THE OXIDATION OF EXOGENOUS AND ENDOGENOUS CYTOCHROME C IN MITOCHONDRIA

A Biochemical and Ultrastructural Study

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ABSTRACT

The effect of the nonionic detergent Lubrol on the oxidation of endogenous and exogenous cytochrome c by cytochrome oxidase in intact and fragmented mitochondria was studied. Mitochondria and mitochondrial fragments from liver, kidney, heart, and skeletal muscle have been used. Negatively stained preparations of intact mitochondria showed the particles of Fernández-Morán on the matrix side of their inner membrane system: under these conditions, the oxidation rate of externally added cytochrome c was very high, and it was stimulated very poorly by Lubrol. Mechanical fragmentation of liver mitochondria yielded vesicles with a smooth external profile: also under these conditions, the oxidation of externally added cytochrome c was very high, and poorly stimulated by Lubrol. The oxidation of endogenous cytochrome c was also unaffected by Lubrol. On the other hand, fragmentation of heart and skeletal muscle mitochondria yielded vesicles having numerous particles of Fernández-Morán on their external profiles. Under these conditions, the oxidation of exogenous cytochrome c was low and was markedly stimulated by Lubrol. On the contrary, no activation of the oxidation of endogenous cytochrome c was induced by the detergent. The results indicate a difference in the permeability properties of the two faces of the inner mitochondrial membrane: a permeability barrier for cytochrome c is suggested to exist at the inner face.

INTRODUCTION

In the last 3 yr, a great deal of interest has been focused on the topography of the mitochondrial enzymes. New methods of subfractionation have led to the separation of the inner and outer mitochondrial menbranes (18, 17, 13, 22, 24), and of the 90-A particles, first described by Fernández-Morán (3), from the inner membranes (21, 9). General consensus has not yet been reached on the nature of the enzymic activities associated with the outer membrane (4, 13, 19, 22, 24): however, it is agreed that neither cytochromes *a*, *b*, *c*, and c_1, nor succinic dehydrogenase, are associated with this membrane. As for the "headpieces" associated with the inner membrane and with the cristae, evidence coming from experiments of Racker and coworkers (21, 9) suggests that they are the structural equivalent of Racker's F_1 ATPase. The inner membrane, in addition to other protein and lipid components, contains the respiratory chains: they accept the reducing equivalents from the Krebs cycle at one end, and react with oxygen, via cytochrome oxidase, at the other end.

No direct information is yet available on the spatial arrangement of the individual respiratory carriers within the inner membrane. Recently, emphasis has been placed on the portion of the

inner membrane that lines the matrix surface, mostly because of the connection between the 90-A particles, which protrude into the matrix, and the so called "base-pieces" of the inner membrane.

The present study is a combined biochemical and ultrastructural attempt to demonstrate that the two faces of the inner membrane are functionally not equivalent. For the study, advantage has been taken of the fact that the 90-A particles are present only on the matrix side of the inner membrane, and can thus be used as markers of the side. Although some authors still maintain that the 90-A subunits could be produced artificially under the conditions of the negative staining, and would thus not preexist as such in intact mitochondria (25), general agreement, indeed, exists on the fact that the subunits are observed only on the matrix side of the inner membrane and of the cristae. The oxidation of externally added cytochrome c by cytochrome oxidase has been selected as the subject of this study following the observation, made in this Institute,[1] that nonionic detergents activated very poorly the high cytochrome oxidase associated with the oxidation of externally added cytochrome c of intact muscle mitochondria and, on the other hand, had a very marked stimulatory effect on the low activity exhibited by muscle submitochondrial preparations. Preliminary electron microscopic investigations pointed to the structural asymmetry of the inner membrane as a possible explanation for the observation.

The results presented indeed indicate that the permeability of the two faces of the inner membrane for cytochrome c is different.

A preliminary account of these investigations has been communicated elsewhere (2).

MATERIAL AND METHODS

Preparation of Mitochondria and of Mitochondrial Fragments

Mitochondria were prepared from liver, kidney, and heart muscle of male, Wistar-strain rats, and from the masseter muscle of young rabbits. The animals were fasted 12 hr before the sacrifice, and killed by decapitation (rats) or by a strong blow on the head (rabbits). The tissues were excised as quickly as possible, and dropped into 0.25 M sucrose at 0°C. All subsequent operations were carried out at about 2°C.

[1] E. Carafoli and P. L. Patriarcha, unpublished observations.

Liver and kidney mitochondria were isolated in 0.25 M sucrose, according to Schneider (23), and washed once. Before isolation, the kidneys were freed of the medullas.

For the isolation of heart mitochondria, the hearts from six to eight rats were pooled. The hearts were freed of blood by repeated soaking in 0.25 M sucrose, minced exhaustively with a pair of scissors, and homogenized with a rather loose lucite Potter homogenizer; the homogenate was at 10% in 0.40 M sucrose. Nuclei, myofibrils, and cell debris were discarded at 770 *g* for 10 min; mitochondria were spun down at 14,000 *g* for 10 min, and washed once. For the isolation of mitochondria from masseter muscle, the muscle was freed from its abundant connective tissue with a pair of scissors, and minced exhaustively as in the case of the heart. It was then homogenized in a Waring-Blendor, at 10% in 0.1 M KCl, buffered at pH 7.0 with 0.005 M histidine. The gravitational forces employed were the same as in the case of heart.

Submitochondrial fragments were obtained by mechanical fragmentation of mitochondria in a Servall Omni-Mixer homogenizer, used at full speed for 4 min, at 0°C. The suspensions were then centrifuged at 14,000 *g* for 10 min for collecting the fragments. Mechanical fragmentation was used routinely for obtaining large amounts of submitochondrial fragments; however, fragments having the same morphological appearance and the same biochemical properties (see below) could be obtained in a much milder way, *i.e.*, by collecting from the postmitochondrial supernatant the material sedimenting at 37,000 *g* for 20 min. In this case, the fragments evidently were derived from the breakage of a number of mitochondria during the homogenization of the tissue.

Sonic fragments were obtained from liver mitochondria in a Blackstone sonicator. Liver mitochondria were suspended in 0.25 M sucrose, at an approximate concentration of 50 mg protein/ml. Sonication was carried out for 2 min, at 0°, with the power indicator set at 50. The fragments were harvested as described for the mechanical fragments.

Analytical Methods

Oxidation of externally added cytochrome c by cytochrome oxidase was routinely assayed with a polarographic Clark oxygen electrode in a medium of the following composition: 0.033 M phosphate buffer, pH 7.4; 0.0004 M $AlCl_3$; 0.00067 M cytochrome c, and 0.1–1.0 mg of enzyme protein. The final volume was 1.8 ml, and the temperature 25°C. The reaction was started by adding 0.01 M Na-ascorbate, and 2 mg of Lubrol were added at the points indicated in the figures. In the experiments shown in Figs. 5, 11, 15, and 17, no cytochrome c was added to the medium: endogenous cytochrome c was kept

reduced by the addition of 0.02 M Na-ascorbate and 0.0002 M N,N,N′,N′-tetramenthyl-*p*-phenylenediamine (8). Succinate–cytochrome c reductase was determined spectrophotometrically at 550 mμ, in a Zeiss spectrophotometer. The method used was that of Sottocasa et al. (24) with minor modifications. The reaction was started by the addition of 3 mM Na-succinate.

Protein concentration was determined with a biuret reaction.

Electron Microscopy

Mitochondria and mechanical mitochondrial fragments, suspended either in the isolation medium at 0°C or in the medium for cytochrome oxidase assay at 25°C, were immediately processed for negative staining according to the procedure suggested by Horne (7). Solutions of ammonium molybdate were routinely used as the electron-opaque material; their osmolarity was made exactly equal to that of the media used for suspending mitochondria or mitochondrial fragments in each single case. Adjustment of the osmolarity of ammonium molybdate to that of the suspending medium was found to be essential in order to preserve the tridimensional organization of these membranous systems and thus to obtain the homogeneous appearance of the preparations studied. In essence, the procedure used was that recently described by Muscatello and Horne (16). Various attempts were made to obtain penetration of the negative stain into the submitochondrial vesicles, so as to visualize the internal profile of the membrane: a partial success was obtained by incubating the vesicles, at room temperature, in the phosphate-containing medium, in the presence of 2% ammonium molybdate. After 4 min of incubation, the excess stain was washed away with the phosphate medium.

For the experiment described in Fig. 18, sonic fragments from liver mitochondria were stained in a 1% solution of phosphotungstic acid, brought to pH 7.4 with KOH.

A Siemens Elmiskop IA was used with a double condenser and objective lens diaphragm apertures of 20 μ. The primary magnifications varied from 10,000 to 40,000.

