OSMOTICALLY-INDUCED ALTERATIONS IN VOLUME AND ULTRASTRUCTURE OF MITOCHONDRIA ISOLATED FROM RAT LIVER AND BOVINE HEART

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ABSTRACT

Detailed studies correlating changes in mitochondrial optical density, packed volume, and ultrastructure associated with osmotically-induced swelling were performed. Various swelling states were established by incubating mitochondria (isolated in 0.25 M sucrose) at 0°C for 5 min in series of KCl and sucrose solutions ranging in tonicity from 250 to 3 milliosmols. Reversibility of swelling was determined by examining mitochondria exposed to 250 milliosmols media after they had been induced to swell. Swelling induced by lowering the ambient tonicity to approximately 130 (liver mitochondria) and 90 (heart mitochondria) milliosmols involves primarily swelling of the inner compartment within the intact outer membrane. Decreasing the ambient tonicity beyond this level results in rupture of the outer membrane and expansion of the inner compartment through the break. The maximum extent of swelling, corresponding with complete unfolding of the cristae and an increase in over-all mitochondrial volume of approximately 6-fold (liver mitochondria) and 11-fold (heart mitochondria), is reached at approximately 15 (liver mitochondria) and 3 (heart mitochondria) milliosmols. Exposure of liver mitochondria to media of lower tonicity results in irreversibility of inner compartment swelling and escape of matrix material. These changes appear to result from increased inner membrane permeability, possibly due to stretching.

INTRODUCTION

It is generally accepted that with regard to ultrastructure the mitochondrion consists of an outer membrane and an inner membrane, the outer membrane completely enclosing the inner membrane and the inner membrane completely enclosing an electron-opaque matrix. Thus, within the mitochondrion are the following two closed compartments: (*a*) the space between the outer and inner membranes (outer compartment) and (*b*) the space bounded by the inner membrane and occupied by the matrix (inner compartment). While suspended isotonically, the inner membrane is invaginated at several points to form cristae that protrude far into the matrix space (1).

It was recognized early in the study of isolated mitochondria that the structural and functional integrity of the mitochondrion is dependent upon the tonicity of the ambient medium (2). When ex-

posed to extremely hypotonic conditions the mitochondrion swells rapidly and extensively (3). During the swelling process, the outer membrane ruptures, the density of the matrix decreases, and the cristae of the inner membrane unfold and disappear as the inner compartment expands (4, 5). Extreme swelling results in partial release of the matrix components (5, 6,) indicating either rupture or increased permeability of the inner membrane.

Although the course of these osmotically-induced changes in mitochondrial ultrastructure has not previously been studied in detail, Tedeschi and Harris (3, 7) have thoroughly characterized the associated changes in mitochondrial volume. These workers demonstrated that mitochondria undergo reversible volume changes in response to alterations in the tonicity of the suspending medium, and showed that these changes occur in accordance with osmotic (Boyle-van't Hoff) law.

The results of the present study confirm the above findings and describe in detail the changes in mitochondrial ultrastructure associated with osmotically-induced changes in mitochondrial optical density and packed volume.

MATERIALS AND METHODS

Liver mitochondria were isolated according to a procedure modified from that of Schneider (8). Large male rats (200 g, Wistar strain) were fasted overnight, stunned with a sharp blow on the head, and sacrificed by cervical dislocation. The livers were rapidly removed, chilled in ice-cold 0.25 M sucrose, blotted, weighed, minced with a scissors in cold 0.25 M sucrose (3 ml/g tissue), and homogenized in a glass-Teflon homogenizer. The homogenate was diluted to 10 ml/g tissue with 0.25 M sucrose and centrifuged for 10 min at 600 *g* (maximum force) in a refrigerated centrifuge maintained at −5°C. The supernatant was saved, and the sediment was resuspended in 0.25 M sucrose (10 ml/g original tissue) and centrifuged as before. The supernatants were combined and centrifuged at 8500 *g* for 10 min. The upper "fluffy" layer of the resulting sediment was separated with a glass rod and removed by washing with fresh suspending medium. The middle layer, consisting primarily of the mitochondria, was separated from a relatively small bottom layer, was resuspended in 0.25 M sucrose, — 0.001 M ethylenediaminetetraacetate (EDTA), — 0.01 M Tris(hydroxymethyl) aminomethane, (Tris)-HCl, pH 7.5 (2.5 ml/mitochondria from 1 g of tissue), and was centrifuged at 8,500 *g* as before. The sediment was resuspended and resedimented in 0.25 M sucrose—0.01 M (Tris)-HCl, pH 7.5, as described in the preceding step. Final resuspension of the mitochondrial fraction was with the sucrose-Tris-HCl solution to give a final protein concentration of from 50 to 80 mg/ml, estimated by the biuret procedure of Gornall et al. (9). This suspension was adjusted to 50 mg mitochondrial protein/ml and was used immediately in the studies of osmotically-induced mitochondrial swelling and contraction.

Bovine heart mitochondria were isolated according to a slight modification (10) of the Nagarse procedure of Hatefi et al. (11). Final suspension of the isolated mitochondria was exactly as described above.

Determination of Optical Density and Packed Volume

Optical density was estimated at 520 mμ in a Model 6A Coleman spectrophotometer (Coleman Instruments Division, Perkin-Elmer Corporation, Maywood, Ill.). Packed volume was determined by using a modified centrifuge tube containing a narrow cylindrical reservoir of uniform bore into which mitochondria were sedimented from a much larger upper reservoir. A diagram of this tube, positioned as it was used in a Sorvall SS-34 centrifuge rotor, (Ivan Sorvall Inc., Norwalk, Conn.) is presented in Fig. 1. The tube was constructed by embedding a 0.065- in. piano wire, lightly coated with silicone grease, in a 1:7:28:35 mixture by weight of 2, 4, 6, Tris(dimethylaminomethyl)phenol (DMP-30): Dow epoxy resin 732 (DER 732): Dow epoxy resin 332 (DER 332) (Dow Corning Corp., Midland, Mich.): dodecenylsuccinic anhydride (DDSA) in a transparent 50 ml polycarbonate centrifuge tube.

Suspensions of mitochondria (0.5 mg protein/ml) were sedimented in the modified tube at 25,000 *g* (maximum force) at 0°C for 5 min. Packed volume (mm^3/mg mitochondrial protein) was calculated from the known amount of mitochondrial protein placed in the tube and the known diameter and the measured height of the column (corrected for slant of upper surface) of packed mitochondria in the small reservoir. Accurate estimates of column height were easily obtained by using a spring divider.

Electron micrographs of mitochondria sedimented at 25,000 *g* indicated that the contribution of extramitochondrial space to mitochondrial packed volume determined by the above procedure is very small, particularly with regard to swollen mitochondria. Although the mitochondria necessarily become extremely distorted in achieving this condition, there was no indication that mitochondrial volume is altered. It is assumed, therefore, that mitochondrial packed volume, determined as described, is a reasonably accurate measure of true mitochondrial volume.

Osmotically-Induced Swelling

Osmotically-induced swelling was studied at 0°C in suspensions containing 0.5 mg mitochondrial protein/

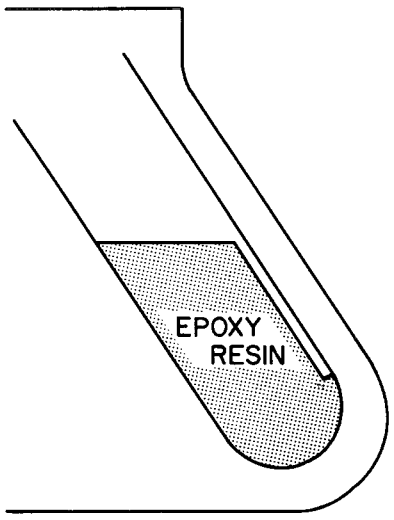

FIGURE 1 Diagram of a modified centrifuge tube of the type used in the determinations of mitochondrial packed volume.

ml and varying levels of KCl or sucrose. Suspension tonicities ranged from 3 to 243 millisomols, the lower limit being the osmotic contribution of the solutes (sucrose-Tris-HCl) added with the mitochondria. Swelling was initiated by rapidly mixing 0.1 ml stock mitochondrial suspension (50 mg mitochondrial protein/ml) with 9.9 ml of test solution. The test suspension was incubated for 5 min, after which the optical density was estimated and the determination of packed volume was initiated.

Ordinarily the mitochondria had been suspended 8–10 min by the time a centrifugal force of 25,000 *g* was reached in the determination of packed volume. Preliminary studies showing that there is little change in mitochondrial optical density after 5 min indicated that this disparity in time between the optical density and packed volume determinations is not important.

The pH of the test suspension varied within the range 6–7. Preliminary experiments established that varying the suspension pH over the range 6–8 has little affect on osmotically-induced swelling indicated by optical density and packed volume. Therefore, it was permissible to avoid the use of buffers and the difficulties in establishing the suspension tonicity that would have resulted therefrom.

