Effects of Hormones and N^6O^2' -Dibutyryl-Adenosine 3':5'-Cyclic Monophosphate, Administered *in vivo*, on Phosphate Transport and Metabolism in Isolated Rat Liver Mitochondria

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1. The administration of glucagon or N^6O^2' -dibutyryl cyclic AMP to fed rats by intraperitoneal injection was associated with a 2-fold increase in the amounts of endogenous P_i and ATP, and an increase in the rate and extent of transport of exogenous P_i (measured in either the presence or the absence of Ca^{2+}) in mitochondria subsequently isolated from the liver. No change was observed in either the maximum rate of transport of exogenous P_i or in the rate of $^{32}P_i$ exchange. 2. The changes induced by glucagon and dibutyryl cyclic AMP were markedly decreased by the co-administration of cycloheximide. 3. The administration of insulin to rats resulted in an increase of about 1.3-fold in the concentration of endogenous mitochondrial P_i . 4. The amounts of endogenous P_i in mitochondria isolated from the livers of starved rats were 3 times those in mitochondria isolated from fed animals. 5. It is concluded that the liver mitochondrial phosphate-transport system may be an important site of hormone action. 6. In the course of these experiments, it was shown that Ca^{2+} markedly stimulates mitochondrial phosphate transport.

We have shown that the administration of insulin to starved rats by intraperitoneal injection is associated with an altered ability of mitochondria subsequently isolated from the liver to transport Ca^{2+} *in vitro* (Dorman *et al.*, 1975; Barritt *et al.*, 1975). The transport of Ca^{2+} by mitochondria is closely linked to the transport of phosphate ions. These anions enhance the rate of Ca^{2+} transport, increase the maximum amount of Ca^{2+} transported and alter the time for which Ca^{2+} ions are retained by isolated mitochondria (Lehninger, 1974; Reed & Bygrave, 1975; Bygrave, 1977). We therefore considered it important to investigate the effect of insulin administration on the properties of the liver mitochondrial phosphate-transport system, and to test the specificity of any effect on phosphate transport for the nature of the hormone administered. We show here that the administration of glucagon, dibutyryl cyclic AMP or insulin to fed rats by intraperitoneal injection is associated with an increase in the concentration of endogenous P_i in isolated liver mitochondria, as well as an increase in the initial rate of mitochondrial phosphate transport.

Experimental

Administration of hormones and N^6O^2' -dibutyryl cyclic AMP

Male hooded Wistar rats, weighing 150-200g

(Institute of Medical and Veterinary Science, Adelaide, South Australia) and maintained on a standard laboratory diet, were used in all experiments. Except for some experiments in which insulin was administered to starved rats (see the Results section), all tests were conducted with rats fed *ad libitum*. When used, rats were starved for 15 h by the removal of food at 16:00 h on the day before the administration of hormone. The hormones, N^6O^2' -dibutyryl cyclic AMP, and cycloheximide were dissolved or suspended in NaCl/albumin [0.9% (w/v) NaCl, pH 7.4, containing 0.05 g of bovine serum albumin/litre], and administered at about 09:30 h by intraperitoneal injection (cf. Hems *et al.*, 1975) as described previously (Dorman *et al.*, 1975). Control rats received NaCl/albumin. After 1 h the rats were killed and liver mitochondria isolated. The experiments were performed in groups of two (control and hormone) or three (control, hormone and hormone in the presence of cycloheximide) rats, matched as closely as possible by weight. The amounts of hormones or dibutyryl cyclic AMP administered (expressed per 100 g body wt.) were: Novo Actrapid insulin (40-100 μ g), glucagon (25 or 75 μ g), adrenaline (50 μ g) and dibutyryl cyclic AMP (0.75-1 mg). When used, cycloheximide (250 μ g/100 g body wt.) was co-injected with the agent under test.

