Biochimica et Biophysica Acta 894 (1987) 507–514
Elsevier

BBA 42655

Freeze-fracture study of heart mitochondria in the condensed or orthodox state

Robert C. Hertsens and Willem A. Jacob

University of Antwerp (UIA), Department of Medicine, Antwerp-Wilrijk (Belgium)

(Received 19 June 1987)

Key words: Freeze-fracture; Mitochondrial membrane; (Rat heart mitochondrion)

Rat heart mitochondria were isolated and forced in a well-defined metabolic state. After freeze-fracturing, the intramembrane particle dimension and density on both fracture faces of the inner mitochondrial membrane were measured. No significant differences could be calculated between the diameter of the membrane particles in the five different states. However, the particle density on the fracture faces of the inner mitochondrial membrane in the condensed configuration is significantly smaller than in the orthodox configuration on the 99.5% level of confidence. These results are compared with the literature, where conflicting data have been published about these particle densities.

Introduction

The multiplicity of configurational states in which mitochondria can exist when isolated from tissue is well documented [1–8] and correlates to the metabolic state of the mitochondrion, or to the osmolarity of the buffer medium. Four configurational states are generally accepted: orthodox, condensed, swollen and contracted. The orthodox and condensed configuration are used to describe the structure of mitochondria in suspension as a function of the metabolic state [8].

To understand the ultrastructural basis for the function of the mitochondrial inner membrane, information is required about the organisation of membrane components. In earlier studies we have shown, with the freeze-fracture technique, that stimulation of the energy metabolism with catecholamines or inhibition with barbiturates affects the macromolecular structure of the membranes [9–11].

In the present study we report the existence of characteristic intramembrane particle densities on the freeze-fracture faces of the inner membrane of heart mitochondria as a function of the metabolic state.

Also, Packer et al. [12] and Melnick and Packer [13] considered the influence of the metabolic or the energetic state on the ultrastructure of the mitochondrial inner membrane, but could not detect a correlation. However, their studies, are contradictory. We will show that, in contrast to Packer et al. [12], a clear discrimination between the conventionally defined respiratory states [8,14,15] can be made on the basis of results from standardised freeze-fracture experiments.

Materials and Methods

Ten female Wistar rats (250–300 g bodyweight) were anaesthetised with chloralosane (100 mg/kg, BDH Chemicals). The hearts were quickly removed and put in an ice-cold isolation medium containing 0.5 mM EDTA, 0.15% bovine serum

Correspondence: R.C. Hertsens, University of Antwerp (UIA), Department of Medicine, EM Laboratory, B 2610 Antwerp-Wilrijk, Belgium.

albumin (fraction V) and 250 mM saccharose. After mincing the hearts, mitochondria were prepared by standard methods [16]. Protein concentration was measured with crystalline bovine serum albumin as a standard [17]. Respiration of the mitochondria was measured with a Clark electrode and succinate as substrate [16–18]. The p/o ratio was calculated as described by Estabrook [19].

The mitochondria were forced in a well-defined metabolic state [8,14,15]. Mitochondria in state I are freshly isolated and suspended in a respiration medium containing 0.3 M mannitol, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.45% bovine serum albumin in 10^{-3} M phosphate buffer and 10^{-2} M Tris/HCl buffer (pH 7.4). Addition of 2 μl ADP (stock solution of 0.125 μMol/μl) to the suspension, results in state II mitochondria. The oxygen consumption is slightly increased. If substrate (1 μl succinate, stock solution of 2.5 μMol/μl) is added to state II mitochondria, a strong increase in oxygen consumption is measured and they become state III mitochondria. If all the ADP is metabolized by the mitochondria they merge into state IV, until all the oxygen is used. At that moment they are in state V.

