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MITOCHONDRIAL FRAMEWORK (RETICULUM MITOCHONDRIALE) IN RAT DIAPHRAGM MUSCLE

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Summary

Reconstitution of rat diaphragm mitochondria has been carried out with the use of the serial section technique.

It is shown that mitochondrial material is organized as networks transpiercing the I band regions of the muscle near the Z-discs. Each network forms tubules, oriented perpendicular to its plane, and branches, connecting the network with mitochondrial clusters in the fiber periphery. Such a system, defined as mitochondrial reticulum, is found to be characteristic of the diaphragm of adult animals. It is absent in the diaphragm of rat embryos and new-born rats.

The junctions of the branches of mitochondrial reticulum are described. In the junction site, the outer membranes of two mitochondrial branches are in contact, and spaces between outer and inner membranes are filled with an osmiophilic substance. No junctions were found in the embryos and in newborn animals whose diaphragm contains single, elliptical or worm-like mitochondria.

The hypothesis is put forward that the mitochondrial reticulum serves as a system for transport of energy, oxygen and fatty acid residues along mitochondrial membranes over distances commensurable with the muscle fiber diameter.

Introduction

According to Mitchell's theory of membrane bioenergetics [1], energization of the inner membrane of mitochondria results in the appearance of a transmembrane difference in electrochemical potential of H^+ ($\Delta\bar{\mu}_{H^+}$), composed of electric ($\Delta\psi$) and chemical (ΔpH) gradients. Electrogenic activity of enzymes isolated from inner mitochondrial membrane was recently directly measured, and several independent bodies of evidence for the existence of about 250 mV

$\Delta\bar{\mu}_{H^+}$ in intact mitochondria were obtained (for review, see ref. 2). $\Delta\bar{\mu}_{H^+}$, when formed by a membrane enzyme molecule, can spread along the membrane since the electric conductivity of the solution on both sides of the membrane is very high, surpassing that of the membrane by many orders. So, $\Delta\mu_{H^+}$ may play the role of a transportable form of energy in the cell as was earlier postulated by Skulachev [3—5].

To transfer $\Delta\bar{\mu}_{H^+}$ over a distance commensurable with the cell size, very long mitochondria, stretching from one end of the cell to the other, are necessary.

There are some observations demonstrating the existence of giant mitochondria in unicellular eukaryotes. A single giant mitochondrion was found in unicellular alga *Micromonas* [6], in a flagellate *Polytomella* [7—9], yeats (*Pitorosporum orbiculare* [10], *Saccharomyces cerevisiae* [11] and *Candida utilis* [12]), as well as in *Leishmania donovani* [13], *Chlorella* [14], a water mold (*Blastocladiella emersonii* [15]). Several huge branched mitochondria were revealed in *Chlamydomonas* [16—19] and *Euglena gracilis* [20—22], *Tripanosoma* [23—25] and some fungi [26].

Recently Brandt et al. [27] described two types of mitochondria in normal adult rat liver cells, namely, small spherical and large branched organelles, resembling giant mitochondria from unicellular organisms.

In insect flight muscle, slab-like mitochondra of about the same length as the muscle fiber radius, i.e. 10 μm, were described [28]. Thread-like 10 μm long mitochondria were found in exocrine cells of pancreas [29]. Similar mitochondria seem to form the chondriom of spermatozoon [29—31].

Very large mitochondria hage also been observed in columnar cells of the iris [32].

In the last and some other papers quoted above, the technique of serial sections has been used. Unfortunately, there are no studies of this type dealing with skeletal muscle tissue which might be one of the most interesting approaches for elucidating intracellular energy transport mechanisms.

Multinucleated muscle cells (fibers) are very large and their energy requirements are extremely high. Gradients of oxygen and oxidizable substrates between the periphery and the core of the cell should arise under conditions of maximal contractile activity. $\Delta\bar{\mu}_{H^+}$ transport from the cell edge to its core along mitochondrial membranes might facilitate energy delivery to the inner parts of muscle fiber. If this is the case, the dimensions of a mitochondrial system in a muscle cell should be extremely large. The first indications of the existence of a mitochondrial system penetrating the muscle fiber body were obtained in electron microscope studies of diaphragm [33—35] and musculus semitendinosus [36]. Ultrastructural investigations in diaphragm mitochondria were pioneered by Palade [37] who observed circular and semicircular mitochondrial profiles in this tissue. Later Bubenzer [33,34] and Gauthier and Padykula [35] showed that there are three types of mitochondrial profiles in rat diaphragm muscle: (1) thin transverse tubules forming networks in isotropic regions of muscle fiber oriented parallel to the Z-discs, (2) longitudinal tubules crossing several Z-discs, and (3) spherical bodies localized close to the fiber edges, with branches leading to the fiber core. It was speculated that these structures are connected, forming a united system [33]. All these data were obtained using random sections through tissue.

Reconstitution of different parts of rat diaphragm by means of the serial sections technique, which will be described below, gave direct evidence of the existence of an unitary mitochondrial system in diaphragm fiber of adult rats. It will also be shown that there is no such system in the diaphragm of rat embryos and new-born rats.

Methods

Electron microscope studies of rat diaphragm muscle were carried out in Hitachi HU-11B and HU-12 microscopes. The tissue was fixed with 5% glutaraldehyde and 2% OsO_4, dehydrated in alcohol with uranyl acetate and embedded in Epon-812. The 600—700 Å sections were prepared with an LKB-4800 ultramicrotome. To reconstitute the three-dimensional structure of diaphragm muscle mitochondrial system, photographs of mitochondrial profiles were transposed onto dental wax plates of thickness proportional to the thickness and magnification of the original sections.

Results

An electron micrograph of a longitudinal section of rat diaphragm muscle is shown in Fig. 1A. One can see mitochondrial profiles that look like small spherical or elliptical bodies localized close to Z-discs. Sometimes, however, mitochondrial material crosses a sarcomere (or several sarcomeres, no shown in the figure), forming bead-like structures stretching alone myofibrils. In some cases mitochondrial profiles are seen to be stretched parallel to Z-discs.

In Fig. 1B a transverse section across an isotropic region of diaphragm muscle is shown. In this micrograph, mitochondrial profiles seem to be united in a net-like system localized parallel to a Z-disc.

Figs. 2A and 2B demonstrate transverse sections at a lower magnification. One can see networks formed by mitochondrial profiles in the light (isotropic) regions of sarcomeres. This network seems to transpierce the whole body of the cell. In the dark (anisotropic) regions, there are rare islands of mitochondrial material, which apparently correspond to the "beads" crossing the sarcomeres. Clusters of mitochondria near the fiber edges can also be seen. They are connected with the network.

Light spheres are droplets of fat. Note that mitochondrial material partially covers these droplets.

In agreement with the original findings by Bubenzer [33] and Gauthier and Padykula [35], we observed that the thin fibers (according to the above authors, red fibers) contain more mitochondrial material than thick (white) fibers. It was a fiber of the first type that is shown in Fig. 2. A fiber of the second type is given in Fig. 3. In this figure, the plane of the section is inclined at a very small angle towards the plane of the Z-disc structure. The figure shows that the mitochondrial profiles forming the network in the I band near the Z-disc, become thinner and thinner as they approach the A band. Note that there are no clusters of mitochondria at the fiber periphery and no mitochondrial material in the A band regions (cf. Fig. 2).

