BBA 45689

ULTRASTRUCTURE OF SONIC AND DIGITONIN FRAGMENTS FROM BEEF HEART MITOCHONDRIA

A. N. MALVIYA, BAHRAM PARSA, RALPH E. YODAIKEN* AND W. B. ELLIOTT

Departments of Biochemistry and Pathology, State University of New York at Buffalo, and the Buffalo General Hospital, Buffalo, N.Y. 14214 (U.S.A.)

(Received March 20th, 1968)

SUMMARY

Examination of OsO_4-fixed, imbedded and sectioned specimens by the electron microscope showed that both sonic and digitonin fragments of mitochondria were vesicular structures. Brief OsO_4 fixation followed by negative staining showed that most of the 'inner membrane subunits' of sonic fragments were on the exterior of the fragment while most of the inner membrane subunits of digitonin fragments were on the inside of the vesicle.

Since the orientation of the membrane in the digitonin fragments is the same as that in intact mitochondria, it is not surprising that many properties of digitonin fragments are quite similar to those of mitochondria while sonic fragments show quite different behavior.

INTRODUCTION

Although there is still controversy about the structure of the mitochondrial membrane[1–6], it is generally accepted that the mitochondrion consists of an inner and outer membrane[7,8]. It has been suggested that the inner membrane is thicker than the outer membrane and is less permeable[9]. The negative staining technique[3,10,11] used in electron microscopy, supports these findings[12–14]. The inner membrane folds into cristae which are found in a variety of shapes[15].

Subunits called inner membrane subunits[14], or elementary particles[16] have been shown on the inner membrane by negative staining of disrupted mitochondria and have also been shown in intact OsO_4-fixed mitochondria by use of a low temperature technique[17]. FERNÁNDEZ-MORÁN *et al.*[17] described elementary particles as the structures consisting of (i) the headpiece, (ii) the cylindrical stalk and (iii) the basepiece. In spite of the controversies[6,18] over the nature of these structures, it is generally accepted that the headpieces are characteristic of the inner membrane[19]. The existence of the stalk and the basepiece is questioned[20,21]. It is generally believed at the present moment[22] that the headpieces are not involved in mitochondrial electron transport.

Mitochondria have been resolved into submitochondrial units, mainly by sonication or digitonin treatment to give sonic fragments[23] or digitonin fragments[24,25].

* Present address: Department of Pathology, University of Cincinnati Medical Center, Cincinnati, Ohio 45229, U.S.A.

Although both types of fragments show phosphorylation coupled to respiration[26–28], the reported differences[29–35] in the behavior and function of sonic and digitonin fragments tempted the authors to look for the morphological differences between these preparations.

MATERIAL AND METHODS

Preparation of mitochondria

The beef-heart mitochondria used in making Figs. 1 and 3 were isolated by the large-scale technique[36].

Preparation of mitochondria for visualizing the elementary particles. FERNÁNDEZ-MORÁN's procedure was used as described in ref. 17, except that mitochondria were isolated by batch centrifugation in an angle centrifuge (Serval RCII).

Preparation of digitonin fragments. Digitonin fragments were prepared as described by ELLIOTT AND HAAS[36].

Preparation of sonic fragments. Mitochondria obtained by the large-scale technique[36] were treated as described by LOW AND VALLIN[37], except that the ATP concentration was not adjusted, the sonication time was increased to 8–10 min to compensate for the use of a different sonicator (Biosonic model G-21, Blackstone Corporation, Jamestown, N.Y.) and the oscillating chamber containing mitochondria was cooled by circulating a mixture of ethanol and dry ice through the cooling coil. The pellet finally obtained was suspended in 0.25 M sucrose–0.01 M $MgCl_2$.

Preparation of sonicated digitonin fragments. The pellets obtained after ultracentrifugation of the supernatant which remained after digitonin treatment of the mitochondria were suspended in 0.25 M sucrose–0.01 M $MgCl_2$; and sonicated as described above.

Electron microscopy

Fixation with OsO_4. The mitochondrial, or submitochondrial fragment suspension, was fixed in the cold for 15 min with equal amounts of 1 % OsO_4 phosphate buffer (pH 7.2). 2 % agar solution[40] was mixed with the suspension and rapidly solidified. The material was cut into 1–2-mm^3 pieces and dehydrated in 100 % alcohol for 15 min, in propylene oxide for 15 min, and embedded in Epon. This method has been used for virus preparations but has not been previously employed for mitochondrial studies. Digitonin fragments suspended in Tris buffer could not be preserved intact by this procedure.

After ultrasectioning with Porter Blum or LKB microtomes, the specimens were examined either unstained or stained with lead citrate[41] and uranyl acetate[42] and examined in an electron microscope (Jem 7).

Negative staining. The surface spreading method[38] was employed for negative staining. 2 % phosphotungstic acid solution (adjusted to pH 6.8 with 1 M KOH) with 0.01 % bovine serum albumin was placed in a small plastic dish. A clean sewing needle was dipped about 0.5–1 cm in the mitochondrial preparations and then dipped slowly into the phosphotungstic acid solution and the 400-mesh electron-microscope specimen grids coated with a film of carbon were floated briefly, film side down on its surface. The grids were picked up in 30 sec. The excess phosphotungstic acid was soaked up by the torn edge of filter paper (Whatman No. 1). The grids were further

TABLE I

RESPIRATORY ACTIVITY OF MITOCHONDRIA AND SUBMITOCHONDRIAL FRAGMENTS

Substrate	*Additions*	*Oxygen uptake** (*mμM/sec*)	*Respiratory control ratio***
Mitochondria (ELLIOTT AND HAAS)			
Succinate	—	640	
	ADP	1207	1.9
β-Hydroxybutyrate	—	149	
	ADP	210	1.4
Mitochondria (FERNÁNDEZ-MORÁN AND GREEN)			
Succinate	—	506	
	ADP	604	1.2
β-Hydroxybutyrate	—	152	
	ADP	200	1.3
Digitonin fragments			
Succinate	—	253	
	ADP	316	1.2
β-Hydroxybutyrate	—	126	
	ADP	253	2.0
NADH	—	69	
	ADP	95	1.4
Succinate	—	188	
	ADP	279	1.6
β-Hydroxybutyrate	—	209	
	ADP	390	2.0
Sonic fragments			
Succinate	—	791	
	ADP	1257	1.5
β-Hydroxybutyrate	—	126	
	ADP	158	1.3
Succinate	—	524	
	ADP	734	1.4
β-Hydroxybutyrate	—	391	
	ADP	495	1.3
Sonicated digitonin fragments			
Succinate	—	155	
	ADP	195	1.3
β-Hydroxybutyrate	—	117	
	ADP	155	1.3
Succinate	—	152	
	ADP	212	1.4
β-Hydroxybutyrate	—	179	
	ADP	237	1.3

* Calculated to 1 mg protein/ml.