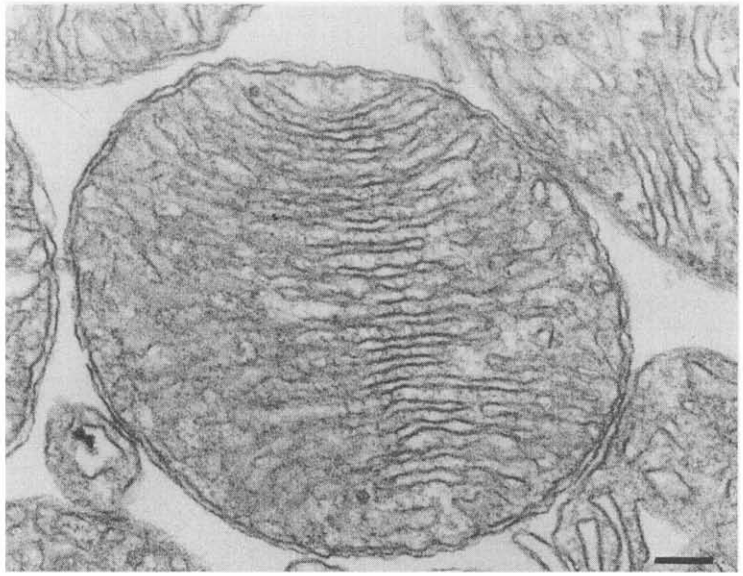
After forcing the mitochondria in to the different states, the suspensions were centrifuged for 30 s at $15000 \times g$. Part of the pellet was fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h, followed by a postfixation with 2% OsO_4 in distilled water for 1 h. After dehydratation in a graded alcohol series, the pellet was embedded in epoxy resin. Thin sections were post-stained with 3% uranyl acetate for 3 min and with 1% lead citrate for 1 min.

The remaining part of the pellet was quickly frozen in nitrogen slush at $-210\,^{\circ}$C between two golden plates, without any chemical fixation or cryoprotection. If not to be used immediately, the frozen samples were stored in liquid nitrogen. The samples were fractured at $-150\,^{\circ}$C and $2 \cdot 10^{-6}$ Torr in a Balzers BAE 121 coating device, shadowed with 3 nm Pt/C and coated with 20 nm of carbon using electron guns. The thickness was measured with a swinging quartz cristal thickness meter. The replicas were floated off on cold technical bleach. The lipid material was removed from the replicas by saponification in 50% NaOH at 60 °C. Finally, the replicas were washed several times with distilled water [20]. Micrographs of thin sections and replicas were taken in a Jeol 1200 EX electron microscope.

The number of orthodox and condensed mitochondria in a defined metabolic state was determined. Electron micrographs were taken at an enlargement of 10000-times. Measurements were done on pictures with a final magnification of 28000-times. For each metabolic state, 500 mitochondrial profiles were classified in three groups: orthodox, intermediate or condensed configuration. Each experiment was repeated five times with different animals. In this way a mean percentage of each configuration in the different metabolic states was calculated from at least 2500 mitochondrial profiles.

The surface density of mitochondrial inner membrane per unit mitochondrial volume was measured according to the method of Weibel and Bolender [21]. To calculate the mitochondrial volume an orthogonal grating with a periodicity of 1 cm and a total area of 10×10 cm was used. The same grating was used as a line grating for calculating the surface density of the inner mitochondrial membrane. It contained ten lines of 10 cm. By counting the number of points falling on a mitochondrial profile and the number of intersections with the inner membrane or cristae membranes, the surface density of mitochondrial inner membrane per unit mitochondrial volume was calculated. This was done for 500 mitochondria in a condensed or orthodox configuration. The measurements were repeated five times on mitochondrial suspensions prepared from different hearts. A mean value from 2500 profiles was obtained.

The diameter and density of the intramembrane particles on the fracture faces of the mitochondrial inner membrane were measured on pictures with a final magnification of 280000 times with a computerised digitising tablet (Videoplan, Kontron, F.R.G.). The diameter of the intramembrane particles was measured as the width of the shadow cap perpendicular to the direction of shadowing. Throughout this study the terminology of Branton et al. [22] was used. At least 400 intramembrane particles were measured for each fracture face from 20–30 different fields. The



