Isolation and Characterization of a Rough Microsomal Fraction from Rat Kidney that is Enriched in Lysosomal Enzymes

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1. A special population of rough microsomal material (microsomes) rich in lysosomal acid hydrolases was separated by isopycnic centrifugation as a discrete fraction (RM_2) from the bulk of rough microsomal material in rat kidney because of its greater density. 2. The specific activities of five acid hydrolases in the RM_2 fraction were approximately one-half those of a purified lysosomal (L) fraction and 10- to 30-fold greater than those of an ordinary rough microsomal (RM_1) fraction. 3. These special rough microsomes have a distinctive ultrastructure and electron-cytochemical properties. Their cisternal content resembles the matrix of lysosomes in that it is electron-dense, osmiophilic and plumbo-philic and gives a positive reaction for acid phosphatase activity. 4. Polyacrylamide-gel electrophoresis of soluble proteins from the L fraction resolved nine anionic glycoproteins, most of which exhibit acid hydrolase activities (Goldstone & Koenig, 1970, 1973; Goldstone *et al.*, 1971*a*). The most anionic glycoprotein is the acidic lipoglycoprotein of the lysosomal matrix (Goldstone *et al.*, 1970). 5. Polyacrylamide-gel electrophoresis of soluble proteins from the RM_2 fraction resolved two cationic glycoproteins with acid hydrolase activities (Goldstone & Koenig, 1973) and an anionic glycoprotein with the same electrophoretic mobility as the lysosomal lipoglycoprotein, but without its lipid constituents or capacity to bind the basic fluorochrome Acridine Orange. These constituents are considered to be the precursors of the lysosomal glycoproteins.

The lysosomal acid hydrolases are generally thought to be synthesized on the ribosomes of the rough endoplasmic reticulum and transported to the Golgi apparatus for packaging into primary lysosomes (de Duve & Wattiaux, 1966; Cohn & Fedorko, 1969) in a similar manner to proteins manufactured for extracellular secretion, e.g., pancreatic enzymes (Caro & Palade, 1964; Jamieson & Palade, 1967; Ericksson, 1969) and thyroid hormones (Nadler *et al.*, 1964). The biochemical investigation of the synthesis, intracellular migration and storage of the acid hydrolases has been hampered by the fact that only a small portion of the protein-synthesizing machinery of most mammalian cells is directed toward the production of these enzymes. Although lysosomal fractions can be obtained from several mammalian tissues by centrifugation procedures, those portions of the endoplasmic reticulum and Golgi apparatus that are involved in the synthesis and processing of lysosomal enzymes have not been isolated before.

We have recently shown that the acid hydrolases present in purified lysosomal fractions from rat kidney and liver are glycoprotein enzymes which contain *N*-acetylglucosamine, mannose, glucose and *N*-acetylneuraminic acid (Goldstone & Koenig, 1968, 1970; Koenig, 1969*a,b*). These lysosomal fractions

also contain a soluble acidic lipoglycoprotein as a major constituent of the internal matrix of lysosomal particles, which may serve as an inhibitor of the bound enzymes (Barrett & Dingle, 1967; Goldstone & Koenig, 1968; Goldstone *et al.*, 1970; Koenig, 1962, 1969*a,b*) and as an acidic buffering system to maintain an acid pH in heterolysosomes (Mego, 1971). We now report that a special population of rough microsomal material (microsomes) with distinctive ultrastructural and cytochemical features and notably enriched in lysosomal acid hydrolase activities can be separated as a discrete fraction from the bulk of rough microsomes in rat kidney because of its greater density. Isotope-incorporation studies indicate that the lysosomal glycoproteins are synthesized in these special rough microsomes (Goldstone *et al.*, 1971*b*; Goldstone & Koenig, 1972; Nayyar & Koenig, 1972). The soluble glycoproteins of this fraction differ in certain significant respects from those in a lysosomal fraction.

Materials and Methods

Chemicals

Sodium β-glycerophosphate, phenolphthalein glucuronic acid and *p*-nitrocatechol sulphate (as

dipotassium salt) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 4-Methylumbelliferyl β-D-galactopyranoside and N-acetyl-β-D-glucosaminide were purchased from Pierce Biochemical Co., Rockford, Ill., U.S.A. Triton X-100 was from Rohm and Haas, Philadelphia, Pa., U.S.A. Reagents for polyacrylamide-gel electrophoresis were purchased from Canalco Co., Rockville, Md., U.S.A. All other chemicals were of the best available analytical grade.

Fractionation of tissues

Kidneys were obtained from young adult Sprague–Dawley rats (Holtzman Co., Madison, Wis., U.S.A.) weighing about 200g. Animals were killed by decapitation, then the kidneys were removed and homogenized in 0.3M-sucrose (4ml per g) with a Potter–Elvehjem-type homogenizer. All operations were at 4°C. The homogenate was centrifuged at 750g for 10min to deposit nuclei and cell debris. The nuclear supernatant was centrifuged at 3300g for 10min to give a heavy mitochondrial fraction. A lysosomal (L) fraction was prepared from the latter fraction as described by Goldstone & Koenig (1972). Briefly, the 3300g pellet was resuspended in 8ml of 0.3M-sucrose, layered over 16ml of 1.65M-sucrose and centrifuged for 2h at 68000g in a Spinco SW25.1 rotor in a model L ultracentrifuge to deposit the L fraction as a pellet. The 3300g supernatant was centrifuged at 16800g for 20min and the light mitochondrial pellet discarded.

