

CYTOCHEMICAL IDENTIFICATION OF PHOSPHATASE ACTIVITY IN THE CONTRACTILE VACUOLE OF *ACANTHAMOEBA CASTELLANII*

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INTRODUCTION

We have found that alkaline phosphatase, an enzyme often associated with active transport, is present in the contractile vacuole of the amoeba, *Acanthamoeba castellanii*. Earlier biochemical studies (1, 2) had suggested that this enzyme and a 5'-nucleotidase might be associated with the plasma membrane. Highly purified plasma membranes were isolated from *Acanthamoeba* by Schultz and Thompson (1) and by Ulsamer et al. (2). These preparations were 12- to 19-fold enriched over cell homogenates in the ability to hydrolyze AMP, and Goodall et al. (3) utilized that activity as a specific marker for the plasma membrane. Ulsamer et al. (2), however, observed a parallel enrichment in the ability to hydrolyze β -glycerophosphate and *p*-nitrophenylphosphate and, because all three substrates were hydrolyzed maximally at alkaline pH and since AMP was the poorest substrate of the three even at neutral pH, they raised the possibility that a nonspecific alkaline phosphatase catalyzed all three reactions. Ulsamer et al. (2) further suggested that the

plasma membrane might not be the source of the phosphatase(s) since cell fractions less enriched in plasma membranes sometimes had a higher specific activity towards all three substrates than did the highly purified plasma membrane fraction. We felt that cytochemical methods might serve to clarify some of these ambiguities.

In the work reported here we have been unable to demonstrate by cytochemical methods any phosphatase activity associated with either the isolated plasma membranes or with plasma membranes of intact cells. The nature of the cytochemical procedures, however, and the inherent ambiguities of "negative" results do not allow us unequivocally to rule out phosphatase activity in the plasma membrane. There was an unexpected finding: the cytochemical data definitely establish the presence of an alkaline phosphatase in the contractile vacuole. Identical results were obtained with α -naphthylphosphate, *p*-nitrophenylphosphate, and AMP as substrates. Within the limits of cytochemical methodology, then, these data suggest that contamination of the

plasma membranes by a small percentage of previously undetected contractile vacuole membrane could explain the phosphatase activity detected in those preparations.

MATERIALS AND METHODS

Acanthamoeba castellanii (Neff strain) was grown axenically in a proteose-peptone, glucose solution (4). Plasma membrane fractions were isolated as described by Ulsamer et al. (2).

Amebas were fixed for 1 h in 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in 0.1 M sodium phosphate buffer, pH 6.8, rinsed overnight in 0.1 M Tris-HCl, pH 7.4, or in 0.1 M sodium phosphate buffer, pH 6.8, then rinsed twice in a large volume of 0.1 M Tris-HCl, pH 9.5, before cytochemical incubation. The use of phosphate-buffered fixative followed by thorough rinsing resulted in no higher background than that found after cacodylate-buffered fixative, and the latter gave much poorer morphological preservation. Isolated plasma membranes for the cytochemical studies were either unfixed or fixed in 4% formaldehyde (prepared from depolymerized paraformaldehyde) with 2% calcium acetate, pH 7.5, for 30 min. and rinsed in 0.1 M Tris-HCl buffer, pH 9.5.

Three substrates were used: *p*-nitrophenylphosphate, α -naphthylphosphate (both from Calbiochem, La Jolla, Calif.), and AMP, Type II (Sigma Chemical Co., St. Louis, Mo.). The incubation medium contained 100 mM Tris-HCl buffer and 10 or 20 mM substrate. The medium pH was adjusted to 9.5 for the first two substrates and to 7.5 for the last. Three capture reagents were used in the medium: CaCl₂ (40 mM), SrCl₂ (20 mM), or lead citrate (3 mM). The lead citrate (5) was made by adding sodium citrate to a concentration of 3.3 mM and then adding lead nitrate dropwise. When calcium (6) or strontium (7) were used as capture reagents, the cells or membranes were rinsed after incubation in 100 mM Tris-HCl buffer of appropriate pH and reincubated in 2% Pb(NO₃)₂ for 5 min. Preparations that used strontium as capture reagent are not illustrated. This reagent was generally less inhibitory than lead or calcium and the same pattern of precipitate was seen as with the latter two capture ions, but in cells the background precipitate over the cytoplasm and especially over the nucleus was much higher. The presence of magnesium enhanced activity in the biochemical assay of phosphatase activity; however, some activity was present with each of the three substrates in the absence of added magnesium. Since addition of magnesium caused a slight precipitate to form in media containing lead, it was omitted in most incubations. The specific composition of medium is given in each figure legend. Incubations

were carried out for 30 min at room temperature. As a control, cells were incubated in medium containing calcium, strontium, or lead but without substrate.

