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On the Stabilization by Fixation of Configurational States in Beef Heart Mitochondria

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Abstract

The energized configuration of the cristal membrane of beef heart mitochondria can be maintained only as long as oxygen is available for electron transfer. When the oxygen supply is exhausted, the membrane undergoes a transition to the nonenergized configuration. Since the exhaustion of the available oxygen supply is complete in 5–20 sec, 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 the most effective way of freezing the configurational state of the cristal membrane. Fixation with glutaraldehyde appears to be complete within 1–2 sec even at 0°. 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). The freezing by fixation of the cristal membrane in the aggregated configuration is best accomplished by the sequential use of glutaraldehyde and osmium tetroxide. When the levels of glutaraldehyde and osmium tetroxide are respectively too low or too high, the mitochondrion will undergo a transition from the aggregated to the orthodox configuration before fixation is complete. Light-scattering studies provide an independent method for monitoring configurational changes in mitochondria; these light-scattering measurements confirm that the conditions for fixation which lead to stabilization of the energized state as judged by electron microscopy, also show maintenance of configuration as judged by absence of light-scattering changes after the fixatives are introduced. Reagents used in negative staining will induce the geometrical form of the energized configuration of the mitochondrion even under nonenergizing conditions. These reagents are thus unsuitable for use in studies of configurational transitions in mitochondria.

Introduction

The cristal membrane of the mitochondrion undergoes a rapid and extensive configurational change when energized and the rapid reversal of this change when the energy source is depleted.[1,2] The problem that is posed, is how to "freeze" the energized configuration by fixation before discharge can take place. The standard techniques of electron microscopy have been designed for perserving stable structures of the cell.

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When the ultrastructure is in dynamic transition, then new problems of fixation arise. Perhaps the phenomenon of cell division comes closest in character to energized configurational transitions in mitochondria. Ordinarily cell division is a relatively slow process in the minute range. But if cell division were speeded up to the second range, the same sort of technical difficulties posed by configurational transitions would arise. One could no longer assume that standard fixatives under standard conditions would in fact stabilize the nucleus or the mitotic system in the ultrastructural state obtaining at the moment of the introduction of fixative. The present communication is addressed to some experimental facets of the problem of preserving configurational states which are in rapid transition. It is our hope that these studies by focusing attention on the problem will stimulate interest in wider exploration of methods for rapid stabilization of configurational states.

Materials and Methods

Preparation of Mitochondria

Heavy beef heart mitochondria were prepared by the method of Crane *et al.*[3] as described by Hatefi and Lester.[4] 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/ml in a medium which was 0·25 M in sucrose, 10 mM in Tris-Cl, and 1 mM in EGTA, pH 7·5.

Procedures for Electron Microscopy

All solutions of glutaraldehyde or osmium tetroxide (from 0·002% to 4%) were 0·25 M in sucrose and 0·05 M in cacodylate (K^+ salt), at a final pH of 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 0·0001% to 2%). The mixture was kept for 1–3 h at 25°. The mitochondria were then sedimented in a clinical centrifuge for 30 min—the pellets then being washed in a medium, 0·25 M in sucrose and 0·05 M in K-cacodylate (at 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 twice to absolute propylene oxide for 10 min.

For embedding, the dehydrated samples were exposed first to a mixture of equal parts by volume of propylene oxide and Epon (for 20 min) and finally to 100% Epon (for 4 h).[5] Specimens were sectioned with a diamond knife, and were examined in a Hitachi HU-II B electron microscope operated at 75 kV.

Biochemical Assays and Probes

Rate of oxygen consumption. The rate of consumption of oxygen was measured at 30° with a Clark-type electrode (Beckman Co., Fullerton, California).[6] The experiments

were carried out in 4 ml of a medium, 0·25 M in sucrose, 0·01 M in Tris-Cl of pH 7·4, and 3 mM in $MgCl_2$, and containing 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.

Light-scattering changes. A Brice-Phoenix light-scattering photometer (Universal 2000 series) with a Phoenix normal-ratio recorder (−0·25 to +0·25 mV) was used to measure light scattered at 90 to the incident beam by mitochondrial suspensions.[1, 7] The measurements were carried out in a 3 ml volume in a medium, 0·25 M in sucrose, 0·01 M in Tris-Cl (pH 7·4) and 3 mM in $MgCl_2$, and containing 6 μg of rotenone and 3 mg of HBHM. The reagents which initiate the light scattering were introduced by rapid injection with a micro-syringe or by pipette with quick stirring after addition. The order of additions 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 concentrations from 0·001%–0·25%. To avoid excessive dilution the stock solutions were made concentrated (10% glutaraldehyde and 5% OsO_4). The stock solutions were also 0·25 M in sucrose and 0·05 M in K-cacodylate, pH 7·4.

Uptake of Pi during energizing of mitochondria. When mitochondria are energized by electron transfer in presence of inorganic phosphate they undergo a transition to the energized-twisted configuration. It has been shown that the extra uptake of inorganic phosphate during energizing is a reliable index of the generation of the energized-twisted configuration. For such measurements of augmented Pi uptake, the mitochondria (1 mg protein) were incubated in a medium (1 ml final volume), 0·25 M in sucrose, 0·01 M in Tris-chloride of pH 7·4, and containing 2 μg of rotenone. ^{32} Pi with a specific activity of 500–1000 cpm per μmole was then introduced with the desired amount of cold phosphate. The experiment was carried out both in presence and absence of added succinate. The uptake of Pi can be determined in one of several ways—by millipore filtration of the mitochondria,[1] by rapid centrifugation of the mitochondria in a Misco centrifuge (complete within 15 sec),[8] and finally by addition of fixatives to the mitochondrial suspension before centrifugation. The first two procedures[1, 8] have been shown to be satisfactory for demonstrating energized uptake of Pi as measured by increased radioactivity in the filter or in the pellet. The third procedure is one we have introduced to determine whether fixatives can 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 presence of succinate and Pi was rapidly mixed with glutaraldehyde or with osmium (see experiment A in Table II). After 20 sec, the suspension was centrifuged in a high-speed Misco centrifuge capable of reaching full speed (22,000 rpm) in less than 10 sec.[9] Glutaraldehyde-osmium double fixation was also carried out as follows. The mitochondrial suspension was first exposed to glutaraldehyde for 20 sec; then it was exposed to osmium for 20 additional seconds; the suspension was finally centrifuged as described above (see experiment B in Table II). The surfaces of the mitochondrial pellets were washed three times with a chilled solution, 0·25 M in sucrose and 0·01 M in Tris-Cl of pH 7·4. The pellets were then extracted at 50° with 1% sodium dodecyl sulfate for 5 h after homogenization in a Teflon homogenizer. The measurement of radioactivity was carried out in a Tri-carb scintillation counter with Brays fluid.

In all such experiments, both a control without added substrate and a control with substrate but without fixative were also carried through. 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. Stabilization of the Energized Configuration

Figure 1 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

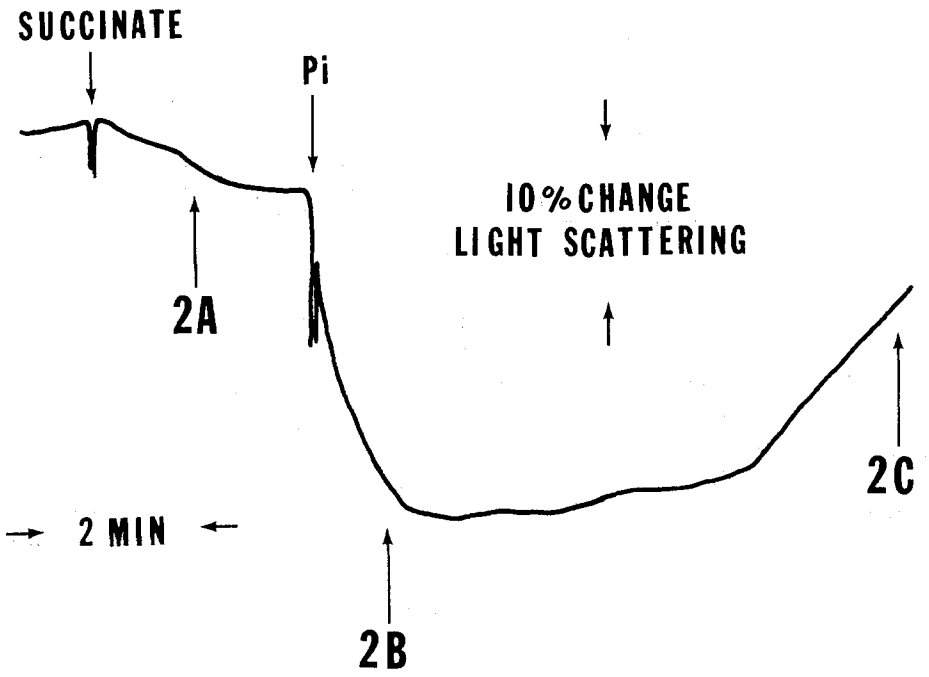


Figure 1. Light-scattering changes and the energy state of beef heart mitochondria. The incubation medium was 0·25 M in sucrose and 0·01 M in Tris-Cl, pH 7·4, and the additions were rotenone (2 μg/mg mitochondrial protein), succinate (5 mM), Pi (10 mM), and beef heart mitochondria (1 mg of protein per milliliter of medium). Samples were removed at the time indicated by arrows at 2A, 2B, and 2C, and after processing of the samples, sections were examined electron microscopically (see Figs. 2A, 2B, and 2C).

