ISOLATION AND STUDY OF MUTANTS LACKING A DEREPRESSIBLE PHOSPHATASE IN *CHLAMYDOMONAS REINHARDI*

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ABSTRACT

In the green alga *Chlamydomonas reinhardi*, removal of inorganic phosphate from the culture medium results in the increase of phosphatase activity (derepression) in the wild-type (WT) strain as well as in a double mutant (P_2P_a) lacking the two main constitutive acid phosphatases. Following treatment of WT and P_2P_a with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), mutants were recovered which display very low phosphatase activities when grown in the absence of phosphate; as shown by electrophoresis, they lack one non-migrating phosphatase (PD mutants). This enzyme is active over a wide range of pH with an optimum at pH 7.5. The comparison of electropherograms from WT and mutants grown on media with or without phosphate allowed us to provide a tentative definition of the pool of derepressible phosphatases in *Chlamydomonas*: in addition to the neutral phosphatase lacking in PD mutants, *Chlamydomonas* produces two electrophoretic forms of alkaline phosphatase showing an optimal activity at pH 9.5.

IN a previous paper (LOPPES and MATAGNE 1973), an original method for detecting acid phosphatase activity in colonies of *Chlamydomonas reinhardi* was described. This method allowed the isolation of two classes of mutants defective for two different enzymes which were shown to be coded by two different genes. These experiments were performed with cells routinely grown on media containing a high concentration of inorganic phosphate (6.75 mM). We mentioned that when phosphate was removed from the medium the activity of acid phosphatase increased considerably in the wild type as well as in the mutant strain lacking the two main constitutive enzymes. In an independent study, LIEN and KNUTSEN (1973) gave strong evidence of the *de novo* synthesis of a phosphatase in *Chlamydomonas* grown in the absence of phosphate: this derepressed enzyme was active over a wide range of pH with an optimum at pH 7.0. The present paper deals with the isolation and characterization of mutants lacking this derepressible neutral phosphatase. It gives further information on the number and properties of derepressible phosphatase species in *Chlamydomonas reinhardi*.

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MATERIALS AND METHODS

Strains

WT: wild-type strain number 137 C, mating type minus (—).

P_2P_a: double mutant lacking the main constitutive acid phosphatases (LOPPES and MATAGNE 1973).

Media

The media used throughout this study are modifications of the Tris-minimal-phosphate (TMP) medium given by SURZYCKI (1971).

(+ P) medium: TMP containing 5 mM phosphate

(— P) medium: TMP deprived of phosphate

(+ P) cells are those grown on (+ P) medium, (— P) cells those grown on (— P) medium.

Mutagenesis

Mutants lacking derepressible phosphatase were recovered after treatment of WT and P_2P_a by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Cells in the logarithmic phase of growth were treated with 50 μg/ml MNNG in 0.02 potassium phosphate buffer, pH 7, 22°, 30 minutes. Survival was about 10% in these conditions.

Genetic analysis

The genetic analysis was carried out according to the methods described in LEVINE and EBERSOLD (1958).

Enzyme assays

The assays were carried out either in suspensions of whole cells or in crude extracts according to a method modified from MACINTYRE (1971) and detailed in a previous paper (LOPPES and MATAGNE 1973). The reaction mixture (0.1 ml cell suspension or extract + 0.3 ml 0.2 M acetate or tris-maleate buffer + 0.9 ml H_2O + 0.2 ml 2 mg/ml sodium naphthylphosphate in H_2O) was incubated at 37° for 30 minutes, after which 1 ml of the post-coupling solution (2 mg/ml tetrazotized-o-dianisidine in 4% sodium dodecyl sulfate in 0.2 M acetate buffer pH 4.8) was added. The extinction at 540 nm was read against a blank in which the substrate was omitted during incubation.

For the determination of phosphatase activity as a function of pH, acetate buffer was used at pH's from 3.5 to 5.5, tris-maleate buffer at pH's from 5.5 to 9.0 and glycine-NaOH at pH's from 8.5 to 10.5.

The values of enzyme activities are expressed as follows: in crude extracts, μmole naphthol/mg protein-hr-37°; in cell suspensions, the activity (E_{540}) was related to the extinction of the suspension at 750 nm (E_{750}).

