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TRANSPORT OF ANIONS IN STORED BOVINE ADRENAL CORTICAL MITOCHONDRIA

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SUMMARY

Bovine adrenal cortical mitochondria may be stored for as long as 7 days at −20 °C without loss of citrate, malate or succinate transport activities. Storage in a medium containing 15% dimethyl sulfoxide, 1% bovine serum albumin and isotonic sucrose results in least loss of transport and respiratory activities. The tricarboxylate transport properties of these mitochondria differ from those of rat liver mitochondria with respect to the efficacy of the dicarboxylic acids on stimulation of citrate uptake.

INTRODUCTION

It has been reported that cryoprotective agents such as dimethyl sulfoxide and glycerol protect against decrease in respiration and oxidative phosphorylation in isolated rat liver and plant mitochondria when stored below freezing temperatures[1–5]. No studies have appeared on retention of transport properties in stored mitochondria. In recent years, an increasing amount of effort has been devoted to measuring the fluxes of substrates, particularly the tricarboxylic acid cycle intermediates, across the rat liver mitochondrial membrane[6,7]. Adrenal cortical mitochondria present and added complexity because these mitochondria, besides having quite different morphological fine structure[8], contain a second electron transport chain responsible for steroid hydroxylation reactions in addition to the normal respiratory chain[9–11]. Recently, we have shown that isolated beef adrenal cortical mitochondria are capable of transporting di- and tricarboxylic acids and that they possess distinctive transport properties not associated with other mammalian mitochondria[12,13].

In this communication we wish to report that we have been able to store beef adrenal cortical mitochondria for several days −20 °C without substantial loss of tri- and dicarboxylate transport activity. In addition the preparation of mitochondria retains some controlled respiratory activity. To the best of our knowledge this is the first report of citrate transport activity in adrenal cortical mitochondria and that transport activity can be fully retained in stored mammalian mitochondria. The details of the different anion translocations in bovine adrenal cortical mitochondria will be described in a subsequent publication.

METHODS

Beef adrenals, obtained from a local slaughter house, were cleaned of adhering fat, bissected and the adrenal medulla cut away cleanly with sharp scissors. The cortex was scraped from the capsule with a spatula and homogenized in the following manner.

The minced adrenal cortex was suspended in a medium containing 0.25 M sucrose, 5 mM $MgCl_2$ and 5 mM Tris–HCl, pH 7.4, and homogenized for 20 s with a Polytron homogenizer (Kinematica G.M.B.H., Lucerne, Switzerland) with the resister setting at 3. The homogenate was subjected to differential centrifugation as described by Crammer and Estabrook[14].

Mitochondria were incubated at 20 °C for 10 min with labelled metabolites in a medium containing 80 mM NaCl, 80 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris–HCl, at 7.4, in the presence of 1 μg/ml rotenone and/or 1 μg/ml antimycin A, final volume being 1.5 ml. Both rotenone and antimycin A were added as ethanolic solutions. The experiments were started by adding mitochondria (approx. 5 mg mitochondrial protein per ml incubation medium) and terminated by filtration under suction, using glass fiber filters (grade 934 AH Reeve Angel, N.J.). The filtration was followed by a wash with 10 ml of ice-cold incubation medium. Radioactivity retained on the filters was determined by liquid scintillation counting. Protein was determined by the method of Lowry *et al.*[15] on the original mitochondrial suspension. Matrix space was determined routinely with ^3H_2O and [^{14}C]sucrose[16].

Measurements of respiration[17] were carried out polarographically in a medium described by Crammer and Estabrook[14], using a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) with a closed reaction vessel of 3.0-ml volume.

Swelling experiments were conducted according to the procedure of Chappell[7].

