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Hormonal stimulation of mitochondrial pyruvate carboxylation in filipin-treated hepatocytes

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A method is described for measuring rates of mitochondrial pyruvate carboxylation in hepatocytes treated with the polyene antibiotic, filipin, to render the plasma membrane permeable to substrates. With this approach it was possible to demonstrate that treatment of cells with glucagon or catecholamines results in a stimulation of mitochondrial CO_2 fixation measured *in situ* comparable with that observed in the isolated mitochondria, in terms of time of onset of the response, hormone selectivity and sensitivity. In addition, angiotensin II and vasopressin were shown to enhance the activity of pyruvate carboxylase in both the intact mitochondria and filipin-treated cells, thus strengthening the postulate that this site is a major locus of hormone action in the control of gluconeogenesis. Addition of 3-mercaptopicolinic acid, to inhibit gluconeogenesis at the level of phosphoenolpyruvate carboxykinase, had no significant effect on the stimulation of pyruvate carboxylation by adrenaline, suggesting that the effect of the hormone at this site is independent of changes in activity of other enzymes further on in the pathway. The data presented preclude the possibility that acute effects of hormones on mitochondrial metabolism are solely artifacts of the preparation procedure.

Adam & Haynes (1969) first demonstrated that mitochondria isolated from rats treated with glucagon, adrenaline (epinephrine) or cortisol exhibited higher rates of pyruvate carboxylation and oxidation than did mitochondria prepared from control animals. Further work has indicated that the alterations in pyruvate carboxylase activity satisfy all the necessary criteria for it to be considered an important locus in the control of gluconeogenesis with respect to time of onset, sensitivity to hormone concentrations, the involvement of cyclic AMP in the glucagon response and the α-adrenergic nature of the stimulation by catecholamines (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979). Studies into the molecular mechanisms by which these hormones stimulate the pyruvate carboxylase reaction have indicated that acute treatment of either isolated hepatocytes or intact animals by gluconeogenic hormones results in a generalized stimulation of mitochondrial metabolism, and to date approximately 15 mitochondrial functions have been reported to be altered (Yamazaki, 1975; Titheradge & Coore, 1976*a*,*b*; Halestrap, 1978*a*,*b*; Siess & Wieland, 1979; Titheradge & Haynes, 1979). From these studies it has become apparent that severe restraints are imposed on pyruvate carboxylase activity within the mitochondria and that the action of gluconeogenic hormones is to relieve this restraint through alterations in the intramitochondrial concentrations of known effectors, particularly ATP and the [ATP]/[ADP] ratio, the supply of pyruvate, glutamate and acetyl-CoA.

Although the above studies strongly suggest that glucagon and catecholamines have acute effects on mitochondrial metabolism in the intact cell, interpretation of the data is not unequivocal, as the possibility that some or all of these effects might be artifacts introduced by the lengthy mitochondrial preparation procedure cannot be eliminated. Indeed, Siess *et al.* (1981) have suggested that glucagon treatment results in an increased stability of the mitochondria against inactivation during the preparation procedure, rather than inducing a true activation of the mitochondria *in vivo*.

In order to clarify the situation, we have used the polyene antibiotic filipin to render hepatocytes permeable to substrates, thus allowing the rate of mitochondrial pyruvate carboxylation to be measured *in situ* after hormone treatment. Filipin is known to form multimolecular complexes with cholesterol in membrane systems in such a way that the permeability of the membrane is increased (for

review see Norman *et al.*, 1976). This technique was used to study oxidative phosphorylation in intact sperm cells (Morton & Lardy, 1967), and has more recently been used to measure glucose 6-phosphatase activity *in situ* in isolated hepatocytes (Jorgenson & Nordlie, 1980).

Using this approach, we have been able to demonstrate increased rates of mitochondrial pyruvate carboxylation in cells made permeable with filipin within very short periods after hormone treatment. The response to gluconeogenic hormones was essentially identical with that of mitochondria isolated by conventional means in terms of hormone selectivity, dose sensitivity and time of onset. This strengthens the hypothesis that, for the rate of pyruvate carboxylation at least, the increased metabolism measured in the isolated mitochondria after hormone treatment is a true reflection of a physiological event occurring within the intact cell and not an artifact of preparation.

Experimental

Materials

Type IV collagenase, bacitracin, [arginine]vasopressin, angiotensin II, phenylephrine hydrochloride, bovine serum albumin (fraction V) and sodium pyruvate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). α-Cyano-4-hydroxycinnamic acid was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Glucagon was given by Novo Laboratories (Basingstoke, Hants, U.K.), and filipin by Upjohn (Crawley, E. Sussex, U.K.). 3-Mercaptopicolinate was a gift from Smith, Kline and French (Philadelphia, PA, U.S.A.). All other chemicals were AnalaR grade from BDH Chemicals (Enfield, Middx., U.K.).

