Rat-Kidney Lysosomes: Isolation and Properties

By S. SHIBKO* AND A. L. TAPPEL

Department of Food Science and Technology, University of California, Davis, Calif., U.S.A.

(Received 14 July 1964)

1. The activities of lysosomal enzymes in the cortexes and medullas and the principal subcellular fractions of rat kidney were measured. 2. A method is described for the isolation of rat-kidney lysosomes and a detailed analysis of the enzymic composition of the lysosomes is reported. Enzyme analysis of the other principal subcellular fractions is included for comparison. 3. Studies of the distribution of α -glucosidase showed that the lysosomal fraction contained only 10% of the total enzyme activity. The microsomal fraction contained most of the particulate α -glucosidase. Lysozyme was concentrated mainly in the lysosomal fraction with only small amounts present in the microsomal fraction. Lysosomal α -glucosidase had optimum pH 5 whereas the microsomal form had optimum pH 6. Both lysosomal and microsomal lysozyme had optimum pH 6.2. 4. The stability of lysosomal suspensions was studied. Incubation at 37° and pH 7 resulted in first an increased availability of enzymes without parallel release of enzyme. This was followed by a second stage during which the availability of enzymes was closely related to the release of enzymes. These changes were closely paralleled by changes in light-scattering properties of lysosomes. 5. The latent nature of the α -glucosidase and lysozyme of intact kidney lysosomes was demonstrated by their graded and parallel release with other typical lysosomal enzymes. 6. Isolated lysosomes were unstable at pH values lower than 5, most stable at pH 6–7 and less stable at pH 8–9. Lysosomes were not disrupted when the osmolarity of the suspending medium was decreased from 0.6M to 0.25M. 7. The discussion compares the properties and composition of kidney lysosomes, liver lysosomes and the granules of macrophages. 8. The possible origin of the lysozyme in kidney lysosomes by reabsorption of the lysozyme in blood is discussed.

The isolation of kidney droplets from normal rats was first reported by Strauss (1954, 1956), who demonstrated that they had an enzymic composition similar to that of the lysosome fraction isolated from liver homogenates (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). However, since these reports were published knowledge of the number of acid hydrolases associated with lysosomes has considerably increased. Because of the importance of lysosomes in the catabolic processes occurring in the cell, a detailed study has been made of the enzymic composition and stability of isolated kidney lysosomes. A method is described for isolating intact kidney lysosomes in a high degree of purity. Similar studies have been reported for liver lysosomes (Sawant, Shibko, Kumta & Tappel, 1964) and the cytoplasmic granules of macrophages (Cohn & Hirsch, 1960), so that a comparison of the enzymic composition and stability properties of

these three lysosomal particles is possible. In addition, since in the rat the kidney has been shown to have the highest activity of α -glucosidase (α -D-glucoside glucosylhydrolase, EC 3.2.1.20) (Goldberg, Tsou, Rutenber, Rutenber & Seligman, 1958) and lysozyme (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17) (Litwack, 1957), and since these enzymes have been shown to be associated with lysosomes in other tissues (Lejeune, Thinès-Sempoux & Hers, 1963; Cohn & Hirsch, 1960), a special study was made of the intracellular localization and properties of these enzymes in the kidney cell.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats weighing 250–350 g. were used (Berkeley Pacific Laboratories, Berkeley, Calif., U.S.A.).

Homogenization and suspending media. Demineralized water and analytical-grade reagent were used for the preparation of all sucrose solutions. The solutions, containing EDTA (tetrasodium salt) (0.34 mM) and EDTA (disodium salt) (0.34 mM), were adjusted to pH 7.

* Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139, U.S.A.

Tissue fractionation. Rats that had previously been starved for 24 hr. were killed by stunning and decapitation, and their kidneys were removed immediately and washed with cold 0.45M-sucrose. After being minced, the chilled kidney was homogenized in 0.45M-sucrose (1:8, w/v). Homogenization was for 20 sec. in a Waring Blendor at top speed. The homogenate was filtered through muslin and fractionated in the SS3 Servall centrifuge. All operations were carried out in a cold room at 4°.

For the study of the distribution of enzymes in kidney, the fractionation was designed to separate the homogenate into the principal subcellular fractions. Four particulate fractions and a soluble fraction were obtained by centrifuging the homogenate successively for 2½ min. at 650g (unbroken cells and nuclei), 1 min. at 10000g (lysosomal-mitochondrial fraction), 20 min. at 12000g (mitochondrial-microsomal fraction) and 60 min. at 100000g (microsomal fraction). The remaining supernatant solution was used for assay of the enzymes in the soluble fraction. The time taken to accelerate to the speed required to sediment the lysosomal-mitochondrial fraction was 20 sec., and for the mitochondrial-microsomal fraction it was 90 sec. Deceleration was achieved by using the automatic braking device, and the time taken was 1½ min. for the lysosomal-mitochondrial fraction and 4 min. for the mitochondrial-microsomal fraction. The particulate fractions were suspended in 0.45M-sucrose. For enzyme assays the suspensions were diluted with 0.45M-sucrose so that the protein concentration was 0.5 mg./ml.

Rat-kidney homogenate (12.5%, w/v) in 0.45 M-sucrose-0.68 mM-EDTA, pH 7

Scheme 1. Isolation of rat-kidney lysosomes.