To determine whether the added carboxylic acid is converted to other substances, the carboxylates were extracted from the mitochondria with 60% $HClO_4$ and neutralized with concentrated KOH at 0 °C. The clear supernatant of the neutralized extract was applied to silica gel G 254 thin-layer chromatographic plates and separated in a solvent system consisting of diethyl ether–formic acid–water (18:5:9, v/v/v)[18], or chromatographed on Whatman No. 1 filter paper with *tert*-butanol–formic acid–water (76:5:19, v/v/v)[19]. All radioactive materials which were taken up by the mitochondria migrated as discrete spots in both systems and were identified by cochromatography with authentic standards.

All radioisotopes except malate were purchased from New England Nuclear Corp., Boston, Mass. Rotenone was obtained from Sigma, St. Louis, Mo., and antimycin A was purchased from Schwarz/Mann, Orangeburg, N.Y. All other chemicals used were of reagent grade. ^{14}C-Malate was purchased from Amersham/Searle, Arlington Heights, Ill., U.S.A.

The following activities were measured: (1) oxygen utilization, including respiratory-control ratio; (2) citrate transport; (3) malate and succinate transport.

RESULTS

The data in Table I show that after storage in 1% albumin and 15% dimethyl sulfoxide, at −20 °C for 24 h, beef adrenal mitochondria retain some coupled respi-

TABLE I

STABILITY OF THE CITRATE TRANSPORT SYSTEM IN BOVINE ADRENAL CORTICAL MITOCHONDRIA UPON STORAGE IN VARIOUS MEDIA AT −20 °C

The citrate accumulation values represent the means ± S.D. of 4 experiments. Details as described in the text.

Storage medium and time stored (days)	*Citrate accumulation in matrix space (mM)*		*Respiratory control ratio with succinate as substrate*
	− Succinate	*+ Succinate (1 mM)*	
(I) Buffered sucrose			
0	0.6 ± 0.1	4.0 ± 0.3	2.4
1	0.5 ± 0.05	1.0 ± 0.1	1.1
(II) + bovine serum albumin (1%)			
0	0.7 ± 0.1	4.0 ± 0.2	2.3
1	0.6 ± 0.1	2.0 ± 0.1	1.6
(III) + dimethyl sulfoxide (15%)			
0	0.7 ± 0.1	3.8 ± 0.4	2.5
1	0.7 ± 0.1	1.5 ± 0.2	2.0
(IV) + bovine serum albumin (1%) + dimethyl sulfoxide (15%)			
0	0.5 ± 0.1	3.6 ± 0.4	2.4
1	0.6 ± 0.1	3.7 ± 0.3	1.9
2	0.6 ± 0.1	3.6 ± 0.3	1.7
7	0.6 ± 0.1	3.5 ± 0.4	1.5

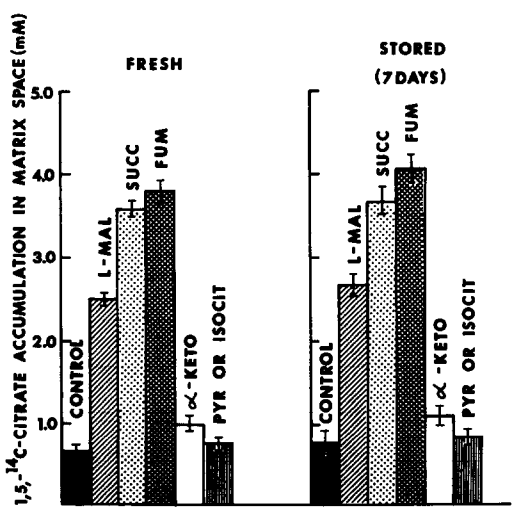


Fig. 1. Citrate accumulation in fresh and stored mitochondria. The experimental details are given in the text. The values given are means of four experiments. The vertical bars represent the S.D. The concentrations of all carboxylic acids was 1 mM. Succ, succinate; fum, fumarate; α-keto, α-ketoglutarate; pyr, pyruvate; isocit, isocitrate.

ratory activity. It should be noted that even with our best and freshest preparations, the respiratory control is 2.4 which is considerably less than the value obtained with other mammalian mitochondria. These data are consistent with previous observations on the low respiratory control ratio of adrenal cortical mitochondria[14] and may reflect the presence of two discrete electron transport chains.

