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A Lattice Structure in Beef Heart Mitochondria Induced by Phosphotungstic Acid

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Abstract

Ordered arrays of structured material were visualized in the intracristal space of isolated beef heart mitochondria in two ways. Under standard conditions of fixation, structured material in the intracristal space appeared as paracrystalline arrays nestled between two apposing membranes. When mitochondria were preincubated with phosphotungstic acid (PTA) prior to fixation, the structures in the mitochondrial intracristal space took on an open lattice structure. Such structures, either paracrystalline or lattice, could not be demonstrated in the mitochondrial matrix space under these conditions.

Pretreatment with PTA prior to fixation increased greatly the frequency with which structured material was observed within the mitochondrial intracristal space. Visualization of the PTA-induced lattice structures appeared to be pH dependent, being most clearly seen between pH 7·0 and 7·5. Above pH 7·5, lattice structures could not be seen, whereas at pH values below 7·0, the observed structures in the intracristal space no longer retained an organized lattice structure but became amorphous. Increasing the concentration of PTA from 0·1% to 3·5% or the incubation time from 5 sec to 1 h did not significantly alter the frequency of observation of lattice structures, as long as the mitochondrial preincubation with PTA was carried out between pH 7·0 and 7·5.

Introduction

Crystalline structures or inclusion bodies in the mitochondrion have been reported by numerous laboratories.[1–21] In many cases, structured material was demonstrated within the matrix space of the mitochondrion. In other reports [3,6,8,9,20,21] paracrystalline structures were observed within the intracristal space of the mitochondrion. In this communication, attention will be focused on the visualization of structured material within the intracristal space of beef heart mitochondria.

Although there has been much work done on the localization of enzymes within the different mitochondrial compartments, relatively little is known about the composition and function of the proteins in the mitochondrial intracristal space. It would appear that adenylate kinase, and nucleoside diphosphokinase are located in the intracristal

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space of rat liver mitochondria,[22,23] whereas general agreement about the composition of the intracristal space in heart mitochondria is lacking.[24,25]

When isolated mitochondria are exposed to sucrose, the intracristal space expands and the matrix space becomes condensed. As a result the intracristal space is transparent and the matrix space is electron opaque in electron microscopy. It would be wrong to assume that because the intracristal space appears transparent, protein or other materials do not exist in the intracristal space. In this communication experiments are described in which PTA was introduced to detect and visualize materials which might exist within the intracristal space. PTA has long been used as a protein precipitant at acidic pH, and it was concluded that PTA acts as an anionic stain for positively charged groups of proteins.[26–29] Selective reactivity of PTA to certain amino acids and albumins was extensively studied by Silverman *et al.*[26]

Materials and Methods

Heavy beef heart mitochondria were prepared by the method of Crane *et al.*[30] as described by Hatefi and Lester.[31] Mitochondria were suspended at a final concentration of 50 mg per ml in a medium which was 0·25 M in sucrose and 10 mM in Tris-chloride, pH 7·5.

PTA solutions were prepared in 0·25 M sucrose and buffered to different pH values, ranging from 3·5 to 10·0. The concentrations of PTA tested were from 0·1% to 3·5%.

0·2 Ml of a mitochondrial suspension (10 mg of total protein) was mixed with 4·8 ml of a PTA solution of specified concentration at a given pH. Incubation was carried out at 25° for varying lengths of time, from 5 sec to 1 h. After incubation with PTA, 0·1 ml of 50% glutaraldehyde solution (Fischer Co.) was added to the sample. To post-fix the sample 1% osmium tetroxide was used. Dehydration was initiated by 1% uranyl acetate in 25% ethyl alcohol and followed by a graded series of increasing ethyl alcohol concentrations. The samples were embedded in Epon.[32] Thin sectioning was carried out with a diamond knife and stained in lead citrate. Sections were examined in a Hitachi-HU-11B electron microscope operated at 75 kV.

Protein was determined by the method of Gornall *et al.*[33] Samples were solubilized by the addition of 0·03 ml of a 10% solution of potassium deoxycholate, pH 13 in a final volume of 3 ml.

Results

I. *Visualization of Paracrystalline Structures under Standard Conditions of Fixation*

When beef heart mitochondria are isolated in 0·25 M sucrose, the intracristal space is expanded and appears transparent in electron microscopy under usual conditions of fixation with glutaraldehyde followed by osmium tetroxide. With careful electron microscopic examination and at high magnification, paracrystalline structures can be observed in the mitochondrial intracristal space. Usually the structured material appears as a continuous line (Fig. 1) or has an array of subunits having a periodicity of 110–120 Å, as is illustrated in Fig. 2. In rare cases, parallel rods can be seen (Fig. 3).

Within the intracristal space paracrystalline structures can be observed either (i) between the inner and outer membranes (Figs. 1 and 4) or (ii) where paired cristal

