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Rat liver mitochondria prepared in mannitol media demonstrate increased mitochondrial volumes compared with mitochondria prepared in sucrose media

Relationship to the effect of glucagon on mitochondrial function

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1. Liver mitochondria isolated from glucagon-treated rats by using both mannitol- and sucrose-based media showed enhanced uncoupled succinate oxidation, pyruvate metabolism and citrulline synthesis. 2. Mitochondria prepared in mannitol medium showed some stimulation of these parameters compared with those prepared in sucrose medium. This was accompanied by an increase in matrix volume of about 20%. 3. Some [^{14}C]mannitol became permanently associated with mitochondria during preparation. It is suggested that mannitol may enter mitochondria during their preparation and cause swelling. 4. The presence of 4mM-phosphate in the sucrose isolation medium stimulated the same parameters as did glucagon treatment, and also caused an increase in matrix volume of about 20%. 5. These results confirm the conclusion that the mitochondrial volume may be important in the regulation of mitochondrial metabolism. They contradict the conclusion of others [Siess (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 279–290, 835–838] that mannitol rather than sucrose should be used when studying hormonal effects on mitochondrial metabolism. 6. Reasons for the discrepancies in the results between groups studying the effects of hormones on mitochondrial metabolism are discussed.

Liver mitochondria isolated from rats treated with glucagon demonstrate changes in many parameters, of which stimulation of pyruvate metabolism, citrulline synthesis, respiration and glutaminase activity and an increase in mitochondrial volume can be demonstrated in the intact cell or perfused liver [for reviews, see Halestrap (1981) and Halestrap *et al.* (1983)]. Nevertheless it has been suggested that some of the effects of glucagon treatment which persist on isolation of mitochondria are artefacts of mitochondrial preparation, perhaps involving stabilization of the mitochondria against the action of endogenous phospholipase A_2 (Halestrap, 1981; Siess *et al.*, 1981). Siess and co-workers have suggested that all effects of glucagon treatment of the rat which persist on isolation of the liver mitochondria may be artefacts produced by the use of sucrose media for the preparation of the mitochondria, and are not seen when mitochondria are prepared in mannitol media (Siess *et al.*, 1981; Siess, 1983*a*,*b*). This view has been challenged by others (Verhoeven *et al.*, 1982; Jensen *et al.*, 1983), who find enhanced rates of citrulline synthesis in mitochondria prepared in both media. Jensen *et al.* (1983) were also able to demonstrate significant activation of the respiratory chain by glucagon when mitochondria were prepared in mannitol medium. This was not observed by Siess *et al.* (1981) or Verhoeven *et al.* (1982). An effect of glucagon treatment of rats or isolated hepatocytes on mitochondrial behaviour in crude homogenates (Jensen *et al.*, 1983) or permeabilized cells (Allan *et al.*, 1983) has also been used to indicate that hormone effects are not artefacts of mitochondrial preparation.

Previous research from our laboratory has demonstrated that many of those effects of glucagon on rats may be mimicked by small increases in the matrix volume (Halestrap, 1982; Armston *et al.*, 1982), and that such volume changes are induced in mitochondria *in situ* when hepatocytes are treated with glucagon (Quinlan *et al.*, 1983). Siess (1983*a*,*b*) has shown that preparation of mitochondria in hypo-osmotic sucrose media, or in iso-osmotic sucrose media containing 4mM-phosphate, produces mitochondria similar to those prepared in iso-osmotic mannitol media.

This suggests that the differential effects of mannitol and sucrose isolation media on mitochondrial metabolism may reflect differences in mitochondrial matrix volume. In the present paper we demonstrate that this is the case. The preparation of liver mitochondria in both mannitol and sucrose media containing 4mM-phosphate causes an increase in the matrix volume associated with some stimulation of mitochondrial metabolism. In agreement with Jensen *et al.* (1983), we report effects of glucagon treatment persisting in both sucrose and mannitol media.

Experimental

Materials

All chemicals, biochemicals and radiochemicals were purchased from the sources described previously (Armston *et al.*, 1982; Halestrap & Quinlan, 1983).

Rat liver mitochondria were prepared from the livers of female Wistar rats (250–300g body wt.) allowed free access to food and water. Rats were anaesthetized by intraperitonal injection of Sagatal (1ml/kg) at least 30min before being killed, and when required glucagon was injected 15min before death, as described previously (Halestrap. 1978). Livers were rapidly removed from the animal and divided in half, each half being homogenized in 40ml of the required (ice-cold) isolation buffer, with several strokes of a Dounce Potter homogenizer. All isolation buffers contained 2mM-EGTA and 10mM-Tris/HCl (pH7.4 at 0°C) and also either 300mM-sucrose or -mannitol. In some experiments 4mM-potassium phosphate was added to the sucrose buffer. Subsequent differential centrifugation of the homogenate as described previously (Halestrap, 1978) yielded a mitochondrial fraction, which was washed once with ice-cold isolation buffer.

