Studies on Bone Enzymes

THE ASSAY OF ACID HYDROLASES AND OTHER ENZYMES IN BONE TISSUE

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1. Nine acid hydrolases, cytochrome oxidase, alkaline phenylphosphatase and catalase were demonstrated in 0.25 M-sucrose homogenates of newborn-rat calvaria. The acid hydrolases were: acid phenylphosphatase, acid β -glycerophosphatase, β -glucuronidase, β -N-acetylglucosaminidase (β -N-acetylaminodeoxyglucosidase), acid ribonuclease and acid deoxyribonuclease, showing optimum activity at about pH 5; cathepsin, β -galactosidase and hyaluronidase, with optimum activity at about pH 3.6. 2. The main kinetic characters of these enzymes have been studied and methods for their quantitative assay have been worked out. The activities present in bone are given and compared with those found in liver. 3. Acid-phosphatase activity was assayed with phenyl phosphate and β -glycerophosphate as substrates: activities with these two substrates appeared to be due to two different enzymes. Acid phenylphosphatase is particularly labile and is readily inactivated by various physical or chemical agents.

Active tissue destruction occurs in bone during normal development or as a consequence of the action of parathyroid hormone (McLean & Urist, 1961). In this process both mineral and organic matrix seem to be removed almost simultaneously (McLean & Urist, 1961; Cameron, 1963; Hancox & Boothroyd, 1963). It is currently thought that the production and secretion of organic acids by bone cells (Borle, Nichols & Nichols, 1960; Vaes & Nichols, 1961, 1962) render the bone mineral soluble (Nordin, 1957; Neuman & Neuman, 1958; Schartum & Nichols, 1962). Enzymes are believed to be the agents of the removal of the organic matrix, but so far data about hydrolytic enzymes in bone cells have been limited to only a small number of enzymes and obtained mostly through histochemical studies.

A study of hydrolytic enzymes in bone cells was therefore undertaken. Effort was directed towards the demonstration of hydrolases active in the acid range, since a low pH is believed to exist at resorption sites. Nine acid hydrolases were investigated. Their demonstration, kinetics and methods of assay are the object of the present paper; studies on their intracellular distribution, activation properties and physiological role are reported by Vaes & Jacques (1965) and Vaes (1965b). Preliminary accounts of this work have already been published (Vaes, 1964, 1965a).

EXPERIMENTAL

Tissue fractionations

The experiments were carried out on infant Wistar rats of either sex and up to 7 days of age (2–5 days in most cases). The animals were killed by decapitation; their calvaria (bones of the cranial vault, including the frontal, the occipital and the two parietals) were carefully dissected out, cleaned free of any adhering muscular or connective tissue and immersed in ice-cold 0.25 M-sucrose. The pooled calvaria were finely minced with scissors and then homogenized in 2–3 ml. of 0.25 M-sucrose with a motor-driven all-glass homogenizer (Potter & Elvehjem, 1936). The homogenate was centrifuged at 6000g-min. at 0° in an International refrigerated centrifuge, yielding a cell-free supernatant ('cytoplasmic extract') and a sediment containing most of the nuclei, together with unbroken cells, erythrocytes, gross cell debris, connective-tissue elements and solid bone mineral (N fraction). The cytoplasmic extract was used for the studies on the kinetic properties and assays of the enzymes. The whole unfractionated homogenate was used only for the determination of the total enzyme activities present in calvaria.

Further fractionation of the cytoplasmic extract by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) was done in some experiments to yield particulate fractions sedimenting at 250 000g-min. (M + L fraction) or 3000 000g-min. (M + L + P fraction); the supernatant of the latter centrifugation was called the S fraction.

In a few comparative experiments, metaphyseal and diaphyseal bones were used: metaphyses from the distal

end of the femur and from the proximal end of the tibia and diaphyses from these two bones were carefully dissected and freed as much as possible from marrow and blood cells before being washed in 0.25 M-sucrose and homogenized.

