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THE ACID AND ALKALINE PHOSPHATASES, INORGANIC PYROPHOSPHATASES AND PHOSPHOPROTEIN PHOSPHATASE OF BONE

I. CHARACTERIZATION AND ASSAY

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SUMMARY

1. Phosphatase activities exerted on phosphoprotein (casein), inorganic pyrophosphate, β-glycerophosphate, *p*-nitrophenylphosphate and on various phosphomonoesters were demonstrated in homogenates of newborn-rat calvaria. The activity exerted on phosphoprotein was optimal below pH 5.8; with all the other substrates, two peaks of activity were observed, one between pH 4.8 and 5.8 and the other between pH 9 and 11.

2. The main kinetic characters of these phosphatases have been studied and methods for their quantitative assays have been developed.

3. Kinetic properties, stability characteristics and competitive inhibition experiments are interpreted as an indication of at least three different acid phosphatases in bone cells: phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), acid β-glycerophosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and acid inorganic pyrophosphatase, the latter enzyme being apparently responsible for the acid *p*-nitrophenylphosphatase activity of the homogenates.

INTRODUCTION

It has long been known that alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is particularly abundant in osteoblasts and that its activity in bone is associated with osteogenesis whereas acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) is most active in osteoclasts and its activity reflects osteolytic processes[1–3]. Recent theories have suggested that alkaline phosphatase acts at the sites of bone formation by destroying inorganic pyrophosphate, an inhibitor of the calcification of collagen[4,5] and that acid phosphatase, a lysosomal enzyme, could be considered as an indicator for the presence, at the bone-

resorption sites, of other lysosomal hydrolases active in the degradation of organic components of the bone matrix[3,6]. Furthermore, it was still suggested that acid inorganic pyrophosphatase[4,5] and phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16)[7–9] may also have a direct solubilizing action on the bone mineral.

A better knowledge of the various phosphatase activities present in bone cells could thus contribute to the understanding of important physiological processes related to bone metabolism. A study of these activities was therefore undertaken. Acid and alkaline phosphatase activities exerted on several substrates, including inorganic pyrophosphate and casein (a phosphoprotein) were investigated in cytoplasmic extracts of bone cells. Their demonstration, kinetics, method of assay and possible association with various enzymes are the object of the present paper. Studies on their intracellular distribution and latency properties are reported in the accompanying paper[10]. Part of the work has already been presented as a preliminary note[11].

EXPERIMENTAL

Preparation and fractionation of homogenates of bone tissue

Pooled calvaria from infant rats up to 7 days of age were prepared and homogenized as described elsewhere[12]. The homogenates were centrifuged at 600 × *g* for 10 min at 0 °C, to yield a cell-free supernatant ("cytoplasmic extract") and a sediment containing most of the nuclei together with cell debris, connective tissue elements and solid mineral (N fraction). In some experiments, the N fraction was resuspended in 0.25 M sucrose containing 0.3 M KCl and centrifuged again in the same way in order to release enzyme activities that were adsorbed on sedimentable components of that fraction into the soluble form (see below); the supernatants were then pooled to constitute the cytoplasmic extract.

Enzyme assays and analytical procedures

The enzymic tests were carried out at 37 °C, in a total volume of 0.5 ml. For the acid phosphatases, the incubation mixtures contained 0.1 M acetate buffer, 5 mM ascorbic acid (see below) adjusted at the pH of the buffer and 0.1% Triton X-100[10,12]. The assays were run at pH 5.0 for α- and β-glycerophosphate, pH 5.8 for casein and pH 5.4 for the other substrates. They were all run at pH 5.8 in comparative experiments that required a similar pH in all the assays. The substrate concentrations were 50 mM for α- and β-glycerophosphate and for phosphoserine; 40 mM for phosphoethanolamine; 20 mM for phenylphosphate; 10 mM for inorganic pyrophosphate; 8 mM for *p*-nitrophenylphosphate; 4 mM (in phosphate equivalent) for casein.

