EFFECT OF LOSS OF CYTOCHROME *c* FOLLOWING STORAGE OF MITOCHONDRIA *IN SITU*

K.S. CHEAH

Agricultural Research Council, Meat Research Institute
Langford, Bristol, U.K.

Received 1 October 1971

1. Introduction

Recent investigation [1] of the post-mortem changes in the structure and function of ox-neck mitochondria indicates that intact mitochondria were still preserved *in situ* after 144 hr storage at 4°. Mitochondria isolated from this muscle at various time intervals post-mortem showed a decline in state 3 respiration but these mitochondria still retained their capacity for oxidative phosphorylation, showing an acceptable respiratory control index. Furthermore, a relationship between the rate of the fall in muscle pH and the time of storage, and the "critical" storage time for the oxidation of succinate, pyruvate *plus* malate and ascorbate *plus* tetramethyl-p-phenylenediamine (TMPD) was established. The decline in the state 3 respiration observed in the ox-neck muscle mitochondria following storage *in situ* further substantiates the general phenomenon reported for the decrease in respiration for aged mitochondria [2] and mitochondria isolated from ischemic livers [3–4].

This communication reports the loss of mitochondrial cytochrome *c* from mitochondria isolated from skeletal muscles after storage at 1° for various time intervals post-mortem and the successful re-establishment of the state 3 respiration in these cytochrome *c*-deficient mitochondria by re-addition of cytochrome *c* and ADP.

2. Materials and methods

Antimycin A, horse heart cytochrome *c* (Type III), rotenone and the sodium salts of ADP and succinate were obtained from Sigma; sodium ascorbate and TMPD-2HCl from British Drug Houses; crystalline *Bacillus subtilis* proteinase (Nagarse) from Teikoku Chemical Co., Osaka; all other reagents were of AnalaR grade.

Mitochondria from the ox-neck and the Pietrain pig back-muscles were isolated as previously described [5] using crystalline *B. subtilis* proteinase. For post-mortem investigations at various time intervals 20–25 g of muscle stored at 1° in a polythene bag was used at a time. Oxygen uptake was determined polarographically with a Clark oxygen electrode at 25°. The cytochromes were detected with an Amin-co-Chance Dual-wavelength/Split-beam spectrophotometer at liquid nitrogen temperature (−196°) using 2.0 mm light-path cells. Concentration of cytochrome *c* ($A_{548\,nm} - A_{540\,nm}$) was estimated from difference spectra (dithionite-reduced *minus* ferricyanide-oxidized) at −196° using the millimolar extinction coefficient of 19.0 [6], and an enhancement factor of 8. The latter represents an average value from 3 separate determinations calculated from difference spectra recorded at room temp and at −196°. Protein was determined by Folin-phenol reagent [7] with bovine serum albumin as standard.

3. Results and discussion

Fig. 1 illustrates the decline of the state 3 respiratory rates for the oxidation of pyruvate *plus* malate (■), ascorbate *plus* TMPD (●) and succinate (▲) by

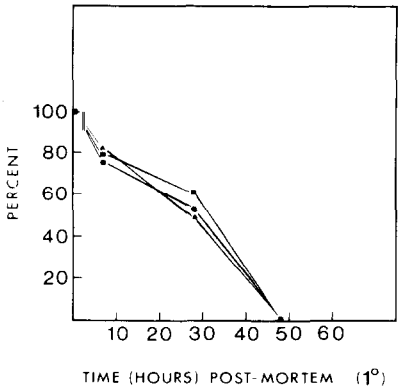

Fig. 1. Post-mortem changes in the state 3 respiration of the Pietrain pig back-muscle. All the state 3 rates were measured polarographically in 2.5 ml at 25°. The data was an average of 3 separate experiments. The sequence of addition, all referring to final concentrations for the three separate oxidase systems was: 8 mM malate, 8 mM pyruvate and 400 μM ADP for the oxidation of pyruvate *plus* malate (■); 4 mM ascorbate, 0.1 μg antimycin A per mg protein, 0.1 mM TMPD and 200 μM ADP for the ascorbate-TMPD oxidase system (●); 2 μM rotenone, 8 mM succinate and 300 μM ADP for the succinoxidase system (▲). Reaction medium (mM): EDTA, 1.0; KCl, 30.0; $MgCl_2$, 6.0; sucrose, 75.0 and KH_2PO_4, 20.0. Final pH 7.20. The 100% values refer to the state 3 rates observed for the mitochondria isolated from 20 min post-mortem tissue. ‖, data after this refers to rates observed for mitochondria obtained from muscle previously kept at 1°.

