A METHOD FOR INCREASING CONTRAST OF MITOCHONDRIAL INNER MEMBRANE SPHERES IN THIN SECTIONS OF EPON-ARALDITE EMBEDDED TISSUE

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The particles of 90 Å diameter on the inner membranes of mitochondria, first discovered by negative staining by Fernández-Morán (2) and later described in various other mitochondria by Parsons (9), Stoeckenius (20), Smith (18), and Sjöstrand (16), have been the center of controversy for nearly a decade. Doubts as to the identity of these inner membrane spheres have been expressed by Sjöstrand et al. (17) and Wainio (22).

The isolation of inner membrane spheres and the morphological and biochemical reconstitution of the membranes led to the identification of these spheres as mitochondrial ATPase, which is also called coupling factor 1 (F_1)[1] (7, 13, 14). The supporting evidence was the concomitant loss of radioactive F_1, ATPase activity, and number of spheres from membranes treated with several agents which dissociate F_1 molecules into subunits (7). F_1 has a mol wt of 284,000 (10) and the content of F_1 was estimated to be about 10 percent of the total proteins of the inner membrane. Ogawa and Mayahara (8) supported the above biochemical data by reporting the intramitochondrial localization of ATPase activity by a histochemical method and by demonstrating its relationship to the inner membrane spheres in rodent cardiac mitochondria.

Reports of the presence of the spheres on cristae of mitochondria embedded in plastic have been few (1, 4). Fleischer et al. (3) have described 90-Å particles in thin sections of cristae of bovine heart mitochondria depleted of neutral lipids. The embedded submitochondrial particles had few if any of the spheres, although the membrane itself was still intact.

The ability to study submitochondrial particles in thin sections has several advantages. These particles do not contain the soluble matrix enzymes which may interfere with the staining process. Since membranes are not flattened when embedded in plastic, as is the case for negatively stained membranes, quantitative assessment of the spheres becomes possible in thin-sectioned material.

This paper describes a method to increase contrast in pictures of bovine heart submitochondrial membranes in thin-sectioned material. A comparison of "stripped" submitochondrial membranes (no spheres on the membranes) and reconstituted membranes (spheres visible on the membrane) in thin-sectioned material further supports the contention that these spheres are functional components of the inner mitochondrial membrane.

MATERIALS AND METHODS

Submitochondrial particles were prepared from bovine heart heavy layer mitochondria (6, 11). F_1-depleted particles (Sephadex-urea-treated particles [SU], alkali-Sephadex-urea-treated particles [ASU]) were prepared according to the method described by Racker and Horstman (13). Reconstitution of the F_1-depleted particles was accomplished as follows: 1 ml of an F_1 suspension in ammonium sulfate (3.95 mg/ml protein) was centrifuged to obtain an F_1 pellet. The supernatant was discarded, and the inside of the centrifuge tube was wiped out to remove any remaining ammonium sulfate. The pellet was resuspended in 0.5 ml of STV-ATP solution (250 mM sucrose, 10 mM Tris-SO_4, pH 7.1, 1 mM EDTA, 2 mM ATP) at room temperature to give approximately 8 mg protein/ml. Aliquots of F_1 solution (160 µl) were mixed with 1 ml of ASU particles (6.5 mg/ml) or 1 ml of SU particles (5.3 mg/ml). These solutions were incubated at room temperature for 15 min. Comparable controls were run at the same time, but lacking F_1.

The particles were fixed in 1 percent glutaraldehyde (50 mM Na-cacodylate, pH 7.3, 250 mM

[1] *The following abbreviations were used in this paper:* ASU, alkali-Sephadex-urea-treated particles: submitochondrial particles depleted of F_1 by sonic oscillation of mitocohondria in dilute ammonia followed by gel filtration through a Sephadex column and urea treatment; F_1, coupling factor 1, mitochondrial oligomycin-insensitive ATPase; SU, Sephadex-urea-treated particles: submitochondrial particles depleted of F_1 by gel filtration through a Sephadex column and urea treatment.

sucrose, 4 mM $CaCl_2$, 4 mM $MgCl_2$) for 1.5 h at room temperature. The suspension was centrifuged at 50,000 *g* for 30 min (last half hour of fixation) in a #40 rotor in a L4 Spinco centrifuge. After several rinses in the buffer solution, the pellets were postfixed in 1 percent OsO_4 dissolved in the same buffer for 1.5 h at 4°C. After several rinses in distilled water, the pellets were placed in 2 percent uranyl acetate for 24 h at 4°C. After the staining with uranyl acetate, all pellets were dehydrated through an ascending series of ethanol, two changes of propylene oxide, and infiltrated and embedded in an Epon-Araldite epoxy resin mixture as described by Telford and Matsumura (21).

