Studies on Bone Enzymes

DISTRIBUTION OF ACID HYDROLASES, ALKALINE PHENYLPHOSPHATASE, CYTOCHROME OXIDASE AND CATALASE IN SUBCELLULAR FRACTION OF BONE TISSUE HOMOGENATES

By G. VAES AND P. JACQUES

Department of Physiological Chemistry, University of Louvain, Belgium

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1. When bone homogenates were fractionated according to the scheme developed for liver by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), all the enzymes assayed except cytochrome oxidase were found to occur partly in soluble and partly in particulate fractions. Among the particle-bound enzymes, the highest specific activity was found in the heavy-mitochondrial fraction for cytochrome oxidase, in the microsomal fraction for alkaline phenylphosphatase and in the light-mitochondrial fraction for eight acid hydrolases and for catalase. 2. Combined heavy-mitochondrial and light-mitochondrial fractions were sub-fractionated by isopycnic centrifugation in density gradients of sucrose or glycogen. In the various systems tried, cytochrome oxidase showed a relatively narrow distribution range with a sharp peak; the acid hydrolases and catalase showed flat and irregular distribution patterns, differing slightly in shape from one enzyme to the other. However, it was not possible to achieve a marked separation between the various enzymes under study. 3. It is concluded from these results that the acid hydrolases belong to special cytoplasmic particles, probably lysosomes, and that these particles are physically and enzymically heterogeneous. Catalase appears to be non-mitochondrial and could also belong to the lysosomes; but the possibility of an association with another type of particle must be kept in mind in view of what is known of liver catalase. Alkaline phenylphosphatase is largely attached to microsomal elements.

In the preceding paper (Vaes & Jacques, 1965), the demonstration of nine acid hydrolases, alkaline phenylphosphatase, cytochrome oxidase and catalase in homogenates of bone was reported and methods for the quantitative assay of these enzymes were described. In the present investigations, the intracellular distribution of these enzymes has been studied by differential centrifugation of bone homogenates and by density equilibration in various density gradients of particulate fractions isolated from these homogenates.

The results of these studies resemble those obtained with rat liver (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and extend to bone the concept of lysosomes (de Duve, 1959), indicating that the acid hydrolases of this tissue are associated with a special group of cytoplasmic particles, distinct from heavier granules containing cytochrome oxidase (mitochondria) and from lighter particles ('microsomes') bearing alkaline phenylphosphatase. They also stress the heterogeneity of the enzyme content of these

lysosomes. The study of the latency of lysosomal hydrolases in bone homogenates is reported separately (Vaes, 1965b).

EXPERIMENTAL

Homogenates of calvaria from newborn rats were prepared in ice-cold 0.25 M-sucrose as described by Vaes & Jacques (1965). They were centrifuged at 6000g-min. at 0°, yielding a cell-free supernatant and a sediment containing most of the nuclei, together with unbroken cells, erythrocytes, gross cell debris, connective-tissue elements and solid bone mineral. This sediment was resuspended in 0.25 M-sucrose, rehomogenized and centrifuged again in the same way. The supernatants were pooled and constituted the cytoplasmic extract (E). The sediment, or nuclear fraction (N), was resuspended in 0.25 M-sucrose and filtered through gauze, to eliminate gross fragments whose presence complicated the analytical measurements. This caused a small loss of enzyme activity, but removed a considerable amount of protein, presumably belonging to extracellular non-enzymic constituents of bone matrix.

Further fractionation of the cytoplasmic extract was carried out according to a scheme identical with that adopted

for rat liver by de Duve *et al.* (1955). Three particulate fractions were successively isolated in the Spinco model L preparative ultracentrifuge (rotor no. 40) by integrated forces of respectively 33 000g-min. (heavy-mitochondrial or M fraction), 250 000g-min. (light-mitochondrial or L fraction) and 3 000 000g-min. (microsomal or P fraction), leaving a final supernatant (S fraction). Each particulate fraction was washed once and then taken up in 0.25M-sucrose to give the desired dilution (expressed in g. original wet wt. of tissue/ml.). In each case, the washing fluid was combined with the first supernatant to be processed for the isolation of the next fraction.

