OBSERVATIONS ON THE ACID PHOSPHATASES OF *EUGLENA GRACILIS*

JACOB J. BLUM, Ph.D.

From the Department of Physiology and Pharmacology, Duke University Medical School, Durham, North Carolina

ABSTRACT

When a bleached strain of *Euglena* is maintained in a medium containing very low con centrations of phosphate, the acid phosphatase activity increases. The increase in acid phosphatase activity is prevented by Actinomycin D and by *p*-fluorophenylalanine (PFA), indicating that the increased activity is due to *de novo* synthesis of acid phosphatase. When phosphate is replenished, the acid phosphatase activity decreases to the level characteristic of uninduced cells before there is any appreciable cell division. When cell division resumes in the presence of PFA, the level of acid phosphatase activity remains approximately constant. This indicates that there are two different phosphatases: a constitutive enzyme, whose synthesis is insensitive to the presence of PFA, and an induced enzyme, whose synthesis is sensitive to PFA. These enzymes are not equally sensitive to changes in pH and in fluoride concentration, thus permitting them to be assayed individually in whole toluene-treated cells. Induced cells also acquire the ability to remove phosphate from the medium very rapidly.

INTRODUCTION

The finding by Price (1) that phosphate deprivation of *Euglena* caused a large increase in acid phosphatase (E.C.3.1.3.2) activity suggested that this was an example, in protozoa, of induced enzyme synthesis. Price further noted that upon replenishing the phosphate, enzyme activity disappeared more rapidly than could be accounted for by repression of synthesis with subsequent dilution by cell growth and cell division. Since many different kinds of cells must have "active" means of removing enzymes which are no longer required, a study of the mechanism by which acid phosphatase activity disappeared in *Euglena* would be of considerable interest. Before this problem could be attacked in detail, however, it was necessary to find out whether the increase in acid phosphatase activity caused by phosphate deprivation was due to *de novo* synthesis (i.e., whether it was an induced enzyme), and whether there were any other phosphatases in this organism. In this paper, we present evidence that the increase in acid phosphatase activity is due to *de novo* synthesis, and that there is at least one constitutive acid phosphatase, whose activity does not change significantly either during induction or after the replenishment of phosphate. It has also been found that phosphate-starved cells develop the ability to rapidly take up large amounts of orthophosphate. In an accompanying paper (2) the cellular localizations of enzyme activity, as determined by histochemical observations with the light and with the electron microscope, are analyzed.

MATERIALS AND METHODS

Although Price (1) used *Euglena gracilis* (Klebs) strain in his work, a similar strain in our laboratory did not show an appreciable increase in enzymatic activity

TABLE I

Composition of One Liter of Culture Medium

$CoCl_2 \cdot 6H_2O$	1.3 mg
$Na_2MoO_4 \cdot 2H_2O$	0.2 mg
$MnCl_2 \cdot 4H_2O$	1.8 mg
$CuSO_4 \cdot 5H_2O$	0.02 mg
$ZnSO_4 \cdot 7H_2O$	0.4 mg
$Fe_2(SO_4)_3 \cdot XH_2O$	3.0 mg
$CaCl_2 \cdot 2H_2O$	20.0 mg
$MgSO_4 \cdot 7H_2O$	0.20 gm
Sodium citrate	0.65 gm
Vitamin B_1	0.02 mg
Vitamin B_{12}	0.01 mg
NH_4Cl	0.80 gm
Maleic anhydride	1.96 gm
Ethanol	3.0 ml

The maleic anhydride was half-neutralized with 10 N KOH and the pH was then adjusted to 6.6 with NaOH.

upon phosphate deprivation. A streptomycin-bleached strain, *Euglena gracilis* var. *bacillaris*, strain SML-1, however, gave fairly large increases in acid phosphatase activity and, since the absence of chloroplasts eliminated a complex set of variables, all experiments were performed on this bleached strain.

The cells were grown axenically in an incubator at 25°C, using a modified Cramer-Myers medium (3), specified in Table I, with ethanol as the sole source of carbon and energy. Unless otherwise specified, cells were grown in Erlenmeyer flasks without shaking. The total volume of liquid in the flask never exceeded half the nominal capacity of the flask. Cells to be induced were transferred into medium containing 2×10^{-5} M orthophosphate. In this medium, the cells grew with a normal doubling time (about 24 hours) up to a density of about 200,000 cells/ml, and then stopped at about 275,000 cells/ml. At about this time, fresh medium containing 2×10^{-6} M orthophosphate was added, thus ensuring that the only ingredient lacking was phosphate. The 2×10^{-6} M phosphate was rapidly taken up by the cells and did not cause any diminution of phosphatase activity, but it did permit the cells to remain fully viable for longer periods of time. Cells which were to be studied in the absence of induced enzyme were grown in 2×10^{-2} M potassium phosphate (pH 6.6) and were kept below 300,000 cells/ml by suitable additions of complete medium containing 2×10^{-2} M phosphate.

Cell counts were made with a Coulter counter (Coulter Co., Hialeah, Florida).

Cells were collected for assay by centrifugation at 1400 *g* in a refrigerated centrifuge for about 5 minutes, washed once in cold 0.5 per cent saline, and recentrifuged. After the saline was decanted by suction, 5 ml of 0.05 M malate buffer, pH 5.0, and 2 ml of toluene were added, and the cells were shaken vigorously by hand for 90 seconds. The suspension was centrifuged for about 3 minutes, the toluene and malate phases were drawn off by suction, and the pellet was frozen until ready for assay. This procedure was adopted when it was found that unless the cells were treated with toluene the activity appeared to steadily increase during the assay. Treatment with toluene increased the initial activity to its maximal level, thereby resulting in an assay which was linear with time.