Osmotic Reversal of Swelling

The osmotic reversal of osmotically-induced mitochondrial swelling was achieved by adding KCl or sucrose to test suspensions of mitochondria that had been allowed to swell in hypotonic media at 0°C for 5 min. In the study with sucrose, 0.1 ml of the stock mitochondrial suspension was added to 8.9 ml of test sucrose solution. At the end of the 5-min incubation period, 1 ml of cold sucrose solution of appropriate concentration to give a final suspension tonicity of 243 milliosmols was rapidly added and mixed thoroughly with the test suspension. The volume of the suspension and the concentration of mitochondrial protein in the suspension were thus brought to 10 ml and 0.5 mg/ml, respectively. The optical density of the suspension was determined one minute after the sucrose was added, followed immediately by determination of the packed volume. Preliminary studies established that changes in optical density induced by adding sucrose or KCl to suspensions of swollen mitochondria are complete within 1 min. The companion study, in which KCl served as the osmotic agent, was conducted in the same way as described above except that the stock mitochondrial suspension was added to 9.4 ml of KCl solution, and the addition of KCl at the end of the 5-min incubation period was made in 0.5 ml of solution.

Electron Microscopy

Mitochondria were fixed first with glutaraldehyde and then with OsO_4 while in suspension at 0°C. In the study of osmotically-induced swelling the mitochondria were suspended in KCl or sucrose as described before, except that all but the suspensions with the lowest and the next to lowest tonicities contained either 5 mM potassium barbital, pH 7.7, or 5 mM of the potassium salt of N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid (K-HEPES), pH 7.55. The suspensions with the lowest tonicities did not contain buffer other than that supplied with the mitochondria, and the suspensions with the next to lowest tonicities contained 2.5 mM buffer.

The barbital stock solution (0.05 M) was prepared by titrating barbituric acid with KOH to pH 7.7. Since the pK of barbital is approximately 7.7, the osmotic contribution of 5 mM of this buffer was assumed to be 7.5 milliosmols. The K-HEPES stock solution (0.05 M) was prepared by titrating HEPES with KOH to pH 7.55. Since at this pH all of the sulfonic acid groups and half of the amine groups are titrated (12), the osmotic contribution of 5 mM of this buffer was assumed to be 12.5 milliosmols.

Fixation was initiated by adding 0.1 ml of 15% glutaraldehyde to 10 ml of mitochondrial suspension (0.5 mg mitochondrial protein/ml) after the mitochondria had been allowed to swell at 0°C for 5 min (final glutaraldehyde concentration was approximately 5 mM). The mitochondria were allowed to undergo fixation for 5 min in the glutaraldehyde, after which the optical density was estimated. This was followed immediately by the addition of 0.1 ml of 4% OsO_4 to 3 ml of the suspension in order to initiate secondary fixation (final OsO_4 concentration was approximately 5 mM). A second addition of 0.1

ml of 4% OsO_4 was made about 15 min later. The mitochondria were then allowed to fix for an additional 30–60 min before they were sedimented, dehydrated, and embedded.

In preparing mitochondria for the study of osmotic reversibility of osmotically-induced swelling, mitochondria were allowed to swell in KCl or sucrose for 5 min, after which the swelling was reversed by adding sufficient solute to take the final suspension tonicity to 250 milliosmols, as described before except that 5 mM of either potassium barbital (pH 7.7) or K-HEPES (pH 7.55) was included in the suspending media. Sufficient buffer to take the final concentration to 5 mM was included in the KCl or sucrose solutions added after 5 min to reverse mitochondrial swelling in the suspensions with initial tonicities too low to accommodate 5 mM buffer initially. Glutaraldehyde was added 1 min after reversal of swelling was initiated and the mitochondria were allowed to undergo fixation for 5 min. This was followed by the determination of the optical density and secondary fixation with OsO_4, as described before.

Fixed mitochondria were sedimented in 400 μl polyethylene tubes in a Model 152 Microfuge (Beckman Instruments, Inc.) controlled by a variable transformer. The Microfuge was operated in a chest-type refrigerator maintained at −5°C. It was necessary to sediment extensively swollen mitochondria at very low centrifugal forces in order to prevent excessive damage due to packing. Thus, all of the suspensions having tonicities lower than 50 milliosmols were centrifuged while operating the Microfuge at 35 v. All other suspensions were centrifuged at 115 v.

The mitochondrial pellets were extremely fragile, necessitating the use of special handling techniques. Thus, the conical polyethylene Microfuge tubes were modified so that mitochondria could be easily dehydrated while still in the tubes and could be easily removed from the tubes without being deformed. This was achieved by polymerizing a small amount of epoxy resin mixture in the bottom of a tube, removing the epoxy plug, cutting away the curved upper portion of the plug, and replacing the plug in the tube with the flat side up. A mitochondrial pellet was formed on the flat epoxy surface in this tube by centrifuging 4–6 aliquots of mitochondrial suspension successively, each for 2–5 minutes. Sedimentation was complete within the time allotted. The size of the aliquots was adjusted so that the final pellet was not more than 1 mm thick. In this way a pellet consisting of several thin layers, each representing the entire mitochondrial preparation, was formed so that the entire range of particle types and particle sizes could be examined in a single thin section.

The pellet was rapidly dehydrated at 0°C with a graded series of ethanol solutions and was allowed to warm to room temperature while being removed from the tube. The pellet was removed by cutting away the upper portion of the tube just above the pellet and the lower portion of the tube and epoxy plug a few millimeters below the pellet. The remaining portion of the epoxy plug, on which the pellet rested, was pushed out of the remaining portion of the tube while immersed in ethanol, and the pellet was gently separated from the epoxy surface with a thin spatula. The ethanol was replaced with a propylene oxide and the pellet was embedded in a 1:11:17:28 mixture by weight of DMP-30:DER 732:DDSA:DER 332. The embedding procedure was essentially as described by Lockwood (13), except that polymerization was achieved by incubating the resin at 60°C for 3 days. Final embedding was in a polyethylene flat embedding mold (LKB Instruments, Inc.).

Thin sections were cut on an LKB Ultrotome I with glass knives. The sections were mounted on grids coated with carbon-stabilized parlodion film, stained with uranyl acetate (by floating the grids on a freshly prepared 1:1 mixture of 4% aqueous uranyl acetate and absolute ethanol, in the dark for 15 min) and lead citrate (14), and examined in a JEM-7 electron microscope (Japan Electron Optics Laboratory Co., Ltd., Medford, Mass.) operating at 80 kv, and with a 20 μ molybdenum objective aperture.

Glutaraldehyde was prepared by vacuum distillation of 50% biological grade glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) and quantitated spectrophotometrically as described by Anderson (15). The pH of the buffered mitochondrial suspensions before glutaraldehyde was added was about 7.5; the pH after glutaraldehyde was added ranged from 7.2 to 7.4. The pH of the buffered suspensions 30–60 min after the second addition of osmium tetroxide ranged from 6.7 to 7.0.

The adequacy of 0.15% glutaraldehyde to fix mitochondria suspended at a concentration of 0.5 mg mitochondrial protein/ml was established in preliminary experiments in which the concentration-effectiveness of the fixative in preventing osmotically-induced mitochondrial swelling was determined. This was achieved by (1) suspending mitochondria at 100 milliosmols tonicity, (2) adding various amounts of glutaraldehyde to aliquots of this suspension, (3) diluting the suspensions 2-fold with water after a suitable incubation period in the presence of fixative, and (4) estimating the optical densities of the diluted suspensions. Fixation was considered to be best in suspensions having the highest optical densities. It was found that optical densities were maximal in suspensions in which the mitochondria had been exposed to initial concentrations of glutaraldehyde as low as 0.15%. A similar study showed that maximum fixation (as indicated by resistance to optical density change upon diluting the suspension) of mitochondria

exposed to 0.15% glutaraldehyde is achieved in less than 1 min.

RESULTS

Osmotically-Induced Swelling

Fig. 2 shows typical changes in mitochondrial optical density and packed volume associated with osmotically-induced swelling of mitochondria from rat liver and bovine heart in KCl and in sucrose. In general, swelling induced osmotically by decreasing the tonicity of the suspending medium over the range 243 to 3 milliosmols is associated with decreases of about 80 and 55% in mitochondrial optical density and increases of 5- to 6-fold and 10- to 12-fold in mitochondrial packed volume with mitochondria from liver and heart, respectively. Most of these changes occur over the range 100–10 millisomols in liver mitochondria and over the range 80–3 milliosmols in heart mitochondria. With regard to liver mitochondria, packed volume reaches a maximum within the approximate range 6–10 milliosmols and then declines slightly as tonicity is lowered further, possibly indicating escape of intramitochondrial solutes.

Other than differences in optical density due to the unequal effect of equiosmol levels of KCl and sucrose on the ability of the mitochondria to scatter light (3), no significant differences were noted between packed volume and optical density results obtained in KCl and sucrose. Different preparations of liver mitochondria varied considerably in maximum extent of swelling as indicated by packed volume. The extent of this variation was established in 10 determinations (5 each in sucrose and in KCl), averaging 14.0 mm^3/mg mitochondrial protein and varying within the extremes of 12.0 and 15.5 mm^3/mg. With regard to differences in the sucrose and KCl optical density results of Fig. 2, correcting the observed values for differences in the refractive indices of the two media largely eliminates the differences, particularly at high tonicities (cf. Fig. 10).