Phosphatase activity in the colonies was detected on replicas of these colonies on filter paper (LOPPES and MATAGNE 1973). The papers were immersed in the naphthylphosphate-tetrazotized-o-dianisidine mixture then incubated on a sheet of paper soaked with the appropriate buffer until the appearance of the brown-red staining of the colonies.

Electrophoresis

Cell extracts for electrophoresis were prepared in distilled water. After disruption of the cells with ultrasound (MSE Ultrasonic Disintegrator), the extracts were centrifuged at 900 g for 3 minutes to eliminate the unbroken cells. The supernatant, containing 15–20 mg protein per ml was used for electrophoresis (5–10 μl of extract per assay). The phosphatases were separated on polyacrylamide gel in Tris-Glycine buffer (4.5 g Tris (hydroxymethyl) aminomethane + 21.8 g glycine in 1000 ml distilled water, pH 8.3), using the Beckman microzone apparatus. Electrophoresis was carried out by applying a current of 30–35 mA (400 V constant) for 90 minutes. After electrophoresis, the gel was washed overnight at 4° in an appropriate buffer, then stained for phosphatases at pH 4.8 (0.2 M acetate buffer) or pH 7.0 (0.2 M tris-maleate buffer) using sodium naphthylphosphate (1 mg/ml) and Fast Garnet GBC as a diazonium salt (1 mg/ml) (37°; 1–3 hrs).

RESULTS

1. *Detection of derepressible phosphatase in colonies*

Wild-type colonies grown on agar phosphate medium (+ P) were replica-plated on the same medium (control) and on medium deprived of phosphate (− P). After a 4-day incubation in the light (during which the absence of phosphate supply did not prevent the alga development), the colonies were again replicated and tested for phosphatase activity at pH 4.8 as described in MATERIALS AND METHODS. Colonies grown on (− P) medium stained dark brown red within 1–2 minutes at room temperature, whereas control colonies grown on (+ P) medium stained poorly and very slowly. This indicates that the derepressible phosphatase activity can be revealed in whole cells, as was the constitutive phosphatase activity.

As stated by LIEN and KNUTSEN (1973), the deprivation of phosphate leads to an increase in neutral phosphatase activity. The staining of (− P) colonies was tested at various pH's (acetate or tris-maleate buffer). A very fast staining of the colonies was observed at pH 4.6 and 5.6 but the reaction was much slower at pH 7.3, probably due to poor permeability of the cell to tetrazotized-o-dianisidine at this pH.

In order to determine whether the derepressible enzyme was different from the two main constitutive enzymes, the staining reaction was performed on the double mutant P_2P_a lacking these two enzymes (LOPPES and MATAGNE 1973). P_2P_a colonies grown on (− P) medium displayed a very high phosphatase activity comparable to that found in WT. The contrast between colonies grown on (+ P) or (− P) medium was much stronger for P_2P_a than for WT; this is not surprising since the double mutant has an extremely low phosphatase activity when grown on (+ P) medium.

2. *Activity and electrophoretic pattern of the derepressible phosphatases*

That the derepressible phosphatase was different from the two main constitutive enzymes was also suggested from measurements of phosphatase activities in WT and P_2P_a at various pH's (Figure 1). The highest activity in WT grown in liquid (+ P) medium was found at pH 4.8–5.0. The activity in P_2P_a grown under similar conditions was negligible.

When WT and P_2P_a were grown in (− P) medium, the phosphatase appeared to be very active in both strains over a wide range of pH's, with an optimum near neutrality. Phosphate-starved P_2P_a displayed lower activity than WT: this result was found in some other experiments but the values of enzyme activities were variable from one experiment to another, probably depending on the degree of derepression of the cells. The lower activity found in the mutant could be related to differences in phosphate utilization.

Crude extracts of (+ P) and (− P) WT cells were analyzed by electrophoresis (Figure 2). In extracts of (+ P) cells two main enzymes, more active at pH 4.8 than at pH 7.0, were identified: one (band 1) was probably bound to particles and remained at the starting line (LOPPES and MATAGNE 1973); the second one