Isolation and incubation of hepatocytes

Fed male Sprague–Dawley rats weighing 180–240g were anaesthetized by intraperitoneal injection of sodium pentobarbital (60mg/kg). Hepatocytes were prepared by a modification of the procedure of Berry & Friend (1969), essentially as described by Hutson *et al.* (1976), except that washed aged erythrocytes were excluded from the perifusion medium. The hepatocytes were resuspended in Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), containing 1.5% gelatin, 2mM-glucose and 0.1% (w/v) bacitracin. The final protein concentration of the cell suspension was approx. 15mg/ml. Samples (10ml) of the cell suspension were transferred to 125ml Erlenmeyer flasks, gassed with O_2/CO_2 (19:1) and preincubated for 30min. After the preincubation period, hormones or vehicle were added, together with a pyruvate/lactate mixture to give final concentrations of 2.5mM-pyruvate and 2.5mM-lactate, and the incubation was continued for a further 10min. The cells were then added to 25ml of ice-cold isolation buffer containing 0.3M-sucrose, 5mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid) and 1mM-EDTA, pH 7.4, and sedimented by allowing the centrifuge to accelerate up to 500*g* followed by immediate deceleration with the brake on. This wash procedure took less than 1min to complete. The cell pellet was resuspended in a further 5ml of isolation buffer and the washing procedure repeated to remove all trace of ions and hormones. The final cell pellet was resuspended in 1.5ml of isolation buffer, a sample was removed for the immediate determinations of cellular pyruvate carboxylation, and the remainder was used to isolate mitochondria.

Isolation of mitochondria

The mitochondrial isolation procedure was based on that of Titheradge & Haynes (1980). The cell suspension was homogenized with four strokes in a Dounce homogenizer, diluted with a further 5ml of isolation medium and centrifuged at 4000*g* for 1min. The supernatant was then removed, and the pellet was re-homogenized and centrifuged as described above. The two supernatants from each pellet were combined and centrifuged at 9000*g* for 10min, and the mitochondrial fraction was washed with 2 × 5ml of isolation medium. The resultant mitochondrial pellets were resuspended in isolation medium to a final concentration of 5–10mg of protein/ml and assayed immediately.

Mitochondrial pyruvate carboxylation was assayed by the incorporation of $NaH^{14}CO_3$ into acid-stable products by the method of Adam & Haynes (1969). The rate of cellular pyruvate carboxylation was assayed as described above, except that filipin was added to a final concentration of 100μM (unless stated otherwise). Filipin was added as a solution in dimethyl sulphoxide to give a final concentration of dimethyl sulphoxide of 1% (v/v). Both the cell and mitochondrial protein concentrations were adjusted to give a final value of approx. 1mg/ml in the assay.

Analysis of metabolites was performed on neutralized $HClO_4$ extracts by paper chromatography in ethanol/conc. NH_3 (sp.gr. 0.880)/water (16:1:3, by vol.) as described by Seakins & Ersser (1976), and two-dimensional t.l.c. on cellulose plates (Myers & Huang, 1969). Citrate and malate were measured as described by Williamson & Corkey (1969).

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as the standard.

Results are expressed as means ± S.E.M., with the numbers of different cell preparations in parentheses. Analysis of results was performed by a paired *t* test.

Results and discussion

From the initial work of Adam & Haynes (1969), the concept has evolved that acute treatment of intact animals or isolated hepatocytes with glucagon or catecholamines results in a general stimulation of mitochondrial energy-conserving mechanisms, which is reflected in increased rates of both pyruvate carboxylation and gluconeogenesis.

Although there is universal agreement that these effects are apparent in the isolated mitochondria, a report by Siess *et al.* (1981) has cast some doubts as to whether mitochondrial function is in fact enhanced *in vivo*. They have shown that the effect of treatment of intact animals or isolated hepatocytes with dibutyryl cyclic AMP or glucagon on pyruvate carboxylation, State-3 rates of respiration and succinate dehydrogenase activity is dependent on the isolation procedure used to prepare the mitochondria. Replacement of sucrose with mannitol, addition of cinchocaine or chloroquine (agents known to decrease the fragility of the mitochondrial membrane), or omission of the washing step, were all shown to decrease the effect of the hormone by increasing the basal activities in the control mitochondria. From this, they have proposed that the effects of the hormones are to stabilize the mitochondria against inactivation during the preparation procedure, possibly as a result of inhibition of phospholipase activity.

The objectives of this study were therefore twofold: first, to develop a method for measuring rates of mitochondrial pyruvate carboxylation *in situ* with minimal interference by other pyruvate-independent CO_2-fixation reactions; second, if a suitable method could be found, to investigate whether hormone treatment of cells resulted in a stimulation of pyruvate carboxylation *in situ* comparable with that obtained in subsequently isolated mitochondria, thus eliminating the lengthy mitochondrial isolation procedure and possible artifacts associated with it. From these data it should be possible to confirm or refute the premise that acute alterations in mitochondrial function are of physiological importance in the regulation of gluconeogenesis in the intact cell.

Effect of filipin treatment on pyruvate carboxylation by intact hepatocytes

The method chosen uses the polyene antibiotic filipin, and is based on the observation that filipin binds to cholesterol in membranes, forming complexes that render the membrane permeable to low-molecular-weight molecules (Kinsky *et al.*, 1967). Owing to the relatively high concentration of cholesterol in the plasma membrane, as compared with the mitochondrial membranes of hepatocytes (Colbeau *et al.*, 1971), filipin can be used to increase cell permeability selectively without disturbing mitochondrial integrity (Morton & Lardy, 1967).

Table 1. *Effect of filipin concentration on pyruvate carboxylation in isolated cells*

Cells were incubated as described in the Experimental section and resuspended in isolation medium to a final concentration of approx. 5 mg/ml. Incorporation of $^{14}CO_2$ into acid-stable products was measured as described by Adam & Haynes (1969) in the presence and absence of 5 mM-pyruvate and various concentrations of filipin or vehicle. Results are expressed as means ± S.E.M. for four different cell preparations.

