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COMPARATIVE X-RAY DIFFRACTION AND ELECTRON MICROSCOPE STUDIES OF ISOLATED MITOCHONDRIAL MEMBRANES

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SUMMARY

Inner membranes and outer membranes from rat liver mitochondria were isolated using monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* oxidoreductase as marker enzymes for outer membrane and succinate dehydrogenase for inner membrane. X-ray diffraction studies have allowed a comparison of structural parameters of the two membranes. Serial diffraction patterns obtained during controlled dehydration of the membrane preparations have been interpreted with the aid of corresponding electron micrographs in terms of an initial close packing of the membranes into lamellar arrays followed by molecular rearrangement in the system as structurally essential water is removed. Comparisons of corresponding patterns from inner and outer membrane preparations have emphasized a fundamental similarity in membrane structure with differences only in detail. No evidence for a subunit structure was obtained for either membrane.

INTRODUCTION

The development of techniques for subfractionation of mitochondria[1–3] has made possible comparative studies of isolated inner and outer membranes. Studies of composition have revealed, for example, that cholesterol is associated primarily with the outer membrane[4] whereas cardiolipin is localized in the inner membrane[5]. In studies designed to compare structural parameters, inner and outer membranes have been prepared using marker enzyme criteria to assess purity and these preparations have been examined by X-ray diffraction and electron microscopy.

Methods for isolating mitochondrial outer membrane, together with a biochemical assessment of these methods, were described in a previous communication[6]. Some preliminary X-ray diffraction data were also presented. In the present paper a method for the preparation of purified inner membrane is reported and comparative studies by X-ray diffraction and electron microscopy of inner membranes and outer membranes are presented in full.

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MATERIALS AND METHODS

Preparation of inner membrane

Intact mitochondria were prepared from liver tissue of 6–8-week-old rats as previously described[6]. For preparation of inner membrane the mitochondria from 30 g of rat liver were initially suspended by pipette in 30 ml of deionized water to allow bursting of the outer membrane and then centrifuged at 80000 × *g* for 25 min. (All sucrose solutions and deionized water were adjusted to pH 7.4 with either sodium bicarbonate or, in a few cases, trihydroxyamino methyl methane.) The resulting pellet was resuspended by pipette in 30 ml of buffered solution (15 mM bicarbonate, 2 mM magnesium sulphate, 2 mM adenosine triphosphate, pH 7.2) in order to contract the swollen inner bags. The suspension was centrifuged at 80000 × *g* for 20 min and the resulting pellet resuspended by pipette in 30 ml of the same medium, incubated at 0° for 10 min and treated in an M.S.E. blendor-type homogenizer at maximum speed for 15 sec to promote husking of the broken outer membrane from the contracted inner bag. After centrifugation at 80000 × *g* for 20 min, the final pellet was suspended in 17 ml of 0.3 M sucrose with a loose-fitting pestle-type homogenizer.

Separation into subfractions was then achieved by density gradient centrifugation at 100000 × *g* for 2 h through a discontinuous sucrose gradient in a 3 × 20 ml M.S.E. swing-out rotor. The gradient consisted of 5 ml each of payload (input of treated mitochondria to the sucrose gradient), 1.37 M sucrose and 1.60 M sucrose. After centrifugation, layers were present at each of the interfaces and there was a pellet at the bottom.

Enzyme assays

The layers from the gradient tubes were each centrifuged and the resulting pellets suspended in deionized water neutralized with sodium bicarbonate. (The pellets from the gradient tubes were suspended directly.) Aliquots of these suspensions were used for enzyme assays. Glucose-6-phosphatase, succinate dehydrogenase, monoamine oxidase and NADH-cytochrome *c* oxidoreductase were assayed as reported previously[6]. This pellet was the final preparation of inner membranes.

X-ray diffraction

Preparations of isolated membrane were washed 5 times in 5 mM ethylenediaminetetraacetic acid (pH 7.4), by successive centrifugation and resuspension. After a final packing at 100000 × *g* for 15 min, samples of the membranes were mounted in a controlled humidity chamber on the diffraction camera as previously described[7,8]. These suspensions were allowed to dry slowly at 95 % relative humidity in an atmosphere of nitrogen over a period of 10–12 h, and during this time low-angle diffraction patterns were recorded from a slit-focused X-ray beam. Point-focus low-angle patterns and wide-angle patterns were recorded from fully dried specimens. In heating experiments, fully dried specimens were allowed to equilibrate at various controlled temperatures up to about 120° in a heating cell mounted on the camera and diffraction patterns were recorded at each temperature.

