Stimulation of mitochondrial pyruvate metabolism and citrulline synthesis by dexamethasone

Effect of isolation and incubation media

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Hepatic mitochondria isolated in 0.3 M-sucrose or 0.3 M-mannitol from rats treated for 3 h with dexamethasone displayed stimulated rates of pyruvate carboxylation and decarboxylation and citrulline synthesis when compared with organelles from control animals. Mitochondria isolated in mannitol also displayed elevated rates of pyruvate carboxylation and decarboxylation when compared with those isolated in sucrose, and this stimulation was shown to be independent of the lengthy isolation procedure. Citrulline synthesis proceeded at similar rates in mitochondria isolated in either sugar. The concentration of exchangeable adenine nucleotides was identical in mitochondria isolated in sucrose or mannitol, suggesting that those prepared in the former sugar are not more permeable to metabolites than those prepared in the latter. The matrix volume of mitochondria isolated in mannitol was greater than that of mitochondria isolated in sucrose, and the effect of mannitol on pyruvate metabolism was mimicked by swelling the organelles in hypo-osmotic sucrose. Measurements of the extra-matrix volume by using [^{14}C]sucrose or [^{14}C]mannitol suggest that mannitol can permeate mitochondria to a greater extent than can sucrose. The possibility that mannitol elicits its effect by entering the mitochondrial matrix and so initiating swelling is discussed.

Mitochondria isolated from either the livers of rats treated with a variety of gluconeogenic hormones or from hormone-treated hepatocytes display increased rates of pyruvate carboxylation and oxidation, citrulline synthesis, State-3 rates of respiration (Chance & Williams, 1956) and an increase in protonmotive force in comparison with untreated organelles (Adam & Haynes, 1969; Yamazaki, 1975; Titheradge & Coore, 1976a,b; Yamazaki & Graetz, 1977; Bryla *et al.*, 1977; Triebwasser & Freedland, 1977; Wakat & Haynes, 1977; Halestrap, 1978). Most of these studies have used sucrose-based media for the isolation of the mitochondria; however, Siess (1983a,b) has proposed that mitochondria isolated in sucrose are inferior to those isolated in mannitol, sorbitol or glucose. Mitochondria prepared in the conventional manner in disaccharides contain lower intramitochondrial concentrations of ATP, glutamate, citrate, malate and *N*-acetylglutamate, and display lower rates of pyruvate carboxylation, State-3 rates of respiration and succinate dehydrogenase activity than those prepared in mono-

saccharides, suggesting a decreased mitochondrial integrity during preparation in sucrose. Similarly it has been shown that, after treatment of hepatocytes with dibutyl cyclic AMP, the percentage stimulation of these rates of reaction is smaller in mitochondria prepared in mannitol than it is in those prepared in sucrose (Siess *et al.*, 1981), and it was suggested that hormone treatment stabilizes the organelles, minimizing damage to them during preparation. Consequently it was proposed that mitochondria isolated in sucrose display an artificial stimulation of metabolism after hormone treatment, as the function of controls is selectively impaired.

It is now apparent that short-term treatment of rats with glucocorticoids results in a stimulation of mitochondrial pyruvate metabolism comparable with that by glucagon (Adam & Haynes, 1969; Wakat & Haynes, 1977; Martin *et al.*, 1984). As the work of Siess implies that most of the effects of hormone treatment at the level of the mitochondria may be interpreted as being due to an inhibition of these parameters in control mitochondria rather

than an activation by hormones *in vivo* (Siess *et al.*, 1981; Siess, 1983a,b), we have further characterized the difference in activity caused by suspension of mitochondria prepared from normal and dexamethasone-treated animals in either sucrose or mannitol. We demonstrate that the hormonal stimulation of pyruvate metabolism and citrulline synthesis occurs in both sugars and that the differences between sucrose and mannitol are independent of the isolation procedure and prior treatment with hormones.

Experimental

Materials

[1-¹⁴C]Mannitol and [2-¹⁴C]sodium acetate were obtained from Amersham International (Amersham, Bucks., U.K.). Bovine serum albumin (fraction V), sodium succinate and ornithine were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). α -Cyano-4-hydroxycinnamic acid was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Lumax was obtained from LKB Wallac (Turku, Finland). All other chemicals were either obtained as described previously (Martin *et al.*, 1984) or were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.).