Enzyme assays and analytical procedures

Most of the techniques used for the enzyme assays were based on those used for liver tissue by de Duve and collaborators (Appelmans & de Duve, 1955; Gianetto & de Duve, 1955; de Duve *et al.* 1955; Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960; Baudhuin *et al.* 1964), due account being taken of the kinetic properties of the calvaria enzymes. The enzymic tests were carried out at 37° for all the enzymes, except for cytochrome oxidase, which was determined at 25°. Acetate or citrate buffers were generally used: they were prepared by mixing in various proportions equimolar solutions of acetic acid or citric acid and sodium acetate or sodium citrate. Activities are expressed in units/g. of tissue, one unit referring to the decomposition of 1 μ mole of substrate/min. unless stated otherwise.

The experiments reported by Vaes (1965b) show that the acid hydrolases and catalase are largely latent in the cytoplasmic extract and in particulate preparations and that they are fully unmasked by 0.1% Triton X-100. In the present experiments, which deal exclusively with the measurements of total enzymic activities, Triton X-100 (0.1%) has been added in all assays of these enzymes. It was verified that this detergent was without effect at this concentration on the soluble enzymes of the S fraction. The detergent must of course be omitted in studies on latency.

Appropriate blanks, in which the enzyme (or, for acid β -glycerophosphatase, the substrate) was added to the incubation flasks only after the enzymic reaction was stopped, were always run in parallel to the tests and subtracted from the observed values. When the incubation period exceeded 4 hr., a bacteriostatic agent was added to the flasks: this was 0.01% sodium merthiolate, except for β -galactosidase for which 0.1% NaN₃ was used. It was always verified that, with the method used, the measured activities were proportional within reasonable limits to both enzyme concentration and incubation time. The ranges of validity of the assays are shown in Table 1.

DNA was determined by the method of Schneider (1957), and protein by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Acid phosphatase. Determinations of acid-phosphatase activity were done by two different methods.

In one, the amount of phosphate liberated from β -glycerophosphate (β -glycerophosphatase) was measured. This activity was assayed in a total volume of 0.5 ml. in the presence of 0.05 M- β -glycerophosphate and 0.1 M-acetate buffer, pH 5.0. The reaction was stopped by the addition of 2.5 ml. of 8% (w/v) trichloroacetic acid. Inorganic phosphate was determined on 0.5–1.5 ml. of filtrate (by the method of Marinetti, Aldrecht, Ford & Stotz, 1959). The extinction coefficient of the inorganic phosphate standard was 2.7×10^7 cm.²/mole at 820 m μ .

Owing to the high tissue blanks, the assay could not be used with large amounts of enzyme preparation. In the latter case, phenyl phosphate was used as substrate ('phenylphosphatase') and the phenol liberated was determined. This phenylphosphatase activity was measured in 0.1 M-

Table 1. *Range of validity of enzyme assays*

The values given represent maximum amounts of tissue and maximum incubation times for which linearity with respect to both variables was observed. When followed by 'at least', the limits indicated correspond to the highest values tested experimentally.

Enzyme	Amount of calvaria (cytoplasmic extract)	Duration of incubation
Acid phenylphosphatase	20 mg. at least	30–60 min.
Acid β -glycerophosphatase	12 mg.	4–6 hr.
β -Glucuronidase	40 mg. at least	26 hr. at least
β -N-Acetylglucosaminidase	30 mg. at least	6 hr.
β -Galactosidase	40 mg. at least	6 hr.
Acid deoxyribonuclease	33 mg.	28 hr. at least
Acid ribonuclease	60 mg.	5 hr.
Cathepsin	8 mg.	6 hr.
Hyaluronidase	200 mg. at least	20 min.
Alkaline phenylphosphatase	0.6 mg.	20 min.
Cytochrome oxidase	10 mg. at least	3 min. at least
Catalase	8 mg.	30 min.

acetate buffer, pH 5.0, with 0.02 M-phenyl phosphate in a total volume of incubation of 1 ml. The reaction was stopped by the addition of 0.5 ml. of 0.5 N-NaOH containing EDTA (0.01 M). The tubes were then kept at room temperature for at least 15 min. to allow complete destruction of the alkaline phosphatase present in the enzyme preparations. They could then be stored, if necessary, for several hours at 0° before the determination of free phenol by its reaction with aminoantipyrine (Kind & King, 1954). The extinction of the final coloured product was read at the maximum absorption wavelength, 510 m μ , exactly 5 min. after addition of the ferricyanide, as the final colour appeared to fade with increasing time. The extinction coefficient of a phenol standard treated under these conditions was 1.3×10^7 cm.²/mole.