Osmotic Reversal of Swelling

Fig. 3 shows the extent to which the optical density and packed volume changes associated with osmotically-induced swelling of heart and liver mitochondria can be reversed. In contrast to what was observed in the study of mitochondrial swelling, results of studies on reversibility of swelling differed considerably depending on whether sucrose or KCl was the suspending solute. In sucrose, reversal of packed volume changes associated with swelling are relatively incomplete and, in the case of heart mitochondria, the optical density of mitochondria exposed initially to hypotonic solutions having tonicities within the range 60–140 milliosmols is higher than that of mitochondria exposed initially to sucrose solutions of tonicity higher than 140 milliosmols.

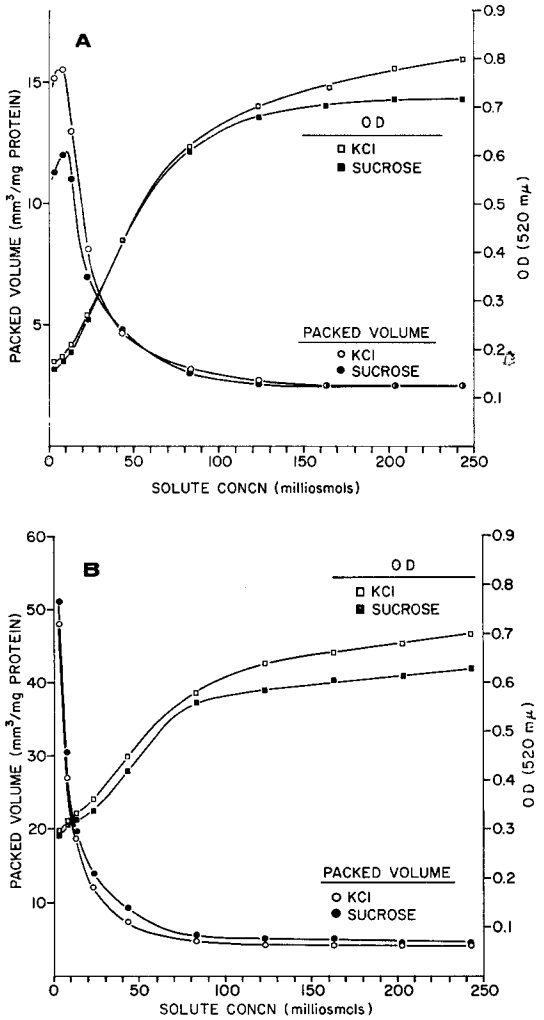

FIGURE 2 *a* and *b* Mitochondrial optical density and packed volume as functions of the tonicity of the suspending medium. Each set of optical density and packed volume data represents duplicate determinations on mitochondria from one animal. (*a*) rat liver mitochondria; (*b*) bovine heart mitochondria.

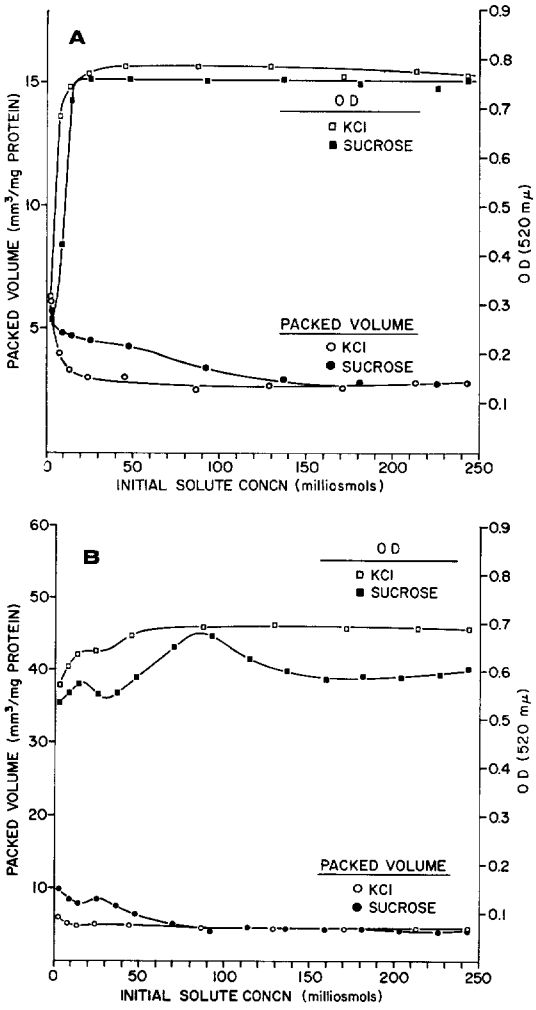

FIGURE 3 *a* and *b* Reversibility of optical density and packed volume changes associated with osmotically-induced mitochondrial swelling (Fig. 2). Each set of optical density and packed volume data represents duplicate determinations on mitochondria prepared from one animal. (*a*) rat liver mitochondria; (*b*) bovine heart mitochondria.

Companion studies revealed that when a small amount (5 mM) of potassium barbital (pH 7.7) or K-HEPES (pH 7.55) is included in the sucrose medium results similar to those obtained in KCl are observed, suggesting that the results obtained with sucrose-suspended mitochondria are in error. It will be shown subsequently that even a slight increase in overall mitochondrial volume, as indicated by packed volume, results in rupture of the outer mitochondrial membrane. Perhaps this irreversible change modifies the peripheral charge characteristics of the mitochondria in such a way as to make them more resistant to centrifugal packing and/or more susceptible to aggregation in media of low ionic strength.

Osmotically-Induced Changes in Ultrastructure

In preparing the mitochondria for electron microscopy it was necessary to include a small amount of buffer in the suspending medium to counter the acid released during osmium tetroxide fixation. Of the buffers tested (barbital, HEPES, and phosphate), all appeared to penetrate the mitochondria as evidenced by a slight shift to the right in the optical density and packed volume curves. This shift is evident in the curves (Fig. 4) defining the swelling state of the glutaraldehyde-fixed mitochondria from which electron micrographs were obtained. Glutaraldehyde alone at the low concentration used (0.15%) appeared to have no effect on mitochondrial volume. However, when fixation was carried out in KCl, glutaraldehyde prevented packed volume determinations by making the mitochondria too sticky to sediment into the capillary of the modified centrifuge tube.

Several mitochondrial preparations from both liver and heart suspended in both sucrose and KCl at the 12 tonicities indicated by the points defining the "swelling not reversed" and "swelling reversed" curves of Fig. 4 were examined in the electron microscope. Minor differences in mitochondrial ultrastructure were noted, depending on whether the mitochondria were suspended in sucrose or KCl. These differences account for the selection of KCl-suspended liver mitochondria and sucrose-suspended heart mitochondria for presentation here.

KCl was the preferred medium for detailed ultrastructural studies in liver mitochondria solely because the residual cristae of swollen mitochondria were slightly more dilated when prepared in sucrose, giving them a slightly less "orthodox" appearance. The selection of sucrose as the preferred medium for detailed ultrastructural studies on heart mitochondria was based on more severe differences. Thus, the ultrastructural characteristics of heart mitochondria prepared in KCl were generally less distinct than comparable sucrose preparations, and in highly contracted mitochondria prepared in KCl, large variations occurred in

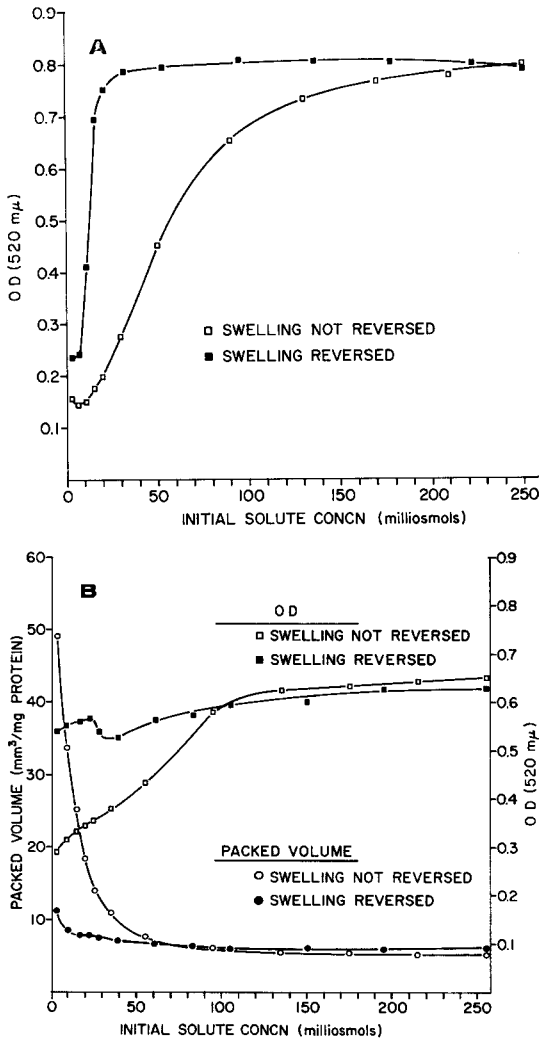

FIGURE 4 *a* and *b* Estimates of mitochondrial optical density and packed volume as functions of the initial tonicity of the suspending medium obtained after the mitochondria had been exposed to 0.15% glutaraldehyde. Liver mitochondria (*a*) were suspended in KCl buffered with potassium barbital (pH 7.7). Heart mitochondria (*b*) were suspended in sucrose buffered with K-HEPES (pH 7.55). Each set of data represents single determinations on mitochondria from one animal. These preparations were subsequently used for determinations on mitochondrial ultrastructure (Figs. 5–7, 9).

the size of the intracristal spaces. Some of the larger of these spaces contained large vesicles which appeared to have derived from the inner membrane as a result of localized expansion of the inner compartment. Apart from this apparent artifact of fixation, however, the ultrastructural characteristics of mitochondria prepared in the two media differed very little.