In Fig. 4, serial reconstitution of a part of the mitochondrial network is

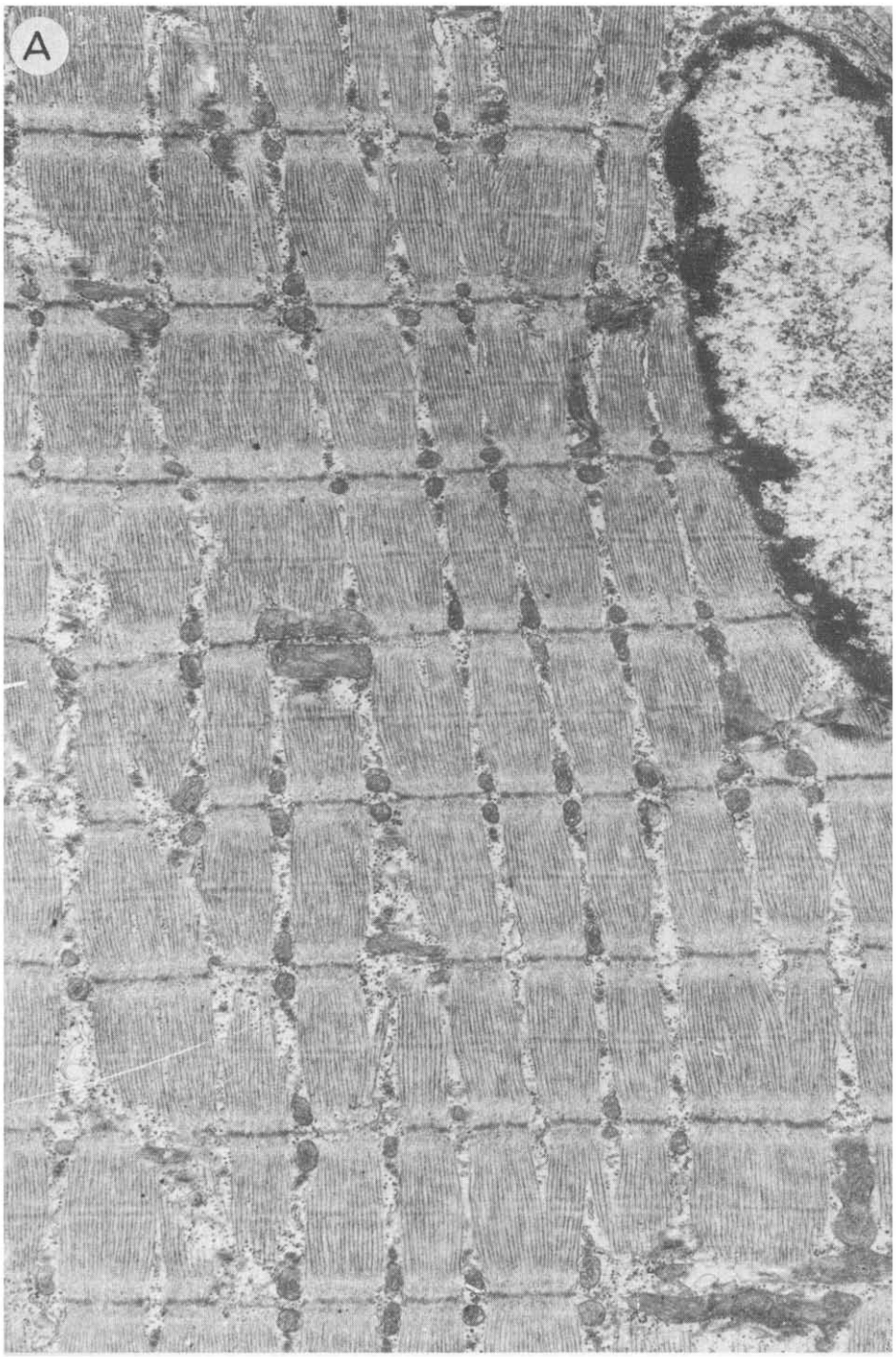

Fig. 1A.

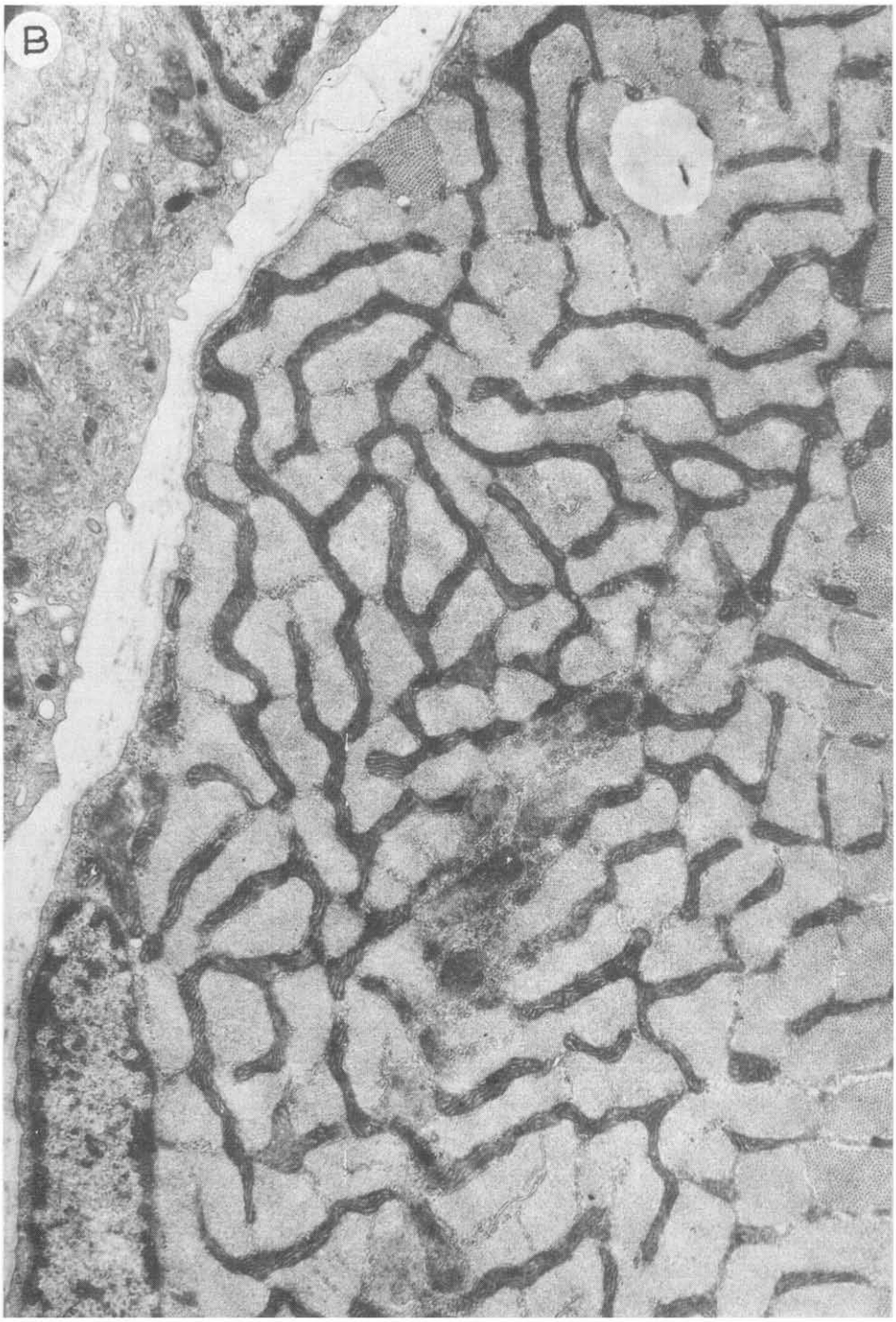

Fig. 1. Longitudinal (A) and transverse (B) sections through a rat diaphragm muscle; ×19 800.