β -Glucuronidase. β -Glucuronidase was measured in a total volume of 1 ml. of 0.1 M-acetate buffer, pH 5.0, with 1.5 mm-phenolphthalein glucuronidate as substrate. The reaction was stopped by the addition of 3.0 ml. of a solution containing glycine (0.133 M), NaCl (0.067 M) and Na₂CO₃ (0.083 M), pH 10.7. The mixture was filtered and its phenolphthalein content was determined at its maximal absorption wavelength, 555 m μ . All readings were made at the same time after the addition of the glycine-carbonate buffer as the colour appeared to fade slowly with time (about 5% loss in 1 hr.). Phenolphthalein standards were treated identically in the presence of the other components of the test mixture since some of these (Triton X-100, sodium merthiolate) modified slightly the extinction of free phenolphthalein. The extinction coefficient of phenolphthalein thus varied somewhat according to the conditions of the test: values obtained were about 2.6×10^7 cm.²/mole.

β -N-Acetylglucosaminidase. Determinations of β -N-acetylglucosaminidase (β -N-acetylaminodeoxyglucosidase) were carried out in a total volume of 1 ml. of 0.1 M-citrate buffer, pH 5.0, with 8 mm-o-nitrophenyl N-acetyl- β -D-glucosaminide as substrate. The reaction was stopped by

the addition of 1.5 ml. of 2.75% (w/v) trichloroacetic acid and the liberated *o*-nitrophenol was measured as done by Sellinger *et al.* (1960). The colour exhibited by *o*-nitrophenol was stable for at least 2 hr.; it was read at 420 m μ , the maximal absorption wavelength. The extinction coefficient of *o*-nitrophenol was under these conditions 4.24×10^6 cm.²/mole.

β -*N*-Acetylglucosaminidase and β -glucuronidase could be measured simultaneously in a single test tube by adding 8 mm-*o*-nitrophenyl *N*-acetyl- β -D-glucosaminide to the incubation mixture of β -glucuronidase and by reading at both 420 and 555 m μ the colour obtained after the addition of the glycine-carbonate buffer, pH 10.7, and filtration: *o*-nitrophenol did not absorb at 555 m μ , and suitable corrections could easily be made for the absorption of phenolphthalein at 420 m μ which amounted to about 1–3% of its absorption at 555 m μ .

β -Galactosidase. β -Galactosidase was assayed like β -*N*-acetylglucosaminidase. The enzyme was incubated in a total volume of 1.0 ml. of 0.1M-citrate buffer, pH 3.6, with 3.75 mm-*o*-nitrophenyl β -D-galactoside as substrate. The reaction was stopped by the addition of 1.5 ml. of 2.75% trichloroacetic acid and the *o*-nitrophenol was measured as for the preceding enzyme.

Acid deoxyribonuclease. Determinations of acid deoxyribonuclease were done in a total volume of 1 ml. of 0.2M-acetate buffer, pH 5.0, with 0.9 mg. of purified deoxyribonuclease as substrate. The reaction was stopped by the addition of 1 ml. of ice-cold 10% (w/v) HClO₄. Assay of the breakdown products and expression of enzyme activity were as done by de Duve *et al.* (1955).

Increasing the duration of incubation or the concentration of enzyme gave the same kinetic curve characterized by a slight initial lag in attaining a constant rate (Fig. 1).

Fig. 1. Kinetics of acid deoxyribonuclease. Results of different experiments in which enzyme concentration (●) or incubation time (○) were varied are shown.

The results became less reliable when hydrolysis proceeded beyond a certain point. For these reasons, the assay gave the best results when between 0.1 and 0.8 μ mole of mononucleotides had been set free.

Acid ribonuclease. Acid ribonuclease was assayed in the same manner as deoxyribonuclease (de Duve, *et al.* 1955) by incubation of enzyme in a total volume of 1 ml. of 0.1M-citrate buffer, pH 5.0, with 3 mg. of purified ribonuclease as substrate. The reaction was stopped by the addition of 1 ml. of ice-cold 20% (w/v) HClO₄ containing 0.25% of uranyl acetate.

