EXPANSION OF THE INNER MEMBRANE COMPARTMENT AND ITS RELATION TO MITOCHONDRIAL VOLUME AND ION TRANSPORT

L. PACKER, J. M. WRIGGLESWORTH, P. A. G. FORTES, and B. C PRESSMAN

From the Department of Physiology-Anatomy, University of California, Berkeley, California 94720, and Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

ABSTRACT

Glutaraldehyde has been used to fix mitochondria undergoing rapid volume changes associated with energized ion transport under oscillatory state conditions and valinomycin-induced potassium uptake. Fixation was found to prevent structural changes which normally occur during ion accumulation or loss. By correlating packed volume measurements with electron microscopy, it is shown that changes in volume associated with ion movements reflect changes in the inner membrane compartment and that this compartment can be related to the sucrose inaccessible space. The method can therefore be used to accurately determine volume changes that arise from ion translocation.

INTRODUCTION

Light scattering is frequently used to follow volume changes accompanying metabolically-linked ion transport in mitochondria (1–3). However, quantitative measurements of changes in volume are difficult to make from light scattering studies since the light changes do not distinguish between volume alterations in different mitochondrial compartments (4, 5). It is necessary to know the relative contribution of these compartments to total mitochondrial volume changes in order to calculate ionic concentrations.

Packed-volume measurement has been applied successfully to follow the osmotic response of the inner membrane compartment in mitochondria suspended in solutions of different osmolality (6). In many systems, however, the rapid time course of volume change indicated by light scattering would make it difficult to apply this method unless the measurements could be carried out in a few seconds. Volume changes subsequent to sampling and before packing into a pellet cause difficulties in relating the metabolic state of the mitochondria to packed volume. Similar difficulties have been encountered in attempts to measure ionic concentrations directly by rapidly separating the mitochondria from the reaction medium, using either Millipore filtration (7), or centrifugation through a layer of silicone fluid (5, 8).

In the present work, glutaraldehyde has been used to fix mitochondria in defined structural states during the course of metabolically-linked ion transport. By this method, it is possible to trap the mitochondrial structure so that further measurements by methods such as electron microscopy, packed volume, and light scattering can be made without fear of subsequent volume changes. Two systems showing relatively large and rapid changes in light scattering have been studied, the oscillatory state (9) and valinomycin-induced K^+ uptake (10). The results from these systems are compared with similar measurements on mitochondria which have been osmotically swollen in sucrose solutions.

METHODS

Rat liver mitochondria were isolated in sucrose-Tris-EDTA at pH 7.4 and put into the oscillatory state as

previously described (9). Kinetics of volume changes during an oscillation or during valinomycin-induced K^+ uptake were simultaneously followed using transmission and 90° light-scattering measurement at 546 mμ.

Glutaraldehyde was purified by distillation from 50% commercial stock solution (Fisher Scientific Company, Pittsburgh, Pa.), and the initial distillate below pH 3.5 was discarded. The concentration was determined from refractive index measurements, and solutions were made up in 10 mM phosphate buffer to a final pH of 7.8.

Packed Volume

Mitochondria were fixed in various structural states by addition of glutaraldehyde to give a final concentration of 0.8% in the reaction mixture. The samples were concentrated by centrifugation and resuspension to one-tenth of their original volume in distilled water. Packed volume was determined in sealed capillary tubes by the mitocrit method (6). Unfixed samples were placed directly in the capillary tubes for packed-volume determination without the preliminary centrifugation procedure.

Electron Microscopy

Glutaraldehyde-fixed mitochondria were centrifuged to form a pellet, were washed in distilled water, and placed in 2% osmium tetroxide for 2 hr. After dehydration in ethanol, the samples were embedded in Epon for thin sectioning.

Multiparameter Ion Uptake Measurements

These measurements were carried out in the apparatus described by Pressman (11).

