OPTICAL DIFFRACTION STUDIES OF CRYSTALLINE STRUCTURES IN ELECTRON MICROGRAPHS

II. Crystalline Inclusions in Mitochondria of Human Hepatocytes

IRMIN STERNLIEB and JACOB E. BERGER

From the Department of Medicine, Albert Einstein College of Medicine and Bronx Municipal Hospital Center, Bronx, New York 10461, and the Center for Crystallographic Research, Roswell Park Division of Health Research, Inc., Buffalo, New York 14203

ABSTRACT

Unit cell dimensions of mitochondrial crystals were determined by optical diffraction analysis of electron micrographs of human liver biopsy specimens. Identical unit cells were found in pathologic material obtained from six patients with Wilson's disease, from one patient with sickle-cell hepatitis, and from two normal subjects. These measurements led to the conclusion that the crystals observed in patients and in normal subjects were probably chemically identical. Furthermore, the relatively large size of the unit cell limits the choices for its constituents to phospholipid micelles or to relatively large protein molecules.

INTRODUCTION

Mitochondria of human hepatocytes from diseased livers frequently contain crystalline inclusions (1, 9, 12, 13, 16–18, 20–22, 25–27, 29, 31–33). Occasionally, the latter have also been observed in normal liver biopsy specimens (19, 34). In either case the inclusions are generally aligned parallel to the long axis of the ellipsoidally-shaped or elongated mitochondria, although in spherical giant mitochondria they are randomly oriented in the matrix. The origin and significance of the inclusions are unclear because their occurrence has not yet been related to any single factor. However, numerous toxic, pathologic, and possibly physiologic stimuli have been postulated as responsible for their genesis. The greater frequency with which the inclusions are found in mitochondria of diseased hepatocytes and their common association with other marked alterations of mitochondrial morphology (for example, with the changes occurring in hepatocytes poisoned by copper in patients with Wilson's disease) suggest that the inclusions are degenerative (31). However, very similar, if not identical, crystalline inclusions are also found in the giant mitochondria of normal-appearing, binucleated hepatocytes which are obviously in a phase of increased metabolic activity.

In electron micrographs, these inclusions may appear as a group of parallel filaments which are straight (Fig. 1), or tightly coiled (31) and packed close together. They may also look like regularly spaced single dots (Fig. 2), groups of dots (26), or as alternating rows of dots and undulating lines or crescents (32). Their dimensions vary considerably, even throughout a single specimen; they may measure as much as 2 μ in length and 0.5 μ in

width (19). The diameters of the component filaments range from 30 to 120 A with center-to-center separations of 50 to 200 A (16–18, 22, 29, 32, 34). Apart from the lengthening and marked increase in volume that the affected mitochondria undergo, one can frequently observe in them a peculiar orientation of the mitochondrial cristae with respect to neighboring inclusions. Instead of the usual, random distribution in the mitochondrial matrix, the cristae are arranged in parallel stacks which are inclined at an angle of about 120–140° to the long axis of the inclusions (29). Interestingly, this pattern is not unique for mitochondria of human hepatocytes, because a similar arrangement of the cristae is seen in adipose cells of a cockroach (*Periplaneta americana*) (10).

There are several interpretations of the rather broad range of measurements obtained for the inclusions by different investigators. This may be accepted as evidence of heterogeneity or as a result of inaccurate measurements obtained from electron micrographs. Such inaccuracies are known to occur as a result of artifactual swelling or shrinking occurring during fixation, from dehydration and processing of specimens, from fluctuations in electron microscope magnification, and from the limitations of visual measurements made on photographic prints. In order to compensate for the last two of these causes, we have analyzed, by optical diffraction techniques (3), the mitochondrial crystalline inclusions observed in identically processed liver biopsy specimens obtained from patients and control subjects.

We have tried in this study (*a*) to characterize the unit cell, (*b*) to compare and contrast these inclusions found in different specimens and in different subjects, and (*c*) to correlate our results with in vitro data from known crystalline forms of some cytoplasmic components.

