Short Communications

Hydrolytic Activity within Intact Lysosomes *in vitro*

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In living cells, the degradative enzymes within lysosomes are separated from their potential substrates in the cell by the lysosomal membrane (de Duve, 1963). One mechanism that has been described for bringing these enzymes and their substrates together is fusion of lysosomes with cell structures containing materials to be digested (Strauss, 1964; Hirsch, 1962). However, alternative mechanisms may also exist by which lysosomal hydrolases are able to react with substrates within cells. One such is suggested by the experiments reported below, namely passage of small molecules from the cytoplasm into the granules through the lysosomal membrane. Our findings also imply that once within the granule such molecules are accessible to the lysosomal enzymes and can be hydrolysed, provided that the enzymes are in an active state.

Methods. (1) Preparation of lysosome-containing suspensions. (a) From whole liver (rat). Sprague-Dawley rats (200g.) were killed by cervical dislocation. The livers were immediately removed and homogenized with a Teflon mortar and pestle in ice-cold mammalian Ringer-Locke solution (pH 7.4) containing 0.25M-sucrose. After removal of nuclei and unbroken cells by low-speed centrifugation, a large-granule fraction containing the lysosomes was sedimented by centrifugation at 17000g for 20 min. This large-granule fraction was resuspended in 25 ml. of Ringer-Locke solution containing 0.25M-sucrose and its protein concentration measured with the Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951). (The results reported below were observed to occur when sucrose alone was used to resuspend the granules. However, stability of lysosomes was improved in the presence of Ringer-Locke solution, and the latter was therefore employed as a routine.)

(b) From isolated hepatocytes (rat). A large-granule fraction prepared from homogenates of whole liver contains granules derived from Kupffer cells as well as from hepatocytes. In view of the distinctly different morphology and physiology of these two cell types it was decided to study the large-granule fraction obtained from hepatocytes alone. This was accomplished as follows. Rats were

anaesthetized with ether and their livers perfused with ice-cold citrate-Locke solution, adjusted to pH 7.4 with CO₂ (Jacob & Bhargava, 1962). The livers were immediately removed and immersed in ice-cold Ca²⁺-free Ringer-Locke solution containing 0.25M-sucrose. The tissue was finely minced and a dispersed cell suspension was prepared in a loose-fitting Dounce mortar and pestle. During this dispersion almost all the Kupffer cells were ruptured, whereas many hepatocytes remained intact. The latter cells were collected by low-speed centrifugation and were homogenized in Ca²⁺-containing Ringer-Locke solution containing 0.25M-sucrose. A large-granule fraction was prepared from this hepatocyte homogenate and assayed for protein as before.

(c) From exudate polymorphonuclear leucocytes (rabbit). A granule fraction was obtained from peritoneal exudate leucocytes by the techniques described by Cohn & Hirsch (1960). The granules were resuspended in 10 ml. of Ca²⁺-free Ringer-Locke solution containing 0.34M-sucrose, to which 50 units of heparin/ml. had been added. Protein concentration was determined as before.

(2) Incubation of lysosomal suspensions. Samples of the three different lysosomal suspensions were placed in flasks containing the appropriate type of Ringer-Locke-sucrose solution and the final concentration of granule protein was adjusted to 2 mg./ml. Some flasks received phenolphthalein glucuronide (Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 μ mole/ml.), a substrate for β -glucuronidase. Other flasks (controls) received no substrate. Flasks containing liver lysosomes were incubated for 1 hr. at 37.5° with slow shaking; rabbit polymorphonuclear-leucocyte granules were incubated at room temperature. After incubation the flasks were cooled in ice and their contents centrifuged (20000g for 20 min. for liver large-granule fraction; 8000g for 20 min. for leucocyte granules). Supernatants were carefully removed, and the pellets were resuspended to their original volume in 0.9% NaCl.

(3) Assays. (a) Distribution of free phenolphthalein. After incubation, the granules were separated from the medium as indicated above.

One ml. of the medium was added to 1 ml. of 1M-glycine-NaOH buffer, pH 10.4, and the extinction due to free phenolphthalein read at 540m μ . This value was called S_1 .