RESULTS AND DISCUSSION

Volume Changes Associated with Ion Transport

Light-scattering traces of mitochondria in the oscillatory state and during valinomycin-induced K^+ uptake are shown in Fig. 1. At the points indicated in the figure, samples were taken for packed-volume determination and electron microscopy. These points are known from earlier studies (12) to have uniformity of population. Values of packed volume are given in Table I and show that, under the experimental conditions used, a decrease in light scattering is accompanied by an approximately equivalent rise in packed volume. Examination of the electron micrographs for the oscillatory state (Fig. 2 *a–c*) and for the valinomycin system (Fig. 3) shows that mitochondria giving

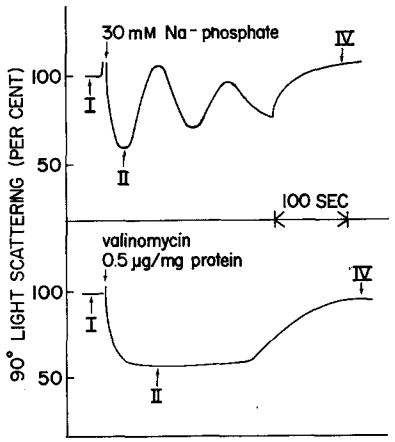


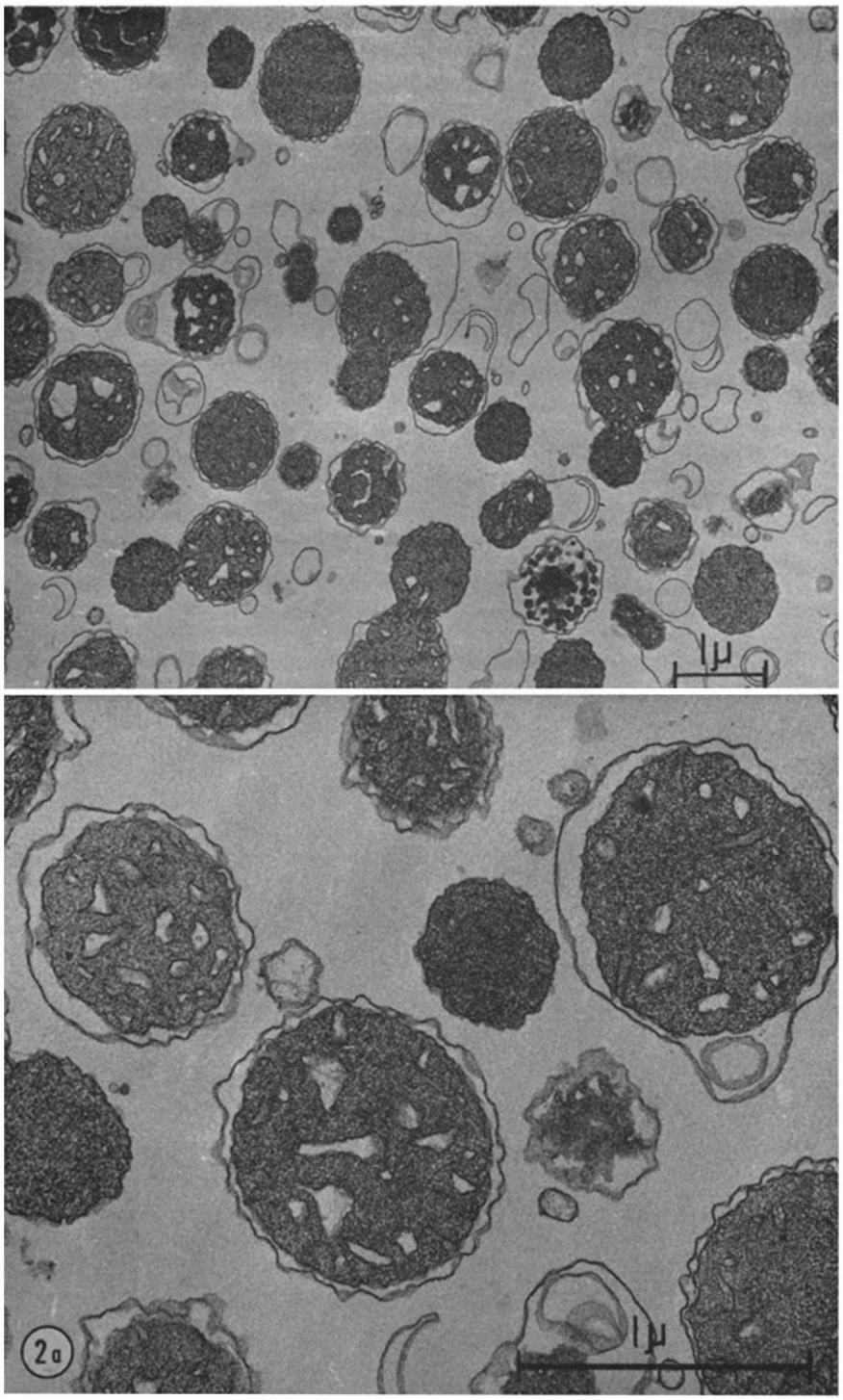
FIGURE 1 Light-scattering traces of the oscillatory state and valinomycin-induced K^+ uptake. Oscillatory state conditions were 3.3 ml of reaction mixture containing sucrose (0.1 M), EDTA (0.5 mM), Tris-HCl buffer (3.0 mM, pH 7.8), sodium succinate (1.5 mM), and rat liver mitochondria (1.4 mg protein/ml) at 25°. Oscillations were induced by the addition of sodium phosphate (pH 7.8) as indicated in upper curve. Conditions for the lower trace were 3.0 ml of reaction mixture containing sucrose (150 mM), sodium succinate (1.5 mM), KCl (5.0 mM), Tris-sodium phosphate buffer (20 mM, pH 6.8), and mitochondria (1.54 mg protein/ml); K^+ uptake was induced by the addition of valinomycin as indicated. An aliquot of glutaraldehyde was added to a final concentration of 0.8% at states I, II, and IV for direct fixation in the cuvette.

