JOURNAL OF BACTERIOLOGY, May 1976, p. 937-950

Vol. 126, No. 2
Printed in U.S.A.

Phosphatases of *Chlamydomonas reinhardi*: Biochemical and Cytochemical Approach with Specific Mutants

R. F. MATAGNE,* R. LOPPES, AND R. DELTOUR

Laboratoire de Génétique Moléculaire et Laboratoire de Morphologie Végétale, Département de Botanique, Université de Liège, B-4000 Liège, Belgium

Received for publication 19 January 1976

The unicellular alga *Chlamydomonas reinhardi* produces two constitutive acid phosphatases and three derepressible phosphatases (a neutral and two alkaline ones) that can utilize naphthyl phosphate as a substrate. Specific mutants defective in either of the two constitutive phosphatases or in the neutral derepressible phosphatase were used to investigate biochemical properties and the cytochemical localization of these enzymes. The two constitutive phosphatases show similar pH optima (about 5.0) and K_m values (2×10^{-3} to 3.3×10^{-3} M) but differ in their heat sensitivity and affinity for glycerophosphate. One is soluble, whereas the other is attached to cell debris. As demonstrated by cytochemical analysis in electron microscopy, the activity of the soluble acid phosphatase is found in the vacuoles. The neutral derepressible phosphatase differs from the alkaline ones by its pH optimum (about 7.0), resistance to heat, and low K_m value (3.6×10^{-5} M). The two alkaline phosphatases are similar in their properties but can be easily distinguished on the basis of their electrophoretic mobility. Only the neutral phosphatase was cytochemically detected: it is localized at the level of the cell wall and in the vacuoles. The reasons why the constitutive insoluble phosphatase and the derepressible alkaline phosphatases have not been cytochemically visualized are discussed.

Several studies have recently been devoted to the control of phosphatase activity and synthesis in the green alga *Chlamydomonas reinhardi*. Lien and Knutsen (9) demonstrated that the cells grown in a medium containing inorganic phosphate displayed phosphatase activity with an optimum at pH 4.5 to 5.0 (constitutive enzyme). These authors also showed that the growth of the cells in a medium deprived of phosphate led to a drastic increase of phosphatase activity, which resulted from a de novo synthesis of phosphatase (derepressible enzyme). This latter phosphatase was shown to be active over a very wide range of pH values (from 3.5 to 11.0) with an optimum at pH 7.0.

In an independent study primarily concerned with the genetic control of phosphatase synthesis in *Chlamydomonas*, we isolated different types of mutants impaired in phosphatase activity (12, 17). The genetic and biochemical analysis of these mutants allowed us to define a "phosphatase pool" in *Chlamydomonas*: indeed, the wild-type strain produces five main different phosphatases, namely, two constitutive and three derepressible phosphatases, some properties of which were previously described (12, 17).

It seemed of interest to use well-defined mutations (or combinations of mutations) to analyze further the biochemical properties of the enzymes and to investigate their ultrastructural localization. This paper illustrates how specific mutants impaired in a definite function are powerful tools in the study of gene-enzyme-cell structure relationships in eukaryotes.

MATERIALS AND METHODS

Strains. The different strains used in this study are described in Table 1.

Media and culture conditions. The media used were modifications of the tris(hydroxymethyl)aminomethane(Tris)-medium (TMP) given by Surzycki (23); (+P) medium is TMP medium containing 5 mM phosphate and (−P) medium is TMP medium deprived of phosphate. The cultures were grown in Erlenmeyer flasks aerated with sterile air under continuous illumination (5,000 lx, 25 C).

Measurements of growth and cell size. The kinetics of growth in liquid cultures was estimated by measuring the absorbance at 750 nm or by counting the number of cells with a hemacytometer. To evaluate the size of cells in different growth conditions, cells were sampled at various times and microphotographed (objective, 40×; Olympus Photomax microscope). After magnification of the photographs, the large and small diameters were measured in 20 cells taken at random. The sum of these two parameters gives an estimation of the cell size.

TABLE 1. *Description of strains*

Designation	Characteristics	Origin
WT	WT 137C	R. P. Levine (Harvard Univ.)
P_2	Single mutant with low or no activity of the insoluble constitutive phosphatase	Treatment of WT with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (12)
P_2P_a	Double mutant impaired in the activities of both constitutive phosphatases	Treatment of P_2 with MNNG (12)
P_a	Single mutant with low activity of the soluble constitutive phosphatase	Product of the cross WT × P_2P_a (12)
$P_2P_aPD_4$	Triple mutant impaired in the activities of both constitutive phosphatases and of the neutral derepressible phosphatase	Treatment of P_2P_a with MNNG (17)

Preparation of extracts. Cells were harvested by centrifugation, washed twice, suspended in distilled water, and disrupted with ultrasound (MSE ultrasonic desintegrator). Undestroyed cells were eliminated by centrifugation at 900 × *g* for 5 min. The supernatant fluid (crude extract) was used for enzyme assays and electrophoresis.

Assays. (i) Phosphatase activity. Phosphatase activities were determined either in suspensions of whole cells or in crude extracts according to a method modified from MacIntyre (16) and described elsewhere (12) using sodium-α-naphthyl phosphate as a substrate and tetrazotized-*o*-dianisidine (Fast Blue B) as a postcoupling agent.

In the experiments where naphthyl phosphate and glycerophosphate were compared as substrates, the phosphatase activity was determined by measuring the amount of inorganic phosphate (P_i) released during incubation (21). The assays were carried out as follows. To 0.2 ml of cell suspension or crude extract were successively added 1.1 ml of 0.2 M acetate buffer, pH 4.8, and 0.2 ml of sodium-α-naphthyl phosphate or sodium-β-glycerophosphate (final concentration in assay, 5×10^{-3} M). The mixture was incubated at 37 C for 30 min and then placed in an ice bath; 1.5 ml of cold 10% trichloroacetic acid was added, and the pellet was discarded after centrifugation; 2.5 ml of the supernatant fluid was mixed with 0.5 ml of an ammonium molybdate solution (1.25% in 2.5 N H_2SO_4) and 0.25 ml of Fiske and Subbarow reducer (Sigma) (0.08 g/ml). The absorbance at 660 nm (A_{660}) was read after 10 min against a blank in which the substrate was added after incubation. In this assay, $A_{660} = 1$ corresponds to 25 μg of P_i.

(ii) Protein. Protein was determined by the method of Lowry et al. (13), using crystalline egg white lysozyme as a standard.

(iii) Starch. Four milliliters of ethanol was added to 1 ml of crude extract in water. The mixture was left for 3 to 4 h at 45 C. After centrifugation at 900 × *g* for 10 min, the amount of starch present in the pellet was estimated by the phenol-sulfuric acid method of Liu et al. (10).

Electrophoresis. Crude extracts containing 15 to 20 mg of protein per ml were used for electrophoresis (4 to 20 μl/assay). The phosphatases were separated on a 7% polyacrylamide gel in a Beckman Microzone apparatus, as previously described (17). Disc electrophoresis according to gel system 1a of Maurer (18) was also used. After electrophoresis, the gels were washed overnight at 4 C with 0.2 M acetate buffer (pH 4.8) or 0.2 M Tris-maleate buffer (pH 7.0) and then stained for phosphatases using naphthyl phosphate and Fast Garnet GBC as a diazonium salt (17).

