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Alkaline Inorganic Pyrophosphatase Activity of Mammalian-Cell Alkaline Phosphatase

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Alkaline phosphatase prepared from mammalian cell cultures was found to have alkaline inorganic pyrophosphatase activity. Both of these activities appear to be associated with a single protein, as demonstrated by: (1) concomitant purification of alkaline phosphatase and alkaline inorganic pyrophosphatase; (2) proportional precipitation of alkaline phosphatase and inorganic pyrophosphatase activities by titrating constant amounts of an enzyme preparation with increasing concentration of antibody; (3) immune electrophoresis, which showed that precipitin bands that have alkaline phosphatase activity also have pyrophosphatase activity; (4) inhibition of pyrophosphatase activity by cysteine, an inhibitor of alkaline phosphatase activity; (5) similar subcellular localization of the two enzyme activities as demonstrated by histochemical methods; (6) hormonal and substrate induction of alkaline phosphatase activity in mammalian cell cultures, which produced a nearly parallel rise in inorganic pyrophosphatase activity.

Inorganic pyrophosphate is produced as a by-product in many essential biosynthetic reactions. It has been suggested by Stetten (1960) and Kornberg (1962) that the presence of one or more inorganic pyrophosphatases (pyrophosphate phosphohydrolase, EC 3.6.1.1) in the cell provides a control mechanism whereby reactions producing pyrophosphate, e.g. those catalysed by nucleic acid polymerases, are rendered irreversible owing to the hydrolysis of inorganic pyrophosphate.

The object of this study was to continue investigating the suggestions (Russell, 1965), supported by investigations (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1966, 1967; Fernley & Walker, 1966), that mammalian alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is an alkaline pyrophosphatase.

MATERIALS AND METHODS

Cell lines. The methods used in this Laboratory for monolayer cell cultures have been described (Cox & MacLeod, 1962; Griffin & Cox, 1966*a*). Two different HeLa cell lines were studied: HeLa S_3, a cell line with low alkaline phosphatase activity that can be increased 4–20-fold by growth in a medium containing prednisolone (Δ^1-hydrocortisone); and HeLa Ch, a cell line in which high activities of alkaline phosphatase are constitutive (Griffin & Cox, 1966*a*,*b*). A third cell line, BSC-1, an established African green monkey kidney-cell line with low alkaline phosphatase activity that can be increased 3–12-fold by growth in a medium containing phenyl phosphate (M. J. Griffin and R. P. Cox, unpublished work), was used for studies on substrate induction.

Methods of culture. Cells were grown in monolayer culture with Waymouth's (1959) medium containing calf serum (10%) and supplemented with kanamycin (30μg./ml.), streptomycin (50μg./ml.) and penicillin (50μg./ml.). Studies on enzyme induction were carried out by adding prednisolone (1·0μg./ml.) or phenyl phosphate (13mM) to the medium 24hr. after subculturing.

Enzyme preparation. Partially purified alkaline phosphatase was prepared by homogenizing about 5×10^7 cells in 6ml. of 0·05M-tris–HCl, pH7·4, with 2ml. of butan-1-ol (Morton, 1954; Griffin & Cox, 1966*b*). The homogenate was centrifuged at 900*g* for 15min. and the aqueous layer was removed. The frothy butan-1-ol layer was re-extracted with 1·0ml. of 0·05M-tris–HCl, pH7·4, and the aqueous extracts were combined. The recovery of alkaline phosphatase activity was about 90% when compared with cells lysed in 0·5% sodium deoxycholate.

The partially purified enzyme was dialysed for 36hr. against 40vol. of 0·05M-tris–HCl, pH7·4, and was then concentrated by filtration and dialysis in collodion bags with a porosity of less than 5mμ (Kollodiumhulsen, Göttingen, Germany). The concentrated enzyme preparation was placed on a Sephadex G-200 column (1·5cm. × 15 cm.) and eluted with 0·05M-tris–HCl, pH7·4. The alkaline phosphatase and most of the inorganic pyrophosphatase

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activity were eluted together just after the void volume and came off the column within the first internal volume. The peak activity represented a further four- to six-fold purification of alkaline phosphatase, with a recovery of 65–75%. This column-purified enzyme was further concentrated by filtration and dialysis in collodion bags.

