PREPARATION OF ISOLATED RAT LIVER MITOCHONDRIA FOR ELECTRON MICROSCOPY

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ABSTRACT

A comparative study of the fixation of isolated rat liver mitochondria was undertaken. If the criterion is adopted that after processing, the mitochondria should resemble as closely as possible rat liver mitochondria *in situ*, the procedure found to produce such preservation was that of fixation in suspension in veronal-buffered 2% potassium permanganate. Fixation in osmium tetroxide produced variable results, while mitochondria fixed in glutaraldehyde were contracted. We suggest that in cases where fixation procedures modify the morphological appearance of mitochondria, the significance of such changes must be treated with caution.

INTRODUCTION

Before it is possible to study the correlation of structure and function or biochemical activity of any biological system, it is necessary to establish basic criteria for assessment of the results. In any technique which requires disruption of cells it is necessary to ensure that the organelle isolated is, in fact, that which is to be studied. Further, as the organelle has been removed from its normal habitat it is necessary to decide upon criteria by which the organelle can be identified and the degree of distortion due to isolation procedures assessed. In this study the morphologic criterion chosen for assessment of adequate preservation of rat liver mitochondria was that after isolation and fixation procedures the mitochondria in sectioned material should resemble as closely as possible the form seen in fixed and sectioned whole liver. It is also necessary to demonstrate that the unfixed isolated organelles satisfy certain functional criteria which, in the case of mitochondria, are adequate respiratory control and normal oxidative phosphorylation. This comparative study of fixation of isolated mitochondria was therefore undertaken.

METHODS

Mitochondrial Preparations

Whole rat livers were passed through a stainless steel press which separated connective tissue from pulp. The latter was homogenized in a hand-operated Dounce homogenizer (Blaessig Glass Co., Rochester, N.Y.) in 0.44 M sucrose and the suspension was made to 10 g liver/100 ml homogenate. Nuclei and debris were centrifuged at 1000 *g* for 7.5 min. The supernatant was centrifuged at 7800 *g* for 10 min. The pellet was washed twice more and made to a final volume of about 1 g original liver/ml of suspension. All centrifugations were done at 2°C in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The conditions for measuring oxidative phosphorylation are given in Table I.

The fixatives used in this study are listed in Table II.

FIXATION IN PELLET: Approximately 1 ml of mitochondrial suspension was added to 10 ml of suspending medium and centrifuged at 10,000 *g* for 5 min. The supernatant was poured off and replaced by the appropriate fixative. This preparation was left for about 1 hr in ice. The centrifuge tube was cut down and the pellet was gently removed and diced in such a

TABLE I

Oxidative Phosphorylation and Electron Micrographic Appearance of Isolated Mitochondria

Exp. No.	O_2 consumed	ATP formed	P/O	Control ratio (state 3/state 4)
	μat/g dry wt	*μmoles*		
1	48	101	2.1	7.5
2a	67	171	2.55	7.0
2b	67	169	2.5	6.9
3	142	440	3.1	4.8

Mitochondria were isolated in 0.44 M sucrose and incubated in a medium (final vol 3.0 ml) containing inorganic orthophosphate, pH 7.40, 5 mM; KCl, 10 mM; $MgCl_2$, 5 mM; Na-β-hydroxybutyrate, 5 mM (Exps. 1, 2a, 2b); K-pyruvate and K-fumarate, 10 mM (Exp. 3); Tris-HCl, pH 7.4, 20 mM; sucrose, 210 mM; 10 mg dry wt mitochondria in Exps. 1 and 2a; 5 mg dry wt mitochondria in Exp. 2b; and 7.8 mg dry wt mitochondria in Exp. 3. Temp 22 C O_2 uptake measured by O_2 electrode. The transition from state 4 to state 3 was affected by addition of 1 μmole ADP (Exps. 1 and 2a), 1.7 μmole ADP (Exps. 2b), and 0.5 μmole (Exp. 3). Samples for electron microscopy (1.0 ml) taken during the initial and final state 4 condition and in state 3 were fixed in suspension in Veronal-buffered $KMnO_4$, pH 7.4.

way as to preserve the orientation of the blocks. The dice were further immersed in the fixative for 1 hr, washed in buffer, dehydrated through graded alcohols, and embedded in Epon 812.

FIXATION IN SUSPENSION: Approximately 1 ml of mitochondria suspension was poured into approximately 10 ml of fixative, left in ice for approximately 10–20 min, and centrifuged at 10,000 *g* for 5 min. The tube was then cut down and the pellet was gently removed and diced in buffer also in such a way as to preserve the orientation. The blocks were dehydrated through graded alcohols and embedded in Epon 812 (9). It was found that during these procedures the blocks remained intact except for those fixed in phosphate buffer with added glucose. In those cases the blocks disintegrated in 70% alcohol. The blocks were sectioned on an LKB ultratome (LKB Instruments Inc., Rockville, Md.) with either diamond or glass knives and examined in either an RCA EMU3 or an AEI EM6.

RESULTS

Metabolic Studies

These studies are summarized in Table I, from which it can be seen that the mitochondria have adequate respiratory control and normal P/O ratios.

As a result of the findings set out in the following section, samples of mitochondria in the differing states of respiration were fixed in suspension in Veronal-buffered potassium permanganate. The conformation of the mitochondria in state 3 is shown in Fig. 11. No change in conformation was seen in any in state of respiration.