After incubation the cells were rinsed once in 100 mM Tris-HCl buffer, pH 7.4, enrobed in agar, postfixed in 1% OsO₄, and embedded in Epon 812 (8). Membranes were postfixed as a pellet.

RESULTS

The cytochemical tests were carried out under conditions similar to those previously used for biochemical assays of alkaline phosphatase activity in *Acanthamoeba* homogenates and membrane fractions (2). The two additional conditions necessary for cytochemistry, namely fixation and addition of lead ions as a capture reagent, are both well known to be deleterious to enzyme activity to varying degrees. The effects of these two parameters were tested in the biochemical assays shown in Table I and the results indicate that under conditions of the cyto-

TABLE I
Effects of Fixation and Lead Citrate on Phosphatase Activity of *Acanthamoeba* and Isolated Plasma Membranes

| | Isolated plasma membranes | Cells |
|-----------------------------|--|---|
| | $\mu\text{mol}/\text{mg protein}/\text{h}$ | $\mu\text{mol}/10^6 \text{ cells}/\text{h}$ |
| Unfixed | 7.5 (100)‡ | 2.6*(100) |
| Unfixed + 3 mM lead citrate | 1.6 (21) | 0.5*(19) |
| Fixed | 7.1 (95) | 1.4, 1.8*(69) |
| Fixed + 3 mM lead citrate | 2.2 (29) | 0.5, 0.3*(12) |

Isolated plasma membranes or cells washed free of growth medium were incubated in 0.1 M Tris-HCl, pH 9.5, 10 mM (cells) or 20 mM (plasma membranes) *p*-nitrophenylphosphate, and 5 mM MgCl₂ for 15 min at 37°C. Activity was measured with isolated plasma membranes by determination of inorganic phosphate (9) and with cells by determination of *p*-nitrophenol spectrophotometrically at 400 nm. The data have been corrected for non-enzymatic hydrolysis as measured in incubations with boiled cells or membranes. Fixation was carried out as described under Materials and Methods (cells in glutaraldehyde, membranes in formaldehyde-calcium acetate).

* Medium also contained 0.05% Triton X-100.

‡ Numbers in parentheses indicate percentage.

chemical assay there is retention of 12–30% of the initial enzyme activity.

Three substrates, *p*-nitrophenylphosphate, α -naphthylphosphate (both at pH 9.5), and AMP (at pH 7.5), were used in the cytochemical procedures. The ratio of phosphatase activity toward these substrates measured in unfixed plasma membrane fractions was 7:2:1. Highest activity with each substrate was obtained in the presence of magnesium (2 or 5 mM). The decrease in activity in the absence of magnesium was variable in different assays possibly because no effort was made to remove magnesium (by adding EDTA) that might have been present in the tissue or reagents. The localization with or without added magnesium and with each of the substrates used was the same. Typical results with cells are illustrated in Figs. 1–3. Regardless of the substrate used and of the capture reagent present, the contractile vacuole and its associated vesicles and tubules contained dense deposits of reaction product (see reference 10 for morphology of contractile vacuole in *Acanthamoeba*). A few small cytoplasmic vesicles not clearly associated with the contractile vacuole contained reaction product, and occasionally a large vacuole was also labelled. The plasma membrane showed a variable, usually light, deposit of lead precipitate on the inside surface. This appears to be part of a general background of lead precipitate that was present after most incubations (Figs. 1 and 2) and was also present in control incubations without substrate (Fig. 4). The contractile vacuole contained no reaction product in the control preparations (Fig. 5). The background precipitate and accumulation of lead deposits along the inside of the plasma membrane were not found when calcium was used as capture ion and later substituted with lead for purposes of microscopy (Fig. 3). These results clearly indicate that a nonspecific phosphomonoesterase of high ac-

tivity is found in the contractile vacuole complex in *Acanthamoeba*. There was an indication that other vesicular compartments may also contain some activity, but the plasma membrane appears to be devoid of phosphatase activity.

