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Post-mortem Changes in Structure and Function of Ox Muscle Mitochondria. 1. Electron Microscopic and Polarographic Investigations

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Abstract

Electron microscopy shows that intact mitochondria can be isolated from neck-muscle stored at 144 h post-mortem at 4°. Isolated mitochondria, all in the condensed configuration, have clearly defined outer and inner membranes, outer compartments and intracristal spaces; a larger proportion of swollen ones was isolated from the 144 h than from the 120 h post-mortem tissue.

Mitochondria from 96 h tissue still retained the following % of the initial values for the ADP/O ratio, respiratory control index (RCI) and state 3 respiratory rate observed for 0·5 h tissue: malate + pyruvate, 100, 72 and 53; succinate, 80, 30 and 74; ascorbate + tetramethyl-*p*-phenylenediamine (TMPD), 92, 88 and 72.

Both the succinate and ascorbate-TMPD oxidase systems appear to have a "critical" storage time of about 70 h, whereas the malate + pyruvate system has one of about 96 h. A sharp decline of the ADP/O ratio, RCI and the state 3 respiratory rate occurred after this time, but these three parameters were better preserved in the ascorbate-TMPD oxidase system.

The oxidation of the citric acid cycle intermediates in the neck-muscle mitochondria thus shows a higher sensitivity to post-mortem ageing with respect to cytochrome oxidase activity. This is probably due to post-mortem muscle acidification.

Introduction

Mitochondria are normally isolated from tissues immediately after slaughter of the animals, although there are no data available to substantiate the necessity for such rapid isolation. Reports of preservation of some of the functions of stored and aged mitochondria from rat-liver have been made by a few groups of investigators. Greiff and Meyers[1] found that about 64 and 69% of the initial value of ADP/O ratio was retained in freeze-dried and frozen mitochondria. Walton *et al.*[2] stored mitochondria at −196° for 60 days with 40% loss in respiratory control. Carafoli and Gazzotti[3] found that most of the energy-linked functions were lost after 2 to 4 days with mitochondria aged at 2°, but the ATP-induced mitochondrial contraction was still preserved after 10 to 14 days. These authors[3] also observed that ADP no longer stimulated succinate oxidation after 2 days of ageing, with the RCI decreasing by about 50% after 1 day.

Ozawa *et al.*[4] working with mitochondria from ischemic livers, observed a 40% decline in phosphorylation rates during 1 h of ischemia at 22°. This was suggested to be due to mitochondrial damage partly caused by free fatty acids released during lipolysis of microsomal lipids,[5] resulting in a complete loss of rat liver RCI observed during 4 h of ischemia[6] at 24°. These authors[6] also reported that approximately 50% of the RCI was preserved by bovine serum albumin (BSA) with mitochondria isolated from rat liver ischemic up to 13 h at 24° with no decline in the ADP/O ratio. However, at 37° even in the presence of BSA complete loss of respiratory control and ADP/O ratio was observed from 1½ h of ischemia.[6]

This paper reports some of the biochemical changes in the mitochondria isolated from ox neck-muscle (*M. sternomandibularis*) following storage, using electron microscopic and polarographic techniques.

Materials and Methods

Chemicals

Antimycin A (Type III), oligomycin and the sodium salts of ADP, ATP, succinate and pyruvate were obtained from Sigma; sodium salts of L-ascorbate and EDTA, and TMPD from British Drug Houses; all other reagents were of analytical grade. *p*-Trifluoromethoxy-carbonylcyanide-phenylhydrazone (FCCP) was a gift from Dr. P. Heytler.

Methods

Isolation of mitochondria. Mitochondria from ox neck-muscle, both fresh and stored at 4° for various time intervals, were isolated as previously described for other skeletal muscle.[7] The mitochondrial-containing 7000 × *g* pellet, free from the top pale "fluffy" layer, was then washed three times before use.

Storage of tissue. The neck-muscle, free of fatty tissue, was kept in a polythene bag at 4°. For post-mortem studies at different time intervals, about 25 *g* tissue was used. No bacterial growth was visible up to 144 h storage under these conditions, but any possible surface contamination was avoided by removing the external 2–3 mm of tissue.