Cells to be assayed for phosphatase activity were thawed and then incubated at 30°C in 5 ml of malate buffer (0.05 M, pH 5.0) for 1 hour. Five ml of 10^{-2} M *p*-nitrophenylphosphate in the same buffer were then added, and aliquots were taken at known times and pipetted into an equal volume of 0.5 M Tris. The final pH was greater than 9 and, at this pH, there was no change in absorbance for at least 1 hour, thus demonstrating the absence of any significant amount of alkaline phosphatase (E.C. 3.1.3.1) activity. The cell suspension was briefly centrifuged in a clinical centrifuge, and the absorbance of the clear supernatant was measured at 410 mμ. The activity was computed from the rate of change of absorbance, using the value of 1.62×10^4 for the molar absorbancy index of *p*-nitrophenol. Similarly, the hydrolysis of *o*-carboxyphenylphosphate was computed from the rate of change of absorbance at 300 mμ, using the value 3500 for the molar absorbancy index of salicylic acid.

Phosphate concentration was measured by the method of Bartlett (4).

In later stages of this work, it was desired to harvest large quantities of cells for preliminary work on enzyme characterization. For this purpose cells were grown in 10-gallon carboys, with constant agitation by a magnetic stirrer. Aeration was provided by an aquarium pump. The cells were collected by centrifugation, treated with proportionately large volumes of toluene and malate buffer as already described, and frozen. After thawing, the cells were suspended in a solution of 0.05 M malate, pH 5.0, containing 0.5 per cent BRIJ 35 SP, a non-ionic detergent manufactured by Atlas Chemical Industries, Wilmington, Delaware. The presence of this concentration of detergent was found not to influence enzyme activity, but to greatly increase the amount of activity released into solution by the next step, which was exposure to 20 kc ultrasound from a Branson Model LS-75 Sonifier. The cells were kept in a Rosett cell and sonicated for a total of 7 minutes at temperatures between 0° and 10°. Centrifugation at 13,000 *g* for 30 minutes removed cell fragments, paramylon granules, and most mitochondria. The supernatant was then centrifuged in a No. 30 rotor at 25,000 RPM for 1 hour in a Spinco Model L centrifuge, resulting

in a brown pellet, a clear supernatant, and an upper layer of fat. Much of the fat was removed by a glass rod and the supernatant fraction was then ready for further purification, to be described elsewhere. In the present work, this supernatant was layered on top of a linear sucrose gradient, as described by Martin and Ames (5), and the acid phosphatase activity of the fractions collected by puncture of the tube was assayed. Further details are given in the text.

Actinomycin D was the gift of Merck, Sharp, and Dohme Co., Rahway, New Jersey; *p*-nitrophenyl-phosphate and *p*-fluorophenylalanine were purchased from the California Biochemical Co., Los Angeles. *O*-carboxyphenylphosphate was purchased from Worthington Biochemical Co., Freehold, New Jersey. All other reagents were reagent grade.

RESULTS

Two approaches were used to ascertain whether the appearance of acid phosphatase during phosphate deprivation was due to *de novo* synthesis or to the acquisition of enzyme activity by preexisting protein units. The first approach was the use of Actinomycin D, which has been shown to prevent the synthesis of messenger RNA in many cell types (6), probably including *Euglena* (7). Early experiments showed that relatively low concentrations of Actinomycin D (7) prevented further increase of phosphatase activity. In one such experiment, for example, 4.7 μg/ml of Actinomycin D were added to one of a pair of cultures which had nearly ceased division because of the depletion of the phosphate in the medium. At the time of addition of the actinomycin, the level of phosphatase activity in both cultures had risen from about 5 units (one unit corresponds to 1 mμ mole *p*-nitrophenylphosphate hydrolyzed per minute per 10^6 cells at pH 5.0 and at 30°C) to 8.5 units. The activity of the control culture increased to 17.5 units during the next 120 hours, while the activity of the actinomycin-treated culture increased to 9.5 units in this time interval. From this and similar experiments, it was apparent that the increase in phosphatase activity could be prevented by low concentrations of Actinomycin D, and, therefore, that the increase in activity was due to *de novo* synthesis. In the experiment shown in Fig. 1, a higher concentration of Actinomycin D was added to cells which had already tripled their phosphatase activity as a result of phosphate depletion of the medium. It can be seen that the two cultures to which actinomycin was added did not show any further increase in phosphatase activity, while in the two cultures which did not receive actinomycin the phosphatase activity continued to increase. The cultures to which 0.8 μM phosphate was then added did not show any loss of phosphatase activity. The cultures to which 16 mM phosphate was added rapidly lost their extra phosphatase activity and dropped, in about 24 hours, to the activity level characteristic of uninduced cells (about 5 units) before any appreciable amount of cell division had occurred. It will be

