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

II. DISTRIBUTION IN SUBCELLULAR FRACTIONS OF BONE TISSUE HOMOGENATES AND STRUCTURE-LINKED LATENCY

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SUMMARY

1. In subcellular fractions of bone homogenates, obtained by differential centrifugation, the distribution patterns of phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), acid inorganic pyrophosphatase, acid β-glycerophosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and of various other acid phosphatase activities are closely similar to those reported by G. Vaes and P. Jacques [*Biochem. J.* 97, 389–392 (1965)] for the other acid hydrolases of this tissue. The highest specific activity of these enzymes is found in the light-mitochondrial fraction. The highest specific activity of alkaline inorganic pyrophosphatase and of various other alkaline phosphatase activities (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is found in the microsomal fraction.

2. In cytoplasmic extracts of bone, the various acid phosphatase activities occur in latent form to the extent of 50–60% of their total activity. The latent enzymes are unmasked by a number of treatments (Triton X-100, Ultra-Turrax, low osmotic pressure, freezing and thawing) that unmask the lysosomal β-glucuronidase (EC 3.2.1.31) in a closely parallel manner.

3. These results support the concept of an association of phosphoprotein phosphatase, acid inorganic pyrophosphatase, acid β-glycerophosphatase and the other acid phosphatase activities with lysosomes in bone. Alkaline inorganic pyrophosphatase and the other alkaline phosphatase activities are largely attached to microsomal elements.

INTRODUCTION

In the preceding paper[1], the demonstration in bone homogenates of phosphatase activities exerted at either acid or alkaline pH on inorganic pyrophosphate, phospho-

protein and several phosphomonoesters was reported and methods for the quantitative assay of these activities were described. Some of these enzymes are thought to be involved in either bone formation or bone resorption[2-7]. It was therefore important to investigate their intracellular localization in order to obtain a better understanding of the cellular physiology of these processes. This was done by studying both the distribution of the phosphatases in subcellular fractions of bone homogenates isolated by differential centrifugation and the structure-linked latency of these enzymes.

The study allows one to conclude that the various acid phosphatase activities investigated, including acid inorganic pyrophosphatase, phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) and acid β-glycerophosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), are all associated with the lysosomes in bone and that the alkaline phosphatase activities (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) are largely attached to microsomes.

EXPERIMENTAL

Homogenates of calvaria from newborn rats were prepared in ice-cold 0.25 M sucrose and fractionated as described by Vaes and Jacques[8] according to a scheme identical with that adopted for rat liver by de Duve *et al.*[9], isolating successively the so-called nuclear (N), heavy-mitochondrial (M), light-mitochondrial (L), microsomal (P) and final supernatant (S) fractions. The fractions were assayed for the various acid and alkaline phosphatase activities[1] as well as for other reference enzymes (β-glucuronidase, EC 3.2.1.31, and cytochrome oxidase, EC 1.9.3.1) and for proteins according to the methods previously described[10].

Latency studies were performed at 37 °C on cytoplasmic extracts (cell-free supernatants obtained after centrifugation of the homogenates at 600 $\times$ *g* for 10 min) as reported by Vaes[11], free activities being measured in the presence of 0.25 M sucrose and at pH 5.8 for all the acid hydrolases and total activities being assayed similarly in the additional presence of 0.1% Triton X-100 to disrupt the particles[12]; the incubation, time was usually limited to 60 min. It was verified that the soluble acid hydrolases were neither activated nor inhibited by Triton X-100 at the concentration used. For several of these latency studies, the cytoplasmic extracts were obtained after washing of the N fraction with 0.25 M sucrose containing 0.3 M KCl as described in the preceding paper[1]; these extracts will be further refered to as "KCl–cytoplasmic extracts". Free activity measurements were then done in the presence of 0.3 M KCl in the incubation flasks.

RESULTS

Fractionation by differential centrifugation

The results obtained in the fractionation experiments are listed in Table I. The corrected distribution patterns are shown in Fig. 1 in the manner proposed by de Duve *et al.*[9].

The distribution of the various acid phosphatase activities between the subcellular fractions is similar to that of β-glucuronidase and of the other lysosomal acid hydrolases previously studied in bone tissue[8,13]; their highest relative specific activity was found in the light-mitochondrial (L) fraction. This distribution contrasted with

TABLE I

INTRACELLULAR DISTRIBUTION OF ENZYMES

Absolute values are in mg/g for protein and in units/g for enzymes. Statistics refer to the means ± S.D. E, cytoplasmic extract; N, nuclear fraction; M, heavy-mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, final supernatant.

