Journal of Bioenergetics and Biomembranes (1977), 9, 105–115

Inclusions in Aged Mitochondria

K.S. Cheah and A.M. Cheah

Agricultural Research Council,
Meat Research Institute, Langford,
Bristol, BS18 7DY, United Kingdom

Received 22 October 1975

Abstract

The formation of paracrystalline inclusions in mitochondria following postmortem aging in situ is further investigated. Inclusions are found to localize either in the mitochondrial intracristal space or between the inner and outer membrane. The greater majority of the inclusions are of the intracristal type. pH is not an important factor in the formation of the inclusions, since lowering the pH of the mitochondrial suspensions does not increase the number of mitochondria with inclusions. It is concluded that changes associated with aging during the prolonged preservation of the mitochondria both in situ and in in vitro experiments are probably the principal cause for the formation of the mitochondrial inclusions.

Introduction

Recent investigation showed that postmortem aging of skeletal muscle mitochondria in situ induced the formation of paracrystalline inclusions in the mitochondrial intracristal spaces [1, 2]. No explanation was given for the induction of the formation of these inclusions, although it was realized at that time that the increase in the number of mitochondria with inclusions paralleled the increase in the acidity of the muscle as a result of postmortem storage. Our initial observation [1] was followed by two reports [3, 4] describing similar types of mitochondrial inclusions, suggested to be due to the effect of the postmortem fall of pH [3]. The latest report of Saito et al. [4] suggests that the paracrystalline inclusions are junctions of two adjoining membranes which could be between the outer and inner membranes (designated as "outer–inner membrane junctions") or between two adjacent cristal

membranes (designated as "intracristal membrane junctions"). Lowering the pH increased the number of the outer–inner membrane junctions following aging of isolated mitochondria in vitro [4] but no intracristal membrane junctions were observed under these conditions.

Our previous data [1, 2] also suggest that the mitochondrial inclusions are not responsible for the gradual over-all decline in normal mitochondrial function in terms of oxidative phosphorylation and respiratory control index. This conclusion was also reached by Saito et al. [4].

This paper describes further studies on the formation of the paracrystalline inclusions in the mitochondrial intracristal spaces, referred to as "intracristal membrane junctions" [4], and shows that low pH is not an important factor for inducing the formation of these inclusions.

Materials and Methods

Reagents

Sodium salts of ADP, ATP, malate, pyruvate, and succinate and rotenone were obtained from Sigma; sodium salts of L-ascorbate and tetramethyl-*p*-phenylenediamine from the British Drug Houses. All other reagents were of analytical grade. Crystalline *Bacillus subtilis* proteinase (Nagarse) was purchased from Teikoku Chemical Co., Osaka.

Methods

Mitochondria from pig diaphragm, both fresh and stored at 1° C, were isolated using 20 g of muscle in the presence of *B. subtilis* proteinase [5]. Aging of mitochondria in situ was carried out by keeping the muscle in a polythene bag at 1° C.

Oxygen uptake was measured with the Clark oxygen electrode [Yellow Spring Biological Oxygen Monitor (model 53)] at 25° C in a total volume of 2.50 ml. The reaction medium (pH = 7.2) contained 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose, and 20 mM KH_2PO_4. The ADP/O ratio and the respiratory control index were calculated from the electrode traces as described by Chance and Williams [6]. Protein was estimated with the Folins–phenol reagent using bovine serum albumin as standard [7].

Electron microscopy was carried out as described by Allmann et al. [8] without using acrolein. Thin sections of diaphragm and also mitochondria isolated from this muscle at various time intervals postmortem were examined. The diaphragm was cut into approximately 0.5 mm cubical blocks and then fixed for 3 h at 0° C with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH = 7.4) containing 180 mM sucrose; it was post-fixed with 1.0% osmium tetroxide in the same buffer for 1 h. The samples were embedded in