For the further characterization of lysosomes and other subcellular fractions a fractionation scheme was devised so that these fractions could be isolated in relatively pure form. Scheme 1 shows the centrifugation procedure used for the preparation of lysosomes. It is emphasized that the characteristic sticky brown lysosomal pellets must be re-suspended by directing a stream of sucrose from a pipette on to them. This method yields a suspension of undamaged lysosomes. Use of the Potter-Elvehjem-type homogenizer for suspending the lysosomal pellet results in considerable mechanical damage to the lysosomes with concomitant release of lysosomal enzymes. For enzyme assay the lysosomal suspensions were diluted with 0.6M-sucrose so that the protein concentration was about 0.1 mg./ml. Mitochondria were prepared from the middle buff-coloured layer of the pellet that separated in the first 5 min. at the 5900g centrifugation stage in the preparation of lysosomes. The layer was carefully removed and resuspended in 0.6M-sucrose and centrifuged for 5 min. at 5900g. The mitochondrial pellet was resuspended in 0.6M-sucrose, care being taken not to include the small amount of dark-brown lysosomal material present at the bottom of the pellet. For the preparation of the microsomal fraction, the supernatant solution remaining after removal of the lysosomal-mitochondrial fraction was centrifuged first for 20 min. at 6600g and then for 30 min. at 14 800g. The second pellet was always found to be the fraction containing the highest glucose 6-phosphatase and alkaline-phosphatase activities and was used as the microsomal fraction. The supernatant solution remaining after the separation of the microsomal fraction was centrifuged for 60 min. at 100000g. The pellet, which showed low glucose 6-phosphatase and alkaline-phosphatase activities, was discarded and the supernatant solution used for subsequent enzyme analysis of the soluble fraction. For enzyme assay the mitochondrial, microsomal and soluble fractions were diluted so that the final protein concentration was 0.5 mg./ml.

Enzyme assays. The enzyme activities of the subcellular fractions were measured after disruption of lysosomal particles by freezing and thawing ten times. Triton X-100 was not used because it interfered with the precipitation of protein at the termination of enzyme reactions and thereby caused difficulties in measuring the products of reaction.

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was measured by the method of Gianetto & de Duve (1955), with sodium β -glycerophosphate as substrate. Released P_i was measured by the method of Lowry, Roberts, Wu, Hixon & Crawford (1954). When *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used as substrate for acid-phosphatase determinations, the subcellular fractions were incubated for 5 min. in 0.2M-sodium acetate buffer, pH 5, before the addition of substrate, to inactivate microsomal phosphatases (Beaufay & de Duve, 1954). Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was measured with *p*-nitrophenyl phosphate as substrate in 0.1M-glycine-NaOH buffer, pH 9.5. The enzyme action was stopped by the addition of 2% (w/v) phosphotungstic acid in 0.1N-HCl. After removal of the precipitated protein the *p*-nitrophenol released was determined after the addition of NaOH to the supernatant. Arylsulphatase (aryl sulphate sulphohydrolase, EC 3.1.6.1) was determined by the method of Roy (1953), with 2-hydroxy-5-nitrophenyl sulphate (Sigma Chemical Co.) as substrate. β -Galactosidase (β -D-

galactoside galactohydrolase, EC 3.2.1.23) was determined by the method of Sellinger, Beaufay, Jacques, Doyen & de Duve (1960), with *o*-nitrophenyl β -D-galactoside (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) as substrate. β -Glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31) was determined by the method of Gianetto & de Duve (1955), with phenolphthalein glucuronide as substrate and with the modifications described by Tappel, Zalkin, Caldwell, Desai & Shibko (1962). Ribonuclease [polyribonucleotide 2-oligonucleotidotransferase (cyclizing), EC 2.7.7.16] was determined by the method of de Duve *et al.* (1955). Cathepsin was determined by the method of Gianetto & de Duve (1955), with denatured haemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) as substrate. The released tyrosine was measured by the method of Miller (1959). The cathepsins were differentiated by using specific substrates. For cathepsin A, *N*-benzyloxycarbonyl- α -glutamyl-L-tyrosine was used as substrate; for cathepsin B, benzoyl-L-argininamide was used; and for cathepsin C, glycyl-L-tyrosinamide (Mann Research Laboratories Ltd., New York, N.Y., U.S.A.) was used. For maximum activity of the cathepsins, mercaptoethanol was added to the enzyme digests to a final concentration of 0.125 M. The buffers used to study the pH optima of the cathepsins were 0.2 M-sodium phosphate-citrate in the range pH 3–7 and 0.2 M-sodium phosphate in the range pH 7–8. The products of hydrolysis were measured by the ninhydrin assay method of Whitaker (1961). Lysozyme was determined with Bacto-Lysozyme-substrate (Difco Laboratories, Detroit, Mich., U.S.A.), a standardized ultraviolet-killed dried culture of *Micrococcus lysodeikticus*. The rate of decrease of the extinction at 450 m μ of the substrate in 0.1 M-sodium phosphate buffer, pH 6.2, for the first 2 min. of the enzyme action was used to calculate the lysozyme activity. Bacto-Lysozyme (Difco Laboratories), a crystalline egg-white lysozyme, was used as a standard. For the measurement of free lysozyme activity in intact preparations of lysosomes, the substrate was suspended in buffered 0.6 M-sucrose. The presence of sucrose did not alter the rate of enzyme action. The buffers used to study the pH optimum of lysozyme were 0.2 M-sodium phosphate-citrate in the range pH 3–7, 0.2 M-sodium phosphate in the range pH 7–8 and 0.2 M-glycine-NaOH at pH 9.