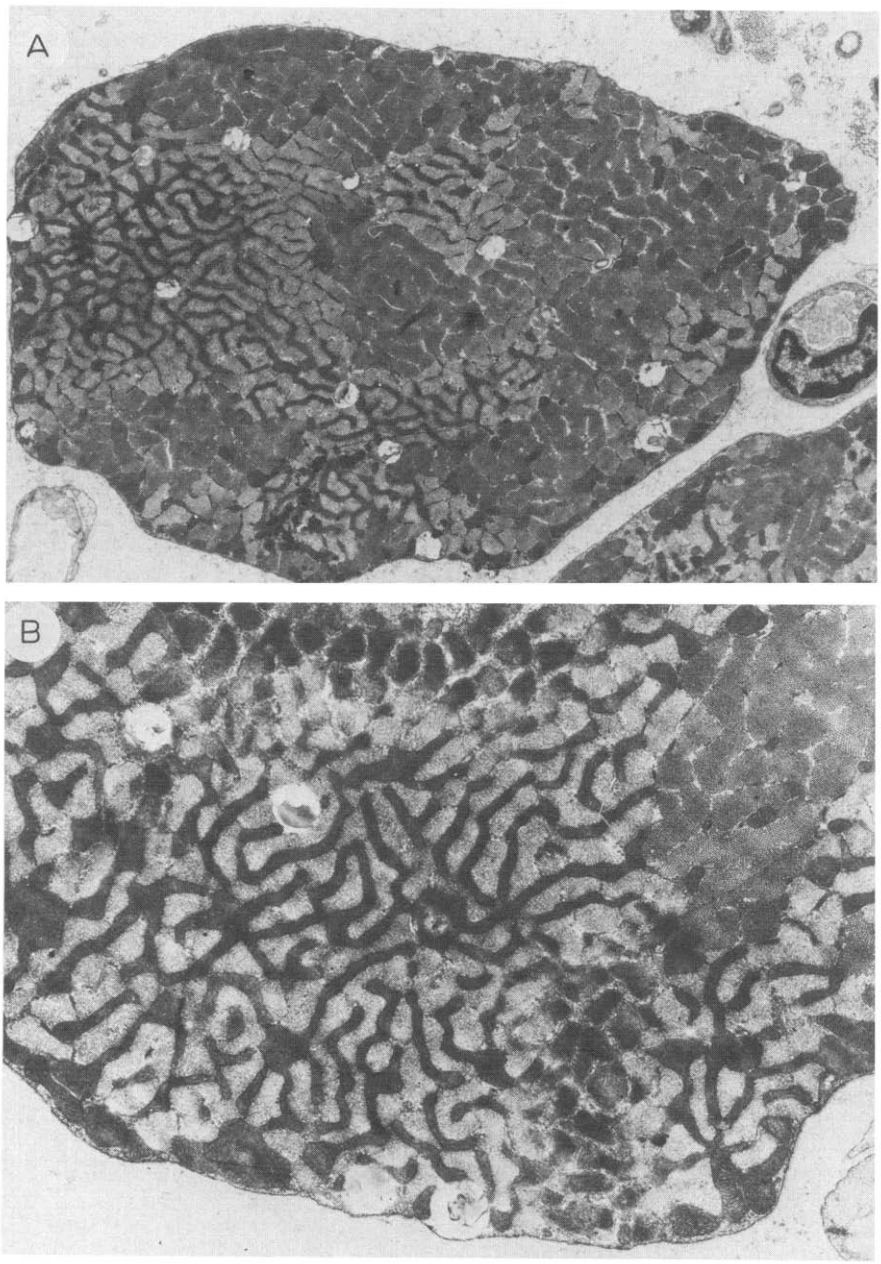

Fig. 2. Transverse sections through a rat diaphragm muscle. A, ×7000; B, ×11 200.

shown. A region of the fiber periphery was reconstituted. Two stages of reconstitution are shown, corresponding to 17 (Fig. 4A) and 34 (Fig. 4B, C) parallel sections. It can be seen that the network has branches oriented at right angles to its plane. At the 17 section stage of reconstitution, some branches look like separate tubules (see the tubule on the extreme left of Fig. 4A). However,

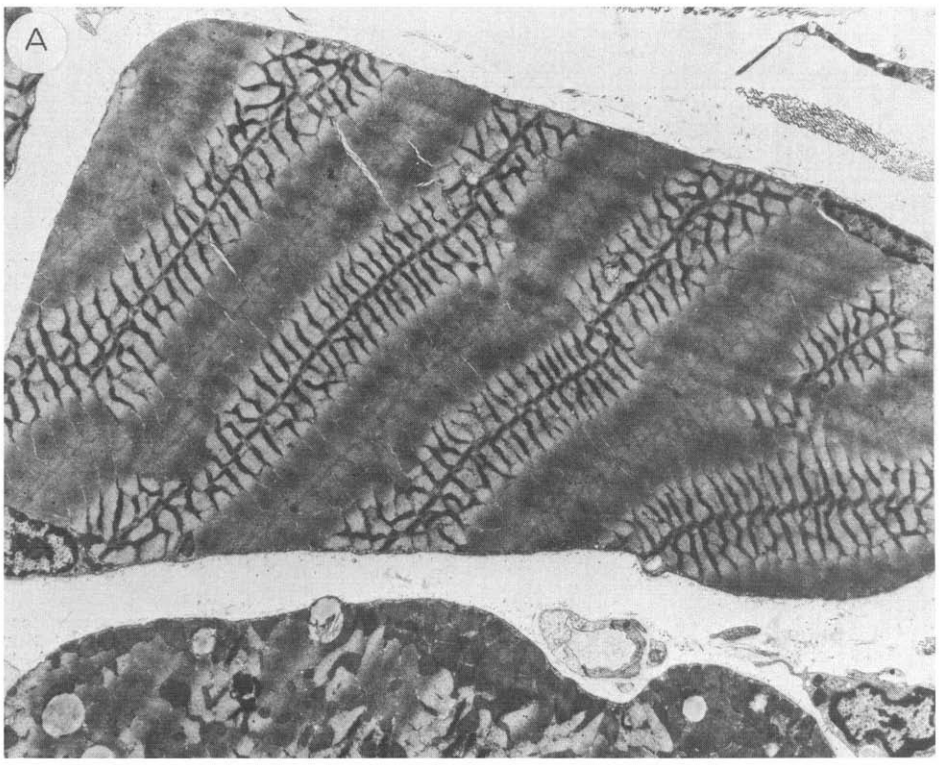

Fig. 3A. See overpage for legend.

further reconstitution revealed connections between these tubules and the network (see Fig. 4B). It is remarkable that in the part of the diaphragm muscle used for reconstitution, not a single mitochondrion that is not connected with the framework was found. As for the bead-like mitochondrial structures stretching along myofibrils (see Fig. 1A), they proved to be twisted tubules.

It should be taken into account that the model shown in Fig. 4 reproduces only a very small part of the mitochondrial system of a diaphragm muscle cell. In reality this framework is much larger, especially horizontally. As for vertical (longitudinal) tubules, most of them go as far as the A band. However, some tubules were found to cross the A band and form connections between networks. Such vertical tubules are characteristic of small (red) fibers rather than of large (white) fibers which contain smaller amounts of mitochondrial material. There is an impression that the major portion (or even all) of the mitochondrial material in red fibers is united into a common system composed of the following elements: (1) the networks situated in the I band regions close to Z-discs; (2) flat cisternae penetrating the I bands, (3) tubules penetrating both I and A bands and connecting adjacent networks, (4) clusters of mitochondrial material near the fiber borders, branching into the networks, and (5) bulges of the network, covering fat droplets. The system in toto may be defined as reticulum mitochondriale. The mitochondrial reticulum being three-dimensional in large fibers. To see a two-dimensional mitochondrial reticulum

