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The Action of Digitonin on Rat Liver Mitochondria

ELECTRON MICROSCOPY

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1. Rat liver mitochondria were examined in the electron microscope by using negative staining in the presence of 0·3M-sucrose. The intact outer membrane does not appear to be freely permeable to the stain. Where the stain penetrated through a tear it was seen that the inner membrane had randomly oriented grooves, many of which contained round structures varying between 200 and 900 Å in diameter. Laminar structures containing two to five layers of approx. 50 Å each were found at the periphery. 2. When the outer membrane was removed by treating the mitochondria with digitonin several types of inner-membrane complexes were formed and they showed a general correlation with those observed in sectioned samples of the same preparations. The main types were: (*a*) a condensed form looking very much like the intact mitochondrion without the outer membrane (this still showed the grooves, some of which contained the round structures, and the laminar whirls at the edges); (*b*) a more transparent form containing tubules of uniform width and various lengths (some of these appeared to terminate in a hole at the surface of the inner membrane); (*c*) a large torn sac, probably the inner membrane, containing some tubules and vesicles. 3. When the inner-membrane complex was further treated with digitonin it was disrupted and the resulting material consisted of pieces of membrane, doughnut-shaped units and lamellar structures. Most of these pieces varied in size between 500 and 1000 Å.

The chief purpose of the work described below and in the accompanying papers is to identify the source and properties of submitochondrial particles isolated after the treatment of rat liver mitochondria with digitonin. Such particles have been previously described and have been utilized to study oxidative phosphorylation (Cooper & Lehninger, 1956). Very little was known about the structure of the preparations and the only published electron micrograph was presented by Siekevitz & Watson (1957). They concluded that the preparation consisted of units 200–500 Å in diameter in various stages of aggregation along with a considerable amount of ill-defined material. More recently Levy, Toury & Andre (1966) and Schnaitman, Erwin & Greenawalt (1967) have reported that digitonin preferentially removes the outer membrane as demonstrated by enzymic analysis and electron micrographs. Hoppel & Cooper (1968) have confirmed that digitonin treatment results in the quantitative removal of the outer membrane. They have also shown that the resulting inner mitochondrial complex can be further disrupted by digitonin and that the action of digitonin on the inner mitochondrial complex is specific and does not result from the hypo-osmotic conditions used.

The photographs presented below show the structures obtained at various stages of repetitive treatment of mitochondria with digitonin.

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EXPERIMENTAL

Mitochondria and preparations A and B were prepared as described in the preceding paper (Hoppel & Cooper, 1968).

Electron microscopy

Sectioned material. The samples (mitochondria and preparation A) were sedimented into a small pellet and the supernatants replaced with 2·5% glutaraldehyde in 0·3M-sucrose buffered with 0·03M-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid adjusted to pH 6·8 with NaOH. The pellets were fixed for 2 hr. with one change of fixative and the fixative was then replaced with 0·3M-sucrose. They were post-fixed with 1% osmium tetroxide buffered to pH 7·2 with veronal acetate, dehydrated in ethanol and embedded in Araldite. Sections were cut with

a glass knife on a Porter Blum MT-2 ultramicrotome. The sections were stained with saturated 50%-ethanolic uranyl acetate (Watson, 1958*a*,*b*) and lead hydroxide (Watson, 1958*a*,*b*; Millonig, 1961).

Negative staining. Carbon-coated Formvar films on 200-mesh cooper grids were used. Unfixed samples (3·6 mg. of protein/ml.) were applied as a small drop to the grid and the excess was removed by touching the edge of the grid with filter paper. A drop of 2% phosphotungstate, pH 6·8 (neutralized with KOH), in 0·05% bovine serum albumin was then applied to the grid and the excess again removed by blotting. The grids were then dried *in vacuo*. In our hands, applying large structures such as mitochondria or preparation A to grids by spraying resulted in fragmentation.

Electron micrographs were taken on an RCA EMU 3H microscope operating at 50 or 100 kv with a double condenser.

RESULTS

Whole mitochondria. Plate 1(*a*) is an electron micrograph of sectioned mitochondria isolated in 0·3 M-sucrose. Two forms were seen consistently, but the predominant one was the condensed type of Hackenbrock (1966). This form is characterized by a heavily stained matrix containing large vacuoles, a small matrix volume and a large outer compartment. The other type is a transitional one that has a lightly stained matrix containing a variable number of cristae and a large matrix volume. The outer membrane was seen on both types, although it was occasionally broken on the transitional form.

Most of the electron micrographs made were with negatively stained preparations. Plates 1(*b*), 2(*a*), 2(*b*) and 3 are photographs of mitochondrial preparations stained with phosphotungstate. Plate 1(*b*) is a unique photograph of a mitochondrion in which a small portion of the outer membrane became dislodged. This flap has the same granular transparent appearance described by Parsons, Williams & Chance (1966) for outer membrane, whereas the covered portion has an almost featureless appearance and is virtually unstained. In contrast, the small portion of the interior visible in the area of the torn flap contains many deeply stained grooves that appear to vary in width and be randomly oriented. Their relationship to the cristae and vacuoles seen in sectioned preparations is not clear. They may have resulted from the accumulation of phosphotungstate in surface folds of the inner membrane.