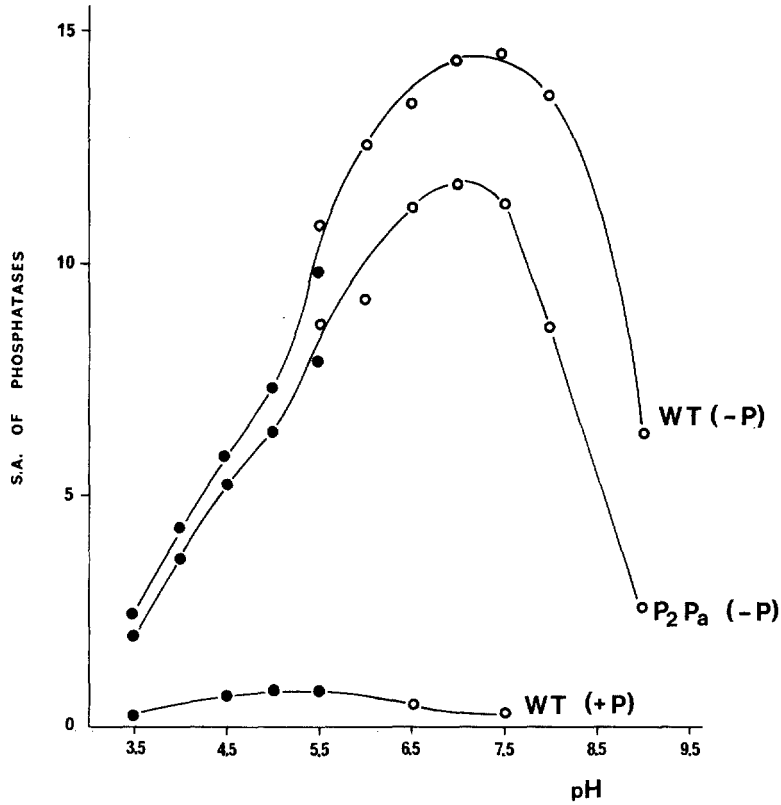

FIGURE 1.—Specific activity of phosphatase (μmole naphthol/mg protein-hr-37°) as a function of pH in extracts of WT grown on (+ P) and (— P) media and of P_2P_a grown on (— P) medium. Assays in acetate (●—●) or tris-maleate (○—○) buffer.

(band 3) was soluble and migrated toward the anode. In (— P) cells two additional enzymes, at least, were found: an enzyme which did not migrate (band 1) and was very active at acid and neutral pH and a migrating enzyme (band 2), very active at pH 7.0 but inactive (or poorly active) at pH 4.8. Sometimes band 2 was lightly visible at low pH.

Under derepressed conditions, two fractions, both active at pH 7.0, were also detected in the strain P_2P_a. This result indicates that the derepressed enzyme present in the band 1 is different from the non-migrating constitutive enzyme since P_2P_a is defective for this latter one. Finally, let us mention that similar conclusions could be drawn when the electrophoreses were conducted at pH 4.5, as previously described (LOPPES and MATAGNE 1973).

3. Isolation of mutants having low derepressible phosphatase activity

WT(—) cells were treated with MNNG as described in MATERIALS AND METHODS and plated on (+ P) medium. After a 10-day growth in the light, colonies were replica-plated on (— P) medium. Four days later, they were again replicated on a filter paper and tested for phosphatase activity at pH 5.0. This

