Studies on Bone Enzymes

THE ACTIVATION AND RELEASE OF LATENT ACID HYDROLASES AND CATALASE IN BONE-TISSUE HOMOGENATES

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1. Eight distinct acid-hydrolase activities present in cytoplasmic extracts from bone tissue occur in latent form to the extent of 50–70% of their total activity, depending on the enzyme. 2. This latency can be decreased or suppressed by exposure to Triton X-100 or to media of low osmotic pressure, by treatment in the Waring Blendor, and by freezing and thawing, but not by increasing the substrate concentration in the assay medium up to 10-fold the Michaelis constant of the enzymes. 3. Latency is the property of the particle-bound enzymes, and treatments that suppress latency simultaneously cause solubilization of the enzymes. Most enzymes show an excess of free over soluble activity; the magnitude of this excess seems to depend largely on the nature of the enzyme, and sometimes also on the kind of treatment suffered by the preparations; it is attributed mainly to adsorption artifacts. 4. In preparations subjected to graded activating treatments, seven of the eight acid hydrolases studied are released in closely parallel fashion, suggesting that they are associated with particles possessing similar properties. Acid phenylphosphatase is released less readily than the other enzymes by Triton X-100 and by exposure to media of low osmotic pressure. 5. It is concluded from these and previous published fractionation experiments that, with the possible exception of part of the acid-phenylphosphatase activity, the eight acid hydrolases studied belong to lysosome-like particles. Bone lysosomes exhibit a relatively high degree of biochemical and physical heterogeneity. Their possible functions are discussed. Part of the acid-phenylphosphatase activity could be linked to another group of particles. 6. Catalase is also partly (30%) latent in cytoplasmic extracts of bone. Latent catalase can be released by some of the treatments that suppress the latency of the lysosomal enzymes, but differs from the latter by a greater resistance to Triton X-100, and, especially, by a complete insensitivity to exposure to media of low osmotic pressure. It is concluded from these results that the catalase-containing particles are probably different from lysosomes, as they are in liver. 7. Cytochrome oxidase, which is presumably associated with the mitochondria, and alkaline phenylphosphatase, an enzyme occurring predominantly in the microsomal fraction, exhibited no latency under the conditions of the present experiments.

Nine acid hydrolases have been demonstrated in homogenates of bone (Vaes & Jacques, 1965*a*), where they have been shown by means of centrifugation techniques to be associated with cytoplasmic particles resembling the lysosomes of liver in their main physical properties (Vaes & Jacques, 1965*b*).

Structure-linked latency is an important characteristic of lysosomes in liver and several other tissues. The results described in the present paper show that the bone particles also exhibit this property. Other enzymes such as cytochrome oxidase or alkaline phenylphosphatase did not display any latency. Particulate catalase was found to occur in partly latent form; it could be activated by several of the treatments that release the acid hydrolases, but, unlike the latter, not by exposure to media of low osmotic pressure. This difference, which has also been observed in liver, supports the concept that bone catalase is associated with cytoplasmic particles distinct from lysosomes, as is the case in liver (de Duve *et al.* 1960; Baudhuin *et al.* 1964).

EXPERIMENTAL

Cytoplasmic extracts of particulate fractions were prepared in ice-cold 0·25 M-sucrose from homogenates of newborn-rat calvaria, as reported by Vaes & Jacques (1965*a*,*b*).

All enzyme assays were performed according to the techniques described by Vaes & Jacques (1965*a*), but always at pH 5·0 for the acid hydrolases. In conformity with the criteria set forth by Appelmans & de Duve (1955) and Gianetto & de Duve (1955) and followed in all subsequent work on hepatic lysosomes in this Laboratory, free activities of acid hydrolases were measured in the presence of 0·25 M-sucrose at pH 5·0 and 37° and total activities were assayed similarly in the additional presence of 0·1% Triton X-100 to disrupt the particles (Wattiaux & de Duve, 1956). The incubation time was first limited to 10 min., as is required for the correct measurement of free activities on liver particles, but was later extended to 2 hr. when it was found that bone particles remain stable for this length of time in cytoplasmic extracts under the assay conditions. An exception had to be made for acid phenylphosphatase, which was assayed in 10 min. owing to the instability of the enzyme itself (Vaes & Jacques, 1965*a*). These procedures are justified by the data presented in the first parts of the Results section.

For the assay of catalase, total activity was obtained by preincubating the enzyme at 0° for 10 min. in 0·25 M-sucrose containing 1% (w/v) of Triton X-100, as done by Baudhuin *et al.* (1964). The incubation was then started by the addition of the substrate solution. Free catalase activity was assayed similarly after preincubation in 0·25 M-sucrose.

RESULTS

Latency of enzymes. The values listed in Table 1 show that all eight acid hydrolases under study displayed considerable latency in cytoplasmic extracts. Significant differences were observed between the free activities of the various enzymes, indicating a fairly considerable degree of heterogeneity, either at the particle or at the enzyme level. Catalase was also partly latent, though to a smaller degree than the acid hydrolases. In contrast, the free activity of alkaline phenylphosphatase was not significantly different from its total activity. Cytochrome oxidase exhibited no latency.

As shown in Table 2, β-glucuronidase and acid phenylphosphatase were partly latent in all particulate fractions, especially in fraction L, which is known to show the highest specific activity of these enzymes (Vaes & Jacques, 1965*a*). No latency was observed for the enzymes in the final supernatant. Detailed experiments of this sort were not carried out on the other enzymes, but it was verified that none of them displayed any latency in the soluble fraction. Latency therefore may safely be considered a property of the particle-bound enzyme.