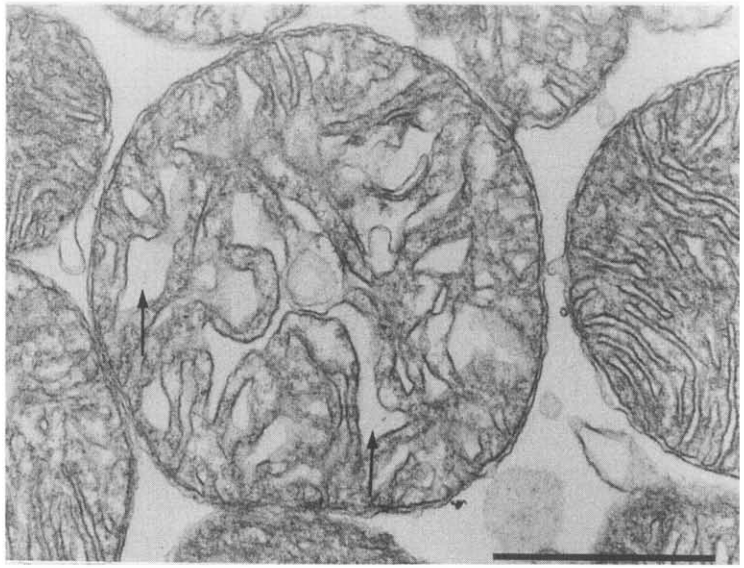
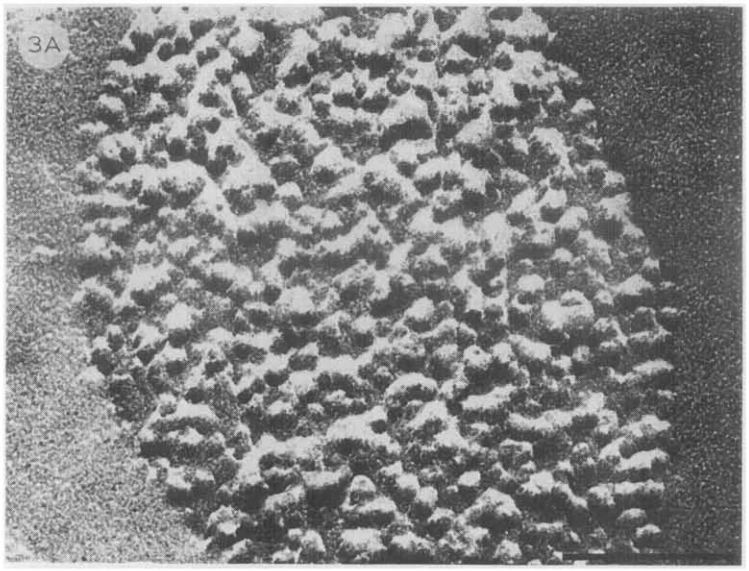


Fig. 1. Section through an isolated mitochondrion in a characteristic orthodox configuration. The cristae are aligned and the outer and inner membrane are clearly visible. (Bar, 0.1 μm).

Fig. 2. Section through an isolated mitochondrion in a characteristic condensed configuration. Large intracristal spaces (arrow) and a small matrix compartment are seen. (Bar, 0.5 μm).



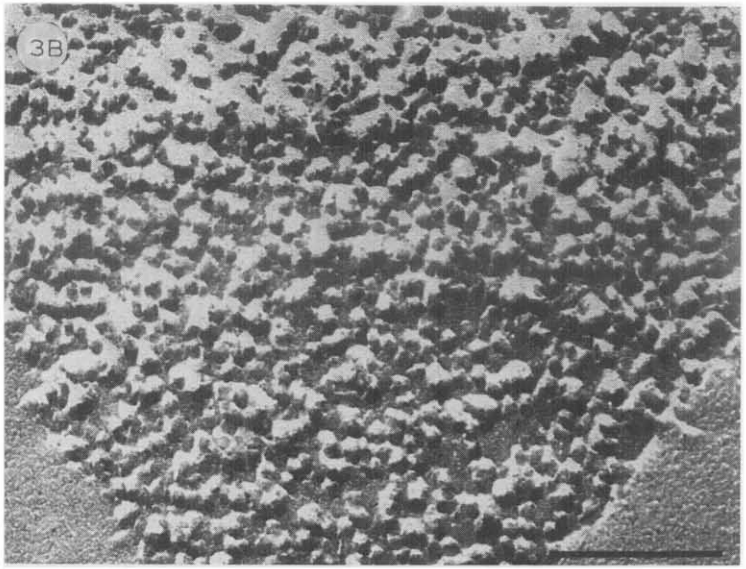
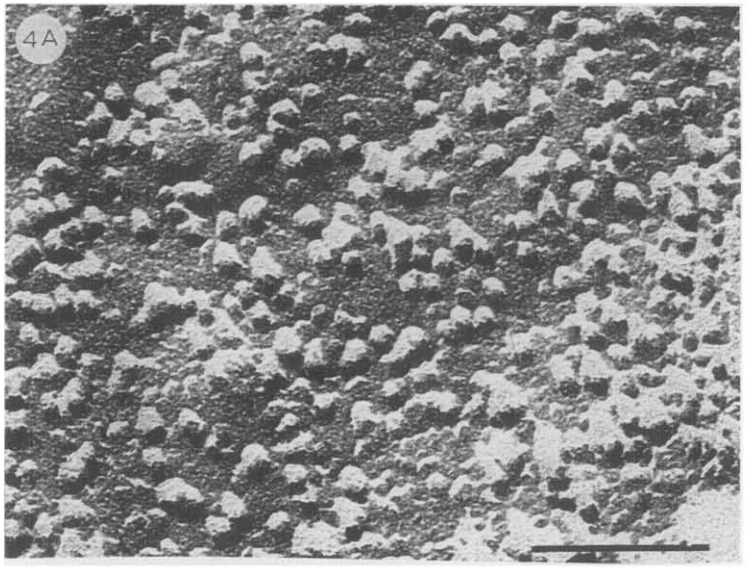