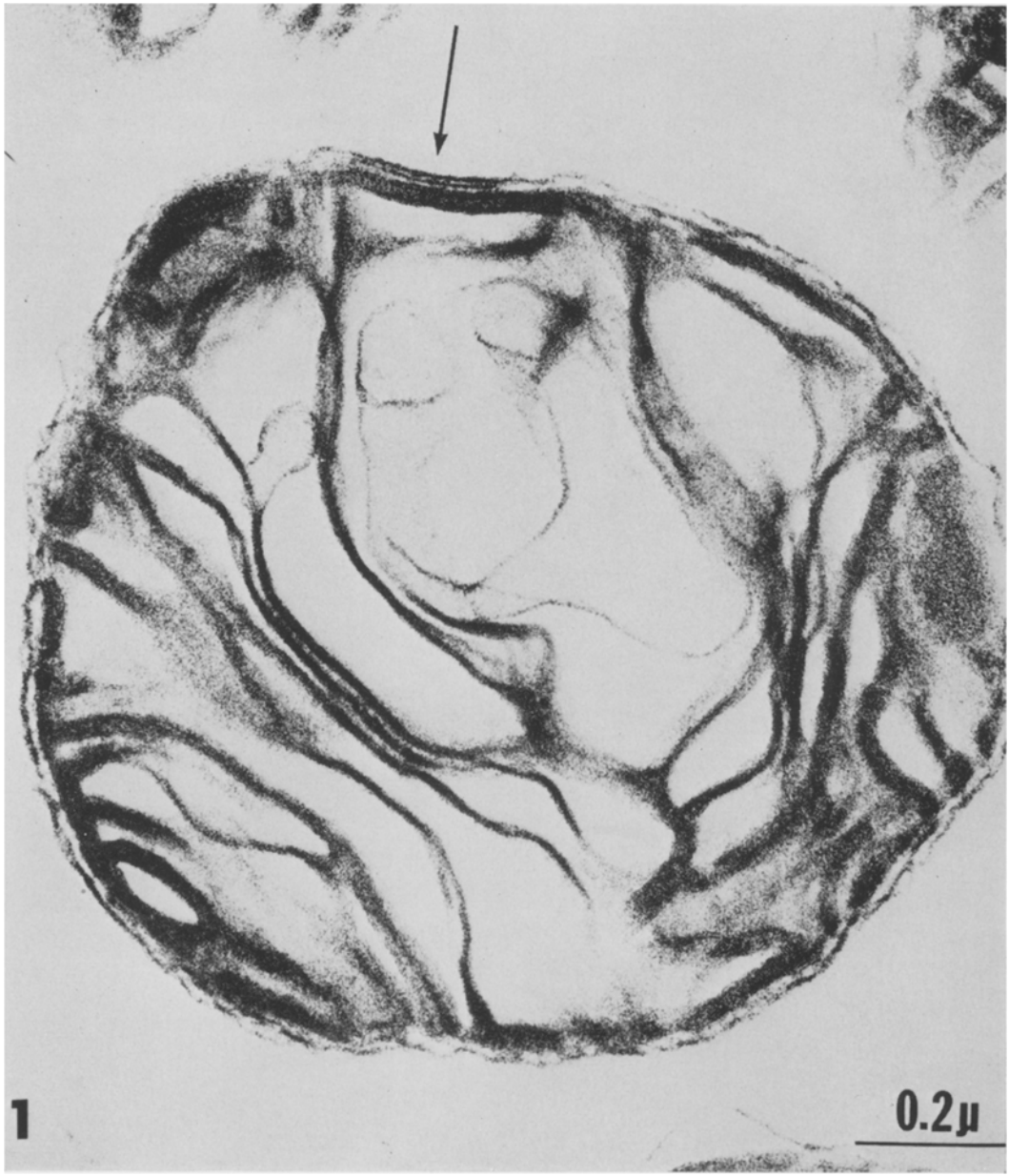

Figure 1. Mitochondria in the aggregated nonenergized configuration showing paracrystalline structures as a continuous line between the outer and inner membranes. × 142,500.

membranes are parallel and close together (Figs. 1, 2, 3, 5 and 6). The energy state of the mitochondrion does not appear to influence the visualization of the paracrystalline structures, for they can be seen in all mitochondrial configurations,[34] whether orthodox (Fig. 5) or aggregated, nonenergized (Figs. 1 and 4), aggregated, energized (Figs. 2 and 3)

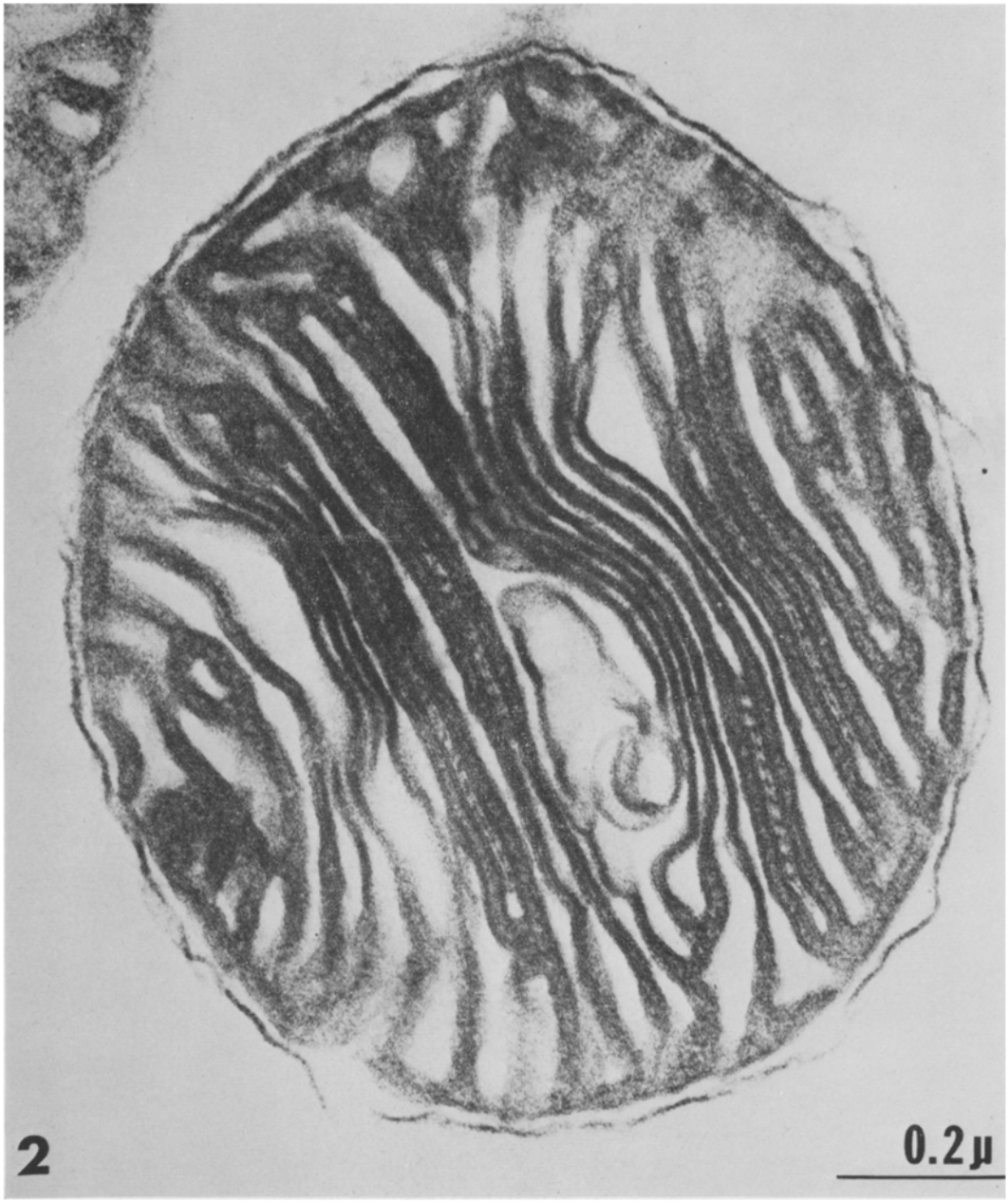

Figure 2. Mitochondria in the aggregated energized configuration showing paracrystalline structures within paired cristal membranes. Note the subunit periodicity of the paracrystalline structures. × 197,500.

or twisted-energized (Fig. 6). The only apparent requirement for the visualization of paracrystalline structures in the intracristal space is that paired membranes be parallel and close together, approximately 100 Å one from another.

