LOCALIZATION OF PRODUCTS OF ENDOGENOUS PROTEOLYSIS IN LYSOSOMES OF PERFUSED RAT LIVER*

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SUMMARY

The subcellular distribution of endogenous proteolysis was determined in perfused rat liver prelabeled with L-[1-^{14}C]leucine; most of the activity was found in the mitochondrial-lysosome (M + L) fraction. Further separation by isopycnic centrifugation of the M + L fraction from unincubated homogenates revealed a peak of trichloracetic acid soluble radioactivity which overlaid the lysosomal marker enzyme peak and was separate from mitochondria. When lysosomal density was decreased by giving Triton WR-1339 *in vivo*, both the enzyme and radioactivity peaks moved to the lighter gradient fractions. Since unlabeled M + L material failed to take up radioactivity from labeled supernatants, the results indicate that the lysosome is a site of endogenous proteolysis.

INTRODUCTION

It is known that protein turnover in liver is a continuous process. While many steps in the sequence of protein synthesis have been delineated, the mechanism of protein degradation is not well understood. The possibility that the lysosomal system is involved in proteolysis is suggested by the localization of cathepsins within lysosomes (1) and by the fact that isolated lysosomal proteases are capable of degrading large proteins to free amino acids or small peptides (2). Moreover, it is known that lysosomes are capable of digesting exogenous protein after its endocytic uptake by liver (3).

It is reasonable to suppose that if endogenous proteins are degraded intralysosomally, lysosomes would contain products of digestion derived from these

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proteins. Previous studies have disclosed the existence of free amino acid pools within lysosomal (4) and mitochondrial (+ lysosomal) fractions of cell homogenates (5). However, the relationship of these pools to the breakdown of endogenous protein in the lysosome was not made clear. In the present report we have attempted to demonstrate labeled products of proteolysis within the lysosome under conditions where endogenous protein was the primary source of label.

METHODS

Male rats (120-135 g) of the Lewis strain (Microbiological Associates) were maintained on standard laboratory chow and water ad libitum. Liver protein was labeled in vivo by the intraperitoneal injection of L-[1-^{14}C]leucine, without carrier, in 0.5 ml of 0.85% sodium chloride 18 and 4 hours prior to perfusion (6). The method of perfusion and composition of the perfusion medium were the same as those employed previously (6,7). At the termination of the experiments segments of liver were quickly excised and homogenized (1:10, w/v) in 0.25 M sucrose containing 10^{-3} M sodium ethylenediamine tetraacetate (8).

In the experiments of Figure 1 the homogenate was fractionated by centrifugation using a method patterned after the scheme of de Duve (9). We eliminated washing of the pellets since control studies indicated that lysosomes were damaged by these extra manipulations and resolution among the major fractions was not greatly improved. In this modification the distributions of acid phosphatase activity and proteolysis among the nuclear (N), mitochondrial-lysosomal (M + L), microsomal (P), and supernatant (S) fractions were calculated by difference from activities in the parent homogenate and in the supernatants after centrifugation at 0.1, 2.5, and 23 x 10^5 g·min. Total acid phosphatase was determined as reported earlier (8). Endogenous proteolysis was estimated from the increase in trichloracetic acid soluble radioactivity after incubation (pH 6.9) at 37° for 30 min. Details of the method were discussed previously (7).

In the remainder of the studies the M + L fractions obtained from the above scheme were further separated by density gradient centrifugation. The pellets were resuspended by gentle agitation and layered on linear 24-70% sucrose gradients; the tubes were then placed in a Beckman SW-50L rotor and spun in a Beckman L3-50 centrifuge at 50,000 rpm for 60 min. Under these conditions the lysosomal marker enzyme and the radioactive peaks were fully equilibrated within the gradient. Fractions were collected on ice by gravity drainage and Triton X-100 (final concentration, 0.1%) was added to each fraction to ensure disruption of the lysosomes. Trichloracetic acid soluble radioactivity was determined in an aliquot of each

