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Mitochondrial Inner Membrane Particles Seen in Sections of *in situ* Large Amplitude Swollen Mitochondria in Rhizodermal Cells of Cress (*Lepidium sativum* L.)

K. Zaar

Lehrstuhl für Zellbiologie
Universität 78 Freiburg i.Br.
Schänzlestr. 9-11
Germany

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Abstract

Addition of 5-40 mM sodium acetate to root explants of cress (*Lepidium sativum* L.) growing in a nutrient medium causes large amplitude swelling of mitochondria in rhizodermal cells. On the average, 10 mM sodium acetate causes a fourfold increase in mitochondrial volume, with 40 mM sodium acetate producing an up to tenfold increase in mitochondrial volume. During swelling, however, the mitochondrial membranes remain predominantly intact, and only the outer membrane occasionally appears to be broken. Two types of swelling can be observed: an overall swelling of mitochondrial matrices and a less frequent local swelling which leads to clearly different matrical regions. These regions may sometimes be separated by a septum formed from the inner mitochondrial membrane. After large amplitude swelling in 10 or 40 mM sodium acetate, the visibility of lollipop-like particles on the matrix side of inner mitochondrial membranes is strongly enhanced. These particles are suggested to be identical with mitochondrial inner membrane particles as visualized by negative staining. The distribution of these particles is described. Possible mechanisms which may cause enhanced visibility are discussed.

Introduction

Lollipop-like structures lining the surface of the inner mitochondrial membrane may be demonstrated routinely in the electron microscope in negatively stained specimens. These structures, originally termed

elementary particles [1, 2], show a spherical headpiece of 80-100 Å diameter and a stalk of 45-50 Å length and 35-40 Å width, which connects the particle to the membrane surface. Kagawa and Racker [3] first presented evidence that headpieces of particles isolated from beef heart mitochondria are identical with a protein component called the F_1 coupling factor, which exhibits ATPase activity. A protein factor (OSCP) conferring oligomycin sensitivity to F_1 has been purified [4] and tentatively identified with the stalk connecting the ATPase to the membrane [5]. In recent years the oligomycin sensitive ATPase complex has been shown to consist of nine different subunit proteins [6, 7] which seem to have their origin in different systems of ribosomal protein synthesis [8, 9, 10].

The existence of inner membrane particles (IMP's) can thus hardly be doubted. Routine procedures for fixation, embedding and sectioning of specimens for the electron microscope have, however, in most cases failed to demonstrate IMP's. Considering the variety of investigations that have been done on sectioned mitochondria, there are only few authors who report on particles being attached to the surface of mitochondrial cristae [11, 12, 13]. Rupec [14] showed particles which seem to be connected by a stalk to the cristal membranes of mitochondria in epidermal cells of man and guinea pig. Previously Telford and Racker [15] demonstrated stalked particles in thin sectioned sub-mitochondrial preparations. They presented a suitable method for enhancing the contrast of these particles in epon araldite embedded specimens.

The present communication reports on lollipop-like particles occurring regularly on cristal membranes within the mitochondria of rhizodermal cells of cress (*Lepidium sat.* L.) after in situ swelling of mitochondria in a nutrient medium containing various amounts of sodium acetate. Dimensions and the arrangement of lollipop-like structures suggest that these particles are morphologically related to IMP's well known from negatively stained preparations of isolated mitochondria. Although IMP's may be detected sporadically in untreated thin sectioned preparations of mitochondria from rhizodermal cells of cress, high amplitude swelling of mitochondria seems to greatly enhance the visibility of these particles. Possible mechanisms which may cause enhanced visibility are discussed. This is the first time that lollipop-like particles could be demonstrated in sectioned preparations after in situ swelling of mitochondria. The ultrastructure of in situ swollen mitochondria is discussed.