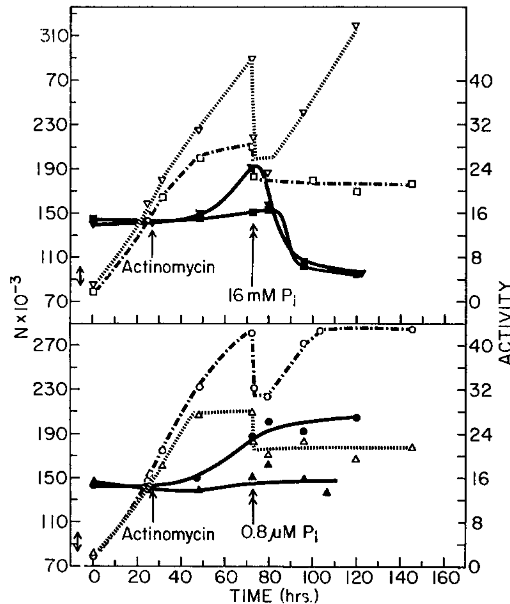

FIGURE 1 Effect of Actinomycin D on acid phosphatase formation and disappearance. Cells were grown in a medium originally containing 2×10^{-5} M phosphate. Dashed lines, with open symbols, refer to the left hand ordinate, and show the number of cells per ml of culture. Solid lines, with solid symbols, refer to the right hand ordinate, and show the phosphatase activity, measured at pH 5.0 and 30°C, in mμ moles of *p*-nitrophenylphosphate hydrolysed/min/10^6 cells. Zero time for the experiment was arbitrarily chosen when the cell count reached about 75,000 cells/ml. Actinomycin (9.8 μg/ml) was added at $T = 27$ hours to two of the cultures, (□------□, ■——■, △------△, ▲——▲), as indicated by the arrow. At $T = 72.3$ hours, 50 ml of culture media containing either 4×10^{-6} M phosphate (lower graphs) or 8×10^{-2} M phosphate (upper graphs) were added to each flask, thus increasing the volume in each flask from about 200 to about 250 ml. An appropriate amount of actinomycin was added to maintain the level of actinomycin unchanged. The level of acid phosphatase activity of these cells when grown in high phosphate media is shown by the double headed arrow to the left of the $T = 0$ abscissa.

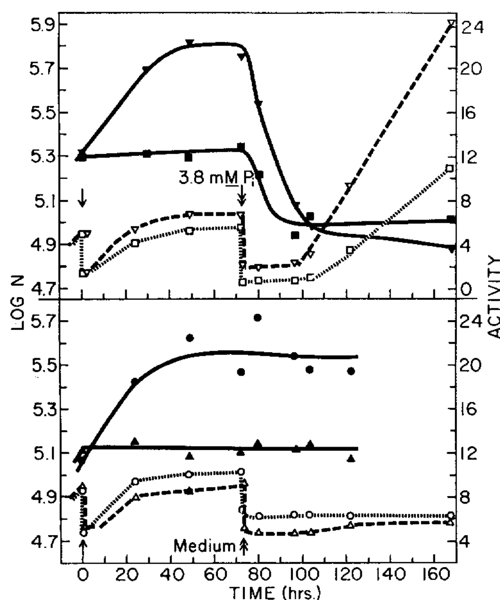

FIGURE 2 Effect of *p*-fluorophenylalanine on acid phosphatase induction and disappearance. Cells were grown in a medium originally containing 10^{-5} M phosphate. Dashed lines, with open symbols, refer to the left hand ordinate, in which is plotted the common logarithm of the number of cells per ml of culture. Solid lines, with solid symbols, refer to the right hand ordinate and show the phosphatase activity as described in the legend to Fig. 1. Zero time for the experiment was arbitrarily chosen when the culture attained a level of about 85,000 cells/ml. At $T = 0.5$ hours, as shown by the arrows, 100 ml of fresh culture medium containing 2 mM *p*-fluorophenylalanine were added to two of the cultures (□------□, ■——■, △------△, ▲——▲), so that the final concentration of *p*-fluorophenylalanine was 1.33 mM. At $T = 72.5$ hours, 100 ml were added of fresh medium containing either no phosphate (lower graphs) or enough phosphate to give a final concentration of 3.8 mM (upper graphs). An appropriate amount of *p*-fluorophenylalanine was added to maintain its concentration constant.

noted that even the presence of a high concentration of Actinomycin D—more than enough to prevent the increase in phosphatase activity—did not noticeably alter the rate of disappearance of enzyme activity. It appears that whatever the mechanism by which the induced activity is destroyed after phosphate is replenished, this mechanism does not require the synthesis of new messenger RNA.

To further check the conclusion that the increase in enzyme activity was due to *de novo* synthesis, one would like to interfere with protein synthesis. The growth rate of *Euglena*, however, is not affected by chloramphenicol, 5-methyltryptophan, 8-azaguanine, or puromycin (7). It was found that *p*-fluorophenylalanine does inhibit the growth of *Euglena*. Since this analogue is generally believed not to interefere with protein synthesis, but instead to substitute into newly formed proteins in place of phenylalanine (8), its effect on acid phosphatase activity in *Euglena* was studied. In the experiment shown in Fig. 2, a concentration of *p*-fluorophenylalanine was used which inhibited the growth rate of *Euglena* by only about one-half. It can be seen that this concentration of analogue (1.33 mM) caused an immediate cessation of the increase of phosphatase activity. It is also evident from these data that there is no appreciable loss of activity due to turnover for at least 120 hours after the addition of *p*-fluorophenylalanine. In conjunction with the data presented in Fig. 1, it is clear that the increase in activity represents induced enzyme synthesis. It should be noted that neither actinomycin (Fig. 1) nor *p*-fluorophenylalanine (Fig. 2) appreciably alters the rate of loss of induced enzyme after phosphate is replenished.

It was also of interest to see whether uncoupling oxidative phosphorylation would influence either the rate of synthesis of the induced enzyme or its rate of destruction when phosphate was replenished. In the experiment shown in Fig. 3, a low concentration of 2,4-dinitrophenol was used, so that no interference with the rate of cell division occurred after phosphate was replenished (upper part of Fig. 3), although the cells deprived of phosphate grew more slowly in the presence of dinitrophenol than the phosphate-starved controls (lower part of Fig. 3). Despite its small effect on the overall rate of population increase, the addition of dinitrophenol greatly reduced the rate of synthesis of phosphatase activity in the phosphate-deprived cells (lower part of Fig. 3), but it did not alter the rate of loss of phosphatase activity following the replenishment of phosphate (upper part of Fig. 3). It is evident from this experiment that the synthesis of the induced enzyme is more sensitive to the action of dinitrophenol than is the destruction of the induced activity.

