The stimulation of mitochondrial pyruvate carboxylation after dexamethasone treatment of rats

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Treatment of rats for 3 h with dexamethasone was shown to stimulate both pyruvate carboxylation and decarboxylation in the subsequently isolated mitochondria. The effect of hormone treatment on pyruvate carboxylation was also apparent in liver homogenates assayed within minutes of killing the animal and was independent of the temperature at which the assay was performed, suggesting that it was not an artifact of the mitochondrial preparation procedure. The stimulation of both aspects of pyruvate metabolism in the intact organelle was independent of the induction of either pyruvate carboxylase or pyruvate dehydrogenase. Similarly, there was no change in the percentage of pyruvate dehydrogenase in the active form, indicating that the effect of steroid treatment on pyruvate oxidation was not via changes in the degree of phosphorylation of the enzyme. Adrenalectomizing the animals for a period of 14 days before the experiment had no effect on either parameter. Glucocorticoid treatment of the animals increased the rate of pyruvate uptake into the mitochondria, as measured by the titration of pyruvate metabolism with α -cyano-4-hydroxycinnamate, a specific inhibitor of the pyruvate translocator. It also increased the intramitochondrial concentrations of acetyl-CoA and ATP and led to an elevated [ATP]/[ADP] ratio within the mitochondria. It is suggested that both enzymes of pyruvate metabolism exist in the mitochondria under considerable restraint and that glucocorticoids act to relieve this restraint by alterations in substrate supply and the intramitochondrial concentrations of effector molecules.

Acute treatment of rats with glucocorticoids is known to stimulate hepatic gluconeogenesis and increase plasma glucose concentrations (Long *et al.*, 1940; Haynes, 1962; Rinard *et al.*, 1969; Exton *et al.*, 1970, 1973). Crossover studies of gluconeogenic intermediates in perfused liver and liver slices after such short-term treatment have implicated the first reaction of the gluconeogenic pathway, the mitochondrial carboxylation of pyruvate to form oxaloacetate, as a possible site of control (Rinard *et al.*, 1969; Exton *et al.*, 1973). In support of this, Adam & Haynes (1969) demonstrated that mitochondria isolated from rats treated with glucocorticoids for 3 h displayed enhanced rates of pyruvate carboxylation and pyruvate uptake into the mitochondria. Further work indicated that the stimulation of mitochondrial CO₂ fixation correlated well with the known effects of the steroids on gluconeogenesis (Wakat & Haynes, 1977) and that there was a

direct correlation between the increased rates of pyruvate carboxylation and the increase in plasma glucose after administration of the hormone to the rat (Wakat & Haynes, 1977). A requirement for protein synthesis *de novo* in the mechanism of action of the steroids was indicated by the time of onset of the response and the abolition of the effect by co-administration of cycloheximide with the steroid, although it was suggested that this was not the result of induction of pyruvate carboxylase (Wakat & Haynes, 1977).

The experiments in the present study were designed to characterize further the effects of glucocorticoids on pyruvate metabolism in rat liver mitochondria. The results confirm the findings of Wakat & Haynes (1977) in that acute treatment of rats with dexamethasone led to a stable increase in the rate of pyruvate metabolism in the subsequently isolated mitochondria. This was a consequence of an increased supply of pyruvate to the enzyme, an elevated intramitochondrial concentration of both acetyl-CoA and ATP and an

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increased [ATP]/[ADP] ratio, but not the induction of either pyruvate carboxylase or pyruvate dehydrogenase. A preliminary account of part of this work has been published (Martin & Titheradge, 1983).

Experimental

Materials

Sodium pyruvate, alanine, 2-oxoglutarate, amino-oxyacetate, dexamethasone, cycloheximide, NADH, NAD⁺, ATP, ADP, acetyl-CoA, CoA and thiamin pyrophosphate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Glucagon was a gift from Novo Chemicals (Basingstoke, Hants., U.K.). [1-¹⁴C]Pyruvate, [4-¹⁴C]aspartate, [³H]water, [U-¹⁴C]sucrose and NaH¹⁴CO₃ were obtained from Amersham International (Amersham, Bucks., U.K.). Bromododecane and bromododecane were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All other chemicals were of AnalaR grade from BDH Chemicals (Enfield, Middx., U.K.).

