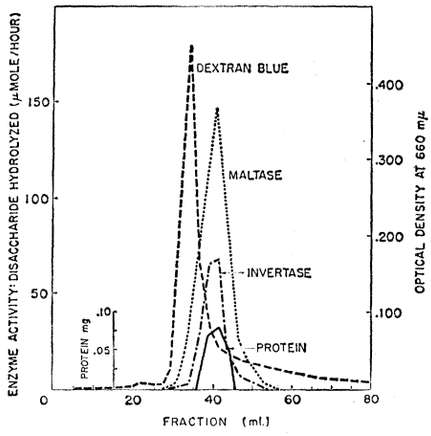

Fig. 3. The result of filtration through a Bio-Gel P-200 column (2 by 50 cm). The marker, dextran blue, was read at 600 mμ. Activities of invertase and maltase and the protein were similarly retarded in the column and were recovered in the same fractions.

and the grid was air dried. Examination in a Philips EM 200 at 80 kv was carried out within 3 hours of preparation.

The specific activities of invertase and maltase for the whole brush border, sediment 1, sediment 2, and the final supernatant are shown in Fig. 2. The highest specific activities are in sediment 2. The specific activity for invertase here is two times that of the brush-border preparation and 20 times that of sediment 1. Clearly the enzymes have been concentrated in this sediment.

Figure 1b reveals the appearance of the first sediment after its digestion with papain. Papain digestion has denuded the microvilli of the knobs, as indicated by the smooth appearance of the edge. Unit membranes are identified after osmium fixation of this sediment, confirming that this sediment contains the plasma membrane. Since relatively little specific enzyme activity was present in this sediment, the plasma membrane of the microvillus appears not to possess significant invertase or maltase activity.

Further, digestion with papain without cysteine, with cysteine, or with trypsin resulted in almost total recovery of the enzyme specific activities in sediment 1. For example, specific activity of invertase in sediment 1 was 100 times that in sediment 2 after digestion with papain not activated with cysteine. In these instances, as judged by electron-microscopic examination, sediment 1 contained the microvilli with the knobs still attached.

Sediment 2 contains numerous small particles, probably globular, 60 Å in diameter (Fig. 1c). The particles tend to aggregate. These particles are similar in size to those seen attached to the plasma membrane of the microvillus in the brush-border preparation. The specific activities of invertase and maltase were highest in this sediment.

Examination of negatively stained preparations of the final supernatant revealed no similar concentration of particles. Analysis also revealed very low specific activities of the enzymes.

Finally, sediment 2, which contains the knobs and enzyme activity, was suspended in a final volume of 1.0 ml of 0.1*M* phosphate buffer, *p*H 6.1, and applied to a Bio-Gel P-200 column (Fig. 3).

There is slight retardation of the enzyme activities and protein during passage through the gel column. The amounts of retardation are similar; the enzyme activities and protein appear in the same fractions collected. Electron microscopy of the sediment recovered from the tubes with these maximum activities reveals the presence of the previously described knobs. Negative staining of other fractions demonstrated no similar particles.

I conclude that digestion, with activated papain, of epithelial-cell brush borders isolated from hamster intestine removes knobs 60 Å in diameter from the plasma membrane. They are attached to the plasma membrane but are not an integral part of it. The activities of invertase and maltase in the brush border are associated with them. I believe these knobs and enzyme activities are contained in the glycocalyx or fuzzy coat, external to the plasma membrane of the microvillus.

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Glutaraldehyde Activation of Nuclear Acid Phosphatase in Cultured Plant Cells

Abstract. *Cultured tobacco cells exhibited a nuclear reaction in the cytochemical test for acid phosphatase after exposure to 2 percent glutaraldehyde. A slight preference for adenosine monophosphate over beta glycerophosphate as substrate was noted, but the enzymatic activity was not typically 5′-nucleotidase. Other aldehydes tested did not elicit the same enzyme response. Nucleolar localization was distinct from that in the rest of the nucleus. Assay for spectrophotometric measurements of enzymatic activity in vitro was developed as a modification of the Burstone procedure with naphthol AS-B1 phosphate and diazo blue B.*

The use of glutaraldehyde for routine fixation of biological materials for electron microscopy has stimulated study of the nature of its interaction with protoplasmic constituents, along empirical as well as theoretical lines (*1*). Chemical fixation generally causes a loss of histochemical staining ability (see *2*). It has been reported that glutaraldehyde does not alter acid phosphatase localization in some organisms (*3*).