Figs. 5 *a–f* present electron micrographs of liver mitochondria that were fixed while suspended at tonicities ranging from 250 to 6 milliosmols. While suspended at 250 milliosmols (Fig. 5 *a*), liver mitochondria have intact outer membranes and extremely dilated cristae that appear in transverse section as angular clear spaces in background of dense matrix material. The inner compartments of some, if not all, are separated from the outer membrane by considerable distances, leaving clear spaces, which in some cases appear to make up about a third of the total mitochondrial volume.

In agreement with the packed volume data of Fig. 2, very little change occurs in the overall volume of liver mitochondria exposed to media of tonicity as low as 130 milliosmols (Fig. 5 *b*). However, it is evident that a considerable increase occurs in the volume of the inner compartment (matrix space) at the expense of a decrease in the volume of the outer compartment (space between the inner and outer membranes, including the intracristal space). In approximately half of the mitochondria suspended at 130 milliosmols outer compartment volume is minimal, and it is apparent that further expansion of the inner compartment would result in rupturing or stretching the outer membrane.

Fig. 5 *c* shows that the outer membranes of mitochondria suspended at 50 milliosmols are ruptured and that the inner compartments are expanded through the breaks to form bleb-like protrusions outside the bounds originally delineated by the outer membranes. Also the number and size of the cristae are distinctly diminished, indicating that expansion of the inner compartment outside the original bounds of the mitochondrion occurs by means of unfolding of the cristae. At 30 milliosmols (Fig. 5 *d*) the inner compartments are expanded into ellipsoidal vesicles, with only a few residual cristae remaining. The ruptured outer membranes of some of the mitochondria are very loosely attached, and it appears that the outer membranes of some are about to become detached.

The inner compartments of liver mitochondria suspended at 16 milliosmols (Fig. 5 *e*) are grossly expanded. Cristae are essentially absent, and slight differences in matrical density are evident. At 6 milliosmols (Fig. 5 *f*) matrical density is consider-

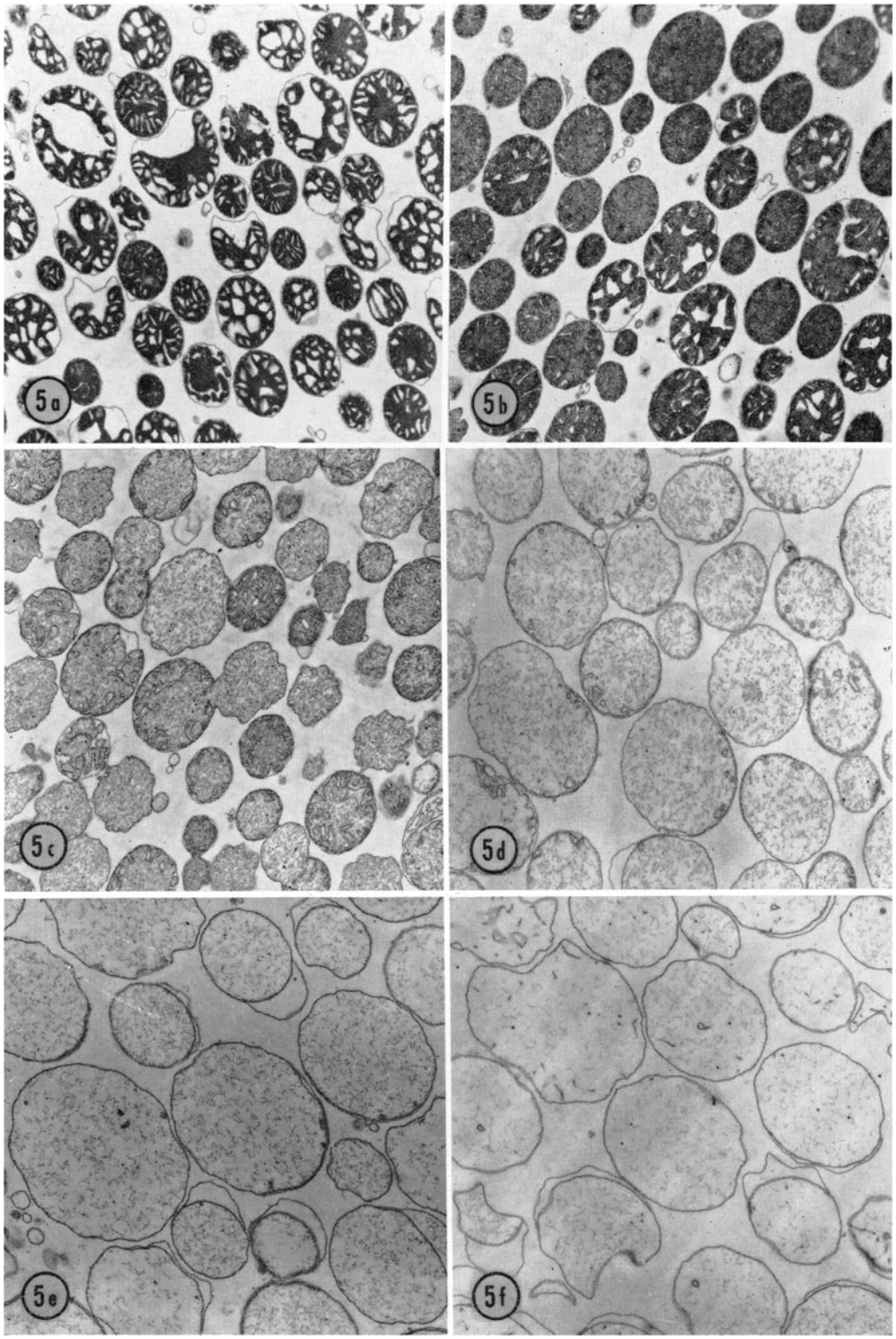

FIGURE 5 *a–f* Ultrastructural changes associated with osmotically-induced swelling of rat liver mitochondria. Mitochondria were fixed while suspended in buffered KCl solutions (Fig. 4) of the following tonicities: (*a*) 250 milliosmols; (*b*) 130 milliosmols; (*c*) 50 milliosmols; (*d*) 30 milliosmols; (*e*) 16 milliosmols; and (*f*) 6 milliosmols. × 10,000.

ably diminished. Escape of matrix material from extremely swollen liver mitochondria was indicated in Fig. 2 where it was shown that a decrease in ambient tonicity from about 10 to 3 milliosmols results in a slight decrease in mitochondrial packed volume. In agreement with these findings are the results of ultrastructural studies on the irreversibility of osmotically-induced swelling. Thus, Fig. 6 shows that liver mitochondria exposed to 250 milliosmols tonicity, after undergoing initial swelling in 3 milliosmols media, are largely devoid of matrix material. In addition, the inner membranes of those mitochondria that have lost most of their matrix material have obviously been rendered permeable to the suspending solute as indicated by a low degree of contraction. Figs. 6 *b* and 6 *c* show that as the initial tonicity is raised to 21 milliosmols the proportion of mitochondria undergoing this transition decreases to a low level.

The KCl packed volume curve of Fig. 3 indicates that reversal of swelling of liver mitochondria suspended at initial tonicities greater than about 25 milliosmols is complete. In good agreement with this observation, electron micrographs of liver mitochondria reversed from swelling in media of 32, 95, and 136 milliosmols tonicity (Figs. 5 *d*, *e*, and *f*, respectively) show that inner compartment swelling is completely reversed. However, it is clear from Figs. 6 *d* and 6 *e* that despite complete reversibility of inner compartment swelling, the swelling changes which occur at tonicities below 130 milliosmols (Figs. 5 *c–f*) involving rupture of the outer membrane and extrusion of the inner compartment through the break are not osmotically reversible. Swelling of the inner compartment within the intact outer membrane at tonicities as low as about 130 milliosmols is, however, completely reversible. This is demonstrated by Fig. 6 *f* showing mitochondria exposed initially to media of 136 milliosmols tonicity.

Figs. 7 *a–f* present electron micrographs of bovine heart mitochondria which were fixed while suspended at tonicities ranging from 255 to 9 milliosmols. While suspended at 255 milliosmols (Fig. 7 *a*), heart mitochondria have extremely dilated cristae. The matrix is sandwiched between the cristae to form what appear at low magnification to be thin, dense ribbons of material which intersect and traverse the interior regions of the mitochondria. Many of the mitochondria appear to be completely circumscribed by a thin band of matrix that is continuous with the dense ribbons of matrix material traversing the interior region.

