Tissue Fractionation Studies

9. ENZYMIC RELEASE OF BOUND HYDROLASES*

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Numerous earlier publications from this Laboratory (for reviews, see de Duve, 1958, 1959) have been devoted to the characterization of a new group of cytoplasmic particles called lysosomes. In rat liver, lysosomes can be distinguished from mitochondria, though not completely separated from them, thanks to a somewhat slower rate of sedimentation in 0·25 M-sucrose. They behave in freshly isolated preparations as essentially inert little bags which, upon injury by any of a variety of means, release into the medium a collection of soluble hydrolytic enzymes with an acid pH optimum. Amongst these are the five hydrolases studied in this laboratory, acid phosphatase, β-glucuronidase, 'cathepsin' [probably a mixture of cathepsins A, B (Rademaker & Soons, 1957) and C (Finkenstaedt, 1957)], acid ribonuclease (ribonuclease II) and acid deoxyribonuclease (deoxyribonuclease II), to which may be added the arylsulphatases A and B (Viala & Gianetto, 1955; Roy, 1958), phosphoprotein phosphatase (Paigen & Griffiths, 1959) and *N*-acetyl-β-glucosaminidase (Sellinger & Doyen, 1959).

The means so far known to effect the release of lysosomal enzymes depend on mechanical breakage (in a Waring Blendor), osmotic rupture (in media of low tonicity or, more slowly, in solutions of substances such as sodium chloride, potassium chloride or glycerol, which afford only transient osmotic protection), physicochemical disruption (by repeated freezing and thawing or by exposure to surface-active agents such as saponin, deoxycholate or Triton X-100) or some kind of autolytic breakdown (thermal activation) which occurs when the particles are incubated at 37° and which is favoured by a lowering of pH. Sonic vibrations have also proved effective in some preliminary experiments. It is probable that all of these treatments act by causing a primary injury to the particle membrane, which then allows the external substrates to penetrate within the particles as well as the internal enzymes to leak out of them. This accounts for the simultaneous activation and solubilization of the bound hydrolases.

In the present studies, the effects on the state of lysosomal hydrolases of pretreating the particles with various enzyme preparations have been investigated. The mechanism of thermal activation has also been studied. The results, some of which have been reported in a preliminary communication (Beaufay, 1957), are in essential agreement with the membrane hypothesis and demonstrate further that the lysosomal membrane is of lipoprotein nature.

METHODS

All experiments were performed on mitochondrial fractions isolated from rat liver as described by Appelmans & de Duve (1955). Adequate amounts of the mitochondrial preparations, corresponding in most cases to 0·2 g. of fresh liver, were pre-incubated for 10 min. or more at 25° in a total volume of 1 ml. containing 0·25 M-sucrose, 15 mM-glyoxaline hydrochloride buffer, pH 7·4, and the enzymic agent under study together with its activator or inhibitor as required.

The enzymes applied during pre-incubation included: a purified sample of lecithinase (a gift from Dr B. Cinader) isolated from *Clostridium welchii* cultures by fractionation with $(NH_4)_2SO_4$ and dialysis, and furnished as a concentrated solution in aq. 50% (v/v) glycerol; crystalline trypsin, donated by the Novo Laboratories, Copenhagen; crystalline chymotrypsin, prepared from five-times recrystallized chymotrypsinogen according to Kunitz & Northrop (1935) and recrystallized three times; a commercial concentrate of pancreatic enzymes (Absolute Pancreatin powder, Merck); crystalline ribonuclease, purified according to the method of Kunitz (1940) as modified by McDonald (1949) and recrystallized three times; crystalline lysozyme, purchased from the Armour Co. Eastbourne, Sussex. When lecithinase was studied, the medium was supplemented with 4 mM-$CaCl_2$ to activate the enzyme, and special controls were set up, containing either 4 mM-$CaCl_2$ and as much glycerol as was added with the enzyme or the complete active mixture together with 8 mM-ethylenediaminetetra-acetic acid (EDTA), which inhibits the phospholipase completely. When not present in the pre-incubation medium, this inhibitor was added together with the substrate mixtures to prevent the lecithinase from acting during the subsequent enzymic assays. In some of the experiments with trypsin, crystalline soya-bean inhibitor (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used in analogous controls.

