PROTEIN ABSORPTION BY RENAL CELLS

II. Very Rapid Lysosomal Digestion Of Exogenous Ribonuclease In Vitro

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INTRODUCTION

In vitro digestion by intact lysosomes was first demonstrated by Mego and McQueen (1965 *a*, 1967) using mouse liver preparations. More recently, similar observations have been made with other organisms and tissues, for instance with preparations of rat yolk sac (Williams et al, 1972) and frog and toad liver and kidney (Mego, 1971). The mechanism of lysosomal protein digestion has been examined using extracts of purified lysosome preparations by Misaka and Tappel (1971), Goettlich-Riemann et al. (1971), Huang and Tappel (1971), and Coffey and de Duve (1968).

A paper of Davies et al. (1969) suggests the possibilities for studying the mechanism of protein degradation with intact particles; these workers measured the inhibition of such degradation by trypan blue when the latter was within the particles. The kinetics and metabolism of uptake and breakdown have also been studied, using intact cells, by Bowers and Olszewski (1972) with amebas and Ehrenreich and Cohn (1967) with macrophages.

Intravenously injected, iodine-labeled bovine ribonuclease is rapidly absorbed by mouse kidney (Schultze et al., 1966; Davidson et al., 1971). Large amounts can be taken up. Some or all of the ribonuclease is then degraded, and the label leaves the kidneys very rapidly. Comparably high lysosomal activity has not been reported. Because the disappearance of radioactivity from kidneys is so rapid, it was important to establish whether lysosomal degradation could be adequate to account for it (Gregoire and Hughes, 1966; Davidson et al., 1971). In this paper, we describe very rapid digestion of intravenously injected ribonuclease by subcellular kidney particles in vitro and report some of the properties of the digestion process. We present evidence that this rapid digestion, as in previously reported instances of protein degradation, occurs in lysosomes. Finally, we discuss the use of in vitro digestion by lysosomal preparations as a tool to study the stability and properties of these particles and their membranes.

MATERIALS AND METHODS

Unlyophilized ribonuclease A was obtained from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill., sodium iodide-125 from New England Nuclear, Boston, Mass., and *N*-bromosuccinimide, technical grade, from Arapahoe Chemicals, Syntex Corp., Boulder, Colo. Other compounds were reagent grade. Glass-distilled water was used throughout.

Labeling of Ribonuclease

The method of iodinating ribonuclease as previously described (Davidson et al., 1971) was modified by including an amount of carrier iodide equivalent, in moles, to the protein. This doubled the labeling efficiency.

10–15 mg of bovine ribonuclease A was mixed with an equivalent molar amount of sodium iodide and up to 2 mCi of sodium iodide-125 in about 1 ml of 0.1 M sodium phosphate buffer, pH 6.8. One equivalent of *N*-bromosuccinimide solution was then added slowly with stirring. A yellow-orange

iodine color appeared, then faded gradually over about 3 min. 0.1 ml of 0.1 M sodium iodide, then 0.1 ml of 0.1 M sodium sulfite were added. Omission of sulfite in some preparations did not change the result. The reaction mixture was dialyzed twice against 250 ml of 0.9% NaCl. For use, the dialyzed, iodinated ribonuclease was diluted to 2 mg/ml in 0.9% saline. Labeling efficiency was 75–82%. In rate and extent of absorption by mouse kidneys, these were indistinguishable from previous preparations. Ribonuclease activity was undiminished.

Kidney Preparations

These were made essentially as previously described (Davidson et al., 1971). In summary, 50–100 μl of the iodinated ribonuclease A solution described above were injected into the tail veins of two to six mice. The mice were sacrificed by cervical fracture 20 min after injection unless otherwise indicated, and the kidneys were immediately excised and chilled in ice-cold, isotonic sucrose. All subsequent operations were performed in the cold. The cut-up kidneys were homogenized in 0.25 M sucrose, usually 2 ml per pair, by 4–10 slow strokes of a plastic motor-driven pestle. The homogenate was centrifuged for 10 min at 1,000 rpm (250 *g*), in a refrigerated International PR-2 centrifuge (no. 269 rotor) (International Equipment Company, Needham Heights, Mass.) To improve yield, the precipitate was often rehomogenized and recentrifuged in the same way and the 250 *g* supernates were pooled.

