ENERGY-LINKED ULTRASTRUCTURAL TRANSFORMATIONS IN ISOLATED LIVER MITOCHONDRIA AND MITOPLASTS

Preservation of Configurations by Freeze-Cleaving Compared to Chemical Fixation

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ABSTRACT

An investigation was carried out in which microsamples of isolated rat liver mitochondria and freshly prepared mitoplasts in defined energy states were freeze-cleaved. Parallel microsamples were fixed with osmium tetroxide and with glutaraldehyde followed by osmium tetroxide as previously used in this laboratory for the preservation of energy-linked mitochondrial configurations. The details of the *orthodox* configuration of energized mitochondria and the *condensed* configuration of de-energized mitochondria, as revealed previously by chemical fixation, are confirmed in this report for nonfixed, freeze-cleaved mitochondria. The precise agreement in preservation of configuration obtained by the physical fixation of rapid freezing and by chemical fixation establishes unequivocally that mitochondria undergo energy-linked ultrastructural transformation between the condensed and the orthodox configurations which are thus natural structural states related to the metabolic activity of the mitochondrion. Configurations observed by freeze-cleaving and by chemical fixation reveal that mitoplasts also undergo a specific and dramatic ultrastructural transformation with the induction of oxidative phosphorylation. The transformation appears to be isovolumetric and therefore is thought to be mediated through energized conformational activity in the surface electron-transport membrane of the mitoplast. Passively swollen, spherical, osmotically active mitoplasts could not be fixed rapidly enough by chemical fixatives as normally used without altering the spherical form. In this special case preservation of configurational form required rapid freezing or chemical fixatives of low osmolar concentration.

INTRODUCTION

Previous investigation in this laboratory established that transformations in ultrastructural configuration occur in isolated liver mitochondria which parallel induced changes in metabolic state. These transformations are known to be reversible (1), are energy dependent (2), and occur under conditions which are favorable for maintaining the integrity of oxidative phosphorylation (2, 3). Identical energy-linked ultrastructural transformations occur in mitochondria within the intact cell when oxidative phosphorylation is induced (4). Although there is currently no general agreement as to a basic mechanism by which energy metabolism is coupled to ultrastructural trans-

formations in mitochondria (5), there is considerable evidence that such transformations occur during steady state as well as oscillatory state electron transport and during oxidative phosphorylation (1–14).

The question of the contribution of the effects of chemical fixation on the final configurational image was dealt with early in these studies (1). However, several reports have recently appeared which are addressed to further consideration of the chemical fixation effect. Stoner and Sirak (15) found that heart mitochondria which were swollen in hypoosmotic media and then fixed with glutaraldehyde could subsequently undergo osmotically induced contraction, resulting in a condensed type mitochondrial configuration. Butler and Judah (16) found that freshly isolated liver mitochondria displayed the usual condensed (1) configuration after fixation with glutaraldehyde and, in some cases, after fixation with osmium tetroxide. However, they observed that fixation with potassium permanganate revealed an orthodox (1) configuration. More recently, Hunter and Brierley (17) observed extensive increases in light-scattering to occur in swollen heart mitochondria after the addition of glutaraldehyde fixative which can be interpreted as a decrease in the volume of the inner compartment during fixation.

Two reports have also appeared in which freeze-cleaving was used to detect changes in mitochondrial configuration linked to energy metabolism (18) and to passive osmotic activity (19). In both reports, unfixed, freeze-cleaved, isolated liver mitochondria, which had been cross-fractured through the matrix, did not reveal the inner electron-transport membrane, and therefore the inner compartment could not be distinctively resolved from the outer compartment. Only when freeze-cleaved mitochondria were fixed with osmium tetroxide (18) or glutaraldehyde (19) *before* freezing could the specific details of condensed and orthodox (1) configurations of chemically fixed liver mitochondria be resolved. Thus, transformations of mitochondrial configuration, whether linked to energy metabolism or passive osmotic activity, as revealed by chemical fixation, have not been satisfactorily confirmed by freeze-cleaving alone.

With these results in mind, an investigation was carried out in this laboratory in which microsamples (1) of isolated rat liver mitochondria and mitoplasts[1] in defined energy states were freeze-cleaved without previous chemical fixation. Parallel microsamples were fixed with osmium tetroxide and with glutaraldehyde followed by osmium tetroxide as routinely used in this laboratory for the purpose of preserving energy-linked mitochondrial configurations. The configurational details of mitochondria, as well as mitoplasts, from parallel freeze-cleaved and chemically fixed microsamples, are compared here.

The observations reveal that osmium tetroxide preserves energy-linked configurations of mitochondria and also of mitoplasts. The energy-linked configurations are identical in structural detail to the configurations of parallel microsamples preserved by freeze-cleaving alone. Although the results with glutaraldehyde fixation followed by osmium tetroxide are not presented here, preservation of energy-linked configurations was found to be identical to preservation by osmium tetroxide and by freeze-cleaving. In one procedure, in which mitoplasts were experimentally passively swollen to near perfect spheres, neither osmium tetroxide nor glutaraldehyde, as routinely used, could preserve configuration. In this case freeze-cleaving or fixatives of low osmolar concentrations were required for preservation of the spherical form.

The results of freeze-cleaving presented in this report are confined to gross mitochondrial and mitoplast configurations. The contribution of freeze-cleaving to visualization of energy-linked ultrastructural transformation at the level of the molecular organization of mitochondrial membranes will be presented in forthcoming communications.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of male Sprague-Dawley rats at 0°C using 0.25 M sucrose according to the method of Schneider (21). For the preparation of mitoplasts, mitochondria were isolated at 0°C by the sucrose-mannitol-HEPES-

[1] The mitoplast fraction is an outer membrane-free mitochondrial preparation that carries out oxidative phosphorylation efficiently while maintaining acceptor control and was originally referred to as the inner membrane-matrix fraction (20). The term "mitoplast" was first suggested by A. L. Lehninger (personal communication) and subsequently adopted by Greenawalt (55) and Pedersen and Schnaitman (56).

BSA[2] method of Schnaitman and Greenawalt (20). Mitoplasts were prepared by a controlled digitonin method followed by differential centrifugation according to Schnaitman et al. (22). Mitoplasts prepared in this way contained less than 2% monoamine oxidase, an outer membrane enzyme marker (20, 22).

Respiratory rates were monitored with Clark oxygen electrodes (23). Mitochondria gave ADP/O ratios of two for succinate and acceptor control ratios of 5 to 7. Mitoplasts gave ADP/O ratios of 1.5 to 2 for succinate and acceptor control ratios of 2 to 4. Respiratory rate was used to determine energization by succinate, de-energization by antimycin, and oxidative phosphorylation as previously described (2).