Enzymatic activity	*Number of expts*	*Absolute values (E + N)*	*Percentage values (E + N = 100%)*					
			N	*M*	*L*	*P*	*S*	*Recovery*
Protein	12	37.22 ± 4.9	50.1 ± 7.5	11.0 ± 2.6	6.9 ± 2.2	7.0 ± 2.1	26.1 ± 3.0	101.1 ± 5.9
Cytochrome oxydase	11	0.61 ± 0.19	36.4 ± 16.8	32.7 ± 8.6	16.2 ± 7.1	4.7 ± 2.1	0	90.0 ± 9.4
β-Glucuronidase	11	0.27 ± 0.08	24.5 ± 6.7	14.7 ± 5.7	15.8 ± 6.5	3.1 ± 0.8	32.0 ± 5.6	90.1 ± 4.8
Acid α-Glycerophosphatase	6	0.85 ± 0.23	36.5 ± 17.3	14.6 ± 8.3	21.9 ± 9.7	12.3 ± 1.7	15.7 ± 1.1	101.0 ± 4.0
Acid β-Glycerophosphatase	11	1.21 ± 0.30	26.7 ± 8.5	17.3 ± 3.2	24.0 ± 5.6	12.9 ± 2.7	19.0 ± 4.6	99.9 ± 5.1
Acid phosphoprotein phosphatase	11	2.98 ± 0.46	32.3 ± 13.9	20.3 ± 3.6	22.9 ± 4.3	6.6 ± 2.0	6.7 ± 3.5	88.8 ± 6.9
Acid phenylphosphatase	11	9.9 ± 2.3	29.5 ± 7.5	17.5 ± 3.3	21.7 ± 5.3	5.6 ± 1.3	9.7 ± 3.0	84.0 ± 5.9
Acid *p*-nitrophenylphosphatase	12	10.8 ± 1.1	29.3 ± 10.2	17.2 ± 3.9	21.4 ± 5.5	7.3 ± 2.5	11.8 ± 4.4	87.0 ± 10.2
Acid phosphoserine phosphatase	5	1.48 ± 0.21	33.2 ± 15.4	19.3 ± 5.4	27.0 ± 1.6	7.0 ± 2.8	6.3 ± 0.8	92.8 ± 13.5
Acid phosphoethanolamine phosphatase	5	0.48 ± 0.07	46.1 ± 18.9	17.5 ± 5.4	25.6 ± 9.6	7.0 ± 3.6	5.7 ± 3.9	101.9 ± 8.8
Acid pyrophosphatase	7	4.1 ± 1.1	27.3 ± 10.6	17.9 ± 1.7	23.1 ± 3.3	6.7 ± 2.1	7.1 ± 4.0	82.1 ± 5.7
Alkaline α-glycerophosphatase	3	16.5 ± 2.9	40.8 ± 6.2	10.2 ± 1.6	11.2 ± 1.6	14.8 ± 1.2	21.6 ± 1.6	98.6 ± 2.4
Alkaline β-glycerophosphatase	3	18.6 ± 2.2	30.8 ± 7.2	10.5 ± 1.9	14.7 ± 3.9	19.2 ± 6.9	22.1 ± 1.6	97.3 ± 4.0
Alkaline phenylphosphatase	11	21.0 ± 2.6	30.3 ± 8.9	10.1 ± 2.0	14.0 ± 4.2	21.5 ± 5.1	21.1 ± 3.3	97.0 ± 2.1
Alkaline *p*-nitrophenyl-phosphatase	3	17.9 ± 3.0	36.2 ± 14.4	8.8 ± 1.5	13.9 ± 5.4	17.9 ± 4.5	19.9 ± 3.2	96.7 ± 7.0
Alkaline phosphoserine phosphatase	4	16.6 ± 1.3	35.4 ± 11.0	10.1 ± 1.8	14.7 ± 4.4	19.4 ± 6.2	21.7 ± 1.3	101.3 ± 3.8
Alkaline phosphoethanolamine phosphatase	6	11.9 ± 1.6	37.9 ± 8.1	11.5 ± 2.6	13.8 ± 3.7	19.0 ± 8.8	22.6 ± 1.7	104.8 ± 7.2
Alkaline pyrophosphatase	6	3.7 ± 0.96	33.9 ± 6.6	12.6 ± 3.5	15.9 ± 4.4	22.7 ± 5.5	25.3 ± 3.4	110.4 ± 9.4