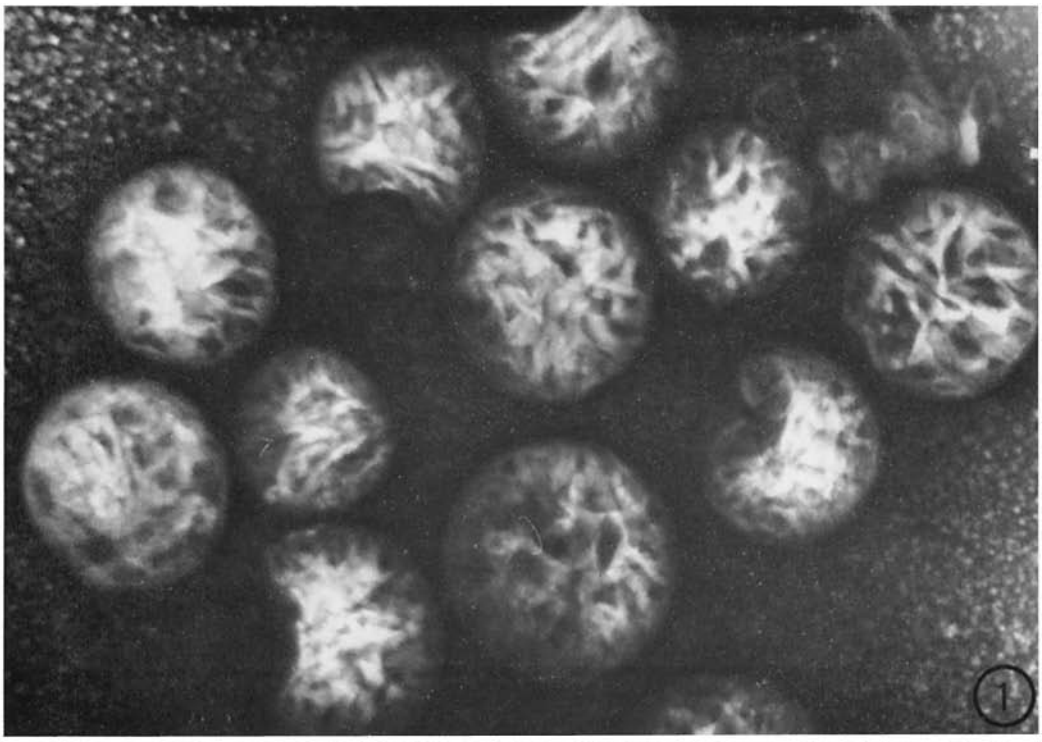
RESULTS

Liver Mitochondria

When cytochrome oxidase was assayed in the medium described in Methods, mitochondria were subjected to strongly hypotonic conditions. They thus underwent considerable osmotic shock during the incubation for the enzyme assay. This is shown in Figs. 1–4, which compare liver mitochondria as they appeared under the isotonic conditions used for their isolation and during incubation in the hypotonic, phosphate-containing medium used for estimation of cytochrome oxidase, respectively. When isolated in 0.25 M sucrose and negatively stained under isotonic conditions with ammonium molybdate, liver mitochondria appeared as spheres of comparable diameter, ranging from 1.4 to 1.9 μ (Fig. 1). As mentioned in Methods, the high degree of homogeneity of the mitochondrial profiles illustrated in Fig. 1 was observed only when solutions of ammonium molybdate isotonic with the suspending medium were used. Under these conditions, the negative stain penetrated only into the space between the outer and the inner membranes as indicated by the presence of a thin layer of electron-opaque material between the two membranes (Fig. 2). This also indicated that the stain did not penetrate into the matrix compartment. Thus, the external surface of the inner membrane and the complex infoldings of the cristae were made clearly visible (Fig. 2). When examined in the electron microscope after incubation in the medium for the assay of cytochrome oxidase, liver mitochondria appeared considerably

FIGURE 1 The appearance of the mitochondria isolated from rat liver in 0.25 M sucrose and negatively stained with an isotonic solution of NH_4 molybdate. Under these conditions, the mitochondria appear as spheres, 1.4–1.9 μ in diameter, "embedded" in the negative stain. The complex system of the inner membrane infoldings is made clearly visible by the penetration of the stain into the external compartment. × 39,000.

FIGURE 2 The appearance of a liver mitochondrion in finer detail isolated in 0.25 M sucrose and negatively stained with NH_4 molybdate under isotonic conditions. The stain has penetrated into the external compartment and the intracristal space, but not into the matrix space. Thus, both the outer membrane and the infoldings of the inner membrane are made visible. No 90-A subunits are seen on the external surface of the inner membrane. × 80,000.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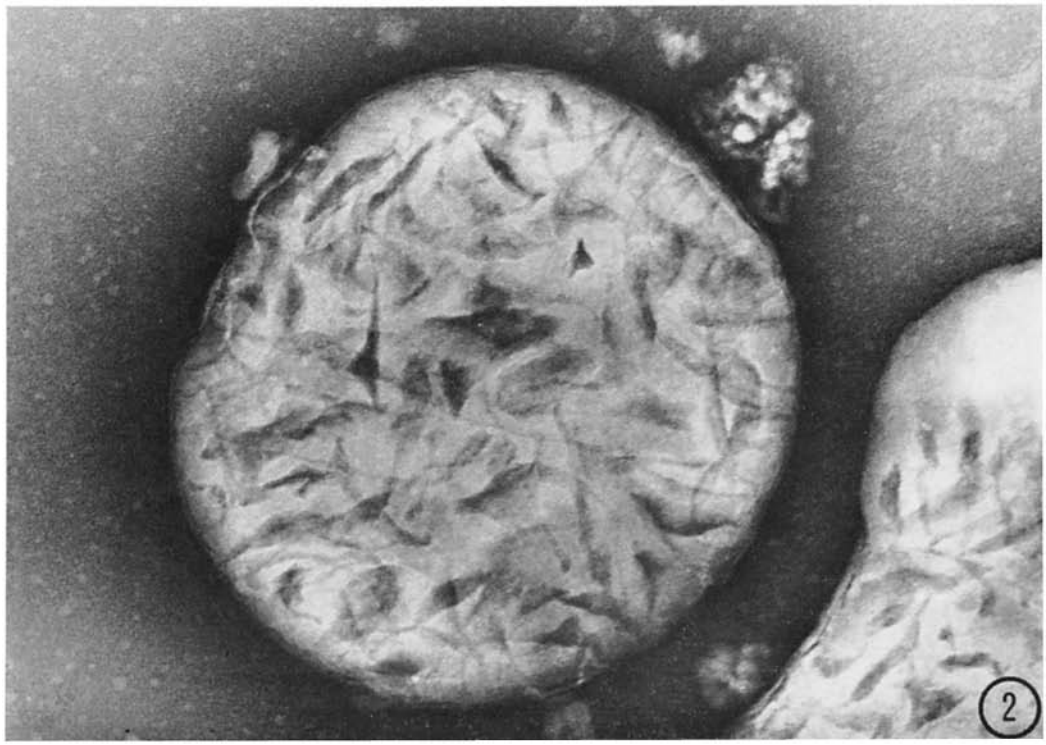
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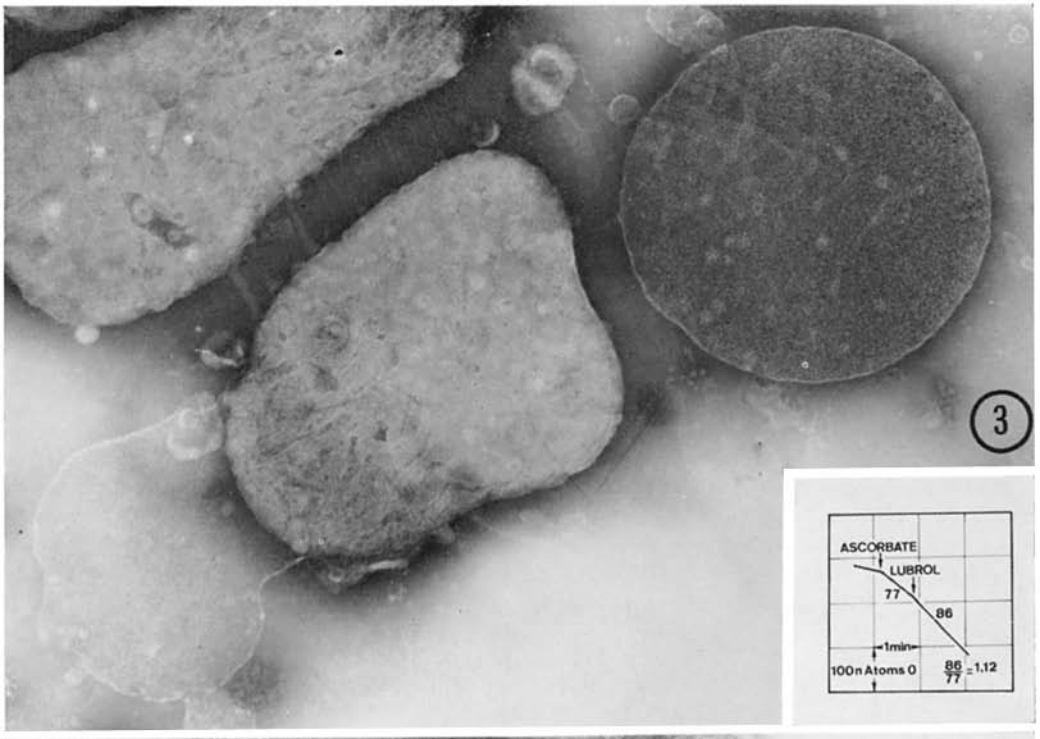
swollen (Figs. 3 and 4). The outer membrane was broken up and partially detached from the inner membrane. The penetration of water into the matrix compartment resulted in a progressive flattening of the cristae, most likely associated with a modification of the inner membrane organization, as indicated by the penetration of some negative stain into the matrix space (Fig. 4). In most cases, however, despite the enormous swelling, the mitochondrion did not break. As a result, the matrix surface of the inner membrane remained inside the large vesicle derived from the swelling of the mitochondrion. In some cases, loss of matrix material was observed, owing to a particularly large swelling of the mitochondrion; however, even in this extreme case, the swelling did not result in the turning of the internal surface inside out. This fact could be easily established since the 90-A particles were not visible on the external profile of the inner membrane, and were, on the other hand, clearly recognizable on the inside (Fig. 4). It could, therefore, be concluded from the above observations that, in the case of liver mitochondria, the incubation in the hypotonic, phosphate-containing medium for the assay of cytochrome oxidase resulted in a large swelling of the organelles; the resulting vesicular structures still presented to the medium the external surface of the inner membrane. The oxidation of externally added cytochrome c by cytochrome oxidase in these swollen mitochondria was rather high: 990 nAtoms of 0 were consumed per mg of protein per min. As it appears from Fig. 3, there was little or no activation of the oxidation rate by the addition of the nonionic detergent lubrol. The oxidation of endogenous cytochrome c in these swollen mitochondria was also studied, as shown in Fig. 5. In this case, only 79 nAtoms of oxygen were consumed per mg of protein per min: addition of lubrol under these conditions had no effect on the rate of O_2 consumption. As shown in Fig. 6, electron microscopic examination of Lubrol-treated preparations showed that practically all mitochondrial membranes were dissolved and only aggregates of granular material of different size were seen.