TABLE I

Packed Volume and Light Scattering Levels of Glutaraldehyde-Fixed Mitochondria

Sample	Relative packed volume	Relative light scattering at time of fixation	Relative light scattering of fixed sample resuspended in water
	%	%	%
Oscillatory state			
I	100	100	100
II	141 ± 7	67	68
IV	103 ± 5	113	112
Valinomycin state			
I	100	100	100
II	149 ± 5	66	64
IV	97 ± 1	101	95

Mitochondria were fixed in oscillation and during valinomycin-induced K^+ uptake as described in Fig. 1.



Figs. 2*a–c* are electron micrographs of rat liver mitochondria during the oscillatory state. Conditions as in Fig. 1.

FIGURE 2*a* State I, mitochondria prior to induction of oscillation. upper × 12,000, lower × 40,000

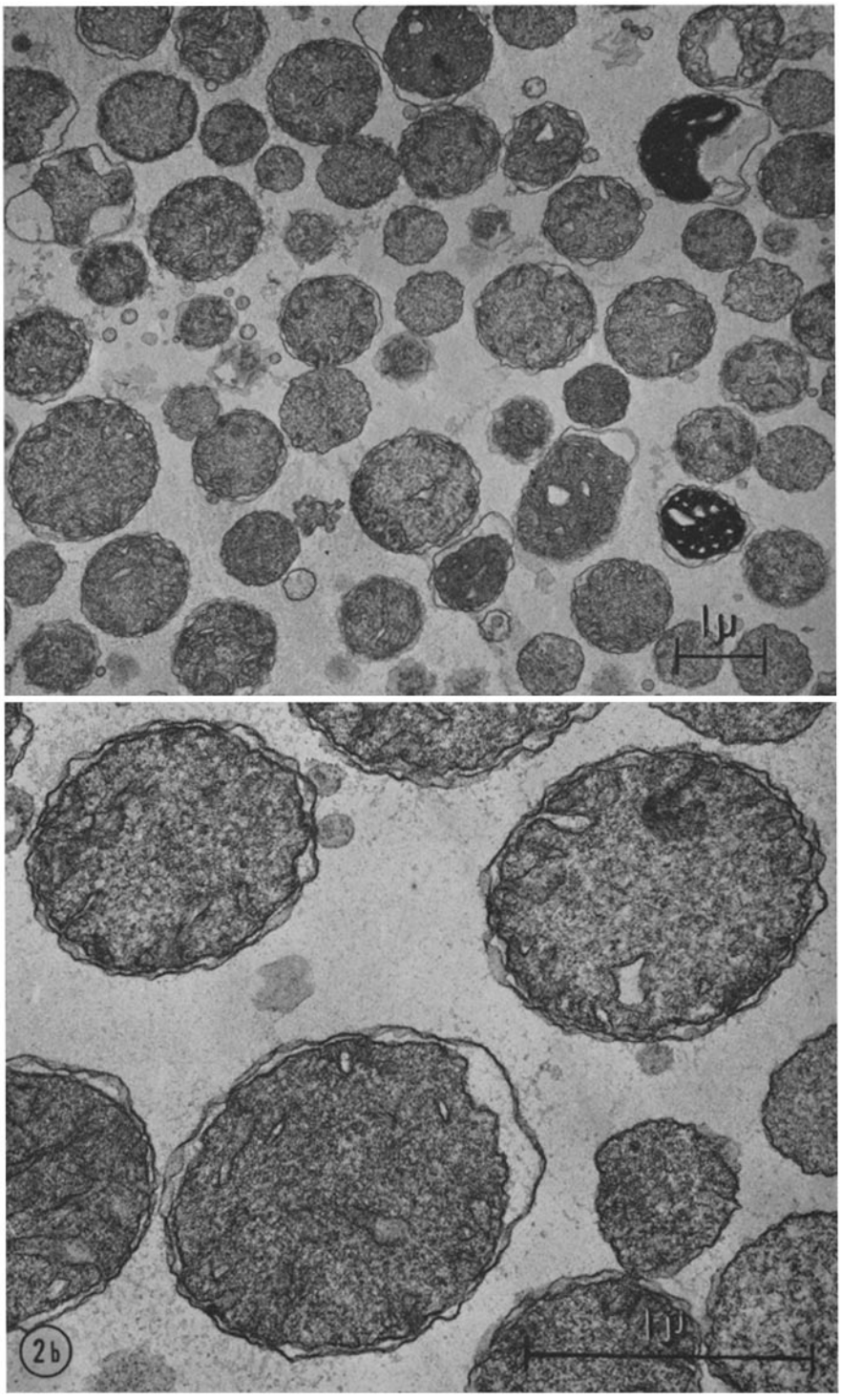


FIGURE 2*b* State II, mitochondria at trough of first light scattering cycle. See legend under Fig. 2*a*. upper × 12,000, lower × 40,000

