Biochem. J. (1984) **223**, 677–685
Printed in Great Britain

A re-evaluation of the role of mitochondrial pyruvate transport in the hormonal control of rat liver mitochondrial pyruvate metabolism

Andrew P. HALESTRAP and Annie E. ARMSTON
Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

(*Received 16 April 1984/Accepted 16 July 1984*)

1. The inhibitor of mitochondrial pyruvate transport α-cyano-β-(1-phenylindol-3-yl)-acrylate was used to inhibit progressively pyruvate carboxylation by liver mitochondria from control and glucagon-treated rats. The data showed that, contrary to our previous conclusions [Halestrap (1978) *Biochem. J.* **172**, 389–398], pyruvate transport could not regulate metabolism under these conditions. 2. This was confirmed by measuring the intramitochondrial pyruvate concentration, which almost equilibrated with the extramitochondrial pyruvate concentration in control mitochondria, but was significantly decreased in mitochondria from glucagon-treated rats, where rates of pyruvate metabolism were elevated. 3. Computer-simulation studies explain how this is compatible with linear Dixon plots of the inhibition of pyruvate metabolism by α-cyano-4-hydroxycinnamate. 4. Parallel measurements of the mitochondrial membrane potential by using [^{3}H]triphenylmethylphosphonium ions showed that it was elevated by about 3 mV after pretreatment of rats with both glucagon and phenylephrine. There was no significant change in the transmembrane pH gradient. 5. It is shown that the increase in pyruvate metabolism can be explained by a stimulation of the respiratory chain, producing an elevation in the protonmotive force and a consequent rise in the intramitochondrial ATP/ADP ratio, which in turn increases pyruvate carboxylase activity. 6. Mild inhibition of the respiratory chain with Amytal reversed the effects of hormone treatment on mitochondrial pyruvate metabolism and ATP concentrations, but not on citrulline synthesis. 7. The significance of these observations for the hormonal regulation of gluconeogenesis from L-lactate *in vivo* is discussed.

Liver mitochondria isolated from rats pretreated with glucagon show enhanced rates of pyruvate carboxylation and decarboxylation (Adam & Haynes, 1969). Since the activity of neither pyruvate carboxylase nor pyruvate dehydrogenase was altered by hormone treatment, it was suggested that pyruvate transport into the mitochondria was the site of hormonal stimulation. Subsequent work in our and other laboratories has supported this conclusion and led to the suggestion that transport is stimulated by an increase in transmembrane pH gradient (Titheradge & Coore, 1976*a*,*b*; Halestrap, 1977, 1978*b*) caused by the well-documented stimulation of respiratory-chain activity (Yamazaki, 1975; Halestrap, 1978*a*,*b*, 1982). This conclusion was largely based on the linear Dixon plots for α-cyano-4-hydroxycinnamate inhibition of pyruvate metabolism, which were taken to indicate that mitochondrial pyruvate transport was the rate-controlling step for pyruvate metabolism (Halestrap, 1978*b*; Halestrap *et al.*, 1980; Thomas & Halestrap, 1981; Armston *et al.*, 1982; Rognstad, 1983).

However, Martin *et al.* (1984) report non-linear Dixon plots under their conditions for measuring mitochondrial metabolism. Furthermore, Groen *et al.* (1982, 1983) have pointed out that linear Dixon plots might occur even if pyruvate carboxylase rather than pyruvate transport is the controlling step for mitochondrial pyruvate metabolism. In support of this view, carboxylation of pyruvate generated inside the mitochondria from alanine is also stimulated in liver mitochondria from rats treated with glucagon (Chan *et al.*, 1979). Moreover, subsequent studies by Groen *et al.* (1983) have indicated that, contrary to the conclusions of Thomas & Halestrap (1981) and Rognstad (1983), gluconeogenesis from L-lactate by hepatocytes

from starved rats cannot be regulated by mitochondrial pyruvate transport. They point out that the apparent K_i for inhibition of pyruvate metabolism or gluconeogenesis by α-cyano-4-hydroxycinnamate should match the K_i found for pyruvate transport in isolated mitochondria before a regulatory role for the transporter can be asserted. Since the K_i of α-cyano-4-hydroxycinnamate for the pyruvate transporter is dependent both on the pH gradient and on the presence of other monocarboxylates, such as ketone bodies (Halestrap, 1978*a*; Halestrap *et al.*, 1980; Thomas & Halestrap, 1981), this comparison is not possible.

In the present paper we circumvent this problem by using the exceptionally potent inhibitor of the pyruvate transporter, α-cyano-β-(1-phenylindol-3-yl)acrylate ($K_i < 10^{-8}$ M). As indicated in the preceding paper (Shearman & Halestrap, 1984), this inhibitor binds so tightly that it titrates out the carrier stoichiometrically until about 65% inhibition is reached. Titration of pyruvate metabolism with this inhibitor and measurement of intramitochondrial pyruvate concentrations during metabolism have been used to assess the potential of the transporter for controlling the rate of pyruvate metabolism. It is demonstrated that, contrary to our earlier conclusions (Halestrap, 1977, 1978*b*), mitochondrial pyruvate metabolism is not controlled by the rate of pyruvate transport. This conclusion is discussed in relation to the stimulation of net flux from pyruvate to phosphoenolpyruvate induced by hormones in the intact cell during gluconeogenesis from L-lactate. An important role for stimulation of the respiratory chain and consequent activation of pyruvate carboxylase is proposed.