Treatment of animals

Male Sprague-Dawley rats weighing 160–200 g were killed by decapitation; the livers were rapidly removed and mitochondria prepared in either 0.3M-sucrose or 0.3M-mannitol by differential centrifugation as described previously (Titheradge & Haynes, 1979). In experiments examining the effect of dexamethasone, the hormone (150 μ mol/kg) was injected intraperitoneally and the rats were maintained at 21–24°C for 3 h before being killed.

Assays

Pyruvate carboxylation, decarboxylation and utilization were measured as described previously

(Martin *et al.*, 1984). Citrulline synthesis was measured by the method of Yamazaki & Graetz (1977) in a medium containing 203mM-sucrose, 12.5mM-KHCO₃, 12.5mM-MgCl₂, 2.5mM-K₂SO₄, 8mM-potassium phosphate, 3.4mM-Tes (2 - {[2 - hydroxy - 1,1 - bis(hydroxymethyl)ethyl] - amino}ethanesulphonic acid), 0.7mM-EDTA, 5mM-sodium succinate, 5mM-ornithine and 5mM-NH₄Cl, pH 7.4. In experiments where the effect of mannitol on mitochondria during incubation was examined, 203mM-sucrose was replaced by 203mM-mannitol in all incubation media. The content of adenine nucleotides was measured by a bioluminescence technique as described previously (Chisholm *et al.*, 1983) in neutralized HClO₄ extracts of mitochondria incubated under the conditions used to measure pyruvate carboxylation. The mitochondrial volumes were determined after incubation of the organelles in the media used to measure pyruvate carboxylation, except that [¹⁴C]bicarbonate was replaced by 2 μ Ci of ³H₂O/ml and either [¹⁴C]sucrose or [¹⁴C]mannitol (0.2 μ Ci/ml; sp. radioactivity of stock solution 552Ci/mol and 60.1Ci/mol respectively). The organelles were separated from the incubation mixture by centrifugation through a layer of bromododecane/bromodecane (2:3, v/v) into 1M-HClO₄ and a sample was then analysed by liquid-scintillation counting in toluene/Lumax (2:1, v/v). 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 $\pm$ S.E.M., with the numbers of different mitochondrial preparations given in parentheses.

Results

Rates of pyruvate metabolism and citrulline synthesis in mitochondria isolated in sucrose or mannitol

Siess *et al.* (1981) have proposed that the stimulation of mitochondrial metabolism in liver seen after hormonal treatment of rats or hepato-

Table 1. Effect of isolation of mitochondria in sucrose or mannitol on the rates of pyruvate carboxylation and citrulline synthesis after dexamethasone treatment of rats

Mitochondria from control and dexamethasone-treated rats were isolated in sucrose or mannitol (both 0.3M), and pyruvate carboxylation, decarboxylation and citrulline synthesis were measured as described in the Experimental section. **P* < 0.05, ***P* < 0.02 and ****P* < 0.001 in comparison with appropriate control with the number of pairs of rats given in parentheses.

Parameter	Isolation medium	Rate (nmol/min per mg of protein)	
		Control	+ Dexamethasone
Pyruvate carboxylation (13)	Sucrose	18.2 $\pm$ 1.2	29.8 $\pm$ 2.2***
	Mannitol	28.8 $\pm$ 2.6	38.6 $\pm$ 3.1***
Pyruvate decarboxylation (6)	Sucrose	16.7 $\pm$ 1.5	21.7 $\pm$ 1.3*
	Mannitol	23.5 $\pm$ 2.9	29.2 $\pm$ 1.8**
Citrulline synthesis (8)	Sucrose	10.4 $\pm$ 1.5	17.2 $\pm$ 2.1**
	Mannitol	11.2 $\pm$ 1.8	17.2 $\pm$ 2.4**