Concentration of Preparative Solutions

In all the fixation procedures to be discussed the mitochondria were prepared in either 0.25 or 0.44 M sucrose. When prepared in 0.25 M sucrose the mitochondria were uniformly round and the cristae tended to be irregularly dilated. This effect was seen when the mitochondria were fixed in suspension in either osmium tetroxide or potassium permanganate (Figs. 1, 2). The mitochondria prepared in 0.44 M sucrose and fixed in suspension were frequently elongated or oval in form. The character of the cristae and matrix varied with the fixative used and will be described under the different fixatives. Following pellet fixation with either concentration of sucrose in any of the fixatives described, no consistent cristal arrangement was seen.

Concentration of Fixative

The concentrations of the components of the fixatives are shown in Table II. With any given fixative there appeared to be no significant difference in the appearance of the mitochondria caused by the variation in the concentration.

Osmium Tetroxide Fixation of the Pellet

Sections cut through the depth of the pellet demonstrated that the morphology of the mitochondria was dependent upon the zone of the pellet. Fig. 3 shows three levels of the same pellet of mitochondria prepared in 0.25 M sucrose and fixed in 1% osmium tetroxide with Veronal buffer, pH 7.2. These micrographs were taken from the same section cut perpendicular to the surface. The most superficial mitochondria show great distortion with dense contracted matrix. The density of the matrix is similar to that of the inner mitochondrial membrane. The space between the outer and inner membranes and the cristal space are greatly increased. In the middle zone the matrix is less dense and the cristal and outer membranes are difficult to distinguish. In

TABLE II

Summary of Condition of Fixatives

Fixative	Amount of fixative	Buffer	pH	Molarity of fixative	Molarity of preparative sucrose	Method of fixation
	%					
Osmium tetroxide	1	Veronal*	7.2	0.14	0.25 0.44	Pellet and suspension
		phosphate	7.2	0.14	0.44	Suspension
		chromate*	7.2	0.14	0.44	Suspension
Glutaraldehyde (postfixation osmium tetroxide)	6	cacodylate*	7.2	0.74	0.25 0.3	Suspension
	3	cacodylate	7.2	0.44	0.3	Suspension
	3	—	7.2	0.3	0.3	Suspension
	3	—	3.4	0.3	0.3	Suspension
Glutaraldehyde	6	cacodylate	7.2	0.3	0.25	Suspension
Potassium permanganate	2	Veronal*	7.2	0.17 0.2 0.25	0.25 0.3 0.44	Pellet and suspension

* See references 14, 2, 17, and 8, respectively.

the lower zone the mitochondrial profiles appear larger. The outer, inner and cristal membranes can be seen while the matrix appears clumped. In the deepest layers of the pellet it is not possible to see intact mitochondria. These changes correspond to the observation that during fixation the superficial layers of the pellet rapidly blacken but after 1-hr immersion the deepest layers are pale brown in color and tend to disintegrate upon dicing. These changes are presumably the result of slow penetration of the large pellet by the osmium tetroxide.

Osmium Tetroxide Fixation of the Suspension

After fixation in suspension followed by pelleting, the appearance of the mitochondria was uniform throughout the pellet and varied with the buffer systems used and the osmolarity of the initial suspending medium. As mentioned previously, with 0.25 M sucrose as the suspending medium, all the mitochondria were round with irregular cristae (Fig. 1). The appearance of the mitochondria was similar in either Veronal or phosphate buffer but not consistent, from preparation to preparation, in the preservation of the matrix. In Fig. 1 the matrix is moderately well preserved, but in other preparations there was considerable loss of matrix material. The outer membrane is also poorly preserved with this fixation, as many breaks can be seen (Fig. 1). A similar appearance was seen when the mitochondria were fixed in chromate-buffered osmium tetroxide (2) but the matrix had a rather coarse granular appearance.

Glutaraldehyde Fixation of the Suspension

No mitochondria were examined following glutaraldehyde fixation of a pellet. Mitochondria prepared in 0.25 M and 0.3 M sucrose were fixed in suspension in glutaraldehyde with cacodylate buffer, pH 7.2, or in unbuffered glutaraldehyde, as in Table I. After centrifugation the pellet was diced and postfixed in 2% osmium tetroxide, either cacodylate- or Veronal-buffered, pH 7.2, for 2 hr.

There appeared to be no significant difference in the appearance of mitochondria fixed with the various concentrations of glutaraldehyde as listed in Table I. The mitochondria prepared in 0.3 M sucrose were fixed in suspension in 0.3 M glutaraldehyde, pH 7.2, either buffered in cacodylate or unbuffered. In each case the appearance of the mitochondria was similar to that seen following 0.6 M glutaraldehyde, buffered or unbuffered. Following these fixation procedures the mito-

