CHANGES IN THE ACTIVITY OF ACID HYDROLASES DURING RENAL REABSORPTION OF LYSOZYME

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Biochemical (1, 2) and morphological (3–5) studies indicate that proteins being reabsorbed from proximal tubules of the kidney are concentrated within cells in lysosome-like bodies. However, little is known about the quantitative relationship between the activity of individual lysosomal enzymes and the uptake of proteins by tubular cells. It has been shown that, 18 hr after the administration of egg white, the activity of some, but not all, hydrolytic enzymes is increased in the rat kidney (1). In present experiments the time course of lysozyme reabsorption by the kidney was studied, and the activities of three lysosomal enzymes were measured at different times following administration of lysozyme; actinomycin D was administered in an attempt to inhibit the increase in enzyme activity induced by the protein. Lysozyme has a low molecular weight of 14,000, can be measured with accuracy and reproducibility, and in the doses administered it has no manifest pathological effects upon the organism or the kidney. Injected egg-white lysozyme is reabsorbed by the kidney and accumulates in the form of "droplets" in the proximal tubular cells (6, 7). It has been shown recently (8) that lysozyme, in the normal rat kidney, is associated with the lysosomal fraction. The above properties make lysozyme a suitable molecule to be used as a tracer protein in the studies of renal uptake and metabolism of injected proteins.

METHODS

Male mice, 25–30 g in weight, were used as experimental animals. Lysozyme 2X crystallized (Worthington Corporation, Harrison, N.J.), or lysozyme chloride 3X crystallized (Sigma Chemical Co., St. Louis, Mo.), was dissolved in 0.9% NaCl and injected intraperitoneally. Control animals received an equivalent volume of 0.9% NaCl (1 ml). At several time intervals following the administration of lysozyme, the animals were sacrificed by a blow on the head, the kidneys were removed, and fine slices of cortical tissue were cut out with a razor blade. The slices were washed in ice-cold saline, blotted with a filter paper, and weighed on a torsion balance. Homogenates were made in distilled water and were frozen and thawed three times to ensure release of the enzymes. Lysosomal enzymes and lysozyme were determined in each homogenate. Acid ribonuclease (acid RNase) activity (9) was assayed at pH 5.0 (acetate buffer) with purified RNA (10) as substrate. Cathepsin was measured (11) with denatured hemoglobin as substrate. Acid phosphatase was assayed either with disodium phenylphosphate (12) or β-glycerophosphate (13) as substrate. All enzyme assays were tested for linearity. Protein was determined by the method of Lowry et al. (14). Lysozyme was assayed by a modification of the turbimetric procedure of Shugar (15). Homogenates were centrifuged at 2,000 rpm for 30 min at 4°C; 0.1 ml of a conveniently diluted supernatant was added, with immediate mixing, to 1 ml of a freshly prepared bac-