Swollen, spherical mitoplasts were prepared by slowly titrating freshly prepared mitoplasts in their isolation medium with distilled water until phase-contrast microscopy showed all mitoplasts to be in the spherical form, which was determined to be at 40 mosmolar.

The reaction medium for mitochondria and for mitoplasts is given for each experiment in the figure legends. For freeze cleaving, all reaction systems were incubated for 5 min at 25°C with 25% glycerol, to establish the metabolic steady state. For chemical fixation, the reaction systems were incubated for 5 min at 25°C without addition of glycerol, to establish the metabolic steady state. Protein was determined by a biuret method (24).

Microsamples were chemically fixed in two ways: as micropellets fixed with osmium tetroxide or in suspension fixed with glutaraldehyde. Microsamples of 0.1 mg mitochondrial or mitoplast protein (50–100 μl) were rapidly removed from reaction systems at specified times for electron microscopy via a quick sampling–micropelleting method described earlier (1). Osmium tetroxide fixative was 2% in 0.1 M Na-phosphate buffer, giving a pH of 7.4 and a mosmolar concentration of 300. Microsamples fixed in glutaraldehyde were ejected directly into the fixative, left for 10 min, then centrifuged into micropellets. The final concentration of glutaraldehyde was 2% in 0.1 M Na-phosphate buffer, giving a pH of 7.4 and a mosmolar concentration of 475. Micropellets were left in either fixative for 1 hr. Micropellets fixed in glutaraldehyde were then washed for 1 hr with 0.2 M sucrose in 0.1 M Na-phosphate buffer, giving a pH of 7.4 and a mosmolar concentration of 435, followed by osmium tetroxide as prepared above for 1 hr.

Microsamples of swollen spherical mitoplasts were fixed as outlined above and also with fixatives of low osmolarity as follows: (*a*) 1% osmium tetroxide in 5 mM Na-phosphate buffer, giving a pH of 7.4 and a mosmolar concentration of 45, and (*b*) 0.3% glutaraldehyde in 1 mM Na-phosphate buffer, giving a pH of 7.4 and a mosmolar concentration of 45, followed by a phosphate wash and postosmication at pH 7.4 and mosmolar concentration of 45.

All micropellets were dehydrated, embedded, oriented, and sectioned as reported earlier (1, 2). Gray sections of Epon-embedded micropellets were cut on diamond knives and stained 20 min at 60°C in 1.0% sodium borate solution saturated with uranyl acetate followed by lead hydroxide $\frac{1}{40}$ dilution for 5 min as described earlier (2).

For freeze-etching, 2-μg samples of mitochondrial or mitoplast protein (0.5–1.0 μl) were transferred onto gold-nickel specimen discs directly from the reaction systems, or larger microsamples were removed from the reaction systems, centrifuged into micropellets (1), and then transferred onto gold-nickel specimen discs. Freezing was carried out immediately by plunging the specimen-containing discs into liquid Freon 22 at −150°C. Fracturing, vacuum sublimation (heat etching), and platinum-carbon replication was carried out at −100°C at 10^{-6} torr in a Balzers BA 360 freeze-microtome high-vacuum unit (Balzers AG. Balzers, Liechtenstein) (25).

Electron micrographs were taken on Kodak $3\frac{1}{4} \times 4$ inch contrast plates (for thin sections) or medium plates (for platinum-carbon replicas) at initial magnifications of 7000 to 50,000 with an RCA 3G electron microscope operated at 50 kv and equipped with an anticontamination cold trap and double condenser.

RESULTS

Mitochondria

Freshly isolated, metabolically efficient mitochondria show a condensed (1) configuration after fixation with either osmium tetroxide or glutaraldehyde followed by osmium tetroxide (1–3, 6–10, 12, 14, 15, 17–19, 26–30, 32–34). The condensed configuration is also characteristic of isolated mitochondria during oxidative phosphorylation and of mitochondria in low energy states induced by inhibitors of electron transport or anaerobiosis (1, 2, 5–10, 12, 14, 18). Fig. 1 shows a field of freeze-cleaved mitochondria de-energized by the electron-transport inhibitor antimycin. It is clearly evident that rapid freezing alone preserves the condensed configuration typical of the low energy state of mitochondria observed after chemical

[2] *Abbreviations used:* ADP/O, adenosine diphosphate/oxygen; BSA bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid.

FIGURE 1 Freeze-cleaved, de-energized mitochondria showing the condensed configuration. Reaction system: sucrose (120 mM); Na-phosphate buffer (10 mM; pH 7.4); $MgCl_2$ (5 mM); Na-succinate (10 mM); antimycin (0.5 μg/mg protein); glycerol (25%); mitochondrial protein (2.0 mg/ml). × 17,000.

FIGURE 2 Freeze-cleaved, de-energized mitochondria showing the condensed configuration. The spatial folding of the inner, electron-transport membrane is random. The volume of the inner compartment is decreased while the volume of the outer compartment and intracristal space is increased. A membrane-membrane contact site is observed (arrow). Reaction system as in Fig. 1. × 61,000.

fixation. At higher magnification (Fig. 2) the distinguishing features of the condensed configuration are well preserved, namely the random spatial folding of the inner, electron-transport membrane, the decreased volume of the inner compartment, and the increased volume of the outer compartment and intracristal space (2). In addition, the preservation of contact sites between the electron-transport membrane and outer membrane (arrow) confirms the recent findings of such sites in chemically fixed, condensed mitochondria (18).

Energization of freshly isolated condensed mitochondria, initiated by succinate-supported electron transport, results in a condensed to orthodox ultrastructural transformation (1, 2) and is confirmed by freeze-cleaving in Figs. 3 and 4. Fig. 4 demonstrates the details of the orthodox configuration which distinguish the latter from the condensed configuration, namely the organized infoldings of the electron-transport membrane into discrete cristae, the increased volume of the inner compartment, and the decreased volume of the outer compartment and intracristal space (2).