Cytochemical localization of the phosphatases. After growth for 3 days in (−P) or (+P) liquid medium, the cells were collected by centrifugation, washed three times in distilled water, and resuspended in 2 ml of 0.1 M cacodylate buffer at pH 7.0; 2 ml of 4% glutaraldehyde in the same buffer was slowly added to the cell suspension. After 1-h fixation, the cells were washed twice in water and then incubated at 37 C for 45 min (cells grown in −P medium) or 120 min (cells grown in +P medium) in a reaction mixture containing 5 mg of β-glycerophosphate per ml and 1 mg of lead nitrate per ml in buffer (method of Gomori). The phosphatase reaction was performed at three different pH values in the following 0.1 M buffers: sodium acetate (pH 4.8), Tris-maleate (pH 7.0), or glycine-NaOH (pH 9.5). The reaction mixture was routinely heated at 60 C for 30 min before use to remove the possible lead phosphate precipitate.

After incubation, the cells were centrifuged, rapidly washed in 0.2 M acetic acid and then in buffers, and finally postfixed in 2% osmic acid in water at 4 C for 2 h. The samples were dehydrated through an alcohol series and embedded in Epon. Ultrathin and 1-μm-thick sections were cut for electron microscopy.

Ultrathin sections were observed without staining by electron microscopy (Siemens Elmiskop 101). Sections (1-μm-thick) were treated with 0.1% $(NH_4)_2S$ for 20 min, dehydrated, and mounted in Canada balsam for light microscopy.

Naphthyl phosphate could not be used in the Gomori reaction because of the poor solubility of this substrate in the presence of lead nitrate.

RESULTS

Constitutive phosphatases. As described earlier (12), the wild-type (WT) strain grown on (+P) medium has two constitutive phosphatases under the control of two different unlinked genes: one is always found in the soluble fraction and the other one in the insoluble fraction of crude extracts prepared in water. These two fractions can be separated by centrifugation at 27,000 × *g* for 20 min. P_2 and P_a mutants lack most of the CS (constitutive soluble) and of

the CI (constitutive insoluble) enzyme activities, respectively; the double P_2P_a mutant exhibits very low activity of both enzymes. An electropherogram summarizing these data is shown in Fig. 1.

In the following experiments, different strains were used as enzyme sources: P_2 for CS, P_a for CI, and WT for both enzymes.

The activities of CS and CI were measured at different pH values (Fig. 2). Both phosphatases were active from pH 4.4 to 7.2, with a maximum of activity at pH 5.2 for CS and pH 4.8 for CI. At pH 5.6, the activity was significantly higher in acetate buffer than in Tris-maleate buffer.

The K_m values for naphthyl phosphate, determined from the Lineweaver-Burk reciprocal plots (Fig. 3), were 3.3×10^{-3} M for CS and 2.0×10^{-3} M for CI.

CI enzyme was found to be much more resistant than CS to the treatment at 60 C (Fig. 4). The denaturation curve for CS was not exponential. This could be due to the low activity of a thermostable enzyme (probably the residual activity of CI) still present in the P_2 strain.

Hence, CI and CS have quite similar pH optima and affinity for naphthyl phosphate but clearly differ in their heat sensitivity. Furthermore, it should be emphasized that the two enzymes are found in two different fractions of the crude extracts. This observation may indicate that they have quite different localizations inside the cell.

The cytochemical localization of the constitutive phosphatases was first investigated with the light microscope using 1-μm sections of the WT, P_2, P_a, and P_2P_a strains. Glycerophosphate was used as a substrate of phosphatase, and the phosphate formed during the enzymatic reaction was localized by the Gomori reaction (see Materials and Methods). A lead sulfur precipitate, corresponding to phosphatase activity, was found inside WT and P_2 cells. No lead precipitate was detected in P_a and P_2P_a strains. This result was fully confirmed with ultrathin sections examined by electron microscopy. A heavy precipitate was apparent in the vacuoles of WT and P_2 strains only (Fig. 5). The light labeling found in the chloroplast lamellae and around the pyrenoid could correspond to the presence of free phosphate: granules indeed were found in cells of all strains incubated with or without glycerophosphate. It therefore appears that most of the CS enzyme, present in WT and P_2 strains, is located inside the vacuoles.

The question now arises as to why the CI phosphatase was not detected in WT and P_a strains. A possible explanation is that glycerophosphate is a poor substrate for this enzyme. Glycerophosphate and naphthyl phosphate were compared as substrates for the two constitutive enzymes. Crude extracts of WT, P_2, and P_a strains were prepared, and the phosphatase activities were measured in the supernatants (soluble enzyme) and pellets (insoluble enzyme)

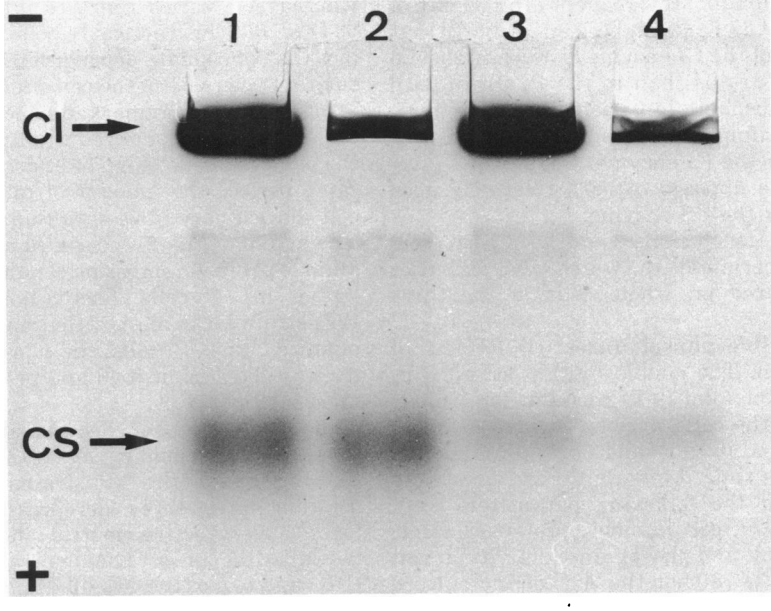

FIG. 1. *Electropherogram of phosphatases present in extracts of WT (1), P_2 (2), P_a (3), and P_2P_a (4) strains grown in (+ P) medium. The gel was stained for phosphatase activity at pH 4.8.*

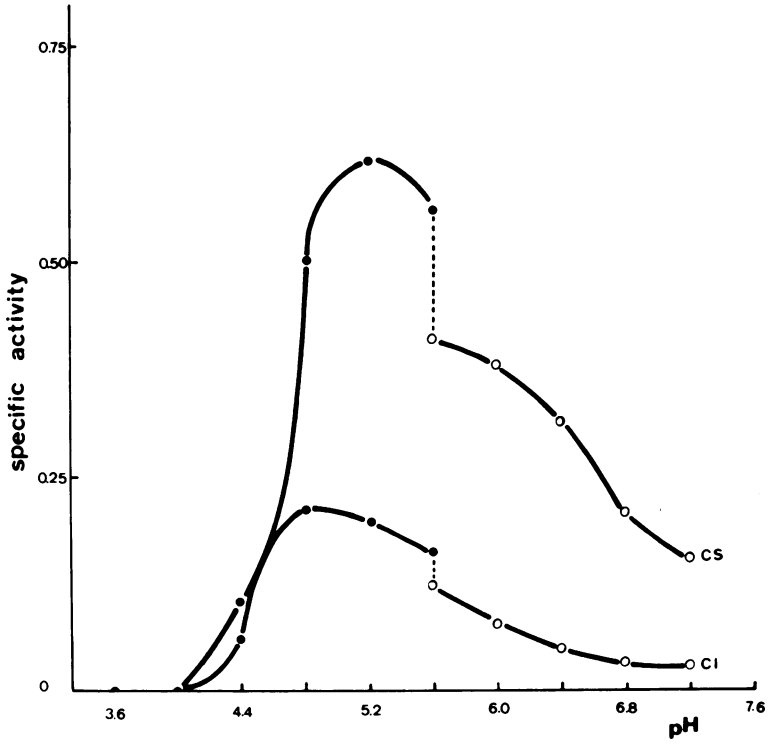

FIG. 2. *Specific activity of constitutive phosphatases (micromoles of naphthol per milligram of protein per hour, 37 C) as a function of pH in crude extracts of P_2 (CS enzyme) and P_a (CI enzyme) strains. Assays were done in 0.05 M acetate (●) or Tris-maleate buffer (○).*

after centrifugation; the inorganic phosphate formed during the reaction was estimated with the Fiske and Subbarow reagent after 30 min of incubation at 37 C. It can be seen (Table 2) that the activity of the CI enzyme is much higher in WT and P_a strains than in the P_2 strain with naphthyl phosphate but not with glycerophosphate. Accordingly, glycerophosphate is a poor substrate for the CI enzyme. Conversely, glycerophosphate appears to be a relatively good substrate for the CS enzyme.