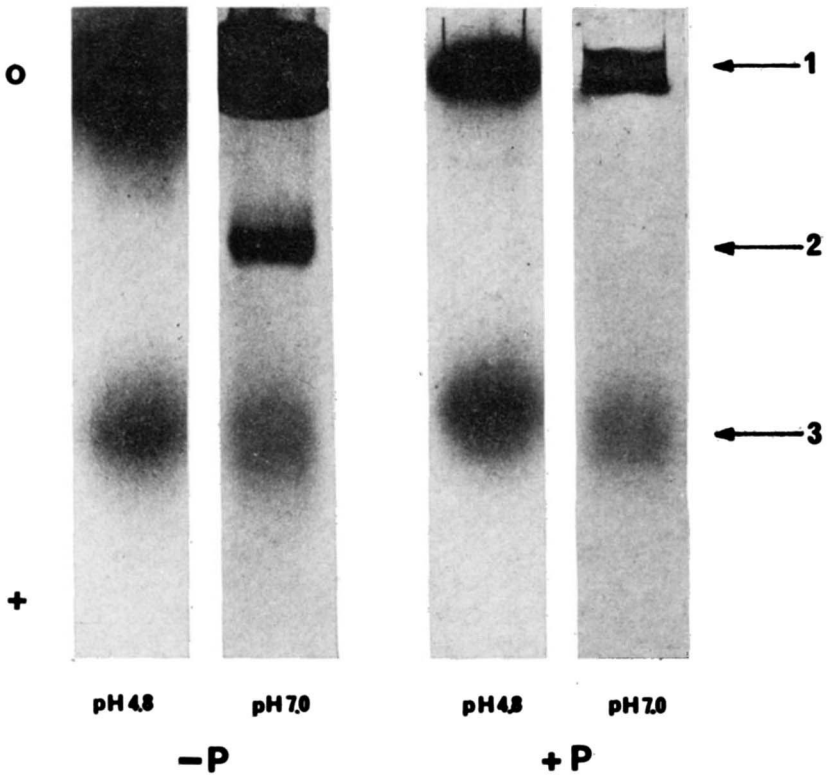

FIGURE 2.—Electropherograms of phosphatases present in extracts of WT cells grown for 3 days in (+ P) or (— P) medium. The gels were stained at pH 4.8 or 7.0 (see MATERIALS AND METHODS).

low pH was chosen because of the improved staining of the colonies (see above) and also because only one derepressible phosphatase is active in acid conditions (as revealed by electrophoresis); this would permit the isolation of mutants defective for this specific derepressible enzyme. Most colonies present on the papers stained very rapidly, indicating a derepressible phosphatase activity. Out of 4,904 colonies tested in two independent experiments, 3 failed to stain rapidly and were suspected to lack the derepressible phosphatase responsible for the fast staining of the colonies. These possible mutants were isolated, tested again for their slow staining reaction and called PD_1, PD_2 and PD_3.

A similar experiment was performed with the double P_2P_a mutant, which lacks the two main constitutive phosphatases. The isolation of mutants from this strain was much easier than from WT since the triple mutant did not stain at all even after 20 minutes' incubation of the replica papers. Out of 926 colonies, two mutants with apparently null activity were isolated (P_2P_a PD_4 and P_2P_a PD_5). Three other colonies, showing a slower staining reaction than the mother strain, were detected.

TABLE 1

Phosphatase activities measured at pH 4.8 and pH 7.5 in cells (E_{540}/E_{750}) and in crude extracts (μmole naphthol/mg protein-hr-37°) of WT, PD_1 and PD_2 strains after growth for 3 days in liquid (+ P) or (— P) medium

		(+ P) pH 4.8	(+ P) pH 7.5	(— P) pH 4.8	(— P) pH 7.5
Cells	WT	4.0	0.74	53	84
	PD_1	4.0	0.76	4.5	10.4
	PD_2	3.7	0.80	4.3	11
Crude extracts	WT	0.86	0.38	16.69	31.47
	PD_1	0.83	0.37	2.10	3.52
	PD_2	0.76	0.32	1.68	3.65

4. *Comparison of phosphatase activities and electrophoretic patterns in WT, P_2P_a and derived* PD *mutants*

Mutants derived from WT

Phosphatase activities at pH 4.8 and 7.5 in cell suspensions and crude extracts of WT, *PD_1* and *PD_2* were measured after a three-day growth in liquid (+ P) or (— P) medium (Table 1). When cells were grown in (+ P) medium, no difference could be found between WT and *PD* mutant activities, measured in cell sus-