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 sequentially fixed with 2% glutaraldehyde and 2% osmium tetroxide, the energized-twisted configuration is found only in the sample which has sustained the rapid fall in light scattering (Figs. 2A and 2B). Moreover the energized-twisted configuration is no longer found in a sample to which both succinate and Pi has been added but which had been allowed to decay to the baseline level of light scattering (see Fig. 2C). This simple experiment estab-

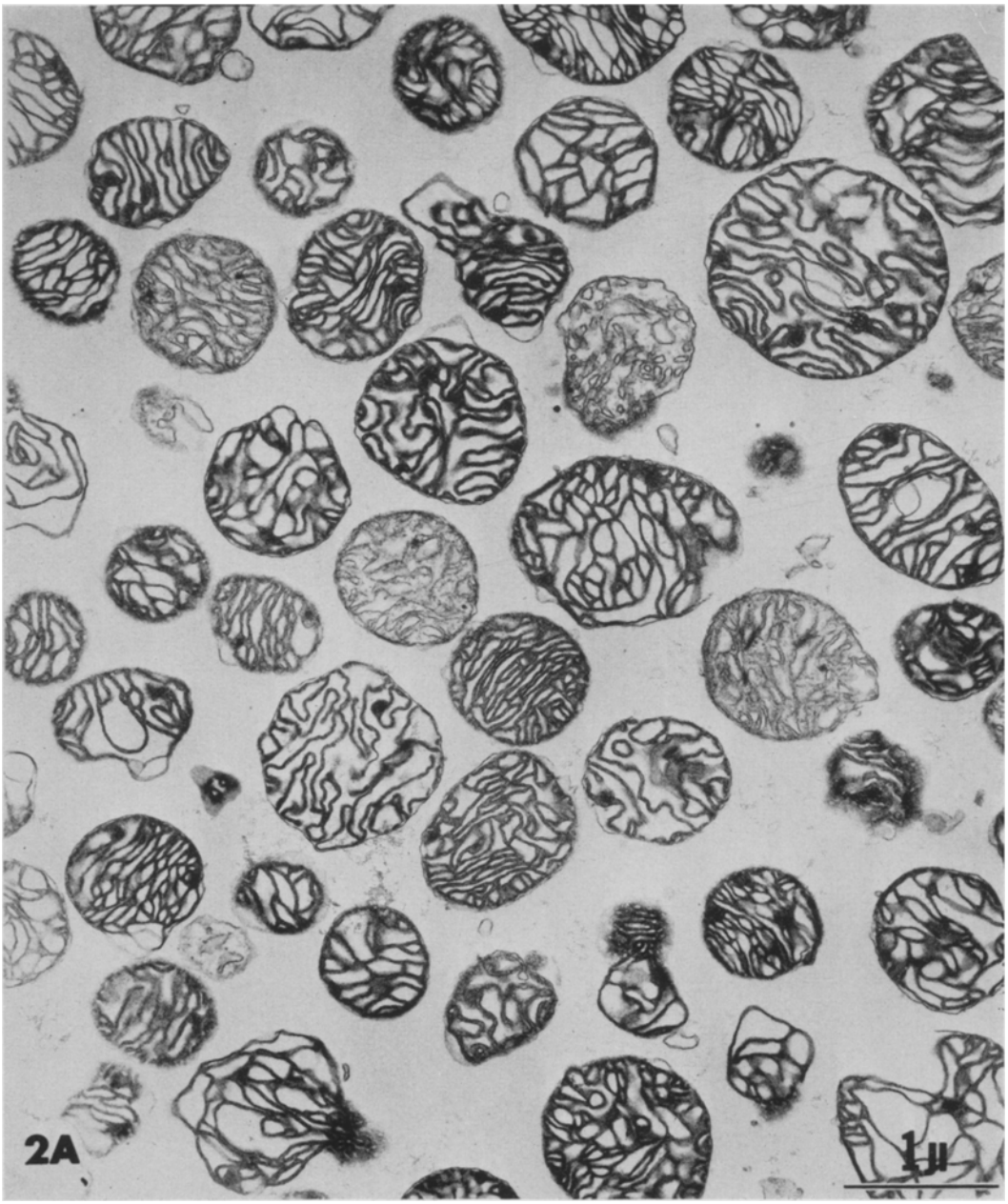
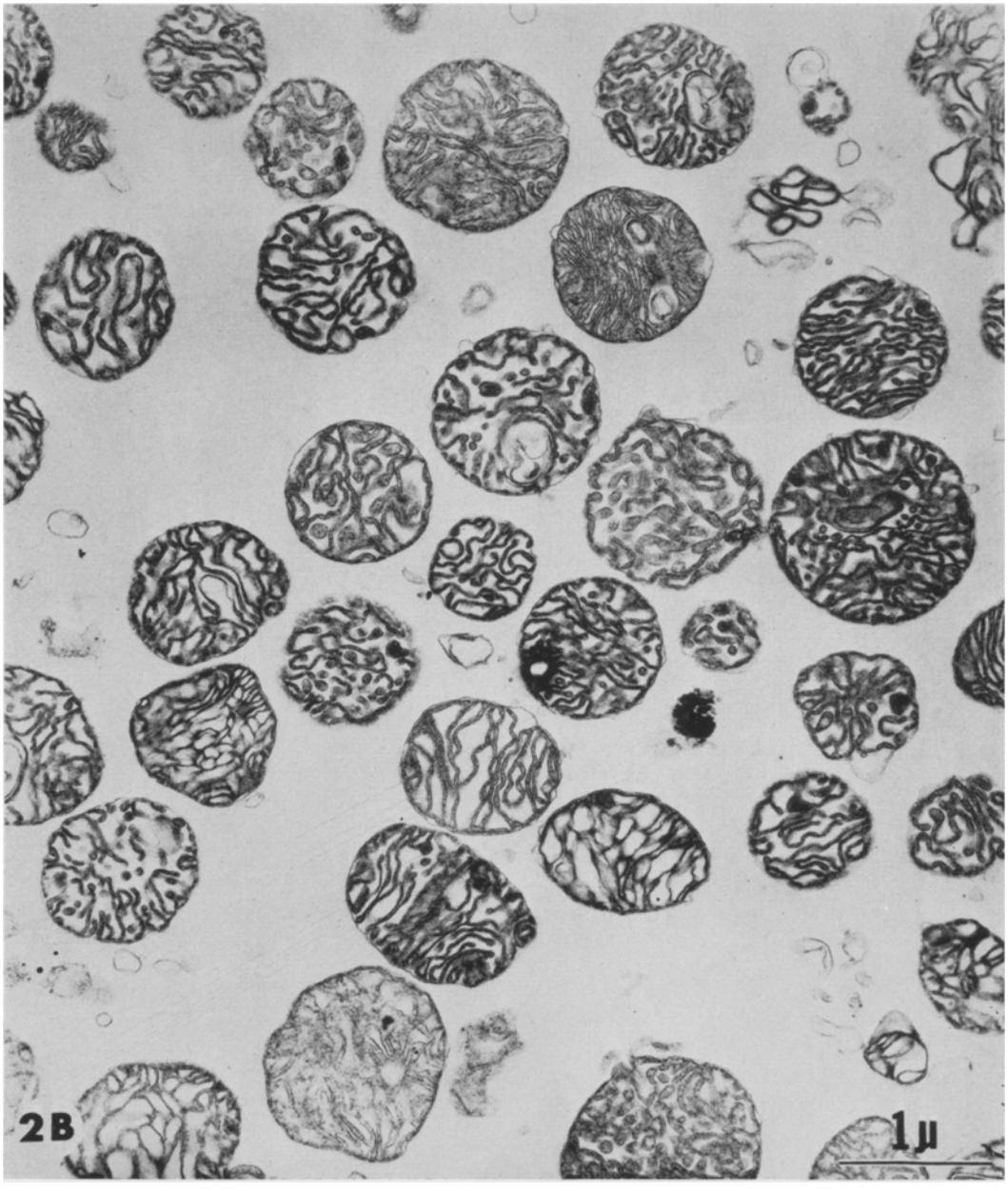
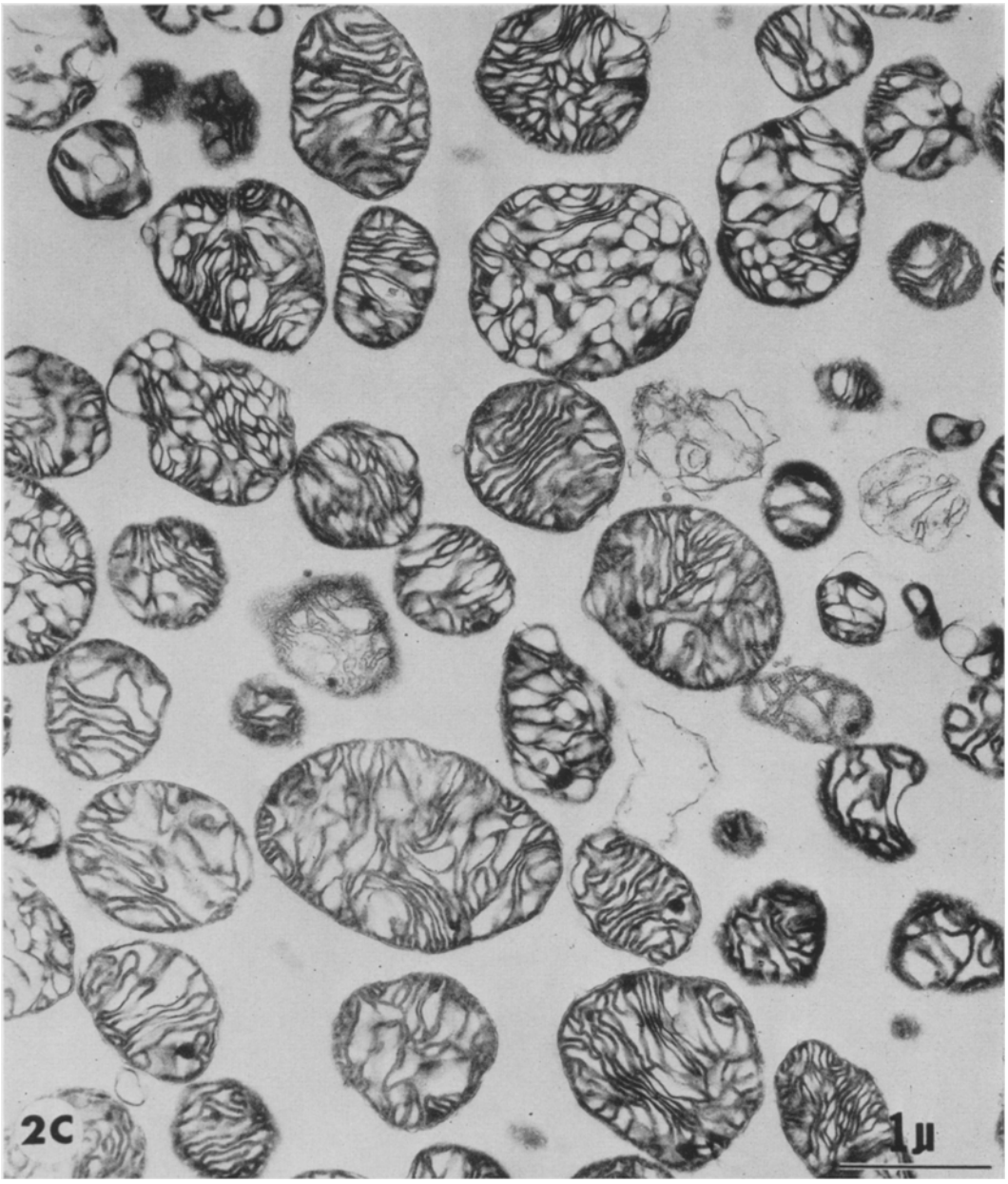