For the alkaline phosphatases, the incubation mixtures contained 0.1 M triethanolamine buffer, pH 9.5. The substrate concentration was 15 mM except for pyrophosphate, used at a 2.5 mM concentration. Assays were done either without or with added $MgCl_2$ (50 mM for alkaline *p*-nitrophenylphosphatase and 1 mM for alkaline pyrophosphatase).

The assays were usually stopped by the addition of 2.5 ml of 5% (w/v) trichloroacetic acid and inorganic phosphate was determined in the filtrate[13,14]. For the studies on substrate competition, made by incubating the enzyme with both *p*-

nitrophenylphosphate and another substrate, *p*-nitrophenylphosphatase was assayed by measuring the absorbance of the liberated *p*-nitrophenol at 405 nm[6]; simultaneous measurement of the P_i released allowed the determination, by difference, of the phosphatase activity exerted on the other substrate. Appropriate blanks[12] were subtracted from the observed values. Activities are expressed in units/g of tissue, one unit referring to the decomposition of 1 μmole of substrate (or to the release of 1 μmole of casein-bound phosphate) per min.

Substrate competitions among acid phosphatases

These experiments were done at an ionic strength rendered constant by the addition of NaCl. *p*-Nitrophenylphosphate was added to either β-glycerophosphate, inorganic pyrophosphate or casein in the incubation flasks. K_m and maximal velocities *(V)* were measured on the same cytoplasmic extract for both acid *p*-nitrophenylphosphatase and the acid phosphatase acting on the other substrate. This allowed, whenever a competition was observed between the two substrates, one to determine whether or not it could be due to the hydrolysis of both substrates by a single enzyme. Then the velocities *(v)* of hydrolysis of one of the substrates in the presence of the other substrate acting as a competitive inhibitor would in fact be the same whether observed experimentally or whether calculated according to the formula (see ref. 15).

$$v = \frac{V}{1 + \frac{K_s}{S}\left(1 + \frac{i}{K_i}\right)}$$

Materials

Phenylphosphate (disodium salt), β-glycerophosphate (disodium salt), and ascorbic acid were obtained from E. Merck, A.G., Darmstadt, Germany; *p*-nitrophenylphosphate (disodium salt), *O*-phospho-L-serine and *O*-phosphoethanolamine were from Sigma Chemical Co., St Louis, Mo., U.S.A.; α-glycerophosphate (disodium salt) was from Koch–Light Laboratories Ltd. Colnbrook, Bucks., England; Triton X-100 was from Rohm and Haas Co., Philadelphia, Pa., U.S.A.; sodium merthiolate was from Eli Lilly and Co., Indianapolis, Ind., U.S.A. Casein (Hammersten) was obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., and prepared according to Revel[16].

RESULTS

Effect of pH

Two peaks of phosphatase activity, one in the acid and the other in the alkaline pH range, were observed with all the substrates used, except with casein that was attacked only at acid pH (Fig. 1). High levels of activity were observed on all substrates in the alkaline pH range, with optimum activity between pH 9.0 and 11.0, according to the substrate. In the acid zone, the activities observed on α-glycerophosphate and phosphoethanolamine were distinctly lower than with the other substrates. The optimum activity could not be determined for phosphoprotein phosphatase, due to the precipitation of casein below pH 5.6; it was obtained between pH 4.8 and 5.8 for all the other activities.