Separation of Labeled Particles

The particles were ribonuclease were prepared from the 250 *g* supernates by one of several alternate ways (see Results). Sometimes they were sedimented by centrifugation at 15,000 rpm (20,400 *g*, max) for 15 min (300,000 *g* × min) in a Spinco Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), with a no. 40 rotor. This is a higher speed than we found necessary with a no. 40.3 rotor (Davidson et al., 1971), presumably because the tubes were not filled when the no. 40.3 rotor was used and because of the greater tilt of the tubes in the no. 40 rotor. Alternatively, for greater digestive activity, 4-ml volumes of 250 *g* supernate were centrifuged at 3,000 rpm (2,250 *g*) for 20 min (45,000 *g* × min) in an International PR-2 centrifuge with no. 269 rotor. The precipitates were resuspended in 0.25 M sucrose or in 0.025 M sodium acetate buffer and 0.25 M sucrose, pH 5.5. Particle stability is greater in this buffer than in pure sucrose solution (Davidson et al., 1971), but the 15,000-rpm precipitates were difficult to resuspend in buffer.

Still another method of sedimentation was by centrifugation at 1,000 *g* for 15 min (15,000 *g* × min) after adding ⅓ vol of 0.1 M acetate buffer, 0.25 sucrose, pH 5.5, to the 250 *g* supernate. The acetate was added dropwise with stirring, and the mixture then allowed to stand for about 15 min before centrifugation at 2,000 rpm in the International PR-2 centrifuge. Precipitates were resuspended as described above. In most experiments, ½ ml of suspension medium was used per kidney pair. Protein concentrations of some suspensions were determined by the method of Lowry et al. (1951).

Incubation

Unless otherwise indicated, incubation was carried out in 10 ml of 0.025 M sodium acetate buffer in 0.25 M sucrose, pH 5.5. Media were warmed to 37°C in a water bath before addition, at zero time, of 0.1–0.2 ml of cold particle suspension or 250 *g* supernate. Similar mixtures were sometimes kept on ice to provide zero time samples. 1-ml samples were removed at intervals during incubation and added to mixtures of 1.35 ml of water with 0.15 ml of phosphotungstic acid (PTA) reagent (6.25% PTA, 9% hydrochloric acid; Davidson et al., 1971), which precipitates intact but not degraded ribonuclease. Zero time samples were made by adding 10–20 μl of particle suspension to 2.35 ml of cold water or 1 ml incubation medium and 1.35 ml water, plus 0.15 ml PTA reagent or else by taking 1-ml samples from a mixture identical to the incubated one, but on ice. All of these acidified mixtures were made up in plastic tubes to avoid quenching of the low energy ^{125}I radiation. Radioactivities were determined by manual counting with well-type Tracerlab (LFE Electronics, Tracerlab Div., Richmond, Calif.) scintillation spectrometers. Usually, at least 2,000 counts were recorded which required 0.2–0.5 min. After counting, the samples were centrifuged in the International PR-2 for 15 min at 2,000 rpm, and the supernates separated and counted on a Tracerlab counter with automatic sample changer. Again, 2,000 counts were recorded per sample. With these less active supernates, this same to at least 1,500 counts above background.

The counting rate for each supernate was divided by that measured before centrifugation. Digestion was shown by the increase in PTA-soluble label as incubation proceeded. A program was developed for use with the Olivetti Programma 101 computer (Olivetti Corp. of America, New York) which included subtraction of backgrounds, correction for isotope decay, and calculation of the quotients described above.

RESULTS

Ribonuclease Digestion

After intravenous injection of radioiodinated ribonuclease, mouse kidneys become labeled by

ribonuclease absorption. If the protein is injected about 15 or more min before death, kidney homogenates prepared in isotonic sucrose digest some of the ribonuclease rapidly upon incubation at 37 C. As previously shown, most of the radioactivity in these preparations can be readily centrifuged down, showing that it is bound to subcellular particles (Davidson et al., 1971). The very rapid digestive activity belongs to the sedimented, ribonuclease-bearing organelles; supernates have little digestive activity. Incubation is performed by adding small samples of kidney preparation to acetate buffer in isotonic sucrose at 37°C. Release of the radioactivity in a form which, unlike intact ribonuclease, is soluble in PTA reagent (see Materials and Methods) indicates digestion. At 0°C, no digestion occurs. At 37°C, rapid release of acid-soluble label continues for about the 1st min (Fig. 1). Then the rate usually falls off sharply. In the 1st min, 5–14% of the label is released; in 5 min, the figure may reach 20–25%.