Mitoplasts

The mitoplast fraction is the only inner membrane-matrix preparation, verified to be biochemically and structurally free of outer membrane, that carries out oxidative phosphorylation and maintains acceptor control. Since the mitoplast is composed of a single osmotically active compartment containing the mitochondrial matrix and is surrounded only by the electron-transport membrane (Fig. 5), it is presumably more sensitive to extrinsic variations in osmotic pressure than is the intact mitochondrion. Characteristic of freshly prepared mitoplasts, as observed after chemical fixation, are outfoldings or finger-like protuberances of the surface electron-transport membrane and the absence of mitochondrial cristae projecting into the matrix compartment (Fig. 5). These unusual features are readily preserved in chemically unfixed mitoplasts by rapid freezing as shown in the freeze-cleaved preparation in Fig. 6.

With initiation of oxidative phosphorylation chemical fixation shows the electron-transport membrane of mitoplasts to undergo a dramatic infolding, resulting in large intracristal spaces projecting into the matrix (Fig. 7). There is no apparent change in mitoplast volume during this transformation and, therefore, by definition, the transformation is not osmotically induced. A freeze-cleaved preparation of phosphorylating mitoplasts is shown in Fig. 8 and confirms the configurational details of phosphorylating mitoplasts observed after chemical fixation.

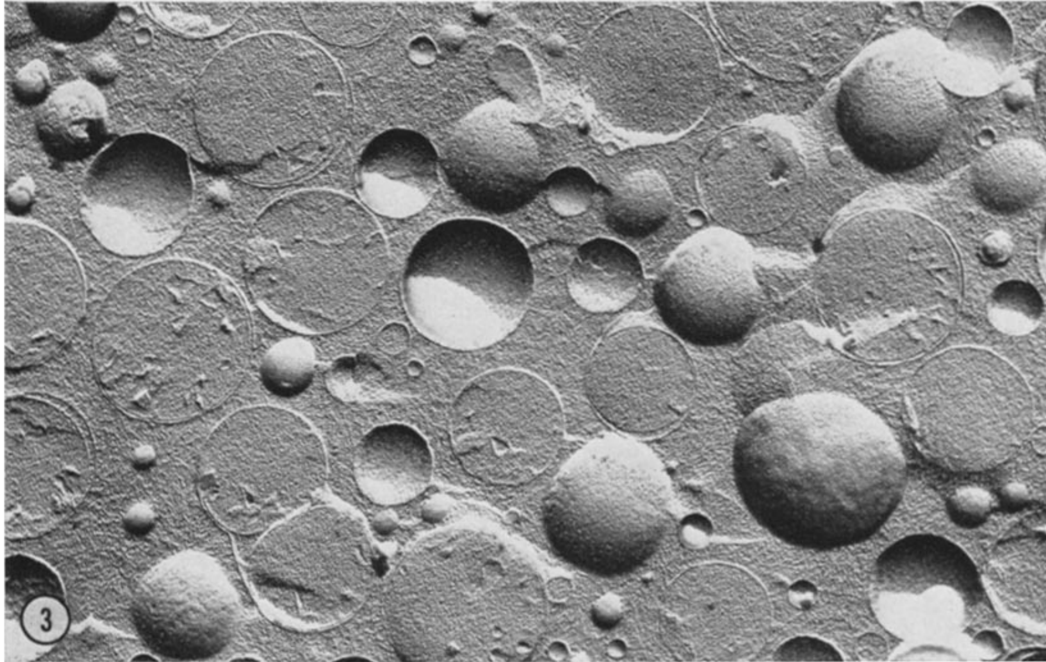
Examination of the topography of chemically unfixed, freeze-cleaved, phosphorylating mitoplasts also reveals the large infolded cristae of the electron-transport membrane (Fig. 9). The particles seen in the fracture face of the electron-transport membrane will be considered in a forthcoming communication.

Swollen, Spherical Mitoplasts

Freshly prepared mitoplasts, which are customarily isolated in a medium having an osmolarity of 300 mosmolar (Fig. 5), were subsequently swollen by slowly decreasing the osmolar concentration of the isolation medium with distilled water while monitoring the volume increase by phase-contrast microscopy. When the mitoplasts were converted to perfect spheres, which occurred at 40 mosmolar as detected by phase-contrast microscopy, samples were fixed with chemical fixatives of various osmolarities while other samples were rapidly frozen for freeze-cleaving. The routine osmium tetroxide (300 mosmolar) and glutaraldehyde (475 mosmolar) fixatives caused an osmotic reversal of volume which occurred before fixation, such that the swollen, spherical mitoplasts were converted to a configuration of diminished volume identical to that of chemically fixed, freshly prepared mitoplasts (Fig. 5). In order to preserve the spherical form of the swollen mitoplasts, which clearly maintained osmotic activity, it was necessary to reduce the osmolar concentration of the fixatives to 45 mosmolar (Fig. 10). It is observed that at 40 mosmolar a few short finger-like protuberances of the electron-transport membrane persist on the surface of the mitoplasts. The tips of these residual protuberances may represent the loci of sites originally in contact with the outer mitochondrial membrane (18). Also persisting in the widely dispersed protein of the matrix are the classical electron-opaque intramitochondrial granules.

Rapid freezing of spherical mitoplasts preserved the spherical form including the short finger-like protuberances (Fig. 11) as preserved by chemical fixation at 45 mosmolar (Fig. 10).

The spherical mitoplast carries out coupled electron transport and is currently being used