The embedded material was sectioned with a diamond knife mounted on a Sorval Porter-Blum MT-2 ultramicrotome. The sections were picked up on uncoated 400-mesh copper Athene grids, and stained with freshly prepared solutions of 2 percent aqueous or 2 percent methanolic (absolute) uranyl acetate, followed by poststaining in concentrated Reynolds' lead citrate (15). Care was taken to ensure that the staining solutions were compared on sections from the same block, sectioned the same day, and stained at the same time. Comparison of ASU particles and SU particles with ASU + F_1 and SU + F_1 were done in the same stain for the same length of time. All grids were completely immersed in the staining solutions.

The tissue was examined in an AEI EM6B electron microscope operated at an accelerating voltage of 60 kV.

RESULTS

During the initial glutaraldehyde fixation, a sample of submitochondrial particles was examined after 1 h in the fixation using the negative staining technique. All particles showed very good preservation of intact spheres around the edge of the membrane, confirming the observations of Stiles and Crane (19) that glutaraldehyde fixation does not interfere with negative staining.

Because there were problems in obtaining good contrast in submitochondrial membranes, the preparation was initially stained in 2 percent aqueous uranyl acetate. This is known to help stabilize as well as add contrast to various membrane components (5). In order to determine the effectiveness of aqueous or methanolic uranyl acetate in the poststaining of the sectioned material, a comparison of the staining solutions was performed. Fig. 1 shows sections (submitochondrial particles) stained in 2 percent methanolic uranyl acetate for 30 min followed by staining in concentrated Reynolds' lead citrate for 10 min at room temperature. Fig. 2 shows sections stained under similar conditions but with 2 percent aqueous uranyl acetate.

Inner membrane spheres are clearly seen in Fig. 1. They project out from the membrane as do those observed in negative staining. Only occasionally were projections of very low contrast seen when aqueous uranyl acetate was used. Increasing the time of staining did not improve the results by either method.

There are still some investigators who maintain that the inner membrane spheres are lipid micelles resulting from a breakdown of the mitochondrial membranes (17, 22). Evidence for the identity of the inner membrane spheres has been summarized by Racker (12). F_1 was stripped off the submitochondrial membranes using Sephadex and urea. These stripped SU particles were then incubated with F_1, and projections were observed on the membranes when examined in negatively stained material. ASU and SU particles were therefore examined in thin-sectioned material which had previously been incubated in the presence and in the absence of F_1. No projections can be seen on the membranes incubated in the absence of F_1 (Figs. 3 and 4). When the particles were incubated with F_1 before fixation, characteristic projections were readily seen in the thin-sectioned material (Figs. 5 and 6).

The inner membrane spheres were also seen in partially disrupted mitochondria (Figs. 7 and 8). Fig. 7 represents bovine heart mitochondria fixed in OsO_4 without prefixation in glutaraldehyde. It apparently does not matter whether the tissue is fixed in OsO_4 only or fixed in glutaraldehyde followed by postfixation in OsO_4. The spheres are still present and can be seen if methanolic uranyl acetate is used in the final staining of the thin sections. Fig. 8, prepared by the double fixation method (glutaraldehyde followed by OsO_4), represents a mitochondrion from bovine brain. Both figures illustrate the presence of spheres on the inner mitochondrial membrane (see arrows).