Fig. 3. (A) Freeze-fracture replica of the P-face of the inner membrane of a state I mitochondrion. (Bar, 0. 1μm). (B) Freeze-fracture replica of the P-face of the inner



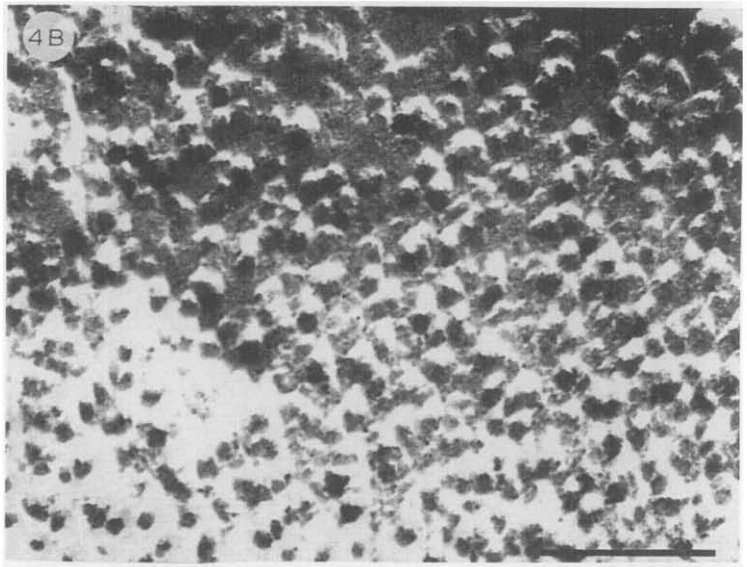


Fig. 4. (A) Freeze-fracture replica of the E-face of the inner membrane of a state I mitochondrion. (Bar, 0.1 μm). (B) Freeze-fracture replica of the E-face of the inner membrane of a state V mitochondrion. (Bar, 0.1 μm).

TABLE I

PERCENTUAL DISTRIBUTION OF DIFFERENT MITOCHONDRIAL MORPHOLOGIES IN A SUSPENSION

Metabolic state	Condensed	Intermediate	Orthodox
I	94	2	4
II	87	10	3
III	71	–	29
IV	35	5	60
V	28	10	62

measurements were done in areas with no curvature so that errors from this side are excluded.

To measure the density of the intramembrane particles on the different fracture faces a standard square corresponding to 1 μm^2 was put on the fracture faces. The number of intramembrane particles/μm^2 was counted on 40–50 fracture faces of either P- or E-face. The measurements were repeated five times and were done 'double-blind'. Student's *t*-test was used to check for significant differences.

Results

The quality of the mitochondrial suspension is indicated by the *ADP/O* ratio and the respiratory control rate. If the suspensions had a *ADP/O* ratio lower than 1.95 or a respiratory control rate lower than 2.5 they were not used.