cytes reflects a stabilization of the organelle rather than an activation of metabolism and have argued that preparation of mitochondria in sucrose leads to selective damage in controls. Therefore we have investigated the effect of isolating mitochondria in 0.3M-sucrose or -mannitol on the stimulation of pyruvate carboxylation (CO_2 fixation), decarboxylation and citrulline synthesis seen after treatment of rats for 3h with the synthetic glucocorticoid dexamethasone. The results are shown in Table 1. The data confirm the report by Siess *et al.* (1981) that mitochondria from control or hormone-treated animals isolated in mannitol exhibit significantly higher rates of pyruvate carboxylation than do those isolated in sucrose ($P < 0.001$ in 13 pairs of animals). The rate of pyruvate decarboxylation was also significantly increased ($P < 0.02$, $n = 6$), indicating a general enhancement of pyruvate metabolism in a mannitol-based medium. However, although the effect of using mannitol as the isolation medium decreased the percentage stimulation of pyruvate metabolism by dexamethasone, the absolute difference in the rates of both pyruvate oxidation and carboxylation in organelles prepared from control and steroid-treated animals was not significantly influenced by the isolation medium ($P > 0.05$). Table 1 also shows that after treatment of animals with dexamethasone there was also a stimulation of citrulline synthesis in the subsequently isolated mitochondria, thus adding a further hormone to the growing list which influence ureogenesis at this site (Yamazaki & Graetz, 1977; Titheradge & Haynes, 1980; Corvera & Garcia-Sainz, 1982). In contrast with the effects on pyruvate metabolism, replacing sucrose with mannitol had no effect on either basal or the hormone-stimulated rate of citrulline synthesis ($P > 0.05$). Verhoeven *et al.* (1982) have also demonstrated that the effect of glucagon on mitochondrial citrulline synthesis is independent of the use of mannitol in the incubation medium. Although the mechanism of stimulation of citrulline synthesis after hormone treatment is unclear, it has been suggested that it may be the result of an increased intramitochondrial concentration of *N*-acetylglutamate, a positive effector of carbamoyl phosphate synthetase (Meijer & Van Woerkom, 1978; Hensgens *et al.*, 1980; Stewart & Walser, 1980; Wanders *et al.*, 1981; Rabier *et al.*, 1982), or an elevated intramitochondrial concentration of ATP (Yamazaki & Graetz, 1977; Titheradge & Haynes, 1980). Therefore the data in Table 1 do not support the hypothesis that mitochondria prepared in sucrose are inferior to those prepared in mannitol, as leakage of *N*-acetylglutamate or adenine nucleotides in the former would be expected to lead to lower rates of citrulline synthesis.

Amounts of adenine nucleotides in mitochondria after isolation in sucrose or mannitol

The data of Siess (1983a,b) have suggested that mitochondria isolated in a sucrose-based medium have lower contents of ATP, glutamate, citrate, malate and *N*-acetylglutamate than do those isolated with mannitol as osmotic support, whereas there was no change in the ADP content. Owing to the potential significance of alterations in the [ATP]/[ADP] ratio to the control of pyruvate carboxylase activity (Stucki *et al.*, 1972; von Glutz & Walter, 1976) and citrulline synthesis (Yamazaki & Graetz, 1977; Titheradge & Haynes, 1980), we have re-examined the effect of the composition of the isolation media on the contents of ATP and ADP in mitochondria incubated under the conditions used for the determination of the rates of pyruvate carboxylation and decarboxylation. Unlike Siess (1983a), we have been unable to demonstrate a decrease in the total content of exchangeable adenine nucleotides (ATP+ADP) after preparation of mitochondria in sucrose (Table 2). Isolation in mannitol did lead to a small, although insignificant, increase in ATP after incubation with 5mM-pyruvate, but this was counteracted by a decrease in ADP, and the total adenine nucleotide pool size remained unchanged. The difference in adenine nucleotide distribution is probably a consequence of the increased rate of pyruvate oxidation (Table 1) in mitochondria prepared in mannitol. The reason for the discrepancy between our results and those of Siess (1983a) is not known, but may reflect the energetic state of the mitochondria. In our experiments, the adenine nucleotides reflect near-State-4 conditions of respiration (Chance & Williams, 1956), with a high [ATP]/[ADP] ratio, whereas those used by Siess (1983a) are essentially de-energized, with a low [ATP]/[ADP] ratio; thus a small change in ATP would become more significant.