Experimental

Materials

Liver mitochondria were prepared from normal fed female Wistar rats (200–250g body wt.) as described previously (Halestrap, 1975). Where hormone treatment was required, rats were anaesthetized by injection (intraperitoneal) of Sagatal (1ml/kg body wt.) and after 30min, where required, glucagon (0.4mg/kg) or phenylephrine (2.0mg/kg) dissolved in saline was injected (intraperitoneally), and the rats were left for a further 8min (phenylephrine) or 15min (glucagon) before removal of the liver.

Unless otherwise stated, all chemicals and biochemicals were obtained from the sources cited in Halestrap (1975) and Armston *et al.* (1982). Radiochemicals were purchased from Amersham International, Amersham, Bucks, U.K. Glucagon was obtained from Serva Feinbiochemica, Heidelberg, West Germany, and L-phenylephrine from Sigma Chemical Co., Poole, Dorset, U.K.

Methods

Measurement of pyruvate metabolism and carboxylation. The rates of pyruvate carboxylation and total pyruvate metabolism at 37°C were measured simultaneously in the same buffer as for oxygen-electrode studies but containing 12.5mM-$KHCO_3$ gassed with O_2/CO_2 (19:1) before addition of $H^{14}CO_3^-$ (100d.p.m./nmol) and pyruvate (2mM). Details have been published elsewhere (Whipps & Halestrap, 1984). Incubations of mitochondria (2.5–5mg of protein/ml) were continued for 6min before termination by addition of $HClO_4$ (final concn. 2%, w/v) and analysis of the supernatant for pyruvate, ATP and acid-stable ^{14}C. Citrulline synthesis was studied similarly (Whipps & Halestrap, 1984), but pyruvate was omitted from the buffer, and 5mM-NH_4Cl, 5mM-glutamate, 1mM-L-malate and 20mM-L-ornithine were added.

Measurement of intramitochondrial pyruvate concentration. The intramitochondrial concentration of pyruvate during metabolism was measured by an adaptation of the method described by Halestrap & Denton (1974). The medium used for pyruvate-carboxylation studies (excluding $H^{14}CO_3^-$) was supplemented with D-[1-^{14}C]-mannitol (1mM; 0.1μCi/ml) and ^3H_2O (1μCi/ml). To 8ml of medium in a 20ml sealed vessel was added approx. 30mg of mitochondrial protein before gassing with O_2/CO_2 (19:1) and incubation for 2min at 37°C. At this time, six 1ml samples were layered on top of silicone oil (sp.gr. 1.02; 200μl) overlaying 50μl of $HClO_4$ (5%, w/v) in a 1.5ml tube before rapid centrifugation at 10000*g* for 1min. The supernatant was carefully removed and acidified with $HClO_4$ (final concn. 2%, w/v), and then the oil was removed and discarded. The remaining mitochondrial extract was diluted to 0.5ml with water, and samples of the supernatant were assayed for ^3H, ^{14}C and pyruvate as described previously (Halestrap & Denton, 1974; Halestrap & Quinlan, 1983). From these measurements, the intramitochondrial volume (^3H_2O–[^{14}C]mannitol space) and the amount of intramitochondrial pyruvate (expressed as the pyruvate–[^{14}C]mannitol space) could be calculated (Halestrap & McGivan, 1979; Halestrap & Quinlan, 1983). Two additional 1ml samples from the original incubation were centrifuged in the absence of silicone oil and $HClO_4$, and the resulting mitochondrial pellet was assayed for protein by a modified biuret reaction standardized with bovine serum albumin (Gornall *et al.*, 1949).

Measurement of the protonmotive force. Mitochondrial membrane potentials and transmembrane pH gradients were determined by using

[^{3}H]phenylmethylphosphonium ions (1.0μM; 0.1 μCi/ml) and [^{14}C]5,5'-dimethyloxazolidine-2,4-dione (0.2mM; 0.1μCi/ml) respectively (see Rottenberg, 1979; Holian & Wilson, 1980). Three parallel incubations were set up under conditions similar to those used for measuring intramitochondrial pyruvate concentrations. All incubations were well gassed with O_2/CO_2 (19:1) and also contained either [^{3}H]triphenylmethylphosphonium iodide or [^{14}C]5,5'-dimethyloxazolidine-2,4-dione and ^3H_2O (1μCi/ml) or D-[1-^{14}C]mannitol (1mM; 0.1μCi/ml) and ^3H_2O (1.0μCi/ml). Mitochondria (approx. 4mg of protein/ml) were incubated for 2min at 37°C before removal of five 1ml samples, which were centrifuged at 10000*g* for 1min. One pellet was assayed for protein, and the remaining pellets were assayed for ^{14}C and/or ^{3}H in order to determine the uptake of [^{3}H]triphenylmethylphosphonium or [^{14}C]5,5'-dimethyloxazolidine-2,4-dione and the intramitochondrial volume (see Halestrap & McGivan, 1979; Halestrap & Quinlan, 1983). From these the mitochondrial membrane potential and transmembrane pH gradient were calculated (Rottenberg, 1979). Non-specific binding of [^{3}H]triphenylmethylphosphonium was determined after addition of uncoupler. It was found to be less than 5% of the energized uptake and could be ignored without significant effect on the final result.

