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ULTRASTRUCTURE AND FUNCTIONAL STATES OF MITOCHONDRIA

—EFFECT OF FIXATIVES ON THE STABILIZATION OF UNSTABLE CONFIGURATION*—

TAKASHI WAKABAYASHI

1st Department of Pathology, Nagoya University School of Medicine
(Director: Prof. Hisashi Tauchi)

ABSTRACT

Effect of fixatives on the unstable functional states of mitochondria was studied. The energized configuration of the cristal membrane of beef heart mitochondria can be maintained and visualized by the electron microscope if fixatives are introduced before the oxygen supply is exhausted. Since the exhaustion of the available oxygen supply is completed in 5-20 seconds, it is impossible to apply the method of sedimenting the mitochondria prior to fixation for studying the energized configurational states of mitochondria. The direct addition of glutaraldehyde followed by osmium tetroxide to the mitochondrial suspension is most effective for freezing the configurational state of cristal membrane. Fixation with glutaraldehyde appears to be complete within 1-2 seconds even at 0°C. Osmium tetroxide alone can also "freeze" the energized configuration by fixation but the concentration of the fixative is critical. The problem of capturing the configurational state applies not only to energized transitions (nonenergized to energized) but also to nonenergized transitions (orthodox to aggregated). When the level of glutaraldehyde or osmium tetroxide is respectively too low or too high, the mitochondria will undergo a transition from the aggregated to the orthodox configuration before fixation is complete. Light-scattering measurements which provide an independent method for monitoring configurational changes in mitochondria confirm that the conditions for fixation which lead to stabilization of the energized state as judged by electron microscopy, also show maintenance of configuration.

INTRODUCTION

Various intracellular organelles have been shown to undergo configurational and conformational changes concomitant with energy cycle. For example, mitochondria change their configuration in different energy states[1)2)]. The cristal membrane of adrenal cortex mitochondria undergoes a transition from the orthodox to the aggregated configuration concomitant with steroidogenesis[3)]. Sarcoplasmic reticulum is also known to undergo configurational

若林　隆

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changes under various conditions[4)~9)]. It may be generalized that in the living cell every single intracellular organelle undergoes dynamic changes configurationally and functionally.

Thus, electron microscopic technique has played a key role in visualizing functional aspects of living systems. However, there have been raised serious questions[10)11)] about the repeatability of visualizing so-called "energized-twisted" configuration proposed by Green *et al.*[2)].

The problem of "energized-twisted" configuration is only an example of the many problems which electron microscopists are and should be concerned about. Question of existence and the nature of gap junction between cells is another example. Is there a true fusion in a gap junction? Moreover, tungstate which is one of the most popular reagents for negative staining has been shown to modify the configuration of osmotically sensitive membrane systems[12)13)].

Since glutaraldehyde in conjunction with osmium tetroxide was introduced as a fixative for electron microscopy and cytochemistry[14)], structural differences of biological systems by different fixatives (glutaraldehyde-osmium double fixation, osmium simple fixation, and potassium permanganate fixation) have been pointed out by several workers[1)14)~17)]. However, little attention has been paid on the problem of why there are structural differences by different fixatives or fixation methods. It must be pointed out that most electron microscopists have been too much concerned on only how to preserve the fine structures which are visualized with the electron microscope, neglecting the possibility that every element composing the biological systems might have polymorphism concomitant with its functional states.

Thus, it seems urgent to seek and establish the fixation conditions by which unstable functional states of biological systems are "frozen". Since the mitochondrion is known to change its configuration concomitant with its functional states, as described above, this organelle was used as a tool to study and clarify the problem in the present communication.

MATERIALS AND METHODS

Preparation of mitochondria

Heavy beef heart mitochondria were prepared by the method of Crane *et al.*[18)] as described by Hatefi and Lester[19)]. Special care was taken to remove as much of the light mitochondrial fraction as possible. Mitochondria were suspended at a final concentration of 50 mg per ml in a medium containing 0.25 M sucrose and 10 mM Tris-Cl, pH 7.5.

Preparation of tissue

The heart of the rat or the beef was rapidly removed from an animal. The tissue was sliced and minced into as small pieces as possible with a razor

blade.

Procedures for electron microscopy

All solutions of glutaraldehyde or osmium tetroxide (from 0.002% to 4%) were prepared in 0.25 M sucrose and 0.05 M cacodylate (K^+ salt), at a final pH 7.4. A mitochondrial suspension containing 4 mg of mitochondria in 4 ml of reaction mixture was mixed with an equal volume of the glutaraldehyde solution (final concentrations covering the range from 0.001% to 2%). The mixture was kept for 1–3 hours at 25°C. The mitochondria were then sedimented in a clinical centrifuge for 30 minutes—the pellet then being washed in a medium containing 0.25 M sucrose and 0.05 M K-cacodylate of pH 7.4. After interaction with glutaraldehyde the mitochondrial pellets were then exposed to osmium tetroxide (final concentration from 0.01% to 2%). In some experiments osmium was the only fixative used. The procedure for the interaction of the mitochondria with osmium was identical with the procedure described above for the interaction with glutaraldehyde.

After fixation, all samples were first exposed to 25% ethanol containing 1% uranyl acetate, before dehydration with ethanol solutions of gradually increasing ethanol concentration. The exposure to 100% ethanol was repeated three times. Dehydrated samples were then exposed to absolute propylene oxide for 10 minutes. For embedding, the dehydrated samples were exposed first to a mixture of equal parts by volume of propylene oxide and Epon (for 20 minutes) and finally to 100% Epon[20]. Specimens were sectioned with a diamond knife or a glass knife and were examined with a Hitachi HU-11 B electron microscope operated at 75 kV.

Biochemical assays and probes

1. Rate of oxygen consumption

The rate of oxygen consumption was measured at 30°C with a Clark-type electrode (Beckman Co., Fullerton, California)[21]. The experiments were carried out in 4 ml of medium containing 0.25 M sucrose, 0.01 M Tris-Cl of pH 7.4, 3 mM $MgCl_2$, 8 μg of rotenone and 4 mg of heavy beef heart mitochondria. The oxidizable substrate was potassium succinate (final concentration 5 mM). Where indicated, glutaraldehyde or osmium tetroxide or both were introduced into the reaction flasks to determine the extent to which respiration was affected.

2. Light-scattering changes

A Brice-Phoenix light-scattering photometer (Universal 2,000 ssries) with a Phoenix normal-ratio recorder (−0.25 to +0.25 mV) was used to measure light csattered at 90° to the incident beam by mitochondrial suspension[2)22)]. The measurements were carried out in 3 ml volume of medium containing 0.25 M sucrose, 0.01 M Tris-Cl (pH 7.4), 3 mM $MgCl_2$, 6 μg of rotenone and 3

mg of heavy beef heart mitochondria. The reagents which initiate the light scattering were introduced by rapid injection with a micro-syringe or pipette with quick stirring after addition. The order of addition was first succinate (5 mM) and then inorganic phosphate (10 mM). In some of the light-scattering studies, fixatives were also introduced into the reaction mixture to final concetrations from 0.001% to 0.25%. To avoid excess dilution the stock solutions were made concentrated (10% glutaraldehyde and 5% osmium tetroxide). The stock solutions also contained 0.25 M sucrose and 0.05 M K-cacodylate, pH 7.4.

3. Uptake of Pi during energization of mitochondria

When mitochondria are energized by electron transfer in the presence of inorganic phosphate they undergo a transition to the energized-twisted configuration. It has been shown that the extra uptake of inorganic phosphate duringe nergizing is a reliable index of the generation of thee nergized-twisted configuration. For such measurement of augmented Pi uptake, the mitochondria (1 mg protein) were incubated in a medium (1 ml final volume) containing 0.25 M sucrose, 0.01 M Tris-Cl of pH 7.4 and 2 μg of rotenone. Pi^{32} with a specific activity of 500–1,000 cpm per μmole was then introduced with the desired amount of cold phosphate. The experiment was carried out both in the presence and absence of added succinate.