Cathepsin. Haemoglobin-splitting cathepsin activity (referred to as 'cathepsin') was assayed at pH 3.6 in a total volume of 0.5 ml. of 0.55 mm- (or 3.75%, w/v) haemoglobin in 0.35N-acetic acid. The reaction was stopped by the addition of 1.5 ml. of ice-cold 4.6% (w/v) trichloroacetic acid; the mixture was immediately cooled in ice and filtered in the refrigerator. Aromatic degradation products were measured in 0.5 ml. of filtrate by means of the Folin-Ciocalteu reagent as in the original method of Anson (1937) and the final coloured product was estimated spectrophotometrically at the maximal absorption wavelength, 730 m μ . Tyrosine was used as standard and the molarity of the products of cathepsin action was expressed conventionally in terms of tyrosine equivalents of the colour developed with the Folin-Ciocalteu reagent. An extinction coefficient of 1.26×10^7 cm.²/mole was found for tyrosine under these conditions.

Greater sensitivity was obtained by measuring the products of the reaction by the protein method of Lowry *et al.* (1951). This modification made it possible to measure cathepsin at pH 5.0, as is required for instance for the assay of latent activity. Haemoglobin was denatured by incubating for 30 min. at 37° a solution brought to pH 3.6 by the addition of acetic acid; it was subsequently freed of acetic acid by dialysis against water. The catheptic activity was assayed by incubating the enzyme in a total volume of 0.5 ml. of 0.1M-acetate buffer, pH 5.0, with 0.34 mm- (or 2%, w/v) denatured haemoglobin as substrate. The reaction was stopped by the addition of 1 ml. of ice-cold 5.2% (w/v) trichloroacetic acid. The mixture was filtered in the refrigerator; 0.2 ml. of 1.25N-NaOH was then added to 0.2 ml. of filtrate and the degradation products of haemoglobin were measured by the method of Lowry *et al.* (1951).

Hyaluronidase. The viscosimetric method of Tolksdorf (1954) served as the basis for the assay of hyaluronidase.

The enzyme was added to 3 ml. of an incubation medium containing potassium hyaluronate (0.2–0.3%), acetate buffer, pH 3.7 (0.1M), and NaCl (0.15M). Then 2.5 ml. samples of this mixture were quickly transferred by means of a long Pasteur pipette to miniature U-tube viscosimeters (BS/U/M; size M4) preheated at 37° in an all-glass water bath. Flow times, f_x , were read at intervals of 2–3 min. for 20–30 min.: dividing f_x by the flow time, f_0 , obtained in the same viscosimeter with the incubation medium without hyaluronate gave the relative viscosity, η_{rel} . Values of η_{rel} at 0 and 20 min. were obtained graphically from a plot of η_{rel} against incubation time (corrected for the time needed for the viscosity measurement by adding $f_x/2$). One unit of activity was defined as the amount of enzyme which under the conditions of the test reduces the viscosity of the substrate to half its value in 20 min. The reaction rate remained constant for at least 20 min. if the enzyme activity was lower than 0.25 unit; with higher enzyme activities, the

activity was measured by extrapolating the initial slope of the plot of relative viscosities against time to 20 min. Controls were incubated in the absence of enzyme with the substrate and other constituents of the incubation mixture: flow times of these controls remained constant for up to 12 hr. Heating the cytoplasmic extract at 60° for 15 min. resulted in a complete loss of the capacity of the enzyme preparation to decrease the flow time of the substrate.

Confirmation of the presence of hyaluronidase in bone was also obtained by determination of the *N*-acetylhexosamines (Reissig, Strominger & Leloir, 1955) or of reducing sugars (Park & Johnson, 1949) liberated from hyaluronate by a cytoplasmic extract. These methods are, however, much less sensitive than the viscosimetric method chosen for the assays. With both the viscosimetric method and the two chemical methods, the activity of a cytoplasmic extract obtained from 1 g. of bone tissue was equivalent to approx. 10 U.S.P. units of purified hyaluronidase.

Alkaline phenylphosphatase. Alkaline phenylphosphatase was assayed like acid phenylphosphatase in 1 ml. of incubation mixture containing 0.02 M-phenyl phosphate in 0.1 M-glycine buffer brought to pH 9.9 with NaOH. Under these conditions of assay the enzyme activity was not affected by the ionic strength.