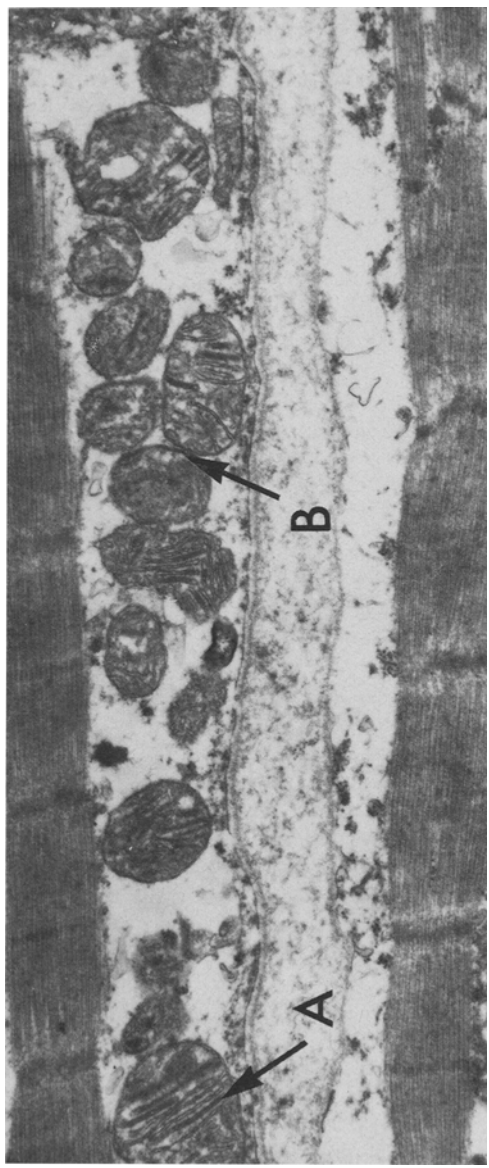


Figure 1. A thin section showing the formation of inclusions in mitochondria after aging in situ at 1°C for 3 days postmortem. Two types of inclusions were observed. A: Intracristal inclusion; B: outer-inner membrane inclusion. Most of the inclusions were of the intracristal type. Final magnification: 20,000x.

Epon 812. The thin sections were cut with a glass knife and stained with 2.0% uranyl acetate and lead citrate [9] before examination with an AEI (model EM6-B) electron microscope.

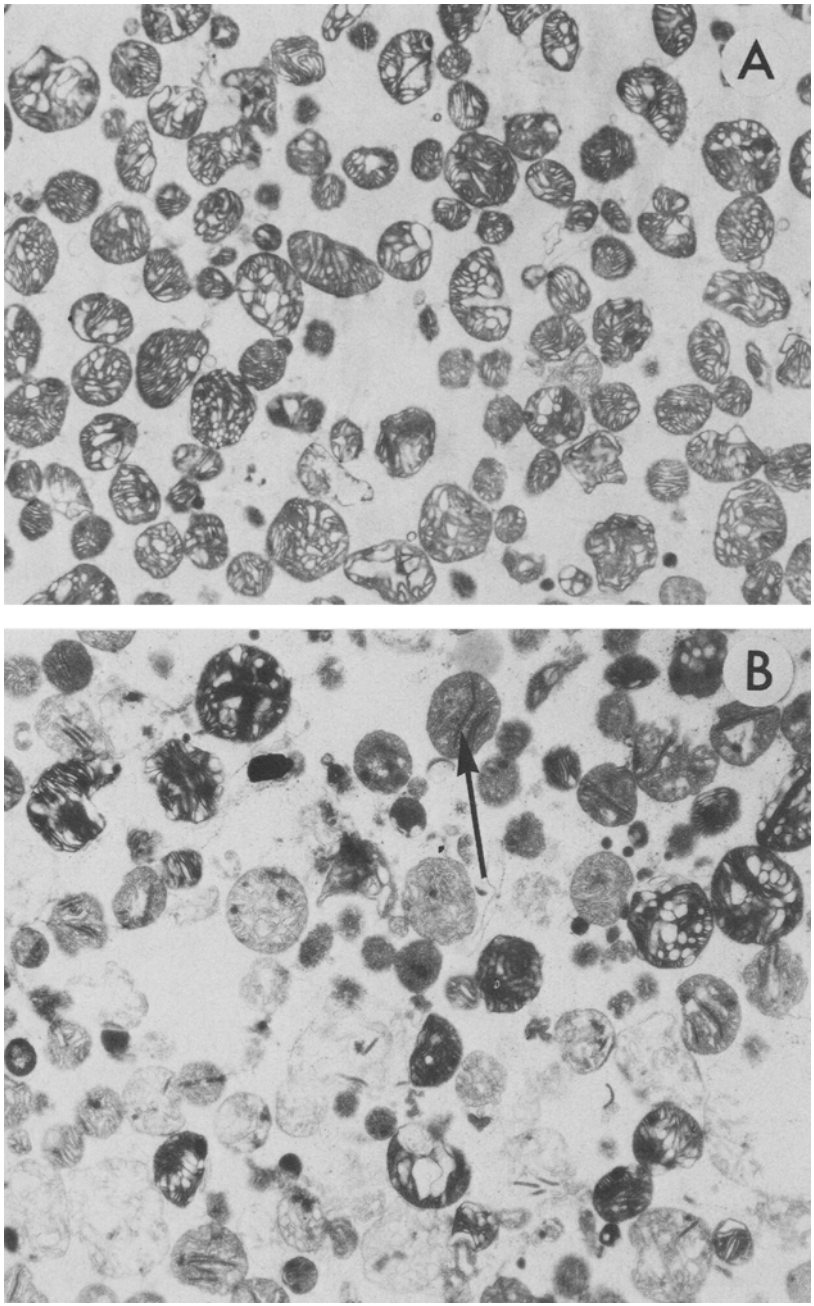
Induction of paracrystalline formation was carried out with freshly isolated mitochondria from pre- and postrigor muscle, and also with mitochondria isolated at 30 min postmortem, and with mitochondria aged in the pellet form for 24 h at 1° C. The effect of pH on the formation of paracrystalline inclusions was studied by adding 0.2 ml of mitochondrial suspension in 250 mM sucrose to 2.0 ml of medium of a varying pH containing 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose, and 20 mM KH_2PO_4. The mitochondrial suspension was kept in an ice-bath in a 1° C cold room for 2 h before being fixed for 30 min by adding 1.0 ml of 2.5% glutaraldehyde.