** The respiratory control ratio is the ratio of oxygen uptake in the presence of ADP to oxygen uptake in the absence of ADP.

dried in the air and were examined in a Jem 7 electron microscope. For sonic and digitonin fragments, 5 min exposure to 1 % OsO_4 buffered with acetate–veronal buffer (pH 7.0) (PALADE's osmium[39]) was followed by the phosphotungstic acid staining.

Respiratory activity

The respiratory activity of these specimens was measured in the Clark oxygen electrode with a polarographic recorder, using the closed cuvette (3.0 ml) described by STRICKLAND, ZIEGLER AND ANTHONY[43]. The reaction mixture consisted of 20 ml 0.02 M phosphate buffer (pH 7.4), 20 ml of the medium (75 mM sucrose, 225 mM mannitol, and 0.1 mM EDTA), 4 ml 1 M KCl, and 2 ml 1 M $MgCl_2$, made up to 100 ml.

Cytochrome oxidase activity

Cytochrome oxidase activity was determined by the procedure described by SMITH AND CAMERINO[45]. The turnover number was calculated using the extinction coefficient for the heme *a* recommended by YONETANI[46].

RESULTS

Respiratory ability of preparations

The respiratory activities of the preparations (Table I) are consistent with reports in the literature with respect to respiratory control ratio and respiratory activity with the substrate used. The rate of respiration of the sonicated digitonin fragments was markedly decreased as compared to that of digitonin fragments,

TABLE II

PIGMENT CONCENTRATION IN DIGITONIN FRAGMENTS AND SONICATED DIGITONIN FRAGMENTS

Cytochromes	*Concentration (moles/mg protein)**	
	Digitonin fragments	*Sonicated digitonin fragments*
$a + a_3$	$17 \cdot 10^{-10}$	$38 \cdot 10^{-10}$
b	$11 \cdot 10^{-10}$	$23 \cdot 10^{-10}$
$c + c_1$	$11 \cdot 10^{-10}$	$22 \cdot 10^{-10}$

* These calculations are according to the wavelength pairs and absorbance indices reported by ESTABROOK AND HOLOWINSKY[52].

TABLE III

CYTOCHROME OXIDASE ACTIVITY

This calculation is with respect to the heme *a* at infinite dilution of the particles. The concentration of heme *a* in these fragments were measured spectrophotometrically (Cary 11) using wavelength pairs 605–630 mμ.

Fragments	*Turn-over number* (sec^{-1})
Digitonin fragments	200
Sonicated digitonin fragments	95

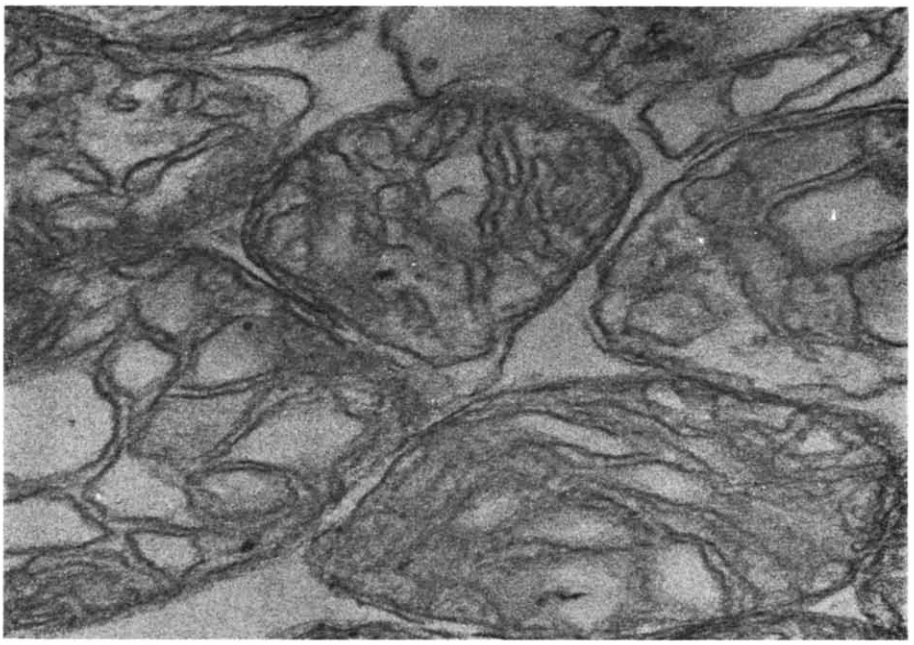

Fig. 1. Intact mitochondria fixed in OsO_4, stained with lead citrate and uranyl acetate. The inner and outer membranes of the mitochondrial envelope are clearly seen. Magnification 91 500 ×.

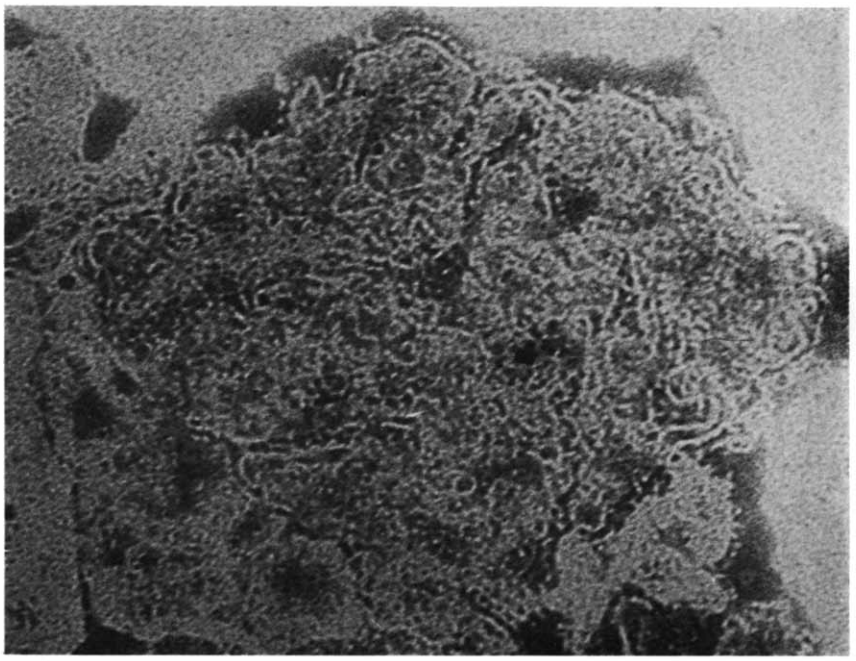

Fig. 2. Mitochondria spread and stained with phosphotungstic acid (FERNÁNDEZ-MORÁN's method showing the elementary particles. Magnification 160 000 ×.

without appreciable change in respiratory control ratio. However, the sonicated digitonin fragments contained about double the concentration of respiratory pigments present in digitonin fragments (Table II).