Assay procedures. Enzyme activities were assayed at 37°.

Alkaline phosphatase activity. This was measured spectrophotometrically at 410mμ by the method of Lowry (1957), with as substrate 8mM-*p*-nitrophenyl phosphate (Sigma 104) in 0·5M-2-amino-2-methylpropane-1,3-diol hydrochloride–1mM-$MgCl_2$, pH 10·5.

Alkaline inorganic pyrophosphatase activity. This was determined with as substrate 3·3mM-sodium pyrophosphate in 1·0M-tris–HCl–1mM-$MgCl_2$, pH 8·5 (Cox & Griffin, 1965). The procedure consisted of incubating 0·1ml. of enzyme with 1·0ml. of substrate for 60min., stopping the reaction by immersing the tubes in ice and adding 1·0ml. of cold 30% (w/v) trichloroacetic acid. Phosphate release was assayed immediately by a modification of the method of Fiske & Subbarow (1925) (Cox & Griffin, 1965).

Total protein. This was determined by the Lowry modification of the method of Folin (Lowry, Rosebrough, Farr & Randall, 1951).

Histochemical procedures. Histochemical studies on the subcellular distribution of the enzyme activities were carried out on cover-slip cultures of HeLa Ch cells. Alkaline phosphomonoesterase activity was demonstrated with sodium α-naphthyl phosphate as substrate, by a modification (Kaplow, 1955) of the method of Menten, Junge & Green (1944). Alkaline inorganic pyrophosphatase activity was demonstrated with sodium pyrophosphate as substrate by the method of Kurata & Maeda (1956). The pyrophosphatase staining reaction faded and was studied immediately after staining.

Preparation of antibody. The antigen was a phosphatase and pyrophosphatase prepared from HeLa Ch cells as described above. The final purification of this antigen was about 250-fold, based on total cell protein. Two white New Zealand rabbits were injected subcutaneously in four sites, interscapularly and into the lateral thighs, with a 1:1 emulsion of enzyme preparation (about 125μg. of protein) in complete Freund's adjuvant (Griffin & Cox, 1966*b*). They were given two further injections at 2-week intervals and bled several days later. Further booster injections were repeated at 3–4-week intervals and bleedings done 7–9 days thereafter. The serum used for these studies was from the fourth post-inoculation bleeding. For certain experiments the antiserum was heated to 56° for 30min., to produce partial inactivation of its endogenous alkaline phosphatase activity.

Immunoelectrophoresis. This was carried out on 8cm. × 10cm. glass plates covered with 1% agar in 0·05M-barbital buffer, pH 8·2, by the method of Grabar & Williams (1953). Immune precipitation was complete after 48hr. at room temperature. The precipitation bands were cut out for enzyme assay. Homogenized slices of agar were assayed in the standard alkaline phosphatase and alkaline inorganic pyrophosphatase assays.

Determination of the antibody concentration necessary for precipitation of alkaline phosphatase and inorganic pyrophosphatase. Antibody precipitation of the enzyme was carried out by adding a constant amount of antigen (0·2ml.) to serial dilutions of antibody (0·2ml.) and incubating at 37° for 1hr. The reaction mixture was kept at 4° for 24hr. The preparation was centrifuged at 900*g* and the supernatant decanted. The precipitate was washed in ice-cold 0·9% NaCl and resuspended in a volume of 0·05M-tris–HCl, pH 7·4, equal to the volume of the decanted supernatant. Both the supernatant and the resuspended precipitate were assayed for alkaline phosphatase and alkaline inorganic pyrophosphatase activity. The enzyme activities remaining in the supernatant were calculated by subtracting the endogenous alkaline phosphatase and inorganic pyrophosphatase activities of each antibody dilution from these activities in the supernatant of the reaction mixture.