	Pyruvate carboxylation (nmol/min per mg of protein)	
Filipin concn. (μM)	−pyruvate	+pyruvate
None	4.6 ± 0.6	8.1 ± 0.6
1% (w/v) dimethyl sulphoxide	4.6 ± 0.6	7.9 ± 0.6
25	2.8 ± 0.2	13.3 ± 0.8
50	2.2 ± 0.2	14.8 ± 1.3
75	1.9 ± 0.2	15.2 ± 1.2
100	1.7 ± 0.3	15.3 ± 0.9
200	1.6 ± 0.3	15.2 ± 1.0

Table 1 shows the effect of increasing concentrations of filipin on the incorporation of $NaH^{14}CO_3$ into acid-stable products by isolated hepatocytes. In the absence of filipin a significant rate of CO_2 fixation was observed, which increased by 75% on addition of exogenous pyruvate as substrate. The relatively high rate of CO_2 fixation in the absence of exogenous pyruvate is probably a reflection of significant concentrations of pyruvate or lactate in the cytosol as a result of the preincubation period of the cells with these substrates. The inclusion of filipin in the assay medium produced a distinct effect on cell permeability as indicated by the rate of CO_2 fixation. Increasing the concentration of filipin resulted in a progressive decline in the rate of endogenous CO_2 fixation, presumably as a result of dilution of the intracellular substrates in the assay medium as cell permeability increased. In contrast, in the presence of 5 mM-pyruvate, CO_2 fixation was progressively stimulated with increasing filipin concentrations until, at a final concentration of 75 μM, it reached a value almost double that measured in its absence and almost 10-fold greater than that measured in the presence of filipin but in the absence of pyruvate. In all further studies 100 μM-filipin was used, as this gave the optimal increase in activity in the presence of pyruvate, together with the lowest activity in its absence.

Analysis of the products of the CO_2 fixation reaction by paper chromatography (Seakins & Ersser, 1976) and two-dimensional t.l.c. (Myers & Huang, 1969) indicated that most of the ^{14}C label

incorporated was found in both citrate and malate, with other tricarboxylic acid-cycle intermediates contributing to a lesser extent. Enzymic measurement of citrate and malate concentrations in the deproteinized extracts showed that these two intermediates accounted for approx. 70% of the pyruvate carboxylated. Similar results have been documented for the products of pyruvate carboxylation in isolated mitochondria (Haynes, 1965; Adam & Haynes, 1969). These data suggest that the assay is specific for an intracellular pyruvate-dependent carboxylation reaction, namely the mitochondrial pyruvate carboxylase reaction, with minimal interference from alternative substrate-dependent reactions.

Effect of protein concentration on the rate of pyruvate carboxylation in filipin-treated hepatocytes

As filipin forms multimolecular complexes with membrane cholesterol, it was necessary to determine whether the concentration chosen to produce optimal rates of CO_2 fixation was dependent on cell density. In both the absence and presence of pyruvate, a linear response was obtained over a 4-fold concentration range, the final concentrations being 0.8–32.mg of protein/ml in the assay. This suggests that the filipin/cholesterol ratio is not critical. In all further experiments the protein concentration in the assay was maintained between 1.0 and 1.6mg of cell protein/ml.

Effect of time of incubation on pyruvate carboxylation in filipin-treated cells

The results in Table 1 confirm previous reports that filipin-treated hepatocytes provide a suitable model for studying enzymes and organelles in their macromolecular environment *in situ* (Jorgensen & Nordlie, 1980; Gankema *et al.*, 1981). However, before meaningful conclusions could be drawn from the results, it was necessary to investigate the time of onset of the filipin response and the effect of prolonged incubation periods on the integrity of both the cell and the mitochondrial membrane. The kinetics of the pyruvate carboxylation reaction are shown in Fig. 1. In the absence of exogenous pyruvate there was an initial rapid phase of CO_2 fixation during the first 2min, followed by a much decreased linear accumulation of ^{14}C-labelled acid-stable material over the next 8min. It is suggested that the initial rapid phase was the result of the utilization of endogenous intramitochondrial pyruvate. The slower linear phase was a possible reflection of either the low endogenous pyruvate concentration in the incubation after an increased membrane permeability, or a low rate of pyruvate-independent CO_2 fixation. In the presence of pyruvate, the time course of the incorporation of

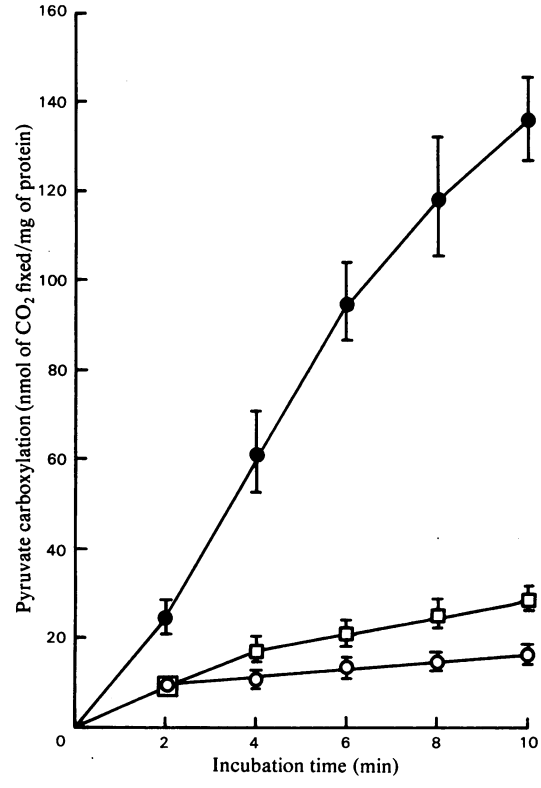

Fig. 1. *Time course of the pyruvate carboxylation reaction in filipin-treated cells*