Cytochrome oxidase activity, determined as turn-over number (sec^{-1}) with respect to heme *a* (Table III) showed a marked decrease in the sonicated digitonin fragments when compared to digitonin fragments.

Electron microscopy

Intact mitochondria. Material fixed with OsO_4, stained with lead citrate and uranyl citrate and sectioned, showed mostly images of intact mitochondria along with some ruptured mitochondria and ghosts of mitochondria. The two membranes (outer and inner) of the mitochondria were easily discernible as were the cristae (Fig. 1).

Mitochondria stained with phosphotungstic acid by the method of FERNÁNDEZ-MORÁN *et al.*[17] showed ruptured mitochondria with the 'elementary particles' such as described by FERNÁNDEZ-MORÁN and PARSONS (Fig. 2). Mitochondria, suspended in 0.25 M sucrose and stained with phosphotungstic acid using the surface spreading

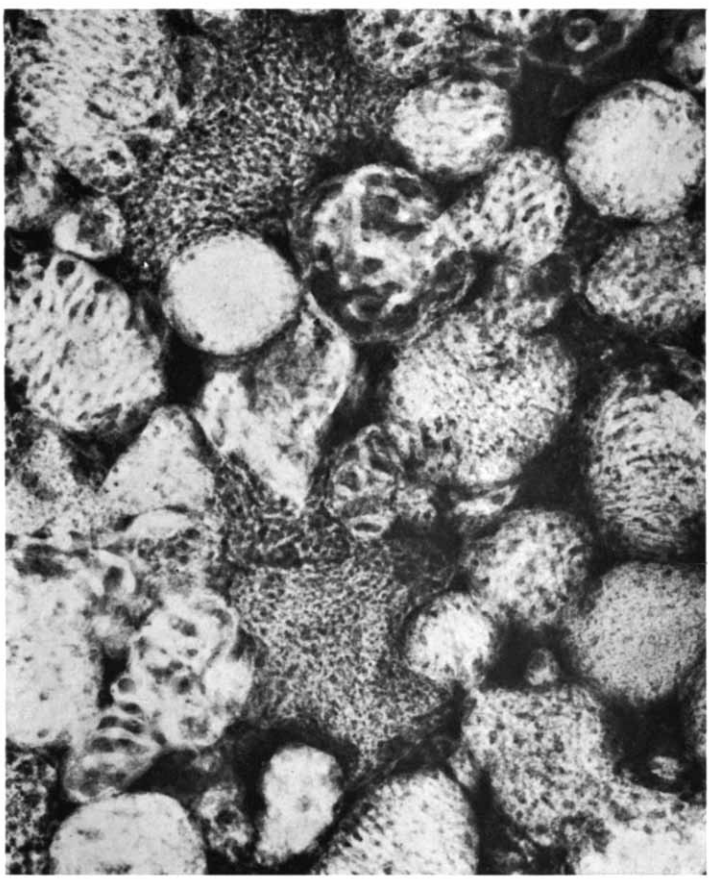

Fig. 3. Intact negatively stained mitochondria. Inner membrane subunits are not seen. Magnification 23100 ×.

technique, appeared as almost intact mitochondria with very distinct cristae (Fig. 3). When suspension in 5 mM phosphate for 15 min was used to swell the mitochondria, before phosphotungstic acid staining[14], ruptured mitochondria with cristae lined by elementary particles were observed (Fig. 4).

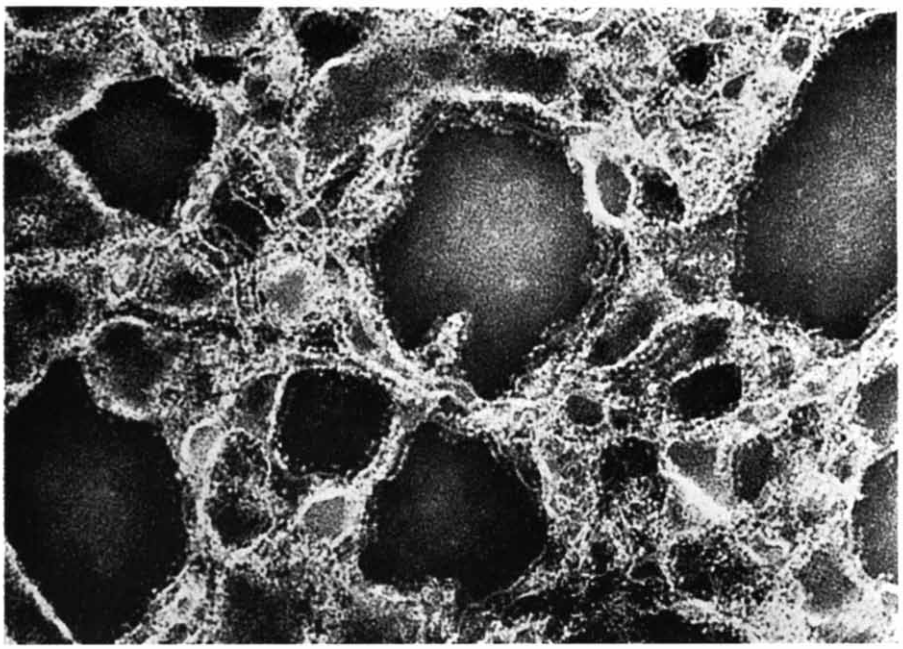

Fig. 4. Ruptured mitochondria showing effect of suspension in 5 mM phosphate. Inner membrane subunits are clearly visible. Magnification 130000 ×.

