Turnover Studies on Proteins of Rat Liver Lysosomes*

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SUMMARY

The turnover of rat liver lysosomal proteins was studied by a double isotope-labeling technique. The cellular fractions investigated included soluble lysosomal proteins, lysosomal membrane proteins, highly purified lysosomal \( \beta \)-glucuronidase, and for comparison, microsomal proteins and soluble cytoplasmic proteins. Both “normal” lysosomes and Triton WR-1339-filled lysosomes (tritosomes) were studied, with similar results. It was found that (a) the turnover rate of lysosomal proteins, of both the soluble and membranous compartments, was very similar to that of the proteins of the microsomal and soluble cytoplasmic fractions. and (b) the turnover rate of lysosomal proteins was asynchronous. The latter conclusion was based on two lines of evidence: (a) lysosomal \( \beta \)-glucuronidase had a distinctly slower turnover rate than the average rate of the soluble lysosomal proteins, and (b) subunits of the proteins of the soluble lysosomal fraction as separated by sodium dodecyl sulfate. Sephadex G-200 gel filtration showed different rates of degradation.

Lysosomes are present in almost all cells and are involved in important biological processes, including developmental processes apparently dependent on the extensive catabolic capacity of these organelles. The possibility that lysosomes and their proteases play key roles in the normal turnover of tissue proteins is currently a subject of experimentation and discussion (1-3). Whereas the turnover of the constituents of several cell organelles has been studied extensively, little is known about the normal dynamics of lysosomes, for example, the comparative turnover rate of lysosomal proteins in relation to that of other organelles and whether lysosomal proteins turn over heterogeneously, as has been shown recently for liver endoplasmic reticulum, plasma membranes, ribosomes, and mitochondria (4-11).

In the present work an attempt has been made to elucidate the turnover pattern of the protein components of rat liver lysosomes. The apparent turnover rates of the total soluble lysosomal and membranous lysosomal proteins as well as ly-

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activity determination. The supernatant from the centrifugation of the microsomes constituted the soluble cytoplasmic fraction. The relative specific activities of the marker enzymes, glucose-6-phosphatase, β-glycerophosphatase, and aroylsulfatase with respect to the homogenates were 4, 20, and 45 for the microsomal, lysosomal, and Tritosomal fractions, respectively.

Antibodies to female rat preputial gland β-glucuronidase were prepared in New Zealand white rabbits. A 1.0 ml portion of an antigen-antidextran mixture, prepared by emulsifying equal volumes of complete Freund's adjuvant and a solution of the enzyme (2 mg of β-glucuronidase per ml) (10) in 6 ml Tris-Cl, pH 8.0, was injected intraperitoneally into each animal at 2- to 3-week intervals. Four injections were given. Three weeks after the final dose, the animals were killed and antisera were collected.

To purify β-glucuronidase from the microsomes, 1 ml of saturated ammonium sulfate was added to every 1 ml of ammonium sulfate fractionation as described (18). The precipitate was collected by centrifugation. The sediment was dissolved in a small volume of 0.1M NaCl, and the globulin fraction was again precipitated with an equal volume of saturated ammonium sulfate at 0°C. The precipitate was collected by centrifugation. The sediment was dissolved in a small volume of 0.1M NaCl, and the globulin fraction was again precipitated with an equal volume of saturated ammonium sulfate at 0°C. This process was repeated at least three times. The γ-globulins obtained were further purified by chromatography on DEAE-Sephadex A-50 (20) with 0.1M NaCl, pH 7.4, at 4°C (10, 17). The eluate was concentrated under N2 in an Amicon ultrafiltration cell with a Diaflo XM-50 membrane.

Partial Purification of Lysosomal β-Glucuronidase—The lysosomal β-glucuronidase was partially purified essentially according to the method of Stahl and Touster (18), except that the nuclear fraction, which contains a considerable amount of lysosomal β-glucuronidase, was produced by centrifugation of the mitochondrial-lysosomal fraction to serve as the source of the enzyme. These combined fractions were dialyzed for 16 hours against 4 liters of 5 mM Tris-HCl, pH 7.8, and the dialyzed suspension was centrifuged in a Spinco filtration cell with a Diaflo XM-50 membrane.