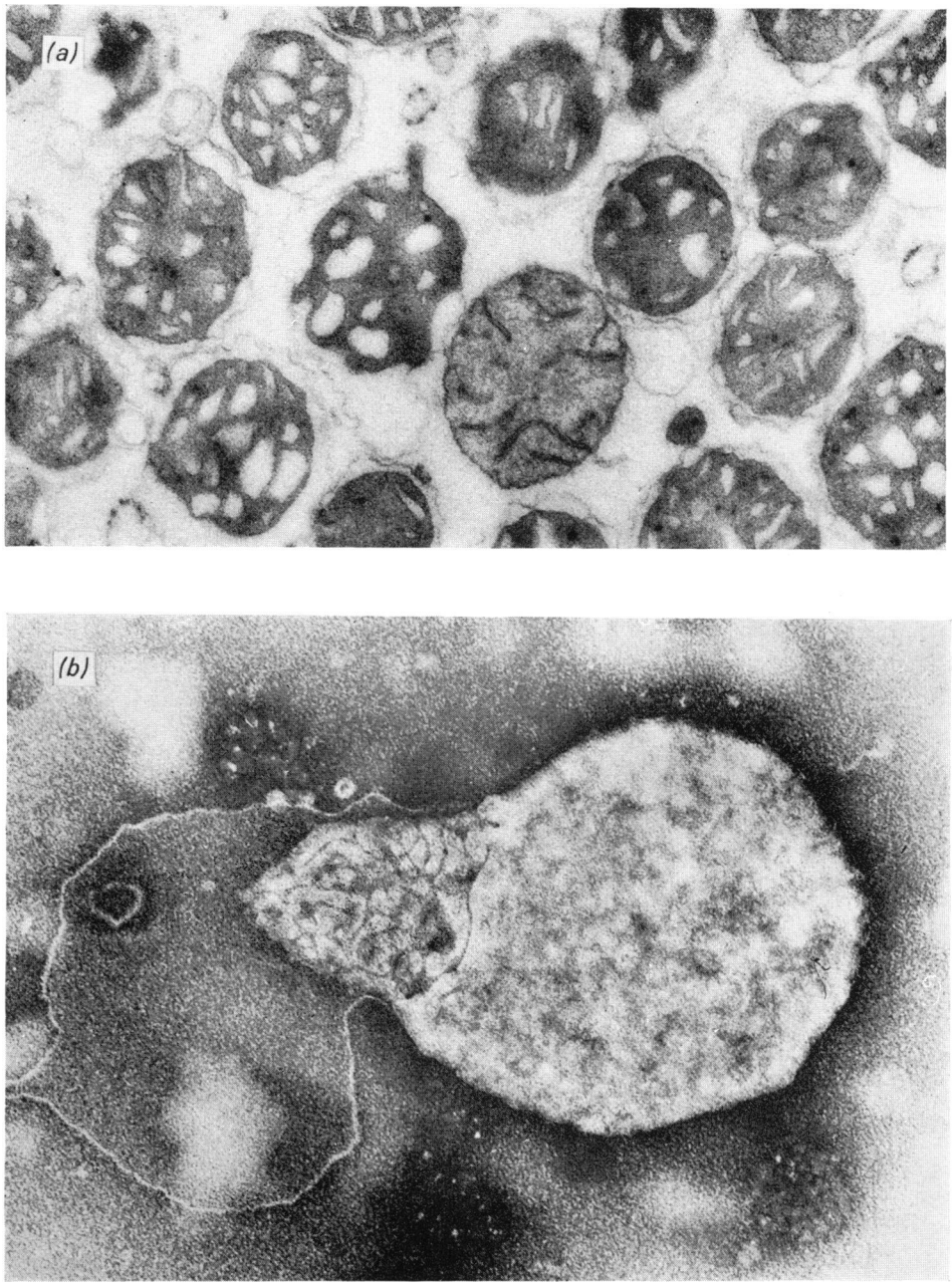
Plate 2(*a*) is a more typical example of what was seen with a negatively stained mitochondrial preparation. Many of the general features of the sectioned material were retained, although it seems likely that some mitochondrial damage occurred during the negative staining. The outer membrane, which is presumed to be the thin white line surrounding at least a portion of the mitochondrion, can be readily identified. Laminar structures were frequently found associated with this area and were also seen in the 'interior' portion (arrow). The latter may have actually been on the surface of a membrane covering the mitochondrion. It was also a common finding that one portion of the mitochondrion was poorly stained and had the same featureless appearance, with only slight indication of either underlying or surface detail, that was assigned to the outer membrane in Plate 1(*b*). The laminar structures were rarely associated with the smooth portion, and the thin white outer line was absent.

Plate 2(*b*) is a photograph of a mitochondrion that is very similar in size to the one shown in Plate 2(*a*), but its appearance is quite different. The stain has penetrated more completely and tubules of varying widths are seen rather than grooves. Some of the tubules end in clearly defined holes (arrow). The tubules may be cristae and the holes their opening into the inner membrane or they may be completely enclosed folds in the inner membrane. The tubule-containing portion (*A*) is surrounded by a thin white line (presumably outer membrane) whereas the remaining portion (*B*) appears to be almost empty and to be a continuation of the outer membrane surrounding part *A*.

Plate 3 shows a group of negatively stained mitochondria in which there is a measure of symmetry between the smooth areas and the remainder of the mitochondrion, and appears to result from the inner portion of the mitochondrion having almost completely emerged from within the outer membrane.

