Alkaline Phosphatase in *Dictyostelium discoideum*

BY KERSTIN GEZELIUS* AND BARBARA E. WRIGHT

From the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University, at the Massachusetts General Hospital, Boston, Mass, U.S.A.

(Received 26 May 1964)

SUMMARY

Several phosphatases have been demonstrated in *Dictyostelium discoideum*. The alkaline phosphatase, pH optimum 9.0, is a 5'-nucleotidase, attacking adenosine monophosphate and deoxyadenosine monophosphate, but catalyses also hydrolysis of *p*-nitrophenyl phosphate. In the absence of exogenous nutrients this enzyme increases about 6-fold *in vitro* during differentiation. Its activity *in vivo* may in part be controlled through end-product inhibition by orthophosphate which has been found to accumulate in the cells during sporulation. Exogenous orthophosphate and glucose repress the levels of alkaline phosphatase in the spores. The data support the conclusion that end product inhibition and repression collaborate to ensure maximal alkaline phosphatase activity *in vivo* during culmination. Exogenous adenosine and deoxyadenosine increase the alkaline phosphatase levels in the sorocarps.

INTRODUCTION

Histochemical and *in vitro* studies on the differentiating cellular slime mould *Dictyostelium discoideum* have shown that the enzyme alkaline phosphatase changes in activity during the development of the organism. The activity increases until spore formation but decreases to a minimum in the mature fruiting bodies or sorocarps (Bonner, Chiquoine & Kolderie, 1955; Krivanek, 1956). These studies were performed with β -glycerophosphate as substrate at pH 9.3. With adenylic acid at pH 8.3, Krivanek & Krivanek (1958) found a similar activity pattern for 5'-nucleotidase. It has been shown (Wright, 1960) for other slime mould enzymes that apparent increases in *in vitro* activities during differentiation may be due to rapid inactivation in extracts prepared at the early stages of development and thus do not represent true increases in enzyme concentrations. In the event that true increases are shown to occur, these do not necessarily reflect increased enzyme activities *in vivo*, where, e.g. changes in substrate and inhibitor concentrations may control the reactions. The present investigation was, therefore, undertaken to determine if the observed changes in alkaline phosphatase activity *in vitro* reflect the conditions inside the living cells.

During this work a number of phosphatases have been detected, distinguishable from each other through pH optima, Mg^{2+} requirements and their *in vitro* activities at the various stages of differentiation. They include, besides the alkaline phosphatase, one acid phosphatase, optimum about pH 3.5, one ATP (see p. 312) attacking enzyme, two pyrophosphatases with optima at pH 6.2 and 8.8 and one enzyme,

* Present address: Institute of Physiological Botany, University of Uppsala, Uppsala, Sweden.

active on 5'-GMP, with optimum at pH 7.5–8.5. The present paper will describe the alkaline phosphatase and only include the other enzymes when necessary for distinguishing purposes. The other phosphatases will be dealt with in subsequent papers. For a full description of the life cycle of the slime mould the reader is referred to Bonner's monograph, *The Cellular Slime Molds* (1959). A summary is presented in Fig. 1.

Fig. 1. Life cycle of *Dictyostelium discoideum*.

METHODS

The myxamoebae of *Dictyostelium discoideum* strain NC-4 were grown at 23° on SM agar (Sussman, 1961) with *Escherichia coli* strain ML 304d, washed and transferred to 2% plain agar for starvation and multicellular differentiation at 17° as described by Liddell & Wright (1961). Compounds studied in induction and repression experiments were included in the 2% agar. For comparison, in one experiment the slime mould was allowed to differentiate on the complex medium in the presence of residual food bacteria.

The enzyme activities were studied in crude cell-free extracts. At the desired stage of development the slime mould was harvested from the agar with 0.01 M-tris

buffer (pH 7.2). The suspension was homogenized in an Aminco (American Instrument Corporation, Silver Spring, Md, U.S.A.) French pressure cell at 5000 lb./sq. in. and the homogenate centrifuged at 3000 g. for 10 min. All these procedures were undertaken in the cold. The protein content in the extracts was usually about 10 mg./ml.

Protein was determined by the modified micro-biuret method (Zamenhof, 1957) with crystalline serum albumin as a standard. The measurements were performed in a Zeiss spectrophotometer Model PMQ II. The determinations were made either on the crude extracts or after precipitation of the protein in 3.5% (w/v) perchloric acid followed by dissolving of the precipitate in 0.5 M-NaOH. The latter procedure was necessary when the experiments involved a comparison between different developmental stages because of the varying size of the amino acid pool and of the small peptide pool during differentiation (Wright, 1964).

Intracellular orthophosphate. To avoid washing out phosphate from the cells during the harvesting procedure, at different stages the organism was removed from the agar surface with a glass slide and suspended in cold 0.01 M-tris (pH 7.2). At the sorocarp stage determinations were made both on the whole fruiting bodies and on the spores.

A sample of the fresh suspension was immediately extracted for 30 min. in cold 5% trichloroacetic acid (TCA). After centrifugation the orthophosphate in the supernatant fluid was determined according to the method of Fiske & SubbaRow (1925) as modified by Dryer, Tammes & Routh (1957).

Another sample of the suspension was centrifuged for 10 min. at 1000 g, giving the corresponding volume of packed organism (Wright & Bard, 1963). The intracellular phosphate concentration was expressed as molarity in terms of packed cell volume.

Enzyme assays. The alkaline phosphatase activity was assayed in diluted extracts (about 0.1 mg. protein/ml.) with *p*-nitrophenyl phosphate (NPP) as substrate (Torriani, 1960) unless specified otherwise. The reaction was followed spectrophotometrically (Beckman DU or Zeiss PMQ II) at 23° by the formation of nitrophenol (NP) at 420 m μ .