Electron microscopy

Samples of freshly prepared membrane fractions were fixed in 6.5 % glutaraldehyde in 0.2 M cacodylate buffer, post-fixed in 1 % osmium tetroxide (pH 7.4), and

embedded in araldite. Specimens at various intermediate stages of drying were prepared for electron microscopy by fixation in 1 % osmium tetroxide and embedding in araldite. Thin sections were post-stained with uranyl acetate (25 % in methanol), or lead citrate[9] and sometimes with both successively. Negatively stained specimens were prepared from a suspension in 2 % phosphotungstic acid (pH 7.4).

RESULTS

Purity of the membrane preparations

Contamination of the mitochondrial fraction by endoplasmic reticulum, expressed as the ratio of recovered glucose-6-phosphatase to recovered succinate dehydrogenase was about 5 %. Separation of the mitochondrial subfractions by density gradient centrifugation resulted in a distribution of this glucose-6-phosphatase activity so that the contaminations in the separated outer and inner membrane fractions were still only 5 % and less than 5 %, respectively.

Inner membrane was identified by the presence of bound succinate dehydrogenase and 26–50 % increases in the specific activity of this enzyme with respect to payload were obtained in the separated fraction. The preparation of inner membrane was approx. 90 % pure with respect to contamination with outer membrane as indicated by a comparison of recovered succinate dehydrogenase and monoamine oxidase or rotenone-insensitive NADH-cytochrome *c* oxidoreductase in the fraction (Table I). The preparative procedure for inner membrane did not cause any detectable solubilization of these marker enzymes.

TABLE I

ENZYME DATA FOR AN ISOLATED FRACTION OF MITOCHONDRIAL INNER MEMBRANE

Numbers in parentheses are ratios with respect to succinate dehydrogenase.

Expt.	*Activities in the fraction as % of payload activities*		
	Succinate dehydrogenase	*Monoamine oxidase*	*Rotenone-insensitive NADH-cytochrome c oxidoreductase*
A	38	2.6 (0.069)	4.9 (0.129)
	48	3.8 (0.079)	5.5 (0.114)
C	54	5.3 (0.098)	7.8 (0.144)

Increases of about threefold with respect to payload were obtained for the specific activities of monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* oxidoreductase in preparations of outer membrane[6]. With respect to succinate dehydrogenase the purity of the outer membrane fraction, as an average for the two methods of preparation employed, was 90–95 % (ref. 6).

Electron micrographs of thin sections through pellets prepared from the isolated membrane fractions are shown in Figs. 1 and 2. The fraction rich in outer membrane was a clean preparation of vesicular material (Fig. 1) and the inner membrane fraction consisted of single membrane bags containing some residual matrix material (Fig. 2).

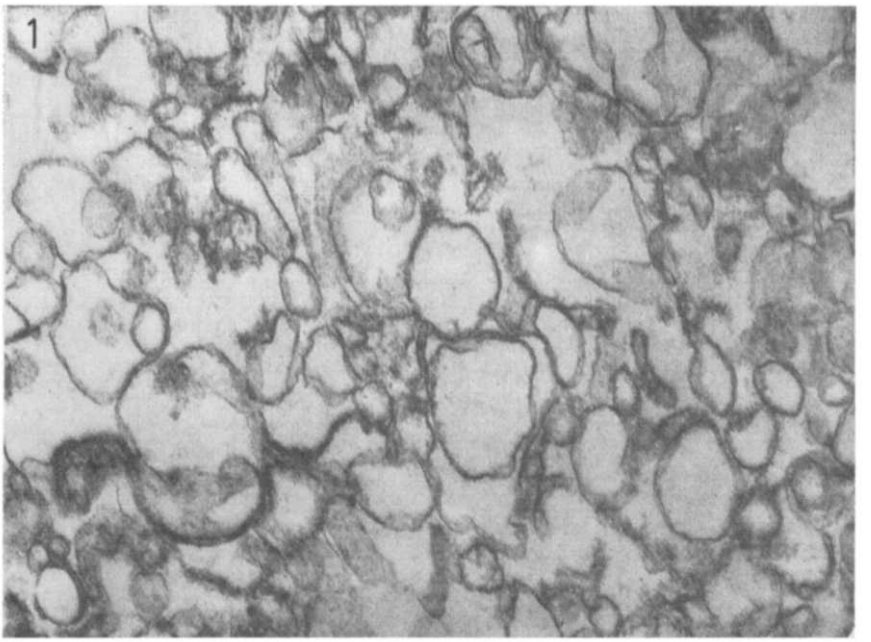


Fig. 1. Outer membrane—thin section through membrane pellet, × 60000.