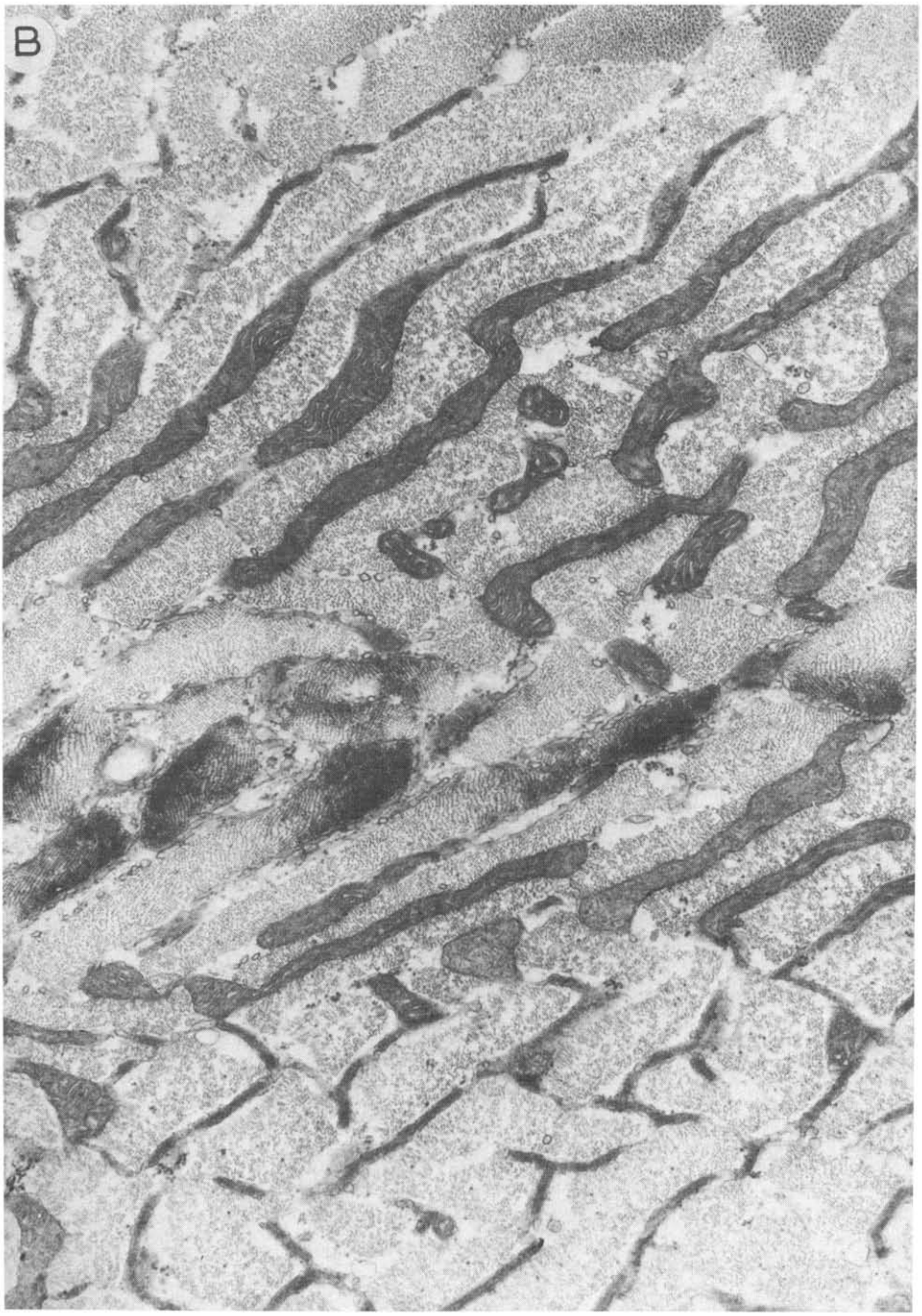

Fig. 3. The section through a rat diaphragm muscle inclined at a small angle to the plane of Z-disc. A, ×5400; B, ×24 000.

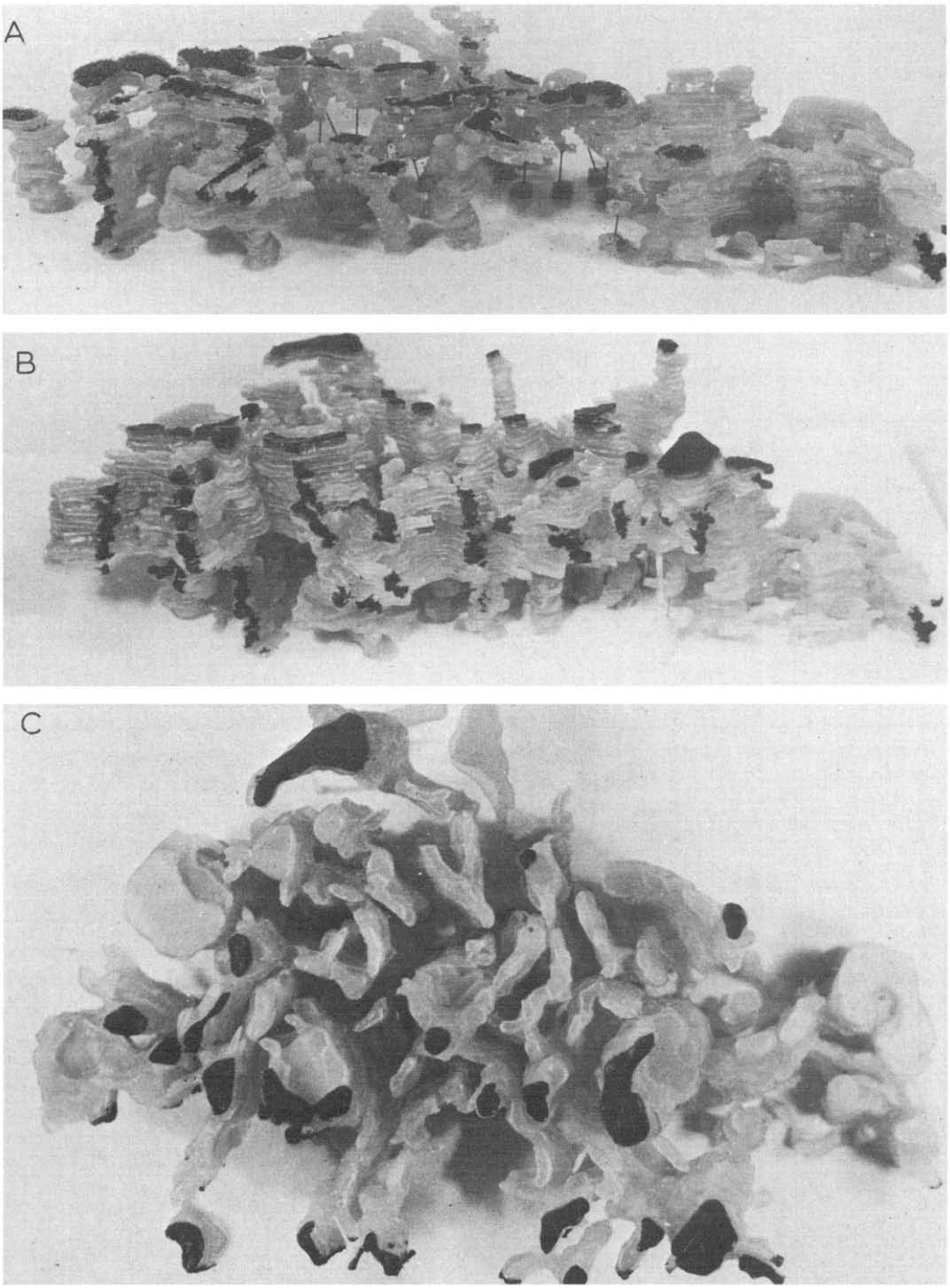

Fig. 4. Three-dimensional reconstitution of a part of the mitochondrial system of rat diaphragm. Dental wax models of a mitochondrial system derived from serial transverse sections are shown. Here and below, the cuts in mitochondrial branches are blackened. A, 17 sections (side view). B, C, 34 sections (B, side-view; C, top-view).

on a single section, one should comply with two conditions, i.e. (1) the plane of the section must be parallel, or inclined at a very small angle, to the plane of the Z-disc structure, and (2) the section must cross the I band near the Z-disc.

It was shown that mitochondrial reticuli can be observed in diaphragm muscle of adult rats whatever (1) the tissue fixation procedure, (2) diaphragm treatments in vitro, e.g. incubation in the presence of uncouplers or inhibitors, (3) in vivo conditions (season, environment temperature, etc.). On the other hand, in diaphragms from embryos or new-born rats, containing smaller amounts of mitochondrial material than adult animals, no mitochondrial framework has been found. In these cases, single elliptical or worm-like mitochondria were observed (Fig. 5).

In all previous studies on reconstitution of giant mitochondria, no systematic investigation was undertaken to elucidate whether the studied structure is really a single mitochondrion surrounded with continuous outer and inner

Fig. 5A. See page 360 for legend.