The intraperitoneal route of hormone adminis-

tration was chosen because most intravenous methods that are used with rats require anaesthesia of the animals to facilitate reliable cannulation of the vein (see Yamazaki, 1975). General anaesthetics have been shown to cause marked changes in metabolism, including a significant increase in the concentration of blood glucose and altered responses of tissues to hormones (Biebuyck, 1973). Therefore the anaesthetized rat was considered to be an unsuitable system in which to study the effects of the hormones on hepatic mitochondria.

Isolation of liver mitochondria

Liver mitochondria were isolated, and their respiratory activities measured, as described by Reed & Bygrave (1974). Mitochondrial integrity was monitored by measuring the ratio (respiratory-control ratio) of the rate of ADP-stimulated respiration to the rate of respiration obtained after the ADP had been depleted, after the addition of limiting amounts of ADP to the incubation medium (Estabrook, 1967). In the presence of 12.5 mM-succinate, respiratory-control ratios of 4–6 were obtained. Mitochondrial protein concentration was determined by a modification of the biuret method (Reed & Bygrave, 1974).

Endogenous intramitochondrial P_i and ATP

Samples of freshly isolated mitochondria (0.1 ml, 2.5–4 mg of protein) were extracted once with 0.4 ml of 10% (w/v) trichloroacetic acid; the extracts were left at 0°C for 30 min, then centrifuged at 7000g for 2 min (Eppendorf centrifuge 3200). The supernatant was removed and immediately stored overnight at –20°C until the amount of P_i present was measured by the method of Baginski *et al.* (1967). Four to six separate determinations of the concentration of endogenous P_i were performed for each preparation of mitochondria. The amounts of ATP present in neutralized $HClO_4$ extracts of mitochondria (prepared 15 min after completion of isolation of the mitochondria) were measured spectrophotometrically as described by Williamson & Corkey (1969).

Mitochondrial phosphate transport

The transport of exogenous P_i into mitochondria was measured at 0°C by a modification of the method of Coty & Pedersen (1974). Mitochondria (10 mg/ml) were equilibrated for 1 min in a medium that contained, in a final volume of 2.0 ml, 250 mM-sucrose, 2.5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] adjusted to pH 7.4 with KOH, and, when present, 0.5 mM- $CaCl_2$. Phosphate transport was initiated by the addition of 50 μ l of the appropriate concentration of potassium phosphate (pH 7.4). Samples of the reaction mixture (200 μ l) were withdrawn at the times indicated in

the Figures and immediately mixed with 1.0 ml of 100 μ M-*p*-chloromercuribenzoate in 250 mM-sucrose/2.5 mM-Hepes/KOH, pH 7.4, at 0°C. A further 10 ml of sucrose/Hepes medium was added within 1 min, and the tubes were centrifuged at 10000g for 5 min. The supernatants were removed by aspiration, the walls of the tubes wiped with tissue paper, and 0.5 ml of 10% (w/v) trichloroacetic acid was added to the mitochondrial pellet, which was then mechanically broken up. After 30 min at 0°C, the extracts were transferred to smaller tubes and centrifuged at 7000g for 2 min. The supernatants were removed and kept overnight at –20°C until the P_i present was measured as described above. To determine the amount of P_i in the mitochondria before the addition of exogenous P_i , two samples (200 μ l) were withdrawn from the incubation mixture before this step, and treated as described above. All estimates of the time course for phosphate transport were performed in duplicate. Good agreement between duplicate time-courses was observed.

Control experiments have shown that similar values are obtained for the amounts of endogenous mitochondrial P_i , and the P_i present in mitochondrial extracts obtained after phosphate transport, when the samples are assayed immediately after the addition of trichloroacetic acid or after the trichloroacetic acid extracts have been stored at –20°C for 24 h. Further experiments, in which known amounts of ATP were added to trichloroacetic acid and the mixtures stored at –20°C, showed that the amount of P_i liberated over a period of 7 days was 1.7% of the amount of ATP present. Calculations have shown that this would make a negligible contribution to the total amount of P_i . These experiments also showed that there is negligible hydrolysis of ATP by the reagents used in the assay for P_i .