Results

Figure 1 illustrates the paracrystalline inclusions in the mitochondria of ox diaphragm after aging the mitochondria in situ for 3 days postmortem at 1° C. The two types of inclusion described by Saito et al. [4] were observed, but the majority of the inclusions were of the intracristal type previously reported [1]. The electron micrographs also show that the intracristal inclusions were randomly distributed with some mitochondria containing more than three inclusions. No inclusions were observed in mitochondria in situ at 30 min postmortem. The frequency of occurrence of these inclusions was known to depend on the time of postmortem aging of the mitochondria in situ [1, 2], and it was therefore quite logical for other workers [3] to conclude that pH was contributing to the formation of these inclusions within the mitochondria of skeletal muscle.

To distinguish between the effects of pH and postmortem aging on the formation of paracrystalline inclusions, experiments were conducted under various conditions using isolated mitochondria. Figure 2 illustrates thin sections of the mitochondrial pellet obtained from diaphragm at 30 min postmortem (A) and from muscle after storage for 144 h at 1° C (B). The electron micrographs show that only the mitochondria isolated after prolonged aging contained intracristal inclusions. A high proportion of the inclusions occurred in intact mitochondria displaying either the "condensed" or "intermediate" ultrastructural appearance observed also with isolated rat liver mitochondria [10].

Figure 2. Thin sections of mitochondria isolated from pig diaphragm at 30 min postmortem (A) and from muscle after aging for 6 days (B) at 1° C postmortem. Inclusions were only present in the mitochondria isolated from tissue aged for 6 days at 1° C. Final magnification: 10,000 x.