The data in Figs. 1 to 3 also demonstrate another point of interest concerning the metabolic properties of *Euglena*. It has been shown elsewhere (9) that when *Euglena* are deprived of acetate, there is a lag period after the replenishment of acetate during

which the cells resynthesize the proteins, ribonucleic acids, and paramylum which were utilized for survival during the period of acetate starvation. After phosphate deprivation, too, there is a lag period which depends on the duration of the preceding period of phosphate deprivation. In the experiment shown in Fig. 1, phosphate was replenished just as the cells began to decrease their growth rate due to a lack of phosphate, and, in this case, the lag period was only about 8 hours. In the experiments shown in Figs. 2 and 3, the cells had stopped dividing for about 30 hours before the phosphate was replenished, and the lag period between the replenishment of phosphate and the resumption of cell division was about 24 to 36 hours. It is clear from the data in Figs. 2 and 3 that most of the destruction of the induced phosphatase activity occurs during this lag period.

It has already been mentioned that, after the addition of phosphate to the fully induced cells, the phosphatase activity drops to the level characteristic of cells grown in the presence of high concentrations of phosphate. This suggests that the basal level of phosphatase activity observed is due to a constitutive enzyme (or enzymes). The data in Fig. 2 show that although *p*-fluorophenylalanine prevents the synthesis of active induced phosphatase, the level of phosphatase activity remains constant after the lag period and during the subsequent period of cell division. In the experiment shown in Fig. 2, the cell number increased from 55,000 to 176,000 cells/ml without any change in the level of phosphatase activity. Since the concentration of *p*-fluorophenylalanine was sufficient to prevent the synthesis of active induced enzyme, it can be concluded that the phosphatase activity of cells grown in the presence of high concentrations of phosphate is due to a constitutive enzyme(s). Unlike the induced enzyme, the constitutive phosphatase presumably does not have a phenylalanine residue located at a crucial point of the enzyme structure.

Properties of the Induced and Constitutive Enzymes

The observation that constitutive phosphatase activity was not interfered with by *p*-fluorophenylalanine suggested that the active sites of the two phosphatases might have different properties. The establishment of different properties would, in turn, be of use for assaying each enzyme instead of only the sum of their activities. Furthermore, if different enzymatic properties could be ascribed to the different enzymes, such differences might prove of great value in establishing the precise cellular localization of these enzymes, as discussed in the accompanying paper (2).

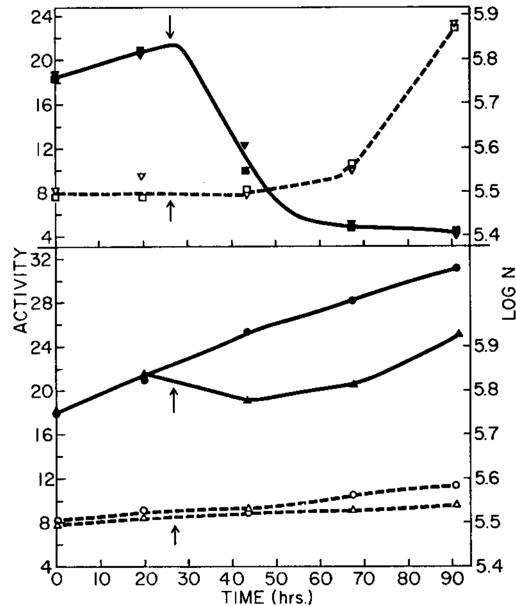

FIGURE 3 Effect of 2,4-dinitrophenol on acid phosphatase activity. Cells were grown in a medium originally containing 2.5×10^{-5} phosphate. Dashed lines, with open symbols, refer to the right hand ordinate, on which is plotted the logarithm of the number of cells/ml. Solid lines, with filled symbols, refer to the left hand ordinate and show the phosphatase activity in mμ moles *p*-nitrophenylphosphate/min/10^6 cells. At the time indicated by the arrows, the following additions were made: ●——●, ○------○, culture medium; ▲——▲, △------△, 0.12 mM dinitrophenol; ■——■, □------□, 0.12 mM dinitrophenol and 15 mM phosphate; ▼——▼, ▽------▽ 15 mM phosphate. The volume change caused by these additions was too small to cause a noticeable decrease in log *N*. In the upper part of this graph, only one solid line and one dashed line are drawn through the data.

Heat Stability

Cells were collected from an induced and from an uninduced culture of *Euglena* and, after toluene treatment, freezing, and thawing, aliquots were added at known times to tubes containing 4 ml of 0.05 M malate buffer, pH 5.0, at 60°C. When an appropriate time interval at 60°C had elapsed, the cells were transferred to an ice bath, thus terminat-

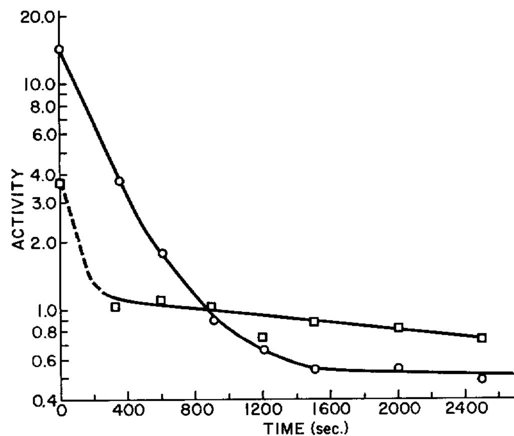

FIGURE 4 Thermal denaturation of phosphatase activity. Cells were heated at 60°C for the times shown on the abscissa, and then assayed at 30°C. Symbols: O——O, induced cells; □——□, cells grown in high phosphate medium. Phosphatase activity is shown on the ordinate in units of mμ moles *p*-nitrophenylphosphate/min/10^6 cells.