The outer membranes are severely fragmented, apparently due to collisions between the particles after they had been fixed with osmium tetroxide and before they had been formed into a stable pellet. Thus, outer membranes are intact in mitochondria fixed with osmium tetroxide after sedimentation. This is demonstrated in Fig. 8, which presents an electron micrograph of heart mitochondria prepared in the same way as those of Fig. 7 *a* except that osmium tetroxide fixation was brought about by exposure of the mitochondria to OsO_4 vapors after they had been sedimented. Although, as Fig. 8 shows, this procedure is suitable for demonstrating intact outer mitochondrial membranes, it was not suitable for general use in these studies because of the inability to provide sufficient buffer to neutralize the acid released during osmium tetroxide fixation without significantly changing the tonicity of the ambient medium. Osmium tetroxide fixation under acidic conditions was found to induce extreme distortions in the inner mitochondrial compartments, particularly in relatively fragile, swollen mitochondria. Some distortion, and perhaps also disruption, is evident in the inner compartments of the mitochondria of Fig. 8.

Figs. 7 *b* through 7 *f* show that qualitatively the same osmotically-induced swelling changes take place in the ultrastructure of heart mitochondria as was described above for liver mitochondria. Thus, swelling initially involves primarily swelling of the inner mitochondrial compartment within the intact outer membrane without appreciably changing overall mitochondrial volume (Figs. 7 *b* and 7 *c*). This is followed by rupture of the outer membrane and expansion of the inner compartment through the break, with concomitant dilution of the matrix and unfolding of the cristae (Figs. 7 *d–f*).

In agreement with the packed volume data of Fig. 2, Figs. 7 *a–c* show that very little change in the overall volume of heart mitochondria occurs as the ambient tonicity is decreased to levels as low as about 95 milliosmols. However, upon lowering the tonicity to 55, 20, and 9 milliosmols (Figs. 7 *d–f*, respectively), overall mitochondrial volume increases by factors of approximately 1.5, 4.0, and 6.0, respectively. During the swelling process, residual cristae tend to be localized on the side of the inner compartment with which the ruptured

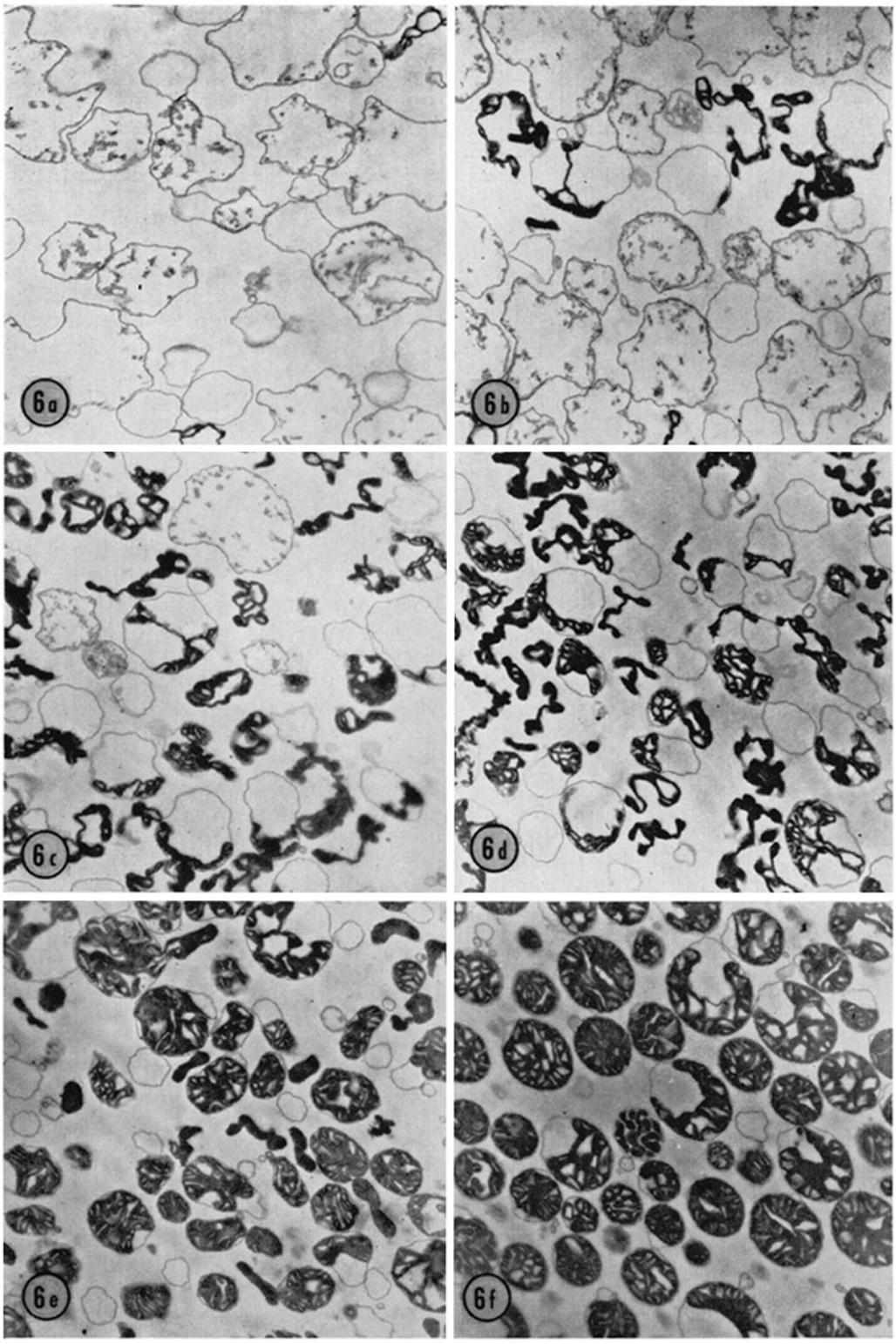

FIGURE 6 *a–f* Reversibility of ultrastructural changes associated with osmotically-induced swelling of rat liver mitochondria (Fig. 5). Various swelling states were established initially by incubating the mitochondria in buffered KCl solutions ranging in tonicity from 3 to 250 milliosmols. The mitochondria were then induced to contract by rapidly adding sufficient solute to take the final ambient tonicity to 250 milliosmols (Fig. 4). Initial tonicities were: (*a*) 3 milliosmols; (*b*) 11 milliosmols; (*c*) 21 milliosmols; (*d*) 32 milliosmols; (*e*) 95 milliosmols; and (*f*) 136 milliosmols. × 10,000.

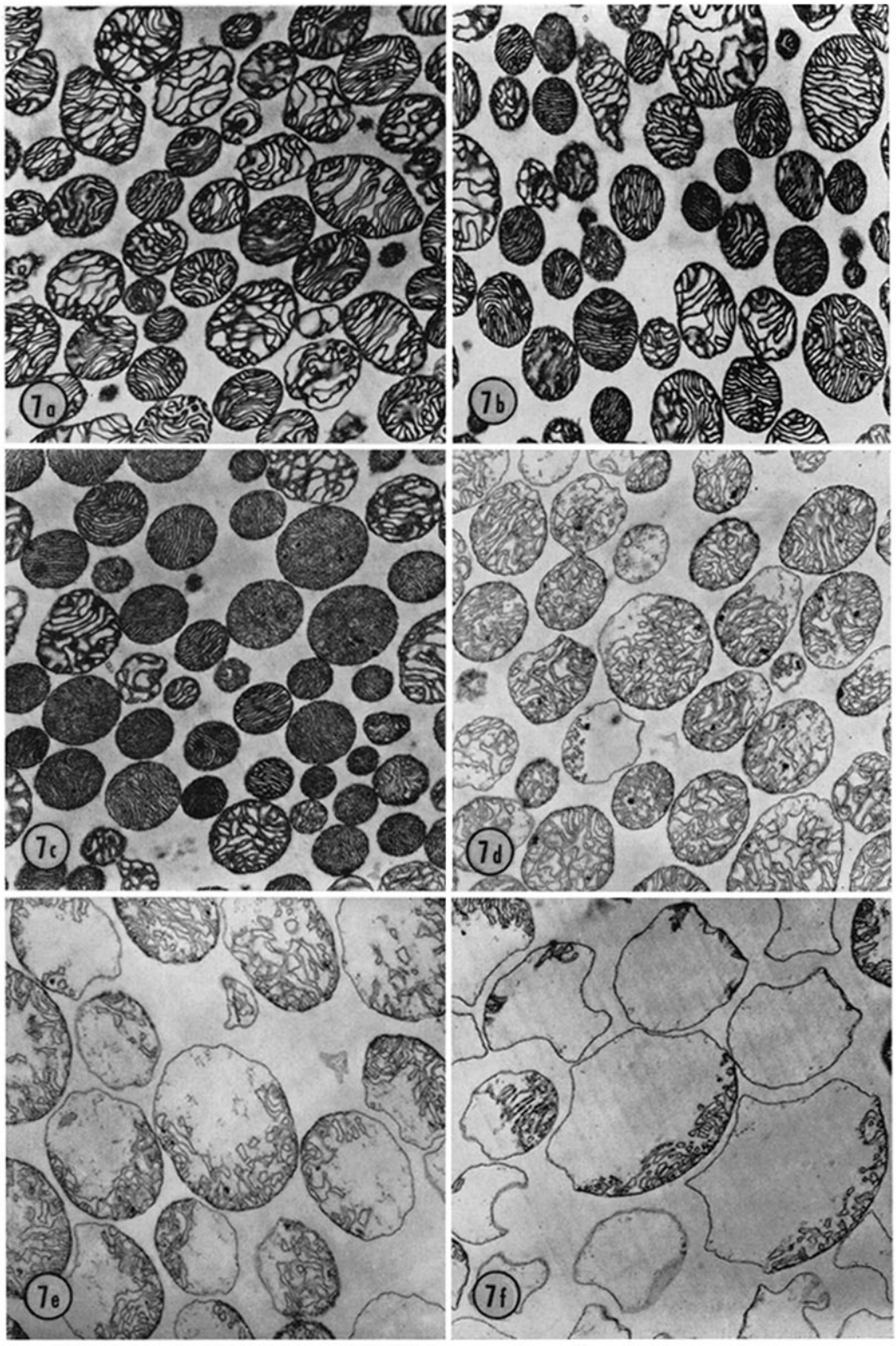

FIGURE 7 *a–f* Ultrastructural changes associated with osmotically-induced swelling of bovine heart mitochondria. Mitochondria were fixed while suspended in buffered sucrose solutions (Fig. 4) of the following tonicities: (*a*) 255 milliosmols; (*b*) 135 milliosmols; (*c*) 95 milliosmols; (*d*) 55 milliosmols; (*e*) 20 milliosmols; and (*f*) 9 milliosmols. × 10,000.