More striking, however, is the fact that dicarboxylate-stimulated citrate uptake is maintained after seven days storage without any significant decrease in activity (Fig. 1 and Table I). The only consistent difference in behaviour that we have observed between fresh and 7-day stored mitochondria is that the matrix space (the space impenetrable by sucrose) increased by about 10%upon storage, suggesting that there may be some changes in permeability. This conclusion was made earlier concerning plant and rat liver mitochondria stored at −18 °C and −15 °C, respectively[1,4]. That transport of other anions is also maintained upon storage is shown in Table II with malate and succinate whose uptake is unimpaired after 48 h at −20 °C.

TABLE II

STABILITY OF THE SUCCINATE AND MALATE TRANSPORT SYSTEM IN BOVINE CORTICAL MITOCHONDRIA UPON STORAGE AT −20 °C

*Time stored** (*days*)	*Accumulation in matrix space (mM)***	
	Succinate	*Malate*
0	5.7 ± 0.3	5.0 ± 0.4
2	5.4 ± 0.4	5.1 ± 0.3

* Mitochondria were stored in a medium containing 15% dimethyl sulfoxide, 1% bovine serum albumin and buffered sucrose. Succinate and malate added at 1 mM.

** The dicarboxylate accumulation values represent the means ± S.D. of 4 experiments.

It may be noted that with adrenal cortical mitochondria, succinate stimulates the uptake of citrate to a greater extent than does malate (Fig. 1). Indeed our data show that fumarate and succinate are equally effective and stimulate uptake by nearly 50% more than does malate at equivalent medium concentrations. This result is in direct contrast to the data with rat liver mitochondria where it is believed that malate is the primary dicarboxylate exchanger with the citrate carrier[6,7]. Our data show that at concentrations from 0.5 to 3 mM succinate and fumarate stimulate citrate uptake by nearly 50% more than does L-malate at any equivalent concentration in that range. (Concentrations of succinate greater than 5 mM are inhibitory.) Since these experiments were conducted in the presence of rotenone and antimycin A at a concentration sufficient to block over 90% of the respiratory activity[20], it is unlikely that the succinate could have been converted to malate to bring about the increased uptake.

Since these data are different from those anticipated with rat liver mitochondria, we carried out swelling experiments in isotonic ammonium citrate, malate and succinate with mitochondria from the two sources to verify the difference in behaviour.

The results in Fig. 2 show that in adrenal cortical mitochondria there is no swelling in ammonium citrate unless a small amount of succinate (malate or fumarate) is added. As before, rotenone and antimycin A were present. Addition of P_i was not

required. Preliminary results have shown that absence of a requirement for added P_i may be due to the presence of sufficient intramitochondrial P_i. Measurement of P_i content after incubation in presence and absence of malate shows that the dicarboxylate causes a decrease in mitochondrial P_i. (Preliminary observations have also shown that these mitochondria swell in ammonium phosphate indicating the presence of a phosphate translocator.)