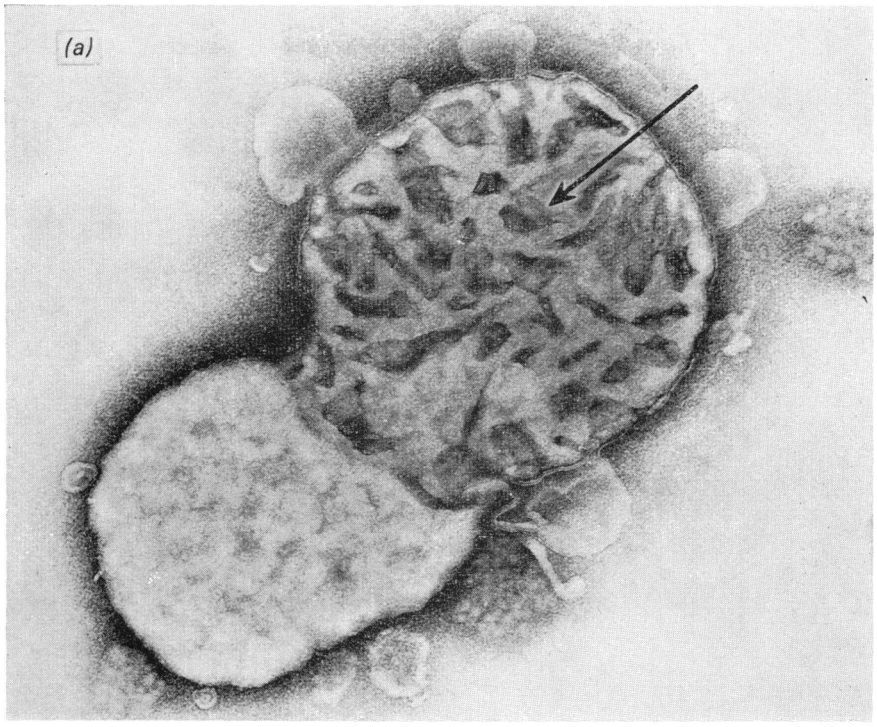
Many of the grooves seen in mitochondria and the condensed form of preparation A (see below) frequently contained numerous round structures. These are seen most clearly in the mitochondrion (*C*) in the upper left portion of Plate 2(*b*). They varied in diameter between 200 and 900 Å and are shown in Plate 4(*a*) in high magnification. It is difficult to be certain whether they are outpouchings or discrete structures, but the former seems more likely.

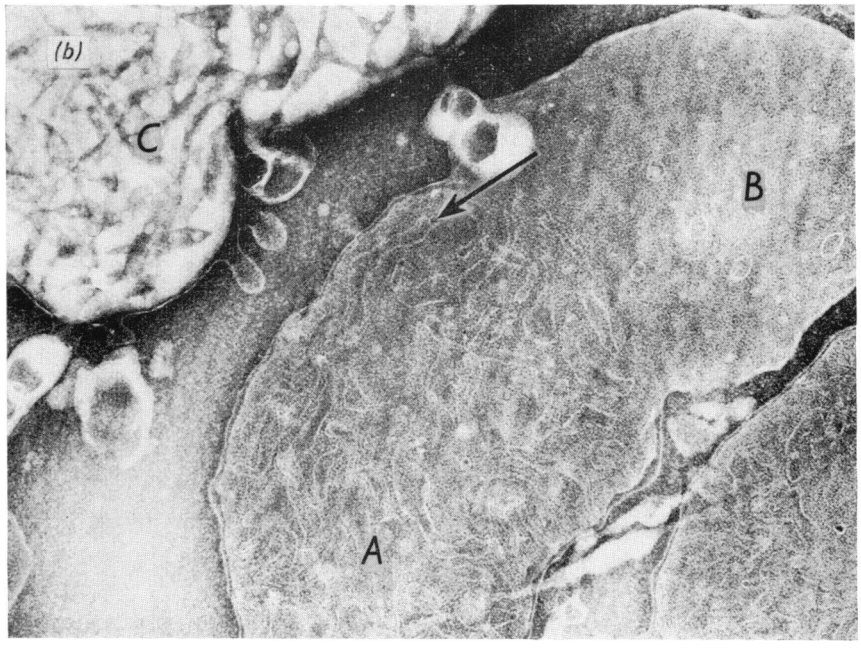
Preparation A. The removal of the outer membrane can be evaluated enzymically by measuring the loss of an enzyme found only in this portion of the mitochondrion (Sottocasa, Kuylenstierna, Ernster & Bergstand, 1967; Parsons, Williams, Thompson, Wilson & Chance, 1967). The preparations described below were found to contain less than 5% of the original NADH–cytochrome *c* reductase (rotenone-insensitive) of the whole mitochondria and therefore were virtually free of outer membrane (Hoppel & Cooper, 1968). Plate 4(*b*) is a sectioned preparation and shows three types of morphology. We refer to these as condensed, intermediate and open types of configuration, in conformity with the nomenclature of Hackenbrock