The same conclusions could be drawn from another experiment in which the activities were measured on whole cells of the three strains.

Derepressible phosphatases. (i) Effects of P_i starvation. The double mutant strain P_2P_a was grown on solid (+P) medium for 3 days, after which the cells were suspended in water and inoculated in (+P) and (−P) liquid media.

At various time intervals the cultures were sampled and the following parameters were measured: A_{750}, cell number, protein content, starch content, and phosphatase activity. It can be seen in Fig. 6 that the A_{750} of the culture increases much faster in (+P) than in (−P) medium. The number of cells growing in (−P) medium remains constant after 48 h (Fig. 7), whereas A_{750} varies from 0.18 at h 48 to 0.32 at h 96 (Fig. 6). This can easily be explained by the fact that phosphate deprivation results in considerable increase of the cell size (Fig. 7), which is parallel to a remarkable accumulation of starch (Fig. 8), visualized in ultrathin sections of P_i-starved cells (Fig. 14 and 15). During the same period, the amount of total protein per cell showed very little variation. The phosphatase activity (Fig. 9) measured at pH 7.0 (optimum of pH in derepression conditions) was negligible in (+P) cells. This is normal since the P_2P_a strain lacks most of the constitutive phosphatases. In (−P) cells, the phosphatase activity was detected at h 48 and greatly increased until h 72.

(ii) Properties of derepressible phosphatases. In a previous paper (17), we mentioned that the WT and the P_2P_a strains grown in (−P) medium have three derepressible phosphatases: a very active neutral phosphatase and two alkaline ones. The neutral phosphatase (DN) and one of the two alkaline phosphatases (DA_1) did not migrate in a 7% acrylamide gel.

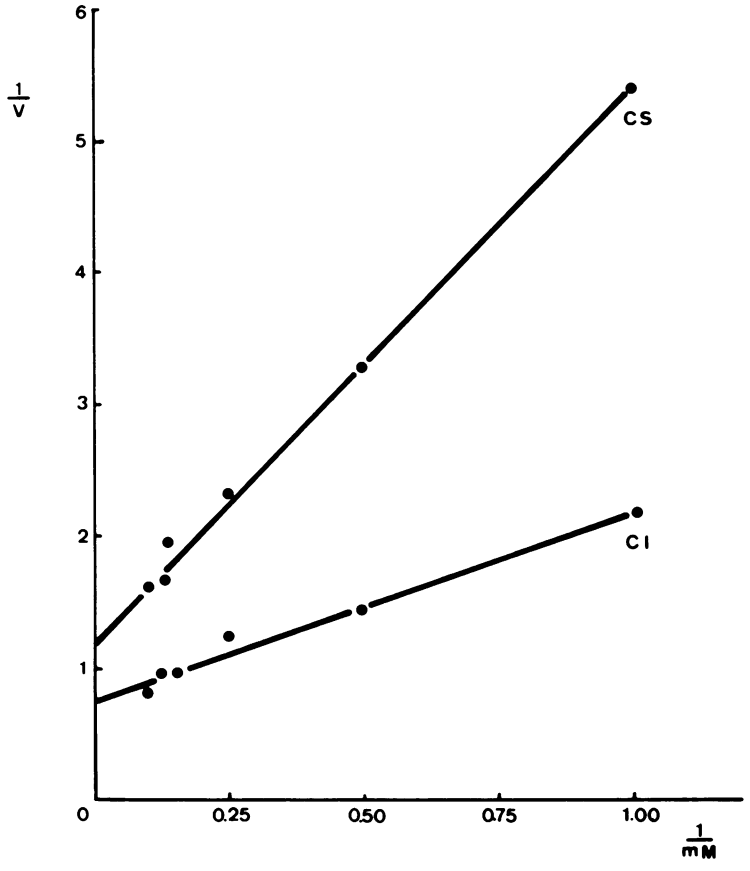

FIG. 3. *Lineweaver-Burk plots of phosphatase activity (pH 4.8) in crude extracts of P_2 (CS enzyme) and P_a (CI enzyme) strains using naphthyl phosphate as a substrate. The initial velocity (v) was measured at 37 C as A_{540}/15 min.*

The other derepressible phosphatase was called DA_2. The distinction between the two enzymatic forms present at the starting line (DN and DA_1) could be made through phosphatase derepressible mutants lacking the neutral phosphatase but still having the two alkaline ones.

The properties of the alkaline phosphatases can be studied as follows: the two enzymes present in a crude extract of strain $P_2P_aPD_4$ grown in (−P) medium are separated by electrophoresis. The gel is cut out at the level of each enzyme, homogenized in distilled water, and centrifuged; the supernatant fluids are used as enzyme sources.

As previously shown, both enzymes exhibited maximal activity at pH 9.5 (glycine-NaOH buffer). The enzymes were still active at pH 7.0 (Tris-maleate buffer) but not at pH 4.8 (acetate buffer). In contrast, the neutral phosphatase present in strain P_2P_a was still very active at pH 4.8: the DN phosphatase could then be studied independently of the two DA phosphatases by using crude extracts of strain P_2P_a at pH 4.8.

Figure 10 gives the Lineweaver-Burk reciprocal plots of the DN phosphatase at pH 4.8 (P_2P_a strain) and of the DA_1 and DA_2 phosphatases at pH 9.5 ($P_2P_aPD_4$ strain), with naphthyl phosphate as a substrate. The K_m values are 3.6×10^{-5}, 4.8×10^{-4}, and 3.0×10^{-4} M, respectively for DN, DA_1, and DA_2 phosphatases. No significant difference was found between the DA_1 and DA_2 regression lines: K_m values for these two enzymes can thus be considered as being very similar. The same sources of enzymes were used for determining their heat sensitivity. The DN enzyme was stable at 55 C, whereas both DA enzymes were much more sensitive at this temperature (Fig. 11). It can be seen in Fig. 12 that the DN enzyme still retains about 20% of its initial activity after 60 min of treatment at 70 C. The denaturation curve for