A
B

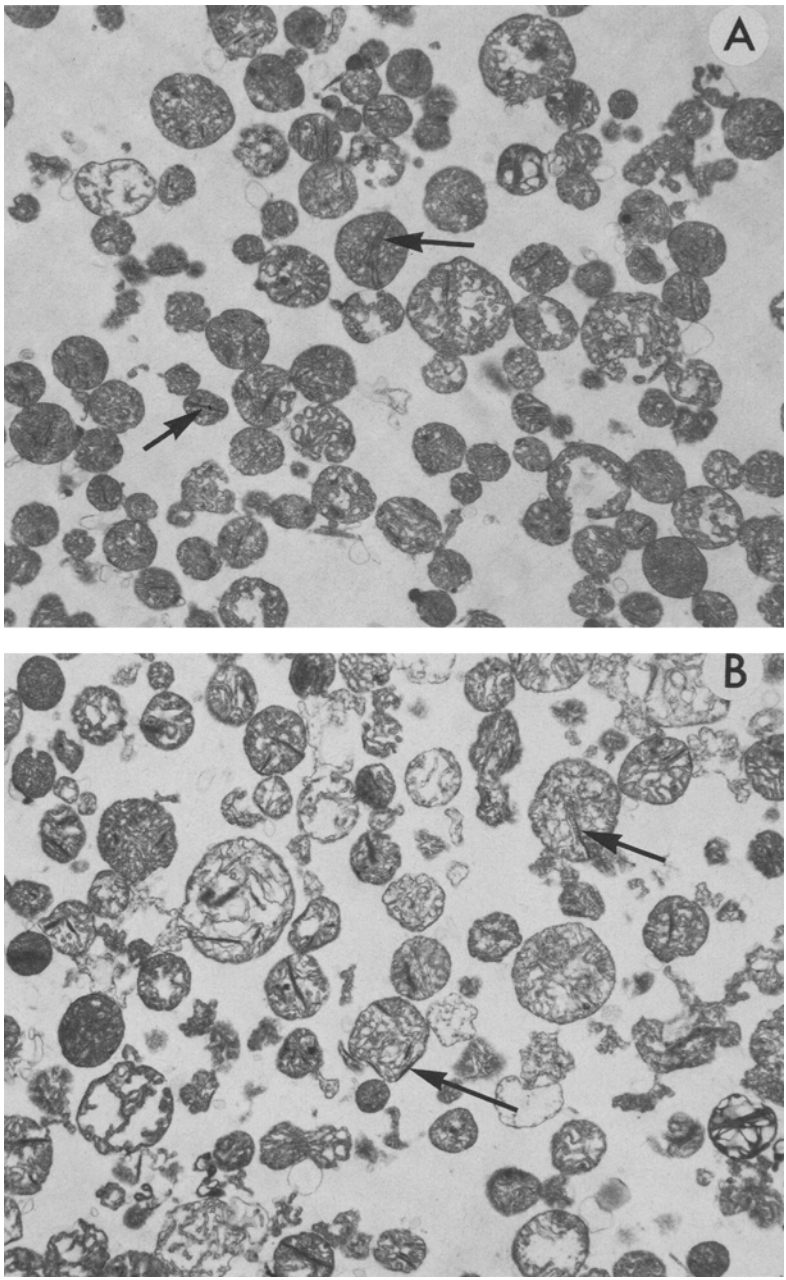

A
B

Figure 1 suggests that the formation of inclusions could be due to low pH, or to postmortem aging in situ, or to a combination of both of these factors. The pH fell from 6.90 at 30 min to 5.85 after 144 h aging postmortem at 1° C. In order to determine the main factor responsible for the formation of these inclusions, experiments were carried out by suspending mitochondria isolated from fresh and aged tissue in media of different pH values.

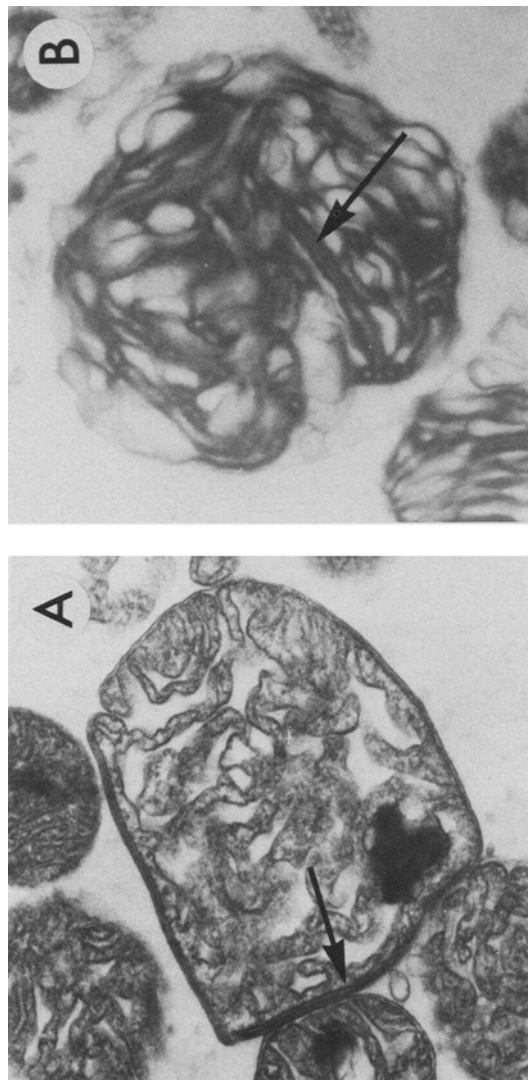
Figure 3 shows the effect of suspending freshly isolated mitochondria from 30 min postmortem tissue in media of pH 5.5 (A) and of pH 7.0 (B). The mitochondria used for these experiments showed normal properties with respect to oxidative phosphorylation and respiratory control index (Table I). The high value of the respiratory control index indicated that the isolated mitochondria were tightly coupled. Furthermore, no inclusions were observed in the mitochondria (see Fig. 2A) prior to suspending them in a different pH medium. Inclusions were first observed 2 h after suspending the mitochondria in media of pH 5.5 (A) and of 7.0 (B), prior to fixation with glutaraldehyde. The percentage of mitochondria with inclusions was about the same in both cases even though the difference in pH value was 1.5 units. Similar results were obtained with mitochondria isolated from tissue stored at 1° C for 144 h postmortem. Table II illustrates the accumulative data suggesting that aging is probably the most important factor inducing formation of inclusions.