α -Glucosidase was determined by incubating the preparation with 0.3% maltose in 0.2 M-sodium acetate buffer, pH 5, as substrate in a total volume of 2 ml. The final concentration of sucrose in the reaction mixture (derived from the sucrose medium used for suspending the subcellular fractions) was not more than 45 mM. The reaction was run at 37° for 15 or 20 min., and stopped by the addition of 2% (w/v) ZnSO₄ followed by an equivalent amount of 1.8% (w/v) Ba(OH)₂. The glucose in 1 ml. samples of the supernatant was determined with the Glucostat reagent (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.). The glucose oxidase was dissolved in 0.2 M-tris-HCl buffer, pH 7, to inhibit any maltase activity present in the preparation (Hers, 1963). The chromogenic agent was dissolved in water and added to the test mixture after the addition of the glucose oxidase. The mixture was incubated for 10 min. at 37° and the reaction stopped by the addition of 4 N-HCl. The extinction of the reaction product was measured at 410 m μ .

Glucose 6-phosphatase (D-glucose 6-phosphate 6-phosphohydrolase, EC 3.1.3.9) was measured by the method of

de Duve *et al.* (1955). Succinoxidase was determined by polarographic measurement of the oxygen consumed when succinate was added to suspensions of the subcellular fractions in 0.1 M-sodium phosphate buffer, pH 7.2, containing KCl (0.1 M). Cytochrome *c* (10 μ M) was added to the reaction mixture to ensure maximum reaction rates. The polarographic apparatus used was that described by Hamilton & Tappel (1963). Glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] was determined by direct spectrophotometric measurement of the oxidation of NADH (Hogeboom & Schneider, 1953).

Determination of protein. Protein was determined by the method of Miller (1959).

Enzyme units. Specific activities are expressed as μ M-moles of substrate hydrolysed/min./mg. of protein. The products of cathepsin digestion (haemoglobin substrate) are expressed in terms of tyrosine equiv. of the colour developed with the Folin-Ciocalteu reagent; cathepsins A, B and C are expressed in terms of leucine equiv. of the colour produced with the ninhydrin reagent; the ribonuclease reaction is expressed in terms of liberated mononucleotide. Lysozyme is expressed as the mg. equiv. of a crystalline egg-white lysozyme preparation causing a rate of change in extinction at 450 m μ , with a suspension of *M. lysodeikticus*, equal to that with 1 mg. of protein of the subcellular fraction. Glutamate dehydrogenase is expressed as μ M-moles of NADH oxidized/min./mg. of protein, and succinoxidase as μ M-moles of O₂ utilized/min./mg. of protein.

Stability of kidney lysosomes. For studies on the stability of kidney lysosomes, suspensions of lysosomes in 0.6 M-sucrose–0.34 M-EDTA buffer, pH 7, were incubated at 0° and 37°. At appropriate time-intervals the available, soluble and total acid phosphatase, acid ribonuclease and arylsulphatase, and also the soluble protein and extinction (light-scattering) at 540 m μ , were determined. The available enzyme activities were determined by incubating the lysosomes with substrate in 0.6 M-sucrose. Incubation was for 10 min. at 37°. The soluble enzyme was determined by assaying the enzyme activity of the supernatant solution obtained after centrifuging the lysosomal preparation for 10 min. at 15000g. The supernatant solution was also used for determination of the soluble protein. Total enzyme activity was determined after freezing and thawing suspensions of lysosomes ten times.

Effect of pH on the stability of the lysosomes. Suspensions of lysosomes in 0.6 M-sucrose in 0.1 M-buffer were incubated at 37° and the extinctions at 540 m μ measured at appropriate time-intervals. The buffers used were sodium phosphate-citrate in the range pH 4–7 and tris-HCl or sodium barbitalurate-HCl in the range pH 7–9.

Electron microscopy. For morphological studies of the composition of the lysosome fractions, the pellets were fixed with buffered 1% (w/v) osmic acid, dehydrated in ethanol, embedded in epoxy resin, sectioned and studied with the RCA EMU-3 electron microscope.

RESULTS

Distribution of lysosomal enzymes in rat-kidney tissue. The specific activities of lysosomal enzymes in homogenates of the cortex and medullas of kidney are shown in Table 1: they are similar to each other and also to those of whole kidney homogenate.

These results indicate that the lysosomal enzyme activities are distributed throughout the kidney and not concentrated in any of the clearly defined morphological regions. For this reason whole kidney tissue was used for further studies. The patterns of distribution of typical lysosomal enzymes (acid phosphatase and arylsulphatase), mitochondrial enzyme (succinoxidase) and microsomal enzyme (glucose 6-phosphatase) in the fractions obtained by differential centrifugation of kidney homogenate are shown in Fig. 1. Fraction 2 (the lysosomal-mitochondrial fraction) showed the highest concentration of lysosomal enzymes in the particulate fractions and contained 33% of the lysosomal acid phosphatase. The lysosomes in this fraction can be readily separated from the mitochondria and other subcellular particles by centrifugal procedures. The mitochondrial-microsomal fraction (fraction 3) contained 22% of the acid phosphatase and the microsomal fraction (fraction 4) 6%. Included in Fig. 1 are the distributions of α -glucosidase and lysozyme. The lysosome-rich fraction has the lowest α -glucosidase activity and contained only 8% of the total cellular α -glucosidase activity. The microsomal fraction contained 21% of the total cellular α -glucosidase activity. Lysozyme separated mainly in the lysosome-rich fraction. This fraction contained 40% of the total lysozyme activity. Only 11% of the total lysozyme activity was present in the microsomal fraction.