Electron microscopy. Thin sections of the various mitochondrial preparations were examined. The mitochondria were fixed in 2% glutaraldehyde and treated with 2% osmium tetroxide for 1 h prior to dehydration with ethanol followed by propylene oxide, before being embedded in Epon 812.[8] The sections were cut with a glass knife and stained with uranyl acetate and lead citrate before examination with an AEI (Model EM6-B) electron microscope.

Biochemical analyses. Oxygen uptake was measured polarographically with a Clark oxygen electrode at 25°. The ADP/O ratio and RCI were calculated from the electrode traces as described by Chance and Williams.[9] Protein was determined by Folin-phenol reagent[10] with BSA as standard.

Results

Figure 1 clearly illustrates the structure of mitochondria which were obtained from the neck-muscle before (A) and after storage at 4° for various time intervals (B–D). Except

Figure 1. Thin sections showing the ultrastructure of isolated ox neck-muscle mitochondria. Mitochondria were isolated at 0·5 (A), 96 (B), 120 (C) and 144 (D) h post-mortem from fresh (A) and stored (B–D) tissue at 4°. With the exception of a few swollen (S) mitochondria (C–D), all the isolated mitochondria are in the condensed configuration, having clearly defined outer (OM) and inner (IM) membranes, outer compartment (OC) and intracristal spaces (IS). Magnification: ×20,000. Electron micrographs were carried out in collaboration with Mr. C. A. Voyle.

TABLE I. pH of the ox neck-muscle at various time intervals post-mortem

Hours post-mortem	pH of tissue homogenate
0·5	6·85
24	6·20
48	5·80
70	5·75
96	5·75
120	5·75
144	5·70

The pH of the neck-muscle was determined by homogenizing 1 g of tissue in 10 ml of 150 mM KCl-10 mM iodoacetate (pH 7·0). The data represents an average value from two experiments. The 24 to 144 h tissue was stored in a polythene bag at 4°.

for a small proportion of swollen ones, all the isolated mitochondria were in the condensed configuration.[11] At 144 h post-mortem (D), there appeared to be rather more swollen isolated mitochondria than at 120 h (C). Practically all the mitochondria obtained from 0·5 h post-mortem were intact, with very clear definition of outer and inner membranes, outer compartments and intracristal spaces. Similar features were also observed with the intact mitochondria isolated from tissues stored up to 144 h. In contrast to the isolated

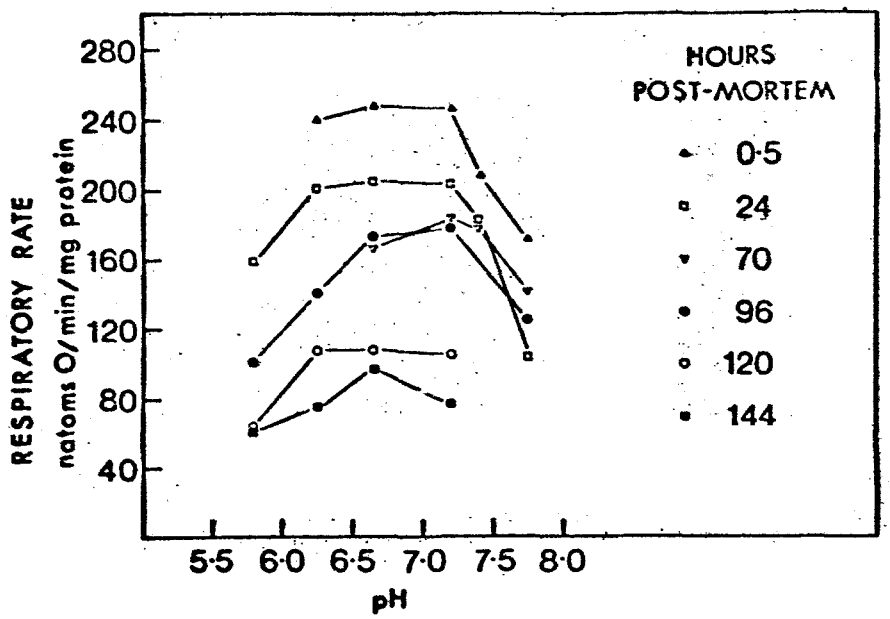

Figure 2. Effect of pH on the state 3 respiratory rate for malate + pyruvate oxidation of the ox neck-muscle mitochondria isolated at various hours post-mortem. All the state 3 respiratory rates were measured polarographically with a Clark oxygen electrode in 2·5 ml at 25°. The sequence of addition, all referring to final concentrations, was: 8 mM malate, 8 mM pyruvate and 400 μM ADP. The data was an average value of at least 3 separate state 3 respiratory rates induced by ADP addition. Reaction medium (mM): EDTA, 1·0; KCl, 30·0; $MgCl_2$, 6·0; sucrose, 75·0 and KH_2PO_4, 20·0. Total protein 1·2 mg.

ones, all the mitochondria *in situ* were in the orthodox configuration,[11] and also a large proportion of intact mitochondria was observed even in the 144 h post-mortem tissue (unpublished data).