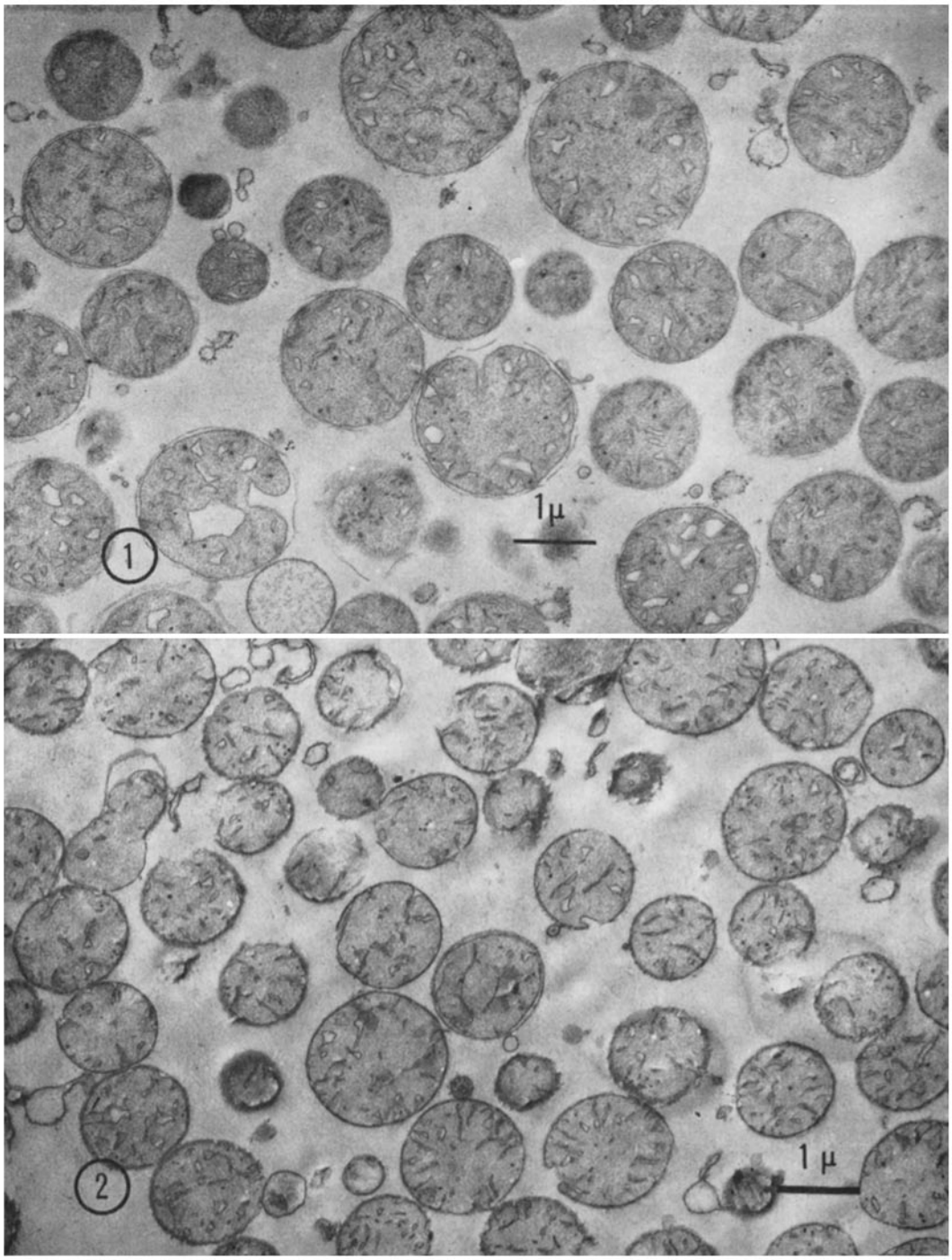

FIGURE 1 Mitochondria prepared in 0.25 M sucrose and fixed in suspension in Veronal-buffered osmium tetroxide. Uranyl acetate. × 11,900.

FIGURE 2 Same preparation of mitochondria as in Fig. 1 fixed in suspension in Veronal-buffered potassium permanganate. Uranyl acetate. × 11,900.