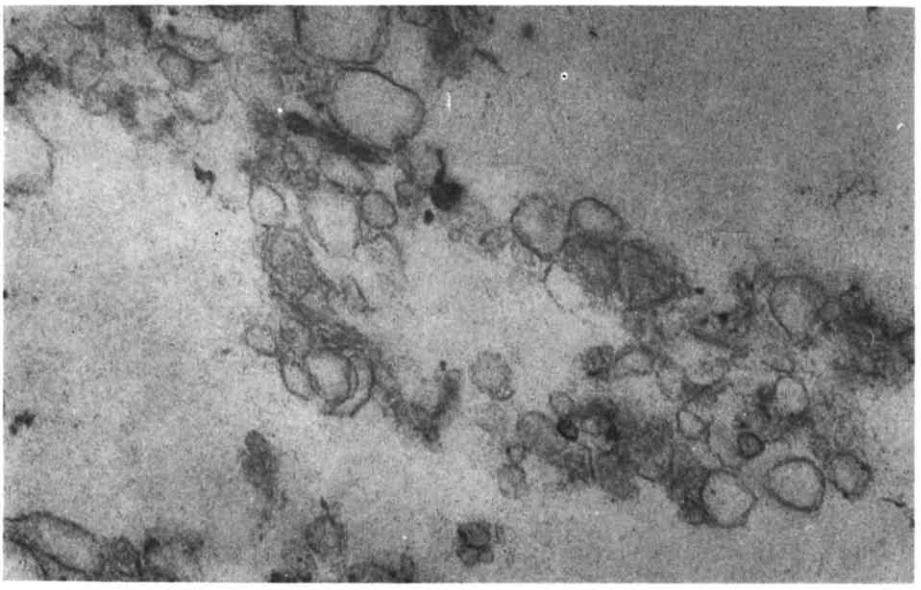

Fig. 5. Sonic fragments after OsO_4 fixation. Magnification 46000 ×.

If Tris were used as the buffer in the mitochondrial suspension medium, the entire morphology of the mitochondria was obscured on fixation with OsO_4.

Sonic fragments. The OsO_4-fixed sonic fragments showed rounded membraneous

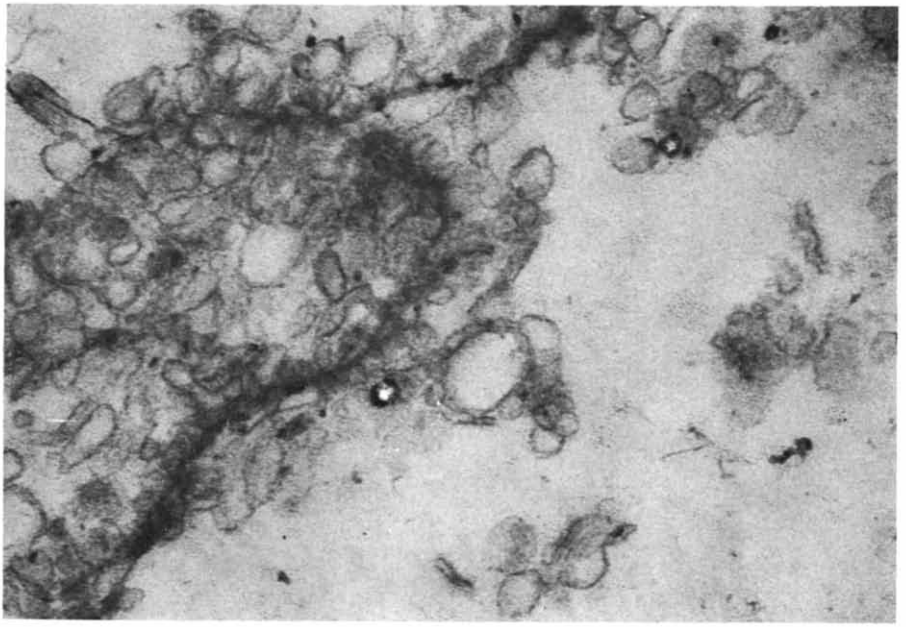

Fig. 6. Sonicated mitochondria, OsO_4-fixed and sectioned showing vague mitochondrial outlines suggesting the cristae as source of the rounded membraneous structures seen in Fig. 5. Magnification 46000 ×.

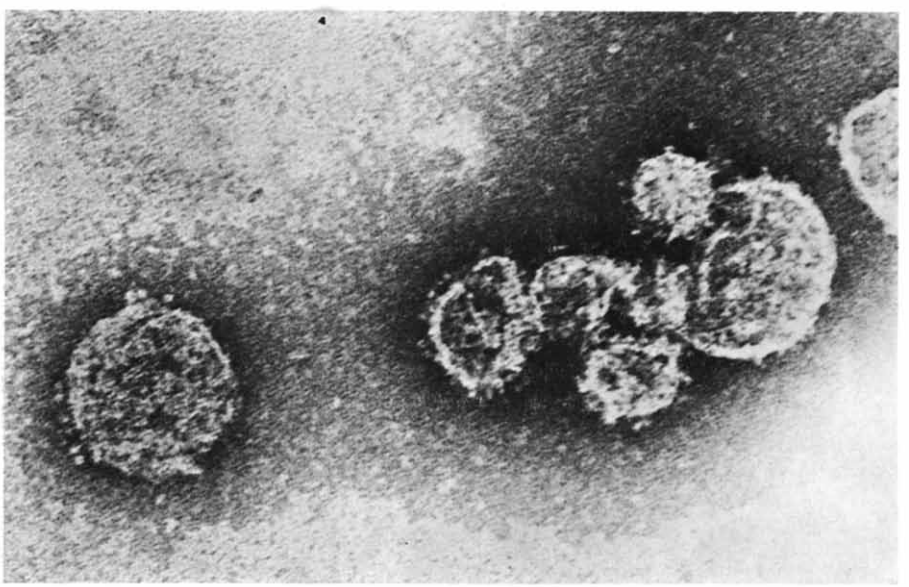

Fig. 7. Vesicular structures, as seen in Figs. 5 and 6, fixed for 5 min in OsO_4 and stained with phosphotungstic acid, show inner membrane subunits. Magnification 164500 ×.

structures (Fig. 5). In some areas, vague mitochondrial outlines were observed with swollen cristae-like profiles suggesting that the rounded membranes were derived from these swollen structures. The membrane measured from 50 to 100 Å in thickness (Fig. 6).

Brief OsO_4 fixation followed by negative staining showed vesicular figures (Fig. 7). A majority of these vesicles were observed to have 'inner membrane subunits' attached to the outer surface (Table IV).