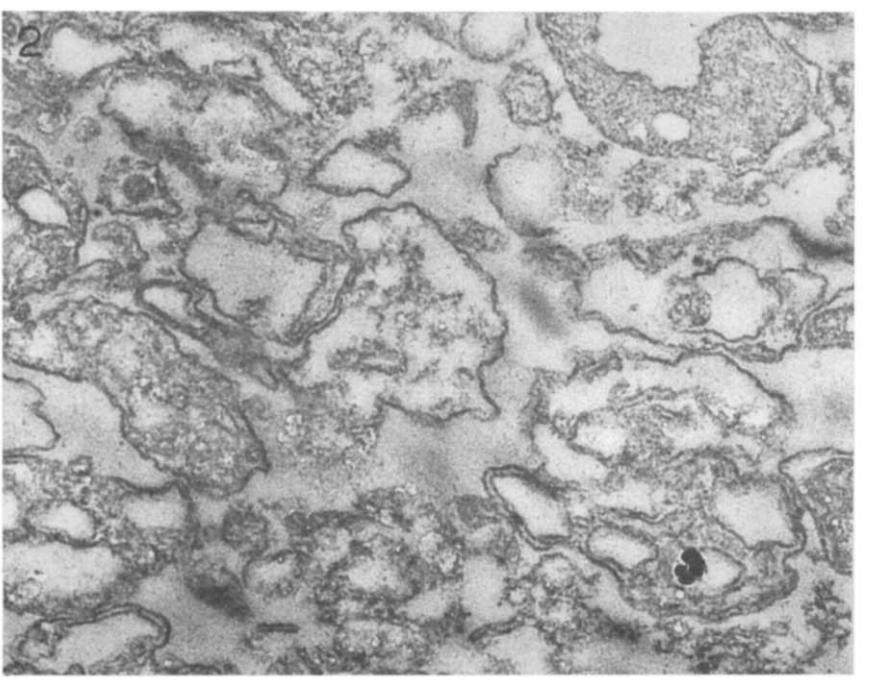


Fig. 2. Inner membrane—thin section through membrane pellet, × 60000.

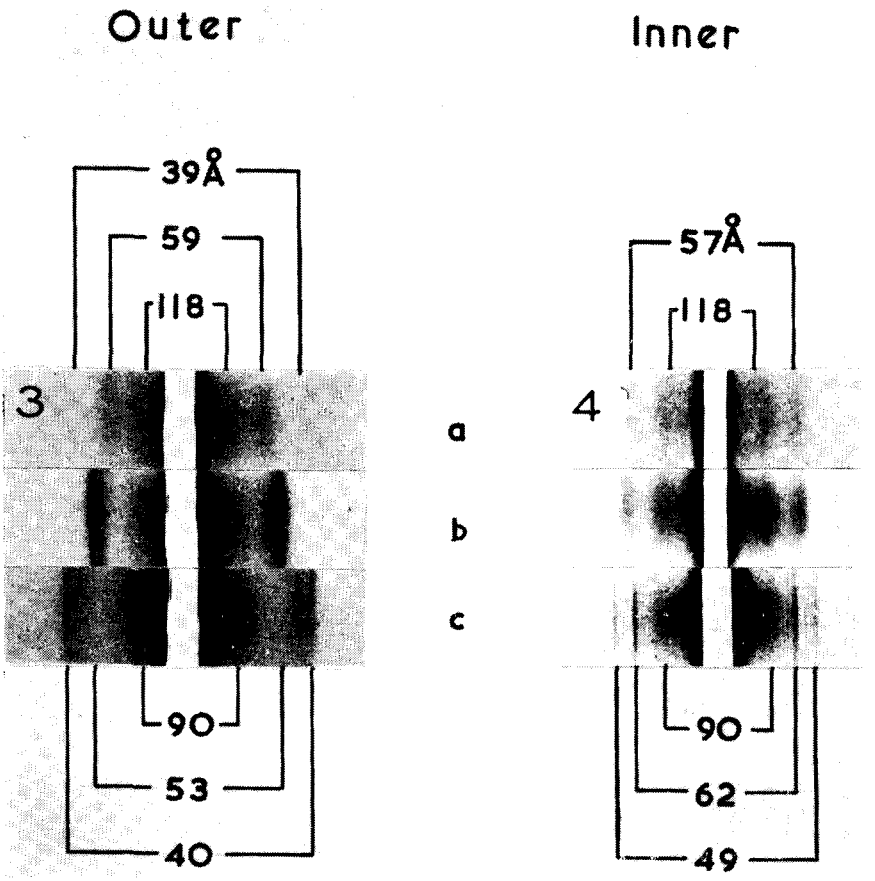
Figs. 1 and 2. Electron micrographs of isolated mitochondrial membranes.