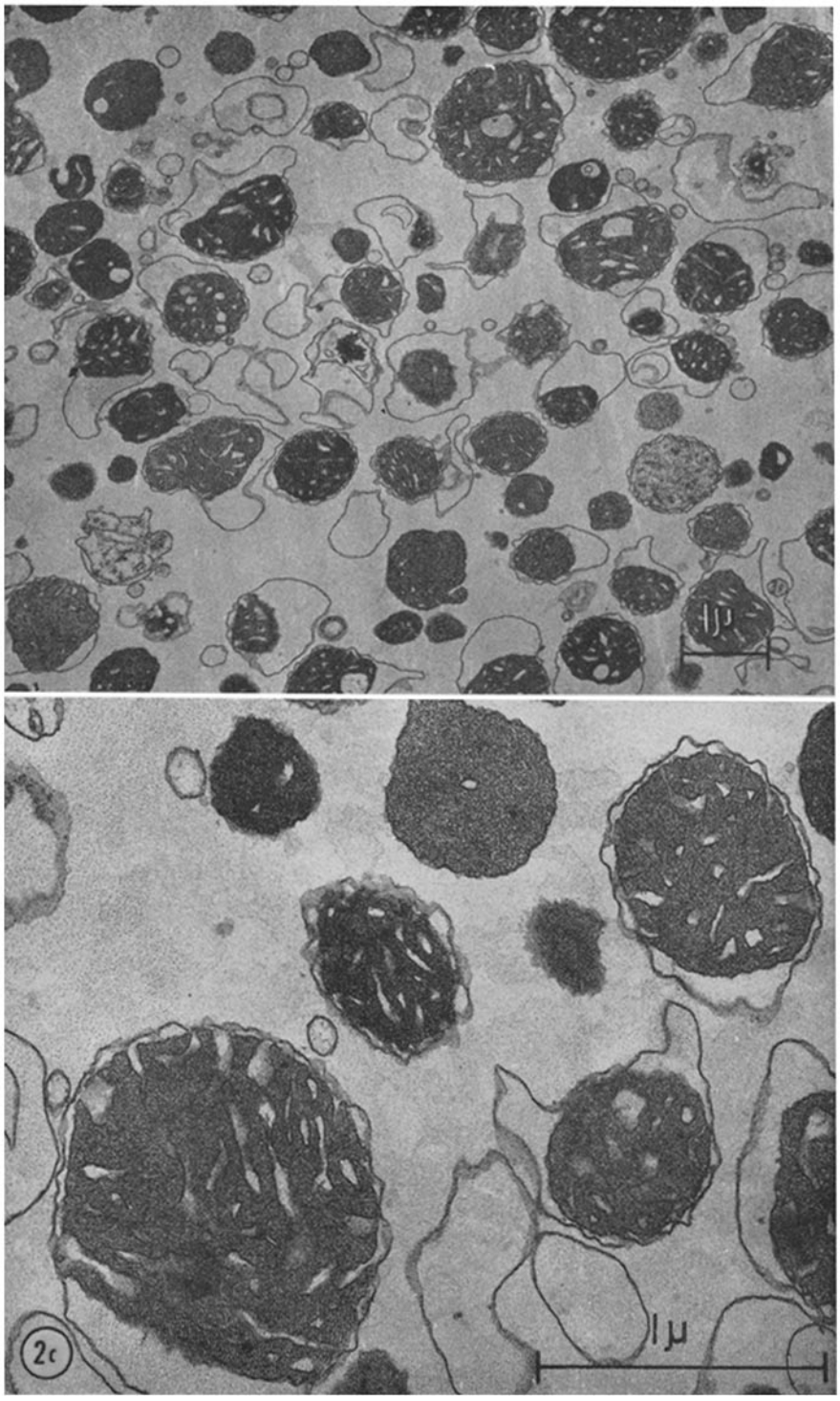