TABLE IV

VESICULAR STRUCTURES WITH AND WITHOUT INNER MEMBRANE SUBUNITS ON THE OUTER SURFACE

Inner membrane subunits outside	*Sonic fragments* (%)	*Digitonin fragments* (%)	*Sonicated digitonin fragments* (%)
With	64	14	42
Without	36	86	58

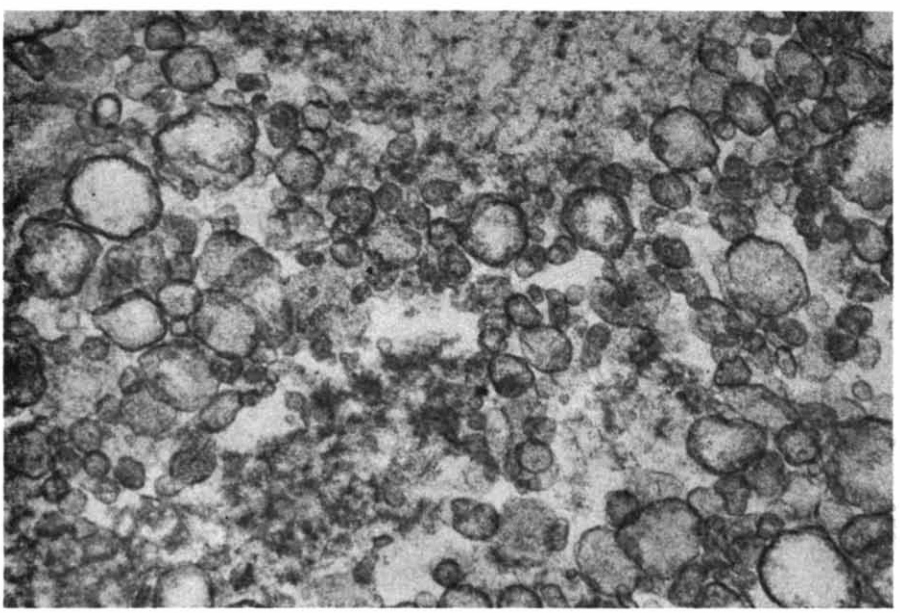

Fig. 8. Digitonin fragments fixed in OsO_4 show a rounded membraneous structure. The fibrillar material is possibly actin from the muscle fibers. Magnification 46000 ×.

Digitonin fragments. OsO_4-fixed, Epon-embedded and sectioned digitonin fragments showed vesicular structures similar to the sonic fragments (Fig. 8).

Brief OsO_4 fixation (5 min) followed by negative staining revealed vesicular structures of various sizes; but in contrast to the sonic fragments, few digitonin fragments had inner membrane subunits on the outer surface of the vesicular structures (Fig. 9 and Table IV). Vesicular structures without visible 'inner membrane

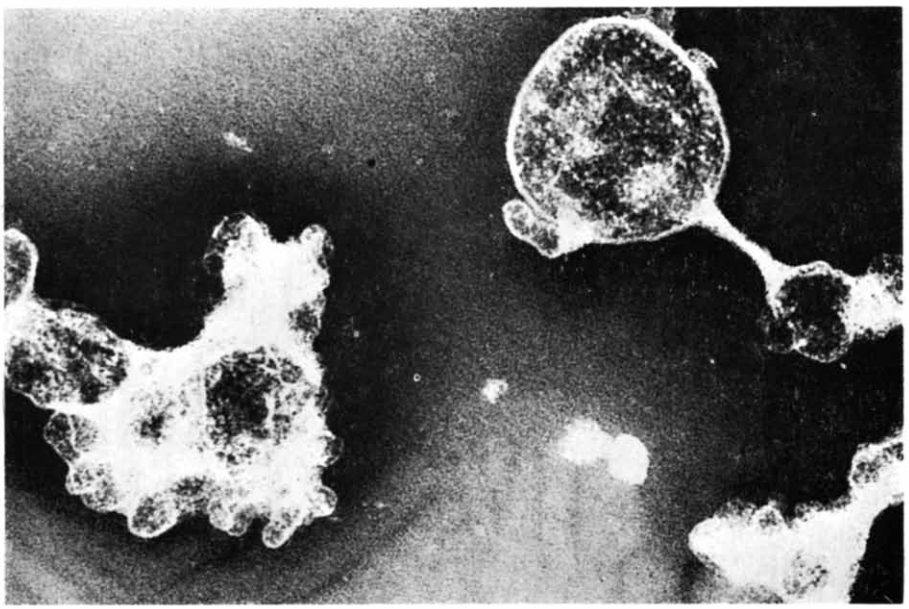

Fig. 9. Digitonin fragments negatively stained after brief OsO_4 fixation. Inner membrane subunit structures are seen within the membranes but there are few such structures on the outer surface. Magnification 152000 ×.

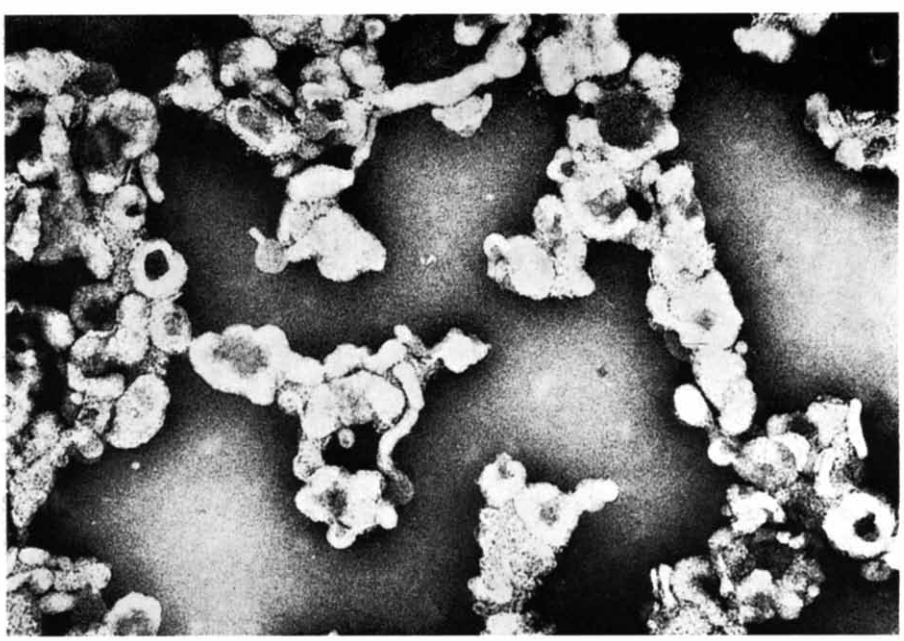

Fig. 10. Digitonin fragment preparation negatively stained without prior OsO_4 fixation shows vesicular structures, often collapsed, with a suggestion of inner membrane subunits inside. Magnification 94000 ×.