With unfixed plasma membrane fractions, the controls (medium without substrate but containing capture ion) showed much precipitate associated with both sides of the membranes. When substrate was included in the incubation medium the amount of precipitate on the plasma membrane was not increased. These preparations differed from the controls only in having dense deposits in some small vesicles. The affinity of unfixed plasma membranes for lead ion precluded cytochemical studies of their phosphatase content.¹ After fixation of membranes however, nonspecific binding of lead was less pronounced, and an experiment with fixed plasma membrane fraction is shown in Fig. 6. In this preparation reaction product was associated not with plasma membranes, but with clusters of small tubules from the contractile vacuole, other unidentified small vesicles, and an occasional vacuole. The images seen with isolated membranes closely parallel the findings in intact cells.

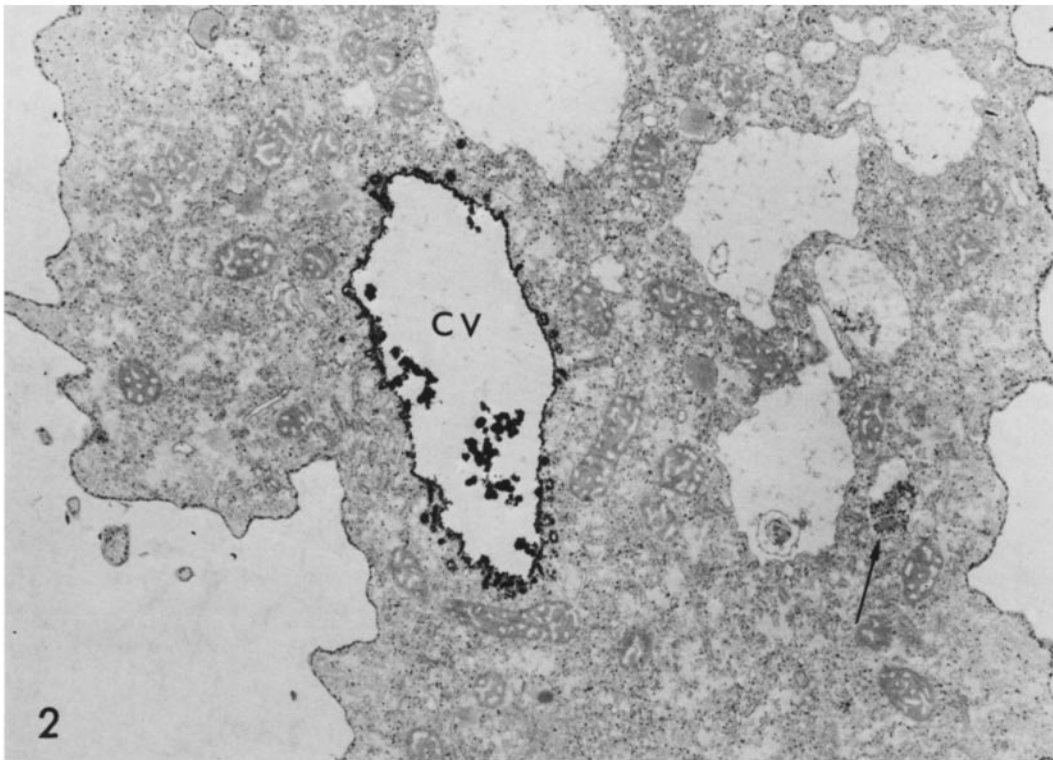
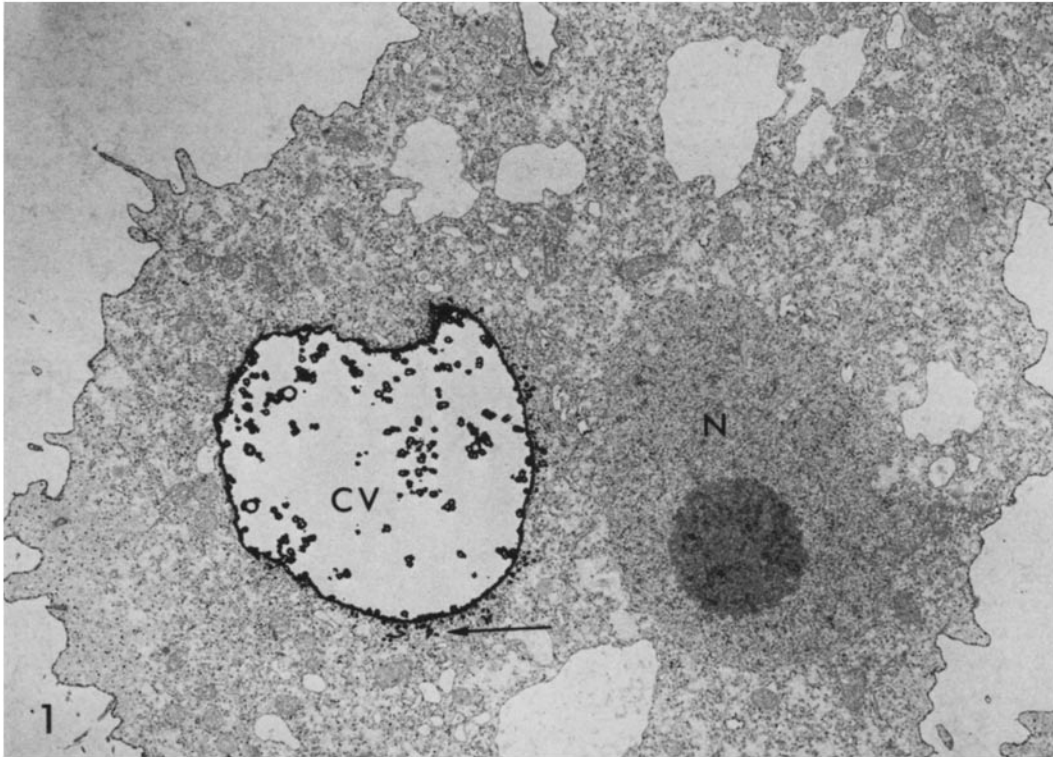
DISCUSSION