Isolation of kidney lysosomes and enzymic properties. Scheme 1 shows the method devised for the isolation of a purified kidney lysosomal preparation. A 45g. sample of rat-kidney tissue fractionated by this procedure yielded 20–30mg. of lysosomal protein. The lysosomal enzymes in this fraction had the highest specific activity of all the fractions studied. However, the yield of lysosomal enzymes in the purified preparation was low, being approx. 1.1% for acid phosphatase and 2.4% for arylsulphatase.

Enzyme characteristics of the kidney lysosomes are shown in Table 2. Increase in the specific activities of the lysosomal enzymes showed considerable variation, e.g. acid phosphatase, acid ribonuclease and β -galactosidase showed a similar increase in specific activity of about 14 times that of the homogenate, whereas arylsulphatase and β -glucuronidase showed increases of 30 and 60 times respectively. Table 2 also shows the enzyme characteristics of the other principal subcellular fractions, and enables some estimate to be made of the contamination of the lysosomes by these organelles. Because the method of purification of the subcellular fractions was designed to obtain fractions that showed the highest specific activity of their characteristic enzymes, rather than a complete fractionation of the tissue, only 36% of the protein

Table 1. *Distribution of lysosomal enzymes in rat kidney tissue*

	Protein %	Acid phosphatase		Cathepsin		Acid ribonuclease		β -Glucuronidase		Arylsulphatase	
		(% of total)	(sp. activity)	(% of total)	(sp. activity)	(% of total)	(sp. activity)	(% of total)	(sp. activity)	(% of total)	(sp. activity)
Whole kidney	100		51 (46)		11 (40)		150 (32)		175 (32)		1.42 (41)
Cortex	78	69	43 (34)	81	11.2 (36)	77	132 (14)	88	224 (35)	72	1.14 (37)
Medulla	22	31	64 (63)	22	10.8 (40)	23	177 (45)	12	128 (27)	38	1.5 (44)

The cortexes and medullas of rat kidneys were homogenized in 0.45 M-sucrose. After freezing and thawing ten times to release lysosomal enzymes, enzyme activities were determined by the methods described in the text. Specific activities of enzyme are given as μ moles of substrate hydrolysed/min./mg. of protein. Values in parentheses represent the percentage latent enzyme activities (percentages of total enzyme activity that is not available to act on the substrate until the lysosomes have been disrupted).

Fig. 1. Distribution pattern of enzymes in rat kidney. Fractions are plotted in order of their isolation. Fraction 1, unbroken cells and nuclei; fraction 2, lysosomal-mitochondrial fraction; fraction 3, mitochondrial-microsomal fraction; fraction 4, microsomal fraction; fraction 5, final supernatant. Each fraction is represented separately on the ordinate by its specific activity. On the abscissa each fraction is represented by its percentage of the total protein in the homogenate. (a) Acid phosphatase; (b) arylsulphatase; (c) succinoxidase; (d) lysozyme; (e) α -glucosidase; (f) glucose 6-phosphatase.

of the homogenate was recovered. Although care was taken not to include any obvious lysosomal material in the mitochondrial fraction during its preparation, the final fraction, which had the highest glutamate-dehydrogenase and succinoxidase activities, always showed considerable lysosomal contamination, as judged by its content of lysosomal enzymes (Table 2). The succinoxidase and glutamate-dehydrogenase activities of the lysosomal fraction indicated a 5-8% contamination with mitochondria. The presence of alkaline phosphatase and glucose 6-phosphatase in the lysosomal fraction suggested that the preparation was also contaminated with microsomal material. However, when the morphological composition of the preparations was studied by electron microscopy (Fig. 2) they were seen to contain intact lysosomes and a few mitochondria. Counts of ten typical fields each containing at least 150 particles indicated that the lysosomal preparation contained about 5% of mitochondria. Microsomal fragments were seldom visible.

Because of the importance of lysosomes in tissue autolysis and the role of cathepsins in this process a

Fig. 2. Electron-microscope picture of section through pellet of rat-kidney lysosomes.

special study was made of the distribution of the cathepsins in kidney lysosomes. Cathepsin A was the major form present, with smaller amounts of

Table 2. *Specific activities of some enzymes of rat-kidney homogenate and subcellular fractions*

Results are given as specific activities of the enzyme (see the text for units). Details of the preparation of the subcellular fractions are given in the text. The number of experiments in the groups for which the s.d. is given was eight. All other results are the mean values of three experiments. Values in parentheses indicate the ratios of the specific activity of each enzyme in the subcellular fraction to that in the homogenate.