After pre-incubation, the free and total enzymic activities of the preparations were determined by adding 1 ml. of substrate mixture and incubating further for 10 min. at

* Part 8: Wattiaux, Baudhuin, Berleur & de Duve (1956).

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37°. For the measurement of free activities, the substrate mixtures were made up so as to realize the assay conditions described by Gianetto & de Duve (1955) and by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955); they contained in addition all the components of low molecular weight present in the pre-incubation medium. As observed before (Berthet, Berthet, Appelmans & de Duve, 1951) and again here in some preliminary experiments, lack of the last-named precaution may be responsible for the osmotic rupture of the particles, if they are permeable to one or more of the omitted components. The methods used for stopping the reactions and performing the subsequent analyses were those described previously (de Duve *et al.* 1955). The total activities were measured as the free activities, but in the presence of 0·1% of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), according to the techniques of Wattiaux & de Duve (1956).

The chemical changes suffered during pre-incubation by the particles themselves were followed on separate samples with the aid of the methods described by Beaufay & de Duve (1954).

RESULTS

Enzymic activation

In Table 1 is given a summary of the main results obtained on mitochondrial fractions pretreated with various enzyme preparations. The experiments were generally run in parallel on acid phosphatase and on one other lysosomal hydrolase, with the result that more data are available for the former enzyme than for the others. For obvious reasons, cathepsin could not be measured in mixtures containing large amounts of proteolytic enzymes; nor could the endogenous acid ribonuclease be assayed after treatment with the homologous crystalline enzyme.

As a rule, the total activities of lysosomal hydrolases suffered only minor changes under the influence of the various treatments applied, with the exception of acid deoxyribonuclease, which exhibited considerable variations. The great sensitivity of this enzyme to its ionic environment (Rotherham, Schottelius, Irvin & Irvin, 1956; Shack, 1957) may explain the moderate activation produced by the lecithinase medium, whereas the inhibitions observed in the presence of ribonuclease and lysozyme are probably attributable to the binding of substrate by these basic proteins. We are unable to account for the striking activation of the deoxyribonuclease by trypsin. This phenomenon was found to depend both on the concentration of trypsin and on the duration of pre-incubation and must therefore involve the actual digestion of some material. Whether the latter process modifies the enzyme molecule, destroys an inhibitor or causes an activator to appear has not been ascertained.

Of more immediate interest to the object of the present study are the increases in free activity observed under the influence of several of the enzyme preparations applied. Further data concerning these effects are given in Table 2 and in Figs. 1 and 2.

As already shown by MacFarlane & Datta (1954), treatment of the mitochondrial fractions with *C. welchii* phospholipase in the presence of Ca^{2+} ions caused practically complete activation of the masked acid phosphatase. This effect, which was accompanied by a considerable solubilization of the enzyme (Table 2), was reproduced to only a very small extent by Ca^{2+} ions alone and was completely inhibited by EDTA (Table 1); it could be achieved with much smaller amounts of lecithinase than were used in the experiments of Table 1, and, when rendered partial by the use of submaximum concentrations of phospholipase, was found to parallel the amount of acid-soluble organic phosphate formed (Fig. 1). It may therefore be safely attributed to the hydrolysis of choline-containing phospholipids present in the preparation. Essentially similar findings were made on β-glucuronidase and on the two nucleases, with the exception that the last-named enzymes, though completely activated, were solubilized to a smaller extent. Cathepsin was activated in the same specific manner by phospholipase, but this activation did not exceed a maximum of approximately 40% and was not accompanied by any appreciable solubilization of the enzyme.