Cytochrome oxidase. Cytochrome oxidase was assayed at pH 7.4 and 25° by the spectrophotometric method of Cooperstein & Lazarow (1951) in the manner described by Appelmans, Wattiaux & de Duve (1955), but in total volumes of either 2 or 2.2 ml. One unit of activity is defined as the amount of enzyme causing the logarithm of the concentration of reduced cytochrome *c* to decrease by 1 unit/min. in 100 ml. of incubation mixture.

Catalase. Catalase was assayed at 37° in 0.02 M-imidazole buffer, pH 7.0, in the manner described by Baudhuin *et al.* (1964); the total volume of the incubation mixture was 3.6 ml. The unit of activity is defined as the amount of enzyme causing the logarithm of the concentration of H₂O₂ to decrease by 1 unit/min. in 50 ml. of incubation mixture.

Materials

Phenyl phosphate (disodium salt), β -glycerophosphate (disodium salt), NaNO₃ and aq. H₂O₂ were obtained from E. Merck A.-G., Darmstadt, Germany; naphthol-AS-BI-phosphate was from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.; cytochrome *c*, *o*-nitrophenyl β -D-galactopyranoside, potassium hyaluronate and the cinchonidine salt of phenolphthalein glucuronide were from Sigma Chemical Co., St Louis, Mo., U.S.A.; Triton X-100 was from Rohm and Haas Co., Philadelphia, Pa., U.S.A.; RNA was from L. Light and Co. Ltd., Colnbrook, Bucks.; sodium merthiolate was from Eli Lilly and Co., Indianapolis, Ind., U.S.A.; purified hyaluronidase was from U.S.P. Reference Standards, New York, N.Y., U.S.A.

Phenolphthalein glucuronide was prepared from its cinchonidine salt according to the method described by Gianetto & de Duve (1955). Haemoglobin was prepared from washed ox erythrocytes (Gianetto & de Duve, 1955). The preparation of DNA and the purification of RNA were as described by de Duve *et al.* (1955). The *o*-nitrophenyl *N*-acetyl- β -glucosaminide was synthesized by the method of Leaback & Walker (1957); the terminal deacetylation was carried out as described by Westphal & Schmidt (1952).

Histological and histochemical controls

Histological examinations of the calvaria used in the present experiments showed abundance of preosseous cells and of osteoblasts, less abundant osteocytes and a few osteoclasts. They contained in addition some cells of non-osseous nature: connective-tissue cells, vascular parietal cells, blood cells and a few cartilage cells. Attempts to localize acid phosphatase (pH 5.2) histochemically were made with naphthol-AS-BI-phosphate as substrate (Burstone, 1962). In both fixed (Holt & Hicks, 1961) and unfixed cryostat sections of undecalcified calvaria, strongly positive reactions were obtained after short periods of incubation in the osteoblasts, in the layer of cells situated directly below the osteoblasts (preosteoblasts), and in the osteoclasts; osteocytes appeared to be negative. These observations suggest that acid phosphatase is not specific to any particular type of bone cell in this material.

RESULTS

Effect of pH. All nine hydrolases investigated displayed significant activity in the acid pH range (Fig. 2). Optimum activity was obtained between pH 3.3 and 3.9 for cathepsin, hyaluronidase and β -galactosidase, and between pH 4.5 and 5.5 for acid phenylphosphatase, acid β -glycerophosphatase, β -glucuronidase, β -*N*-acetylglucosaminidase, acid deoxyribonuclease and acid ribonuclease. Phenylphosphatase, β -glycerophosphatase and ribonuclease were the only three hydrolases showing a second significant peak of activity at alkaline pH (Fig. 2).

The pH range over which hyaluronidase was active was particularly narrow: optimum activity was obtained at pH 3.7 and no activity could be detected at pH 5.0; this was found also when the liberated *N*-acetylhexosamines were measured for the assay. A similar pH-activity curve was found by using the viscosimetric assay technique with purified hyaluronidase.

Effect of substrate concentration. The *K_m* values were determined for the acid hydrolases under their standard assay conditions. The following values were obtained: 0.046 mM for β -glucuronidase; 0.093 mM for cathepsin; 0.37 mM for β -galactosidase; 1.3 mM for β -*N*-acetylglucosaminidase; 5 mM for acid β -glycerophosphatase; 0.96 g./l. for acid ribonuclease.