Treatment of animals

Male Sprague-Dawley rats weighing 160–200 g were injected intraperitoneally with dexamethasone (150 µmol/kg) suspended in iso-osmotic saline, or with vehicle alone. The rats were maintained at a temperature of 21–24°C as described by Wakat & Haynes (1977), and after 3 h were killed by decapitation. The livers were rapidly removed and mitochondria prepared in 0.3 M-sucrose as described previously (Titheradge & Haynes, 1979). Sucrose was used as the osmotic support during the preparation, as opposed to mannitol (Siess, 1983a,b), as there is evidence to suggest that mannitol can enter the mitochondrial matrix (Halestrap & Quinlan, 1983), and preliminary studies in our laboratory have suggested that this leads to mitochondrial swelling, with a consequent increase in pyruvate utilization (A. D. Martin & M. A. Titheradge, unpublished work). Addition of either EDTA or EGTA to the isolation medium was found to have no significant effect on the rates of pyruvate metabolism when compared with mitochondria measured in 0.3 M-sucrose (results not shown). In experiments in which the effects of cyclohexamide were examined, the drug was injected intraperitoneally (3.5 µmol/kg) in iso-osmotic saline, together with the dexamethasone or vehicle. Glucagon (0.14 µmol/kg) was injected intraperitoneally 20 min before removal of the livers. For some experiments animals were bilaterally adrenalectomized under diethyl ether anaesthesia 14 days before the experiment and maintained with 0.9% NaCl in their drinking water. Controls received a sham operation.

Assays

Pyruvate carboxylation and decarboxylation were measured by the method of Adam & Haynes (1969) in a medium containing 203 mM-sucrose, 12.5 mM-MgCl₂, 2.5 mM-K₂SO₄, 8 mM-potassium phosphate, 3.4 mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid), 0.7 mM-EDTA and 12.5 mM-KHCO₃, pH 7.4. Carboxylation was measured by the incorporation of H¹⁴CO₃⁻ (sp. radioactivity 0.06 Ci/mol) into acid-stable products, and decarboxylation was measured by the production of ¹⁴CO₂ from [1-¹⁴C]pyruvate (sp. radioactivity 0.02 Ci/mol). The loss of ¹⁴CO₂ from the medium during the incubation period in the absence of mitochondria was 7.3 ± 0.4% (S.E.M.; *n* = 3). There was no change in the pH during this period. In a series of experiments, the sum of the rates of pyruvate carboxylation and decarboxylation was 103 ± 3% (*n* = 10) of the rate of disappearance of enzymically assayable pyruvate from the medium. Total pyruvate carboxylase activity was measured in mitochondria lysed by freeze-thawing as described by Weinberg & Utter (1979). Pyruvate decarboxylation activity was measured in mitochondria preincubated under the conditions used to measure pyruvate oxidation. After a 5 min preincubation period the mitochondria were diluted with an equal volume of isolation medium containing 50 mM-Tris/HCl, pH 7.0, 5 mM-EDTA and 1 mM-dithiothreitol at 0°C and immediately sedimented in an Eppendorf micro-centrifuge for 15 s. The supernatant was discarded and the mitochondria were rapidly frozen in liquid N₂. Just before assay, they were resuspended in isolation medium and assayed at 30°C as described by Leiter *et al.* (1978). Total pyruvate dehydrogenase activity was estimated by the inclusion of 50 µM-2,4-dinitrophenol in the preincubation medium (Walajtys *et al.*, 1974). The intramitochondrial concentration of glutamate was measured in neutralized HClO₄ extracts prepared by separating mitochondria from the incubation medium by centrifugation through bromododecane/bromodecane (2:3, v/v) into 2.9 M-perchloric acid, with an Eppendorf micro-centrifuge. The results were corrected for the amount of glutamate in the extramitochondrial volume by measurement of the extra-matrix space with [U-¹⁴C]sucrose and the total mitochondrial volume with [³H]water. The intramitochondrial volume was 0.71 ± 0.04 µl/mg of mitochondria protein and the extramitochondrial volume 2.66 ± 0.07 µl/mg of mitochondrial protein (*n* = 16). Glutamate was determined by the method of Lowry & Passonneau (1972). All other assays were performed as described as previously (Chisholm *et al.*, 1983).