Trypsin also caused a specific and important release of the four acid hydrolases which could be studied. This effect was completely suppressed by a slight excess of soya-bean inhibitor (Table 1) and accompanied the initial rapid phase of proteolytic attack (Fig. 2). Chymotrypsin, though similarly able to release the acid hydrolases, was much less effective in this respect than trypsin. Pancreatin powder showed a strong releasing activity, attributable for its larger part to EDTA-sensitive enzymes, presumably lipases or phospholipases or both, partly also to an EDTA-insensitive component, possibly trypsin. Finally, no significant effects were obtained with ribonuclease or lysozyme. The former result contradicts a report by Allard, de Lamirande & Cantero (1955), but these authors did not control the pH of their pre-incubation medium and used a commercial preparation of ribonuclease, which may have been contaminated by proteolytic enzymes.

Thermal activation

The observation that proteolytic enzymes can cause the release of lysosomal enzymes prompted a re-investigation of the possible role played by the particles' own cathepsin in this process as it occurs spontaneously. Such a role has been suspected before on the basis of temperature and pH effects (Berthet *et al.* 1951; Appelmans & de Duve, 1955).

Table 1. *Influence of various enzymic treatments on activity of lysosomal hydrolases*

When more than one experiment has been performed, means are given ± S.E.M.; figures in parentheses: numbers of experiments. APase, acid phosphatase; β-Gase, β-glucuronidase; RNase, acid ribonuclease; DNase, acid deoxyribonuclease.

Pre-incubation at 25° in 0·25 M-sucrose + 15 mM-glyoxaline HCl buffer, pH 7·4						
Addition to pre-incubation medium	Time (min.)	APase	β-Gase	RNase	DNase	Cathepsin
		Total enzymic activities (% of control)				
None	10	100	100	100	100	100
Glycerol (5%) + 4 mM-$CaCl_2$	10	91 ± 3·9 (5)	108	90	128	90
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10)	10	97 ± 4·3 (5)	105	89	125	97
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10) + 8 mM-EDTA	10	104 ± 4·5 (5)	110	87	128	—
Chymotrypsin (10 mg./ml.)	10	93 ± 2·0 (5)*	96	79	105	—
Pancreatin (10 mg./ml.)	10	100 ± 3·1 (2)	—	100	97	—
Pancreatin (10 mg./ml. + 8 mM-EDTA)	10	97	—	—	96	—
Ribonuclease (1 mg./ml.)	10	91 ± 3·5 (6)*	103	—	74	100
Lysozyme (10 mg./ml.)	10	103 ± 4·3 (6)	94	97	47	96
None	30	100	100	100	100	—
Trypsin (1·0 mg./ml.)	30	93 ± 1·5 (5)†	94 ± 2·3 (3)	115 ± 1·7 (2)	229	—
Trypsin (1·0 mg./ml.) + soya-bean inhibitor (1·25 mg./ml.)	30	92	98	—	—	—
Chymotrypsin (10 mg./ml.)	30	87	—	80	—	—
None	60	100	—	100	—	—
Chymotrypsin (10 mg./ml.)	60	88	—	77	—	—
		Free enzymic activities (% of total)				
None	10	11 ± 0·4 (6)	13	15	13	0
Glycerol (5%) + 4 mM-$CaCl_2$	10	14 ± 0·9 (5)*	12	20	8	10
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10)	10	91 ± 3·9 (5)‡	107	90	108	44
Glycerol (5%) + 4 mM-$CaCl_2$ + lecithinase (1:10) + 8 mM-EDTA	10	12 ± 0·8 (5)	10	18	10	6
Trypsin (0·2 mg./ml.)	10	34§	—	—	—	—
Trypsin (1·0 mg./ml.)	10	51§	—	—	—	—
Chymotrypsin (10 mg./ml.)	10	18 ± 3·3 (5)	23	30	27	—
Pancreatin (10 mg./ml.)	10	64 ± 6·0 (2)‡	—	69	61	—
Pancreatin (10 mg./ml. + 8 mM-EDTA)	10	31	—	—	19	—
Ribonuclease (1 mg./ml.)	10	14 ± 1·7 (6)	11	—	6	0
Lysozyme (10 mg./ml.)	10	10 ± 0·6 (6)	14	28	10	2
None	30	12 ± 2·2 (5)	17 ± 8·5 (2)	11	—	—
Trypsin (0·2 mg./ml.)	30	61§	—	—	—	—
Trypsin (1·0 mg./ml.)	30	76 ± 5·7 (5)‡	62 ± 9·0 (3)*	95 ± 8·8 (2)	101	—
Trypsin (1·0 mg./ml.) + soya-bean inhibitor (1·25 mg./ml.)	30	12	19	—	—	—
Chymotrypsin (10 mg./ml.)	30	33	—	53	—	—
None	60	24	—	42	—	—
Chymotrypsin (10 mg./ml.)	60	60	—	74	—	—

