DEMONSTRATION OF MITOCHONDRIAL ATPase ACTIVITY IN FORMALIN-FIXED COLONIC EPITHELIAL CELLS

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INTRODUCTION

The intracellular distribution of adenosine triphosphatase (ATPase) activity has been extensively studied by means of two histochemical methods in which the inorganic phosphate (Pi) produced upon the enzymatic hydrolysis of adenosine triphosphate (ATP) is trapped as an insoluble salt (26, 40). The application of these methods in light microscope studies on a variety of unfixed tissues revealed the ATPase activity on the cell membranes, microvilli, and mitochondria (13, 15, 19, 23, 24, 27, 39, 41).

The mitochondrial activity was in no case demonstrable in tissue which had been chemically fixed for more than 90 minutes, and usually for shorter periods (13, 18, 23, 24, 35, 39, 40, 41). Fixation of cell fractions from liver homogenates reduced the mitochondrial ATPase activity by 80 to 90 per cent of the original activity (24). Even very brief fixation in OsO4 completely inhibited the mitochondrial activity, while not all structural details were adequately preserved for electron microscopy (9). Shortly thereafter the preservation of ultrastructure and enzymatic activity in general was greatly improved by fixation in formalin preceding incubation (16, 38), and subsequently a large number of other aldehydes have been employed for enzyme histochemistry (32). However, prolonged fixation in the aldehydes also inhibits the mitochondrial ATPase. Thus the electron microscope studies on tissues subjected to prolonged chemical fixation have not revealed significant deposits within the mitochondria after incubation for ATPase activity (1, 8, 9, 16, 19, 21, 22, 29, 32, 33, 37, 38).

A reaction product within mitochondria has been observed after incubation of fresh frozen sections (1, 19, 33) and of sections very briefly fixed in formalin (1, 19, 39) or osmium tetroxide (1, 29). In these studies, some ultrastructural details of the tissues have been compromised in order to retain maximal enzymatic activity.

In this note we report some unusual observations on the mitochondria of rat colonic mucosa incubated to reveal ATPase activity after overnight fixation in 4 per cent formaldehyde.

MATERIALS AND METHODS

The descending colon from stock male Sherman rats was opened, cut into pieces about 5 X 5 mm, and fixed overnight (approximately 16 hours) at 4°C in 4 per cent formaldehyde in 0.05 M cacodylate buffer and 1 per cent CaCl2 at pH 7.4 to 7.6. (The fixative had been prepared 4 months before this study was carried out, but the pH was checked immediately before use.) Ten per cent sucrose was added to the fixative for some specimens. Fixation was followed by rinsing in 0.05 M cacodylate buffer with 10 per cent sucrose at pH 7.4 to 7.6 at 4°C for at least six changes (2 hours total). Some tissues were used immediately, but others were kept in the buffer for 1 month at 4°C. Since the ATPase of kidney has been extensively studied, pieces of this tissue were fixed in the same manner for use as controls.

Tissues were then frozen in a mixture of 95 per cent alcohol and dry ice at --70°C, and frozen sections were cut in a cryostat at 30 to 40 μ and collected in buffered sucrose plus 0.05 M MgCl2 at 4°C. The sections were then transferred into the incubation mixture modified from that of Wachstein and Meisel (40) to the following composition:

<table>
<thead>
<tr>
<th>Test</th>
<th>Control</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP disodium salt (Pabst) 125 mg/100 ml</td>
<td>20 ml</td>
<td>8.3 X 10^-4 M</td>
</tr>
<tr>
<td>Tris-Maleate, pH 7.3</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Pb (NO3)2 2%</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MgCl2 0.5 M</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3</td>
<td>23</td>
</tr>
</tbody>
</table>

1 The importance of the purity of maleic acid has been recently discussed (3). In these experiments, we have used No. 2927 from Mathieson, Coleman and Bell as recommended by Barka (personal communication).
The components were added in the order listed. The lead solution was added dropwise while the medium was constantly stirred. The final solution was clear or only very slightly cloudy; in either case, it was filtered. Media with any considerable cloudiness or precipitate yielded negative results. The final pH was adjusted to 7.3.

The sections were incubated at 37°C for various lengths of time (5 to 90 minutes). The incubation was followed by rinses in distilled water, very briefly initially examined unstained, but for most of the studies the sections were stained for 30 to 60 minutes in a saturated solution of uranyl acetate in 50 per cent ethanol. Preparations were examined in an RCA EMU 2E.

RESULTS AND DISCUSSION

Lead deposits can be seen on the brush border and the lateral membranes of the rat colonic mucosa (Fig. 1), as reported by previous workers for a variety of other tissues (1, 9, 16, 18). The interdigitations between lateral membranes are greatly accentuated by the lead deposits. All mitochondria exhibit a banded array of lead deposits. X 10,000, unstained.