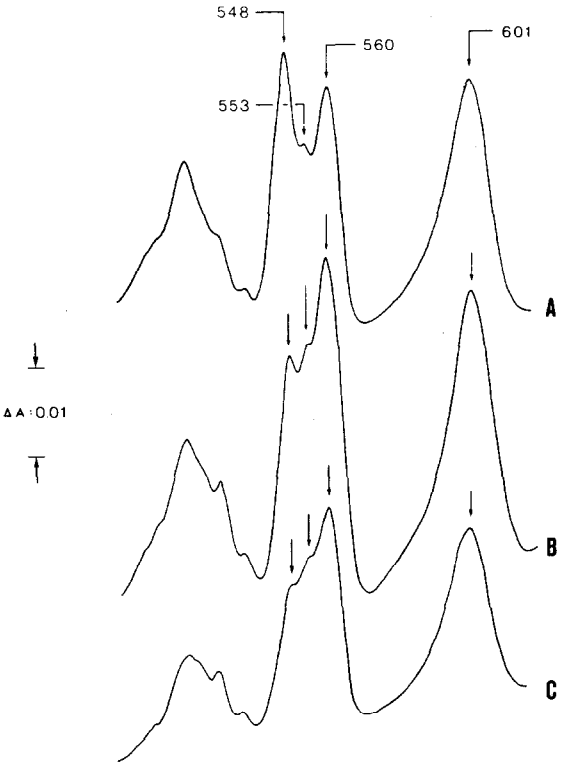

Fig. 2. Difference spectra (−196°) of mitochondria isolated from the Pietrain pig back-muscle at various hours post-mortem. The cytochromes were reduced with dithionite (sample cuvette) and oxidized with ferricyanide (reference cuvette).

A: mitochondria isolated from 20 min post-mortem tissue;
B: mitochondria isolated from 24 hr post-mortem tissue after storage at 1°;
C: mitochondria isolated from 48 hr post-mortem tissue after storage at 1°.

Difference spectra were recorded with the Aminco-Chance Dual-wavelength/Split-beam spectrophotometer in 2.0 mm light-path cuvettes using 0.15 mm slit. Speed of recording: 0.5 nm per sec. Protein concentration (mg/ml): A, 3.0; B, 4.2; C, 2.72.

the Pietrain pig back-muscle mitochondria following their storage *in situ* at 1°. The muscle pH was 6.45 at 10 min post-mortem. Following storage at 1°, the muscle pH decreased to 6.0 at 7 hr post-mortem, to pH 5.50 at 28 hr and subsequently to pH 5.45 at 48 hr. Mitochondria isolated from muscle with a pH of 5.45 failed to exhibit the classical state 3 to state 4 transition [8] for the oxidation of either pyruvate *plus* malate, succinate or ascorbate *plus* TMPD.

The difference spectra (−196°) of mitochondria (fig. 2) isolated from fresh muscle (A) and from muscle after storage at 24 (B) and 48 (C) hr post-mortem at 1° clearly shows a decline in cytochrome *c* (548 nm) concentration. No detectable loss in cytochromes aa_3 (601 nm), *b* (560 nm) and c_1 (553 nm) was observed. The average loss of cytochrome *c* estimated from three sets of experiments using three different pieces of muscle, was 33 and 47% at 24 and 48 hr, respectively, post-mortem at 1°, taking the content of mitochondrial cytochrome *c* isolated from 20 min post-mortem muscle as 100%. The pH of the muscle plays an important part in the decline of mitochondrial cytochrome *c*. The ox-neck muscle has a much slower rate of fall of tissue pH. This muscle has an ultimate pH of about 5.80 after 48 hr storage at 1° and mitochondria isolated from it, up to 48 hr post-mortem, showed no apparent loss in cytochrome *c*.