Fig. 1 shows a mitochondrion with a typical orthodox morphology. The intermembrane and intracristal space are small. The two surrounding membranes lie close together. The cristae are more or less parallel to each other. The matrix demonstrates a low electron density.

Fig. 2 represents a mitochondrion with a condensed morphology. Here, the intermembrane space is enlarged and the matrix is very electron dense.

The percentual distribution of orthodox, intermediate and condensed configurations in mitochondrial suspensions in different metabolic states is shown in Table I.

The surface density of the inner mitochondrial membrane in the condensed configuration ($41.2 \pm 9.2\ \mu m^{-1}$) is significantly lower ($P < 0.005$) compared to the surface density in the orthodox configuration ($24.9 \pm 5.3\ \mu m^{-1}$).

Characteristic histogram distributions of the intramembrane particle diameter on the P- and E-face of the inner mitochondrial membrane have a range of diameters between 5 and 17 nm (not shown). No significant differences between the mean intramembrane particle diameters on the P- or the E-face are calculated in the different states. Between the mean intramembrane particle densities in states I, II and III or between the densities in states IV and V, no mutual significant differences are calculated on P- or E-face. However, between the mean intramembrane particle densities of the condensed configuration (state I, II and III) and the values of the mean intramembrane particle densities in the orthodox configuration (states IV and V) a significant difference is calculated ($P < 0.01$) for the P-face as well as for the E-face.

TABLE II

MEAN INTRAMEMBRANE PARTICLE DIAMETER AND DENSITY ON THE P- AND E-FACE OF THE INNER MEMBRANE OF MITOCHONDRIA IN DIFFERENT METABOLIC STATES

The numbers in parentheses represents the number of intramembrane particles counted.

Metabolic state	P-face		E-face	
	diameter (nm)	density (μm^{-2})	diameter (nm)	density (μm^{-2})
I	10.4 ± 1.7 (864)	2.615 ± 20	10.5 ± 1.6 (1057)	2.025 ± 20
II	10.2 ± 1.7 (758)	2.695 ± 20	10.5 ± 1.6 (950)	2.020 ± 20
III	10.1 ± 1.7 (719)	2.733 ± 20	10.5 ± 1.6 (771)	2.055 ± 20
IV	10.5 ± 1.6 (448)	1.767 ± 20	10.5 ± 1.6 (536)	1.483 ± 20
V	10.9 ± 1.8 (686)	1.872 ± 20	10.9 ± 1.7 (458)	1.472 ± 20

The mean diameters and densities of the intramembrane particles on the P- and E-face are summarized in Table II.

Characteristic replicas of the mitochondrial inner membrane are shown in Figs. 3 and 4 for, respectively, the P- and E-face in states I and V.

Discussion

Mitochondria isolated from any tissue can be put in well-defined energy states. Chance and Williams [14,15] introduced a classification in different metabolic states on the basis of oxygen consumption of a mitochondrial suspension, while Hackenbrock [7,8] described these states morphologically.

Several techniques can be used to investigate the structure of membrane proteins in the function of external factors, such as substrates, ADP or ions. Spectrophotometric techniques will give a global idea of the alterations in the membrane structure [23–27], but with these techniques it is not possible to gain information about the organisation of the membrane components on a molecular level.

Electron microscopical investigation of chemically fixed mitochondria can give additional information about the macromolecular and morphological differences as a function of these external factors. In this way the conversion from an orthodox to a condensed morphology was characterised [5–8].

Heart mitochondria in the orthodox configuration showed well organised inner membrane and cristae membranes. The cristae are arranged parallel to each other. No pressure is put to the membranes by an enlarged intracristal or intermembrane space, nor by a strong bending of the membrane itself. In the condensed configuration, however, such forces do exist. The cristae are bent strongly and the intracristal space is enlarged. We have quantified the amount of different morphological states in the function of the energy state of the mitochondria (Table I). The alterations in the morphology and orientation of the membranes in the mitochondria must have repercussions on the mutual interactions of the membrane components. With the thin sectioning technique, however, it is not possible to get an idea of these differences.

Direct information and observation of the membrane ultrastructure is only possible with the freeze-fracture technique. With this technique it is easy to get some insight in the distribution, dimension and organisation of the membrane components, as a function of the metabolic state.