Methods

Rates of O_2 uptake by mitochondria were measured in a Clark-type oxygen electrode at 25°C as described previously (Halestrap, 1978; Armston *et al.*, 1982). The medium contained 125mM-KCl, 10mM-Mops (4-morpholinepropanesulphonic acid), 7mM-Tris base, 2.5mM-potassium phosphate, 2.5mM-$MgCl_2$, 1mM-EGTA, rotenone (1μg/ml) and 5mM-Tris succinate, and was adjusted to pH7.2. Mitochondria were added at about 1.5mg of protein/ml, and the rates of O_2 uptake before and after addition of 1mM-ADP and 0.1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were measured. Antimycin (0.1μg/ml) was then added, followed by 10mM-ascorbate and 0.3mM-*NNN'N'*-tetramethyl-*p*-phenylene-diamine, and the new rate of O_2 uptake was measured.

Rates of pyruvate carboxylation and citrulline synthesis were estimated by measuring the incorporation of ^{14}C from $H^{14}CO_3^-$ into acid-stable products essentially as described previously (Armston *et al.*, 1982). In both cases the basic incubation medium was the same as that used for the oxygen-electrode studies, but lacked succinate and rotenone and contained 12.5mM-$KHCO_3$. For pyruvate carboxylation the medium also contained 4mM-pyruvate, and for citrulline synthesis glutamate (5mM), malate (1mM), NH_4Cl (5mM) and ornithine (20mM) were also present. The buffers were pre-equilibrated with O_2/CO_2 (19:1) to give a final pH of 7.2 before addition of carrier-free $H^{14}CO_3^-$ to a final specific radioactivity of about 100d.p.m./nmol. Four 0.5ml samples of the buffer were transferred to closed 1.5ml plastic centrifuge tubes and equilibrated at 37°C before addition of about 2.5mg of mitochondrial protein. After further incubation for 6min, the reaction was stopped by addition of 50μl of $HClO_4$ (20%, w/v). The protein was removed by centrifugation at 5000*g*-min and 250μl portions were added to scintillation vials. The excess $^{14}CO_2$ was removed from the sample by exposure to an overdraught for 12h before addition of 1ml of water followed by 10ml of Unisolve E (Koch–Light Laboratories, Slough, Berks., U.K.). Acid-stable ^{14}C was then determined by scintillation counting. In parallel incubations blanks lacking pyruvate or ornithine were taken through the assay. Mitochondrial protein concentrations were determined after sedimentation of the mitochondria by centrifugation at 10000*g*-min and assay of the protein in the pellet by a biuret reaction as described previously (Armston *et al.*, 1982). In assays of pyruvate carboxylation, total pyruvate metabolism was measured by enzymic assay of pyruvate in the supernatant (Halestrap, 1978).

Mitochondrial volumes were measured at 0°C in the KCl medium used for oxygen-electrode studies, but without added succinate. The technique was exactly as described previously (Halestrap & Quinlan, 1983) and utilized D-[1-^{14}C]-mannitol and ^3H_2O. The values reported in the present study (0.75μl/mg of mitochondrial protein) are slightly higher than reported previously (0.5μl/mg of mitochondrial protein; Halestrap & Quinlan, 1983), but are still substantially lower than the values of about 1.3μl/mg of protein obtained when [^{14}C]sucrose rather than mannitol was used as extra-mitochondrial marker. The larger values reported here appear to be the result of some variation in the status of the rats used, since all other factors have been eliminated. In experiments performed after the submission of

this paper, volumes have again returned to about 0.5 μl/mg of protein, without any changes in technique or materials used. We conclude that some seasonal, nutritional or hormonal factors outside our control have influenced the absolute value of the mitochondrial volume. However, on any one day mitochondrial volumes measured for mitochondria from several different rats are extremely similar.

Light-scattering of mitochondria under pressure

Mitochondria were suspended at 2mg of protein/ml in either sucrose or mannitol isolation medium and placed in the pressure cells of a pressure-jump apparatus (Davis & Gutfreund, 1978). The A_{520} was continuously recorded and pressure rapidly applied or released as required. Pressures of up to 150atm (15.2MPa) could be applied to the cell within about 2s, and released again in less than 100ms.

Statistical comparisons of control versus experimental parameters were performed by Student's *t* test. Where the same liver was homogenized in two different media, comparison between the two resulting mitochondrial preparations utilized a paired *t* test.

