STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XI. CORRELATION OF THE MORPHOLOGY AND ENZYMIC PROPERTIES OF MITOCHONDRIAL AND SUB-MITOCHONDRIAL PARTICLES

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In previous communications of this series, the isolation and properties of the sub-mitochondrial electron transport particle (ETP)[1] and the phosphorylating electron transport particle (PETP)[2] have been described. Both these particles were prepared from beef-heart mitochondrial preparations. ETP catalyzes the rapid oxidation of DPNH and succinate by molecular oxygen and is relatively free of the pyridino-protein dehydrogenases of the citric acid cycle. PETP is a submitochondrial particle with many of the properties of ETP, but it still retains the citric acid cycle dehydrogenases and is capable of carrying out oxidative phosphorylation.

By the procedures employed in these earlier studies only a relatively small proportion of the total mitochondrial protein could be recovered as ETP or PETP. It was suggested[2] that the mitochondrial suspensions which were the starting points for the preparation of ETP and PETP were already mixtures of particles, and that the methods used in the isolation procedures merely separated the mixture into the component particles.

More recently HATEFI AND LESTER[3] have shown that suspensions of beef-heart mitochondria in 0.25 *M* sucrose can be readily separated by differential centrifugation into two well defined fractions. While these fractions are distinctly different in gross appearance, they are qualitatively similar in their enzymic characteristics.

In the present communication, the results of electron-microscopic examination of beef-heart mitochondria and various derivative particles are reported. Furthermore, evidence bearing on the functional and morphological interrelationships of these particles is presented. These studies may throw light on how the various sub-mitochondrial or mitochondrial particles arise from the original unmodified mitochondrion.

METHODS

Unless otherwise stated, beef-heart mitochondrial suspensions were prepared by the large scale method of CRANE *et al.*[1] and then sub-fractionated into the heavy and light particle fractions by the procedure described in full by HATEFI AND LESTER[3]. ETP was prepared from the light mitochondrial fraction according to a method proposed by GREEN *et al.*[2]. The details are as follows.

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The light mitochondrial suspension (in 0.25 *M* sucrose) was diluted with 0.25 *M* sucrose to adjust the protein concentration to 50 mg/ml. The diluted suspension was mixed at 0° with an equal volume of a 30% ethanol solution containing per ml 300 μmoles of potassium phosphate (pH 7.8) and 1 μmole of versene EDTA. After homogenization the suspension was centrifuged for 5 min at 15,000 r.p.m. in the number 30 head of the Spinco preparative ultracentrifuge. The supernatant fluid was decanted and then centrifuged for 30 min at 30,000 r.p.m. The sediment was suspended in 20 vol. of 0.25 *M* sucrose with thorough homogenization. The suspension was centrifuged for 30 min at 30,000 r.p.m. The washing procedure was then repeated exactly. Finally the sediment was suspended in 5 vol. of 0.25 *M* sucrose. This will be referred to hereinafter as a suspension of ETP prepared by the alcohol–phosphate procedure.

The particles of the heavy and light mitochondrial fractions obtained by the procedure of HATEFI AND LESTER[3] by fractionation of the original mitochondrial suspensions are reasonably free from cross contamination and are not to be confused with the heavy and light mitochondrial fractions prepared from the original mitochondrial suspension by the earlier method of GREEN *et al.*[2]. PETP was prepared by the alcohol–phosphate procedure[2] from the heavy fraction described in the earlier method of GREEN *et al.*[2]. It cannot however be prepared by this procedure from the heavy fraction of the later method of HATEFI AND LESTER[3]. The heavy fraction of the earlier method is a mixture of particle types including PETP, whereas the heavy fraction of the later method consists basically of one particle type and contains no PETP.

DPNH and succinic dehydrogenase activities were measured by methods previously described[1].

For electron microscope studies* the suspension of particles was treated in either of two ways. The first procedure was followed when information on the shape of the intact particles was needed. In order to avoid deformation of the material during air drying, the critical-point method of ANDERSON[4] was used. Drops of the suspension were placed on a perforated brass plate carrying specimen grids covered with a formvar film. They were then exposed for 5 min to osmium tetraoxide vapor and dried according to the schedule proposed by ANDERSON. Stereoscopic photos were prepared with the special holder for the RCA EMU2 microscope.

In the second and alternative procedure, ultrathin sections through the various particles were prepared. Suspensions of particles were fixed for 20 min in a 1:1 (v/v) mixture of suspension and osmium tetraoxide solution (2 g of osmium tetraoxide per 100 ml of the desired medium, usually 0.25 *M* sucrose). The mixture was then centrifuged, and small pieces of the pellet were embedded in methacrylate for sectioning.

Small pieces of fresh beef heart were fixed in PALADE's[5] buffered osmium tetraoxide (pH 7.8) containing 0.25 *M* sucrose and sectioned for a study of mitochondria in intact heart muscle. All sections were prepared with a Porter-Blum microtome. The electron micrographs were taken with a RCA EMU2 electron microscope on Ilford N-40 process plates. An objective aperture of 50 μ diameter was used.

RESULTS

1. *Mitochondria in situ*

In Fig. 1 is shown a section through fresh beef-heart muscle. The mitochondria are found in groups between the myofibrils. They appear as elongated bodies with a more or less cylindrical structure. They have a continuous outer membrane filled with a complex system of tightly packed double membranes (cristae). The overall structure of the beef-heart mitochondria is similar to that of mitochondria from other tissues[6,7].

2. *Mitochondria isolated in 0.88 M sucrose*

A section through mitochondria isolated in 0.88 *M* sucrose is shown in Fig. 2. The mitochondria appear essentially normal. Special precautions had to be taken to obtain these morphologically intact mitochondria. Only a small fraction of the total heart mitochondria could be isolated in this unaltered state. Minced heart mucle was washed twice with 0.88 *M* sucrose containing 10^{-4} *M* EDTA** versene. The pH was

* All of the work with the electron microscope was done in the Electron Microscope Laboratory of the Department of Biochemistry, University of Wisconsin.

** The following abbreviations will be used: EDTA (ethylenediamine tetraacetic acid); DPNH (reduced diphosphopyridine nucleotide); ETP (electron transport particle); PETP (phosphorylating electron transport particle); ETP_H (electron transport particle derived from the heavy mitochondrial fraction).