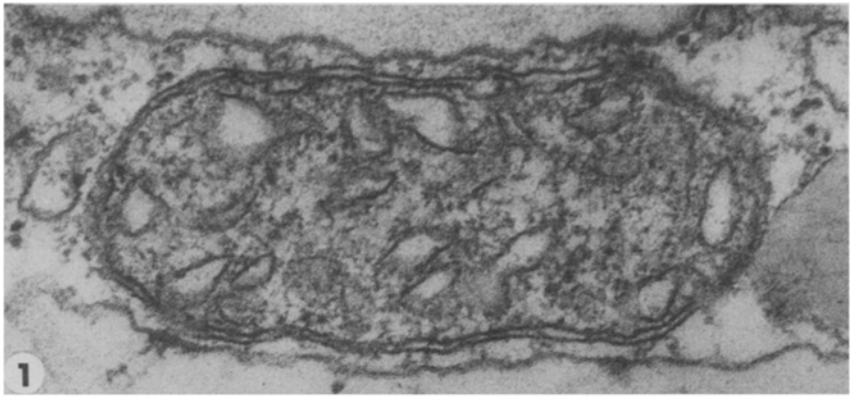
Material and Methods

Seeds of cress (*Lepidium sativum* L.) were grown in agar. About 36 h after planting the seeds, root hair bearing zones were removed and cut to

slices with a razor blade. These slices were incubated for 1,5-5 h in 10 ml vessels containing approximately 2 ml of a modified nutrient medium according to White [16], to which 5, 10 or 40 mM sodium acetate were added. Following incubation, the specimens were fixed for 30-60 min at room temparature in 2% glutaraldehyde in 50 mM cacodylate buffer (pH 6,8) and post-fixed in 2% osmium tetroxide similarily buffered. Post-fixed specimens were soaked overnight with cold 0,5% aqueous uranyl acetate. Slices were embedded in an epon araldite mixture according to Mollenhauer [17]. Grey to silver sections cut from root hairs and rhizodermal cells were double stained with methanolic uranyl acetate [15, 18] and lead citrate [19] and viewed in the Elmiskop I A and 101 at 80 and 100 kV accelerating voltage. With the Elmiskop 101 the anticontamination device was used routinely. Magnification was checked with a diffraction grating replica.