Mitochondrial $^{32}P_i$ exchange

Rates of $^{32}P_i$ exchange were measured as described by Coty & Pedersen (1974) in the absence of added Ca^{2+} in a medium of the same composition as that described for the transport of exogenous P_i . The mitochondria were equilibrated with 0.5 mM-phosphate for 2 min before the addition of $^{32}P_i$ (0.25 μ Ci). Samples of the reaction mixture (200 μ l) were withdrawn at the times indicated and treated as described above. The mitochondrial pellets were finally dissolved in 0.5 ml of 2% (w/v) deoxycholate and the amount of radioactivity present was determined as described previously (Foldes & Barritt, 1977). The amounts of $^{32}P_i$ associated with the intermembrane and extramitochondrial spaces were determined by the separate addition of $^{32}P_i$ to two samples withdrawn from the central incubation mixture before the addition of $^{32}P_i$. The data were analysed as described by Coty & Petersen (1974) to determine the maximum extent of $^{32}P_i$ exchange

(P_m), the time constant of equilibration, k , and the initial rate of transport, $v_0 = kP_m$.

Plasma glucose

Glucose was measured in HClO_4 extracts of plasma by the glucose oxidase/peroxidase coupled enzyme assay (Werner *et al.*, 1970).

Chemicals

Novo Actrapid insulin was obtained from Novo Industri A/S, Copenhagen, Denmark; glucagon, adrenaline, dibutyryl cyclic AMP, Hepes, *p*-chloromercuribenzoic acid and cycloheximide were from Sigma Chemical Co., St. Louis, MO, U.S.A.; CaCl_2 , ammonium molybdate, sodium citrate and sodium arsenite were from Ajax Chemicals, Sydney, Australia; reagents ('GOD-Perid' diagnostic test kit) for the assay of plasma glucose were from Boehringer Mannheim G.m.b.H., Mannheim, Germany; $\text{NaH}_2^{32}\text{PO}_4$ was from the Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia. All other reagents were of analytical grade.

Results

Effects of glucagon and dibutyryl cyclic AMP on endogenous mitochondrial P_i

The administration of glucagon to fed rats by intraperitoneal injection was associated with a 2-fold increase in the concentration of endogenous P_i in mitochondria subsequently isolated from the liver (Table 1). The amount of endogenous P_i increased with increasing age of the mitochondria isolated from rats treated with glucagon, but a smaller increase with aging was observed in control mitochondria (Table 2). Isolation and suspension of the mitochondria in the presence of *p*-chloromercuribenzoate resulted in (a) a small increase in the amounts of endogenous P_i in both control mitochondria and mitochondria isolated from rats treated with glucagon and (b) a difference between the two sets of mitochondria which is similar to that observed when the isolation is conducted in the absence of *p*-chloromercuribenzoate (Table 2). The administration of glucagon *in vivo* was also associated with an increase in the concentration of endogenous mitochondrial ATP from 2.3 ± 0.3 nmol/mg of protein (mean $\pm$ S.E.M.) for control mitochondria to 3.9 ± 0.5 nmol/mg of protein for mitochondria isolated from rats treated with glucagon ($n = 10$, $P < 0.001$, by using Student's *t* test for paired samples).

A significant increase in the concentration of endogenous P_i was also observed in mitochondria isolated from rats treated with dibutyryl cyclic AMP (Table 1). The effects of the administration of both glucagon and dibutyryl cyclic AMP on the concentration of mitochondrial endogenous P_i were

Table 1. *Effects of hormones, administered in vivo, on the concentration of endogenous P_i in isolated liver mitochondria*

The administration of hormones and isolation of mitochondria were performed as described in the Experimental section. Rats were killed 1 h after the administration of 100 μg of insulin, 25 μg of glucagon, 0.75 mg of dibutyryl cyclic AMP or 50 μg of adrenaline per 100 g body wt. When present, cycloheximide (250 μg /100 g body wt.) was co-injected with glucagon or dibutyryl cyclic AMP. The amount of endogenous P_i present in isolated mitochondria was measured as described in the Experimental section. The values shown are the means $\pm$ S.E.M. of the numbers of determinations shown. The degrees of significance, P , were determined by using Student's *t* test for paired samples, with the value obtained for each test set of mitochondria being paired with the value obtained for the corresponding control set of mitochondria. Values of $P > 0.05$ were considered to be not significant.