The major proportions of negatively stained specimens of the inner membrane preparation showed the characteristic stalks and knobs, but in samples of the outer membrane preparation only a few fragments showed this feature.

X-ray diffraction and electron microscopy

For both membrane types, discrete low-angle diffraction bands were apparent after 3–5 h of controlled dehydration. At this point the amount of water remaining in the preparation was of the order of 20–30 % with respect to final dried weight. The series of patterns obtained could be identified in terms of three stages[10], an initial lamellar stage, an intermediate stage which featured a number of modifications of pattern, and a fully dried stage.

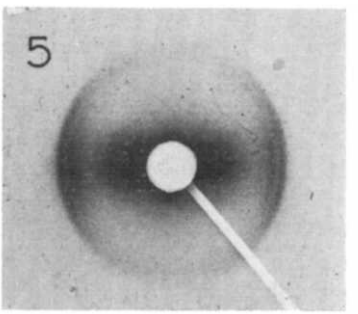
For outer membrane, the first pattern obtained in the series featured three orders of a lamellar repeat measuring about 118 Å. This lamellar spacing gradually decreased to about 95 Å whilst an intense reflexion developed at about 50 Å. This latter spacing appeared unrelated to the lamellar spacing. Patterns of the lamellar phase were not of sufficient intensity for reproduction as illustrations. Fig. 3a shows a pattern of the transition between the lamellar stage and the intermediate stage



Figs. 3 and 4. Examples from sequences of low-angle X-ray diffraction patterns recorded during dehydration of samples of mitochondrial outer and inner membranes.

and Fig. 3b shows a pattern representative of the fully developed intermediate stage. Finally, patterns from fully dried specimens showed two apparently unrelated bands at 90 Å and 40 Å (Fig. 3c).

The first pattern obtained from inner membrane showed two orders of what could be a lamellar repeat, measuring 115–120 Å, but the bands were rather faint and difficult to measure accurately. Again patterns of the lamellar phase were not reproducible as illustrations and Fig. 4a shows a pattern of the transition from the lamellar stage to an intermediate stage of dehydration. In some experiments, the dehydration series was reversed before the specimen had reached complete dryness by placing a drop of water in contact with the specimen. During rehydration, it was possible to identify three orders of a lamellar repeat, but accurate measurement was still difficult. During stage II (Fig. 4b), two rather broad reflexions became apparent at about 100 Å and 58 Å and in many cases additional faint reflexions were apparent in the region of 30–40 Å. The two broad bands were not orders of a lamellar repeat. The 58-Å band appeared to have two components in that a somewhat sharpened outer



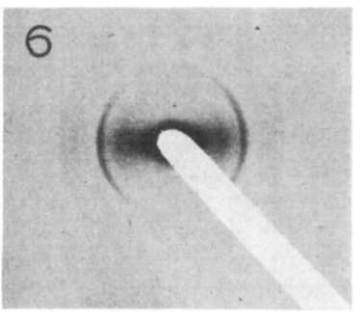


Fig. 5. Example of point-focus low-angle X-ray diffraction pattern recorded from a sample of mitochondrial outer membrane at the final dried stage. Spacings: 91 Å, 39 Å.