We have found that treatment with 2 percent glutaraldehyde (*4*) specifically activates acid phosphatase in the nuclei of WR-132 culture cells. The suspension culture was derived from tobacco stem explant (*5*). Cytochemical localization of acid phosphatase in untreated cells is primarily at the cross-walls (Fig. 1A). This agrees with reports that a substantial portion of acid phosphatase activity in plant cells is associated with the walls, particularly those from tissue cultures (*6*). We observed that if the Gomori reaction for phosphatase (*7*) is performed subsequent to glutaraldehyde treatment, there is no longer a detectable wall reaction, but instead an intense positive reaction in the cell nucleus (Fig. 1B). The glutaraldehyde effect on acid phosphatase activity is not limited to experiments *in situ* since activation has been achieved with isolated nuclei. As observed, the phenomenon is unique for glutaraldehyde. Neither the monoaldehydes—formaldehyde, glyceraldehyde, propionaldehyde, crotonaldehyde, and isovaleraldehyde—nor the dialdehydes glyoxal and hydroxyadipaldehyde can serve as substitutes for glutaraldehyde. Substrates other than beta

glycerophosphate (β-GP) are hydrolyzed. The cytochemical results indicate a preference for nucleotide monoesters (Table 1).

The specificity for selected substrates is in the order: adenosine monophosphate $\geqq$ beta glycerophosphate > glucose-6-phosphate > adenosine triphosphate > cytidine diphosphate (Fig. 1, C–F). Negative results were obtained with *p*-nitrophenyl phosphate and thiamine pyrophosphate (Fig. 1G). The range of effective glutaraldehyde concentration was fairly broad; positive results have been observed with concentrations of 0.5 and 10 percent. The effectiveness of the concentration of glutaraldehyde in promoting the reaction was apparently dependent upon other factors, such as age of the culture and the substrate subsequently used.

Because of the periodic fluctuation of certain enzyme activities in WR-132 cultures (*5*), the stage of culture development was an obvious complication. Maximum activity was observed in 3- to 5-day-old cultures (3-day-old meaning 3 days after inoculation); a general decline in nuclear acid phosphatase response was observed after the 5th day. The duration of glutaraldehyde treatment had a noticeable effect upon the degree of nuclear staining up to a period of 30 minutes. As the time was increased, nuclear staining became more intense and uniformly distributed throughout the cell population. After 30 minutes, over 90 percent of the nuclei of 3-day-old cells were stained by the Gomori reaction. The labile wall reaction was invariably lost after glutaraldehyde treatment for 2 to 5 minutes.

Table 1. Comparison of substrates in cytochemical determination of nuclear acid phosphatase.

Substrate	Relative activity
Adenosine monophosphate	++++
β-Glycerophosphate	++++
Glucose-6-phosphate	+++
Adenosine triphosphate	++
Cytidine diphosphate	+
p-Nitrophenyl phosphate	None
Thiamine pyrophosphate	None

None of the other aldehydes tested produced a positive nuclear reaction for acid phosphatase (Fig. 1H). We included $MgCl_2$ in the incubation mixture (*7*), although experiments with substrates of isolated nuclei and naphthol phosphate have shown negligible effects with addition of Mg^{++}. The nuclear activity did not show inhibition by NaF in vivo, but slight inhibition was detected in vitro. A cell-to-cell staining gradient was sometimes observed in cell chains. The localization was especially pronounced in the nucleolus when the overall activity was low, such as with less-preferred substrates or during periods in the growth cycle when activity was not maximum (Fig. 1, E and F). The nucleolar activity was often confined to vesicular bodies which appeared refractile in living, untreated cells under phase contrast. These bodies were observed to pulsate and undergo changes in size and shape during microscopic examination.