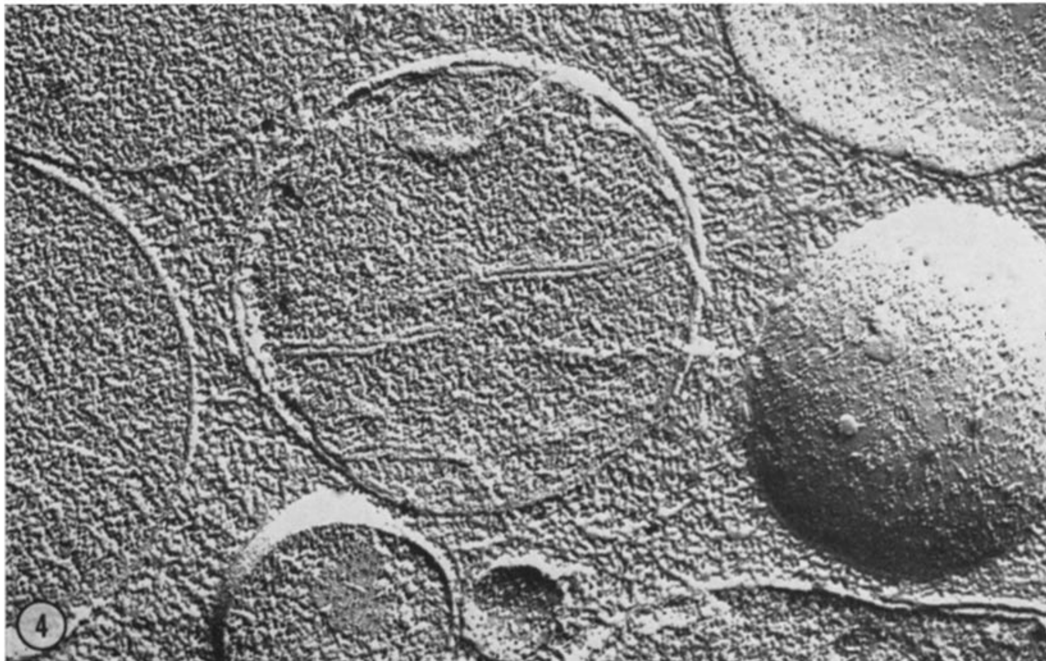


FIGURE 3 Freeze-cleaved, energized mitochondria showing the orthodox configuration. Reaction system as in Fig. 1 minus antimycin. × 17,000.

FIGURE 4 Freeze-cleaved, energized mitochondria showing the orthodox configuration. The inner, electron-transport membrane shows organized infoldings of discrete cristae. The volume of the inner compartment is increased while the volume of the outer compartment and intracristal space is decreased. The orthodox configuration is to be compared with the condensed configuration shown in Fig. 2. Reaction system as in Fig. 1 minus antimycin. × 61,000.

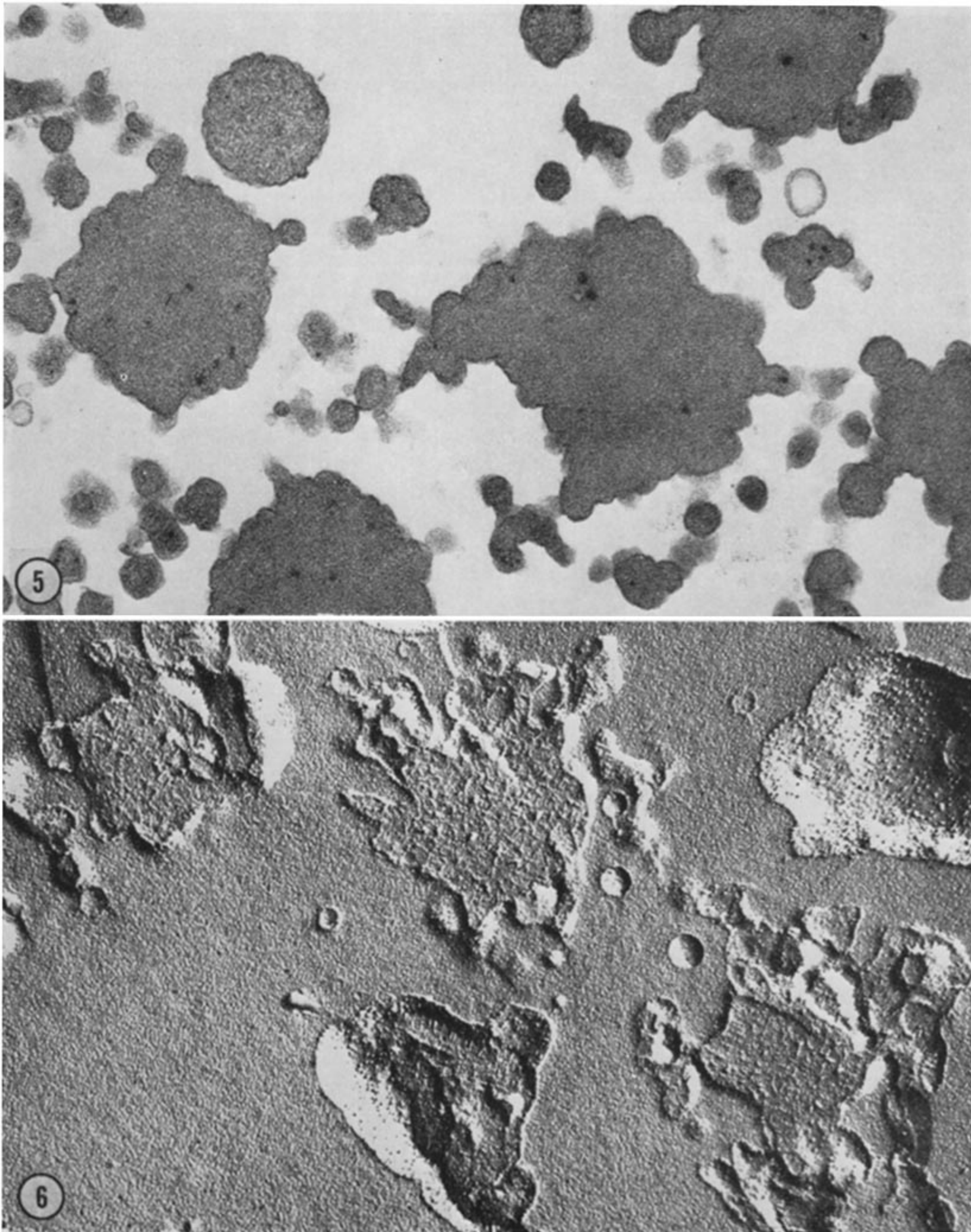


FIGURE 5 Thin section of osmium tetroxide-fixed, freshly prepared, nonphosphorylating mitoplasts. The surface, electron-transport membrane shows many outfoldings or finger-like protuberances. Mitochondrial cristae are not observed projecting into the matrix. Intramitochondrial electron-opaque granules are observed in the matrix. Mitoplasts in low energy state isolation medium. × 51,000.

FIGURE 6 Freeze-cleaved, freshly prepared, nonphosphorylating mitoplasts. Cross fracture through the matrix of three mitoplasts shows finger-like protuberances of the surface electron-transport membrane and a lack of intracristal spaces. To be compared to chemically fixed freshly prepared, nonphosphorylating mitoplasts shown in Fig. 5. Mitoplasts in low energy state isolation medium as in Fig. 5 plus 25% glycerol. × 43,700.