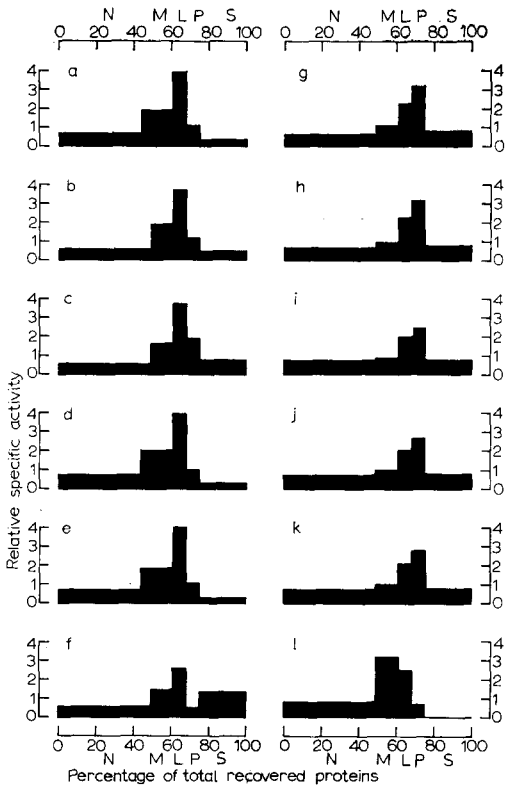

Fig. 1. Distribution patterns of enzymes or enzyme activities: (a)–(e): acid phosphatase activities assayed on (a) inorganic pyrophosphate, (b) *p*-nitrophenylphosphate, (c) *β*-glycerophosphate, (d) phosphoprotein (casein) and (e) phospho-L-serine; (f) *β*-glucuronidase; (g)–(k): alkaline phosphatase activities assayed on (g) inorganic pyrophosphate, (h) phenylphosphate, (i) *β*-glycerophosphate, (j) phosphoethanolamine and (k) phospho-L-serine; (l) cytochrome oxidase. The results are presented in the manner used by de Duve *et al.*[9]. The ordinate shows the mean relative specific activity of the fractions. On the abcissa the fractions are represented by their mean relative protein content, in the order in which they are isolated, *i.e.* from left to right: N, M, L, P and S (see Table I).

that of cytochrome oxidase, a marker for mitochondria, which shows the highest specific activity in the heavy-mitochondrial (M) fraction, whereas alkaline inorganic pyrophosphatase and all the other alkaline phosphatase activities are particularly concentrated in the microsomal (P) fraction.

The recovery values are satisfactory in most cases although they are usually somewhat lower for the acid phenyl-, *p*-nitrophenyl- and inorganic pyrophosphatase, presumably because of the lack of stability of the enzyme that is responsible for these activities[1]. A still lower recovery (67%) was obtained for acid phenylphosphatase in previous experiments[8], when this enzyme was assayed in the absence of ascorbic acid; addition of ascorbic acid to the incubation flasks brought the mean recovery to 84% but did not change significantly the distribution pattern of the enzyme.

Structure-linked latency of acid phosphatase activities

In the presence of ascorbic acid[1], the reaction rates remained linear for at least

1 h in a cytoplasmic extract incubated under the conditions of both the free and total activity assays. The free activity measurements could thus be extended over one hour.

All the acid phosphatase activities under study displayed an important degree of latency in cytoplasmic extracts. In the absence of washing of the N fraction with KCl, a higher proportion of free activity (51%) was obtained for acid β-glycerophosphatase than for the other acid phosphatase activities exerted on inorganic pyrophosphate, *p*-nitrophenylphosphate or phosphoprotein (33–36%). However, a significant amount of soluble activity of these other acid phosphatases was released from the N fraction into the cytoplasmic extract when the N fraction was washed with 0.25 M sucrose supplemented with 0.3 M KCl ("KCl–cytoplasmic extract"), as reported in the preceding paper[1]. Thus this raised the proportion of free activity found in the cytoplasmic extracts for these phosphatases up to values comparable to those found for acid β-glycerophosphatase. The percentage of free activity of this last enzyme was not significantly affected by the KCl washings. The free activities of alkaline *p*-nitrophenyl- and of inorganic pyrophosphatase were not significantly different from their total activities.