Cytochemical studies of alkaline phosphatase activity in fixed *Acanthamoeba* cells and plasma membrane fractions demonstrate that alkaline phosphatase activity is associated with the contractile vacuole and not with the plasma membranes. Some other vesicles which also contain activity could be dispersed elements of the con-

¹ This affinity is presumably related to the presence in *Acanthamoeba* plasma membrane of a complex polymer containing aminophosphonates (11) that would confer an especially marked negative charge on the membrane at high pH.

FIGURE 1 Glutaraldehyde-fixed *Acanthamoeba* incubated with α -naphthylphosphate. Only the contractile vacuole (CV) and associated vesicles and tubules (arrow) contain dense deposits of lead. There is a random scatter of precipitate over the cytoplasm and nucleus (N) that appears to pile up slightly at the plasma membrane but this background labelling is indistinguishable from that seen in cells incubated without substrate (Fig. 4). Incubation medium contained 10 mM substrate and 3 mM lead citrate. $\times 5,400$.

FIGURE 2 Glutaraldehyde-fixed *Acanthamoeba* incubated with AMP as substrate. Activity is restricted to the contractile vacuole (CV). The precipitate in the digestive vacuole (arrow) is considered to be nonspecific since the dense content of vacuoles was observed to bind lead in control sections without substrate. Medium contained 20 mM substrate and 3 mM lead citrate. $\times 11,600$.