Fractionation of Lysosomal β-Glucuronidase—The lysosomal β-glucuronidase was partially purified essentially according to the method of Stahl and Touster (18), except that the nuclear fraction, which contains a considerable amount of lysosomal β-glucuronidase, was produced by centrifugation of the mitochondrial-lysosomal fraction to serve as the source of the enzyme. These combined fractions were dialyzed for 16 hours against 4 liters of 5 mM Tris-HCl, pH 7.8, and the dialyzed suspension was centrifuged in a Spinco filtration cell with a Diaflo XM-50 membrane.

Specific Radioactivity of Lysosomal β-Glucuronidase—The amount of the antibody required to precipitate quantitatively the β-glucuronidase was determined from the precipitation curve as described in Fig. 1. Then, to 5-ml portions of the β-glucuronidase solutions was added the equivalent amount of the antibody solution. The antigen-antibody mixtures were incubated at 37°C for 1 hour, then at 4°C for 18 hours. The mixtures from each experiment were then centrifuged, and the precipitates were washed three times with 10-ml portions of 0.15 M NaCl-5 mM Tris-Cl, pH 7.4. The washed enzyme-antibody precipitates were solubilized and reduced by treatment at 100°C for 5 min with 0.5 ml of 1M sodium phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulfate, 1% Triton X-100, 5 mM EDTA, and 1% mercaptoethanol. The resulting solutions were divided into 6 equal parts and subjected to electrophoresis.

**SDS Electrophoresis**—The electrophoresis was carried out according to the method of Weber and Osborn (24) with slight modifications. The polyacrylamide concentration was 5%, and the gel dimensions were 0.5 × 11 cm. Electrophoresis was performed for 8.5 hours at a constant current of 3.5 mA/gel with the positive electrode in the lower chamber. Each gel was stained for 12 hours with 60 ml of a solution prepared by dissolving 0.5 g of Coomassie brilliant blue in a mixture of 100 ml of glacial acetic acid, 200 ml of methanol, and 800 ml of H2O. The gels were destained for 10 hours with two changes of a 60 ml solution containing 10% each of glacial acetic acid and methanol. The stained protein bands were identified by comparison with control gels, upon which dissociated and reduced samples of the purified preputial gland β-glucuronidase, which has same molecular weight as the lysosomal counterpart, or antibodies, or both, had been run. In each pattern (Fig. 2) the leading and middle bands represented the light and heavy chains of the antibody, respectively; the trailing bands contained the enzyme subunits. Five gels used for the fractionation of a single antigen-antibody precipitate were each sliced into 4.8 mm wide segments beginning from the top of the gel. Corresponding slices from the five gels were combined, dissolved in 0.5 ml of 30% H2O at 37°C or 55°C, and prepared for counting. Slice 7 corresponded to β-glucuronidase subunits. The remaining gel was put aside for photography.

**Characterization of β-Glucuronidase Subunits on SDS Polyacrylamide Gels**—Both rat liver lysosomal β-glucuronidase (18) and preputial gland β-glucuronidase (15) have been shown to be tetramers with a similar subunit molecular weight of about 70,000. In addition, both enzymes were found to have very similar mobility under the present electrophoresis conditions. Thus it was feasible to use the β-glucuronidase from preputial gland as a control to identify the β-glucuronidase from rat liver lysosomes.

**Radioactivity Determination**—The H2O2 solutions of the gel segments were counted by liquid scintillation counting in 10 ml of PCS solution (Amersham/Searle, Arlington Heights, Ill) with the aid of 0.5 ml of NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.).

Other protein fractions (0.2 mg of protein) were precipitated with trichloroacetic acid (8%) final concentration), filtered through glass membranes (Whatman GF/C), and dissolved in 0.5 ml of NCS solubilizer. Radioactivity was determined in 10 ml of a solution containing 4 g of 2,5-diphenyloxazole and 0.05 g of p- bis(2-5-phenyloxazolyl)benzene (Research Products International Corp., Elk Grove Village, Ill.) per liter of toluene.

In the channels used there was ~20% spillover of 14C counts into HI-labeled window. Efficiencies were 42 to 50%; 14C and 14 to 18% for H for all samples.

**RESULTS**

**Characterization of β-Glucuronidase—Antibody Reactions**—The precipitation curve for β-glucuronidase-anti-β-glucuronidase reactions (Fig. 1) was typical for the precipitin reaction. The antibodies were prepared against β-glucuronidase isolated from the preputial gland of the female rat, since it is difficult to prepare sufficient quantities of the enzyme from rat liver. Because the liver and preputial gland enzymes reacted similarly with the antibody (Fig. 1), it was feasible to use antibody directed against preputial gland β-glucuronidase to precipitate the lysosomal enzyme from rat liver.