RESULTS

Purification of alkaline phosphatase and inorganic pyrophosphatase. Table 1 shows the purification of alkaline phosphatase and alkaline inorganic pyrophosphatase activities from HeLa Ch cells. Fig. 1 shows the elution pattern of alkaline phosphatase activity, inorganic pyrophosphatase activity and protein concentration resolved by chromatography on Sephadex G-200. The major peaks of alkaline phosphatase and alkaline inorganic pyrophosphatase activities passed through the column together, appearing after the void volume in tubes

Table 1. *Purification of alkaline phosphatase and inorganic pyrophosphatase activities from HeLa Ch cells*

Specific activities are expressed as μmoles of *p*-nitrophenyl phosphate or inorganic pyrophosphate hydrolysed/30min./mg. of protein at 37°. The percentage recovery is the mean of the values found for the two activities.

	Specific activity		Purification factor		
	Alkaline phosphatase	Inorganic pyrophosphatase	Alkaline phosphatase	Inorganic pyrophosphatase	Recovery (%)
Cell suspension	4·3	0·13	(1)	(1)	(100)
Butan-1-ol extract	73·4	2·30	17·0	17·7	95
Dialysis residue	160	5·25	37·2	40·4	93
Column fractions	575	19·5	134	150	68

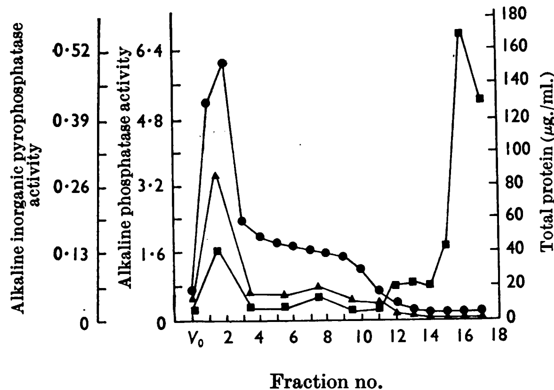

Fig. 1. Purification of alkaline phosphatase and inorganic pyrophosphatase activities by Sephadex G-200 gel filtration. V_0, The first tube after eluting the internal volume of the column; ●, alkaline phosphatase activity (μmoles of p-nitrophenyl phosphate hydrolysed at 37° in 60min. by 0·1ml. of enzyme preparation); ▲, alkaline inorganic pyrophosphatase activity (μmoles of inorganic pyrophosphate hydrolysed at 37° in 60min. by 0·1ml. of enzyme preparation); ■, protein concentration (μg./ml.).

1 and 2 with the first minor peak of protein. There was a second peak of alkaline inorganic pyrophosphatase activity in tubes 7 and 8, corresponding to a second minor peak of protein. Most of the protein appeared in tubes 15–17, well separated from the combined peak of alkaline phosphatase and alkaline inorganic pyrophosphatase activities. Thus by gel filtration the alkaline phosphatase and most of the alkaline inorganic pyrophosphatase were eluted together and conveniently separated from most of the protein. The presence of a single minor peak of alkaline inorganic pyrophosphatase activity may be due to the presence of a second inorganic pyrophosphatase.

Heat-lability of enzymes. Table 2 shows the alkaline phosphatase and alkaline inorganic pyrophosphatase activities of column-purified enzyme after heating at various temperatures. The enzyme was heated for 60min. and then cooled for 3min. in an ice bath before activities were assayed. Both enzyme activities began to diminish proportionately at 55°, but at higher temperatures the alkaline phosphatase activity of the enzyme preparation appeared to be rather more labile.

Inhibition of enzymes. The response of the enzyme activities to selective inhibitors provides a convenient means of discriminating between certain characteristics of these activities. Table 3 shows that L-phenylalanine and L-tryptophan are inhibitors of alkaline phosphatase activity, but did not inhibit HeLa-cell inorganic pyrophosphatase activity at the concentrations tested. On the other hand, L-cysteine markedly diminished both of these enzyme activities.