Cells were prepared and preincubated as described in the Experimental section and resuspended in isolation medium. The pyruvate carboxylation assay was performed in the presence of 100μM-filipin for the indicated times with no additional substrate (○), 5mM-pyruvate (●) or 5mM-pyruvate plus 200μM-α-cyano-4-hydroxycinnamate (□). Each time point is the mean ± S.E.M. for five different cell preparations.

radioactivity exhibited an initial lag phase during the first 2min, possibly owing to temperature equilibration or generation of acetyl-CoA via the pyruvate dehydrogenase reaction, and a tailing-off at later time points. As the time course shown is comparable with that found when the assay is performed with isolated mitochondria (Garrison & Haynes, 1975; Wakat & Haynes, 1977), the sigmoidicity is not thought to be a property of the filipin treatment. The rate of CO_2 fixation independent of exogenous pyruvate was less than 5% of that in the presence of pyruvate.

Measurements of the rate of pyruvate carboxylation in isolated mitochondria in the presence of vehicle or filipin confirm that neither filipin nor dimethyl sulphoxide has any significant direct effect

on either the activity of pyruvate carboxylase itself or mitochondrial integrity under the conditions used for the assay ($P > 0.1$ for both treatments). The rates of pyruvate carboxylation, in nmol/min per mg of mitochondrial protein, were 24.1 ± 1.7 (5), 21.4 ± 1.1 (5) and 25.7 ± 2.3 (5) for control, control plus 1% dimethyl sulphoxide, and control plus 100 μM-filipin respectively. Addition of dimethyl sulphoxide alone resulted in a small inhibitory effect, but this was not significant over the range of experiments. Similar conclusions were drawn by Morton & Lardy (1967), using both hepatic and spermatozoan mitochondria.

Effect of α-cyano-4-hydroxycinnamate on CO_2 fixation in filipin-treated cells

To substantiate the claim that the pyruvate-dependent CO_2 fixation was in fact due to intramitochondrial pyruvate carboxylase activity, and that at the concentration of filipin used there was no effect on mitochondrial integrity over the time period studied, the time course of the CO_2 fixation reaction was investigated in the presence of α-cyano-4-hydroxycinnamate. At the concentration used, this agent has been shown to inhibit carrier-mediated pyruvate transport completely in isolated mitochondria, while having a minimal effect on pyruvate carboxylase activity in the presence of the concentrations of pyruvate used in the CO_2-fixation assay (Halestrap, 1975). Fig. 1 shows that, in the presence of 5 mM-pyruvate, 200 μM-α-cyano-4-hydroxycinnamate caused a rapid inhibition of pyruvate carboxylation. During the first minutes of the incubation the rate of CO_2 fixation was identical with that observed in the absence of external pyruvate. This declined to a linear rate of pyruvate carboxylation less than 10% of that in the absence of α-cyano-4-hydroxycinnamate. The ability of α-cyano-4-hydroxycinnamate to inhibit CO_2 fixation indicates that pyruvate transport across the mitochondrial inner membrane is an obligatory step in the CO_2-fixation reaction as measured and is further evidence to indicate that, under the assay conditions used, filipin has little effect on mitochondrial integrity, as this might be expected to be manifest as carrier-independent pyruvate carboxylation. The low rate of CO_2 fixation insensitive to α-cyano-4-hydroxycinnamate has also been demonstrated in isolated mitochondria and has been ascribed to a slow rate of carrier-independent diffusion of pyruvate across the membrane, owing to the high concentrations of pyruvate used (Halestrap & Denton, 1975).

Effect of amino-oxyacetate on CO_2 fixation

Although the above results strongly suggest that the pyruvate-dependent incorporation of $^{14}CO_2$ into acid-stable material is a measure of pyruvate carboxylase activity, the results are not conclusive. A further mechanism that might account for a mitochondrial CO_2-fixation reaction with similar products could be via a stimulation of the isocitrate dehydrogenase reaction with the carboxylation of 2-oxoglutarate to yield isocitrate. The apparent dependence on pyruvate in this system might be twofold: first, to maintain the concentration of 2-oxoglutarate within the mitochondria as a result of transamination of glutamate, and second, to supply the ATP necessary to sustain isocitrate formation by pyruvate oxidation. This mechanism would also appear to be sensitive to α-cyano-4-hydroxycinnamate, as intramitochondrial pyruvate would be required for both transamination and oxidation.