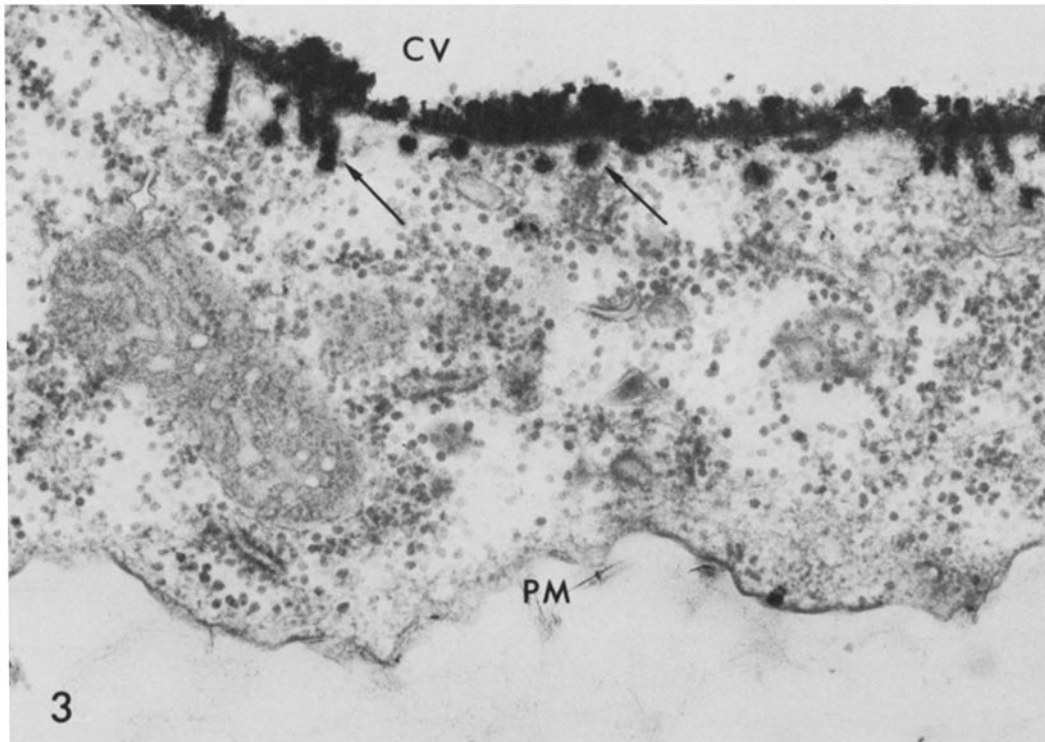


FIGURE 3 Higher magnification of a portion of the contractile vacuole (*CV*) of a cell incubated with α -naphthylphosphate and calcium as capture ion. Note the heavily labelled small tubules and vesicles that are characteristically associated with the contractile vacuole (arrows). The plasma membrane (*PM*) is free of precipitate. This variation of the incubation procedure resulted in very low background of lead precipitate. Incubation medium contained 10 mM substrate and 40 mM CaCl_2 . $\times 44,300$.

tractile vacuole system but at present we have no firm basis for their identification. It is of interest that the 5'-nucleotidase activity, examined at pH 7.5, shows the same localization, supporting the suggestion of Ulsamer et al. (2) that this activity represents the alkaline phosphatase enzyme(s) operating at suboptimal pH.

We found no evidence that the plasma membrane itself or that any substantial number of internal vacuoles, which undoubtedly derive in part from the plasma membrane (12), have alkaline phosphatase activity. Of course we cannot rule out the possibility that a plasma membrane phosphatase might have been selectively and completely inactivated by fixation or by capture reagents. There is no way to control for the latter possibility, and the high level of nonspecific binding to unfixed membranes also makes the former difficult to eliminate. The phosphatase activity previously reported (1, 2) in highly purified preparations of isolated plasma membranes

can probably be explained by the contamination of those preparations by a small amount of highly active contractile vacuole membrane or vesicles. This interpretation is consistent with the observation that fractions less pure in plasma membranes by electron microscope, chemical, and enzymatic criteria often contained a higher specific activity of alkaline phosphatase (2).

To our knowledge this is the first report of an enzyme localization in an ameba contractile vacuole. It seems clear that the protozoan contractile vacuole plays a major role in osmotic regulation (13), and this has been documented recently for *Acanthamoeba* (14). Two micropuncture studies (15, 16) on larger amebas have shown that the contractile vacuole content is hypotonic to the cytoplasm. Riddick (16) measured the ionic content of the vacuole and found it high in sodium and low in potassium, with these two ions accounting for essentially all of the osmotically active content of the vacuole. *Acanthamoeba*