A standard rough microsomal (RM$_1$) fraction was prepared by a modification of the method of Dallner et al. (1963). Samples (5ml) of the 16800g supernatant containing 0.01M-MgCl$_2$ were layered over 4.5ml of 1.5M-sucrose containing 0.01M-MgCl$_2$ and centrifuged in the Spinco no. 50 rotor in the model L2 ultracentrifuge at 150000g for 1h. The RM$_1$ fraction was recovered as a pellet from 1.5M-sucrose. A special rough microsomal (RM$_2$) fraction rich in lysosomal enzymes was isolated from the 16800g supernatant by isopycnic centrifugation over a discontinuous sucrose gradient. MgCl$_2$ was omitted from the gradient as it causes rough microsomes and some smooth microsomes to aggregate, thereby increasing their buoyant density (Dallner et al., 1963; Dallner & Nilsson, 1966). This method was based on the observation that a 'light lysosomal' fraction obtained as a pellet from 1.65M-sucrose on isopycnic centrifugation contained numerous rough microsomes which were rich in lysosomal enzymes (Goldstone & Koenig, 1972). The 16800g supernatant was placed over a gradient consisting of 1.4, 1.5 and 1.6M-sucrose, or over 1.6M-sucrose alone, and centrifuged at 68000g for 2h in a Spinco SW25.1 rotor in a model L ultracentrifuge. The RM$_2$ fraction was recovered as a pellet from 1.6M-sucrose.

Electron microscopy and cytochemistry

Fractions were pelleted and fixed for 2–15h at 4°C in a solution containing 2% glutaraldehyde, 0.1M-sodium cacodylate buffer, pH7.4, and sucrose in the same concentration as that present in the density gradient in which the fractions were equilibrated. In several experiments, pellets were fixed in the buffered glutaraldehyde solution containing 1.0M-sucrose for 30min at 4°C and transferred to the same fixative without sucrose for several more hours to avoid excessive shrinkage of vesicles due to hyperosmotic sucrose media and disruption by osmotic shock. Fractions were postfixed in buffered 2% (v/v) OsO$_4$, dehydrated in a graded ethanol series and propylene oxide and embedded in Araldite. Ultrathin sections were viewed unstained or after staining with lead hydroxide and/or uranyl acetate. Acid phosphatase activity was displayed by incubating small fragments of pellets in a β-glycerophosphate medium (Gomori, 1950), rinsing, treating with OsO$_4$, and processing for electron microscopy as already described. Sections were photographed in an RCA EMU3F electron microscope.

Analytical procedures

Proteins, phospholipid and RNA were precipitated from fractions by addition of trichloroacetic acid to a final concentration of 10% (v/v). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. Lipids were extracted from fractions with chloroform–methanol (2:1, v/v) (Folch et al., 1957). Lipid phosphorus was determined according to the method of Bartlett (1959) and multiplied by 25 to give phospholipid values. RNA was determined by u.v. spectrophotometry by a modification of the Schmidt–Thannhauser procedure (Hutchinson & Munro, 1961).

Enzyme assays

All acid hydrolase activities were assayed in the presence of 0.2% Triton X-100 to give total enzyme activities (Wattiaux & de Duve, 1956). Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity was assayed with sodium β-glycerophosphate as a substrate (Gianetto & de Duve, 1955). Tissue samples were incubated with 0.5ml of 0.1M-sodium acetate buffer, pH5.0, and 1.0ml of 0.1M-β-glycerophosphate in a total volume of 2ml for 15min at 37°C. The reaction was terminated by adding 0.5ml of 30% (w/v) trichloroacetic acid to the reaction mixture before incubation. The precipitate was removed by filtration through two thicknesses of Whatman no. 3 filter paper and P$_i$ was measured in filtrates by the Fiske & SubbaRow (1925) method. β-Galactosidase (β-D-galactoside