The reaction mixture, 1 ml., was 0.05 or 0.5 M-tris (pH 9), 0.02 M-MgCl₂, 0.01 M-NPP. In the early experiments, where 0.5 M-tris was used, no MgCl₂ was added, and it was also omitted in the experiments on phosphate inhibition. The amount of crude enzyme added corresponded to 5–50 μ g. protein/ml. The enzyme-specific activity was calculated from the initial rapid reaction rate and expressed as a μ mmole NP/min./mg. protein. Figs. 7–11 express activity in these terms; comparative data on substrate specificity, pH optima, etc., are expressed as per cent of maximal activity.

The hydrolysis of other phosphate compounds was determined as the amount of orthophosphate (Pi) released, and the specific activity expressed as μ mmole Pi/min./mg. protein. The reaction mixture was 0.05 M-tris, 0.02 M-MgCl₂ (except as noted) and 0.01 M with respect to substrate concentration. The incubation time was 15 min. to 1 hr, occasionally longer, and the temperature was 23°. The reaction was stopped through addition of cold 30% TCA to final concentration 6%. The Pi was determined according to Dryer *et al.* (1957). Control tubes without enzyme were incubated in all experiments.

The hydrolysis of 5'-AMP was determined also as the amount of adenosine formed (Herman & Wright, 1959). The reaction was stopped by addition of 10% TCA to final concentration 2%. After centrifugation, samples (0.03 ml.) of the supernatant fluid were assayed with adenosine deaminase in a total of 1.1 ml. of 0.05 M-phosphate buffer (pH 7.5). The reaction was followed as the decrease in extinction at 265 m μ in the Zeiss (Kornberg & Pricer, 1951).

Reagents. NPP, NP, β GP, G6P, G1P, FDP, 5'-UMP, 5'-CMP, GTP (type II, 95–98%), GDP, UDP, adenosine, deoxyadenosine, and adenosine deaminase (type II, crude powder) were obtained from Sigma Chemical Company, St Louis, Mo, U.S.A.; 5'-GMP, 5'-TMP, ADP and UTP from Pabst Laboratories, Inc., Milwaukee, Wisc., U.S.A.; 5'-AMP and ATP from Sigma and Pabst; 5'-IMP and 5'-dAMP from California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A.; and 3'-AMP from Schwarz BioResearch, Inc., Mount Vernon, N.Y., U.S.A. With the exception of GTP and adenosine deaminase, all compounds were reagent grade.

The following abbreviations are used throughout this paper: NPP, *p*-nitrophenyl phosphate; NP, nitrophenol; Pi, orthophosphate; β GP, β -glycerophosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; FDP, fructose-1,6-diphosphate; 5'-AMP, 5'-GMP, 5'-CMP, 5'-UMP, 5'-TMP, and 5'-IMP, the 5'-monophosphates of adenosine, guanosine, cytidine, uridine, thymidine and inosine respectively; 5'-dAMP, deoxyadenosine-5'-monophosphate; 3'-AMP, adenosine-3'-monophosphate; ATP, GTP, UTP, ADP, GDP, UDP, the tri- and diphosphates of adenosine, guanosine and uridine; PPi, pyrophosphate; tris, tris(hydroxymethyl)amino-methane; and TCA, trichloroacetic acid.

RESULTS

The stages usually studied were amoeba (starved for 2–3 hr), pre-culmination, culmination and sorocarp (about 10–15 hr after sorocarp formation). The time required for sporulation varied between the experiments depending on the status of the amoebae at the time of transfer from the rich medium, variations in washing and plating procedures, and so on.

pH optimum. As shown in Fig. 2 optimal phosphatase activity against NPP was obtained at pH 9.0 in extracts prepared either at the amoeba or sorocarp stage. The optimum on 5'-AMP was also pH 9, whereas activity on 5'-GMP had a broader optimum around pH 8. This fact and other data (see below) indicate that 5'-GMP is not a substrate for alkaline phosphatase.

Substrate concentration. The effect of increasing substrate concentration on phosphatase activity against NPP and 5'-AMP is shown in Fig. 3. The optimal concentration range was the same for both compounds.

Mg²⁺ activation. The phosphatase was more dependent on Mg²⁺ ions when assayed in a final concentration of 0.05 M-tris than when 0.5 M-tris was used. With dialysed extracts about 75% of maximal activity against NPP was obtained in 0.5 M-tris without addition of Mg²⁺ compared to 50% at the lower buffer concentration (Fig. 4). A similar relationship between buffer concentration and Mg²⁺ activation has been reported for the alkaline phosphatase of *Escherichia coli* (Garen & Levinthal, 1960). Optimal Mg²⁺ concentration in 0.05 M-tris was 0.02–0.05 M at

amoeba and sorocarp stages, with either NPP or 5'-AMP as substrate. (In the curve for AMP the activity without Mg^{2+} is higher than that usually obtained as in Table 3.) In contrast, activity on 5'-GMP was completely dependent on Mg^{2+} implicating another enzyme (see below).

Fig. 2. Effect of pH on phosphatase activity against NPP (bottom), 5'-AMP and 5'-GMP (top). The activities against NPP were assayed in 0.5 M-tris without $MgCl_2$, those against 5'-AMP and 5'-GMP in 0.05 M-tris, 0.02 M- $MgCl_2$. They are expressed in percent of maximal activity obtained.

Distribution of enzyme activity in vitro during differentiation. The alkaline phosphatase activity *in vitro* increased about 5–7 times during differentiation. The activity was highest in the sorocarps and the major increase took place during culmination (Table 5).