* Difference from controls significant at $P < 0·05$. † Difference from controls significant at $P < 0·01$. ‡ Difference from controls significant at $P < 0·001$.
§ Values expressed as percentages of total activity before pre-incubation with trypsin.

Table 2. *Influence of lecithinase on solubility of lysosomal hydrolases*

A mitochondrial preparation was pre-incubated for 10 min. at 25° in 0·25 M-sucrose in the presence of 1:10 lecithinase, 4 mM-$CaCl_2$ and 15 mM-glyoxaline HCl buffer, pH 7·4. EDTA, to a final concentration of 8 mM, was added after pre-incubation. After removal of a sample for the measurement of total enzymic activities, the suspension was centrifuged at 3 000 000 *g*-min. in the no. 40 rotor of the Spinco Model L preparative ultracentrifuge and the supernatant was assayed similarly to determine the unsedimentable activities. The control was treated in an identical manner, except that EDTA was added at the beginning of pre-incubation.

	Enzymic activities		
		Unsedimentable (% of total)	
Hydrolase	Total (% of control)	Control	Treated
Acid phosphatase	93·2	17·8	67·7
β-Glucuronidase	91·2	21·0	86·0
Acid ribonuclease	87·4	30·1	50·7
Acid deoxyribonuclease	104·0	23·6	35·6
Cathepsin	110·0	20·4	21·6

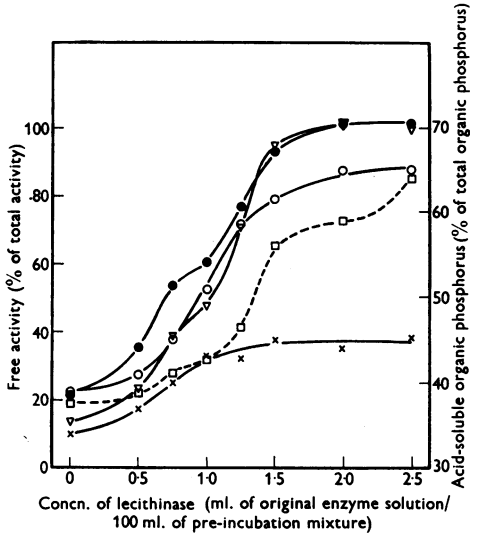

Fig. 1. Influence of lecithinase concentration on release of acid hydrolases (left ordinate: ●, acid phosphatase; ○, β-glucuronidase; ▽, acid deoxyribonuclease; ×, cathepsin) and on appearance of acid-soluble organic phosphate (right ordinate: □). Pre-incubation for 10 min. at 25°.

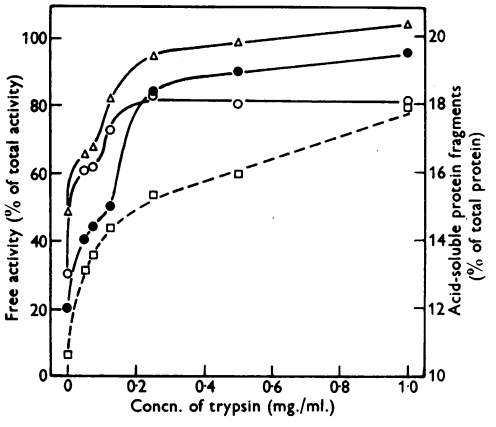

Fig. 2. Influence of trypsin concentration on release of acid hydrolases (left ordinate: ●, acid phosphatase; ○, β-glucuronidase; △, acid ribonuclease) and on appearance of acid-soluble aromatic fragments (right ordinate: □). Pre-incubation for 30 min. at 25°.