fraction after precipitation of the protein by cold 5% trichloracetic acid. The acid then was extracted 3X with water-saturated diethyl ether and the remaining solution counted in Aquasol (New England Nuclear Corp.) with the use of a Beckman LS-150 liquid scintillation spectrometer. Counts were corrected for quenching by external standards. The lysosomal enzyme, N-acetyl-β-D-glucosaminidase (EC 3.2.1.30), was measured in each fraction as described by Barrett (10), modified by a reduction of the citrate buffer concentration according to Woolen et al. (11). Because of the small sample volumes, this enzyme was easier to assess than acid phosphatase. Protein was determined by the Lowry procedure (12).

RESULTS AND DISCUSSION

In an earlier report we presented evidence suggesting that increases in free leucine + isoleucine during the incubation of liver homogenates at 37° may reflect the final exergonic phase of an ongoing process of general protein degradation in the intact liver (7). As shown in Figure 1 the subcellular distribution of this proteolytic phase in homogenates of control perfused liver follows closely the distribution of a typical lysosomal marker enzyme, acid phosphatase. It is of interest to note that very little proteolytic activity was found in the microsomal or supernatant fractions. Proteolytic activity in association with micro-

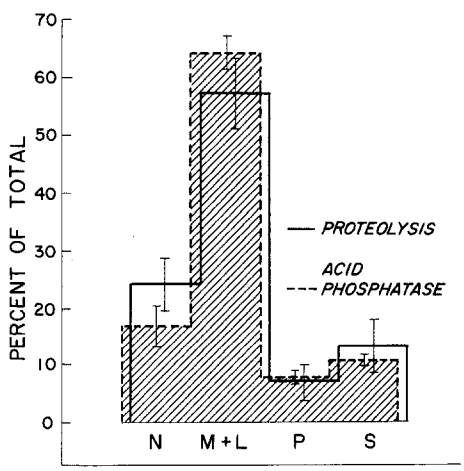

Figure 1. Distributions of endogenous proteolysis and acid phosphatase activity in subcellular fractions of perfused rat liver. Livers previously labeled with 10 μCi of 1-[1-^{14}C]leucine were perfused without additions to the medium (control) for 60 min, homogenized and then fractionated as described in METHODS. Abbreviations are: N, nuclear; M + L, mitochondrial + lysosomal; P, microsomal; S, supernatant. Values shown are means ± one S.E. of 6 sets of proteolysis and 5 sets of acid phosphatase analyses.

somes has been noted (13), but it was appreciably less than lysosomal activity. Since the trichloracetic acid soluble radioactivity fraction that was measured in this study included small peptides as well as free amino acids (7), the results of Figure 1 would not have revealed peptide degradation by peptidases known to be in the supernatant phase (2,14,15). It is thus reasonable to assume that Figure 1 reflects the distribution of catheptic activity which is largely lysosomal in location (1).

On the assumption that the proteolysis demonstrated in the foregoing experiments is an intralysosomal event, an attempt was made to demonstrate labeled products of proteolysis in association with lysosomes in livers prelabeled with L-[1-^{14}C]leucine. Separation of the M + L fraction was carried out by the use of equilibrium density gradient centrifugation. It is apparent from Figure 2 that the distribution of trichloracetic acid soluble radioactivity closely