Table 2. *Effect of heat on HeLa Ch alkaline phosphatase and alkaline inorganic pyrophosphatase activities*

The enzyme was prepared by gel filtration on Sephadex G-200 as described in the Materials and Methods section. The enzyme preparation was heated for 60min. at the temperature indicated. Enzyme activities are expressed as μmoles of p-nitrophenyl phosphate or inorganic pyrophosphate hydrolysed at 37°/30min./0·1ml. sample. Mean values of the enzyme activities and the ranges for quadruplicate assays are shown.

	Alkaline phosphatase		Alkaline inorganic pyrophosphatase	
Temp.	Activity	Activity remaining (%)	Activity	Activity remaining (%)
23°	4·27±0·09	100	0·16±0·01	100
55	3·99±0·08	93	0·13±0·00	82
60	2·94±0·05	69	0·12±0·00	75
65	1·26±0·08	30	0·08±0·01	50

Table 3. *Effect of amino acid inhibitors on HeLa Ch alkaline phosphatase and alkaline inorganic pyrophosphatase activities*

The inhibitor was diluted to 1·0mM and was added to the enzyme preparation 1min. before diluting with substrate. Enzyme activities are expressed as described in Table 2.

Inhibitor	Final concn. of inhibitor (mM)	Alkaline phosphatase activity	Alkaline inorganic pyrophosphatase activity
Sucrose (control)	0·2	2·76±0·12	0·094±0·007
L-Phenylalanine	0·2	1·14±0·09	0·090±0·004
L-Tryptophan	0·2	1·65±0·06	0·092±0·010
L-Cysteine	0·2	0·25±0·05	0·034±0·003

Induction of alkaline phosphatase and alkaline inorganic pyrophosphatase activities in cell cultures. Hormonal induction in HeLa S_3 cells. Prednisolone induces an increase in alkaline phosphatase activity in certain HeLa cell lines (Cox & MacLeod, 1962). Table 4 shows that in HeLa S_3 cells there is also an induction of inorganic pyrophosphatase activity. Both alkaline phosphatase specific activity and alkaline inorganic pyrophosphatase specific activity were increased about fivefold.

Substrate induction in BSC-1 cells. Phenyl

Table 4. *Induction of alkaline phosphatase and alkaline inorganic pyrophosphatase activity in mammalian cell cultures*

Specific enzyme activities are expressed as described in Table 1. The enzyme preparation was a butan-1-ol extract of the cells. Each average specific enzyme activity and the range of each activity are based on three assays carried out on each of four replicate cell cultures. For details see the text.

Cell line	Alkaline phosphatase specific activity	Alkaline inorganic pyro-phosphatase specific activity
HeLa S_3 control	0.50 ± 0.03	0.027 ± 0.004
HeLa S_3 with prednisolone (1·5μg./ml.)	2.60 ± 0.04	0.142 ± 0.001
BSC-1 control	0.101 ± 0.035	0.005 ± 0.002
BSC-1 with phenyl phosphate (13mM)	0.802 ± 0.033	0.054 ± 0.006