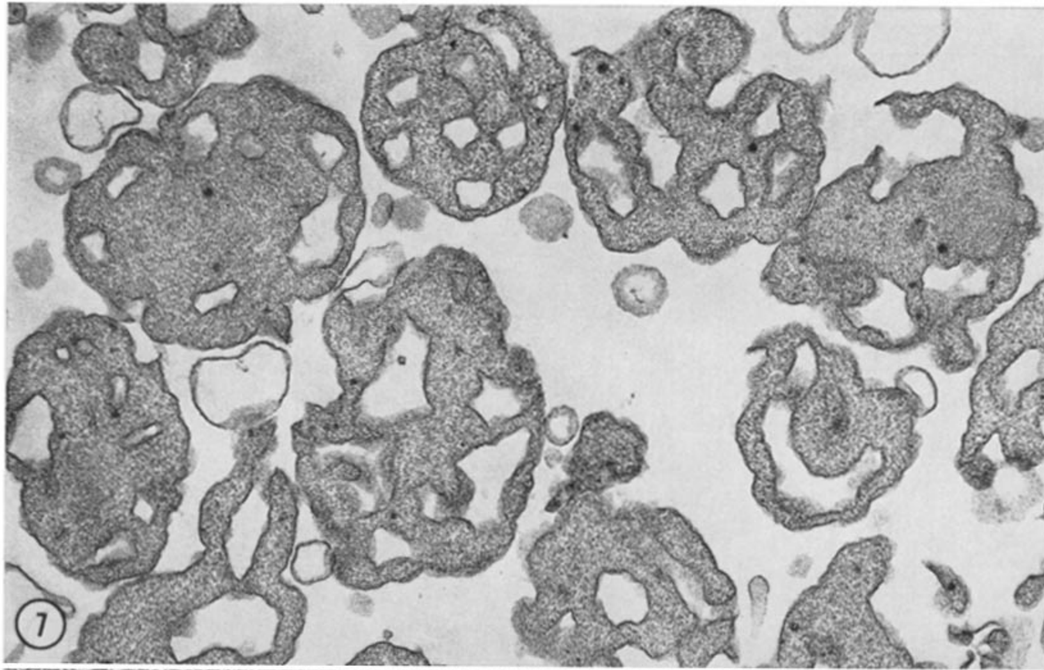


FIGURE 7 Thin section of osmium tetroxide–fixed mitoplasts during oxidative phosphorylation. The surface electron-transport membrane shows many infoldings, resulting in large intracristal spaces projecting into the matrix. This configuration is to be compared to the configuration of nonphosphorylating mitoplasts shown in Fig. 5. Reaction system: sucrose (70 mM); mannitol (220 mM); HEPES buffer (2 mM; pH 7.4); Na-succinate (10 mM); K-phosphate (2.5 mM); EDTA (1 mM); ADP (250 μmoles/mg); mitochondrial protein (2 mg/ml). × 51,000.

FIGURE 8 Freeze-cleaved mitoplasts during oxidative phosphorylation. Cross fractures through the matrix of three mitoplasts show infolding of the electron-transport membrane into the matrix, resulting in large intracristal spaces. To be compared to chemically fixed, phosphorylating mitoplasts shown in Fig. 7 and to freeze-cleaved, nonphosphorylating mitoplasts shown in Fig. 6. Reaction system as in Fig. 7 plus 25% glycerol. × 43,700.

FIGURE 9 Freeze-cleaved mitoplast during oxidative phosphorylation. Surface fracture reveals the topography of the electron-transport membrane. Two arrows point to large intracristal spaces which are infoldings of the surface electron-transport membrane projecting into the matrix. To be compared to infoldings in phosphorylating mitoplasts after chemical fixation as shown in Fig. 7. Reaction system as in Fig. 7 plus 25% glycerol. × 100,000.

in this laboratory for investigation of the molecular organization of the electron-transport membrane and in studies of energy-linked transitions in surface charge density and transmembrane potential.

DISCUSSION

Ultrastructural Transformation and Freeze-Cleaving

The results of this investigation reveal that isolated rat liver mitochondria, as well as mitoplasts, undergo energy-linked ultrastructural transformation as determined independently by the rapid freezing procedure of freeze-cleaving and by chemical fixation.

This is the first study to demonstrate that the ultrastructural details of the condensed configuraton of the low energy state and the orthodox configuration of the high energy state can be preserved for electron microscope examination by means other than chemical fixation. Precise structural details were found to be preserved by freeze-cleaving, including such subtle features as the membrane-membrane contact sites of the condensed configuration described in low energy states after chemical fixation (18).

In a previous report freeze-cleaving of mitochondria in different energy states, without previous chemical fixation, failed to preserve the structural details observed in chemically fixed mitochondria, presumably because cryoprotecting agents were not used (18). These agents, such as glycerol and dimethyl sulfoxide, which enhance structural preservation by compressing the crystallization interval during rapid freezing, were omitted since they were also found to depress the efficiency of oxidative phosphorylation and acceptor control (18). In the present investigation the problem of protecting ultrastructure from ice crystal damage with a cryoprotecting agent while preserving metabolic efficiency was, in retrospect, solved very simply by exposing mitochondria to glycerol for short periods of time (5 min or less) at