In some experiments fixatives were introduced to a reaction mixture to determine whether fixatives could stabilize the energized-twisted configurational state as measured by prevention of the efflux of bound Pi. This procedure was carried out as follows. The mitochondrial suspension which had been incubated in the presence succinate and Pi was rapidly mixed with glutaraldehyde or with osmium. After 20 seconds, the suspension was centrifuged in a high-speed Misco centrifuge capable of reaching full speed (22,000 rpm) in less than 10 seconds[23]. Glutaraldehyde-osmium double fixation was carried out as follows. The mitochondrial suspension was first exposed to glutaraldehyde for 20 seconds; then it was exposed to osmium for the additional 20 seconds; the suspension was finally centrifuged as described above. The surfaces of the mitochondrial pellets were washed three times with a chilled solution of 0.25 M sucrose and 0.01 M Tris-Cl of pH 7.4. The pellets were then extracted at 50°C with 1% sodium dodecyl sulfate for 5 hours after homogenization in a Teflon homogenizer. The measurement of radioactivity was carried out in a Tri-carb scintillation counter with Brays fluid.

In all experiments, both a control without added substrate and a control with substrate but without fixative were carried out. In such controls, the sucrose-Tris medium was added in lieu of the solution containing the missing component. Measurements of energized Pi uptake were usually monitored by electron microscopic examination of the identical suspensions.

RESULTS

I. Effect of concentration of fixatives on the configuration of mitochondria either isolated or in situ

A. Transition of the cristal membrane of mitochondria *in situ* from the orthodox to the aggregated configuration by sucrose.

When slices of tissue from beef heart were fixed with 2% glutaraldehyde containing 0.25 M sucrose and 0.05 M K-cacodylate of pH 7.4, mitochondria *in situ* invariably show the orthodox configuration (Fig. 1 A), where the intracristal space is minimal and the matrix space is maximal. On the contrary, when slices of heart tissue were first exposed to 0.25 M sucrose solution and then fixed with 2% glutaraldehyde, mitochodria show the aggregated configuration (Fig. 1 B), where the intracristal space is maximal and the matrix space is minimal. The above described transition of configuration can be simply explained by the fact that sucrose is impermeable to the matrix space through the cristal membrane. Thus, mitochondria isolated in 0.25 M sucrose solution show the aggregated configuration (Fig. 1 C).

B. Determination of the minimum glutaraldehyde concentration at which isolated mitochondria exhibit the aggregated configuration.

Fig. 2 A shows an electron micrograph in which beef heart mitochondria were fixed with 0.01% glutaraldehyde. Mitochondria remain in the aggregated configuration. However, transition from the aggregated to the orthodox configuration takes place when isolated mitochondria are fixed with glutaraldehyde at concentrations lower than 0.01% (Fig. 2 B).

Effectiveness of low concentration of glutaraldehyde (0.01%) is also demonstrated by the following simple experiment. When isolated mitochondria are incubated with Krebs-Ringer phosphate solution (KRP), transition from the aggregated to the twisted configuration takes place (Fig. 2 C), but once isolated mitochondria are fixed with glutaraldehyde at concentrations as low as 0.01%, incubation of mitochondria with KRP solution does not change the original mitochondrial configuration (Fig. 2 D).

C. Determination of the minimum glutaraldehyde concentration at which mitochondria *in situ* exhibit the orthodox configuration.

As has been described above, mitochondria *in situ* stay in the orthodox configuration when they are fixed with 2% glutaraldehyde. The lowest concentration of gultaraldehyde at which mitochondria *in situ* exhibit the orthodox configuration is as low as 0.05% (Fig. 3 A). When tissue slices were fixed with glutaraldehyde containing 0.25 M sucrose at concentrations lower than 0.05%, most of the mitochondria showed the aggregated configuration (Fig. 3 B). Moreover, when slices of tissue were exposed to KRP solution before fixation with glutaraldehyde, the twisted configuration is induced (Fig. 3 C),

but prefixation with 0.05% glutaraldehyde prevents this transition in the configuration of mitochondria *in situ*. Effect of concentration of fixatives on mitochondrial configuration is summarized in Table 1.

TABLE 1. Effect of Concentration of Glutaraldehyde on Configuration of Mitochondria

Concentration of glutaraldehyde	Mitochondria, isolated	
	Glutaraldehyde prepared in sucrose	Glutaraldehyde prepared in KRP
lower than 0.01%	orthodox	twisted
higher than 0.01%	aggregated	aggregated
	Mitochondria *in situ*	
	Glutaraldehyde prepared in sucrose	Glutaraldehyde prepared in KRP
lower than 0.05%	aggregated	twisted
higher than 0.05%	orthodox	orthodox

II. Stabilization of the energized configuration

A. Comparison of mixing and centrifugation methods in relation to aerobic-anaerobic transition in the energized configurational states of mitochondria.

Fig. 4 shows a light-scattering trace for mitochondria first energized by succinate and then supplemented with inorganic phosphate (Pi). It is only after addition of Pi that a rapid decrease in light-scattering takes place. As oxygen is gradually being consumed, the light-scattering increases to the level before addition of Pi. When samples are removed before and after the rapid fall in light scattering and then subsequently fixed with 2% glutaraldehyde,

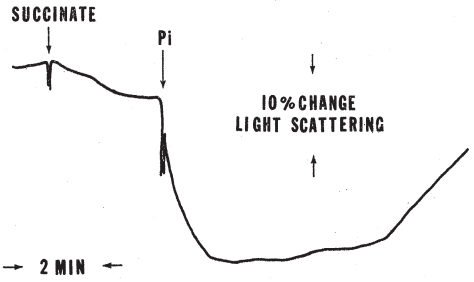

FIG. 4. Light-scattering changes and the energy state of beef heart mitochondria. The incubation medium contained 0.25 M sucrose and 0.01 M Tris-Cl, pH 7.4, and the additions were rotenone (2 μg per mg mitochondrial protein), succinate (5 mM), Pi (10 mM), and beef heart mitochondria (1 mg of protein per milliliter of medium).

the energized-twisted configuration is found only in the sample which has sustained the rapid fall in light scattering (Fig. 5 A). Moreover, the energized-twisted configuration is no longer found in a sample to which both succinate and Pi had been added but which had been allowed to decay to the baseline level of light scattering (Fig. 5 B). This simple erperiment established first that the phosphate-induced light-scattering change is an indicator of the generation of the energized-twisted configuration and second that the light-scattering change decays with exhaustion of oxygen and is paralleled by the disappearance of the energized-twisted configuration.

An exactly same type of experiment can be used to correlate the light-scattering change with the level of Pi uptake. If the same three samples are withdrawn as described above, and assayed for increased Pi uptake by the rapid sedimentation method, it is only in the sample which showed the light-scattering decrease that Pi uptake was demonstrable (Table 2). In the light-scattering experiment, we assumed that the reversal of the light-scattering increase was a consequence of oxygen exhaustion. Fig. 6 is an experiment

TABLE 2. Correlation of Increased Pi Uptake with the Generation of the Energized-Twisted Configuration

Status of light-scattering	Increment in phosphate uptake* (mμ moles/mg protein)
During phase of decrease (after 60 sec)	13
During phase of decrease (after 120 sec)	17
During phase of increase (after 60 sec)	2
During phase of increase (after 240 sec)	0

* The Pi content of the mitochondrial sample has been corrccted for the Pi taken up in a control without substrate.

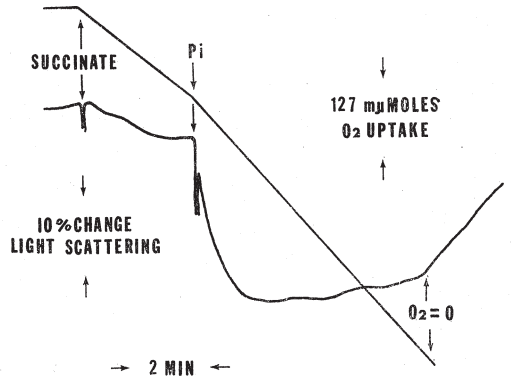

FIG 6. Correlation of the reversal of light-scattering change with exhaustion of oxygen in the system. The system was the same as that described in the legend of Fig. 4.

run in parallel with light scattering measurements and under identical conditions in which the oxygen consumption is measured. The amount of oxygen in the system introduced initially by aeration is limited and the system becomes anaerobic after addition of substrate as respiration proceeds. If the mitochondria in the energized configuration are sedimented before fixation as in the method of Hackenbrock, then given the usual rate of oxygen uptake, a decay not exceeding 10 seconds is permissible before addition of fixatives. Electron microscopic examination of such pelleted mitochondria after fixation will invariably show the nonenergized configuration (Fig. 7 A).