**Turnover Studies on Lysosomal Proteins**—To determine the turnover of lysosomal β-glucuronidase and of other protein fractions, the double isotope technique of Arias et al. (5) was used. The experimental ease of this method in the study of protein degradation has been discussed previously (5, 7, 9). In Experiments 1 and 2, [4,5-3H]leucine administered initially was allowed to decay for 4 days, followed by the administration of the second isotope, [14C]leucine. The animals were killed 2 or 5 days after the second isotope injection. The β-glucuronidase and other fractions were prepared, and their radioactivities counted as described under "Methods." The apparent half-lives were calculated according to the following equation (1).

$$t_{1/2} = \frac{0.693}{k}$$

1. Micromolar β-glucuronidase was not studied because the enzyme has been shown to lose radioactivity rapidly after labeling (23).
2. The abbreviation used is: SDS, sodium dodecyl sulfate.
were obtained by centrifugation at 2,600 rpm for 25 min in the DEAE-cellulose column.

Triton X-100 portions of 0.15 M NaCl-5 mM Tris-HCl buffer, pH 7.4, containing varying amounts of antibody. The resulting mixtures were incubated for 60 min at 37°C, then 18 hours at 4°C. The precipitates (pH 7.8) and 0.1 M NaCl were used to elute the enzyme from the column.

Experiment 2: Triton X-100 portions of 0.15 M NaCl-5 mM Tris-HCl buffer, pH 7.4, containing varying amounts of antibody. The resulting mixtures were incubated for 60 min at 37°C, then 18 hours at 4°C. The precipitates (pH 7.8) and 0.1 M NaCl were used to elute the enzyme from the column.

Table I gives the isotopic ratios and apparent half-lives for the microsomal and lysosomal fractions in the turnover of lysosomal β-glucuronidase. The similarity in the 14C:3H ratios for β-glucuronidase were 1.1 and 1.2 in the two different experiments, the low extent of incorporation of isotopes limiting the precision of the calculated isotopic ratios. From the isotopic ratios, the apparent half-lives were calculated to be 29 and 15 days, respectively. The large deviation in the half-life values was apparently due to the fact that the 14C:3H ratio approaches the theoretical limit of 1.0, which value corresponds to a half-life of infinity. As can be seen, no gross difference was obtained between the soluble lysosomal and lysosomal membrane fractions in the turnover of their total proteins. On the other hand, the soluble β-glucuronidase definitely had a slower turnover rate, a result indicating that lysosomal proteins had similar turnover rates to soluble cytoplasmic proteins. The physiological significance of this correlation is difficult to assess at the present time. The 14C:3H ratios for β-glucuronidase were 1.1 and 1.2 in the two different experiments, the low extent of incorporation of isotopes limiting the precision of the calculated isotopic ratios. From the isotopic ratios, the apparent half-lives were calculated to be 29 and 15 days, respectively.

Relative Turnover of Soluble Lysosomal Proteins—The finding by Schimke and his co-workers of a general correlation between the rate of degradation and the subunit molecular weight of lysosomal β-glucuronidase, lysosomal soluble fraction, lysosomal membranes, tritosomal soluble fraction, microsomal fraction, soluble cytoplasmic fraction, and lysosomal β-glucuronidase...
FIG. 2. SDS-polyacrylamide gel electrophoresis of antigen, antibody, or both. Methods are given in the text. A, left, antigenantibody complex from Experiment 1; center, the precipitate of preputial gland β-glucuronidase with antibody; right, mixture of preputial gland β-glucuronidase and antibody. B, left, antigen-antibody complex from Experiment 2; left center, mixture of preputial gland β-glucuronidase and antibody; right center, preputial gland β-glucuronidase; right, antibody. In the experimental gel (B, left), there is a protein band which moves slightly faster than the β-glucuronidase subunit band. Although the identity of this protein remains to be determined, it is possible that it is a degradation product of P-glucuronidase, since it is smaller in size and is precipitable by the antibody. (Segment 8 in Fig. 3B corresponds to this band.)

FIG. 3. Distribution of radioactivity in SDS-polyacrylamide gel electrophoretograms of lysosomal β-glucuronidase labeled in vivo. The gels were obtained from the turnover experiments described in Table I. The upper (A) and lower (B) graphs represent Experiments 1 and 2, respectively. After staining with Coomassie brilliant blue and destaining, the gel segments were dissolved in 30% HzO2 (25). Segment 7 corresponds to the β-glucuronidase subunit bands.