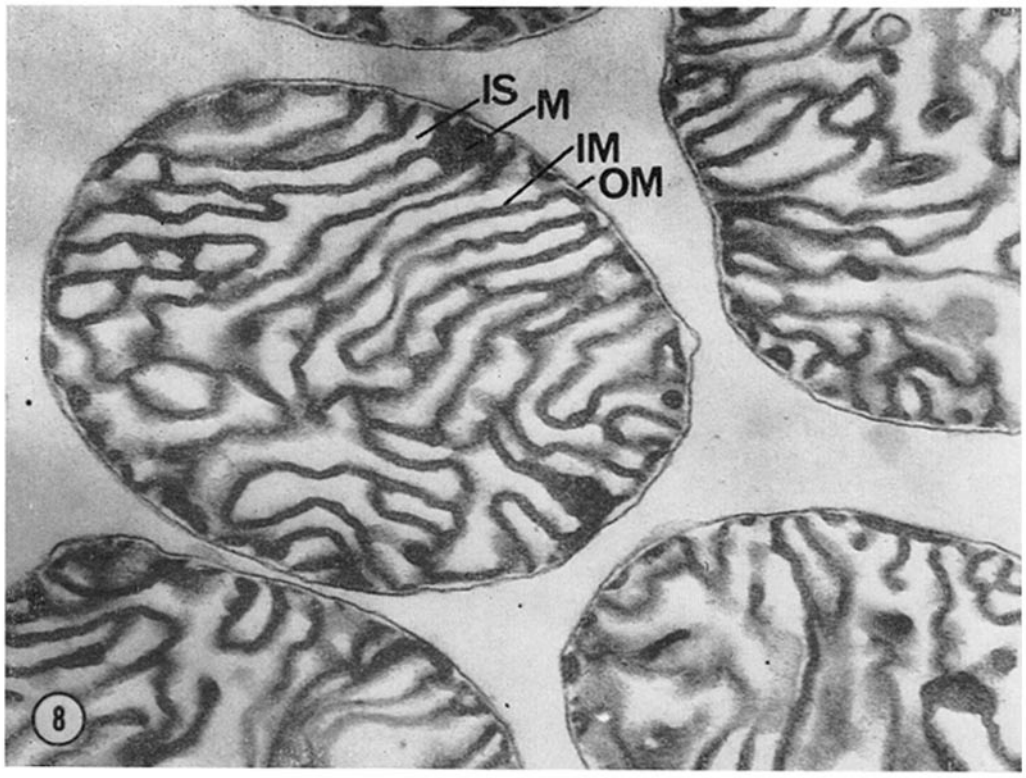

FIGURE 8 Demonstration of intact outer membranes in bovine heart mitochondria that were suspended and fixed in the same way of those of Fig. 7 *a* except that osmium tetroxide fixation was brought about by exposure of the mitochondria to OsO_4 vapors for 30 min at 0°C after they had been sedimented. Intracristal space, *IS*; matrix (inner compartment space), *M*; inner membrane, *IM*; outer membrane, *OM*. × 40,000.

outer membrane is associated, indicating that the affinity of the outer and inner membranes for each other is sufficient to provide resistance to unfolding of the cristae.

Although not shown, electron micrographs of mitochondria fixed while suspended at 3 milliosmols were also examined. It was evident that the extremely swollen mitochondria obtained in this medium (volume increased 10- to 12-fold) had been grossly distorted by collisions between the extremely fragile particles during sedimentation and possibly also by acid released during osmium tetroxide fixation. It could be seen, however, that the cristae had completely unfolded and that the ruptured outer membranes had not become dissociated from the inner membranes.

In contrast to what was observed with liver mitochondria, the inner membranes of extremely swollen heart mitochondria appear to retain their normal permeability characteristics. This is indicated in Figs. 9 *a–f* showing electron micrographs of heart mitochondria exposed to initial tonicities ranging from 3 to 105 milliosmols, followed by exposure to 255 milliosmols media. Thus, retention of matrix material and reversibility of inner compartment swelling appear to be complete, even in mitochondria exposed initially to 3 milliosmols media. At low magnification mitochondria exposed initially to the 3 milliosmols medium (Fig. 9 *a*) appear in cross-section to be disorganized strands or ribbons of matrix material identical to those observed in mitochondria suspended at 255 milliosmols (Fig. 7 *a*), except for the high degree of disorganization. Portions of some of these strands are organized in somewhat the same way as those of intact mitochondria, and it can be seen that some of these relatively organized portions are associated with the ruptured outer membrane.

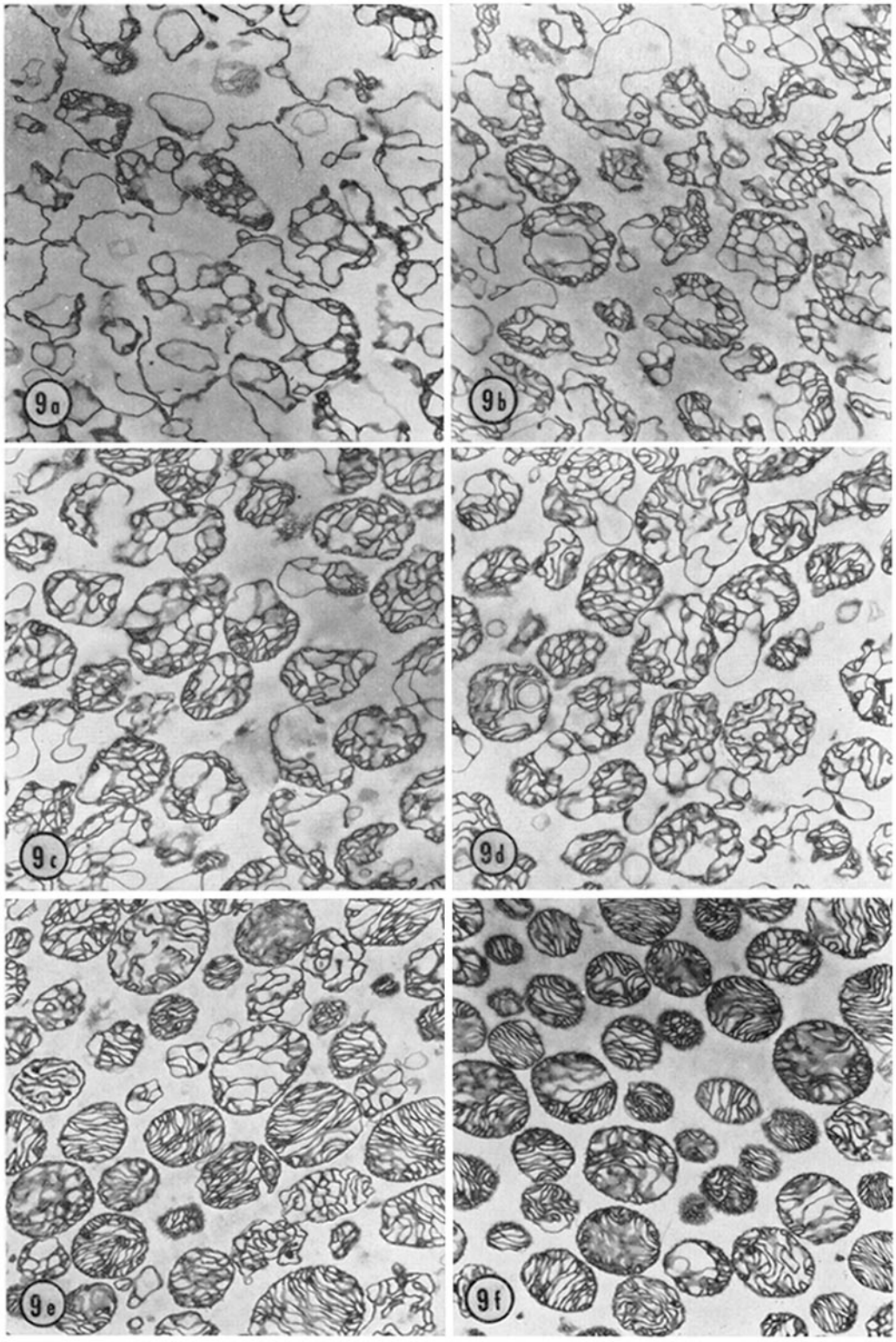

FIGURE 9 *a–f* Reversibility of ultrastructural changes associated with osmotically-induced swelling of bovine heart mitochondria (Fig. 7). Various swelling states were established initially by incubating the mitochondria in buffered sucrose solutions ranging in tonicity from 3 to 255 milliosmols. The mitochondria were then induced to contract by rapidly adding sufficient solute to take the final tonicity to 255 milliosmols (Fig. 4). Initial tonicities were: (*a*) 3 milliosmols; (*b*) 10 milliosmols; (*c*) 22 milliosmols; (*d*) 39 milliosmols; (*e*) 83 milliosmols; and (*f*) 105 milliosmols. × 10,000.