Sedimentation Properties

Different modes of preparation were developed in the hope that these differences might prove useful in developing purification methods. High yields of labeled, particulate material were obtained from 250 *g* supernates either by centrifugation of 300,000 *g* × min in 0.25 M sucrose or by acidifying with dilute sodium acetate buffer in isotonic sucrose, pH 5.5, to a final acetate concentration of 0.025 M and spinning at 1,000 *g* for 15 min. The smallness of the force required for sedimentation in acetate buffer indicates that such preparations were aggregated. Both types of preparation were digestively active (Table I). By either method, intact particles sedimented in the presence of sodium chloride (Davidson et al., 1971), and sedimentation in sodium chloride was sharply reduced after suspension of the preparation in hypotonic medium. Repeated freezing and thawing, like hypotonic medium, either lyses the particles or makes them liable to lysis in the saline centrifugation solution (Table II). A concentration of 0.25 M sodium chloride was found to give better discrimination of particle damage than the lower concentration used previously (Fig. 2).

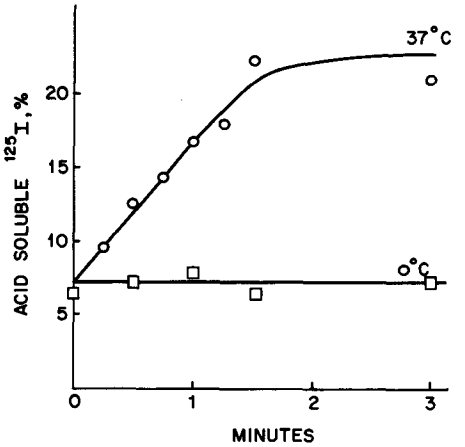

FIGURE 1 Ribonuclease digestion by kidney particles at 37°C. 0.2-ml portions of a resuspended 300,000 *g* × min precipitate were added to 10-ml portions of 0.025 M sodium acetate buffer, 0.25 M sucrose, pH 4.7, at 0° (□) and 37°C (○) for incubation as described in Materials and Methods.

Digestively active particles are centrifuged down more readily than other labeled particles. This was shown by multiplying the amount of radioactivity sedimented by the percentage of radioactivity made acid soluble during 1 min of incubation at pH 5.5 and 37°C. Preparations centrifuged with an integrated force of 45,000 *g* × min in 0.25 M sucrose can release a total of PTA-soluble radioactivity as great as that of preparations brought down under more vigorous conditions, although the latter contain 30–70% more particle-bound radioactivity (Table I). In consequence, the 45,000 *g* × min preparations are digestively more active relative to the radioactivity they contain.

The Location and Nature of Digestion

Rapid digestion requires intact particles. The damage caused by dilution to 0.05 M sucrose or repeated freezing and thawing before incubation almost completely destroys digestive activity in the dilute suspensions studied here.

Moreover, digestion occurs within particles. When labeled ribonuclease is added to the incubation buffer rather than being injected, only very slow digestion occurs. This means that macromolecules in the medium cannot gain access to the digestive enzymes. If the ribonuclease being digested were adsorbed to particle surfaces, ribonuclease in the medium should also be able to reach these surfaces, become adsorbed, and undergo digestion. The results are similar whether or not the mice have been previously injected with unlabeled ribonuclease.

It was necessary to demonstrate also that digestion, rather than release of the products of previous digestion, is the cause of the increase in PTA-

TABLE I

Comparison of Sedimentation between Digestively Active and Total Ribonuclease-Containing Particles

Centrifugation	Sedimentation of particles *a*	Digestive activity of precipitates: Acid soluble cpm/min *b*	Digestive activity of precipitates: Total sedimented *c*
g × *min*	*cpm, fraction of total*	*% of total*	*a* × *b* (*arbitrary units*)
45,000 (6)	0.41 (±0.051)	9.3 (±1.8)	3.8 (±1.1)
300,000 (5)	0.54 (±0.051)*	7.2 (±2.1)	3.9 (±1.2)
15,000 (6) (in acetate, pH 5.5)	0.67 (±0.072)‡	5.5 (±0.61)*	3.7 (±0.38)

*, ‡ Significance levels of differences from top value in same column: * 0.01, ‡ 0.002.

Numbers of experiments and standard deviations are in parentheses. 250 *g* kidney supernates were centrifuged as described (Materials and Methods). The radioactivity of each precipitate was divided by the amount centrifuged to give the fraction sedimented (column *a*). The precipitates were resuspended and portions incubated at pH 5.5; the percentage of the incubated radioactivity which was made PTA soluble in 1 min at 37°C is given in column *b*. The total digestive activity sedimented (column *c*) was calculated by multiplying the values for columns *a* and *b*.