All these enzymes displayed Michaelis-Menten kinetics; reciprocal plots of activity and substrate concentration gave straight lines. For acid phenylphosphatase, however, reciprocal plots of activity (measured at constant ionic strength) and substrate concentration gave a curve of progressively decreasing slope. Half-maximum activity of acid phenylphosphatase was obtained at a substrate concentration of 0.5–1.0 mM (apparent *K_m*). In the assays of all these enzymes, excess of substrate was

Fig. 2. Influence of pH on hydrolytic activities in cytoplasmic extract: (a) phenylphosphatase; (b) deoxyribonuclease; (c) cathepsin; (d) β -glycerophosphatase; (e) β -N-acetylglucosaminidase; (f) hyaluronidase; (g) ribonuclease; (h) β -glucuronidase; (i) β -galactosidase. Incubations were carried out in 0.10 M or 0.05 M concentrations of the following buffers: glycine-HCl or glycine-NaOH (○); sodium citrate-citric acid (▲); sodium acetate-acetic acid (■), sodium cacodylate-HCl (●); glycylglycine-NaOH (Δ). The haemoglobin used in the cathepsin assays was first denatured by heating for 30 min. at 37° at pH 3.6 and then brought to the required pH; undenatured haemoglobin was used for the assays at or above pH 7, owing to the lack of solubility of the denatured protein in neutral or alkaline solution.

provided to keep the reaction rate almost constant (zero-order reaction).

Standard K_m could not be measured for hyaluronidase or for acid deoxyribonuclease. For hyaluronidase, the substrate concentration used in the assay was chosen to give relative viscosities of about 3 at zero time; less activity was obtained at lower relative viscosities and the sensitivity of the assay decreased at higher relative viscosities. For acid deoxyribonuclease, the activity (amount of perchloric acid-soluble nucleotides made free by the reaction) appeared to decrease in the presence of excess of substrate. Under the conditions chosen to measure this enzyme, lowering the substrate concentration from 0.9 to 0.3 mg./ml. had little effect on the reaction rate.

Effect of ionic strength. The activities of acid phenylphosphatase, acid deoxyribonuclease and acid ribonuclease depend on the ionic strength of the incubation mixture (sodium chloride, potassium chloride or acetate buffer, pH 5.0). Phenylphosphatase activity was optimum at about 1.025–0.35. Acid-deoxyribonuclease activity, measured with a substrate concentration of 0.45 mg./ml., was increased about 2.5-fold when the concentration of acetate buffer was changed from 0.05 to 0.3 M; it decreased at higher concentrations; optimum activity in the presence of 0.9 mg. of substrate/ml. was obtained in 0.2 M-acetate buffer. Acid-ribonuclease activity was optimum in 0.1 M-citrate buffer, pH 5.0. Hyaluronidase activity was also increased by 50% by the addition of sodium chloride (0.15 M) to the

Table 2. Comparison of acid-phenylphosphatase and acid- β -glycerophosphatase activities

	Phenylphosphatase	β -Glycerophosphatase
Optimum pH	4.7-5.4	4.7-5.0
Michaelis constant	0.5-1.0 mM (apparent K_m)	5.0 mM
Activity $\pm$ s.d. in cytoplasmic extract (units/g. of tissue)	7.38 $\pm$ 3.06 (29 assays)	1.12 $\pm$ 0.22 (9 assays)
Stability at 37° under standard assay conditions	30-60 min.	4-6 hr.
Treatment in a Waring Blender for 15 min.	Strong inactivation (-59%)	Slight inactivation (-16%)
Freezing and thawing (once)	Slight inactivation (-11%)	Unaffected
Preincubation without substrate at pH 5.0 and 37° for 3 hr.	Strong inactivation (-71%)	Slight inactivation (-15%)
Effect of ionic strength (for conditions of experiments see the text)	Marked	None
Effect of 16.5 mM-formaldehyde	Strong inhibition (-75%)	Slight inhibition (-16%)
Effect of 1 mM-sodium (+)-tartrate	Slight inhibition (-19%)	Strong inhibition (-81%)
Effect of 5 mM-NaF	Strong inhibition (-85%)	Strong inhibition (-73%)

test medium. The effect of increased ionic strength on cathepsin activity could not be tested since excess of salt caused the precipitation of the haemoglobin substrate. The other enzymes were unaffected by changes in ionic strength (sodium chloride).