Effect of incubation at pH 5 *and* 37°. As illustrated for three enzymes in Fig. 1, the reaction rates remained linear for at least 2 hr. in a cytoplasmic extract incubated under the conditions of both the free and the total activity assays. The same observation was made on the other acid hydrolases except phenylphosphatase, which suffers progressive inactivation at pH 5 and 37° (Vaes & Jacques, 1965*a*). It forms the basis of our free activity measurements on cytoplasmic extracts which were frequently extended over as much as 2 hr. (see the Experimental section).

Effect of Triton X-100. The influence of this detergent on the free activity of the enzymes was investigated in two different ways. In one type of experiment, illustrated by the results of Fig. 2, Triton X-100 was added to the substrate mixture in various concentrations and the free activity assays were started by the addition of appropriate amounts of cytoplasmic extract to this mixture.

Table 1. *Latency of enzymes in cytoplasmic extract*

Free activities, measured in 2 hr. assays, except for acid phenylphosphatase (10 min. assays), are expressed as percentages of the total activities measured in the same extract. Statistics refer to the means ± S.D. The numbers of individual determinations are given in parentheses.

Enzyme	Free activity (% of total)
β-Glucuronidase	44·8 ± 2·1 (18)
β-Galactosidase	42·0 ± 2·9 (12)
β-*N*-Acetylglucosaminidase	31·5 ± 3·2 (11)
Acid β-glycerophosphatase	48·6 ± 4·7 (14)
Acid phenylphosphatase	26·6 ± 2·9 (8)
Acid deoxyribonuclease	33·9 ± 4·7 (13)
Acid ribonuclease	45·2 ± 5·5 (12)
Cathepsin	36·4 ± 5·7 (12)
Catalase	68·9 ± 5·7 (8)
Alkaline phenylphosphatase	94·3 ± 4·3 (3)

Table 2. *Distribution of latent activity in subcellular fractions*

Fractionation was carried out as described by Vaes & Jacques (1965*b*). Free and total activities were measured on all fractions in 10 min. assays.

Fraction	Free activity (% of total)	
	β-Glucuronidase	Acid phenylphosphatase
Cytoplasmic extract E	37·9	28·7
Nuclear fraction N	53·6	54·9
Fraction M	54·7	41·8
Fraction L	36·8	22·2
Fraction P	41·6	64·4
Final supernatant S	103	96·7

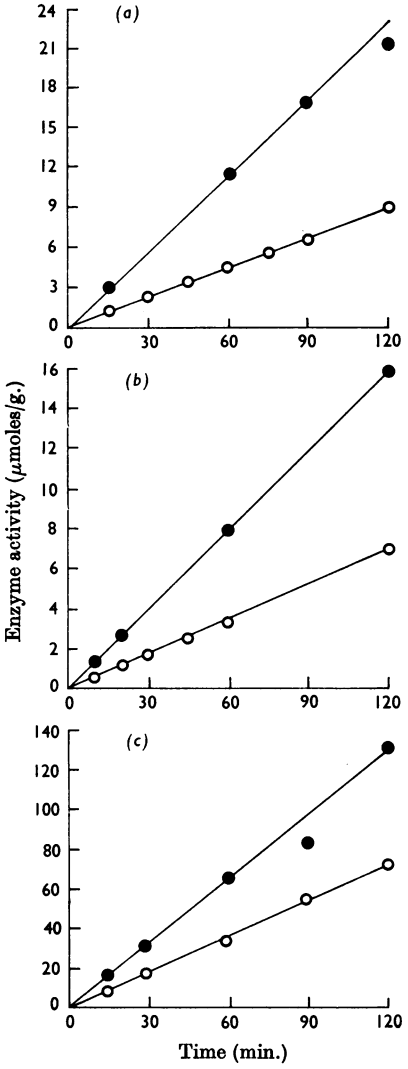

Fig. 1. Influence of time of incubation on free (○) and total (●) activities of (*a*) β-galactosidase, (*b*) β-glucuronidase and (*c*) acid β-glycerophosphatase in cytoplasmic extract of bone.

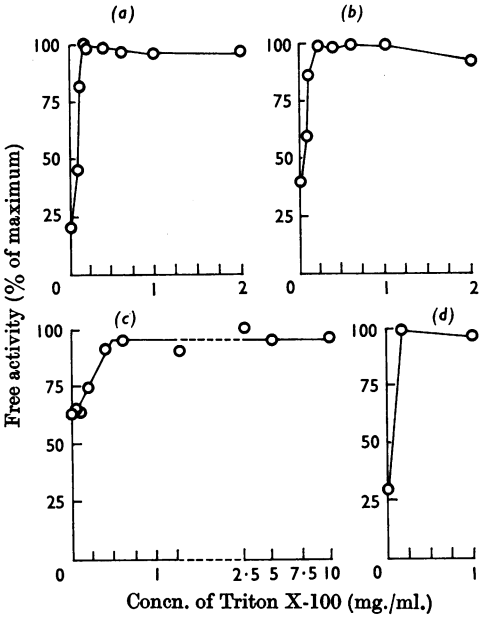

Fig. 2. Influence of Triton X-100 concentration in incubation medium on free activities of (*a*) acid phenylphosphatase, (*b*) β-glucuronidase, (*c*) catalase and (*d*) β-*N*-acetylglucosaminidase. Free activities were measured in 10 min. assays in the presence of Triton X-100 at the concentration shown on the abscissa. The amounts of cytoplasmic extract added corresponded to 0·5, 10·0, 16·7 and 28·5 mg. of original calvaria/ml. of incubation medium for acid phenylphosphatase, β-*N*-acetylglucosaminidase, catalase and β-glucuronidase respectively. Results are expressed as percentages of the highest observed activities.