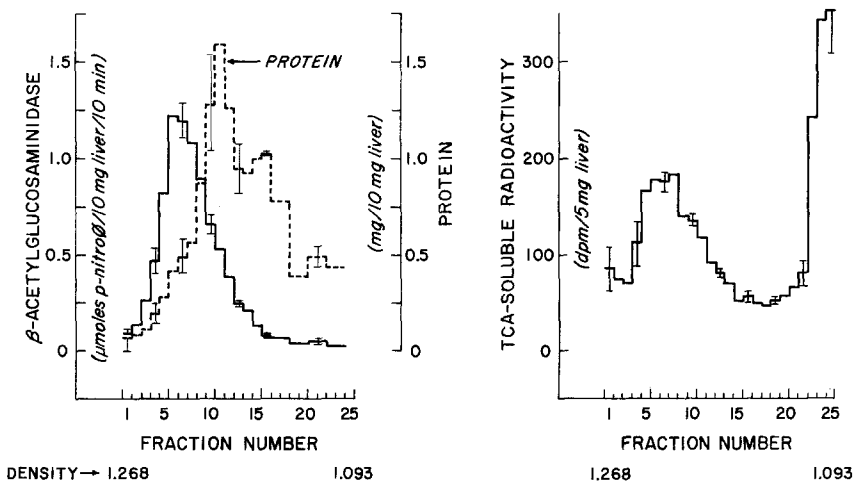

Figure 2. Equilibrium density distributions of N-acetyl-β-D-glucosaminidase, protein and trichloracetic (TCA) soluble radioactivity from M + L fractions of perfused rat liver. Liver protein was previously labeled in vivo with 100 μCi of L-[1-^{14}C]leucine. After 60 min of control perfusion M + L fractions from each liver were centrifuged in 24-70% linear sucrose gradients as described in METHODS. Mean values (± one S.E.) of the individual fractions were normalized to the average total values of the respective groups. The numbers of experiments were: β-acetylglucosaminidase, 9, protein, 4; trichloracetic acid soluble radioactivity, 4.

parallels the distribution of the lysosomal marker, β-acetylglucosaminidase, and differs significantly from the distribution of protein, which is known to reflect mitochondria largely. The sharp peak of radioactivity in the uppermost gradient fractions represents soluble label, most of which was probably initially present in the cytosol.

As shown in Figure 3 a decrease in lysosomal density following the adminis-

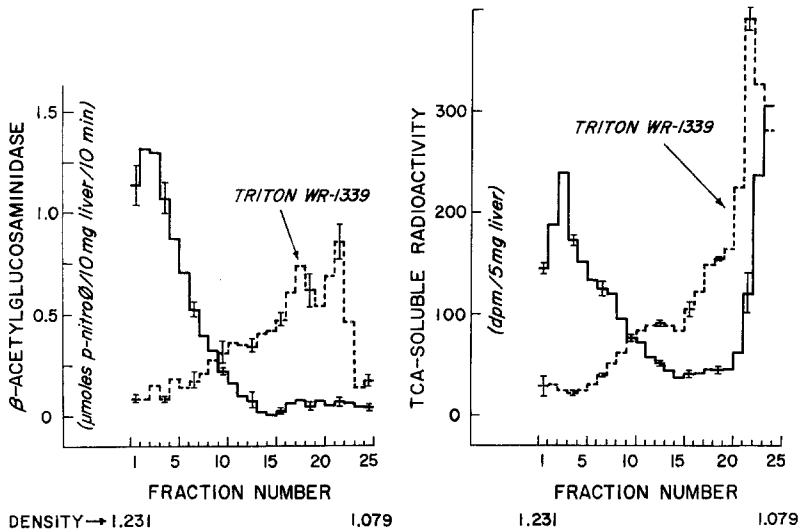

Figure 3. Effect of Triton WR-1339 loading on the equilibrium density distribution of N-acetyl-β-D-glucosaminidase and trichloracetic acid (TCA) soluble radioactivity from M + L fractions of perfused rat liver. The experiments were carried out and reported as in Figure 2 except for the intraperitoneal administration of Triton WR-1339 (85 mg/100 g body weight in 0.5 ml 0.85% sodium chloride) to one group of animals 3-1/2 days prior to perfusion (16). The concentration limits of the sucrose gradient were reduced from 24-70% to 20-60% because of the lower density of Triton-containing lysosomes. Values in left panel are means ± S.E. of 4 experiments for each group; right panel, 2 experiments.