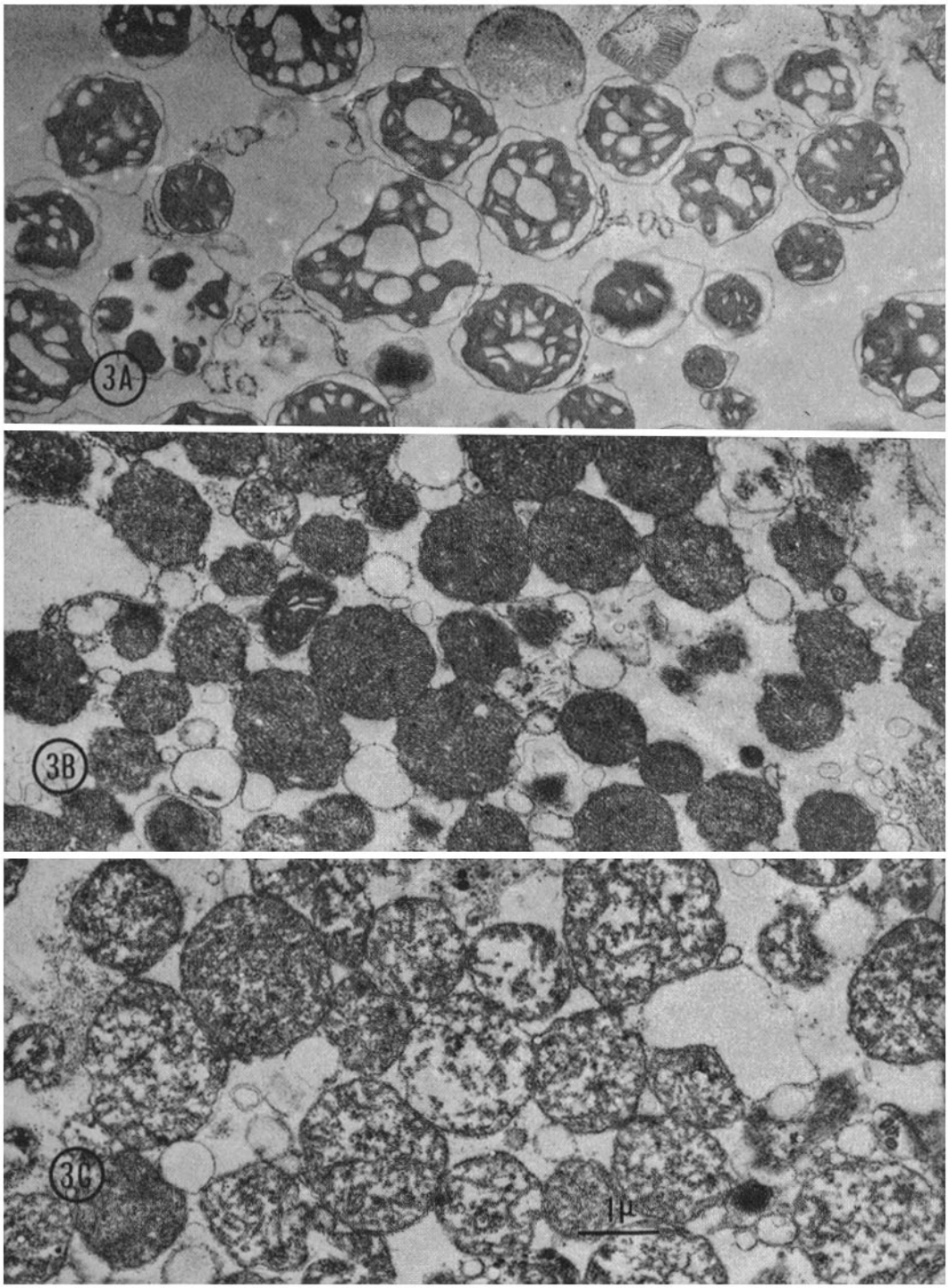

FIGURE 3 Same preparation of mitochondria as in Figs. 1 and 2, fixed as a pellet in Veronal-buffered osmium tetroxide. The micrographs are taken from the same section cut perpendicular to the surface of the pellet. *A*, superficial; *B*, middle; and *C*, deep. Uranyl acetate. × 11,900.

chondria were fairly uniform in appearance throughout the pellet and consisted of mitochondria with a dense contracted matrix. The electron opacity of the matrix was similar to that of the inner mitochondrial membrane. The space between the inner and outer membranes was irregularly enlarged, and the otuer membranes showed frequent breaks (Fig. 4). In the most superficial layers of the pellet some of the mitochondria had a less contracted appearance, with only a small space between the inner and outer membranes.

Potassium Permanganate Fixation of the Pellet

Veronal-buffered $KMnO_4$, pH 7.2, was used throughout. A pellet of mitochondria fixed as a pellet usually darkened rapidly on addition of the fixative. On section, the form of the mitochondria was similar throughout the pellet. The mitochondria appeared swollen and angular (Fig. 5), with only a few peripheral cristae remaining.

Potassium Permanganate Fixation of the Suspension

Mitochondria prepared in 0.44 M sucrose and fixed in suspension in $KMnO_4$ are shown in Fig. 6. Oval and elongated profiles can be seen.

In many mitochondria the outer membrane is intact whereas in others there are breaks in the outer and inner membranes. This degree of rupture is consistent with that seen in the preparations of other workers (4, 13). Little increase in the space between the outer and inner membranes is seen. The cristae are regular and can be seen to be continuous with the inner membrane. The matrix is evenly granular but the intramitochondrial dense bodies are not seen. In these preparations a few swollen distorted profiles are present, with clumping of the remaining cristae and loss of matrix. The microsomal contamination seen in Fig. 6 is of the usual level seen in these experiments. The derivation of the smaller membrane bodies lying between inner and outer membranes (Fig. 7) is not clear. The character of both the inner and outer membranes is similar (Fig. 8). Both are unit membranes, approximately 80–100 A, composed of dense granules.

In preparations in 0.25 M sucrose the mitochondria are uniformly round and the cristae are somewhat irregular (Fig. 2); a similar appearance is seen in mitochondria prepared in 0.3 M sucrose. Otherwise, the characteristics of the membranes and matrix are similar to those seen in mitochondria prepared in 0.44 M sucrose.

Resuspension in KCl

Mitochondria prepared and suspended in ice-cold sucrose were resuspended in ice-cold KCl (154 mM). They were kept at 0° C for 10 min, and their appearance was compared with that in the original sucrose suspension, similarly kept, with $KMnO_4$ or glutaraldehyde as fixatives.

With $KMnO_4$, the mitochondria prepared in KCl were seen to be uniformly round and swollen, as compared to the mitochondria prepared in sucrose, with few irregular cristae (Fig. 9). The outer membrane was frequently broken.

With glutaraldehyde as fixative, the mitochondria prepared in KCl again presented an appearance compatible with swelling (Fig. 12). The main point to be noted, however, is the loss of the contracted state seen in the mitochondria prepared in sucrose and similarly fixed (Fig. 4).

The swelling of the mitochondria in KCl was further established by determining the water content, which was about 4.0 g/g dry wt, whereas in 0.25 M sucrose the same preparation had a water content of 2.85 g/g dry wt. Agents which block swelling (e.g. 0.1 mM dinitrophenol) prevent the increase in water content as well as the observed increase in size (Fig. 10).