Enhancement of pyruvate metabolism in mannitol is independent of the isolation procedure

The above data show that there is a difference in the behaviour of mitochondria suspended in

Table 2. Adenine nucleotide content of mitochondria isolated in sucrose or mannitol

Mitochondria were prepared in sucrose or mannitol (both 0.3M) and incubated with 5mM-pyruvate as described in the Experimental section. After deproteinization with HClO_4 , the content of exchangeable adenine nucleotide was measured. * $P < 0.01$.

Isolation medium	ATP (nmol/mg)	ADP (nmol/mg)	ATP+ADP (nmol/mg)
Sucrose (6)	10.5 ± 0.7	1.7 ± 0.2	12.1 ± 0.8
Mannitol (7)	12.0 ± 0.4	$0.4 \pm 0.3^*$	12.4 ± 0.3

Table 3. *Effect of incubation in sucrose or mannitol-based media on the rates of pyruvate carboxylation and decarboxylation by mitochondria prepared in sucrose or mannitol*

Mitochondria were isolated in 0.3M-sucrose or 0.3M-mannitol and incubated in media which were identical apart from containing either sucrose or mannitol as described in the Experimental section. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ compared with mitochondria isolated and incubated in the presence of sucrose.

Isolation medium	Sugar present during incubation	Pyruvate carboxylation (nmol/min per mg of protein)		Pyruvate decarboxylation (nmol/min per mg of protein)	
		sucrose	mannitol	sucrose	mannitol
Sucrose (7)		20.6 ± 1.7	25.8 ± 1.6*	17.1 ± 1.5	24.0 ± 2.7***
Mannitol (7)		30.4 ± 3.9**	31.0 ± 3.1**	27.8 ± 3.6**	30.7 ± 3.4***

Table 4. *Pyruvate carboxylation in homogenates of livers prepared in sucrose or mannitol*

Pyruvate carboxylation was measured in homogenate of livers immediately after preparation in 0.3M-sucrose or 0.3M-mannitol. * $P < 0.05$ and ** $P < 0.001$ compared with control.

Isolation medium	Pyruvate carboxylation (nmol/min per mg wet wt.)			
	Control	+ Dexamethasone	Increase in rate owing to hormone treatment	(%) Stimulation
Sucrose (7)	2.61 ± 0.27	3.48 ± 0.32**	0.87 ± 0.07	34 ± 3
Mannitol (7)	3.29 ± 0.26	4.32 ± 0.30**	1.03 ± 0.12	32 ± 5

sucrose or mannitol. However, it is not evident that this is a result of deleterious effects of sucrose occurring during the isolation process. To examine the possibility that the difference in the rate of pyruvate metabolism may be due to the mere presence of the sugars in the incubation, mitochondria were isolated in sucrose and the rates of pyruvate carboxylation and oxidation determined in either a sucrose-based or a mannitol-based medium. Table 3 shows that there was a significant stimulation of the rates of both CO₂ fixation and pyruvate decarboxylation in mitochondria incubated in mannitol compared with those incubated in sucrose. This demonstrates that the effect of the different sugars on pyruvate metabolism does not require the lengthy isolation process to be manifest and implies that it is not due to leakage of metabolites and ions incurred as a result of damage during isolation in sucrose, as it is unlikely that such damage could be rectified by contact with mannitol for 5 min. These data suggest either that the presence of mannitol produces a rapid stimulation of pyruvate metabolism or that sucrose inhibits it. The latter possibility has been postulated by Morikofe-Zwez *et al.* (1973), who showed that the rate of pyruvate carboxylation by isolated mitochondria could be decreased by successive replacement of mannitol by sucrose in the incubation medium. To examine these two possibilities, reciprocal experiments were performed in which mitochondria were prepared in mannitol and then

assayed in both the sucrose- and mannitol-based media. In contrast with the findings of Morikofe-Zwez *et al.* (1973), there was no significant decrease in the rates of CO₂ fixation or pyruvate decarboxylation when the organelles were incubated in the presence of sucrose, suggesting that either the inhibitory effect of sucrose is slow in onset or that mannitol has a stimulatory effect on the rates of pyruvate metabolism which is not readily reversed.