The centrifugation method requires at least 30 seconds before fixative can be added to the pellet, and during this time the mitochondrial pellet becomes anaerobic particularly at 25°C. At this temperature 1 mg of mitochondrial protein consumes oxygen at a rate of at least 0.25 mμatoms oxygen per second[24]. Since the pellet of mitochondria contains at most 3 μl of water per mg protein in isotonic media, the amount of oxygen dissolved in the pellet cannot maintain aerobic conditions for longer than 12 seconds[24]. At 30°C the prevention of anaerobios is during sedimentation is not easy to achieve because of the rapid rate of oxygen consumption. But at 1°C, the rate of oxygen uptake is reduced to 1-2% of the rate at 30°C. All changes are slower but at least the onset of anaerobiosis during centrifugation is avoided. The rate of oxygen uptake at 1°C is slow enough for the pellet collected after 20 seconds of centrifugation in the Misco centrifuge to be still aerobic. Thus, when the centrifugation method is applied at 1°C and the additional precaution is taken of adding H_2O_2+catalase to ensure aerobiosis during the centrifugation and when the pellet thus obtained by centrifugation is resuspended in the glutaraldehyde-containing fixative solution, the electron micrographs clearly show that the configuration is twisted as would be predicted (Fig. 7 B).

B. Speed of fixation of the energized configuration.

In the previous section it was clearly demonstrated that the demonstration of the energized configuration requires direct addition of the fixative to the mitochondrial suspension at 25–30°C to avoid the aerobic-anaerobic transition. This immediately raises the question of how long it takes for the fixatives to be effective.

The following very simple experiment showed that glutaraldehyde at a final concentration of 2% can freeze the configuration of the mitochondria within seconds after addition. In one tube, mitochondria were first energized with succinate, then gutaraldehyde and Pi were added simultlaneously. In the other tube, glutaraldehyde was added 5 seconds after addition of Pi. The electron micrographs showed no energized-twisted configuration in the former and predominantly energized-twisted in the latter (Fig. 8 A and 8 B). This experiment suggests that the fixation by glutaraldehyde is at least as fast as

the generation of the energized-twisted configuration. Time sequence of the generation of the energized-twisted configuration after addition of Pi is shown in Table 3. The point at stake is merely that fixation by glutaraldehyde is in the second range—fast enough to avoid the complication of the medium becoming anaerobic before fixation is complete.

TABLE 3. Correlation of time durations of the addition of Pi with the generation of the energized-twisted configuration

Time (sec)	Population of the energized-twisted configuration* (%)
0	0
5	76
10	75
20	83
40	80
60	86

* The incubation medium contained 0.25 M sucrose, 0.01 M Tris-Cl, and 3 mM $MgCl_2$, pH 7.4, and the additions were rotenone (2 μg per mg mitochondrial protein), succinate (5 mM), and beef heart mitochondria (1 mg of protein per milliliter of medium). After certain incubation times with Pi specified in the Table, a mitochondrial suspension was fixed with 2% glutaraldehyde, postfixed in 1% osmium tetroxide.

C. Concentration of glutaraldehyde and osmium required for rapid fixation of the energized configuration.

When the final concentration of osmium tetroxide was kept constant at 2%, and the concentration of glutaraldehyde was varied, it appeared that at a concentration of glutaraldehyde as low as 0.01%, fixation of the energized-twisted configuration was complete (Fig. 9 A). A similar series was carried out with the concentration of glutaraldehyde kept constant at 0.01%, and the concentration of osmium tetroxide varied. Even at a final concentration of 0.03% osmium tetroxide fixation of the energized configuration is as complete as with the standard concentration of 2%. When mitochondrial suspension was fixed by addition only of osmium tetroxide without prefixation with glutaraldehyde the electron micrographs showed poor contrast but none the less the configuration of the mitochondria was unambiguously energized-twisted even a tafinal concentration as low as 0.01% (Fig. 9 B). At higher concentrations of osmium tetroxide, *e.g.*, at 2%, the energized-twisted configuration was no longer observed (Fig. 9 C). From these studies it might be concluded that osmium alone at low concentrations is as effective as the combination of glutaraldehyde followed by osmium in freezing the energized configuration of the cristal membrane. But when prefixation with glutaraldehyde is carried out, higher concentrations of osmium tetroxide do not

affect the preservation of the energized-twisted configuration.

III. Correlation between the preservation of the energized-twisted configuration by fixatives and the effect of these fixatives on the light-scattering changes and increased Pi uptake induced by energizing in presence of Pi.

Theoretically, it would require that fixatives which preserve the phosphate-induced energized configuration would not materially change the light-scattering pattern on the increased Pi uptake of the mitochondrial suspension, *i.e.*, mitochondria in the energized configuration should retain their light-scattering properties and increased Pi uptake capacity even after exposure to fixatives if indeed these fixaiives preserve the energized configuration. The supporting data for these predictions are shown in Fig. 10 and Table 4 respectively. Glutaraldehyde at a concentration of 0.1% maintains fairly closely the light-scattering level which was obtained before addition of glutaraldehyde, whereas a concentration of 0.01% is somewhat less effetive. Osmium at a concentration of 0.01% is fairly effective in stabilizing the light-scattering pattern, whereas a concentration of 0.1% osmium induces extensive changes in the light-scattering pattern.

A similar correlation was established, shown in Table 4, between the

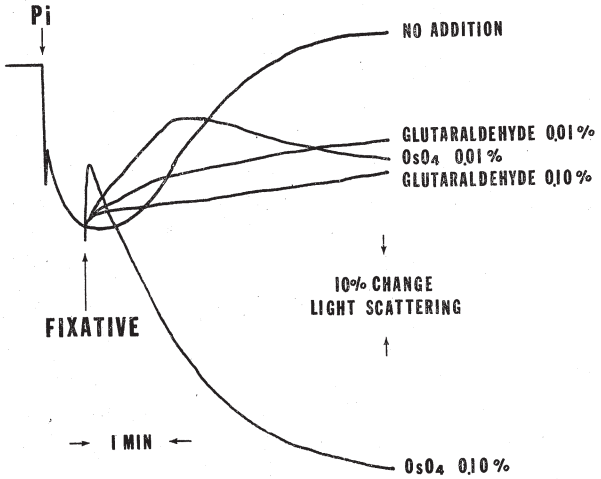

FIG. 10. Effects of fixatives on light scattering of mitochondria. The incubation medium is described in the legend of Fig. 4. After energizing of the mitochondrial suspension in the presence of succinate, rotenone and Pi, the effect of fixatives on the light-scattering properties of the system were recorded. The final concentrations of the fixatives are indicated in the Figure. Glutaraldehyde and osmium tetroxide were added as concentrated solutions to minimize dilution effects (see text). Change in pH during the measurement of light scattering was less than 0.2–0.3 pH units.

TABLE 4. Correlation of Increased Pi Uptake with the Generation of the Energized-Twisted Configuration

Conditions and additions	Increment in phosphate uptake* (mμ moles/mg protein)
A. No fixative (20 sec)	
Glut. (20 sec) 0.01%	13.0
2.0%	15.8
OsO_4 (20 sec) 0.01%	4.6
2.0%	12.4
B. No fixative (40 sec)	
Glut. (2%) (20 sec) + OsO_4 (2%) (20 sec)	5.3
No fixative (20 sec) + OsO_4 (2%) (20 sec)	0

* The Pi content of the mitochondrial sample has been corrected for the Pi taken up in a control without substrate. After incubation with Pi (2 mM), both in presence and absence of succinate, the mitochondrial suspension was mixed with an equal volume of the appropriate fixative, allowed to stand 20 sec, and was then spun down in a Misco centrifuge. The pellets were washed with chilled sucrose-Tris solution and assayed for ^{32}Pi. In the control, sucrose-Tris was added instead of fixatives. In experiment B, the mitochondria were fixed with glutaraldehyde for 20 sec and then fixed with osmium for 20 sec.

concentrations of glutaraldehyde or osmium tetroxide required for retention of the extra Pi uptake and the concentrations required for preservation of the energized configuration.