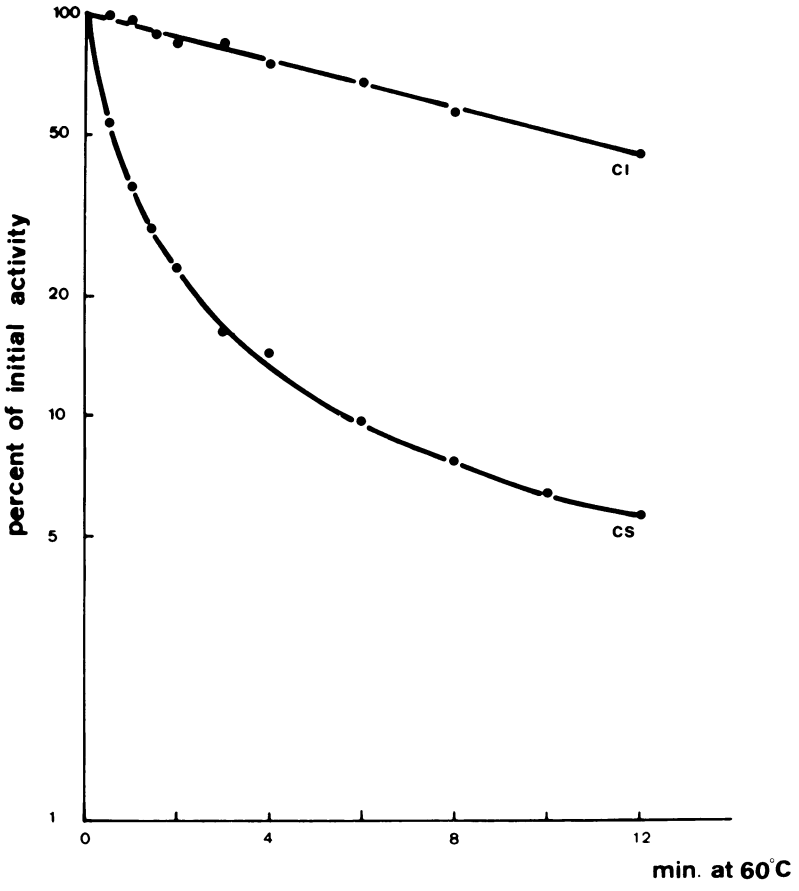

FIG. 4. *Decrease in phosphatase activity in crude extracts of P_a (CI enzyme) and P_2 (CS enzyme) strains in relation to the time of treatment at 60 C; samples were removed at various times and transferred into cold test tubes. The remaining acid phosphatase activity was assayed at pH 4.8 in acetate buffer with naphthyl phosphate as a substrate.*

DN was not exponential, suggesting that this enzyme could exist under several enzymatic forms with different heat sensitivities (see also Fig. 13).

Another problem is whether the DA_1 and DN phosphatases are insoluble enzymes attached to cell structures (as the CI enzyme) or are high-molecular-weight proteins too large to migrate through the pores of a 7% acrylamide gel. In crude extracts prepared in distilled water, both enzymes were always found in the supernatant fluids after centrifugation at 27,000 × *g*, whereas the pellets retained almost no activity. Phosphatases present in the supernatant fluids were separated by disc electrophoresis using two successive separating gels (pH 8.9) with 4.5 and 7.0% acrylamide, according to gel system 1a of Maurer (18). The gel was stained for phosphatase activity at pH 7.0 as previously described (17). In strain P_2P_a, three migrating enzymes could be visualized: the DN and the DA_1 phosphatases in the 4.5% gel and the DA_2 phosphatase in the 7% gel (Fig. 13). Only the DA_1 and DA_2 bands were present in strain $P_2P_aPD_4$. It can thus be concluded that the three derepressible phosphatases are soluble. The cytochemical localization of the derepressible phosphatases by electron microscopy was analyzed by the lead nitrate method of Gomori in the WT, P_2P_a, and $P_2P_aPD_4$ strains. As with the constitutive enzymes, the substrate used was glycerophosphate, but the incubation time was limited to 45 min. The enzymatic reactions were carried out at three different pH values: 4.8 for the DN phosphatase and 7.0 and 9.5 for the DN and DA phosphatases. In each series, blanks were prepared in which the substrate was omitted during incubation.

After incubation of WT cells at pH 4.8 (Fig. 14), the reaction product appeared as small

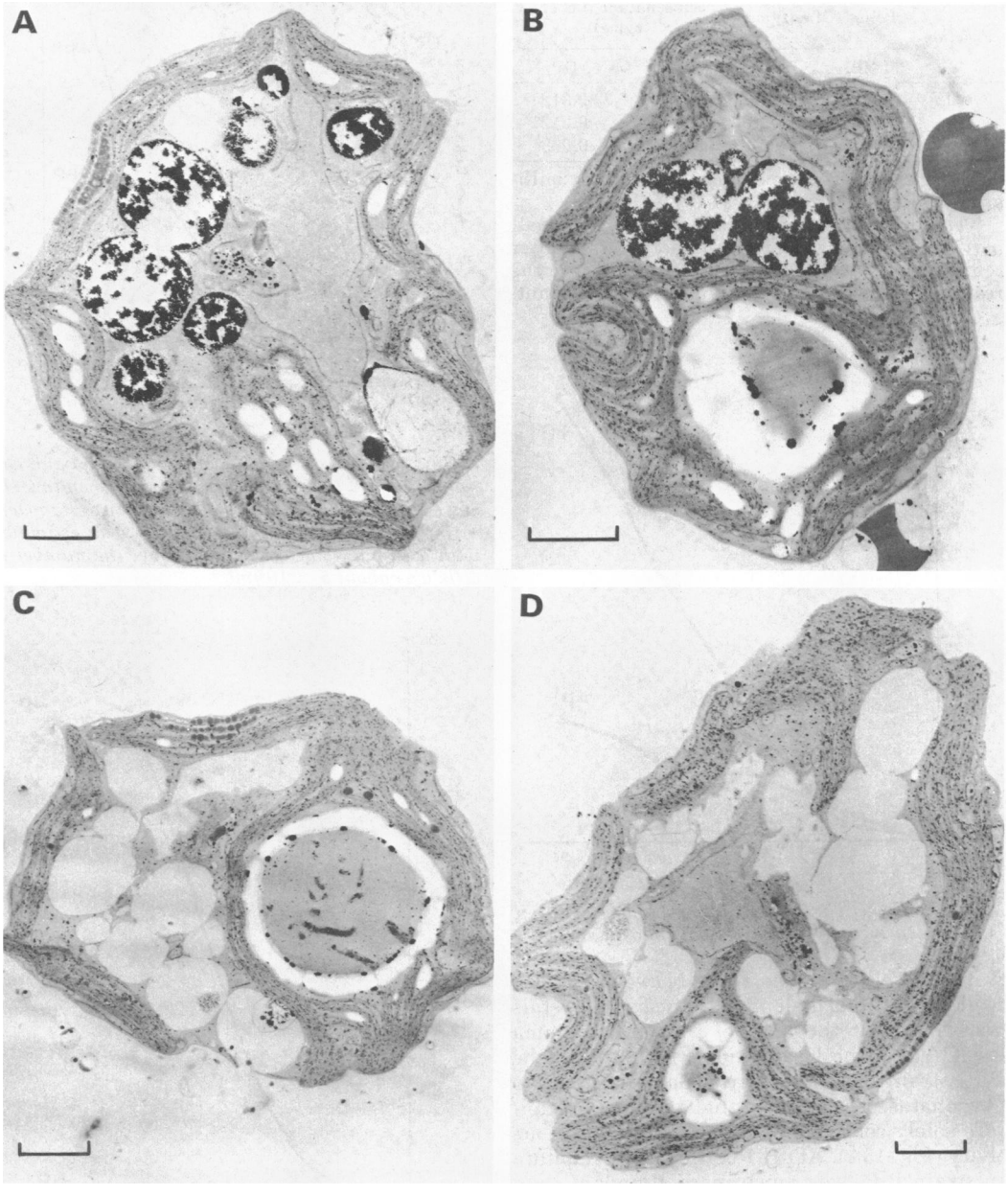

FIG. 5. *Reaction product localization of acid phosphatases in cells of WT (A) and P_2 (B) strains. Note the absence of crystals in vacuoles of strains P_a (C) and P_2P_a (D) grown in the same conditions. Bar, 1 μm.*

TABLE 2. *Phosphatase activities in pellets and supernatants of crude extracts of WT, P_2, and P_a strains using 5 × 10^{-3} M naphthyl phosphate (NP) or glycerophosphate (GP) as a substrate for the enzyme*[a]

Fraction	Pellet (CI enzyme)		Supernatant (CS enzyme)	
	NP	GP	NP	GP
WT	0.473	0.070	0.647	0.312
P_2	0.038	0.029	0.424	0.212
P_a	0.288	0.041	0.153[b]	0.068[b]

[a] Results are given in micromoles of P_i per milligram of protein per hour at 37 C.