Statistical significance was calculated by using a paired *t* test.

Results and discussion

Effect of dexamethasone on pyruvate metabolism in hepatic mitochondria and homogenates

Haynes and co-workers have demonstrated that mitochondria isolated from the livers of rats treated with a variety of glucocorticoids display increased rates of pyruvate metabolism and State-3 and uncoupled rates of respiration with succinate and β -hydroxybutyrate as the substrate (Adam & Haynes, 1969; Wakat & Haynes, 1977). Although it has been proposed that these alterations in mitochondrial metabolism reflect a physiological change in mitochondrial function *in vivo* related to the stimulation of gluconeogenesis, a report by Siess *et al.* (1981) has suggested that hormone treatment stabilizes the mitochondria against inactivation during the preparation procedure rather than causing a true activation in the intact cell. Therefore we have examined the effect of dexamethasone treatment of the rat on pyruvate carboxylation in homogenates compared with the effect of hormone treatment in the isolated mitochondria. The results are shown in Table 1. In agreement with the above studies by Haynes and co-workers, treatment of the rat with the glucocorticoid resulted in a stable parallel increase in the rate of both pyruvate carboxylation and decarboxylation in the subsequently isolated mitochondria. The increased incorporation of $^{14}\text{CO}_2$ into acid-stable products was also observed in liver homogenates assayed within minutes of removing the tissue from the animal, although the degree of stimulation of CO_2 fixation by the homogenates

was less than observed in the isolated mitochondria (31%, compared with 52%). This may suggest that some enhancement of the effect occurred during the isolation procedure, as described by Siess *et al.* (1981), or alternatively there may have been a significant component of the $^{14}\text{CO}_2$ fixation that was insensitive to hormone treatment and presumably was not due to pyruvate carboxylase activity. Although the latter cannot be excluded, more than 90% of the CO_2 fixed was in response to exogenous pyruvate (results not shown) and the remaining 10% also showed a significant stimulation ($P < 0.05$), thus minimizing this possibility. However, despite the decreased degree of stimulation in the homogenate CO_2 fixation, the increase in pyruvate carboxylation was still highly significant and comparable with the degree of stimulation of gluconeogenesis after glucocorticoid treatment (Rinard *et al.*, 1969; Wakat *et al.*, 1977).

Effect of incubation temperature on the rate of pyruvate carboxylation by mitochondria prepared from control and dexamethasone-treated animals

The data of Siess *et al.* (1981) indicated that the effect of glucagon pretreatment of the rat on the rate of pyruvate carboxylation was highly dependent on the assay temperature, and from this it was argued that the effect of hormone treatment must be an artifact. It was suggested that, if a true activation of the steps leading to CO_2 fixation occurred *in vivo*, the degree of stimulation would be independent of the assay temperature. The results from similar experiments using dexamethasone as the gluconeogenic hormone are shown in Table 1. In contrast with the studies by Siess *et al.* (1981), there was a parallel decrease in the rate of CO_2 fixation in mitochondria prepared from both the

Table 1. *Effect of dexamethasone treatment of the rat on hepatic pyruvate metabolism*

Dexamethasone (150 $\mu\text{mol/kg}$ body wt.) or saline (0.9% NaCl) was administered intraperitoneally to fed rats 3 h before removal of the liver. The livers were homogenized in 0.3 M-sucrose, and the homogenate (approx. 10 mg wet wt.) was assayed immediately to determine the rate of pyruvate carboxylation in the presence and absence of 5 mM-pyruvate. The remainder of the homogenates were used to prepare mitochondria as described previously (Titheradge & Haynes, 1979). The rates of mitochondrial pyruvate carboxylation and decarboxylation were determined as described in the Experimental section. Mitochondrial pyruvate carboxylation was measured at 25° and 37°C. The results are expressed as means $\pm$ S.E.M. for the numbers of preparations given in parentheses: * $P < 0.01$ and † $P < 0.05$ compared with the control.