Since some bacteria were always transferred to the plain agar with the amoebae, and since the amoebae, even after starvation for 2–3 hr, still might contain engulfed *Escherichia coli* in various states of disintegration, the phosphatase activity measured in the amoeba extracts might have been derived in part from bacterial enzyme. No phosphatase activity, however, was demonstrable in the *E. coli* mutant strain used when it was grown on the rich medium. This was consistent with the

finding that alkaline phosphatase formation in *E. coli* strain ML was repressed (Torriani, 1960) by orthophosphate, a component in the SM agar.

In cell-free extracts prepared from the amoebae immediately after removal from the complex medium, as well as after washing and after starvation for 3 hr on the plain agar, the alkaline phosphatase activities were 11, 13 and 19 $\mu\text{M}/\text{min.}/\text{mg.}$ protein, respectively. In a separate experiment, the amoebae were grown on dead

Fig. 3. Phosphatase activity against NPP and 5'-AMP (top) as a function of substrate concentration (same abscissa for each substrate). The activities (expressed as per cent of maximal activity) were assayed in 0.5 M-tris without MgCl_2 (NPP) and in 0.05 M-tris, 0.02 M- MgCl_2 (AMP).

Escherichia coli. No control with living bacteria was possible because of the lower growth rate on the dead organism (Raper, 1951). The phosphatase activity obtained was 18 $\mu\text{M}/\text{min.}/\text{mg.}$ protein, i.e. of the same magnitude as observed for amoebae grown on live bacteria. It is therefore concluded that the alkaline phosphatase activity measured in the amoeba extracts was derived from *Dictyostelium discoideum*.

Remnant bacteria, possibly de-repressed on the plain agar, did not contribute significantly to the alkaline phosphatase activity in the sorocarp extracts since spores separated from stalks, debris and bacteria gave an extract higher in specific

activity than the complete sorocarps. Apparently the stalks contained relatively little of the enzyme. All experiments were performed with cell-free extracts. However, the enzyme-specific activity against NPP was the same whether the homogenate or the extract was used.

Stability. Storage of the cell-free extracts at $+4^{\circ}$ resulted in loss of activity, especially in those prepared at the early stages of development (Table 1). However,

Fig. 4. Mg^{2+} activation of phosphatase activity against NPP (bottom), 5'-AMP and 5'-GMP (top). The tris buffer concentration was 0.05 M. The activity of the sorocarp enzyme against NPP was also assayed in 0.5 M-tris. The activities are expressed as per cent of maximal activity.

Table 1. *Activity with time of undiluted extracts*

Stage	Specific enzyme activities			
	Fresh	1 day	2 days	8 days
Amoeba	10	4	2	1
Preculmination	14	7	3	4
Sorocarp	60	63	37	31

The concentrated extracts were stored at $+4^{\circ}$ and assayed without dilution in 0.5 M-tris without $MgCl_2$. Specific enzyme activity is defined in Methods.

when the extracts were diluted immediately by a factor of 100, i.e. to a concentration of about 0.1 mg. protein/ml. and stored as such at $+4^{\circ}$, no decrease in activity appeared in 48 hr (Table 2). There was, on the contrary, a pronounced increase in alkaline phosphatase activity during the first day, especially in the sorocarp extract (see below). Because of this release of enzyme activity and the stability of the enzyme thereafter, the phosphatase activities were always assayed after storage of

the diluted extracts at $+4^{\circ}$ for 20–24 hr. Dialysis of the diluted extracts did not increase the alkaline phosphatase activities beyond what was obtained through dilution only.

The crude extracts tolerated a temperature of 50° for 5 min.; prolonged treatment (30 min.) inactivated the alkaline phosphatase. At 80° all activity was lost within 1 min. both at the sorocarp and the amoeba stage. Because of the undefined conditions in the extracts, no further experiments on heat stability were performed.

Table 2. *Effect of dilution and stability of diluted enzyme*

Stage	Specific enzyme activities			
	Undiluted extract	Diluted extract		
		0 hr*	20 hr*	48 hr*
Amoeba	13	16	18	18
Preculmination	28	32	38	37
Sorocarp	52	69	95	95

The fresh extracts were diluted to a concentration of *ca.* 0.1 mg. protein/ml. and stored at $+4^{\circ}$. The activities were measured in 0.5 M-tris without MgCl_2 .

* Hours after initial dilution.

Table 3. *Relative substrate specificities in an extract of sorocarps*

	0.02 M- MgCl_2	No MgCl_2		0.02 M- MgCl_2	No MgCl_2
NPP	100	52	5'-UMP	1	—
5'-AMP	41	22	5'-TMP	5	—
5'-GMP	160	0	5'-IMP	1	—
ATP	64	33	3'-AMP	8	—
ADP	5	0	TPN	5	—
GTP	7	0	G6P	5	—
GDP	77	0	G1P	5	—
UTP	45	0	FDP	0	—
UDP	6	0	β GP	5	—
5'-CMP	1	—			

The activities were measured at pH 9.0 in 0.05 M-tris, at 0.01 M substrate concentration.

Substrate specificity

A large number of phosphate esters were tested as substrates for the alkaline phosphatase at the sorocarp and amoeba stages (Tables 3, 4). The tables show that, for the compounds tried at both stages, the substrate specificity was the same in the amoeba and sorocarp extracts. Since the pH optimum (Fig. 2) and the Mg^{2+} activation (Fig. 4) also were the same at both stages, it seemed probable that the amoeba and sorocarp alkaline phosphatases were identical.

β GP, used by Bonner *et al.* (1955) and by Krivanek (1956) was a poor substrate for the enzyme. It required prolonged incubation times (1–3 hr) and higher substrate concentration than did NPP. Although the results for β GP are therefore difficult to interpret, the specific activity did increase from amoeba to sorocarp in the experiments, thus resembling the activity pattern against NPP.