In the other type, exemplified by the results of Fig. 3, the cytoplasmic extract was pretreated at 0° with various concentrations of Triton X-100, and appropriate amounts of this pretreated preparation were added to the substrate mixture for the measurement of free activity. The results obtained in both types of experiments were largely similar. With the exception of acid phenylphosphatase, the acid hydrolases were released in a closely parallel fashion, the free activity approaching the total activity at a detergent concentration of about 0·02%. Higher concentrations of Triton X-100 up to 0·1% were not inhibitory to the enzymes and the shape of the curves indicated clearly that the detergent concentration of 0·1% chosen for the assays of total activity was amply sufficient to unmask the latent hydrolases completely. Acid phenylphosphatase behaved differently in the two types of experiments. Its release occurred in parallel with that of the other hydrolases when Triton X-100 was added to the incubation mixture, but required higher detergent concentrations than did the other hydrolases when the cytoplasmic extract was pretreated with Triton X-100. Owing to the higher activity of phenylphosphatase, much smaller amounts of cytoplasmic extract were used for the assay of this enzyme than for that of the others. Consequently, the ratio of detergent to cytoplasmic extract was much higher in the former than in the latter type of experiments. Thus it appears that the release of acid phenylphosphatase requires a higher concentration of Triton X-100, other things being equal, than that of the other acid

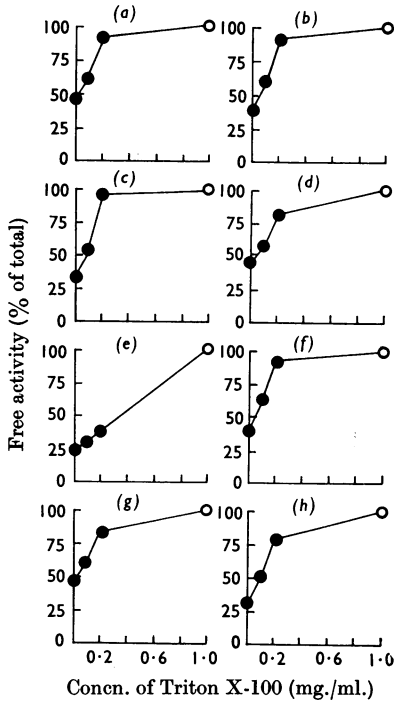

Fig. 3. Influence of pretreatment of cytoplasmic extract with Triton X-100 on free activities of (*a*) β-glucuronidase, (*b*) β-galactosidase, (*c*) β-*N*-acetylglucosaminidase, (*d*) acid β-glycerophosphatase, (*e*) acid phenylphosphatase, (*f*) acid deoxyribonuclease, (*g*) acid ribonuclease and (*h*) cathepsin. The detergent was added to the cytoplasmic extract of 0° up to a concentration of either 0·1 or 0·2 mg./ml. Appropriate amounts of these mixtures and of an untreated cytoplasmic extract were then used for the measurement of free activity (2 hr. assays). Total activities (shown by ○ on the graphs) were measured in the usual manner, in the presence of 1 mg. of Triton X-100/ml. in the incubation mixture. In all these experiments the total activities were essentially unaffected by the treatments.

hydrolases. As indicated by the results of Fig. 2, catalase shares this property since it required three times more detergent than β-glucuronidase to be fully released, even though it was assayed on little more than half the amount of cytoplasmic extract used for the hydrolase.

The results listed in Table 3 show that treatment of a cytoplasmic extract with 0·1% Triton X-100 caused the transfer of a considerable proportion of the acid-hydrolase activities from the particulate to the soluble phase. The proportion of the total activity found in soluble form in fully activated preparations varied from one enzyme to the other. As shown in Fig. 4, this proportion was directly correlated with the ratio of soluble to free activity

Table 3. *Influence of Triton X-100 on sedimentability of acid hydrolases*

Triton X-100, dissolved in ice-cold 0·25 M-sucrose, was added to a cytoplasmic extract to give a final concentration of 0·1%; another portion of the same extract was similarly diluted with 0·25 M-sucrose containing no Triton, to serve as control. Samples of both preparations were centrifuged at 3000000*g*-min. The supernatants were assayed for their total enzyme activities; these are given as percentages of the corresponding activities measured on non-centrifuged samples. Essentially the same total activities were recovered in both preparations, except for acid phenylphosphatase, which suffered a 30% inactivation in the Triton-treated extract.