The severe limitation of this method of visualization is that under our standard con-

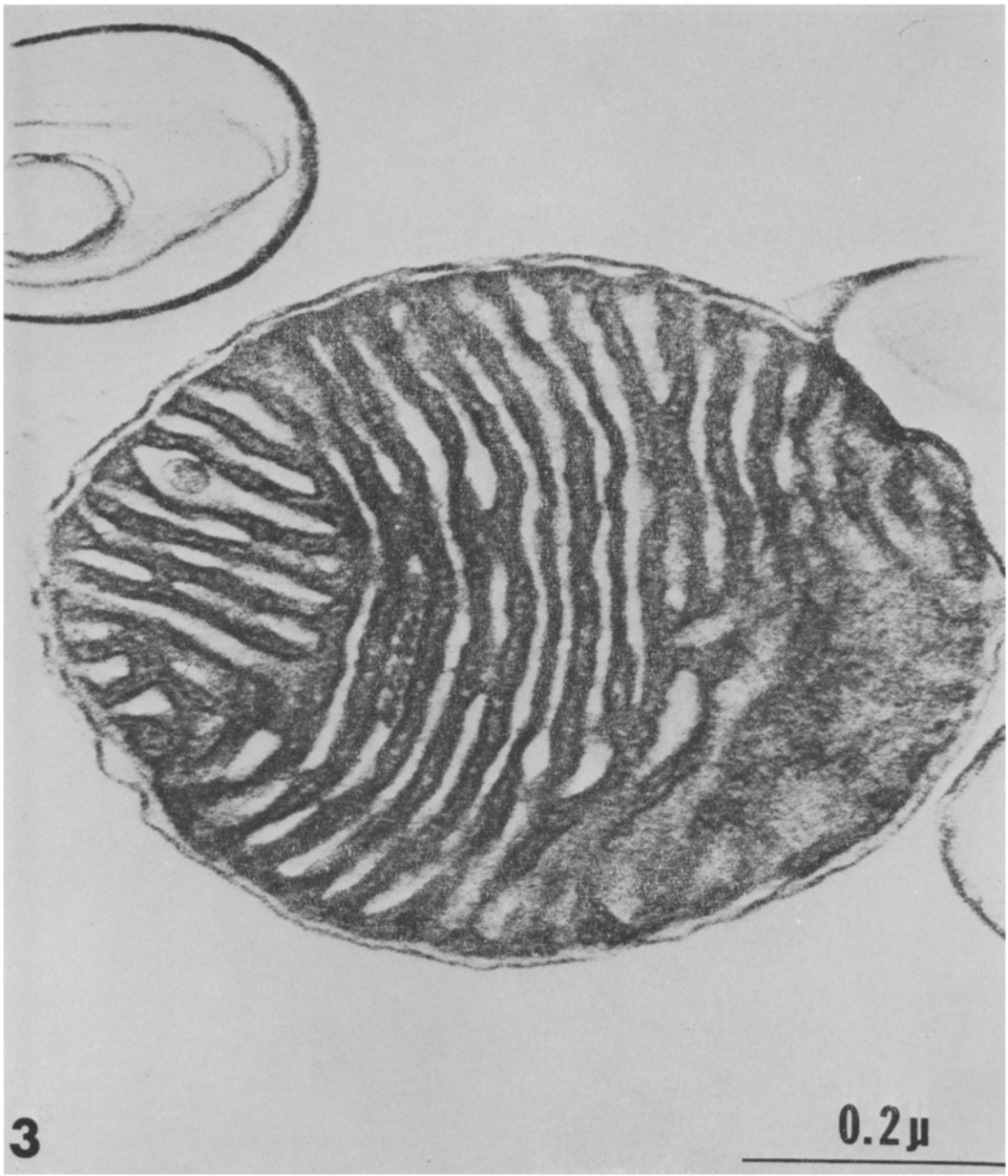

Figure 3. Mitochondria in the aggregated energized configuration having paracrystalline structures appearing as parallel rods within paired cristal membranes. × 210,000.

ditions of fixation, the frequency of observation of paracrystalline structures in the intracristal space is low. In a given field of mitochondria, careful scrutinization at high magnification is required in order to find paracrystalline structures nestled between parallel membrane systems.