galactohydrolase, EC 3.2.1.23) and β-N-acetylglucos-aminidase (β-2-acetamido-2-deoxy-D-glucoside acet-amidodeoxyglucohydrolase, EC 3.2.1.30) activities were assayed fluorimetrically with 4-methylumbelli-feryl β-D-glycosides as substrates (Robinson et al., 1967). For β-galactosidase, tissue samples were incu-bated with 0.5 ml of 0.1 M-sodium acetate buffer, pH 3.7, and 1 ml of 0.001 M-4-methylumbelliferyl β-D-galactopyranoside in a final volume of 2 ml at 37°C for 15 min. For β-N-acetylhexosaminidase the reac-tion mixture consisted of 0.5 ml of 0.1 M-sodium acetate buffer, pH 4.6, and 0.5 ml of 0.001 M-4-methylumbelliferyl N-acetyl-β-D-glucosaminide in a total volume of 2 ml. Incubation was also at 37°C for 15 min. The enzyme reaction was terminated by add-ing 3 ml of 0.5 M-glycine–NaOH buffer, pH 10.4. The liberated 4-methylumbelliferone was determined in an Aminco–Bowman spectrophotofluorimeter at a wavelength of activation of 360 nm and a wavelength of emission of 440 nm with 4-methylumbelliferone as standard. β-Glucuronidase (β-D-glucuronide glucuro-nohydrolase, EC 3.2.1.31) was assayed with phenol-phthalein glucuronic acid as substrate (Fishman & Bernfeld, 1955). Enzyme samples were incubated with 50 μmol of sodium acetate buffer, pH 5.0, and 0.1 ml of substrate solution (Sigma Chemical Co.) in a total volume of 1 ml for 20 min at 37°C. The reaction was terminated with 2 ml of 0.5 M-glycine–NaOH buffer, pH 10.4, then the precipitate was removed by centri-fugation and the E_{540} of the supernatant was measured against a zero-time blank. Arylsulphatase (aryl sulphate sulphohydrolase, EC 3.1.6.1) was assayed with p-nitrocatechol sulphate as substrate (Roy, 1958). The enzyme sample, 0.5 ml of 0.1 M-sodium acetate buffer, pH 5.5, and 0.2 ml of 0.1 M-substrate in a total volume of 1 ml were incubated at 37°C for 15 min. The reaction was stopped by addi-tion of 2 ml of 1 M-NaOH, then the precipitate was removed by centrifugation and the E_{515} of the super-natant was measured against a zero-time blank. Glutamate dehydrogenase (L-glutamate–NAD oxido-reductase, EC 1.4.1.3), a mitochondrial reference enzyme, was assayed according to Schachter et al. (1970). The incubation mixture contained enzyme, 0.25 μmol of NaOH, 5 μmol of ammonium chloride, 10 μmol of EDTA, 50 μmol of potassium phosphate buffer, pH 7.4, 1 μmol of α-oxoglutarate and 1 μl of Triton X-100 in a final volume of 1 ml. The E_{340} was measured for 5 min at 25°C in a recording spectro-photometer and enzyme activities were calculated from the initial slopes. Control incubations lacking α-oxoglutarate were run and corrections applied when necessary. Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9), a microsomal reference enzyme, was assayed according to Swanson (1955). The incubation mixture, contain-ing enzyme, 10 μmol of glucose 6-phosphate and 60 μmol of sodium acetate buffer, pH 6.5, in a final

volume of 1 ml, was incubated for 15 min at 37°C. The reaction was stopped by adding 1 ml of 10% trichloro-acetic acid. The P_i was measured in protein-free filtrates by the Fiske & SubbaRow (1925) method. For a control the trichloroacetic acid was added at zero time. Alkaline β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), a plasma-membrane reference enzyme, was measured by a modification of the method of Robinson et al. (1967). The incubation mixture, containing enzyme, 0.1 ml of 0.01% 4-methylumbelliferyl β-D-galactopyranoside and 160 μmol of sodium borate buffer, pH 8.6, in a final volume of 2 ml, was incubated for 15 min at 37°C. The reaction was stopped with 2 ml of 0.5 M-NaOH and the liberated 4-methylumbelliferone measured fluorimetrically. Uricase (urate–oxygen oxidoreduc-tase, EC 1.7.3.3) activity was assayed as a peroxisomal marker enzyme (Worthington Enzyme Manual). The incubation mixture contained 0.2 μmol of uric acid, 220 μmol of sodium borate buffer, pH 8.5, and enzyme in a final volume of 3 ml. The decrease in E_{290} was followed spectrophotometrically for 6 min at 25°C. For a control 0.2 ml of 0.1% KCN was added to the incubation mixture at zero time. The specific activity of acid β-galactosidase and β-N-acetylhexosaminidase is expressed as nmol $\times$ 10, and of all other enzymes as μmol of substrate hydrolysed/h per mg of protein.

Disc electrophoresis

The RM$_2$ and L fractions were sonicated in 0.2% Triton X-100 and 0.1 M-sodium acetate buffer, pH 5.2, to solubilize proteins. The sonicates were clarified by centrifugation at 100000 g for 60 min and subjected to electrophoresis in 5% (w/v) polyacryl-amide gels at pH 8.8 or 4.3 for about 45 min at 5 mA/tube (Davis, 1964). Gels were stained for protein with Amido Schwarz (1% in 7% (v/v) acetic acid), for carbohydrate with the periodic acid–Schiff reagent (Scott & Harbinson, 1967), for lipid with Sudan Black B (0.5% in propylene glycol with 2% formaldehyde), and for acidic sites with the basic fluorochrome Acridine Orange (0.003% in 0.01 M-sodium glycinate buffer, pH 8, with 2% formalde-hyde), as described previously (Goldstone et al., 1970). Gels stained for protein and carbohydrate were scanned with a Gilford 2410 linear-transport and a Gilford 2000 absorbance recorder.

Results

Ultrastructure and cytochemistry of fractions

The L fraction consisted almost entirely of typical lysosomal dense bodies with infrequent (approx. 5%) contaminating mitochondria (Plate 1a). The RM$_1$ fraction contained numerous ordinary rough microsomes with a sparse electron-lucent content,

many free ribosomes, some smooth vesicles and microvilli, and occasional small mitochondria, dense bodies and peroxisomes. Some anastomosing tubules with an electron-dense content resembling those of a Golgi fraction (Goldstone & Koenig, 1972) were also present (Plate 1b). Approximately one-half of the RM_2 fraction was made up of an unusual type of rough microsome filled with an electron-dense osmiophilic and plumbophilic content. Some smooth vesicles, small mitochondria and occasional dense bodies and peroxisomes also were present as well as a few anastomosing tubules of the Golgi type (Plates 1c and 1d). Most dense bodies in the L fraction and about 35–50% of the special rough microsomes in the RM_2 fraction had a positive acid phosphatase reaction that was absent in controls incubated without substrate (Plate 1e). The ordinary rough microsomes in the RM_1 fraction were non-reactive, but some of the Golgi tubules in the RM_1 and RM_2 fractions contained acid phosphatase reaction product (Plate 1f).