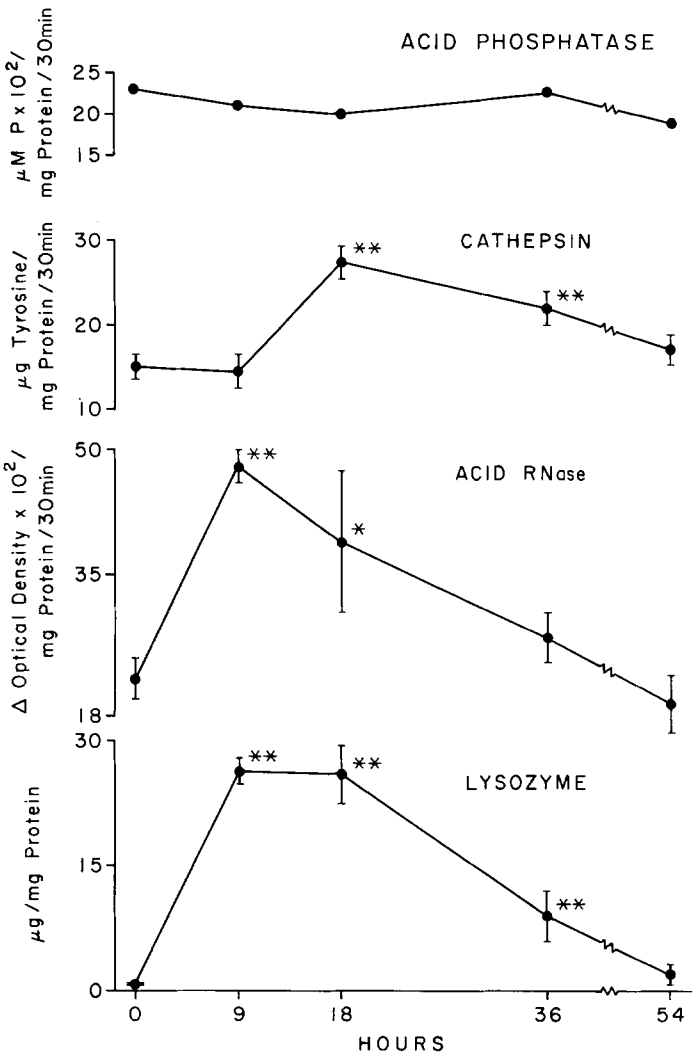

FIGURE 1 Specific activities of lysozyme, acid RNase, cathepsin, and acid phosphatase in mouse kidney cortex homogenates at several time intervals following an intraperitoneal injection of 1 mg/g body weight mouse of 3× crystallized lysozyme chloride. At each time interval a group of five animals was used to assay the activities of lysozyme, acid RNase, and cathepsin. Dots and bars represent mean ± standard deviation. Values indicated at time 0 are those from the five animals receiving 0.9% NaCl and sacrificed at 9 hr. Significance of the difference between the means of treated and control animals (time 0) was tested by the t test and are indicated in the figure by: * significant ($0.01 < P < 0.05$); ** highly significant ($P < 0.01$). Acid phosphatase values (β glycerophosphate as substrate) were obtained from a separate set of animals and activities at each time interval represent those of kidney cortices pooled from four mice. The solid lines connecting the points are drawn for illustrative purposes only.

terial solution containing 30 mg/100 ml of lyophilized *Micrococcus lisodeikticus* cell walls (Worthington Corporation) and 100 mg/100 ml NaCl in phosphate buffer 0.06 M (pH 6.60). The increase in transmittance was determined at 475 mμ at a temperature of 24 ± 1°C and was registered by means of a recorder attached to the spectrophotometer. The linear region of the curve was related to that of lysozyme standards and the activity was expressed as equivalent micrograms lysozyme per milligrams protein. Under the conditions used, increase in transmittance is linear between ½ and 3 min or more when 1–10 μg of

TABLE I

Concentration of Injected Lysozyme in Blood Serum

Hours	0	3	6	9	12	24	36
Lysozyme	1.5	302.0	96.5	22.8	3.0	2.3	1.2

Serum lysozyme (μg/ml) at several time intervals after the intraperitoneal administration of 1 mg/g body weight of lysozyme. Each value was obtained by pooling serum of two mice.

lysozyme is added. The reproducibility was better than 5%, and the average recovery, by adding lysozyme to the samples, was 96%. When used, actinomycin D (Merck, Sharp & Dohme, West Point, Pa.) was injected intraperitoneally in buffered saline (0.9% NaCl in buffer phosphate 0.04 M, pH 7.4). Dosages and schedules are given in the results. Blood was collected by cardiac puncture and serum activities of lysozyme, and the acid hydrolases were measured in both control and experimental animals.

RESULTS

Data from a typical experiment showing the time course of lysozyme reabsorption and disappearance from renal cortex and the corresponding specific activities of acid RNase, cathepsin, and acid phosphatase are given in Fig. 1. The peak of lysozyme activity is reached around 9 hr after the administration of the protein. From 9 to 18 hr (and 24 hr in experiments not shown in Fig. 1) values of lysozyme remained at a fairly constant level. At about 54 hr activity reached control levels. Table I gives the serum lysozyme values of similarly treated animals. As can be seen, the maximum activity of lysozyme in renal cortex is attained at the time of its very sharp exponential fall in blood.