Results

Comparison of mannitol and sucrose isolation media for the preparation of control and glucagon-treated mitochondria

Table 1 shows the effects of glucagon pre-treatment of rats on various parameters of liver mitochondria prepared in sucrose or mannitol media. In agreement with Jensen *et al.* (1983), the percentage stimulation by glucagon of pyruvate carboxylation and total metabolism, citrulline synthesis and uncoupled respiration in the presence of succinate was similar in both media. In all cases, however, the mannitol medium gave slightly higher values than the sucrose medium. This was accompanied by a significant increase in the intramitochondrial volume in the mitochondria prepared in mannitol medium. The percentage increase (mean ± S.E.M. for ten observations) was 20.2 ± 4.4 for control mitochondria and 14.7 ± 3.8 for mitochondria from glucagon-treated rats.

Effects of phosphate addition to sucrose isolation media on mitochondrial function

Table 1 also presents data confirming the observations by Siess (1983*a*) that the presence of 4mM-phosphate in sucrose isolation medium enhances uncoupled succinate oxidation, pyruvate carboxylation and total metabolism and citrulline synthesis. In addition, we show that this is also accompanied by an increase of 20.9% in intramitochondrial volume (means ± S.E.M. of four observations) from 0.776 ± 0.008 to 0.938 ± 0.019 μl/mg of protein. Previous work from our laboratory has shown that such an increase in mitochondrial volume could account for the stimulation of the other parameters (Armston *et al.*, 1982).

Uptake of mannitol into mitochondria during isolation

We have shown previously (Halestrap & Quinlan, 1983) that at 0°C mannitol does not enter liver mitochondria. However, some very slow uptake may occur at higher temperatures, which increases dramatially during prolonged incubation at 37°C. We decided to see whether the increased volume of mitochondria isolated in mannitol medium could be accounted for by slight leakage of mannitol into the matrix during isolation and subsequent osmotic swelling. To investigate this, mitochondria were isolated conventionally in sucrose or mannitol media as described in the Experimental section, but in the presence of either [^{14}C]sucrose or [^{14}C]-mannitol respectively at 0.08 μCi/ml. The first wash of the mitochondria also contained either [^{14}C]-sucrose or [^{14}C]mannitol, and a second wash was performed without radioisotope. Samples of the resulting mitochondria (15mg of protein) were then suspended in 0.5ml of the appropriate isolation medium containing 0.025 μCi of ^3H_2O/ml and sedimented by centrifugation at 10000*g* for 1min. From an analysis of the amounts of ^{3}H and ^{14}C in both the pellet and the supernatant (similar to that performed for measuring the matrix volume), it was possible to calculate the amount of mannitol or sucrose bound to or trapped inside the mitochondria. Expressed as μl of the original extraction medium persisting in association with 1mg of mitochondrial protein, the values (means ± S.E.M. for seven separate mitochondrial preparations) were 0.071 ± 0.006 and 0.117 ± 0.014 for sucrose and mannitol respectively. The difference in values (0.046 ± 0.011 μl/mg of protein) is highly significant. The [^{14}C]mannitol associated with the mitochondria is likely to be trapped within the matrix rather than bound to the exterior, since any extramitochondrial ^{14}C should be removed by the wash with unlabelled mannitol.

It has been suggested that the pressures exerted on mitochondria during high-speed centrifugation may cause them to become permeable to sucrose and mannitol (Sitaramam & Sarma, 1981; Sambasivarao & Sitaramam, 1983). This was not found to be the case. Additional centrifugation of mitochondria resuspended in [^{14}C]sucrose or [^{14}C]-mannitol media resulted in no further increase in ^{14}C associated with the mitochondria even when the process of resuspension and centrifugation was repeated three times (result not shown). Further-

Table 1. *Influence of glucagon treatment of rats and various isolation media on the properties of liver mitochondria*

Mitochondria were prepared in either sucrose or mannitol media as described in the Experimental section. Where appropriate, 4mM-potassium phosphate was present. Measurements of all parameters shown were performed as described in the Experimental section and are given as means ± S.E.M. for the numbers of different mitochondrial preparations shown (or half that number where both 'control' and 'glucagon' values are given). Statistical significance of the difference between control and experimental conditions was calculated by Student's *t* test: $*P<0.05$; $**P<0.02$; $***P<0.01$.