Results

After incubation of root explants for 1,5-5 h in a nutrient medium [16] containing 5, 10 or 40 mM sodium acetate, mitochondria from rhizodermal cells were of irregular shape and showed large amplitude swelling in situ. Stereological measurements according to Weibel [20] revealed after 3 h of incubation, on the average, a doubling of mitochondrial volume with 5 mM sodium acetate, with 10 mM sodium acetate causing a four times increase of mitochondrial volume and 40 mM sodium acetate increase the mitochondrial volume up to tenfold. High amplitude swelling of mitochondria in nutrient media containing 10 and 40 mM sodium acetate was characterized by an excessively expanded state of mitochondrial matrix compartments (Fig. 2). The space between the inner and outer mitochondrial membrane did not seem to be markedly altered. In most planes of sectioning, saccular to tubular cristae, with only slightly enlarged intracristal spaces, were predominantly restricted to the periphery of the swollen matrical compartments. In spite of this remarkable swelling of mitochondria in various concentrations of sodium acetate, only a few mitochondria showed local disruptions of outer membranes. No such disruptions could be detected with inner mitochondrial membranes. An examination of swollen mitochondrial matrices revealed electron opaque materials which consisted of a few typical large intramitochondrial granules 250-400 Å in diameter, small spherical granules approximately 50-100 Å in diameter and granulo-fibrillar material which seemed to be interconnected to some extent. Thus, this interconnecting sometimes introduced a loose sponge-like appearance of matrical materials (Fig. 2 and 3). Corresponding granulo-fibrillar materials were occasionally found outside the mitochondria in the surrounding cytoplasm.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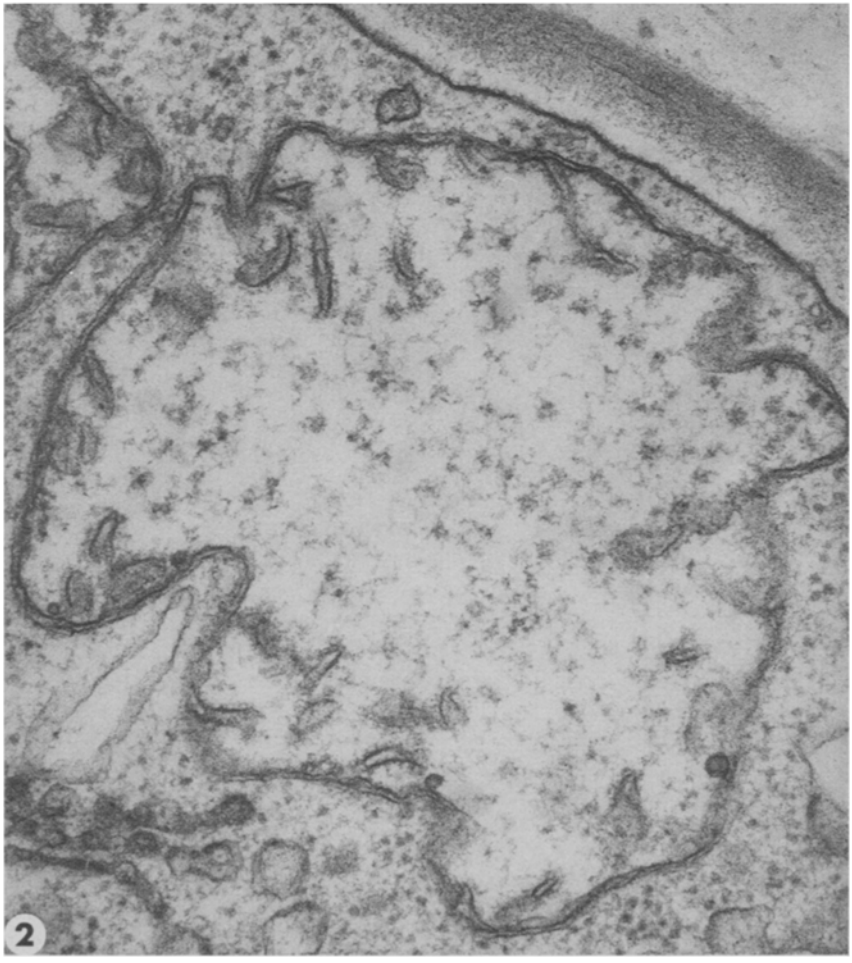
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Usually, high amplitude swelling of mitochondria comprised the whole mitochondrial bodies. In some preparations, especially after incubation in 40 mM sodium acetate, the mitochondrial swelling seems to have begun as a localized process leading to swollen regions in mitochondrial bodies, although other regions of the bodies may remain normal (Fig. 3). Local swelling of mitochondrial bodies produced two clearly discernible matrical regions which differed in their expansion, in the density of matrices, and in the number of cristae recognizable (Fig. 3). These domains within a single mitochondrion, in some cases, were separated from each other by a septum formed by the inner mitochondrial membrane (Fig. 3).

Observation of the surface of cristae of high amplitude swollen mitochondria showed that many of the membranes were outlined by parallel layers of electron dense materials facing the mitochondrial matrix at a distance of approximately 70-100 Å (Fig. 4). Pictures taken from sections through selected section planes revealed that these electron dense layers consist of groups or arrays of lollipop-like particles regularly and periodically lined up on the membrane surface (Fig. 4 and 5). On close examination, these particles showed an approximately spherical headpiece with an average diameter of 70-90 Å. The headpieces seemed to be connected to the membrane surface by a narrow stem (in the range of 30-40 Å width and 30-50 Å length) with low electron density (Fig. 4, 5 and 6). The center to center distance between two particles approximates 100-120 Å . Large arrays of periodically recurring particles could be predominantly demonstrated in sections which cut a long axis to sacculiform or longitudinally to elongated mitochondrial cristae. In cross sections, particularly of elongated cristae, most particles appeared not to be periodically arranged (Fig. 6). Many of the cristae profiles within swollen mitochondrial bodies showed only small groups of periodically arranged particles or individual particles with heterogeneous center to center distances. Many of the cristae profiles were also totally devoid of particles. No significant subunit structure of particle bearing membranes could be detected. The thickness of profiles of inner mitochondrial membranes was not markedly altered after swelling. However, the profiles of the outer mitochondrial membranes were sometimes considerably thinner than in preparations of unswollen mitochondria.