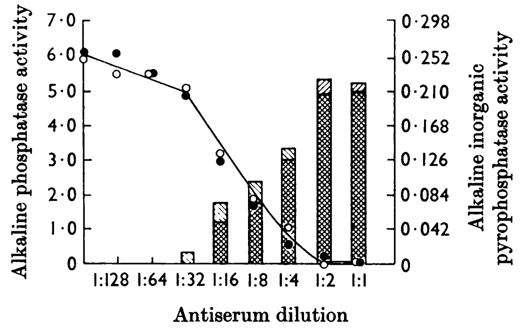

Fig. 2. Precipitation of the enzyme preparation by increasing concentrations of antibody. The antigen was prepared from butan-1-ol extracts of HeLa Ch cells and was further purified by gel filtration on Sephadex G-200. The final purification was about 250-fold. Antigen (0·2ml.) was incubated in 0·2ml. of antiserum for 2hr. at 37°. The reaction mixture was then incubated at 4° for 24hr. The precipitate was removed by centrifuging and the supernatant was decanted. The precipitate was washed twice with ice-cold 0·9% NaCl and then resuspended in 0·4ml. of 0·05M-tris–HCl, pH7·4. ○, Alkaline phosphatase activity in the supernatant; ●, alkaline inorganic pyrophosphatase activity in the supernatant; ▩, alkaline phosphatase activity in the precipitate; ▨, alkaline inorganic pyrophosphatase activity in the precipitate. Alkaline phosphatase and inorganic pyrophosphatase activity are expressed as μmoles of substrate hydrolysed in 30min. at 37° by 0·1ml. (see Fig. 1). The endogenous alkaline phosphatase and inorganic pyrophosphatase of the antiserum at each dilution was subtracted from the enzyme activities in the supernatant of the reaction mixture.

phosphate, a substrate of alkaline phosphatase, causes increased enzyme activity in certain fibroblastic cell lines such as BSC-1 monkey-kidney cells (M. J. Griffin & R. P. Cox, unpublished work). As shown in Table 4, phenyl phosphate increases the alkaline phosphatase specific activity eightfold and increases the alkaline inorganic pyrophosphatase specific activity about tenfold.

Histochemical localization of alkaline phosphatase and alkaline inorganic pyrophosphatase activities in HeLa Ch cell cultures. Histochemical staining of HeLa Ch cells indicated a similar localization of alkaline phosphatase and alkaline inorganic pyrophosphatase activities within the cell. Both activities were present rather diffusely in the cytoplasm, but were markedly greater in the nucleus. Further, it appeared that both enzyme activities were highest in the nucleolus.

Precipitation of alkaline phosphatase and inorganic pyrophosphatase preparations by increasing concentrations of antibody. Fig. 2 shows the amounts of alkaline phosphatase and inorganic pyrophosphatase activity precipitated and the amounts of these activities remaining in the supernatant when constant amounts of the HeLa cell enzyme preparation were treated with increasing concentrations of antiserum. Both enzyme activities were completely precipitated at an antiserum dilution of 1:2. Moreover, at lower antibody concentrations the proportion of phosphomonoesterase and pyrophosphatase activity in the supernatant and in the precipitate was nearly the same. This evidence suggests that both of these enzyme activities are associated with the same protein.

Immunoelectrophoretic resolution of enzyme activities. On immunoelectrophoresis, the enzyme preparation purified by chromatography on Sephadex G-200 was resolved into three bands (see Fig. 3). The two overlapping cathodic bands contained the major portion of the alkaline phosphatase, as well as the alkaline inorganic pyrophosphatase activities (Table 5). The anodic band contained enzyme activities not appreciably greater than the background enzyme activity in the gel.

DISCUSSION

Recent publications (Cox & Griffin, 1965; Moss *et al.* 1966, 1967; Fernley & Walker, 1966) have indicated that mammalian alkaline phosphatase has inorganic pyrophosphatase activity. The purification of human liver, human intestinal (Moss *et al.* 1966, 1967) and calf intestinal alkaline phosphatase (Fernley & Walker, 1966) was accompanied by a concomitant increase in inorganic pyrophosphatase activity. Further, Moss *et al.* (1967) showed that the alkaline phosphatase isoenzymes separated on