FIG. 4. Fractionation of rat liver soluble tritosomal proteins on Sephadex G-200 column in the presence of 0.1% sodium dodecyl sulfate. The soluble tritosomal proteins were derived from Experiment 3. The experimental and control soluble tritosomal fractions (18 ml) were concentrated in an Amicon ultrafiltration cell under N2 to 4.75 ml, which contained about 10 mg of protein. The sample, in which sodium dodecyl sulfate was present at a final concentration of 1%, was incubated at 37° for 3 hours and applied to a Sephadex G-200 column (2.5 × 90 cm) equilibrated with 50 mM Tris-Cl, pH 7.8-0.1% sodium dodecyl sulfate. The column was run at 25°. Column flow rate was 4 ml/hour and ~5-ml fractions were collected. Much of the optical absorption in the main peak may be due to Triton WR-1339, since parallel experiments indicated that the detergent was eluted at that position. The migration rates of marker proteins were determined in a separate run. The fractions in which the marker substances emerged were dextran blue, 31; conalbumin (MW 78,000), 37; ovalbumin (MW 45,000), 44; cytochrome c (MW 13,000), 56. The protein in each fraction was precipitated with trichloroacetic acid and processed for radioactivity determination as described under “Methods.” The broken lines represent the 99% tolerance limits for variation in H:3C ratios based on the control experiment, the results of which are shown in the lower curve. Any fraction in which the H:3C ratio exceeds the bracketed values is significantly different from the mean with a p value of 0.01 or less.

soluble cytoplasmic proteins were fractionated according to subunit molecular weight by Sephadex G-200 column chromatography in the presence of sodium dodecyl sulfate, and the ratios of the isotopes in the eluted fractions were determined. Although carbohydrate moieties might affect the determination of glycoprotein molecular weight, the SDS-gel filtration method is the method of choice since it is superior to SDS electrophoresis and others available (26).

Fig. 4 shows that the relative degradation rates of the soluble tritosomal proteins were generally not proportionate to subunit molecular size. A similar lack of proportionality was obtained when the proteins were fractionated in the absence of SDS (data not shown). On the other hand, the soluble cytoplasmic proteins exhibited a general correlation between the relative rates of degradation and the molecular sizes of the proteins (Fig. 5), a
Fig. 5. Fractionation of rat liver soluble cytoplasmic proteins on Sephadex G-200 column in the presence of 0.1% sodium dodecyl sulfate. The experimental and control soluble cytoplasmic proteins were from the same source as in Fig. 4. Fractionation conditions are also the same as in Fig. 4. The samples (10 ml, 45 mg of protein) were each gel-filtered through a Sephadex G-25 column (1.5 × 22 cm), equilibrated with 50 mM Tris-Cl buffer, pH 7.8, and concentrated to 4.75 ml before fractionation on Sephadex G-200 as described in Fig. 4.

finding consistent with the results of Dehlinger and Schimke (4), who utilized non-Triton-treated animals.

The use of Triton WR-1339 in the isolation of lysosomes raises the question of the extent to which measurements of protein turnover in lysosomes reflect the passage of serum proteins into the lysosomes. The amount of radioactivity in the lysosomes which might be of serum origin was determined with the use of an antiserum against rat serum. The results indicated that at most 6% of the radioactivity in tritosomes represented serum proteins (Fig. 6, upper curve). The antiserum used was capable of precipitating labeled serum proteins to at least 90% of the radioactivity in the serum (Fig. 6, lower curve). These experiments, then, suggest that serum proteins, which are presumed to form a complex with Triton WR-1339 and are trapped in the lysosomes, constituted only a small portion of the proteins being studied.

The lack of general correlation between the rate of degradation and subunit molecular size was also found for the soluble fraction of lysosomes isolated by the method of Ragab et al. (10) (Fig. 7).

The ^14C:^3H ratios should be the same in all protein fractions if all soluble proteins of the lysosomal fraction were synthesized and degraded at the same rate. As shown in Figs. 4 and 7, there were significant differences in such ratios, a result indicating, again, that lysosomal protein turnover was not synchronous.