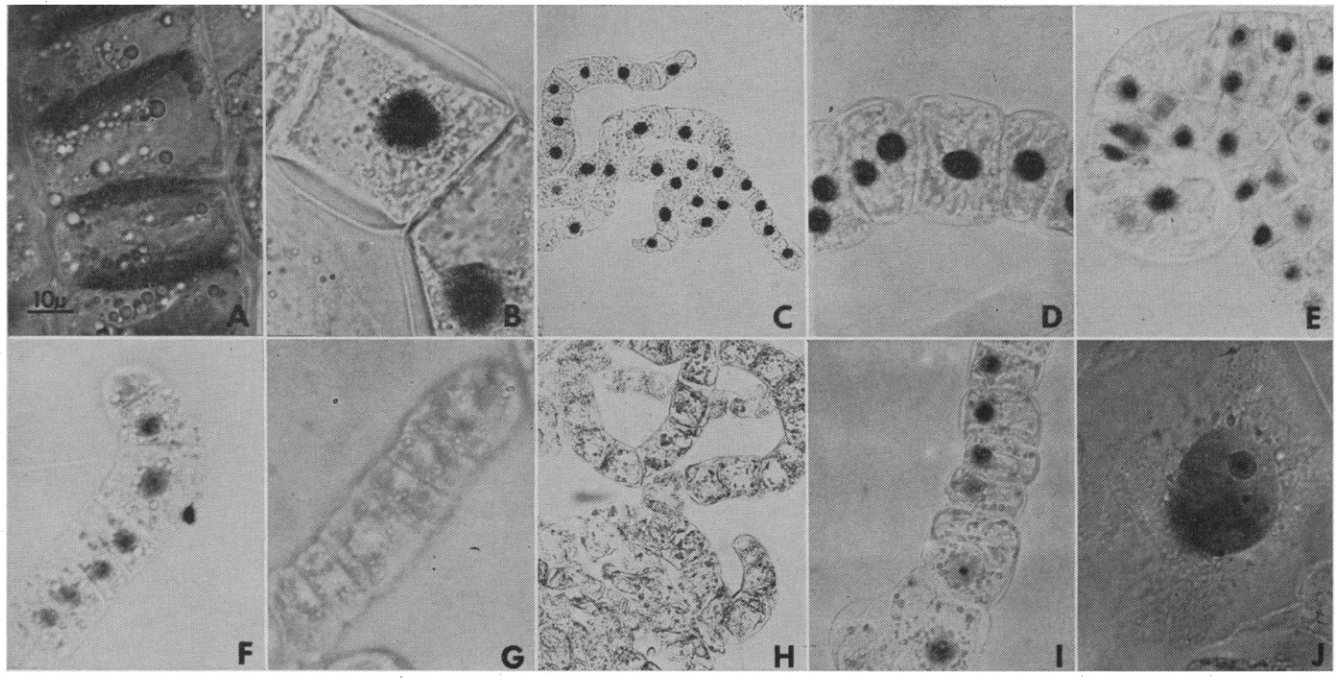

Fig. 1. Cytochemical localization of acid phosphatase in whole cells of WR-132 culture by the Gomori lead sulfide method. (A) Cross-wall reaction in untreated cells (with β-GP); (B) nuclear reaction in cells treated with glutaraldehyde (with β-GP); (C) inhibition with sodium fluoride not detectable (with β-GP); (D) nuclear reaction (with AMP); (E) nuclear reaction with (G-6-P); (F) nuclear reaction (with CDP); (G) negative (with TPP); (H) formaldehyde treatment yielding negative results (with β-GP); (I) cell-to-cell differences in 4-day-old cultures (with β-GP); (J) positive nucleolar vesicles in abnormally large nucleus of 4-day-old cell (with β-GP). Magnifications in A, B, and J are equivalent; in C and H, 0.2 of A; in D, E, F, G, and I, 0.4 of A. Cytochemical assay performed as follows: (i) cells concentrated by brief low-speed centrifugation to packed volume of 0.5 ml; (ii) treatment with 2 percent glutaraldehyde for 30 minutes at 4°C (0.5 ml added); (iii) cells rinsed twice; (iv) incubation for 2 hours at 37°C in standard mixture of 0.1*M* β-GP (buffered in 0.2*M* acetate at *p*H 5.0) 0.005*M* $Pb(NO_3)_2$, 0.05*M* $MgCl_2$ in ratio of 6:2:1; (v) cells rinsed four times; (vi) incubation with 0.5 percent $(NH_4)_2S$ for 5 minutes; (vii) cells rinsed twice; (viii) cells transferred to microscope slides for examination. Abbreviations: β-GP, beta glycerophosphate; AMP, adenosine monophosphate; CDP, cytidine diphosphate; TPP, thiamine pyrophosphate; G-6-P, glucose-6-phosphate.

When the effect of glutaraldehyde on isolated nuclei was studied, precipitation of lead sulfide in the Gomori reaction was found in glutaraldehyde-treated nuclear material but not in untreated controls (Fig. 2). Nuclei fractionated in tris-sucrose osmoticum (*8*) by gentle grinding with a Potter-Elvehjem homogenizer (four strokes) were used for rate studies. Although the supernatant contained an enzyme which hydrolyzed *p*-nitrophenyl phosphate at an appreciable rate, this substrate was not attacked by the activated nuclear enzyme.

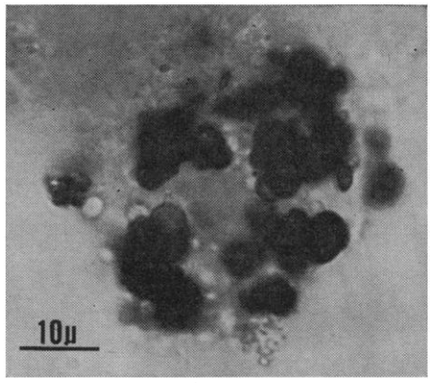

Fig. 2. Gomori reaction with isolated nuclear fraction (with β-GP after glutaraldehyde); the smaller intact bodies in this preparation are nucleoli; many nuclei were ruptured during the water rinses.