Dietary state	Agent administered	Endogenous phosphate (nmol/mg of protein)	No. of rats	P
Fed	Control	4.3 ± 0.5	11	
	Glucagon	8.9 ± 1.0	11	<0.0005
	Glucagon+cycloheximide	6.1 ± 0.2	4	
	Control	5.3 ± 0.9	4	
	Dibutyryl cyclic AMP	8.7 ± 1.7	4	<0.05
	Dibutyryl cyclic AMP + cycloheximide	7.2 ± 1.1	3	<0.025
	Control	5.0 ± 0.6	4	
	Cycloheximide	6.4 ± 1.8	4	
	Control	8.0 ± 2.6	3	
	Adrenaline	7.1 ± 2.1	3	
	Control	3.6 ± 0.4	3	
	Insulin	4.6 ± 0.4	3	<0.05
Starved	Control	12.7 ± 1.7	9	
	Insulin	18.8 ± 2.2	9	<0.0005

markedly decreased when cycloheximide was co-administered with the agent under test (Table 1). The administration of cycloheximide alone had no effect on the concentration of endogenous mitochondrial P_i (Table 1).

Effects of glucagon and dibutyryl cyclic AMP on phosphate transport and exchange in the absence of exogenous Ca^{2+}

The effect of glucagon administration on the *p*-chloromercuribenzoate-sensitive transport of phosphate into mitochondria which follows the addition of exogenous P_i to the incubation medium was investigated. The results obtained at 0.5 mM-exogenous P_i , a concentration below the apparent K_m of 1.8 mM for phosphate transport (Coty &

Pedersen, 1974), indicate that mitochondria isolated from rats treated with glucagon exhibit (a) an increase of $72 \pm 24\%$ (mean $\pm$ S.E.M., $n = 4$) in the initial rate of phosphate transport (Fig. 1a) and (b) greater net transport of phosphate (Fig. 1a, inset) compared with control mitochondria. Similar

Table 2. *Effects of age of isolated mitochondria and inclusion of p-chloromercuribenzoate during isolation on the concentration of endogenous mitochondrial P_i*

The experimental details are described in the legend to Table 1. When present, $50 \mu\text{M}$ -p-chloromercuribenzoate was included in the suspension media at all stages of isolation of the mitochondria and in the final mitochondrial suspension. The age of the mitochondria refers to the time elapsed after their isolation. The data are the means $\pm$ S.E.M., or the mean and range of the values obtained for two experiments.

Agent administered to rat	Additions to isolation media	No. of rats	Endogenous phosphate (nmol/mg of protein)	
			20 min age	85 min age
Control	None	6	7.3 ± 0.4	8.1 ± 0.6
Glucagon		6	10.8 ± 1.4	14.3 ± 2.3
Control	p-Chloromercuribenzoate	2	9.4 ± 0.5	10.2 ± 0.4
Glucagon		2	13.5 ± 2.0	17.8 ± 2.1

results were obtained at 0.25mM-phosphate for mitochondria isolated from rats treated with glucagon, and at 0.5mM-phosphate with mitochondria isolated from rats treated with dibutyryl cyclic AMP (results not shown). The values obtained for the concentration of endogenous P_i in the transport experiments (Fig. 1a) are lower than those measured directly (Tables 1 and 2). This difference was observed in all experiments, and may be due to dilution and preincubation of the mitochondria in the medium used for the measurement of phosphate transport (see the Experimental section).