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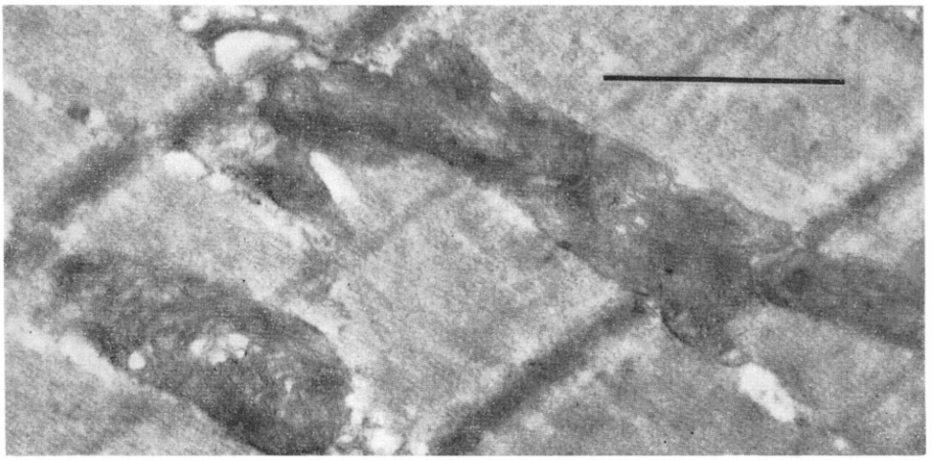

Fig. 1. Electron micrograph of a section through fresh beef-heart muscle. The mitochondria appear as elongated structures embedded between the myofibrils. × 32,500. The length of the bar equals 1 μ.

maintained at 7.2. by addition of 0.1 *M* KOH whenever necessary. The washed heart muscle was homogenized in 0.88 *M* sucrose (10 g wet weight tissue per 100 ml of suspension) with a loose-fitting Potter-Elvehjem homogenizer. It was important not to homogenize too thoroughly as excessive homogenization produced a preponderance of mitochondrial fragments which were difficult to separate from the intact forms. The suspension was centrifuged in an International Refrigerated Centrifuge for ten minutes at 1,000 × *g*. The supernatant fluid was decanted and centrifuged at 12,000 × *g* for 10 min in the Spinco Model L preparative centrifuge. The mitochondrial pellet was resuspended in twenty volumes of 0.88 *M* sucrose and again sedimented at 12,000 × *g* for 10 min.

At this stage the sediment showed clear evidence of two layers. The light colored, loosely packed material on top was sloughed off by layering a small volume of sucrose on the sediment and then gently swirling the centrifuge tube. The well packed residue was washed and finally resuspended in 0.88 *M* sucrose. This fraction consisted largely of morphologically intact mitochondria.

We have carried out some experiments designed to estimate the percentage of the total protein of beef-heart muscle accounted for by the mitochondria. The specific succinoxidase and DPNH-oxidase activities of the whole homogenate (0.88 *M* sucrose) were compared with those of the isolated (presumably homogeneous) mitochondrial fraction also prepared in 0.88 *M* sucrose. If there are no complicating factors, then the increase in specific activity should be a direct measure of the concentration of mitochondria in the original homogenate. The increase is 4 to 5 fold (*cf.* Table I). Therefore on this basis mitochondria should account for 20 to 25% of the total protein. In point of fact the experiment could not be carried out in just this fashion. The homogenate has such a high endogenous oxygen uptake that it is unreliable to determine the rate of succinic or DPNH oxidation by subtracting the control from the experimental rate. The particles of the homogenate were washed three times with 15 vol. of the 0.88 *M* sucrose solution by alternate high-speed centrifugation and resuspension. Care was taken to ensure that no particles were lost

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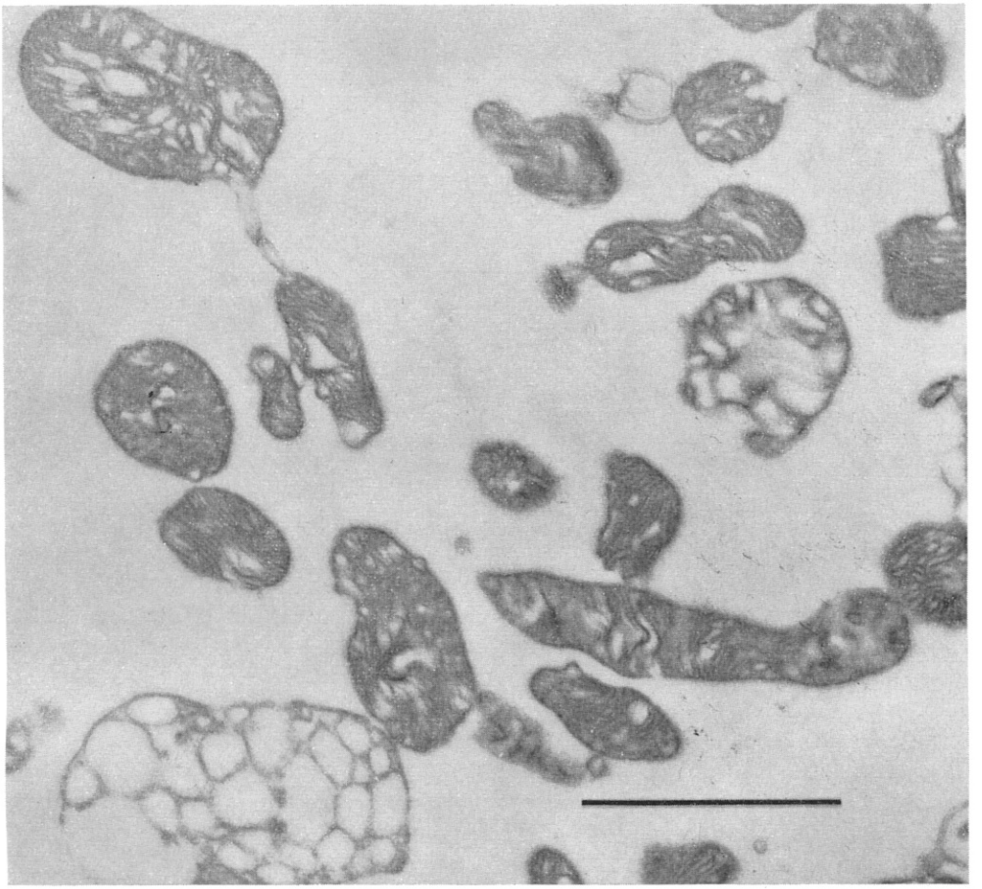

Fig. 2. Electron micrograph of a section through beef-heart mitochondria isolated in 0.88 *M* sucrose. The mitochondria show a well defined outer membrane surrounding a dense network of branching double membranes. Most of the mitochondria in this fraction show only slight morphological changes when compared to those *in situ* (*cf.* Fig. 1). However, in a few mitochondria the spaces between the cristae are enlarged and nearly spherical. × 33,500. The length of the bar equals 1 μ.

during the washing procedure. The protein content of the final washed suspension was then corrected for the soluble protein extracted during the washing procedure. The estimate of the proportion of mitochondrial protein in beef-heart muscle thus rests on the further assumption that the washed particle suspension is equivalent to the original homogenate on a specific enzymic basis. Succinoxidase activity is generally accepted as a reliable measure of mitochondrial activity[8].