MATERIALS AND METHODS

Needle biopsy specimens of liver were obtained for diagnostic or therapeutic reasons from four boys, one girl, and one woman, all with Wilson's disease and ranging in age from 12 to 40 years. Specimens were also obtained from one 19 year old girl with sickle-cell hepatitis and from two female subjects, 12 and 51 years old, who proved not to have any liver disease. Samples were fixed in Caulfield's solution (7) and were processed identically according to published methods (31). Fine sections of Araldite- or Epon-embedded blocks of tissue, stained with 10% uranyl acetate in methyl alcohol (30) and lead citrate (23), were examined in an RCA EMU-3H electron microscope at an acceleration potential of 100 kv and at magnifications ranging from 4,600 to 14,500. Selected contact prints of original micrographs, all made on DuPont Cronar Ortho A Litho film (duPont, E. I., de Nemours & Co., Inc., Wilmington, Del.), were analyzed with the oil cell diffractometer previously described (4), with a Helium-Neon laser (Spectra-Physics, Palo Alto, Calif.) as light source (5). Diffraction patterns were recorded on Kodak Panatomic X film (Eastman Kodak Co., Rochester, N. Y.) with exposure times which ranged from 5 to 35 seconds. The constant of inverse proportionality for our diffractometer is 1.83. Measurements on diffraction patterns were converted to spacings in reciprocal Ångstroms by the equation $A^{-1} = M \times D/(1.83 \times 10^7)$, where A = spacing in Ångstroms corresponding to a measured distance D (in mm) between spots on the diffraction pattern, and M is the magnification of the electron micrograph.

RESULTS

Electron Microscopy

For this study we used only two of the types of patterns apparent on electron micrographs of intramitochondrial inclusions: (*a*) a linear array of parallel strands about 80 A in width, with beaded profiles (Fig. 1), and (*b*) a punctate, two-dimensional lattice (Fig. 2). Most of the mitochondria which displayed patterns of the first type could be distinguished easily from normal mitochondria because they were larger, unusually elongated, and elliptical in section. In contrast, mitochondrial sections with lattice patterns were more difficult to find unless high magnifications were used, because most of them were of normal size and had circular or oval outlines indistinguishable from those of unaltered mitochondria. Consequently, mitochondria containing inclusions appeared as elongated ellipsoids with the major axes approximately parallel to the strands, while the punctate patterns were transverse sections through similar inclusions. Measurements of spacings common to both types of patterns were compatible with this conclusion.

In many mitochondria, cristae were stacked and formed angles ranging from 110° to 156° with respect to the long axes of the crystals (Fig. 3). Although the average of seven individual mean values was 129.8°, the measurements in each individual tended to fall about a different and