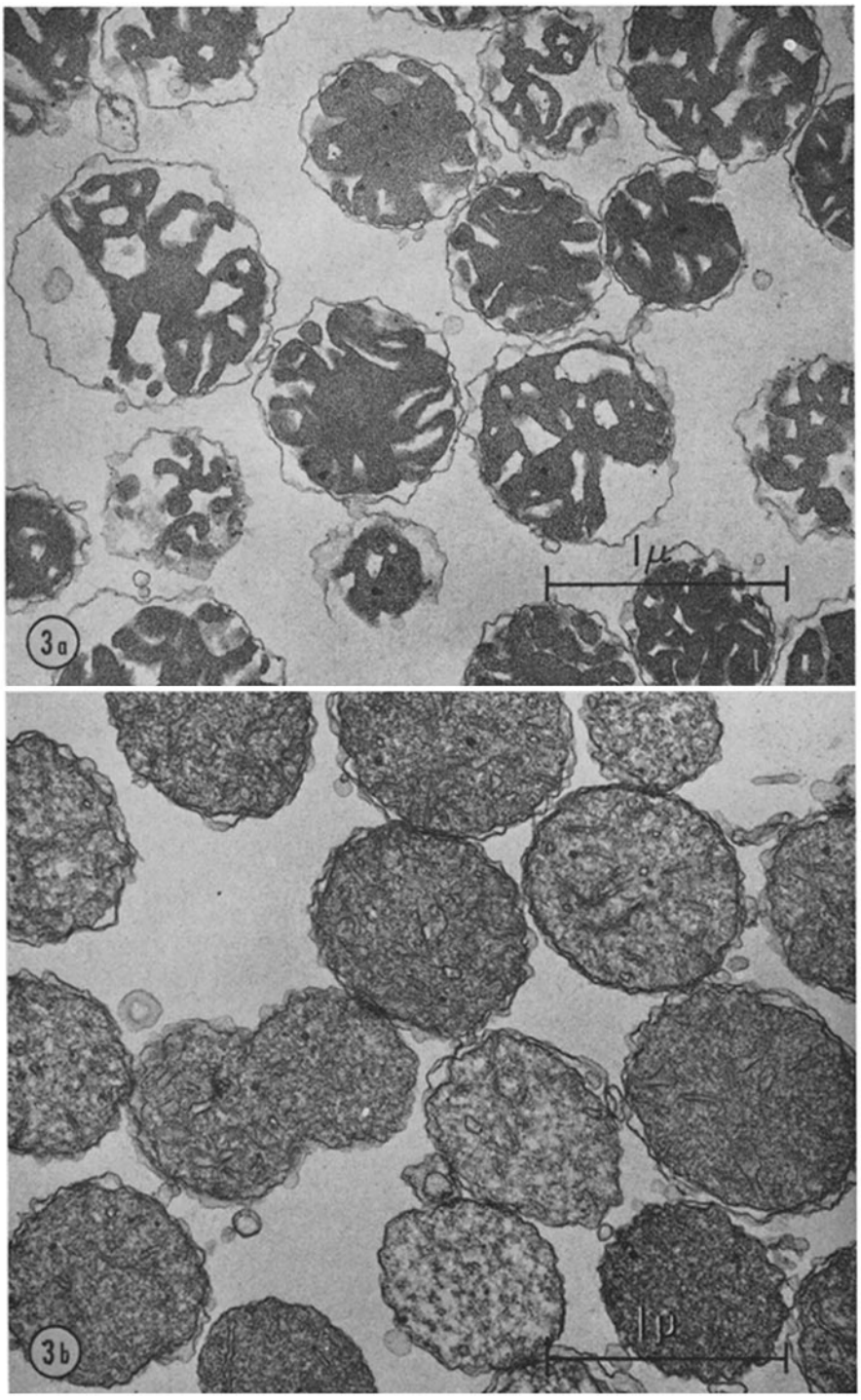