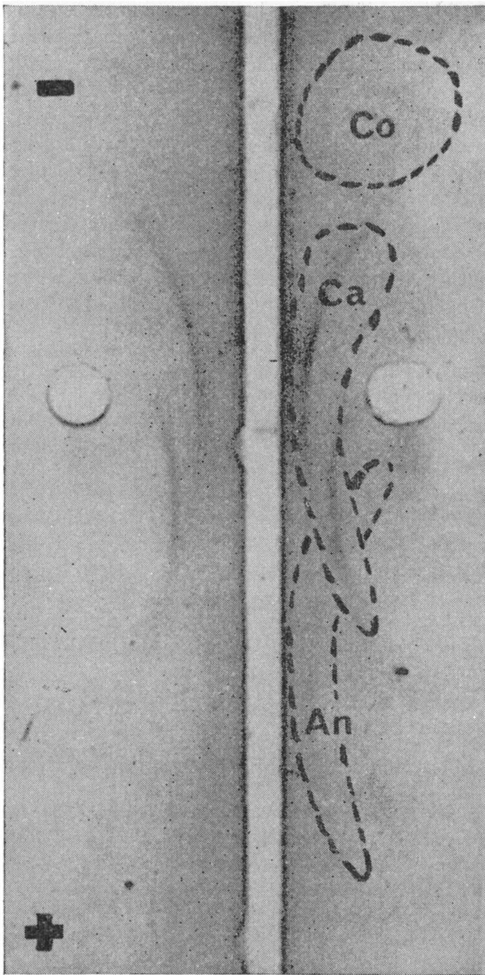

Fig. 3. Immunoelectrophoresis on agar of HeLa-cell alkaline phosphatase prepared by chromatography on Sephadex G-200. The two cathodic precipitin bands had alkaline phosphatase and inorganic pyrophosphatase activities. The anodic band had neither of these enzymic activities (see Table 5). The right-hand side of the photograph shows the areas that were cut out of the gel for the assays shown in Table 5. Co, The slice of agar used as a control; Ca, the slice of agar that contains the two cathodic precipitin bands; An, the slice of agar that contains the anodic precipitin band.

starch-gel electrophoresis coincide with the pyrophosphatase isoenzymes. Moss *et al.* (1967) also found that hydrolysis of each type of substrate was inhibited by the presence of the other, and that in human liver and intestinal enzyme preparations the two activities showed a constant ratio during heat inactivation at 55°. The present study indicates that the alkaline phosphatase and inorganic pyrophosphatase activities are carried out by the same enzyme by the criteria of enzyme purification, immunological reactivity, subcellular localization and the induction in two tissue-culture cell lines of an increase in both enzyme activities with both a hormone and a substrate as an inducer.

Table 5. *Enzyme activities of precipitin bands separated by immunoelectrophoresis*

A concentrated enzyme preparation (10 μl.), purified by gel filtration on Sephadex G-200, was placed in each well and the proteins were separated by electrophoresis as described in the Materials and Methods section. The precipitin bands were developed by filling the trough with antibody and allowing the bands to develop overnight at room temperature. The two cathodic bands and the anodic band were cut out and homogenized in 0·05 M-tris–HCl, pH 7·4. A slice of agar of approximately the same size from another area of the gel was used as control. Enzyme activities are expressed as described in Table 2 except that these assays were carried out in triplicate.

Band assayed	Alkaline phosphatase activity	Alkaline inorganic pyrophosphatase activity
Expt. I		
Control	0·006 ± 0·003	0·0009 ± 0·0004
Cathodic	0·146 ± 0·005	0·0162 ± 0·0009
Anodic	0·017 ± 0·007	0·0027 ± 0·0006
Expt. II		
Control	0·002 ± 0·003	0·0005 ± 0·0003
Cathodic	0·137 ± 0·006	0·0151 ± 0·0006
Anodic	0·018 ± 0·003	0·0024 ± 0·0005