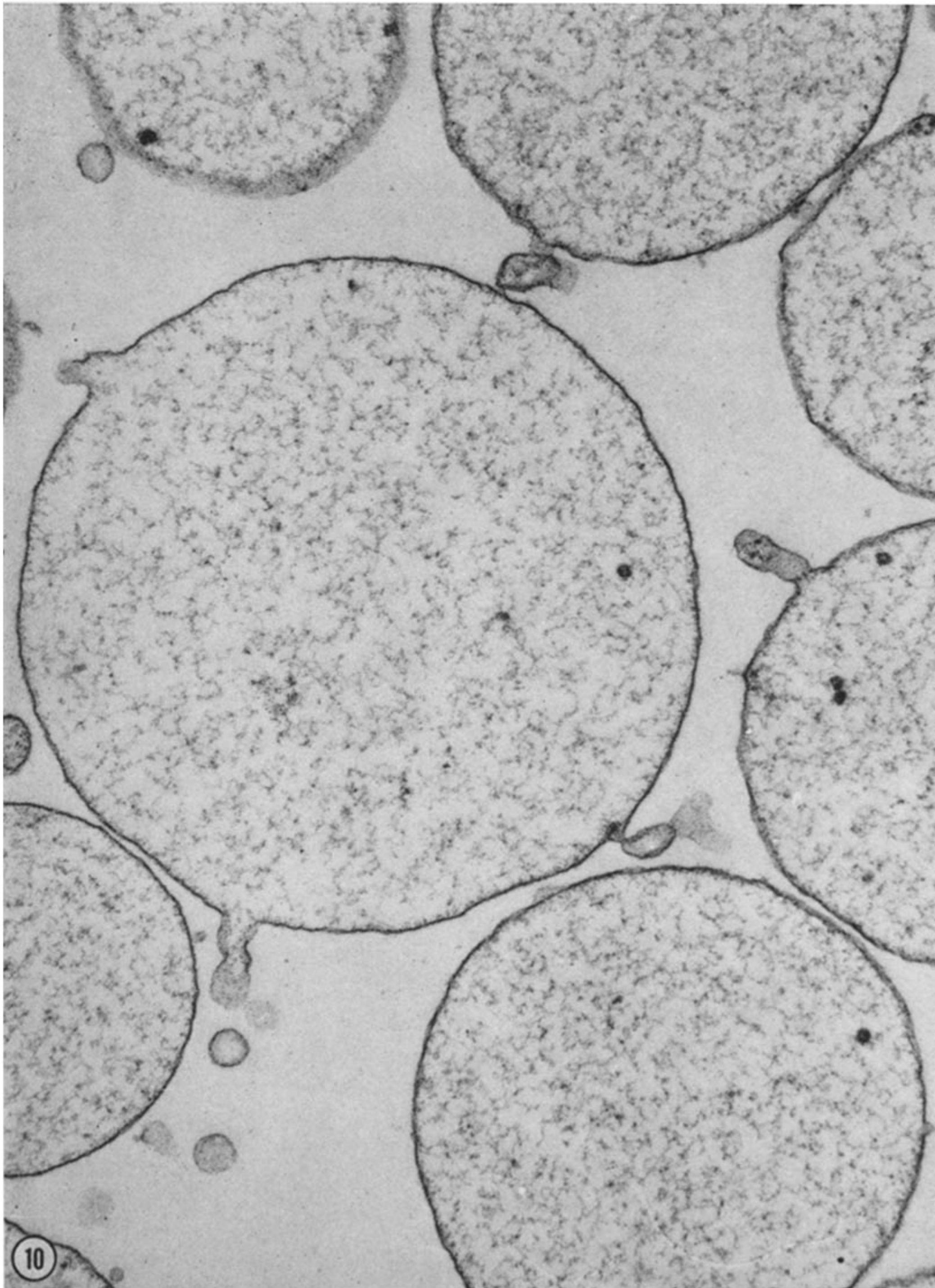


FIGURE 10 Thin section of osmium tetroxide–fixed, swollen, spherical mitoplasts. Mitoplasts in 40 mosmolar low energy state preparation medium show the persistence of short finger-like protuberances of the surface electron-transport membrane and the classical electron-opaque intramitochondrial granules. To be compared to nonswollen freshly prepared mitoplasts shown in Fig. 5. Fixative mosmolar concentration was 45. × 51,000.

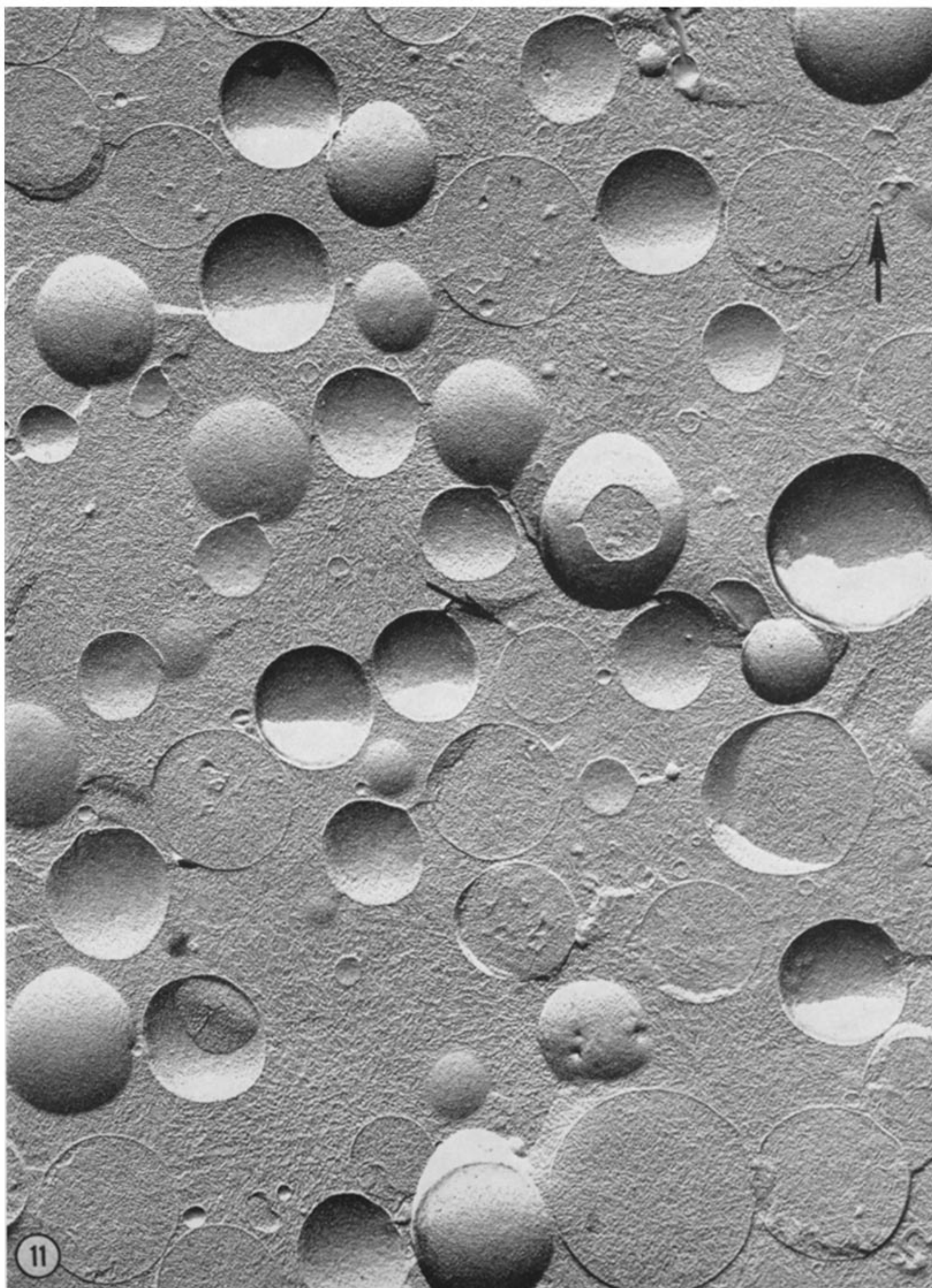


FIGURE 11 Freeze-cleaved, swollen, spherical mitoplasts. Mitoplasts in 40 mosmolar low energy state preparation medium as in Fig. 10. The short finger-like protuberances of the surface electron-transport membrane can be observed (arrows). To be compared with chemical fixation at low osmolarity shown in Fig. 10 at higher magnification. × 17,000.