Effect of pH on Mitochondrial Function

Table I shows the relationship between pH of the tissue and storage time at 4° (post-mortem). The fall in pH of the tissue is due to lactic acid formation.[12] Mitochondria

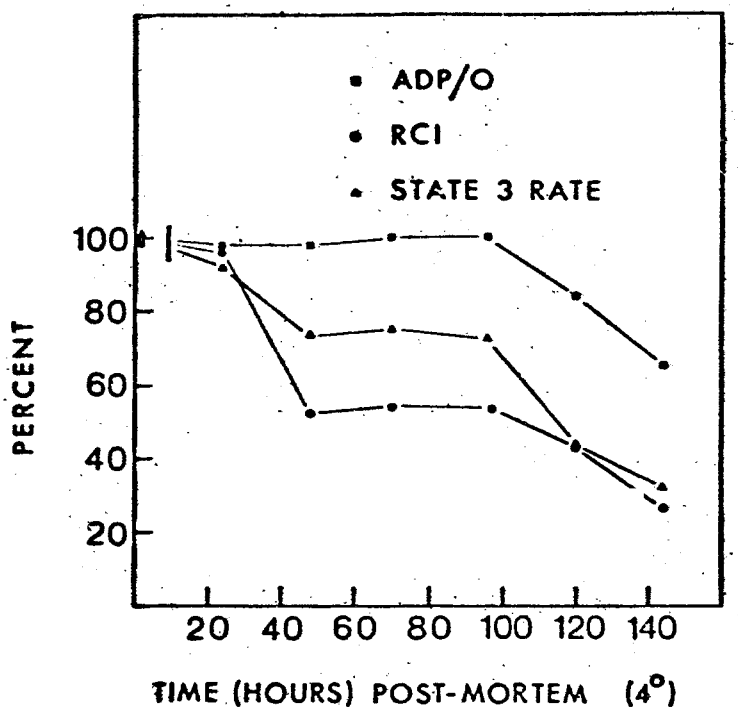

Figure 3. Post-mortem changes in the mitochondrial oxidation of malate + pyruvate. Experimental details as described in Fig. 2. The ADP/O ratio, RCI and the state 3 respiratory rate were expressed as a percentage of those values observed for mitochondria isolated from fresh muscle at 0·5 h post-mortem. The 100% values of these three parameters for the mitochondria from 0·5 h tissue are: ADP/O ratio, 2·77; RCI, 11·39; state 3 respiratory rate, 246·0 natoms O per min per mg protein at 25° at pH 7·2. , data after this indicates parameter values observed for mitochondria isolated from muscle previously stored at 4°.

showing a capacity for oxidative phosphorylation could still be isolated by the above method, as long as the pH in the tissue was not lower than 5·50 (unpublished data).

Figure 2 illustrates the pH effect on the state 3 respiratory rate induced by malate + pyruvate using intact mitochondria isolated from neck-muscle, stored at 4° for various time intervals post-mortem. The malate + pyruvate oxidation had a broad pH optimum between 6·6 and 7·2 with mitochondria isolated from 0·5 and 24 h tissue. The rather pronounced optimum pH of 7·2 observed with mitochondria from 70 and 96 h post-mortem shifted to about pH 6·6 with the 144 h tissue. The succinoxidase activity, on the

other hand, had a clear cut optimum at about 7·3 up to 70 h. With both systems, the state 3 respiratory rate decreased post-mortem.