Certain amino acid inhibitors distinguish between the alkaline phosphatase and pyrophosphatase activities of the HeLa-cell enzyme preparation. Cysteine is a good inhibitor of both alkaline phosphatase and inorganic pyrophosphatase activity, but L-phenylalanine or L-tryptophan selectively inhibits the HeLa cell phosphatase activity without affecting the pyrophosphatase activity. The mechanism of the cysteine inhibition is believed to be chelation of the zinc atom near the active site of the enzyme (Agus, Cox & Griffin, 1966). Both D- and L-cysteine are equally effective as inhibitors and the kinetics of the inhibition are of the mixed competitive and non-competitive type. The mechanism of inhibition of alkaline phosphatase by L-tryptophan and L-phenylalanine is unknown; however, inhibition is stereospecific for the L-isomer (Fishman, Green & Inglis, 1962). The kinetics of this inhibition are of the non-competitive type (Ghosh & Fishman, 1966; R. S. Baltimore, R. P. Cox & M. J. Griffin, unpublished work). The selective inhibition of alkaline phosphatase by L-phenylalanine and L-tryptophan provides a useful

experimental method of separating the two catalytic activities of the HeLa-cell enzyme, but cannot be used as evidence for deciding whether these two enzyme activities are carried out by the same or different catalytic sites. However, the inhibition of both enzyme activities by cysteine, whose mechanism of inhibition is better understood, may implicate zinc-dependent catalytic site(s) in both enzyme reactions. In contrast with the HeLa-cell enzyme, both the alkaline phosphatase and inorganic pyrophosphatase activities of the human intestinal enzyme are inhibited by L-phenylalanine (Eaton & Moss, 1966). However, Eaton & Moss (1966) found that neither the alkaline phosphatase nor the inorganic pyrophosphatase activities of human liver enzyme were affected by this amino acid. These observations probably indicate organ-specific differences among alkaline phosphatases from various sources.

The physiological substrate and function of alkaline phosphatase is unknown. Patients with a deficiency of cytoplasmic alkaline phosphatase in certain cells (hypophosphatasia) have pyrophosphaturia, suggesting that this inborn error of metabolism is associated with an impaired metabolism of inorganic pyrophosphate (Russell, 1965). Pyrophosphate is a product of many biosynthetic reactions. It is possible that the physiological role of alkaline phosphatase is that of an inorganic pyrophosphatase in one or more of these biosynthetic pathways, which are thus rendered effectively irreversible (Stetten, 1960; Kornberg, 1962). Inorganic pyrophosphatase is found in all cell extracts examined, but inorganic pyrophosphate, when detectable in tissue, is present in extremely small amounts (Kornberg, 1962).

Inorganic pyrophosphatases previously described appear to be predominantly cytoplasmic (Nordlie & Lardy, 1961*a*; Pynes & Younathan, 1964). There are at least two different pyrophosphatases in the cytoplasmic fraction of rat liver (Nordlie & Lardy, 1961*a*) and pigeon pancreas (Pynes & Younathan, 1964). One pyrophosphatase requires Mg^{2+} and has optimum pH 7·5 when the substrate concentration is 1·5mM (Nordlie & Lardy, 1961*a*,*b*; Nordlie & Gehring, 1963; Swanson, 1952); the other is an acid pyrophosphatase not requiring Mg^{2+} and with optimum pH about 5·4 (Nordlie & Lardy, 1961*a*,*b*; Nordlie & Gehring, 1963). It is noteworthy that the Mg^{2+}-requiring inorganic pyrophosphatase in rat liver is induced when hydrocortisone is administered to adrenalectomized rats (Nordlie & Schultz, 1964).

Alkaline phosphatase activity is known to be present in the nuclei of most plant and animal cells and is concentrated on the chromosomes during mitosis (Danielli, 1953). The physiological function of this nuclear alkaline phosphatase is not known. The present indications that a nuclear HeLa-cell enzyme is a bifunctional protein with both alkaline phosphatase and inorganic pyrophosphatase activities suggest that this enzyme may play a role in nucleic acid synthesis by hydrolysing pyrophosphate, an inhibitory product of the reaction. The alkaline phosphatase activity, with its broad specificity, may be involved in the phosphorylation and dephosphorylation of nuclear proteins. These phosphoproteins have recently been implicated in the control of gene activation, presumably by affecting the binding of histones to DNA (Pogo, Allfrey & Mirsky, 1966; Kleinsmith, Allfrey & Mirsky, 1966).

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