DISCUSSION

The configuration of freshly prepared mitochondria varies with the nature of the fixation. Using $KMnO_4$ fixation, we have shown that the appearance of isolated rat liver mitochondria can be made to resemble that of mitochondria in the liver cell. These mitochondria are capable of efficient oxidative phosphorylation prior to fixation. Nevertheless, we find none of the structural changes said to accompany variations in mitochondrial respiration from state 4 to state 3 and vice versa. The mitochondria, in our hands, never depart from the configuration said to be that of state 4.

Furthermore, the appearance of freshly prepared mitochondria, in our experiments with $KMnO_4$ fixation, is no different from that seen in states 3 and 4. These results are in disagreement with the results of other work.

In this study on the same preparation of mitochondria, it has been possible to demonstrate contracted mitochondria in samples suspended in

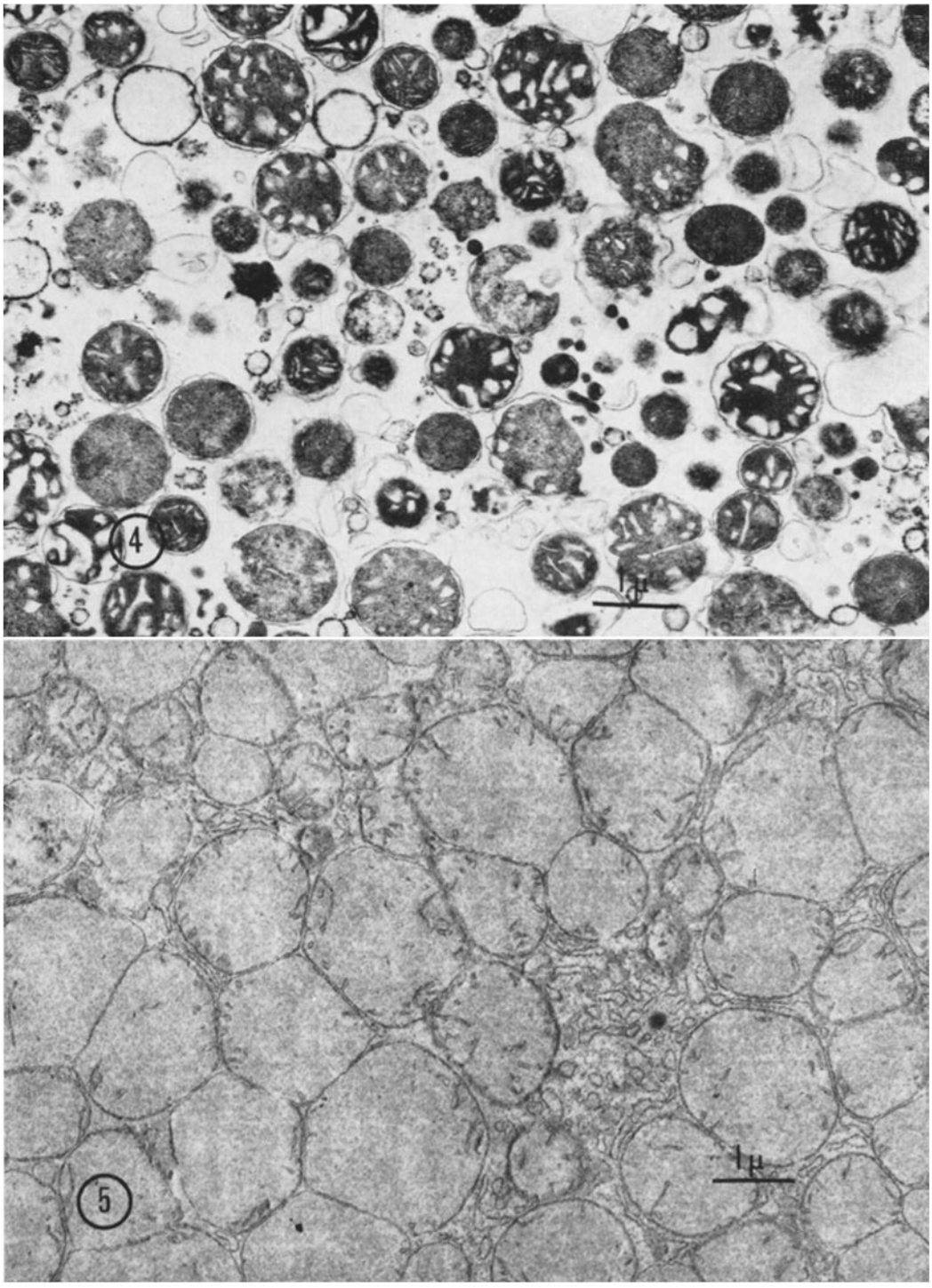

FIGURE 4 Mitochondria prepared in 0.3 M sucrose and fixed in suspension in 0.3 M glutaraldehyde unbuffered pH 7.2 and postfixed in osmium tetroxide. A similar appearance was found when the sample of mitochondria shown in Figs 1–3 was fixed in 0.6 M glutaraldehyde. Uranyl acetate and lead citrate. × 11,900.

FIGURE 5 Same preparation of mitochondria as in Figs. 1–3, fixed as a pellet in Veronal-buffered potassium permanganate. Uranyl acetate. × 11,900.