Further evidence that lengthy isolation procedures are not necessary to elicit the stimulation of CO₂ fixation caused by exposure to mannitol is shown in Table 4. Pyruvate carboxylation was measured in homogenates of livers immediately after preparation in iso-osmolar sucrose or mannitol. In addition to effects at the level of the isolated mitochondria, homogenates prepared from both control and dexamethasone-treated rats displayed a significant stimulation of CO₂ fixation in mannitol compared with those in sucrose ($P < 0.001$), although neither the percentage nor the absolute stimulation by dexamethasone was significantly different in either of the media.

Effect of mannitol on the matrix volume of mitochondria prepared from control and dexamethasone-treated animals

Adam & Haynes (1969) demonstrated that induction of mitochondrial swelling by incubating mitochondria in hypo-osmotic media led to a

Table 5. *Volume of the matrix of mitochondria isolated and incubated in sucrose or mannitol*
Mitochondria were prepared and the matrix volume was measured as described in the Experimental section.

Extra-matrix volume measured with Mitochondria isolated and incubated in the presence of	Matrix volume ($\mu\text{l}/\text{mg}$ of protein)			
	$[^{14}\text{C}]\text{Sucrose}$		$[^{14}\text{C}]\text{Mannitol}$	
	Control	+ Dexamethasone	Control	+ Dexamethasone
Sucrose (8)	0.69 ± 0.05	0.72 ± 0.07	0.20 ± 0.05	0.23 ± 0.05
Mannitol (8)	1.21 ± 0.02	1.19 ± 0.05	0.68 ± 0.05	0.71 ± 0.03

stimulation of the rate of CO_2 fixation compared with that measured in iso-osmotic media. This has since been confirmed and extended to include other aspects of mitochondrial metabolism by Halestrap and co-workers, who have suggested that an increase in matrix volume may be the mechanism by which gluconeogenic hormones act to enhance mitochondrial metabolism (Armston *et al.*, 1982; Quinlan *et al.*, 1983). Jensen *et al.* (1983) have postulated that mannitol may not be excluded from the mitochondrial matrix and that the resultant swelling may explain the increased rates of metabolism compared with those isolated in sucrose. To test this possibility we have measured the intramitochondrial volume in organelles isolated and incubated in sucrose- or mannitol-based buffers and metabolizing 5 mM-pyruvate. The total water space was measured with $^3\text{H}_2\text{O}$ and the extra-matrix space with either $[^{14}\text{C}]\text{sucrose}$ or $[^{14}\text{C}]\text{mannitol}$. Table 5 shows that, when $[^{14}\text{C}]\text{sucrose}$ was used as the extra-matrix marker, the mitochondria isolated in mannitol showed an increased intramitochondrial volume compared with those isolated and incubated in sucrose. The total water space was not significantly different between both groups of mitochondria ($P > 0.05$), the effect of mannitol being to decrease the extra-mitochondrial space and increase the sucrose-impenetrable volume. This was observed in both control and dexamethasone-treated mitochondria, the difference in matrix volume as measured in the two media being 0.52 ± 0.06 ($P < 0.001$) and 0.48 ± 0.06 ($P < 0.001$) $\mu\text{l}/\text{mg}$ respectively. When $[^{14}\text{C}]\text{mannitol}$ was employed to measure the extra-matrix space, the difference in intramitochondrial volume in mitochondria prepared and assayed in sucrose-based media as opposed to mannitol was also highly significant and comparable with those measured with $[^{14}\text{C}]\text{sucrose}$ as the marker, the absolute values being 0.47 ± 0.06 ($P < 0.001$) and 0.48 ± 0.04 ($P < 0.001$) $\mu\text{l}/\text{mg}$ for mitochondria prepared from control and dexamethasone-treated animals respectively. These results confirm the suggestion by Jensen *et al.* (1983) that mannitol is capable of gaining access into the mitochondrial matrix, causing it to become hyper-osmotic in comparison with the

external medium and consequently leading to swelling. This is supported by measurements of the intramitochondrial volume by using $[^{14}\text{C}]\text{mannitol}$ to measure the extra-matrix space. The apparent intramitochondrial volume measured under these conditions in either sucrose- or mannitol-based media was considerably lower than that measured with $[^{14}\text{C}]\text{sucrose}$ as the marker ($P < 0.001$) as a result of an apparent increased extra-matrix space, presumably owing to the $[^{14}\text{C}]\text{mannitol}$ leaking into the matrix of the mitochondria.