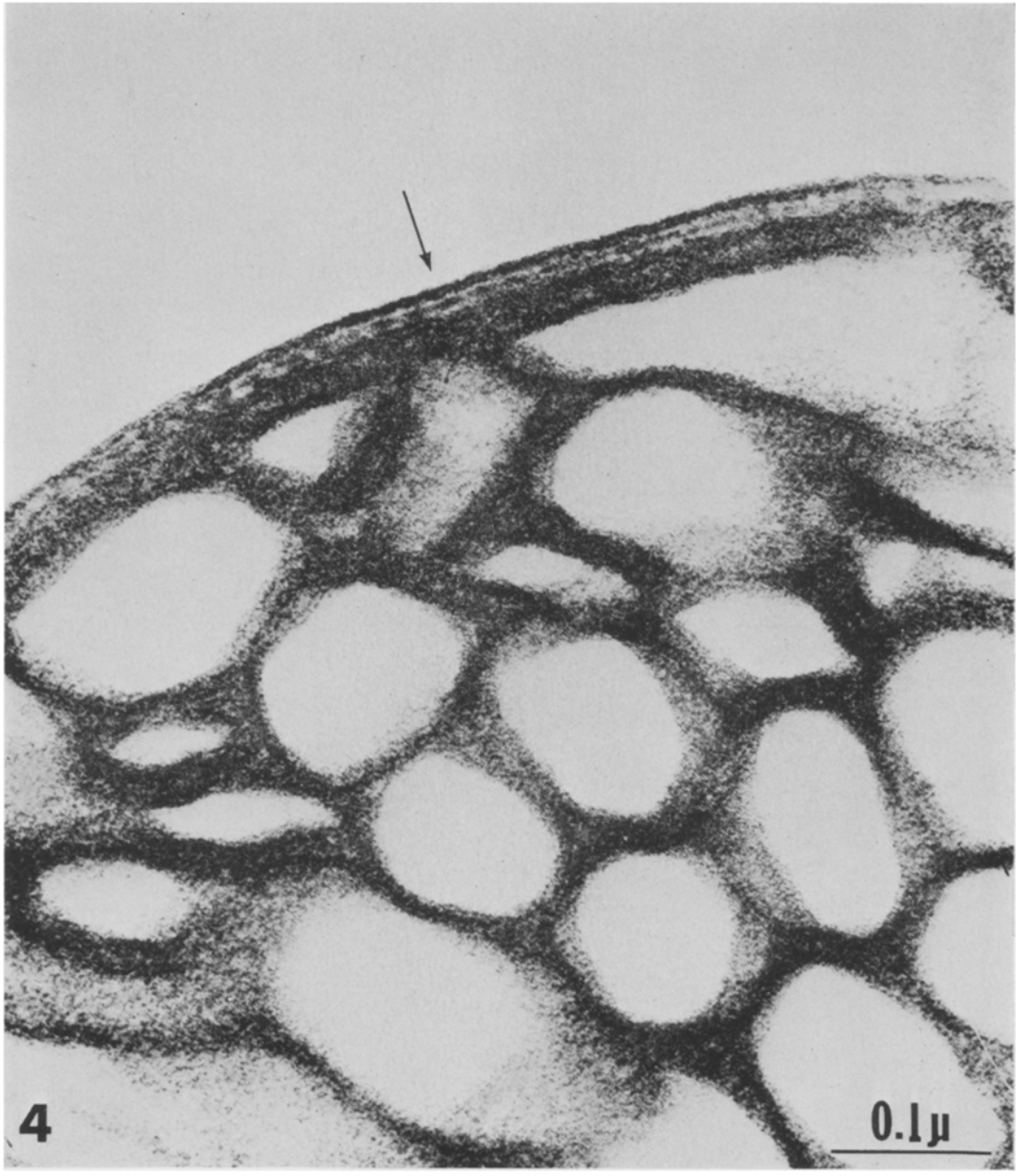

Figure 4. Mitochondria in the aggregated nonenergized configuration having paracrystalline structures with periodic substructure lying between the outer and inner membranes. × 300,000.

II. *Induction of Lattice Structures in Mitochondria Exposed to PTA.*

Fig. 7 is an electron micrograph of mitochondria which were incubated with 1% PTA at pH 7·5 for 20 minutes at room temperature prior to fixation with glutaraldehyde followed by osmium tetroxide. Lattice structures were seen in about 50% of the mito-

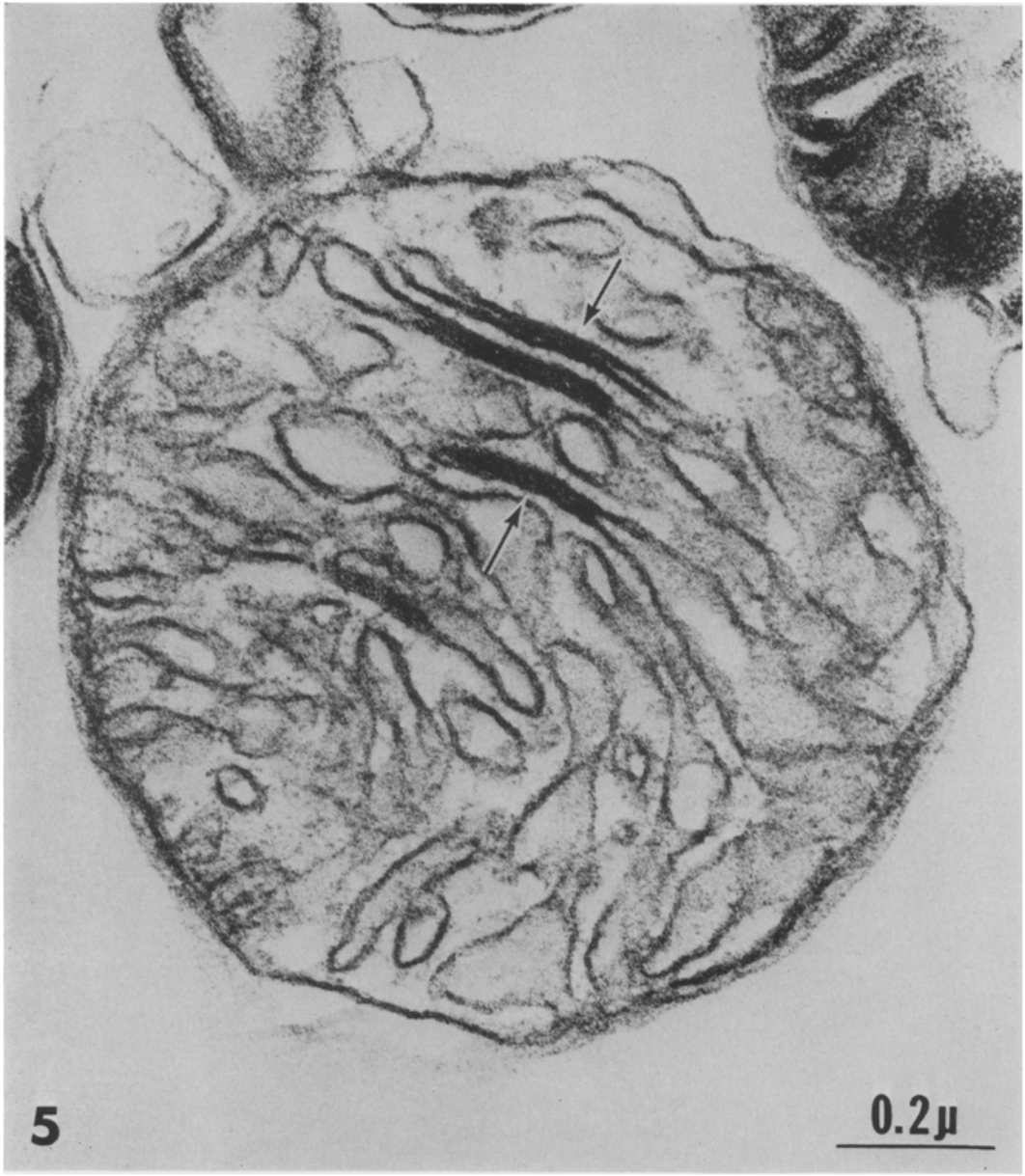

Figure 5. Mitochondria in the orthodox configuration having paracrystalline structures within paired cristal membranes. × 150,000.

chondria—a much greater frequency of observation than that observed with our standard fixation technique. Again, these ordered structures were observed in the intracristal space and not in the mitochondrial matrix space, which is electron opaque or condensed. At higher magnification, the details of the lattice structure can be discerned clearly (Fig. 8). The size of each knob or particle is 160 Å, and individual particles are connected