TABLE II

Effect of Hypotonic Medium, Freezing and Thawing, and Dilute Acid upon the Integrity of Labeled Particles

Treatment	Precipitate cpm
	% of total
None	69.2
Freezing and thawing	26.8
Hypotonic medium	26.8
Centrifugation in 0.0125 M HCl	3.0

1,000 *g*, pH 5.5, precipitate from mouse kidney was resuspended in 0.025 M sodium acetate buffer, 0.25 M sucrose, pH 5.5. A portion was diluted 1:10 with water and stored cold for about 20 min. Another portion was rapidly frozen and thawed five times with methanol in dry ice, then diluted 1:10 with 0.25 M sucrose. 0.9-ml samples of each dilution were mixed with 0.9 ml of 0.5 M NaCl, 0.25 M sucrose plus 0.4 ml of 0.25 M NaCl, and 0.25 M sucrose for centrifugation. 0.05-ml samples of the original suspension were added to 2 ml of either 0.25 M NaCl, 0.25 M sucrose, or 0.0125 M hydrochloric acid for centrifugation. The mixtures were centrifuged in uncapped tubes for 15 min at 8,900 *g* (Spinco no. 40.3 rotor). Duplicate tubes were made up for each treatment. Values from duplicates agreed to within 2½% of the total radioactivity centrifuged.

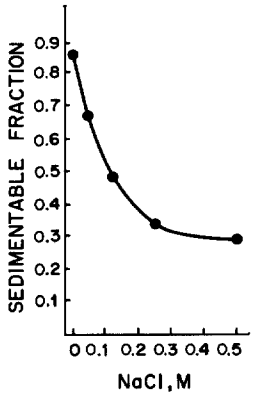

FIGURE 2 Effect of varying saline concentrations upon sedimentation of label from damaged particles. Labeled particles were precipitated from a kidney preparation with pH 5.5 acetate and resuspended in 0.025 M sodium acetate, 0.25 M sucrose pH 5.5. The particles were damaged by freezing. 0.5-ml aliquots were placed in Oak-Ridge type centrifuge tubes and counted and the tubes were filled with 8.9–9.4 ml of 0.25 M sucrose containing sodium chloride at the concentrations indicated on the abscissa. These mixtures were centrifuged for 15 min at 15,000 rpm (Spinco no. 40 rotor). The radioactivity of each precipitate was divided by that in the original aliquot. The preparation used for this curve came from mice killed 5 min after injection; the results were very similar from a 20-min preparation.

soluble label at 37°C. Particle preparations were treated with dilute hydrochloric acid which releases their entire content of label (Table II). The material so released was completely precipitated by PTA reagent, showing the particle-bound ribonuclease to be undegraded by this criterion.

To establish that the degradation reported here is a true proteolysis, not a deiodination, the

TABLE III

Iodide in the Digestion Products as Measured by Extraction with I_2 in Toluene

Time of incubation	PTA-soluble cpm	Extractable cpm
min	*% of total*	*% of total*
0	5.5	1.4
1	14.2	2.0
2	16.6	1.9
5	22.4	2.6
Na I^{125}		
1		90.7
2		95.9

Incubation samples were added to dilute PTA reagent and centrifuged at 1,000 *g*, as usual. At the same time, a drop of sodium iodide-125 was mixed with incubation buffer and dilute PTA reagent in the same proportions as for the incubation samples. All of these mixtures were extracted with a solution of iodine in toluene, with similar intervals of mixing. The radioactivities of the aqueous phases were determined and the extractable radioactivities were calculated by difference and divided by the total radioactivities in each original sample. As a control for the quenching of radioactivity, a similar mixture was agitated with two drops of iodine-toluene solution; the aqueous phase acquired a color similar to that of the iodine controls, but showed no detectable quenching.

PTA-soluble supernatants obtained after incubation were extracted with I_2 in toluene (Table III). Unlike sodium iodide controls, the digestion products partitioned into the organic phase to only a small extent with each extraction.