Expt. no.	Control	+Dexamethasone	Stimulation (%)
1. Homogenates			
Pyruvate carboxylation (nmol/min per mg wet wt.)	2.78 $\pm$ 0.26 (14)	3.63 $\pm$ 0.29* (14)	31
Mitochondria			
Pyruvate carboxylation (nmol/min per mg of protein)	22.5 $\pm$ 1.2 (14)	34.1 $\pm$ 2.3* (14)	52
Pyruvate decarboxylation (nmol/min per mg of protein)	25.0 $\pm$ 2.1 (10)	38.2 $\pm$ 3.5* (10)	53
2. Mitochondria			
Pyruvate carboxylation (nmol/min per mg of protein) at:			
25°C	9.69 $\pm$ 1.07 (6)	17.27 $\pm$ 2.27 †(6)	78
37°C	15.37 $\pm$ 1.93 (6)	28.26 $\pm$ 4.29 †(6)	84

control and the steroid-treated animals when the temperature of the assay was lowered from 37°C to 25°C; thus there was no significant difference between the degree of stimulation at either temperature ($P > 0.05$). Similar results have been shown by Jensen *et al.* (1983) after treatment of animals with glucagon. These data, together with the finding that mitochondrial pyruvate carboxylation measured *in situ* in hepatocytes made permeable by treatment with the polyene antibiotic filipin is also under hormonal control (Allan *et al.*, 1983a), argue in favour of a true activation of mitochondrial function *in vivo* after treatment of animals or hepatocytes with gluconeogenic hormones.

Effect of cycloheximide treatment on mitochondrial pyruvate metabolism

The work of Wakat & Haynes (1977) has suggested that the effect of cortisol on mitochondrial pyruvate carboxylation occurs via a protein-synthetic mechanism, on the basis of the time of onset of the response and the ability of cycloheximide to inhibit the effect completely. We have examined the effect of treatment of the rat for 3 h with cycloheximide on pyruvate metabolism by mitochondria prepared from control, dexamethasone-treated and glucagon-treated animals. Injection of dexamethasone 3 h or of glucagon 20 min before decapitation resulted in a significant stimulation of pyruvate carboxylation ($P < 0.01$) and decarboxylation ($P < 0.001$), similar to those observed by Adam & Haynes (1969), the results being 28.0 ± 2.7 , 44.9 ± 2.4 and 48.4 ± 4.5 nmol/min per mg of protein for pyruvate carboxylation and 17.3 ± 1.1 , 27.1 ± 1.7 and 27.3 ± 1.5 nmol/min per mg for pyruvate decarboxylation by mitochondria from control, dexamethasone-treated and glucagon-treated animals respectively ($n = 6$). Treatment with cycloheximide had no effect on the rate of pyruvate oxidation, but led to a significant inhibition of the basal rate of pyruvate carboxyl-

ation ($P < 0.05$). It completely abolished the effect of both the glucocorticoid and glucagon on both aspects of pyruvate metabolism, the rates in the presence of cycloheximide being 20.1 ± 1.1 , 24.5 ± 1.4 and 19.2 ± 1.7 nmol/min per mg for pyruvate carboxylation and 17.2 ± 1.1 , 17.5 ± 0.9 and 18.3 ± 1.7 nmol/min per mg for pyruvate decarboxylation in mitochondria prepared from control, dexamethasone-treated and glucagon-treated rats respectively. Although the abolition of the effect of dexamethasone by cycloheximide agrees with the data of Wakat & Haynes (1977), the finding that the stimulation by glucagon was also inhibited suggests that this may be a toxic effect of the drug rather than a specific inhibition of protein synthesis, as the effect of glucagon is believed to occur via a rapid cyclic AMP-dependent mechanism which is independent of protein synthesis (Garrison & Haynes, 1975; Titheradge *et al.*, 1979; Siess & Wieland, 1980). Neither hormone altered the total pyruvate carboxylase activity, despite the significant effects on CO₂ fixation in the intact mitochondria, though treatment with cycloheximide for 3 h resulted in a slight but insignificant decrease in the total pyruvate carboxylase activity, which may have been reflected in the decreased basal rates of pyruvate carboxylation in the intact organelle (results not shown). If the action of glucocorticoids in the short term is to stimulate pyruvate carboxylation via a protein-synthetic mechanism, then it is apparent that it is not through induction of pyruvate carboxylase. This is at variance with the results of Henning *et al.* (1966), but supports those of Walter & Anabitarte (1973).