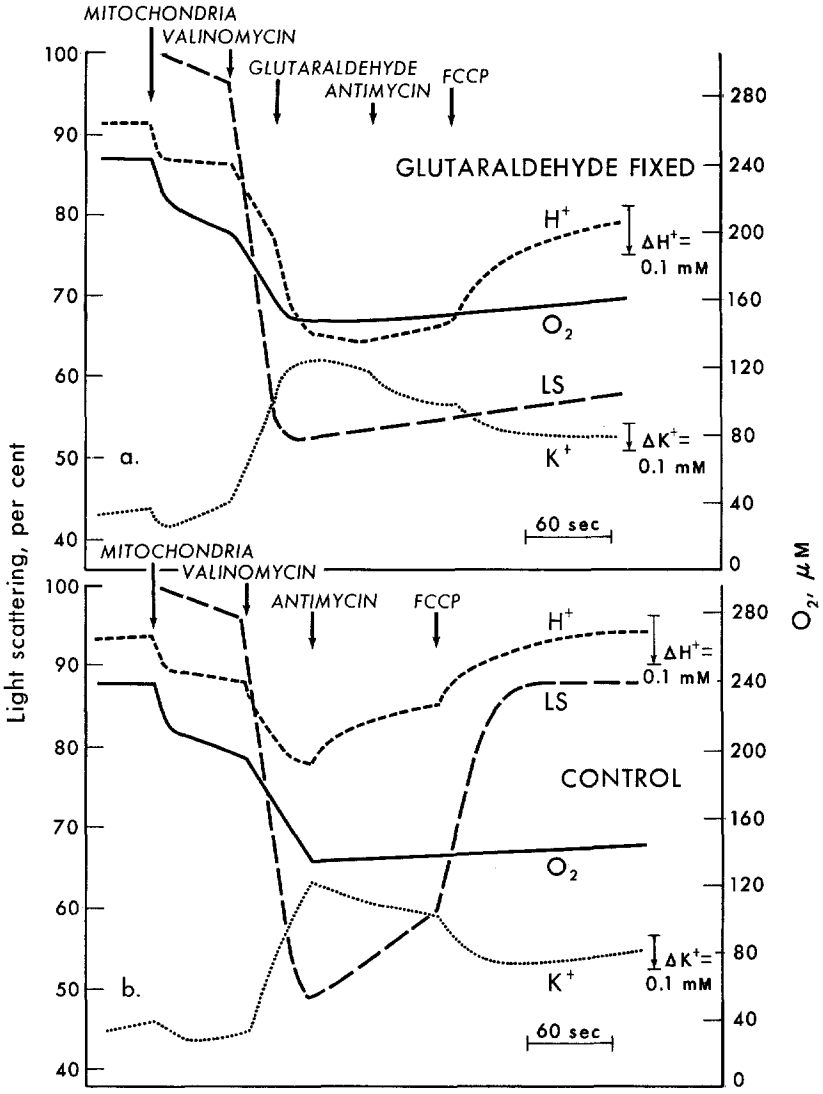