To eliminate this possibility, the rate of CO_2 fixation was examined when supported by a variety of substrates in the presence of either 1 mM-amino-oxyacetate or 200 μM-α-cyano-4-hydroxycinnamate. This concentration of amino-oxyacetate has been shown to inhibit alanine metabolism completely in cells at the level of alanine aminotransferase (Edmondson *et al.*, 1977; Joseph *et al.*, 1978). The results are shown in Table 2. When 2-oxoglutarate was used as the substrate, no significant incorporation of $^{14}CO_2$ was observed above the endogenous rate, indicating that the formation of 2-oxoglutarate is not the mechanism by which pyruvate stimulates this reaction. This is further confirmed by the finding that addition of amino-oxyacetate had no significant effect on the rate of CO_2 fixation with pyruvate as the substrate, although it completely abolished the increased incorporation of label when pyruvate was generated intramitochondrially by transamination of alanine. The rate of CO_2 fixation with alanine plus 2-oxoglutarate as the substrate was significantly lower than that measured in the presence of exogenous pyruvate ($P < 0.001$) and is probably a reflection of the increase in concentration of glutamate within the mitochondria, a known inhibitor of pyruvate carboxylase (Scrutton & White, 1974). Addition of α-cyano-4-hydroxycinnamate resulted in a small but insignificant decrease in the rate of CO_2 fixation with alanine plus 2-oxoglutarate as the substrate, indicating that transamination to pyruvate occurred almost exclusively within the mitochondrial matrix under the assay conditions used. Similar observations have been obtained by using isolated mitochondria to measure CO_2 fixation with alanine (Chan *et al.*, 1979; Martin & Titheradge, 1983).

To exclude the possibility that pyruvate oxidation within the mitochondria is acting solely to supply ATP to support an otherwise pyruvate-independent reaction, the effect of the addition of succinate plus palmitoylcarnitine and arsenite was examined in the presence and absence of α-cyano-4-hydroxycinnamate. When added together with pyruvate, these agents have been demonstrated to inhibit pyruvate

Table 2. *Effect of amino-oxyacetate and α-cyano-4-hydroxycinnamate on CO_2 fixation in filipin-treated cells*
Cells were preincubated as described in the Experimental section and resuspended to a final concentration of approx. 5 mg of protein/ml. CO_2 fixation was determined with the substrates shown in the presence of 100 μM-filipin and either 1 mM-amino-oxyacetate or 200 μM-α-cyano-4-hydroxycinnamate as indicated. The results are expressed as means ± S.E.M. for five different cell preparations. $^{**}P < 0.01$, $^{*}P < 0.05$, for difference from control without addition of either amino-oxyacetate or α-cyano-4-hydroxycinnamate.

Substrate	Amino-oxyacetate	α-Cyano-4-hydroxycinnamate	CO_2 fixation (nmol/min per mg of protein)
None	−	−	1.67 ± 0.29
5 mM-Pyruvate	−	−	13.41 ± 0.91
	+	−	12.09 ± 0.43
	−	+	3.32 ± 0.21**
1 mM-2-Oxoglutarate	−	−	2.02 ± 0.57
5 mM-Alanine + 1 mM-2-oxoglutarate	−	−	7.58 ± 0.76
	+	−	1.94 ± 0.35**
	−	+	7.14 ± 0.89
5 mM-Pyruvate + 1 mM-succinate + 30 μM-palmitoylcarnitine + 0.2 mM-arsenite	−	−	12.17 ± 1.04
	−	+	3.19 ± 1.16*

oxidation completely, while at the same time providing sufficient ATP and acetyl-CoA to support pyruvate carboxylation (Yamazaki & Haynes, 1975). In the absence of pyruvate oxidation, CO_2 fixation in filipin-treated cells was not significantly altered when supported by the oxidation of succinate and palmitoylcarnitine. However, in the presence of α-cyano-4-hydroxycinnamate to prevent pyruvate entry into the mitochondria, CO_2 fixation was completely abolished, indicating that a supply of intramitochondrial pyruvate is essential to sustain the reaction independent of the supply of ATP. The incorporation of $^{14}CO_2$ into acid-stable products in these cells therefore displays all the characteristics expected of the pyruvate carboxylase reaction, and it is concluded that this assay is specific for pyruvate carboxylation, with little or no interference from other cellular CO_2-fixation reactions.

Effect of adrenaline on CO_2 fixation in filipin-treated cells

Previous studies have demonstrated that adrenaline treatment of intact hepatocytes results in a significant stimulation of the rate of pyruvate carboxylation in subsequently isolated mitochondria, and that there is an excellent correlation between the enhanced rate of gluconeogenesis and the increased enzyme activity (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979). Fig. 2 shows the effect of preincubating hepatocytes with 1 μM-adrenaline on the rate of cellular pyruvate carboxylation measured in the presence of 100 μM-filipin. Cells that have been pretreated with adrenaline show a similar time course to cells incubated with vehicle alone. However, at all time points examined, it was apparent that adrenaline pretreatment resulted in a significant stimulation. In the absence of pyruvate, no significant difference between the control and hormone-treated cells was observed (results not shown). The time elapsing from the termination of the incubation with hormone until the CO_2-fixation assay was begun was no longer than 3 min, thus minimizing potential artifacts introduced by the normal isolation procedure of the mitochondria.

Table 3. *Stability of the effect of adrenaline on cellular pyruvate carboxylation*
After a 30 min preincubation period, adrenaline (final concn. 1 μM) or vehicle was added, and the incubation continued for a further period of 10 min. The cells were then washed and resuspended in isolation medium at a protein concentration of approx. 5 mg of cell protein/ml. The cells were maintained at 0°C for the periods indicated before the rate of pyruvate carboxylation was determined in the presence of 100 μM-filipin. Results are the means ± S.E.M. for four different cell preparations. $^{*}P < 0.01$.