No distinct peak of pyrophosphatase activity could be demonstrated in the

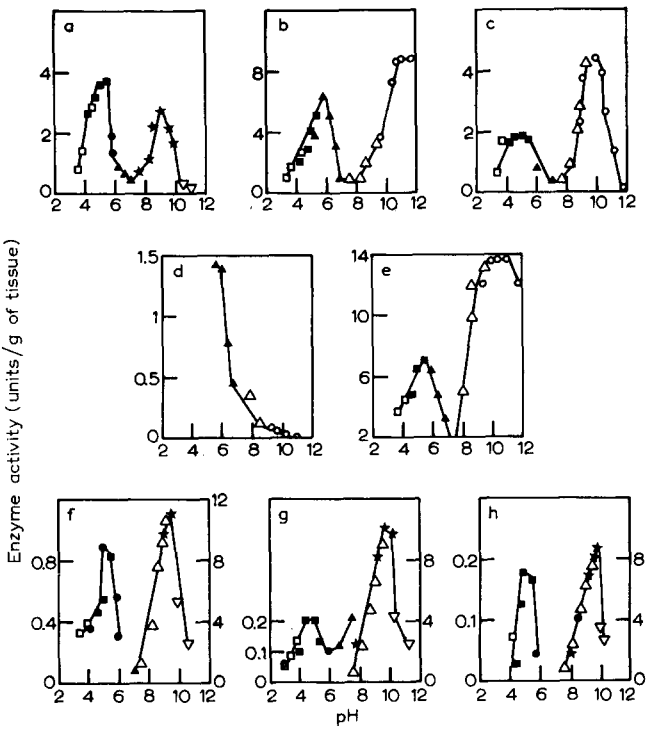

Fig. 1. Influence of pH on phosphatase activities in cytoplasmic extract. The following substrates were used: (a) inorganic pyrophosphate; (b) phenylphosphate; (c) β-glycerophosphate; (d) phosphoprotein (casein); (e) *p*-nitrophenylphosphate; (f) phospho-L-serine; (g) α-glycerophosphate; (h) phosphoethanolamine. Incubations were carried out in 0.1 M concentrations of the following buffers: glycine–HCl or glycine–NaOH (○); formic acid–sodium formiate (□); sodium acetate–acetic acid (■); sodium citrate–citric acid (●); Tris–HCl (△); triethanolamine hydrochloride–NaOH (*); sodium borate–NaOH (▽). For (f), (g) and (h), the scale on the left refers to the values observed at acid pH and the scale on the right, for those observed at alkaline pH. The assays were done in the absence of added $MgCl_2$.

neutral pH range (pH 5.8–8.4) despite several attempts where 2.5 mM pyrophosphate was used either without or with 2 or 10 mM $MgCl_2$ in either cacodylate, Tris–maleate or Tris–HCl (0.1 M) buffer.

Effect of substrate concentration

The K_m values were determined for the various phosphatase activities under their standard assay conditions (Table I). All these enzymes displayed Michaelis–Menten kinetics; reciprocal plots of activity and substrate concentration gave straight lines. Excess of substrate did, however, slightly inhibit phosphoprotein phosphatase and acid pyrophosphatase but caused an important inhibition of alkaline pyrophosphatase. On the other hand, for acid *p*-nitrophenylphosphatase and for the acid pyrophosphatase, reciprocal plots of activity and substrate concentration gave curves of progressively decreasing slope. as observed already for acid phenylphosphatase in previous studies[12]. Half-maximum activity of acid *p*-nitrophenylphosphatase and of acid pyrophosphatase were obtained at substrate concentrations of 2–5 mM (apparent K_m). However, a strictly linear reciprocal plot

TABLE I

K_m VALUES OBTAINED FOR THE HYDROLYSIS OF VARIOUS PHOSPHATASE SUBSTRATES AT EITHER ACID OR ALKALINE pH BY BONE CYTOPLASMIC EXTRACTS UNDER THE STANDARD ASSAY CONDITIONS (WITHOUT ADDITION OF $MgCl_2$)

Substrate	K_m *(mM)*	
	pH 5–5.8	*pH 9.5*
β-Glycerophosphate	5.0 (at $I \leqslant 0.20$)	3.6
α-Glycerophosphate	25	3.3
Phenylphosphate	—	4.0
p-Nitrophenylphosphate	10 (at $I = 0.39$)	3.3
Phosphoethanolamine	250	4.3
Phospho-L-serine	200	1.3
Casein (in P_i equivalent)	0.5 (at $I = 0.23$)	—
Inorganic pyrophosphate	7.2 (at $I = 0.25$)	1.7

of activity and substrate concentration with slightly higher K_m was obtained when both activities were measured at constant ionic strength (Table I).