Enzyme	Homogenate	Lysosomal fraction	Mitochondrial fraction	Microsomal fraction	Soluble fraction
Protein (% of total homogenate)	100	0.36 ± 0.12	11	6	28
Acid phosphatase (sodium β -glycerophosphate substrate)	52.8 ± 8.9	847 ± 197 (16)	212 (4.1)	74 (1.4)	15.8 (0.3)
Acid phosphatase (<i>p</i> -nitrophenyl phosphate substrate)	52	507 (9.8)	146 (2.8)	82 (1.6)	40 (0.8)
Alkaline phosphatase	98	25 (0.25)	31 (0.31)	395 (4.0)	5.6 (0.06)
Glucose 6-phosphatase	97	31 (0.32)	23 (0.24)	284 (2.9)	4 (0.04)
Arylsulphatase	1.46 ± 0.4	51.8 ± 15.6 (35)	14 (9.5)	0.9 (0.6)	0.9 (0.6)
Cathepsin (haemoglobin substrate)	11.3 ± 1.91	65 ± 6.1 (5.8)	22 (1.9)	7.2 (0.6)	3.8 (0.34)
Cathepsin A	—	4.25	—	—	—
Cathepsin B	—	0.56	—	—	—
Cathepsin C	—	0	—	—	—
β -Glucuronidase	16.4 ± 1.2	966 ± 119 (60)	176 (11)	6.6 (0.4)	102 (6.2)
β -Galactosidase	3.8 ± 0.9	67.7 ± 7.4 (18)	14 (3.7)	1.8 (0.5)	2.9 (0.75)
α -Glucosidase	124.5 ± 12.5	108 ± 23.8 (0.9)	43 (0.35)	205 (1.7)	8 (0.06)
Lysozyme	0.018 ± 0.002	0.131 ± 0.066 (7.2)	0.03 (1.7)	0.022 (1.2)	0.007 (0.4)
Acid ribonuclease	7.9 ± 0.6	139.6 ± 32.1 (17)	23 (2.9)	6.8 (0.86)	5.2 (0.65)
Glutamate dehydrogenase	17	11 (0.62)	180 (10.5)	31 (0.18)	63 (3.7)
Succinoxidase	—	12	138	7.6	0
Catalase	375	23 (0.06)	47 (0.12)	450 (1.2)	1460 (4)

Fig. 3. pH-dependence curve of hydrolysis of *N*-benzyloxy-carbonyl- α -glutamyl-L-tyrosine (cathepsin A) and benzoyl-L-arginamide (cathepsin B) by rat-kidney lysosomes. $\circ$, Cathepsin A; $\bullet$, cathepsin B.

cathepsin B. Cathepsin C was not detected (Table 2). Fig. 3 shows the pH optima and relative activity of the cathepsins in the lysosomal preparation. Cathepsin A had optimum pH 5 and cathepsin B a much broader pH optimum with maximum activity at pH 6.

Properties of lysosomal and microsomal α -glucosidase and lysozyme. Since α -glucosidase and lysozyme are mainly concentrated in the lysosomal and microsomal fractions some properties of these enzymes in these fractions were studied. Fig. 4 shows the pH optima of lysosomal and microsomal α -glucosidase. Lysosomal α -glucosidase has a sharp peak at pH 5, whereas the microsomal fraction shows optimum activity at pH 6. The fractions did not release glucose from glycogen or soluble starch. The microsomal fraction showed some invertase activity. Lysosomal and microsomal lysozyme showed optimum activity at pH 6.2 (Fig. 5). Lysozyme from these fractions was completely inhibited by 0.5 mM-iodine.

Spectral characteristics of kidney lysosomes. Suspensions of kidney lysosomes exhibit a typical dark-olive-green colour. Disruption of the lysosomes resulted in the release of some of the pigment into the soluble fraction. Absorption spectra of the

Fig. 4. Influence of pH on the hydrolysis of maltose by rat-kidney fractions. $\bullet$, Lysosomal fraction; $\circ$, microsomal fraction.

Fig. 5. Influence of pH on lysozyme activity of rat-kidney fractions. $\bullet$, Lysosomal fraction; $\circ$, microsomal fraction.

soluble fraction in the visible-light region (340–700 mμ) did not show any characteristic pigment peaks. Fluorescent spectra showed the presence of protein and small amounts of flavine, but no other characteristic peaks were observed.

Stability of kidney lysosomes. Freshly prepared lysosomal preparations suspended in 0.6M-sucrose-0.68mM-EDTA buffer, pH 7, showed typical latent enzyme activities characteristic of intact lysosomes. Fig. 6 (zero time) shows that under these conditions lysosomal enzymes were latent to the extent of 90–95%. When suspensions were incubated at 37°, changes occurred in their light-scattering properties and these were paralleled with the availability and release of enzymes and protein into a soluble form (Fig. 6). The first changes observed were a decrease in light-scattering properties and an increase in the amount of lysosomal enzyme available. Only small amounts of protein and enzyme were released into the soluble fraction during this period. This initial period was followed by a continuous decrease in the light-scattering properties of the suspension and a rapid release of enzyme and protein into the soluble fraction. After 2 hr. the availability of enzymes and release of soluble enzymes paralleled one another and were almost equal. After 4 hr. the release of soluble enzymes was virtually complete. About 10% of the lysosomal enzyme activities remained associated with the lysosomal membrane, which represented 15–20% of the total lysosomal protein. There was little difference in the release pattern, or the total amounts released, of the various enzymes studied. No loss of total enzyme activity was observed during the period of incubation. This pattern has been observed for a number of preparations, although the time-interval for complete release of the enzymes varies. In general, lysosomes from rats 12–20 weeks old (350g.) were disrupted more readily than those from rats 6–8 weeks old (250g.). Increase in concentration of lysosomes also increased the rate of disruption. After 4 hr. at 0° there was little change in the light-scattering properties of the suspensions, and only small changes in the amount of enzymes available or soluble. After 24 hr. at 0°, 40% of the arylsulphatase was available and after 48 hr. all the arylsulphatase was available. No loss in total enzyme activity was observed during this period.