G6P, G1P and FDP were only very slightly hydrolysed at pH 9.0, if at all. Of these compounds, at least G6P and FDP served as substrates for the acid phos-

phatase, which was not activated by Mg^{2+} . The low activities against these three phosphate esters obtained at pH 9.0 were probably due to this latter enzyme.

Of the 5'-nucleotides listed in Table 3, only 5'-AMP and 5'-GMP were readily hydrolysed at pH 9.0; GMP, however, was only attacked in the presence of Mg^{2+} .

With NPP and 5'-AMP there was a 6-fold increase in enzyme concentration during differentiation (Table 5). Hence the ratio between the specific activities in AMP and NPP was constant during development. The specific activity on 5'-GMP was entirely different: it was about the same at all stages. The ratio 5'-GMP:NPP was therefore highest at the amoeba and lowest at the sorocarp stage. Together

Table 4. *Relative substrate specificities in an extract of amoebae*

NPP	100
5'-AMP	42
5'-UMP	0
5'-TMP	0
5'-IMP	0
3'-AMP	0
G6P	0
G1P	0
FDP	9
β GP	14

The activities were assayed at pH 9.0 in 0.05 M-tris, 0.02 M with respect to $MgCl_2$, at 0.01 M substrate concentration.

Table 5. *Phosphatase specific activities on NPP, 5'-AMP and 5'-GMP at four stages of development*

Stage	NPP	5'-AMP	Ratio 5'-AMP		Ratio 5'-GMP	
			NPP	5'-GMP	NPP	
Amoeba	29	11	0.38	187	6.43	
Preculmination	56	23	0.41	178	3.17	
Culmination	76	29	0.38	157	2.06	
Sorocarp	157	66	0.42	176	1.12	

The activities were measured at pH 9.0 in 0.05 M-tris, 0.03 M with respect to $MgCl_2$, at 0.01 M substrate concentration.

Table 6. *Phosphate specific activities on NPP, 5'-AMP and 5'-dAMP at two stages of development*

Stage	NPP	5'-AMP	5'-dAMP
Amoeba	24	12	11
Sorocarp	168	79	79

The extracts were assayed in the presence of 0.05 M-tris and 0.02 M- $MgCl_2$, at 0.01 M substrate concentration.

with the absolute Mg^{2+} requirement for phosphatase activity against GMP, the enzyme activity pattern during development provided strong evidence that 5'-GMP was hydrolysed by an enzyme other than the alkaline phosphatase. This conclusion is supported further by the fact that the hydrolysis of GMP has a different pH optimum than that of NPP (Fig. 2).

The phosphatase activity was the same whether 5'-AMP or 5'-dAMP was used

as substrate at pH 9. For both these compounds, as well as for NPP, there was a 7-fold increase in enzyme concentration during differentiation (Table 6). The hydrolysis of 5'-dAMP showed the same Mg^{2+} activation as that of 5'-AMP and NPP. Both AMP and dAMP must thus be considered as probable substrates for the alkaline phosphatase.

Of the di- and trinucleotides studied (Table 3), phosphatase activity was obtained against GDP, ATP and UTP. GDP and UTP were, however, only hydrolysed in the presence of Mg^{2+} . They must therefore be excluded as substrates for the alkaline phosphatase.

Fig. 5

Fig. 6

Fig. 5. Pi inhibition of alkaline phosphatase activity in a dialysed sorocarp extract. The activities were assayed in 0.5 M-tris without $MgCl_2$ and expressed as per cent of the activity obtained in the absence of Pi. Enzyme activity was determined by nitrophenol formation.

Fig. 6. Alkaline phosphatase activity against NPP in a dialysed sorocarp extract assayed with and without added K_2HPO_4 . The activities are plotted as v versus v/s (v in $\mu M/min./mg.$ protein; s in mm). The activities were measured in 0.5 M-tris without $MgCl_2$.

Table 7. *Phosphatase specific activities of a fresh and aged dialysed extract of sorocarps on NPP, 5'-AMP and ATP*

	Fresh	Aged	Residual activity (%)
NPP	138	131	95
5'-AMP	55	55	100
ATP	37	9	24

The activities were assayed in the presence of 0.05 M-tris, and 0.02 M- $MgCl_2$. The diluted and dialysed extract was aged at -20° for 3 days before re-assaying.

ATP was hydrolysed even in the absence of Mg^{2+} (Table 3). The activity varied immensely, however, between the experiments. As shown in Table 7, the enzyme attacking ATP is very unstable, and it is for this reason not identical with the alkaline phosphatase.

Sodium pyrophosphate, PPi, was hydrolysed at alkaline pH values with a sharp optimum at pH 8.8. The reaction was completely Mg^{2+} dependent and required a Mg:PPi ratio of unity for optimal activity. The specific activity hardly changed during differentiation. (Under optimal conditions the values 1020, 770, 1050 and 970 m μ M/min./mg. protein were obtained for the amoeba, pre-culmination, culmination and sorocarp stages, respectively.) Hence the pyrophosphatase and the alkaline phosphatase must be two different enzymes.

Of all naturally occurring compounds studied, only 5'-AMP and 5'-dAMP were possible substrates for the alkaline phosphatase.

With respect to enzyme pattern during differentiation (Table 5) stability (Table 7), pH optimum (Fig. 2), effect of substrate concentration (Fig. 3), and Mg^{2+} activation (Fig. 4) the characteristics for phosphatase activity against NPP and 5'-AMP were the same. This suggests the same enzyme. It is hence concluded that 5'-AMP and 5'-dAMP as well as NPP are hydrolysed by the alkaline phosphatase in *Dictyostelium discoideum*.