**DISCUSSION**

The experiments reported in this paper relate to the broad question as to how lysosomes are formed and degraded. Funda-
mental to the problem is the question of the turnover rate of the proteins in both soluble and membrane fractions of the particles. In the determination of the apparent half-lives for the various protein fractions, it was assumed that lysosomal protein degradation follows first order kinetics and that both isotopes were in the state of degradation when the animals were killed. These assumptions are probably valid in view of the fact that (a) the degradation of all proteins thus far reported, with the exception of hemoglobin, are degraded according to first order kinetics, (b) similar isotopic ratios were obtained whether the animals were killed 2 or 5 days after the second isotope injection, and (c) Triton WR-1339 has no effect on the apparent isotopic ratios, which is consistent with the assumption that both isotopes in a given protein fraction are in a state of decay. Triton WR-1339 is known to inhibit protein synthesis and protein incorporation into membranes (28). Had the second isotope still been in the process of incorporation into the lysosomal proteins when the detergent was administered, different $^{14}C:$H ratios should have been obtained. In previous studies (5) the turnover rates of lysosomal proteins were investigated by measuring the decrease in specific radioactivities at various times after pulse labeling in vivo with L-$\text{L}$-glutamino-$^{14}C$arginine. The decay in specific radioactivity was found to follow first order kinetics, from which the mean halflife of the lysosomal fraction was estimated to be 7.1 days. The higher value than obtained in the present study may have resulted from the different procedure employed. In addition, the lysosomal fraction was prepared by a method different from those used in the present study.

Our results show that the turnover rate of soluble lysosomal proteins is, on the average, very similar to that of lysosomal membrane: However, that the lysosomes are not synthesized and degraded as a whole is suggested by two lines of evidence: (a) lysosomal soluble $\beta$-glucuronidase has a distinctly slower turnover rate than the average of total soluble lysosomal proteins, and (b) subunits of the proteins of the soluble lysosomal fraction, as separated by the SDS-Sephadex G-200 column, have different rates of degradation. The identity of the proteins displaying a heterogeneity of turnover in the lysosomes is, of course, presently unknown, except for $\beta$-glucuronidase.

It is interesting that our finding of a rather long half-life for lysosomal $\beta$-glucuronidase is similar to the 20 days estimated for the acid hydrolyses of rat-liver based on the amount of enzymes excreted in the bile (29). It is also consistent with the very slow rate of synthesis of lysosomal $\beta$-glucuronidase found by Van Lanker and Lentz (23) but is, however, grossly different from the rapid synthesis and degradation of microsomal $\beta$-glucuronidase (23), which makes unlikely the possibility of the microsomal form being the precursor of lysosomal form. Indeed, Swank and Paigen (30) have recently presented biochemical and genetic evidence suggesting that, in the mouse, the microsomal and lysosomal forms of $\beta$-glucuronidase are independently formed from a common enzymatically active precursor. The equal reactivity of lysosomal and microsomal $\beta$-glucuronidase against prepland $\beta$-glucuronidase-antibody (Fig. 1) suggests that lysosomal and microsomal enzymes have very similar, if not identical, immunological properties.

In order to explain the correlation between molecular size and degradation rates of proteins associated with various membrane fractions (4), ribosomes (11), chromosomes (9), fatty acid synthetase complex (31), and rat liver cytoplasm (32), it has been suggested (9, 11) that protein oligomers may be in a state of continual dissociation and association in which their subunits rapidly exchange with free subunits and that the protein subunits are the basic units of degradation. If this idea is valid, our finding of the lack of a rather simple correlation between rates of degradation and molecular size of lysosomal protein might suggest that protein subunits are not the basic units of degradation in lysosomes. However, one cannot rule out the possibility that the lack of correlation of degradation rates and subunit size results from partial proteolytic digestion during the isolation of lysosomes, since it has been shown that proteins turning over rapidly in vivo are more rapidly degraded by exogeneous proteases (9).

Finally, it should be mentioned that the underlying basis for the selective turnover of cellular proteins is not understood (1). Although there are difficulties in accepting the idea that lysosomes and their proteases play central roles in this phenomenon, Haider and Segal (2) and, very recently, Neely and MORTIMORE (3) have presented work in favor of such an involvement of lysosomal proteases. Our finding that lysosomal and nonlysosomal proteins generally have similar turnover rates will have to be borne in mind in further consideration of this problem. Indeed, the present results are consistent with the concept that lysosomes are acting as sieves, as proposed by Haider and Segal (2).

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REFERENCES

Turnover studies on proteins of rat liver lysosomes.
Wang C-C and O Touster


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