Figure 2. Correlation of configurational changes with light-scattering changes. The incubation medium contained 1 mg of mitochondrial protein per milliliter and was 0·25 M in sucrose and 0·01 M in Tris-Cl, pH 7·4. Samples were mixed with glutaraldehyde (final concentration 2%) and post fixed with 2% OsO_4.

A. Energized configuration obtaining in the presence of rotenone (2 μg/mg of protein) and succinate (5 mM). The sample was removed at the point indicated by arrow 2A in the light-scattering trace in Fig. 1. ×30,000.



B. Energized-twisted configuration obtaining in the presence of rotenone, succinate, and Pi (10 mM), pH 7·4. The sample was removed at the point indicated by arrow 2B in the light-scattering trace in Fig. 1. ×30,000.



C. Discharge of energized-twisted configuration by anaerobiosis. The sample was removed at the point indicated by the arrow 2C in the light-scattering trace in Fig. 1.

TABLE I. Correlation of increased Pi uptake with the generation of the energized-twisted configuration

Status of light-scattering change	Increment in phosphate uptake* (mμmoles/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 corrected for the Pi taken up in a control without substrate.

lished 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.

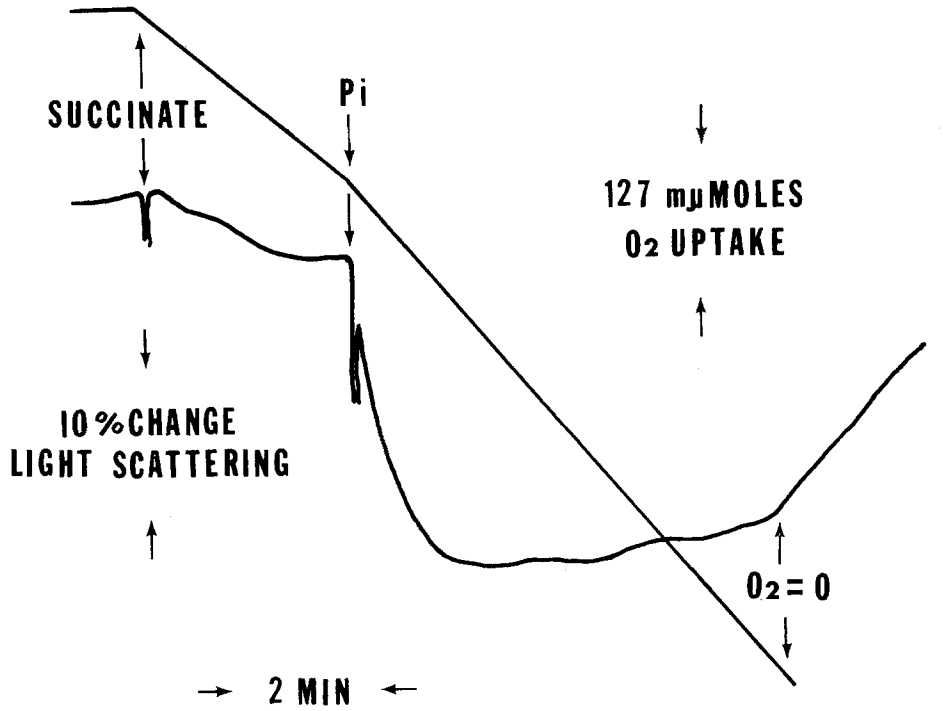


Figure 3. Correlation of the reversal of light-scattering change with exhaustion of oxygen in the system. The system was the same as that described in the legend of Fig. 1. The upper trace records oxygen consumption and the lower trace records the light scattering observed.