tration of Triton WR-1339 *in vivo* (16) moved both the peaks of β-acetylglucosaminidase and trichloracetic acid soluble radioactivity to the upper end of the gradient. Owing to the lower sucrose concentrations employed in these experiments, the lysosome peaks from non Triton-treated rats were lower in position than those in Figure 2. As revealed in separate control experiments, the position of protein was not affected by Triton loading. The results of these experiments and those

of Figure 2 clearly demonstrate the presence of a pool of trichloracetic acid soluble radioactivity in close relationship with lysosomes. The lack of association between the peaks of acid soluble radioactivity and protein appears to eliminate the possibility that the acid soluble radioactivity fraction was nonspecifically adsorbed to or sequestered by particulate fractions such as mitochondria.

We also considered the possibility that a significant portion of the lysosomal acid soluble radioactive pool was acquired artifactually after homogenization by the adsorption or uptake of amino acids or small peptides from the supernatant. In further experiments (Figure 4) we observed that when unlabeled M + L fractions

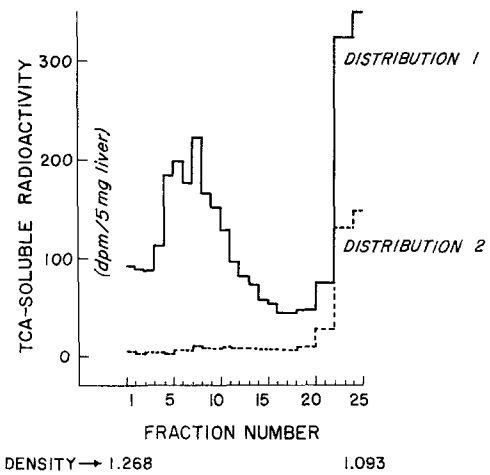

Figure 4. Equilibrium density distribution of trichloracetic acid (TCA) soluble radioactivity from mixtures of labeled and unlabeled M + L and supernatant fractions. Rats were previously labeled and perfused as described in Figure 2. In Distribution 1, the M + L fraction applied to the gradient consisted of an M + L pellet from a previously labeled liver resuspended in unlabeled supernatant. Distribution 2 was obtained from a mixture of unlabeled M + L material and labeled supernatant. Density gradient fractionation was carried out as described in METHODS.

were mixed with the supernatants from labeled livers, no trichloracetic acid soluble radioactivity appeared in the lysosome region after subsequent density gradient centrifugation. The opposite mixture, however, did reveal the expected labeled lysosome peak. Thus it is unlikely that the lysosomal radioactivity was derived directly from the supernatant of the homogenate. A more reasonable

source of the acid soluble label, it seems to us, would be its proteolytic generation within the lysosome. While it is possible that acid soluble label might have penetrated the lysosome in the intact cell, there is no reason to believe that lysosomes within the cell would be more permeable to amino acids and small peptides than they would be in homogenates. It is also possible that some uptake of label might have been demonstrated if the mixtures were allowed to stand longer than 15 to 20 min before centrifugation. However, owing to the risk of further lysosomal damage, the significance of such uptake might be difficult to assess.

Assuming then that the labeled proteolytic products associated with lysosomes were formed intralysosomally, one may ask how the lysosomes acquired the labeled protein substrate. It is conceivable that protein or protein fragments penetrated the lysosomal membrane after tissue homogenization. The lysosomal membrane, however, is thought to be impermeable to particles greater than 200-300 M.W. (17). Thus the direct entry of protein by simple diffusion would not be a likely possibility. In previous studies we have observed a correlation between relative rates of proteolysis in perfused rat liver and physical alterations of the lysosomal system indicative of lysosomal enlargement (7,8,18). This relationship has suggested that the lysosomal system may sequester endogenous protein as an ongoing, regulated process under physiological conditions. While the mechanism of sequestration is not known, the present data support this notion and underscore the need for further investigation of the role of the lysosomal system in general protein turnover.

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