Freezing and thawing the lysosome suspension ten times resulted in complete disruption of the lysosomal particles. Although all the enzymes became completely available only 50–60% was soluble, the remainder being associated with insoluble membranous material (Table 3).

Effect of pH on the stability of the lysosomes. Because changes in light-scattering properties of lysosomes are closely related to the release of lysosomal enzymes, measurement of the extinctions of suspensions of lysosomes at various pH values was used to study the effect of pH on the lability of lysosomes. Fig. 7 shows the effect of pH on the light-scattering properties of suspensions of lysosomes in 0.6M-sucrose in 0.1M-buffer at various pH values.

Fig. 6. Changes in the light-scattering properties of rat-kidney lysosomes, and availability and release of lysosomal enzymes and of protein into a soluble form, when lysosomal suspensions were incubated at 0° (----) and 37° (—). Results are expressed as percentages of the total at commencement of experiment. ○, Available enzyme; ●, soluble enzyme. (a) $E_{540m\mu}$; (b) release of protein; (c) acid phosphatase; (d) arylsulphatase; (e) acid ribonuclease.

Lysosomes were most labile at pH 4–5, showed maximum stability at pH 6–7 and were less stable at pH 8–9.

Effect of the molarity of the suspending medium on the stability of the lysosomes. Decreasing the molarity of the suspending medium from 0.6M to 0.45, 0.3 or 0.25M did not result in any significant change in the light-scattering properties of suspensions of rat-kidney lysosomes. Incubation for 30 min. at 37°

Table 3. Release of lysosomal enzymes from rat-kidney lysosomes after disruption by freezing and thawing

A suspension of rat-liver lysosomes was frozen and thawed ten times and then centrifuged at 100000g for 60min. Enzyme activities of the insoluble pellet and soluble fraction were determined by the methods described in the text. Results represent percentages of total enzyme found in soluble and insoluble fractions.

	Protein	Acid phosphatase	Acid ribonuclease	Arylsulphatase	β-Glucuronidase
Soluble fraction	82	52	58	54	67
Insoluble fraction	18	48	42	46	23

Table 4. Comparison of availability and release of arylsulphatase, lysozyme and α-glucosidase of rat-kidney lysosomes and microsomes

Suspensions of rat-kidney lysosomes or microsomal fraction in 0.6M-sucrose were incubated at 37°. At appropriate time-intervals samples were removed and available enzyme activities determined by the methods described in the text. For measurement of released enzyme the lysosome suspensions were centrifuged at 20000g for 15min. and the microsomal fractions at 100000g for 60min., and the activities of the supernatant solutions determined. The results given represent the percentages of total enzyme activity of the lysosomal preparation.

Time (hr.)	Arylsulphatase		Lysozyme				α-Glucosidase*	
	Lysosomal fraction		Lysosomal fraction		Microsomal fraction		Lysosomal fraction	Microsomal fraction
	Available	Soluble	Available	Soluble	Available	Soluble	Soluble	Soluble
0	2.4	0	8	0	98	2	0	0
1	35	26	29	17	97	0	12	0
2	72	64	76	43	94	1	48	1
3	90	78	85	59	95	4	65	2

* Method of assay precludes determination of available enzyme.

Fig. 7. Effect of pH on the light-scattering properties of suspensions of rat-kidney lysosomes. Suspensions of lysosomes in 0.6M-sucrose in 0.1M-buffer were incubated at 37° and $E_{540m\mu}$ values measured. ○, pH4; ●, pH5; △, pH6; ■, pH7; □, pH8; ▲, pH9.

under these conditions did not result in any significant changes.

Latent forms of α-glucosidase and lysozyme associated with purified kidney lysosomes. Freshly prepared lysosomes assayed with substrate in 0.6M-sucrose showed only 8% of the lysosome activity compared with lysosomes that had been disrupted by freezing and thawing (Table 4). It was not possible to measure the α-glucosidase activity of intact preparations directly because: (1) the addition of sucrose (0.6M) to the substrate to maintain the integrity of the particles interferes with the method of assay of the liberated glucose; (2) owing to the low specific activity of the enzyme in the lysosomal preparation, a 30min. incubation period with the substrate at pH 5 was required, which would result in complete disruption of the lysosomes. However, the gradual and parallel release of lysosomal enzymes from suspensions of kidney lysosomes in 0.6M-sucrose-0.68 mM-EDTA, pH7, at 37°, as described in the present paper appears to be a suitable method for demonstrating the lysosomal nature of α-glucosidase. The release of α-glucosidase from lysosomal preparations incubated under these conditions was similar to that observed for arylsulphatase, a typical lysosomal enzyme (Table 4). Similar results were obtained for studies on the release of lysozyme from these preparations (Table 4). Microsomal α-glucosidase and lysozyme did not show any latent properties (Table 4).