The yield of intact mitochondria from beef-heart muscle prepared according to the method described above is about 2% of the total mitochondrial protein assuming the ratio of mitochondrial protein to total protein to be 0.20. The preponderant proportion remains occluded in the myosin fraction which is discarded.

3. *Mitochondria isolated in 0.25 M sucrose*

Mitochondria appear to be extensively damaged from the standpoint of structure

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TABLE I

COMPARISON OF THE SUCCINIC- AND DPNH-OXIDASE ACTIVITIES OF BEEF-HEART MUSCLE "HOMOGENATE" AND BEEF HEART MUSCLE MITOCHONDRIA

	μmoles Substrate oxidized/min/mg at 38°	
	*Succinic-O_2***	*DPNH-O_2****
"Homogenate"*	0.11	0.25
Mitochondria**	0.52	0.97

* Fresh beef-heart muscle was homogenized in 6 volumes of 0.88 *M* sucrose for 90 sec in the Virtis high-speed blendor at maximum speed and at 0°. The total protein of the suspension was determined. Then the homogenate was centrifuged in the Spinco ultracentrifuge for 30 min at 40,000 r.p.m. The well-packed residue was washed three times with 15 vol. of 0.88 *M* sucrose. The washed residue was suspended in 6 vol. of 0.88 *M* sucrose. The protein contents of both the suspension and washings were determined. The specific activity of the "homogenate" has been corrected for the soluble protein lost during the washing of the particles.

** The mitochondria were prepared by the method described above in Section 2 of Results.

*** Cytochrome *c* (0.3 mg/ml) was added to the assay mixture.

when isolated in 0.25 *M* sucrose. Fig. 3 shows a whole-mount electron micrograph of such a mitochondrial suspension.

Some of the mitochondria are swollen and appear similar to the "crescent" forms described by HARMAN[9] and by CLELAND AND SLATER[10]. In these crescent forms the outer membrane is pulled away from the cristae and appears as a simple transparent film.

In many of these ballooned, crescent mitochondria the outer membrane is pulled away from the cristae and forms individual vesicles or strings of vesicles. This is clearly shown in Fig. 3 (E), where part of the outer membrane in one of the crescent forms adheres to the formvar film. The string of small vesicles appears to be continuous with the outer membrane.

In Fig. 3 (H) dense spherical particles are seen which are too opaque to show internal structure. When seen in section (Fig. 4), these particles resemble intact mitochondria with respect to the branching double membrane structures. The electron-transparent areas between the cristae are swollen and often spherical. These mitochondrial particles have a closely adhering outer membrane in contrast to the crescent mitochondrial forms in which the mass of cristae is pushed to one side (*cf.* Figs. 3 and 5).

Part of the morphological changes exhibited by the mitochondria isolated in 0.25 *M* sucrose can be attributed to the low tonicity of the medium. In sections through heart muscle exposed to 0.25 *M* sucrose before fixing, the mitochondria are swollen and show many of the changes seen in the isolated mitochondrial suspensions.

Mitochondria isolated in 0.25 *M* sucrose can be separated by the method of HATEFI AND LESTER[3] into fast sedimenting or heavy mitochondria, and slower sedimenting or light mitochondria. Sections through heavy mitochondria (Fig. 4) show that this fraction is composed almost entirely of the large, densely staining particles, described previously, which were characterized by their closely adhering outer membrane and the enlarged intercristal spaces. The light mitochondrial fraction, on the other hand (Fig. 5), consists predominantly of crescent forms plus the numerous small vesicles which were present in the original mitochondrial suspension.

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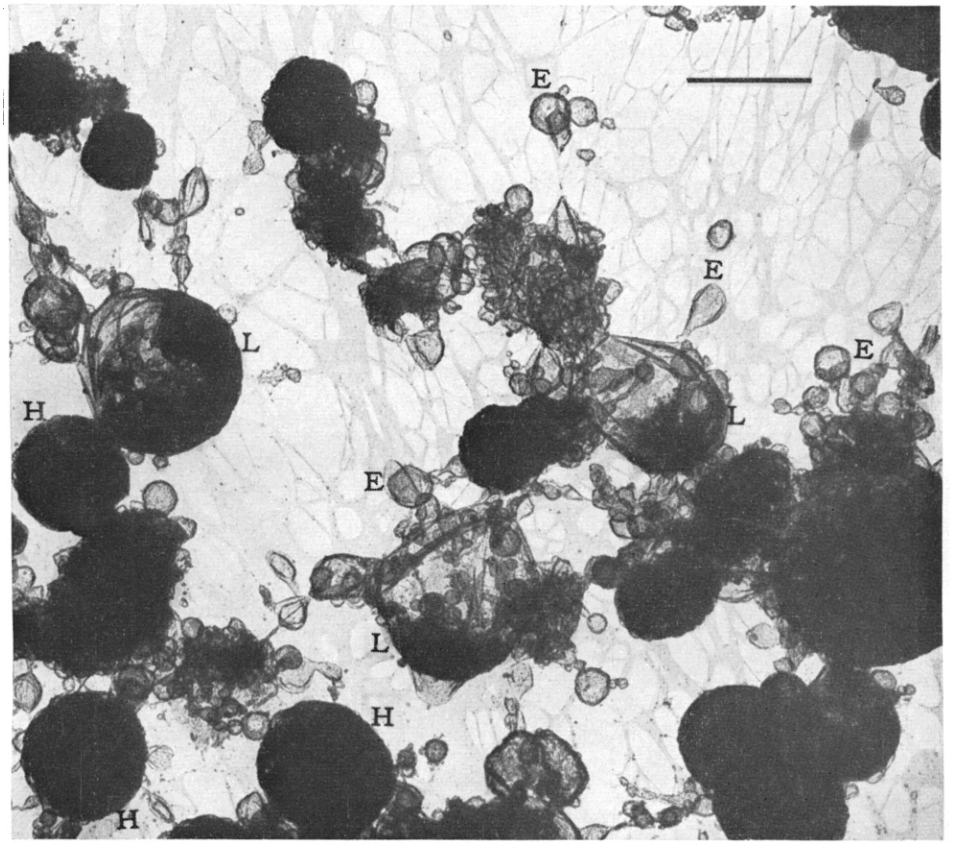