A graded release of the latent acid phosphatases could be evoked by exposing cytoplasmic extracts to various treatments injurious to the particles. The release of the latent acid *p*-nitrophenylphosphatase, acid pyrophosphatase and phosphoprotein phosphatase was closely parallel to that of acid β-glycerophosphatase providing that the cytoplasmic extracts included washings of the N fraction with 0.3 M KCl and that KCl had been added at the same concentration to the incubation flasks for the assays; when this was not done, their release was slower than that of β-glycerophosphatase, as already observed with acid phenylphosphatase in previous experiments[11]. These observations suggest that part of the acid phenyl-, pyro- and phosphoprotein phos-

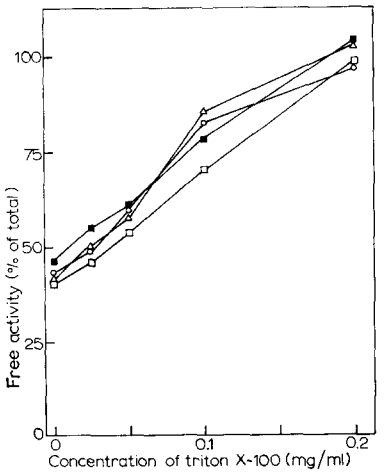

Fig. 2. Influence of pretreatment of KCl–cytoplasmic extract with Triton X-100 on free activities of acid β-glycerophosphatase (△), acid phenylphosphatase (○), acid inorganic pyrophosphatase (□) and phosphoprotein phosphatase (■). The detergent was added to the cytoplasmic extract at 0 °C up to the concentration indicated on abcissa. Appropriate amounts of these mixtures and of untreated cytoplasmic extract were then used for the measurement of free activity. Total activities were measured in the usual manner, in the presence of 1 mg of Triton X-100 per ml in the assays. Results are expressed as percentages of the corresponding total activities; in this experiment the total activities were unaffected by the treatments.

phatase is bound by adsorption on subcellular particles of the cytoplasmic extract as well as on particulate components of the N fraction in a manner that prevents the action of the enzymes on their substrates, but that this action becomes possible when the enzymes have been desorbed by KCl.

The release of the latent enzymes could be achieved by exposure of the KCl–cytoplasmic extracts to increasing concentrations of a detergent, Triton X-100 (Fig. 2). Total release of latent activity was obtained in all cases with less than 0.1% Triton X-100, thus establishing the validity of the assays for total activity. Treatment of a cytoplasmic extract with 0.1% Triton X-100 caused the transfer of a considerable proportion of the acid phosphatase activities from the particulate to the soluble phase. Progressive release of the latent phosphatase activities with a simultaneous solubilization of the enzymes was still obtained by treating the KCl–cytoplasmic extract with an Ultra-Turrax homogenizer for increasing lengths of time (Fig. 3), by exposing it to

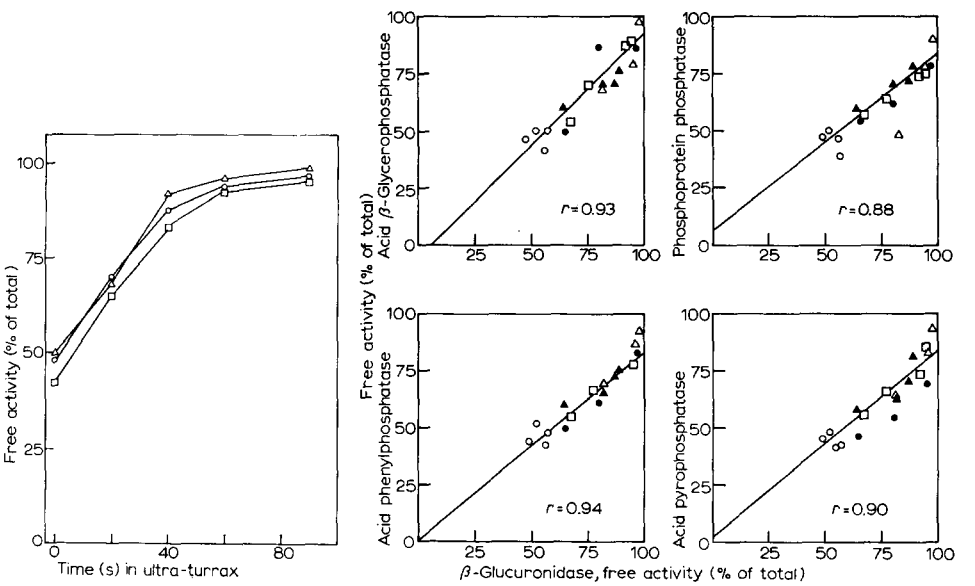

Fig. 3. Influence of treatment for increasing periods of time in the Ultra-Turrax on free activities of acid β-glycerophosphatase (△), acid phenylphosphatase (○) and acid inorganic pyrophosphatase (□) in KCl–cytoplasmic extract. Values are expressed as percentages of the corresponding total activities. These were essentially unaffected by the treatment for acid β-glycerophosphatase; the other activities suffered a 20–30% inactivation in the treated extracts.