Effect of ionic strength

Increasing the ionic strength by addition of NaCl or of KCl to the incubation flasks (Fig. 2) caused an important stimulation of the acid *p*-nitrophenylphosphatase and pyrophosphatase activities of cytoplasmic extracts but inhibited the phosphoprotein phosphatase activity; acid β-glycerophosphatase was not affected. The alkaline pyrophosphatase activity was rapidly inhibited; alkaline *p*-nitrophenylphosphatase was not influenced.

Also, washing of the N fraction with concentrated KCl solutions released acid phosphatase activity that was adsorbed on the sedimentable components of that fraction into soluble form (Fig. 3). This treatment released only a small amount of

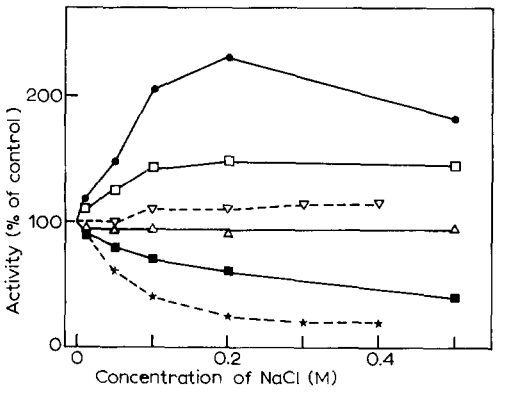

Fig. 2. Influence of the ionic strength (NaCl) on the phosphatase activities in cytoplasmic extract. NaCl was added to the incubation flasks at the concentration indicated on abcissa. ●, acid *p*-nitrophenylphosphatase; □, acid inorganic pyrophosphatase; ▽, alkaline *p*-nitrophenylphosphatase; △, acid β-glycerophosphatase; ■, phosphoprotein phosphatase; ∗, alkaline inorganic pyrophosphatase. The alkaline phosphatase activities were assayed in the presence of $MgCl_2$.