elevated temperatures (25°C). In practice, this was accomplished simply by including 25% glycerol in the reaction media used for inducing the desired energy state. Stock mitochondria were not pretreated with cold glycerol nor were mitochondria further treated with glycerol once the energy state was established. Since glycerol has a high temperature coefficient of permeation, at least in living cells (38), it is presumed that the ice crystal protection effect of glycerol in the experiments discussed here occurred very rapidly during induction of the energy state since the reaction media were thermostated at 25°C. In separate experiments it was found that glycerol did not afford protection against ice crystal damage when mitochondria were exposed for 5 min at 0°C. The use of short exposure to glycerol at 25°C resulted in no significant depression of oxidative phosphorylation efficiency or aceptor control although the rate of electron transport was decreased somewhat, which was anticipated and desirable.

The use of glycerol simply as part of the reaction media has also permitted excellent preservation of configurational states linked to energy metabolism in chemically unfixed, freeze-cleaved mitoplasts. Freeze-cleaving was anticipated to be a particularly useful approach for preserving energy-linked configurations of mitoplasts since, unlike the mitochondrion, the mitoplast is a one membrane–one compartment system with an osmotic dead space composed only of its molecular solids. Since the outer membrane of the mitochondrion is absent in the mitoplast, swelling is less restricted. The capacity of passive reversible swelling in mitoplasts, without rupture of the electron-transport membrane, is exemplified in the results shown in Figs. 5 and 10 which reveal, by calculation, an approximately 8-fold difference in mitoplast volume related to a 7.5-fold difference in external osmotic pressure.

The results reveal that ultrastructural transformation occurs in freshly prepared mitoplasts with the induction of oxidative phosphorylation. Greenawalt has observed a similar ultrastructural transformation in a mitochondrial preparation of this type (57). The effectiveness of chemical fixation in preserving the configurational details of this energy-linked ultrastuctural transformation (Figs. 5 and 7) is verified by freeze-cleaving (Figs. 6, 8, and 9). Although the ultrastructural transformation which occurs during oxidative phosphorylation is a rather dramatic one, it is of interest that the transformation appears to be isovolumetric, i.e., it occurs without apparent change in the mitoplast volume (cf. Figs. 5 and 7, 6 and 8). This observation suggests energized conformational activity rather than energized ion-induced osmotic activity (5) as the mediator of this gross configurational change.

Ultrastructural Transformation and Chemical Fixation

The use of chemical fixatives in the past has been instrumental in studies regarding energy-linked ultrastructural transformations in isolated mitochondria. The possible influence of the chemical fixative on the final conformational image was recognized very early in these studies; thus, the initial survey of ultrastructural transformation in mitochondria during change in energy state was carried out while monitoring changes in mitochondrial light-scattering signals (1). For the most part the direction and amplitude of the light-scattering signal are found to be in congruence with the energy-linked configurational state of isolated mitochondria as observed by electron microscopy after fixation in either osmium tetroxide or glutaraldehyde (1, 3, 5, 6, 9, 12, 28, 35). A most critical correlation between direction and amplitude of the light-scattering signal and energy-linked ultrastructural transformation has been reported recently for mitochondria in the intact cell (4). The fact that an increased light-scattering signal is correlated with a condensed configuration, whereas a decreased light-scattering signal is correlated with an orthodox configuration, is consistent with the mathematical treatment of solution optics and mitochondrial turbidity (39). Also, the finding that mitochondria can transform between a condensed and an orthodox configuration has contributed significantly to the understanding of change in light-scattering signals related to energized ion-inducing osmotic changes in mitochondria (3, 5, 6, 12, 17, 28, 35).

The reality of the condensed configuration recently has been challenged by Butler and Judah (16) since they contend a priori that isolated mitochondria should resemble as closely as possible that configuration most often observed in intact cells, namely the orthodox configuration. Empirically, however, an ever increasing number of investigators have confirmed the condensed configuration in freshly isolated liver mitochondria, heart mitochondria, kidney mitochondria, skeletal

muscle mitochondria, and brain mitochondria after fixation in osmium tetroxide or glutaraldehyde followed by osmium tetroxide (1–3, 6–10, 12, 14, 15, 17–19, 26–30, 32–37). A condensed configuration also has been observed in isolated mitochondria by the use of negative staining (13, 31) and in isolated mitochondria exposed to osmium tetroxide vapors (30). In addition, the condensed configuration has been shown in mitochondria in the intact cell to be linked to induced change in physiological state as observed after either osmium tetroxide or glutaraldehyde fixation (4, 11, 13).

Butler and Judah (16) found that the appearance of freshly isolated mitochondria and those in various energy states "can be made to resemble that of mitochondria in the liver cell," provided potassium permanganate was used as the fixative. The authors thus recommend potassium permanganate as the choice fixative in investigations involving isolated mitochondria and suggest caution regarding the use of fixation procedures which modify the configuration of mitochondria. Since Butler and Judah (40) could not identify the condensed configuration after fixation with potassium permanganate, their results regarding ion movements related to ultrastructural volume changes are in direct disagreement with those of Deamer et al. (6), Packer et al. (12), and Hackenbrock and Caplan (3) who previously showed by osmium tetroxide and glutaraldehyde fixation that the volume change which occurs in mitochondria during ion uptake at physiological levels occurs in the inner compartment while the total mitochondrial volume remains relatively constant. Thus, these studies show that mitochondria transform from a condensed to an orthodox configuration as ions and osmotic water move into the inner compartment. Butler and Judah (40) found that the uptake of K+ at levels of 400 μmoles/g of mitochondrial dry weight caused a large increase in *total* mitochondrial volume. However, the capacity for intact mitochondria to accommodate osmotic water was exceeded in these experiments since it is to be noted that the outer membranes disrupted. This is not surprising since the physiological level of K+ in intact freshly isolated liver mitochondria is approximately 150 μmoles/g of mitochondrial protein (41–45) and, together with its counteranions, chiefly total phosphates, accounts for the maintenance of the osmotic equilibrium of the inner compartment of liver mitochondria isolated in 0.25 M sucrose (46). Packer et al. (12) have shown quite convincingly, by using electron microscopy, light scattering, and packed volume measurements, that K+ uptake at physiological levels is linked to an increase in the volume of only the inner compartment of freshly isolated condensed mitochondria. In agreement with this finding are the correlative data of Hackenbrock and Caplan (3) who quantitated Ca^{2+} uptake and per cent volume increase of the inner compartment of condensed mitochondria by electron microscopy after osmium tetroxide fixation. Packer et al. (12) also showed by light-scattering that the addition of glutaraldehyde to K+-loaded mitochondria did not change but rather stabilized the light-scattering signal and therefore did not cause change in the mitochondrial configuration during fixation. Lastly, only the condensed configuration of freshly isolated mitochondria, as observed after fixation in osmium tetroxide or glutaraldehyde, is compatible with the fact that freshly isolated mitochondria contain a compartment of sucrose-inaccessible water which is equal in volume to the inner compartment of the condensed configuration but is approximately one-half the volume of the inner compartment of the orthodox configuration (18, 26, 46–48). Passive osmotic volume changes also show conversion of the condensed to the orthodox configuration and its reversal by the controlled use of sucrose (30).