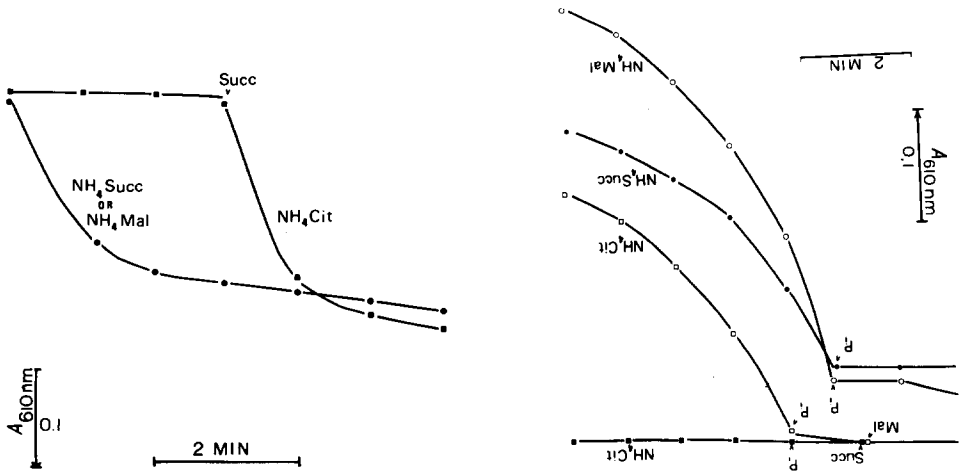


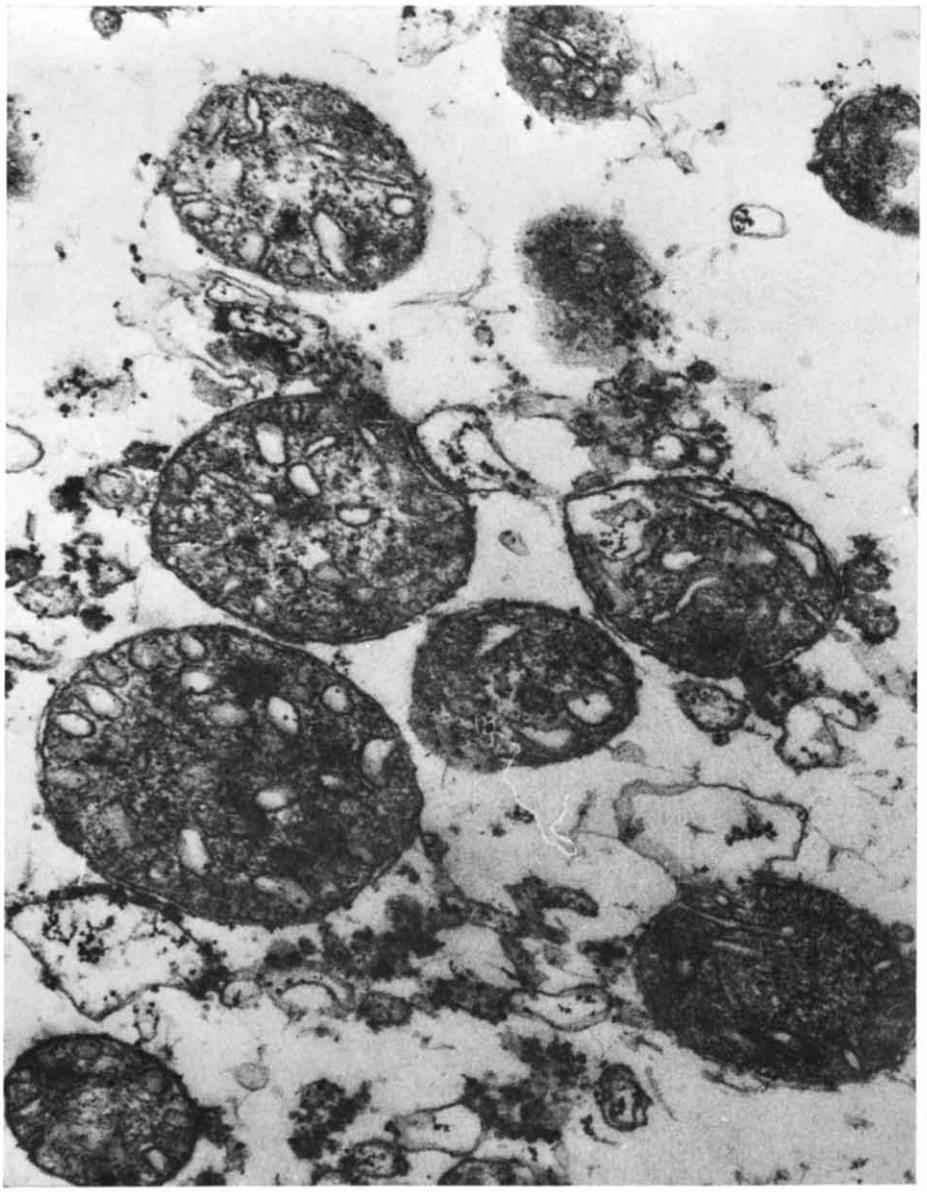
Fig. 2. The swelling of isolated bovine adrenal cortical mitochondria suspended in 75 mM ammonium citrate or 100 mM ammonium succinate. Mitochondria (0.1 mg of protein) were suspended at 20 °C in 1 ml of the ammonium salt, containing 5 mM Tris–HCl (pH 7.4). Swelling was monitored at 610 nm using a Beckman DB spectrophotometer. In all cases, respiration was blocked with rotenone (1.0 μg/ml) and antimycin A (1.0 μg/ml). Where indicated succinate was added at a final concentration of 2 mM. ■—■, ammonium citrate + 2 mM succinate; ●—●, ammonium succinate or malate. Similar results were obtained with addition of malate or fumarate (data not shown) to ammonium citrate.