The effect of storage on the ADP/O ratio, RCI and state 3 respiratory rate is clearly shown in Figs. 3–5. The ADP/O ratio of malate + pyruvate oxidation (Fig. 3) remained constant up to 96 h post-mortem, while a decline in the RCI and the state 3 respiratory rate were observed as follows: RCI, 4% (24 h) and 48% (48 to 96 h); state 3 respiratory rate, 8% (24 h) and 27% (48 to 96 h). The "critical" storage time appears to be about 96 h. All the three parameters started to decline rather sharply after this period, ultimately

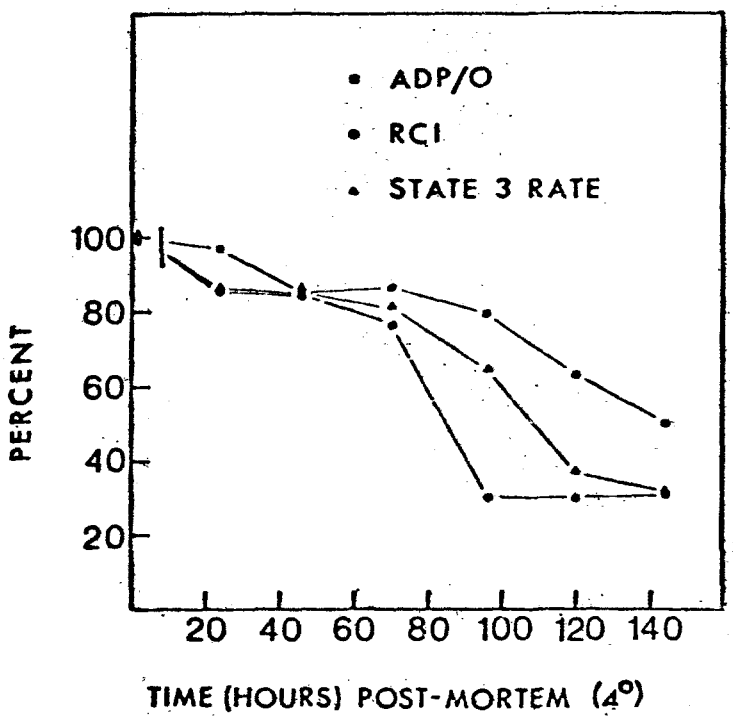

Figure 4. Post-mortem changes on the succinoxidase activity of the ox neck-muscle mitochondria. All the experimental details are described in Figs. 2 and 3 except that the 100% values of the three parameters are as follows: ADP/O ratio, 1·72; RCI, 5·83; state 3 rate, 301·2 natoms of O per min per mg protein at pH 7·3. The sequence of addition, all referring to final concentrations, was 2 μM rotenone, 8 mM succinate and 300 μM ADP.

reaching values of about 65% for the ADP/O ratio, 32% for the state 3 respiratory rate and 27% for the RCI, of the original values for mitochondria from 0·5 h tissue.

The succinoxidase system (Fig. 4) appears to have a "critical" storage time of about 70 h. The ADP/O ratio, RCI and the state 3 respiratory rate decreased quite markedly after this time, showing the following % retention: ADP/O ratio, from 86% (70 h) to 50% (144 h); RCI, 77% (70 h) to 31% (144 h); state 3 respiratory rate, 82% (70 h) to 32% (144 h).

The ascorbate-TMPD oxidase activity (Fig. 5), like the succinoxidase system, started to decline after 70 h. In contrast to the succinoxidase activity, the % decline in the three parameters was much smaller. The mitochondria isolated from the 144 h tissue still

retained 77, 85 and 65% of the values originally observed for the ADP/O ratio, RCI and state 3 respiratory rate of mitochondria isolated from 0·5 h post-mortem tissue.

pH had an irreversible inhibitory effect on the respiratory activity of mitochondria obtained from heart and liver of rats.[13] This observation is supplemented by the present data on the effect of storage on mitochondrial function. Mitochondria isolated from 144 h tissue had already been subjected to a pH 5·8 environment for about 96 h *in situ*. The effect of pH in this range appears to be irreversible with the NAD^+-linked substrate,