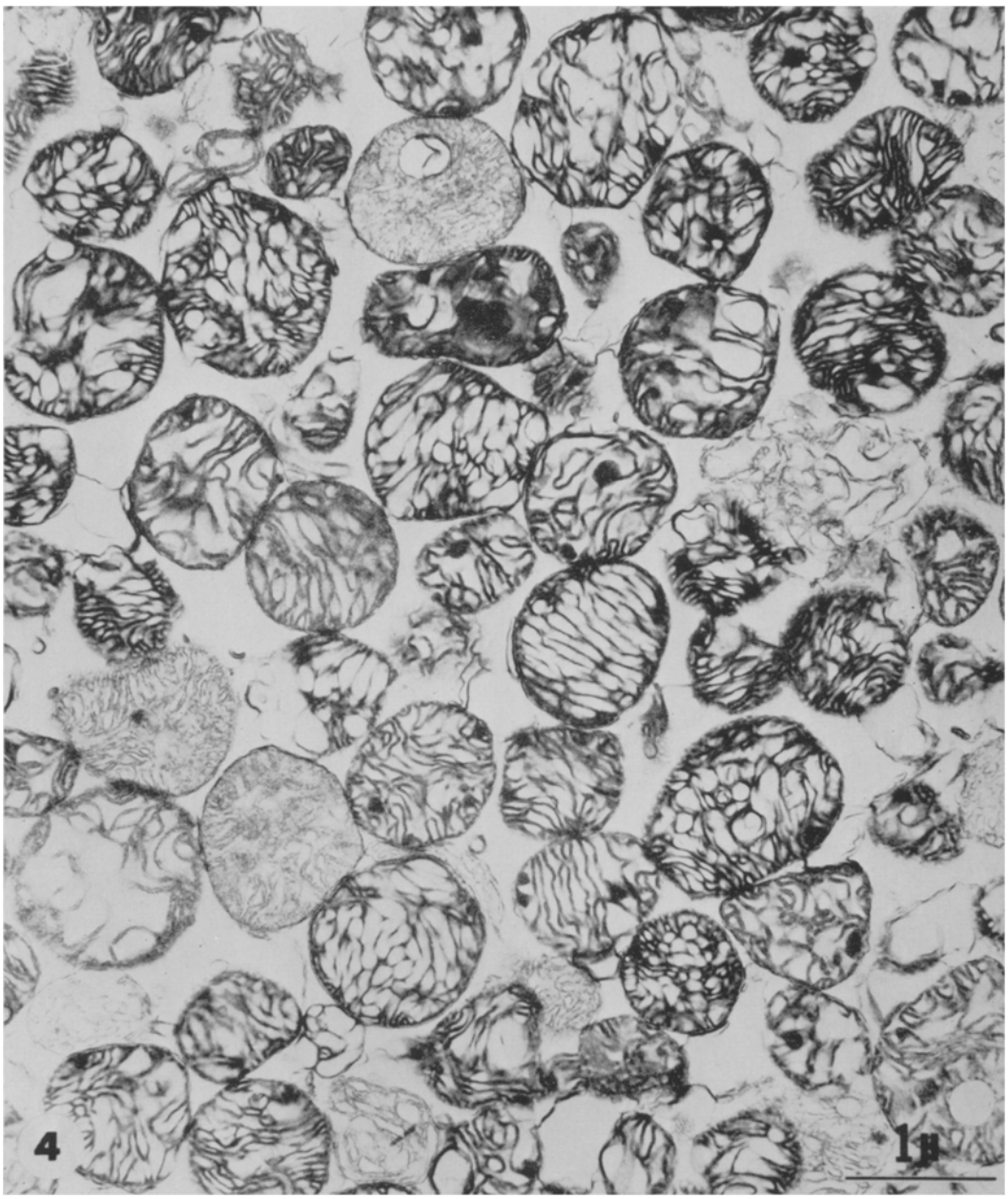


Figure 4. Discharge of energized-twisted configuration in mitochondria which had been sedimented before fixation. The mitochondrial suspension which had been energized in the presence of rotenone, succinate and Pi was spun down in a Misco centrifuge; the pellet was then fixed with 2% glutaraldehyde and post fixed with 2% OsO_4. ×30,000.

The exact 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 increased Pi uptake was demonstrable (see Table I). From these two types of experiments we can now correlate three attributes of the energized-twisted configuration—the electron microscopic pattern, the decreased light scattering of the mitochondrion and the increased uptake of inorganic phosphate. There is thus provided a baseline for evaluating the effect of fixatives on the preservation of the energized-twisted configuration. The biochemical probes can be used to specify that the configuration was energized-twisted at the time of addition of fixatives and the electron microscopy can be used to determine whether the fixatives were effective in preserving the configurational pattern.

In the light-scattering experiment, we have assumed (see also ref. 1) that the reversal of the light-scattering increase was a consequence of oxygen exhaustion. Figure 3 is an experiment run parallel with the light-scattering measurements and under identical conditions, in which the oxygen consumption is measured. It will be noted that the oxygen is exhausted at precisely the same time that the reversal of light-scattering change is complete. 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. Pulsing the system with additional oxygen can of course preserve the energized state for even longer periods. The point to be made is that the energized state is stable only as long as oxygen is present in the medium for electron transfer to take place.

If the mitochondria in the energized configuration are sedimented before fixation as in the method of Hackenbrock,[13] then given the usual rate of oxygen uptake, a delay not exceeding 10 sec is permissible before addition of the fixative. Under optimal conditions, the delay in Hackenbrock's procedure is close to 60 sec. By this time, the sedimented mitochondria will no longer be in the energized configuration. Electron microscopic examination of such pelleted mitochondria after fixation will invariably show the non-energized configuration (see Fig. 4). Clearly then the demonstration of the energized configuration requires direct addition of the fixative to the mitochondrial suspension. This immediately raises the question of how long it takes for the fixatives to be effective and of which concentrations of fixatives are most effective for rapid fixation.

II. Speed of Fixation of the Energized Configuration

The following very simple experiment established that glutaraldehyde at a final concentration of 2% can freeze the configuration of the mitochondrion within seconds after addition. In one tube, mitochondria were first energized with succinate; then glutaraldehyde and Pi were added simultaneously. In the other tube, glutaraldehyde was added 5 sec after addition of Pi. The electron micrographs showed no energized-twisted configuration in the former and predominantly energized-twisted in the latter (see Fig. 5). This experiment suggests that the fixation by glutaraldehyde is at least as fast as the generation of the energized-twisted configuration. According to the measurements of Penniston *et al.*[10] the time for generation of the energized state as measured by the speed of proton release is about 100 msec. We are not suggesting that our quasi-kinetic measurements have a comparable degree of accuracy. 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.

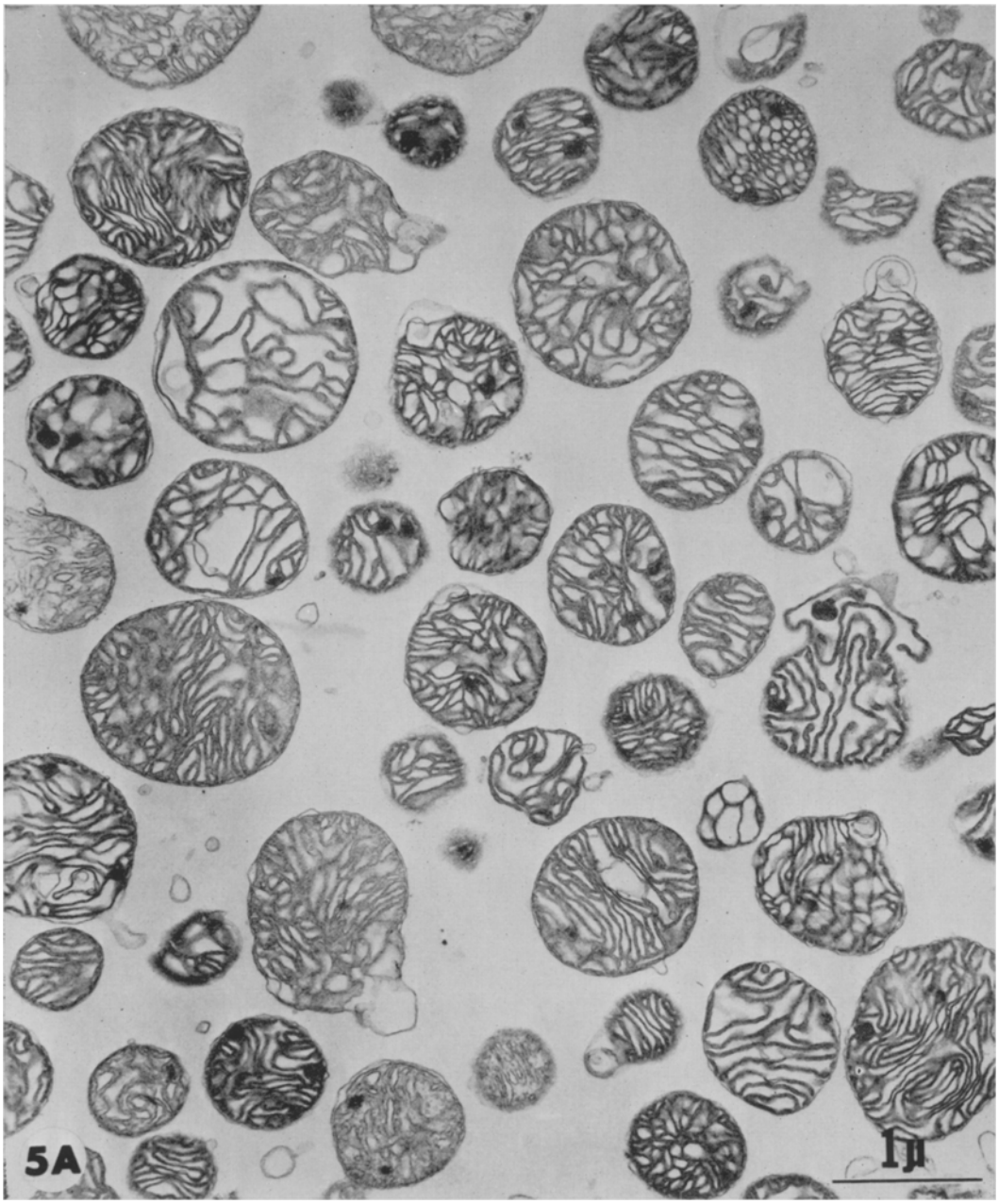
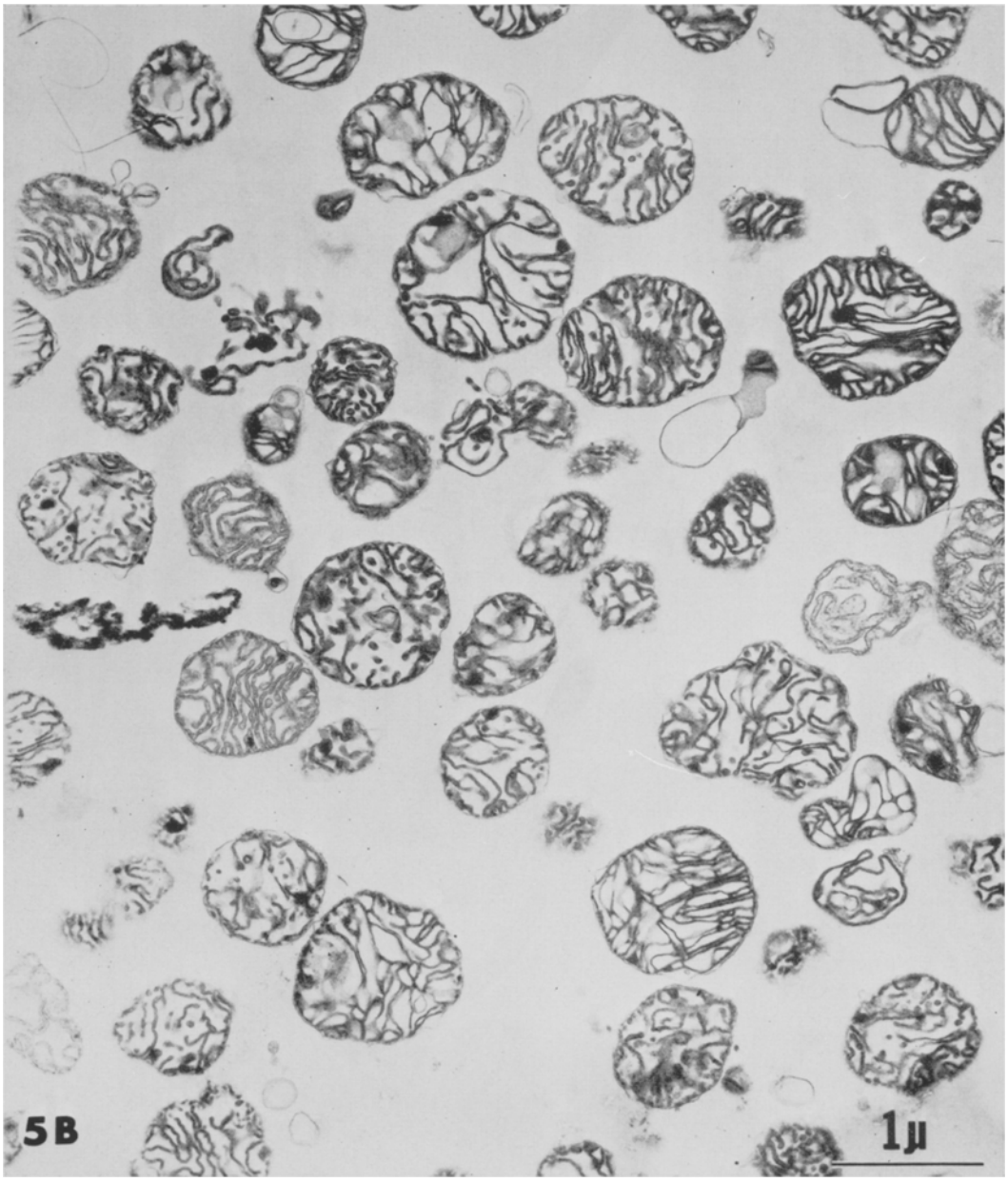