In the present experiments, an attempt was made to assess in a quantitative manner the temperature coefficients of acid phosphatase release and of endogenous proteolysis.

For the former determination, samples of a freshly prepared mitochondrial fraction were incubated at 19, 28, 37 and 45° in a medium containing 0·25 M-sucrose and 0·05 M-sodium acetate–acetic acid buffer, pH 5·0. Measurements of the free and total acid phosphatase activities were made at regular intervals. As found before, the release of acid phosphatase followed a sigmoid curve, which, however, had the same shape at all temperatures, so that the relative rates of activation could be estimated from the time necessary to reach a given arbitrary value (40 %). Endogenous proteolysis was studied by measuring the trichloroacetic acid-soluble aromatic fragments set free under the same conditions of pH and temperature in a Blendor-treated preparation.

In Fig. 3 are shown Arrhenius plots of the values found. From these plots, activation energies of 19 000 and 17 200 cal. mol.$^{-1}$ were calculated for the activation of acid phosphatase and for the autolysis of proteins respectively.

DISCUSSION

The experiments described in this paper have established that pretreatment of mitochondrial preparations with lipolytic or proteolytic enzymes, but not with ribonuclease or lysozyme, causes a

gradual release of all lysosomal hydrolases. These phenomena are most readily interpreted as consequences of an enzymically or chemically induced disorganization of a lipoprotein barrier, restricting the physical freedom of the acid hydrolases as well as their ability to interact with their substrates. These two restrictions are obviously related, but not in an entirely rigid fashion, since the former concerns the molecules of enzyme, whereas the latter may conceivably involve only those of the substrate. Also the effectiveness of the barrier in imposing them does not necessarily depend only on its own properties, but may also be influenced by characteristics of the individual enzymes and of their respective substrates.

Such factors could account for the differences in behaviour of individual enzymes, which were occasionally encountered in the present investigations and which contrast with the close parallelism observed previously with grosser activating procedures. Considering, for instance, the effects of lecithinase, an enzyme which removes phosphorylcholine from phospholipids, it is obvious from the data of Tables 1 and 2 and of Fig. 1 that the injury inflicted on the lysosome structure is sufficient to allow a considerable part of acid phosphatase and of β-glucuronidase to diffuse into the medium and to enable the totality of these hydrolases to react freely with their respective substrates under the conditions of the free activity assays. The other extreme is represented by cathepsin, which is not solubilized at all and shows a maximal activation of 40%, whereas the nucleases are intermediate, being released into the medium to a fairly small extent, although reacting completely in the free activity measurements.

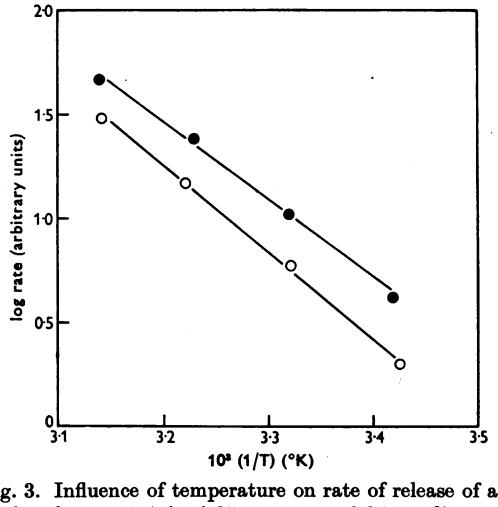

Fig. 3. Influence of temperature on rate of release of acid phosphatase (○) in 0·25 M-sucrose + 0·05 M-sodium acetate–acetic acid buffer, pH 5·0, and on rate of spontaneous proteolysis (●) in 0·05 M-sodium acetate–acetic acid buffer, pH 5·0, after disintegration in the Blendor.