IV. Application of glutaraldehyde fixation on negative staining

Another facet of the problem of configurational transition is shown by the effect of polyanions such are used as negative stains in changing mitochondrial configuration.

When mitochondria are incubated with 1% silicotungstate prepared in distilled water the mitochondrial outer membrane became ruptured and the tubularization of the inner membrane takes place (Fig. 11 A). This phenomenon cannot be attributed simply to an osmotic effect since mitochondria which had been exposed to 1% silicotungstate containing 0.25 M sucrose remain intact but the original configuration is no longer observed (Fig. 11 B). Modification of the original configuration of mitochondria by tungstate can be prevented by prefixation of mitochondrial suspension with glutaraldehyde before exposure to tungstate. In Fig. 11 C, a mitochondrial suspension was first fixed with 0.01% glutaraldehyde and then exposed to 1% silicotungstate. Mitochondria remained in the original aggregated configuration.

DISCUSSION

Correlations between function and ultrastructural changes of various intra-

cellular organelles have been extensively studied by many workers. Among the components in the cell, the mitochondrion has been one of the structures most extensively studied[1)2)12)24)-32)]. Since the early studies of Hackenbrock[25)] and Green *et al.*[2)], it has been established that mitochondria undergo configurational changes concomitant with changes in their energy states. It might be gernerally stated that one configurational state of a particular intracellular organelle corresponds to one of its functional states. For example, when adrenal cortex mitochondria carry out steroidogenesis, their cristal membranes are in the orthodox configuration, *i.e.*, in the form of "scalloped" tubes[29)]. Under certain conditions, the tubular orthedox configuration is known to undergo a transition to the aggregated configuration[28)-30)], where the cristal membrane is arranged as flat opposed sheets. At the same time, the mitochondria switch from steroidogenesis to ATP synthesis as their major measured biochemical function.

Thus, it is essential to establish the conditions by which unstable functional states of various intracellular organelles can be "frozen" and visualized with the electron microscope. Since mitochondria are known to change their configuration concomitant with their functional states, as described above, the mitochondrion was used as a tool to examine the effect of fixatives on various intracellular organelles in the present communication.

Since glutaraldehyde was introduced as a fixative for electron microscopy glutaraldehyde-osmium double fixation method is replacing osmium simple fixation method because of its excellent cytological fixation[14)]. Glutaraldehyde is also known to preserve some enzymatic activities[33)], whereas osmium tetroxide completely surpresses enzyme activities[34)]. However, there is little data available concerning how to "freeze" dynamic aspects of biological systems. Thus, it is the main purpose of the present communication to establish the conditions by which unstable functional states of intracellular organelles can be stabilized.

Since with very few exceptions, electron microscopists have not been concerned with a correlative study, having been only interested in preserving the fine structures of biological systems, they processed the excised tissues in such a way that anaerobiosis was inevitable by the time the fixative was added to the specimen. This accounts for the fact that the vast majority of electron micrographs of mitochondria *in situ* show the cristae in the non-energized, orthodox configuration. However, a few investigators, notably Fernández-Morán[35)], Revel *et al.*[36)], Luft *et al.*[37)], Sternger and Spiro *et al.*[38)], Slautterback[39)], Fawcett and McNutt[40)] took precautions with respect to the speed of processing the excised tissues or to the speed of fixation, or both. All these investigators recognized unusual configurational patterns of the cristal membrane. It is only recently that a method has been devised for the study of configurational changes in mitochondria *in situ* during the transition

from the nonenergized to energized conditions[32].

Since the conformational thesis was proposed by Green *et al.*[2] the criticism of several workers has been focussed on the existence and demonstration of so-called "energized-twisted" configuration which is visualized by electron microscopic technique[10,11]. It became clear from the data presented in this communication that energized-twisted configuration could be inevitably demonstrable if an appropriate fixation method, namely a mixing method with glutaraldehyde-osmium tetroxide fixation is applied. Data also are presented in this communication concerning the minimum effective concentration of glutaraldehyde to freeze functional states of mitochondria. Effectiveness of unexpectedly low concentrations of glutaraldehyde might open a new facet for electron microscopic enzyme cytochemistry since glutaraldehyde at low concentrations can leave some of the enzymatic activities unaffected[33,41].

Finally, the necessity of glutaraldehyde prefixation for negative staining is stressed. The oxidizable form of cytochrome oxidase which is one of the complexes of electron transfer chain yields crystalline arrays as visualized by negative staining with phosphotungstate[42]. When cytochrome oxidase was reduced with dithionite in the presence of cyanide or azide, the characteristic herring-bone pattern of the preparation was completely abolished and replaced by a pattern with no recognizable order[42]. However, such a striking difference in surface structure will not be clear if prefixation with glutaraldehyde is not applied for negative staining.

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REGENDS TO FIGURES

FIG. 1 A. Slices of tissue from beef heart were fixed with 2% glutaraldehyde, post-fixed in osmium tetroxide. ×30,000

FIG. 1 B. Slices of tissue from beef heart were exposed to 0.25 M sucrose solution for 5 minutes at 25°C before fixation with 2% glutaraldehyde. ×30,000

FIG. 1 C. Heavy beef heart mitochondria were fixed with 2% glutaraldehyde, post-fixed in 2% osmium tetroxide. ×30,000

FIG. 2 A. Heavy beef heart mitochondria were fixed with 0.01% glutaraldehyde, post-fixed in 2% osmium tetroxide. ×30,000

FIG. 2 B. Heavy beef heart mitochondria were fixed with 0.001% glutaraldehyde, post-fixed in 2% osmium tetroxide. ×30,000

FIG. 2 C. Heavy beef heart mitochondria were exposed to KRP solution for 10 minutes prior to fixation with 0.01% glutaraldehyde. ×30,000

FIG. 2 D. Heavy beef heart mitochondria were first fixed with 0.01% glutaraldehyde and then exposed to KRP solution. ×30,000

FIG. 3 A. Slices of tissue from beef heart were fixed with 0.05% glutaraldehyde, post-fixed in 2% osmium tetroxide. ×30,000

FIG. 3 B. Slices of tissue from beef heart were fixed with 0.01% glutaraldehyde which had 0.25 M sucrose, and post-fixed in 2% osmium tetroxide. ×30,000

FIG. 3 C. Slices of tissue from beef heart were exposed to KRP solution prior to fixation with 0.05% glutaraldehyde. ×30,000

FIG. 5 A. Energized-twisted configuration obtaining in the presence of rotenone, succinate, and Pi (10 mM), pH 7.4. The sample was removed after the rapid fall in light scattering and then subsequentl yfixed with glutaraldehyde. ×30,000

FIG. 5 B. Discharge of the energized-twisted configuration by anaerobiosis. A sample to which both succinate and Pi have been added but had been allowed to decay to the baseline level of light scattering. ×30,000

FIG. 7 A. Discharge of the energized-twisted configuration in mitochondria which has been sedimented before fixation. The mitochondrial suspension which had been energized in the presence of rotenone, succinate and Pi at 25°C was spun down in a Misco centrifuge; the pellet was then fixed with 2% glutaraldehyde and post-fixed with 2% osmium tetroxide. ×30,000

FIG. 7 B. Experimental conditions are the same as those described in the legend of Fig. 7 A except that the experiment was carried out at 1°C. Just before centrifugation H_2O_2 was added in presence of catalase. ×30,000

FIG. 8 A. A mitochondrial suspension was first energized with succinate, then 2% glutaraldehyde and Pi were added simultaneously. ×30,000