Chemical and enzymic composition of fractions

The RM_2 fraction comprised 1.0–2.0% of the protein in the RM_1 fraction (Table 1). The RNA and phospholipid content of the RM_1 and RM_2 fractions was similar. The L fraction contained one-fourth the phospholipid (0.11 mg/mg of protein) of the latter fractions and negligible RNA. The bulk of the phospholipid in the latter occurs in the soluble acidic lipoglycoprotein of the matrix of lysosomal particles (Goldstone et al., 1970), whereas the phospholipid in the RM_1 and RM_2 fractions is associated mainly with the insoluble membrane fraction (A. Goldstone & H. Koenig, unpublished work). The glucose 6-phosphatase activity of the RM_1 and RM_2 fraction was elevated about sevenfold over that of the whole homogenate, indicating that these fractions were similarly enriched in microsomal membranes. Contamination with mitochondria, as judged by the glutamate dehydrogenase activity, was about 3% for the RM_1 fraction and 7% for the RM_2 fraction. (The specific gluatamate dehydrogenase activity of the mitochondrial fraction from rat kidney, not shown in Table 1, was 13.6.) Peroxisomal and plasma-membrane contamination in the RM_1 and RM_2 fraction was negligible as indicated by the low uricase and alkaline β-galactosidase activity respectively.
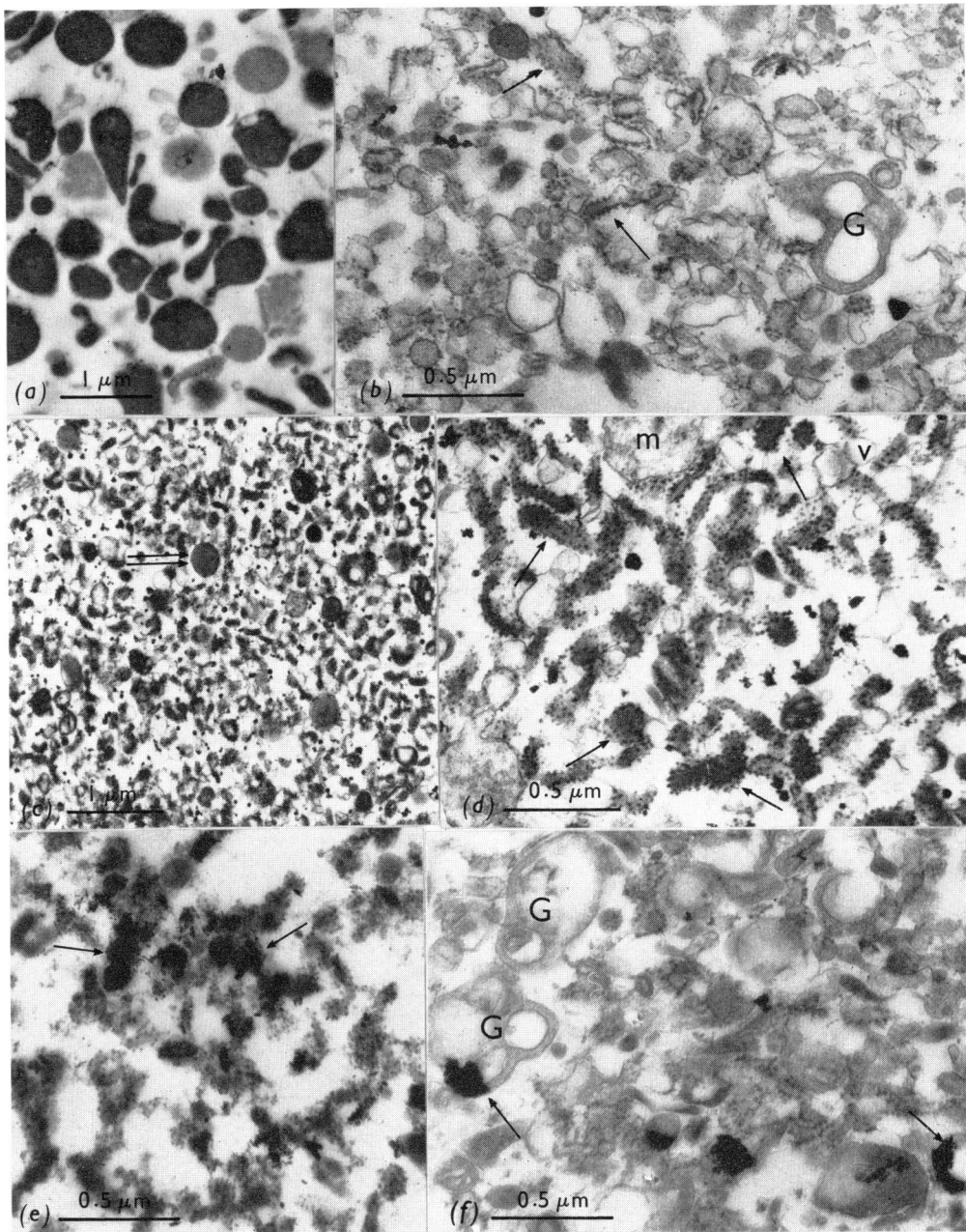
Acid hydrolases of fractions