Tonicity, pH, and Digestion

Digestion is most active in isotonic, buffered sucrose (Fig. 3). A requirement for a nearly isotonic digestion medium is to be expected for digestion within particles. The particles are fairly tolerant of variations in tonicity, however, from 0.2 M to 0.35 M sucrose the rate of label release is not significantly affected. In still more concentrated sucrose, the rate falls off slowly. Three experiments gave an average digestion rate in 1 M sucrose of 78% of the rate in 0.25 M sucrose. Sodium chloride does not provide complete osmotic protection, but neither does it penetrate rapidly

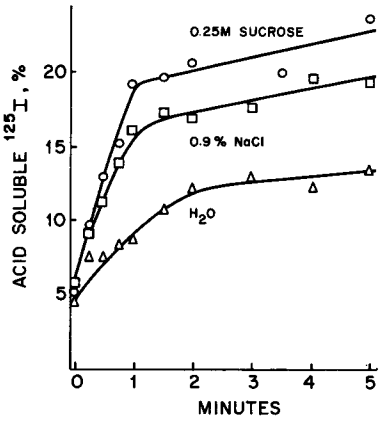

FIGURE 3 Osmotic protection of digestively active lysosomes by isotonic sucrose and partial protection by saline. Portions of particle preparation were incubated at 37°C in aqueous solution (△), 0.25 M sucrose (○), or 0.9% saline (□), each at pH 4.7 with 0.025 M sodium acetate buffer, as described in Materials and Methods.

because, in buffered physiological saline, the extent of digestion was diminished with no great effect on the initial rate (Fig. 3).

Characteristically for the reactions of lysosomal proteases, digestion is more rapid at moderately acid pH and shows a very broad pH optimum. The most vigorous digestion occurs in the pH range 4.5–5.5. The label solubilized in 1 min at 37°C at neutral pH averaged 76% of that solubilized at pH 5.5. Phosphate buffer was slightly inhibitory compared to sodium acetate. This was not due to greater ionic strength because 0.1 M and 0.025 M sodium acetate buffers, pH 5.5, gave the same rate.

Particle Lysis and Incubation

Under the conditions described, digestion is rapid at the beginning of an incubation, then slows down rather sharply. This falloff in rate is not due to lysis of the particles under incubation conditions. If it were, centrifugation of the particulate suspensions in saline sucrose after 1 or 2 min of incubation should leave most of the radioactivity in the supernate as is seen when particles are extensively damaged. When samples were removed from incubation mixtures, then centrifuged in this way, only modest breakdown was seen in the first few minutes of incubation. In Fig. 4, the upper curve shows these results; most radioactivity is still particle-bound when digestion

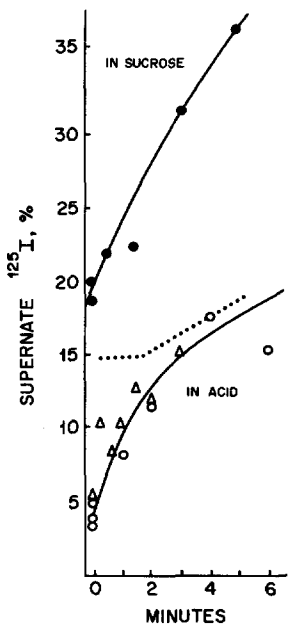

FIGURE 4 Slowdown in digestion precedes extensive lysis of lysosomes. Two identical particle dilutions were incubated at pH 5.5, 37°C. At 0, ½, 1, 3, and 5 min, 1-ml samples were removed from one mixture, diluted with 1 ml of cold 0.5 M NaCl–0.25 M sucrose, and centrifuged at 8,900 *g* for 15 min (Spinco no. 40.3 rotor). The dark circles give proportion of radioactivity which was not sedimented. At 1, 2, 4 and 6 min, 1-ml samples were removed from the same incubation mixture and treated with PTA as usual (light circles). All samples from the duplicate incubation were treated with PTA (triangles). The difference between the upper and lower curves (dotted line) indicates ribonuclease release because of particle breakdown.

slows down. The actual degree of particle breakdown is the difference between total noncentrifugable label and acid-soluble label (Mego and McQueen, 1965 *b*). This is shown in the middle curve of the figure. Breakdown in the 1st min is very moderate and insufficient to account for the fall in digestion rate.

DISCUSSION

Liver clearance of label from denatured bovine serum albumin (BSA) has a half time of 40–45 min (Mego and McQueen, 1965 *a*). Disappearance of peroxidase activity from kidney lysosomes takes days (Strauss, 1964). In these instances, lysosomes were shown to be the sites of protein degradation. In contrast, labeled exogenous ribonuclease leaves mouse kidneys with a half time of about 14 min (Davidson et al., 1971). The question arises, therefore, whether breakdown of the ribonuclease proceeds by the same general mechanisms as that of other proteins. This must be asked because the ultimate functional capacities of lysosomes have not yet been ascertained. The results presented here show that ribonuclease degradation does indeed occur in subcellular particles, i.e., in lysosomes. They also demonstrate that it is possible to observe very high lysosomal activity in vitro. These conclusions follow from the following facts: (*a*) when incubated at 37°C, particles isolated by centrifugation of kidney homogenates degrade the ribonuclease at a rate which is initially very high and comparable to that in vitro, (*b*) rapid ribonuclease digestion occurs only within the particles.