Computer simulation of the inhibition of mitochondrial pyruvate metabolism by α-cyano-4-hydroxycinnamate. In order to assess the effects of α-cyano-4-hydroxycinnamate on mitochondrial pyruvate metabolism under conditions where pyruvate transport was not rate-limiting, computer simulations were performed by the procedure outlined previously (Thomas & Halestrap, 1981) to calculate the intramitochondrial pyruvate concentration. Eqn. (5) of Thomas & Halestrap (1981) was modified by inclusion of a diffusion constant for carrier-independent pyruvate transport rather than a fixed rate. From the estimated intramitochondrial pyruvate concentration, the rates of pyruvate carboxylation and oxidation were calculated from the relevant K_m and $V_{max.}$ values.

Results and discussion

Inhibition by α-cyano-β-(1-phenylindol-3-yl)acrylate of liver mitochondrial pyruvate carboxylation

The inhibition by α-cyano-β-(1-phenylindol-3-yl)acrylate of pyruvate carboxylation and total pyruvate metabolism by liver mitochondria from control and glucagon-treated animals is shown in Fig. 1. A linear relationship between [inhibitor] and rate of pyruvate carboxylation or total pyruvate metabolism was observed only after the first inhibitor addition. The amount of inhibitor required to give 50% inhibition was dependent on the protein concentration (results not shown), and extrapolation of the linear portion of the plot to zero rate of carrier-dependent metabolism (dotted line in Fig. 1) was used to estimate the concentration of carrier sites. The extrapolated value was the same for both pyruvate carboxylation and total pyruvate metabolism and was shown to be proportional to the protein concentration. In 17 separate experiments on different mitochondrial preparations the mean ± S.E.M. was 121.9±4.12pmol of carrier/mg of protein (range 71–179). As expected, this value is similar to that obtained from the oxygen-electrode studies in the preceding paper (Shearman & Halestrap, 1984).

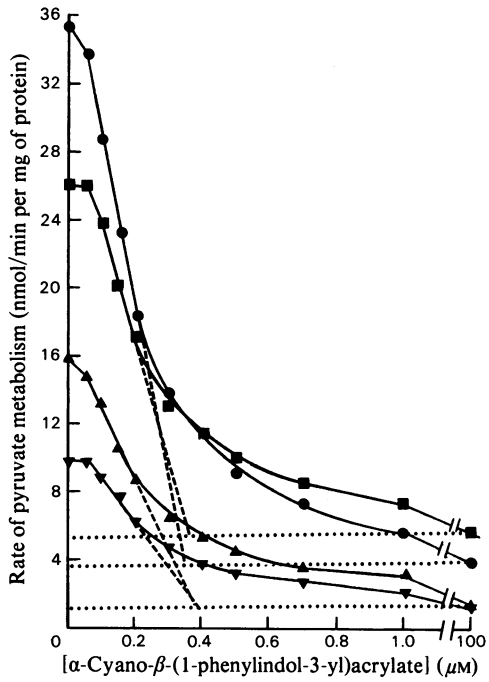

Fig. 1. *Inhibition by α-cyano-β-(1-phenylindol-3-yl)acrylate of pyruvate metabolism by liver mitochondria from control and glucagon-treated rats, in bicarbonate medium*

Total pyruvate metabolism (●, ■) and pyruvate carboxylation (▲, ▼) by liver mitochondria from control (■, ▼) and glucagon-treated (●, ▲) rats were measured as described in the Experimental section. Each data point is the mean of duplicate observations. The mitochondrial protein concentrations were 3.59 (control) and 3.57 (glucagon) mg/ml. The dashed and dotted lines represent the extrapolated linear portion of the plots and the inhibitor-insensitive rate of metabolism respectively. From the intercepts, the concentrations of carrier molecules per mg of protein, estimated as described in the Experimental section, were 118 for control and 111 for glucagon-treated mitochondria.

The lack of inhibition of pyruvate metabolism by low inhibitor concentrations, especially in control mitochondria, indicates that pyruvate transport does not limit pyruvate metabolism in the absence of inhibitor under the conditions used in the present experiments. This contrasts with previous conclusions derived from the linear Dixon plots observed with α-cyano-4-hydroxycinnamate (Halestrap, 1978*b*; Armston *et al.*, 1982). We have repeated these experiments on numerous occasions and continue to obtain such linear plots, despite data to the contrary obtained by Martin *et al.* (1984). Such apparent linearity under conditions where pyruvate carboxylase rather than pyruvate transport limits metabolism can be predicted by using computer simulations similar to those used previously to investigate the effects of α-cyano-4-hydroxycinnamate on gluconeogenesis (Thomas & Halestrap, 1981), as shown in Fig. 2. Actual data (the mean values from five separate experiments) are plotted on top of simulated plots for mitochondria from control and glucagon-treated rats. For both the carboxylation and total metabolism of pyruvate the fit is very good. The simulation uses a value of the K_m for pyruvate dehydrogenase of 175 μM, a value higher than that used previously (Thomas & Halestrap, 1981), but reflecting the apparent K_m for the enzyme under appropriate conditions of effectors and cofactors (Walsh *et al.*, 1976). This higher K_m value was required to account for the equivalent decrease in both pyruvate carboxylation and total pyruvate metabolism at increasing inhibitor concentration. Although not strictly linear, the deviation from linearity predicted would be insufficient to be detected under our experimental conditions. There is also an increase in the apparent K_i from the real value (about 6 μM; Halestrap, 1975, 1978*a*) to the observed values of about 40 μM. The effects of glucagon treatment have been mimicked by both an increase in the $V_{max.}$ and a decrease in the K_m of pyruvate carboxylase. An increase in ATP/ADP ratio (see below) would be expected to increase the $V_{max.}$, and a fall in the K_m would be predicted from the decrease in glutamate concentration after glucagon treatment (Siess *et al.*, 1977; Siess & Wieland, 1978), since this metabolite is a competitive inhibitor of pyruvate carboxylase (Scrutton & White, 1974).