The effect of administration of glucagon on the exchange of $^{32}\text{P}_i$ between the extra- and intra-mitochondrial pools of phosphate was also investigated. The results of one of six separate experiments are shown in Fig. 2. In each experiment, plots of the amount of $^{32}\text{P}_i$ exchanged as a function of time revealed differences between control mitochondria and mitochondria isolated from rats treated with glucagon (Fig. 2a). However, no consistent trend was observed. Moreover, when the data were plotted according to the integrated form of the first-order rate equation (Fig. 2b) and the initial rate of $^{32}\text{P}_i$ exchange was calculated (see the Experimental section), no significant difference between the rates obtained for the two sets of mitochondria was observed. Rates of 13.3 ± 1.9 ($n = 6$; mean $\pm$ S.D.) and 13.2 ± 3.8 ($n = 7$) nmol/min

Fig. 1. *Effects of the administration of glucagon to fed rats (a) and of insulin to starved rats (b) on the initial rate of phosphate transport in mitochondria subsequently isolated from the liver*

Administration of glucagon ($75 \mu\text{g}/100\text{g}$ body wt.) and insulin ($100 \mu\text{g}/100\text{g}$ body wt.), and isolation of mitochondria from control rats (○) and rats treated with hormone (●), were performed as described in the Experimental section. Phosphate transport was measured (see the Experimental section) in a medium containing 250mM-sucrose, 2.5mM-Hepes/KOH (pH 7.4), 0.5mM-potassium phosphate (pH 7.4) and mitochondria (10mg of protein/ml). The insets show the net amount of P_i transported by the mitochondria as a function of time. In each case, the data represent the results of one of four experiments which gave similar results.

Fig. 2. Effect of glucagon on $^{32}\text{P}_i$ exchange in isolated liver mitochondria

The administration of glucagon ($75\text{ }\mu\text{g}/100\text{ g}$ body wt.) and isolation of mitochondria from control rats ($\circ$) and rats treated with glucagon ($\bullet$) were performed as described in the Experimental section. $^{32}\text{P}_i$ exchange was measured in a medium of the same composition as that described in the legend of Fig. 1 (see the Experimental section).

(a) Plots of the amount of $^{32}\text{P}_i$ exchanged as a function of time. (b) Plots of $-\ln\left(\frac{P_m - P_t}{P_m}\right)$ against time, where P_m and P_t represent the maximum extent of $^{32}\text{P}_i$ exchange (measured at 3 min) and the extent of exchange at time t min respectively. The data are the results of one of six similar experiments performed with separate preparations of mitochondria.

Fig. 3. Stimulation by Ca^{2+} of the initial rate and extent of phosphate transport by liver mitochondria isolated from control rats

Phosphate transport was measured (see the Experimental section) in a medium containing 250 mM -sucrose, 2.5 mM -Hepes/KOH ($\text{pH} 7.4$), 4 mM -potassium phosphate and mitochondria (10 mg of protein/ml) in the absence ($\circ$) and presence (Δ) of 0.5 mM - CaCl_2 .

Effects of glucagon and dibutyryl cyclic AMP on phosphate transport in the presence of exogenous Ca^{2+}

The addition of CaCl_2 (0.5 mM) to mitochondria isolated from control rats markedly increased both the initial rate of phosphate transport as well as the amount of phosphate transported at 4 mM - (Fig. 3) or 0.5 mM - (results not shown) exogenous P_i . The effect of increasing concentrations of Ca^{2+} (0.1 – 1 mM) on the initial rate of phosphate transport at 0.5 mM -phosphate was investigated. Half-maximal stimulation of the initial rate of phosphate transport was observed at 0.25 mM - Ca^{2+} . The lowest concentration of Ca^{2+} (0.5 mM) that gave maximal stimulation was used in all subsequent experiments.