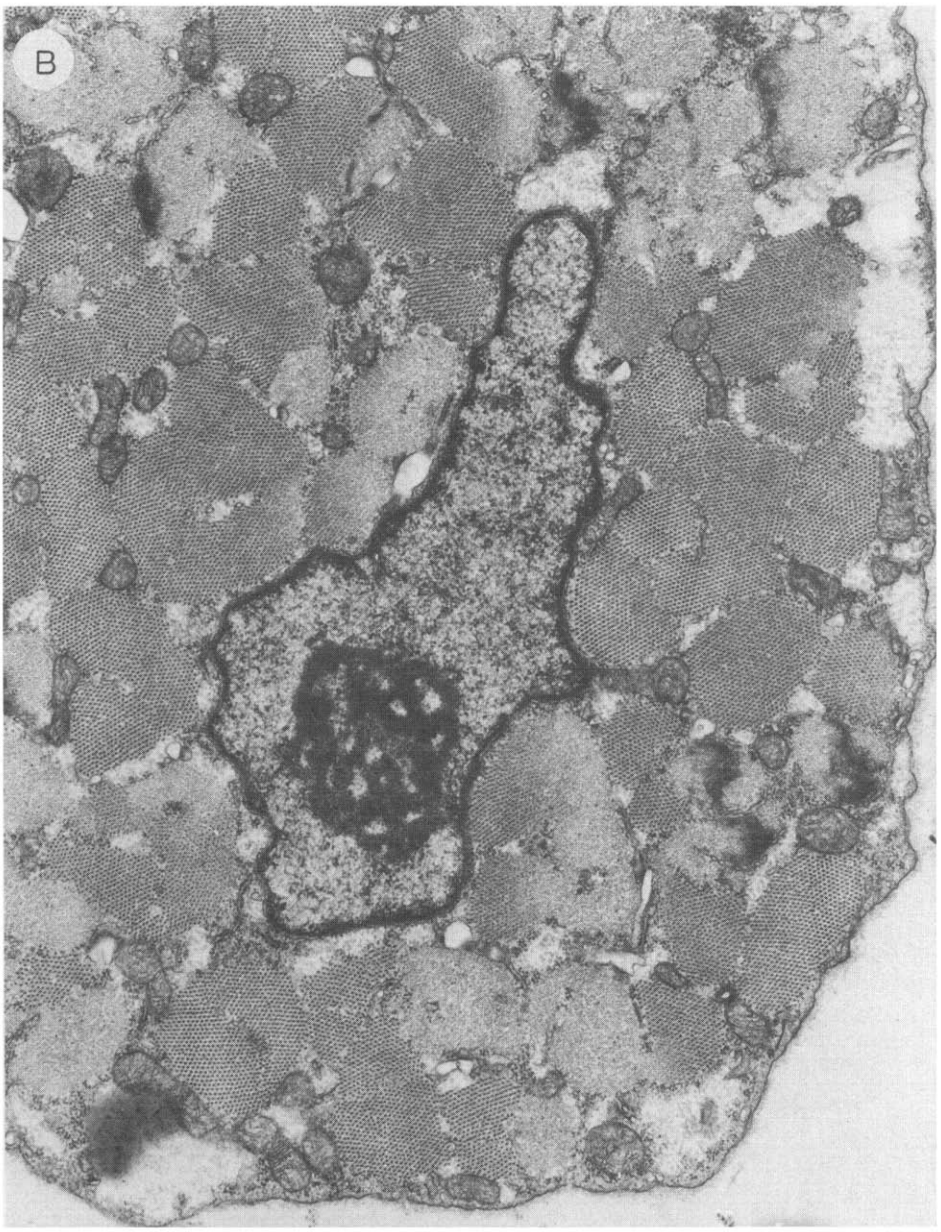

Fig. 5B. See page 360 for legend.

membranes, or whether it is an assembly of several, end-to-end, associated mitochondria. Our ultrastructural analysis of mitochondrial reticuli in diaphragm muscle revealed that sometimes reticulum-forming tubules are crossed by dark partitions built of several membranes, the intermembrane space being filled with an osmiophilic material. Examples of the structure of this type are shown in Figs. 6 and 7.

Apparently, the structure in question is a junction of two branches of mitochondrial reticulum. These branches might belong to two different

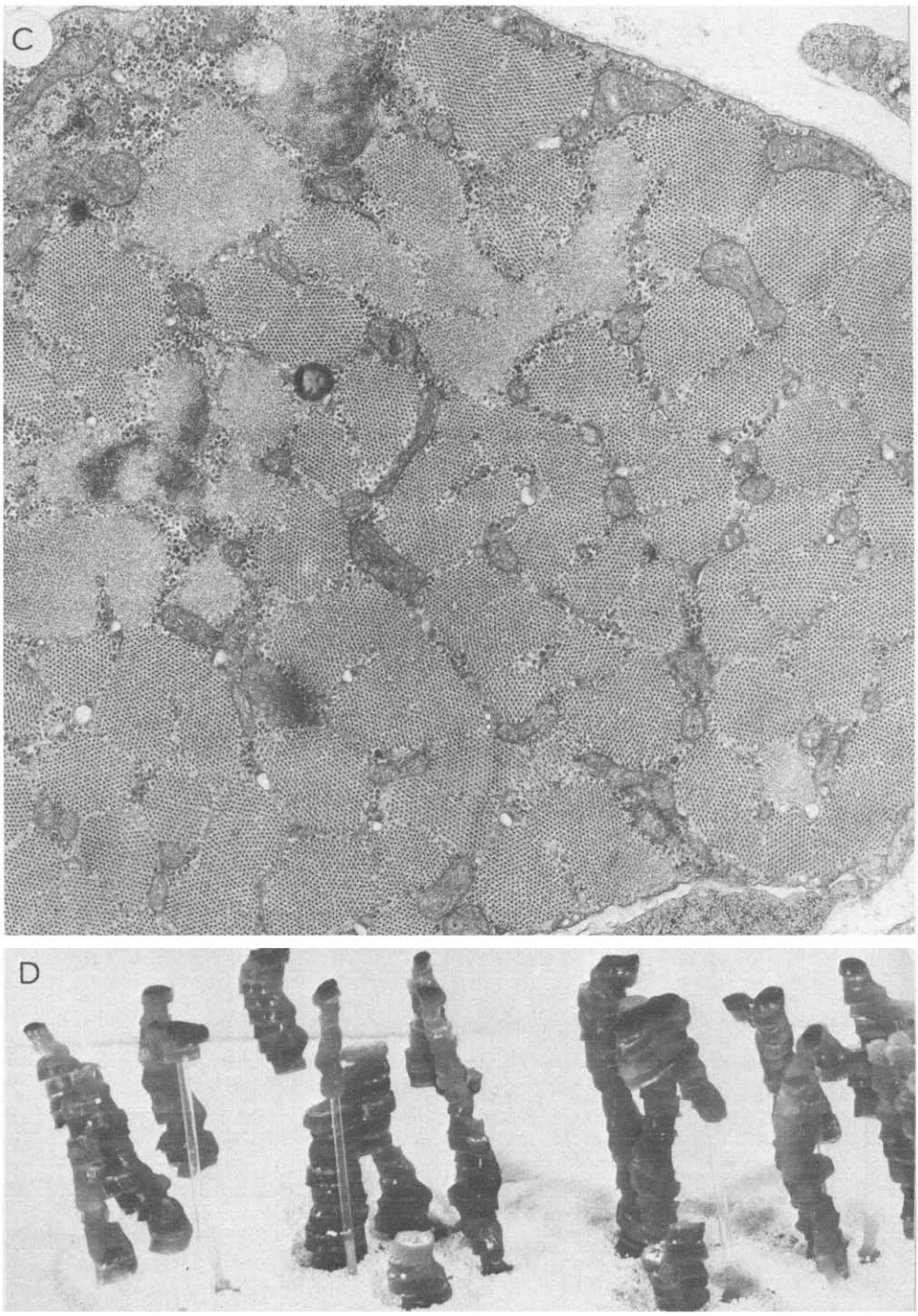

Fig. 5. Ultrastructure of diaphragm muscles of a rat embryo (A, B, D) and a newly-born rat (C). A, C transverse sections; B, longitudinal section, ×16 800; D, side-view of a 19-section model.

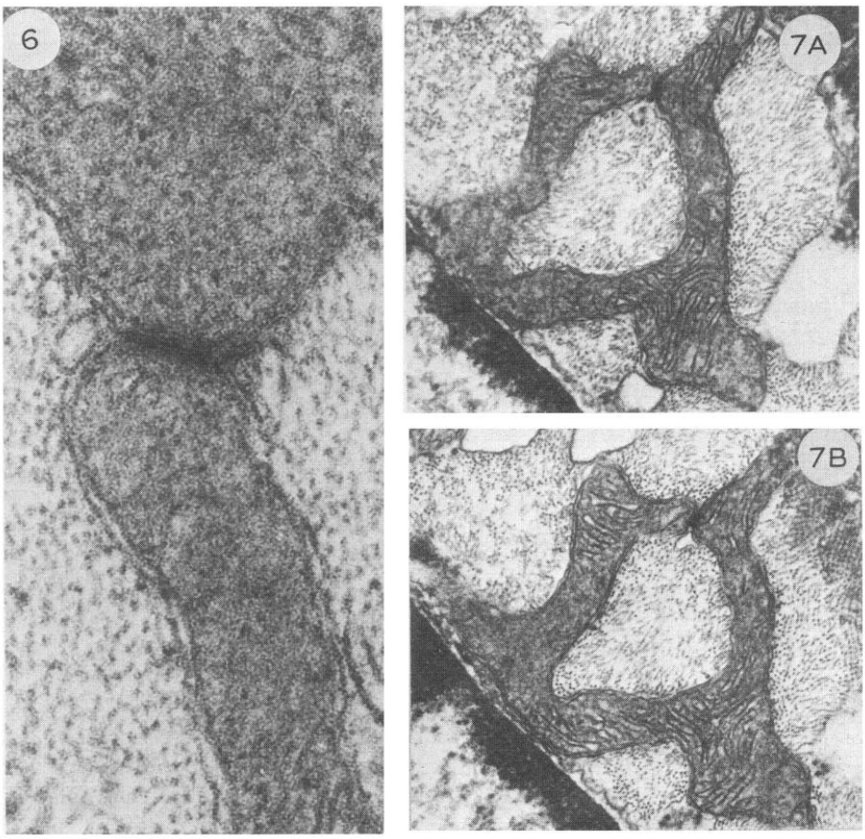

Fig. 6. A junction site formed by two branches of mitochondrial reticulum in a rat diaphragm muscle, ×108 000.