Figure 5. Speed of fixation with glutaraldehyde.

A. Mitochondria were first energized with succinate; then glutaraldehyde (final concentration 2%) and Pi (10 mM final concentration) were added to the medium at the same time. None of the mitochondria in the electron micrograph are in the energized-twisted configuration. ×30,000.



B. Mitochondria energized in presence of rotenone and succinate were fixed with glutaraldehyde (final concentration 2%) 5 sec after addition of Pi (10 mM). The mitochondria in this electron micrograph are predominantly in the energized-twisted configuration. ×30,000.

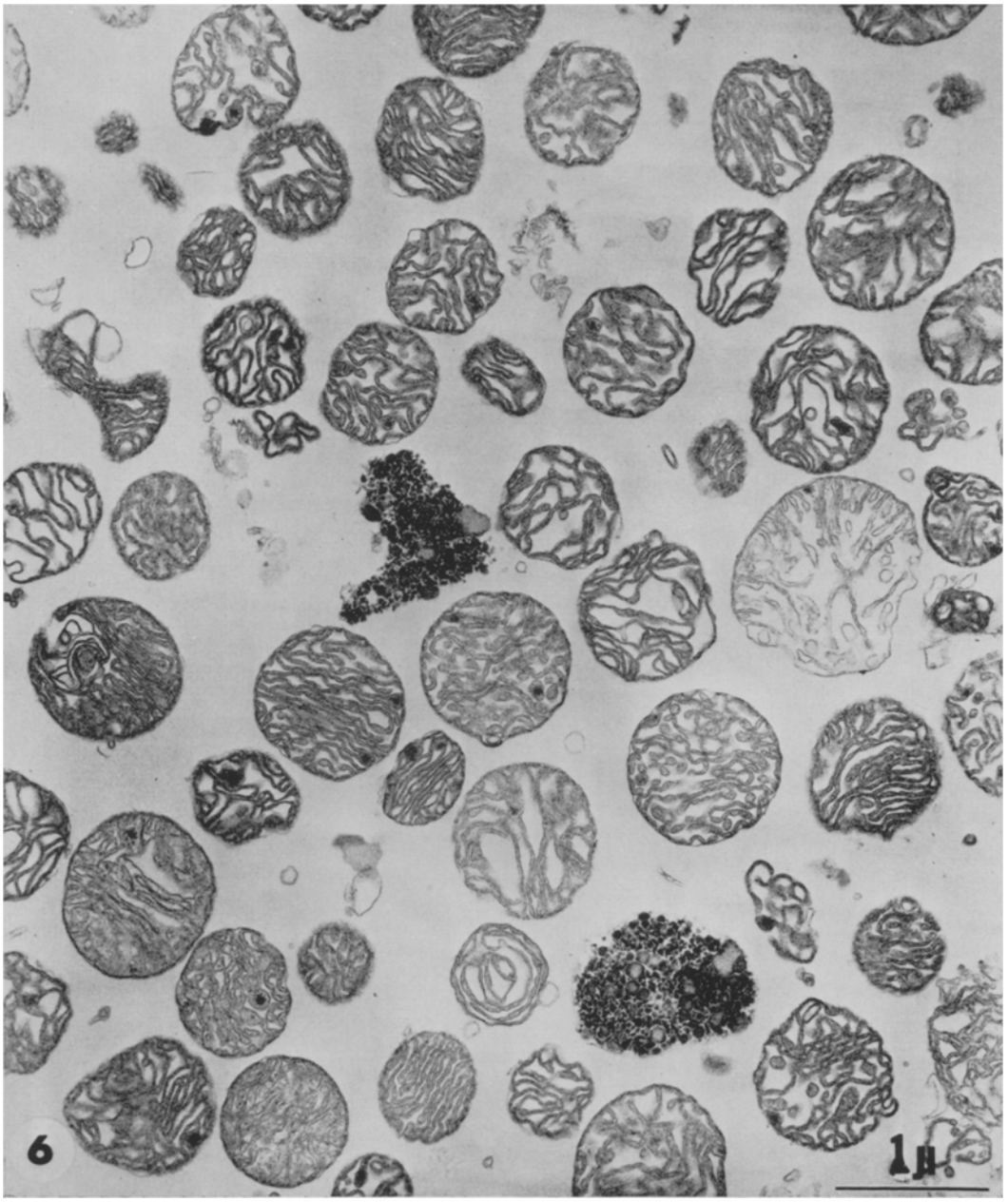


Figure 6. Preservation of the energized-twisted configuration when mitochondria are fixed with glutaraldehyde at low concentrations. The mitochondrial suspension in the presence of rotenone, succinate and Pi was mixed with glutaraldehyde (final concentration 0·01%) and post fixed with 2% OsO_4. ×30,000.

III. 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 (see Fig. 6). 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 was as complete as with the standard concentration of 2%. It would appear that there is no advantage in using concentrations of glutaraldehyde higher than 0·01% and concentrations of osmium tetroxide higher than 0·03%.

When mitochondrial suspensions were fixed by addition only of osmium without prefixation with glutaraldehyde, the electron micrographs showed poor contrast but nonetheless the configuration of the mitochondria was unambiguously energized-twisted even at a final concentration as low as 0·01%. At higher concentrations of osmium tetroxide, e.g., at 2%, the energized-twisted configuration was no longer observed (see Fig. 7). From all these studies, we have 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 do not affect the preservation of the energized-twisted configuration.

IV. Correlation between the Preservation of the Energized 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

Theory would require that fixatives which preserve the phosphate-induced energized configuration would not materially change the light-scattering pattern or the increased Pi uptake of the mitochondrial suspension. That is to say mitochondria in the energized configuration should retain their light-scattering properties and increased Pi uptake capacity even after exposure to fixatives if indeed these fixatives are preserving the energized configuration. The supporting data for these predictions are shown in Fig. 8 and Table II 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 effective. 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. It is to be noted that in this experiment only one fixative at a given concentration was tested. No combinations of fixatives were used. The stabilization of the light-scattering level correlates well with the stabilization of the energized configuration as determined electron microscopically. A similar correlation is established in Table II between the concentrations of glutaraldehyde or osmium required for retention of the extra Pi uptake and the concentrations required for preservation of the energized configuration.