Consideration of the above evidence taken together with the freeze-cleave results presented in this report leaves little doubt that potassium permanganate, at least as used by Butler and Judah (16, 40), fails to preserve the natural configuration of mitochondria related to freshly prepared preparations or to specific metabolic states, but rather causes a severe swelling during fixation. The severity of the swelling, as well as structural fragmentation caused by the fixative, is exemplified in Figs. 6 and 11 in the paper by Butler and Judah (16) which reveal at least 75% of the mitochondria to contain fragmented outer as well as inner membranes and a consistent loss of large chunks of matrix protein. Identical swelling and structural fragmentation can be observed in Fig. 1 of their more recent paper (40).

Potassium permanganate is not a general purpose fixative and should be used with care, if at all, for fixation of isolated mitochondria. Permanganates are well known to cause modification

in the gross arrangements of membranes along with breakage and fragmentation (49) while the swelling and extraction of the matrix of mitochondria in intact cells was reported very early in their use (50, 51). That permanganates are deficient in their ability to optimally preserve mitochondrial matrix (52) is consistent with the unsuitability of permanganates for fixing proteins (53, 54).

In the present investigation the use of freeze-cleaving confirms not only that the condensed configuration of mitochondria, as observed after fixation with osmium tetroxide or glutaraldehyde, exists as a natural structural state but that it transforms to the orthodox configuration during energization.

Configurational Preservation after Induced Swelling: Chemical Fixation versus Rapid Freezing

The use of mitoplasts swollen passively to near perfect spheres has permitted an evaluation of the ability of chemical fixatives to preserve the configuration of a simplified version of the highly labile inner osmotic compartment of the mitochondrion.

Neither osmium tetroxide nor glutaraldehyde of routine osmolarity, i.e. at or above 300 mosmolar, could preserve the spherical form of swollen mitoplasts prepared at 40 mosmolar. Apparently, the osmotic response of spherical mitoplasts to the fixatives is more rapid than the penetration and binding of the fixatives to the mitoplast membrane. Freezing, however, if rapid enough, and in the presence of cryoprotectors, should not induce osmotic alteration (although slow freezing will). Thus, rapid freezing was found to preserve the osmotically labile spherical mitoplasts (Fig. 11). Since the freezing rate in the routine freeze-cleaving procedure is in the millisecond time range, the rate of vitrification far surpasses that of chemical fixation, thus preventing volume changes in highly labile osmotic biological compartments.

Obviously, care must be taken when attempting to fix exceptionally swollen, osmotically labile, biological compartments with fixatives as routinely used. The spherical form of the swollen mitoplasts, which was determined by phase-contrast microscopy, was well preserved by either osmium tetroxide or glutaraldehyde, provided the fixatives were adjusted to equal the internal osmolar concentration of the mitoplasts (Fig. 10).

Stoner and Sirak (15) and Hunter and Brierley (17) have recently reported configurational instability in isolated heart mitochondria during and after fixation with glutaraldehyde. In both instances mitochondria were first induced to swell before addition of glutaraldehyde. Stoner and Sirak (15) found that mitochondria swollen passively in 50 mosmolar–buffered sucrose and then mixed with buffered glutaraldehyde-sucrose at final osmolarities of approximately 250 mosmolar followed by osmium tetroxide showed the configuration of a condensed form. Hunter and Brierley (17) found that heart mitochondria incubated in a phosphate-succinate swelling medium in 0.25 M sucrose resulted in swelling as indicated by a precipitous decrease in the light-scattering signal. The subsequent addition of glutaraldehyde, however, resulted in a reversal of this light-scattering decrease, indicating a transformation of the swollen mitochondria to a contracted or condensed form during and after fixation. An interesting observation by these investigators was that the addition of glutaraldehyde to swollen mitochondria in sucrose-free media did not result in a reversal of the light-scattering signal, a result which can be observed in their earlier paper (35). Deamer et al. (6), and Packer et al. (12), however, had found earlier that glutaraldehyde added to mitochondria swollen to *low-amplitude* levels did not result in a reversal of the light-scattering signal although 100 mosmolar sucrose was present.

In the investigations of Stoner and Sirak (15) and Hunter et al. (17, 35) the amplitude of swelling was quite high as indicated by both the electron micrographs and the light-scattering decreases. Discontinuous outer membranes are a major criterion identifying high-amplitude swelling (1). In another investigation Stoner and Sirak (30) found it necessary to use low osmolar concentrations of glutaraldehyde and osmium tetroxide in order to preserve the swollen form of high-amplitude passively swollen mitochondria. These findings, and the results presented in this report regarding fixation of swollen, spherical mitoplasts, suggest that preservation of the configuration of high-amplitude swollen mitochondria by chemical fixation requires adjustment of the fixative media to lower osmolarities than routinely used. Apparently, special care must also be taken when extramitochondrial impermeants are present during fixation.

Those configurational transformations, however,

related to low-amplitude light-scattering changes, indicative of transformation between the condensed and the orthodox configurations, are well preserved by routinely prepared osmium tetroxide and glutaraldehyde fixatives of high osmolar concentrations (1–8, 11–14, 18) and are documented by the freeze-cleave results reported in this paper.

In conclusion, freeze cleaving which depends on vitrification for rapid physical fixation establishes unequivocally that isolated mitochondria undergo energy-linked gross ultrastructural transformation between the condensed and the orthodox configurations. These configurations which heretofore have been identified primarily by chemical fixation are thus natural structural states related to the functional activity of mitochondria.

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