The acid hydrolase activities of these fractions are shown in Table 2. All five lysosomal marker enzymes showed a similar subcellular distribution. The L fraction contained about 13–25% of the acid hydrolase activities and only 1% of the protein in the whole homogenate. In accord with previous findings (Gold-

Table 1. Chemical and enzymic composition of rat kidney subcellular fractions

Subcellular fractions were prepared and analysed for protein, RNA, phospholipid and enzyme activities as described in the text. Each value for protein, RNA and phospholipid is the mean of three or four separate experiments with ranges in parentheses. The specific enzyme activities are expressed as μmol of substrate hydrolysed/h per mg of protein. The relative specific activity was obtained by dividing the specific activity of the fraction by the specific activity of the whole homogenate. Each value is the average of two determinations. N.D., not determined.

Fraction	Protein (% of total)	Protein (mg/g of tissue)	RNA (mg/mg of protein)	Phospholipid (mg/mg of protein)	Glutamate dehydrogenase Specific activity	Glutamate dehydrogenase Relative specific activity	Glucose 6-phosphatase Specific activity	Glucose 6-phosphatase Relative specific activity	Alkaline β-galactosidase Specific activity	Alkaline β-galactosidase Relative specific activity	Uricase Specific activity	Uricase Relative specific activity
Whole homogenate	100	58.7 (47.0–70.0)	0.05 (0.04–0.06)	0.08 (0.07–0.09)	1.84	1.0	0.45	1.0	6.45	1.0	0.95	1.0
RM_1	9.0	5.26 (3.40–7.35)	0.26 (0.23–0.29)	0.41 (0.34–0.48)	0.43	0.23	3.26	7.3	1.5	0.23	0.47	0.45
RM_2	0.17	0.10 (0.07–0.15)	0.20 (0.19–0.22)	0.40 (0.33–0.48)	0.94	0.51	2.90	6.5	1.6	0.23	0.76	0.8
L	1.1	0.64 (0.44–0.78)	0.003 (0.000–0.007)	0.11 (0.10–0.12)	0.87	0.47	0.22	0.49	N.D.	N.D.	1.0	1.06

EXPLANATION OF PLATE I

Electron micrographs of rat kidney fractions

Uranyl acetate–lead citrate stain was used. (*a*) L fraction. This fraction is made up almost exclusively of lysosomal dense bodies. Magnification ×12600. (*b*) RM_1 fraction. This fraction demonstrates one profile of anastomosing tubules with an electron-dense content from the Golgi system (G) and many ordinary rough microsomes (→). Magnification ×34200. (*c*) and (*d*) RM_2 fraction. This fraction consists predominantly of special rough microsomes (→) with an electron-dense content. A few smooth vesicles (v), mitochondria (m) and dense bodies and/or peroxisomes (⇉) also are present. Magnification for (*c*) ×13200, for (*d*) ×34200. (*e*) RM_2 fraction incubated in lead β-glycerophosphate medium. Acid phosphatase reaction product (→) is present over the cisternae of some rough microsomes. Magnification ×34200. (*f*) RM_1 fraction incubated for acid phosphatase activity. Reaction product (→) is present in several smooth tubular profiles from the Golgi apparatus (G), but is absent from the ordinary rough microsomes. Magnification ×34200.

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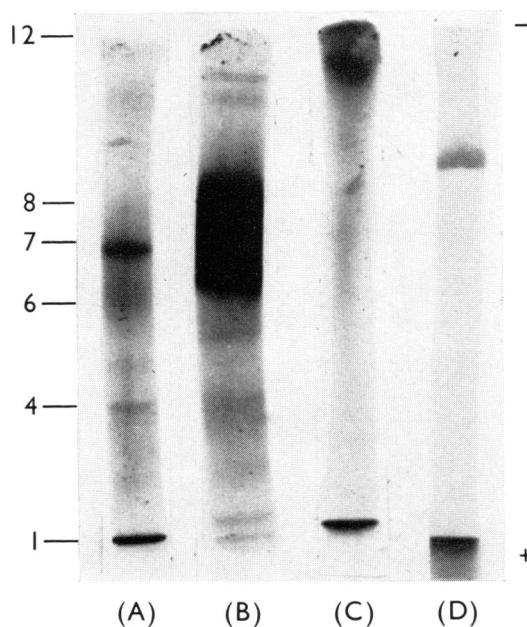
EXPLANATION OF PLATE 2(*a*)

Polyacrylamide-gel electrophoretograms of 0.2% Triton X-100 extracts of the L fraction