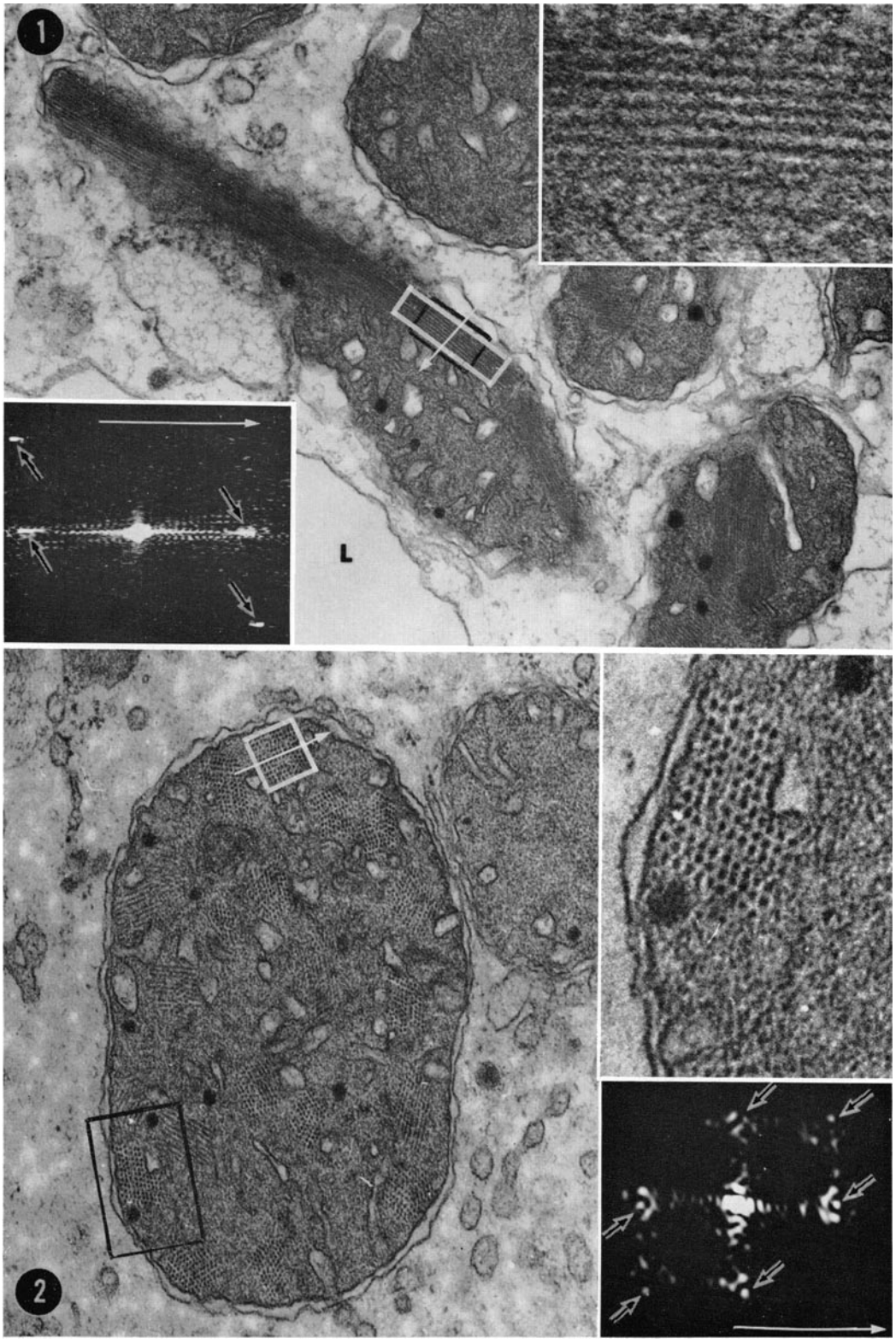
fairly characteristic value for him (Table I). This was particularly marked for the two subjects with the lowest and the highest mean values, M.K. and H.F.

Optical Diffraction

Results of optical diffraction measurements in the series of electron micrographs are presented in Table II. Dimensions are given in reciprocal Angstroms (A^{-1}). For reasons discussed in the previous paper (3), periodicities which are in fact identical cannot, when measured on different specimens, be expected to agree to within better than about 10%. All measurements on samples from the control subjects agreed with those from patients within this limit. Therefore, data from these two groups were pooled. The initial step in the analysis was to find two transforms with a common spacing (3). Transforms *1* and *4* met this requirement with diffraction maxima at 1/156 A^{-1} and 1/160 A^{-1}, respectively. These two reciprocal lattice sections must, therefore, intersect along a row of reciprocal lattice points of periodicity approximately 1/160 A^{-1}. Both *1* and *4*, the latter averaged with the essentially identical *5*, have a characteristic angle of about 90°. Therefore, roughly perpendicular to the common row are the two other reciprocal lattice vectors of the transforms, 1/156 A^{-1} and 1/130 A^{-1}. For the reason discussed above, we placed the transform of the punctate section *2* (Fig. 2) at right angles to the plane of both *1* and *4*. Identifying the 1/160 A^{-1} vector of *2* with vector 1/156 A^{-1} of *1* and identifying the 1/140 A^{-1} vector of *2* with vector 1/130 A^{-1} of *4* established a complete set of three vectors defining the reciprocal lattice. The mean value for each of these vectors, calculated from data in Table II, was 1/130 A^{-1}, 1/156 A^{-1}, and 1/156 A^{-1}. The best value for the face angle was 84°. A cell with sides 1/130, 1/156, 1/156 has a diagonal (101) section measuring 1/100 × 1/156. The observed optical transforms *9*, *8*, and *6* all showed two-dimensional arrays compatible with this measurement. Averaging the two transforms from a single specimen, *10* and *11* gave dimensions of 1/97 A^{-1} and 1/87 A^{-1} with an included angle of 57°. The (111) plane through the postulated reciprocal lattice would have dimensions 1/100 A^{-1} and 1/95 A^{-1} with an included angle of 59°. It should be noted that none of the measurements on inclusions from the patients with sickle-cell hepatitis was incompatible with those from patients with Wilson's disease. Conversion from the reciprocal cell to the object cell was accomplished as outlined in the previous paper (3). The final result, a monoclinic cell with dimensions 130 × 156 × 156 A and a face angle 96°, is shown in Fig. 4. As indicated, the long axis of the elongated mitochondrion is perpendicular to the rhomboidal face of the unit cell.