Fig. 3. Swelling of isolated rat liver mitochondria in ammonium citrate (75 mM), malate (100 mM) or succinate (100 mM). Conditions as for Fig. 2. At the points indicated malate, succinate or phosphate were each added at a final concentration of 2 mM. ■—■, ammonium citrate + succinate (2 mM); □—□, ammonium citrate + malate (2 mM); ●—●, ammonium succinate; ○—○, ammonium malate.

In contrast, in rat liver mitochondria (Fig. 3), malate, but not succinate, induced swelling with ammonium citrate and addition of P_i was required to obtain swelling in either ammonium citrate + malate or ammonium malate. The results are consistent with those in Fig. 1 and indicate that the transport properties of adrenal cortical mitochondria are different from those of rat liver.

The results of the swelling experiments and the stimulated uptake of citrate with fumarate suggest that fumarate also may be transported by adrenal cortical mitochondria. However, the possibility that some of the action of fumarate may be due to conversion to malate through contaminating fumarase is not eliminated.

The data of Chappell[7] indicated that malate was specific amongst the dicarboxylic acids in stimulating an exchange with citrate in rat liver mitochondria. Ferguson and Williams[24] showed some years ago that a number of structural analogues of



malate would stimulate citrate uptake. The effect of succinate was ascribed, at least in part, to its conversion to malate. It has recently been reported that other dicarboxylates including succinate will stimulate exchange with citrate in rat liver mitochondria[21] and in beef heart mitochondria[22]. The data of Kleineke *et al.*[21] suggest that the specificity for the dicarboxylate in liver is rather low, with malate being best exchanged.

Although some biochemical properties of the mitochondria appear to be retained upon storage, the actual appearance of the mitochondria under the electron micros-

cope is different from that of a fresh preparation (Fig. 4). First, the dense matrix appears to disappear. Second, the cristae are less evident and third, the external mem-