Skeletal and Heart Muscle Mitochondria

The basic mitochondrial structure, *i.e.*, two concentric compartments bound by membranes, was observed also in the case of skeletal and heart muscle mitochondria. However, the structural organization of the cristae differed in significant details from that of the cristae observed in rat liver mitochondria. As can be seen from Figs. 7 and 8, in the case of skeletal and heart muscle mitochondria, the cristae formed very frequently complete septa. Although it is fairly possible that the continuity of the septa was interrupted by orifices, as suggested by Whittaker for the case of brain mitochondria (26), the matrix space was evidently compartmentalized and the resulting compartments were probably only incompletely communicating with each other. Because of this structural organization, during the incubation in the medium for the assay of cytochrome oxidase, the swelling of

FIGURE 3 Rat liver mitochondria after incubation in the phosphate medium for the assay of cytochrome oxidase. The mitochondria appear considerably swollen, and the inner membrane infoldings are almost completely flattened. The outer membrane is constantly detached. Under these conditions, penetration of some negative stain into the matrix space takes place and thus the 90-A subunits of the inner membrane become visible on the matrix surface of the inner membrane. In the insert, the effect of the addition of Lubrol on the cytochrome oxidase activity is illustrated. General conditions for the enzyme assay reported in this figure and in Figs. 9, 10, 13, 14, 16, and 18 are described in Methods. As it appears from the trace, the addition of lubrol has only a negligible effect on the rate of oxidation. × 42,000.

FIGURE 4 The ultrastructure at a higher magnification of a rat liver mitochondrion treated as in Fig. 3. The mitochondrion appears as a large, flattened vesicle without an outer membrane. No 90-A subunits are seen on the external surface of the inner membrane, as can be appreciated by examination of the external profile of the vesicle. The subunits are clearly recognizable on the internal surface of the membrane, that is, on the surface facing the matrix space. In the insert, the presence of the elementary particles on the internal surface of the inner membrane is illustrated at higher magnification. × 80,000. Insert, × 160,000.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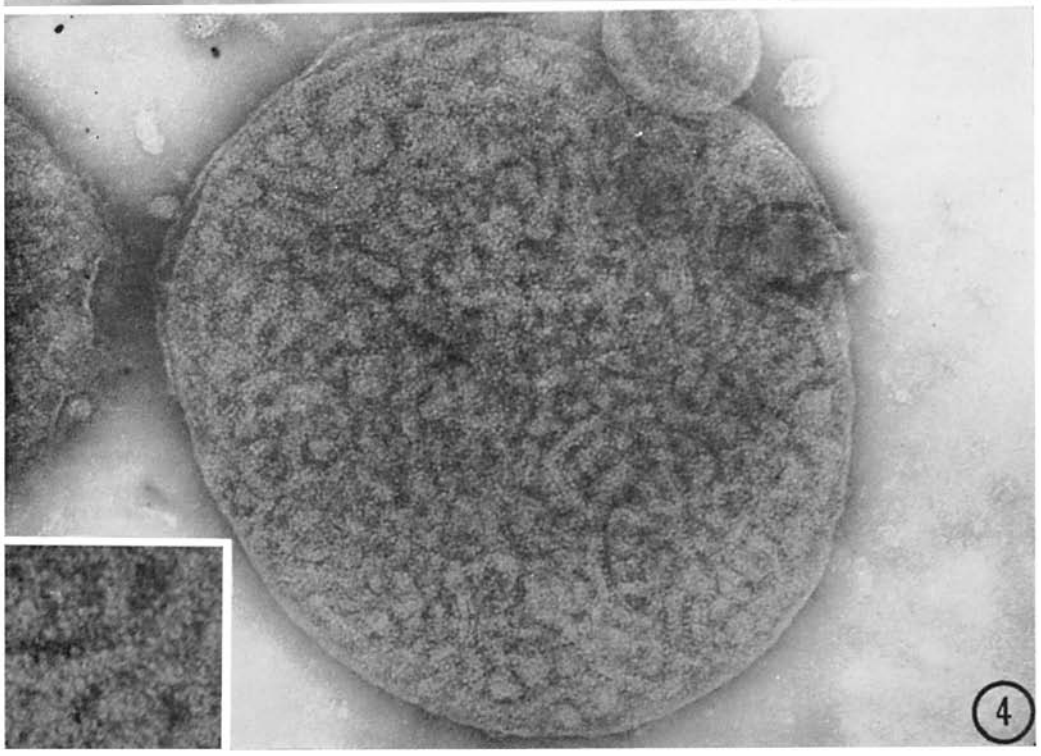
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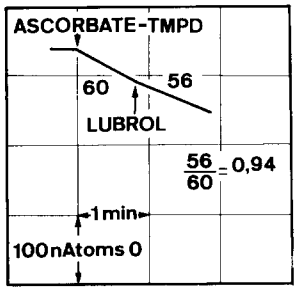


FIGURE 5 Oxidation of endogenous cytochrome c by cytochrome oxidase in rat liver mitochondria. Effect of the addition of Lubrol. It appears that the rate of cytochrome c oxidation is considerably lower than that observed in the presence of added cytochrome c and that the addition of Lubrol has no effect on the rate of oxidation. General conditions for the enzyme assay as illustrated in Methods; no cytochrome c was added to the medium; endogenous cytochrome c was kept reduced by Na-ascorbate and tetramethyl-*p*-phenylendiamine.

the mitochondria was partially prevented (Fig. 9) and the penetration of water into the different compartments could result in the breaking of the whole structure (Fig. 10). As a consequence, the mitochondria were disrupted into large fragments, which appeared void of outer membrane, but still maintained remnants of the cristal organization (Fig. 10). Such fragments presented to the medium the external surface of the inner membrane, as shown by the absence of the 90-A subunits from the outer profiles. In addition, as a further consequence of the water penetration into the matrix space under hypotonic conditions, a certain number of cristae became occasionally detached; probably by a process of pinching off, tubules were then formed from them. In this case, the external surface of the tubules was clearly the matrix surface of the inner membrane, as shown by the presence of the 90-A subunits on their external profiles. Thus, the skeletal and heart muscle mitochondrial fraction, incubated in the hypotonic, phosphate-containing medium, consisted of a variable mixture of mitochondrial fragments of differing structural organization; the majority of the fragments presented to the medium the external surface of the inner membrane, while a few others, derived from the fragmentation of the cristae, presented to the incubation medium the matrix face. When tested for the oxidation of exogenous cytochrome c, these mixed populations from skeletal and heart muscle mitochondria exhibited a very high rate of oxygen uptake (670 and 1970 nAtoms of 0 per mg of protein per min, respectively) which was only modestly activated by the nonionic detergent lubrol, as illustrated in Figs. 9 and 10. In a series of determinations, the values for the activation were found to fall in the range 1.3–1.7 times greater than the values obtained in the absence of lubrol. Fig. 11 illustrates the oxidation of endogenous cytochrome in heart mitochondria. The rate of O_2 consumption was much lower than with exogenous cytochrome c (166 nAtoms of 0 per mg of protein per min) and was not affected by the addition of lubrol.

Kidney Mitochondria

Kidney mitochondria were characterized by the presence of a much higher number of cristae than in liver mitochondria. However, at variance with skeletal and heart muscle mitochondria, as a rule the cristae did not form complete septa (Fig. 12). As a consequence, when incubated in the hypotonic, phosphate-containing medium for the assay of cytochrome oxidase, kidney mitochondria tended to swell as liver mitochondria did; however, some tubules with the external surface encrusted with elementary particles were also formed, as in the case of muscle mitochondria (Fig. 13). The rate of oxidation of externally added cytochrome c in kidney mitochondria was intermediate between that of liver mitochondria and that of heart mitochondria, and was moderately increased by the addition of Lubrol, as illustrated in Fig. 13.