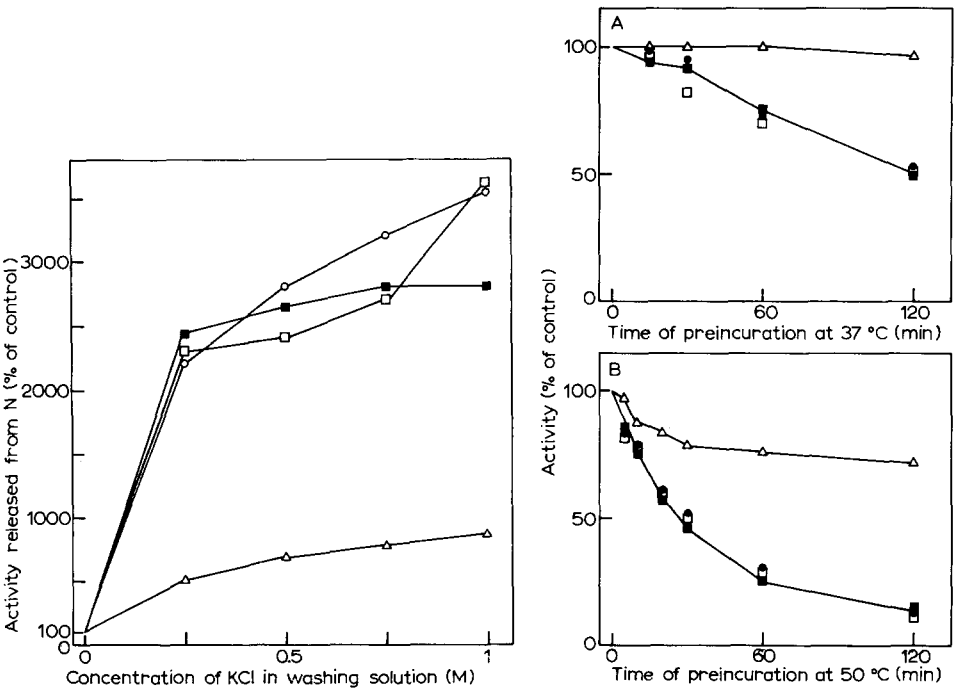

Fig. 3. Release of adsorbed acid phosphatase activities from the N fraction by KCl. The N fraction was resuspended and washed in 0.25 M sucrose containing KCl at the concentration indicated. Acid phosphatase was assayed in the supernatant obtained after recentrifugation of the fraction on the following substrates: phenylphosphate (○); inorganic pyrophosphate (□); phosphoprotein (casein) (■); β-glycerophosphate (△). The enzyme activities released from the N fractions are expressed as percentage of those released from the controls by washing the fraction with 0.25 M sucrose only.

Fig. 4. Effect on the acid phosphatase activities of the preincubation of cytoplasmic extract at either 37 °C (A) or 50 °C (B) and at pH 7.5 for various periods of time in the absence of substrate. The activities were then measured on the different substrates (△, β-glycerophosphate; □, inorganic pyrophosphate; ●, *p*-nitrophenylphosphate; ○, phenylphosphate; ■, casein) in the absence of ascorbic acid. The incubation time was 60 min.

acid β-glycerophosphatase but much larger quantities of the acid pyro-, phenyl- or phosphoprotein phosphatase activities.

Activations, inhibitions and stability

When assayed in cytoplasmic extracts, the activities of acid pyro-, *p*-nitrophenyl- and phosphoprotein phosphatases remained constant with time for only 20 min. They remained stable for a longer period when ascorbic acid (0.5–10 mM) was added to the incubation flasks. Similar phenomena were observed for the acid phosphatase activities exerted on phenyl phosphate, phosphoserine and phosphoethanolamine. On the contrary, the activity of acid β-glycerophosphatase remained constant for more than 4 h and was not affected by ascorbic acid.

The rapid levelling off of the phosphatase activities exerted on substrates other than β-glycerophosphate is due to their lability at 37 °C. Experiments in which the cytoplasmic extract was preincubated without substrate at 37 or 50 °C and at either

pH 5.8 or 7.5 showed increased loss of activity with longer times of preincubation. Acid β-glycerophosphatase was, however, much more stable (Fig. 4), as were the other acid phosphatase activities when ascorbic acid was added to the preincubation flasks. Part of the activities of the thermolabile group of phosphatases that had been lost during a preincubation in the absence of ascorbic acid at 37 °C could be recovered by the further addition of ascorbic acid to the assays.

Sodium (+)-tartrate caused a strong inhibition of acid β-glycerophosphatase but had only a slight effect on acid pyro-, *p*-nitrophenyl- or phosphoprotein phosphatase (Fig. 5). NaF inhibited markedly all the acid phosphatase activities investigated (−60 to −75% at 2.5 mM) as did *p*-chloromercuribenzoate (−50 to −80% at 1 mM). EDTA (0.25–2.5 mM) inhibited acid β-glycerophosphatase (−60%) and phosphoprotein phosphatase (−35%) but caused a slight stimulation of acid *p*-nitrophenylphosphatase (+10%) and acid inorganic pyrophosphatase (+35%).

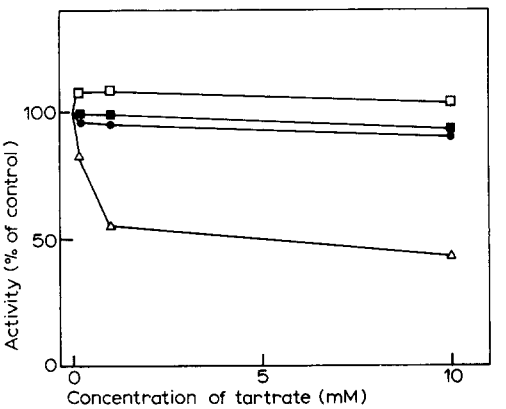

Fig. 5. Effect of (+)-tartrate on the acid phosphatase activities. The inhibitor was added directly to the assays at the concentrations indicated. The substrates were β-glycerophosphate (△); inorganic pyrophosphate (□); *p*-nitrophenylphosphate (●); casein (■). The incubation time was 60 min.