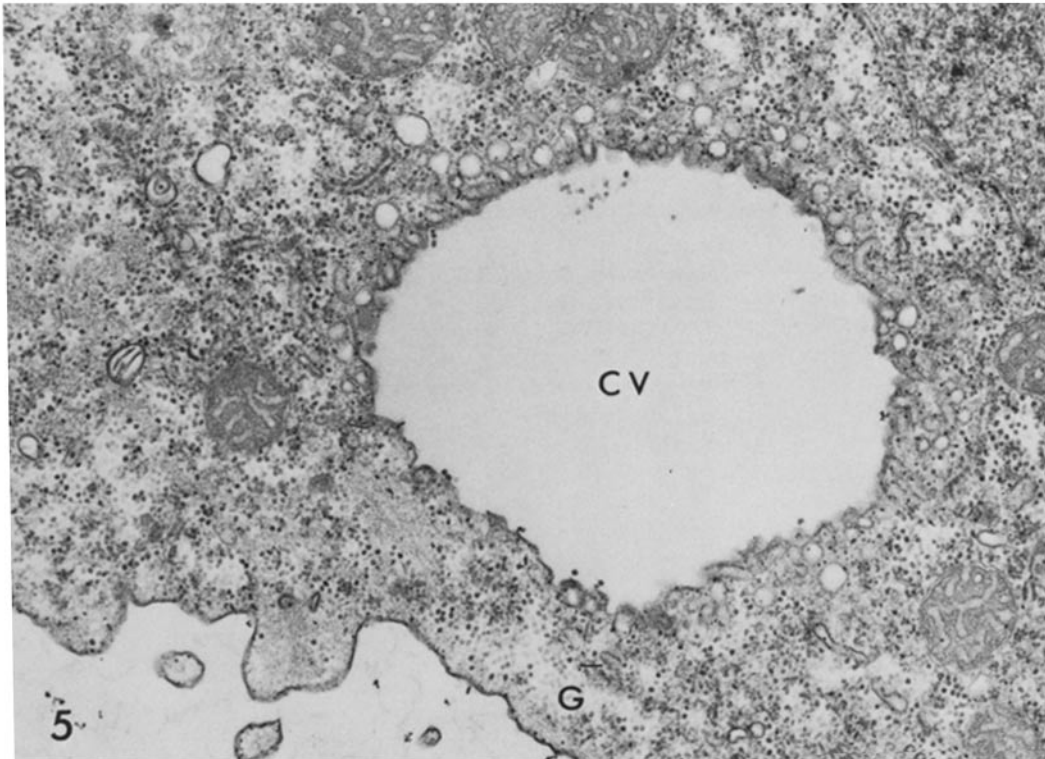
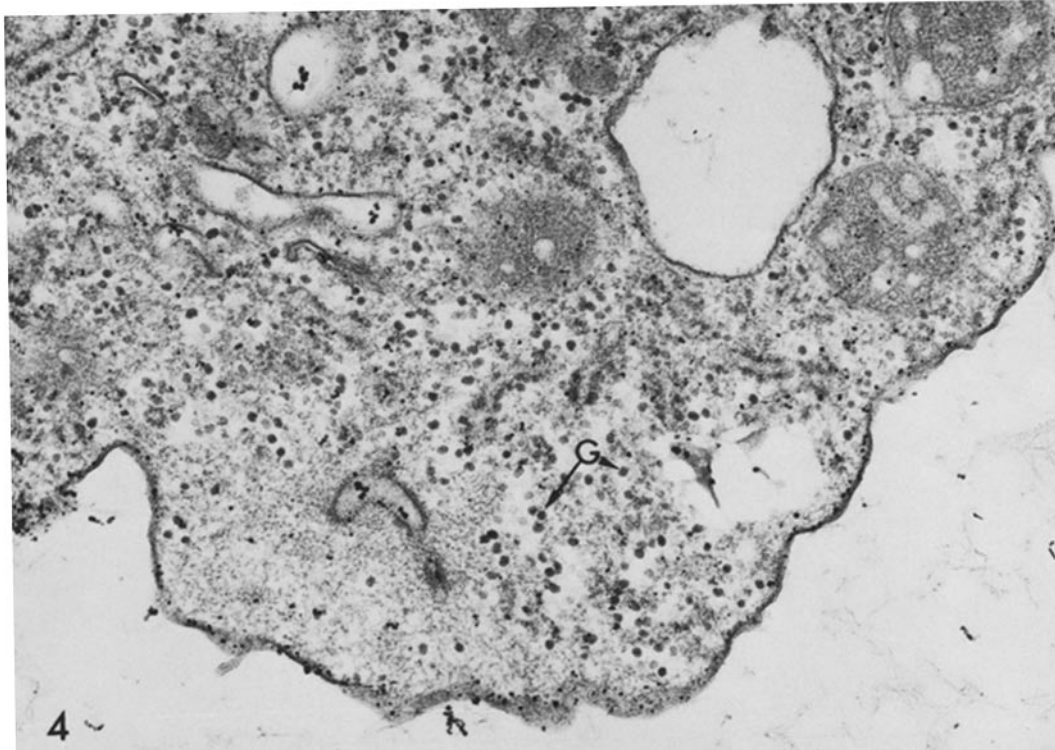


FIGURE 4 Control cell incubated in 3 mM lead citrate without substrate. Note the general precipitate in cytoplasm and accumulation of precipitate along the plasma membrane. Glycogen (*G*). $\times 43,800$.