Table 1
Restoration of the state 3 respiration in cytochrome *c*-deficient mitochondria from the Pietrain pig back-muscle.

System	Oxygen uptake (%)	
	24 Hr post-mortem	48 Hr post-mortem
Succinate + ADP	28	22
Succinate + cytochrome *c*	53	44
Succinate + ADP + cytochrome *c*	80	82
Ascorbate + TMPD + ADP	39	21
Ascorbate + TMPD + cytochrome *c*	57	80
Ascorbate + TMPD + ADP + cytochrome *c*	100	100

All the respiratory rates were estimated as described in the legend to fig. 1. 3.0 nmole of horse heart cytochrome *c* was added. The 100% values refer to the state 3 rates observed for the mitochondria isolated from 20 min post-mortem muscle.

Fig. 3. Thin sections of mitochondria from the Pietrain pig back-muscle. The mitochondria were isolated at 20 min (A) and 24 hr post-mortem from fresh (A) and stored muscle (B) at 1°. The sections were stained with uranyl acetate and lead citrate before examination with an AEI (Model EM6-B) electron microscope. Magnification: × 20,000. Electron micrographs were carried out in collaboration with Mr. A. George.

The decline in cytochrome *c* content was not due to using damaged mitochondria. The electron micrographs (fig. 3) of mitochondria, isolated from fresh muscle (A) and from muscle kept at 24 hr post-mortem (B) show that the isolated mitochondria were intact. No marked difference was observed in the mitochondrial structure except that the preparation from the 24 hr post-mortem tissue tends to have a few slightly swollen mitochondria, a result similarly reported for mitochondria isolated from the ox-neck muscle after prolonged storage *in situ* [1].

Table 1 summarizes the data on the restoration of the state 3 rate for the oxidation of succinate and ascorbate *plus* TMPD with mitochondria stored *in situ* at 24 and 48 hr post-mortem at 1°. With

both substrates the addition of both cytochrome *c* and ADP is essential for obtaining high percentage restoration of the state 3 respiration. With mitochondria isolated from 48 hr post-mortem ADP could no longer induce a state 3 respiration (i.e. oxidative phosphorylation), a property affected by the ultimate pH of the tissue. As long as the pH is maintained at or above 5.50, mitochondria capable of carrying out oxidative phosphorylation can still be isolated.

This communication suggests that 47% of the mitochondrial cytochrome *c* in the Pietrain pig back-muscle is labile and is also essential for oxidative phosphorylation. An important aspect of the present work left unanswered is the manner in which cytochrome *c* is lost. This hemoprotein could possibly be transported out of the mitochondria by the same unsolved mechanism associated for transporting newly synthesized cytochrome *c* from the endoplasmic reticulum into the mitochondria [9, 10].

Acknowledgement

The author would like to thank Mrs. A.M. Cheah for expert technical assistance.

References

[1] K.S. Cheah and A.M. Cheah, J. Bioenergetics 2 (1971) 85.
[2] E. Carafoli and P. Gazzotti, Biochem. Biophys. Res. Commun. 39 (1970) 842.
[3] I. Boime, E.E. Smith and F.E. Hunter, Jr., Arch. Biochem. Biophys. 128 (1968) 704.
[4] I. Boime, E.E. Smith and F.E. Hunter, Jr., Arch. Biochem. Biophys. 139 (1970) 425.
[5] K.S. Cheah, FEBS Letters 10 (1970) 109.
[6] D.F. Wilson and D. Epel, Arch. Biochem. Biophys. 126 (1968) 83.
[7] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[8] B. Chance and G.R. Williams, in: Advances in Enzymology Vol. 17, ed. F.F. Nord (Intersciences Publischers, Inc., New York, 1956) p. 65.
[9] B. Kadenbach, Biochim. Biophys. Acta 138 (1967) 651.
[10] R. Penniall and N. Davidian, FEBS Letters 1 (1968) 38.