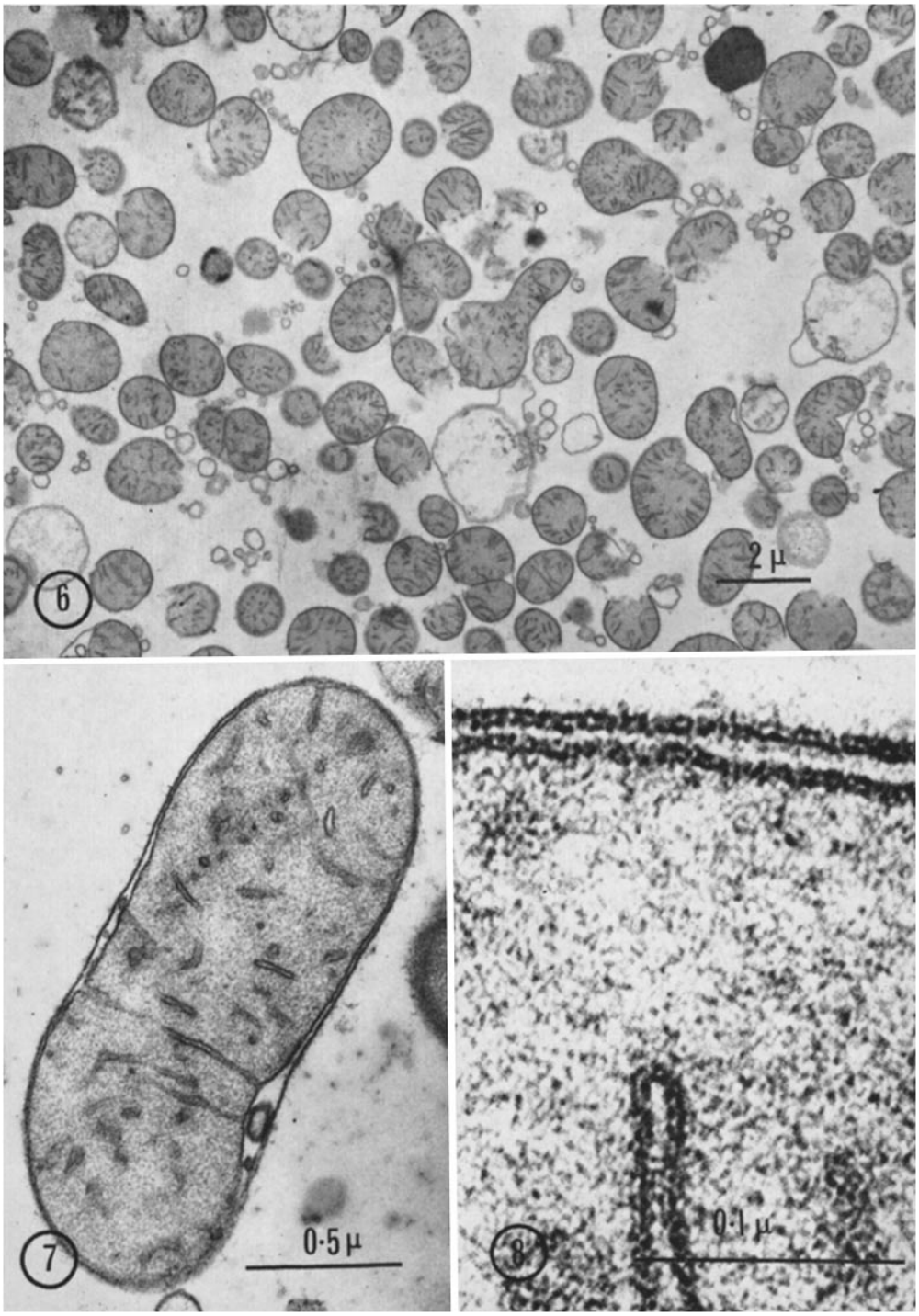

FIGURE 6 Mitochondria prepared in 0.44 M sucrose and fixed in suspension in VERONAL-buffered potassium permanganate. Uranyl acetate. × 6900.

FIGURE 7 Same preparation of mitochondria as in Fig. 6. The outer and inner membranes are intact with regular cristae. Note the small membrane-bounded vesicles lying between the inner and outer membranes. Uranyl acetate. × 45,000.

FIGURE 8 Same mitochondria as in Fig. 7, showing the globular character of the membranes. Uranyl acetate. × 392,000.