In sectioned specimens, the resolution of particles was dependent upon the plane of sectioning, as outlined above. Therefore, it was hard to

Figure 1. Orthodox mitochondrion from a rhizodermal cell of a root explant from cress grown in White's nutrient medium. × 90,000.

Figure 2. Grossly swollen mitochondrion (40 mM sodium acetate, 3 h). The cristae are restricted to the periphery of the swollen matrix. Within the matrical compartment granulo-fibrillar materials. × 78,000.

quantitate the number of particles seen on the cristal membrane surfaces. Nevertheless, the frequency of particles does not seem to be seriously altered by the experimental conditions used, e.g. by prolonged sodium acetate incubation (1,5-5 h) or varying sodium acetate concentrations (10, 40 mM).

Discussion

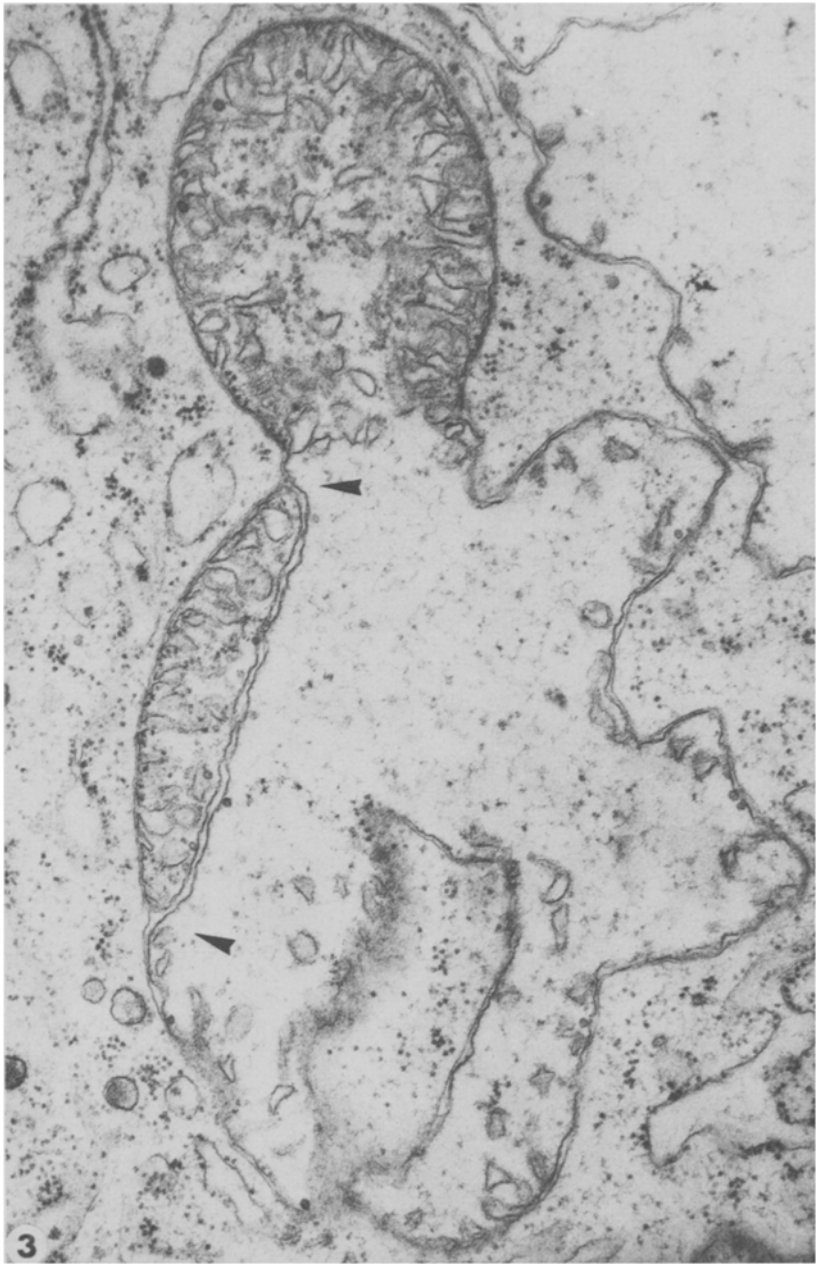
Mitochondria from various sources may undergo reversible energy-linked low amplitude volume changes. Distinct from low amplitude swelling and contraction, is the high amplitude swelling phenomenon which is characterized by a considerable and in most cases irreversible increase in mitochondrial volume. Many investigators have shown that rapid high amplitude swelling of mitochondria may occur when isolated mitochondria are suspended in media containing ammonium, alkali or earth-alkali salts of acetic acid [21, 22, 23, 24, 25, 26, 27]. In 1972 Ruigrok and Elbers [27] demonstrated an in situ 50% volume increase of mitochondria from rat liver perfused with calcium acetate.