Time (min)	Pyruvate carboxylation (nmol/min per mg of cell protein)	
	Control	+ Adrenaline
0	13.3 ± 0.7	20.3 ± 1.7*
5	13.4 ± 0.7	19.2 ± 0.9*
10	14.3 ± 1.2	20.4 ± 1.3*
15	13.4 ± 0.8	20.1 ± 0.7*
20	13.1 ± 1.1	19.5 ± 1.6*
30	12.8 ± 1.0	17.8 ± 1.4*

Table 3 demonstrates the stability of the effect of adrenaline on cells that have been preincubated with hormone for 10 min, followed by a rapid wash and resuspension in isolation medium at 0°C. Zero time

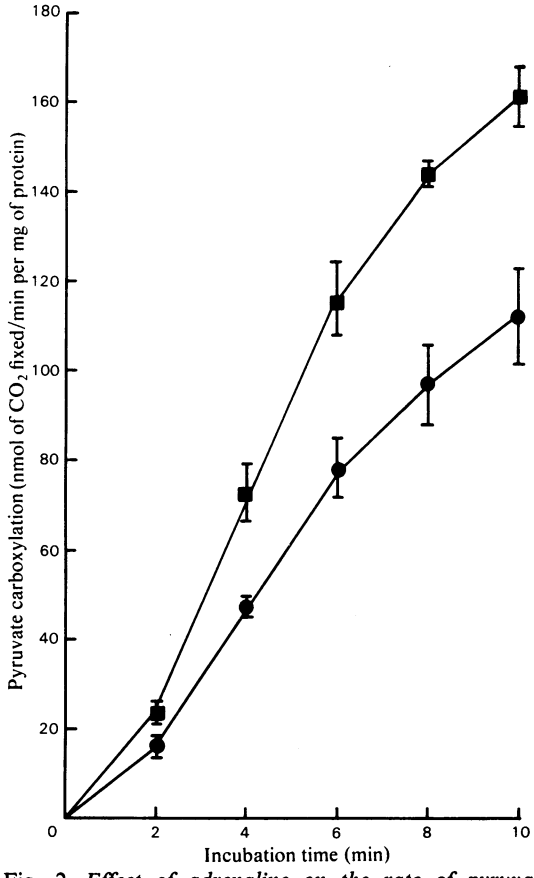

Fig. 2. *Effect of adrenaline on the rate of pyruvate carboxylation in filipin-treated cells*

Cells were incubated as described in the Experimental section for 30min before adrenaline was added (final concn. 1μM). After a further 10min of incubation, the cells were separated from the medium by centrifugation, washed and resuspended in isolation medium. The rate of cellular CO_2 fixation was assayed immediately in the presence of 100μM-filipin for the times indicated. Means ± S.E.M. for five different cell preparations are shown at each time point. ●, Control; ■, +adrenaline.

is designated as the time at which the cells were resuspended after the washing procedure. The control cells showed no significant decrease in the rate of CO_2 fixation over a 30min storage period on ice. Adrenaline treatment produced a significant stimulation at all times investigated, although a marked decrease was apparent after 30min. All further assays were therefore performed within 10min of resuspension of the cells.

Effect of 3-mercaptopicolinate on the adrenaline-induced stimulation of pyruvate carboxylation

Although it is apparent that after treatment of cells with adrenaline there is an increase in the flux through pyruvate carboxylase, it cannot be concluded that this is necessarily a prime locus of hormone action, as the increased flux may be secondary to alterations in the activity of other enzymes of the gluconeogenic pathway, for example pyruvate kinase, phosphofructokinase and fructose 1,6-bisphosphatase (for review see Claus & Pilkis, 1981). To resolve this question, the effect of adrenaline on the rate of pyruvate carboxylation was measured in the presence of 150μM-mercaptopicolinate, to inhibit gluconeogenesis at the level of phosphoenolpyruvate carboxykinase and thus to eliminate the involvement of enzymes distal to this in the pathway. The results are shown in Table 4. Although this concentration of 3-mercaptopicolinate was sufficient to decrease the rate of gluconeogenesis in intact cells from pyruvate + lactate to less than 15% (results not shown), it had no significant effect on the magnitude of the adrenaline-induced stimulation of pyruvate carboxylation with either alanine plus 2-oxoglutarate or pyruvate as the substrate.

Table 4. *Effect of 3-mercaptopicolinate on the adrenaline-induced stimulation of cellular pyruvate carboxylation*

Isolated hepatocytes were incubated as described in the Experimental section with 0.1μM-adrenaline. After washing the cells, the rate of cellular pyruvate carboxylation was immediately determined in the presence of 100μM-filipin and presence and absence of 150μM-3-mercaptopicolinate. The results shown are the means ± S.E.M. for five separate cell preparations. *$P < 0.01$ for difference from control.

Substrate	Treatment of cells	3-Mercaptopicolinate	CO_2 fixation (nmol/min per mg of protein)
5mM-Pyruvate	Control	–	13.19 ± 0.53
		+	12.54 ± 0.59
	Adrenaline	–	18.14 ± 0.46*
		+	17.46 ± 0.47*
5mM-Alanine + 1mM-2-oxoglutarate	Control	–	8.20 ± 0.39
		+	7.80 ± 0.14
	Adrenaline	–	12.48 ± 1.0*
		+	11.21 ± 0.75*

Comparison of the effects of gluconeogenic hormones on pyruvate carboxylation measured in situ and in isolated mitochondria

Although the effects of glucagon and adrenaline on gluconeogenesis and mitochondrial pyruvate carboxylation have been well documented (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979), if alterations in the rate of pyruvate carboxylation are to be considered a major site at which gluconeogenesis is regulated, then other gluconeogenic hormones such as angiotensin II and vasopressin (Hems & Whitton, 1973; Whitton *et al.*,

Table 5. *Effect of gluconeogenic hormones on cellular and mitochondrial pyruvate carboxylation*

Isolated hepatocytes were incubated as described in the Experimental section. After 30 min, the hormones were added to the final concentrations indicated and the incubation was continued for a further 10 min. After washing of the cells in isolation medium, a sample was removed for the immediate determination of the rate of pyruvate carboxylation in the presence of 100 μM-filipin, and the remainder were used in the preparation of mitochondria for the determination of the rate of mitochondrial pyruvate carboxylation. Results are expressed as means ± S.E.M. for six different cell preparations. *$P < 0.001$.