FIGURE 1 Portion of a hepatocyte from a 9 year old asymptomatic girl with Wilson's disease, J.S. (31). This section shows mitochondria with dark matrices containing arrays of parallel filaments; the cristae of these mitochondria are irregularly shaped and their outer membranes are loose. *L*, lipid droplet. × 36,000. Right upper insert is a higher magnification of the area outlined in black. × 217,000. Left lower *insert* illustrates the optical transform obtained from the area outlined by the white rectangle (Specimen No. 4, Table II). The white long arrows in the electron micrograph and transform indicate their alignment. Distances between diffraction maxima (short arrows) are inversely proportional to the corresponding periodicities within the crystal lattice. The bright, segmented, horizontal arms which extend from the center of the diffraction pattern arise from the shape of the analyzed specimen and are unrelated to the internal structure of the crystal.

FIGURE 2 Portion of a hepatocyte from a 40 year old woman with Wilson's disease, A.C., treated with D-penicillamine. In this section some patterns are formed by regularly spaced dots and others by short, parallel filaments scattered in the matrix of an enlarged mitochondrion. × 46,000. Right upper insert corresponds to rectangle outlined in black. × 147,000. Optical transform (Specimen No. 2, Table II) corresponds to the square outlined in white with alignment indicated by long white arrows. Short arrows point to diffraction maxima. The small number of diffracting points accounts for the increased background.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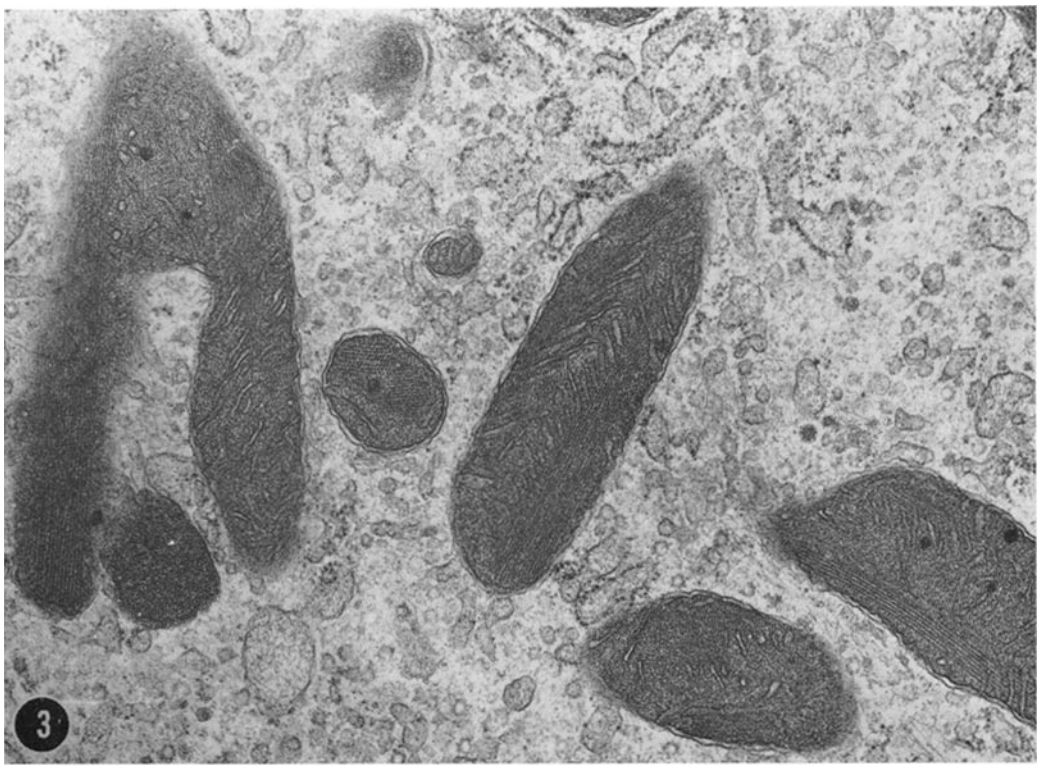