The role of particles appears from the isolation of digestive activity in the precipitate after centrifugation. Most of the digestively active particles can be isolated by rather gentle centrifugation: 45,000 *g* × min (Table I) and after 300,000 *g* × min the supernate contains almost no digestive activity.

That degradation occurs upon incubation follows because the contents of the particles, which are quantitatively released by dilute acid (Table II), are insoluble in PTA reagent and are therefore undegraded before incubation. The digestion is an actual proteolysis because, although the digestion products have not yet been studied in the detail they deserve, they are not simply free iodide arising from deiodination. This was shown by extraction of incubation media with iodine in toluene after a period of digestion (Table III). The proof that digestion occurs within the particles was that ribonuclease added to the incubation medium was not digested.

The subcellular particles in which exogenous proteins are digested are lysosomes (de Duve and Wattiaux, 1966; Mego and McQueen, 1965 *a*; Strauss, 1962) and it is therefore lysosomes labeled by uptake of labeled ribonuclease and active in degrading this ribonuclease with which we are dealing here. Like lysosomes (de Duve, 1963), these particles are damaged by freezing and thawing and by osmotic shock as shown by their loss of digestive activity. The degradative enzymes are contained within an impermeable barrier so that protein freely dissolved in the incubation medium is not degraded. This is related to latency, another characteristic of lysosomal enzymes. A characteristic of at least some kidney lysosomes is very easy sedimentability. Strauss (1962) and Shibko and Tappel (1965) isolated lysosomes from rat kidney

at low speeds, and most of the particle-bound ribonuclease in our preparations also came down very readily. The digestively active particles were especially readily sedimented (Table I). Digestively active particles were also sedimented from mouse liver preparations at low forces by Mego and McQueen (1965 a).

The acid pH optimum characterizes lysosomal "acid hydrolases" (Tappel, 1969; de Duve, 1963) and a broad pH optimum is characteristic of lysosomal proteases (Huang and Tappel, 1971; Mego and McQueen, 1965 *a*; Goettlich-Riemann et al., 1971; Coffey and de Duve, 1968). It may be significant for lysosomal function that the pH-activity optimum range is even broader in intact lysosomes than with extracts. The intact particles may maintain an internal pH significantly lower than that outside, or the environment or binding of hydrolases within lysosomes may alter their catalytic properties. Our preparations released all of the particle-bound ribonuclease in dilute acid, suggesting dissolution (Table II), as has been seen before with lysosomes (Zeya, 1969).

The notable features of ribonuclease absorption by mouse kidneys are organ specificity, massive and rapid uptake, and then rapid loss of label (Schultz et al., 1966; Davidson et al., 1971). Once the kidney content of exogenous ribonuclease has reached a maximum, about 7 min after injection, the label decreases logarithmically with a half time of about 14 min (Fig. 5). This decrease is associated with degradation of the exogenous ribonuclease in the kidneys as shown by incubation experiments with kidney slices (Gregoire and Hughes, 1966) and homogenized preparations and by the appearance of breakdown products in the kidneys (Davidson et al., 1971).

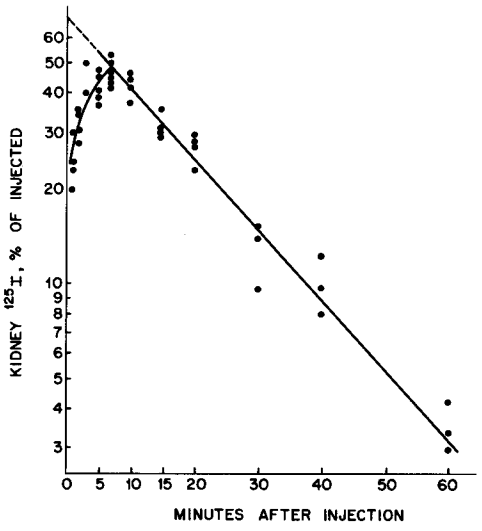

FIGURE 5 Logarithmic decay of mouse kidney radioactivity after intravenous injection of 100 μg of [^{125}I]-ribonuclease A (●). Experiments were performed as described in Davidson et al. (1971).