Mechanical Fragmentation of Mitochondria

It appeared conceivable at this stage of the research that the differential effects of lubrol on the oxidation of exogenous and endogenous cytochrome c were related to the structure and the origin of the two surfaces of the vesicles. Since it has often been suggested that sonic oscillation or other means of fragmentation yields particles that are turned "inside-out" with regard to the intact mitochondria they are derived from (14, 10), attempts were made to prepare from mitochondria populations of fragments very homogeneous as to the surface facing the incubation medium. This was easily achieved by mechanical fragmentation of mitochondria under isotonic conditions as described in Methods. Exhaustive examination of the fragments in the electron microscope allowed the external surface of the fragments to be distinguished with respect to its origin from the intact

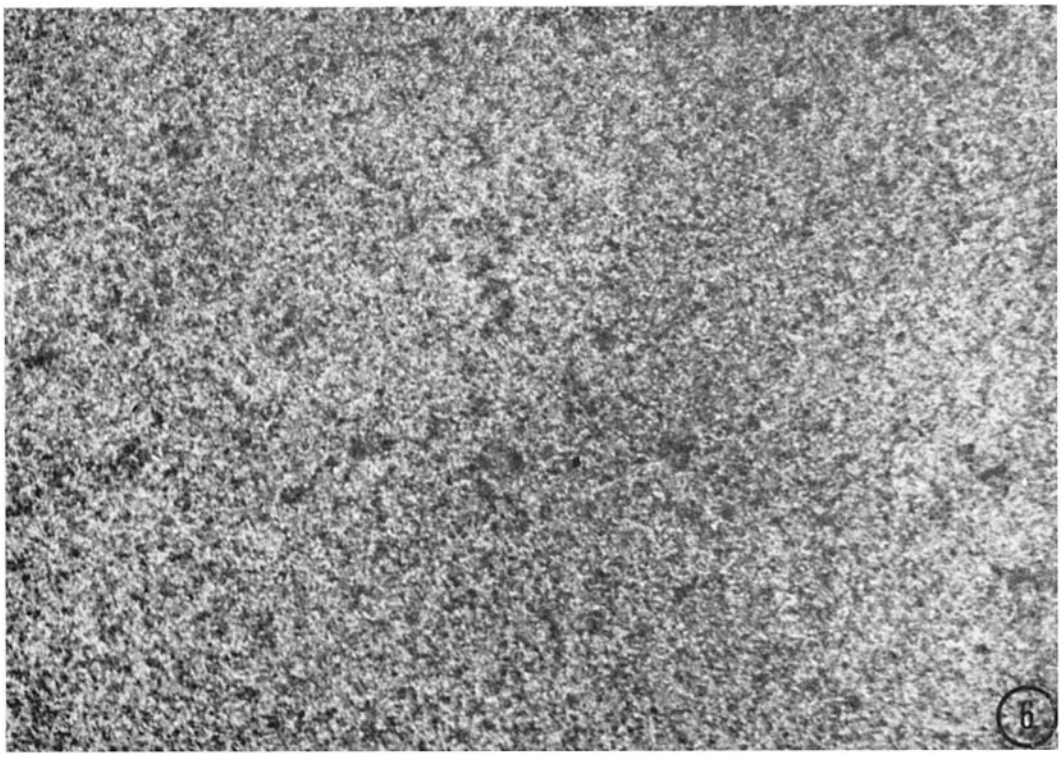


FIGURE 6 The appearance of liver mitochondria suspended in the medium for cytochrome oxidase assay, and treated with the nonionic detergent lubrol. It is apparent that the original structure of mitochondria is completely disrupted and that the membrane organization is dissolved into granular material. This forms aggregates of variable size. × 120,000.

mitochondrial inner membrane, with the use of the 90-A particles as markers.

A fraction composed of a very homogeneous population of fragments was thus obtained from skeletal or heart muscle mitochondria. As shown in Fig. 14, which illustrates the ultrastructural features of a fraction derived by mechanical fragmentation from masseter muscle mitochondria, practically all the vesicles and tubules seen in the fraction were encrusted with elementary particles on their external profiles; this finding indicated that their external surface corresponded to the original matrix face of the inner membrane. When these preparations were tested for oxidation of externally added cytochrome c, they showed a very high degree of activation by Lubrol, as illustrated in Fig. 14. In a large group of experiments, activation ratios ranging from 3.0 to 5.4 were measured. Similar results were obtained with fragments prepared from heart mitochondria. On a protein basis, the rate of O_2 consumption by the fragments in the absence of Lubrol was much lower than that observed in intact mitochondria. After addition of lubrol, the rate of O_2 consumption was generally greater than that observed under the same conditions in the intact mitochondria from which the fragments were prepared. This was to be expected since fragmentation of mitochondria involved loss of matrix. When oxidation of endogenous cytochrome c was studied in these fragments, a very slow rate of O_2 consumption was found (164 nAtoms of 0 per mg of protein per min); no acceleration of the rate of O_2 consumption was observed upon addition of lubrol (Fig. 15). On the other hand, mechanical fragmentation of liver mitochondria resulted in the formation of smooth-surfaced vesicles having no 90-A subunits on the profiles facing the medium (Fig. 16). When assayed for oxidation of externally added cytochrome c, the vesicles obtained from liver mito-

chrondria by mechanical fragmentation were only modestly activated by addition of lubrol, and the activation in no case exceeded that found in the unfragmented mitochondria. Similarly, no activation by Lubrol was observed when the oxidation of endogenous cytochrome c was studied (Fig. 17). The possibility was considered that liver mitochondria were more labile to mechanical fragmentation than muscle mitochondria, and would thus lose the 90-A particles to the medium during mechanical fragmentation, at variance with muscle mitochondria. The vesicles would thus appear smooth; yet the surface presented to the medium would be the same as in the fragments derived from muscle mitochondria. This possibility was rendered unlikely by two experimental findings: (*1*) particles having the same appearance as those obtained by exhaustive mechanical fragmentation were obtained from liver mitochondria by much milder procedure (see Methods); (*2*) the low oxidation rate of exogenous cytochrome c was powerfully stimulated by Lubrol in sonic fragments from liver mitochondria (Fig. 18). As suggested by data in the literature (14, 10), the presence of the 90-A knobs on the external profile of the sonic vesicles permitted the conclusion that the vesicles were turned inside out. Attempts were also made to visualize the 90-A knobs on the inner profile of the vesicles derived from liver mitochondria by mechanical fragmentation; the penetration of the stain inside the vesicles was obviously necessary for the visualization. The procedure described in Methods indeed permitted the penetration of the stain into some of the vesicles. One of these vesicles is shown in Fig. 19: quite clearly, its outer profile is smooth, whereas the knobs are recognizable inside the vesicle. When kidney mitochondrial fragments were assayed, intermediate results were obtained; the activation of exogenous cytochrome c oxidation by Lubrol appeared to be higher in those fractions in which a higher number of tubules and vesicles with the external surface lined with elementary particles were seen.

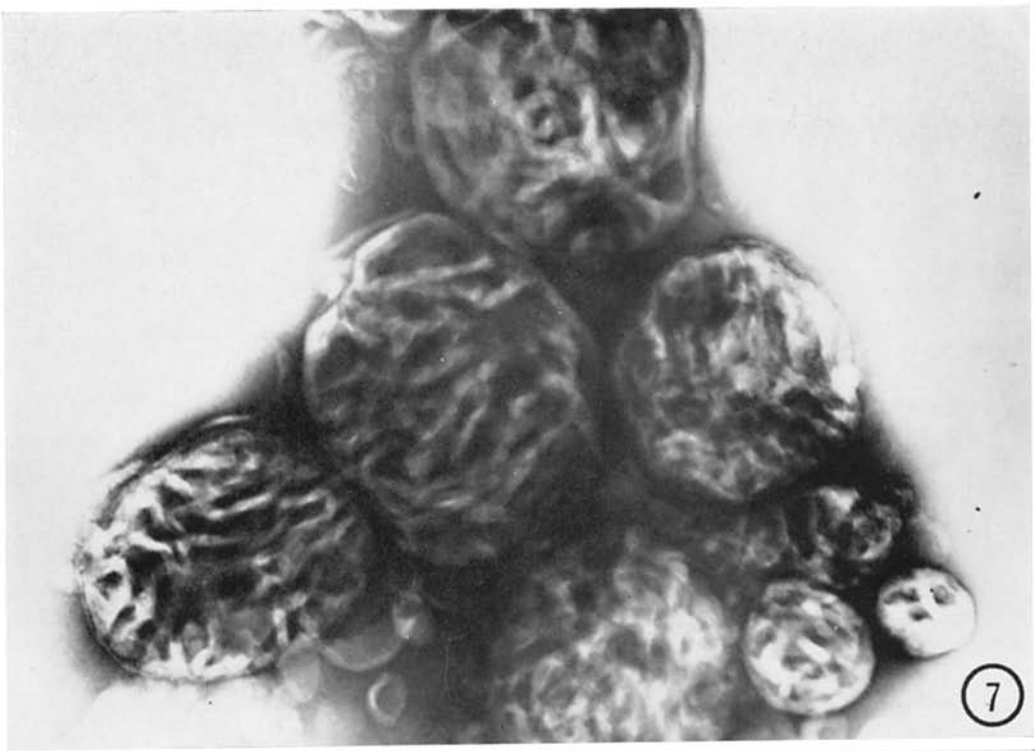
Fig. 20 *A* and *B* shows the effect of Lubrol on the succinate-cytochrome c reductase of heart mitochondria and submitochondrial fragments. Lubrol had no effect on the rate of reduction of cytochrome c in intact heart mitochondria, but it stimulated the rate considerably in the submitochondrial fragments: thus reduction by succinate-cytochrome c reductase behaved quite like the oxidation of external cytochrome c with respect to the effect of Lubrol. Since no cytochrome oxidase was involved in the assay, the experiment ruled out the possibility that the effects of Lubrol on the oxidation of cytochrome c described above were due to an asymmetrical location of cytochrome oxidase itself in the inner mitochondrial membrane. In this connection, it is pertinent to mention that a very recent study involving the electron microscopic visualization of antibodies against specific mitochondrial components has failed to reveal any cytochrome a type compounds at, or near, the matrix side of the inner membrane (1).