[b] These activities are due to the presence of small particles contaminating the supernatant fluids after centrifugation at 27,000 × *g* for 20 min and to the residual activity of the CS enzyme in the P_a mutant.

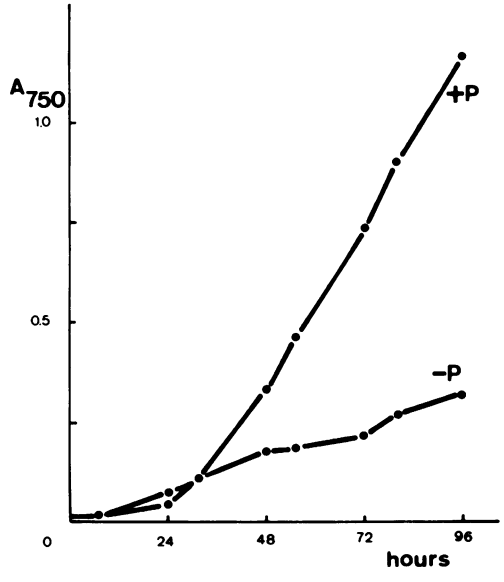

FIG. 6. *Growth of strain P_2P_a in media containing (+P) or deprived of (−P) inorganic phosphate.*

wall, as well as in the space between the cell wall and the plasmalemma. Almost no crystals were found at the level of the plasmalemma itself. The same localization was found in the P_2P_a strain lacking the two main constitutive phosphatases, indicating that the lead precipitate solely corresponds to DN phosphatase activity (Fig. 15A). At pH 7.0, the lead precipitate in strain P_2P_a was much more abundant than at pH 4.8; crystals were also observed inside the vacuoles (Fig. 15B). At pH 9.5, the reaction product appeared as dark spots heterogeneously distributed at the periphery of and inside the cells (Fig. 15C). In the P_2P_a strain incubated at pH 7.0 without substrate (Fig.

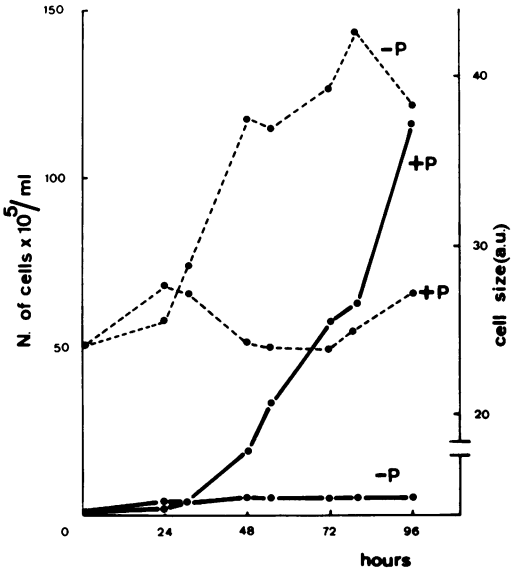

FIG. 7. *Changes in cell size (dotted line) and cell number (solid line) in the P_2P_a strain grown in (+P) or (−P) medium. Cell size = sum of two diameters (arbitrary units). The cell number was estimated with a hemacytometer. At zero time, the number of cells was about 5 × 10^4/ml.*

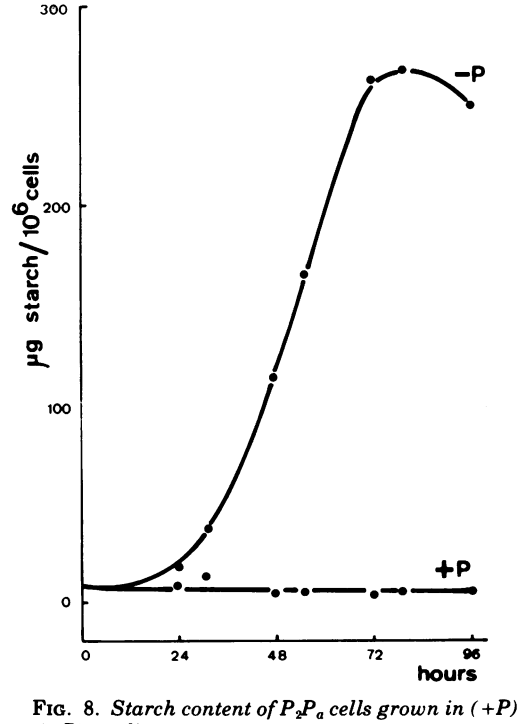

FIG. 8. *Starch content of P_2P_a cells grown in (+P) or (−P) medium.*

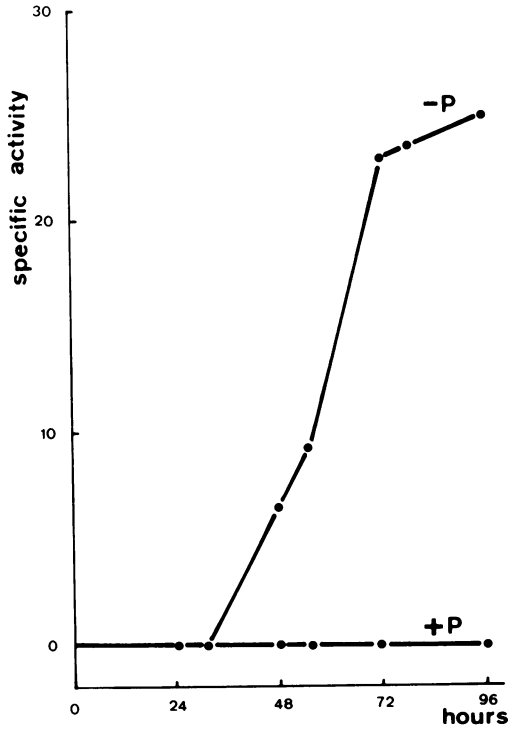

FIG. 9. *Phosphatase specific activities (micromoles of naphthol per milligram of protein per hour, 37 C) measured at pH 7.0 on whole cells of strain P_2P_n grown in (+P) or −P) medium.*

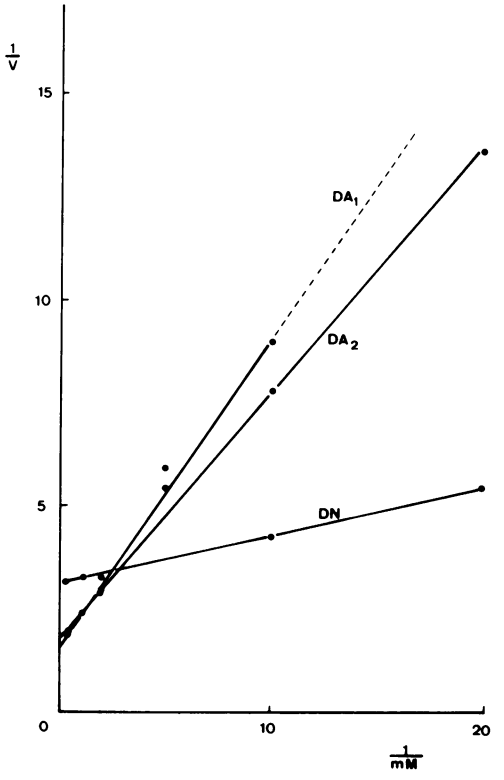

FIG. 10. *Reaction velocity of DN (pH 4.8) and DA_1 and DA_2 (pH 9.5) derepressible enzymes as a function of naphthyl phosphate concentration (Lineweaver-Burk plots). The initial velocity (v) was measured at 37 C as A_{540}/15 min.*