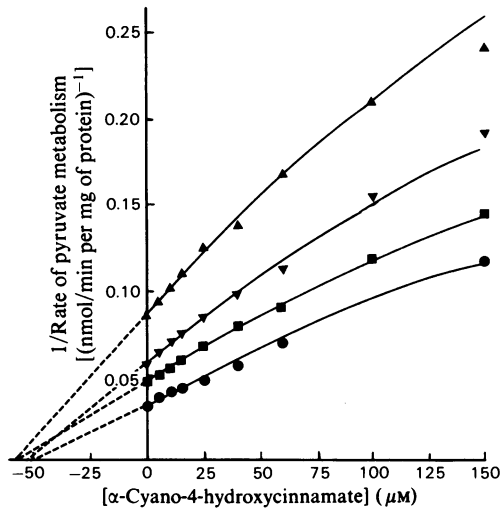

Fig. 2. *Computer simulation of the inhibition of liver mitochondrial pyruvate metabolism by α-cyano-4-hydroxycinnamate*

The data points represent the reciprocal of the mean rates of pyruvate carboxylation (▲,▼) and total pyruvate metabolism (■, ●) from five experiments on liver mitochondria from five different control (■, ▲) and glucagon treated (●,▼) rats. The solid lines are derived by computer simulation as described by Thomas & Halestrap (1981), but with the modifications outlined in the Experimental section. For both control and glucagon-treated mitochondria, the $V_{max.}$ and K_m for pyruvate transport were set at 200 nmol/min per mg and 150 μM respectively, the K_i for α-cyano-4-hydroxycinnamate at 6 μM, the K_m for pyruvate dehydrogenase at 175 μM and the external pyruvate concentration at 2 mM. Other parameter values were changed between control (first value) and glucagon-treated (second value) conditions as follows: K_m of pyruvate carboxylase 150 μM to 100 μM, $V_{max.}$ of pyruvate carboxylase 14 to 21 nmol/min per mg of protein, $V_{max.}$ of pyruvate dehydrogenase 12.8 to 19.5 nmol/min per mg of protein and carrier-independent diffusion constant for pyruvate 0.001 to 0.0015 min^{-1}. Justification for the values used is given in the Results section.

Measurement of intramitochondrial pyruvate concentration during metabolism

The data of Table 1 confirm that pyruvate transport was not rate-limiting for pyruvate metabolism in the absence of inhibitor. In these experiments the intramitochondrial pyruvate concentration was determined by measuring the ^3H_2O, [^{14}C]mannitol and enzymically assayable pyruvate present in the mitochondrial pellets. The intramitochondrial pyruvate concentration may then be calculated by multiplying the external pyruvate concentration by the ratio of the intramitochondrial pyruvate space to the intramitochondrial volume (Halestrap & McGivan, 1979). Table 1 shows that the control mitochondria support an intramitochondrial pyruvate concentration about equal to the extramitochondrial concentration, whereas mitochondria from the glucagon-treated animals have a significantly lower con-

Table 1. *Intramitochondrial pyruvate concentration during pyruvate metabolism*

Measurements of mitochondrial pyruvate metabolism and mitochondrial pyruvate, [^{14}C]mannitol and ^3H_2O spaces were performed as described in the Experimental section. Where indicated, 250 μM-α-cyano-4-hydroxycinnamate was added to the incubation medium. Three separate experiments are shown, each value being the mean ± S.E.M. for six observations on the same mitochondrial preparation. Statistical significances were calculated by Student's *t* test: glucagon compared with control: $*P<0.01$, $**P<0.02$; glucagon + α-cyano-4-hydroxycinnamate compared with glucagon: $\dagger P<0.01$, $\dagger\dagger P<0.02$.

Expt. no.	Rate of pyruvate metabolism (nmol/min per mg of protein)			Mitochondrial 'spaces' (μl/mg of protein)					
				^3H_2O–[^{14}C]mannitol			Pyruvate–[^{14}C]mannitol		
	Control	Glucagon	Glucagon + α-cyano-4-hydroxycinnamate	Control	Glucagon	Glucagon + α-cyano-4-hydroxycinnamate	Control	Glucagon	Glucagon + α-cyano-4-hydroxycinnamate
1	23.8 ± 0.54	33.2 ± 1.10*	5.60 ± 0.21†	0.570 ± 0.032	0.554 ± 0.031	0.516 ± 0.034	0.558 ± 0.098	0.133 ± 0.088*	−0.048 ± 0.047
2	20.6 ± 1.03	34.3 ± 0.90*	7.75 ± 0.30†	0.591 ± 0.026	0.567 ± 0.027	0.596 ± 0.035	1.092 ± 0.151	0.609 ± 0.053**	0.077 ± 0.172††
3	20.7 ± 0.68	36.6 ± 0.25*	9.3 ± 0.35†	0.535 ± 0.033	0.517 ± 0.038	0.535 ± 0.012	0.893 ± 0.077	0.720 ± 0.098	0.227 ± 0.076†

centration. This is consistent with an activation of intramitochondrial pyruvate metabolism without stimulation of transport, as demonstrated in the simulation presented in Fig. 2. As expected in the presence of 250 μM-α-cyano-4-hydroxycinnamate, the pyruvate concentration detected inside the mitochondria was not significantly different from zero. This result validates the use of mannitol rather than sucrose as an extramitochondrial marker (Halestrap & Quinlan, 1983). It should be noted that the changes in mitochondrial volume observed *in situ* and after rapid cell disruption (Quinlan *et al.*, 1983) are not observed consistently in mitochondria isolated by conventional techniques (Armston *et al.*, 1982; Whipps & Halestrap, 1984). In both glucagon-treated and control mitochondria the intramitochondrial concentration of pyruvate was considerably higher than the K_m values for either pyruvate carboxylase (100–150 μM) or pyruvate dehydrogenase (175 μM) (see above), which confirms that pyruvate transport cannot limit pyruvate metabolism under these conditions.