When phosphate transport was measured at 0.5 mM -phosphate in the presence of Ca^{2+} , mitochondria isolated from rats treated with glucagon exhibited a significant increase in the amount of phosphate transported as well as an apparent increase in the initial rate of transport, compared with mitochondria from control rats (Fig. 4). The magnitude of the increase in net phosphate transport observed after treatment with glucagon was greater than that observed in the absence of Ca^{2+} (cf. Fig. 1a). In these experiments the mitochondria were incubated with Ca^{2+} for 1 min before the addition of P_i . Other experiments (results not shown) have shown that the simultaneous addition of Ca^{2+} and P_i to the incubation medium does not alter the observed differences between mitochondria from control and glucagon-

per mg of protein were obtained for control mitochondria and mitochondria isolated from rats treated with glucagon respectively.

Fig. 4. Effect of glucagon on phosphate transport in isolated liver mitochondria measured in the presence of exogenous Ca^{2+}

The administration of glucagon (25 μ g/100 g body wt.) and isolation of mitochondria from control rats ($\circ$) and rats treated with glucagon ($\bullet$) were performed as described in the Experimental section. Phosphate transport was measured (see the Experimental section) in a medium containing 250 mM-sucrose, 2.5 mM-Hepes/KOH (pH 7.4), 0.5 mM- $CaCl_2$, 0.5 mM-potassium phosphate (pH 7.4) and mitochondria (10 mg of protein/ml). The inset shows the net amount of phosphate transported by the mitochondria as a function of time. The data represent the results of one of six experiments which gave similar results.

treated rats. The difference in the rates of phosphate transport between the two sets of mitochondria was also observed when phosphate transport was measured in the presence of 4 μ g of oligomycin/ml (results not shown).

In all transport experiments performed in the presence of Ca^{2+} , values for the concentration of endogenous mitochondrial P_i (cf. Fig. 4) were found to be greater than those obtained by direct deter-

Fig. 5. Effect of glucagon on the initial rate of phosphate transport in isolated mitochondria, measured in the presence of Ca^{2+}

The administration of glucagon (25 μ g/100 g body wt.) and isolation of mitochondria from control rats ($\circ$) and rats treated with glucagon ($\bullet$) were performed as described in the Experimental section. Phosphate transport was measured (see the Experimental section) in a medium containing 250 mM-sucrose, 2.5 mM-Hepes/KOH (pH 7.4), 0.5 mM- $CaCl_2$, 0.1 mM-potassium phosphate (pH 7.4) and mitochondria (10 mg of protein/ml). The data represent the results of one of three experiments which gave similar results.

mination (Table 1). This may be due to an enhancement by Ca^{2+} of the transport of phosphate from the medium into the mitochondria before the addition of exogenous P_i (cf. Fig. 3).

The apparent increase in initial rate of phosphate transport observed in mitochondria isolated from rats treated with glucagon was examined more closely by using shorter sampling times and lower concentrations of exogenous P_i . Data obtained at 0.1 mM-phosphate show an increase of about 55% in the initial rate of phosphate transport for mitochondria isolated from rats treated with glucagon (Fig. 5). Measurement of the initial rate of phosphate transport in the presence of increasing concentrations of exogenous P_i showed that mitochondria isolated from rats treated with glucagon exhibit a lower

Fig. 6. Effect of glucagon on the apparent K_m for exogenous phosphate in the mitochondrial phosphate-transport system (a) The initial rate of phosphate transport plotted as a function of the concentration of added P_i for mitochondria isolated from control rats ($\circ$) and rats treated with glucagon ($\bullet$). The initial rate of phosphate transport was measured (see the Experimental section) in the presence of 0.5 mM-Ca^{2+} under the conditions described in the legend of Fig. 4 at the indicated concentrations of added P_i . Initial rates were calculated from the values obtained for the amounts of phosphate transported at 5 and 10 s after the addition of exogenous P_i . The data represent the combined results of three separate experiments. (b) The data of (a) plotted in the form of the reciprocal of the initial rate of phosphate transport as a function of the reciprocal of the exogenous P_i concentration. The lines were drawn from the constants obtained from a linear-regression analysis of the data.

apparent K_m for phosphate compared with mitochondria isolated from control rats (Fig. 6). There is no change in the maximum rate of phosphate transport (Fig. 6). Regression analysis of the data plotted in double-reciprocal form (Fig. 6b) yields values of 0.15 mM for the apparent K_m for mitochondria treated with glucagon, compared with 0.33 mM for control mitochondria.