Activations and inhibitions. As shown in Table 2, tartrate was much more inhibitory to acid β -glycerophosphatase than to acid phenylphosphatase, whereas the reverse was true for formaldehyde; sodium fluoride was about equally inhibitory to the acid-phosphatase activity with either substrate. Replacement of sodium chloride in the incubation mixtures by β -glycerophosphate without changing the ionic strength depressed markedly the rate of hydrolysis of phenyl phosphate; this inhibition was released by an increase in the concentration of phenyl phosphate, and thus appears to be of a competitive nature.

β -Galactosidase activity was inhibited by 80-90% by 0.1% sodium merthiolate. In 0.1M-acetate buffer, pH 5.0, 0.05M-EDTA stimulated this enzyme activity slightly; replacement of acetate buffer by citrate buffer had a similar effect. β -N-Acetylglucosaminidase activity was also slightly higher in 0.1M-citrate buffer than in 0.1M-acetate buffer. Acid-deoxyribonuclease activity, however, was inhibited by citrate buffer and by EDTA: a 50% inhibition was observed with 0.057M-EDTA.

The addition of sucrose (0.25M) to the test media caused slight inhibitions of β -glucuronidase (-26%), cathepsin (-15%), β -N-acetylglucosaminidase (-10%) and acid β -glycerophosphatase (-6%); its effect on the other acid hydrolases was negligible.

Quantitative assay and stability of enzymes. The limits within which the measured activities were directly proportional to the amount of cytoplasmic

Fig. 3. Activities of phenylphosphatase (○) and of β -glycerophosphatase (●) measured in cytoplasmic extract after preincubation at pH 5.0 and 37° in the absence of substrate for various periods of time.

extract and to the duration of incubation have been presented in Table 1.

β -Glucuronidase and acid deoxyribonuclease appear to be the most stable enzymes measured: direct proportionality between activities and duration of incubation was maintained for more than 24 hr. For most of the acid hydrolases,

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Table 3. *Enzyme activities of bone tissue*

Values are in units/g. for enzymes and in mg./g. for protein. Data on enzyme contents of liver, obtained from de Duve *et al.* (1955), Sellinger *et al.* (1960) and Baudhuin *et al.* (1964), are presented for comparison. Statistics refer to the means $\pm$ s.d. The numbers of individual determinations are given in parentheses. Results listed under E + N are sums of values obtained separately on cytoplasmic extract (E) and on nuclear fraction filtered through gauze (N) from the same tissue; values listed under H were obtained independently on whole unfiltered homogenates. Thus the main difference between the two sets of values is the filtration step which, as shown, removes a considerable amount of protein (in the form of gross fragments presumably originating mostly from the matrix), but little enzyme activity. Sp. act. refers to specific activity (units/mg. of protein).

Enzymes	Calvarium		Metaphysis H	Diaphysis H	Liver	Sp. act. of calvarium (E + N)	
	H					Sp. act. of liver	
	E + N	H					
Acid phenylphosphatase	9.63 $\pm$ 2.6 (7)	10.7 $\pm$ 4.07 (6)	45.7 $\pm$ 0.28 (2)	15.4 $\pm$ 0.79 (2)	—	—	—
Acid β -glycerophosphatase	1.43 $\pm$ 0.28 (5)	—	—	—	5.83	1.4	1.4
β -Glucuronidase	0.260 $\pm$ 0.067 (5)	0.265 $\pm$ 0.043 (6)	0.276 $\pm$ 0.036 (2)	0.202 $\pm$ 0.030 (2)	0.782	1.8	1.8
β -Galactosidase	0.481 $\pm$ 0.072 (5)	0.439 $\pm$ 0.082 (6)	0.355 $\pm$ 0.100 (2)	0.214 $\pm$ 0.022 (2)	0.340	7.8	7.8
β -N-Acetylglucosaminidase	2.52 $\pm$ 0.76 (4)	2.70 $\pm$ 0.78 (6)	2.76 $\pm$ 0.28 (2)	1.76 $\pm$ 0.07 (2)	6.90	2.0	2.0
Hyaluronidase (on E alone)	8.11 $\pm$ 1.43 (8)	—	—	—	—	—	—
Cathepsin	0.264 $\pm$ 0.009 (5)	0.260 $\pm$ 0.063 (10)	0.346 $\pm$ 0.016 (2)	0.250 $\pm$ 0.076 (2)	1.46	1.0	1.0
Acid deoxyribonuclease	0.506 $\pm$ 0.109 (5)	0.680 $\pm$ 0.149 (6)	0.660 $\pm$ 0.090 (2)	0.346 $\pm$ 0.033 (2)	1.31	1.4	1.4
Acid ribonuclease	1.01 $\pm$ 0.18 (5)	1.08 $\pm$ 0.08 (4)	—	—	2.70	2.1	2.1
Cytochrome oxidase	0.634 $\pm$ 0.239 (5)	0.870 $\pm$ 0.122 (4)	0.848 $\pm$ 0.011 (2)	0.799 $\pm$ 0.029 (2)	30.6	0.11	0.11
Alkaline phenylphosphatase	36.7 $\pm$ 6.4 (4)	38.8 $\pm$ 1.72 (4)	—	—	—	—	—
Catalase	0.409 $\pm$ 0.129 (5)	—	—	—	42.7	—	0.05
Protein	36.6 $\pm$ 4.1 (7)	90.9 $\pm$ 13.7 (6)	81.5 $\pm$ 9.0 (2)	85.0 $\pm$ 13.0 (2)	202		