For details see the text. (A) Amido Schwarz stain for protein. (B) Periodic acid–Schiff stain for glycoprotein. (C) Sudan Black B stain for lipoprotein. (D) Acridine Orange metachromasia for acidic groups.
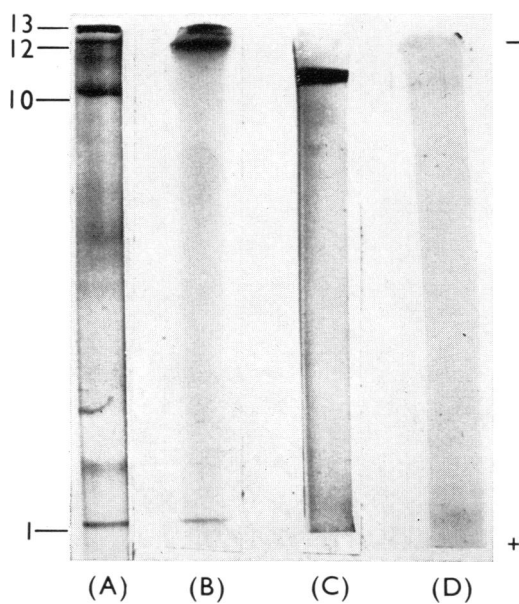
EXPLANATION OF PLATE 2(*b*)

Polyacrylamide-gel electrophoretograms of 0.2% Triton X-100 extracts of the RM$_2$ fraction

For details see the text. (A) Amido Schwarz stain for protein. (B) Periodic acid–Schiff stain for glycoprotein. (C) Sudan Black B stain for lipoprotein. (D) Acridine Orange metachromasia for acidic groups.

A. GOLDSTONE, H. KOENIG, R. NAYYAR, C. HUGHES AND C. Y. LU

stone *et al.*, 1970; Goldstone & Koenig, 1970), the relative specific acid hydrolase activities (whole homogenate = 1) of the L fraction ranged from 15 to 28. The RM_1 fraction contained 4–7% of the total acid hydrolase activities and about 9% of the protein in the whole homogenate. The relative specific acid hydrolase activities in the RM_1 fraction were approximately 0.5. About 25–50% of the acid hydrolase activities and only 1–2% of the protein in the RM_1 fraction was recovered in the RM_2 fraction, the relative specific activities of the latter ranging from 6 to 12. In individual experiments the relative specific activities of these lysosomal enzymes in the RM_2 fraction were 35–70% of the corresponding values in the L fraction, and 10- to 30-fold greater than those in the RM_1 fraction.

Disc electrophoresis of fractions

About 45% of the protein in the RM_2 fraction and 67% of the protein in the L fraction was solubilized by sonicating in 0.2% Triton X-100 and 0.1 M-sodium acetate buffer, pH 5.2 (Goldstone & Koenig, 1973). Approximately 12 protein components were resolved from L fractions by electrophoresis in 7.5% polyacrylamide gels at pH 8.8 (Plate 2a and Fig. 1). Nine of these bands, totalling about 90% of the soluble protein, reacted strongly for carbohydrate by the periodic acid–Schiff method. These glycoprotein components occurred throughout the gels, but the bulk of the periodic acid–Schiff-positive protein (about 55% of the total) migrated as slow-moving anionic components in bands 6 to 8. The fastest-moving anionic component, band 1, contained 19% of the protein and gave a periodic acid–Schiff reaction. In addition, it was coloured by Sudan Black B, and stained metachromatically with Acridine Orange, denoting the presence of protein-bound lipids. This constituent and a minor metachromatic component, band 9 (not labelled) containing 3% of the protein, represent the acidic lipoglycoprotein of lysosomal fractions described previously (Goldstone *et al.*, 1970). Most of the remaining glycoprotein bands coincided with bands of hydrolytic activities (Goldstone & Koenig, 1973; Goldstone *et al.*, 1971a).

Approximately 13 soluble protein components were resolved in gel electrophoretograms of RM_2 fractions (Plate 2b and Fig. 2). Three components, bands 1, 12 and 13, representing about 37% of the soluble protein, were stained by the periodic acid–Schiff method for glycoprotein. The carbohydrate content of these glycoproteins, as measured by densitometry, was about one-half that of the lysosomal glycoproteins (Fig. 2). The cationic glycoproteins, bands 12 and 13, comprising about 30% of the soluble protein, occupied the same position in these gels as a number of lysosomal hydrolases in the RM_2 fraction (Goldstone & Koenig, 1973; A. Goldstone & H.

Table 2. *Acid hydrolase activities of rat kidney subcellular fractions*

Subcellular fractions were prepared and protein and enzyme activities determined as described in the text. The specific activity of acid phosphatase, β-glucuronidase and arylsulphatase is expressed as μmol, and of β-galactosidase and β-N-acetylhexosaminidase as nmol × 10, of substrate hydrolysed/h per mg of protein. The relative specific activity was obtained by dividing the specific activity of the fraction by the specific activity of the whole homogenate. Each value is the mean of 3–4 separate experiments with ranges in parentheses.