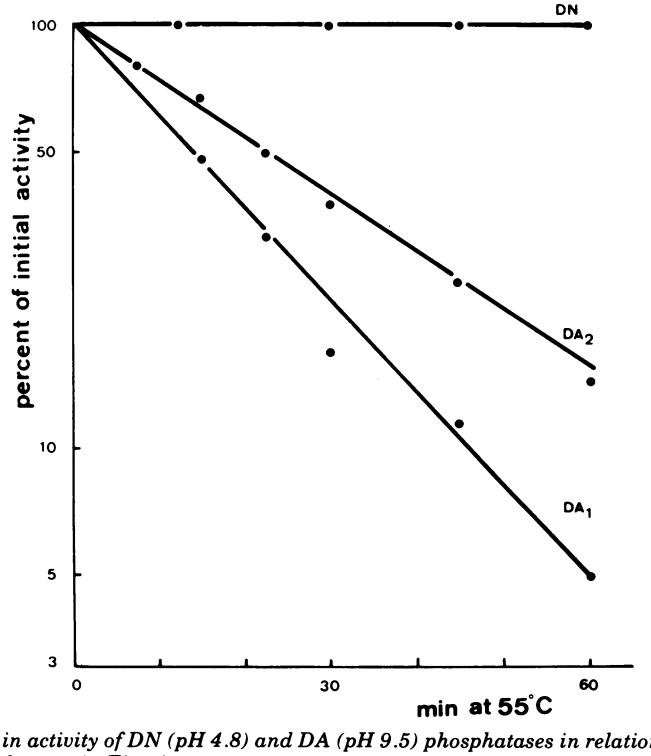

FIG. 11. *Decrease in activity of DN (pH 4.8) and DA (pH 9.5) phosphatases in relation to time of treatment at 55 C. Same procedure as in Fig. 4.*

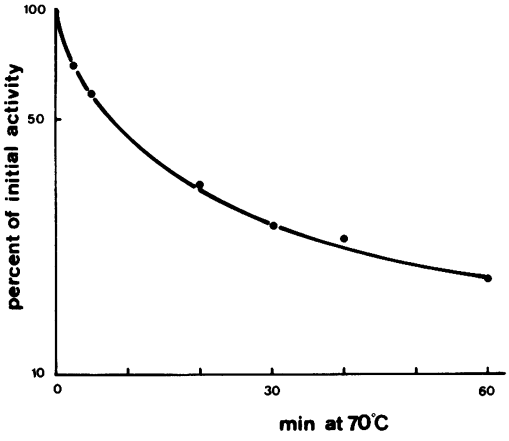

FIG. 12. *Decrease in activity of DN phosphatase pH 4.8) in relation to time of treatment at 70 C. Same procedure as in Fig. 4.*

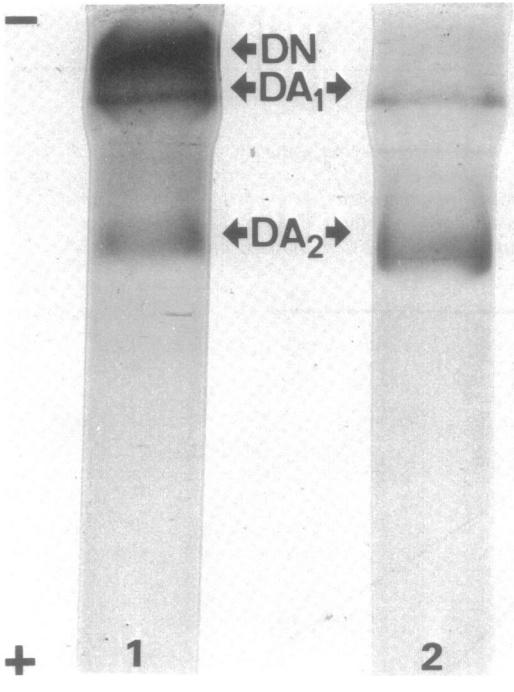

FIG. 13. *Electropherogram of phosphatases present in P_2P_a (1) and $P_2P_aPD_4$ (2) strains grown in (−P) medium. The gels were stained at pH 7.0 for phosphatase activity. Upper part of the gels: 4.5% acrylamide; lower part: 7% acrylamide. The positions of the three derepressible phosphatases (DN, DA_1, and DA_2) are indicated.*

crystals mainly located at the level of the cell 15D), no reaction product was visible except around the pyrenoid, this picture being very similar to that obtained with blanks of (+P) cells.

In $P_2P_aPD_4$ cells (lacking the CS, CI, and DN enzymes) incubated at various pH values, no lead precipitate was visualized except a very faint one visible in cells of all strains incubated without substrate (see for example Fig. 15D). This result confirms that the reaction products found in derepressed WT and P_2P_a cells are due to the activity of the derepressible neutral phosphatase. Moreover, under our experimental conditions, the activities of the two DA phosphatases were not detected by the method of Gomori using glycerophosphate. This could be due to the inability of these enzymes to use glycerophosphate as a substrate or to their more or less specific inactivation by glutaraldehyde. These hypotheses have not been tested so far.

Table 3 summarizes our findings on the properties of the phosphatases present in *Chlamydomonas*.

DISCUSSION

As previously shown (12), *C. reinhardi* produces two constitutive acid phosphatases specified by two different unlinked genes. The present work shows that these two enzymes have similar pH optima (around pH 5.0) and affinities for naphthyl phosphate as a substrate. The K_m for naphthyl phosphate appears to be higher than the K_m for nitrophenyl phosphate (1.9×10^{-4} M), determined with an acetone powder on WT cells (9). The two enzymes, however, differed in some respects, namely, in their heat sensitivity, utilization of glycerophosphate as a substrate, electrophoretic mobility, and solubility. The fact that the insoluble (CI) acid phosphatase is always found in the centrifugation pellet of crude extracts suggests this enzyme to be strongly attached to cellular structures. Our present cytochemical study did not allow us to localize the CI enzyme, probably because it cannot utilize glycerophosphate efficiently as a substrate. Attempts made with naphthyl phosphate in the Gomori reaction failed because of the poor solubility of this substrate in the presence of lead nitrate.

On the other hand, with the specific mutants P_2 and P_a, we could localize the soluble (CS) acid phosphatase in vacuoles.

A similar localization has often been found in various plant organisms and, more particularly, in root tip cells (6–8, 19). Glycerophosphatase activity was also present in vacuoles of the colorless alga *Polytomella caeca* (4).