Fig. 6. Example of point-focus low-angle X-ray diffraction pattern recorded from a sample of mitochondrial inner membrane at the final dried stage. Spacings: 89 Å, 59 Å, 38 Å.

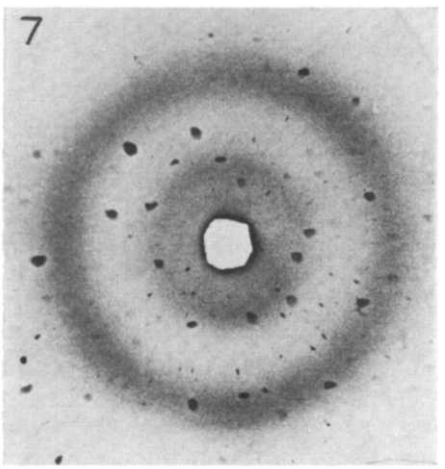


Fig. 7. Example of a wide-angle diffraction pattern from mitochondrial outer membrane. Spacings: 4.7 Å, 10.4 Å. Dense spots are diffractions from the mica window of the controlled humidity cell.

edge was superimposed on a more diffuse portion. At the fully dried stage, the highest spacing had reduced to about 90 Å, but in contrast to outer membrane, there were two additional reflexions at 60 and 50 Å or sometimes at 60 and 40 Å (Fig. 4c).

During heating of fully dried specimens, the 40-Å band featured in the pattern for outer membrane moved slowly out to about 35 Å, while in the corresponding pattern for inner membrane the 60-Å and 50-Å bands disappeared while the 40-Å band became more intense and gradually moved out to about 36 Å. The 90-Å band present in patterns from fully dried specimens remained unaltered during this treatment.

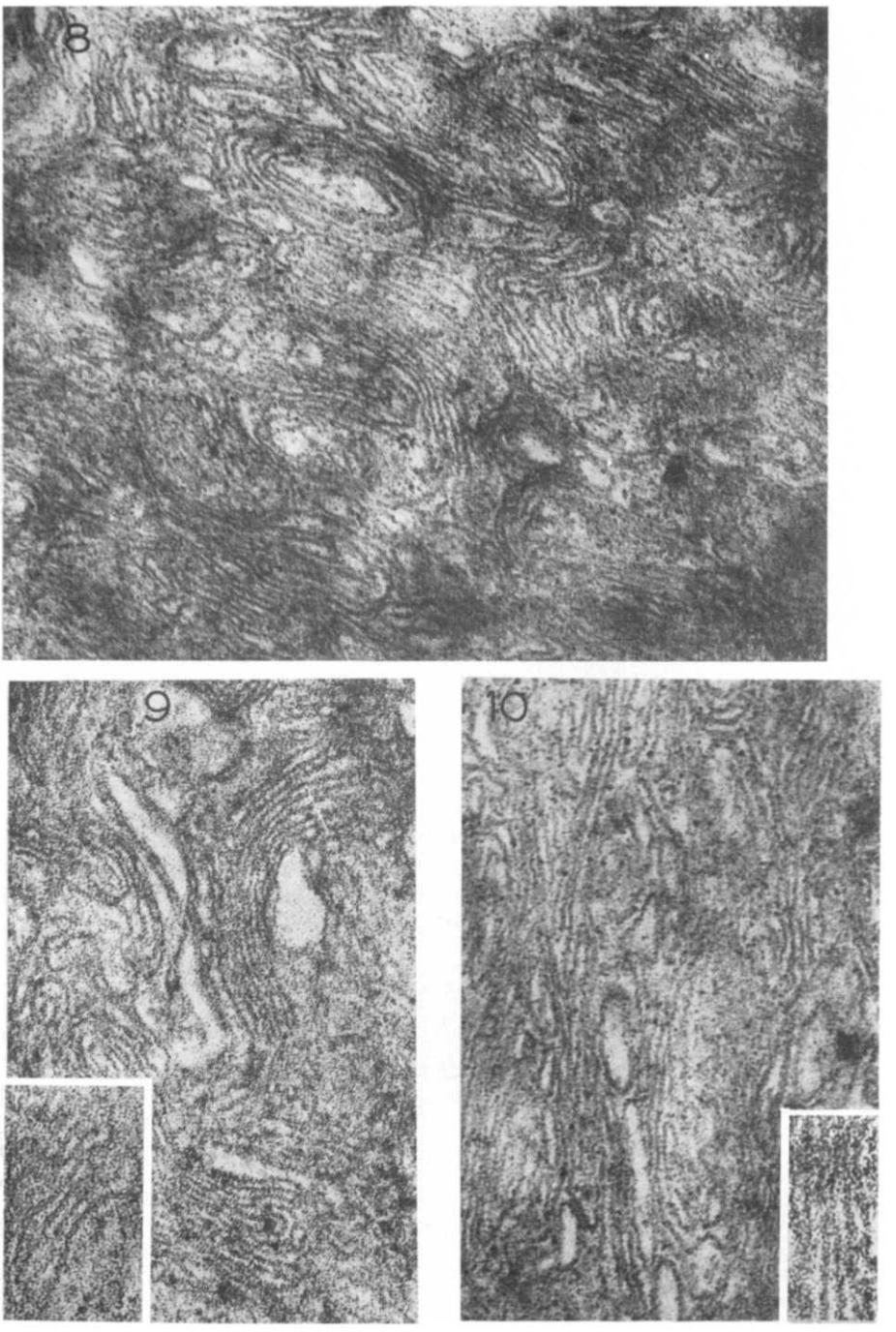
Electron micrographs of the condensed membrane samples fixed at intermediate stages of dehydration are shown in Figs. 8–10. For both systems, it was necessary to post-stain with lead citrate as well as uranyl acetate in order to obtain sufficient contrast and this produced a pronounced granularity which limited resolution. The lamellar arrays evident in preparations of both inner and outer membrane were very similar. The repeating layer measured about 100 Å and included only a single membrane. The trilamellar features of the membranes did not come into close contact. In electron micrographs of samples fixed at the fully dried stage areas of fine layering in which the repeating layer measured about 40 Å could be distinguished but it has proved difficult to obtain good micrographs from specimens fixed at this stage possibly because of poor penetration of the osmium tetroxide fixative into the tightly packed dried membranes. Small areas of fine layering were also present at late intermediate stages of drying.