Mitochondria isolated from rats treated with dibutyl cyclic AMP also exhibited (a) an enhancement of 30% in the initial rate of phosphate transport, and (b) an increase of 30% in the extent of net phosphate transport when these parameters were measured at 0.1 mM -exogenous P_i in the presence of Ca^{2+} . The co-administration of cycloheximide with glucagon markedly lessened the increases in net phosphate transport and the initial rate of phosphate transport induced by this hormone (Fig. 7). Similar results were obtained when cycloheximide was co-administered with dibutyl cyclic AMP. The administration of cycloheximide alone had no effect on the initial rate of phosphate transport (results not shown).

Effects of insulin and adrenaline on endogenous mitochondrial P_i and rates of phosphate transport

The administration of adrenaline *in vivo* had no significant effect on the concentration of endogenous P_i in isolated liver mitochondria under the conditions used (Table 1), although a marked increase in the concentration of plasma glucose (20.8 mM , compared with 7.9 mM for control rats at 1 h after injection of the hormone) was recorded. Insulin administration

produced an increase of about 1.3-fold in the concentration of endogenous mitochondrial P_i (Table 1). Since our previous experiments on the effects of insulin on Ca^{2+} transport in liver mitochondria were conducted with starved rats (Dorman *et al.*, 1975), the effect of insulin on the concentration of mitochondrial P_i in fed rats was compared with that in starved animals. The results (Table 1) indicate that the concentration of endogenous P_i in starved control rats was significantly higher than that present in fed control animals ($P < 0.0005$). The administration of insulin to starved rats was associated with (a) a 1.5-fold increase in the concentration of endogenous mitochondrial phosphate (Table 1) and (b) a stimulation in the initial rate and extent of phosphate transport when this was measured at 0.5 mM - (Fig. 1b) and 0.25 mM -exogenous phosphate (results not shown). No effect was observed at 4 mM -phosphate.

Discussion

Elevated concentrations of P_i in mitochondria isolated from the livers of rats treated with hormones or dibutyl cyclic AMP may have arisen from increased uptake of P_i during isolation of the mitochondria, or from the hydrolysis of organic phosphates during storage of the trichloroacetic acid extracts. However, results of control experiments, in which it was shown that the differences are apparent when the mitochondria are isolated in the presence of *p*-chloromercuribenzoate and that little P_i

Fig. 7. Effect of cycloheximide, co-administered with glucagon to rats *in vivo*, on the stimulation by glucagon of phosphate transport in isolated liver mitochondria

The administration of glucagon (25 µg/100 g body wt.) and glucagon plus cycloheximide (250 µg/100 g body wt.) was performed as described in the Experimental section. Mitochondria were isolated from control rats (○), rats treated with glucagon (●) and rats treated with glucagon and cycloheximide (△) as described in the Experimental section. Phosphate transport was measured in the presence of CaCl_2 under the conditions described in the legend of Fig. 4.

and the Experimental section]. The observation that the amount of P_i associated with mitochondria isolated from rats treated with glucagon increases on aging of the isolated mitochondria indicates that a proportion of the measured P_i may be derived from the hydrolysis of organic phosphates. These compounds may be metabolites (e.g. ATP) or phosphate moieties bound to proteins (cf. Zahlten *et al.*, 1972). Attempts to prevent the hydrolysis of ATP during isolation of the mitochondria by conducting the isolation in the presence of oligomycin were not successful. The resulting mitochondria contained very low concentrations of ATP, presumably because oligomycin-insensitive reactions also contribute to ATP hydrolysis and because oligomycin also inhibits the synthesis of ATP.