The reason why methanolic solutions help the staining of F_1 is not clear. However, it may be pointed out that F_1 is a protein which is more difficult to stain than membranes containing phospholipids. It is also possible that the 90-Å particles are rarely sectioned at the surface of the embedded material. In order to reach a buried and hard-to-stain protein molecule, methanolic solutions must be used which probably make the

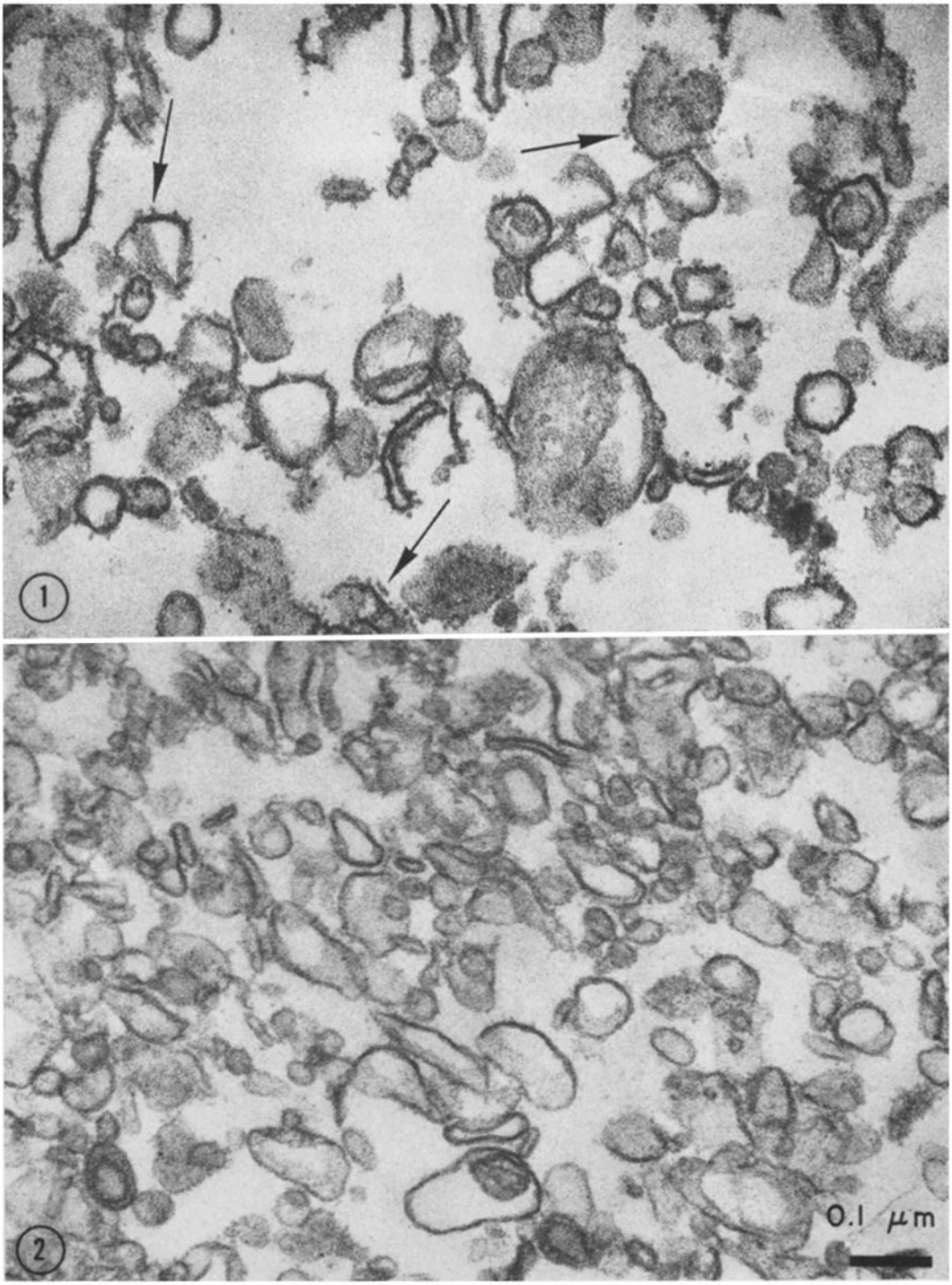

FIGURE 1 Thin section of submitochondrial particles of the inner membrane stained with methanolic 2 percent uranyl acetate (30 min) and concentrated Reynolds' lead citrate (10 min). Arrows point to a few of the spheres projecting from the membranes. × 120,000.

FIGURE 2 Thin section of submitochondrial particles of the inner membrane stained with aqueous 2 percent uranyl acetate (30 min) and concentrated Reynolds' lead citrate (10 min). Very few if any spheres are observed projecting from the membranes. × 120,000.