Starvation of the cells in inorganic phosphate results in: (i) arrest of cell divisions after 48 h; (ii) increase in cell size; (iii) accumulation of starch; and (iv) dramatic increase of phospha-

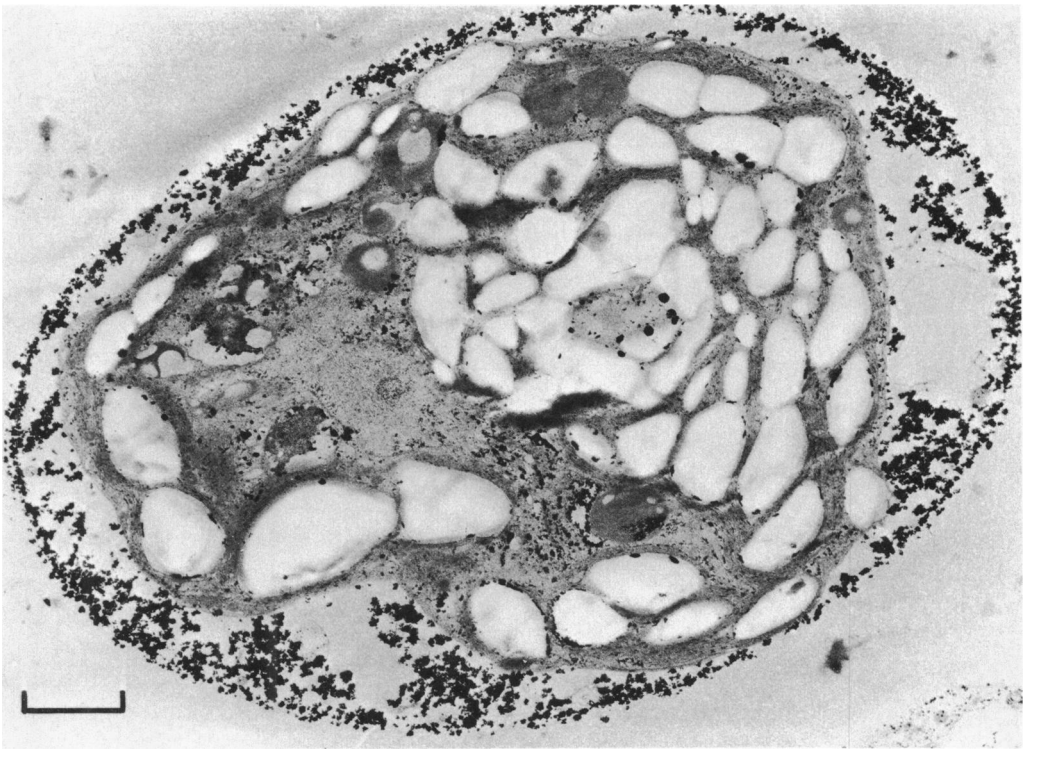

FIG. 14. *Reaction product localization of DN phosphatase in WT cells grown in (−P) medium; pH during incubation, 4.8. Bar, 1 μm.*

tase activity corresponding to a de novo synthesis of enzymes (9). In fact, three different enzyme forms could be detected. The most active phosphatase displays maximum activity at pH 7.0 (DN), whereas the two other enzymes are alkaline phosphatases (DA_1 and DA_2) with optimal activity at pH 9.5. Contrary to what was proposed previously (17), the three enzymes are soluble: all of them are found in the supernatant fluids after centrifugation and migrate in large-pore acrylamide gels. The fact that DN and DA_1 do not migrate in a 7% but do in a 4.5% acrylamide gel suggests that they are high-molecular-weight enzymes. Preliminary attempts at purifying the DN phosphatase by gel filtration indicate that it has a molecular weight higher than 500,000.

The three derepressible phosphatases have K_m values 1/10 to 1/100 the K_m of the two constitutive acid phosphatases (CS, 3.3×10^{-3} M; CI, 2×10^{-3} M), indicating higher affinity for naphthyl phosphate. A very similar situation was found in *Euglena* (2), in which the acid-constitutive phosphatase had a K_m for nitrophenyl phosphate of 1.5×10^{-3} M and the induced acid phosphatase a much lower K_m (about 10^{-5} M).

In addition to the difference in pH optimum, the DN phosphatase differs from the DA phosphatases by its lower heat sensitivity and its higher affinity for naphthyl phosphate. The two DA phosphatases have very similar K_m, thermosensitivity, and pH optimum but differ considerably in their electrophoretic mobility: they could be two different enzymes specified by distinct genes or they could correspond to different enzymatic forms specified by the same structural gene. The problem of the genetic control of the two DA phosphatases is not yet solved, as it has not been possible so far to isolate mutants lacking one or both alkaline phosphatases.

The cytochemical study allowed us to unequivocally localize the DN phosphatase at the level of the cell wall and in the space between the wall and the plasmalemma (this space is generally well defined in our pictures owing to the slight plasmolysis of the cells). No activity was found at the level of the plasmalemma. In several cells, lead phosphate crystals were visualized in the vacuoles (see, for example, Fig. 15B).

As far as the DA phosphatases are concerned, they could not be localized in our experiments. Nevertheless, it can be postulated that the DA enzymes, like the DN enzyme, are mainly lo-

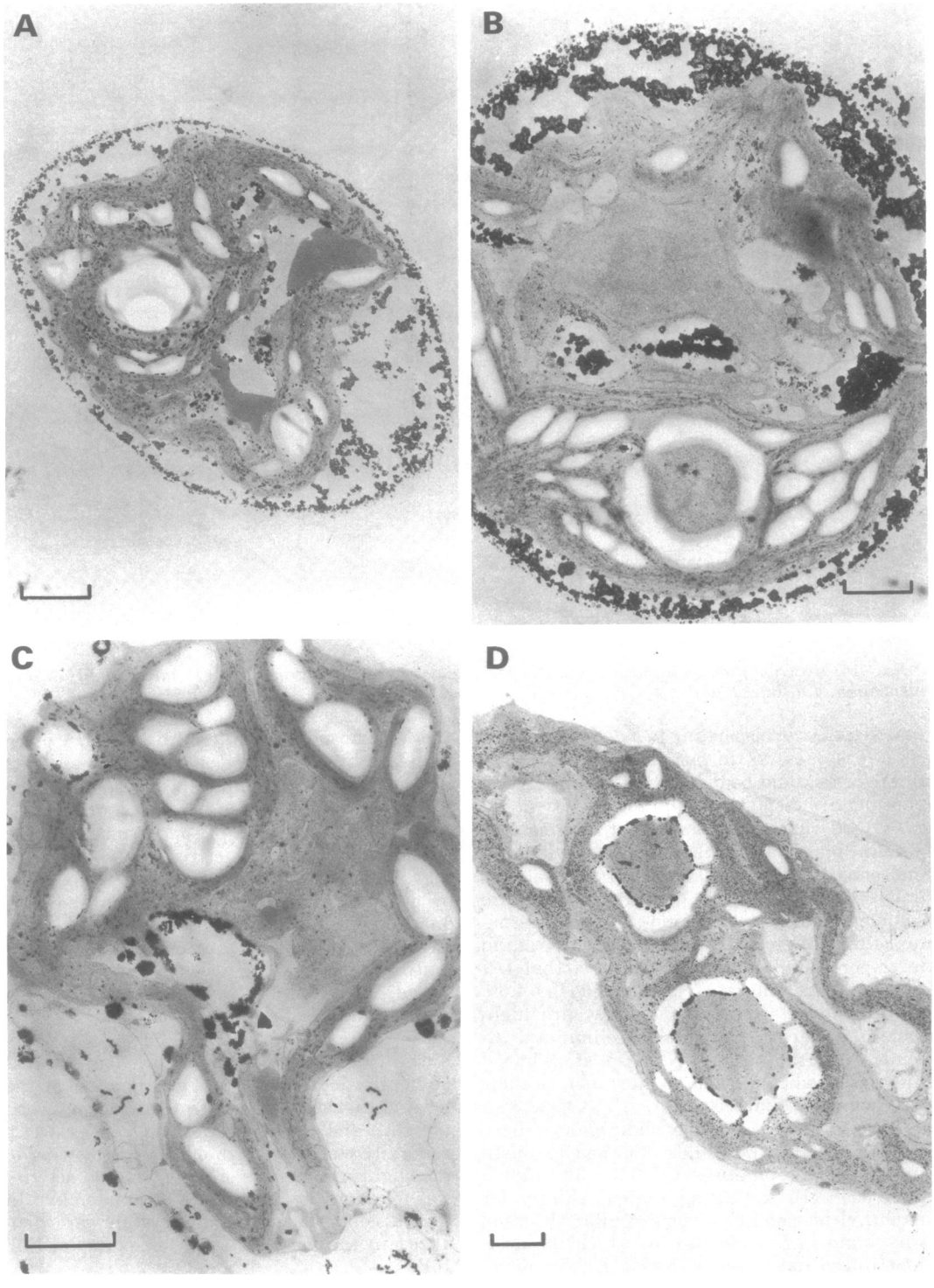

FIG. 15. *Reaction product localization of phosphatases in P_2P_a cells grown in (−P) medium; pH during incubation, 4.8 (A), 7.0 (B), and 9.5 (C). (D) A control incubated without substrate at pH 7.0. Bar, 1 μm.*