Point-focus low-angle diffractions from fully dried specimens of inner and outer membrane are shown in Figs. 5 and 6. Both patterns were obtained by directing the incident X-ray beam parallel to the broad faces of the ribbon-like specimens, but such specimens were also examined with the X-ray beam perpendicular to the face. In this way, the lamellar arrays were examined both parallel to and perpendicular to the membrane layers. In the patterns shown, the reflexions are all markedly intensified in the equatorial direction and there are no reflexions of appreciable intensities along the meridian. Even the low-angle continuous scatter is oriented along the equator. The patterns that were obtained perpendicular to the face of the specimen showed reflexions in very similar positions to those in Figs. 5 and 6, but they were much less intense and poorly oriented. When specimens were examined at intermediate stages of dehydration with a point-focus beam, all of the diffraction was still in the equatorial plane.

Wide-angle patterns (Fig. 7) for both types of preparation were essentially equivalent and featured broad reflexions at 4.7 Å and 10.4 Å.

DISCUSSION

The sequences of X-ray diffraction patterns obtained from mitochondrial membranes showed striking general similarities to those obtained previously from preparations of myelin and of erythrocyte ghosts[8] but the definition of the diffractions was somewhat inferior. This could have been due in part to contamination although the relatively low contamination with other membranous material (10 %) would not have been expected to have any appreciable effect. Contamination of the inner membrane preparation with matrix material remaining in intact inner bags might have



Figs. 8–10. Electron micrographs from thin sections of samples of mitochondrial membranes dried to a stage corresponding to the development of lamellar diffraction before fixation and embedding. Fig. 8. Inner membrane, × 80000. Fig. 9. Outer membrane, × 120000; inset × 160000. Fig. 10. Inner membrane, × 120000; inset × 160000.

been expected to have some effect on the membrane diffraction but the data from inner membrane was only marginally inferior to that from outer where no such contamination was possible. Furthermore the variable matrix content which is almost certainly reflected in the variation (26 % to 50 %) in the increase of specific activities with respect to marker enzyme of different inner membrane preparations did not appear to affect the quality of the diffraction data. In fact, electron micrographs of the condensed membrane systems indicated that matrix material was to a large extent squeezed out from between the membranes in the lamellar arrays. Another factor which might be expected to affect the definition of the diffraction data would be the stability of the membranes and the damage arising from the long preparative procedure involved in isolating these particular membranous components, and in this respect it was noted that the performance of the first part of the preparative procedure in saline solution rather than in sucrose did produce a slight improvement. However, in the absence of a more substantial alternative preparative procedure this effect cannot be accurately assessed. Dehydration in an atmosphere of nitrogen minimized any degradative effects due to oxidation during the diffraction series.

