

# PREVENTION OF PHOSPHATE - INDUCED MITOCHONDRIAL SWELLING

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## ABSTRACT

The prevention of phosphate-induced mitochondrial swelling in the whole retina of the rabbit was studied with the electron microscope. It was found that a mixture of ATP,  $Mg^{++}$ , and bovine serum albumin protected the mitochondria *in vitro*. This finding confirmed the results obtained spectrophotometrically with isolated rat liver mitochondria by Lehninger.

## INTRODUCTION

Numerous studies of mitochondrial swelling *in vitro* have been reported in the biochemical literature. Thyroxine (1-3), inorganic phosphate (4, 5),  $Ca^{++}$  (1, 4), reduced glutathione (6), U-factor (an endogenous uncoupling agent) (7), *p*-chloromercuribenzoate (8), arsenate (5),  $Zn^{++}$  (5), phlorizin (9), and simple hypotonicity have all been shown to cause mitochondrial swelling (10). Since mitochondrial function depends on intact mitochondrial structure (5, 11, 12) any alteration in structural integrity has biochemical significance. The enzymes of electron transport and coupled phosphorylation are currently thought to have an orderly arrangement in the internal membranes and cristae of the mitochondria (13).

The usual means for studying mitochondrial swelling *in vitro* have been the measurement of optical density of suspensions of mammalian hepatic mitochondria. Phase contrast microscopy (14) and gravimetric procedures (15) have also been found to correlate well with the optical density measurements. We approached a study of mitochondrial swelling by direct observation of rabbit retina with the electron microscope. The inner segments of retinal photoreceptors contain many mitochondria. We chose 0.10 M phosphate buffer as the incubating medium because it is commonly employed in histochemical studies for

the electron microscope (16-20). We used the mixture of adenosine triphosphate (ATP), magnesium, and bovine serum albumin (BSA), which Lehninger found protected suspensions of rat liver mitochondria from swelling in a phosphate medium (10).

## MATERIALS AND METHODS

Albino rabbits, weighing approximately 2 kg, were sacrificed by injection of 50 cc of air into an ear vein. One eye was quickly enucleated and opened along the equator. The vitreous was carefully removed and small (approximately 2 mm square) pieces of retina were cut with a sharp razor blade from the area immediately below the optic nerve. The tissue was placed in incubating media for 15 minutes at room temperature. (This time and temperature were selected to duplicate the conditions under which Lehninger (10) found that phosphate-induced swelling approached a maximum.) The experimental medium was 10 ml of 0.10 M sodium phosphate buffer (pH 7.6) to which was added ATP, 0.005 M (adenosine triphosphate, disodium salt from Sigma Chemical, St. Louis, Missouri;  $MgCl_2$ , 0.003 M; and BSA, 2 mg/ml, (crystallized bovine plasma albumin from Armour & Co., Chicago). These substances were added as dry material to avoid volume changes in the incubating mixtures. The control medium consisted solely of 10 ml of the sodium phosphate buffer.

After incubation, the incubating medium was poured off, and cold (4°C) buffered 2 per cent osmium tetroxide (containing sucrose, 45 mg/ml, and 0.002 per cent CaCl<sub>2</sub>) was quickly added for 30 minutes. (The fixative was not added directly to the incubation mixture because this would dilute the fixative.) The tissue was then dehydrated in a graded series of ethanol, and embedded in Epon 812 according to the method of Luft (21). Sections were cut in a Servall ultramicrotome with glass knives, then mounted on bare copper grids, stained in a saturated solution of uranyl acetate in 50 per cent ethanol for 15 minutes and viewed in a JEM 5Y electron microscope.

## RESULTS AND INTERPRETATION

The retina is a favorable tissue for study of *in vitro* effects on mitochondria because: (a) it is an easily obtainable "thin tissue slice," 0.5 mm thick, in which all cells are close to the incubating medium, and (b) mitochondria are found in high concentration in the inner segments of the photoreceptor cells.