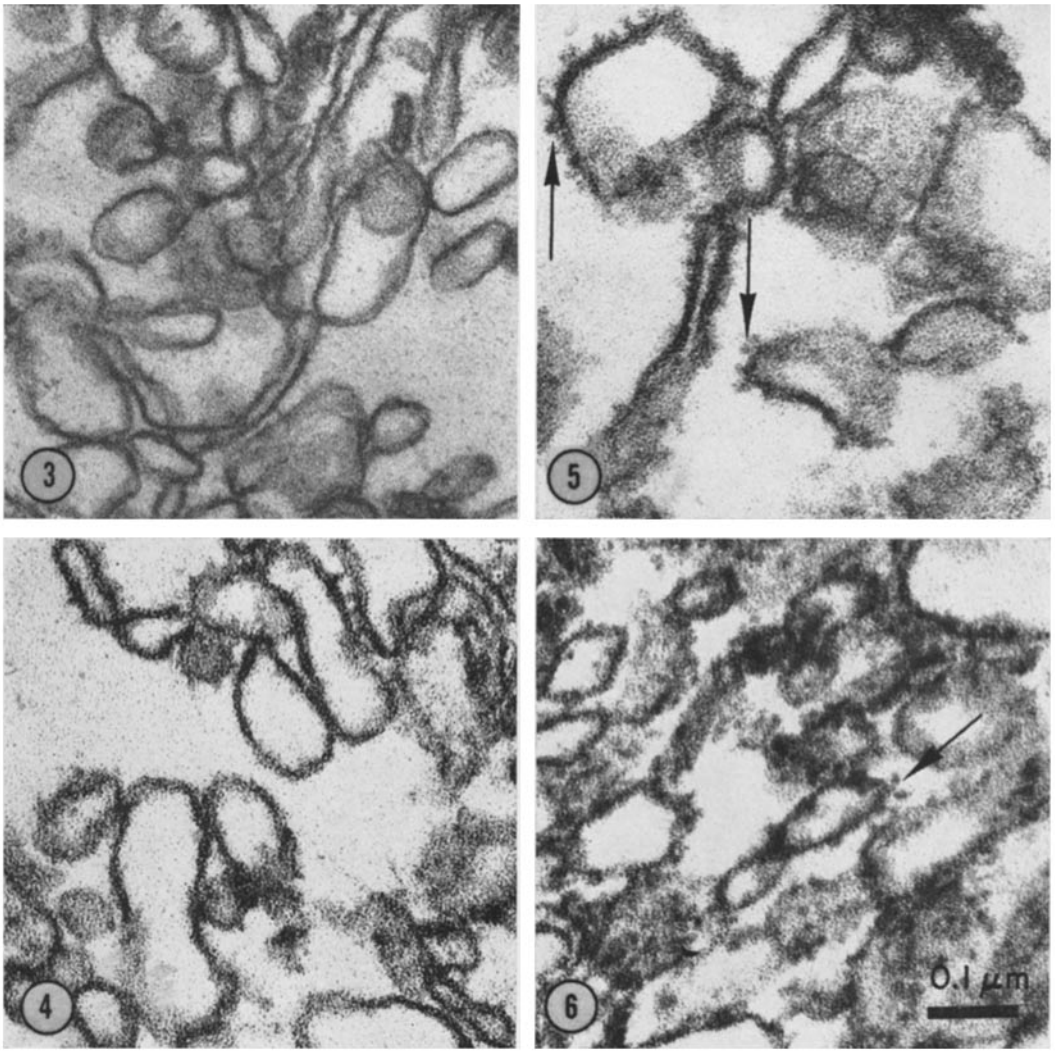

FIGURE 3 Thin section of ASU particles stained with methanolic 2 percent uranyl acetate and concentrated Reynolds' lead citrate. No spheres are present. × 120,000.

FIGURE 4 Thin section of SU particles stained as in Fig. 3. No spheres are present. × 120,000.

FIGURE 5 Thin section of ASU + F_1 particles stained as in Fig. 3. Spheres can be seen projecting from the membranes (arrows). × 120,000.

FIGURE 6 Thin section of SU + F_1 particles stained as in Fig. 3. Arrow points to presence of spheres. × 120,000.

epoxy resin more permeable to the stain than do aqueous solutions.