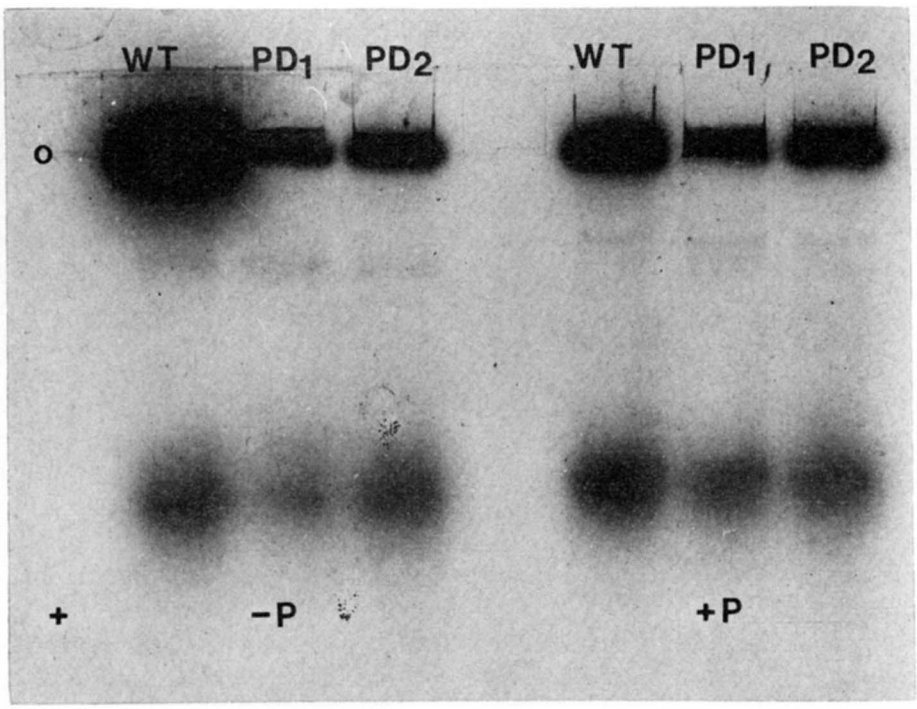

FIGURE 3.—Electropherograms of phosphatases present in extracts of WT, *PD_1* and *PD_2* strains grown for 3 days in (+ P) or (— P) medium. The gels were stained for phosphatase activity at pH 4.8.

pensions as well as in crude extracts. After growth in (− P) medium, the activity at pH 4.8 or 7.5 was about ten times higher in WT than in PD_1 and PD_2. This result strongly suggests that a derepressible enzyme active at acid and neutral pH, is missing in *PD* strains. The optimal pH for this enzyme was accurately calculated as follows: enzyme activity as a function of pH was measured in extracts of WT and PD_1 grown in (− P) medium. At every pH, the difference between the WT and the PD_1 values represents the activity of the unique enzyme lacking in PD_1. The curve obtained in these conditions was very similar to the curves shown in Figure 1 and displayed a maximum at pH 7.5.

The *PD* mutants were then studied by electrophoresis. The gels were stained for phosphatases at pH 4.8 (Figure 3) and pH 7.0 (Figure 4). In (+ P) cells, very little (if any) difference was found between the three strains. After growth on (− P) medium, the *PD* mutants had lower enzyme activity than WT in band 1 (starting line) when the gels were stained at pH 7.0; at low pH, this band was almost absent in the mutants. This confirms the results presented in Table 1. However, the sharp difference in enzyme activity at pH 7.0 between WT and mutants (Table 1) does not appear so clearly in the electrophoretic gels (Figure 4).

Mutants derived from P_2P_a

One triple mutant, $P_2P_a\,PD_4$ was compared to P_2P_a for enzyme activity and electrophoretic pattern, after growth in (+ P) or (− P) liquid medium (Table 2 and Figure 5).

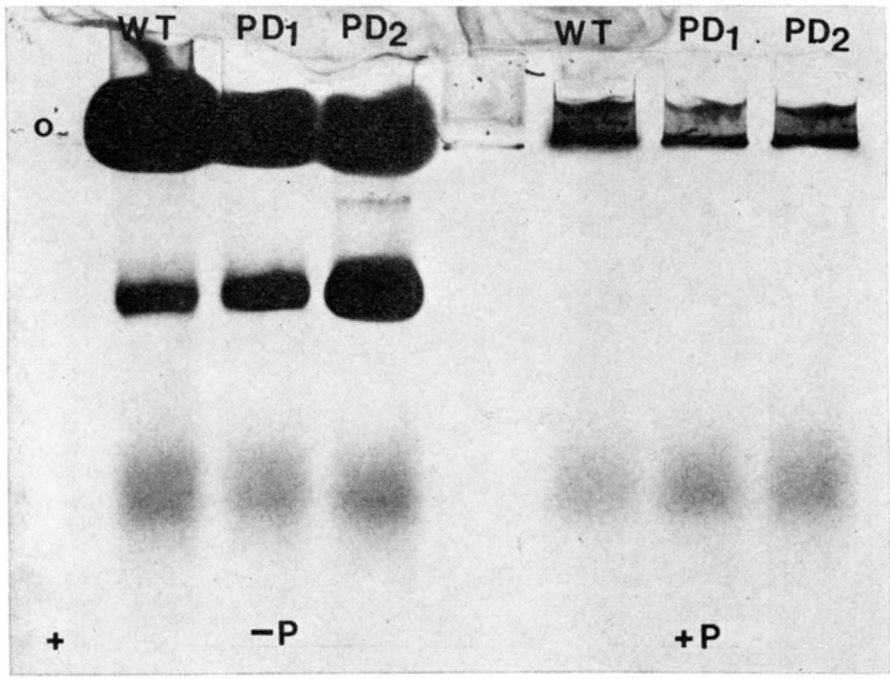

FIGURE 4.—As in Figure 3, except that the gels were stained at pH 7.0.