TABLE 3. *Summary of the properties of the constitutive (C) and derepressible (D) phosphatases*

Phosphatases	Solubility	Electrophoretic mobility in acrylamide gel		Optimum pH activity	K_m (M)	Heat treatment conditions for 50% inactivation	Cytochemical localization
		7%	4.5%				
CS (soluble)	+	+	+	5.2	3.3×10^{-3}	60 C, 0.5 min	Vacuoles
CI (insoluble)	−	−	−	4.8	2.0×10^{-3}	60 C, 10.5 min	?
DN (neutral)	+	−	+	7.0	3.6×10^{-5}	70 C, 7.5 min	Cell wall and vacuoles
DA_1 (alkaline)	+	−	+	9.5	4.8×10^{-4}	55 C, 14 min	?
DA_2 (alkaline)	+	+	+	9.5	3.2×10^{-4}	55 C, 22.5 min	?

cated at the level of the cell wall. Mutants lacking the cell wall are well known in *Chlamydomonas* (5). Such mutants are not able to retain the DA phosphatase activity at the level of the cells, although they normally synthesize the enzymes (11) that are lost in the culture medium. The same is true for the DN phosphatase. On the other hand, *Chlamydomonas* cells with normal cell wall release part of their derepressible enzymes in the medium (Loppes, unpublished data). It can thus be assumed that the cell wall plays the role of a molecular sieve, partly retaining the phosphatases during their transit to the outside of the cell.

Numerous studies have demonstrated the presence of phosphatase activity associated with the cell wall of various organisms: *Escherichia coli* (15), a marine pseudomonad (15, 24), *Saccharomyces cerevisiae* (1), and various higher plants (3, 8, 14). Similarly, high induced phosphatase activity was found in the pellicle of *Euglena* (22).

It would now be of interest to define precisely the actual binding site of the derepressible phosphatases in relation to what is known about the structure and composition of the cell wall in *Chlamydomonas* (20).

ACKNOWLEDGMENTS

We are indebted to Béatrice Sosson for helpful participation in some experiments and to L. Ledoux for constant encouragement and discussions. We also thank R. Bronchart for valuable criticism of the manuscript. The skillful technical assistance of Françoise Cogniaux and Jean-Pierre Schlösser is gratefully acknowledged.

This work was supported in part by a grant (no. 10.118) from the Fonds National de la Recherche Fondamentale et Collective. R. L. is a qualified researcher with the Fonds National Belge de la Recherche Scientifique.

LITERATURE CITED

1. **Arnold, W. N.** 1972. Location of acid phosphatase and β-fructofuranosidase within yeast cell envelopes. J. Bacteriol. 112:1346-1352.
2. **Bennun, A., and J. J. Blum.** 1966. Properties of the induced acid phosphatase and of the constitutive acid phosphatase of *Euglena*. Biochim. Biophys. Acta 128:106-123.
3. **Bieleski, R. L.** 1974. Development of an externally-located alkaline phosphatase as a response to phosphorus deficiency, p. 165-170. *In* R. L. Bieleski, A. R. Ferguson, and M. M. Cresswell (ed.), Mechanisms of regulation of plant growth. Bull. 12, the Royal Society of New Zealand, Wellington.
4. **Cooper, R. A., I. D. Bowen, and D. Lloyd.** 1974. The properties and subcellular localization of acid phosphatases in the colourless alga, *Polytomella caeca*. J. Cell Sci. 15:605-618.
5. **Davies, D. R., and A. Plaskitt.** 1971. Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardi*. Genet. Res. 17:33-43.
6. **Goff, C. W.** 1973. Localization of nucleoside diphosphatase in the onion root tip. Protoplasma 78:397-416.
7. **Hall, J. L., and C. A. M. Davie.** 1971. Localization of acid hydrolase activity in *Zea mays* L. root tip. Ann. Bot. 35:849-855.
8. **Halperin, W.** 1969. Ultrastructural localization of acid phosphatase in cultured cells of *Daucus carota*. Planta 88:91-102.
9. **Lien, T., and G. Knutsen.** 1973. Synchronous cultures of *Chlamydomonas reinhardi*: properties and regulation of repressible phosphatases. Physiol. Plant. 28:291-298.
10. **Liu, D., P. T. S. Wong, and B. J. Dutka.** 1973. Determination of carbohydrate in lake sediment by a modified phenol-sulfuric acid method. Water Res. 7:741-746.
11. **Loppes, R., and R. Deltour.** 1975. Changes in phosphatase activity associated with cell wall defects in *Chlamydomonas reinhardi*. Arch. Microbiol. 103:247-250.
12. **Loppes, R., and R. F. Matagne.** 1973. Acid phosphatase mutants in *Chlamydomonas*: isolation and characterization by biochemical, electrophoretic and genetic analysis. Genetics 75:593-604.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
14. **Lugon, M., R. Goldberg, and J. Guern.** 1974. Evidence for the intervention of cell wall phosphatases in AMP utilization by *Acer pseudoplatanus* cells. Plant Sci. Lett. 3:165-171.
15. **MacAlister, J. J., J. W. Costerton, L. Thompson, J. Thompson, and J. M. Ingram.** 1972. Distribution of alkaline phosphatase within the periplasmic space of gram-negative bacteria. J. Bacteriol. 111:827-832.
16. **MacIntyre, R. J.** 1971. A method for measuring activities of acid phosphatases separated by acrylamide gel electrophoresis. Biochem. Genet. 5:45-56.
17. **Matagne, R. F., and R. Loppes.** 1975. Isolation and study of mutants lacking a derepressible phosphatase in *Chlamydomonas reinhardi*. Genetics 80:239-250.
18. **Maurer, H. F.** 1971. Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. Walter de Gruyter, Berlin.

19. **Poux, N.** 1970. Localisation d'activités enzymatiques dans le méristème radiculaire de *Cucumis sativuus* L. III-Activité phosphatasique acide. J. Microsc. (Paris) 9:407-434.
20. **Roberts, K., M. Gurney-Smith, and G. J. Hills.** 1972. Structure, composition and morphogenesis of the cell wall of *Chlamydomonas reinhardi*. I. Ultrastructure and preliminary chemical analysis. J. Ultrastruct. Res. 40:599-613.
21. **Sigma Chemical Co.** 1965. Sigma technical bulletin no. 670. Sigma Chemical Co., St. Louis, Mo.
22. **Sommer, J. R., and J. J. Blum.** 1965. Cytochemical localization of acid phosphatases in *Euglena gracilis*. J. Cell Biol. 24:235-251.
23. **Surzycki, S.** 1971. Synchronously grown cultures of *Chlamydomonas reinhardi*, p. 67-73. *In* A. San Pietro (ed.), Methods in enzymology, vol. XXIII, part A. Academic Press Inc., New York.
24. **Thompson, L. M. M., and R. A. MacLeod.** 1974. Biochemical localization of alkaline phosphatase in the cell wall of a marine pseudomonad. J. Bacteriol. 117:819-825.