As pointed out before, the two types of measurements made are vitiated to some extent by artifacts which tend to exaggerate the differences between them. Owing to adsorption phenomena, the values for physical release (solubilization) are underestimated; on the other hand, the accessibility of the enzymes (free activities) may be overestimated if further disruption of the particles occurs during the assays themselves. With acid phosphatase, which is known to be fairly strongly adsorbed by mitochondrial sediments (Berthet *et al.* 1951), solubilization may be considered as practically complete and suffices therefore to account for the activation results. This is also true of β-glucuronidase, but not of the acid nucleases, a fair proportion of which appears to remain attached to particles. They do, however, react fully in the free activity assays, either because their release is completed under the combined effects of the acidity and temperature prevailing during these assays, or because their substrates are able to penetrate freely within the particles in which they are trapped. Whichever the reason, it is obvious that all the nuclease-containing particles have been injured by the phospholipase. With cathepsin, we go one stage further; there is no detectable physical release, but a sufficient degree of permeation of the particles occurs to allow the free protease activity to reach 40% of its maximum value under the conditions of the free activity measurements. Although one cannot exclude the alternative possibility that 60% of the cathepsin-containing particles have not been damaged at all by lecithinase, it seems much more likely that all the particles have been damaged, but that a somewhat more drastic injury is necessary to allow the enzyme to diffuse freely out of the particles and possibly also to allow haemoglobin to enter them. It is also possible that less additional activation occurs in the course of the assay when haemoglobin is present in the medium.

How these observations bear upon the already frequently debated problem of the unity or plurality of the lysosomes is difficult to decide at the present time, but it is obvious that they weaken to some extent the argument put forward previously in support of the monistic hypothesis and based on the strict parallelism in the mode of release of all five lysosomal enzymes. However, sedimentation experiments have already made it clear that lysosomes form a heterogeneous population and it is therefore not particularly surprising that, as more data accumulate and new methods of activation are discovered, evidence of this heterogeneity also should appear in studies of the release of the lysosomal enzymes. Reasons for considering

the particles as forming a distinct class still remain overwhelming, but the possibility that the class may contain more than one species cannot be excluded.

With respect to thermal release, the relatively low activation energy of the process suggests that it is due to enzyme action rather than to denaturation. Cathepsin is a very good candidate for this function. As a protease, it is presumably capable of disrupting the particle membrane; it is almost inactive at pH 6·0, where the particles are relatively stable, and increases in activity up to pH 3·6, as does the lability of the particles, which are much more fragile at pH 5·0 (Appelmans & de Duve, 1955) and even more so at lower pH values (Gianetto & de Duve, 1955); finally, the activation energy of catheptic proteolysis as determined in these experiments is of the same order of magnitude as that of the release of acid phosphatase, the small difference observed being easily explained by the fact that the proteins attacked cannot have been the same in the two cases. The rapidity with which lysosomes are disrupted in acid media further suggests that if cathepsin is involved it must exert some type of selective action on the lysosomes, since these constitute only a small part of the particles present in the mitochondrial fractions studied. The possibility thus arises that the internal cathepsin of the lysosomes may actually be the agent of their thermal activation at an acid pH.

SUMMARY

1. The percentage of free activity of five lysosomal enzymes (acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease) has been measured on mitochondrial fractions from rat liver pre-incubated under mild conditions with various enzyme preparations.

2. *Clostridium welchii* lecithinase, trypsin, chymotrypsin and pancreatin all caused a more or less important release of all enzymes which could be assayed. Ribonuclease and lysozyme, on the other hand, were without effect on the state of the acid hydrolases.

3. In confirmation of previous data, these results have been taken to indicate that the release of the lysosomal enzymes is due to the disruption of a lipoprotein barrier restricting their physical freedom and their accessibility to their respective substrates.

4. Further indications have been obtained that the internal cathepsin of the particles may be involved in the spontaneous disruption of the lysosomes which occurs during incubation at pH 5·0 and 37°. On the other hand, it has been found that this enzyme lags behind the others when their release is accomplished by the action of lecithinase.

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