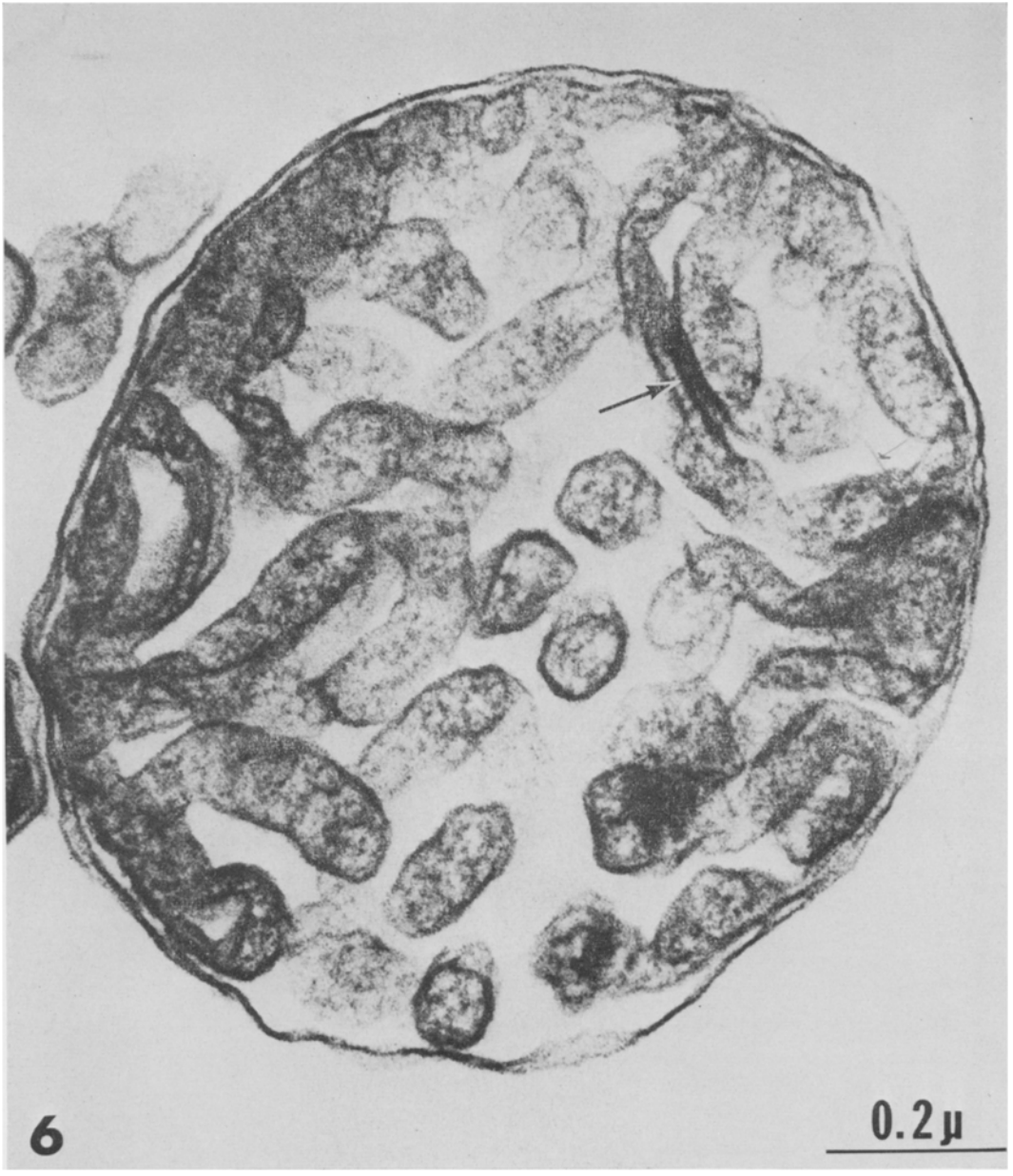

Figure 6. Mitochondria in the twisted-energized configuration showing paracrystalline structures within paired cristal membranes. × 150,000.

by lines which are 300 Å in length, 60 Å in width, having a center to center distance of 460 Å.

Since PTA, prepared in 0·25 M sucrose pH 7·5, induces the pseudoenergized-twisted configuration[35] in mitochondria, no attempt was made to correlate PTA-induced visualization of lattice structures with different energy states of the mitochondrion.