Digestion of injected ribonuclease upon incubation of particles isolated from mouse kidneys, described in this report, is more than adequate to account for the loss of label as seen in vivo, reinforcing the conclusion that ribonuclease is degraded before its label is lost from the kidney. During the first 40 s of incubation, active preparations released 9% of the ^{125}I in PTA-soluble form. This corresponds to a catabolic half time of 5 min, assuming that the initial rate were maintained. Since degradation products do not accumulate to any great extent in the kidneys (Davidson et al., 1971), degradation in vivo must proceed at a suboptimal rate, or a smaller proportion of the protein may be undergoing degradation at any one time. This is the fastest in vitro degradation of protein within particles yet described. Because of the massive accumulation of eoxgenous ribonuclease by mouse kidney, the absolute amounts of ribonuclease which can be digested are impressive. To estimate this, let us assume that the release of one atom of iodide in an acid-soluble form corresponds to the complete degradation of a molecule of ribonuclease (see below). 20 min after injection of 200 μg of ribonuclease, particles isolated from the kidneys of one mouse contain about 55 μg of the enzyme (Davidson et al., 1971); 20 μg of this may be isolated on active particles by centrifugation at 2,250 *g*. These particles could degrade 2.5 μg of ribonuclease per min with a half time in vitro of 5 min. The corresponding figure, derived from the data of Bertini et al. (1967), for the degradation of exogenous BSA by particles from mouse liver is 0.4 μg per min. It should be noted that it is difficult to compare breakdown rates seen in different studies because of the differences in the extent of uptake of the different proteins used and in the quantities administered.

The reason for the rapid digestion of ribonuclease is obscure. It may be a consequence of low molecular weight, facilitating access by proteases and requiring less bond breakage per molecule for complete digestion. Conceivably, release of the iodinated tyrosine could correspond to less than complete digestion. There is, on the

average, one iodine atom per protein molecule, and some iodine atoms may be on a highly exposed tyrosine which is released, alone or on a small peptide, very early. This is unlikely; otherwise, it would be hard to see why the loss of label from kidney does not have a rapid phase, but proceeds instead at a regular semilogarithmic rate (Fig. 5). It seems more likely that ribonuclease breakdown in vivo also continues at a rate proportional to the amount of remaining ribonuclease and that the spurt seen in vitro does not occur in the living animal. The simplest explanation for the rate decrease in vitro, that the particles lyse after short incubation, is untrue because most of the undigested ribonuclease remained sedimentable upon centrifugation in saline sucrose (Fig. 4). It is possible that some structural change short of lysis occurs to affect the digestion. However, published work shows that related lysosome preparations are fairly stable upon incubation. In vitro digestion of BSA in liver lysosomes has been observed for about 1 h at 37°C (Mego and McQueen, 1967). Shibko et al. (1965) incubated lysosomes from rat kidney for several hours at 37°C before observing release of hydrolases. Another possible reason for apparently incomplete digestion is precipitation of some digestion products by PTA reagent. This cannot be the only reason because in experiments newly reported we have observed about 70% digestion as measured by PTA release (Davidson, 1973).

At least a partial explanation for incomplete label release is that only some of the particles are digestively active. The bulk of the digestive activity is sedimented by milder centrifugation conditions than are required to bring down other components of the labeled particles (Table I). Little of the sedimented label represents an adsorption artifact because there is no significant labeling of these particles by adsorption of free ribonuclease (Table IV). The digestive activity observed is not greatly affected by lengthy homogenization with a glass pestle instead of the more usual, briefer homogenization with teflon, so these particles are not very fragile and particle damage is not the explanation for partial digestion (unpublished experiments). We thus have further evidence for the heterogeneity of the particles bearing pinocytosed protein. This reinforces the morphological (Creemers and Jaques, 1971; Goldfischer et al., 1970; Daems et al., 1969; Strauss, 1964) and cell-free studies (Davidson et al., 1971; Mego and McQueen, 1965 *a*) which led to the same conclusion. In the mouse, after uptake of ribonuclease, label loss from the kidney is active for over an hour (Schultz et al., 1966), so that the population of digestively active particles is being replenished, presumably by fusion with primary lysosomes (de Duve and Wattiaux, 1966).

TABLE IV

Adsorption of Iodinated Ribonuclease on Fractions from Mouse Kidney

Step	Centrifugation	cpm in precipitate
	g × *min*	*% of total*
1	2,500	6.6
2	45,000	2.5
3	300,000	4.5
4	3,300,000	12.2

Four kidneys from uninjected mice were homogenized as usual in 4 ml of 0.25 M sucrose containing 5–10 μg of [^{125}I]ribonuclease. An equal volume of 0.25 M sucrose was then added, and the mixture was centrifuged in successive steps as shown in the table. All but the last centrifugation correspond to the usual preparative procedures. Radioactivities in the precipitates were measured and divided by the total radioactivity in the homogenate. Results given are the means of two experiments whose values were all within 1.3% of the averages.