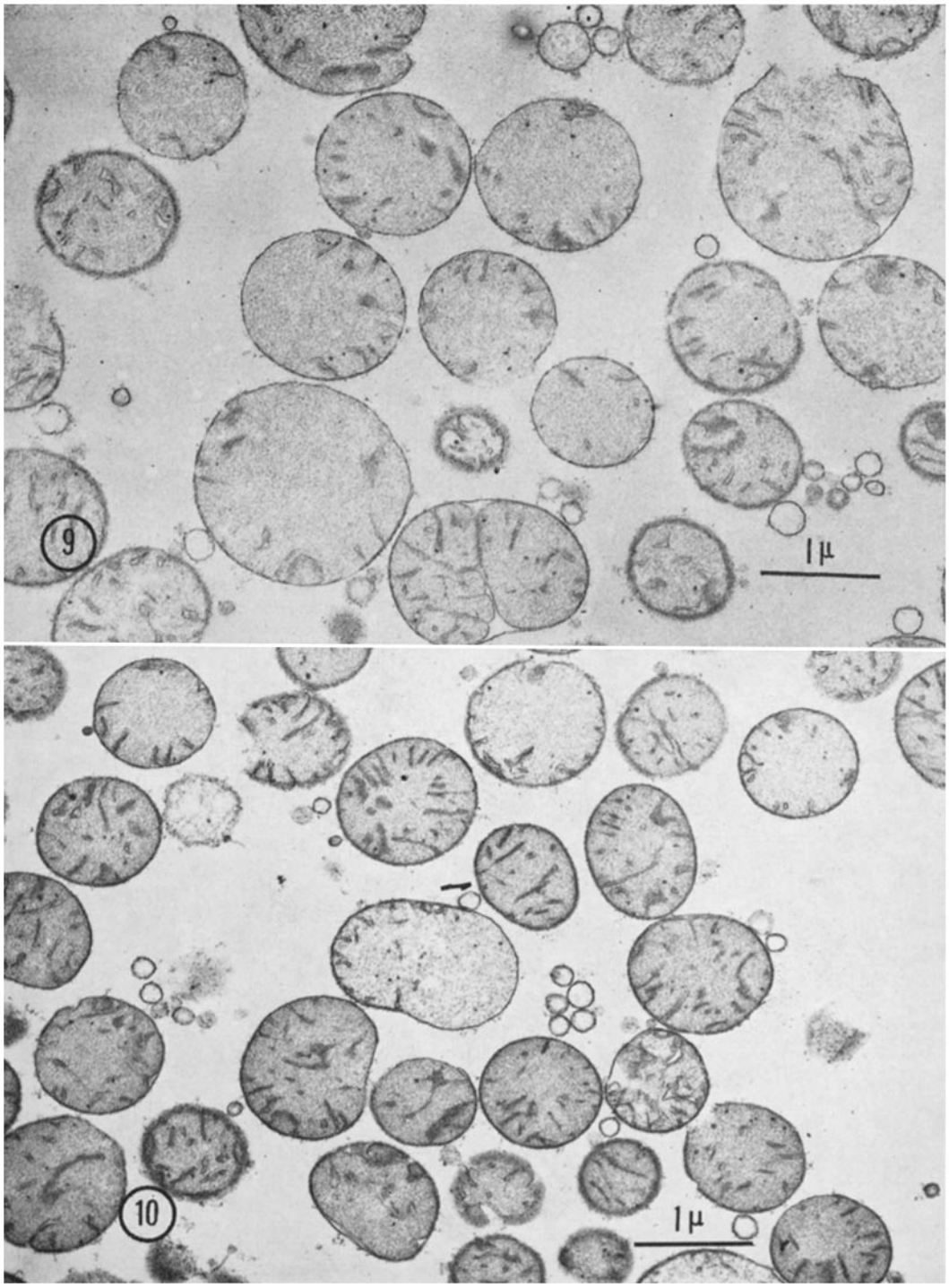

FIGURE 9 Mitochondria prepared in 0.44 M sucrose and resuspended in 0.154 M KCl. Uranyl acetate. × 17,500.

FIGURE 10 Mitochondria prepared in 0.44 M sucrose, resuspended in 0.154 M KCl, and treated with dinitrophenol (0.2 mM). Uranyl acetate. × 17,500.