EXPLANATION OF PLATE 1

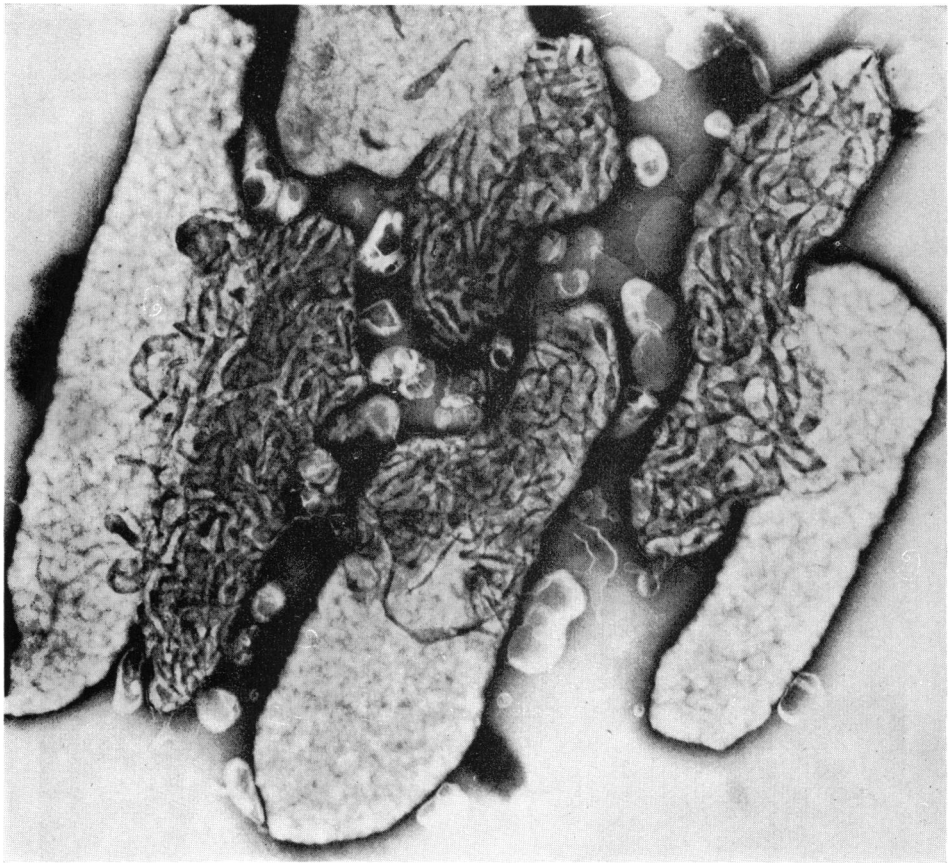
(*a*) Rat liver mitochondria isolated in 0·3 M-sucrose, fixed in glutaraldehyde, post-fixed in osmium tetroxide, sectioned and stained. Magnification 36000×. (*b*) Negatively stained rat liver mitochondrion showing torn flap of outer membrane and deeply stained grooves of the inner-membrane complex. Magnification 58000×.