Fig. 3. Electron micrograph of beef-heart mitochondria isolated in 0.25 *M* sucrose and prepared by the critical-point method of Anderson. It is apparent that extensive fragmentation of mitochondria has occurred. This preparation can be separated, by the method referred to in the text, into several fractions. The large electron opaque particles (H) correspond to the heavy mitochondrial fraction (*cf.* Fig. 4). The swollen, crescent mitochondria (L) with the ballooned outer membrane can be identified with particles present in the light mitochondrial fraction. The small vesicles (E) are identical in appearance with the granules characteristic of the isolated ETP fraction (*cf.* Figs. 6 and 7). Many of the small vesicles (E) are continuous with the outer membrane of the crescent mitochondria (L) and appear to have originated by vesiculation of the outer membrane. The connection of these vesicles with the outer mitochondrial membrane is especially clear in stereoscopic photographs of such preparations. × 16,650. The length of the bar equals 1 μ.

4. *Separation of ETP*

Electron micrographs of ETP preparations (Fig. 6b and 7b) show that it is composed of small vesicles which occur singly, in clumps, or which are joined together to form long chains. These vesicles are identical with the small particles present in the original mitochondrial suspension (*cf.* Fig. 3). The obvious suggestion that the ethanol–phosphate method of GREEN *et al.*[2] for the isolation of ETP merely separates preformed particles is further borne out by the data in Table II, which show that the ethanol–phosphate method for the separation of ETP is only applicable to the light mitochondrial fraction. An examination of the electron microscope photographs of

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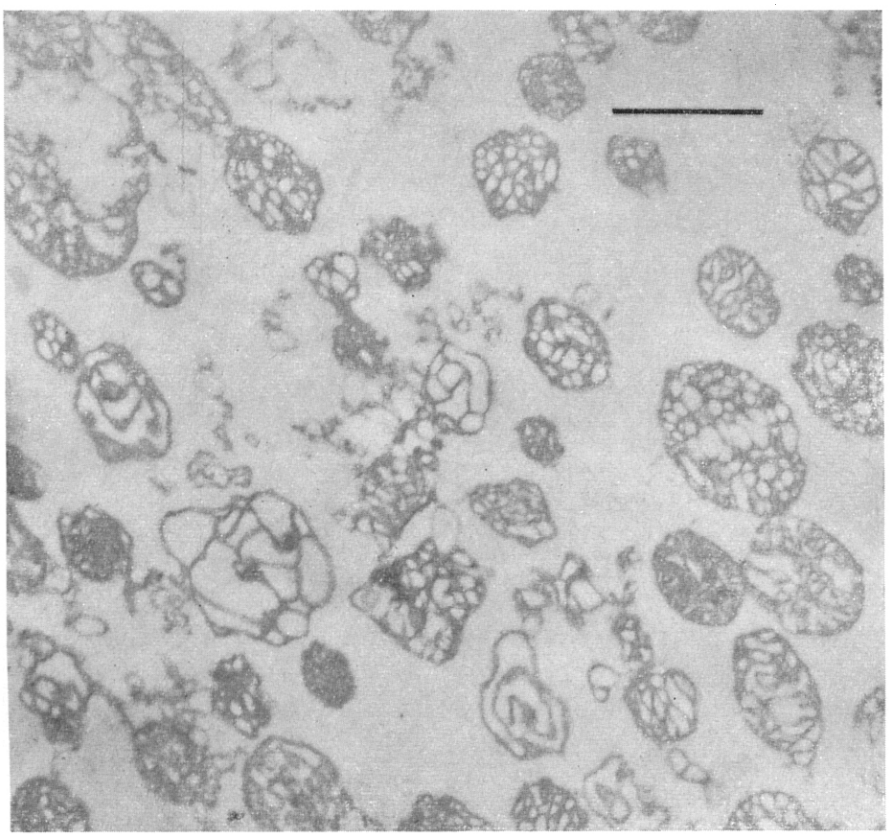

Fig. 4. Electron micrograph of a section through the heavy mitochondrial fraction. These particles resemble intact mitochondria with respect to the branching double membrane system, but the spaces between the cristae are greatly enlarged when compared to mitochondria *in situ*. × 20,250. The length of the bar equals 1 μ.

the light and heavy mitochondrial fractions shows that only the light fraction contains significant numbers of the small vesicles characteristic of ETP.

5. *Formation and separation of PETP*

The form of PETP is shown in Fig. 6a while Fig. 7a shows a section through a pellet of PETP particles. Many of the vesicles are similar to those present in preparations of ETP. The major difference between sections taken from ETP and PETP preparations respectively is the presence of fragments of cristae which are often surrounded by an outer membrane in the PETP preparations. The capacity of the PETP fraction to carry out the reactions of oxidative phosphorylation may be attributed to these bundles of cristae.