Large amplitude swelling of mitochondria in general is thought to be due to an inward transport of ions accompanied by an osmotic equivalent of water. Different permeability properties of the outer and inner mitochondrial membrane thus may lead to a characteristic osmotic swelling of mitochondrial bodies.

In rhizodermal cells of cress considerable swelling of mitochondria in situ may be induced by the addition of sodium acetate (concentrations greater than 5 mM) to the nutrient medium of cultured root explants. The rate of volume increase over the same period of incubation clearly depends upon the concentration of acetate anion. Control preparations of mitochondria from rhizodermal cells growing only in modified White's nutrient medium or nutrient medium containing 40 mM sodium chloride showed no comparable swelling (Fig. 1). Thus influx of salt and concomitant osmotic swelling may be governed by the concentration of acetate anion. There is no conclusive evidence whether acetate acts as a "permeant" anion facilitating active cation transport [e.g. 21, 22, 25], as "reactive" anion undergoing energy-linked transport [26] or, finally, produces "pseudoenergized" swelling [28, 29] of cress mitochondria.

In general in situ swelling of mitochondria affected the whole mitochondrial body. In some cases, however, mitochondrial swelling occured in a special way. A part of a mitochondrion showed specific features of large amplitude swelling, the remaining part seemed to exhibit an unchanged ultrastructure. A similar type of swelling is

Figure 3. Local large amplitude swelling of a mitochondrion. Two clearly different matrical regions may be distinguished. One of the relatively unswollen regions is separated from the remaining matrix compartment by a septum formed from the inner mitochondrial membrane (arrows). x 33,000.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suggested by Bielawsky and Kwinto [30] to begin as a local liquifaction of the matrix then extending gradually over some part of the mitochondrion.

Most descriptions of the ultrastructural changes which occur during swelling of isolated mitochondria have included the interpretation that there is an expansion of the matrix space enclosed by an intact mitochondrial inner membrane. During swelling, the inner mitochondrial membrane is thought to have a constant surface area [31]. The different configurational states of mitochondrial inner membrane during swelling, as shown by several authors [e.g. 32, 33, 34, 35], might be explained as folding or unfolding processes of this membrane [31, 32]. On the other hand, the outer membrane of isolated mitochondria shows no comparable response to changes in osmotic pressure. In most cases the outer membrane is disrupted [36, 37]. In mitochondria of rhizodermal cells of cress swollen in situ, like in swollen isolated mitochondria, an unfolding process of the inner membrane followed by a decrease in the number of cristae may be a valuable concept to account for the expansion of the inner mitochondrial membrane. In contrast to isolated mitochondria, however, the outer membranes of swollen mitochondria within cells of cress showed no significant ruptures even after a tenfold increase in mitochondrial volume. A possible explanation for this apparently greater flexibility of mitochondria swollen in situ is that mitochondrial membranes in situ may be considered active in synthesizing membrane constituents. Especially the outer membrane has been shown to incorporate, e.g. precursors of phospholipids [38]. In many of the sectioned preparations of swollen mitochondria the outer membrane showed a considerably thinner profile than the outer membranes of mitochondria in unswollen tissues. This, possibly, reflects the distension of the outer membrane or an altered ability of these membranes to bind heavy metal salts commonly used for the staining of thin sections.