Measurement of mitochondrial protonmotive force and ATP concentrations during metabolism

Previous data from this and other laboratories suggested that hormones stimulate mitochondrial pyruvate transport by increasing the transmembrane pH gradient (Titheradge & Coore, 1976*a*,*b*; Halestrap, 1977, 1978*b*). In addition, stimulation of pyruvate carboxylase by an increase in the intramitochondria ATP/ADP ratio has been implied (Bryla *et al.*, 1977; Titheradge *et al.*, 1979; Bryla & Niedzwiecka, 1979; Chan *et al.*, 1979; Martin & Titheradge, 1983; Chisholm *et al.*, 1983). In previous experiments from this laboratory (Halestrap, 1977, 1978*b*; Armston *et al.*, 1982), open containers were used for incubation of mitochondria when studying pyruvate metabolism. We have now established that rapid loss of CO_2 during the incubation led to a rise in pH of the medium to 7.8–8.0. This probably accounts for the acid mitochondrial matrix observed under these conditions (Halestrap, 1978*b*) and may have influenced the changes of ΔpH induced by hormones. We have therefore re-examined mitochondrial pH gradients and membrane potentials, using closed containers with an O_2/CO_2 (19:1) atmosphere. In addition, [^{14}C]5,5'-dimethyloxazolidine-2,4-dione was used in order to measure ΔpH without problems associated with metabolism of acetate and binding of methylamine encountered in previous experiments (Halestrap, 1978*b*). To avoid the use of valinomycin in K^+-depleted media, [^{3}H]triphenylmethylphosphonium iodide was added in place of $^{86}Rb^+$ to measure the membrane potential. Phenylephrine-

treated animals were used in addition to glucagon-treated animals to investigate the effects of α-agonists on mitochondrial function. Such α-agonists appear to have the same effects as glucagon on mitochondrial function (Adam & Haynes, 1969; Garrison & Haynes, 1975; Titheradge & Coore, 1976*b*; Garrison & Borland, 1979; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980*a*; Halestrap, 1981; Allan *et al.*, 1983), and we have confirmed this in assays of citrulline synthesis, respiratory-chain activity and Ca^{2+}-induced mitochondrial swelling (A. E. Armston & A. P. Halestrap, unpublished work).

In parallel with their effects on pyruvate metabolism, both hormones increased the ATP content of the metabolizing mitochondria by about 80% from 1.30 ± 0.23 to 2.30 ± 0.16 and 2.38 ± 0.23 nmol/mg of mitochondrial protein for glucagon and phenylephrine treatment respectively (values given as means ± S.E.M. for four separate experiments). This effect was accompanied by a significant increase in the membrane potential of 2.9 ± 0.71 mV and 2.9 ± 0.76 mV in mitochondria from glucagon and phenylephrine-treated rats respectively from a control value of 179.6 ± 2.1 mV (values given as means ± S.E.M. for four separate experiments). No change in the transmembrane pH gradient was detected under these conditions, the values (alkaline inside, and expressed in mV, as means ± S.E.M. for four separate observations) being 21.2 ± 1.45, 22.0 ± 2.21 and 23.68 ± 1.97 for mitochondria from control, glucagon- and phenylephrine-treated rats respectively. These values are similar to those measured *in situ* (Shulman *et al.*, 1979; Hoek *et al.*, 1980; Strzelecki *et al.*, 1984), and more recent data indicate no change in the value after hormone treatment of hepatocytes (Strzelecki *et al.*, 1984). In agreement with others (Bryla *et al.*, 1977; Siess *et al.*, 1977; Prpic *et al.*, 1978; Titheradge *et al.*, 1979; Taylor *et al.*, 1980; Titheradge & Haynes, 1980*a*,*b*; Aprille *et al.*, 1982; Chisholm *et al.*, 1983), the total adenine nucleotide content of mitochondria was shown to rise in liver mitochondria from glucagon- or phenylephrine-treated rats by about 25% (results not shown). As suggested by others (Bryla *et al.*, 1977; Martin & Titheradge, 1983; Chisholm *et al.*, 1983), these data imply that it is the increase in mitochondrial ATP rather than a pH-driven stimulation of pyruvate transport that stimulates mitochondrial pyruvate carboxylation. The increase in ATP/ADP ratio predicted for the observed change in protonmotive force can be calculated if it is assumed that $2H^+$ are required to synthesize 1 molecule of intramitochondrial ATP (Wander *et al.*, 1981; Williamson *et al.*, 1981). A 5 mV increase in protonmotive force would produce a 50% increase in mitochondrial ATP/ADP ratio. Taking into account the increase in total mitochondrial adenine nucleotides induced by hormone treatment, the observed changes in ATP content of metabolizing mitochondria are of the order predicted. Thus it would appear that it is the increase in protonmotive force that induces an increase in mitochondrial ATP/ADP ratio and that this causes the stimulation of pyruvate metabolism observed.