FIGURE 2c State IV, anaerobic mitochondria. See legend under Fig. 2a. upper × 12,000, lower × 40,000

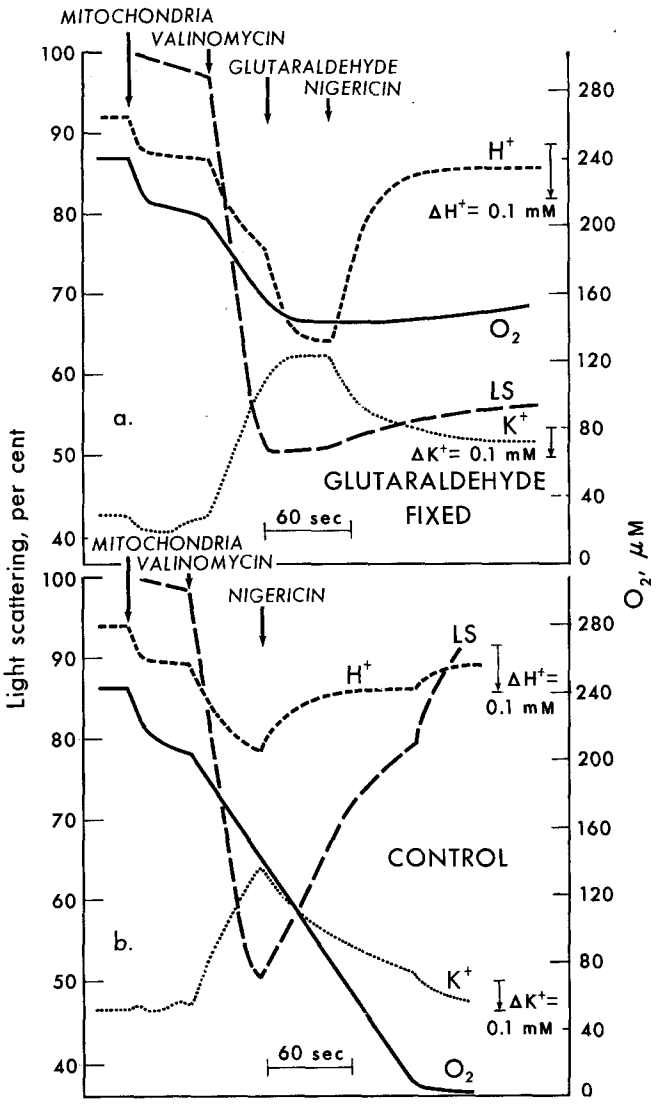


FIGURES 3*a* and *b* Electron micrographs of valinomycin-treated mitochondria. *a*, Prior to induction of K^+ uptake. *b*, At trough of light scattering curve after the addition of valinomycin. Conditions as in Fig. 1. × 32,000

high light-scattering levels, states I and IV, have a highly condensed inner matrix bounded by a folded and convoluted inner membrane. The more condensed form of valinomycin state I (Fig. 3 *a*) compared to the oscillatory state I (Fig. 2 *a*) is probably a reflection of the increased osmolarity of the initial suspending medium, 176 mM compared to 105 mM. When the light scattering is minimal (state II) the inner membrane has unfolded, giving rise to a less electron-opaque matrix and a morphology more typical of mitochondria in vivo. These changes occur synchronously throughout most of the population. There is no apparent change in the folding of the outer membrane. Measurements taken directly from the micrographs show that the volume enclosed by the



FIGURES 4*a* and *b* Multiparameter monitoring of the response of glutaraldehyde-fixed and control mitochondria to an electron transport inhibitor and an uncoupler during K^+ accumulation. Experimental conditions were 10 ml of reaction mixture containing sucrose (0.1 M), sodium phosphate buffer (4 mM, pH 6.8), tetraethylammonium succinate (3 mM), KCl (5 mM), and mitochondria (2.1 mg protein/ml) at 22°. Additions: In *a*, glutaraldehyde (0.5% final concentration) was added at arrow. In both *a* and *b*, valinomycin (18 μg/g protein), antimycin A (90 μg/g protein), and FCCP 9 (2 μM) were added at indicated times.



FIGURES *5a* and *b* Multiparameter monitoring during valinomycin-nigericin cycle of glutaraldehyde-fixed and unfixed mitochondria. Experimental conditions as in Fig. 4. Additions: In *a*, glutaraldehyde (0.5% final concentration) was added at arrow. In *a* and *b*, valinomycin (18 μg/g protein) and nigericin (9 μg/g protein) were added at indicated times.

outer membrane does not change within the limits ± 10%. It would therefore appear that, in the two systems studied, the mitochondria pack into a pellet not according to their total volume but predominantly according to the volume (or density) of the inner matrix compartment, with the outer membrane passively filling the interstitial spaces. Changes in packed volume would thus reflect volume changes in the inner compartment.

Ion Transport in Glutaraldehyde-Fixed Mitochondria

It was of interest to examine the effects of glutaraldehyde fixation on the response of mitochondria to various agents which influence the energy-linked transport of K^+. If glutaraldehyde is added prior to valinomycin with either succinate or ATP as energy sources, no uptake of K^+ is obtained. This appears to be due to the inhibition of energy production rather than impaired permeability, since the aldehyde strongly inhibits both respiration and trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP)-induced ATPase.