| Fraction	Acid phosphatase			β-Galactosidase			β-N-Acetylhexosaminidase			β-Glucuronidase			Arylsulphatase		
	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity
Homogenate	100	17.1 (12.4–24.8)	1.0	100	5.6 (4.7–6.4)	1.0	100	5.7 (4.8–6.1)	1.0	100	12.0 (9.1–16.6)	1.0	100	2.00 (1.07–2.92)	1.0
RM₁	4.0	7.6 (4.8–10.5)	0.4	6.7	4.2 (3.7–5.0)	0.75	4.9	3.1	0.5	4.5	6.0 (2.0–8.0)	0.5	4.2	0.93 (0.67–1.20)	0.5
RM₂	1.3	129.0 (84–173)	7.5	3.4	43.0 (37.0–51.0)	7.7	1.3	42.0 (41.0–44.0)	7.4	1.6	110.0 (97–128)	9.2	1.1	13.1 (8.7–18.7)	6.5
L	13.2	286.0 (230–460)	16.7	20.0	100.0 (82–120)	17.9	18.8	98.0 (88–100.8)	17.2	18.2	205.0 (165–216)	17.1	25.1	46.0 (33.3–70.8)	23.0
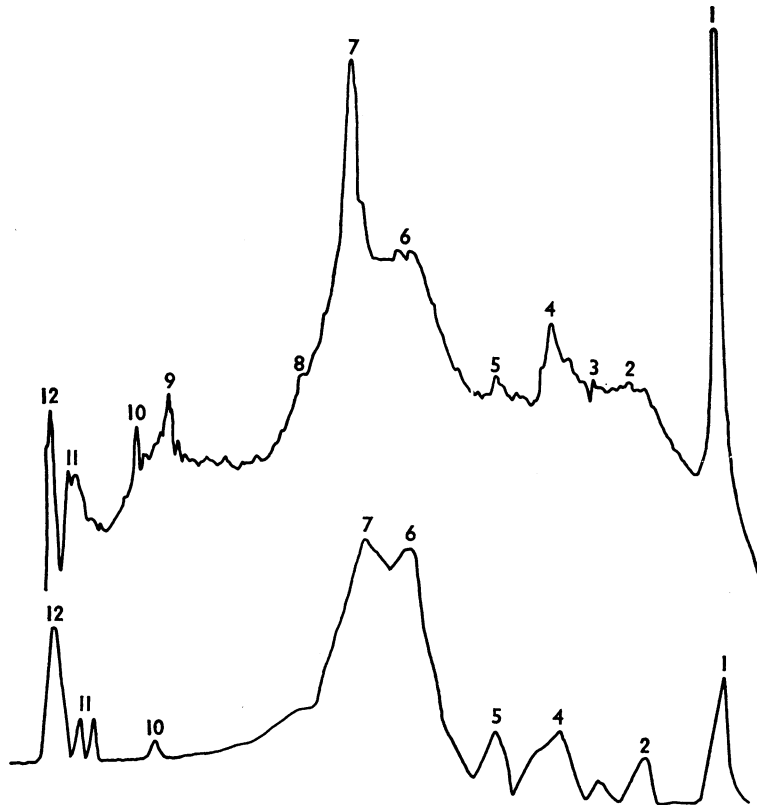
Fig. 1. *Densitometric scan of gel electrophoretograms of the L fraction stained for protein and glycoprotein*

For details see the text. The upper tracing is for the protein bands shown in Plate 2(*a*), gel A, and the lower tracing for the glycoprotein bands shown in Plate 2(*a*), gel B.

Koenig, unpublished work) and are considered to be lysosomal constituents. The fastest-moving anionic component, band 1, contained 7% of the protein and resembled the acidic lipoglycoprotein of the L fraction in its positive periodic acid–Schiff reaction and its electrophoretic mobility. Unlike the acidic lipoglycoprotein, however, this component exhibited only a feeble metachromatic staining with Acridine Orange and was unstained by Sudan Black B, denoting the essential absence of lipid (Goldstone *et al.*, 1970). The electrophoretic mobility of the acidic lipoglycoprotein in polyacrylamide gels is independent of its lipid content (Goldstone *et al.*, 1970; A. Goldstone & H. Koenig, unpublished work). We tentatively conclude, therefore, that band 1 of the RM_2 fraction is an apolipoglycoprotein of the corresponding lysosomal constituent. When extracts of the RM_2 fraction were subjected to electrophoresis at pH 4.3, the two cationic glycoproteins migrated rapidly into the gel to assume

the most cathodic position, whereas the anionic glycoprotein did not enter the gel.

