degree of certainty that no such change occurred. Thus the balance of the data suggests that it is unlikely, but not inconceivable, that glucagon influences the mitochondrial pH gradient *in situ*.

Mechanism of stimulation of mitochondrial pyruvate transport in the hepatocytes

Gluconeogenesis may be inhibited by inhibitors of the respiratory chain or oxidative phosphorylation (Davidoff, 1971; Holland *et al.*, 1973; Meijer & Williamson, 1974; Rognstad, 1976). Since stimulation of the respiratory chain by glucagon and phenylephrine has been suggested to be responsible for enhanced pyruvate transport and metabolism in mitochondria isolated from hormone-treated animals (Titheradge & Coore, 1976a,b; Titheradge *et al.*, 1978; Halestrap, 1978b,c, 1981; Halestrap *et al.*, 1980), the effects of the respiratory chain inhibitor amytal on the sensitivity of gluconeogenesis to inhibition by α -cyano-4-hydroxycinnamate was investigated (Fig. 5b). A level of amytal was chosen (0.6 mM) which inhibited gluconeogenesis by about 20% whilst having little effect on cellular ATP content (Fig. 6). Inhibitor titration under these conditions gave a linear Dixon plot (Fig. 5b) suggesting that pyruvate transport is rate limiting for gluconeogenesis even

Fig. 6. Relative effects of amytal on gluconeogenesis and cellular ATP levels

Hepatocytes were incubated with 1 mM-oleate, 10 mM-lactate and the concentrations of amytal shown, as described in the Experimental section. Glucose output (●) was measured over the period from 20 to 50 min after the addition of inhibitor. The ATP content of the cells (■), which did not change during this period, was measured as described in the Experimental section and is presented as the mean $\pm$ S.E.M. of the values found at the beginning and the end of the incubation in three separate incubations.

though the reduced rate of gluconeogenesis has been induced by inhibiting the respiratory chain. Thus a relationship between pyruvate transport and respiratory chain activity does appear to exist in the intact hepatocyte as well as in the isolated mitochondrion. The involvement or otherwise of the mitochondrial transmembrane pH gradient in this relationship is again difficult to establish from the K_i values. In two such experiments amyltal caused a small decrease in the K_i value for α -cyano-4-hydroxycinnamate from 60.6 ± 9.2 and $54.5 \pm 4.4 \mu\text{M}$ to 40 ± 9.1 and $41.8 \pm 3.1 \mu\text{M}$ (values given $\pm$ S.E. for the data fitted as described in the legend to Fig. 5b). However, it was also observed that the total ketone body output under these conditions dropped from 1.97 ± 0.09 to $1.37 \pm 0.18 \text{ nmol/min per mg of protein}$ (values given as the mean $\pm$ S.E.M. for three observations). This decrease in ketone body output might perturb the K_i value, as outlined above.

General conclusions

The data presented in this paper provide strong evidence for a regulatory role of mitochondrial pyruvate transport in gluconeogenesis from L-lactate in isolated rat liver cells. An additional role of pyruvate carboxylase is likely, since parallel regulatory mechanisms exist for both processes. Elevated mitochondrial ATP/ADP ratios and acetyl-CoA concentrations have been reported in glucagon-treated hepatocytes (Siess *et al.*, 1977; Bryla *et al.*, 1977; Titheradge *et al.*, 1979) and both of these would stimulate pyruvate carboxylase activity (see Denton & Halestrap, 1979). Associated with these changes an increased ketone body output and possibly transmembrane pH gradient, both of which stimulate pyruvate transport, might be expected (Halestrap *et al.*, 1980; Halestrap, 1981). An additional role for pyruvate kinase inhibition in glucagon action seems well established (see Engström, 1980, and Table 2 of the present paper), but the present data suggest this is not the primary stimulatory site of gluconeogenesis, but rather a precautionary measure to prevent excessive recycling of the phosphoenolpyruvate produced. In the case of phenylephrine, inhibition of pyruvate kinase is only reported by some workers and then normally with cells from fed rats (Foster & Blair, 1978; Chan & Exton, 1978; Steiner *et al.*, 1980). Our own data (Table 2) and that from some other workers (Hue *et al.*, 1978; Blair *et al.*, 1979) do not show a persistent effect of phenylephrine on pyruvate kinase activity, although they do not rule out regulation by changes in the concentration of effectors *in situ*. However Rognstad (1976) could find no evidence for inhibition of phosphoenolpyruvate recycling in the intact hepatocyte by phenylephrine. The computer simulation studies show that although pyru-

vate kinase is unlikely to be the only site of hormone action on gluconeogenesis from L-lactate, a coincident activation of pyruvate transport (and pyruvate carboxylase) with an inhibition of pyruvate kinase fits the observed data very well. A role for phosphoenolpyruvate carboxykinase in the acute regulation of gluconeogenesis is not supported by the data presented here. Indeed, inhibition of phosphoenolpyruvate carboxykinase with quinolinate to make it rate limiting for gluconeogenesis demonstrated the validity of the inhibitor titrations to test whether pyruvate transport is a rate-limiting process. Thus, in the presence of quinolinate the Dixon plot was no longer linear (Fig. 5a). Rognstad (1979), however, concluded that phosphoenolpyruvate carboxykinase was rate-limiting for gluconeogenesis from L-lactate by using inhibitor titrations similar to those reported here, but with mercaptopycolinate. The apparent conflict between these two conclusions may reflect a situation in which the activities of all the enzymes involved in the conversion of pyruvate to phosphoenolpyruvate are closely matched such that a change in any one of them can alter the rate of the whole pathway. This conclusion contrasts with the view that only one step in a pathway is rate-limiting and therefore subject to control. However, it does allow great flexibility in the control of a particular segment of a pathway. It also provides an explanation of how the acute control of gluconeogenesis appears to be regulated by pyruvate kinase, pyruvate transport and pyruvate carboxylase, whilst longer term regulation involves induction and repression of phosphoenolpyruvate carboxykinase (see Denton & Halestrap, 1979). A similar conclusion has been reached by Rognstad (1979).

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