From the histogram distributions of the intramembrane particle diameters in Figs. 3 and 4 it is seen that for the P-face as well as for the E-face the diameter is between 5 and 17 nm. This is in agreement with previously published results [28,29]. Discrimination between the ultrastructure of the inner membrane indifferent metabolic states is not possible on the basis of the intramembrane particle diameter. Indeed, no significant differences could be calculated between the mean intramembrane particle diameters on the inner mitochondrial membrane in the different metabolic states.

From Table I it can be concluded that the only morphological difference between the energetic states is the appearance of either a majority of condensed or of orthodox configurations in the suspension. On the other hand, if we consider the intramembrane particle density in different metabolic states (Table II), significant differences are measured between these two extreme configurations. Only states I and V are considered as being homogeneous. Conflicting data have been published about intramembrane particle densities on the fracture faces of the inner mitochondrial membrane. These are summarised in Table III.

Our results are in contradiction with the results

TABLE III

COMPARISON BETWEEN INTRAMEMBRANE PARTICLE DENSITIES ON E- AND P-FACE OF THE INNER MITOCHONDRIAL MEMBRANE

Reference	E-face	P-face
Melnick and Packer [13]	990	2290
Packer [32]	990	2290
Packer [30]	2120	4208
Vial et al. [31]	2554	5500
Sowers and Hackenbrock [29]	1837	2480
Sowers [33]	2609 ± 760	5270 ± 438
This study		
Orthodox	1503 ± 20	1845 ± 20
Condensed	2034 ± 20	2674 ± 20

and conclusions described by Packer [30]. It is also striking that the same laboratory [30,32], using the same isolation procedures for the mitochondria, reported such big differences in densities. This prompted us to re-examine the results by carefully and rigorously standardised methods. The densities in Table II represent mean values of five independent experiments. The measurements were done 'double-blind' on a significant number of fracture faces, so that statistical demands were guaranteed. The results from the five independent experiments were mutually not significantly different.

From these results it can be stated that, in spite of the conclusions of Packer [30], it is possible to characterise the metabolic state of a mitochondrion on the basis of particle densities on the P- and E-face. Mitochondria in a condensed morphology showed a significant higher intramembrane particle density on both fracture faces compared to the orthodox morphology.

A possible explanation can be give in relation to the surface density of the inner mitochondrial membrane per unit mitochondrial volume. A significant lower surface density ($P < 0.005$) is obtained in the condensed configuration (24.9 ± 5.3 μm^{-1}) compared with the orthodox configuration (41.28 ± 9.2 μm^{-1}). If we assume that the protein content in the membrane is constant, since de novo synthesis is hardly possible in the short transition times which are imposed in this study, the higher intramembrane particle density in the condensed morphologic state results from lowering the surface density (Table IV).

The optimum energy production in the mitochondrion is dependent on the exact concentration of substrates, but also on the exact interprotein distance and arrangements of electron transport components in the membrane [28,32,34]. The inner mitochondrial membrane is characterised as a highly fluid membrane. The components can migrate easily in the membrane. If the surface density of the inner mitochondrial membrane rises in the orthodox configuration by stretching the membrane, the interprotein distance enlarges too. This results in a lower intramembrane particle density, because the same number of protein complexes are now dispersed over more membrane surface. So the chance of interaction between enzyme complexes of the electron transport chain decreases and, as a result, the yield of these energy transmitting reactions is also lowered. Thus, the lowering of oxygen consumption in states IV and V is not caused only by a reduction of the ADP and O_2 concentration, but also by an ultrastructural alteration of the membrane.

From these studies it must be concluded that changes in the metabolic state of the mitochondrion do give rise to changes in the distribution of the intramembrane particles on the P- as well as on the E-face of the inner mitochondrial membrane. These characteristic intramembrane particle densities can be used to discriminate between the orthodox and the condensed configuration of mitochondria.