Addition of Mg^{2+} to the incubation flasks inhibited acid pyrophosphatase when its concentration was higher than 0.5–1 mM (up to −60% at 7.5 mM). Alkaline pyrophosphatase activity of fresh cytoplasmic extracts was not affected by the addition of up to 1 mM $MgCl_2$ but it was inhibited at higher $MgCl_2$ concentrations. However, a stimulating effect (+83%) of $MgCl_2$ on alkaline pyrophosphatase, optimal between 1 and 1.5 mM, was demonstrated with cytoplasmic extract that had first been extensively dialysed against 5 mM Tris-buffered (pH 7.5) 0.15 M NaCl; an inhibition of the activity was then also observed at higher $MgCl_2$ concentrations. Alkaline *p*-nitrophenylphosphatase activity of cytoplasmic extracts was stimulated (+60%) by $MgCl_2$ (50 mM); the stimulation was higher (+130%) when the extract had first been extensively dialysed.

Alkaline pyro- and *p*-nitrophenylphosphatase activities were both stable when they were preincubated around neutrality for up to 100 min at 37 °C in the absence of substrate. At 55 °C, both activities were rapidly lost in a closely parallel manner (−40, −70 or −88% after 4, 8 or 12 min, respectively).

Quantitative assays

On the basis of the previous experiments, standard assay methods were devised (see under Experimental), taking care that the measured activities were always proportional to both enzyme concentration and incubation time. The activities investigated varied linearly with the amount of tissue put in the incubation flasks (as cytoplasmic extract) up to a concentration of 6 mg of original tissue per ml for alkaline pyrophosphatase, of 12 mg/ml for the other alkaline phosphatase activities and of at least 16 mg/ml for the acid phosphatases. Linearity with respect to the duration of the incubation was achieved for 1 h for the alkaline phosphatases and for more than 4 h for acid β-glycerophosphatase; in the presence of ascorbic acid, it was achieved for 1 h for acid pyrophosphatase and for 2 h for the other acid phosphatase activities.

Substrate competitions among acid phosphatases

An inhibitory action of the competitive type was exerted by β-glycerophosphate on the hydrolysis of *p*-nitrophenylphosphate at acid pH. The calculations showed that a single enzyme could not be involved in the hydrolysis of both substrates as the reaction rates observed experimentally for the hydrolysis of *p*-nitrophenylphosphate in the presence of β-glycerophosphate were significantly higher than those that would have been observed if a single phosphatase had been involved. For instance, at 8 mM *p*-nitrophenylphosphate, addition of 50 mM β-glycerophosphate to the flasks decreased the reaction rate from 20.3 to 12.5 units/g; v (45.7 units/g), K_s (10 mM) and K_i (10 mM) were measured simultaneously in the same experiment (at $I = 0.39$), so that it could be calculated that the velocity of hydrolysis of *p*-nitrophenylphosphate should have been 4.7 units/g if both substrates had been split exclusively by a single enzyme.

An inhibitory action of the competitive type was also exerted by *p*-nitrophenylphosphate on the hydrolysis of inorganic pyrophosphate at acid pH. In this case, however, the calculations showed that both substrates could be hydrolysed by the same enzyme. For instance, at 10 mM pyrophosphate, addition of 50 mM *p*-nitrophenylphosphate to the flasks decreased the reaction rate from 6.6 to 2.6 units/g, a value close enough to the reaction rate (2.1 units/g) that was calculated on the assumption of the existence of a single enzyme, knowing v (11.4 units/g), K_s (7.2 mM) and K_i (10 mM) from the experiment (at $I = 0.25$).