A section through mitochondria treated with ethanol–phosphate is shown in Fig. 8. The ethanol appears to disperse the cristae within the mitochondria, and in several mitochondria small clumps of the cristae are shown pushed into a small sac formed by the outer membrane. These small packets of cristae are evidently pinched off to form the characteristic particles of PETP during the vigorous homogenization

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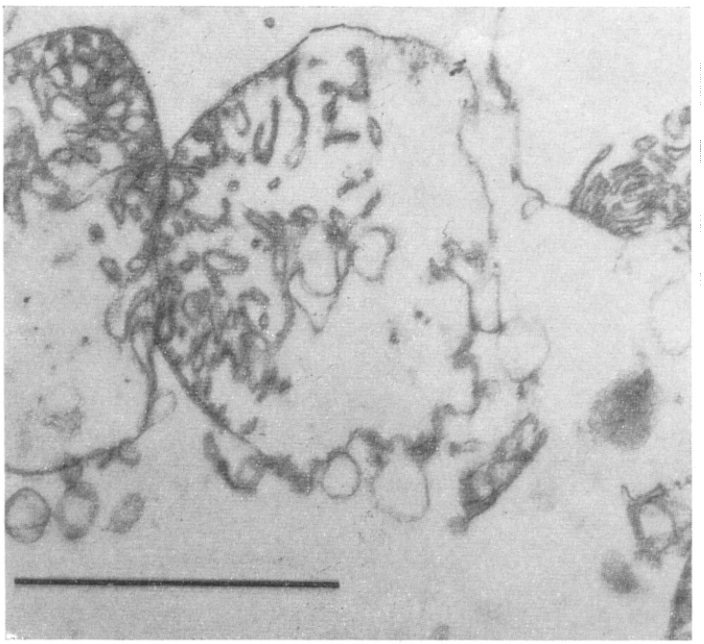

Fig. 5. Electron micrograph of a section through the light mitochondrial fraction. In these mitochondria the outer membrane is pulled away from the cristae on one side and a large electron-transparent space between the outer membrane and the cristae is left. Vesiculation of the outer membrane is apparent in the mitochondrion shown in the center of this photograph. × 43,000. The length of the bar equals 1 μ.

applied in the isolation of PETP. Whether all or only part of the PETP is formed during the isolation, can not be definitely stated at this time. Small granules similar to the PETP entities are occasionally found in the whole mitochondrial suspensions.

It has been reported[2] that only a small fraction of the mitochondrial suspension could be converted to PETP. The hypothesis for the formation of PETP developed in the present communication explains why the conversion is necessarily low. The ethanol–phosphate medium does not disperse all of the cristae into small masses, and the chance that these small bundles of cristae are then pinched off in an enclosed membrane is probably low.

6. *Formation of ETP and a similar particle derived from the heavy mitochondrial fraction*

The standard methods of MACKLER AND GREEN[11] and GREEN *et al.*[2] for the preparation of ETP do not yield a particle like ETP from the heavy mitochondrial fraction. However, heavy mitochondria exposed to sonic irradiation liberate small vesicles similar in appearance but smaller in size than those of ETP (Fig. 9). From the standpoint of enzymic activities, these particles closely resemble those of ETP though some differences have been recognized which will be considered in a later section.

Examination of the electron micrographs points to the origin of ETP from the outer mitochondrial envelope or membrane as distinct from the cristae. That is to say that when the light mitochondrial fraction is exposed to the alcohol–phosphate reagent the vesicles of preformed ETP or those which thereby are formed are derived from

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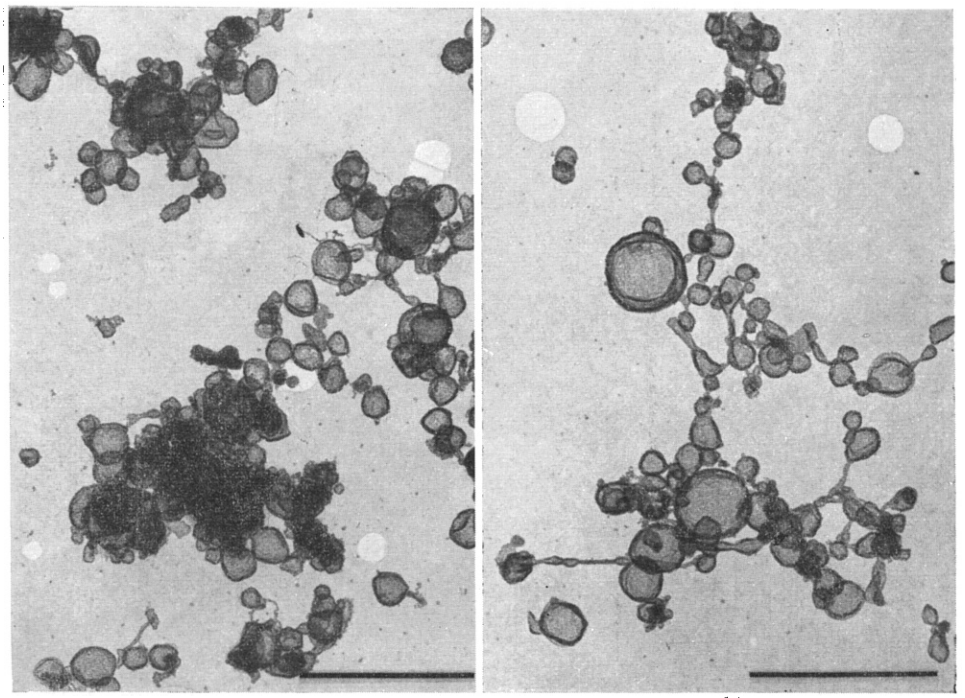

(a) (b)

Fig. 6. Electron micrograph of preparation of PETP on the left (a) and ETP on the right (b). The preparations were made by the critical-point method of Anderson. The small vesicles in both fractions appear to be similar; however, the PETP preparation (a) contains electron-opaque granules that are not found in the ETP (b) fraction. PETP × 23,000. ETP × 24,000. The length of the bars equals 1 μ.

the outer membranous envelope and not from the cristae. Other methods such as sonic oscillation are needed to fragment cristae into vesicles of ETP. It seems likely that under the conditions of our isolation procedures, the particles of our standard ETP preparations originate wholly or in large part from the outer mitochondrial membrane. Some support for this hypothesis is provided by the photographs in Fig. 3 and 5, which show clearly the vesiculation of the outer membrane, the separation of these vesicles from the rest of the outer membrane, and the similarity in size and shape of these extruded vesicles with the characteristic particles of ETP (Fig. 6 and 7). Even though a considerable portion of the outer membrane may be stripped off in this manner, it would appear that enough of the outer membrane may be left to encase the bulk of the cristae in a tightly packed unit such as the heavy mitochondrial particle. A diagrammatic representation of the origin of ETP is given in Fig. 10.

Morphologically intact mitochondria isolated in 0.88 *M* sucrose do not yield ETP when exposed to the standard ethanol–phosphate procedure. Thus clearly, only the outer membrane of mitochondrial particles damaged or modified in a particular way, may give rise to ETP. Perhaps during the tearing away of some mitochondria from the myofibrils in which they are imbedded *in vivo*, the events which lead up to the alteration of the outer membrane and the formation of ETP take place. These special conditions may not readily be reproducible once the mitochondrion has been isolated and the outer membrane has not sustained any damage.