Role of the respiratory chain in hormonal effects on mitochondrial metabolism

The increase in protonmotive force induced by hormone treatment is thought to be secondary to the stimulation of the respiratory chain seen in liver mitochondria from hormone-treated rats (Yamazaki, 1975; Halestrap, 1978*b*, 1982, and references therein), and there is a good correlation between the effects of glucagon on pyruvate metabolism and on the respiratory chain (Halestrap (1978*b*,*c*). Thus it would be predicted that the hormone effects on mitochondrial pyruvate metabolism and ATP concentration could be reversed by mild inhibition of the respiratory chain. The data of Table 2 show that this is what is observed.

Table 2. *Reversal of the effects of glucagon treatment on the rate of mitochondrial pyruvate metabolism by Amytal*

All experimental procedures were performed as outlined in the Experimental section. Where indicated, Amytal (0.15 mM) was added to the incubation. Values, expressed as nmol/min per mg of protein, are given as the means ± S.E.M. for four observations on the same mitochondrial preparation. The experiment given is typical of three such experiments. Statistical significances of the effects of glucagon and Amytal were calculated by Student's *t* test: †$P < 0.01$ (glucagon compared with control); *$P < 0.01$ (glucagon + Amytal compared with glucagon).

Parameter	Control	Glucagon	Glucagon + 0.15 mM-Amytal
Rate of pyruvate carboxylation	9.70 ± 0.10	13.67 ± 0.10†	8.23 ± 0.08*
Rate of total pyruvate metabolism	23.77 ± 0.54	33.20 ± 1.09†	22.71 ± 1.04*
ATP content of mitochondria during pyruvate metabolism	1.93 ± 0.07	2.63 ± 0.06†	1.86 ± 0.07*
Rate of citrulline synthesis	9.86 ± 0.18	15.17 ± 0.18†	13.04 ± 0.11*
ATP content of mitochondria during citrulline biosynthesis	2.69 ± 0.07	4.04 ± 0.08†	2.98 ± 0.11*

Addition of 0.15mM-Amytal to the incubations of mitochondria from glucagon-treated rats decreases pyruvate carboxylation, total pyruvate metabolism and ATP concentrations to control values. We have used other respiratory-chain inhibitors such as 2-heptyl-4-hydroxyquinoline *N*-oxide and 2,5-dibromo-6-isopropyl-3-methylbenzoquinone with similar effect (results not shown), but these inhibitors were too powerful to allow accurate control of the low inhibitor concentration required for reversal of the hormone effects. The effects of Amytal on citrulline synthesis were less pronounced than on pyruvate metabolism, and the effect of glucagon was only partially decreased (Table 2). This is to be expected, since at least part of the hormone's stimulation of citrulline synthesis is caused by elevated *N*-acetylglutamate concentrations (Hensgens *et al.*, 1980; Rabier *et al.*, 1982), in addition to an effect of elevated ATP/ADP ratios (Bryla & Niedzwiecka, 1979; Titheradge & Haynes, 1980*a*,*b*; Goldstein & Aprille, 1982; Jensen *et al.*, 1983; Hamman & Haynes, 1983).

Conclusions

The data in the present paper indicate that our previous conclusion that hormone treatment of rats stimulates pyruvate metabolism in subsequently isolated mitochondria through an effect on pyruvate transport was erroneous. Rather it would seem that it is the effect of glucagon and phenylephrine to stimulate the respiratory chain (Yamazaki, 1975; Halestrap, 1978*b*,*c*, 1982) which leads to an increase in protonmotive force, a consequent rise in ATP/ADP ratio and hence activation of pyruvate carboxylase. It is not clear what causes the stimulation in flux through pyruvate dehydrogenase, although stimulation of pyruvate carboxylase would provide increased oxaloacetate to remove both the acetyl-CoA (as citrate) and NADH (as malate) produced by pyruvate dehydrogenase. Our conclusion concerning the central role played by the respiratory chain is supported by the observed correlation between the hormonal effect on the uncoupled rate of succinate oxidation, the rate of pyruvate carboxylation and the reduction state of the *b* cytochromes under metabolizing conditions (Halestrap, 1978*b*,*c*). It is further confirmed by the effects of adding a low concentration of a respiratory-chain inhibitor to the glucagon-treated mitochondria, which causes them to behave like control mitochondria in relation to their ATP concentration and pyruvate metabolism (Table 2).

The question arises as to whether the stimulation of pyruvate metabolism seen in isolated mitochondria from glucagon-treated rats is relevant *in vivo*. Siess *et al.* (1981) have argued that results obtained in isolated mitochondria are artefacts, but this has been strongly challenged by others (Verhoeven *et al.*, 1982; Jensen *et al.*, 1983; Allan *et al.*, 1983; Whipps & Halestrap, 1984). Groen *et al.* (1983) concluded from experiments with perifused liver cells that, although pyruvate carboxylase has a high control strength, it is not the major site of regulation by glucagon. They ascribe pyruvate kinase to this role. However, the effect of glucagon on pyruvate kinase activity in hepatocytes from starved rats is small, since the enzyme is already greatly inhibited in the control cells under these conditions (Thomas & Halestrap, 1981). Furthermore, α-agonists are without effect on the enzyme activity in starved cells (Thomas & Halestrap, 1981) and yet stimulate gluconeogenesis at the same locus as glucagon (Exton & Park, 1969; Chan & Exton, 1978) and have similar effects on mitochondrial function (see Halestrap *et al.*, 1983; Armston, 1984).