Parameter	Units	Sucrose medium		Mannitol medium		'Mannitol' value as a percentage of 'sucrose' value	No. of observations	Value with sucrose + 4mM-phosphate as a percentage of 'sucrose' value	No. of observations
		Control	Glucagon	Control	Glucagon				
Uncoupled oxidation of succinate	(ng-atoms of O/min per mg of mitochondrial protein)	59.4±1.6	85.3±5.3***	65.2±3.7	88.6±4.5***	108.0±3.9	24	159.4±11.6**	4
Uncoupled oxidation of ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD)	(ng-atoms of O/min per mg of mitochondrial protein)	137.1±7.8	139.0±11.3	126.2±9.2	134.2±9.3	95.6±2.6	24	102.9±6.5	4
Rate of succinate oxidation as a percentage of the rate of ascorbate + TMPD oxidation		44.6±2.2	62.3±2.9***	53.8±3.2	67.1±2.8***	119.3±4.1***	24	157.5±13.2*	4
Rate of pyruvate carboxylation	(nmol/min per mg of mitochondrial protein)	13.4±0.58	22.5±3.5*	16.8±2.0	25.3±2.6*	121.8±8.6*	12	185±17.2**	4
Rate of total pyruvate metabolism	(nmol/min per mg of mitochondrial protein)	24.5±1.75	34.9±3.0**	28.9±2.6	40.4±2.5*	119.0±7.3*	12	170.7±14.6**	4
Rate of citrulline synthesis	(nmol/min per mg of mitochondrial protein)	23.6±0.31	35.6±2.0***	25.3±1.9	42.6±2.5***	116.3±6.6*	20	149.6±18.9	4
Intramitochondrial volume	(μl/mg of mitochondrial protein)	0.745±0.037	0.797±0.045	0.888±0.039	0.902±0.033	117.5±2.9***	20	120.9±3.2***	4

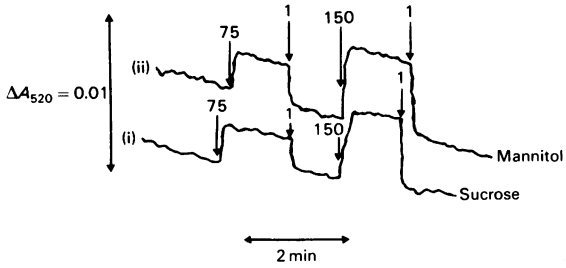

Fig. 1. *Effects of pressure on light-scattering by liver mitochondria*

Rat liver mitochondria were suspended at 2mg of protein/ml in either sucrose (i) or mannitol (ii) isolation medium containing 0.1 μg of antimycin/ml. The A_{520} was then continuously recorded in a pressure-jump apparatus maintained at 5°C and the pressure was changed as required. Separate experiments showed that the apparatus was readily able to detect volume changes of 1% or less. Arrows indicate changes in pressure to the new values indicated (expressed in atm; 1 atm = 1063 kPa). Rises in pressure took several seconds to stabilize, whereas pressure release occurred in less than 100ms.

more, by using a special pressure cell, light-scattering of mitochondria was used to study mitochondrial swelling in sucrose and mannitol media under pressures of up to 150atm. The results are shown in Fig. 1. Pressure produced a rapid reversible light-scattering change in both media (probably representing a shape change), but no evidence for swelling was found. Thus the association of mannitol with mitochondria is unlikely to be dependent on the pressure generated by centrifugation, but on the initial homogenization event.

Discussion

Rat liver mitochondria prepared in mannitol medium demonstrated persistent effects of glucagon similar to those found when 300mM-sucrose was used as isolation medium (Table 1). These data agree well with those of Jensen *et al.* (1983), but are at variance with the results of Siess *et al.* (1981). Verhoeven *et al.* (1982) were able to demonstrate an effect of glucagon on citrulline synthesis, but not on State-3 respiration, by mitochondria isolated in mannitol medium. The data of Table 1 also show that the values of all glucagon-sensitive parameters are slightly higher in mitochondria prepared in mannitol media. This conclusion is similar to that by Siess *et al.* (1981) and Siess (1983*a*,*b*). However, the elevation of glucagon-sensitive parameters in mannitol medium was not as great as that observed by Siess *et al.* (1981), nor was it accompanied by a large decrease in the observed effect of glucagon. An explanation of the greater rates of uncoupled respiration, citrulline synthesis and pyruvate metabolism seen in mitochondria isolated in mannitol medium may be provided by the larger matrix volume that is also observed (Table 1). These parameters are all stimulated by small increases in intramitochondrial volume (Halestrap, 1982; Armston *et al.*, 1982). In this context it is noteworthy that Siess (1983*b*) reports a very considerable (100%) increase in the inulin-impermeable space of mitochondria prepared in media containing monosaccharides rather than disaccharides as osmotic support. This correlated well with the observed increase in succinate dehydrogenase and uncoupler-stimulated ATPase activity. Thus it seems that, under the conditions of mitochondrial preparation used by Siess (1983*a*,*b*), the mitochondria swelled considerably more than reported here. Such large increases in matrix volume may also be produced by using hypo-osmotic media (150mosM), and Siess (1983*a*,*b*) has demonstrated that the use of hypo-osmotic sucrose medium allowed the preparation of mitochondria similar to those produced in iso-osmotic mannitol medium. The use of such hypo-osmotic conditions can greatly decrease or abolish the effect of glucagon on pyruvate metabolism and uncoupled respiration but not on citrulline synthesis (Armston *et al.*, 1982). Thus the large increase in inulin-impermeable space observed by Siess (1983*b*) in mannitol medium or hypo-osmotic sucrose medium may explain the inability of Siess *et al.* (1981) to demonstrate substantial glucagon effects when mitochondria were prepared in such media.