FIG. 8 B. A mitochondrial suspension was first energized with succinate, then glutaraldehyde at a final concentration of 2% was added 5 seconds after addition of Pi. ×30,000

FIG. 9 A. A mitochondrial suspension which had been energized in presence of succinate and Pi was fixed with 0.01% glutaraldehyde, post fixed in 2% osmium tetroxide. ×30,000

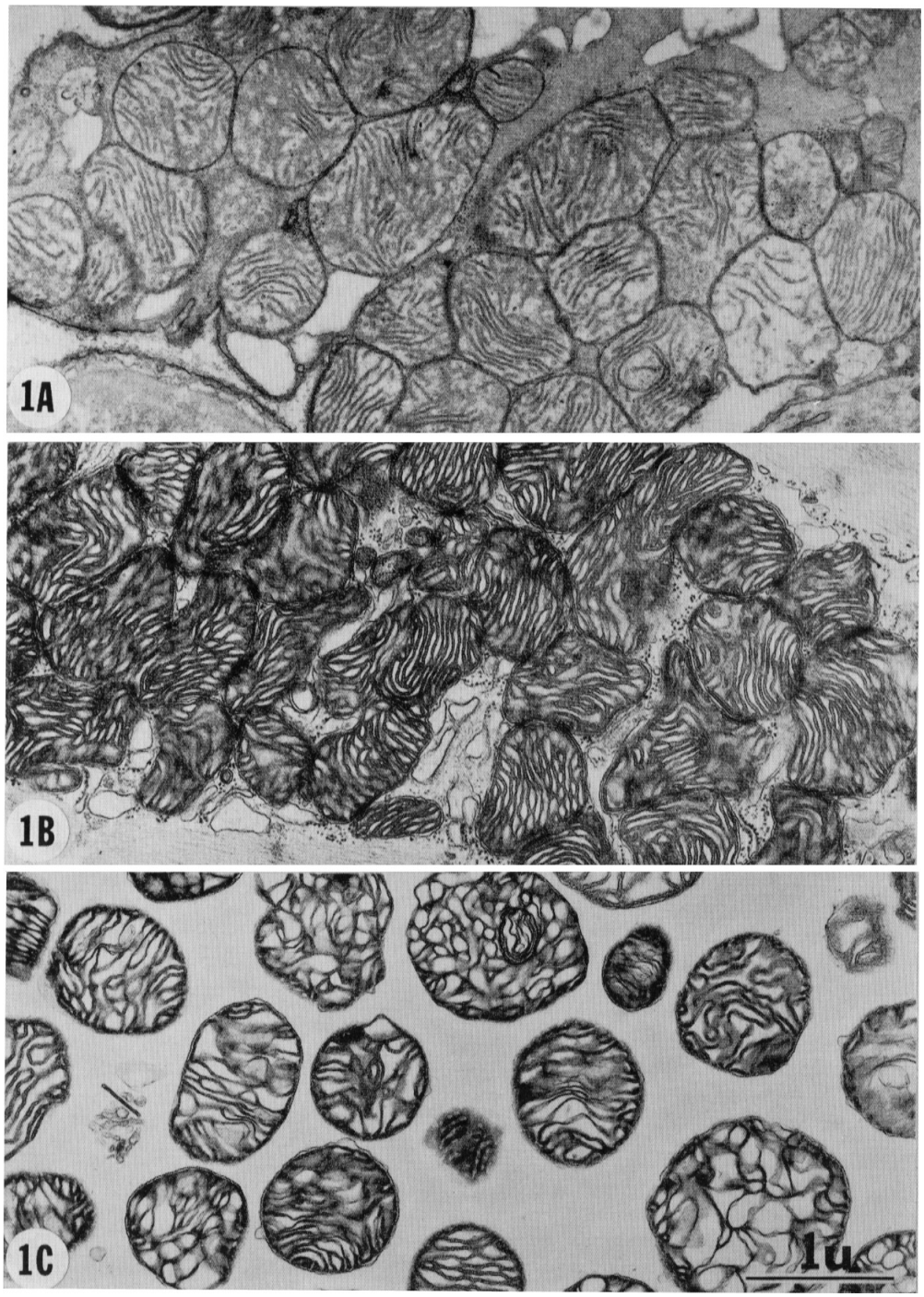
FIG. 9 B. Experimental conditions are the same as those described in the legend of Fig. 9 A except that a mitochondrial suspension was fixed with 0.01% osmium tetroxide without prefixation with glutaraldehyde. ×30,000

FIG. 9 C. Experimental conditions are the same as those described in the legend of Fig. 9 A except that a mitochondrial suspension was fixed with 2% osmium tetroxide without prefixation with glutaraldehyde. ×30,000

FIG. 11 A. A mitochondrial suspension was exposed to 1% silicotungstate prepared in distilled water for 20 minutes prior to fixation with glutaraldehyde. ×30,000

FIG. 11 B. A mitochondrial suspension was exposed to 1% silicotungstate prepared in 0.25 M sucrose solution for 20 minutes prior to fixation with glutaraldehyde. ×30,000