subunits' and with 'inner membrane subunits' on the inner surface were seen when digitonin fragments were suspended in 5 mM phosphate for 15 min, fixed for 5 min in PALADE's OsO_4 and negatively stained. Digitonin fragments without OsO_4 treat-

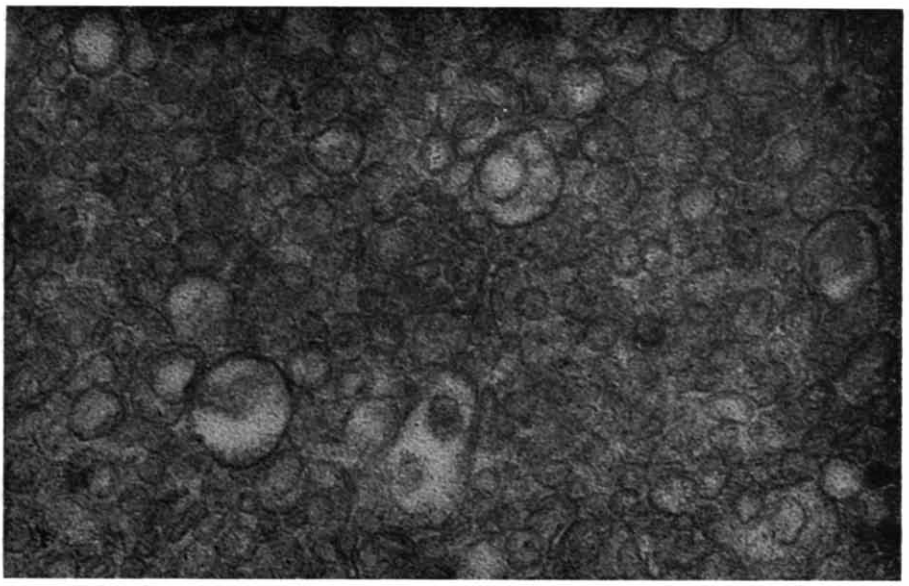

Fig. 11. Sonicated digitonin fragments. Magnification 86000 ×.

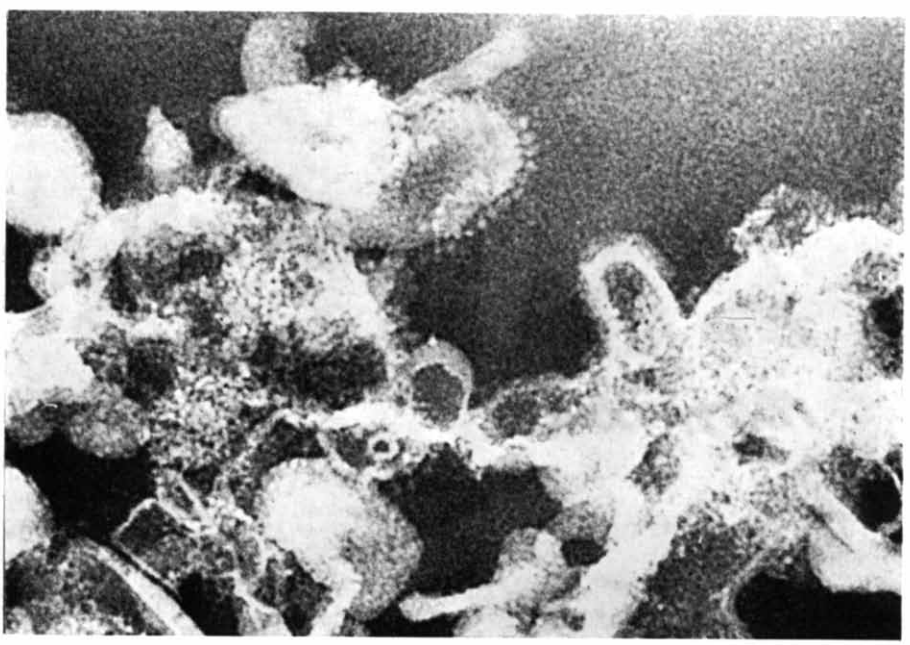

Fig. 12. Negatively stained sonicated digitonin fragments. Inner membrane subunits are seen clearly on the outer surface of some membranes (top, center). Magnification 300000 ×.

ment before negative staining showed collapsed vesicular structures of various dimensions without clearly defined inner membrane subunits (Fig. 10).

Digitonin fragments fixed with OsO_4 for 10 and 15 min prior to phosphotungstic acid staining showed a variety of structures, including many which were not vesicular. All structures were without 'inner membrane subunits'.

Sonicated digitonin fragments. The OsO_4-fixed and sectioned preparations showed a great resemblance to the similarly treated digitonin fragments (Fig. 11). However, negative staining showed that 'inner membrane subunits' were located on the outside of some of the sonicated digitonin fragments (Fig. 12), according to counts on a number of photographs, about one half (Table IV).

DISCUSSION

The respiratory control ratio obtained in the mitochondria prepared by the FERNÁNDEZ-MORÁN AND GREEN method in our laboratory is slightly lower than that obtained with the ELLIOTT AND HAAS method. We believe that the procedure used by ELLIOTT AND HAAS leaves slightly more intact mitochondria. The respiratory control ratio of heart mitochondria made on a large scale is markedly lower than that obtained with rat liver mitochondria, probably because the preparation of heart mitochondria, on a large scale, requires several hours and the structural integrity of the mitochondria is not as well maintained as in the normal one-rat-liver mitochondrial preparation.

Intact mitochondria do not show 'inner membrane subunits', regardless of the method of staining. 'Inner membrane subunits' were not seen in sectioned OsO_4-fixed material. The presence of Tris buffer in the mitochondrial suspending medium during OsO_4 fixation results in very poor visualization of mitochondrial structures[47]. However, in the phosphotungstic acid-stained preparation, the mitochondrial wall is often broken, probably due to swelling, prior to the stress occurring during dehydration of the negatively stained material, and often the cristae are extruded. The 'inner membrane subunits' are distinctly observed when mitochondria are treated with 5 mM phosphate followed by phosphotungstic acid staining.

Sonic fragments fixed with OsO_4 have a membraneous vesicular structure. Negative staining of sonic fragments shows 'inner membrane subunits' attached on the outside of many of the vesicular structures. Probably sonication breaks friable points on the cristae, giving rise to fragments which form vesicles.