The inhibition obtained by the addition of *p*-nitrophenylphosphate (up to 10 mM) to the phosphoprotein phosphatase assays was more complex, mainly of the non-competitive type; at higher (20 mM) *p*-nitrophenylphosphate concentration, an activation of phosphoprotein phosphatase was observed. However, a competitive inhibition was obtained by the addition of casein to the acid *p*-nitrophenylphosphatase assays. In that case, calculations showed that a single enzyme could not be responsible for the hydrolysis of both substrates. For instance, with a 5.6 mM concentration of *p*-nitrophenylphosphate the observed velocity of acid *p*-nitrophenylphosphatase shifted from 18 to 11.6 units/g when 4 mM casein (in phosphate equivalent) was added to the incubation flasks; knowing v (50 units/g), K_s (10 mM) and K_i (0.5 mM) from the experiments (at $I = 0.23$), it was calculated that this velocity should have shifted to 2.9 units/g if a single enzyme had been responsible for the splitting of both substrates.

DISCUSSION

Besides providing adequate tools for the study of the intracellular distribution of bone phosphatases reported in the accompanying paper[10], the present experiments allowed a better characterization of these activities and open the way towards further studies on their possible association with various enzyme molecules.

It was evident that cytoplasmic extracts from bone cells were able to split a variety of phosphate esters at either acid or alkaline pH; the affinity of the phosphatases for some of these substrates (*e.g.* phosphoserine and phosphoethanolamine at acid pH) was, however, very low. In accordance with the observations of others[17,18], extracts from bone cells were found to display only two peaks of inorganic pyrophosphatase activity, one centered on pH 5.4 and the other on pH 9; no distinct peak of activity could be demonstrated in the neutral pH range. However, a neutral inorganic pyrophosphatase has been found in ossifying rat cartilage[19].

It has already been indicated that, in bone, acid β-glycerophosphatase and acid *p*-nitrophenylphosphatase activities are due largely to two different enzymes[12,20]. The present experiments support that view and extend it to other substrates. Acid β-glycerophosphatase was characterized by its great stability at 37 °C and at pH 5; it was not affected by ascorbic acid but was inhibited by tartrate and EDTA. Acid phenylphosphatase, inorganic pyrophosphatase and phosphoprotein phosphatase were much more labile, their activities being lost rapidly and in an almost parallel manner during their incubation at 37 °C and at pH 5. Ascorbic acid increased their stability considerably and even allowed the recovery of part of the activity that had previously been lost. They were not inhibited by tartrate; EDTA inhibited phosphoprotein phosphatase but not acid *p*-nitrophenyl- nor inorganic pyrophosphatase. A partial physical separation of the latter three activities from acid β-glycerophosphatase was obtained when the N fractions were separated from the cytoplasmic extracts during the fractionation of the bone homogenates; soluble acid phenyl-, pyro- and phosphoprotein phosphatase activities were indeed more heavily adsorbed on the particulate components of the N fraction and they could be released from these components by washing with KCl solutions. Substrate-competition experiments confirmed that acid β-glycerophosphatase is largely distinct from acid *p*-nitrophenylphosphatase and that the latter activity is mainly due to the enzyme that is also responsible for the acid inorganic pyrophosphatase activity. They indicated, however, that a large part of phosphoprotein phosphatase activity is not due to the same enzyme as the one acting on *p*-nitrophenylphosphate. This appeared at first surprising in view of the similitudes observed between the two enzymes as far as heat inactivation, protection by ascorbic acid and adsorption properties are concerned. However, preliminary attempts to separate the two activities by gel filtration indicate that part of the phosphoprotein phosphatase can indeed be separated under some conditions from acid *p*-nitrophenylphosphatase. These results are thus best interpreted as indicating the presence of at least three distinct acid phosphatases in bone cells.

In the alkaline pH zone, more attention was directed towards the activities exerted on *p*-nitrophenylphosphate and on inorganic pyrophosphate. The fact that both activities were progressively lost in a closely parallel fashion when cytoplasmic extracts were preincubated at 55 °C around neutrality is compatible with the asso-

ciation of both activities with a single enzyme, as reported by others for bone[21] or teeth[22] extracts, as well as for several soft tissues (for a review, see ref. 23).

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