FIG. 11 C. A mitochondrial suspension was first fixed with 0.01% glutaraldehyde and then exposed to 1% silicotungstate prepared in 0.25 M sucrose solution. ×30,000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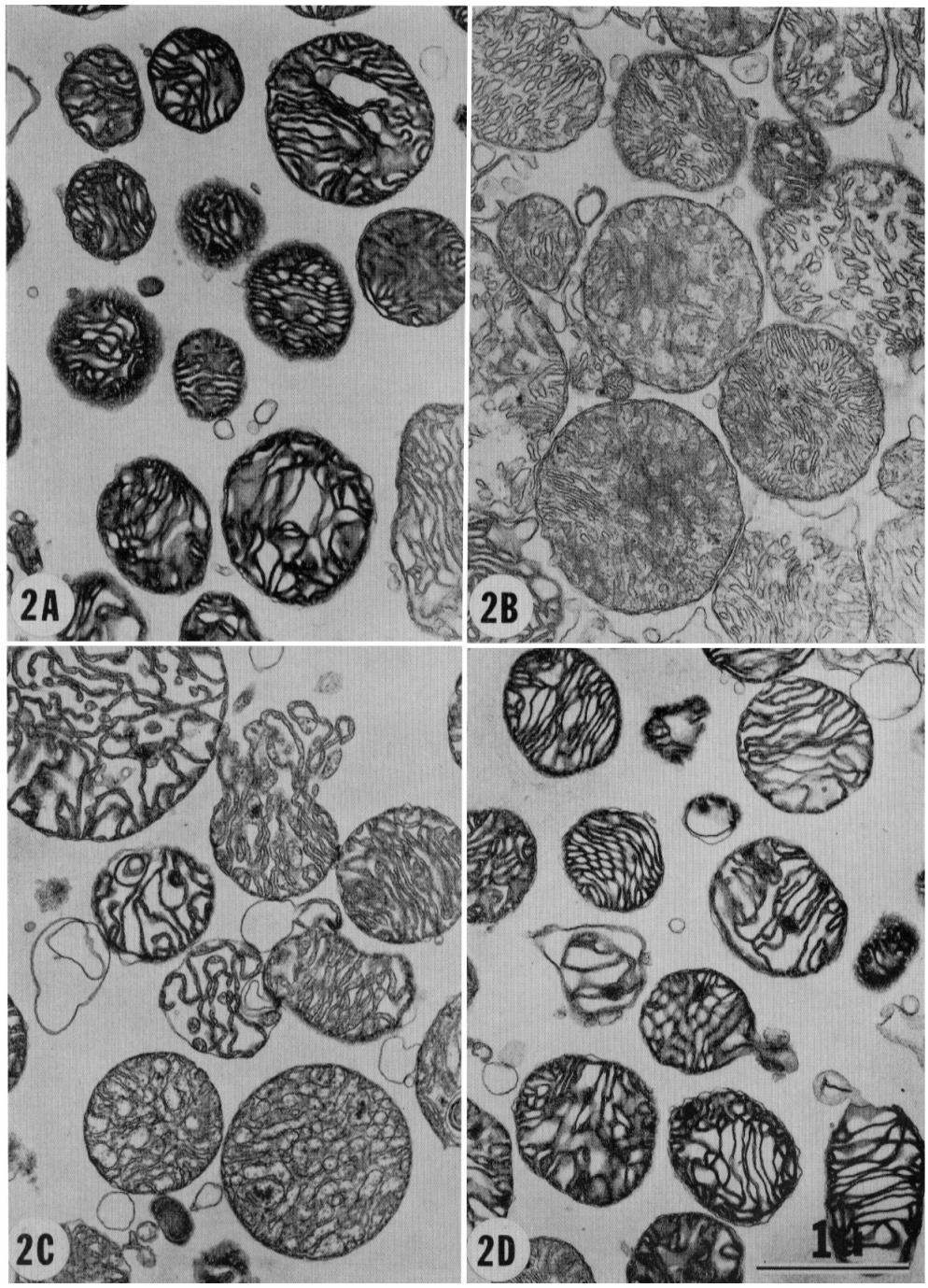