TABLE IV

INTRAMEMBRANE PARTICLE DENSITY AND SURFACE DENSITY OF THE INNER MITOCHONDRIAL MEMBRANE IN DIFFERENT CONFIGURATIONS

Configuration	Intramembrane particle density		Surface density
	P-face	E-face	
Condensed	2674 ± 20	2034 ± 20	24.9 ± 5.3
Orthodox	1845 ± 20	1503 ± 20	41.2 ± 9.2

References

1 Harris, R.A., Penniston, J.T., Asai, J. and Green, D.E. (1968) Proc. Natl. Acad. Sci. USA 59, 830–837
2 Penniston, J.T., Harris, R.A., Asai, J. and Green, D.E. (1968) Proc. Natl. Acad. Sci. USA 59, 624–631
3 Harris, R.A., Williams, C.H., Caldwell, M. and Green, D.E. (1969) Science 165, 700–702
4 Green, D.E. and Harris, R.A. (1969) FEBS Lett. 5, 241–245
5 Hackenbrock, C.R. (1966) J. Cell. Biol. 30, 269–297
6 Hackenbrock, C.R. (1968) J. Cell. Biol. 37, 345–369
7 Hackenbrock, C.R. (1968) Biochemistry 61, 598–605
8 Hackenbrock, C.R. (1972) J. Cell. Biol. 53, 450–465
9 Hertsens, R.C., Jacob, W.A. and Van Bogaert, A. (1984) Biochim. Biophys. Acta 769, 411–418
10 Hertsens, R.C. and Jacob, W.A. (1981) Proc. R. Microsc. Soc. 16, 175
11 Hertsens, R.C. and Jacob, W.A. (1982) Biol. Cell. 45, 224
12 Packer, L., Williams, M.A. and Criddle, R.S. (1973) Biochim. Biophys. Acta 292, 92–104
13 Melnick, R.L. and Packer, L. (1971) Biochim. Biophys. Acta 253, 503–508
14 Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 409–427

15 Chance, B. and Williams, G.R. (1956) Adv. Enzymol. 17, 65–134
16 Lindenmayer, G.E., Sordahl, L.A. and Schwartz, A. (1968) Circ. Res. 23, 439–450
17 Hartree, E.F. (1972) Anal. Biochem. 48, 422–427
18 Clark, L.C. Jr., Wolf, R., Granger, G. and Taylor, L. (1953) J. Appl. Physiol. 6, 189–195
19 Estabrook, R.W. (1967) Methods Enzymol. 10
20 Van den Bosch, M. and Jacob, W.A. (1982) J. Microsc. 128, 239–240
21 Weibel, E.R. and Bolender, R.P. (1973) in Principles and Techniques of Electron Microscopy (Hayat, M.A., ed.), pp. 237–296, Van Nostrand Reinhold Company, New York
22 Branton, D., Bullivant, S., Gilula, N.B., Karnovsky, M.J., Moor, H., Mühlethaler, K., Northcote, D.H., Packer, L., Satir, B., Satir, P., Speth, V., Staehelin, L.A., Steere, R.L. and Weinstein, R.S. (1975) Science 190, 54–56
23 Azzi, A., Chance, B., Radda, G.K. and Lee, C.P. (1969) Proc. Natl. Acad. Sci. USA 62, 612–619
24 Azzi, A., Gherardini, P. and Santato, M. (1971) J. Biol. Chem. 246, 2035–2042
25 Graham, J.M. and Wallach, D.F.H. (1969) Biochim. Biophys. Acta 193, 225–227
26 Graham, J.M. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 241, 180–194
27 Chance, B. (1970) Proc. Natl. Acad. Sci. USA 67, 560–571
28 Wrigglesworth, J.M., Packer, L. and Branton, D. (1970) Biochim. Biophys. Acta 205, 125–135
29 Sowers, A.E. and Hackenbrock, C.R. (1981) Eur. J. Cell Biol. 24, 101–107
30 Packer, L. (1972) in Mechanisms in Bioenergetics (Azzone, G. et al.), eds. pp. 33–52, Academic Press, New York
31 Vial, C., Comte, J. Font, B. and Gautheron, D.C. (1981) Biol. Cell 41, 195–202
32 Packer, L. (1973) Biokhimiya 38, 1288–1293
33 Sowers, A.E. (1983) Biochim. Biophys. Acta 735, 426–428
34 Ferguson-Miller, S., Hochman, J. and Schindler, M. (1986) Biochem. Soc. Trans. 14, 822–824