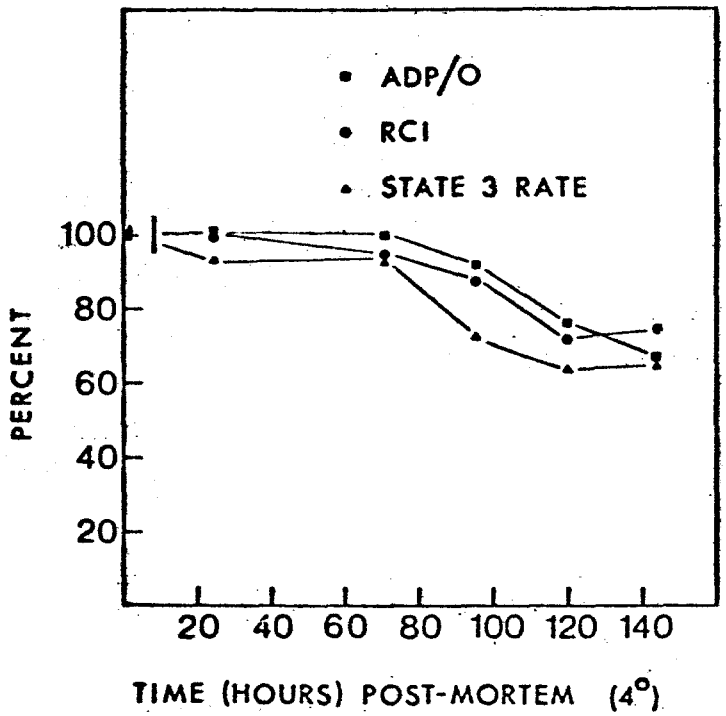

Figure 5. Post-mortem changes on the cytochrome oxidase activity of the ox neck-muscle mitochondria. The sequence of addition, referring to final concentration, in assaying the cytochrome oxidase activity are: antimycin A, 0·1 μg per mg protein; 4 mM ascorbate, 0·2 mM TMPD and 200 μM ADP. The 100% values of the three parameters are: ADP/O ratio, 1·01; RCI, 1·80; state 3 rate, 240·1 natoms O per min per mg protein at pH 7·2. All other experimental details are given in Figs. 2 and 3.

malate + pyruvate oxidation but not with ascorbate + TMPD. At pH 7·2, the following data were obtained: malate + pyruvate, ADP/O ratio, 1·80; RCI, 3·0; ascorbate + TMPD, ADP/O ratio, 0·8; RCI, 1·4. These mitochondria failed to exhibit the classical state 3 to state 4 transition[9] with malate + pyruvate when suspended in a reaction medium of either pH 7·5 or 7·9.

Discussion

Intact mitochondria were still preserved after 144 h storage in the intact tissue at 4° even though they were being subjected to an anaerobic environment and a declining

pH, from 7·0 to 5·70. Isolated mitochondria still retained their capacity for oxidative phosphorylation, showing an acceptable RCI even after storage of 144 h *in situ*. Only the state 3 respiratory rate was markedly affected by storage up to 144 h, decreasing to about 32% for the oxidation of malate + pyruvate and succinate. This decrease was responsible for the corresponding fall in the RCI. However, storage up to 96 h of the ox neck-muscle still did not cause any great change in the three parameters tested with respect to the mitochondrial oxidation of malate + pyruvate, succinate and ascorbate + TMPD. The cytochrome oxidase activity (EC 1.9.3.1) appears to be more stable to storage. Mitochondria isolated from 144 h tissue still preserved 77, 85 and 65% of the originally observed values for the ADP/O ratio, RCI and state 3 respiratory rate with ascorbate + TMPD as substrate. It is tempting to conclude that substrate oxidation in mitochondria involving a dehydrogenase is more sensitive to storage *in situ* as both the state 3 respiratory rate induced by malate + pyruvate and succinate shows a higher % decline than the oxidation of ascorbate + TMPD used for assaying the cytochrome oxidase activity. Ascorbate + TMPD donates electrons directly to the *c*-type cytochrome of the mammalian respiratory chain system.[14]

The success of the present work in isolating functional and intact mitochondria from tissue stored up to 144 h post-mortem is mainly due to the slow fall of pH in ox neck-muscle. As long as the pH of the tissue is maintained at or above 5·5, and the temperature between 2 to 4°, mitochondria capable of oxidative phosphorylation could still be isolated (unpublished data). This observation favours the concept that rapid mitochondria isolation from animal tissue is unnecessary as long as the relationship between the rate of pH fall and the storage time, and the "critical" time of storage for specific substrate oxidation are known. The rather sharp decline in the state 3 respiratory rate after the "critical" storage time, especially with the oxidation of malate + pyruvate and succinate could be due to the falling tissue pH, prolonged storage or the combined effects of pH and the time of storage.

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