Effect of adrenalectomy on mitochondrial pyruvate metabolism

The above results indicate that short-term treatment of rats with glucocorticoids stimulates pyruvate metabolism by a mechanism that is independent of the induction of pyruvate

Table 2. *Effect of adrenalectomy on mitochondrial pyruvate metabolism*

Animals were bilaterally adrenalectomized 14 days before the experiment. Controls received a sham operation. The animals were treated with dexamethasone or vehicle on the day of the experiment, and mitochondria were prepared as described in the legend to Table 1. The assays were carried out as described in the Experimental section. Except for pyruvate dehydrogenase, which was measured at 30°C, all the other assays were performed at 37°C. Results (in terms of nmol/min per mg of mitochondrial protein) are expressed as means $\pm$ S.E.M., for the numbers of preparations given in parentheses: * $P < 0.05$ as compared with control.

Treatment	Pyruvate carboxylation	Pyruvate decarboxylation	Total pyruvate carboxylase activity	Pyruvate dehydrogenase activity	
				Active	Total
Control	33.2 ± 4.7 (8)	22.4 ± 1.2 (9)	220.8 ± 25.1 (6)	15.7 ± 2.3 (5)	15.5 ± 2.4 (5)
Dexamethasone	59.4 ± 8.6 (8)*	29.0 ± 2.8 (9)*	232.2 ± 33.6 (6)	15.7 ± 1.3 (5)	16.4 ± 2.0 (5)
Adrenalectomized	39.0 ± 5.1 (8)	22.5 ± 2.3 (9)	230.9 ± 15.1 (6)	16.6 ± 3.8 (5)	15.7 ± 2.1 (5)

carboxylase. The effect of adrenalectomizing the animals for a period of 14 days was also examined to determine whether circulating concentrations of glucocorticoids are necessary for the maintenance of the enzymes of pyruvate metabolism over a longer duration (Table 2). The results indicate that adrenalectomy had no effect on the rate of CO_2 fixation or pyruvate oxidation in the intact mitochondria, nor did it alter either the total pyruvate carboxylase activity or the total pyruvate dehydrogenase activity. Similarly, there was no change in the percentage of pyruvate dehydrogenase in the active form in the incubation, although this does not preclude an effect of dexamethasone on the active form *in vivo*. Exton *et al.* (1973) have also shown that, in the diabetic animal, adrenalectomy had no effect on the tissue content of pyruvate carboxylase, although in that study the animals were only adrenalectomized for a period of 24 h. Table 2 shows that, despite a significant effect of dexamethasone on pyruvate oxidation in the intact mitochondria, the hormone had no effect on either the mitochondrial concentration or the proportion of pyruvate dehydrogenase in the active form. The high percentage of pyruvate dehydrogenase in the active form is probably a reflection of the high pyruvate concentration used in the assay of pyruvate metabolism, preventing pyruvate dehydrogenase kinase activity (Denton *et al.*, 1975). From these results it is evident that endogenous glucocorticoids are not essential to maintain the concentration of either pyruvate carboxylase or pyruvate dehydrogenase activity in the intact cell and that dexamethasone acts to relieve the restraint imposed on both enzymes of pyruvate metabolism by alterations in the supply of substrate or intramitochondrial concentrations of effector molecules. In contrast with pyruvate carboxylase, the restraint imposed on pyruvate dehydrogenase in the isolated mitochondria was much less, 70% of the activity being expressed as opposed to 10–20%. This may reflect the lower K_m of pyruvate dehydrogenase for pyruvate, $40\text{ }\mu\text{M}$ as opposed to $100\text{ }\mu\text{M}$ (McClure *et al.*, 1971; Denton *et al.*, 1975), or alternatively some degree of compartmentation of pyruvate within the mitochondrial matrix.