DISCUSSION

As mentioned in the Introduction, it is generally accepted that the components of the respiratory chain are housed in the inner mitochondrial membrane. According to Green and coworkers (4, 5), the inner membrane and the cristal infoldings contain a regular succession of the four basic complexes of the respiratory chain: each complex is connected with one of the 90-A particles which protrude into the matrix. Green et al. (6) have shown that the four basic complexes of the respiratory chain could be separated with bile salts. Upon dilution of the bile salt, they tended to reaggregate spontaneously, to form membrane-like structures having a vesicular appearance. McConnell et al. (15) have also shown that membranes

FIGURE 7 Skeletal muscle mitochondria isolated from the masseter muscle of the rabbit in KCl-histidine buffer, and negatively stained with solutions of ammonium molybdate of equal osmolarity. It is apparent that the infoldings of the inner membrane tend to form complete septa across the whole organelle. × 80,000.

FIGURE 8 Mitochondrial fraction isolated from rat heart in 0.40 M sucrose and negatively stained with solutions of ammonium molybdate of equal osmolarity. Heart mitochondria are characterized by the presence of an extremely high number of cristae that frequently form complete septa, as in the case of the masseter muscle mitochondria. × 80,000.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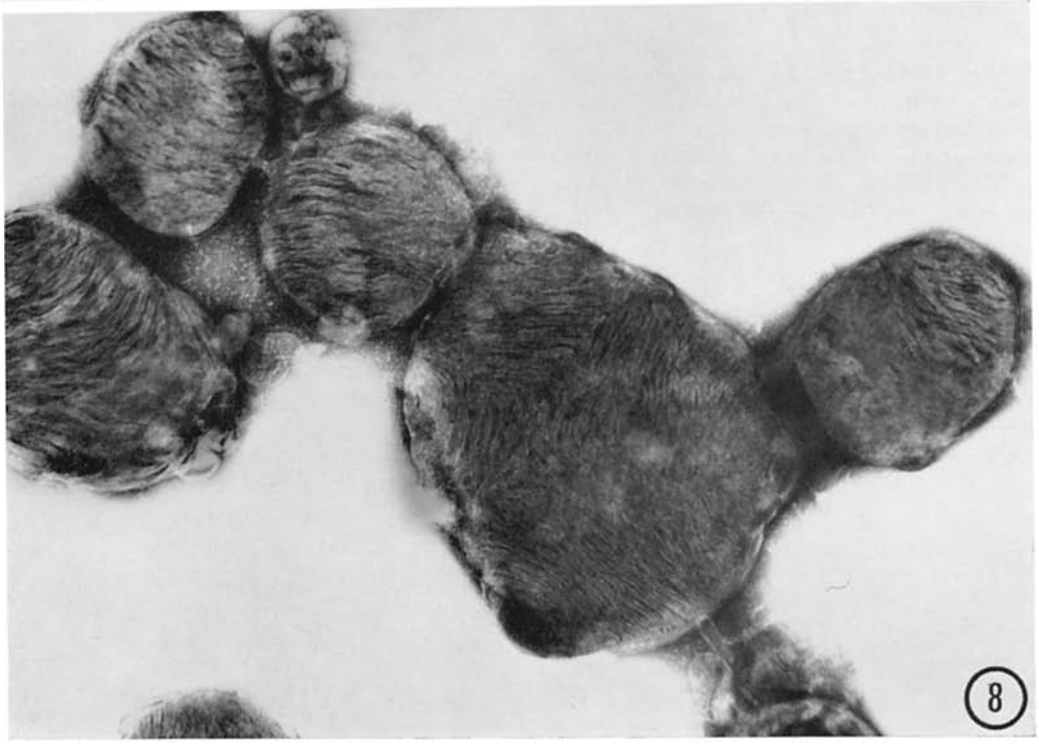
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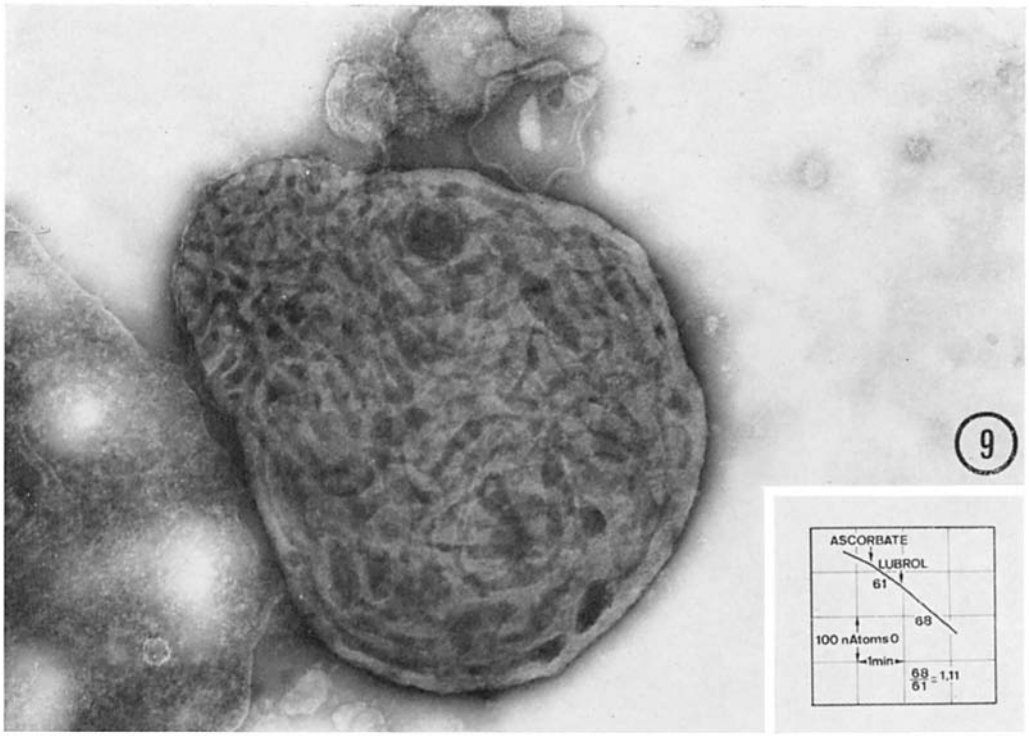


FIGURE 9 Skeletal muscle mitochondria after incubation in the phosphate medium for the assay of cytochrome oxidase. As in the case of liver mitochondria, the outer membrane is detached under these conditions. The mitochondrion appears swollen, although to a lesser degree as compared to liver mitochondria since the presence of complete septa do not allow a large expansion of the inner compartment. No 90-A subunits are seen on the external surface of the mitochondrion. In the insert, the effect of Lubrol on the cytochrome oxidase activity is shown. It is apparent that the addition of detergent has a very limited effect on the oxidation rate. × 80,000.

could be formed by the reaggregation of isolated complex IV (cytochrome oxidase). They concluded that cytochrome oxidase makes a major contribution to the morphology of the inner membrane; according to their estimate, it could account for a very large fraction of the total protein of the inner membrane, up to 20%. According to other authors (19, 20), the presence of a regular succession of complexes in the molecular structure of the inner membrane is still open to question.

As stressed in the Introduction, no direct experimental information is yet available on the geometrical organization of the individual components of the respiratory chain within the inner membrane The location of the link between the Krebs cycle dehydrogenases and the respiratory chain is, for instance, a controversial issue. According to Green and coworkers (4), the Krebs cycle is housed at the level of the outer membrane: the dehydrogenases would thus presumably discharge the reducing equivalents into the outer chamber of the mitochondrion, to be delivered to the NAD end of the respiratory chain at the external face of the inner membrane. According to most other authors, however, the Krebs cycle is located in the matrix, most likely in close functional link with the inner membrane: reduction of NAD would, in this case, take place at the interface between the internal side of the inner membrane and the matrix.