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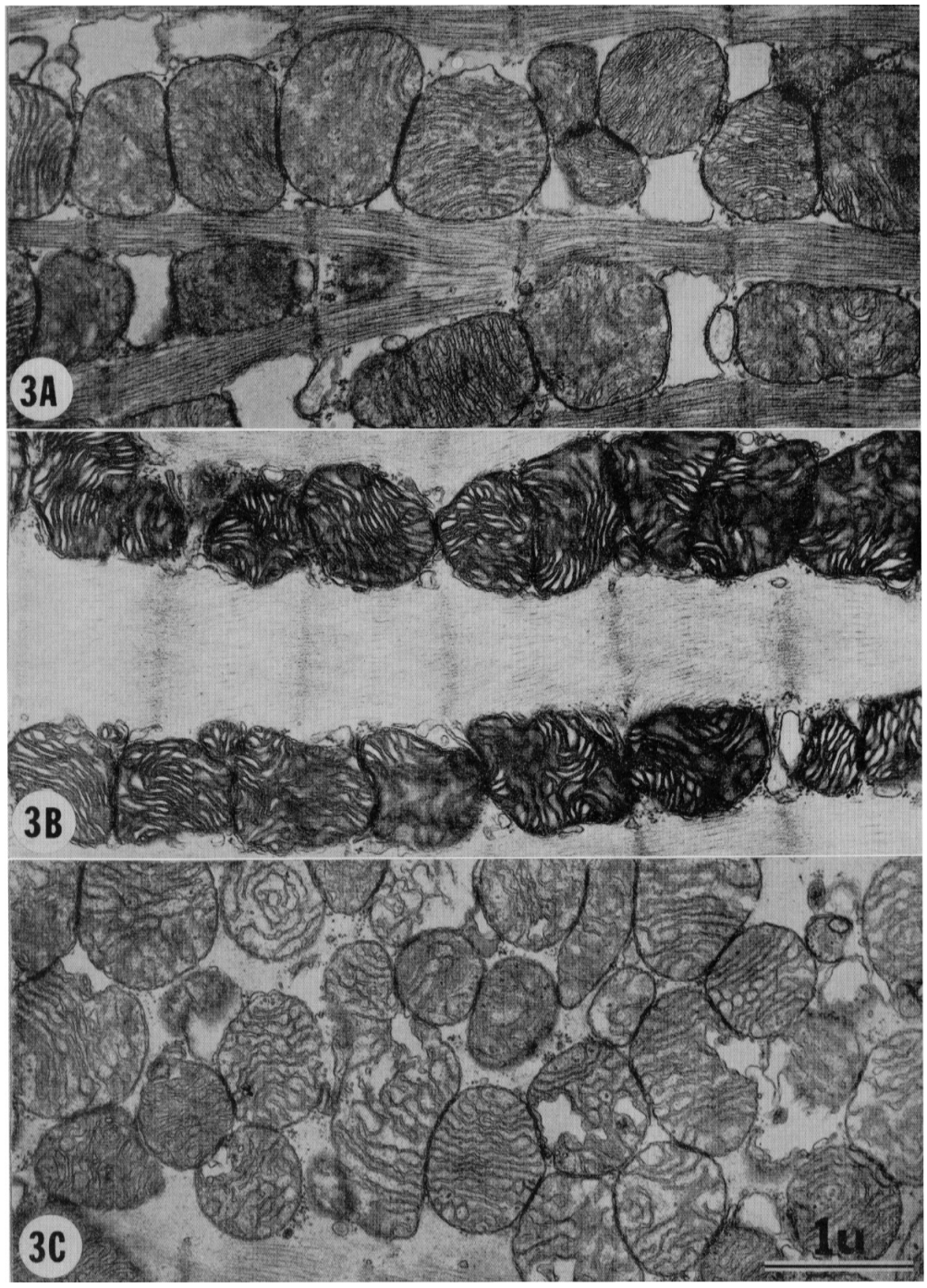
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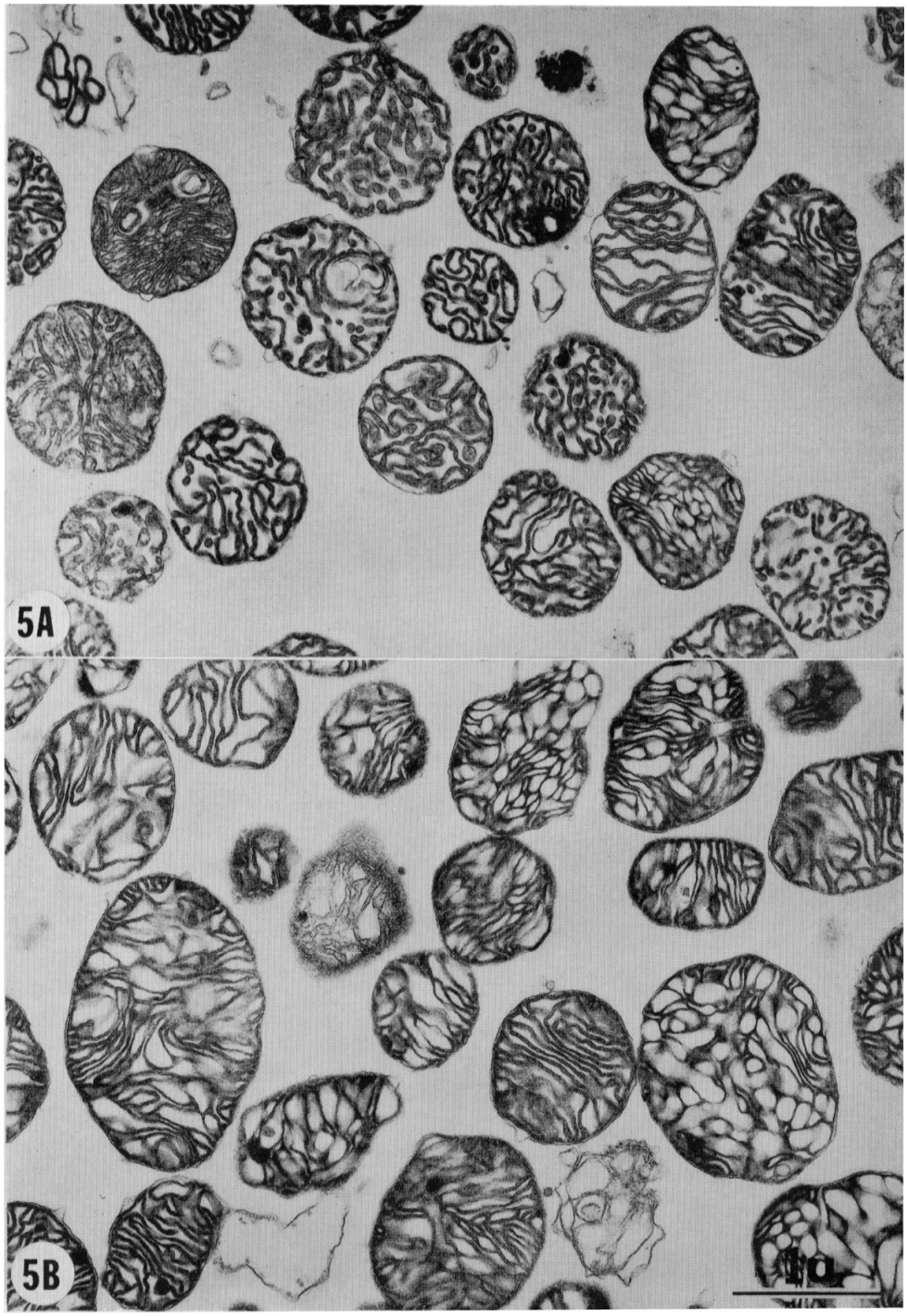
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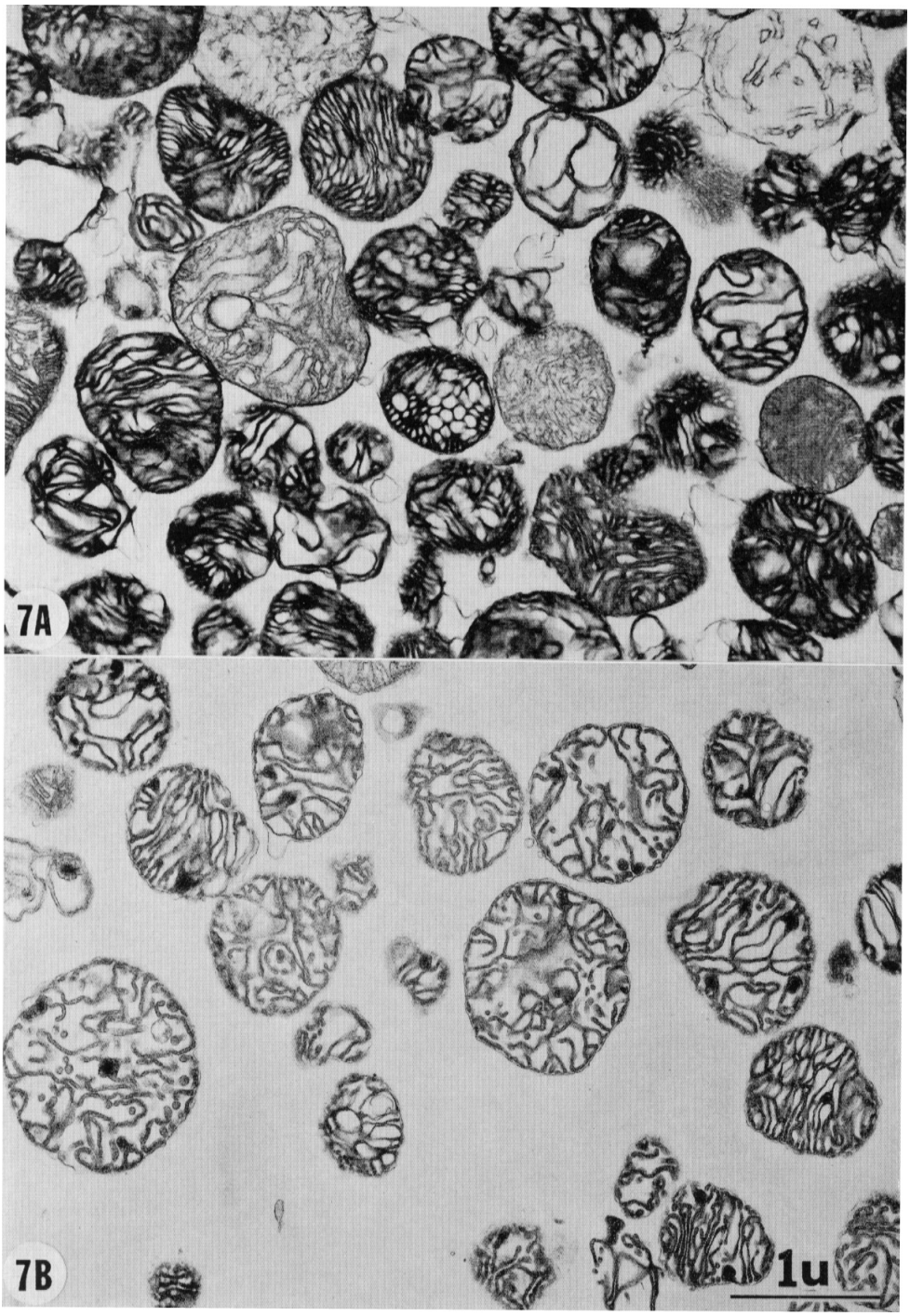