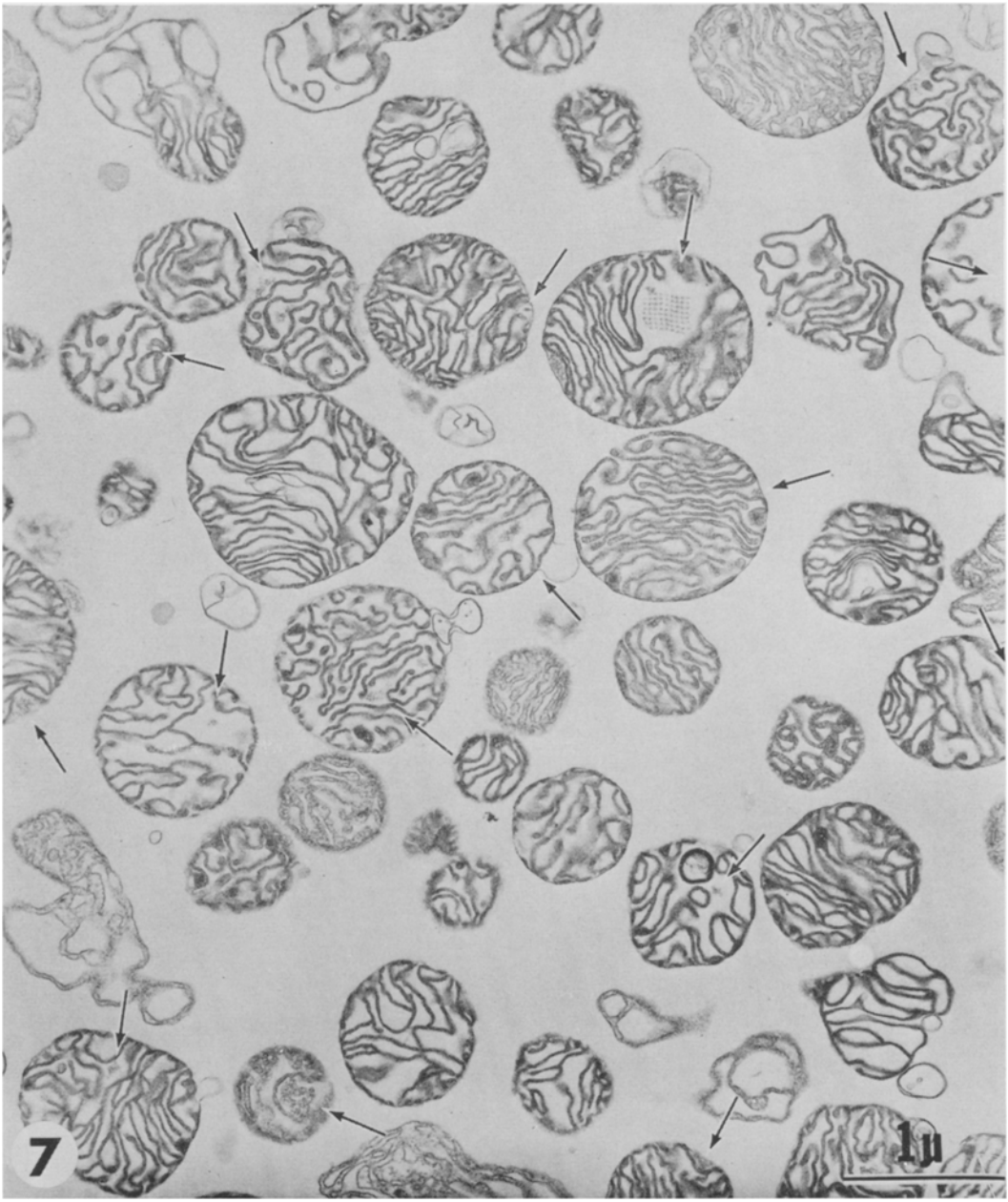

Figure 7. PTA-induced lattice structures in mitochondria treated with 1% PTA, pH 7·5, for 20 min prior to glutaraldehyde fixation. The electron micrograph illustrates the high frequency with which lattice structures were observed. × 30,000.

The pH of the PTA solution used in the mitochondrial preincubation appeared to affect profoundly the organization of the observed lattice structures. When the pH of the PTA solution was dropped below 7·0, the lattice structure lost its regular pattern and

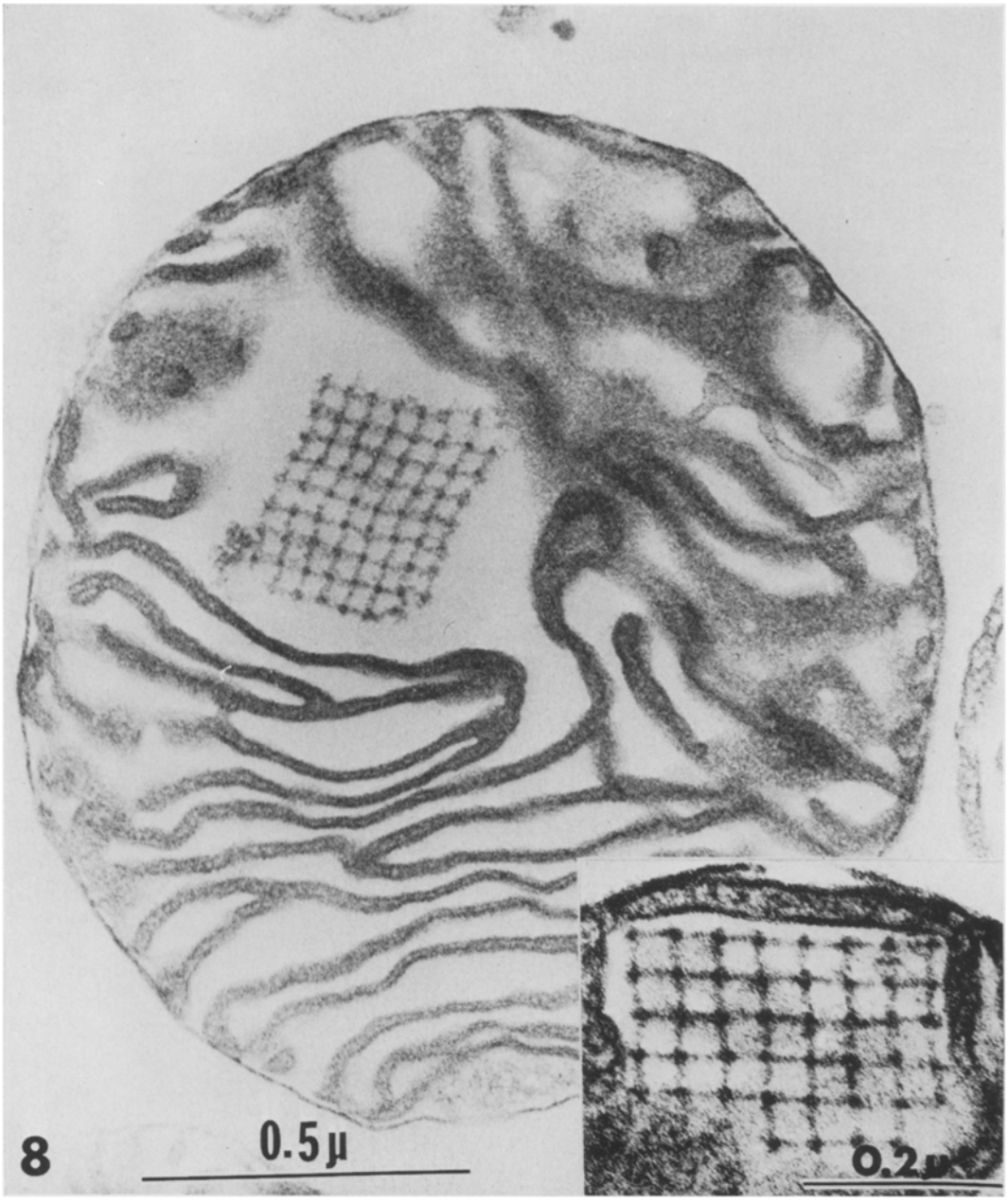

Figure 8. High magnification of the lattice structures demonstrating a highly regular pattern. × 126,000. Inset: × 185,000.

became amorphous. In Fig. 9 are shown mitochondria which were treated with PTA under the same conditions as those shown in Fig. 7, except that the pH was decreased to 6·0. When the pH of the PTA solution was raised above 7·5, lattice structures could no longer be visualized. The results of pH studies are summarized in Table I.