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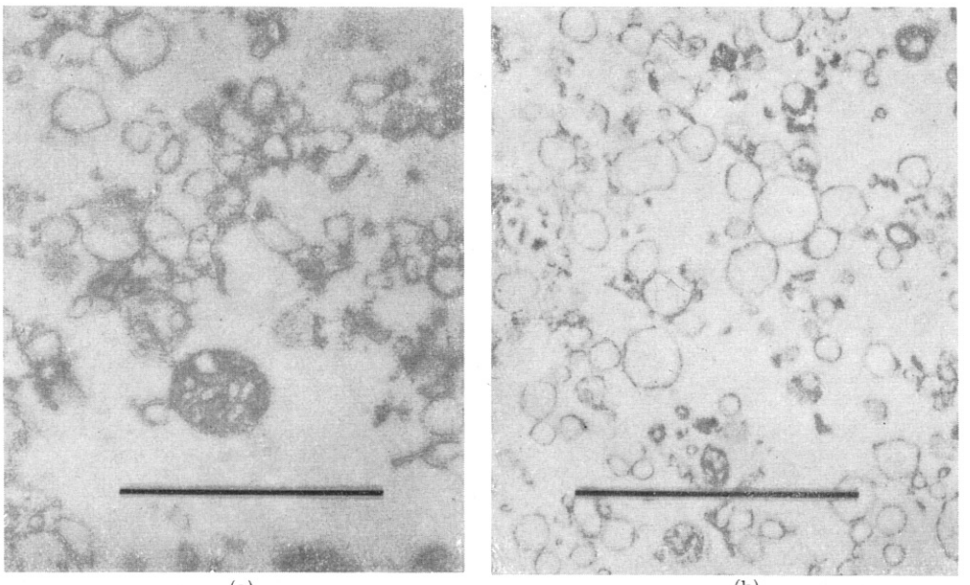

(a) (b)

Fig. 7. **Electron micrographs of sections through PETP on the left (a) and ETP on the right (b). Both fractions consist predominately of small vesicles. In addition the PETP fraction (a) contains vesicles filled with cristae which resemble in structure the particles of the heavy mitochondrial fraction. However, they are much smaller than the heavy particles and presumably originate by fragmentation of mitochondria. PETP × 34,000. ETP × 36,500. The length of the bars equals 1 μ.**

7. Biochemical properties of ETP and of the small particle derived from the heavy mitochondrial fraction

Sonic irradiation of suspensions of the heavy mitochondrial fraction in a 10 K.C. Raytheon oscillator for 2 min at maximum energy leads to quantitative fragmentation into much smaller particles that closely resemble ETP (Fig. 9). Since these derivative particles originate largely, if not exclusively, from the cristae of the heavy mitochondrial fraction, they will be designated as ETP_H to differentiate them from the particles originating from the outer mitochondrial membrane, *viz.* ETP.

Table II contains a summary of some comparative data on the composition of ETP and ETP_H. The heme and flavin analyses were carried out by Drs. R. BASFORD and J. JÄRNEFELT according to previously published methods[12,13]. The concentrations of total flavin as well as of heme are much the same for the two particles. The principal point of difference appears to reside in the cytochrome $c + c_1$ fraction. The chemical method of estimation[13] when applied to a particle containing both cytochromes c and c_1 does not distinguish between these two hemoproteins. These are not separated during the procedure and consequently are estimated together. However, cytochrome c is extractable from some particles under relatively mild conditions, *e.g.* in 0.9% KCl, and not from other particles under these same conditions. About one third of the cytochrome $c + c_1$ fraction of ETP_H can be extracted by exposing the particles to 0.9% KCl whereas none is extracted from ETP under the same conditions. This result does not necessarily mean that ETP contains exclusively c_1 whereas ETP_H contains roughly half as much c as c_1. In our laboratory WIDMER AND CRANE[14] have discovered lipide-soluble forms of cytochrome c which according to BASFORD[15] may be classified

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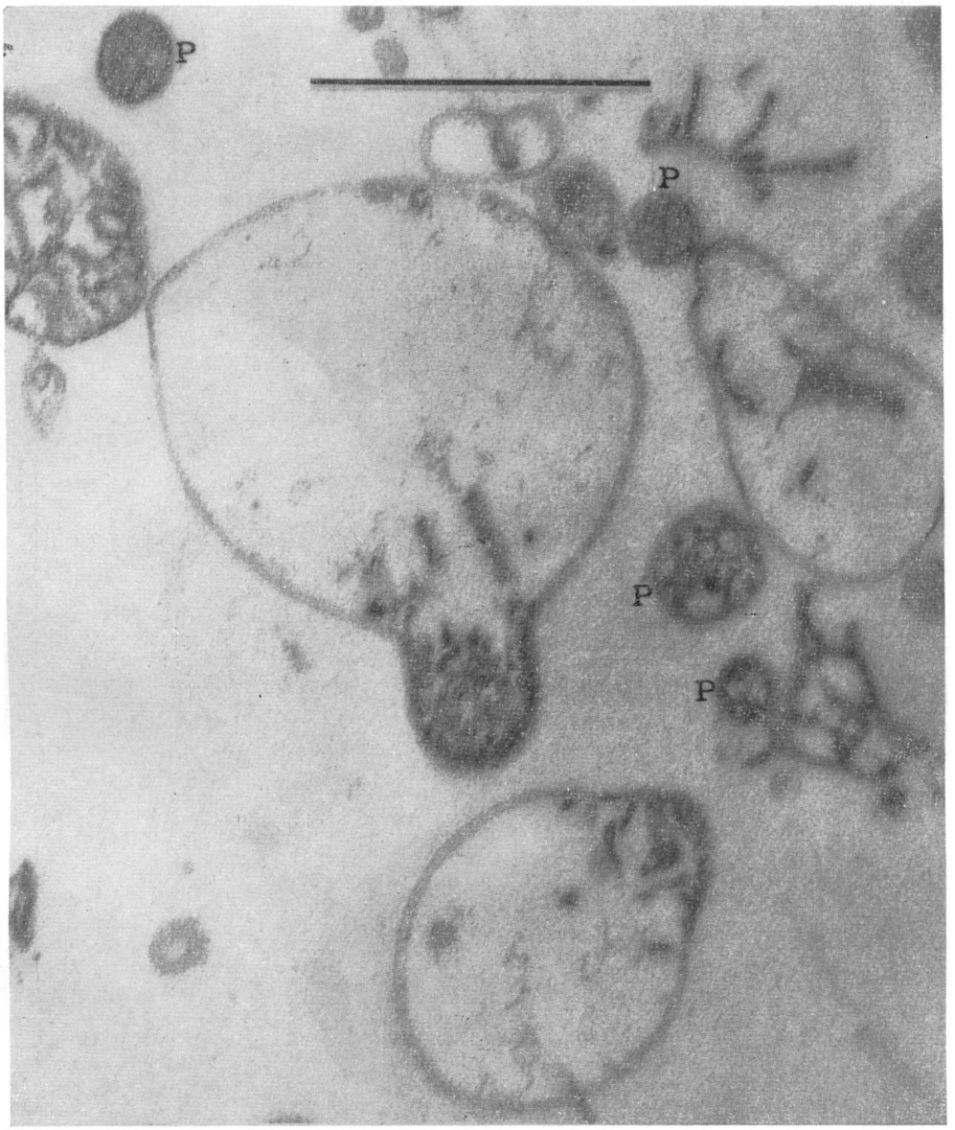