Effect of dexamethasone treatment of the rat on mitochondrial pyruvate transport

Previous studies with glucagon have suggested that the restraint imposed on the enzymes of pyruvate metabolism within the mitochondria may be imposed at the level of pyruvate transport into the mitochondrial matrix, the effect of hormone treatment being to increase pyruvate entry (Adam & Haynes, 1969; Titheradge & Coore, 1976a,b; Halestrap, 1978; Thomas & Halestrap, 1981). By

using the indirect approach of titrating pyruvate metabolism with increasing concentrations of α -cyano-4-hydroxycinnamic acid (Halestrap, 1978), the effect of dexamethasone on pyruvate transport has been examined. The results are shown in Fig. 1. It is apparent that increasing concentrations of α -cyano-4-hydroxycinnamic acid, a specific inhibitor of the pyruvate translocator (Halestrap & Denton, 1974), result in a progressive decline in the rate of pyruvate metabolism in both the control and dexamethasone-treated mitochondria. The inset in Fig. 1 shows the Dixon plot of the dependence of the reciprocal of the rate of metabolism on concentration of inhibitor. In contrast with the findings reported by Halestrap (1978), with mitochondria prepared from both control and dexamethasone-treated animals, the Dixon plot was non-linear at low concentrations of inhibitor, indicating that some factor other than pyruvate transport was limiting the enzymes of pyruvate metabolism under these conditions. This deviation from linearity was noted with all eight mitochondrial preparations. By extrapolation of the linear portion of the Dixon plot to zero concentration of inhibitor, the rates of pyruvate transport were estimated to be 50.1 ± 5.5 and 65.8 ± 6.7 nmol/min per mg of protein ($n = 8$) for the mitochondria from control and hormone-treated animals respectively. This compares with the rates of pyruvate utilization in the absence of inhibitor of 42.5 ± 4.7 and 58.6 ± 6.0 nmol/min per mg of protein ($P < 0.01$) for control and hormone-treated mitochondria. Therefore, although the rates of pyruvate transport exceeded those of metabolism, it is evident that the rate of transport in the control mitochondria is insufficient to cope with the demands of both pyruvate carboxylation and oxidation after stimulation. In agreement with the work of Halestrap (1978), hormone treatment also decreased the K_i of the transport system for α -cyano-4-hydroxycinnamic acid, from 152.7 ± 25.2 to $108.8 \pm 17.2\text{ }\mu\text{M}$. The apparent discrepancy between the dependence of the rate of pyruvate metabolism on the rate of transport in control mitochondria in the present study and that of Halestrap (1978) may reflect the lower concentration of pyruvate used as the substrate in the present study, 1 mM compared with 4 mM. One possible explanation for this could be that in the present study the concentration of pyruvate in the incubation became rate-limiting for the transporter as pyruvate was utilized, and therefore the rate of pyruvate entry into the mitochondria decreased over the incubation period, leading to an underestimation of the rate of pyruvate metabolism. However, this appears unlikely, for several reasons. First, the rate of pyruvate utilization over the 10 min incubation period in the absence of

Fig. 1. *Inhibition of pyruvate metabolism by α -cyano-4-hydroxycinnamic acid*

Mitochondrial pyruvate metabolism was measured as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. for eight different mitochondrial preparations. In the Dixon plots shown in the insets, the reciprocals of the mean rates are plotted against increasing concentration of inhibitor. ■, Control; ●, dexamethasone-treated.

inhibitor was linear with time, with no evidence of tailing-off at the longer time periods, the mean correlation coefficient over five experiments being 0.997. Second, at the end of the incubation period the concentrations of pyruvate remaining in the medium were $486 \pm 55 \mu\text{M}$ and $343 \pm 62 \mu\text{M}$ ($n = 8$) respectively for the mitochondria prepared from control and dexamethasone-treated animals, indicating that the range of pyruvate concentrations experienced by the mitochondria in the assay was near to physiological concentrations found in the cytosol (Siess *et al.*, 1977), and in excess of the K_m for the transport system, which has been estimated to be between 115 and $150 \mu\text{M}$ (Paradies & Papa, 1978; Halestrap *et al.*, 1980). Third, the Dixon plot for the mitochondria prepared from the dexamethasone-treated animals was a better approxi-

mation to linearity than that of the control mitochondria, despite the fact that the rate of pyruvate utilization was enhanced by hormone treatment.