After in situ high amplitude swelling of mitochondria in rhizodermal cells of cress, lollipop-like particles could be detected on the inner surface of mitochondrial cristae membranes. Location, arrangement and dimensions suggest these particles to be morphologically identical with IMP's as visualized by negative staining. In pictures obtained from thin sections of swollen mitochondria, the surfaces of inner mitochondrial membranes showed, besides greater recurring arrays of particles, many small groups or solitary particles and cristae profiles which are devoid of particles. It is easy to see that particles on a crista membrane may be only sufficiently resolved when the plane of sectioning is close to 90° to the plane of the membrane in question. However, the varied direction of section planes may not only account for the distribution of particles mentioned above. One has to suppose a loss of the spheres from the membrane surface during the swelling process. These spheres may then

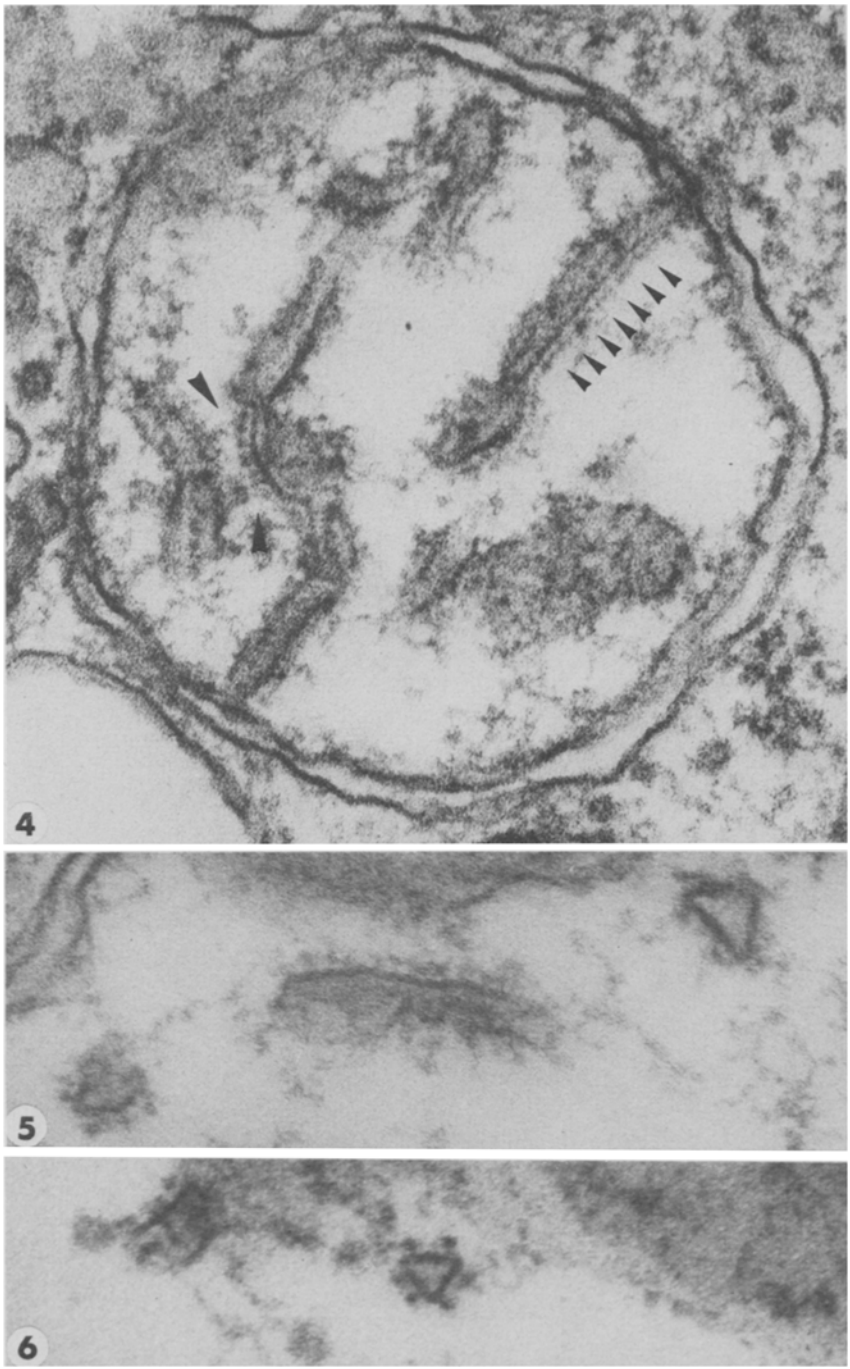
contribute to the granular materials often found in the matrices of large amplitude swollen mitochondria.