TABLE 2

Phosphatase activities measured at pH 4.8 and pH 7.5 in crude extracts (μmole naphthol/mg protein-hr-37°) of P_2P_a *and* P_2P_a PD_4 *strains, after growth for 3 days in (+ P) or (— P) liquid medium*

	(+ P)		(— P)	
	pH 4.8	pH 7.5	pH 4.8	pH 7.5
P_2P_a	0.18	0.02	6.35	11.32
P_2P_a PD_4	0.17	0.01	0.61	1.50

As in *PD* mutants derived from WT, the P_2P_a PD_4 mutant displayed much lower phosphatase activity than P_2P_a when the cells were grown in phosphate-free medium (Table 2).

As the two main constitutive enzymes are almost completely absent in this strain, the electropherogram (Figure 5) gave a very clear picture of the PD mutation: at pH 4.8, there was almost no more enzyme activity in P_2P_a PD_4, contrasting strongly with P_2P_a in which high enzyme activity was present at the starting line; at pH 7.0 two enzymes were still active in P_2P_a PD_4, namely (a) the migrating enzyme which is as active in P_2P_a PD_4 as in P_2P_a and (b) the

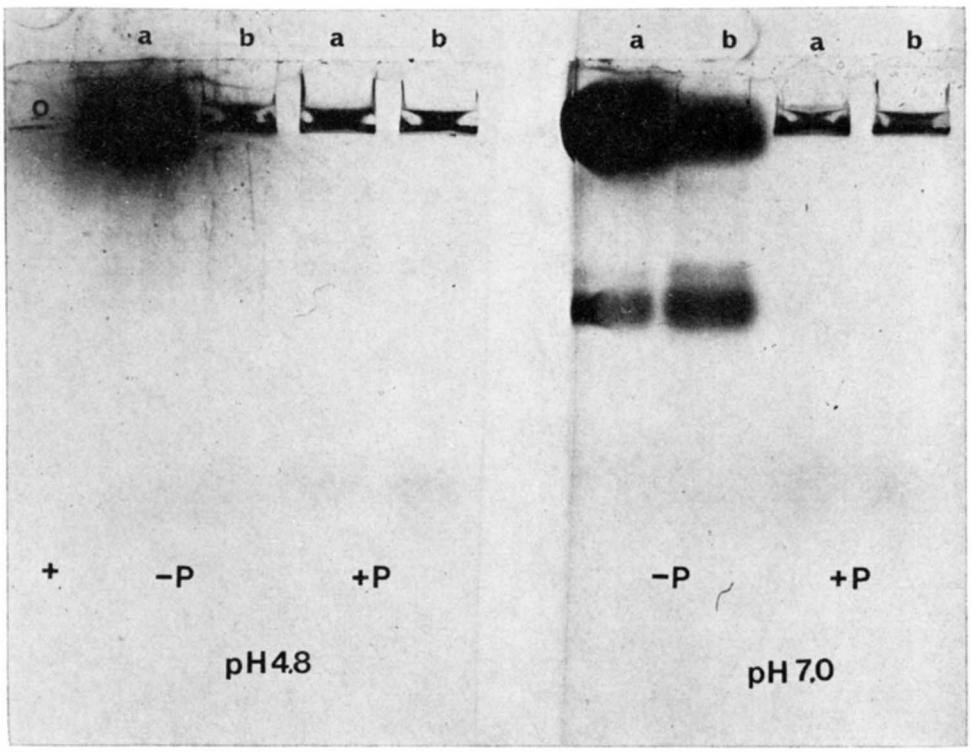

FIGURE 5.—Electropherograms of phosphatases present in extracts of P_2P_a (a) and P_2P_a PD_4 (b) grown for 3 days in (+ P) or (— P) medium. The gels were stained for phosphatase activity at pH 4.8 (left) or pH 7.0 (right).

enzyme present at the starting line that displays much lower activity in P_2P_a PD_4.