Digitonin fragments observed in sectioned OsO_4-fixed specimens bear a strong resemblance to sonic fragments. Phosphotungstic acid-stained preparations of digitonin fragments differ greatly from sonic fragments in that the 'inner membrane subunits' are not seen on the outside of the majority of vesicular structures. The data show that 14 out of a hundred of these structures were similar but not identical in appearance to sonic fragments, while 86 of such structures were devoid of any 'inner membrane subunits' on the outside. The structures observed within some of the vesicles are presumed to be 'inner membrane subunits'. It is possible that during the digitonin treatment of the mitochondria the inner membrane fragments fold into a vesicle with 'inner membrane subunits' inside (Fig. 9).

It should be made very clear here that 'inner membrane subunit' structures of sonic and digitonin fragments could be clearly observed only when the fragments were fixed for 5 min in PALADE's OsO_4 prior to phosphotungstic acid staining.

Prolonging the OsO_4 treatment to 10 or 15 min gave electron micrographs showing vesicular structures of indefinable shape without any distinct structures on the 'membrane' of the vesicles. Thus longer treatment (10–15 min) with OsO_4 tends to destroy the structural assembly, which the milder treatment for 5 min preserves. The OsO_4 fixation safeguards the delicate fragments from being broken when subjected to a comparatively drastic treatment with phosphotungstic acid.

These observations of sonic and digitonin fragments in the electron micrographs support the hypotheses of LOW AND VALLIN[37] and MITCHELL[48] which asserted that the orientation of the membrane of digitonin fragments was similar to the orientation in intact mitochondria while the orientation of the membrane in sonic fragments was inverted in relation to intact mitochondria. The proposed scheme (Fig. 13) showing

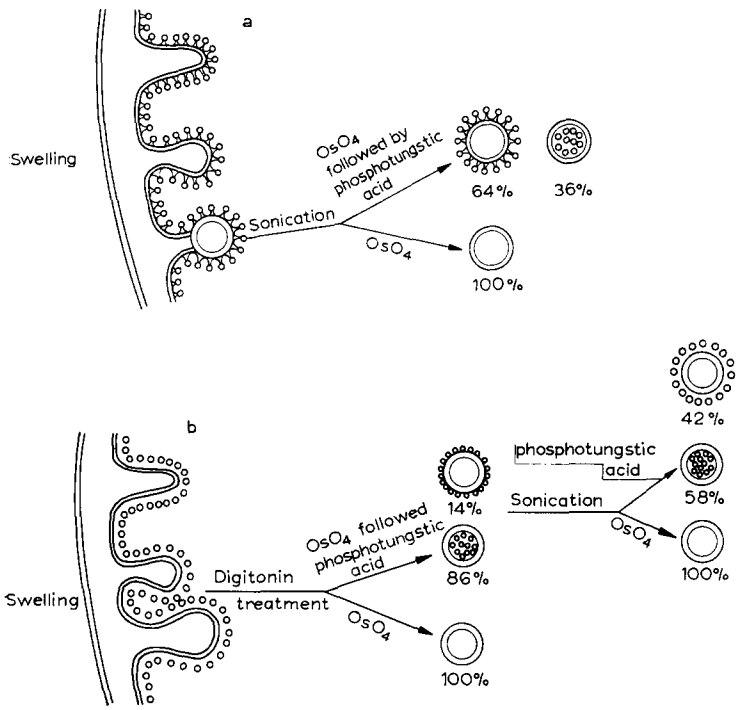

Fig. 13. Diagramatic representation of the production of: (a) sonic fragments; (b) digitonin and sonicated digitonin fragments.

the relationship of the two types of fragment structure, could account for their formation. Thus, the structural differences observed in sonic and digitonin fragments appear to be responsible for the functional differences of these fragments reported in the literature: (a) VASINGTON[29] has shown the energy-linked uptake of Ca^{2+} by digitonin fragments and not by sonic fragments. (b) CHANCE AND FUGMANN[30] and LEE[31] have observed that digitonin fragments oxidize exogenous cytochrome *c*, but reduce endogenous NAD^+; while the sonic fragments oxidize endogenous cytochrome *c* and reduce exogenous NAD^+. (c) Transfer of the high-energy phosphate bonds from ATP at the site of phosphorylation to ADP in the suspension medium is partially inhibited by atractyloside in mitochondria (BRUNI[49]), in digitonin fragments (VIGNAIS, VIGNAIS

AND STANISLAS[33]), but not in sonic fragments (LOW, VALLIN AND ALM[32]). (d) MALVIYA AND ELLIOTT have demonstrated the extractability of cytochrome *c* from digitonin fragments[34] while LENAZ AND MACLENNAN have demonstrated only very limited extraction of cytochrome *c* from sonic fragments under similar conditions[35] (confirmed in this laboratory: A.N. MALVIYA AND W. B. ELLIOTT, unpublished data).

Digitonin fragments, when sonicated, show many 'inner membrane subunits' attached to the outside of the membrane. Thus, it would appear that in the digitonin fragments, the structures which are in the interior of the rounded vesicles, become exposed during sonication (possibly by inversion of the vesicular membrane). Following exposure to ultrasound, the respiratory activity of the sonicated digitonin fragments is decreased to about one half that of digitonin fragments, while the cytochrome concentration per mg protein is doubled. The decrease in the rate of respiration observed in the sonicated digitonin fragments is due to the loss of cytochrome oxidase activity (not loss in cytochrome oxidase) during the sonication of the digitonin fragments. Respiratory pigments were studied in both digitonin fragments and sonicated digitonin fragments in the dual-wavelength spectrophotometer and all the cytochromes were reduced with succinate as well as NADH in both of these preparations. RACKER *et al.*[50] have shown that the further exposure of submitochondrial particles to trypsin and urea does not result in the loss of the respiratory chain. As the trypsin–urea-treated particles in their preparation were devoid of 'inner membrane subunits', these authors concluded that the 'inner membrane subunits' are not essential ingredients for the electron-transport chain. It has also been observed by electron micrographic studies that the prolonged exposure of submitochondrial particles to urea results in the loss of ATPase activity as well as in the loss of inner membrane subunits[51].

Digitonin fragments have properties quite similar to mitochondria, but have (1) no outer (mitochondrial) membrane, (2) higher concentrations of cytochrome pigments, and (3) less light scatter than mitochondria. They provide a system that is intermediate in complexity between mitochondria and the Keilin–Hartree heart muscle particles that should continue to be very useful in the study of energy transformation.