In the density-gradient experiments, a combined M+L fraction was first separated from the cytoplasmic extract by centrifugation at 250 000g-min. in 0.25M-sucrose, and washed once in the same manner. The particles were then subfractionated by density equilibration in density gradients, with the procedures described by Beaufay *et al.* (1964). Linear gradients of sucrose in water containing EDTA (10mM) and of glycogen with as solvent sucrose solutions of several well-defined concentrations (0.125, 0.15, 0.25 and 0.75M) were used. The addition of EDTA to the sucrose gradients was essential; in the absence of this chelating agent most of the particles collected together at the bottom of the tube. Ca^{2+} released from bone mineral is probably responsible for this phenomenon, but its mechanism is not understood. The particles were layered above the sucrose gradients, but uniformly incorporated in the glycogen gradients. About ten subfractions were separated.

Enzyme assays and other analyses were performed according to the methods described by Vaes & Jacques (1965).

RESULTS

Fractionation by differential centrifugation. In Table 1 are listed the means $\pm$ S.D. of the results obtained in the fractionation experiments. The corrected distribution patterns are shown in Fig. 1 in the manner proposed by de Duve *et al.* (1955).

Recovery values are satisfactory in most cases, except for acid phenylphosphatase, which con-

sistently showed a low recovery, presumably owing to the lack of stability of the enzyme (Vaes & Jacques, 1965). The data on this enzyme are accordingly less reliable than the others.

For most enzymes, the observed distributions resemble those recorded for rat liver (de Duve *et al.* 1955; Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960; Baudhuin *et al.* 1964). The amounts found in the nuclear fraction are higher than with liver, but this is not surprising in view of the complex composition of this fraction, which contains many unbroken cells and gross cell debris in addition to numerous fragments of matrix which may serve as sites of adsorption of soluble proteins and of cytoplasmic particles. As in liver, cytochrome oxidase shows the highest specific activity in the heavy-mitochondrial fraction (M), whereas all the acid hydrolases and catalase exhibit a peak of specific activity in the light-mitochondrial fraction (L). Alkaline phenylphosphatase, on the other hand, is particularly concentrated in the microsomal fraction (P). These results suggest strongly that the acid hydrolases of bone cells are localized in lysosome-like particles that, like those of liver, have sedimentation characteristics intermediate between those of the cytochrome oxidase-containing mitochondria and those of the microsomes; the latter, in this tissue, seem to be the main bearers of alkaline phenylphosphatase.

Although qualitatively similar, the distribution patterns of the various acid hydrolases show relatively large individual differences, which are also reflected in the variable amounts of activity recovered in the final supernatant (S). Adsorption and other artifacts may be partly responsible for these differences but it is likely that they are due, at least in part, to an intrinsic enzymic heterogeneity of bone lysosomes or to the existence of more than one population of particles of this type.