V. Stabilization of the Aggregated Configuration

When beef heart mitochondria are isolated in a medium 0·25 M in sucrose, the cristal membranes—which are functionally intact—are invariably found in the aggregated

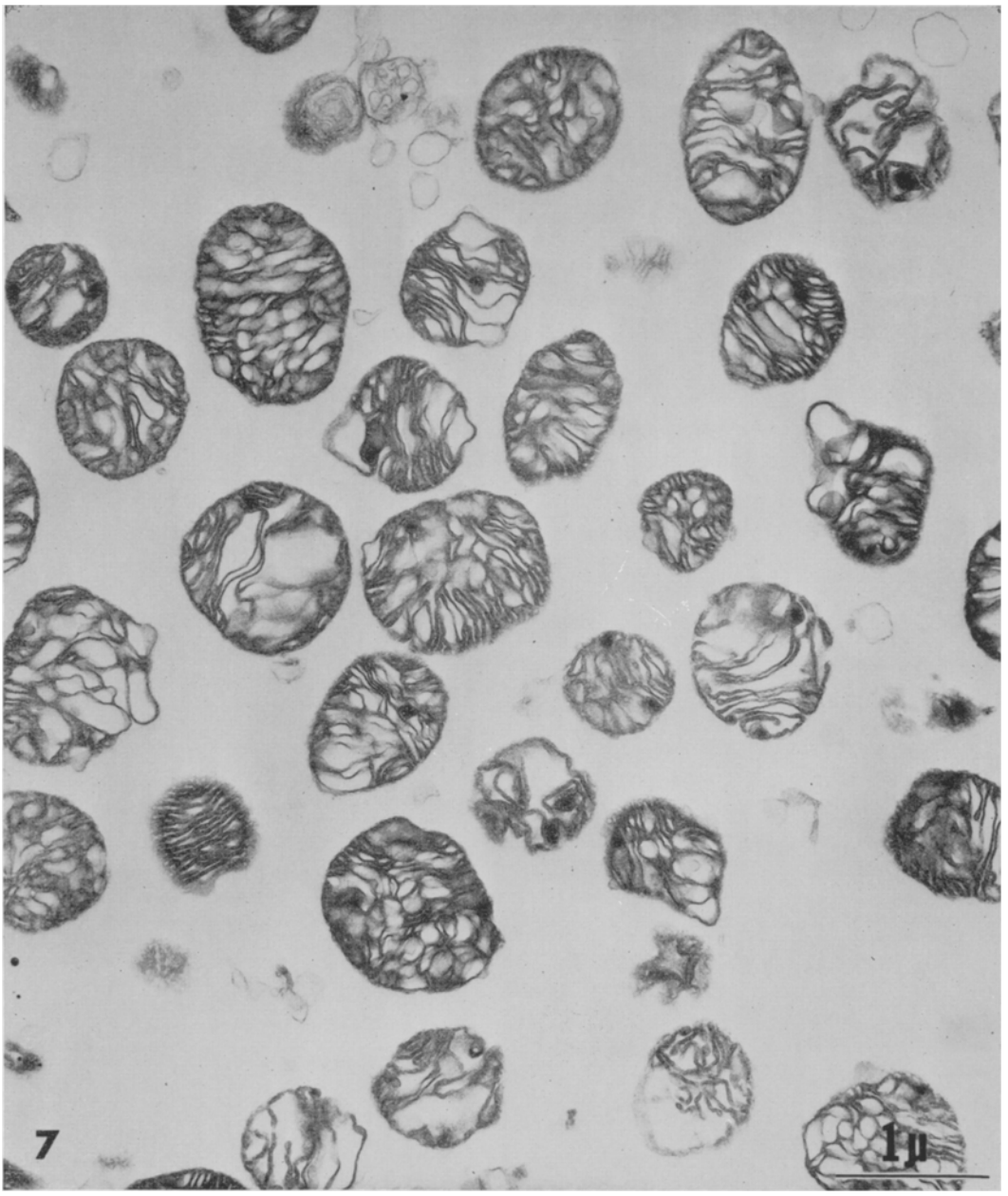


Figure 7. Discharge of the energized-twisted configuration when mitochondria are fixed with OsO_4 at high concentrations. The mitochondrial suspension energized in the presence of rotenone, succinate and Pi was mixed with OsO_4 (final concentration 2%) ×30,000.

configuration when fixed under standard conditions, i.e., fixed initially with 2% glutaraldehyde and post fixed with 2% osmium (see Fig. 9A). *In situ* the cristal membranes of the same mitochondria are invariably found in the orthodox configuration (see Fig.

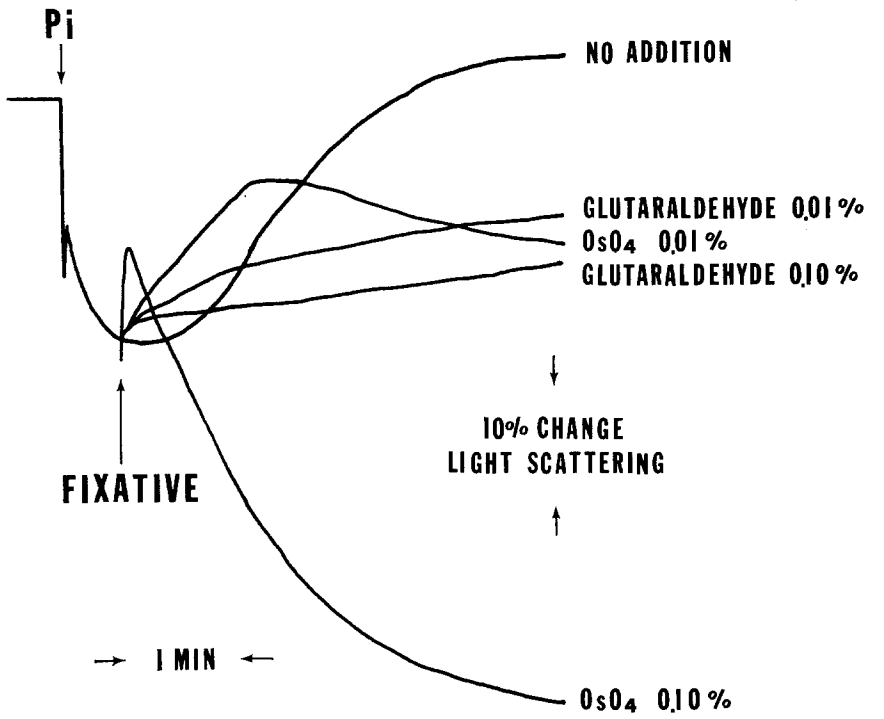


Figure 8. Effects of fixatives on light scattering of mitochondria. The incubation medium is described in the legend of Fig. 1. After energizing of the mitochondrial suspension in the presence of succinate rotenone and Pi, the effect of fixatives on the light-scattering properties of the system were recorded. The final concentrations of the fixatives are indicated in the figure. Glutaraldehyde and osmium were added in concentrated solution 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 II. Correlation of increased Pi uptake with the generation of the energized-twisted configuration

Conditions and additions		Increment in phosphate uptake* (mμmoles/mg protein)
A. No fixative (20 sec)		13·0
Glutaraldehyde (20 sec)	0·01%	15·8
	2·0%	4·6
OsO_4 (20 sec)	0·01%	12·4
	2·0%	0·2
B. No fixative (40 sec)		25·0
Glutaraldehyde (2%) + OsO_4 (2%) (20 sec) (20 sec)		5·3
No fixative + OsO_4 (2%) (20 sec) (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 ^{32}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 spundown 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.