FIGURE 3 Portion of a hepatocyte from a 19 year old girl with sickle-cell hepatitis, N.J. Note parallel stacks of mitochondrial cristae at angles of about 130° with respect to the long axes of the crystals. × 36,000

TABLE I

Measurements of Angles between Stacked Cristae and Elongated Crystals

Subject*	n	Mean ± SD	Range
M. K. (Control)	6	118.5° ± 6.7	110°–130°
A. C. (W.D.)	14	126.0° ± 7.0	113°–134°
A. S. (W.D.)	7	130.1° ± 3.7	124°–135°
S. S. (W.D.)	6	130.6° ± 5.5	124°–137°
R. T. (W.D.)	8	131.2° ± 8.7	126°–137°
N. I. (SS.H)	10	134.6° ±11.4	126°–156°
H. F. (W.D.)	5	138.0° ± 4.0	135°–145°

* Control = normal control subject; W.D. = Wilson's disease; SS.H = sickle cell hepatitis.
n = number of measurements per subject.

DISCUSSION

These mitochondrial inclusions, variously described as filamentous (19), "crystalloid" (12), or "paracrystalline" (26), actually are crystals, i.e., they are solids whose subunits are arranged in an orderly, repetitive three-dimensional array (11). The possibility that they are derived from mitochondrial cristae has been considered (12), but no direct morphologic proof of such a relationship has been obtained as yet. The peculiar spatial orientation apparent in the stacking of cristae at angles, with respect to the long axis of the crystals (Fig. 3), ranging from 110° to 156° with a mean of about 130°, suggests that some relationship may exist between these structures. Available data

TABLE II

Periodicity of Mitochondrial Inclusions Obtained from Optical Transforms

Specimen no.	Subject	Transform type*	Periodicity (A^{-1})	Angle
1	J. F. (W.D.)	L	1/156 × 1/156	90°
2	A. C. (W.D.)	L	1/160 × 1/140	84°
3	A. S. (W.D.)	L	1/160 × 1/142	82°
4	J. S. (W.D.)	L	1/160 × 1/130	93°
5	J. M. (C.)	L	1/158 × 1/135	88°
6	S. S. (W.D.)	L	1/166 × 1/110	86°
7	N. I. (SS.H)	L	1/164 × 1/115	82°
8	J. S. (W.D.)	L	1/160 × 1/112	82°
9	R. T. (W.D.)	L	1/148 × 1/108	88°
10	A. S. (W.D.)	L	1/100 × 1/85	52°
11	A. S. (W.D.)	L	1/94 × 1/88	63°
12	J. M. (C.)	R	1/158	
13	J. M. (C.)	R	1/152	
14	A. C. (W.D.)	R	1/150	
15	A. S. (W.D.)	R	1/146	
16	J. M. (C.)	R	1/135	
17	J. M. (C.)	R	1/135	
18	S. S. (W.D.)	R	1/132	
19	N. I. (SS.H)	R	1/126	
20	S. S. (W.D.)	R	1/124	
21	N. I. (SS.H)	R	1/121	
22	R. T. (W.D.)	R	1/120	
23	R. T. (W.D.)	R	1/120	
24	R. T. (W.D.)	R	1/104	
25	N. I. (SS.H)	R	1/104	
26	J. M. (C.)	R	1/92	
27	J. M. (C.)	R	1/92	

* L = Two dimensional lattice; R = row of points.
C. = Control subject; W.D. = Wilson's disease; SS.H = Sickle cell hepatitis.

are insufficient for a more precise definition of this relationship, although a three-dimensional reconstruction of an affected mitochondrion has been attempted by Spycher and Rüttner (29). Further studies of sections made at known angles to the crystal axes would be helpful.