Fig. 1 is an electron micrograph of a random area of outer retina, the tissue incubated for 15 minutes in phosphate buffer to which was added the ATP-Mg<sup>++</sup>-BSA mixture. Mitochondrial morphology is well preserved.

Figs. 2 and 3 are similar areas of retina from the same eye, incubated for 15 minutes in the control medium (phosphate buffer with no ATP-Mg<sup>++</sup>-BSA mixture added). The following changes may be noted in the mitochondria of some of the inner segments: (a) increase in size to two or three times normal, (b) change in shape from cylindrical to spherical, (c) fragmentation of cristae mitochondriales, and (d) rupture of the photoreceptor cell membrane with disappearance of the usual cytoplasmic components. That the damaged areas are indeed the inner segments of the photoreceptor cells and not, for example, processes of the retinal pigment epithelium is made clear in Fig. 2. It is known that the outer segment of the photoreceptor cell is connected to the inner segment by a connecting cilium containing nine longitudinal fine

filaments arranged in a circle (30-32). Fig. 2 shows two such connecting cilia (CC<sub>1</sub> and CC<sub>2</sub>) in oblique and cross-section, respectively. They both clearly arise from areas which could only be photoreceptor inner segments.

The mitochondrial changes do not occur in all the cells at the same time; indeed it is common to observe a cell with normal-appearing mitochondria adjacent to a cell whose mitochondria are severely damaged. Approximately every fourth or fifth cell was severely damaged. Three stages in the evolution of mitochondrial swelling are seen in Fig. 3, in which cells with intact mitochondria, partially swollen mitochondria, and also severely swollen mitochondria are seen side by side. The reason for the differing rates of mitochondrial swelling from cell to cell may be attributable to differences in cellular permeability to the phosphate ion. In any event, no similar mitochondrial damage was evident in any of the cells of the retina protected by the ATP-Mg<sup>++</sup>-BSA mixture.

The markedly increased electron opacity of the fragmented cristae mitochondriales seen in Figs. 2 and 3 is unexplained. It is known that mitochondrial fragments do not have the same biochemical properties as do intact mitochondria (12). A change, therefore, in electron opacity with fragmentation is less surprising.

## DISCUSSION

### *Phosphate as a Swelling Agent*

In 1953, Raaflaub (4) noted that as little as 10<sup>-3</sup> M inorganic phosphate could induce mitochondrial swelling. Hunter and Ford (5) reported that this swelling was associated with inactivation of DPN-dependent oxidations and a loss of mitochondrial DPN (diphosphopyridine nucleotide). They felt that this was not due to a simple electrolyte effect because Tris, NaCl, KCl, NaNO<sub>2</sub>, NaNO<sub>3</sub>, and NaF were relatively inactive. The mechanism of how phosphate or any swelling agent produces swelling remains unclear, but the substances which uncouple oxidative phosphoryla-

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### FIGURE 1

Outer segments (OS) and inner segments (IS) of photoreceptor cells of the retina of the albino rabbit. The tissue was incubated at room temperature for 15 minutes in 0.10 M phosphate buffer to which was added an ATP-Mg<sup>++</sup>-BSA mixture. The mitochondria (m), in high concentration in the inner segments, are well preserved. dp, dense particles. × 19,000.



tion commonly produce mitochondrial swelling.

It is generally agreed that swelling involves upsetting a dynamic balance between uptake and extrusion of water in favor of the former (3, 15, 22, 24). The electron micrographs in this study provide direct confirmation of this hypothesis. The increase in mitochondrial size, and the change in shape from cylindrical to spherical are compatible with increasing pressure within the organelle. The disorganization of internal structure with swelling correlates with the alterations in function known to occur with swelling (5, 11).