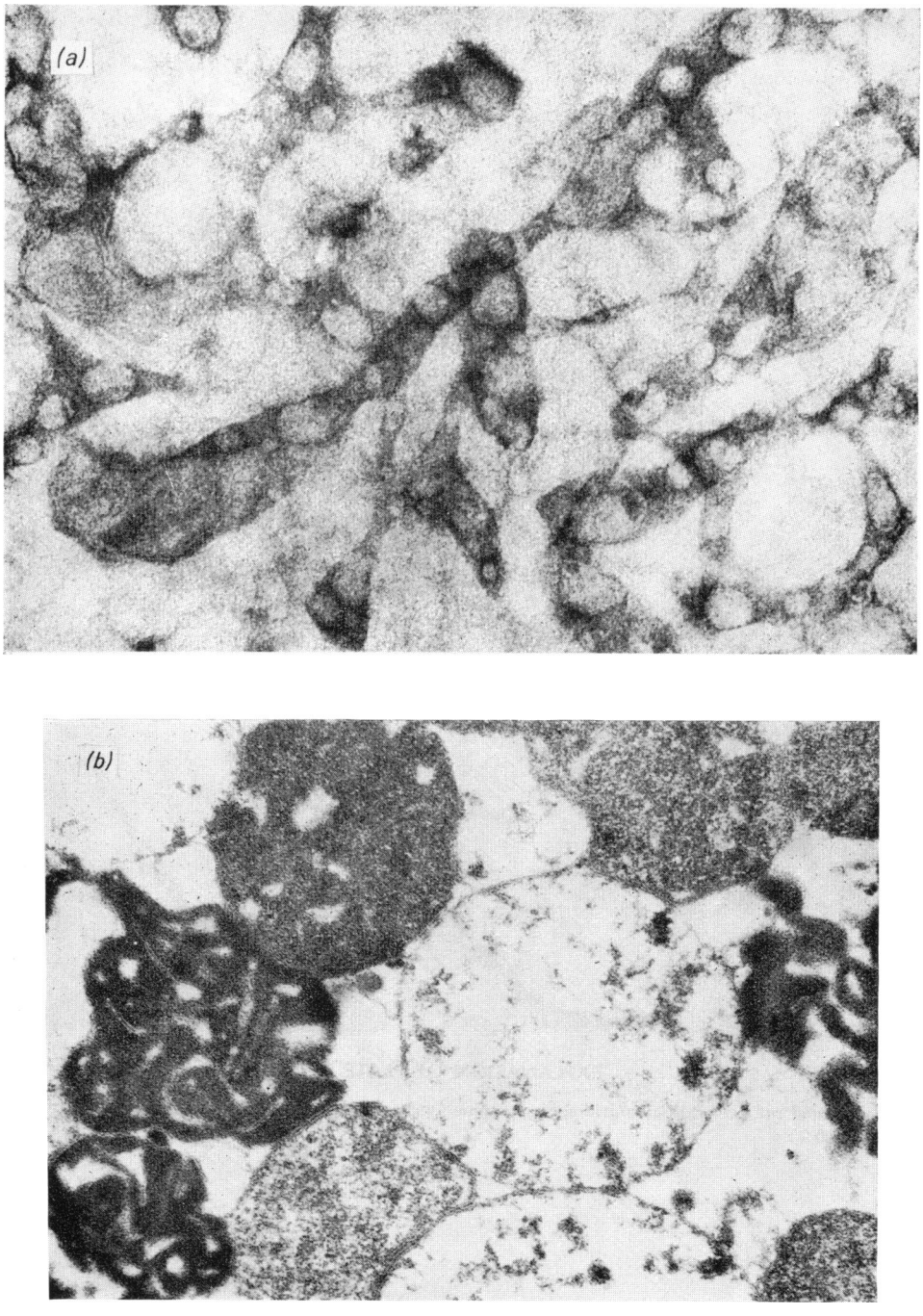
EXPLANATION OF PLATE 2

(*a*) Rat liver mitochondria isolated in 0·3 M-sucrose and negatively stained. Magnification 52000×. (*b*) Rat liver mitochondrion more completely penetrated by negative stain. Arrow indicates hole at end of tubule. Portion *A* contains tubules, whereas portion *B* appears to be an empty continuation of the outer membrane. Portion *C* shows the round structures in the mitochondrial grooves. Magnification 52000×.



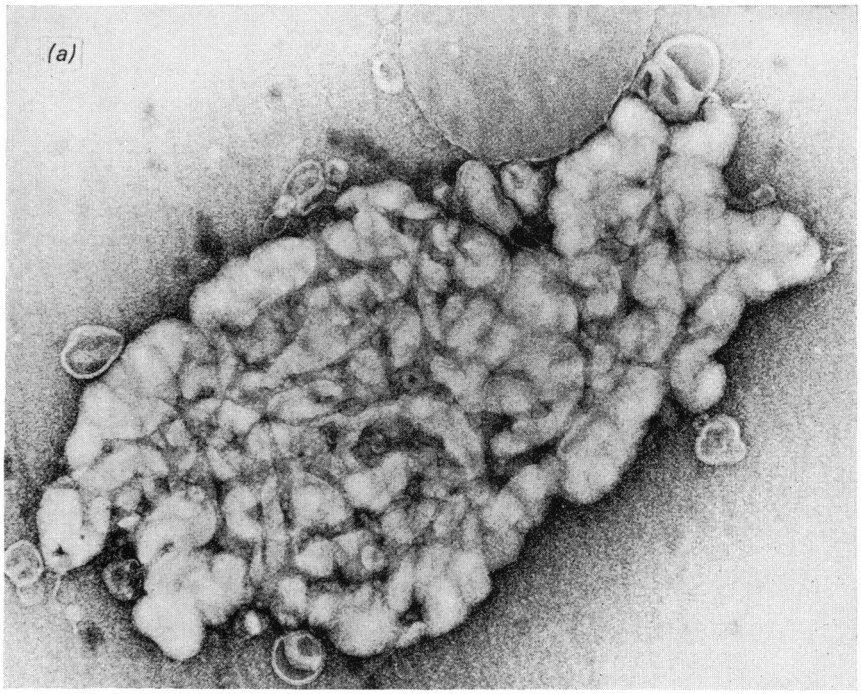
EXPLANATION OF PLATE 3

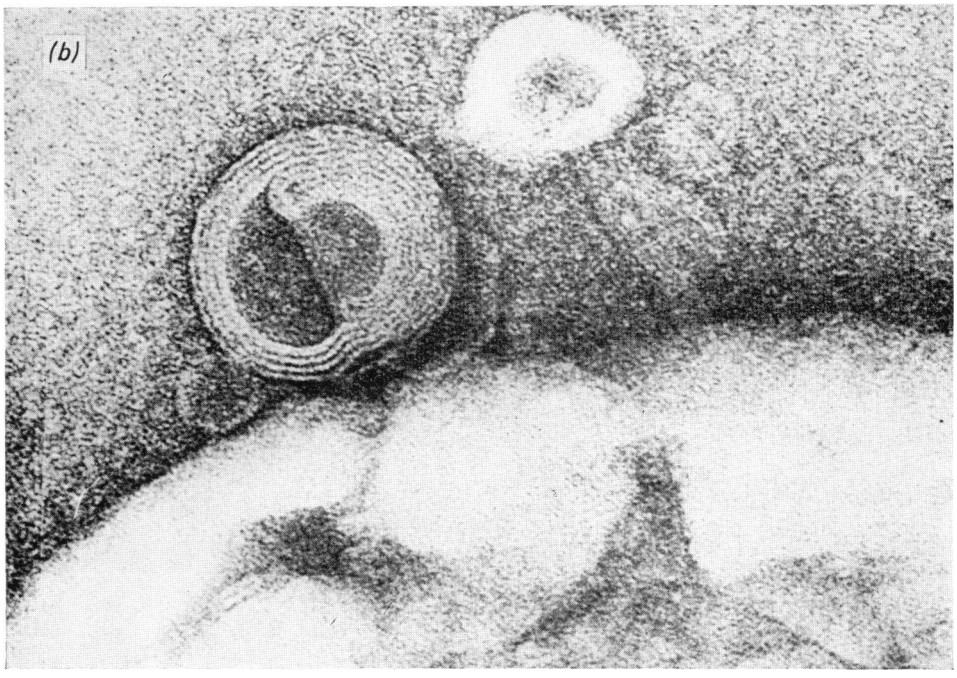
Negatively stained rat liver mitochondria showing some symmetry between smooth and grooved areas. Magnification 31 000×.