Acid-phosphatase activity remained unchanged during the cycle of lysozyme in renal cortex, but specific activities of acid RNase and cathepsin are significantly increased following lysozyme administration. However, cathepsin activity increased at a slower rate and reached a peak of activity later than acid RNase. In three other experiments, not shown in Fig. 1, the specific activity of acid phosphatase, with phenylphosphate as substrate, did not change. Further experiments have confirmed the earlier rise in acid RNase activity compared to cathepsin; increase in acid RNase activity was observed as early as 3 hr after lysozyme administration. The time course of return of acid RNase activity to the control levels is variable; in some experiments the enzyme returned to control levels as early as 18 hr after lysozyme administration,

TABLE II

Dose-accumulation Relationship of Lysozyme and Activities of Acid RNase in Renal Cortex

Lysozyme administered mg/g body wt	Acid RNase Δ OD 10^2/mg protein/30 min	Lysozyme in renal cortex μg/mg protein
0*	26.2 ± 2.3‡	0.7§
0.05	26.9 ± 4.8	10.4
0.20	31.0 ± 3.6‖	18.2
0.50	33.7 ± 4.4‖	22.5
1.00	45.0 ± 4.0¶	25.7

Specific activities of acid RNase and lysozyme in mouse renal cortex 9 hr after the intraperitoneal administration of increasing doses of lysozyme.
* Each group consists of four animals.
‡ mean ± standard deviation.
§ average value.
Difference between means of treated and control animals tested by the t test:
‖ $0.01 < p < 0.05$.
¶ $p < 0.01$.

and in others, at this time, the enzyme activity was similar to that observed at the time point of 9 hr. The experiment illustrated in Fig. 1, however, represents the most frequent finding.

Average values of cathepsin and acid RNase in serum were found to be very low (0.12 μg tryosyne/microliter serum/30 min and 6×10^{-3} Δ optical density/microliter serum/30 min) and did not increase after lysozyme administration.

Table II shows the relationship between dose of lysozyme administered, accumulation of lysozyme in renal cortex, and the specific activity of acid RNase. In the range studied, the dose–accumulation relationship of lysozyme is not linear, and the increase in acid RNase activity could be detected only when the lysozyme present in renal cortex was about 25 times greater than in control animals.

Actinomycin D, an inhibitor of RNA synthesis, was used in an attempt to influence the increased activity of the enzymes induced by lysozyme. The

TABLE III

Action of Actinomycin D upon Lysozyme-Induced Increase in Acid RNase and Cathepsin Activities

Treatment*	Acid RNase (mean ± SD)	Lysozyme
Saline	26.1 ± 2.3	<1.0
Saline + AM	24.2 ± 2.4	<1.0
Lysozyme	43.3 ± 5.9	17.7
Lysozyme + AM I	44.1 ± 4.8	18.2
Lysozyme + AM II	33.3 ± 8.8	21.0
Lysozyme + AM III‡	26.7	24.1
Treatment§	**Cathepsin**	**Lysozyme**
Saline	12.8 ± 1.7	<1.0
Saline + AM	15.3 ± 1.6	<1.0
Lysozyme	23.2 ± 1.7	21.6
Lysozyme + AM IV	28.7 ± 5.2	13.4
Lysozyme + AM V‖	25.0 ± 5.6	6.7

Action of actinomycin D (AM) upon specific activities of mouse kidney, cortex acid RNase, and cathepsin in lysozyme-treated and control animals. Average concentration of lysozyme in kidney cortex for each group is also shown. Except as noted, each group consisted of five animals.

* Animals sacrificed 9 hr following intraperitoneal administration of 30 mg 2× crystallized lysozyme or saline (1 ml).

‡ Two animals alive from a group of 5;

§ Animals sacrificed 18 hr following administration of lysozyme or saline;

‖ Three animals alive from a group of seven.

Units of acid RNase, cathepsin and lysozyme as given in Fig. 1.

Doses and schedules of AM administration were as follows:

AM I, two doses of 5 μg each, given 24 and 2 hr before lysozyme administration;

AM II, two doses of 10 μg each, given as AM I;

AM III, two doses of 15 μg each, given as AM I;

AM IV, four doses of 3 μg each, given at 24, 12, 0 hr before and 9 hr after lysozyme administration;

AM V, four doses of 5 μg each, given as in AM IV.

results are summarized in Table III. Actinomycin D at high doses inhibited a great proportion of the increase in acid RNase activity, but it was not possible to inhibit the increase in cathepsin activity even with doses that killed more than 50% of the animals in the group. Schedules of actinomycin D administration different from those presented in Table III were also tried with similar results: 12.5 μg of actinomycin D given 2 hr before lysozyme administration inhibited 72.5% of the increase in acid RNase activity but did not change the increase in cathepsin activity. The influence of actinomycin D upon the increase in acid RNase activity is not due to its interference with the renal uptake of the protein, as the accumulation of lysozyme in kidney cortex shows the same pattern whether the animals were treated or untreated with actinomycin D (Table III, top). 18 hr after the protein administration, lysozyme activity is significantly less in the animals that also received actinomycin D, and this antibiotic slightly enhances the increase in cathepsin activity that is seen in the animals receiving only lysozyme (Table III, bottom).