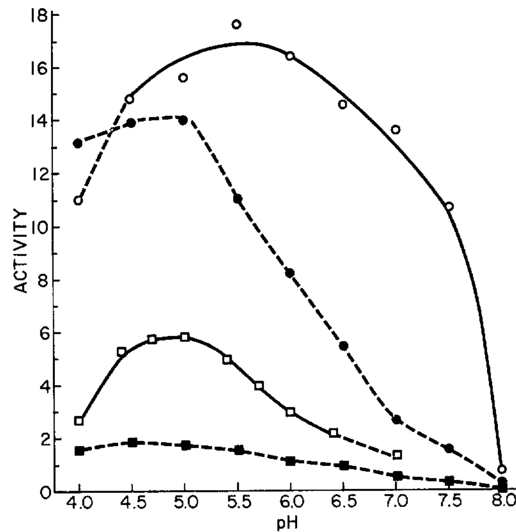

FIGURE 5 Effect of pH on phosphatase activity. Cells were collected from induced (O, ●) and from uninduced (□, ■) cultures, and, after treatment with toluene, freezing, and thawing, were placed in 5 ml of 0.10 M malate adjusted with Tris to the pH indicated on the abscissae. At zero time, 5 ml of 10^{-2} M *p*-nitrophenylphosphate (solid lines) or 5 ml of 5 mM *o*-carboxyphenylphosphate (dotted lines) were pipetted into each tube, and at known times thereafter, aliquots were removed and pipetted into 0.5 M Tris to stop the reaction. The units of activity are mμ moles of *p*-nitrophenol or of salicylic acid liberated per 10^6 cells per minute.

ing the heat treatment. The cells were then brought to 30°C, 5 ml of 10^{-2} M *p*-nitrophenylphosphate (in malate buffer at 30°C) were added, and the phosphatase activity was assayed. The results (Fig. 4) further substantiate the view that there are at least two enzymes in the induced cell. After the destruction of the most heat-labile enzyme, which is obviously the induced enzyme, the remaining enzyme activity of the induced cells is destroyed at approximately the same rate as the phosphatase activity of the uninduced cells. The first order denaturation constant for this process at 60°C is about 1.7×10^{-4} sec^{-1}. There is also some suggestion from this data that there are two constitutive enzymes, since a large fraction of the activity of the uninduced cells disappears in the first 300 seconds of heating.

Effect of pH

The pH *versus* activity curve for uninduced cells grown in high phosphate is a typical bell-shaped curve with a peak at about pH 5 when either *p*-nitrophenyl-phosphate or *o*-carboxyphenylphosphate is the substrate (Fig. 5). The induced cells, however, have a different pH dependence for these two substrates. When *o*-carboxyphenylphosphate is the substrate, the activity drops smoothly from its peak at about pH 5. With *p*-nitrophenylphosphate, however, the activity is relatively insensitive to pH in the range from pH 5 to pH 7, and then drops sharply between pH 7.5 and 8.0.

Effects of Fluoride and of Tartrate

It is well known that fluoride and tartrate are inhibitors of a number of phosphatases. The effect of these substances on the acid phosphatases of *Euglena* is shown in Fig. 6. Tartrate was a rather weak inhibitor of the acid phosphatase activities of both the induced and uninduced cells. Fluoride, however, was a potent inhibitor of both activities and, unlike tartrate, inhibited the activities differentially. At 2 mM fluoride, for example, the phosphatase activity of uninduced cells was reduced to 10 per cent of the activity observed in the absence of fluoride, while the phosphatase activity of induced cells only decreased to about 70 per cent (Fig. 6). This observation suggested that, by the use of fluoride at different pH values, it might be possible to assay the constitutive and the

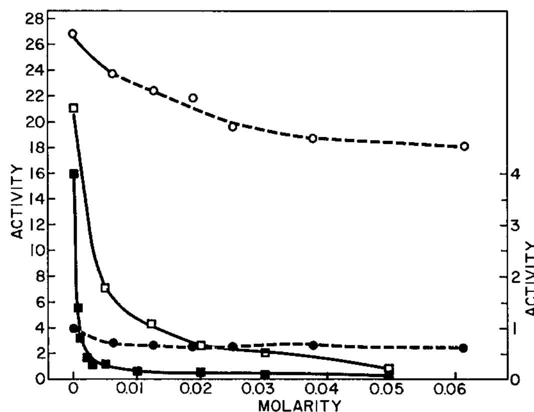

FIGURE 6 Effects of fluoride and of tartrate on acid phosphatase activity. Cells were collected from induced (○, □) and uninduced (●, ■) cultures, and after toluene treatment, freezing, and thawing were placed in 5 ml of 0.1 M malate, pH 5.0, containing either tartrate (○------○, ●------●) or sodium fluoride (□——□, ■——■). At zero time, 5 ml of 10^{-2} M *p*-nitrophenylphosphate in H_2O at pH 5 were added to each tube, and the activity, shown on the ordinate in mμ moles *p*-nitrophenylphosphate hydrolyzed/min/10^6 cells, was assayed at 30°C. The right hand ordinate refers to the uninduced cells treated with fluoride (■——■). The left hand ordinate refers to induced cells treated with fluoride (□——□) and to cells treated with tartrate. In the experiments with fluoride, the sodium ion concentration was adjusted to 0.05 M in each assay.