EXPLANATION OF PLATE 4

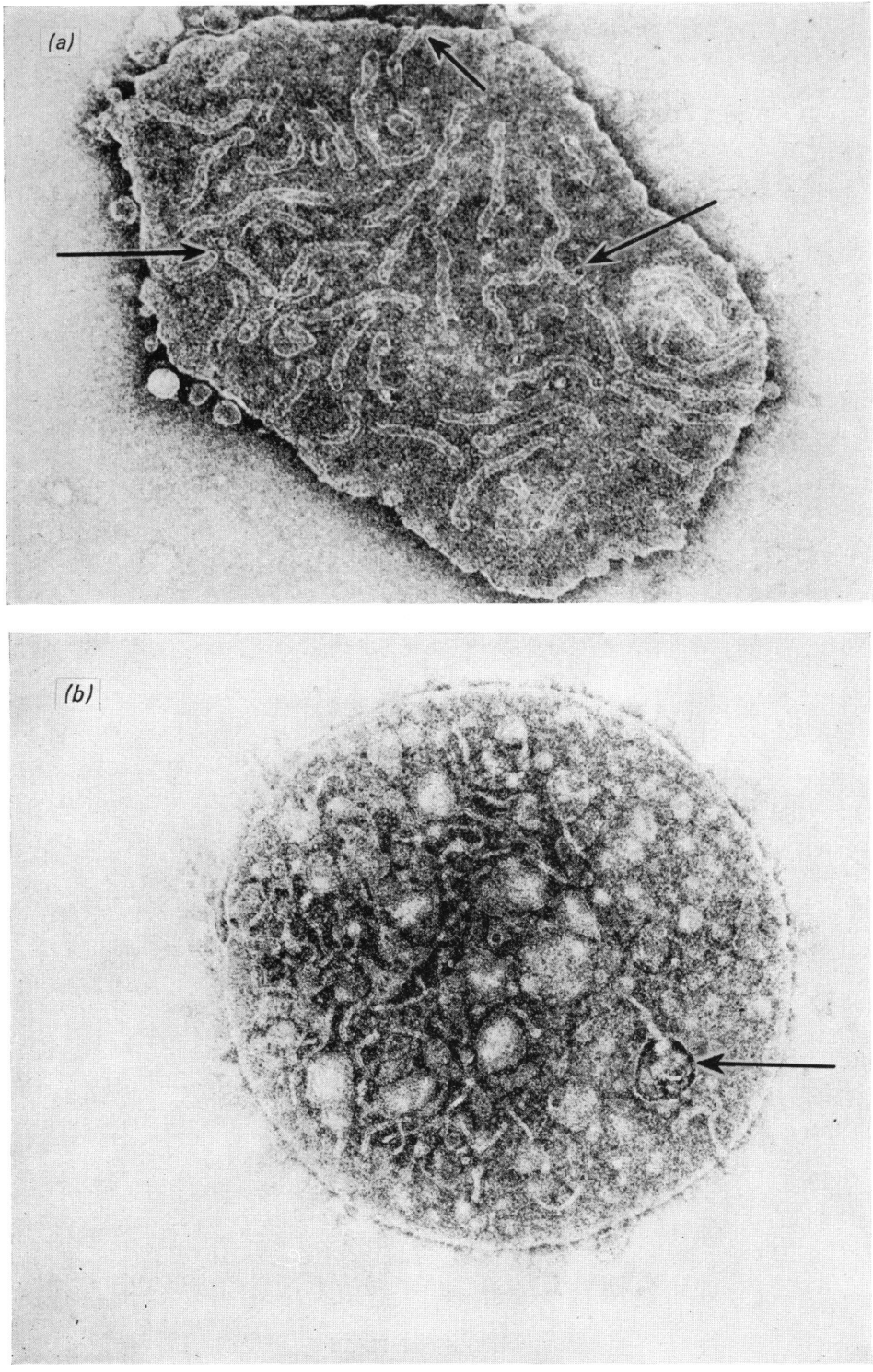
(*a*) High-magnification view of round structures in grooves of negatively stained rat liver mitochondria and preparation A. Magnification 175000×. (*b*) Preparation A isolated in 0·3M-sucrose, fixed in glutaraldehyde, post-fixed in osmium tetroxide, sectioned and stained. There are three types: contracted with a heavily stained matrix and a few cristae; intermediate with a more lightly stained, granular matrix; and open with very little matrix. Magnification 29000×.