	Unsedimentable activity (% of total)	
Enzyme	Control	Triton X-100 added
β-Glucuronidase	37·0	98·9
β-Galactosidase	22·0	67·5
β-*N*-Acetylglucosaminidase	12·0	53·9
Acid β-glycerophosphatase	21·6	94·6
Acid phenylphosphatase	10·9	101·2
Deoxyribonuclease	11·1	59·7
Ribonuclease	29·3	79·8
Cathepsin (pH 3·6)	37·4	97·1

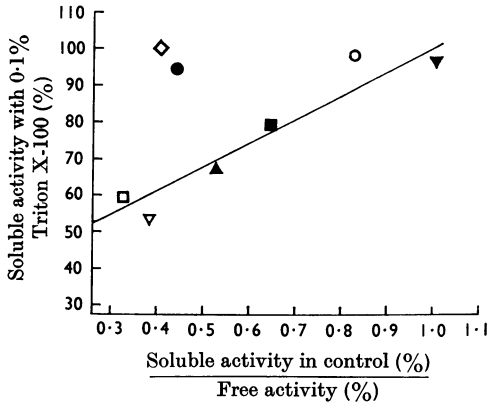

Fig. 4. Correlation between soluble/free activity ratios in preparations treated with 0·1% Triton X-100 and in untreated cytoplasmic extracts. Ordinate values are taken from second column of Table 3; values on abscissa are ratios of values in the first column of Table 3 to corresponding values in Table 1. The enzymes measured are: β-glucuronidase (○), acid β-glycerophosphatase (●), acid phenylphosphatase (◇), β-*N*-acetylglucosaminidase (▽), β-galactosidase (▲), acid deoxyribonuclease (□), acid ribonuclease (■) and cathepsin (▼). The regression line and the correlation coefficient, $r = 0{\cdot}95$, were calculated on all values represented, except those given for the two acid phosphatases.

in an untreated cytoplasmic extract for all the hydrolases except the two acid phosphatases. Such a correlation would be expected if differential adsorption of free enzyme molecules were largely responsible for the deficit of soluble as compared with free activity, and if this phenomenon were largely unaffected by Triton X-100. The results on acid phenylphosphatase are ambiguous since only 70% of the total original activity of this enzyme was recovered in the Triton-treated extract; the equivalence between soluble and free activity in the latter preparation could be spurious. Such is not the case for acid β-glycerophosphatase and it appears either that the adsorbed form of this enzyme is eluted by Triton X-100 or that some 25–30% of its total activity occurs in a particulate form that is both accessible to substrate under the conditions of the free activity measurements and released in soluble non-adsorbed form by Triton X-100.

Effect of lowering the osmotic pressure of the medium. The results in Figs. 5 and 6 illustrate the manner in which the free activity of the various enzymes under study is affected by a temporary dilution of the cytoplasmic extract with distilled water to lower the concentrations of sucrose. With the exception of acid phenylphosphatase, which shows relatively little release even in 0·033M-sucrose, the acid hydrolases all exhibit a progressive loss of latency as the concentration of sucrose is decreased. Their osmotic-activation curves run roughly parallel with each other, except that the enzymes acting on small molecules appear to be unmasked somewhat more readily than those acting on macromolecular substrates. This difference could reflect a gradation in the permeability changes of the osmotically swollen particles. In contrast with the acid hydrolases, the latency of catalase was entirely unaffected by exposure to media of low osmotic pressure. The activity of

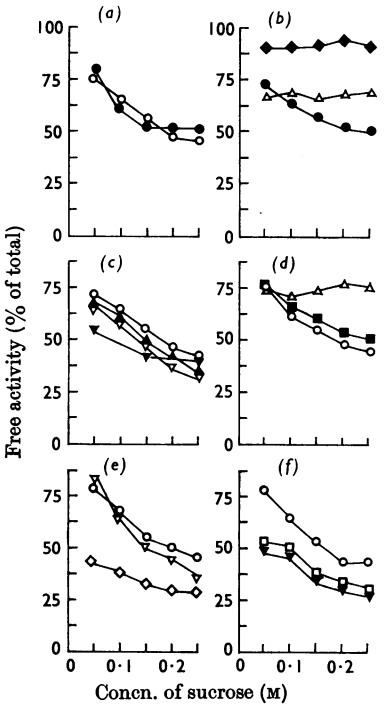

Fig. 5. Influence of decreasing osmotic pressure on free activities of acid hydrolases and of catalase. Cytoplasmic extracts were diluted with distilled water to give the sucrose concentrations shown on the abscissa, kept for 30 min. at 0° and then brought back to the original sucrose concentration by the addition of concentrated sucrose solution. They were then assayed for free and total enzyme activities in 2 hr. assays (10 min. assay for acid phenylphosphatase). Each graph refers to a separate experiment. The enzymes measured are: β-glucuronidase (○), acid β-glycerophosphatase (●), acid phenylphosphatase (◇), β-*N*-acetylglucosaminidase (▽), β-galactosidase (▲), acid deoxyribonuclease (□), acid ribonuclease (■), cathepsin (▼), alkaline phenylphosphatase (◆) and catalase (△). Results are expressed as percentages of the corresponding total activities; in all these experiments the total activities were essentially unaffected by the treatments.

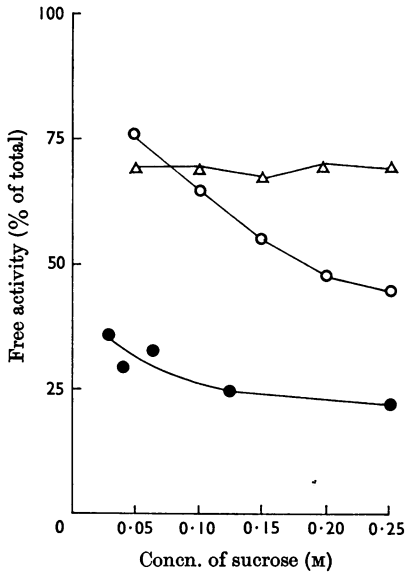

Fig. 6. Differential response to exposure of cytoplasmic extract to media of low osmotic pressure of latent β-glucuronidase (○), acid phenylphosphatase (●) and catalase (△). The experimental procedure was as given in Fig. 5. Values shown are averages of eight, two and three separate experiments for β-glucuronidase, acid phenylphosphatase and catalase respectively.

alkaline phenylphosphatase, which exhibits practically no latency under normal conditions, was also unchanged by this treatment.