The above observations strongly suggest that the inner membrane spheres seen in negatively stained tissue are not artifacts caused by the phosphotungstic acid as suggested by Wainio (22) and others, but represent structures occurring naturally in the inner mitochondrial membrane. The fact that these spheres are not embedded within the membrane but attach to its surface raises interesting questions regarding its mode of action as a coupling agent.

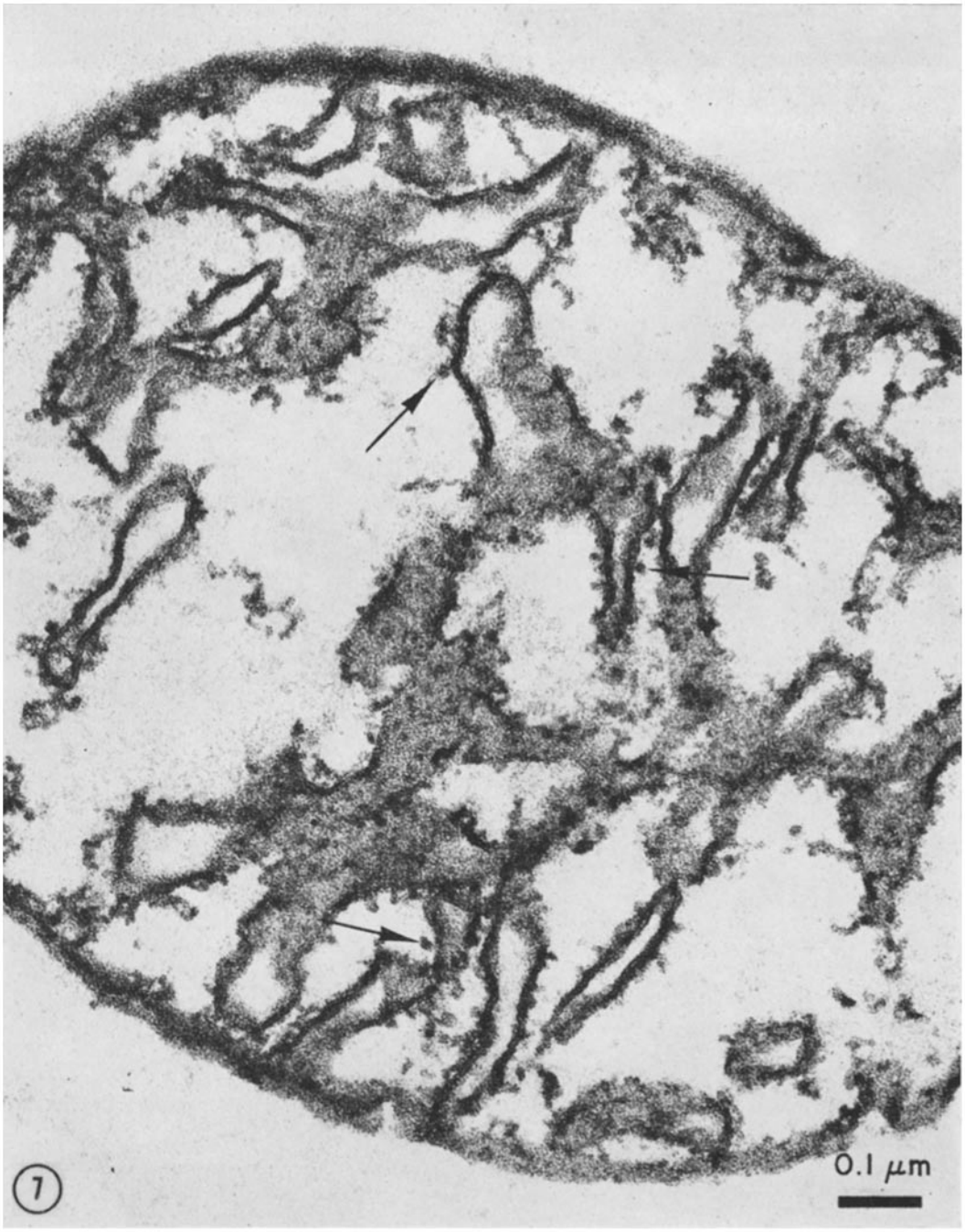

FIGURE 7 Thin section of heavy layer bovine heart mitochondrion, fixed in OsO_4 only. Sections stained with methanolic 2 percent uranyl acetate and concentrated Reynolds' lead citrate. Arrows point to a few of the many spheres observed. × 120,000.