Addition	Pyruvate carboxylation (nmol/min per mg of protein)	
	Cellular	Mitochondrial
Vehicle	11.3 ± 1.0	15.3 ± 1.7
Glucagon (1 nM)	17.8 ± 1.3*	28.7 ± 1.7*
Adrenaline (0.1 μM)	17.5 ± 1.0*	30.7 ± 1.6*
Phenylephrine (0.1 μM)	16.4 ± 0.8*	25.8 ± 1.3*
Angiotensin (1 μM)	17.4 ± 1.7*	32.3 ± 2.5*
Vasopressin (1 munit/ml)	16.4 ± 1.7*	28.6 ± 1.6*

1978) ought also to act at this site. Table 5 shows the effect of treatment of cells with maximal concentrations of gluconeogenic hormones on the rate of pyruvate carboxylation in both filipin-treated cells and mitochondria isolated from those same cells. As described above, both glucagon and adrenaline treatment of the cells resulted in a significant stimulation of mitochondrial pyruvate carboxylation, the effect of catecholamines being through a stimulation of the α-adrenergic receptor, as indicated by the response of the α-agonist, phenylephrine. Treatment of the cells with both angiotensin II and vasopressin also resulted in a significant stimulation of the rate of pyruvate carboxylation in subsequently isolated mitochondria, thus extending the list of hormones known to be effective at this site and strengthening the postulate that it is a major point of regulation of gluconeogenesis.

In all previous studies, the rate of CO_2 fixation in mitochondria prepared from isolated hepatocytes has been 25–50% of the rate obtained in mitochondria isolated from the intact animal, when expressed as nmol/min per mg of mitochondrial protein (Garrison & Haynes, 1975; Titheradge *et al.*, 1979; Siess *et al.*, 1981). This has meant that interpretation of previous results has had to be treated with some caution, as, although they were qualitatively the same as those obtained with mitochondria from the intact animal, reservations concerning mitochondrial integrity in these preparations must remain. In the present study, in which a less vigorous homogenization procedure was used, the rate of CO_2 fixation in mitochondria prepared from hepatocytes was comparable with the rates obtained with mitochondria prepared from the intact animal. The effect of hormone treatment of the cells was still persistent, although the magnitude of the response was slightly less when expressed as a percentage of the control activity (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979). This would tend to vindicate the conclusions drawn from the previous studies.

The effect of the gluconeogenic hormones on mitochondrial pyruvate carboxylation was paralleled by an increased CO_2 fixation in the cells from which the mitochondria were prepared. The magnitude of the response obtained with filipin-treated cells in this series was marginally less than in the isolated mitochondria, suggesting that some enhancement of the hormone effect may have occurred during preparation of the mitochondria, as described by Siess *et al.* (1981). However, the response was still very significant and of a similar magnitude to the stimulation of gluconeogenesis observed under comparable conditions with these agents (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979).

Time of onset and dose–response of the effect of adrenaline on pyruvate carboxylation in filipin-treated cells and isolated mitochondria

Figs. 3 and 4 show a comparison of the effects of adrenaline on pyruvate carboxylation measured in both isolated mitochondria and filipin-treated cells in terms of the time course of the response and sensitivity to adrenaline concentration. In contrast with the results in Table 5, neither of these two parameters showed any significant difference between the magnitude of response to adrenaline in either the isolated mitochondria or filipin-treated cells, nor was there any significant difference in either the time of onset or sensitivity to hormone. After treatment with adrenaline, the rate of CO_2 fixation was rapidly stimulated within 2 min, the response being maximal within 5–10 min, with some fall-off at 15 min. This agrees favourably with previous studies comparing gluconeogenesis and mitochondrial pyruvate carboxylation (Garrison & Borland, 1979; Titheradge *et al.*, 1979). The concentration of adrenaline required to produce a half-maximal response in the isolated mitochondria was 25 nM, and that in the filipin-treated cells was 30 nM.

Therefore, in conclusion, using this assay we have been able to measure rates of mitochondrial pyruvate carboxylation *in situ* within very short time periods after hormonal treatment of hepatocytes. The results indicate that the effect of glucagon or catecholamines on pyruvate carboxylation in the