As for the other end of the respiratory chain, where the oxidation of cytochrome c takes place, a number of interesting reports have recently appeared in the literature. Palmieri and Klingenberg (17) have shown that cytochrome oxidase in liver mitochondria was inhibited by azide only when the inhibitor had permeated the matrix

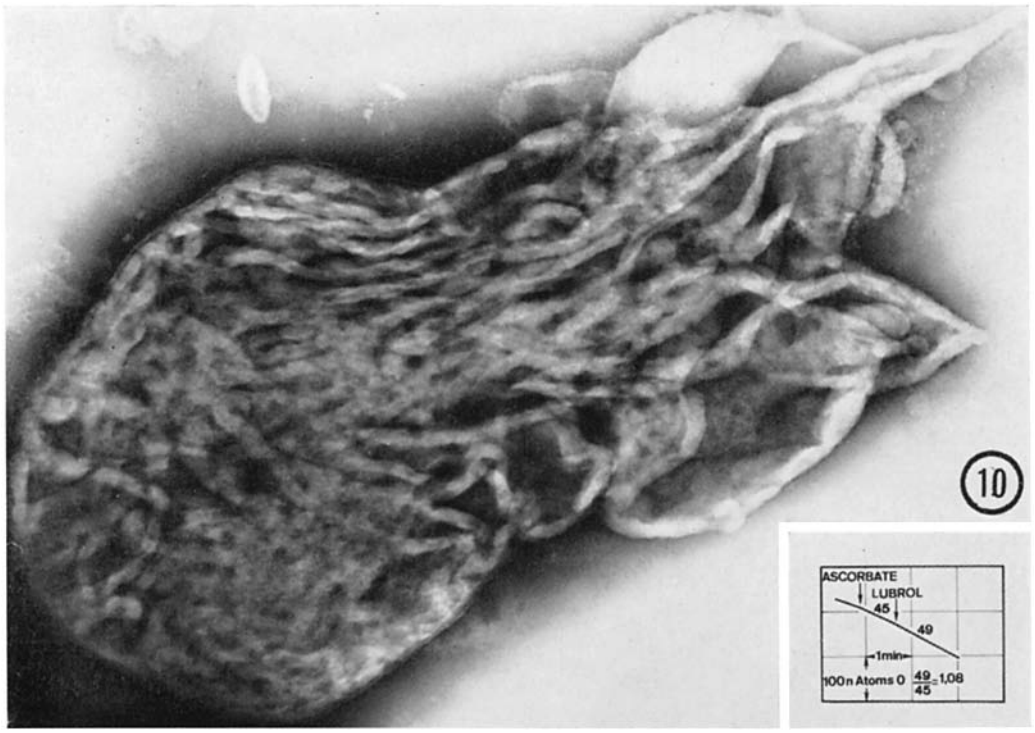


FIGURE 10 Heart muscle mitochondria incubated in the phosphate medium for the assay of cytochrome oxidase. Considerable swelling of the mitochondrion occurs; however, the organization of the inner membrane infoldings does not allow a spherical expansion of the inner membrane and a progressive unfolding of the cristae. However, it has to be noted that, although the mechanism of the swelling differs from that observed in liver mitochondria, also in the case of heart muscle mitochondria the 90-A particles are not seen on the external surface of the swollen mitochondrion. As it appears from the insert, the addition of Lubrol results in a very limited activation of the oxidation of cytochrome c. × 80,000.

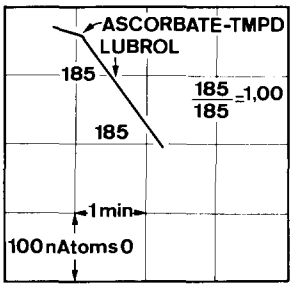
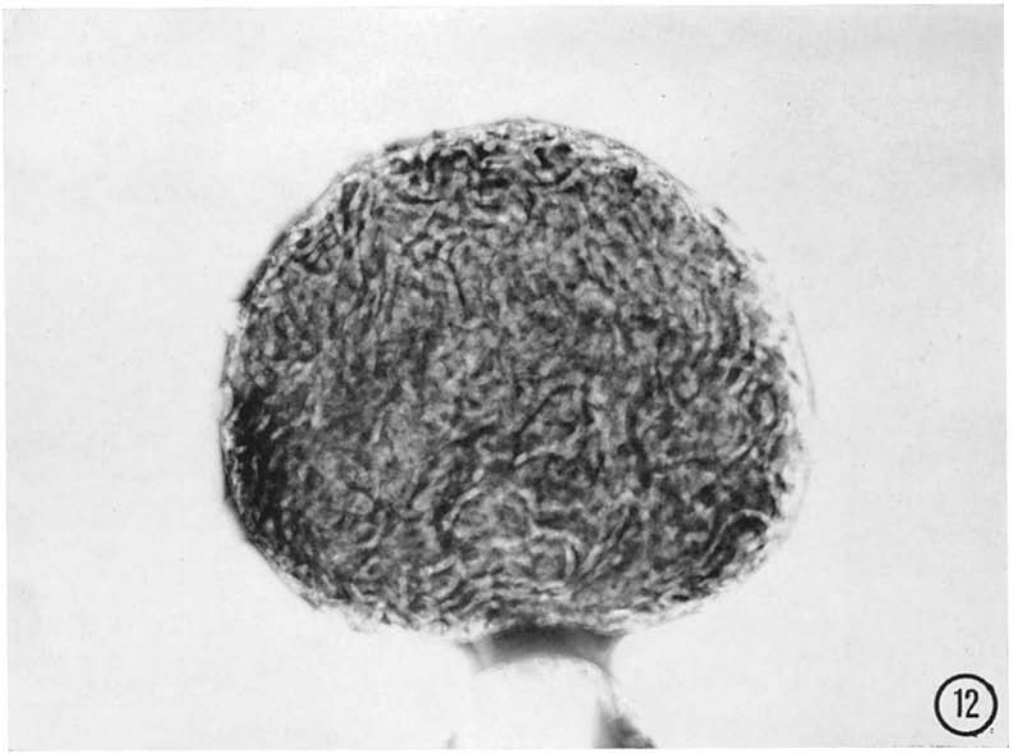


FIGURE 11 The effect of the addition of Lubrol on the rate of oxidation of endogenous cytochrome c by heart muscle mitochondria. Addition of Lubrol has no effect on the rate of oxygen consumption. The conditions for the enzyme assay were as in Fig. 5.

space: the binding of azide to the enzyme molecule would thus presumably take place from the matrix side of the inner membrane. Lenaz and MacLennan (12) and Lee and Carlson (11) have, on the other hand, reported observations suggesting that the binding of externally added cytochrome c took place at the external side of the inner membrane. The results presented in this paper are indeed directly relevant to these problems.

Electron microscopic examination of negatively stained liver, kidney, skeletal, and heart muscle mitochondria, which had been incubated in the highly hypotonic, phosphate-containing medium for assay of cytochrome oxidase, has shown that, in all cases, the incubation induced the loss of the outer membrane, in agreement with previous observations of Parsons et al. (18) on liver mitochondria. In addition, swelling was constantly observed; however, depending on the structural organization of the various mitochondria considered, the degree of swelling varied considerably. Swelling was maximal in liver mitochondria, where the cristae did not form complete septa,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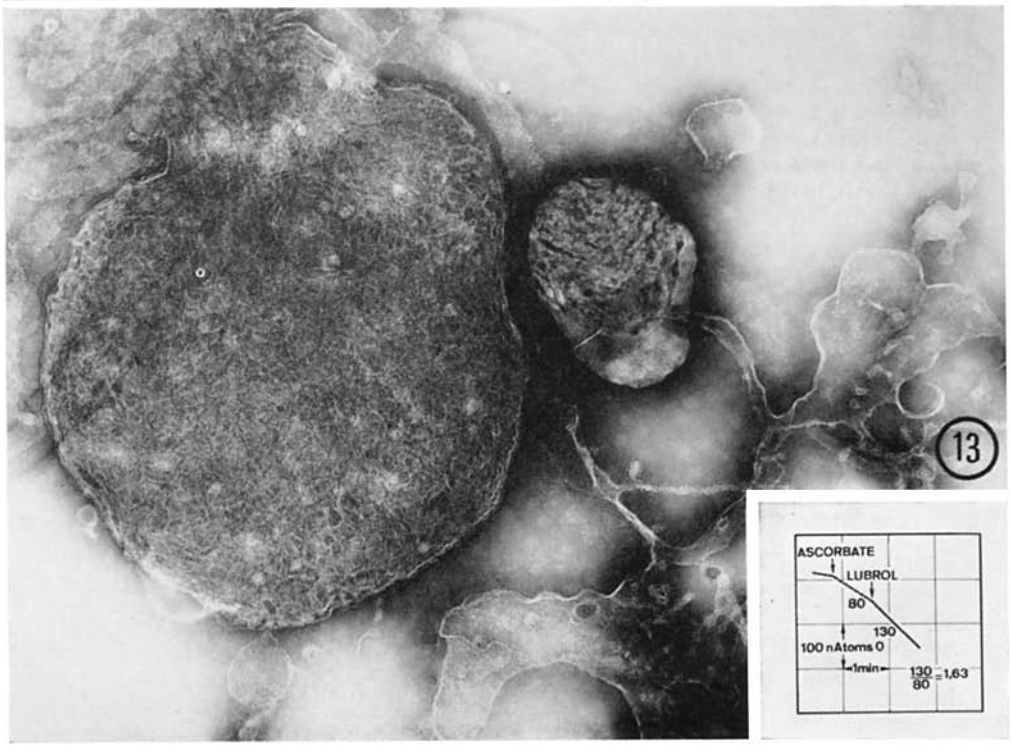