The optimal pH for the activity of the two phosphatases remaining in P_2P_a PD_4 grown in (− P) medium was determined. After electrophoresis, the gel was cut out at the level of the two enzymatic bands; these bands were homogenized in distilled water, then centrifuged. The supernatant was assayed for phosphatase activity at pH's ranging from 5.5 to 10.5 using tris-maleate or glycine-NaOH buffer. For both enzymatic bands, the maximum of activity was found at pH 9.5. The same value was obtained for crude extracts of PD_1 and PD_2 mutants.

5. *Genetic analysis of* PD *mutants*

The five *PD* mutants isolated from wild-type and P_2P_a strains were first crossed with P_2P_a or wild type in order to isolate the five single mutants PD_1, PD_2 . . . PD_5 and the five triple mutants $P_2P_aPD_1$, P_2P_a PD_2 . . . $P_2P_aPD_5$: $PD_1(+) \times P_2P_a$ (−), PD_2 (+) × P_2P_a (−), PD_3 (+) × P_2P_a (−), $P_2P_aPD_4$ (−) × +++ (+), $P_2P_aPD_5$ (−) × +++ (+). The progeny were studied by random spore plating analysis. The genotypes of the meiotic products were determined by staining for phosphatase activity the colonies grown on (+ P) or (− P) agar medium. In all crosses, the PD and corresponding wild phenotypes always segregated in the ratio 1:1, indicating that the character modified in PD mutants is controlled by a single nuclear gene. Moreover, in the two last crosses, the segregation between PD_4 (or PD_5) and P_2 on one hand, and between PD_4 (or PD_5) and P_a on the other hand, was analyzed: the results clearly indicated that the three markers were not linked.

The five triple mutants were then crossed in all combinations to determine whether the PD mutations were allelic or not (Table 3). Surprisingly, only those zygotes involving PD_2 did germinate. Anyway, it can be concluded from the successful crosses that PD_1, PD_2, PD_4 and PD_5 are allelic, whereas PD_3 is unlinked to this group of alleles (the expected segregation in the cross PD_2 (+) ×

TABLE 3

Allelism test between the five P_2P_aPD *mutants*

Crosses (the markers P_2 and P_a are present in each strain)	Germination of zygotes	No. of unstained colonies (P_2P_aPD phenotype)	No. of stained colonies (P_2P_a + phenotype)
PD_1 (+) × PD_2 (−)	+	324	0
PD_1 (+) × PD_3 (−)	0	—	—
PD_1 (+) × PD_4 (−)	0	—	—
PD_1 (+) × PD_5 (−)	0	—	—
PD_2 (+) × PD_3 (−)	+	324	140
PD_2 (+) × PD_4 (−)	+	463	0
PD_2 (+) × PD_5 (−)	+	352	0
PD_3 (−) × PD_4 (+)	0	—	—
PD_3 (−) × PD_5 (+)	0	—	—
PD_4 (+) × PD_5 (−)	0	—	—

PD_3 (—) is 348: 116, i.e. a ratio 3:1). The electrophoretic pattern of $P_2P_aPD_3$ grown in (— P) medium was similar to that of the other *PD* triple mutants.

DISCUSSION

The fact that derepressible phosphatase activity could be detected in colonies grown on phosphate-free medium allowed the isolation of new mutants related to this enzyme system; it was then possible to determine the precise number of phosphatase species present in Chlamydomonas.

In a previous paper (LOPPES and MATAGNE 1973), we described the isolation of two types of phosphatase mutants: in one (P_2) a particle-bound enzyme was absent, while in the other one (P_a) a soluble enzyme was absent. In addition to these two main constitutive acid phosphatases (displaying optimum activity at pH 4.8) a number of minor acid phosphatase species were detected by electrophoresis.

In WT and P_2P_a grown on (— P) medium (at least two divisions can occur in these conditions), we found an increased phosphatase activity with an optimum at pH 7.0–7.5, which is in agreement with the results of LIEN and KNUTSEN (1973). The electrophoretical study, however, provided more details about the number and the nature of phosphatase species in Chlamydomonas. Namely at least two additional enzymes were detected in (— P) cells: one present at the starting line and another one, migrating toward the anode. (Figure 2). The fact that the same banding patterns was obtained after electrophoresis of WT (Figure 2) and P_2P_a (Figure 5) indicates that the derepressible enzyme present at the starting line differs from the non-migrating constitutive enzyme since this latter is lacking in the double P_2P_a mutant.