The phenolphthalein in the granules was determined by adding 0.1 ml. of 20% (w/v) trichloroacetic acid to 1 ml. of the resuspended pellet. The tubes were kept at room temperature for 10 min. Then 10 ml. of the glycine buffer was added and the tubes were centrifuged to remove the trichloroacetic acid precipitate. The extinction due to free phenolphthalein in the supernatant was read at 540m μ .

(b) Reabsorption/release control. In the foregoing incubations, there are two compartments in which substrate is hydrolysed, namely within the granules (by granule-bound enzyme) and, to a smaller extent, in the incubation medium (by enzyme released from the granules). Some of the phenolphthalein produced in the granules may subsequently leak into the incubation medium. Conversely, some of the phenolphthalein produced in the medium may adsorb on the granules. To correct for these effects it became necessary to determine how much phenolphthalein might have been produced during the incubation by enzyme leaking into the medium. This was done by incubating the supernatant from the control granule suspension (originally without substrate) for an additional 1 hr. at 37.5° in the presence of newly added phenolphthalein glucuronide (1 μ mole/ml.). The quantity of phenolphthalein resulting from this incubation was designated S_2 . Comparison of S_2 with S_1 (see above) afforded a measure of phenolphthalein released from or adsorbed by the granules. This correction factor never exceeded $\pm 5\%$ of the total phenolphthalein produced.

(c) Distribution of β -glucuronidase. This assay was carried out to evaluate the integrity of the lysosomes. The percentage of total β -glucuronidase activity that remained granule-bound at the end of the incubation period was determined. This value reflects the stability of granule membranes during the experiments (Janoff & Zweifach, 1963).

(d) Modified β -glucuronidase assay. The method used throughout these experiments for the assay of β -glucuronidase activity was based on that of Fishman, Springer & Brunetti (1948), except that saccharo-(1 $\rightarrow$ 4)-lactone (Calbiochem, Los Angeles, Calif., U.S.A.), a specific β -glucuronidase inhibitor, was always added to the blank tubes.

Results and discussion. The results obtained by the foregoing procedures are summarized in Table 1. Lysosomes from whole liver and isolated hepatocytes show similar levels of intragranular β -glucuronidase activity. In both cases over 80% of the total free phenolphthalein was recovered from the granules. These findings suggest that: (1) phenolphthalein glucuronide penetrated into lysosomes under the conditions of the experiment; (2) a significant quantity of the substrate was hydrolysed by β -glucuronidase within the lysosomes.

The distribution of β -glucuronidase (see Table 1) shows that most of the granules remained intact during the incubation period. Thus at least 90% of the enzyme was still granule-bound at the end of 1 hr. Clearly, the distribution of β -glucuronidase corresponds closely to the distribution of enzyme product (phenolphthalein).

Leucocyte granules, on the other hand, showed significantly less stability than did liver lysosomes (see Table 1). Nevertheless, the results obtained with polymorphonuclear-leucocyte granules indicate that the β -glucuronidase within those leucocyte lysosomes that did remain intact was also active and able to hydrolyse substrate.

Thus the concept of penetration of phenolphthalein glucuronide into lysosomes and the subsequent hydrolysis of this substrate within the granules appears to hold for particles from widely differing cell types. The possibility exists that other small-molecular-weight substrates may similarly diffuse into lysosomes within cells and become accessible to degradation by the enzymes contained within these particles. It may be that a number of physiologically important molecules (e.g. hormone-glucuronic acid conjugates) are metabolized in this manner. Further experimentation, to investigate

Table 1. *Phenolphthalein glucuronidase activity within lysosomes of different cell types*

Values given for phenolphthalein have been corrected for phenolphthalein lost from or adsorbed on the granules. Values given for β -glucuronidase activity serve as indices of granule integrity during incubation.

Source of granules	No. of experiments	Recovery from granules (%)	
		Phenolphthalein	β -Glucuronidase
Whole liver homogenate (rat)	9	83 $\pm$ 4	90 $\pm$ 3
Isolated hepatocyte homogenate (rat)	10	82 $\pm$ 5	94 $\pm$ 2
Polymorphonuclear-leucocyte homogenate (rabbit)	3	67 $\pm$ 5	73 $\pm$ 3

the potential physiological importance of these mechanisms, appears warranted.

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