As shown by the results in Table 4, exposure of bone particles to distilled water led to a partial release of their acid hydrolases in soluble form. As could be expected from the weak responses observed for this enzyme in the experiments shown in Figs. 5 and 6, the proportion of total activity brought into solution in this manner was lowest for acid phenylphosphatase. It varied for the other enzymes roughly according to their ability to occur in a high-speed supernatant from particles resuspended in 0·25 M-sucrose (Table 4) or from cytoplasmic extracts, either untreated or exposed to 0·1% Triton X-100 (Fig. 4). Therefore secondary adsorption is probably mainly responsible for these differences.

Effect of treatment in the Waring Blendor. Treatment of a cytoplasmic extract in the Waring Blendor for increasing lengths of time led to a progressive loss of latency of all eight acid hydrolases (Figs. 7 and 8) and to their partial release in soluble form (Table 4). Except for β-glucuronidase, which behaved unusually in this respect, the proportion of activity rendered unsedimentable by this treatment varied with the degree of adsorbability of the enzymes. Catalase was also released by treatment in the Waring Blendor (Fig. 8). The time-course of the release was essentially the same for all enzymes studied, including catalase.

Effect of freezing and thawing. Freezing and thawing caused a roughly parallel release of all acid hydrolases (Fig. 7) and of catalase (Fig. 9). The activity of alkaline phenylphosphatase was unaffected by this treatment.

Table 4. *Influence of exposure to distilled water and of treatment in the Waring Blendor on sedimentability of acid hydrolases*

These experiments were performed on particulate fractions (M+L) isolated from a cytoplasmic extract as described by Vaes & Jacques (1965*b*). After the last washing, pellets containing equal amounts of particles were resuspended in 0·25 M-sucrose (control sample) or in distilled water for the first experiment. In the other experiment, the particles were taken up in 0·25 M-sucrose and a sample of the suspension was treated for 5 min. in a cooled Waring Blendor. Further manipulations were performed as described in the experiments of Table 3. Treatment with distilled water caused a 35% inactivation of acid phenylphosphatase and appeared to increase the total activity of acid ribonuclease by 25%; it did not significantly affect the total activities of the other enzymes. After treatment in the blender, 5–10% losses were recorded for all enzymes except acid phenylphosphatase, which suffered a 55% inactivation.

	Unsedimentable activity (% of total)			
	Exposure to water		Treatment in Waring Blendor	
Enzyme	Control	Treated	Control	Treated
β-Glucuronidase	43·3	67·1	21·1	35·2
β-Galactosidase	32·9	52·2	20·8	62·6
Acid phenylphosphatase	6·6	22·2	1·5	43·7
Deoxyribonuclease	14·2	29·9	8·5	31·1
Ribonuclease	44·7	62·5	20·5	79·8
Cathepsin	47·8	65·1	29·1	82·5

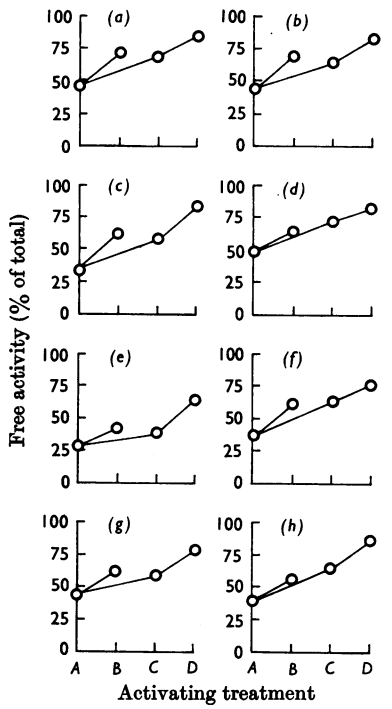

Fig. 7. Influence of treatment in the Waring Blendor and of freezing and thawing on free activities of acid hydrolases in cytoplasmic extract (1 g. of tissue/10 ml.). The enzymes measured are: (*a*) β-glucuronidase, (*b*) β-galactosidase, (*c*) β-*N*-acetylglucosaminidase, (*d*) acid β-glycerophosphatase, (*e*) acid phenylphosphatase, (*f*) acid deoxyribonuclease, (*g*) acid ribonuclease and (*h*) cathepsin. The graphs summarize three separate experiments (B, C and D). Letters on the abscissa refer to: *A*, initial free activity (mean of the three experiments); *B*, free activity measured after freezing the cytoplasmic extracts once in propan-2-ol–solid CO_2 and thawing; *C* and *D*, free activity measured after treatment of the cytoplasmic extract in a cooled Waring Blendor for 1 min. (*C*) and 5 min. (*D*). Values are expressed as percentages of the corresponding total activities. These were essentially unaffected by the treatments, except for acid phenylphosphatase, which suffered 15–25% inactivation according to the experiment.