induced enzymes separately in the toluene-treated cells. The data presented in Table II show that the differential inhibition by fluoride observed at pH 5 (Fig. 6) is also observed at pH 7. Since the *p*-nitrophenylphosphatase activity of uninduced cells was considerably inhibited by increasing the pH from 5 to 7 (Fig. 5 and Table II), while the activity of induced cells was only slightly decreased, it appeared that a large differential inhibition might be observed on comparing the activity at pH 5 *versus* the activity at pH 7 in the presence of 5 mM fluoride. It can be seen (Table II) that the total activity of induced cells assayed at pH 5 is decreased by a factor of only 0.88 when the assay is repeated at pH 7 with 5 mM fluoride, whereas the activity of uninduced cells is decreased by a factor of 0.11. Since it can be assumed that the total phosphatase activity of induced cells is given by the sum of the induced activity plus the constitutive activity, it may be inquired whether the inhibition observed in the induced cells could be accounted for by a 90 per cent inhibition of their constitutive enzyme. This would be equivalent to assuming that the acid phosphatase activity (with *p*-nitrophenylphosphate as substrate) of the induced enzyme is not significantly inhibited by changing the assay conditions from pH 5.0 to pH 7.0 with 5 mM fluoride. If we define T_5 and T_{7F} as the activities observed at pH 5.0 and at pH 7.0 with 5 mM fluoride, respectively, then we may write

$$T_5 = i + c; \quad T_{7F} = i + 0.11c,$$

where i and c are, respectively, the activities of the induced and constitutive enzymes. When applied to the data shown in Table II, these equations would specify that the induced cells at pH 5.0 had 3.1 units of constitutive enzyme activity and 14.2 units of induced phosphatase activity. The equations would predict that a value of 14.5 units of activity would be observed at pH 7 in the presence of fluoride, which is within experimental error of the observed value of 15.3. As a first approximation, therefore, we may use the above equations to compute the activity of the constitutive and of the induced enzymes in cells at any stage of induction. In Fig. 1, we have shown the time course of the total acid phosphatase activity of cells at various times before and after phosphate was replenished. The phosphatase activity was also measured at pH 7 with 5 mM fluoride, and in Fig. 7 the time course of the induced and of the constitutive enzymes is plotted for two of the four experimental conditions shown in Fig. 1. It can be seen that within the limits of error the constitu-

TABLE II

Effect of Fluoride at pH 5 and at pH 7 on Acid Phosphatase Activity

The third and fourth columns show the activities of the cells in 5 mM NaCl and in 5 mM NaF, respectively. The last column shows the ratio of the activity at pH 7 with fluoride to the activity at pH 5.

Cells	pH	Activity NaCl	Activity NaF	Ratio $\frac{\text{pH } 7 + F^-}{\text{pH } 5 + Cl^-}$
Induced	5	17.3	5.37	0.88
	7	15.6	15.3	
Uninduced	5	3.08	0.17	
	7	0.77	0.33	0.11

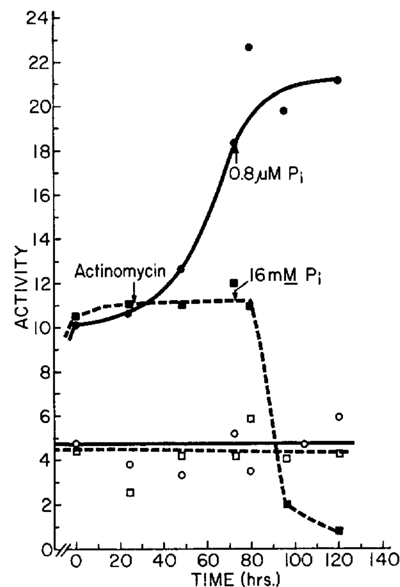

FIGURE 7 Effect of Actinomycin D on induced and constitutive acid phosphatase activities. The cell population and the total phosphatase assay (*i.e.*, measured at pH 5.0) are shown in Fig. 1. From the data shown in Fig. 1, and from measurements of phosphatase activity at pH 7.0 with 5 mM fluoride, the activity due to the induced enzyme (solid symbols) and to the constitutive enzyme (open symbols) was computed as described in the text.

tive enzyme activity was not changed during the course of induction, after the replenishment of phosphate, or in the presence of Actinomycin D. The synthesis of induced enzyme activity is seen to be stopped shortly after actinomycin addition and to decrease to very small values after the phosphate is replenished.

Sucrose Gradient Separation of Phosphatase Activities

The data presented so far demonstrate that the constitutive and induced enzymes have different properties with respect to their pH profile, substrate specificity, sensitivity to fluoride ion, thermal stability, and to the effect of *p*-fluorophenylalanine substitution on their activity. It was also desirable to know whether they had different molecular weights. A simple way to resolve proteins by molecular weight is the sucrose density gradient sedimentation procedure devised by Martin and Ames (5). Cells were treated with ultrasound as described in the section on Methods, and the partially defatted supernatant obtained by centrifuging the homogenate for 1 hour at about 70,000 *g* was used as the starting material.

In the experiment shown in the upper part of Fig. 8, 0.1 ml of the supernatant obtained from normal cells was layered on each of two 5 to 20 per cent sucrose gradients (the sucrose was dissolved in 0.05 M malate, pH 5.0), and centrifuged at an average speed of 36,950 RPM in the SW39 rotor of the Model L centrifuge for 15 hours and 47 minutes. Fractions of ten drops were then collected from each gradient. The tubes from one gradient were assayed at pH 5, while the tubes from the other gradient were assayed at pH 7 with 5 mM fluoride. The results show that most of the phosphatase activity in the supernatant of uninduced cells is strongly inhibited at pH 7 in the presence of 5 mM fluoride, in agreement with our previous conclusion. The value of $S_{20,W}$, computed according to the integration procedure of Martin and Ames (5), is 6.2×10^{-13} seconds.