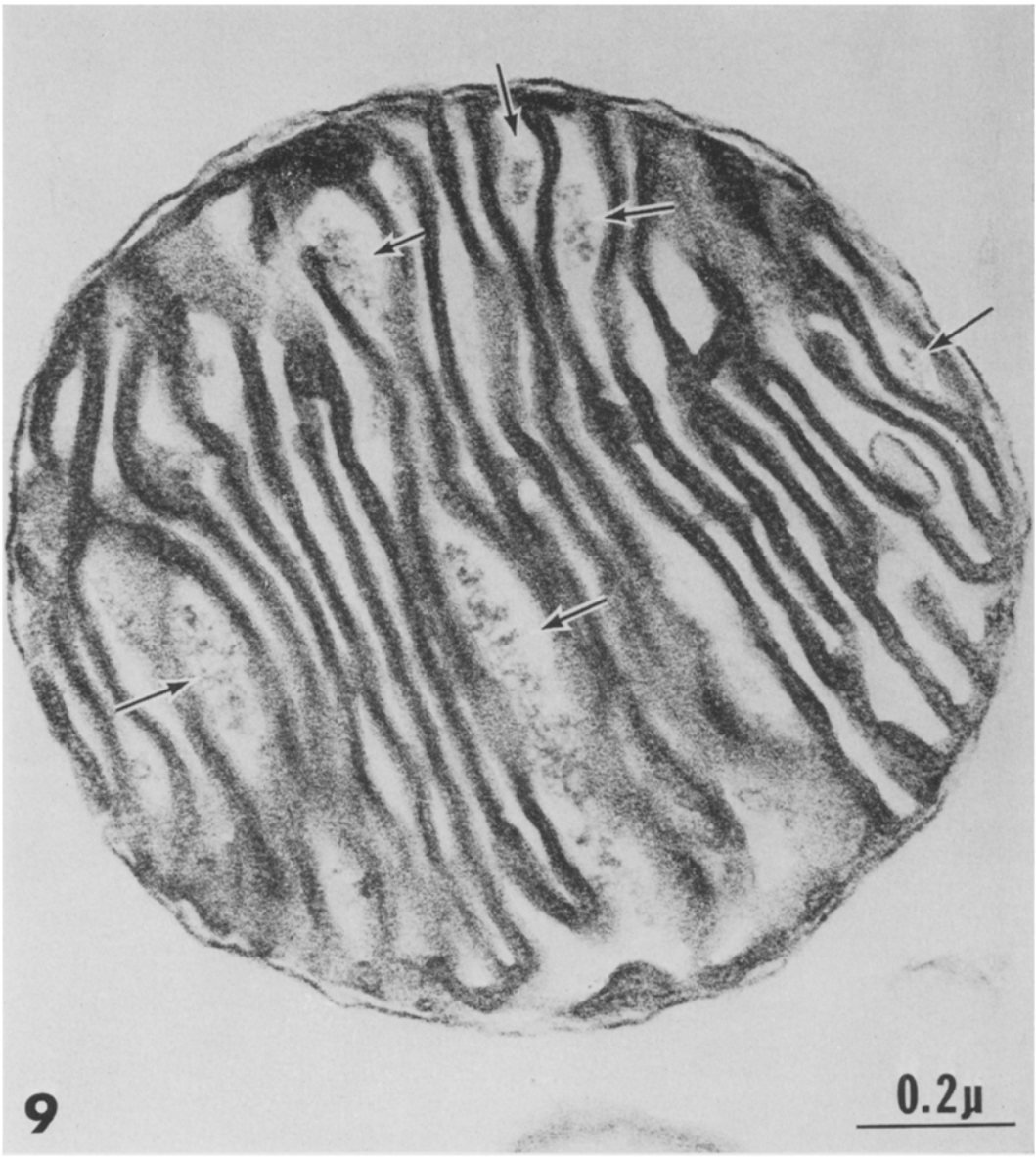

Figure 9. pH Dependency of the lattice structures. Mitochondria were treated as described in legend of Fig. 7, except that the pH of the PTA solution was decreased to 6·0. × 150,000.

Concentrations of PTA between 0·1% and 3·5% and incubation times between 5 sec and 1 h gave essentially the same results. Although the frequency to form lattice structures seemed to increase slightly with higher PTA concentrations and with longer times of exposure, overall, there was no significant change as long as the pH was maintained between 7·0 and 7·5.

Visualization of lattice structures in the intracristal space required preincubation with

TABLE I. Effect of pH on the PTA-induced lattice structures

pH of PTA solution	Visualization of lattice structures
10	–
9·0	–
8·0	–
7·5	+++
7·0	+++
6·5	+*
6·0	+*
5·5	–
5·0	–
4·5	–
4·0	–
3·5	–

– No structure was seen within the intracristal space.
+++ Lattice structures were observed.
+* Amorphous materials and lattice structures were seen within the intracristal space, but amorphous materials were predominant.

PTA prior to glutaraldehyde fixation. When mitochondria were fixed with glutaraldehyde and then exposed to PTA, no lattice structures could be detected. Conceivably, PTA interacts with some components of the intracristal space, and through rearrangement of these components, induces lattice structure. When, however, the mitochondrial proteins are first fixed with glutaraldehyde the rearrangement and the induction of lattice structure by PTA appears to be prevented.

Since PTA is widely used as a negative stain in electron microscopy, we tested another common reagent used in negative staining, namely ammonium molybdate. Pretreatment of beef heart mitochondria with ammonium molybdate (at concentrations from 0·5 to 2·0%, pH 7·5) prior to glutaraldehyde fixation did not induce lattice structures in the mitochondrial intracristal space.

Although PTA-induced lattice structures could be seen with regular frequency in the intracristal space of beef heart mitochondria, we were unable to visualize PTA-induced structures within the intracristal space of beef or rat liver mitochondria. At the present time, no reason can be given for the apparent anomaly.

Discussion

Numerous investigators have reported the visualization of crystalline material in mitochondria from various sources under normal and pathological conditions. Reports of structured material in the mitochondrial intracristal space are relatively few.[3,6,8,9,20,21] Crane *et al.*[21] have recently observed crystalline structures in the intracristal space of mitochondria both in the orthodox and aggregated configurations.

We have shown that the paracrystalline structures in the mitochondrial intracristal space can be found in all configurational states of mitochondria, namely, in the aggregated, twisted, and the orthodox configurations.[34] These data would suggest that these paracrystalline structures are in some way related to components intrinsic to the intracristal space of the mitochondrion.

In every report the frequency of observation of crystalline structures is very low. This fact immediately raises the question of why crystalline structures are not seen in every mitochondrion, if the components of the structures are intrinsic to the mitochondrion. To this question the present communication is addressed.

Silverman *et al.* have reported the specificity of the reactivity of various proteins with PTA.[26] Our data show that lattice structures induced by PTA were found in about 50% of the mitochondria—by far a higher frequency than has been observed to date. Nevertheless, why were lattice structures not observed in every mitochondrion? In our study, thin sections were used, so that only one dimension of the mitochondrion could be visualized under the electron microscope. We would predict that if serial sections were carried out, every mitochondrion would reveal lattice structures. Implicit in this statement is the notion that if, for example, only 5% of the total mitochondrial protein were contained in the intracristal space and this protein formed a lattice structure upon interaction with PTA, then one would not expect the entire intracristal space to display lattice structure, for the relative amount of lattice structure and protein would be small; and, one could not expect to see lattice structures in all mitochondria upon random thin-sectioning but would expect to see at least one lattice structure per mitochondrion, if serial sectioning were carried out.