Effect of mannitol on pyruvate transport into the mitochondria

The data in Table 1 demonstrate that there is an enhancement of both aspects of pyruvate metabolism after exposure of mitochondria to mannitol. The rates of both pyruvate oxidation and carboxylation have been shown to be regulated in part by the rate of entry of pyruvate into the mitochondria (Adam & Haynes, 1969; Titheradge & Coore, 1976a,b; Halestrap, 1978; Thomas & Halestrap, 1981; Armston *et al.*, 1982; Martin *et al.*, 1984), and it is possible that the enhancement of pyruvate metabolism observed in mitochondria suspended in mannitol may reflect an increase in the rate of pyruvate translocation. Therefore we have examined carrier-mediated pyruvate transport in mitochondria isolated and incubated in sucrose- and mannitol-based media as described by Halestrap (1978) (Fig. 1). Pyruvate utilization was titrated with a specific inhibitor of the pyruvate translocator, α -cyano-4-hydroxycinnamic acid (Halestrap & Denton, 1973). The inset in Fig. 1 shows a Dixon plot of reciprocal rate of pyruvate utilization against concentration of inhibitor. By extrapolation of the linear portion of the graph, it is possible to estimate the rate of entry of pyruvate into the mitochondria. When they were isolated and incubated in sucrose, the rate of entry was 38.5 ± 2.8 nmol/min per mg of protein, and this was increased to 55.2 ± 3.1 nmol/min per mg of protein in mitochondria isolated and incubated in mannitol ($P < 0.001$). This increase in transport is sufficient to account for the change in the rates of CO_2 fixation and pyruvate decarboxylation reported in control mitochondria in Table 1.

Fig. 1. Effect of isolation and incubation of mitochondria in sucrose or mannitol on rates of pyruvate utilization in the presence of α -cyano-4-hydroxycinnamic acid

Mitochondria were isolated and incubated in the presence of sucrose (■) or mannitol (●), and pyruvate utilization was measured as described in the Experimental section, with the concentration of α -cyano-4-hydroxycinnamic acid shown. The inset shows a Dixon plot of reciprocal pyruvate utilization against concentration of α -cyano-4-hydroxycinnamic acid. ($n = 10$).

Effect of hypo-osmotic media or mitochondrial volume and metabolism

Although the above data suggest that the increased matrix volume observed in mitochondria prepared and incubated in mannitol may be responsible for the elevated rates of pyruvate metabolism, this seems to be precluded by the lack of effect of mannitol on citrulline formation, which has also been shown to be elevated by hypo-osmolarity (Armstrong *et al.*, 1982). However, this latter effect has been refuted by Jensen *et al.* (1983), although no results were presented. There-

fore we have measured the effect of incubating mitochondria in different concentrations of sucrose or mannitol, to induce swelling, on both pyruvate metabolism and citrulline synthesis. Fig. 2(a) shows that both pyruvate carboxylation and decarboxylation increased when mitochondria were incubated with decreasing concentrations of sucrose. However, citrulline synthesis remained unaffected by this treatment, confirming the conclusion of Jensen *et al.* (1983). The inset to Fig. 2(a) shows that over a range of 180–300 mosM the volume of the mitochondrial matrix increased from 0.9 to 1.37 μ l/mg of protein and there was a