Inhibition by orthophosphate (Pi)

With NPP or with 5'-AMP as substrate the alkaline phosphatase in *Dictyostelium* was inhibited by its end product, orthophosphate. The inhibition at pH 9 was about 50 % at a phosphate concentration of 0.007 M and almost complete in 0.05–0.1 M- K_2HPO_4 (Fig. 5). Similar curves were obtained for both amoeba and sorocarp extracts.

In Fig. 6 the enzyme activities with and without phosphate are plotted as *v* versus *v*/*s* according to the formula

$$v = V_{\max} - v/s \left(1 + \frac{I}{K_I} \right) K_m$$

(Eadie, 1952). The increased slopes in the presence of inhibitor and the fact that the straight lines all have the same intercept ($V_{\max}$) show that orthophosphate was a competitive inhibitor of alkaline phosphatase *in vitro*.

Intracellular orthophosphate

Because of this end-product inhibition of alkaline phosphatase *in vitro*, the intracellular orthophosphate (Pi) concentration was determined at various stages of development. The first column in Table 8 shows that the phosphate concentration increased about 20-fold during the development from amoebae to spores. Calculated for the whole sorocarp the increase was less; the stalks contribute significantly to the packed cell volume but probably not to the phosphate determinations. The main increase in intracellular phosphate occurred at the late culmination and young sorocarp stages. The extent to which the various phosphate concentrations inhibited alkaline phosphatase activity *in vitro* (Fig. 5) is shown in the second column of Table 8.

Phosphate release and phosphate inhibition in vitro

As shown in Table 8 orthophosphate (Pi) was present in the spores in a concentration high enough to inhibit the alkaline phosphatase by more than 90 %. The Pi concentration in the amoebae would cause only a 25 % inhibition.

In the concentrated fresh extracts, the enzyme must also have been inhibited to

some extent. A determination of the Pi contents gave the values 10^{-3}M for the amoeba and $3 \times 10^{-3}\text{M}$ for the sorocarp extract, corresponding to 10 and 25% inhibition of the enzyme (Fig. 5). A 100-fold dilution of the extracts decreased the Pi values to 10^{-5} and $3 \times 10^{-5}\text{M}$ respectively, at which concentrations there is no Pi inhibition (Fig. 5). However, maximal activity was not obtained immediately after dilution and the increase in the sorocarp extract during the first 20 hr was considerably higher than in the extracts from the early stages (Table 2). In an experiment involving two sorocarp extracts, one was prepared in 0.05 M phosphate

Table 8. *Intracellular Pi molarity* during development*

Stage	Molarity Pi	Inhibition <i>in vitro</i> of alkaline phosphatase (%)
Amoeba	0.008	25
Pre-culmination	0.008	25
Culmination	0.006	45
Very young sorocarp (including stalks)†	0.014	67
1 day sorocarp (with stalks)†	0.020	78
Spores	0.065	98

* Expressed in terms of packed cell volume.

† Stalks, present in both these preparations, contribute significantly to the packed cell volumes but not to the phosphate contents of the extracts.

buffer (the approximate Pi concentration in the spores) and the other, as a control, in 0.01 M-tris, both at pH 7.2. The fresh extracts were diluted 100-fold in tris. This brought the Pi concentrations below inhibiting values (Fig. 5) in both extracts. Most of the 90% inhibition caused by the 0.05 M-phosphate was released immediately after dilution. The specific activity was 74 $\text{m}\mu\text{M}/\text{min.}/\text{mg. protein}$ compared to 85 in the control. Maximal activity (105 $\text{m}\mu\text{M}/\text{min.}/\text{mg. protein}$) was obtained in both diluted extracts within 20 hr. When the phosphate-containing thick extract was preincubated 24 hr before dilution, the specific activity immediately after dilution was low, 29 $\text{m}\mu\text{M}/\text{min.}/\text{mg. protein}$, suggesting a binding of phosphate to the enzyme. The final activity, after 20 hr, however, was the same as in the not pre-incubated, 100-fold diluted extracts. This slow recovery from Pi inhibition in the preincubated extract may explain the slow increase in activity obtained during the first 20 hr in the diluted ordinary extracts (Table 2). This might then be due to release from bound phosphate (Schwartz, 1963).

Table 1 showed that the phosphatase activity decreased with time in the concentrated extracts. This decrease was about 80% during the first 2 days at the amoeba stage and 40–50% at the sorocarp stage. During this time the phosphate content increased to about $5 \times 10^{-3}\text{M}$, both in the amoeba and sorocarp extracts. This concentration inhibited the alkaline phosphatase *in vitro* to 40% (Fig. 5), while the Pi concentrations in the fresh extracts corresponded to 10 and 25% inhibition (see above). Hence, only part of the decrease in enzyme activity could be explained through phosphate inhibition. The enzyme was possibly inhibited also by some other phosphate compound(s). Apparently the decrease was not due to instability of the enzyme: a 100-fold dilution of the aged concentrated extracts and storage of the dilutions at $+4^\circ$ for 20 hr did not only restore the enzyme activities

but increased them, at the early developmental stages, above the values obtained in the diluted fresh extracts. The same increase occurred in preparations kept at -20° for 20 hr before dilution. The effect was about 2-fold at the amoeba stage and very low at the sorocarp stage (Fig. 7). The nature of this increase was not studied. It might, however, have involved an activation or release of preformed enzyme protein. Increase in alkaline phosphatase activity *in vitro* has been reported for *Bacillus subtilis* (Whiteley & Oishi, 1963).