#### *Reversal of Phosphate-Induced Swelling*

It was observed in 1953 that adenosine monophosphate (AMP) could maintain a low mitochondrial water content (25), and later that phosphate-induced swelling could be prevented by EDTA (ethylenediaminetetraacetate),  $Mn^{++}$  and  $Mg^{++}$  and to a less extent by citrate, ATP, ADP (adenosine diphosphate), AMP, and DPN (5). Other work has shown that resumption of oxidative phosphorylation could reverse the swelling effects of phosphate, and that restoration of respiration by supplying ADP, substrate and oxygen could also shrink mitochondria (22, 26).

Lehninger (10), however, showed that an ATP- $Mg^{++}$ -BSA mixture had a maximal ability to reverse phosphate-induced mitochondrial swelling and that this was independent of both respiration and coupled phosphorylation. Neither the ATP, the  $Mg^{++}$ , nor the BSA alone had any swelling reversal effect, but ATP +  $Mg^{++}$  produced definite reversal, and adding BSA to the ATP- $Mg^{++}$  mixture produced maximal reversal. No attempt was made in the present investigation to assess quantitatively the relative protective effects of these three substances. Only a limited number of inner segments are seen in a given field in the

electron microscope, and a subjective impression of degree of protection would vary from field to field. It was felt that in optical density studies of large populations of isolated mitochondria one could assess more objectively and accurately the effect of different combinations of the three active substances.

The possible mechanisms which have been suggested to explain the observed effect are: (a) ATP- $Mg^{++}$  may polymerize some solute inside the mitochondrion to the point where the internal osmotic pressure is lowered, (15), (b) an ATP-specific contractile process (4, 10, 15, 22, 23, 27) based upon mitochondrial phosphoprotein (28) and phosphokinase (29) may exist, and (c) BSA may bind an endogenously formed uncoupling agent believed to be a fatty acid and known as U-factor (7).

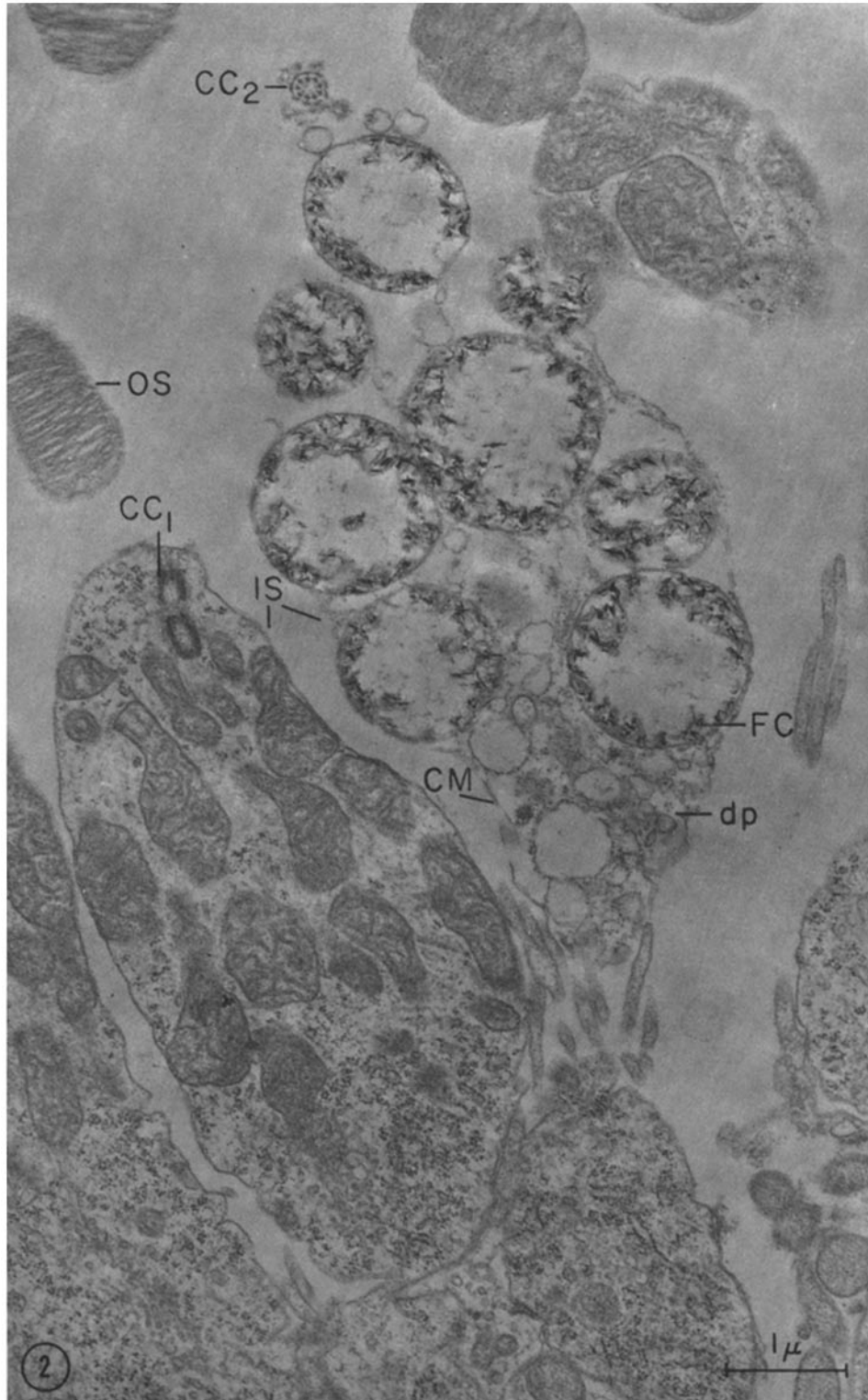
#### *Correlation between Studies of Isolated and Intracellular Mitochondria*