Fig. 8. Electron micrograph of a section through beef-heart mitochondria exposed to 15 % ethanol and 0.15 *M* phosphate. The suspension was homogenized and centrifuged before fixation. The two mitochondria shown in the center of this photo clearly demonstrate the manner in which the PETP granules (P) are formed. Portions of the cristae are forced into a small sac formed by the outer membrane and then pinched off to give the small particles (P). × 43,000. The length of the bar equals 1 μ.

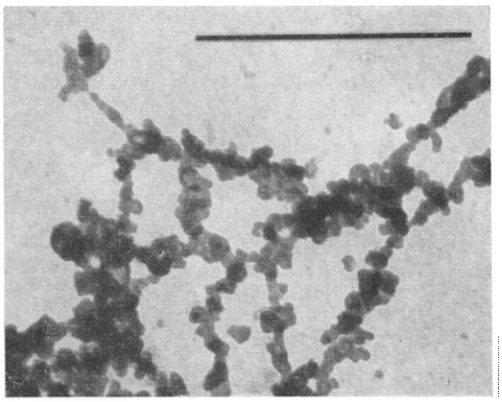

Fig. 9.

Fig. 9. Electron micrograph of the heavy mitochondrial fraction after sonic irradiation and prepared by Anderson's critical-point method. The mitochondria (20 mg protein/ml) were exposed to the sonic vibration for 2 min. Complete destruction of the mitochondrial structure has occurred, and long strings of very small vesicles (average dia. approx. 38 mμ) are formed. × 36,000. The length of the bar equals 1 μ.

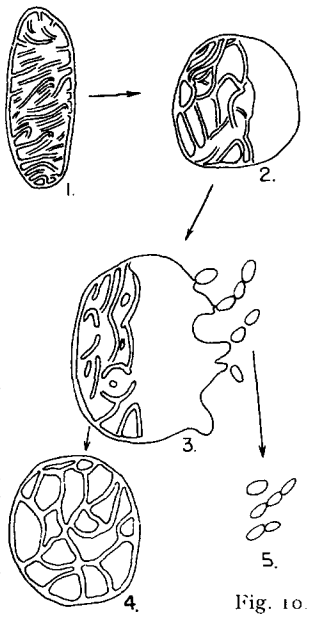

Fig. 10.

Fig. 10. Schematic representation of the sequence of events which lead to the formation of the various particles present in the mitochondrial suspensions prepared in 0.25 *M* sucrose. 1. Heart mitochondria as they appear in normal heart muscle. 2. Mitochondria within the muscle after exposure to 0.25 *M* sucrose. The outer membrane is pushed away from the cristae, and there is progressive swelling of the intercristal areas. 3, 4 and 5. These three forms are present in heart mitochondria isolated in 0.25 *M* sucrose. Number 3 is a reproduction of a crescent mitochondrial form showing the vesiculation of the outer membrane. The transition from 2 to 3 occurs when the mitochondria are stripped from between-the-muscle fibrils. It is during this process that portions of the outer membrane are pinched off to form the vesicles characteristic of the ETP fraction (5). If all or most of the ballooned part of the outer membrane is stripped off, the cristae encased in the remaining portion of the outer membrane give rise to the characteristic particle of the heavy mitochondrial fraction (H).

TABLE II

YIELD OF ETP FROM THE HEAVY AND LIGHT MITOCHONDRIAL FRACTION AFTER TREATMENT WITH THE ETHYL ALCOHOL–PHOSPHATE PROCEDURE

	Per cent of original protein in each fraction		
	*Residue**	*ETP*	*Soluble*
Heavy mitochondrial fraction	83	3	6
Light mitochondrial fraction	60	29	8

* After the mitochondrial suspensions had been exposed to the alcohol–phosphate reagent, they were separated by differential centrifugation into a rapidly sedimenting fraction (residue), a slow sedimenting fraction (ETP) and a soluble supernatant. The details of the procedure are identical with those described under METHODS for the isolation of ETP from the light mitochondrial fraction.

into two categories—one of which is decomposed by exposure to 0.9% KCl while the other is decomposed only under more drastic conditions (*e.g.* in presence of 10% butyl alcohol). ETP and ETP_H thus may differ primarily in the nature of the lipide cytochrome *c*. This difference, furthermore, could be a consequence of the preparative procedure rather than an expression of a basic chemical difference between the electron transport systems of the envelope and cristae.

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It is of interest that ETP_H shows a marked requirement for added cytochrome *c* in the DPNH-O_2 reaction whereas ETP does not (*cf.* Table IV). This result could have been anticipated on the basis of the ready extraction of cytochrome *c* from ETP_H but not from ETP.