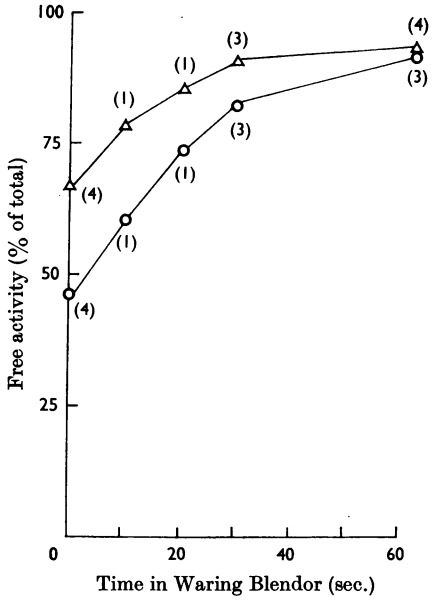

Fig. 8. Influence of time of treatment in the Waring Blendor on free activities of β-glucuronidase (○) and catalase (△) in cytoplasmic extract (0·08–0·1g. of tissue/100ml.). Each point is the average of the number of experiments shown in parentheses on the graph. Values are expressed as percentages of the corresponding total activities. These were essentially unaffected by the treatments.

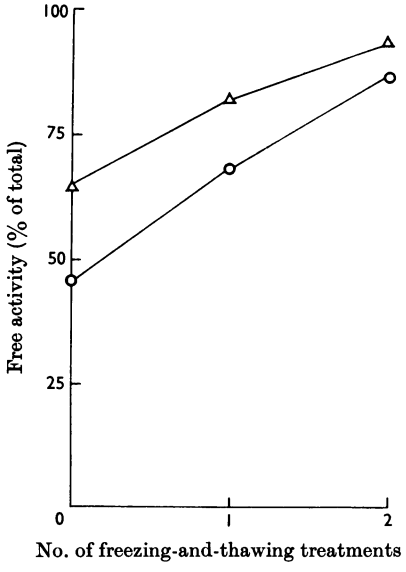

Fig. 9. Influence of freezing and thawing a cytoplasmic extract once or twice on free activities of β-glucuronidase (○) and catalase (△). Values are averages from two experiments and are given as percentages of the corresponding total activities. These were essentially unaffected by the treatment.

Effect of substrate concentration. As shown by the results in Table 5, increasing the substrate concentration over a 10-fold range, corresponding to a number of multiples of the Michaelis constant of the enzymes, did not significantly alter the ratio of free to total activity of the three acid hydrolases studied in this regard, namely acid phenylphosphatase, β-glucuronidase and β-*N*-acetylglucosaminidase (β-*N*-acetylaminodeoxyglucosidase).

DISCUSSION

The experiments described in this paper show that eight distinct acid-hydrolase activities occur in partly latent form in cytoplasmic extracts of bone tissue and that latency belongs exclusively to the particle-bound form of the enzyme. In the preceding paper (Vaes & Jacques, 1965*b*) it was concluded from the results of various fractionation experiments that the acid hydrolases of bone are not associated with the mitochondria as characterized by cytochrome oxidase, nor with the microsomes, which in this tissue are the main bearers of alkaline phenylphosphatase, but with a special group of cytoplasmic particles believed to be analogous to the lysosomes first identified in rat liver and now known to exist in numerous other tissues. Whenever they have been investigated in this respect, lysosomes have been found to exhibit structure-linked latency. As demonstrated here, this is also true of bone lysosomes.

It has been pointed out (see, e.g., Fig. 4) that the free activity of the acid hydrolases in a given preparation generally exceeds the activity recovered in the high-speed supernatant isolated from this preparation, to an extent that varies from one enzyme to the other but appears to be relatively constant for each enzyme irrespective of the treatment to which the preparation had been subjected (see, however, the acid phosphatases after exposure to Triton X-100, and β-glucuronidase after treatment in the blender). This difference could indicate either that all the molecules of particle-bound enzymes are partly reactive or that there exist two types of binding, one suppressing the enzyme activity completely, the other leaving it unaffected. The results in Table 5 show that, if the former hypothesis is correct, the postulated partial restriction imposed on the activity of the enzymes by their association with their host-particles does not depend on a limited accessibility to external substrates since it cannot be overcome by increasing the substrate concentration. Further, to accept this interpretation makes it necessary to postulate a third type of binding without effect on the enzyme activity, to account for the partial sedimentability of several enzymes in preparations fully activated by Triton X-100. For these reasons,

Table 5. *Influence of substrate concentration on free and total activities of acid hydrolases*

Results were obtained in 10 min. assays on cytoplasmic extracts and are expressed in units/g. of tissue.

Concn. of substrate (mM)	Acid phenylphosphatase			β-Glucuronidase			β-*N*-Acetylglucosaminidase		
	Total activity	Free activity	$\frac{\text{Free}}{\text{Total}} \times 100$	Total activity	Free activity	$\frac{\text{Free}}{\text{Total}} \times 100$	Total activity	Free activity	$\frac{\text{Free}}{\text{Total}} \times 100$
0·5	—	—	—	0·134	0·063	47·2	—	—	—
1·0	2·52	0·67	26·7	—	—	—	0·48	0·165	34·6
2·0	2·88	0·78	27·2	—	—	—	—	—	—
2·5	—	—	—	0·150	0·068	45·3	—	—	—
4·0	4·92	1·01	20·6	—	—	—	—	—	—
5·0	—	—	—	0·160	0·070	43·4	0·855	0·242	28·3
6·0	5·81	1·28	22·1	—	—	—	—	—	—
8·0	5·40	1·31	24·3	—	—	—	—	—	—
9·0	—	—	—	—	—	—	1·03	0·295	28·6
10·0	5·44	1·30	23·9	—	—	—	—	—	—

and by analogy with what is known of hepatic lysosomes, it appears much more likely that the enzymes are completely latent in their native particle-bound form and that the excess of sedimentable over latent activity is due to adsorbed fully active enzyme molecules. Though this conclusion is borne out by the type of correlation illustrated in Fig. 4, the possibility must be kept in mind that particles undergoing progressive structural injury may pass through a stage where their enzymes become accessible to outside substrates, especially of low molecular weight, but have not yet been released in soluble form. This possibility has been mentioned in connexion with the results of the osmotic-activation experiments.