In determining the parameters of a crystalline structure from electron micrographic evidence, it is essential to recognize that although the particular obliquity of the plane of sectioning can affect the electron microscopic appearance and lattice

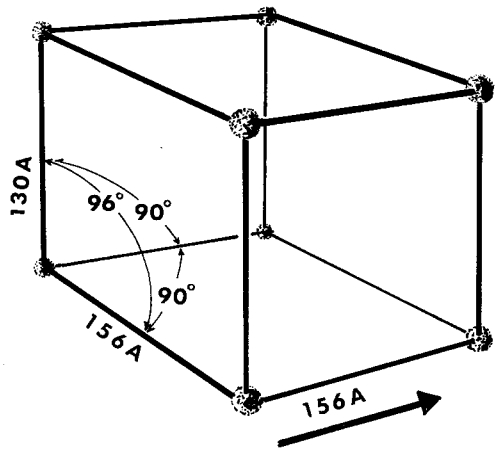

FIGURE 4 Unit cell of intramitochondrial crystal. The arrow indicates the direction of the long axis of the mitochondrion relative to the unit cell.

spacings, the full three-dimensional lattice (unit cell) characterizes the structure unequivocally with respect to its compatibility with any given periodicity. In our case, for example, three quite different two-dimensional nets were observed, but all were compatible with a single three-dimensional structure sectioned at various obliquities. Five different linear periodicities were observed, again all derived from a single three-dimensional lattice (Table II). Further, the dimensions of the unit cell determined only from material obtained from patients with Wilson's disease and the dimensions of the unit cell derived solely from control subject biopsy material were identical within the limits stated above. The dimensions of inclusions from the patient with sickle-cell hepatitis were also compatible with this unit cell. This fact indicated that the crystals found in pathologic and in normal livers were probably chemically identical, because the unit cell parameters of a crystal constitute a highly distinctive set of measurements, and it is unlikely that two chemically dissimilar crystalline materials would share a common set of dimensions.

What can we say of the chemical nature of these intramitochondrial inclusions? Earlier investigators believed these inclusions to be phospholipids (32), since mitochondria contain abundant amounts of phospholipids of which cephalin ordinarily predominates. Proteins, nucleic acids, and various small molecules make up the remainder of the constituents, and possibilities that

the crystals contain "protein-like" material (18) or abnormal metabolites (16) have been considered. The monoclinic unit cell found here has a volume of 3.15×10^6 A^3. This corresponds to a minimum molecular weight for the crystalline material of about 200,000 and a maximum value in the neighborhood of 1.5×10^6 (3). For centrosymmetric micellar structures, these figures would be halved (3). Therefore, we can conclude that the observed structures are not crystalline phospholipid. We cannot exclude, however, the possibility that we are dealing with micelles. Among the several distinct periodic micellar arrays known to occur in lipid-water mixtures is a hexagonal array of long cylinders with diameters of approximately 20–35 A and center-to-center distances of 55–70 A (14). A cylindrical micelle extending the length of the unit cell (156 A) and having a diameter of about 37 A would have a molecular weight of about 100,000. On the other hand, of the various mitochondrial protein components we may exclude cytochrome *c* because of its relatively small molecular weight (mol wt 12,300) (15). Also cytochrome c_1 (mol wt 53,000) (24), mitochondrial structural protein (mol wt 22,500–43,000) (8), and the core protein of Complex III (mol wt 40,000–50,000) (28) probably can be eliminated for the same reason. Complex III of the mitochondrial electron transfer with a molecular weight of 275,000–330,000 (28) and particles with diameters of about 80–100 A (2) cannot be definitely excluded. The smallest icosahedral virus studied to date, ϕX174, has a diameter of approximately 250 A which is too large for the observed unit cell (6).

This study illustrates both the capabilities and the limitations of optical diffraction analysis of crystals in electron micrographs. Although the technique allowed us to distinguish between simple phospholipid and large protein molecules, it did not permit us to identify the substance because of the incompleteness of our knowledge of crystalline patterns of mitochondrial constituents. It permitted us to establish that the intramitochondrial crystals seen in our biopsy material from pathologic livers were probably identical to those in normal livers. We were also able to conclude that these crystals were not simple phospholipids because unit cells of the latter are quite different in size from the crystals observed in our material.

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