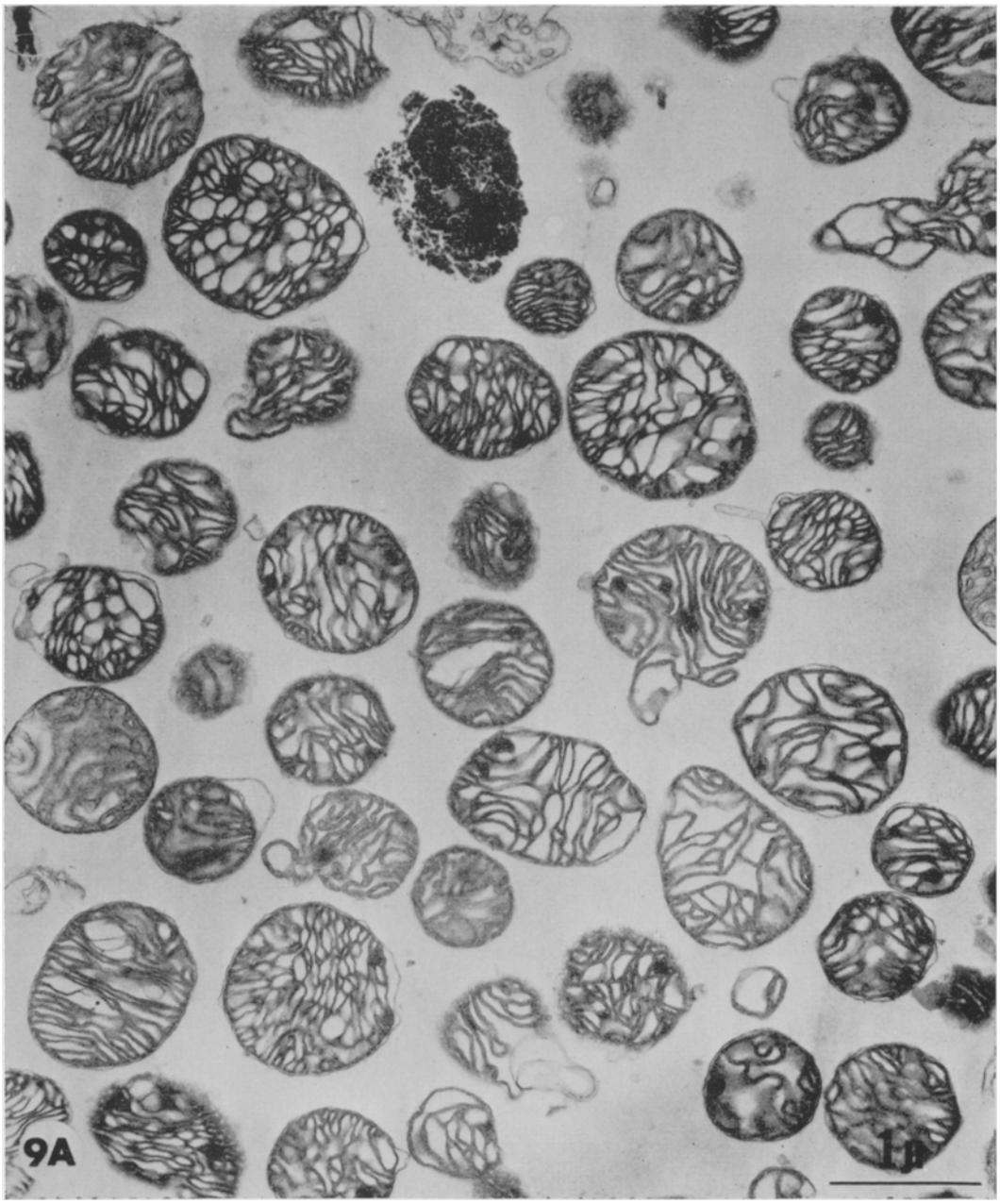
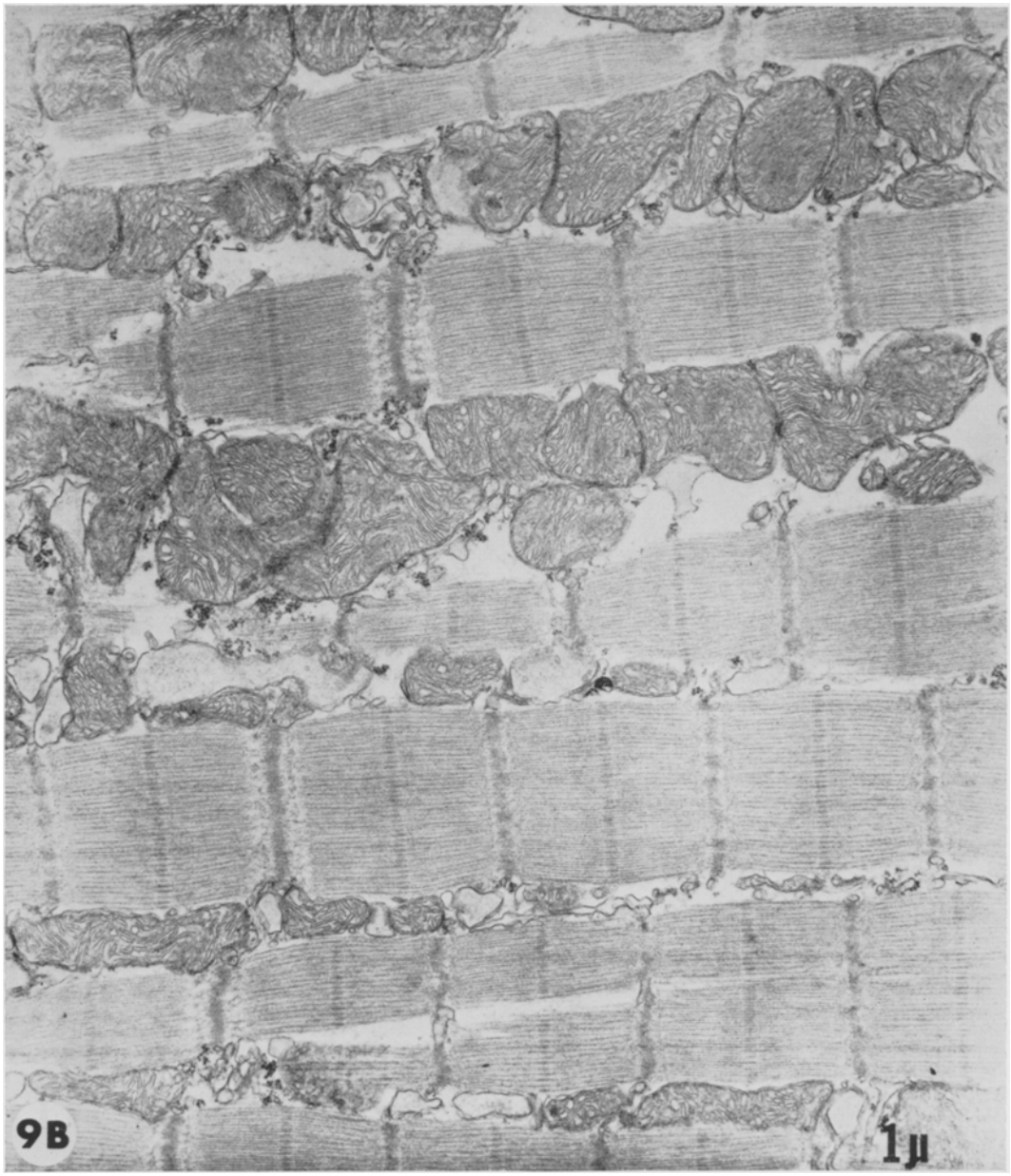
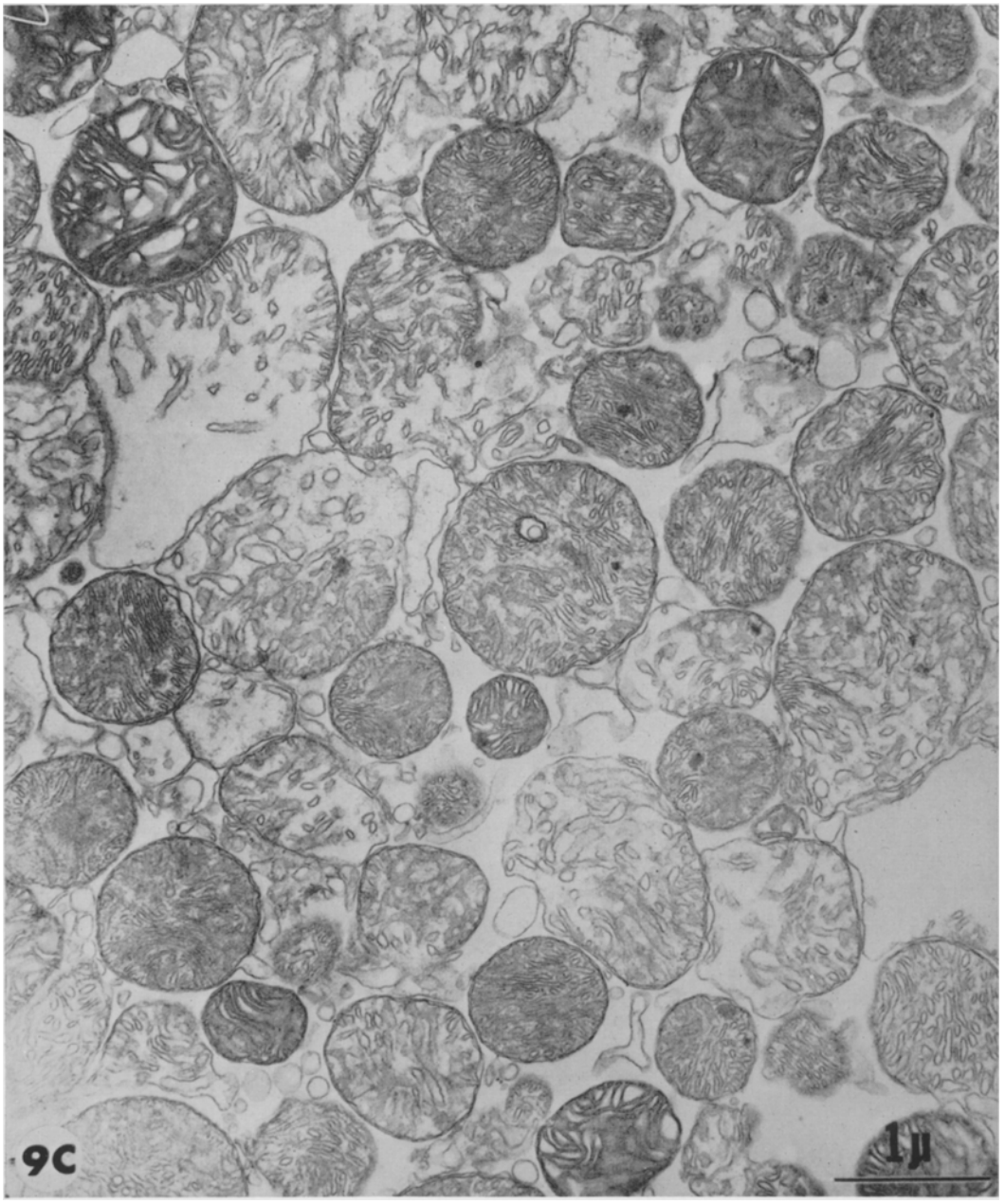


Figure 9. Stabilization of the aggregated configuration.
A. Mitochondrial suspensions prepared in sucrose-Tris solution as described previously were fixed with glutaraldehyde (final concentration 2%) and post fixed with 2% OsO_4. ×30,000.