Another lipoprotein constituent of the RM_2 fraction (band 10) comprised 20% of the soluble protein. This band stained strongly with Sudan Black, but it did not give a periodic acid–Schiff reaction or stain metachromatically with Acridine Orange (Plate 2*b*). A lipoprotein with similar electrophoretic and staining properties has been isolated from rat kidney mitochondria (Goldstone *et al.*, 1970). This lipoprotein and the other carbohydrate-free proteins account for about 63% of the soluble protein in the RM_2 fraction and probably originate from small mitochondria and other contaminating structures in this fraction. This value for non-lysosomal protein agrees with estimates for contamination of the RM_2 fraction that are based on survey electron micrographs, and on the relative specific acid hydrolase activities of the RM_2 fraction when
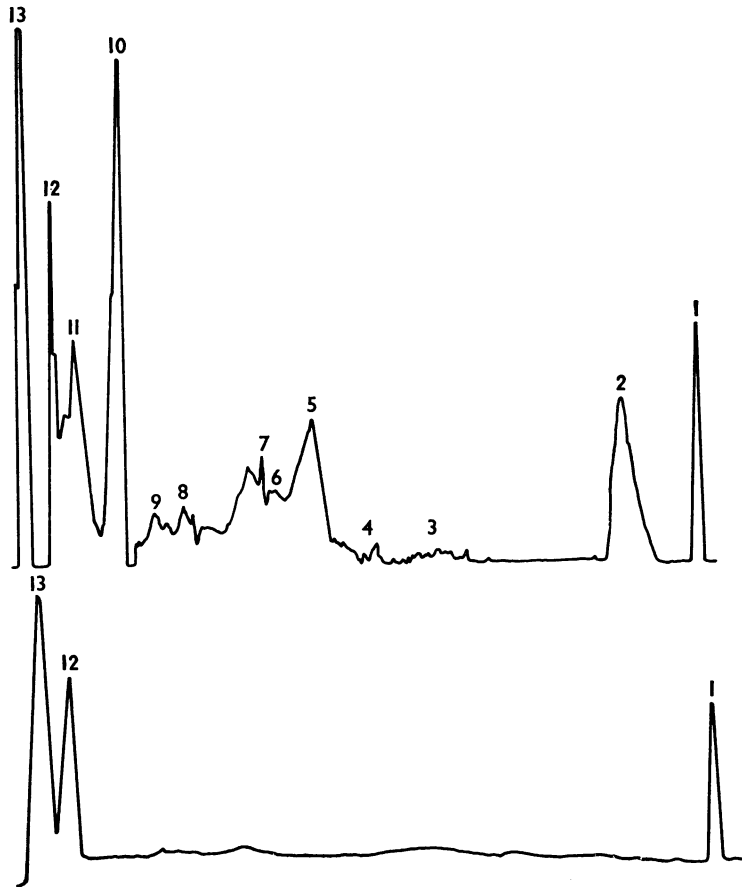
Fig. 2. *Densitometric scan of gel electrophoretograms of the RM₂ fraction stained for protein and glycoprotein*

For details see the text. Upper tracing is for the protein bands in Plate 2(*b*), gel A, and the lower tracing for the glycoprotein bands in Plate 2(*b*), gel B.

compared with the L fraction as the standard of purity.

Discussion

These experiments show that a special population of rough microsomes that are rich in acid hydrolase activities can be separated from the bulk of the rough microsomes by virtue of their unusual sedimentation characteristics. The special rough microsomes and lysosomal dense bodies share certain significant features. Both structural elements have a high density on isopycnic centrifugation, which allows them to be ultracentrifuged through 1.60M-sucrose. The cisternal content of the special rough microsomes resembles the internal matrix of lysosomal dense bodies in that both are electron-dense and osmiophilic and

show electron-cytochemical reactions for glycoprotein and acid phosphatase activity (Goldstone & Koenig, 1972) (Plate 1*e*). The demonstration of glycoproteins in gel electrophoretograms of extracts of these fractions (Plates 2*a* and 2*b*) supports the electron-cytochemical observations and is consistent with the finding that *N*-acetylglucosamine, glucose and mannose are incorporated into polypeptide in these special rough microsomes (Goldstone *et al.*, 1971*b*; Goldstone & Koenig, 1972; Nayyar & Koenig, 1972). It is also noteworthy that some of the Golgi tubules which sediment in the RM₁ fraction, and to a lesser extent with the RM₂ fraction, have a cisternal content which is osmiophilic and give electron-cytochemical reactions for acid phosphatase (Plate 1*f*) and glycoprotein (R. Nayyar, H. Koenig, A. Goldstone & C. Hughes, unpublished work). In accord with these

findings, the RM_1 and RM_2 fractions contain appreciable amounts of the Golgi enzyme markers, N-acetyl-lactosamine, galactosyl transferase and sialyl transferase (Goldstone & Koenig, 1973).

The soluble glycoproteins of the special rough microsomes and lysosomal dense bodies also differ in certain important respects. Only two glycoproteins were resolved in the RM_2 fraction which were associated with acid hydrolase activities (Goldstone & Koenig, 1973); both of these are strongly cationic (Plate 2b and Fig. 2). By contrast the L fraction contains at least eight glycoproteins with enzymic activities (Plate 2a, and Fig. 1) (Goldstone & Koenig, 1970, 1973; Goldstone et al., 1971a). All of these constituents migrate toward the anode under the electrophoretic conditions employed and evidently possess a greater carbohydrate content than the RM_2 glycoproteins. In addition, the anionic glycoprotein band in the RM_2 fraction differs from the corresponding acidic lipoglycoprotein of the L fraction in that it lacks lipids (Plates 2a and 2b).

These special rough microsomes are thought to originate from restricted portions of the rough endoplasmic reticulum where the lysosomal acid hydrolases and apolipoglycoprotein are synthesized. This inference is supported by recent biochemical (Goldstone et al., 1971b; Goldstone & Koenig, 1972) and ultrastructural radioautographic (Goldstone et al., 1971b; Nayyar & Koenig 1972) studies showing that labelled leucine, lysine, N-acetylglucosamine, glucose and mannose are initially incorporated into polypeptide in these special rough microsomes, whereas N-acetylneuraminic acid residues are incorporated in the Golgi apparatus, labelled glycoproteins subsequently appearing in lysosomes. In the accompanying paper (Goldstone & Koenig, 1973) evidence is presented to show that the electrophoretic heterogeneity, anodic mobility and solubility of five lysosomal enzymes, acid phosphatase, β-N-acetylhexosaminidase, β-galactosidase, β-glucuronidase and arylsulphatase, increase as they migrate through the Golgi apparatus, apparently as a consequence of glycosylation reactions involving the attachment of N-acetylneuraminic acid residues.

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