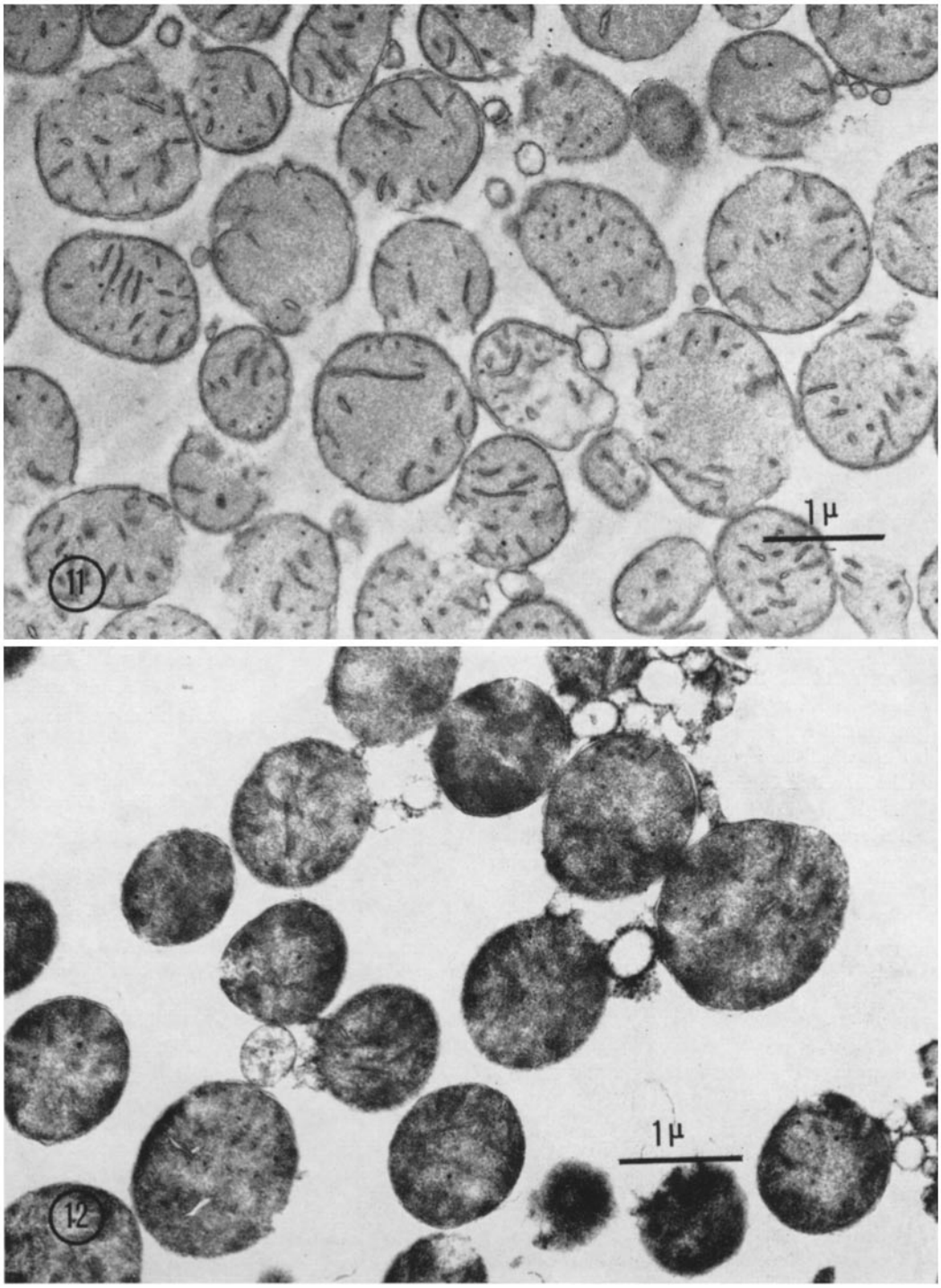

FIGURE 11 Mitochondria in state 3 fixed in suspension in $KMnO_4$. Uranyl acetate. × 17,900.

FIGURE 12 Mitochondria prepared in 0.44 M sucrose and resuspended in 0.15 M KCl. Fixed in suspension in 0.3 M gluteraldehyde and postfixed in osmium tetroxide. Uranyl acetate and lead citrate. × 17,900.

sucrose and fixed in glutaraldehyde, while the very same organelles suspended in 154 mM KCl and fixed in glutaraldehyde lost this contracted form. The contracted form was also seen in the superficial layers of mitochondria fixed as pellets in Veronal-buffered osmium tetroxide (Fig. 3). This contracted form is similar to that seen following fixation in osmium tetroxide of micro pellets in which this mitochondrial conformation is described as occurring through the full thickness of the pellet (4). The same sample of mitochondria fixed in suspension either in Veronal-buffered osmium tetroxide (Fig. 1) or Veronal-buffered potassium permanganate (Fig. 2) produced only "conventional" forms of mitochondria.

It can be seen from Fig. 6 that the mitochondrial membranes are intact and have a unit membrane structure. The granular appearance of these membranes is similar to that described following potassium permanganate or glutaraldehyde fixation of kidney mitochondria *in situ* (18, 19), while the dimensions of the membrane are within the limits described for glutaraldehyde-fixed mitochondria (19). Potassium permanganate has also been shown to be the fixative of choice for isolated synaptosomes (22). $KMnO_4$ has been shown to cause considerable disruption of the helical conformation of proteins in red blood cells (7), but in spite of this the over-all conformation of some isolated organelles is well maintained.

There is good agreement as to the conformation of rat liver mitochondria *in situ* (1, 15, 17). With a variety of fixation procedures and processing schedules (1, 6, 17) the apparent conformation of mitochondria, in vivo, is remarkably constant, but the fine structure of the mitochondrial membrane appears to be modified during fixation and processing (10, 19). When isolated rat liver mitochondria are examined, this constant appearance is not found by most workers, and it has been suggested that this is due to the variations in the functional state of the mitochondria (see 3, 4, 12, 13, 20, 21). However, our experiments indicate that the demonstration of this modification is dependent upon the fixative.

Little is known of the speed at which isolated mitochondria are fixed. However, it would be difficult to explain the preservation of the conventional form as seen following fixation in suspension in osmium tetroxide or potassium permanganate on the grounds that the fixation is slow, allowing swelling to take place from the contracted form.

As well as the obvious different results of fixation with osmium tetroxide between the pellets (Fig. 3) and suspensions (Figs. 1 and 2) of mitochondria, other experiments to be published have shown that the potassium permanganate fixation can preserve the volumes of mitochondria in their state of rapid change in size induced by valinomycin.

The chemical events which constitute fixation of a tissue or organelle by any of the materials used in this study are not well understood (5, 11, 16). In view of this uncertainty it is necessary to be cautious in interpreting structural modifications which can be influenced by the fixation procedure. Reproducibility of the results with a given fixative does not invalidate this cautionary statement.

The authors are indebted to Miss S. Elsey, Miss Gayle Daniels, Mrs. M. R. Nichols, and Miss S. Lear for skilled technical assistance.

Part of this work was done in the Divisions of Oncology and Metabolic Research, Chicago Medical School, and supported by U.S. Public Health Service Contract No. PH 43-65-67 (W.H.B.), National Institutes of Health (Grant AM07226), Life Insurance Medical Research Fund, Burroughs Wellcome Fund, and the Medical Research Council (J.D.J.).

Received for publication 6 April 1969, and in revised form 10 October 1969.

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