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. D. F. PARSONS for his valuable suggestions in fixation techniques and for several discussions during the progress of this work. We thank B. S. KABEL for cytochrome oxidase assays. This investigation was supported by research grants from the National Institutes of Health, AM 08334 to R.E.Y., 1-SO1-FR05400 and GM06241 to W.B.E., the Heart Association of Erie County, Inc. to B.P. A.N.M. is the recipient of a travel grant from C.S.I.R.O., New Delhi and W.B.E. has a U.S. Public Health Service Research Career Program Award (GM-K3-15514).

REFERENCES

1 D. F. PARSONS, *J. Cell Biol.*, 16 (1963) 620.
2 D. S. SMITH, *J. Cell Biol.*, 19 (1963) 115.
3 R. W. HORNE AND V. P. WHITTAKER, *Z. Zellforsch.*, 58 (1962) 1.

4 H. FERNÁNDEZ-MORÁN, in F. O. SCHMITT, *Macromolecular Specificity and Biological Memory*, Mass. Inst. Tech. Press, Cambridge, 1962, p. 39.
5 H. FERNÁNDEZ-MORÁN, *Res. Publ. Assoc. Res. Nervous Mental Disease*, 40 (1962) 235.
6 F. S. SJÖSTRAND, E. ANDERSSON-CEDERGREN AND U. KARLSSON, *Nature*, 202 (1964) 1075.
7 F. S. SJÖSTRAND, *J. Ultrastructure Res.*, 9 (1963) 340.
8 G. E. PALADE, in O. H. GAEBLER, *Enzymes—Units of Biological Structure and Function*, Academic Press, New York, 1956, p. 185.
9 F. S. SJÖSTRAND, *Radiation Res. Suppl.*, 2 (1960) 348.
10 S. BRENNER AND R. W. HORNE, *Biochim. Biophys. Acta*, 34 (1959) 103.
11 V. P. WHITTAKER AND R. W. HORNE, *Abstr. 5th Intern. Congr. Electron Microscopy, Philadelphia, 1962*, Vol. II, Academic Press, New York, 1962, p. 1.
12 D. F. PARSONS, *Intern. Rev. Exptl. Pathol.*, 4 (1965) 1.
13 D. F. PARSONS, W. D. BONNER, JR. AND J. G. VERBOON, *Can. J. Botany*, 43 (1965) 647.
14 D. F. PARSONS, *Science*, 140 (1963) 985.
15 A. L. LEHNINGER, *The Mitochondrion*, Benjamin, New York, 1965, p. 23.
16 H. FERNÁNDEZ-MORÁN, *Circulation*, 26 (1962) 1039.
17 H. FERNÁNDEZ-MORÁN, T. ODA, V. P. BLAIR AND D. E. GREEN, *J. Cell Biol.*, 22 (1964) 63.
18 V. P. WHITTAKER, *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, VIII-S10.
19 W. STOECKENIUS, *J. Cell Biol.*, 17 (1963) 443.
20 W. STOECKENIUS, *Ann. N.Y. Acad. Sci.*, 137 (1966) 641.
21 T. ODA AND G. NISHI, *J. Electron Microscopy (10th Symp. Soc. Electron Microscopy)*, 12 (1963) 290.
22 M. E. PULLMAN AND G. SCHATZ, *Ann. Rev. Biochem.*, 36 (1967) 539.
23 C. T. GREGG, *Biochim. Biophys. Acta*, 74 (1963) 573.
24 C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 489.
25 T. M. DEVLIN AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 507.
26 U. FUGMANN AND R. W. ESTABROOK, *Proc. 5th Intern. Congr. Biochem, Moscow, 1961*, Pergamon Press, Oxford, 1961, p. 472.
27 D. W. HAAS AND W. B. ELLIOTT, *J. Biol. Chem.*, 238 (1963) 1132.
28 W. W. KIELLEY AND J. R. BRONK, *Biochim. Biophys. Acta*, 23 (1957) 448.
29 F. D. VASINGTON, *J. Biol. Chem.*, 238 (1963) 1841.
30 B. CHANCE AND U. FUGMANN, *Biochem. Biophys. Res. Commun.*, 4 (1961) 317.
31 C. P. LEE, *Federation Proc.*, 22 (1963) 2190.
32 H. LOW, I. VALLIN AND B. ALM, in B. CHANCE, *Energy Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 5.
33 P. V. VIGNAIS, P. M. VIGNAIS AND E. STANISLAS, *Biochim. Biophys. Acta*, 51 (1962) 394.
34 A. N. MALVIYA AND W. B. ELLIOTT, *Biochim. Biophys. Acta*, 131 (1967) 210.
35 G. LENAZ AND D. H. MACLENNAN, *J. Biol. Chem.*, 241 (1966) 5260.
36 W. B. ELLIOTT AND D. W. HAAS, in R. ESTABROOK AND M. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 179.
37 H. LOW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
38 D. F. PARSONS, in R. ESTABROOK AND M. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 655.
39 G. E. PALADE, *J. Exptl. Med.*, 95 (1952) 285.
40 E. KELLENBERGER, A. E. RYTER AND J. SECHAND, *J. Biophys. Biochem. Cytol.*, 4 (1956) 671.
41 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
42 M. L. WATSON, *J. Biophys. Biochem. Cytol.*, 4 (1958) 475.
43 E. H. STRICKLAND, F. D. ZIEGLER AND A. ANTHONY, *Nature*, 191 (1961) 969.
44 B. CHANCE AND B. HAGIHARA, *J. Biol. Chem.*, 237 (1962) 3540.
45 L. SMITH AND P. W. CAMERINO, *Biochemistry*, 2 (1963) 1432.
46 T. YONETANI, *J. Biol. Chem.*, 237 (1962) 550.
47 D. F. PARSONS, G. R. WILLIAMS AND B. CHANCE, *Ann. N.Y. Acad. Sci.*, 137 (1966) 643.
48 P. MITCHELL, *Biol. Rev.*, 41 (1966) 445.
49 A. BRUNI, in J. M. TAGER, S. PAPA, E. QUAGLIARELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, B.B.A. Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 275.
50 E. RACKER, D. D. TYLER, R. W. ESTABROOK, T. E. CONOVER, D. F. PARSONS AND B. CHANCE, in T. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, John Wiley and Sons, New York, 1965, p. 1077.
51 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2475.
52 R. W. ESTABROOK AND A. HOLOWINSKY, *J. Biophys. Biochem. Cytol.*, 9 (1961) 19.