B. Freshly obtained beef heart tissue was sliced as thin as possible with a razor blade. Sliced tissues were fixed with 2% glutaraldehyde and post fixed with 2% OsO_4. ×30,000.



C. Mitochondrial suspensions prepared in sucrose-Tris solution as described previously were fixed with glutaraldehyde (final concentration 0·001%) and post fixed with 2% OsO_4. ×30,000.

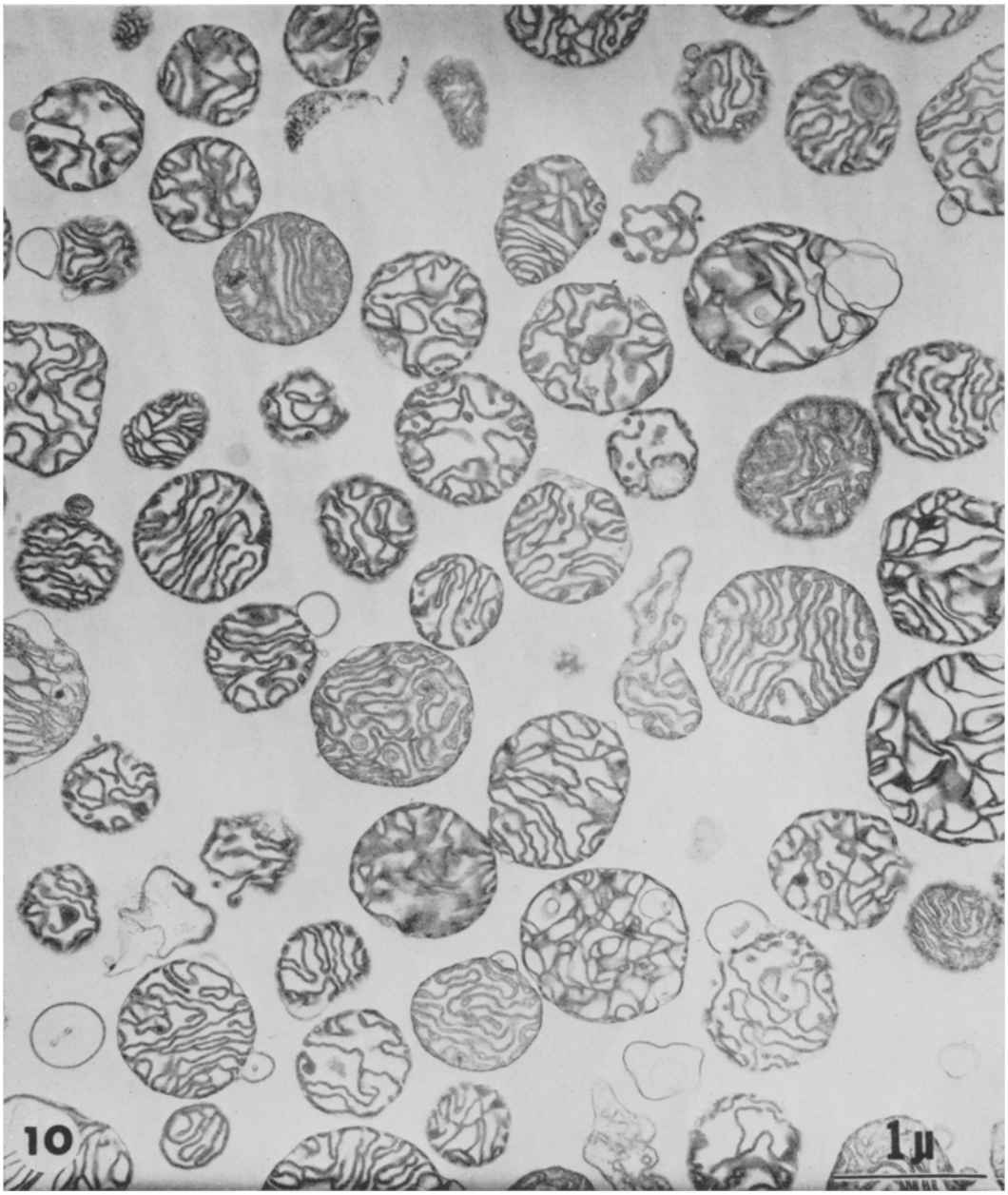


Figure 10. Induction of the energized-twisted configuration by phosphotungstate.
Mitochondria in 0·25 M sucrose were incubated with 1% phosphotungstate (pH 7·5) for 20 min at room temperature, and were fixed first with 2% glutaraldehyde and then later with 2% OsO_4. ×30,000.

9B). On isolation of mitochondria in isotonic sucrose media, the osmotic pressure of sucrose to which the inner mitochondrial membrane is impermeable compels the orthodox (*in situ*) to the aggregated (isolated) transition. This configurational transition like the nonenergized to energized transition can be very rapid. It can be shown that exposure of isolated mitochondria to very low concentrations of glutaraldehyde (0·001%) for some minutes before postfixing with 2% osmium tetroxide leads to the emergence of the swollen orthodox configuration (Fig. 9C). We are not concerned in this communication with the multiple reagents and conditions which can induce the orthodox to aggregated transition. The point at issue here is that when fixation is incomplete as with 0·001% glutaraldehyde, the permeability of the mitochondrial inner membrane can become altered to the point that the osmotic influence of sucrose is attenuated,[11] and then later when osmium is added to the suspension, the configuration of the inner membrane is not the same as the configuration which was obtained prior to the addition of glutaraldehyde. Incomplete fixation by glutaraldehyde leads to a configurational transition before the addition of osmium can stabilize the ultrastructural pattern. This is yet another example of the problem of changing configuration states in mitochondria. The difficulty is avoided by using glutaraldehyde in final concentrations above 0·01%.

VI. Induction of the Energized-Twisted Configuration by Phosphotungstate

Another facet of the problem of configurational transitions in mitochondria is pointed up by the effect of polyanions such as are used in negative staining in inducing the energized-twisted configuration under nonenergizing conditions. When mitochondria are exposed to phosphotungstate in relatively high concentrations, and then fixed in the usual way with glutaraldehyde and osmium, dehydrated, embedded and sectioned, the electron micrographs of such specimens show mitochondria predominantly in the energized-twisted configuration (Fig. 10). This induction does not require either an energizing source or aerobic conditions. The induction of configurational change by this polyanion is similar to the induction of the geometrical form of the energized-twisted configuration by weak acid anions in relatively high concentration (0·15 M) previously reported in our laboratory.[9, 12] The phenomenon is referred to as pseudo-energizing of the mitochondrial cristal membrane. We are not concerned here with the meaning of such inductions of configurational change but rather with the fact of such possibilities and with the problems which the use of reagents selected for negative staining may engender.

Discussion

The data presented in the present communication are relevent to the problem of demonstrating energized configurational changes in mitochondria. Whichever method of demonstration is applied, one absolute requirement has to be met. Fixation must be sufficiently rapid that energizing conditions are still operative when the ultrastructure is completely immobilized by the fixative. By means of biochemical probes, it is now possible to determine by each of several methods exclusive of electron microscopy whether this test is met. Reports in the literature of the failure to observe energized configurational changes by electron microscopy in most cases are based on experiments in which mitochondria were sedimented prior to fixation, and the pellet was fixed with

osmium (1–2%) without glutaraldehyde prefixation.[13,14,15] According to our studies, it would be almost impossible to demonstrate energized configurations by such methods. Anaerobiosis would set in long before the fixative was applied to the pellet. Williams *et al.* in our laboratory has shown that much the same difficulty applies to the demonstration of configurational changes in mitochondria *in situ*.[16] It was only when rapid fixation under energizing conditions was achieved that these configurational changes were consistently demonstrable. There are by now a considerable number of reports in the literature of consistent configurational changes in mitochondria under energizing conditions. The methods used by these investigators in all cases involved rapid fixation by direct, sequential addition of the two fixatives to the mitochondrial suspension.[17,18,19]

In general, our results suggest that a combination of glutaraldehyde as a prefixative and osmium tetroxide as a postfixative is the one of choice for preserving the energized configuration. The concentrations of each of these two fixatives can with profit be two orders of magnitude lower than the concentrations usually recommended (0·01% for glutaraldehyde and 0·03% for osmium tetroxide).

The speed of interaction of glutaraldehyde with mitochondria is exceedingly high (within 1–2 sec). The rate-limiting step may in fact be the rate of mixing rather than the speed of interaction of the fixative with the mitochondrion.

Sabatini *et al.* and Packer *et al.*[18–22] were pioneers in recognizing the remarkable properties of glutaraldehyde in respect to stabilization of mitochondrial structure. They showed that glutaraldehyde at appropriate levels would freeze the ultrastructure without affecting the capacity of the mitochondrion for electron transfer.[18,19]

We have purposely restricted our inquiry to configurational transitions that are easily demonstrable electron microscopically. There is yet another energized transition (the one which takes place in absence of inorganic phosphate) that has yet to be studied systematically from the standpoint of fixation conditions. But it is more than likely that the same rules will apply to this transition as for the nonenergized to energized-twisted configuration.

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