A similar experiment was performed in the cell-free homogenate of induced cells, and gave a large peak at approximately the same position as was observed for a homogenate of uninduced cells, plus a smaller peak further from the meniscus. The shape of this heavy small peak suggested that it might be composed of two peaks, and an effort was made to resolve this peak by repeating the sedimentation in a 10 to 20 per cent gradient. The results (bottom part of Fig. 8) indicate that there are two heavy components, neither of which is appreciably inhibited by changing the assay condi-

tions from pH 5 to pH 7 plus 5 mM fluoride. It should be noted that the inhibition of the constitutive enzyme is not so great as is observed with the enzyme from a homogenate of uninduced cells (upper part of Fig. 8), and the shift in peak location from fraction 21 to fraction 23 appears greater than might be accounted for by the error of collecting the fractions. It is thus possible that the induced enzyme has an S_{20} near that of the constitutive enzyme, but also exists in forms with larger S_{20} values, either as dimers or higher n-mers, or still associated with fragments of the pellicle (2). The difficulty with which the induced enzyme is solubilized is indicated by the fact that although the ratio of induced to constitutive activity in the whole cell is at least 5:1, the ratio of these activities in the homogenate is much less than 5:1. Further characterization of the induced enzyme is obviously necessary, but the sedimentation data (Fig. 8) confirm the view that there are at least two independent enzymes and, in addition, provide some useful, if preliminary, information concerning their physical properties.

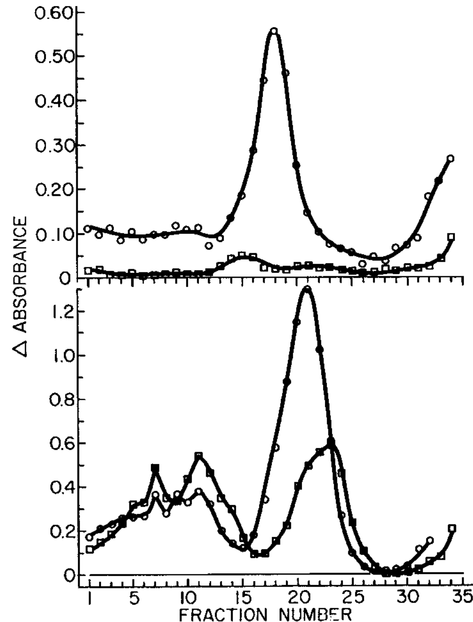

FIGURE 8 Sedimentation behavior of acid phosphatases of *Euglena*. The upper part of the figure shows the sedimentation pattern of acid phosphatase activity from a cell-free homogenate of uninduced cells, after centrifugation in a 5 to 20 per cent sucrose gradient. The lower part of the figure shows the sedimentation pattern obtained from a cell-free homogenate of induced cells after centrifugation in a 10 per cent to 20 per cent sucrose gradient for 15 hours and 26 minutes at approximately 37,000 RPM. To each 10-drop fraction (0.14 ml), was added 0.5 ml of 6.5 mM *p*-nitrophenylphosphate in 0.05 M Tris-malate buffer, pH 5.0 (○——○), or 0.5 ml of 6.5 mM *p*-nitrophenylphosphate plus 6.5 mM NaF in 0.05 M Tris-malate buffer at pH 7.10 (□——□). The tubes were incubated at 30°C for 3 hours (lower part of graph, induced cells), or for 2 hours (upper part of graph, uninduced cells). At the end of the incubation, 0.6 ml of 0.5 M Tris was added, and the change in absorbance at 410 mμ (relative to an appropriate blank) was measured, as shown on the ordinate.

Phosphate Accumulation by Induced Cells

During the course of this study, some experiments were performed to ascertain whether small amounts of phosphate could be added to cells without causing the disappearance of the induced enzyme. Such supplementation of phosphate-starved cells with small amounts of phosphate would avoid problems arising from cell mortality and, if done on a continuous basis, would permit chemostatic growth of induced cells. It was found that if induced cells at, *e.g.*, 250,000 cells/ml were supplied with 2 mμ moles of phosphate/ml, the population would increase to about 300,000 cells/ml after a lag period, with no decline in phosphatase activity. It was also observed that within a few hours, phosphate was no longer present in the medium. This suggested the possibility that cells deprived of phosphate had developed the ability to rapidly accumulate phosphate. The rate of uptake of orthophosphate by induced and by uninduced cells was, therefore, studied.

When cells growing logarithmically in high phosphate medium were briefly centrifuged (approx. 4 minutes) and resuspended in fresh medium, it was found (Fig. 9) that phosphate (or a labile phosphate ester) was released into the medium. The rate of release was dependent on the external phosphate concentration. At low external phosphate (0.2 μ moles/ml or less), the rate was approximately 0.15 mμ moles released/10^6 cells/minute, while at higher external phosphate concentrations (*e.g.*, 1.0 μ moles/ml) there was no appreciable release of phosphate. It is known (10) that brief centrifugation of *Euglena* decreases the oxygen consumption for a few hours, and it is possible that the process of centrifugation is responsible for the temporary loss of phosphate into the medium.

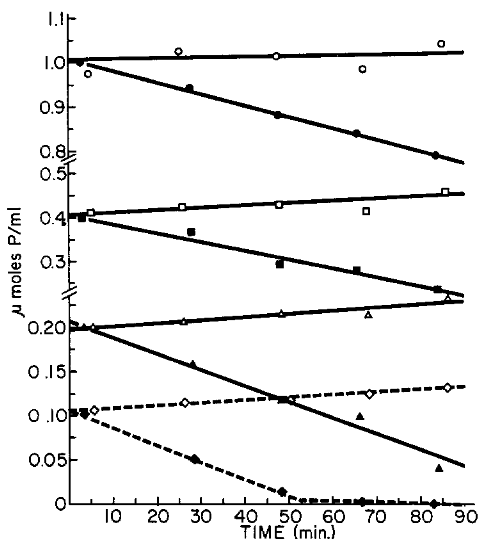

FIGURE 9 Uptake of phosphate by induced cells and release of phosphate by uninduced cells. Cells were collected by centrifugation for 4 minutes at 1000 g and washed three times by mixing them in phosphate-free buffer and recentrifuging. The cells were then suspended in fresh medium at room temperature ($\approx 26°C$) containing the amounts of phosphate shown on the ordinate. At the times shown in the abscissa, 3-ml aliquots were taken and pipetted into centrifuge tubes which were spun for 1 minute at 2,000 g. Samples were immediately taken from the clear supernatant for phosphate analysis. Solid symbols, induced cells; open symbols, cells grown in high phosphate.