Computer simulation studies on the inhibition of gluconeogenesis in hepatocytes from starved animals by α-cyano-4-hydroxycinnamate are incompatible with control of gluconeogenesis residing in pyruvate kinase alone, but would support a role for pyruvate carboxylase on its own or in conjunction with pyruvate transport and pyruvate kinase (Thomas & Halestrap, 1981). The data in the present paper make it unlikely that pyruvate transport is a regulatory step, as do the data of others (Siess *et al.*, 1977; Tischler *et al.*, 1977; Groen *et al.*, 1983), since the measured intramitochondrial pyruvate concentration approaches the equilibrium value in the hepatocyte as well as in isolated mitochondria. Unfortunately, we have been unable to use α-cyano-β-(1-phenylindol-3-yl)-acrylate to confirm this conclusion, since the inhibitor is too rapidly metabolized by liver cells. However, using the computer simulation techniques of Thomas & Halestrap (1981), we have been able to simulate the linear and Dixon plots of α-cyano-4-hydroxycinnamate inhibition of gluconeogenesis under conditions where the $V_{max.}$ for pyruvate transport was set at a value 5–10-fold in excess of that for pyruvate carboxylase (results not shown). The K_i value for the inhibitor used in the simulation was the determined value of 6 μM (Halestrap, 1975), although the apparent K_i value was 40–50 μM, as was observed experimentally (Thomas & Halestrap, 1981). Activation by hormones can be simulated by 45% stimulation of pyruvate carboxylase, which produced a 40% stimulation of gluconeogenesis. At a Hill coefficient of 2.5, changing the K_m of pyruvate kinase from 0.8 to 1.4mM, as occurs after glucagon treatment of starved rats (Thomas & Halestrap, 1981), produced a further increase in gluconeogenesis rate of 13%. This is insufficient to be the sole mechanism by which glucagon activates

gluconeogenesis, as suggested by Groen *et al.* (1982), but it may explain the larger stimulatory effect of glucagon than of phenylephrine on gluconeogenesis. Thus pyruvate carboxylase remains the most likely locus of hormonal control.

Activation of the gluconeogenesis by glucagon or α-agonists occurs in isolated hepatocytes in conjunction with an increase in mitochondrial matrix volume (see Quinlan *et al.*, 1983; Halestrap *et al.*, 1983). In isolated mitochondria, increasing the matrix volume increases both respiratory-chain activity and pyruvate metabolism (Armston *et al.*, 1982; Halestrap, 1982). Thus activation of mitochondrial pyruvate metabolism through an increased respiratory-chain activity would be expected *in vivo*. The observed increase in mitochondrial ATP/ADP ratios and acetyl-CoA concentrations and a fall in glutamate and 2-oxoglutarate concentrations (Siess *et al.*, 1977; Siess & Wieland, 1978, 1980; Bryla *et al.*, 1977; Titheradge *et al.*, 1979; Jensen *et al.*, 1983; Chisholm *et al.*, 1983) could all be explained by a stimulation of the respiratory chain and would lead to activation of pyruvate carboxylase. In support of this, low concentrations of Amytal inhibit gluconeogenesis by isolated hepatocytes without affecting ATP concentration (Thomas & Halestrap, 1981). Preliminary experiments indicate that the concentration of Amytal required to reverse the effects of glucagon on gluconeogenesis is exactly the same as that required to reverse the hormone's effect on the mitochondrial cytochrome spectra in hepatocytes (P. T. Quinlan & A. P. Halestrap, unpublished work).

This work was supported by a project grant from the Medical Research Council.

References

Adam, P. A. J. & Haynes, R. C. (1969) *J. Biol. Chem.* **244**, 6444–6450

Allan, E. H., Chisholm, A. B. & Titheradge, M. A. (1983) *Biochem. J.* **212**, 417–418

Aprille, J. R., Nosek, M. T. & Brennan, W. A. (1982) *Biochem. Biophys. Res. Commun.* **108**, 834–839

Armston, A. E. (1984) Ph.D. Thesis, University of Bristol

Armston, A. E., Halestrap, A. P. & Scott, R. D. (1982) *Biochim. Biophys. Acta* **681**, 429–439

Bryla, J. & Niedzwiecka, A. (1979) *Int. J. Biochem.* **10**, 235–239

Bryla, J., Harris, E. J. & Plumb, J. A. (1977) *FEBS Lett.* **80**, 443–448

Chan, T. M. & Exton, J. H. (1978) *J. Biol. Chem.* **253**, 6393–6400

Chan, T. M., Bacon, C. B. & Hills, S. A. (1979) *J. Biol. Chem.* **254**, 8730–8732

Chisholm, A. B., Allan, E. H. & Titheradge, M. A. (1983) *Biochem. J.* **214**, 451–458

Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 1424–1433

Garison, J. C. & Borland, M. K. (1979) *J. Biol. Chem.* **254**, 1129–1133

Garrison, J. C. & Haynes, R. C. (1975) *J. Biol. Chem.* **250**, 2769–2777

Goldstein, F. C. & Aprille, J. (1982) *Arch. Biochem. Biophys.* **213**, 7–13

Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756

Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. R. H. & Tager, J. M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 9–37, Academic Press, New York