TABLE I. Properties of pig diaphragm mitochondria[a]

Substrate	O_2 uptake (*n*atoms/min/mg protein)	ADP/O	Respiratory control index
Pyruvate + malate	133	2.69	11.07
Rotenone + succinate	258	1.78	7.38
Ascorbate + TMPD	241	0.98	2.01

[a] The data were an average of two separate mitochondrial preparations from muscle at 30 min postmortem. The sequence of additions, all referring to final concentrations for the three separate oxidase systems was: 8 mM malate, 8 mM pyruvate, and 500 μM ADP for the oxidation of pyruvate *plus* malate; 2 μM rotenone, 8 mM succinate, and 300 μM ADP for the succinoxidase system; 4 mM ascorbate, 0.1 μg antimycin A per mg protein, 0.1 mM TMPD for the ascorbate–TMPD oxidase system. All other experimental details are described in Materials and Methods.

Figure 3. Effect of pH on the formation of inclusions in isolated mitochondria. The mitochondria were isolated from pig diaphragm at 30 min postmortem and were suspended in a medium of either pH 5.5 (A) or 7.0 (B). The same percentage of mitochondria with inclusions were observed in both cases. The majority of the inclusions were of the intracristal type. Final magnification: 10,000 x.


B
A

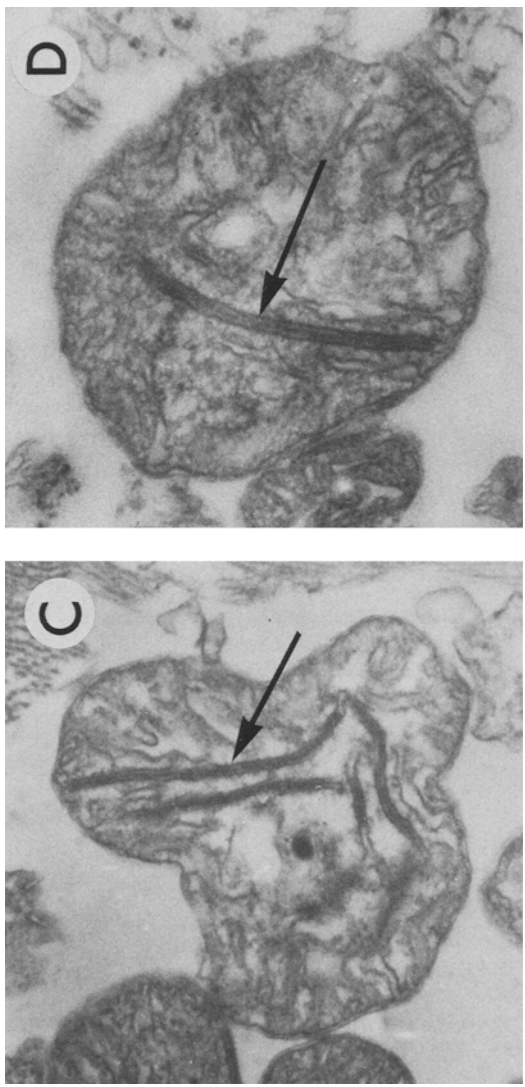


Figure 4. Inclusions in mitochondria of different ultrastructural configurations. Two types of inclusions were observed. (A) shows two outer–inner membrane junctions in a mitochondrion having the twisted configuration. The intracristal inclusion was observed in mitochondria having the condensed (B), orthodox (C and D), and twisted (see Fig. 3) configuration. Final magnification: 40,000x.