13
ASCORBATE
LUBROL
80
130
100 nAtoms O
1min
130/80 = 1,63

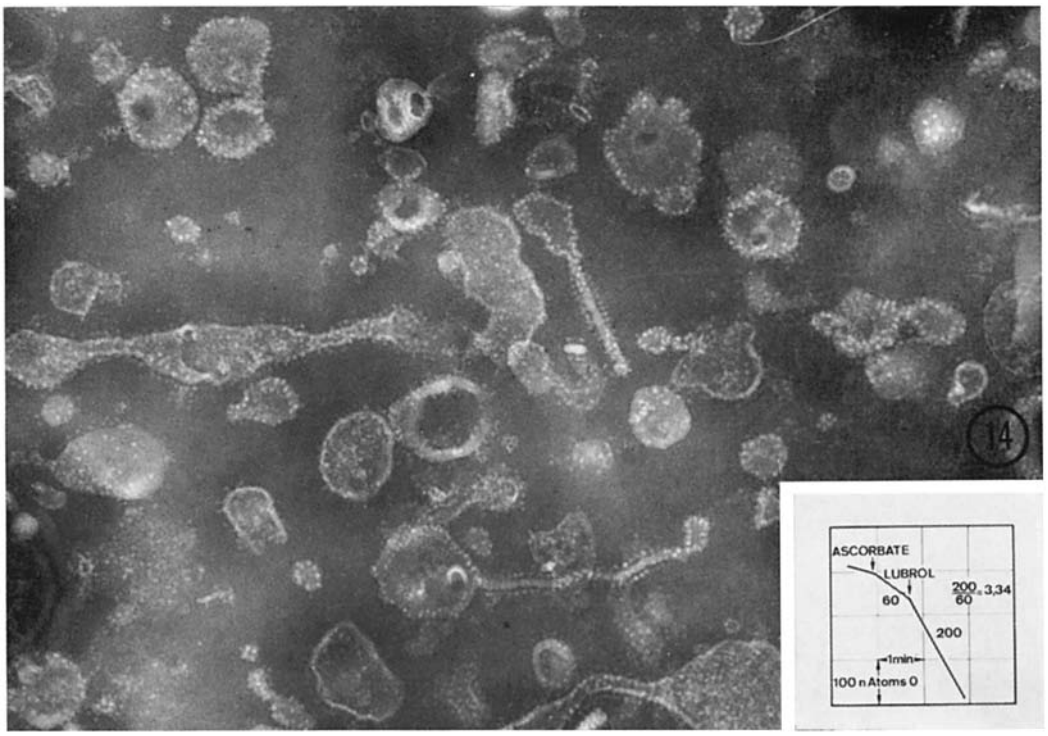


FIGURE 14 The mitochondrial subfraction shown here was obtained by mechanical fragmentation of skeletal muscle (masseter) mitochondria as described in Methods, and was incubated in the medium for the assay of cytochrome oxidase. Practically the whole fraction consists of small vesicular or tubular structures encrusted with 90-A particles. It is thus apparent that the surface of the vesicles and tubules that faces the medium corresponds to the original matrix surface. As shown in the insert, addition of Lubrol to this fraction results in a strong activation of the rate of oxidation of externally added cytochrome c by cytochrome oxidase. × 80,000.

and minimal in heart and skeletal muscle mitochondria in which the presence of these complete septa hindered the expansion of the mitochondria. However, the outer profiles of the swollen mitochondria, isolated from all sources, appeared largely devoid of the 90-A particles which are usually located on the internal surface of the inner membrane. It could thus be concluded that mitochondria from different sources, suspended in the hypotonic phosphate medium for the assay of cytochrome oxidase, invariably presented to the medium the external face of the inner membrane. The results presented have, on the other hand, shown that in these preparations the oxidation of

FIGURE 12 Rat kidney mitochondrion in 0.25 M sucrose and negatively stained under isotonic conditions with ammonium molybdate. Of interest are the high number of cristae and the complex system of inner membrane infoldings. × 45,000.

FIGURE 13 Rat kidney mitochondria in the incubation medium for the assay of cytochrome oxidase. The swelling of the mitochondria is very considerable and occasionally results in the formation of fragments encrusted with 90-A subunits. However, the bulk of of the fraction consists of vesicles that present to the medium the external surface of the inner membrane, as shown by the absence of 90-A particles. In the insert, the effect of lubrol on the cytochrome oxidase activity with external cytochrome c is shown. × 40,000.

externally added cytochrome c was rather high and could not be increased any further by the addition of the membrane-disrupting agent lubrol. The oxidation of endogenous cytochrome c was slower, as expected from data existing in the literature, and was not affected by lubrol. Apparently, then, when mitochondria from all sources considered presented to the external medium the

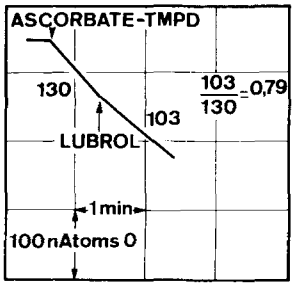


FIGURE 15 Oxidation of endogenous cytochrome c by heart muscle mitochondrial fragments. No acceleration of the oxidation rate is observed upon addition of Lubrol. Conditions for the assay of the cytochrome oxidase were as in Fig. 5.

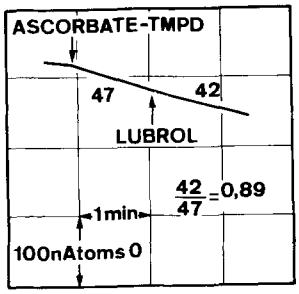
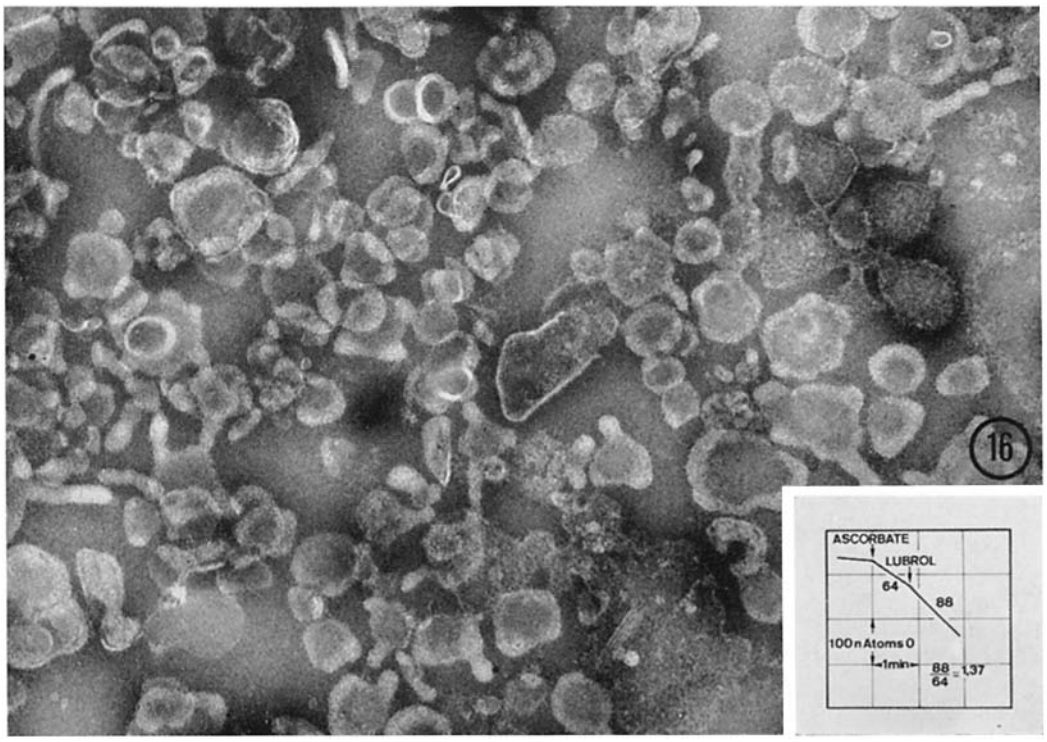


FIGURE 17 Oxidation of endogenous cytochrome c by liver mitochondrial fragments. No acceleration of the rate of oxidation of endogenous cytochrome c is seen upon addition of Lubrol to the medium. The conditions for the enzyme assay were as in Fig. 5.



outer surface of the inner membrane, they were maximally capable of oxidizing added cytochrome c, in the absence of agents that disrupted the membrane structure, thus allowing the internal surface of the inner membrane to come into contact with the external medium.

By contrast, the fragments obtained by mechanical fragmentation of mitochondria displayed a strikingly different structural organization depending on the source from which they were derived. Fragments from liver mitochondria consisted mostly of small vesicles bounded by a single membrane system having no 90-A particles on its outer profile: the conclusion was reached that these vesicles presented to the medium the outer surface of the inner membrane. The fragments obtained from skeletal and heart muscle mitochondria and, to a lesser degree, those from kidney mitochondria, consisted of small vesicles and tubules variously folded and having a very narrow lumen. At variance with liver mitochondrial fragments, the fragments derived from skeletal and heart muscle mitochondria by mechanical fragmentation had numerous elementary particles on their external surface: clearly, they were qualitatively different from the liver mitochondrial fragments in that they presented to the medium the opposite side of the inner membrane. On the other hand, the oxidation of externally added cytochrome c by the fragments obtained from liver mitochondria by mechanical disruption was not activated any further by dissolution of the membrane by Lubrol, while a strong activation was observed upon addition of lubrol to the fragments obtained from heart or masseter muscle mitochondria. A very evident activation could also be observed in the sonic vesicles from liver mitochondria, which are known to be turned inside out. Evidently, the access of cytochrome c to the active sites of cytochrome oxidase was already maximal in the mechanical fragments from liver mitochondria, while dissolution of the membrane organization was required for obtaining maximal accessibility in the mechanical fragments derived from heart and skeletal muscle mitochondria, and in the sonic fragments from liver mitochondria.