Fig. 7. Two parallel transverse sections through a junction region. Note that the junction is formed by two branches of the same mitochondrion. ×52 500.

mitochondria or, alternatively, to one and the same mitochondrion. In Fig. 6 we cannot choose between these two possibilities. In Fig. 7 it is clear that two branches forming the junction are parts of a single mitochondrion. No junctions were found in diaphragms of embryos and new-born rats.

In the next experiment with adult rat diaphragm, we followed a mitochondrial profile without crossing a junction site, assuming that in this case we were dealing with a single mitochondrion in the sense that at least its outer membrane should be continuous. Fig. 8A demonstrates a section through a part of muscle fiber, used to reconstitute a junction-free mitochondrion. The reconstituted model is given in Figs. 8B and 8C. It is shown that a mitochondrial tubule crosses the muscle fiber, stretching from one edge to the other. In the fiber periphery the tubule branches in three dimensions. So, the reconstituted part of the junction-free mitochondrion is composed of two peripheral clusters of mitochondrial material connected by tubule(s) crossing the fiber core. It should be noted that in the tubule the cristae are usually parallel to its long axis.

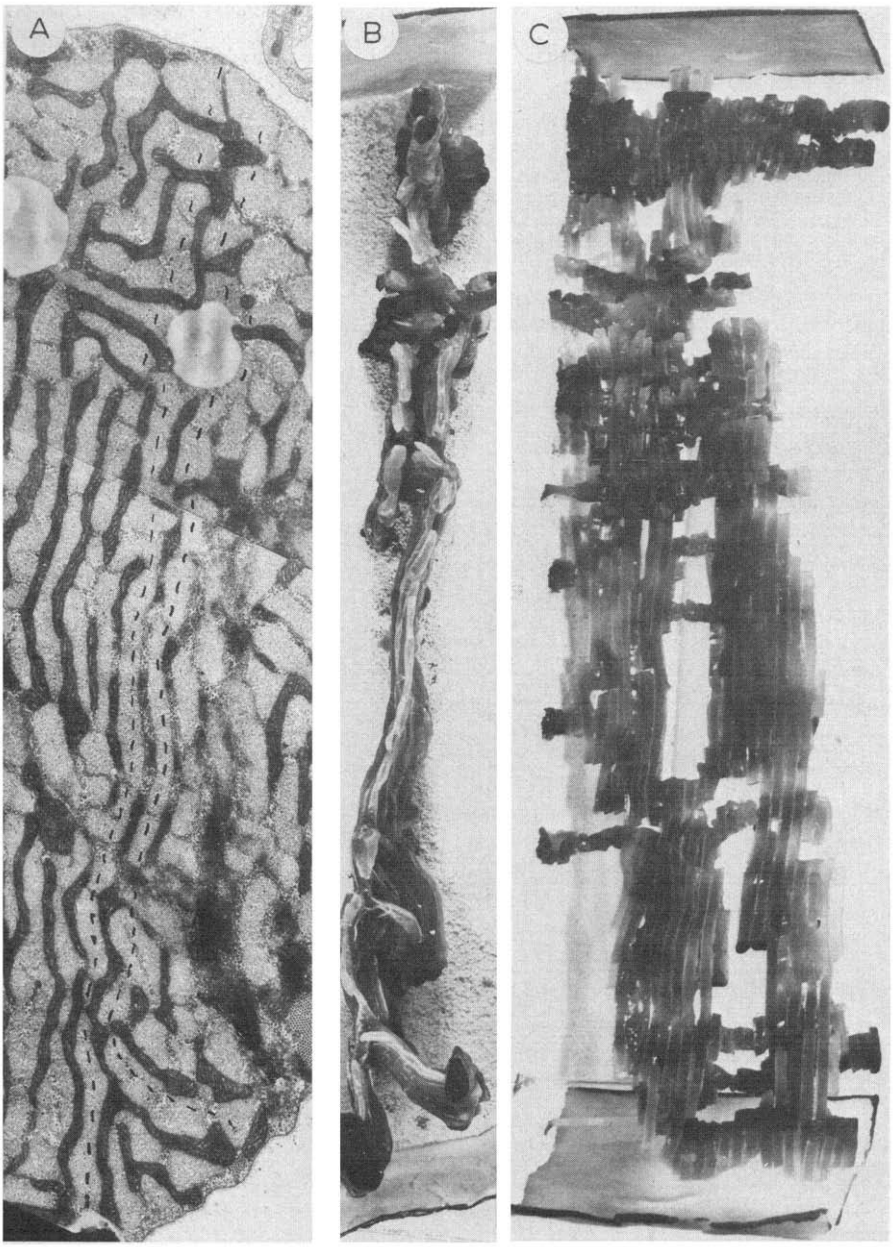

Fig. 8A,B,C.