Groen, A. K., Vervoorn, R. C., Van der Meer, R. & Tager, J. M. (1983) *J. Biol. Chem.* **258**, 14346–14353

Halestrap, A. P. (1975) *Biochem. J.* **145**, 85–96

Halestrap, A. P. (1977) *Biochem. Soc. Trans.* **5**, 216–219

Halestrap, A. P. (1978*a*) *Biochem. J.* **172**, 377–387

Halestrap, A. P. (1978*b*) *Biochem. J.* **172**, 389–398

Halestrap, A. P. (1978*c*) *Biochem. J.* **172**, 399–405

Halestrap, A. P. (1981) in *Short Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G. eds.), pp. 389–409, Elsevier/North-Holland, Amsterdam

Halestrap, A. P. (1982) *Biochem. J.* **204**, 37–47

Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **138**, 313–316

Halestrap, A. P. & McGivan, J. D. (1979) in *Techniques in Metabolic Research* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), vol. B206, pp. 1–23, Elsevier/North-Holland, Amsterdam

Halestrap, A. P. & Quinlan, P. T. (1983) *Biochem. J.* **214**, 387–393

Halestrap, A. P., Scott, R. D. & Thomas, A. P. (1980) *Int. J. Biochem.* **11**, 97–105

Halestrap, A. P., Armston, A. E., Quinlan, P. T. & Whipps, D. E. (1983) *Horm. Cell Regul.* **7**, 185–200

Hamman, H. C. & Haynes, R. C. (1983) *Arch. Biochem. Biophys.* **223**, 85–94

Hensgens, H. E. S. J., Verhoeven, A. J. & Meijer, A. J. (1980) *Eur. J. Biochem.* **107**, 197–205

Hoek, J. B., Nicholls, D. D. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 1458–1464

Holian, A. & Wilson, D. F. (1980) *Biochemistry* **19**, 4213–4221

Jensen, C. B., Sistare, F. D., Hamman, H. C. & Haynes, R. C. (1983) *Biochem. J.* **210**, 819–827

Martin, A. D. & Titheradge, M. A. (1983) *Biochem. Soc. Trans.* **11**, 78–81

Martin, A. D., Allan, E. H. & Titheradge, M. A. (1984) *Biochem. J.* **219**, 107–115

Prpic, V., Spencer, T. L. & Bygrave, F. L. (1978) *Biochem. J.* **176**, 705–716

Quinlan, P. T., Thomas, A. P., Halestrap, A. P. & Armston, A. E. (1983) *Biochem. J.* **214**, 395–404

Rabier, D., Briand, P., Petit, F., Parvy, P., Kamoun, P. & Cathelineau, L. (1982) *Biochem. J.* **206**, 627–631

Rognstad, R. (1983) *Int. J. Biochem.* **15**, 1417–1421

Rottenberg, H. (1979) *Methods Enzymol.* **55**, 547–569

Scrutton, M. C. & White, M. D. (1974) *J. Biol. Chem.* **249**, 5405–5415

Shearman, M. S. & Halestrap, A. P. (1984) *Biochem. J.* **223**, 673–676

Shulman, R. G., Brown, T. R., Ugurbil, K., Ogawa, S., Cohen, S. M. & den Hollander, J. A. (1979) *Science* **205**, 160–166

Siess, E. A. & Wieland, O. H. (1978) *FEBS Lett.* **93**, 301–306

Siess, E. A. & Wieland, O. H. (1980) *Eur. J. Biochem.* **110**, 203–210

Siess, E. A., Brocks, D. G., Lattke, M. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–233

Siess, E. A., Fahimi, F. M. & Wieland, O. H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1643–1651

Strzelecki, T., Thomas, J. A., Koch, C. D. & LaNoue, K. F. (1984) *J. Biol. Chem.* **259**, 4122–4129

Taylor, W. M., Prpic, V., Exton, J. H. & Bygrave, F. L. (1980) *Biochem. J.* **188**, 443–450

Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **198**, 551–564

Tischler, M. E., Hecht, P. & Williamson, J. R. (1977) *Arch. Biochem. Biophys.* **181**, 278–292

Titheradge, M. A. & Coore, H. G. (1976*a*) *FEBS Lett.* **63**, 45–50

Titheradge, M. A. & Coore, H. G. (1976*b*) *FEBS Lett.* **71**, 73–78

Titheradge, M. A. & Haynes, R. C. (1980*a*) *Arch. Biochem. Biophys.* **201**, 44–55

Titheradge, M. A. & Haynes, R. C. (1980*b*) *J. Biol. Chem.* **255**, 1471–1477

Titheradge, M. A., Stringer, J. L. & Haynes, R. C. (1979) *Eur. J. Biochem.* **102**, 117–124

Verhoeven, A. J., Hensgens, H. E. S. J., Meijer, A. J. & Tager, J. M. (1982) *FEBS Lett.* **140**, 270–272

Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J. & Randle, P. J. (1976) *Biochem. J.* **157**, 41–67

Wander, R. J. A., Groen, A. K., Meijer, A. J. & Tager, J. M. (1981) *FEBS Lett.* **132**, 201–206

Whipps, D. E. & Halestrap, A. P. (1984) *Biochem. J.* **221**, 147–152

Williamson, J. R., Steinman, R., Coll, K. & Rich, T. L. (1981) *J. Biol. Chem.* **256**, 7287–7297

Yamazaki, R. K. (1975) *J. Biol. Chem.* **250**, 7924–7930