TABLE II. Induction of mitochondrial intracristal inclusions[a]

Time postmortem	Mitochondria		
	Pellet	Suspension (pH = 7.0)	Suspension (pH = 5.5)
30 min	0%	19–30% (25%)	14–27% (19%)
144 h (1°C)	4–8% (6%)	12–14% (13%)	8–14% (11%)

a The results are expressed as percentage of the mitochondrial population containing the intracristal inclusions. Mitochondria were isolated from pig diaphragm at 30 min postmortem and after being aged in situ for 144 h at 1°C. The pellet refers to the mitochondria sedimented at 7000 g before they were suspended for 2 h in a medium of either pH 5.5 or pH 7.0. All other experimental details are described in Materials and Methods.

The importance of aging in influencing the formation of mitochondrial inclusions was demonstrated in another experiment by aging freshly isolated mitochondria in pellet form for 24 h at 0°C. This resulted in 4% of the mitochondrial population containing inclusions. A further increase in the percentage of inclusions was observed if aged mitochondria were resuspended and further aged for 2 h at 0°C. At pH 5.5, 7–13% of the mitochondrial population contained inclusions as compared with 10–18% at pH 7.0, again suggesting that an increase in acidity was not the essential factor controlling inclusion formation.

Figure 4 illustrates the distribution of both types of intermembrane inclusion [4] observed in intact mitochondria obtained from the diaphragm under various conditions. Inclusions occurred in mitochondria having different ultrastructural configurations including the condensed, orthodox, and twisted conformation. The majority of the inclusions were of the intracristal type. A few of the mitochondria had both types of inclusions previously described by Saito et al. [4].

Discussion

The main aim of this investigation was to try to offer a plausible explanation for the occurrence of the helical paracrystalline inclusions in the intracristal spaces following prolonged aging of mitochondria in situ [1]. The two factors most likely to be associated with the paracrystalline inclusion formation are aging and the pH of the tissue. The present data suggest that aging is more important than low pH, since lowering the pH of the isolated mitochondrial suspension does not increase the number of inclusions.

The inclusions observed in our experiments are localized mostly in the mitochondrial intracristal space and to a lesser extent between the inner and outer membranes. Our present data differ from the recent

observation of Saito et al. [4] in two aspects. First, lowering the pH does not appear to increase the number of mitochondria with inclusions. Secondly, intracristal inclusions are observed after aging isolated mitochondria in vitro, which was not observed by Saito et al. [4].

The inclusions do not appear to be responsible for the gradual decline in mitochondrial function following prolonged aging in situ [1, 2]. However, the presence of inclusions provides a good means of assessing the "freshness" of skeletal muscle mitochondrial preparations since freshly isolated mitochondria from 30 min postmortem tissue do not contain these structures. We agree with Saito et al. [4] that the formation of inclusions is a better indicator of the extent of mitochondrial aging than the respiratory control index.

References

1. K.S. Cheah, A.M. Cheah, and C.A. Voyle, *J. Bioenerg.*, 4 (1973) 383.
2. K.S. Cheah and A.M. Cheah, *Int. J. Biochem.*, 5 (1973) 753.
3. D.J. Morton, R.W.D. Rowe and J.J. Macfarlane, *J. Bioenerg.*, 4 (1973) 445.
4. A. Saito, M. Smigel, and S. Fleischer, *J. Cell Biol.* 60 (1974) 653.
5. K.S. Cheah and A.M. Cheah, *J. Bioenerg.*, 2 (1971) 85.
6. B. Chance and G.R. Williams, *Advances in Enzymology*, Nord, F.F., ed., Interscience, New York (1956), pp. 65–134.
7. O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J.Biol. Chem.*, 193 (1951) 265.
8. D.W. Allman, E. Bachmann, N. Orme-Johnson, W.C. Tan, and D.E. Green, *Arch. Biochem. Biophys.*, 125 (1968) 981.
9. E.S. Reynolds, *J. Cell Biol.*, 17 (1963) 208.
10 C.R. Hackenbrock, *J. Cell Biol.*, 30 (1966) 269.