FIGURE 5 Portion of control cell showing the absence of precipitate in the contractile vacuole (*CV*). The cell has a large number of glycogen granules (*G*) that can be distinguished from lead precipitate by their position, lower density, and larger size. Incubation medium same as in Fig. 4. $\times 24,000$.

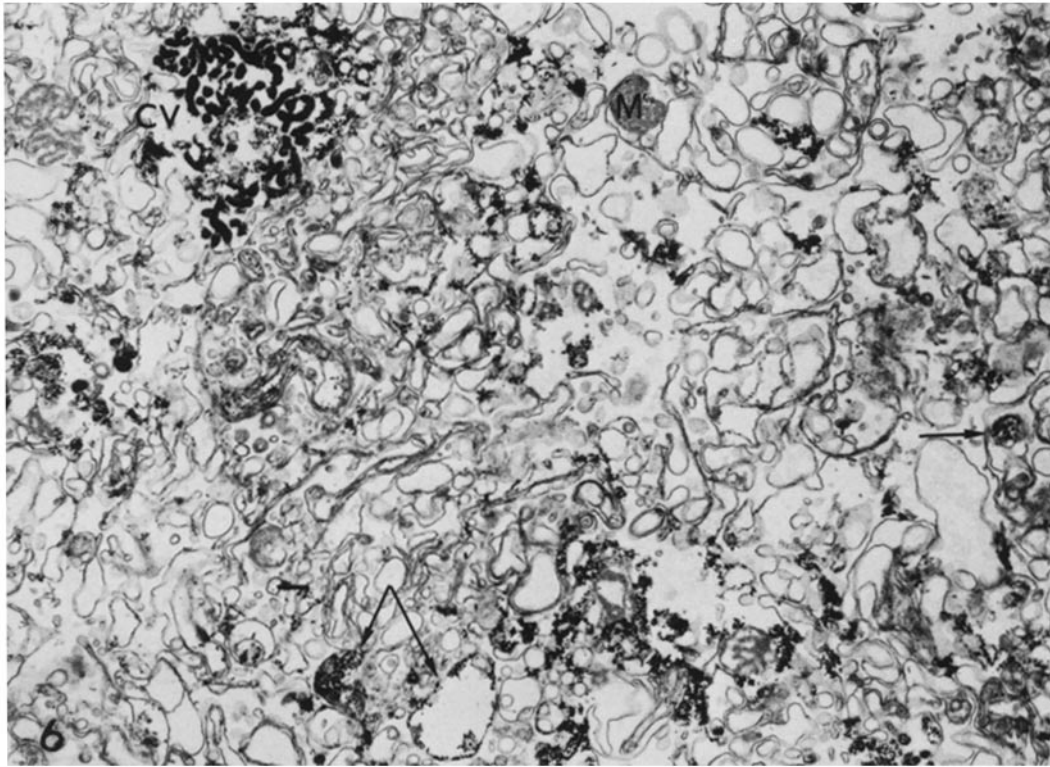


FIGURE 6 A somewhat impure isolated membrane fraction fixed in formaldehyde-calcium acetate before incubation in *p*-nitrophenylphosphate. Despite a high background of nonspecific precipitate in the interstices of the pellet, there is no constant association of precipitate with the large membrane fragments. A cluster of tubules deriving from the contractile vacuole is heavily stained (CV). Unidentified small vesicles and some larger membrane-enclosed components also contain precipitate (arrows). Mitochondrion (M). Medium contained 10 mM substrate, 5 mM MgCl₂, and 3 mM lead citrate. × 10,800.

appears to lack a "sodium pump" similar to that found in many cells since no Na-K ATPase activity was found in whole homogenates or in isolated plasma membranes (2) and since *p*-nitrophenylphosphate activity in homogenates is neither stimulated by potassium nor inhibited by ouabain (unpublished observations). It is probable that the contractile vacuole membranes and enzymes associated with them play some role in ion transport, but we cannot postulate a specific function for alkaline phosphatase. The physiological role of alkaline phosphatase is not understood in other systems either, although it is found in association with epithelia that show active transport (17).

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