Micrographs of mitochondria suspended initially at 10, 22, 39, 83, and 105 milliosmols (Figs. 9 *b* through 9 *f*, respectively) show structures with increasing degrees of organization of the ribbons of matrix material, until at 105 milliosmols mitochondria indistinguishable from those suspended initially at 255 milliosmols are observed.

The degree of reorganization which occurs upon osmotically-induced contraction of extremely swollen heart mitochondria appears to be greatest on the side or portion of the inner compartment with which the ruptured outer membrane is associated. It was shown in Fig. 7, that, in the extremely swollen inner compartment, residual cristae are localized on the side that retains a close association with the outer membrane. From these observations it is apparent that upon contraction the residual cristae provide a pattern for reestablishment of relatively normal inner compartment configuration.

Osmotically-Induced Swelling and Osmotic Law

Tedeschi and Harris (3) have established that mitochondria from rat liver undergo osmotically-induced swelling and contraction in accordance with the Boyle-van't Hoff relation:

$$V = K\ 1/C + b$$

where V is the measured volume, C the osmotic pressure (measured as concentration), b the volume which does not undergo change (osmotic dead space), and K a constant. Thus, they showed mitochondrial volume to be a linear function of the reciprocal of the concentration of nonpenetrating solute in the suspending medium, as the law predicts for a perfect osmometer. Tedeschi and Harris (3) also demonstrated that, under conditions of constant mitochondrial concentration and constant refractive index of the suspending medium, this relationship still holds if the reciprocal of the optical density (1/OD) is substituted for V.

Fig. 10 shows our data of Fig. 2 to be in good agreement with the above findings with regard to both optical density and packed volume estimates. Our data reveal in addition, however, that the slopes of the linear curves describing the relationship of $1/C$ to 1/OD and mitochondrial volume change at specific points over the range of tonicities examined. Thus, changes in slope of the relationship $1/C$ vs. 1/OD occur at about 110 and 17 milliosmols with liver mitochondria and at about 28 milliosmols with heart mitochondria. The increase in slope at approximately 110 milliosmols corresponds fairly well with the tonicity at which the outer membrane of the liver mitochondrion ruptures, and therefore might be explained in terms of the ultrastructural transformations associated with this event (Fig. 5). However, the relatively abrupt decreases in slope of the $1/C$ vs. 1/OD relationship at 17 and 28 milliosmols in mitochondria from liver and heart, respectively, appear to have no ultrastructural basis. Furthermore, contrary to what one might expect, it is clear from the packed volume data that these abrupt changes do not correspond with points at which the mitochondria reach their swelling limit.

Two linear regions appear in the plots of $1/C$ vs. mitochondrial packed volume (Fig. 10); however, only one involves a change in overall mitochondrial volume. A linear region with zero slope is observed between 243 and approximately 140 milliosmols with liver mitochondria and between 243 and approximately 110 milliosmols with heart mitochondria (consistently observed in the KCl medium only). The electron micrographs of Figs. 5 and 7 showed that these are the approximate tonicity ranges over which swelling of the inner compartment occurs without affecting overall mitochondrial volume.

When the ambient tonicity is decreased beyond the point at which expansion of the inner compartment is detectable as an increase in overall mitochondrial volume, mitochondrial packed volume increases as a linear function of $1/C$ to tonicities as low as about 13 milliosmols with liver mitochondria, and as low as about 8 milliosmols with heart mitochondria. With regard to liver mitochondria, the low end of this linear range corresponds closely with the point at which the cristae become completely unfolded as revealed by electron microscopy (Fig. 5 *e*). The cristae of heart mitochondria, however, unfold completely only at tonicities closely approaching 3 milliosmols. Therefore, deviation from linearity in the plot $1/C$ vs. mitochondrial packed volume at approximately 8 milliosmols probably occurs as a result of the inner compartment osmotic pressure not being high enough to completely match resistance to unfolding of the residual cristae.

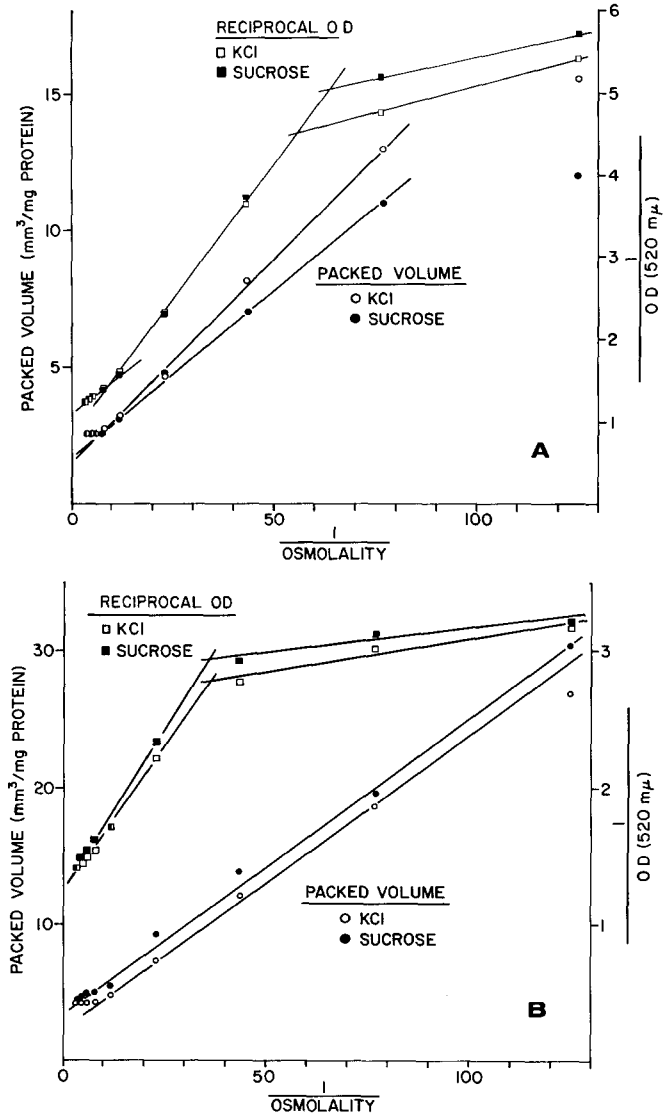

FIGURE 10 *a* and *b* Relationship of mitochondrial packed volume and reciprocal optical density to reciprocal osmolality of the suspending medium. The data presented here are identical to those of Fig. 2 except that optical densities were corrected according to the equation: $ODc = ODobs/(1 - \Delta RI \times 8.6)$ where *ODc* is the corrected optical density, *ODobs* is the observed optical density, and ΔRI is the difference between the refractive indices of the suspending medium and pure water. This correction is based on the observation (3, 7) that the optical density of intact rat liver mitochondria decreases 0.86% per 0.001 increase in ΔRI. (*a*) rat liver mitochondria; (*b*) bovine heart mitochondria.

DISCUSSION

The principal objectives of this study were (*a*) to determine the relationship between mitochondrial optical density, packed volume, and ultrastructure, (*b*) to determine the changes in these parameters during osmotically-induced swelling, and (*c*) to determine the osmotic reversibility of the osmotically-induced swelling changes. Our primary reason for undertaking this work was to provide a firm basis for interpreting energy-linked changes in mitochondrial volume and ultrastructure. The feasibility of achieving this objective by means of the present approach was indicated by the preliminary observation that passive swelling and contraction changes in mitochondrial ultrastructure of the type reported here are very similar to those which occur during energy-linked swelling and contraction. In view of this observation and in view of the observation by Azzi and Azzone (16 that ions are accumulated and extruded in unison with energy-linked swelling and contraction, it seemed likely that active changes in mitochondrial volume and ultrastructure occur by an osmotic mechanism, which, in effect, does not differ appreciably from the mechanism of passive osmoti-