Effect of dexamethasone on the rate of pyruvate carboxylation with alanine plus 2-oxoglutarate as the substrate

Although the above data indicate that a stimulation of pyruvate transport is required to cope with the enhanced rate of pyruvate metabolism after hormone treatment, it is evident that the rate of pyruvate utilization in both the control and stimulated mitochondria is probably the result of a summation of alterations in the intramitochondrial concentrations of effector molecules in addition to transport. Evidence purporting to support this has

Table 3. Effect of dexamethasone on the incorporation of $^{14}\text{CO}_2$ into acid-stable products with alanine plus 2-oxoglutarate as substrate

Rats were treated and mitochondria prepared as described in the Experimental section. CO_2 fixation was measured as described by Adam & Haynes (1969), except that pyruvate was omitted from the medium and the assay supplemented with substrates as shown. Glutamate-pyruvate transaminase activity was measured enzymically in mitochondria freeze-thawed three times (Bergmeyer & Bernt, 1974). Results are means $\pm$ S.E.M. for the numbers of mitochondrial preparations given in parentheses: * $P < 0.001$ as compared with the appropriate control.

Additions	Incorporation of label (nmol/min per mg of mitochondrial protein)	
	Control	+ Dexamethasone
5 mM-Pyruvate	22.0 $\pm$ 1.1 (16)	34.2 $\pm$ 2.0* (16)
5 mM-Alanine + 5 mM-2-oxoglutarate	8.3 $\pm$ 0.5 (16)	13.1 $\pm$ 0.9* (16)
5 mM-Alanine + 5 mM-2-oxoglutarate + 1 mM-amino-oxyacetate	5.3 $\pm$ 0.7 (5)	6.7 $\pm$ 0.6 (5)
5 mM-2-Oxoglutarate	5.1 $\pm$ 0.7 (7)	5.4 $\pm$ 0.2 (7)
Glutamate-pyruvate transaminase activity	79.8 $\pm$ 6.2 (7)	88.8 $\pm$ 6.3 (7)

come from the work of Chan *et al.* (1979), which showed that, after treatment of rats with glucagon, pyruvate carboxylation was enhanced when the requirement for pyruvate transport was obviated by generating intramitochondrial pyruvate through transamination of alanine. Results from similar experiments performed after administration of dexamethasone to rats are shown in Table 3. A significant stimulation of CO_2 fixation was apparent when pyruvate was generated intramitochondrially or when exogenous pyruvate was the substrate. Addition of 1 mM-amino-oxyacetate, an inhibitor of the glutamate-pyruvate transaminase activity (Edmondson *et al.*, 1977; Joseph *et al.*, 1978), resulted in a significant inhibition ($P < 0.01$) of the rate of CO_2 fixation and abolished the effect of hormone treatment, although not the absolute rate of incorporation of label into acid-stable products. The residual rate of CO_2 fixation was independent of pyruvate formation from alanine, as shown by the omission of alanine from the incubation medium, and probably reflects the ATP-dependent incorporation of $^{14}\text{CO}_2$ into 2-oxoglutarate to form isocitrate by isocitrate dehydrogenase. This lack of effect of dexamethasone on CO_2 fixation with 2-oxoglutarate as the substrate is further evidence that the action of hormones at the level of the mitochondria is not solely the result of protection of the mitochondria against damage during the isolation procedures, as this might be expected to be reflected in a change in any energy-dependent reaction (Siess *et al.*, 1981). Therefore, these results indicate that glucocorticoids, like glucagon, are capable of stimulating pyruvate carboxylation by a mechanism which is independent of their effects on mitochondrial pyruvate transport. However, the rates in the presence of alanine plus 2-oxoglutarate as