The first low-angle reflexions to be recorded from both inner and outer membranes of mitochondria during controlled water removal were clearly related to a lamellar system and in each case the repeating layer measured 115 to 120 A. This dimension is very similar to that measured previously for erythrocyte membranes and the water content of 20 to 30 % with respect to dried weight at this stage was again similar to that observed for erythrocytes[8]. However, the electron micrographs showed appreciable differences. The membrane layers showed very close similarities as between the two mitochondrial membranes but the trilamellar feature was much narrower and the separations much wider than was the case with the corresponding erythrocyte ghost preparations.

As in the case of erythrocyte ghosts it is suggested that this single lamellar phase which is detected in both inner membrane and outer membrane preparations of mitochondria represents the intact membrane which retains the water essential to its structural integrity and that the minimum water content required is of the order of 20 to 30 % with respect to dried weight. The subsequent changes in diffraction patterns can be interpreted as reflecting changes in molecular organisation arising from the removal of this water. An indication of the nature of these changes is provided by the studies of the dried membrane preparation and in particular by the demonstration in both types of membrane preparation that diffractions in the 40–60-A region are much more sensitive to temperature changes in the specimen than is the band at the higher spacing. Such observations have previously been suggested to indicate the existence of chemically distinguishable phases in the dried preparations, possibly of separated lipid phases as well as a residual lipoprotein membrane phase[8,10]. The observation of patches of fine (40 A) layering as well as the major (100 A) layering in electron micrographs of sections of mitochondrial membrane preparations would again support such an observation.

This general similarity of low-angle diffraction pattern sequences from inner membranes and outer membranes suggests fundamental structural similarities particularly with respect to the molecular interactions involved. The virtually identical wide-angle patterns recorded from both preparations add further support to this suggestion and stress in particular the similarities in physical states of lipid phases.

In both cases the hydrocarbon regions must be assumed to be in a liquid state.

Some differences between outer membranes and inner membranes of mitochondria are probably reflected in the differences of detail observed in the comparison of diffraction sequences. This is most pronounced in the intermediate stage of drying when it is considered that the removal of water leads to modification of lipid–protein association. The development of a diffraction at about 50 Å which differs clearly in character from the earlier lipoprotein membrane bands is more clear-cut and rapid for outer membrane. This 50-Å diffraction is probably associated with the separation of a lipid phase, and the observed difference may simply reflect differences in the composition which have been shown to exist by lipid analyses[4,5].

Major structural differences such as are suggested by electron micrographs of negatively stained preparations of these membranes[11] are not evident in the X-ray diffraction data. So far there has been no indication of a physically pronounced subunit structure. Such indications would be expected to be most pronounced in low-angle diffraction patterns obtained using point-focused X-ray beams but the positions and orientations of all reflexions recorded in patterns obtained with the incident beam parallel to and perpendicular to the broad face of the ribbon-like specimen are such as to suggest that they arise from the stacking of layers in a lamellar array. Even the low-angle particle scatter shows a pronounced orientation in the direction of the lamellar reflexions and no appreciable intensity at right angles to this.

The X-ray diffraction observations would seem to indicate that a physically pronounced substructure such as is seen in negatively stained preparations viewed in the electron microscope is not a feature of the hydrated membrane or even of the dried membranes in the form used for X-ray diffraction studies. Consequently, although the negative staining technique may demonstrate a real difference between inner and outer mitochondrial membranes it must be concluded that the physical manifestations of this difference are greatly exaggerated by the preparative procedures involved.

Further diffraction studies, possibly with improved membrane preparations, may eventually reveal some kind of differentiation in the plane of the mitochondrial membranes, a differentiation that would be anticipated from chemical and functional data, but it is unlikely that such a differentiation will be found to correspond to a physically pronounced segmentation such as is indicated in many current speculations about membrane structure.

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