DISCUSSION

Since the term 'lysosome' has been applied to most particles containing acid phosphatase (de Duve, 1963), it is necessary to define the type of lysosomes isolated and used in the present study. The sedimentation characteristics of the particles correspond closely to those of the large kidney droplets or phagolysosomes described by Strauss (1954). The origin of the droplets involves the fusion of two separate particles, the protein droplet formed by reabsorption and concentration of protein by cells of the proximal tubule, and an acid-phosphatase-bearing particle that presumably contains the other lysosomal enzymes (Strauss, 1964).

Strauss (1956) has shown that there is a wide variability in the size of these lysosomal particles. In addition, our results on the distribution of lysosomal enzymes in the particulate fractions of kidney (Fig. 1) show that they are present in all the fractions studied, although some of the enzymes present may be due to contamination of each fraction by the next most rapidly sedimenting fraction because the individual fractions were not washed before assay. Because of the large size of some of the lysosomes it is possible to separate them from other contaminating organelles by simple centrifugal procedures. The low yield of lysosomal enzymes recovered in this lysosomal fraction suggests that only a small percentage of the lysosomal particles differs sufficiently in size and density from the other subcellular particles to be separated by this technique.

Detailed enzyme analysis of the kidney lysosomes shows some marked differences from those reported for liver lysosomes. A study of the cathepsins shows that cathepsin C is not present in kidney lysosomes, although this has been reported to be present in liver lysosomes (Finkenstaedt, 1957). However, as the liver preparation used was a mitochondrial fraction, it is possible that this activity may have been related to the neutral-protease activity of mitochondria (Korner & Tarver, 1957; Alberti & Bartley, 1963) and not a true lysosomal enzyme. The presence of alkaline-phosphatase and glucose 6-phosphatase activities in kidney lysosomes, which does not appear to be due to microsomal contamination, as judged by electron micrographs, suggests the presence of another phosphatase in the kidney lysosome in addition to those reported in liver (Neil & Horne, 1962; Shibko & Tappel, 1963). Its presence has also been reported in the cytoplasmic granules of macrophages (Cohn & Hirsch, 1960). α -Glucosidase, which has been shown to be a constituent of liver lysosomes (Lejeune *et al.* 1963), is also present in kidney lysosomes. However, the difference in pH-activity properties of the lysosomal and microsomal forms of the enzyme (optimum pH values 5 and 6) is not as great as that reported for the isoenzymes

of α -glucosidase that occur at the various intracellular sites in liver (optimum pH values 3.8 and 7; Lejeune *et al.* 1963). The role of α -glucosidase in glycogen degradation in the liver has been discussed by Hers (1963). The function of α -glucosidase in the kidney is not known, as the enzyme does not release glucose from glycogen, which is only normally present in kidney in very small amounts (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963). Lysozyme, which has not been reported in liver lysosomes but is present in the macrophage granules (Cohn & Hirsch, 1960), is an important enzyme in high concentration in the kidney lysosomes. On the basis of comparison of lysozymal activity of lysosomes and that of purified egg-white lysozyme, lysozyme constitutes 6–10% of the lysosomal protein.

In any study of the enzymic composition of the large kidney lysosomes it is necessary to examine the available information to decide whether the enzymes present are derived from the protein-absorption droplet or the acid-phosphatase-bearing particle. It has been demonstrated that the accumulation of lysozyme in kidney is directly related to its concentration in the blood (Perri, Faulk & Money, 1964). Further, exogenous and endogenous lysozyme accumulates in the kidney (Perri, Faulk, Mellors & Stock, 1962; Perri & Anigstein, 1960) and does not normally appear in the urine. These facts suggest that lysozyme in the kidney lysosome originates from the lysozyme in the blood which has been reabsorbed in the form of protein droplets, and that the enzyme is not synthesized in the kidney tissue or part of the original acid-phosphatase-bearing particle. The function of lysozyme in kidney is not known. Unlike other lysosomal enzymes it does not appear to be available to act on its natural substrate, as the kidney provides a more favourable environment than other tissues of the body for the growth of bacteria (Beeson & Rowley, 1961). Presumably the lysozyme is being slowly degraded by the catheptic enzymes in the kidney lysosomes, as has been observed with injected peroxidase (Strauss, 1962), and its presence in kidney lysosomes may represent another phase in the body's protein metabolism. At present it is not known if other serum enzymes are reabsorbed in the kidney to form the protein-absorption droplets, and undergo degradation in this manner.

Isolated kidney lysosomes show a high degree of stability at 0° and pH 7, which enables them to be used for studies on the release of lysosomal enzymes. Studies of the release of enzymes from suspensions of lysosomes incubated at 37° suggests that this process occurs in two stages. First, there is a large increase in the amount of enzyme available but not soluble. This change is accompanied by a decrease in light-scattering properties of the suspended par-

ticles that may represent a swelling of the lysosomes due to a change in permeability of the lysosomal membrane. The second stage is characterized by an increasing availability of enzymes and a rapid increase in the amount of soluble enzymes, which eventually is almost equal to the amount of enzyme available. This stage represents the almost complete release of the contents of the lysosomal particles.

Kidney lysosomes, as has been observed for liver lysosomes (de Duve *et al.* 1955) and the cytoplasmic granules from macrophages (Cohn & Hirsch, 1960), are very labile at pH values below 5.

These results suggest that changes in the stability of lysosomes may be dependent on the activation of some system either by incubation at 37° or by lowering of pH.