Fig. 7

Fig. 8

Fig. 7. Alkaline phosphatase activity *in vitro* during differentiation. The lower curve represents the activity in the fresh extracts. The upper curve shows the release of enzyme activity obtained through freezing. The activities were assayed in 0.5 M-tris without MgCl_2 . The symbols along the abscissa in this and subsequent graphs refer to amoeba (A), pre-culmination (PC), culmination (C), and sorocarp (F) stages.

Fig. 8. Specific phosphatase activity against NPP during development. The calculated activity *in vivo* is based on the amount of orthophosphate present in the cells at the various stages of differentiation. The extracts were assayed in 0.05 M-tris, and 0.1 M- MgCl_2 .

Phosphatase activity *in vitro* and *in vivo*

Figure 8 shows both the alkaline phosphatase activities *in vitro* and the predicted enzyme activities *in vivo* based on the intracellular phosphate molarities and assuming that the inhibitor was available to the enzyme. The data indicate an increase during differentiation in enzyme concentration but suggest that the inhibition of the enzyme *in vivo* resulted in highest activity during culmination.

Alkaline phosphatase in cells differentiating in the presence of exogenous metabolites

In all the previous experiments the slime mould was differentiating on plain 2% agar in the absence of any exogenous nutrients. When orthophosphate or

glucose was present in the agar, the rate of differentiation was increased. The optimal stimulating concentrations were 0.01 M with respect to Pi and 0.05 M for glucose (Krichevsky & Wright, 1963). In the presence of 0.01 M Pi the intracellular Pi values were higher than when the slime mould was differentiating on plain agar, but only early in development. The high Pi concentrations in the sorocarps were not further increased (Table 9). On plain agar, the intracellular glucose content was highest during pre-culmination and culmination (Wright, Brühmüller & Ward, 1964). Although direct determinations were not made, it is known that exogenous glucose does enter the cells during differentiation, since it stimulates the rate of development and respiration and also affects the pathways of hexose metabolism (Wright *et al.* 1964).

Table 9. *Pi molarity* in cells differentiating on plain agar and on agar 0.01 M with respect to Pi*

Stage	Molarity Pi*	
	Plain agar	0.01 M Pi agar
Aggregation	0.002	0.005
Pseudoplasmodium	0.002	0.005
Culmination	0.005	—
Very young sorocarp†	—	0.011
1 day sorocarp	0.018	0.018
2 day sorocarp	0.021	0.018

* Expressed in terms of packed cell volume.

† The stalks were included in all the sorocarp preparations.

Figure 9 shows the alkaline phosphatase activity *in vitro* from cells differentiating on plain agar, and on agar 0.01 M with respect to Pi or 0.05 M with respect to glucose. The enzyme was assayed, as always, under conditions of no Pi inhibition. In the presence of exogenous Pi or glucose, the phosphatase values did not increase after culmination, while in the control the enzyme concentration was maximal in the sorocarps. Early in development there was no negative effect of either Pi or glucose. On the contrary, the phosphatase concentration was higher in the cells on Pi agar. This is most likely due to the fact that phosphate is speeding up the rate of development.

No attempts were made to study the synthesis of the alkaline phosphatase protein. However, the data in Fig. 9 suggest that the enzyme was repressed, but only in the sorocarps, in the presence of glucose or Pi. This effect of Pi, and probably also of glucose, appeared to be a delayed phenomenon, since the alkaline phosphatase levels were not affected in the early stages, though the intracellular Pi concentration was increased when the compound was present in the agar (Table 9). This was more strikingly demonstrated when 0.1 M Pi was used. In an experiment with this concentration, the Pi inside the cells was 0.028 M in the young pre-culmination stage as compared to 0.002 M in the somewhat slower control (still pseudoplasmodia). There was thus a 14-fold increase in intracellular Pi, yet the alkaline phosphatase concentration was not affected in the early stages of development.

The SM medium contained, in addition to potassium phosphate (0.016 M) and glucose (0.05 M), peptone and yeast extract. Excluding the yeast extract, this was

the substrate used in the investigations by Bonner *et al.* (1955), Krivanek (1956) and Krivanek & Krivanek (1958). On this medium the effect on the enzyme was much more pronounced than in the presence of Pi or glucose alone; the phosphatase concentration decreased drastically in the sorocarps (Fig. 10). When concentrated extracts were used (as in the experiments by Krivanek, 1956), the phosphatase values obtained for the sorocarps were still lower, due to phosphate inhibition in the extracts.

Fig. 9

Fig. 10

Fig. 9. Specific phosphatase activity against NPP during development for cells differentiating on plain 2% agar without any supplements (control), on 2% agar, 0.01 M-potassium phosphate and on 2% agar, 0.05 M-glucose. The activities of the diluted, dialysed extracts were assayed in 0.05 M-tris, 0.02 M-MgCl₂.

Fig. 10. Specific phosphatase activity against NPP during development for cells differentiating on the SM medium in the presence of residual food bacteria. The diluted, dialysed extracts were assayed in 0.05 M-tris, 0.02 M-MgCl₂.

Table 10. *Specific enzyme activities in extracts from sorocarps formed in the presence of exogenous adenosine and deoxyadenosine*

Stage	Specific enzyme activities				
	Control	Adenosine		Deoxyadenosine	
		5×10^{-4} M	5×10^{-3} M	5×10^{-4} M	5×10^{-3} M
Very young sorocarp	184	174	120	194	200
2-day sorocarp	180	300	308	310	400

The diluted, dialysed extracts were assayed in 0.05 M-tris, 0.02 M with respect to MgCl₂.