DISCUSSION

Data presented demonstrate the independent behavior of the individual acid hydrolases studied in face of the stimuli of lysozyme uptake by the kidney. The increase in activity of acid RNase and cathepsin not only differs in rate but probably also in nature. The complexity of the system used precludes a definitive elucidation of the mechanism by which the enzyme activities are increased, but results of the actinomycin D experiments suggest that the increase in acid RNase activity is due, at least partially, to a *de novo* synthesis of the enzyme while that of cathepsin is not. It is possible that lysozyme enhances cathepsin activity by reducing its rate of degradation rather than by increasing its rate of synthesis; the stabilization of an enzyme by its substrate has already been reported for other enzyme–substrate systems in vivo (see reference 16).

It has been demonstrated (1) that egg white administration increased the activity of acid RNase and cathepsin in the rat kidney cortex, while acid phosphatase remained unchanged. Present results confirm and extend this observation. In view of quantitative considerations, it is unlikely that the results obtained with egg white are due to its lysozyme content. Furthermore, methyl lysozyme and trypan blue also increase acid RNase activity in mouse kidney cortex (unpublished data). The role of kidney acid hydrolases in the fate of reabsorbed proteins is still unclear but it is likely that cathepsin has an important function in the handling of the reabsorbed protein. The relationship between increased cathepsin activity and the disappearance of injected lysozyme from renal cortex, the possibility that lysozyme stabilizes cathepsin

(a type of enzyme–substrate interaction), and the fact that mouse kidney cortex homogenates hydrolyse lysozyme in vitro (unpublished data) add evidence to the assumption that cathepsin is catabolizing the reabsorbed protein. The significance of the early rise in acid RNase needs further investigation to be established. It appears that the increase in acid RNase is not essential for the disposal of the reabsorbed protein because, once it is inhibited by actinomycin D, lysozyme disappears from the renal cortex at a faster than normal rate (Table III).

Although only total activities of enzymes were measured in the present experiments, preliminary data not here reported show that the three acid hydrolases considered have a distribution pattern and latency properties characteristic of lysosomal enzymes. The finding that acid phosphatase remains unchanged when acid RNase increases by *de novo* synthesis poses some questions regarding the relationship between acid hydrolases and lysosomes. Three main possibilities are considered: (*a*) acid RNase, being synthesized at the ribosomes, migrates to preformed lysosomes, and changes the previous balance of enzyme concentration in the particle; (*b*) lysosomes are heterogeneous in respect to their enzyme content, and the increase in acid RNase activity could be an expression of a more rapid turnover of lysosomes rich in this enzyme and poor in acid phosphatase content; and (*c*) increased synthesis of acid RNase not bound to lysosomes occurs. With respect to the last possibility it should be noted that several azo-dyes (17) and some proteins (6), including lysozyme, greatly increase the activity of alkaline RNase, an enzyme not considered to be related to lysosomes. A study, now in process, pertaining to the intracellular distribution of lysozyme and acid hydrolases at several time intervals after lysozyme administration should permit further elucidation of the relationship between the process of renal reabsorption of proteins and the role of lysosomes and acid hydrolases.

SUMMARY

Present studies quantitatively describe the cycle of injected lysozyme and the concomitant changes in the specific activity of acid hydrolases in mouse kidney cortex. The increase in acid ribonuclease activity corresponds to the phase of lysozyme accumulation and that of cathepsin activity to the leveling off and disappearance of the protein from renal cortex. It was demonstrated that actinomycin D inhibits the increase in acid ribonuclease but has no effect on the increase in cathepsin. The results suggest that the increase in acid ribonuclease is due to a *de novo* synthesis of the enzyme and it is possible that the changes in cathepsin activity are a result of the stabilization of the enzyme by its substrate.

The relationship between renal uptake of proteins and the role of acid hydrolases and lysosomes is briefly discussed.

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