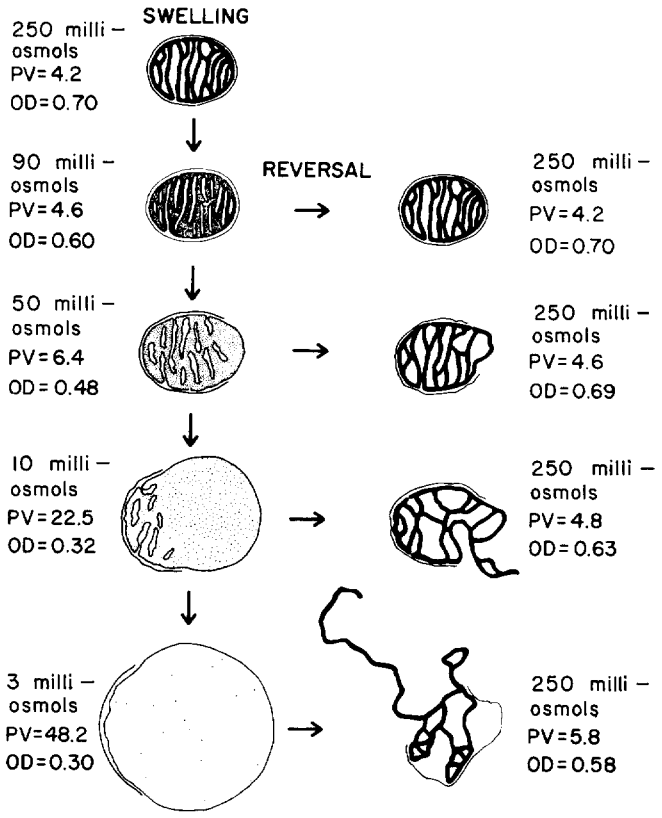

FIGURE 11 Summary of packed volume, optical density, and ultrastructural changes associated with osmotically-induced swelling and contraction of rat liver mitochondria. Packed volume (*PV*) and optical density (*OD*) data were obtained from the KCl curves of Figs. 2 and 3.

cally-induced swelling and contraction. Further support for this interpretation was provided when it was found that the same passive osmotically-induced changes occur in mitochondrial optical density, packed volume, and ultrastructure regardless of whether the mitochondria are activated for maximal energy-linked contraction.

Figs. 11 and 12 summarize the pertinent findings of this investigation with mitochondria from liver and heart, respectively. In general, osmotically-induced changes in mitochondrial ultrastructure were found to be in good agreement with changes in mitochondrial optical density and packed volume. Thus, changes in mitochondrial optical density, indicating changes primarily of matrical density, and mitochondrial packed volume, indicating changes in over-all mitochondrial volume, were found to correspond very well with changes in matrical density and over-all mitochondrial volume detected by means of electron microscopy. This attests to the validity of using the relatively simple optical density and packed volume techniques to follows changes in mitochondrial volume.

In addition to achieving the primary objectives outlined above, this work provides a detailed comparison of mitochondria from liver and heart. This aspect of the investigation is particularly important to the understanding of heart mitochondria because relatively few previous studies have been devoted to the characterization of heart mitochondria with respect to osmotically-induced changes in volume and ultrastruture. From the observations summarized in Figs. 11 and 12, it is clear that the basic architecture of the heart mitochondrion is essentially identical to that of the liver mitochondrion as originally described by Palade (1). Thus, both have an outer membrane that encloses a highly invaginated inner membrane which, in turn, encloses an electron-opaque matrix. The present work further demonstrates that upon being exposed to hypotonic media the two types of mitochondria undergo similar changes in optical density, packed volume, and ultrastructure. Both types have outer membranes that are readily penetrated by sucrose and KCl (indicated by rapid osmotic response of the inner compartments of in-

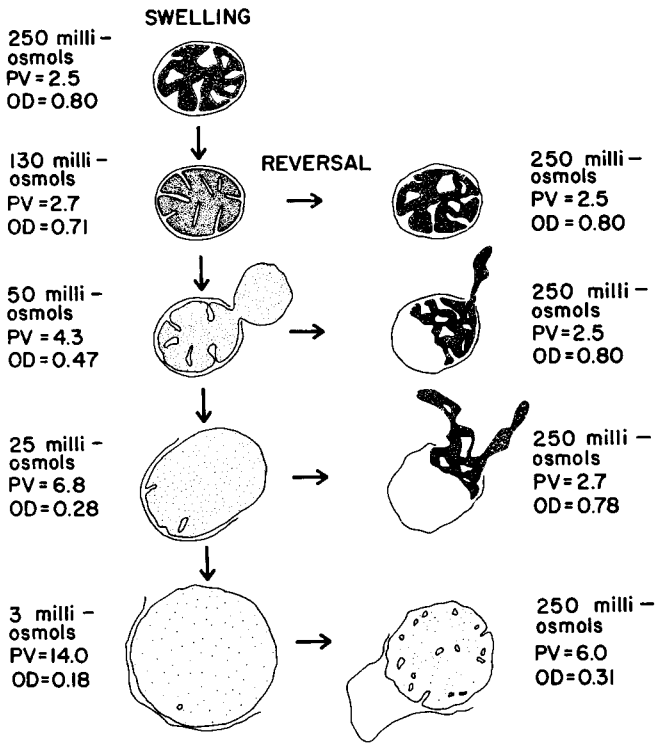

FIGURE 12 Summary of packed volume, optical density, and ultrastructural changes associated with osmotically-induced swelling and contraction of bovine heart mitochondria. Packed volume (*PV*) and optical density (*OD*) data were obtained from the KCl curves of Figs. 2 and 3.

tact mitochondria to changes in external tonicity), and both types have inner membranes that are, in effect, completely impermeable to these solutes under the conditions of low temperature and short-term incubations employed.

From the standpoints taken in this work, the only striking differences observed between heart and liver mitochondria are quantitative in nature. From the electron micrographs of intact mitochondria suspended at 250 milliosmols tonicity it is clearly evident that, although liver and heart mitochondria do not differ appreciably in size, they differ considerably in the amount of inner membrane and matrix material they contain. Heart mitochondria are relatively rich in inner membrane material, giving them relatively great potential for undergoing inner compartment swelling. Thus, upon lowering the ambient tonicity from 243 to 3 milliosmols, heart mitochondria increase 10- to 12-fold in volume, whereas rat liver mitochondria increase only 5- to 6-fold in volume. Heart mitochondria are relatively poor in the amount of matrix material they contain. This difference is manifest, among other ways, as a relatively low mitochondrial density based on the amount of protein per unit packed volume, and as a relatively small change in mitochondrial optical density as the matrix is diluted or concentrated during osmotically-induced swelling or contraction. Thus, the densities of isotonically-suspended miotchondria were found to be 0.24 and 0.40 mg protein/mm^3 and the decreases in optical density occurring as a result of swelling maximally in extremely hypotonic media (3 milliosmols) were found to be 57 and 78% in mitochondria from heart and liver, respectively.

From the results of the studies on the reversibility of osmotically-induced swelling it is clear that the inner membranes of heart mitochondria suffer relatively little damage as a result of being exposed to extremely hypotonic media. This difference can readily be accounted for in terms of the relatively high swelling capacity and the relatively low level of matrix material in heart mitochondria.

It is apparent from the electron micrographs that heart mitochondria also differ from liver mitochondria in that they have more fragile outer membranes. This difference is indicated most convincingly by differences in the way the inner compartments of the two types of mitochondria swell after the outer membrane is ruptured. The hole in

the outer membrane of the liver mitochondrion is initially quite small and is sufficiently resistant to widening to force the inner compartment to expand by extrusion of the inner membrane and enclosed matrix through it, forming a bleb-like protrusion outside the bounds originally delineated by the outer membrane. Inner compartment pressure becomes sufficiently high to widen the hole only after most of the cristae have unfolded. In contrast, the outer membrane of the heart mitochondrion opens up with relative ease, permitting the inner compartment to expand relatively symmetrically. Unlike that of the liver mitochondrion, however, the outer membrane of the heart mitochondrion tends to stay closely associated with the inner membrane throughout the swelling process, apparently providing resistance to unfolding of the cristae. This resistance appears to be responsible for the formation of swollen mitochondria of the "crescent" type, originally described by Harmon (17), in which the remaining cristae are localized on the side of the swollen inner compartment with which the ruptured outer membrane is associated.

Upon being isolated in sucrose or other solute capable of penetrating the outer membrane, mitochondria from both heart and liver transform from elongate to relatively spherical forms. In their normal, intracellular milieu the mitochondia are surrounded by at least a low concentration of solutes that are incapable of penetrating the outer mitochondrial membrane. Removal of these solutes during isolation could account for the above transformation. That this is so is indicated by Novikoff's observation (18) that the rodlike configuration of the rat liver mitochondrion can be preserved during isolation in isotonic sucrose by including a small amount (approximately 2 mM) of polyvinylpyrrolidone (average molecular weight = 40,000) in the isolation medium. In agreement with this observation, we have demonstrated in preliminary experiments that isolated bovine heart mitochondria can be returned to their normal intracellular configuration simply by adding a low concentration of cytochrome c to the suspending medium. Presumably, equiosmol concentrations of other solutes of the same or higher molecular weight would give the same result.

The tendency of mitochondria to assume the spherical configuration upon being isolated in solutes that penetrate the outer membrane, but which maintain the inner compartment in a highly condensed state, suggests that the outer mitochondrial compartment contains at least a low level of solutes that are incapable of penetrating the outer membrane. Outward osmotic pressure exerted by these solutes could also account for the results of Fig. 2, which indicate that the outer mitochondrial compartment does not collapse under the fairly high centrifugal force (25,000 *g*) used in the determination of mitochondrial packed volume.

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