Table 1. *Intracellular distribution of enzymes*

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

Enzyme	No. of expts.	Absolute values (E+N)	Percentage values (E+N=100%)					Recovery
			N	M	L	P	S	
Protein	7	36.6 $\pm$ 4.1	41.5 $\pm$ 11.2	10.7 $\pm$ 2.5	6.9 $\pm$ 2.2	7.5 $\pm$ 2.5	23.6 $\pm$ 7.5	90.2 $\pm$ 7.0
Cytochrome oxidase	5	0.634 $\pm$ 0.239	39.3 $\pm$ 12.1	36.5 $\pm$ 8.4	16.4 $\pm$ 10.0	5.1 $\pm$ 2.6	0	97.2 $\pm$ 9.5
Alkaline phenylphosphatase	4	36.7 $\pm$ 6.4	18.0 $\pm$ 5.3	8.4 $\pm$ 1.6	15.3 $\pm$ 3.1	29.1 $\pm$ 5.4	20.2 $\pm$ 3.6	91.0 $\pm$ 3.5
Catalase	5	0.409 $\pm$ 0.129	18.7 $\pm$ 8.0	11.2 $\pm$ 2.2	16.5 $\pm$ 4.2	8.2 $\pm$ 3.0	41.0 $\pm$ 8.7	95.6 $\pm$ 16.9
Acid phenylphosphatase	7	9.63 $\pm$ 2.6	16.3 $\pm$ 8.6	14.2 $\pm$ 2.8	20.7 $\pm$ 5.2	6.8 $\pm$ 1.4	9.2 $\pm$ 2.8	67.2 $\pm$ 4.6
Acid β -glycerophosphatase	5	1.43 $\pm$ 0.28	13.6 $\pm$ 7.9	16.6 $\pm$ 3.7	23.2 $\pm$ 3.9	14.0 $\pm$ 1.8	25.5 $\pm$ 9.5	92.9 $\pm$ 12.7
β -Glucuronidase	5	0.260 $\pm$ 0.067	20.8 $\pm$ 12.7	19.7 $\pm$ 1.9	21.5 $\pm$ 4.9	3.3 $\pm$ 0.5	26.2 $\pm$ 2.9	91.5 $\pm$ 7.7
β -N-Acetylglucosaminidase	4	2.52 $\pm$ 0.76	32.8 $\pm$ 17.0	26.6 $\pm$ 4.6	29.2 $\pm$ 6.3	7.2 $\pm$ 1.8	3.5 $\pm$ 1.6	99.3 $\pm$ 25.6
β -Galactosidase	5	0.481 $\pm$ 0.072	23.1 $\pm$ 11.6	21.8 $\pm$ 4.9	26.7 $\pm$ 4.5	7.5 $\pm$ 2.7	14.0 $\pm$ 2.9	93.1 $\pm$ 2.37
Cathepsin	5	0.264 $\pm$ 0.009	18.5 $\pm$ 8.1	15.1 $\pm$ 4.0	21.1 $\pm$ 4.3	4.5 $\pm$ 1.9	37.7 $\pm$ 5.5	96.9 $\pm$ 7.3
Acid ribonuclease	5	1.01 $\pm$ 0.18	26.5 $\pm$ 7.7	15.2 $\pm$ 5.1	15.6 $\pm$ 6.4	7.5 $\pm$ 5.7	26.1 $\pm$ 12.5	90.9 $\pm$ 18.7
Acid deoxyribonuclease	5	0.506 $\pm$ 0.109	26.2 $\pm$ 12.2	24.3 $\pm$ 5.7	24.7 $\pm$ 4.3	7.4 $\pm$ 2.4	8.4 $\pm$ 3.0	91.0 $\pm$ 4.1

Fig. 1. Distribution patterns of enzymes: (a) cytochrome oxidase (5); (b) β -galactosidase (5); (c) β -glucuronidase (5); (d) acid ribonuclease (5); (e) cathepsin (5); (f) catalase (5); (g) alkaline phenylphosphatase (4); (h) acid phenylphosphatase (7); (i) acid β -glycerophosphatase (5); (j) acid deoxyribonuclease (5); (k) β -N-acetylglucosaminidase (4). The numbers in parentheses refer to numbers of experiments. The results are presented in the manner used by de Duve *et al.* (1955). The ordinate shows the mean relative specific activity of the fractions. On the abscissa the fractions are represented by their 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 1).

It is also possible that some of the measured activities are due partly to non-lysosomal enzymes. Catalase could be associated with the lysosomes or, more probably, with particles analogous to the 'microbodies' of liver (de Duve *et al.* 1960; Baudhuin *et al.* 1964; Beaufay *et al.* 1964; Baudhuin, Beaufay & de Duve, 1965). The high amount of catalase found in the final supernatant may indicate either that the enzyme is partly present in free form in the cells, or that the particles containing it are particularly fragile. Liver homogenates also contain more catalase than acid hydrolases in soluble form, and this difference has been attributed mainly to a greater fragility of the microbodies (Baudhuin *et al.* 1964; Beaufay *et al.* 1964).

Density equilibration in density gradients. Attempts to resolve particulate fractions (M+L) from bone in gradients of sucrose or of glycogen in different concentrations of sucrose, by taking advantage of possible differences in response to

changes in osmotic pressure, as was done by Beaufay *et al.* (1964) for liver, were not successful. In no case did it prove possible to achieve a marked separation between the three types of particles assumed to be present. However, further evidence was obtained that the acid hydrolases and catalase do not belong to the mitochondria, since the distributions of these enzymes were always broader and more irregular than that of cytochrome oxidase. None of the systems tried allowed a dissociation between catalase and the acid hydrolases comparable with that observed with liver particles. In view of these facts, it did not seem profitable to report the results of these experiments in detail. One set of results has been presented elsewhere (Vaes, 1965a), for illustrative purposes.