the substrate were substantially lower than those measured with pyruvate as the substrate, and therefore it cannot be concluded that the same factors regulate pyruvate carboxylation with the two substrates, as implied by Chan *et al.* (1979). Table 3 also shows the activity of the glutamate-pyruvate transaminase in lysed mitochondria prepared from control and dexamethasone-treated animals. The activity of the enzyme was considerably greater than the rate of pyruvate carboxylase in the intact mitochondria, and was not altered after hormone treatment, suggesting that the rate of formation of pyruvate was unlikely to be limiting the flux through pyruvate carboxylase. Further support for this comes from measurements of pyruvate accumulation in the mitochondria plus medium at the end of the incubation period, which showed values of 166 ± 21 and 196 ± 17 nmol/mg of mitochondrial protein over the 10 min incubation period for the control and dexamethasone-treated mitochondria respectively. The most obvious mechanism to account for the decreased rate of pyruvate carboxylation with alanine plus 2-oxoglutarate as the substrate was via the accumulation of glutamate, an effective inhibitor of pyruvate carboxylase activity (Scrutton & White, 1974). Glutamate accumulated in the mitochondria, and matrix concentrations of 4.39 ± 0.44 and 6.10 ± 1.24 mM ($n = 8$) were found at the end of the incubation for control and dexamethasone-treated mitochondria respectively. Although this concentration of glutamate is of sufficient magnitude to decrease the rate of pyruvate carboxylation significantly, it does not account for the effect of hormone treatment, as there was no significant difference between the two groups of mitochondria ($P > 0.05$), with the dexamethasone-treated mitochondria tending to show the higher values.

Effect of dexamethasone treatment on the intramitochondrial concentrations of effector molecules of pyruvate carboxylase

Table 4 shows the intramitochondrial concentrations of the known effectors of pyruvate carboxylase when mitochondria are incubated under the conditions used for the measurement of pyruvate carboxylation. Dexamethasone treatment resulted in a significant increase in the intramitochondrial concentrations of both acetyl-CoA and ATP. The ADP concentration was unchanged, so therefore the [ATP]/[ADP] ratio was elevated on hormone treatment, in agreement with data from previous studies after treatment of rats and isolated hepatocytes with glucagon and catecholamines (Adam & Haynes, 1969; Halestrap, 1978; Titheradge *et al.*, 1979; Chisholm *et al.*, 1983). The matrix glutamate content was below the detectable limits of the assay when pyruvate was the substrate, the intramitochondrial concentration being less than 1 mM and well below the K_i of pyruvate carboxylase for glutamate (Scrutton & White, 1974). Ca^{2+} has also been proposed as a potent inhibitor of pyruvate carboxylase by competing with Mg^{2+} (McClure & Lardy, 1971; Wimbhurst & Manchester, 1970); however, we have been unable to show any effect of dexamethasone treatment on the content of intramitochondrial Ca^{2+} , although the Mg^{2+} content of the mitochondria was significantly elevated (Allan *et al.*, 1983b).

In conclusion, the results confirm the studies of Haynes and co-workers (Adam & Haynes, 1969; Wakat & Haynes, 1977) in that short-term treatment of rats with glucocorticoids results in an increase in the rates of both pyruvate carboxyl-

ation and oxidation in subsequently isolated mitochondria. The increased activity of the two enzymes of pyruvate metabolism appears to be the result of an increased substrate supply and elevated intramitochondrial concentrations of both acetyl-CoA and ATP, together with an increased [ATP]/[ADP] ratio within the mitochondrial matrix. The evidence in favour of a protein-synthetic mechanism being involved in the action of the glucocorticoids at this level appears to be restricted to the time of onset of the response, as cycloheximide treatment produced equivocal results, and adrenalectomizing the animals for a period of 14 days had no direct effect on the total activity of either pyruvate carboxylase or pyruvate dehydrogenase.

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Table 4. *Effect of dexamethasone treatment of the rat on the intramitochondrial concentrations of adenine nucleotides, glutamate and acetyl-CoA*

Mitochondria were prepared from rats treated with dexamethasone as described in the Experimental section. The mitochondria were incubated under the conditions used for the measurement of pyruvate carboxylation for 5 min, and the metabolites were assayed in neutralized HClO_4 extracts as described in the Experimental section. Results shown (in nmol/mg of protein) are means $\pm$ S.E.M. for the numbers of experiments given in parentheses: * $P < 0.05$ as compared with control.

Metabolite	Control	+Dexamethasone
Acetyl-CoA	0.75 ± 0.02 (6)	0.89 ± 0.05 (12)*
Glutamate	< 1.0 (7)	< 1.0 (7)
ATP	10.8 ± 0.8 (9)	13.9 ± 0.9 (9)*
ADP	2.0 ± 0.5 (8)	2.2 ± 0.5 (9)
ATP/ADP	5.47	6.25

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