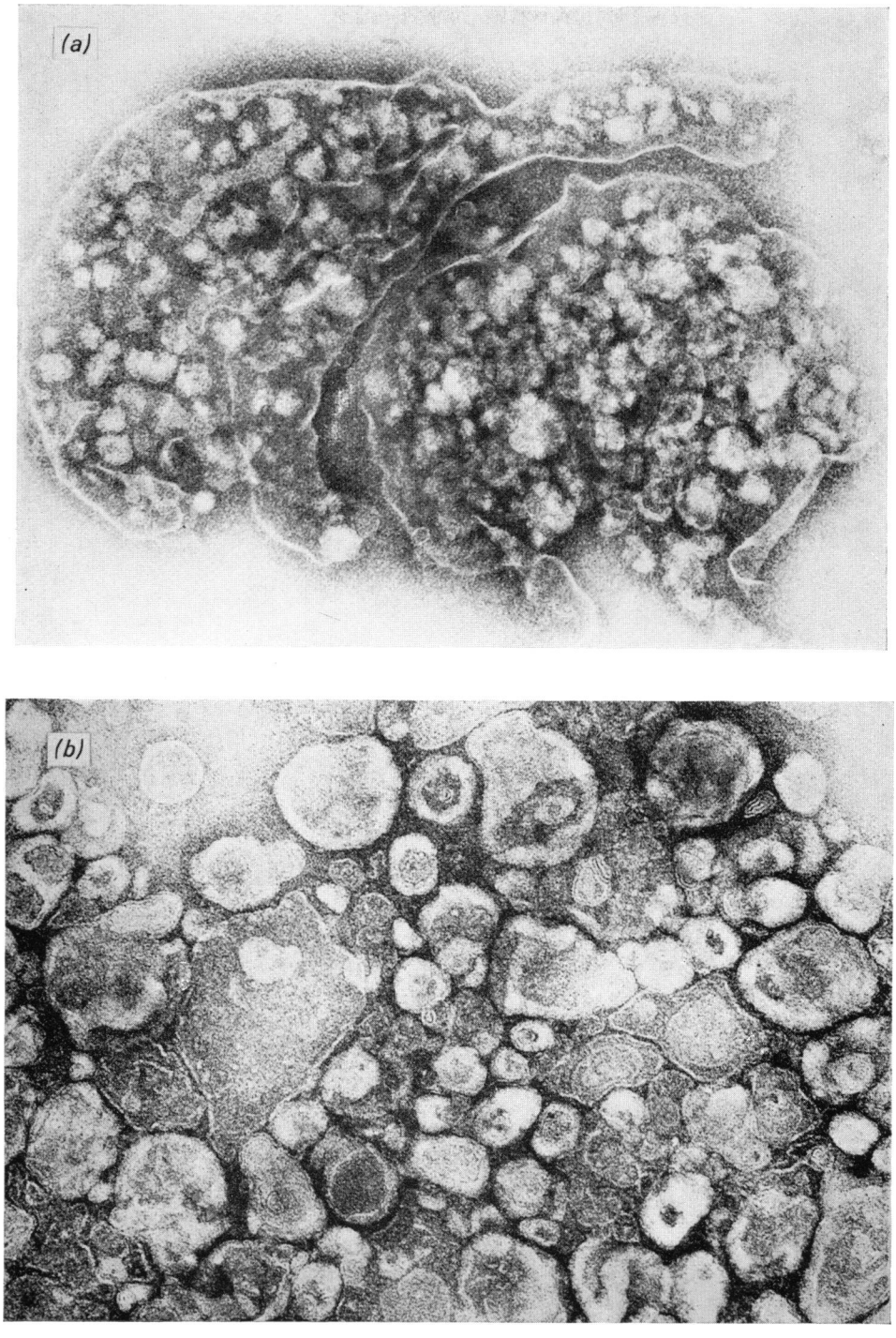
EXPLANATION OF PLATE 5

(*a*) Negatively stained, contracted form of preparation A isolated in 0·3 M-sucrose. Magnification 40000×. (*b*) High-magnification view of peripheral myelin figure. The layers appear to contain spherical sub-units. Magnification 200000×.



EXPLANATION OF PLATE 6

(*a*) Intermediate form of preparation A negatively stained. Arrows indicate tubules that appear to have a hole at one end. Magnification 54000×. (*b*) Intermediate form of preparation A negatively stained. There is a hole (arrow) in the membrane and the matrix contains both tubules and large pieces of undefined material. Magnification 40000×.



EXPLANATION OF PLATE 7

(*a*) Open form of preparation A negatively stained. Membrane is ruptured and is filled with aggregated material. Magnification 36000×. (*b*) Preparation B negatively stained. It contains pieces of membrane, pieces with deeply stained centres and lamellar structures. No 90 Å sub-units are seen. Magnification 120000×.

(1966). The condensed type is heavily stained, compact and contains cristae and vacuoles. The intermediate form is lightly stained and has only occasional cristae and vacuoles, whereas the open form is much larger in diameter than the other two and contains very little matrix material. Absence of the outer membrane is apparent, thus supporting the biochemical evidence on the absence of outer-membrane enzyme activity. The inner membranes are clearly seen where adjacent structures are in contact.

Three types of conformations were seen in the negatively stained preparations also. The condensed form (Plate 5*a*) was characterized by the presence of numerous deeply stained, randomly oriented, grooves and was frequently irregular and somewhat lobular in outline. In many instances it gave the impression of having been formed from a highly convoluted tube whose compactness varied. Laminar structures containing two to five layers of approx. 50Å each were observed at the periphery. Plate 5(*b*) shows one such layered structure at higher magnification. The layers appear to be composed of globular units.

Plate 6(*a*) shows a transition form and it is evident that a major change occurred. This type appears to consist of a large flattened bag whose uniform matrix is composed of granular material containing tubules some of which terminate in holes (arrows). Plate 6(*b*) shows a related form. A tear in the membrane (arrow) is seen and the matrix material appears to be forming clumps. Plate 7(*a*) represents an open form. In this case a limiting membrane is seen and material consisting primarily of aggregated structures appears to be inside or on the surface of the membrane. In general, the open forms of preparation A were devoid of the 'myelin-like' structures at the periphery.