linear relationship between the components of pyruvate metabolism and the matrix space as measured with [^{14}C]sucrose and $^3\text{H}_2\text{O}$. Experiments performed on mitochondria incubated in mannitol showed there was a similar relationship between osmolarity and intramitochondrial volume, although at each osmolarity examined there was a significant increase in the intramitochondrial volume compared with that measured at the same osmolarity of sucrose ($P < 0.002$). However, in contrast with mitochondria incubated in a sucrose-based medium, there was no increase in pyruvate metabolism with decreasing osmolarity, although the matrix volume increased from 1.33 to 1.73 $\mu\text{l}/\text{mg}$ of protein (Fig. 2b). A comparison of the rate of pyruvate metabolism in mitochondria in hypo-osmotic sucrose and hypo-osmotic mannitol (180 mosM) shows that the rate in the former exceeds that in the latter. Although the reason behind this difference is unclear, it is possible that mitochondria in mannitol reach a critical volume above which they begin to leak their substrates and cofactors into the medium. A loss of mitochondrial ATP and ADP has been reported after isolation and incubation of mitochondria in a medium containing predominantly mannitol as osmotic support by Moriköfer-Zwez *et al.* (1973), though their incubation buffer was iso-osmotic.

Discussion

The data in the present paper do not support the hypothesis that isolation of mitochondria in sucrose selectively impairs the function of the organelles through loss of metabolites (Siess *et al.*, 1981; Siess, 1983a,b), since we have been unable to demonstrate any significant effect of the isolation medium on either the total exchangeable adenine nucleotide pool size or the rate of citrulline formation in mitochondria prepared from control and dexamethasone-treated rats. The results in Table 1 confirm that the use of mannitol in the

Fig. 2. Effect of decreasing osmolarity on the rates of pyruvate metabolism and citrulline synthesis in mitochondria isolated and incubated with sucrose (a) or mannitol (b)

Mitochondria were isolated and incubated in the presence of sucrose or mannitol as described in the legend to Table 1, except that the concentration of the sugar present was varied to give the osmolarity shown. The inset shows a plot of mitochondrial volume, measured as described in Table 5, against the rates of pyruvate carboxylation and decarboxylation. ○, Pyruvate carboxylation ($n = 14$); ●, pyruvate decarboxylation ($n = 6$); ■, citrulline synthesis ($n = 8$).

isolation medium enhances the rate of pyruvate utilization as compared with mitochondria prepared in sucrose; however, the effect of dexamethasone treatment of the animals on pyruvate metabolism remained unaltered, although the percentage stimulation was reduced. Similar results have been reported by Jensen *et al.* (1983) after treatment of animals with glucagon, suggesting that the effect of both of these gluconeogenic hormones and mannitol are independent. In Tables 3 and 4 we have shown that the changes in mitochondrial metabolism caused by suspension in the different sugars are rapid in onset and do not involve the isolation procedure. Morikofer-Zwey *et al.* (1973) have proposed that the inclusion of sucrose in the isolation medium leads to an inhibition of pyruvate carboxylase activity. If this were true, the data in Table 1 and Fig. 1 would suggest that this effect is restricted not only to pyruvate carboxylase activity, but also to pyruvate oxidation, presumably as a result of sucrose inhibiting the activity of the pyruvate transporter in the mitochondrial membrane. The increased rate of pyruvate metabolism observed in hypo-osmotic media (Adam & Haynes, 1969; Armston *et al.*, 1982) could similarly be explained by a decrease in the concentration of sucrose. However, several factors refute this hypothesis: first, the titration curve of pyruvate metabolism with increasing concentration of α -cyano-4-hydroxycinnamate indicates that pyruvate metabolism is not rate-limiting under these conditions; second, in Table 3, we have shown that incubation in a sucrose-based medium of mitochondria isolated in mannitol does not lead to an inhibition of pyruvate metabolism; and third, Siess (1983a,b) has shown that the inhibition of metabolism is not restricted to pyruvate alone, but is more generalized. The results in Table 5 suggest that the effect of mannitol may be to gain entry to the mitochondrial matrix, resulting in mitochondrial swelling and an increased matrix volume, as first suggested by Jensen *et al.* (1983). Mitochondrial swelling has been shown to also lead to an increase in some other mitochondrial functions in addition to pyruvate metabolism, including respiration (Johnson & Lardy, 1958; Nicholls & Lindberg, 1972; Armston *et al.*, 1982), uncoupler-dependent ATPase activity (Nicholls & Lindberg, 1972) and glutaminase activity (Lacey *et al.*, 1981), possibly as a result of a change in the conformation of the matrix and so the environment of the enzymes present in it. Support for the hypothesis that mannitol can induce mitochondrial swelling may be derived from the study of Halestrap & Quinlan (1983), which suggests by both light-scattering and isotope techniques that mannitol can enter the mitochondrial matrix at 37°C, albeit slowly,