Kidney lysosomes are not readily disrupted by changes in the osmolarity of the suspending medium. Lowering the molarity of the sucrose medium suspension from 0.6 to 0.25 M did not cause any changes in the light-scattering properties of lysosomes. Strauss (1957) showed that a decrease in molarity of the suspending sucrose medium from 0.88 to 0.097 M resulted in 75% of the lysosomal enzymes becoming available after 15 min. incubation at 37°. Similar drastic changes in the osmolarity of the suspending medium of macrophage granules are also required to cause changes in availability of enzyme (Cohn & Hirsch, 1960). In contrast, liver lysosomes are readily disrupted under these conditions (Sawant *et al.* 1964).

Freezing and thawing suspensions of lysosomes results in complete disruption of the lysosomes (Shibko, Pangborn & Tappel, 1965) and presumably availability of lysosomal enzymes. However, the release of enzymes into the soluble fraction (50–60%) is not as effective as that observed when lysosomes are incubated at 37°.

The non-specific binding of enzymes to the membrane of disrupted kidney lysosomes and the pattern of release of lysosomal enzymes into the soluble form under the conditions reported above do not show the differential pattern of binding and release observed for liver lysosome enzymes (Sawant *et al.* 1964).

In general, the kidney lysosomes show stability properties similar to those described for the cytoplasmic granules of macrophages, but differing in some aspects from those described for liver. Detailed enzyme analyses of the kidney lysosomes show that the complement of enzymes differs from that of liver lysosomes. This may indicate that the lysosomes from each tissue contain, in addition to a common group of hydrolytic enzymes, other enzymes that are specifically related to the metabolism of the organ.

The authors are indebted to Mr J. Pangborn for sectioning and preparing the electron micrographs. This investigation

was supported by U.S. Public Health Service Research Grant AM05609 from the National Institute of Arthritic and Metabolic Diseases and Project WM-33 of the U.S. Department of Agriculture.

REFERENCES

- Alberti, K. G. M. M. & Bartley, W. (1963). *Biochem. J.* **87**, 104.
- Beaufay, H. & de Duve, C. (1954). *Bull. Soc. Chim. biol., Paris*, **11**, 1525.
- Beeson, P. B. & Rowley, D. (1961). In *Biology of Pyelonephritis*, p. 427. Ed. by Quinn, E. L. & Kass, E. H. Boston: Little, Brown and Co.
- Cohn, Z. A. & Hirsch, J. G. (1960). *J. exp. Med.* **112**, 983.
- de Duve, C. (1963). In *Lysosomes*, p. 20. Ed. by de Reuck, A. V. S. & Cameron, M. F. London: J. and A. Churchill Ltd.
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
- Finkstaedt, J. T. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 302.
- Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
- Goldbarg, J. A., Tsou, K.-C., Rutenberg, S. H., Rutenberg, A. M. & Seligman, A. M. (1958). *Arch. Biochem. Biophys.* **75**, 435.
- Hamilton, J. W. & Tappel, A. L. (1963). *J. Amer. Oil Chem. Soc.* **40**, 52.
- Hers, H. G. (1963). *Biochem. J.* **86**, 11.
- Hogeboom, G. H. & Schneider, W. C. (1953). *J. biol. Chem.* **204**, 233.
- Korner, A. & Tarver, H. (1957). *J. gen. Physiol.* **41**, 219.
- Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.
- Lejeune, N., Thines-Sempoux, D. & Hers, H. G. (1963). *Biochem. J.* **86**, 16.
- Litwack, G. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 764.
- Lowry, O. H., Roberts, N. R., Wu, M. S., Hixon, W. S. & Crawford, E. J. (1954). *J. biol. Chem.* **207**, 19.
- Miller, G. L. (1959). *Analyt. Chem.* **31**, 964.
- Neil, M. W. & Horne, M. W. (1962). *Biochem. J.* **84**, 32.
- Perri, G. & Anigstein, R. (1960). *Cancer Res.* **20**, 1054.
- Perri, G. C., Faulk, M., Mellors, J. & Stock, C. C. (1962). *Nature, Lond.*, **193**, 649.
- Perri, G. C., Faulk, M. & Money, W. L. (1964). *Proc. Soc. exp. Biol., N.Y.*, **115**, 185.
- Roy, A. B. (1953). *Biochem. J.* **53**, 12.
- Sawant, P., Shibko, S., Kumta, U. & Tappel, A. L. (1964). *Biochim. biophys. Acta*, **85**, 82.
- Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A. & de Duve, C. (1960). *Biochem. J.* **74**, 450.
- Shibko, S., Pangborn, J. & Tappel, A. L. (1965). *J. Cell Biol.* (in the Press).
- Shibko, S. & Tappel, A. L. (1963). *Biochim. biophys. Acta*, **73**, 76.
- Strauss, W. (1954). *J. biol. Chem.* **207**, 745.
- Strauss, W. (1956). *J. biophys. biochem. Cytol.* **2**, 513.
- Strauss, W. (1957). *J. biophys. biochem. Cytol.* **3**, 933.
- Strauss, W. (1962). *J. Cell Biol.* **12**, 231.
- Strauss, W. (1964). *J. Cell Biol.* **20**, 497.
- Tappel, A. L., Zalkin, H., Caldwell, K. A., Desai, I. & Shibko, S. (1962). *Arch. Biochem. Biophys.* **96**, 340.
- Whitaker, J. R. (1961). *Nature, Lond.*, **189**, 662.