It should be noted that preparation A had a P/O ratio with β-hydroxybutyrate that was only slightly less than that of the intact mitochondria from which it was made (Hoppel & Cooper, 1968).

Preparation B. The further treatment of preparation A with digitonin led to a fragmentation of the inner mitochondrial complex with the formation of particles ranging in size from 300 to 2000Å with most between 500 and 1000Å (Plate 7*b*). Several types of structures were customarily seen. These included flat pieces of membrane with a white outer edge and a granular appearance, doughnut-shaped units whose centre may have been empty or consisted of a depression filled with stain and particles composed of lamellae.

Though both the whole mitochondria and preparation A were subject to osmotic variability, preparation B was most active with regard to oxidative phosphorylation when maintained in distilled water.

DISCUSSION

The photographs presented above provide some information on the structural alterations occurring during various stages of the repetitive treatment of mitochondria with digitonin. We have attempted to correlate the general features of fixed and sectioned material with those seen by negative staining and in general there is fair agreement. Negative staining in fact may give an insight into the structure of the inner mitochondrial complex, though the interpretation of many of the photographs is frequently difficult.

Workers in several Laboratories have examined fixed, negatively stained, mitochondria and published photographs of intact-looking structures showing heavily stained areas interpreted as cistae and surrounded by a thin white line presumed to be outer membrane. On this basis it was suggested that the outer membrane is readily permeable to phosphotungstate (Whittaker, 1966), but it is difficult to evaluate the degree of intactness of the outer membrane under the staining conditions employed. One interpretation of Plate 1(*b*) is that the only portion of the inner mitochondrial complex visible is where the outer membrane had been torn and folded back. This flap then appears to be transparent whereas the portion still intact appears opaque, possibly because the stain had not penetrated the space between the two structures.

The smooth area seen in only a portion of the picture of many 'intact' mitochondria is very similar in appearance to the outer membrane of Plate 1(*b*). It was never found in A preparations that were made by removing the outer membrane, and it therefore seems most likely that it arose from the outer membrane. It may represent an area still covered by outer membrane in which the stain had not penetrated between the membrane and the inner mitochondrial complex. It is also possible, however, that it may have been an empty flap or pocket of outer membrane that remained unstained because of material still adhering to it that was not penetrated by stain. This empty-flap explanation seems to fit the situation seen in Plates 2(*b*) and 3. The *B* portion of the mitochondrion in Plate 2(*b*) appears to be an empty piece of outer membrane, whereas in Plate 3 the outer membrane seems to have been completely removed from some of the mitochondria.

The lamellar structure was a very characteristic feature of the preparations observed with negative staining. Such areas were seen in the interior portion (arrow) and periphery of whole mitochondria (Plate 2*a*), around the condensed form of preparation A that had its outer membrane removed (Plate 5*a*) and in preparation B (Plate 7*b*). They were rarely associated with the intermediate

(Plates 5*b*, 6*a* and 6*b*) or open form (Plate 7*a*) of preparation A seen with negative staining. These laminar structures are very reminiscent of those obtained by Lucy & Glauert (1964) and by Bangham & Horne (1964) with negatively stained lecithin suspensions. It is therefore very difficult to tell whether such structures were present as such in intact mitochondria and the other preparations or arose artificially.

The transition from the closed to open form of preparation A (Plates 5*a*, 6*a*, 6*b* and 7*a*) indicates that a profound change had occurred, and when carried to extreme by treatment of preparation A with water or dilute phosphate buffer resulted in the formation of structures containing 'inner-membrane sub-units' similar to those first described by Fernández-Morán (1962). When liver mitochondria are placed in hypo-osmotic media and stained with phosphotungstate they undergo extensive disruption, and tubules or ribbons of material studded with 90Å sub-units are frequently seen (Parsons, 1965). We have repeated these findings, but wish to point out that when the samples were prepared as described above we did not see these sub-units. In addition it is noteworthy that preparation B, consisting of small units capable of oxidative phosphorylation and related reactions, was never found to contain these structures. This may result from their destruction during sample preparation (Kagawa & Racker, 1966) or the lack of suitable conditions for their adventitious formation (Bangham & Horne, 1964; Sjöstrand, Cedergren & Karlsson, 1964).

The preparation of the negatively stained samples in the presence of sucrose allows much more detail to be observed than with the hypo-osmotic media more usually used. In addition, it appears that fixation procedures are not essential for the maintenance of intact structures. While this manuscript was in preparation Mitchell (1967) published photographs of fixed, negatively stained, ascites-tumour mitochondria in the presence of various concentrations of sucrose. Many similarities can be seen between his photographs and ours. He also observed mitochondria surrounded by a thin white line, which he interpreted to be outer membrane. Various forms corresponding to our condensed and open forms of preparation A were present when the samples were prepared in hypo-osmotic sucrose. He commented on the presence of lamellar structures seen within the mitochondrial matrix of sectioned preparations. Finally, he concluded that the tubules and ribbons associated with swollen preparations appear to arise via a rearrangement of matrix or inner-membrane material. It is apparent from our Plates 6(*b*) and 7(*a*) that rearrangements may account for the formation of the aggregates.

The point on possible rearrangements raises the question whether the enzymes normally considered to be bound to the inner membrane may not actually be bound to structures normally present in the matrix. Presumably this question could be answered by isolation of inner membrane uncontaminated by matrix material. Parsons *et al.* (1967) have reported experiments along this line, but their inner-membrane preparations did not appear to be completely free of matrix.

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