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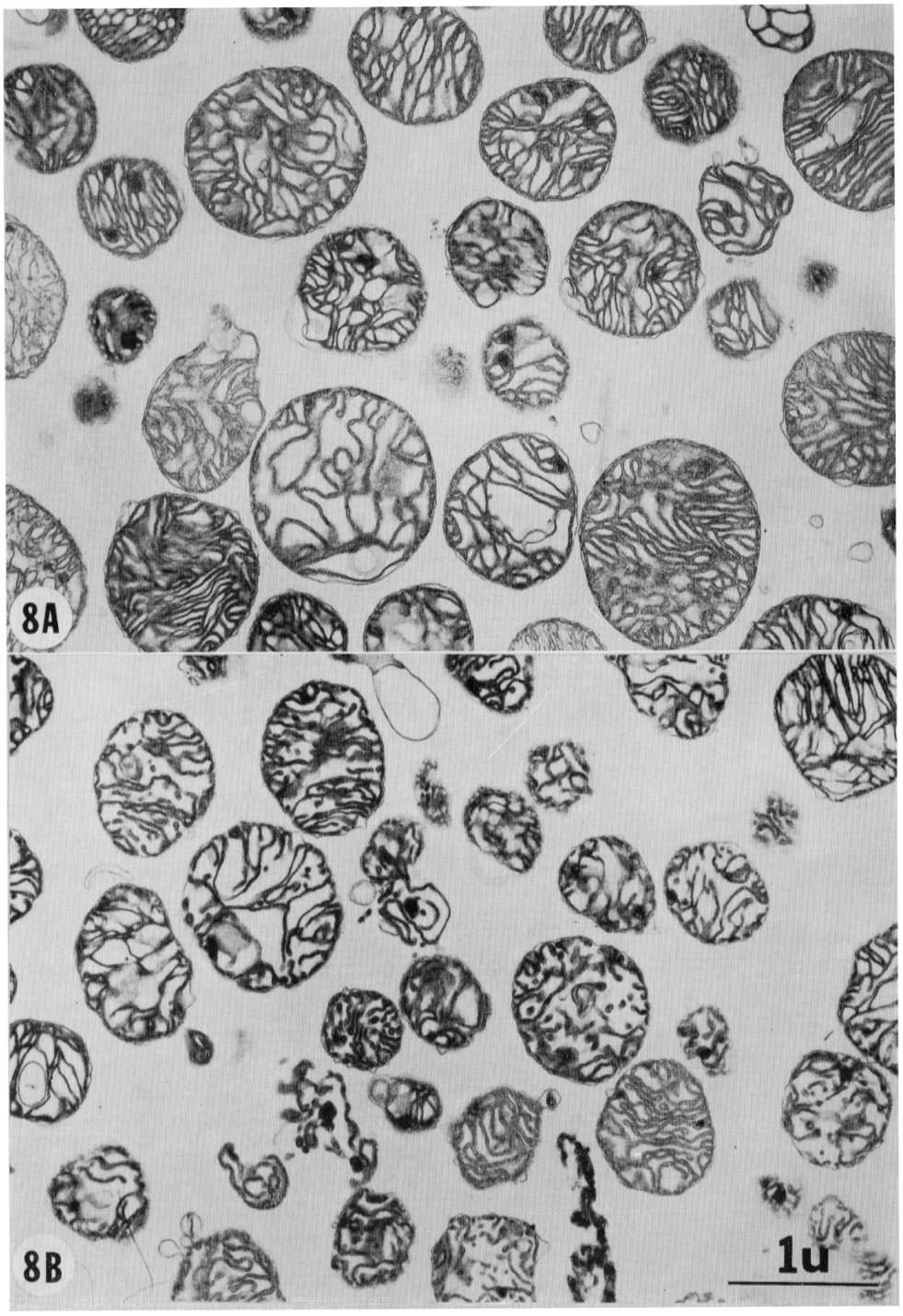
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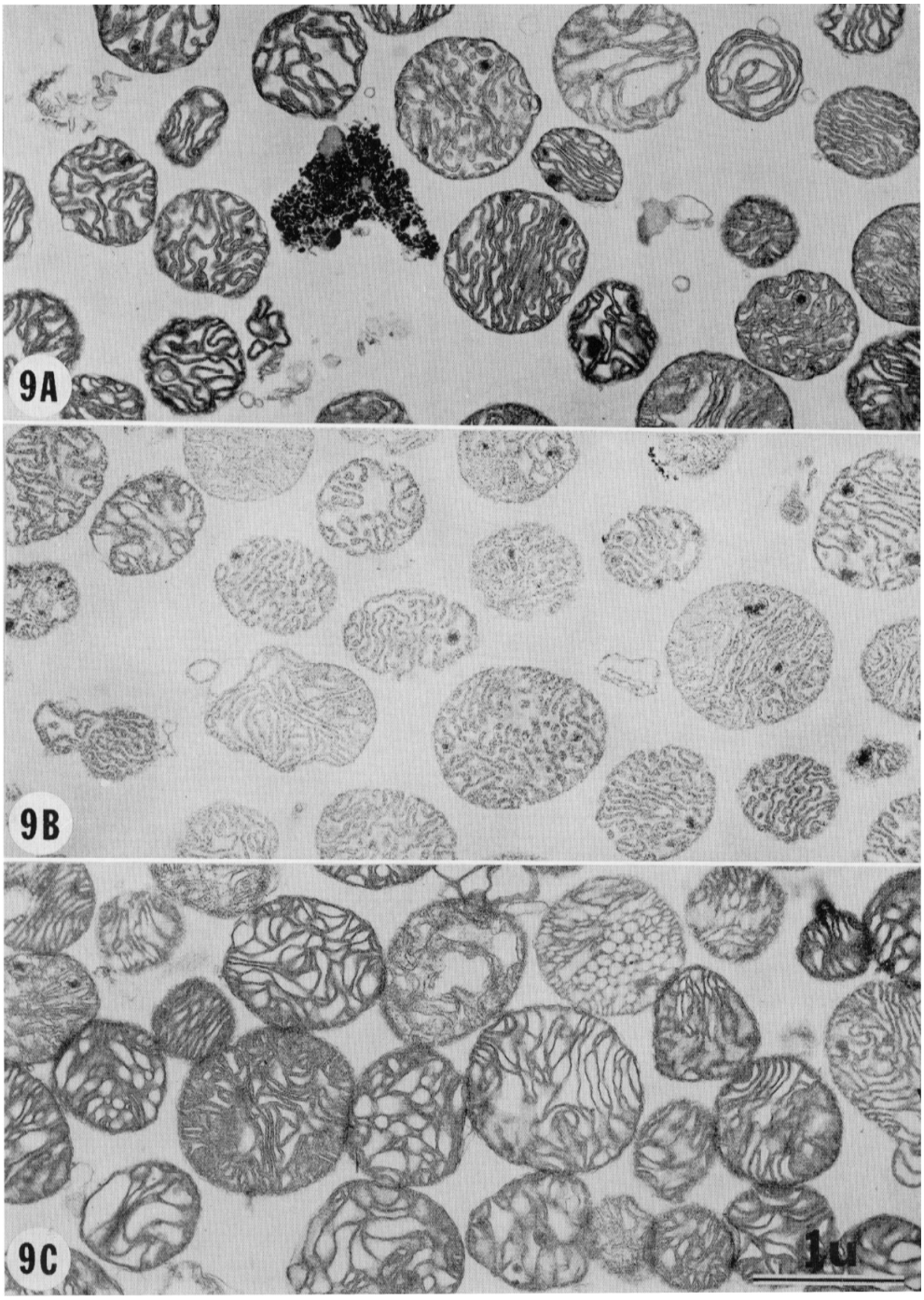
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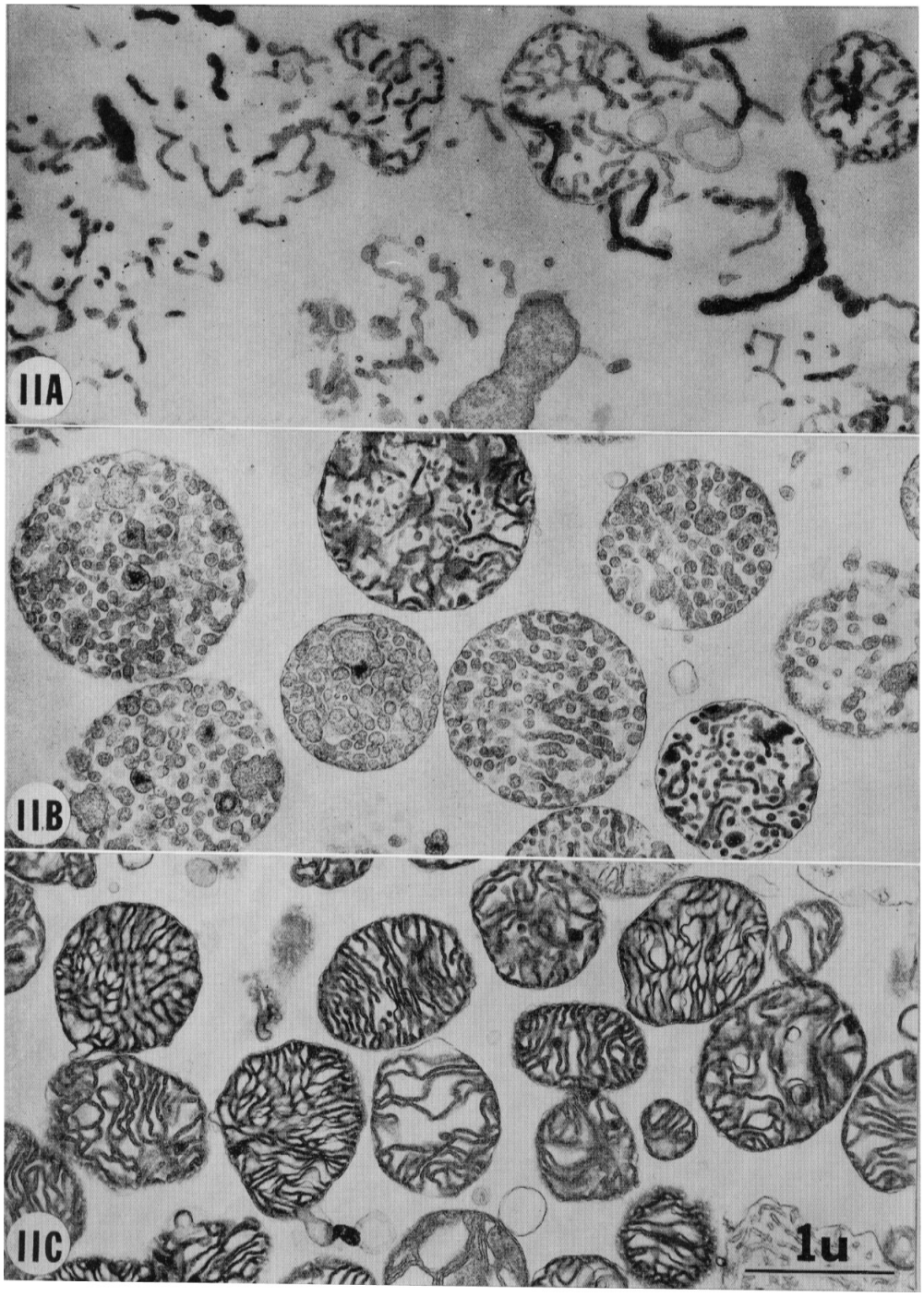
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