The reason for the increased matrix volumes in mannitol medium may be explained by the entry of mannitol into the mitochondria during the preparation procedure. We have demonstrated uptake of a small amount of [^{14}C]mannitol into liver mitochondria during their preparation, sufficient to account for some, but not all, of the increased volume. It is possible that under the conditions used by Siess (1983*a*,*b*) considerably more mannitol entered the mitochondria. This cannot be the result of different centrifugation techniques, since the data of Fig. 1 demonstrate that mannitol does not enter the mitochondria even under a pressure of 150atm. Such a pressure is greater than that maximally exerted during centrifugation at 10000*g*. However, mannitol can enter mitochondria under conditions under which sucrose does not when mitochondria are incubated for 5min at 37°C (Halestrap & Quinlan, 1983). The capacity of mitochondria to become permeable to low-molecular-weight molecules has been extensively studied by others (Hunter & Haworth, 1979; Haworth & Hunter, 1979) and can be induced by very low Ca^{2+} concentrations. The effect is

enhanced in the absence of Mg^{2+}. It is therefore noteworthy that Siess (1983*a*,*b*) used EDTA (1.0mM) in his isolation medium, and it is known that EDTA can cause mitochondrial swelling (see Joseph *et al.*, 1980, for discussion). Verhoeven *et al.* (1982) used neither EDTA nor EGTA in their isolation medium, and a Ca^{2+}-induced leakage of mannitol into the matrix could explain their inability to detect changes in respiratory-chain activity. Citrulline synthesis is less sensitive than the other parameters to volume changes (Armston *et al.*, 1982; Siess, 1983*a*; Jensen *et al.*, 1983) and still demonstrates a glucagen effect in swollen mitochondria (Armston *et al.*, 1982). This may explain why Verhoeven *et al.* (1982) were still able to detect a hormone effect on citrulline synthesis when mitochondria were prepared in mannitol medium. Both Jensen *et al.* (1983) and ourselves used EGTA in the mitochondria isolation medium, and were able to detect glucagon effects on all parameters with both media.

It may therefore be concluded that the expression of persistent effects of glucagon in isolated mitochondria is critically dependent on their matrix volume. In contrast with Siess (1983*a*,*b*), we do not believe that mannitol is a better osmotic support than sucrose for the preparation of mitochondria. Rather it may enter the mitochondria and swell them under some conditions, thus diminishing the hormone effect. The use of EGTA and not EDTA to chelate bivalent metal ions during isolation appears to be advisable to prevent such swelling.

The stimulation of mitochondrial metabolism induced by including 4mM-phosphate in the sucrose isolation medium (Siess, 1983*a*) may also be explained by an increase in matrix volume, as indicated in Table 1. It is likely that the increase in volume involves the uptake of potassium phosphate into, or prevention of K^+ loss from, the mitochondria during isolation. Such an increase in matrix K^+ or phosphate may account for the larger effects of phosphate inclusion in sucrose isolation medium on mitochondrial metabolism than the use of mannitol isolation medium, despite the increase in volume caused by the two treatments being quite similar (Table 1). Hamman & Haynes (1983*a*,*b*) have shown that incubation of mitochondria in sucrose medium containing potassium phosphate and $MgCl_2$ causes activation of many of the parameters stimulated by glucagon treatment. From the results presented in Table 1 it seems likely that this treatment *in vitro* would also be accompanied by an increase in matrix volume, although the authors claim this is not the case. However, our own measurements using [^{14}C]mannitol and ^3H_2O do show that the treatment used by Hamman & Haynes (1983*a*,*b*) produces a 15–20% increase in matrix volume similar to that observed when phosphate is present in the isolation medium (results not shown).

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