In contrast with the results obtained on liver by Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959) and Beaufay *et al.* (1964), density-gradient centrifugation of a washed M+L fraction led to a significant further purification of cytochrome oxidase. In the various experiments done, this enzyme was generally purified 2-2.5 fold over the original particle preparation in the most active subfraction. It follows from this that the mitochondria, if reasonably homogeneous in composition, can constitute no more than 40-50% of the particulate proteinaceous material present in an M+L fraction from bone. To what extent lysosomes contribute to the non-mitochondrial material is not known, but it is striking that the specific activities of the acid hydrolases were not very different in the various subfractions.

DISCUSSION

The analysis of the results described in this paper shows close similarities in the distribution of the acid hydrolases after fractionations of bone homogenates. Like the homologous enzymes in liver homogenates, they occur partly in soluble form and partly in association with cytoplasmic particles. On fractionation by differential centrifugation, the particle-bound acid hydrolases sediment more slowly than the mitochondrial cytochrome oxidase and become concentrated in the L fraction, as do the liver enzymes. Their sedimentation rate is, however, faster than that of alkaline phenylphosphatase, which becomes most concentrated in the P fraction. No significant further resolution of particulate M+L fractions could, however, be obtained by density equilibration in density gradients of sucrose or of glycogen, in contrast with the results obtained with liver (Beaufay *et al.* 1959, 1964).

When considered in the context of what is at present known of lysosomes in general, the results obtained for the bone enzymes may be confidently

interpreted as sufficient proof that the main acid hydrolases of this tissue are, as in all other tissues similarly investigated, largely or totally associated with a special group of particles resembling those described in liver under the name 'lysosomes' (de Duve, 1959, 1963). This is particularly true since the bone enzymes also show typical latency properties, as reported by Vaes (1965b).

The differences in distribution exhibited by the various acid hydrolases must provisionally remain unexplained. As mentioned above, they may reflect the enzymic heterogeneity of the individual members of a single lysosome population, or the existence of more than one population of particles, each of which may or may not be enzymically homogeneous; they could also be due to a greater or smaller extent to artifacts or, in some cases, to the participation of non-lysosomal enzymic species to the hydrolysis of substrate under the assay conditions adopted.

The existence of lysosomes in bone cells is in agreement with cytochemical staining studies for acid phosphatase (Tonna, 1958; Schajowicz & Cabrini, 1958; Burstone, 1959; Rose, 1961) and other acid hydrolases such as β -glucuronidase (Cabrini, 1961), β -galactosidase (Schlager, 1959) and protease (Belanger & Migicovsky, 1963). Further, since bone contains several types of cells, the presence of several groups of lysosomes in the homogenate would not be surprising.

As mentioned above, the results obtained on the distribution of catalase indicate that this enzyme does not belong to the mitochondria, but provide no evidence that it is not a constituent of lysosomes. One may, however, assume, by analogy with liver (Baudhuin *et al.* 1965), that it is present in a different type of cytoplasmic particle, more sensitive than lysosomes to mechanical disruption. The lack of activation of bone catalase by exposure of particles to media of low osmotic pressure, which activate the acid hydrolases (Vaes, 1965b), supports this assumption.

The localization of alkaline phenylphosphatase in microsomes recalls the similar location found for this enzyme in other tissues with high alkaline-phosphatase activity, e.g. intestine and kidney (Hers, Berthet, Berthet & de Duve, 1951). The significance of this observation remains obscure, since the function of alkaline phosphatase is still unknown. Histochemical studies have, however, shown that this enzyme is present mostly in the cells that synthesize bone matrix, namely the osteoblasts (Bourne, 1956). It must be pointed out

that, unlike intestine and kidney homogenates, bone preparations contain a fair amount of soluble alkaline phenylphosphatase: the Mg^{2+} -stimulated alkaline phosphatase of liver is entirely restricted to the final supernatant (see de Duve, Wattiaux & Baudhuin, 1962).

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