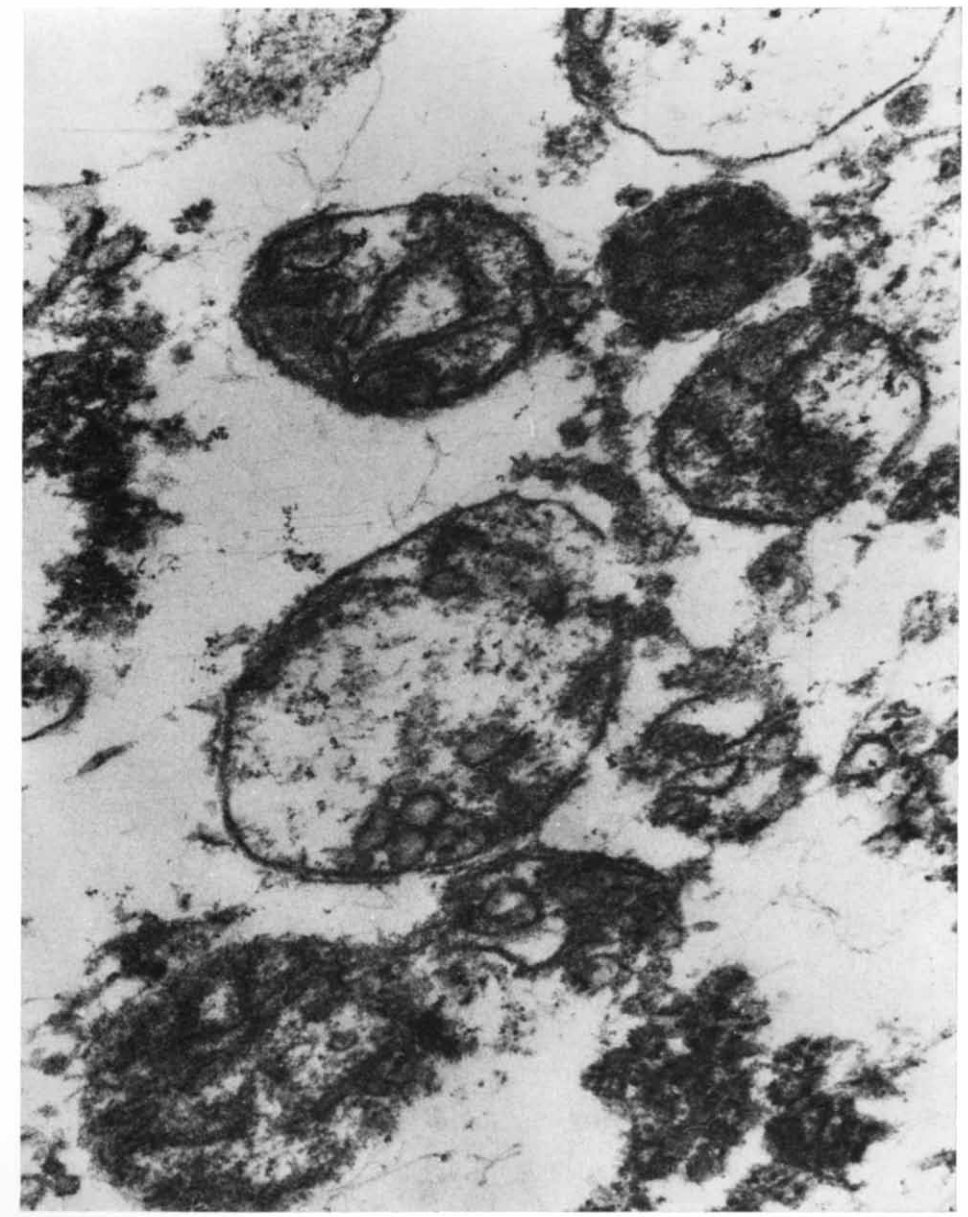


Fig. 4. Electron microscope micrographs of fresh and stored bovine adrenal mitochondria. The mitochondria were fixed in 2% glutaraldehyde in phosphate buffer, pH 7.4, isotonic with our incubation medium and then treated with 1% OsO_4. Lead citrate was used as the contrast agent. A, freshly isolated mitochondria; B, mitochondria stored for 7 days before fixation. Magnification approx. 90000×.

brane appears discontinuous in many areas. We do not know whether these changes in appearance are reversible.

Despite the histological alterations, the functional activity of the anion transport system is maintained. It would appear that the outer membrane does not contribute greatly to the regulation of anion transport activity in these mitochondria. This possibility is currently under investigation.

Thus our findings suggest that adrenal cortical mitochondria contain functional di- and tricarboxylate carriers and that the tricarboxylate carrier shows different selectivity than rat liver mitochondria for dicarboxylic acids.

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