however, this proportionality held only for 5–6 hr.: after this time, the velocity of the reaction decreased progressively owing presumably to slow inactivation of the enzymes.

The phenylphosphatase activity remained linear with time for only 30–60 min. This is due to lability of the enzyme at pH 5 and 37°: experiments in which the enzyme was preincubated without substrate at pH 5 and 37° for increasing periods of time before the addition of the substrate showed increased loss of activity with longer times of preincubation (Fig. 3). Progressive inactivation of the enzyme also occurred in many other circumstances, e.g. storage at 0°, prolonged homogenization, treatment in a Waring Blendor or by ultrasonic waves, freezing and thawing, and exposure of particle-bound enzyme (M + L fraction) to distilled water or to 0.1% Triton X-100.

Comparison of acid phenylphosphatase and acid β -glycerophosphatase. As already indicated, a number of differences were observed between the activities on phenyl phosphate and on β -glycerophosphate. These differences are summarized in Table 2. They make it clear that the activities on the two substrates must be largely due to different enzymes or groups of enzymes.

Enzyme activities. Considerable amounts of acid hydrolases are present in calvaria (Table 3). It is apparent from the data in Table 3 that the sums of the enzymic activities measured separately on the cytoplasmic extract and on the filtered nuclear fraction (E + N) are not much lower than the activities measured on the whole unfiltered and unfractionated homogenate (H). The protein content of the E + N fraction is, however, less than half that of H: this is due mainly to the filtration through gauze of the nuclear fraction which caused only a small loss of enzyme activity but removed a considerable amount of protein, presumably belonging to extracellular non-enzymic constituents of bone matrix. The protein content of the E + N fraction is therefore probably more representative of the true protein content of the cells in bone.

Compared with rat liver, calvaria show lower hydrolase activities/g. of tissue, but generally higher activities/mg. of protein in the E + N fraction. In contrast, they contain much less cytochrome-oxidase and catalase activity than liver. Comparison of activities in calvaria, metaphyses and diaphyses indicates relatively small differences between the three types of bone, except for acid phenylphosphatase, which is remarkably active in metaphyseal bone.

DISCUSSION

The studies described in this paper have provided adequate tools for the investigations reported by

Vaes & Jacques (1965) and Vaes (1965b). They also establish that bone cells show a remarkably high content in acid hydrolases, capable of breaking down some of the most important constituents of the cells themselves and of the extracellular matrix. It is too early to speculate about the possible functions of these enzymes, although it is tempting to link them to the process of bone resorption for the reasons outlined in the introduction.

Knowledge of the distribution of these enzymes between the various types of bone cells could be most useful to the study of their physiological function. Some cytochemical work along this line has already been done (Tonna, 1958; Schlager, 1959; Burstone, 1960; Cabrini, 1961; Belanger & Migicovsky, 1963). However, the lability of some of the enzymes, particularly of acid phenylphosphatase, and the presence in bone of at least two different acid phosphatases, should be remembered in connexion with such studies.

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