Fig. 4. Correlation coefficient, r, between the free activities of the acid phosphatases and of β-glucuronidase, in untreated KCl–cytoplasmic extracts (○), in KCl–cytoplasmic extracts treated with increasing amounts of Triton X-100 (●), subjected to the action of an Ultra-Turrax for increasing lengths of time (△), exposed to decreasing sucrose concentrations (▲) or frozen-and-thawed once or several times (□). The free activities were measured in 1-h assays and expressed as percentage of the corresponding total activities.

decreasing osmotic pressure or by treating it by an increasing number of freezing-and-thawing cycles. In all these experiments, good correlations were obtained between the free activities measured for β-glucuronidase, a lysosomal enzyme in bone cells[11], and those measured for either acid β-glycerophosphatase, acid phenylphosphatase, phos-

phoprotein phosphatase and acid inorganic pyrophosphatase (Fig. 4), suggesting strongly that the latency of these enzymes is due to their association inside typical lysosomes.

DISCUSSION

The observations reported in the present paper should be interpreted at the light of previous studies on the acid hydrolases of bone tissue, bearing either on the distribution of these enzymes in subcellular fractions[8] or on the activation and release of the latent acid hydrolases[11].

In the present study of the fractions obtained by differential centrifugation of bone homogenates, the acid and alkaline phosphatase activities exerted on many different substrates, including inorganic pyrophosphate, phosphoprotein (casein), *p*-nitrophenylphosphate and β-glycerophosphate, were found to occur partly in soluble form and partly in association with cytoplasmic particles. The particle-bound acid phosphatases sediment more slowly than the mitochondrial cytochrome oxidase and become concentrated in the L fraction, as do the other acid hydrolases[8]. The particle-bound alkaline phosphatases sediment at a slower rate than the acid phosphatases and become most concentrated in the P fraction. This strongly suggests that the acid phosphatase activities are associated with lysosomes and that the alkaline activities are bound to the rather poorly characterized "microsomes", a group of particles that, in liver, contains both rough and smooth membranes of the endoplasmic reticulum as well as fragments of the plasma membrane[14]. Thus the cytological nature of the material bearing the alkaline phosphatases in bone cells remains to be elucidated.

Further support for an association of the various acid phosphatases activities within lysosomes comes from the study of their latency in cytoplasmic extracts of bone tissue. The latency belongs exclusively to the particle-bound form of the enzyme and no latent activity was found for the soluble enzyme of the S fraction. It was decreased or suppressed by various treatments that affect the integrity of the lysosomal membrane, such as by exposure to Triton X-100 or to media of low osmotic pressure, by mechanical disruption in an Ultra-Turrax or by freezing and thawing; suppression of the latency was accompanied by the solubilization of the enzymes. The significance of all these properties has been discussed at length previously[11]. Of particular interest were the close correlations found between the progressive release of β-glucuronidase, a lysosomal enzyme in bone tissue[11], and that of the various acid phosphatases observed under a graded application of these membrane-disrupting agents (Fig. 4): it gives a strong support for the association of all these enzymes inside typical lysosomes in bone cells. In liver, only part of acid *p*-nitrophenylphosphatase[15] and of phosphoprotein phosphatase[16] is present in the lysosomes although acid β-glycerophosphatase[9] and acid inorganic pyrophosphatase[17] are lysosomal.

The precise extent into which the various acid or alkaline phosphatase activities are associated with one or more acid or alkaline phosphatase enzyme is at present unknown. However, the experiments reported in the preceding paper[1] indicate that there may exist at least three different acid phosphatases in bone homogenates (acid β-glycerophosphatase, phosphoprotein phosphatase and acid inorganic pyrophosphatase, an enzyme that appears identical to acid *p*-nitrophenylphosphatase). It was

observed also that alkaline inorganic pyrophosphatase could be identical to alkaline *p*-nitrophenylphosphatase.

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