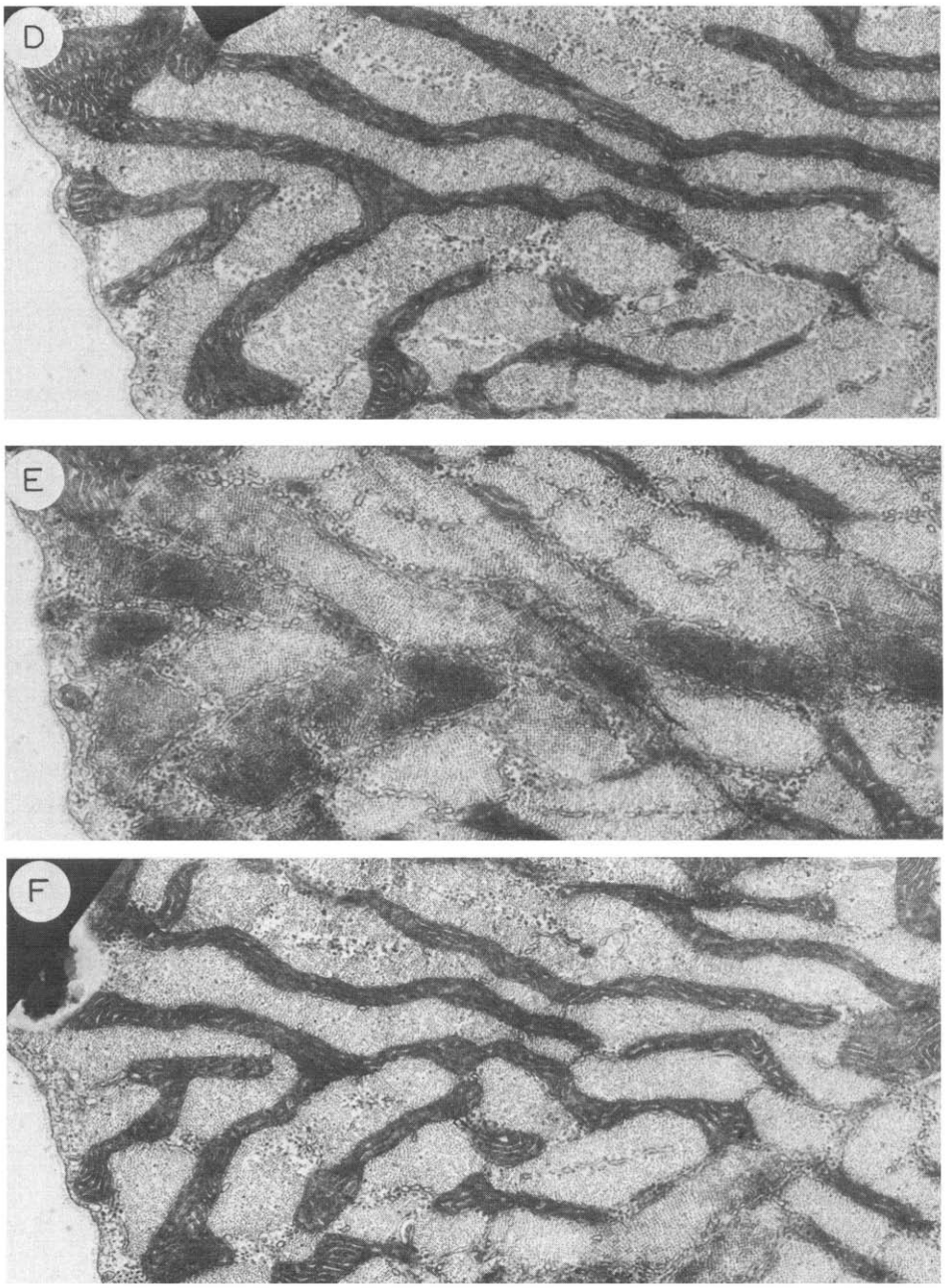

Fig. 8. Reconstitution of a part of a junction-free mitochondrial profile. A, a transverse section of a diaphragm muscle. The part used in reconstitution of the model is surrounded by a dotted line. X16 200. B, C, the model: B, top-view; C, side views, plates along the edges of the model are parts of the outer cell membrane; D—F, serial transverse sections through a peripheral part of the fiber shown in A; X24 300.

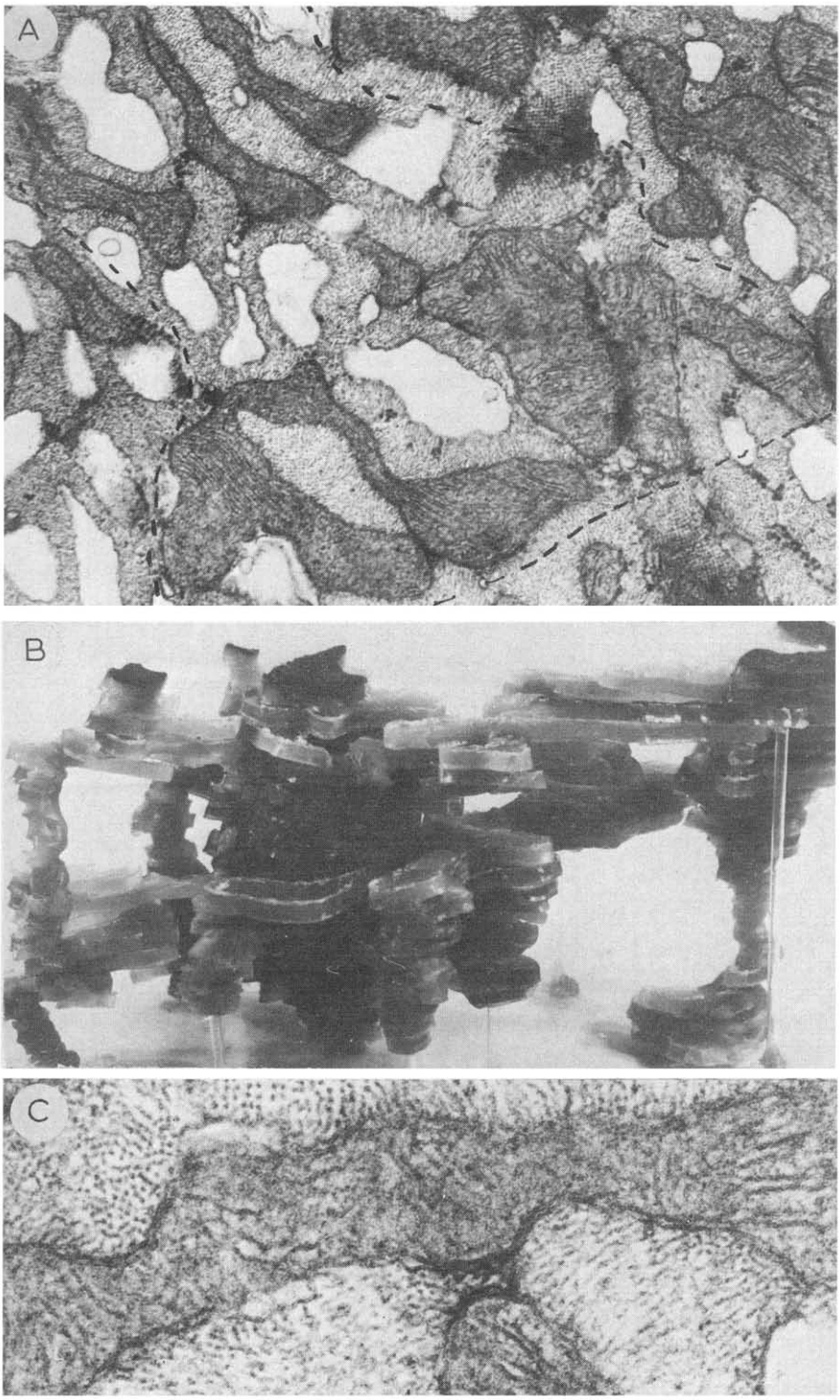

Fig. 9. Reconstitution of a part of a junction-free mitochondrion in an ouabain-treated diaphragm muscle. A, transverse section through the cell studied; X33 000. B, side-view of a 25-section model. C, a part of mitochondrial reticulum which looks like a disrupted junction. Note an osmiophilic substance in the intermembrane spaces in the site where the distance between two mitochondrial branches is minimal; X108 000.

Analysis of the model reveals the symmetry of mitochondrial profiles with respect to the Z-disc. As can be seen in Fig. 8B, the models look like a two-storey construction. The space between the storeys is filled with Z-disc material. There are mitochondrial branches connecting the first and the second storey of the mitochondrial structures. In Figs. 8D—F, sections through a peripheral mitochondrial cluster are shown. The E section crosses the Z-disc region, whereas the D and F sections cross the isotropic regions above and below the Z-disc. Note the symmetrical Y-like mitochondrial profiles situated on the first (D) and second (F) storeys.

For further analysis of the mode of organization of the mitochondrial reticulum, a treatment inducing mitochondrial swelling in situ was applied. A piece of diaphragm was incubated with ouabain, which induces swelling of mitochondria, most probably due to changes in distribution of some ions between cytoplasm and intercellular fluid. Fig. 9 demonstrates a model of a part of a junction-free mitochondrion, derived from serial transverse sections of a diaphragm treated in this way. One can see that the branched two-storey character of the mitochondrion is retained. However, the stretched planar cisternae tend to be transformed to tubules which look like circles in the transverse sections. Sometimes these tubules are very thick (see the tubule in the middle of the model). The network structure and at least certain junctions were found to survive the in situ swelling. Some junctions however, seem to have been destroyed, as one can see in Fig. 9C. Note the osmiophilic material localized in the intermembrane spaces in the regions where two mitochondrial branches come close together. In this connection, we can mention several observations where osmiophilic material was reported to be present between inner and outer membranes [38] or in the intracristal space [39—43] of muscle mitochondria under certain conditions (cf. fusion of two membranes of the same mitochondrion, described by Hackenbrock [44,45]).

Fig. 9 gives some information about interrelationships between mitochondrial and sarcoplasmic reticula in diaphragm muscle fiber. It was found that ouabain treatment results in swelling of the cisternae of the sarcoplasmic reticulum, which look like electron-transparent areas surrounded by a membrane (Fig. 9A). One can see that the reticula are tightly interwoven in the isotropic region of sarcomere. It is also clear that the total amount of mitochondrial membranes (outer, inner and cristal membranes) is much greater than that of the membranes of the sarcoplasmic reticulum.