![Figure 1](image-url)

**Figure 1** Rat colonic mucosa fixed overnight in buffered formaldehyde at 4°C and incubated for 40 minutes in ATP-containing medium. The brush border (BB) and multivesicular bodies (MV) are covered with very heavy deposits. Interdigitations between lateral membranes are greatly accentuated by the lead deposits. All mitochondria exhibit a banded array of lead deposits. × 10,000, unstained.
after incubation in the presence of $\beta$-glycerophosphate at pH 5.0 (2, 25).

The lead deposits encountered within the mitochondria give them a banded appearance like the array of the *cristae mitochondriales* (Figs. 1 to 4). The mitochondrial membranes are not clearly discernible in these preparations, even after prolonged "staining" with lead or uranyl salts. This difficulty in revealing membranes after fixation in the mitochondrial reaction was observed, but the banded pattern was still easily recognizable (Fig. 4).

The mitochondrial deposits were not present in tissues incubated in substrate-free medium or in complete medium plus $10^{-3}$ M N-ethyl-maleimide (NEM). Nor were they observed when thiamine pyrophosphate (TPP), inosine diphosphate (IDP), or $\beta$-glycerophosphate were used as

![Image](image_url)

**Figure 2** Rat colonic mucosa prepared as described in Fig. 1. The pattern of the intramitochondrial deposits suggest that they cover the *cristae mitochondriales*. Multivesicular bodies (MV) and cell membranes also exhibit reaction product. × 31,000. Stained with uranyl acetate for 20 minutes.

with aldehydes has been experienced by other investigators (22, 32). The deposits are apparently confined to the inner mitochondrial membrane and its convolutions, and are lodged upon the cristae (Fig. 3).

ATPase activity of the colonic epithelial mitochondria, but of different intensity, was discerned in three different experiments using the same methods.

When sections cut from pieces of aldehyde-fixed colon, which had been kept for 1 month in buffer at 4°C, were incubated in the medium, a reduction in the substrate (25). This suggests that the reaction has some specificity.

It might be argued that deposits such as these may arise as the result of the diffusion of the product from a highly reactive site outside the mitochondria. That this is not the case here is suggested by the three following observations. (a) The multivesicular bodies are highly reactive towards $\beta$-glycerophosphate, but the mitochondria in their immediate vicinity never show any deposits (2, 25). (b) Similarly, the endoplasmic reticulum of the colonic mucosa which exhibits
high enzymatic activity towards IDP is often seen in very intimate contact with mitochondria, which are always free of deposits (25). (c) The so-called β cytomembranes on the basal portion of the cells in the distal tubule of the kidney are known to delimit compartments containing mitochondria and to exhibit ATPase activity (21, 35), but even after prolonged incubation (90 minutes) in this medium no deposits were apparent in the mitochondria (Fig. 5).

The difference in response between the mitochondria in the kidney and colon is not completely understood. It may be a real difference, or result from different sensitivities of the mitochondrial enzymes to formaldehyde fixation.

The nature and significance of mitochondrial ATPase activity is still debated. This activity is usually attributed to the action of two different enzymes, one of which is enhanced by dinitrophenol (DNP) and another which is stimulated by Mg++ ions. These two enzymes have different pH optima and also exhibit different sensitivities toward inhibitors (4, 5, 14, 34). In most of these studies the activity has been found associated with submitochondrial “particles” obtained by the treatment of mitochondria with digitonin (14), deoxycholate (34), or ultrasonication (7). Electron microscopic examination of the “deoxycholate particles” revealed that these consist of membranous structures (42). From this evidence the mitochondrial ATPase is believed to be membrane bound, presumably to the cristae.

More recently a soluble fraction of the ruptured mitochondria has been reported to exhibit properties which may explain the effects of DNP and Mg++ as the action upon a single enzyme (30). This soluble enzyme may represent a further fractionation of the particles described above. Extensive reviews on this subject have been recently published (20, 31).

The methods for histochemical localization of phosphatases in the electron microscope allow only the detection of the site of deposition of the trapped reaction product, i.e., lead phosphate. This may or may not coincide with the exact site of the enzyme. The importance of this limitation is heightened by the results which are being currently obtained with the “negative staining” techniques as applied to viruses (6, 28) and mitochondria (11, 28, 36), in which a macromolecular aggregate is embedded in an electron-opaque substance which fills all the space available except that
occupied by the aggregate itself. Thus if an active enzyme is part of such a macromolecular aggregate, it may preclude any extensive deposition of the reaction product precisely on the enzyme site, and, consequently, the deposits may produce the effect of a negative stain at high levels of resolution.

In summary, an extensive deposition of reaction product has been observed within the mitochondria in apparent relation to the inner membranes after incubation of formaldehyde-fixed colonic mucosa using ATP as a substrate. Similar results were obtained with these tissues after 1-month storage in buffer at 4°C. The mitochondria of formalin-fixed kidney gave no reaction. The colonic mitochondria showed no deposits in the presence of β-glycerophosphate, IDP, TPP, or ATP plus NEM.

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