In their response to the various treatments that suppress enzyme latency, bone lysosomes behave very much like liver lysosomes. In particular, they are susceptible to osmotic disruption and are sensitive to treatments known to affect the integrity of lipoproteins. These properties suggest a sac-like structure of the type postulated for the liver lysosomes, with a peripheral membrane acting as a permeability barrier between the internal enzymes and external substrates. The results in Table 5 are consistent with this interpretation and indicate further that the membrane must be considered essentially impermeable to the substrates and that this property suffices to account entirely for the latency of the enzyme. Were it not so, raising the substrate concentration by several multiples of the Michaelis constant of the enzymes should, as pointed out by de Duve (1965), increase the free activity in a significant manner. Such an increase failed to occur for three distinct enzymes (Table 5). Bone lysosomes appear to differ from liver lysosomes by a greater stability at pH 5 and 37°. They resemble those of spleen and thymus in this respect (Rahman, 1964).

Considerable heterogeneity has been attributed to bone lysosomes on the basis of the differences in distribution observed for their individual enzymes in fractionation experiments, especially by density-gradient centrifugation (Vaes & Jacques, 1965*b*). Some heterogeneity also shows up in the present experiments, but, on the whole, similarities rather than differences in behaviour appear to characterize the latent hydrolases of bone. This is shown by the correlations shown in Fig. 10. According to these graphs, the closest similarities exist between β-glucuronidase, β-glycerophosphatase, β-galactosidase and, with somewhat greater scattering, acid ribonuclease on one hand, and between cathepsin, β-*N*-acetylglucosaminidase and acid deoxyribonuclease on the other. These two groups differ by the proportion of free activity found in the cytoplasmic extract, which is some 10–15% higher for the former than for the latter (see Table 1 and Fig. 10). These differences, like those observed between the distribution patterns of the enzymes, are most easily explained on the basis of the existence of more than one population of lysosomes in bone, a very likely possibility in view of the known cellular heterogeneity of the tissue. Biochemical and physical heterogeneity within each population may also exist. In addition, the possibility must be kept in mind that bone tissue may contain lysosomal enzymes in free form, especially in the interstitial spaces at the sites of bone resorption; the extracellular hydrolases could occur in unequal amounts owing to differential release, inactivation or clearance of the various enzymes. Finally, some of the activities measured could be due partly to non-lysosomal enzymes present in the cell sap or associated with some other intracellular structure.

The behaviour of acid phenylphosphatase poses a special problem. Studies of this enzyme are greatly complicated by its instability, but this factor alone

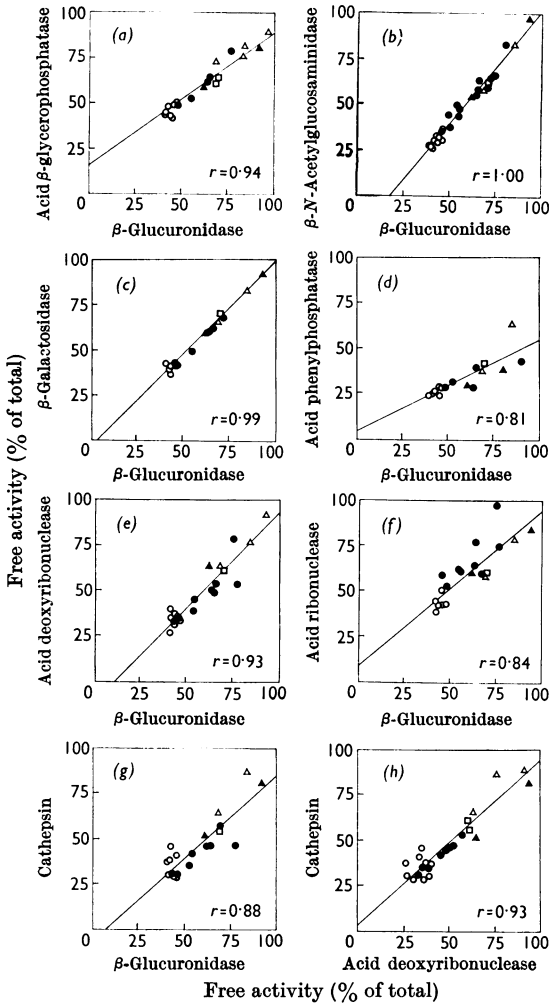

Fig. 10. Correlation coefficients, *r*, between free activities of acid hydrolases in untreated cytoplasmic extract (○) and in cytoplasmic extracts exposed to decreasing sucrose concentrations (●), treated with increasing amounts of Triton X-100 (▲), frozen and thawed once or twice (□) or subjected to the action of a Waring Blendor for increasing lengths of time (△). The free activities were measured in 2hr. assays (10min. assay for acid phenylphosphatase) and expressed as percentages of the corresponding total activities.