Discussion

Reconstitution of the three-dimensional structure of diaphragm muscle mitochondrion

The above data on the reconstitution of three-dimensional structures of mitochondria are sufficient to conclude that mitochondrial material in the cell of rat diaphragm muscle forms a unitary system composed of networks, cisternae and tubules mainly localized in the isotropic regions of sarcomeres, as was proposed previously by Bubenzer who studied random sections of diaphragm [33].

Sometimes the continuity of mitochondrial profiles are interrupted by very

osmiophilic septa which seem to represent the junctions of branches belonging to the same mitochondrion or, maybe, to two individual mitochondria. Reconstitution of a junction-free mitochondrial profile revealed a structure that crosses the muscle cell and is parallel to the Z-disc. This structure was found to branch near the fiber borders, organizing a three-dimensional reticular system in the fiber periphery. Close to the Z-disc, mitochondrial profiles are symmetrical being usually of the same form above and below Z-disc.

The diameter of the tubules forming the mitochondrial reticulum is about 0.2—0.4 μm. Several cristae, which are seen in the tubule interior are, as a rule, parallel to the long axis of the tubule. Near the cell membrane, the tubules enlarge, forming 0.5—1.0 μm bodies. Mitochondrial material partially covers the surface of the intracellular fat droplets, which are usually localized below or above the intersections of the network-forming mitochondrial tubules.

Not a single "free' mitochondrion (that is not connected with the mitochondrial reticulum) was found in adult rat diaphragms. On the contrary, no mitochondrial reticulum structures were observed in diaphragms of rat embryos and new-born rats.

Possible functions of mitochondrial reticulum

The very low resistance of intra- and extramitochondrial media must result in a situation when a membrane potential, if formed in one point of the mitochondrion, is transmitted along its membrane which has a very high resistance. The energy loss of such a transmission can be calculated using the equation for an electric cable. Assuming the mean diameter of the mitochondrial tubule to be 0.3 μm and the membrane resistance $\geqslant 1 \cdot 10^7$ ohm $\cdot$ cm^{-2} [3], one can obtain a 50% energy loss at a distance of $\geqslant 3.5$ cm. Since this value is much higher than real intracellular distances, we can conclude that membrane potential transmission can be used by muscle fiber as an effective mechanism of energy transport. In other words, the mitochondrial reticulum may be considered as a system of "intracellular electric cables" uniting thousands of membrane potential-generating proteins (respiratory chain enzymes and H^+-ATPases) into a common energy-producing system of diaphragm muscle fiber.

Another problem is how the energy, if it moves along the membrane, crosses the junction sites. If there is no electric conductivity through the junction, the energy transport may be described as a "mixed relay" in which the energy is trasmitted over the major part of the distance as $\Delta\bar{\mu}_{H^+}$ (along the membrane of tubules of the mitochondrial reticulum), whereas the short distances at the junctions of two tubules are overcome by ATP diffusion:

respiration $\rightarrow \Delta\bar{\mu}_{H^+} \rightarrow$ ATP $\rightarrow \Delta\bar{\mu}_{H^+} \rightarrow$ ... ATP $\rightarrow$ actomyosin ATPase

The alternative and simpler version can be offered if one assumes that junction conductivity is high. Then direct $\Delta\bar{\mu}_{H^+}$ transmission between the junction-separated parts of the mitochondrial reticulum becomes possible (with no ATP involved). High conductive gap junctions formed by two plasma membranes of neighbouring animal cells can exemplify an ion-permeable membrane contact [46—49].

The latter type of organization of membrane contacts seems to give some functional advantages since the conductivity of a gap junction can be regulated within very wide limits. For instance, swelling of cells connected via gap junctions results in fast and complete disappearance of the conductivity. If the same effect were inherent in junctions of muscle mitochondria, it might be a way to increase the safety of the mitochondrial reticulum as a power-transmitting system, if one assumes that disruption or swelling of one of the mitochondria forming the network entails a decrease in the junction conductivity connecting this mitochondrion with other mitochondria of the system. In this manner one can minimize the risk of de-energization of the reticulum in toto as a consequence of a single damage to one of its branches.

The respiration-supported $\Delta\bar{\mu}_{H^+}$ generation is described as follows:

$$AH_2 + 1/2O_2 + n\,H_i^+ \rightarrow A + H_2O + n\,H_o^+, \qquad (1)$$

where AH_2 and A stand for an oxidizable substrate and the corresponding product, H_i^+ and H_o^+ t for H^+ inside and outside the mitochondria, n for the number of H^+ transported from inside to outside per H_2O molecule formed by the respiratory chain.

The suggestion seems to be attractive that not only H^+ but also two other reactants in Eqn. 1, i.e. AH_2 and O_2, are transported along the membranes of the mitochondrial reticulum.

Fatty acids are the main oxidizable substrates in muscle tissue. They might be transported by lateral diffusion along the mitochondrial membrane in a free form or as fatty acyl esters with CoA [50] and carnitine [51]. One may speculate that fatty acids formed by lipolysis of fat droplets inside the muscle fiber reach places of their oxidation in the same fiber without being diluted in cytosol. In fact, a fatty acid released from a fat droplet should immediately meet the membrane of the mitochondrial reticulum which usually forms "caps" covering fat droplets. According to de Robertis et al. [52], the contact of mitochondria and fat droplets in cells of liver and pancreas can be so tight that only one (inner) mitochondrial membrane is seen between the droplet and the mitochondrial matrix, as if the droplet were localized in the intermembrane space of the mitochondrion. It seems highly probable that a fatty acid molecule as well as fatty acyl-CoA and fatty acyl-carnitine tend to be localized at the membrane/water interface, their charged group(s) facing water and the hydrocarbon chain being immersed in the membrane lipid phase. These compounds, once adsorbed by the membrane surface, diffuse in the plane of the membrane. This results in the formation of interconnections between the sources of fatty acids and the fatty acid-oxidizing systems localized in the same mitochondrial membrane.

Similar consideration may apply to intracellular transport of molecular oxygen. O_2 molecules diffusing from the fiber periphery to the fiber core, should be absorbed by membranes, since O_2 solubility in lipids is almost 20 times higher than in water. The O_2-utilizing enzyme system (cytochrome oxidase), like the system of fatty acid oxidation, is localized in the mitochondrial membrane. So, there is no necessity for membrane O_2 to be released into cytosol to reach its destination.

It should be stressed that, with hard muscle work, intracellular gradients of O_2, fatty acids and ADP + inorganic phosphate, of different directions tend to arise. Maximal concentration of oxygen should be near the capillaries, and that of fatty acids near fat drops. As to ADP and phosphate, their maximal concentrations should be found at a maximal distance from a capillary and a fat drop. Perhaps lateral movement of O_2, fatty acids (acyl?) and $\Delta\bar{\mu}_{H^+}$ along the membranes of the mitochondrial reticulum results in equalizing and buffering all these components.

In terms of the above hypothesis, diffusion of O_2 and substrate in a two-dimensional space, i.e. in the plane of the membrane, substitutes for diffusion in the three-dimensional space of cytosol.

According to Adam and Delbrück [53], the mechanism of lateral diffusion along membranes permits a much faster transfer of a component from cytoplasm to a small target on the membrane than free diffusion in cytosol. Samper and Träuble [50] calculated that there is quite a wide range of parameters of the target and the cell for which lateral diffusion proves favourable. Keith and Snipes [54] have found that a spin-labelled synthetic compound of low molecular weight, whose partition coefficient in a lipid/water system is close to 1, moves preferably in the lipid phase of various types of cells. They concluded that the viscosity of cytosol is higher than that of membranes.

Apparently, there is no need for the formation of a united mitochondrial system when functional activity of the tissue is low and the energy sources are abundant. This can explain why mitochondrial reticulum is absent in the diaphragm of embryo and is developed in postembryonic period. Formation of this structure during ontogenesis may be compared with the phenomenon of the end-to-end aggregation of mitochondria during formation of spermatozoon from spermatide [29—31]. Moreover, this type of aggregation was described by Schabadasch [55—58] and some other authors [59—63] under favourable conditions (e.g. hypoxia) in different tissues. The above hypothesis allows one to explain this phenomenon as an attempt by the cell to unite the activity of individual organelles when their uncoordinated functioning proves insufficient for maintaining the normal rate of energy production. This system may be important to such tissue as muscle which must be always ready to manifest its maximal working capacity.

In any case, the above data are sufficient to conclude that the muscle fiber is transpierced by a framework built of mitochondrial membranes which are known to be under voltage of about a 0.25 V.

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