It is reasonable to assume that during the high amplitude swelling of mitochondria, matrix proteins as well as extrinsic proteins [39] may be diluted. Extrinsic proteins may be washed away to some extent from the membrane surfaces. These proteins may otherwise possibly interfere with staining procedures. The ATPase, although it may be regarded as extrinsic protein [6, 8, 9, 40] is supposed to be attached, in part, by hydrophobic bonds to the membrane [41, 42] and, therefore may be more resistant to washing. For this reason, it might be that high amplitude swelling helps to unveil inner membrane spheres which are possibly buried by membrane surface materials.

However, one has to consider additional mechanisms which may cause enhanced visibility of IMP's after high amplitude swelling of mitochondria. Interest is turning increasingly to dynamic organization of components of the inner membrane and their role in energy coupling. It seems that respiration dependent proton translocation as well as environmentally induced changes may affect the conformational state of functional proteins within inner mitochondrial membranes [43, 44, 45, 46]. Respiration dependent changes in conformational states of components of the ATPase complex e.g. are thought to play a crucial role in energy coupling in mitochondrial inner membrane [e.g. 43, 44]. Hatase *et al* [47] possibly demonstrated that conformational changes of the ATPase complex during coupling and uncoupling may be detected on the level of the electron microscope ("collapsed" or "extended" configuration of ATPase headpiece-stalk complexes in beef heart submitochondrial particles).

High amplitude swelling of isolated corn mitochondria with alkali salts of acetic acid produces uncoupling [26]. Supposing that uncoupling occurs also in mitochondria of cress swollen in situ with sodium acetate, it may be possible that components of the ATPase complex undergo conformational changes visible on the level of electron microscope as suggested by Hatase *et al* [47].

As has been shown above, a particularly valuable procedure for demonstrating possibly uncovered IMP's in sectioned preparations of mitochondria swollen in situ and embedded in epon-araldite mixtures is the introduction of heavy contrast to the specimens by application of, e.g. aqueous uranyl acetate to the hydrated tissue and by a prolonged staining of the thin sectioned specimens especially with methanolic uranyl acetate [15]. The same staining procedure applied to sections of unswollen mitochondria of rhizodermal cells of cress leads to a heavy contrast all over the mitochondrial bodies, even in the mitochondrial matrix materials. In this case, no satisfactory resolution of particles on the membrane surfaces could be attempted.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There is now growing evidence that IMP's may be demonstrated in sectioned preparations of mitochondria from various tissues. The demonstration of IMP's in fixed and sectioned preparations rules out that IMP's are destroyed during fixation with glutaraldehyde and/or osmium tetroxide (see also Stiles and Crane [48]), or that IMP's are artifacts caused by phosphotungstic acid during negative staining of mitochondrial preparations. Breakage of isolated mitochondria [15] as well as swelling of mitochondria in situ, seems to be advantageous for the demonstration of IMP's in sectioned preparations. Both processes may be considered to cause hypotonic conditions in mitochondrial matrix compartments. In negative staining of isolated mitochondria quite similar conditions may result as a consequence of the staining procedure for demonstrating IMP's.

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Figure 4. Thin section through the periphery of a mitochondrion. One crista outlined by a parallel layer of electron dense materials (arrows). Another crista with lollipop-like particles (arrows). × 180,000.

Figure 5. Thin section longitudinally to a crista. On the membrane surface facing the mitochondrial matrix an array of periodically recurring stalked particles may be seen. × 240,000.

Figure 6. Cross section through an elongated crista. Stalked particles on the membrane surface show heterogeneous center to center distances of headpieces. × 200,000.

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