cannot account for the differences that distinguish it from the other acid hydrolases. The simplest explanation suggested by the graph of Fig. 10 is that about half the acid-phenylphosphatase activity of bone is associated with typical lysosomes and that the other half belongs to another group of particles exhibiting similar latency properties, but differing from the lysosomes by a greater resistance to several of the disruptive treatments investigated. It is not incompatible with the present results and with those reported by Vaes & Jacques (1965*a*) that the lysosomal acid phenylphosphatase is identical with the acid β-glycerophosphatase, and that another more labile enzyme species is responsible for the other half of the activity. It is well known and has again been emphasized by Neil & Horner (1964) that in liver phenyl phosphate is susceptible to hydrolysis at pH 5 by numerous enzymes, several of which may be present elsewhere than in lysosomes, whereas the activity of β-glycerophosphate appears to reside mostly in these particles. This seems to be true also for bone.

The existence in bone tissue of lysosome-like particles endowed with a rich complement of acid hydrolases raises the problem of the physiological function of these particles. A role in bone resorption comes to mind immediately, especially since, as pointed out by Vaes & Jacques (1965*a*), this process appears to be associated with the formation of acid. The lysosomal enzymes, possibly including the collagenolytic activity reported by Walker, Lapière & Gross (1964), are probably the agents of an extracellular digestion of the organic matrix of bone. They have indeed been shown to be secreted, together with acid, into the extracellular spaces of bone undergoing active resorption in an organ-culture system under the effect of parathyroid extract (Vaes, 1965). In addition, in other cell types, lysosomes are known to be involved in the intracellular digestion of exogenous materials engulfed by phagocytosis or pinocytosis (de Duve, 1963*a*,*b*); they could function similarly in osteoclasts since these cells show evidence of active phagocytosis of fragments of matrix (Hancox & Boothroyd, 1963; Hancox, 1963). Finally, it must be remembered that the other bone cells besides the osteoclasts contain cytochemically demonstrable acid hydrolases (Tonna, 1958; Schajowicz & Cabrini, 1958; Burstone, 1959; Schlager, 1959; Rose, 1961; Cabrini, 1961); lysosomes could accomplish other specialized functions in these cells.

In contrast with the acid hydrolases, latent catalase was entirely unaffected by exposure to media of low osmotic pressure; it also required higher concentrations of Triton X-100 for its release than the hydrolytic enzymes. These properties recall those of the latent catalase of liver (Baudhuin, 1964, and personal communication; Miller, 1964), which is known to be associated, together with urate oxidase and D-amino acid oxidase, with a special group of particles different from the lysosomes and identified with the so-called 'microbodies' (Baudhuin, Beaufay & de Duve, 1965). A non-mitochondrial localization of catalase in bone has been inferred from the results of

fractionation experiments (Vaes & Jacques, 1965*b*) and the present results therefore raise the possibility that the bone enzyme may be associated with particles related to the hepatic microbodies. However, it must be remembered that the particle-bound catalase of bone does not exhibit the same high equilibrium density in an aqueous sucrose gradient as the liver particles.

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REFERENCES

Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
Baudhuin, P. (1964). *Abstr. 6th int. Congr. Biochem., New York*, vol. 32, p. 296.
Baudhuin, P., Beaufay, H. & de Duve, C. (1965). *J. Cell Biol.* **26**, 219.
Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. & de Duve, C. (1964). *Biochem. J.* **92**, 179.
Burstone, M. S. (1959). *J. Histochem. Cytochem.* **7**, 39.
Cabrini, R. L. (1961). *Int. Rev. Cytol.* **11**, 284.
de Duve, C. (1963*a*). *Sci. Amer.* **208**, 604.
de Duve, C. (1963*b*). In *Ciba Found. Symp.: Lysosomes*, p. 1. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.
de Duve, C. (1965). *Harvey Lect.* **59**, 49.
de Duve, C., Beaufay, H., Jacques, P., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R. & De Coninck, S. (1960). *Biochim. biophys. Acta*, **40**, 186.
Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
Hancox, N. M. (1963). In *Cinemicrography in Cell Biology*, p. 247. Ed. by Rose, G. G. New York: Academic Press Inc.
Hancox, N. M. & Boothroyd, B. (1963). In *Mechanisms of Hard Tissue Destruction*, p. 497. Ed. by Sognaes, R. F. Washington: American Association for the Advancement of Science.
Miller, L. A. (1964). *Exp. Cell Res.* **34**, 283.
Neil, M. W. & Horner, M. W. (1964). *Biochem. J.* **93**, 220.
Rahman, Y. E. (1964). *Biochim. biophys. Acta*, **90**, 440.
Rose, G. G. (1961). *J. biophys. biochem. Cytol.* **9**, 463.
Schajowicz, F. & Cabrini, R. L. (1958). *Science*, **127**, 1447.
Schlager, F. (1959). *Acta histochem., Jena*, **8**, 176.
Tonna, E. A. (1958). *J. Gerontol.* **13**, 14.
Vaes, G. (1965). *Exp. Cell Res.* (in the Press).
Vaes, G. & Jacques, P. (1965*a*). *Biochem. J.* **97**, 380.
Vaes, G. & Jacques, P. (1965*b*). *Biochem. J.* **97**, 389.
Walker, D. G., Lapière, C. M. & Gross, J. (1964). *Biochem. biophys. Res. Commun.* **15**, 397.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.