After MNNG treatment several mutants with low phosphatase activity were isolated.

In the four mutants analyzed here (PD_1, PD_2, P_2P_a PD_3 and P_2P_a PD_4), the derepressible phosphatase showing an optimum at pH 7.5 was not detected. Two hypotheses can be proposed for explaining the phenotype of the *PD* mutants and especially the fact that they retain enzyme activity at the starting line (band 1, Figure 4) in a gel stained at neutral pH:

a) two different derepressible enzymes are present in the band 1 of WT. In the PD mutants, the neutral phosphatase is inactive, which allows one to visualize the second enzyme showing an optimum at pH 9.5. Like the band 2 enzyme, this alkaline phosphatase is active at pH 7.0 but not at pH 4.8 (Figures 3, 4 and 5);
b) in the WT strain, there is only one derepressible enzyme species at the starting line, the neutral phosphatase. The *PD* mutation would result in a considerable decrease of enzyme activity together with a shift of the pH optimum from 7.5 to 9.5.

The second hypothesis seems unlikely, for it is hard to explain (1) why four mutations of independent origin and involving two different loci (Table 3) all result in such a particular phenotype; (2) why no structural mutant lacking

completely band 1 enzyme was obtained. We therefore consider the first hypothesis as the most probable.

In Chlamydomonas, the main derepressible enzyme does not migrate in electrophoresis and is probably linked to small particles present in the crude extract. It can incidentally be remembered that the inducible acid phosphatase of Euglena is also insoluble in extracts and is found located *in vivo* at the surface of the alga (BENNUN and BLUM 1966).

An important question arising from the genetic analysis of the *PD* mutants is why two different mutations involving two unlinked genes apparently lead to the same phenotype. Phenotypically, PD_3 does not differ from PD_1, PD_2, PD_4 and PD_5, having very low phosphatase activity after growth on (— P) medium and a similar electrophoretic pattern. Therefore, it seems that two different genes are involved in the synthesis of the derepressible neutral phosphatase. A possible explanation is that one gene is a structural gene, the other one a regulatory gene. Another possibility is that the enzyme missing in PD mutants is composed of two (or more) non-identical subunits coded by two different genes.

The experiments reported in this paper, and especially the electropherograms obtained from P_2P_a and $P_2P_a\,PD_4$ mutants, allow a tentative definition of the "phosphatase pool" in *Chlamydomonas reinhardi*.

Besides the two main constitutive acid phosphatases (see + P samples in electropherograms of WT), Chlamydomonas produces three additional enzymes (derepressible) when cells are grown in phosphate-free medium:

1) soluble alkaline phosphatase; pH optimum: 9.5 (band 2, present in *PD*)
2) insoluble alkaline phosphatase; pH optimum: 9.5 (band 1, present in *PD*)
3) insoluble neutral phosphatase; pH optimum: 7.5 (band 1, lacking in *PD*). This enzyme has been shown to be derepressible in phosphate-free medium (LIEN and KNUTSEN 1973).

It is not possible by now to state whether the two alkaline phosphatases still present in *PD* mutants correspond to two different enzymes coded by two different genes or rather to a unique enzyme partly soluble and partly linked to particles. The solution to this problem could be found by verifying whether one or both enzyme species are inactivated by a single mutation.

This rather complex picture of the phosphatase pool in Chlamydomonas is not unusual in eukaryotic microorganisms. Neurospora, for example, produces two repressible enzymes: an alkaline phosphatase (NYC, KADNER and CROCKEN 1966) and an acid phosphatase (NYC 1967) and, in addition, three constitutive enzymes: two alkaline phosphatases and an acid prosphatase (KUO and BLUMENTHAL 1961; DAVIS and LEES 1969). In *Aspergillus nidulans*, one constitutive and three repressible phosphatases are found (DORN 1965). In *Coprinus lagopus*, a relatively complex situation has been described in which the control of alkaline phosphatase is mediated by at least three structural genes and five regulator genes (NORTH and LEWIS 1971).

The fair knowledge we have now of the phosphatase pool in Chlamydomonas will make easier the analysis of the mechanisms involved in the regulation of phosphatase activity and synthesis.

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