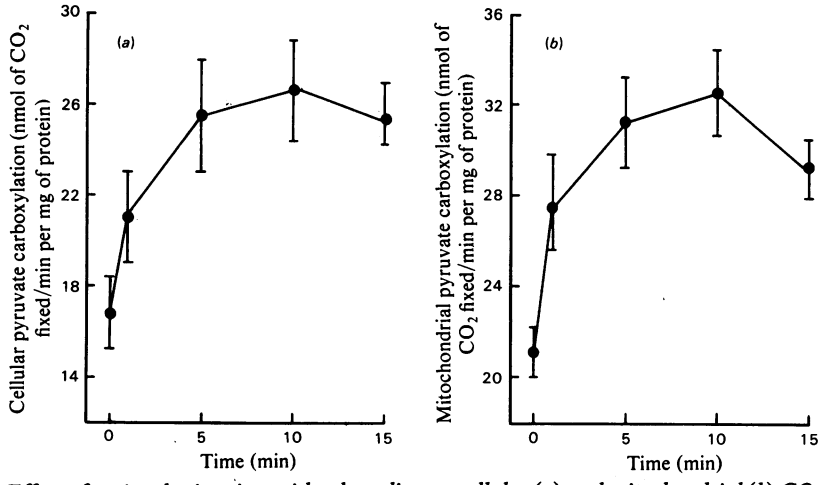

Fig. 3. *Effect of preincubation time with adrenaline on cellular (a) and mitochondrial (b) CO_2 fixation*
Cells were incubated as described in the Experimental section for 30min; then adrenaline was added (final concn. 30nM) and the incubation continued for the times indicated. At the end of the incubation periods shown, the cells were rapidly separated from the medium, washed and resuspended in isolation medium. A sample was immediately assayed for the rate of cellular pyruvate carboxylation in the presence of 100μM-filipin, and the remainder was used to prepare mitochondria. The rate of pyruvate carboxylation was measured over a 5min incubation period. Each point is the mean ± S.E.M. for five different cell preparations.

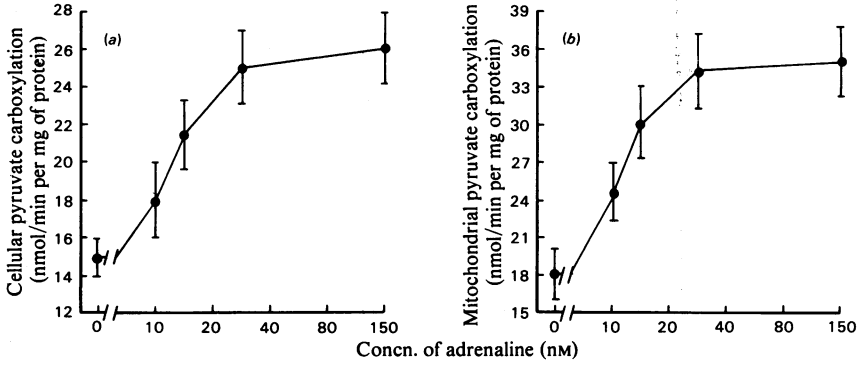

Fig. 4. *Effect of adrenaline concentration on CO_2 fixation in isolated mitochondria (b) and filipin-treated cells (a)*
The methods were the same as in Fig. 3, except that after the 30min incubation period adrenaline was added to the concentration indicated. The incubation with hormone was continued for 10min. Each point is the mean ± S.E.M. for five different cell preparations.

isolated mitochondria is not merely a consequence of a lengthy isolation procedure, with loss of activity in the control preparations, but is a true reflection of an increased rate of CO_2 fixation *in vivo*. The specificity to hormones, time of onset and sensitivity of the response to catecholamines were not significantly different when the rate of CO_2 fixation was measured in either filipin-treated hepatocytes or mitochondria isolated from the hepatocytes, suggesting that no major alterations in activity occurred during the mitochondrial preparation procedure. This contrasts with the observations of Siess *et al.* (1981). It seems possible that the discrepancy in the stability of the hormone effect during isolation of the mitochondria and the magnitude of the response found in this study and that of Siess *et al.* (1981) is a result of the improved isolation procedure. The values for the rates of pyruvate carboxylation reported here are some 3–4-fold higher than those in other studies using mitochondria from isolated hepatocytes. This suggests either that the mitochondrial preparations employed previously have been grossly contaminated with other membrane fractions, or that the integrity of the mitochondrial inner membrane was sufficiently decreased to be unable to support maximal rates of pyruvate carboxylation, possibly as a result of loss of adenine nucleotides or uncoupling of oxidative phosphorylation. If, as Siess

et al. (1981) suggest, glucagon treatment results in a decreased phospholipase activity, with concomitant stabilization of the membrane, then it is conceivable that the effect of the hormone might be enhanced with the more vigorous preparation procedure used in other reports (Garrison & Haynes, 1975). Similarly, addition of cinchocaine or chloroquine to the isolation medium might lead to a decrease in the magnitude of the hormone effect by making the control less resistant to damage during isolation. However, the high rates of pyruvate carboxylation and close similarity between the effects of hormones on CO_2 fixation measured in filipin-treated hepatocytes and in the isolated mitochondria in this study would suggest that the use of a milder preparation procedure to minimize mitochondrial damage results in negligible loss of activity in control mitochondria during isolation and maintenance of any differential effects as they existed *in vivo*. The results presented above do not discount an inhibitory effect of glucagon on phospholipase activity, with alteration in membrane phospholipids, although they do suggest that, if such an inhibition occurs and it is relevant to the effect of hormones on mitochondrial function, then it occurs *in vivo* in response to hormone treatment and it is part of the metabolic mechanism for regulating mitochondrial metabolism. In support of this, Halestrap (1982) has presented some evidence to suggest that perturbation of the mitochondrial phospholipids by aging, addition of benzyl alcohol or treatment with phospholipase A_2 can reverse some of the effects of glucagon on mitochondrial respiratory-chain activity. However, if such changes occur, then they are very localized, as no gross alterations in phospholipid composition have been observed after glucagon treatment (Siess *et al.*, 1981; Halestrap, 1982).

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