TABLE III

HEME AND FLAVIN COMPOSITION OF ETP AND ETP_H

	μmoles X 10⁻³/mg protein	
	ETP	*ETP_H*
Flavin	0.64	0.55
1) Cytochrome *a** (equivalent of extracted *a* heme)	0.81	0.70
2) Cytochrome *b** (equivalent of extracted *b* heme)	0.70	0.60
3) Cytochrome *c* + c_1**	0.60	0.60
4) Cytochrome *c* extractable with 0.9% KCl	0	0.23
Total cytochrome (1 + 2 + 3)	2.11	1.90

* Cytochrome *a*, *b* and *c*, may each represent more than one component of similar or identical spectral properties whose heme components are also identical. These hypothetical components would have different protein moieties (*e.g. cf.* KEILIN[16]).

** The hemes of cytochrome *c* and c_1 are not extracted during the analytical procedure. It appears to be the hemoproteins rather than free heme which react with pyridine to form the pyridine hemochromogen.

TABLE IV

CYTOCHROME *c* REQUIREMENT FOR THE DPNH-O_2 REACTION OF ETP AND ETP_H

	DPNH-O_2	
	+ cytochrome c	*— cytochrome c*
	μmoles DPNH/min/mg at 38°	
ETP	3.76	3.66
ETP_H	1.86	0.56

ETP was prepared from the light mitochondrial fraction by the ethanol–phosphate method of GREEN *et al.*[2]. ETP_H was prepared from heavy mitochondria which had been exposed to sonic irradiation for 2 min. The particles were then washed once in a mixture of 0.15 *M* phosphate and 0.08 *M* sucrose. The particles were resuspended in 0.25 *M* sucrose.

DISCUSSION

The "mitochondrial" suspensions which are the starting points of our preparative procedures are mixtures of particles, both mitochondrial and submitochondrial. By differential centrifugation these can be sorted out into two main fractions: (1) the rapidly sedimenting or heavy fraction; and (2) the slowly sedimenting or light fraction. The latter consists of (a) swollen mitochondria and (b) derivative particles such as ETP. The former contains predominantly dense particles in which the cristae retain the characteristic double-membraned structure of the intact mitochondria. The available evidence from electron microscopy points to a common origin of all the submitochondrial particles present in both the heavy and light fractions, *viz.* the intact mitochondrion. That is to say, the various transitional stages by which the intact mitochondrion could be converted eventually into ETP or PETP or the heavy mitochondrial particles can either be seen or inferred.

The outer extended envelope of the swollen mitochondrial particles in the light

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fraction tends to bud off clusters of small single-walled vesicles. These vesicles have been isolated from the light mitochondrial fraction by the alcohol–phosphate procedure and identified as ETP. The same particle can be obtained essentially quantitatively by the sonic irradiation of the particles in the heavy mitochondrial fraction which contain predominantly cristae material. Thus ETP preparations can be formed from either outer envelope or cristae. Since ETP is the fundamental unit of electron transport, it follows that no basic biochemical distinction can be made between the vesicles derived from the outer membrane and those from the cristae, and insofar as the analytical data permit a conclusion, they have also essentially the same composition and components. Thus neither a structural nor functional difference can be recognized between these two parts of the mitochondrion. The remaining difference is exclusively one of position or geography.

Outer envelope and cristae form a continuous double-membrane structural system. Under the stresses of the isolation procedure cristae may become partly detached from the outer envelope and concentrated in one or more pockets or conversely the outer envelope may lift away from the mass of cristae. The extent to which outer envelope and cristae are damaged or modified in a given preparation will determine the extent to which, or the ease with which they give rise to derivative particles like ETP under a given set of conditions.

PETP is a particle far smaller than the intact mitochondrion which still retains the capacity for citric cycle oxidations and the accompanying oxidative phosphorylation. Whatever mechanism is assumed for the formation of PETP, it is impossible to avoid the necessity for assuming fragmentation or rupture of the outer mitochondrial envelope during the process. Since the characteristic particles of PETP preparations contain intact cristae enclosed by an outer envelope (in fact a miniature mitochondrion) it must be inferred that the outer envelope of PETP particles is only a segment of the original mitochondrial envelope just as the cristae of PETP represent only a small fraction of the original number. If the mitochondrion can be miniaturized in this way then the least common denominator of mitochondrial function must be a smaller unit. The biochemical evidence points to an exact parallelism between the presence of double-membrane structures in a particle preparation and the capacity for citric-cycle oxidations. Single-membrane vesicles like ETP no longer are capable of citric-cycle oxidations.

The concept that the electron transport system of the outer envelope may not be coupled to phosphorylation rests on the observation that ETP derived predominantly from the outer envelope catalyzes the uncoupled oxidation of DPN Hand succinate. Some recent work in our laboratory (unpublished studies of ZIEGLER AND LINNANE) suggest that even ETP derived from outer envelope is capable of coupled phosphorylation providing certain preparative precautions are taken. Non-coupling may be a consequence of the preparative procedure and the state of the starting material rather than a preexisting attribute of the electron transport system.

CLELAND AND SLATER[10,17] were the first to localize the electron transport system specifically in the structural elements of the outer envelope or outer membrane in their terminology. While they assign more significance to the outer envelope than we think necessary, nonetheless there is common ground in considering the double-membrane structure of the mitochondrion as the seat of citric cycle oxidations and of electron transport. WATSON AND SIEKEVITZ[18] more recently present a wealth of data

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which point to much the same conclusion though these authors assign special significance to the outer envelope.

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SUMMARY

The morphological changes which mitochondria and their derivative particles undergo during isolation from beef-heart muscle have been studied by electron microscopy. Mitochondria isolated from the tissue in a 0.25 *M* sucrose medium are grossly modified in structure. By virtue of these modifications they can readily give rise to smaller submitochondrial particles such as ETP and PETP. Morphologically intact mitochondria can be isolated from heart tissue which is comminuted in 0.88 *M* sucrose but only in relatively small yield. Such mitochondria do not give rise to ETP and PETP by the methods successfully applied to the mitochondria isolated in 0.25 *M* sucrose.

The light mitochondrial fraction contains swollen mitochondria whose outer membrane shows a marked tendency to undergo vesiculation leading to the formation of the electron transport particle. Some or all of the cristae of these swollen mitochondria can be pinched off together with a part of the outer mitochondrial membrane to form new particles which, depending upon size, conform either to the particles of the heavy mitochondrial fraction (large particle) or to PETP (small particle).

When the heavy mitochondrial fraction is exposed to sonic irradiation, a smaller particle is liberated which resembles ETP closely both morphologically and chemically. It differs primarily in the respect that bound cytochrome *c* is readily extractable whereas the bound cytochrome *c* of ETP is not.

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