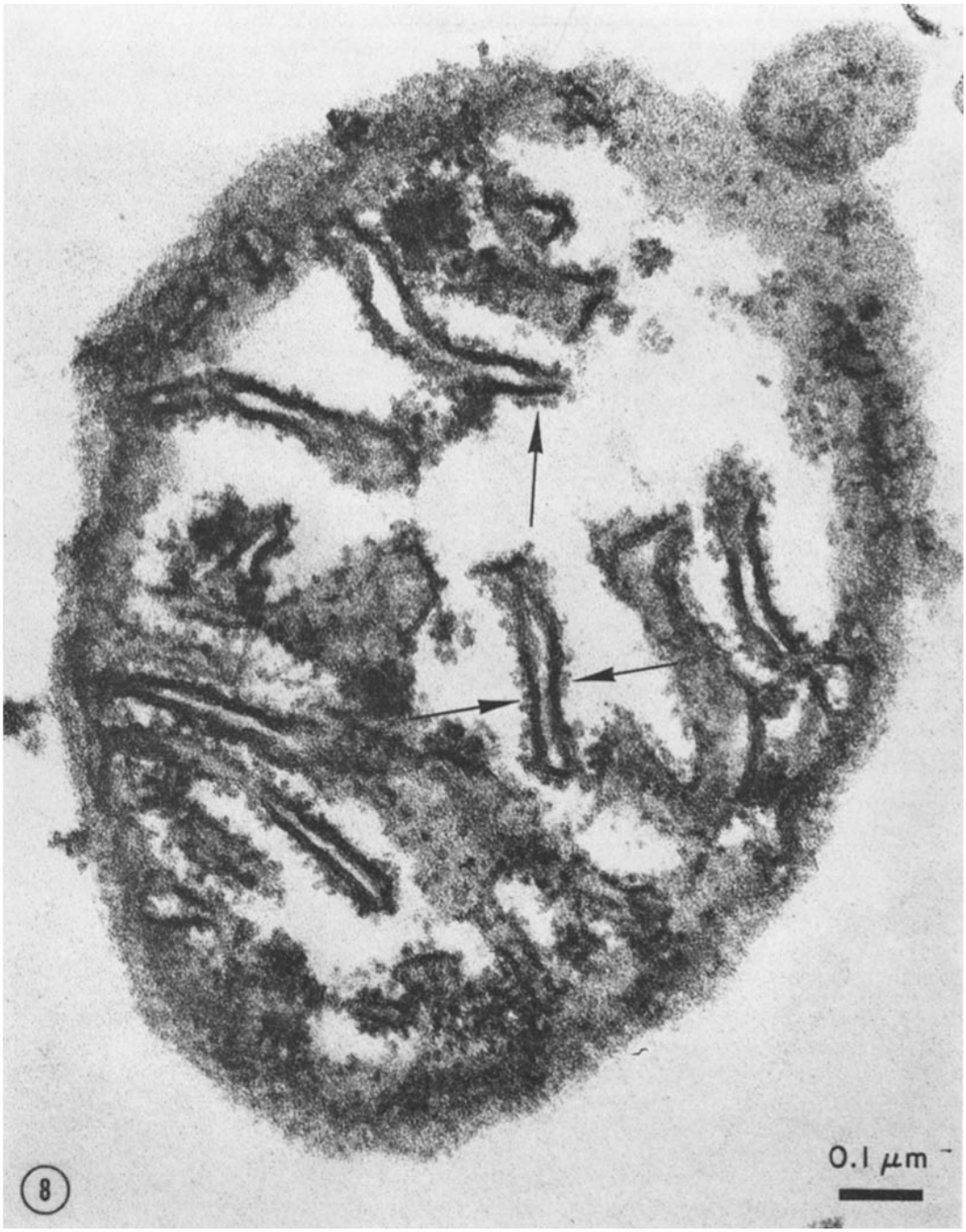

FIGURE 8 Thin section of bovine brain mitochondrion fixed in glutaraldehyde and in OsO_4. Sections stained with methanolic 2 percent uranyl acetate and concentrated Reynolds' lead citrate. Arrows point to rows of spheres observed projecting from the inner membrane. × 120,000.

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