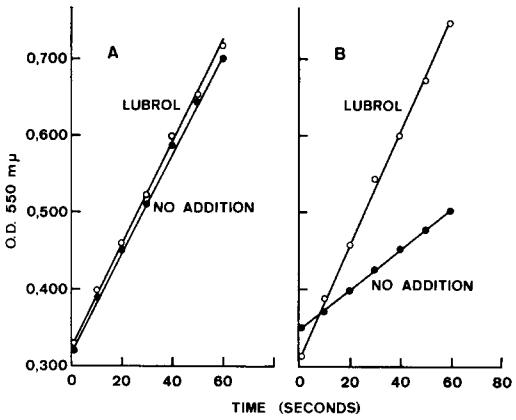
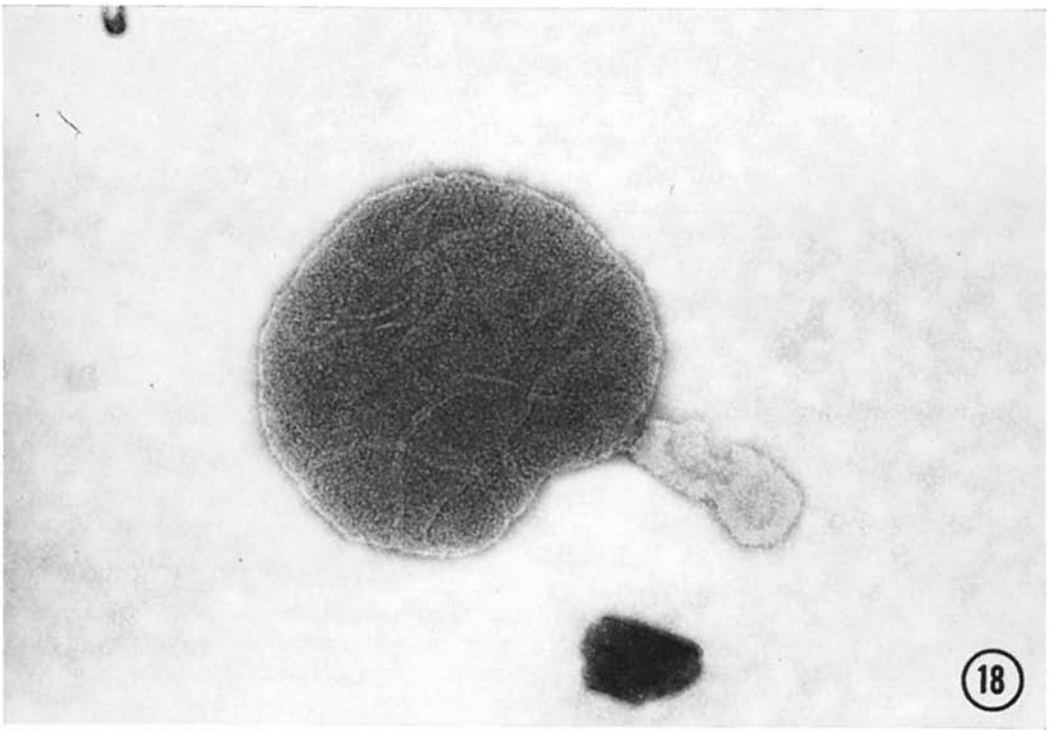


FIGURE 20 Succinate–cytochrome c reductase in heart mitochondria and mechanical submitochondrial fragments. The technical details are found in Methods. Protein concentration was 125 μg. The rate of reduction of cytochrome c in intact mitochondria is not influenced by Lubrol (*A*); a very marked stimulation is, on the other hand, evident in the submitochondrial fragments (*B*).

All the observations thus demonstrated that the maximal activation of the oxidation of externally added cytochrome c by Lubrol required a situation in which mitochondrial fragments presented to the external medium the outer surface of the inner membrane. This could indicate that the binding sites for *exogenous* cytochrome c were housed on the external side of the inner membrane of the mitochondrion or at least closer to it than to the matrix side: taken together with the observation of Palmieri and Klingenberg (17) that azide reacted with cytochrome oxidase only from "inside" the mitochondrion, this could be suggestive

FIGURE 16 Subfraction obtained by mechanical fragmentation of liver mitochondria. The fragments were incubated in the medium for the assay of cytochrome oxidase. The fraction appears to consist of smooth-surfaced vesicles and tubules, and no 90-A subunits are seen on the external surface of the membrane structures. It appears from the insert, the activation of the cytochrome oxidase by Lubrol is rather limited. × 80,000.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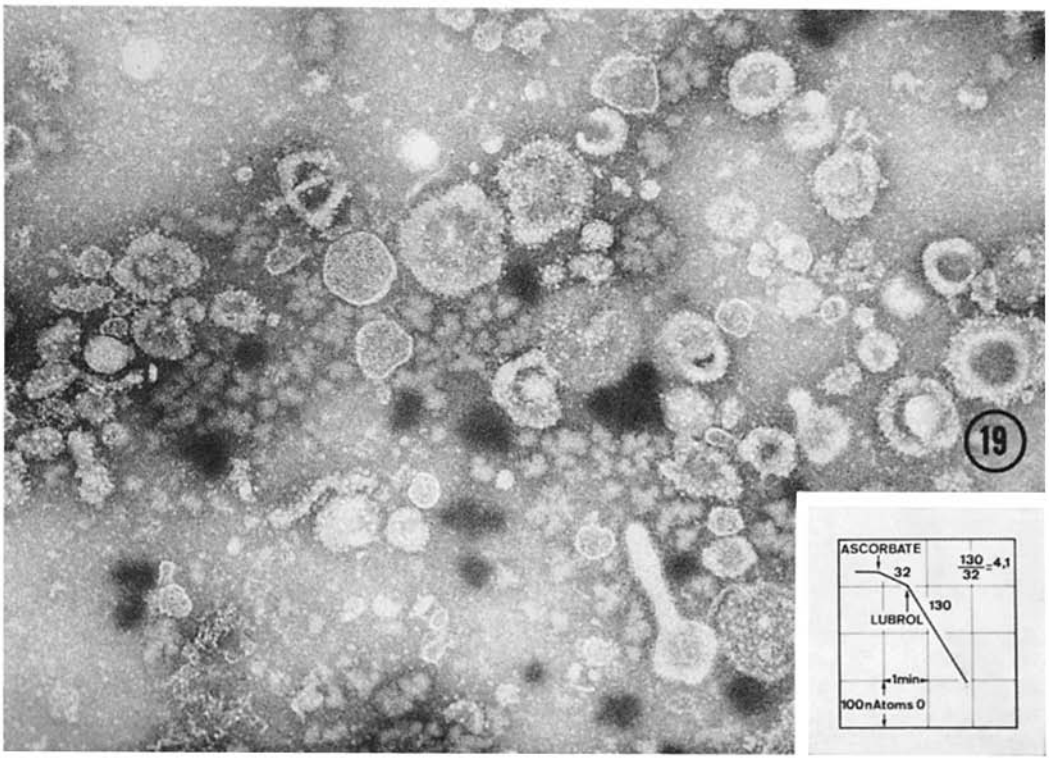
19
ASCORBATE
32
130/32 = 4,1
130
LUBROL
1min
100nAtoms O

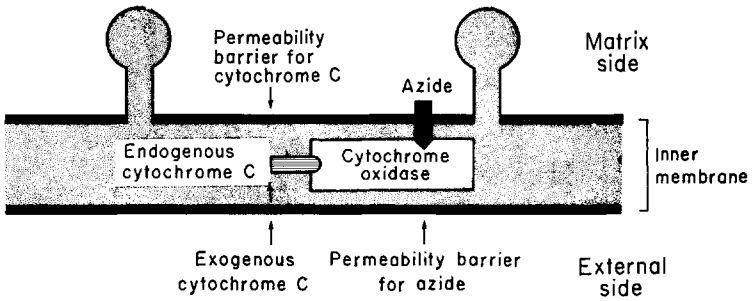

FIGURE 21 Spatial arrangements of the terminal components of the respiratory chain within the inner mitochondrial membrane.

of an anisotropic electron transport across the third phosphorylation side of the respiratory chain. However, when the system ascorbate-TMPD was used to feed electrons on the endogenous cytochrome c, no activating effect of Lubrol was observed, irrespective of which surface the fragments presented to the medium. Presumably, TMPD easily penetrated the inner membrane from both sides. The experiments on the succinate–cytochrome c reductase have, on the other hand, shown that, even when cytochrome oxidase was not involved, the asymmetric behavior of the inner mitochondrial membrane towards exogenous cytochrome c was observed. It thus seems more likely that the phenomena observed reflected a different permeability of the two faces of the inner membrane towards cytochrome c: access of exogenous cytochrome c to cytochrome oxidase would thus have been limited when the internal side of the inner membrane faced the medium: by contrast, a permeability barrier for azide could exist at the outer surface of the inner membrane.

A possible scheme of the spatial arrangement of the terminal components of the respiratory chain within the inner membrane is shown in Fig. 21.

The authors wish to thank Professor P. Buffa for this interest and many stimulating discussions. The research was supported by Public Health Service Grant No. R05-TW00216 (to Dr. Carafoli) and by the National Research Council of Italy.

Received for publication 26 April 1968, and in revised form 28 October 1968.

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FIGURE 18 Subfraction obtained by mechanical fragmentation of liver mitochondria. Conditions for the incubation of the fraction with ammonium molybdate, and for the removal of the excess stain, are described in Methods. The figure illustrates the appearance of a large submitochondrial fragment which has evidently been penetrated by the negative stain. The external profile of the fragment shows no evidence of knobs, which are, on the other hand, recognizable on the interior of the vesicle. × 68,000.

FIGURE 19 Sonic fragments from liver mitochondria. Conditions of sonication and of negative staining are described in Methods. The preparation consists mostly of vesicles surrounded by a single membrane. The 90-A particles are recognizable on the external profiles of many vesicles. As shown in the insert, the addition of Lubrol to this preparation results in a very marked activation of the rate of oxidation of externally added cytochrome c. × 70,000.

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