whereas sucrose was excluded under the same conditions. Similarly, Siess (1983b) has also demonstrated a decreased content of $^3\text{H}_2\text{O}$ in mitochondria after isolation in disaccharide-based media, though it was suggested that this indicated a loss of metabolites from mitochondria with a consequent loss of water by osmosis rather than swelling of mitochondria after isolation in monosaccharides. Examination of mitochondria in iso- and hypo-osmotic sucrose confirms that increasing the volume of the matrix leads to a stimulation of pyruvate metabolism (Adam & Haynes, 1969; Armston *et al.*, 1982; Fig. 2a), but not citrulline synthesis (Fig. 2a), which is comparable with the effect of suspending mitochondria in mannitol. The data in Fig. 1 suggest that the increase in pyruvate metabolism seen after suspension of the mitochondria in mannitol may be in part the result of an increased entry of pyruvate into the matrix by a carrier-mediated mechanism. Preliminary studies have shown that the pH gradient is not increased in mitochondria isolated and incubated in mannitol (A. D. Martin & M. A. Titheradge, unpublished work), suggesting that the increase in volume of the matrix seen after suspension in mannitol causes a change in the conformation of the transporter, increasing its activity and leading to higher rates of pyruvate carboxylation and oxidation.

The finding that liver homogenates prepared from hormone-treated rats display a significant stimulation of CO_2 fixation in comparison with controls when prepared in both sucrose and mannitol suggests that the effect of hormone treatment is independent of the isolation procedure (Jensen *et al.*, 1983; Martin *et al.*, 1984; Table 4). Similarly, measurements of pyruvate carboxylation *in situ* by using the polyene antibiotic filipin (Allan *et al.*, 1983) and examination of the contents of metabolites in the cytosolic and mitochondrial compartments of hepatocytes after digitonin fractionation (Siess *et al.*, 1977; Jensen *et al.*, 1983; Allan & Titheradge, 1984) also favour the hypothesis that treatment of animals with glucagon, glucocorticoids and other gluconeogenic hormones leads to a stimulation of pyruvate carboxylation in mitochondria *in vivo*. The effect of treatment of rats or hepatocytes with gluconeogenic hormones is approximately to double the rate of pyruvate metabolism in subsequently isolated mitochondria (Adam & Haynes, 1969; Haynes *et al.*, 1974; Garrison & Haynes, 1974; Yamazaki & Haynes, 1974; Titheradge & Coore, 1976a,b; Garrison & Borland, 1977; Wakat & Haynes, 1977; Halestrap, 1978; Siess *et al.*, 1981; Armston *et al.*, 1982; Martin *et al.*, 1984). It has been proposed that an increase in the volume of the mitochondrial matrix may be the mechanism of

action of these hormones (Armston *et al.*, 1982; Halestrap & Quinlan, 1983; Quinlan *et al.*, 1983). However, the data in Fig. 2(a) suggest that swelling alone is insufficient to account for the effect of hormone treatment, as a doubling in the rate of pyruvate metabolism would require a similar increase in the volume of the matrix, whereas only changes in mitochondrial volume of up to 15% have been observed with [^{14}C]sucrose as the extra-matrix marker (Armston *et al.*, 1982; Quinlan *et al.*, 1983).

The results described in the present paper confirm the data of Siess *et al.* (1981) that mitochondria isolated in mannitol display enhanced rates of pyruvate metabolism, although we demonstrate that this stimulation is a result not of isolation, but rather of suspension in mannitol-based media. In view of the reports in the literature which suggest that even sucrose can penetrate mitochondrial membranes during centrifugation (Sitaraman & Sarma, 1981; Sambasivarao & Sitaraman, 1983), or after swelling with Ca^{2+} (Hunter *et al.*, 1976), caution should be taken in interpreting experiments on these isolated organelles, particularly when they examine the hormonal stimulation of metabolism.

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