Why then is the frequency of the PTA-induced lattice structures greater than the frequency of the paracrystalline structures, if both structures represent intracristal space components and the random thin-sectioning technique is carried out in both cases? Under standard conditions of fixation, without PTA pretreatment, paracrystalline structures are observed only when paired membrane systems lie parallel approximately 100 Å apart. Thus, such a prerequisite for the pairing of membranes would tend to further decrease the probability of the frequency of observation of structured material in the intracristal space. In the case of the PTA-induced lattice structures, this prerequisite does not exist and, we would like to suggest, only the distribution and amount of the components in the intracristal space would determine the frequency with which crystalline structures are observed in random sections.

In this communication we have presented electron micrographs showing organized structures in the mitochondrial intracristal space. Questions which arise are: (1) Are the lattice structures protein in nature? (2) Did PTA react with components intrinsic to the intracristal space? (3) Did PTA release components from the cristal membrane or from the mitochondrial matrix space?

The possibility that the crystalline structures observed in the mitochondrial intracristal space are in fact organized molecular crystals of protein can be extrapolated from the general electron microscope appearance of known protein crystals—their particle size, the distance between the particles making up the crystal and the complex arrangement of particles within an organized structure. In comparison with our observed mitochondrial lattice structures, several well-defined proteins of known chemical compositions[36–41] show in similar fashion an organized arrangement of particles into

complex three-dimensional structures. For example, native tropoymosin and the troponin-tropomyosin factor, in thin section, have been shown[41, 42] to have lattice structure, and in the troponin-tropomyosin lattice structure the particle size was measured to be 85 Å in diameter and the individual particles were separated by 75 Å (Fig. 10). From such analogy to known protein crystals and from our observations of the highly regular order and the pH dependency of the PTA-induced lattice structures in the mitochondrial intracristal space, we might reasonably speculate that these lattice structures represent protein structures. However, from the size of the particles, it would be difficult to assume that PTA reacted with only a single protein.

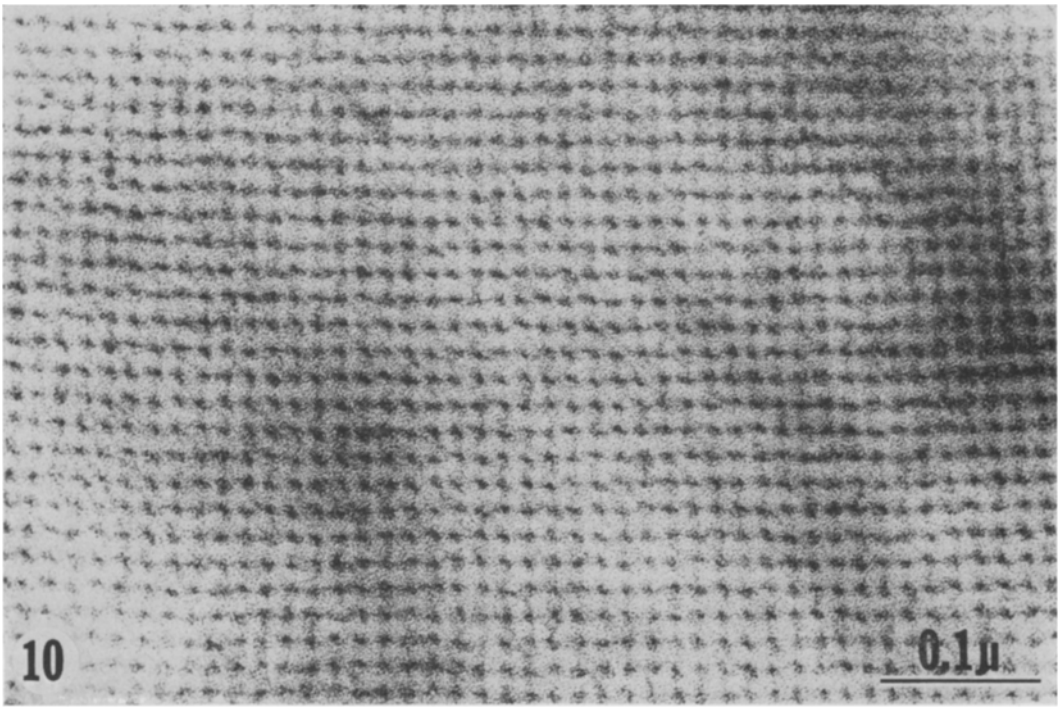

Figure 10. Crystallized troponin-tropomyosin system. Standard conditions of fixation and embedding were carried out. × 300,000.

The possibility was considered that the observed crystalline structures represented mitochondrial DNA in some modified form. Nass *et al.*[43] and Schuster[6] noticed the disappearance of a crystalline structure after DNA-ase treatment. The paracrystalline structures observed in glutaraldehyde-fixed mitochondria and the lattice structures seen in PTA-pretreated mitochondria are too big to be compared with the structures which were identified as DNA. Moreover, PTA cannot precipitate DNA even under acidic conditions.[26] Thus, the structured material observed in the mitochondrial intracristal space must have been derived from components other than DNA.

To answer the question as to the origin of the components visualized as crystalline structures in the mitochondrial intracristal space, we considered the possibility that PTA pretreatment caused a release of components from the mitochondrial matrix space.

We point to the intactness of the mitochondria under the conditions where the crystalline structures are visualized. In all cases where the inner membrane has been damaged to such an extent that matrix proteins are released, the mitochondrial electron microscopic appearance reflects the disorganization and disruption of the membrane systems.[44] Furthermore, chemical analysis of the supernatant after treatment of mitochondria with PTA at pH 7·5 indicates that proteins are not released from the mitochondrion. It has been suggested[45] that PTA buffered to neutral pH does not penetrate the mitochondrial inner membrane. Thus, we feel that the crystalline structures observed originate from components intrinsic to the mitochondrial intracristal space. The exact nature of the components entering into the observed crystalline structures has yet to be determined.

Since we have been able to visualize successfully components in the mitochondrial intracristal space, we are now attempting to visualize electron microscopically the components of the mitochondrial matrix space. Hackenbrock[46] and Phil *et al.*[47] have reported a nonmembranous structured system in the matrix space. We have isolated the proteins endogenous to the mitochondrial matrix space and shown (to be published elsewhere) that the matrix proteins are capable of aggregating into nonmembranous structures—sheets or strands in appearance. The ability of the nonmembranous proteins to undergo changes in conformation and in their state of aggregation, suggests that the nonmembranous structured system assumes physiological significance in controlling configurational function of mitochondria.

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