It is concluded that the elevated concentrations of endogenous P_i result from both an increased formation of organic phosphate and an increase in the amount of free P_i associated with the mitochondria *in vivo* as a result of an increased ability to transport phosphate ions. The range of values obtained for the different groups of control fed rats (Tables 1 and 2) may reflect different lengths of time that have elapsed between removal of the liver from the animal and completion of the isolation procedure, or small differences in the amounts of phosphate lost or taken up by the mitochondria during isolation.

The most pronounced effect of glucagon administration to rats on mitochondrial phosphate transport was observed when this parameter was measured in the presence of Ca^{2+} . Enhancement by Ca^{2+} of phosphate transport in control mitochondria is likely to be due to both the simultaneous movement of these cations with phosphate ions across the inner membrane and the provision of additional sites in the mitochondrial matrix to which phosphate can bind. The increased rate of phosphate transport in mitochondria isolated from rats treated with hormone is not likely to be due primarily to an enhanced rate of Ca^{2+} transport (Dorman *et al.*, 1975) in itself. The effect is observed both in the absence of added Ca^{2+} and after Ca^{2+} has been permitted to accumulate in the mitochondria before the addition of P_i . Since the enhancement after glucagon administration of the rate and extent of phosphate transport measured in the presence of Ca^{2+} was observed in the presence of oligomycin, which should have inhibited both the synthesis and hydrolysis of ATP, it is concluded that the data represent the movement of freely dissociable P_i .

The increase in the rate and extent of transport of exogenous phosphate in mitochondria isolated from rats treated with hormones or dibutyryl cyclic AMP is consistent with an increase in the proton gradient across the mitochondrial inner membrane (cf. Titheradge & Coore, 1976; Halestrap, 1977;

is liberated during storage of the acid extracts, indicate that these possibilities are unlikely. Furthermore, hydrolysis of organic phosphates during assay for P_i should have been negligible [see Baginski *et al.* (1967)

Yamazaki *et al.*, 1977). The magnitude of this gradient appears to be the major driving force for the net transport of phosphate by mitochondria (McGivan & Klingenberg, 1971; Lehninger, 1974; Coty & Pedersen, 1975). The observations that the maximum rate of phosphate transport and the rate of exchange of $^{32}\text{P}_i$ are not altered indicate that the properties of the phosphate transporter itself are not affected.

The present data do not permit a distinction to be made between a direct effect of the hormones or dibutyl cyclic AMP on the liver and a secondary effect which may have arisen from the primary action of the agents on another tissue. Experiments with a perfused liver system have shown that the perfusion of livers with glucagon results in a significant increase in the concentration of endogenous P_i associated with mitochondria subsequently isolated from the liver (B. P. Hughes & G. J. Barritt, unpublished work). This observation is consistent with the direct action of glucagon on the liver to affect the concentration of mitochondrial P_i . The ability of cycloheximide to block the effects of glucagon and dibutyl cyclic AMP indicates that the action of these agents on phosphate transport and metabolism involves a step or steps in which new protein is synthesized (Pestka, 1971), although the possibility that in the presence of both glucagon and cycloheximide the concentration of cyclic AMP is decreased (cf. Wititsuwannakul & Kim, 1977) cannot be excluded.

The data indicate that the mitochondrial phosphate-transport system may be an important site of hormonal action. Changes in mitochondrial phosphate transport may be involved in regulating the supply of P_i to the mitochondrial matrix for the reactions of oxidative phosphorylation. Furthermore, the transport of phosphate is closely linked to that of ADP and Ca^{2+} and is an obligatory requirement for the net transport of malate, α -oxoglutarate and citrate (McGivan & Klingenberg, 1971; Lehninger, 1974; Coty & Pedersen, 1975; Reed & Bygrave, 1975).

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