When induced cells were studied, however, it was observed that they could very rapidly remove phosphate from the medium. In the experiment shown in Fig. 9, the rates of phosphate uptake were 3.86, 2.07, 1.85, and 1.96 mμ moles P/min/10^6 cells, when the initial phosphate concentrations in the medium were 1.00, 0.40, 0.20, and 0.10 μ moles P/ml, respectively. In another experiment, not shown here, the rate of phosphate uptake at pH 7 by induced cells, when the experiment was performed with constant shaking in a 30°C water bath, was 2.08 mμ moles P/min/10^6 cells at an initial phosphate concentration of 0.33 μmoles P/ml. In 30 minutes, then, approximately 0.0625 μmoles of phosphate were removed from the medium into 10^6 cells. The approximate volume of *Euglena* is 0.0017 ml/10^6 cells, so that had the phosphate remained as orthophosphate in the cells, the intracellular to extracellular ratio of phosphate would have been 100 to 1. Preliminary experiments have shown, however, that some of the phosphate in the cell is no longer orthophosphate, so that much more work is required to adequately characterize the transport of phosphate into these cells and its immediate metabolic fate.

DISCUSSION

It has long been recognized that the study of induced enzymes can yield information not only about the mechanism of protein synthesis, but also about the control of the enzyme complement of the cell. For a variety of reasons, most of this work has been done with bacteria and most of the attention has been focused on the induction process. In the relatively few metazoan systems so far studied in detail (11), the induction process seems to be similar to that found in the bacteria, and the data so far obtained on the induced phosphatase of *Euglena* in no way suggest that the process of induced enzyme formation in *Euglena* is different from that in other organisms. The loss of induced enzyme activity after an inducer is removed has been termed "enzymic reversion" by Pollock (12). In those cases which have been most extensively studied (termed Type 2 by Pollock), there is no loss in total activity, and the induced enzyme vanishes by dilution as the cells grow. Destruction of the activity in the absence of appreciable cell multiplication (termed Type I by Pollock) has been observed in several cases (13–15) and could result from a variety of mechanisms. Type I reversion might be expected to occur relatively frequently in cells in which a slow rate of division precludes the possibility of rapid destruction of the induced activity.

The discovery by Price (1) that the acid phosphatase of *Euglena* increased when growth stopped because of phosphate depletion, and that this increased activity largely disappeared before cell division began after phosphate replenishment, suggested that this organism would be useful for studying a mechanism of destruction of an induced enzyme. If one admits that active destruction might, for example, occur by an alteration of the active site, then an increase in enzyme activity need not necessarily result from induced enzyme synthesis. The observations that Actinomycin D (Figs. 1 and 7) and *p*-fluorophenylalanine (Fig. 2) prevent the increase in phosphatase activity normally observed in the absence of phosphate demonstrate that the increased activity is due to

de novo synthesis. It is possible, however, that the protein synthesized is not the induced enzyme itself, but an activator of a preexisting inactive enzyme. Since in the bleached strain of *Euglena* used in this work the level of phosphatase activity in cells grown in high phosphate medium is already fairly high, it becomes necessary to ascertain whether the newly synthesized activity is merely an addition to the already present enzyme or whether it is an entirely different enzyme, perhaps even with a different localization in the cell.

The first observation which showed that there were two enzymes with different properties was that *p*-fluorophenylalanine, while preventing the synthesis of induced activity, did not prevent the synthesis of constitutive activity. Further evidence that these enzymes were different was then obtained by studying the differential effects of pH and of fluoride ion: and it was possible to show that during phosphate deprivation, induced phosphatase was synthesized from an initially very small level, and after phosphate replenishment the induced phosphatase activity disappeared. It has also been found that the induced phosphatase activity is confined to particular regions of the pellicle, while the constitutive phosphatase activity is localized in certain specific intracellular organelles (2).

It has been shown (9) that after a period of acetate deprivation of *Euglena* there is a biosynthetic lag, the duration of which is proportional to the length of the preceding period of acetate deprivation. During this lag there is considerable metabolic activity, including the resynthesis of RNA, protein, and paramylum which were utilized for survival. In the present work, it has been observed that when phosphate is replenished after a period of phosphate deprivation, there is also a lag. It is during this lag that the induced phosphatase activity is destroyed. The observation that Actinomycin D does not inhibit the rate of loss of induced activity (Fig. 1) indicates that the destruction of induced activity does not depend on a "message" from the nucleus. The rate of reversion of thymidine kinase in microspores has been studied by Hotta and Stern (14), who find that the rate of loss of this enzyme is insensitive to 8-azaguanine, fluorouracil, and chloramphenicol, suggesting that in this case also the mechanism for removal of the enzyme activity is not induced in the same way as its formation. Further work on the mechanism of destruction of the induced phosphatase of *Euglena* is in progress.

It has frequently been observed that cells which develop an induced enzyme to meet a particular metabolic challenge also develop a "permease" to enhance the transport of the substrate in question. The subject has been reviewed in detail by Cohen and Monod (16), who point out that the existence of a "permease" cannot be inferred with certainty without the use of labeled non-metabolized inducers. In *Euglena*, the induction process results from the removal of phosphate, presumably causing a decrease in the concentration of a repressor. When phosphate is replenished, it is taken into the cell with great rapidity (Fig. 9), suggesting the possibility that a "permease" was induced during the period of phosphate deprivation. Phosphate, however, is certainly utilized by a variety of metabolic processes, such as conversion to polyphosphate (*e.g.*, reference 17), and it will be necessary to examine the metabolic fate of phosphate in *Euglena* shortly after it is resupplied to the phosphate-starved cell to see whether it is necessary to postulate the development of a phosphate "permease."

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