The nucleosides adenosine and deoxyadenosine, end products of the 5'-nucleotidase reaction, were somewhat inhibitory to the rate of slime mould development. The inhibition in the presence of adenosine was quite obvious at 5×10^{-3} M. As shown in Fig. 11, both compounds increased the specific enzyme activities in the

sorocarps and they seemed thus to induce alkaline phosphatase formation in *Dictyostelium*. The data in Table 10 suggest that deoxyadenosine had a stronger effect than adenosine. All extracts were assayed after dilution and dialysis, i.e. in the absence of any removable inhibitor.

Figure 12, bottom, shows the Pi concentrations in cells differentiating on plain agar and on agar 5×10^{-3} M in respect to adenosine. Early in development the intracellular Pi molarities were the same in both cases, but during sorocarp formation and in the sorocarps the Pi concentration was considerably lower in the presence of

Fig. 11

Fig. 12

Fig. 11. Specific phosphatase activity against NPP for cells differentiating on plain 2% agar (control) or agar 5×10^{-3} M in adenosine and deoxyadenosine respectively. The diluted dialysed extracts were assayed in 0.05 M-tris, 0.02 M-MgCl₂. The symbols indicate amoeba (A), aggregation (AGG), migrating pseudoplasmodium (MP) and sorocarp (F) stages.

Fig. 12. Lower graph: Intracellular Pi molarity (expressed in terms of packed cell volume) during development on 2% plain agar (control) and on agar 5×10^{-3} M-adenosine. Top graph: Plot of the calculated difference in intracellular Pi molarities between cells on plain and adenosine agar during development.

exogenous adenosine. The upper part of Fig. 12 represents a plot of the calculated difference in intracellular Pi between cells on plain and on adenosine agar. The resulting curve rises during sorocarp formation and in the young sorocarps, the stage at which the stimulation of alkaline phosphatase levels occurred in the presence of adenosine.

DISCUSSION

Of naturally occurring compounds the alkaline phosphatase of *Dictyostelium discoideum*, routinely assayed with NPP, hydrolyses AMP and dAMP. It is in this respect completely different from the unspecific alkaline phosphatase of *Escherichia*

coli which hydrolyses mononucleotides and hexosephosphates (Torriani, 1960; Garen & Levinthal, 1960) and such compounds as ATP, ADP and PPi (Heppel, Harkness & Hilmo, 1962). The specific 5'-nucleotidases studied usually attack several mononucleotides but not NPP (Reis, 1951; Heppel & Hilmo, 1951; Sulkowski, Björk & Laskowski, 1963).

The *in vitro* activity pattern obtained on plain agar for β GP, a very poor substrate for the Dictyostelium enzyme, resembled that for NPP. In the presence of nutrients, the activity pattern for NPP (Fig. 10) was the same as that shown for β GP (Bonner *et al.* 1955; Krivanek, 1956). A comparison between the Krivanek's data for alkaline phosphatase (1956) and for 5'-nucleotidase (1958) shows identical distribution of the enzymes. We consider it most probable that these enzymes are one and the same, and that the enzyme studied by us with NPP and 5'-AMP as substrates is identical with the alkaline phosphatase described by Bonner *et al.* (1955) and by Krivanek (1956).

The alkaline phosphatase in Dictyostelium is inhibited by orthophosphate. Data (Fig. 9) suggest that the enzyme is also repressed by Pi, but only in sorocarps. In *Escherichia coli* the alkaline phosphatase is both inhibited and repressed by Pi (Horiuchi, Horiuchi & Mizuno, 1959; Torriani, 1960). Glucose, a known repressor of many enzymes (Neidhart & Magasanik, 1956) causes the same decrease in enzyme concentration in the spores as Pi. The rich medium, which contains glucose, Pi and peptone, is more powerful than glucose or Pi alone. Whether this depends on the combination of Pi and glucose or in part on the presence of some amino acid(s) in peptone (Cox & MacLeod, 1963) is not known.

The alkaline phosphatase activity *in vivo* in *Dictyostelium discoideum* appears to be controlled by at least two systems: (1) End-product inhibition by Pi. This is perhaps most important when the organism differentiates under extreme conditions of starvation. (2) Repression. The accumulation of the enzyme in the sorocarps is inhibited by the presence of exogenous metabolites in the environment during development. Both systems appear to collaborate to ensure maximal alkaline phosphatase activity during culmination, i.e. when cellulose synthesis and the final differentiation into spores and stalk cells takes place. The conclusions are based on the assumption that the Pi concentration determined for the whole cell approximates the concentration for the enzyme in the cell. The significance of *in vitro* data with respect to *in vivo* conditions has been discussed elsewhere (Wright, 1964).

In the presence of exogenous adenosine or deoxyadenosine the alkaline phosphatase concentrations increased, but only in the sorocarps (Fig. 11). If this effect involves increased enzyme synthesis, considerable protein synthesis must occur in the spores. That this is the case was shown by Wright & Anderson (1960).

The effect of the nucleosides could be due to one or both of the following mechanisms: (1) The inducer may not be adenosine or deoxyadenosine *per se* but AMP or dAMP formed inside the cells. (2) Another possibility is that the increase in enzyme concentration is due to the removal of the repressor, Pi, by the formation of AMP or dAMP from the base + Pi. We cannot at present decide between these two interpretations. We can only point out that the time of increase in alkaline phosphatase in the presence of adenosine coincides with the time of decrease in intracellular Pi concentration.

A preliminary report of this work has been published, (Gezelius & Wright, 1963).

This work was supported by grant No. GM-08958-02 from the U.S. Public Health Service and an allocation for the American Cancer Society Institutional Grant (IN-42B) to the Massachusetts General Hospital. This is publication No. 1190 of the Cancer Commission of Harvard University.

One of the authors (K.G.) held a Fulbright Scholarship during the work.