One difficulty encountered in interpreting the results of the present study is that electron micrographs of sections of mitochondria in cells have to be correlated with optical density studies of isolated mitochondria. In view of the reversibility of the swelling reaction in isolated mitochondria, how may one explain the profound morphologic changes observed with swelling within intracellular mitochondria?

In the first place, it is known that swelling does change biochemical properties of mitochondria (5, 11); therefore, intramitochondrial structural changes with swelling should not be surprising. Secondly, electron microscopic studies of the effects of swelling upon isolated mitochondria have recently been reported (33). It was shown that with swelling the cristae undergo change in size and shape and that with extreme swelling

FIGURE 2

An area similar to that in Fig. 1, from control retina of the same eye, in which no ATP- $Mg^{++}$ -BSA mixture had been added to the 0.10 M phosphate incubating medium. The mitochondria of the inner segment of one receptor cell are enlarged, are spherical instead of cylindrical, and contain fragmented cristae (FC) of high density. The cytoplasmic membrane (CM) of the receptor cell is ruptured, and the cell cytoplasm is in free communication with the external environment. Most of the dense particles (dp) in the cell have disappeared.  $CC_1$ , connecting cilium in oblique section.  $CC_2$ , connecting cilium in cross section showing nine fine filaments circularly arranged. Outer segment (OS) and inner segment (IS) of photoreceptor cell. See text for explanation.  $\times 17,000$ .



they disappear. This finding correlates well with the observations in the present study of intracellular mitochondria. It would seem that the spectrophotometer, although it is extremely useful in determining swelling effects upon large populations of isolated mitochondria, must yield to the electron microscope when one desires to know of intramitochondrial alterations.

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#### FIGURE 3

Retina incubated as in Fig. 2. Receptor cell A contains well preserved mitochondria. Receptor cell B contains a mitochondrion (*m*) showing early changes of swelling with increase in size and fragmentation of cristae. Receptor cell C has mitochondria showing severe changes as in Fig. 2.  $\times 19,000$ .