In contrast to ribonuclease, the slower digestion of BSA in particles proceeds at almost the initial rate for about half an hour (Mego and McQueen, 1967). If the extent of ribonuclease digestion in vitro is limited by the proportion of particles containing ribonuclease which are digestively active, this raises the interesting possibility that different exogenous proteins may be treated differently by the vacuolar apparatus. If this should prove true, the question of what causes the differences in treatment will become important.

It is significant that uptake and digestion occur with native ribonuclease. The enzyme is active, and therefore undenatured, when injected. According to the calorimetric studies of Tsong et al. (1970), there is no thermal transition of ribonuclease below 50°C at pH 3.7. Glushko et al. (1972), using carbon-13 NMR, found no evidence of ribonuclease denaturation at 45°C and pH 4.14. A reasonable lower limit for lysosomal pH is 4, which is below optimum for most lysosomal enzymes (Jensen and Bainton, 1973; Tappel, 1969). Therefore, ribonuclease should be in its native form within the lysosomes, although it is

possible that a small amount of a more or less denatured form exists in mobile equilibrium with the native protein and that this is the form undergoing digestion. Also, conditions within lysosomes may favor denaturation at higher pH or lower temperature than otherwise. However, in contrast to ribonuclease, it was necessary to denature BSA with formaldehyde before injection so it could be readily degraded by mouse liver and kidney (Mego and McQueen, 1965 *a*). Bowers and Olszewski (1972) observed breakdown of native bovine serum albumin by *Acanthamoeba castellanii*, in contrast to the findings with mice.

Degradation of exogenous and endogenous materials is a biological activity which is characteristic of organized lysosomes, and it is possible to use such activity both as a criterion for structural integrity of the lysosomes and as a test for the effect upon lysosomes of various agents and conditions, especially when the effects of such treatments upon the free lysosomal enzymes are known. This should be a subtler index of integrity than the release of lysosomal components which represents gross disorganization and is very subject to artifact (Baccino et al., 1971). It should be possible to study the lysosomal membrane in this way, for instance by examining the effect upon digestion of steroids known to affect membranes, and so to ascertain whether the membrane in lysosomes may modulate function rather than merely serve as a barrier. The rapid digestion described here provides a measure of disorganization occurring within the 1st min of incubation. For instance, since we know that after storage in hypotonic medium lysosomes have very little digestive activity, the presence of significant activity upon addition of intact preparations to aqueous buffer (Fig. 3) shows that osmotic degradation of the particles takes at least 1 or 2 min to be complete. The results of BSA digestion by preparations of mouse liver lysosomes in hypotonic medium (Mego et al., 1967) showed that the liver particles survived well enough to retain digestive activity for at least 1 h. The low initial rate reported here suggests that some damage appears almost instantly, but this will remain uncertain until parallel experiments are performed to establish the effect of the medium on digestion by the free lysosomal enzymes. Similarly, while the results of incubation in buffered isotonic NaCl suggest that the salt enters these lysosomes slowly, further experiments of the sort described here will be necessary to confirm this. Some of this work has been briefly presented elsewhere (Davidson, 1973).

SUMMARY

After intravenous injection of bovine ribonuclease, labeled with ^{125}I, into mice, subcellular particles containing the ribonuclease were separated by centrifugation of kidney homogenates. When the mice were killed 20 min after injection and the particles were resuspended in isotonic sucrose, pH 4.5–5.5, and incubated at 37°C, very rapid digestion of ribonuclease occurred for about the 1st min. This was shown by release of label in a form soluble in PTA. The initial rate of digestion was greater than the rate of label loss by kidney in vivo.

The digestively active particles sediment upon mild centrifugation. The [^{125}I] ribonuclease was totally released from the particles in dilute HCl and was shown to be entirely acid-insoluble before incubation. The label released by digestion was not free iodide. Repeated freezing and thawing or suspension of the preparations in hypotonic medium essentially abolishes digestive activity and structurally damages the particles as shown by centrifugation in NaCl-sucrose. Digestion was slow but significant when intact particles were added to hypotonic medium, showing that complete osmotic breakdown takes more than a minute to occur. The effect of isotonic sodium chloride was less marked.

We conclude that the highly active particles are lysosomes containing exogenous ribonuclease absorbed by pinocytosis. The in vitro study of lysosomal digestion is discussed in relation to lysosome structure and the role of the lysosome membrane.

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