REFERENCES

- BONNER, J. T. (1959). *The Cellular Slime Molds*. Princeton, N.J.: Princeton University Press.
- BONNER, J. D., CHIUQUINE, A. D. & KOLDERIE, M. Q. (1955). A histochemical study of differentiation in the cellular slime molds. *J. expt. Zool.* **130**, 133.
- COX, R. P. & MACLEOD, C. M. (1963). Repression of alkaline phosphatase in human cell cultures by cystine and cysteine. *Proc. natn. Acad. Sci., U.S.A.* **49**, 504.
- DRYER, R. L., TAMMES, A. R. & ROUTH, J. I. (1957). The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. *J. biol. Chem.* **225**, 177.
- EADIE, G. S. (1952). On the evaluation of the constants V_m and K_m in enzyme reactions. *Science*, **116**, 688.
- FISKE, G. H. & SUBBAROW, Y. (1925). The colorimetric determinations of phosphorus. *J. biol. Chem.* **66**, 375.
- GAREN, A. & LEVINTHAL, C. (1960). A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. *Biochim. biophys. Acta*, **38**, 470.
- GEZELIUS, K. & WRIGHT, B. (1963). Alkaline phosphatase and inorganic phosphate in *Dictyostelium discoideum*. *Bact. Proc.* p. 68.
- HEPPEL, L. A., HARKNESS, D. R. & HILMOE, R. J. (1962). A study of the substrate specificity and other properties of the alkaline phosphatase of *Escherichia coli*. *J. biol. Chem.* **237**, 841.
- HEPPEL, L. A. & HILMOE, R. J. (1951). Purification and properties of 5'-nucleotidase. *J. biol. Chem.* **188**, 665.
- HERMAN, E. C. & WRIGHT, B. E. (1959). A 5'-nucleotidase activated by ferrous iron. *J. biol. Chem.* **234**, 122.
- HORIUCHI, T., HORIUCHI, S. & MIZUNO, D. (1959). A possible negative feedback phenomenon controlling formation of alkaline phosphomonoesterase in *Escherichia coli*. *Nature, Lond.* **183**, 1529.
- KORNBERG, A. & PRICER, W. E. (1951). Enzymic phosphorylation of adenosine and 2,6-diaminopurine riboside. *J. biol. Chem.* **193**, 481.
- KRICHEVSKY, M. I. & WRIGHT, B. E. (1963). Environmental control of the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **32**, 195.
- KRIVANEK, J. O. (1956). Alkaline phosphatase activity in the developing slime mold, *Dictyostelium discoideum* Raper. *J. exp. Zool.* **133**, 459.
- KRIVANEK, J. O. & KRIVANEK, R. C. (1958). The histochemical localization of certain biochemical intermediates and enzymes in the developing slime mold, *Dictyostelium discoideum* Raper. *J. exp. Zool.* **137**, 89.
- LIDDEL, G. U. & WRIGHT, B. E. (1961). The effect of glucose on respiration of the differentiating slime mold. *Develop. Biol.* **3**, 265.
- NEIDHARDT, F. C. & MAGASANIK, B. (1956). The effect of glucose on the induced biosynthesis of bacterial enzymes in the presence and absence of inducing agents. *Biochim. biophys. Acta*, **21**, 324.
- RAPER, K. B. (1951). Isolation, cultivation and conservation of simple slime molds. *Quart. Rev. Biol.* **26**, 169.
- REIS, J. L. (1951). The specificity of phosphomonoesterases in human tissues. *Biochem. J.* **48**, 548.
- SCHWARTZ, J. H. (1963). The phosphorylation of alkaline phosphatase. *Proc. natn. Acad. Sci., U.S.A.* **49**, 871.

- SULKOWSKI, E., BJÖRK, W. & LASKOWSKI, M., SR. (1963). A specific and non-specific alkaline monophosphatase in the venom of *Bothrops atrox* and their occurrence in the purified venom phosphodiesterase. *J. biol. Chem.* **238**, 2477.
- SUSSMAN, M. (1961). Cultivation and serial transfer of the slime mould *Dictyostelium discoideum* in liquid nutrient medium. *J. gen. Microbiol.* **25**, 375.
- TORRIANI, A. (1960). Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim. biophys. Acta*, **38**, 460.
- WHITELEY, H. R. & OISHI, M. (1963). An increase in alkaline phosphatase in an *in vitro* system derived from *Bacillus subtilis*. *Biochem. biophys. Res. Comm.* **13**, 6.
- WRIGHT, B. E. (1960). On enzyme-substrate relationships during biochemical differentiation. *Proc. natn. Acad. Sci., U.S.A.* **46**, 798.
- WRIGHT, B. E. (1964). Biochemistry of Acrasiales. In *Biochemistry and Physiology of Protozoa*. Ed. by S. Hutner, vol. 3. New York: Academic Press.
- WRIGHT, B. E. & ANDERSON, M. L. (1960). Protein and amino acid turnover during differentiation in the slime mold. II. Incorporation of [³⁵S]methionine into the amino acid pool and into protein. *Biochim. biophys. Acta*, **43**, 67.
- WRIGHT, B. E. & BARD, S. (1963). Glutamate oxidation in the differentiating slime mold. I. Studies *in vivo*. *Biochim. biophys. Acta*, **71**, 45.
- WRIGHT, B. E., BRÜHMÜLLER, M. & WARD, C. (1964). Studies *in vivo* on hexose metabolism in *Dictyostelium discoideum*. *Develop. Biol.* **9**, 287.
- ZAMENHOF, S. (1957). Preparation and assay of deoxyribonucleic acid from animal tissue. *Meth. Enzymol.* **3**, 696.