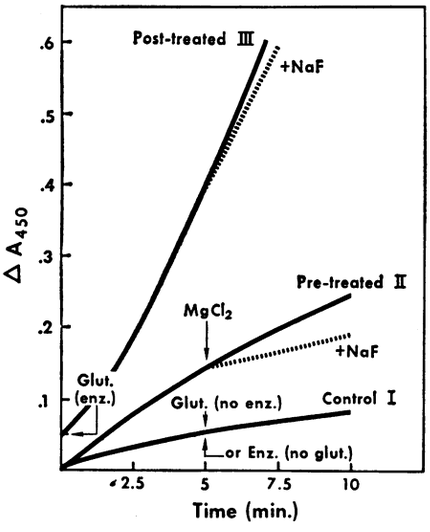

Fig. 3. Kinetic information relative to acid phosphatase activation in nuclear suspensions from WR-132 plant cell culture by glutaraldehyde treatment at 25°C. (*I*) Rate of hydrolysis without either enzyme or glutaraldehyde; (*II*) rate of hydrolysis with enzyme from cells treated with 2 percent glutaraldehyde prior to disruption of cells; (*III*) rate of hydrolysis with nuclei treated after centrifugation (glutaraldehyde added to cuvette at time of assay). Reaction mixture contained 0.2 ml naphthol AS-B1 phosphate (1 mg per milliliter of 2-methoxyethanol); 0.2 ml diazo blue B (1 mg per milliliter of 0.2*M* acetate buffer, *p*H 4.8); 1.0 ml H_2O. Enzyme preparation (0.2 ml containing 0.1 mg protein, according to a bovine serum albumin standard) added after equilibration (for 10 minutes) of naphthyl substrate and diazonium salt. Final concentration of glutaraldehyde in mixture was approximately 0.024 percent; $7.15 \times 10^{-4}M$ NaF and $1.38 \times 10^{-3}M$ $MgCl_2$ were added.

While investigating alternative procedures, we developed an adaptation of the Burstone azo coupling method (*9*) for spectrophotometry in measuring quantitative production of a soluble dye resulting from phosphatase activity. We found that naphthol AS-Bl phosphate was readily hydrolyzed by the activated nuclear preparation and coupled with diazo blue B to form a soluble colored product with an absorption peak at 450 mμ. Cells treated with glutaraldehyde prior to homogenization and centrifugation yielded an active nuclear fraction. Untreated nuclei showed no activity until glutaraldehyde was added to the incubation mixture. The enzyme reaction commenced immediately but did not attain a steady state for approximately 5 minutes. Specific activity in the nuclei treated after fractionation was four times that in the nuclear fraction treated before (Fig. 3). The nuclear enzyme was extremely heat-stable, which is not unusual for acid phosphatase from other sources.

The enzyme latency observed in WR-132 cell nuclei is unlike that reported for acid hydrolases contained in lysosomal and spherosomal organelles (*10*). Acid phosphatase activity in subcellular particles of the latter type requires treatment with lytic agents to rupture the organelle membrane so as to release the enzyme to react with the substrate. The occurrence of acid phosphatase in cell nuclei has been viewed with suspicion because of the possibility of histological artifacts or contamination from other fractions (*11*). No evidence of such contamination is observed in the WR-132 system.

Physiological implications of glutaraldehyde-activated acid phosphatase in WR-132 cell nuclei could have a bearing upon the high turnover rates of nuclear phosphoprotein associated with gene activation (*12*). If so, the enzyme would be involved in some phase of the mitotic cycle. Whether glutaraldehyde activates acid phosphatase in the WR-132 nuclei by removal of an inhibitor, by changing of molecular configuration of the enzyme, or by linking inactive subunits so as to form an active multiple-unit enzyme has not been determined.

The nuclear phosphatase in our study differs from that reported by other workers (*2*, *10*) with respect to the following criteria. (i) Glutaraldehyde is required for the demonstration of activity in WR-132 nuclei with β-GP as substrate; (ii) an overlap of substrate specificity exists pertaining to adenosine monophosphate and β-GP; and (iii) the common lytic agents do not take part in activation of the enzyme in vitro.

Our findings contribute to the knowledge of glutaraldehyde fixation by identifying a particular enzyme reaction which has been modified during the course of treatment. Although glutaraldehyde provides superior structural preservation for electron microscopy, caution in its use as a routine fixative for cytochemistry must be stressed.

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