In Fig. 4 *a* glutaraldehyde addition during valinomycin-induced K^+ accumulation halts all observed metabolic responses. The subsequent addition of antimycin causes a small release of K^+ and uptake of H^+ without any apparent change in

light scattering. This implies that low levels of residual respiration, obscured by the back diffusion of oxygen into the open cuvette, were supplying sufficient energy to maintain the accumulated level of K^+, although incapable of initiating transport. Further de-energizing of the mitochondria by the uncoupler FCCP resulted in more extensive changes in the K^+ and H^+ levels, although the light-scattering signal remained constant. In the control experiment, Fig. 4 *b*, the relaese of K^+ by antimycin and uncoupler was accompanied, as previously documented (2), by mitochondrial shrinkage. In Fig. 5 *a* nigericin was employed to release the accumulated K^+ (13) from glutaraldehyde-fixed mitochondria. Again the extensive release of K^+ is accompanied by negligible light-scattering changes, although, in the control, Fig. 5 *b*, the expected shrinkage is obtained with unfixed mitochondria.

It thus appears that glutaraldehyde fixation dissociates light-scattering changes, presumed to reflect water movements, from ion movements. Further details of the responses of glutaraldehyde-fixed mitochondria to metabolic perturbations are under study.

Relation of Inner Membrane Compartment to Mitochondrial Volume

Additional experiments, the results of which are shown in Fig. 6, make it possible to equate the inner compartment which undergoes volume changes associated with ion transport with the sucrose inaccessible space described by other workers (5, 6, 14, 15). In Fig. 6, the packed volume of mitochondria can be seen to vary inversely with the osmolarity of the suspending sucrose medium. Examination of electron micrographs of mitochondria fixed in 60 mM sucrose and 300 mM sucrose (not shown) indicate that these changes in packed volume brought about by a passive osmotic method reflect volume changes of the inner membrane compartment in the same way as was found for volume changes during oscillation and during valinomycin-induced K^+ uptake (see Figs. 2 and 3).

Before the present method can be used to calculate changes in mitochondrial volume from changes in packed volume, it is necessary to establish that the processes of fixation and subsequent treatment do not affect the results. It can be seen from Table I that the light scattering of fixed mitochondria after centrifugation and resuspension

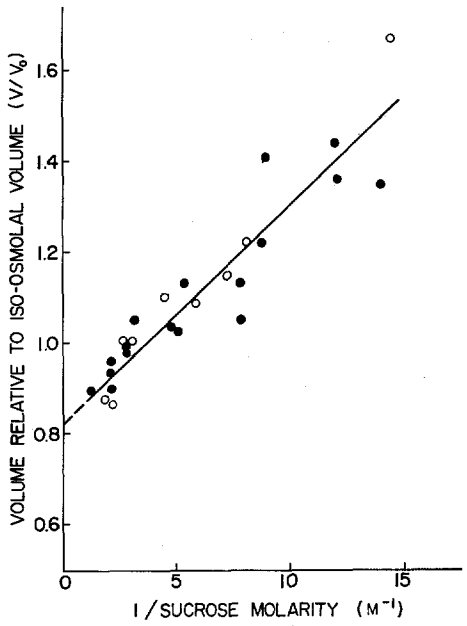


FIGURE 6 Relation of mitochondrial packed volume to osmolarity of suspending medium. Conditions: 3.0 ml of reaction mixture containing sucrose of the desired molarity and rat liver mitochondria (about 3 mg protein/ml). Mitochondrial packed volume was measured on unfixed material suspended in the sucrose medium (○). Mitochondria fixed with glutaraldehyde as described in Fig. 1, centrifuged and resuspended at a × 10 concentration in distilled water before packed volume was measured (●). Each point represents the mean of five measurements. Two independent experiments were carried out, and all points are included.

in distilled water remains at the same level as at the time of fixation. Thus, under the conditions used, no subsequent volume changes detectable by light scattering occur after fixation. Mitochondrial packing during centrifugation was found to be unaffected by glutaraldehyde fixation, and Fig. 6 shows that the relative response of mitochondria to changes in the osmolality of the medium is the same for measurements on both fixed and unfixed mitochondria. The results do not, of course, preclude alteration in membrane structure at the molecular level upon fixation, as suggested by the work of Lenard and Singer (16), but they do show that mitochondrial macrostructure is not altered in any manner which would affect the volume measurements.

The present work therefore indicates that glutaraldehyde can be used to trap mitochondrial macrostructure, during rapid volume changes, for

subsequent measurement. Using this method and correlating electron microscopy, light scattering, and packed volume measurements, it has been shown that a maximum volume change of 41% during oscillation and 49% during valinomycin-induced K^+ uptake occurs by the unfolding and expansion of the inner membrane compartment.

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