

Physicochemical Modifications of Lysosomal Hydrolases during Intracellular Transport

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1. The following fractions were prepared from rat kidney and characterized ultra-structurally, biochemically and enzymically: (a) an ordinary rough microsomal (RM₁) fraction; (b) a special rough microsomal (RM₂) fraction enriched seven- to nine-fold in acid hydrolases over the homogenate; (c) a smooth microsomal (SM) fraction; (d) a Golgi (GM) fraction enriched 2.5-fold in acid hydrolases and 10-, 15- and 20-fold in sialyltransferase, *N*-acetyl-lactosamine synthetase and galactosyltransferase respectively; (e) a lysosomal (L) fraction enriched 15- to 23-fold in acid hydrolases. The frequency of Golgi sacs and tubules seen in the electron microscope and the specific activity of the three glycosyltransferases in these fractions increased in the order: RM₂ < RM₁ < SM < GM. 2. Five lysosomal hydrolases, acid phosphatase, β -*N*-acetylhexosaminidase, β -galactosidase, β -glucuronidase and arylsulphatase, were characterized in these fractions with respect to (a) solubility on freeze-thawing and (b) electrophoretic mobility in polyacrylamide gels. 3. In the RM₂ fraction each of these hydrolases occurred largely or exclusively as a single bound basic form coincident with cationic glycoprotein bands in gels (Goldstone *et al.*, 1973). 4. In the L fraction these hydrolases were present largely as soluble, acidic (anionic) forms. 5. The solubility, electrophoretic heterogeneity and anodic mobility of these hydrolases increased progressively in sub-cellular fractions in the order: RM₂ < RM₁ < SM < GM < L. 6. These findings, together with evidence cited in the text showing that *N*-acetylneuraminic acid residues are responsible for the solubility and electronegative charge of these acidic forms and incorporation of these residues into the Golgi apparatus, support the following scheme for the biosynthesis of lysosomal enzymes. Each hydrolase is synthesized as a bound basic glycoprotein enzyme in a restricted portion of the rough endoplasmic reticulum. The soluble, acidic forms are generated as the nascent glycoprotein enzymes migrate through the Golgi apparatus through the attachment of sugar sequences containing *N*-acetylneuraminic acid.

The lysosomal acid hydrolases occur in multiple molecular forms which differ in electrophoretic mobility, solubility, thermal stability and other physicochemical properties (Barrett, 1969; Tappel, 1969). The biochemical basis of this molecular heterogeneity and its biological significance are obscure. These forms may exhibit divergent substrate specificities and hydrolytic activities *in vivo* as suggested by the finding of an enzymic deficiency in two genetic sphingolipidoses which is restricted to the acidic (A) form of a lysosomal hydrolase, namely, β -*N*-acetylhexosaminidase A in Tay-Sachs disease (Sandhoff, 1969; Okada & O'Brien, 1969) and arylsulphatase A in metachromatic leucodystrophy (Austin *et al.*, 1965; Mehl & Jatzkewitz, 1965). The various acid hydrolases in purified lysosomal fractions from rat kidney and liver tissue appear to be glycoproteins that contain *N*-acetylglucosamine, mannose, glucose and *N*-acetylneuraminic acid (Goldstone &

Koenig, 1970). A number of these hydrolases in rat kidney lysosomes migrate as two to five distinct forms on polyacrylamide-gel electrophoresis (Goldstone & Koenig, 1970; Goldstone *et al.*, 1971a). The acidic forms of these hydrolases are readily solubilized by physical disruption, whereas the basic forms are more firmly bound to the insoluble lysosomal residue (Goldstone *et al.*, 1971a).

Neuraminidase treatment markedly retards the anodic mobility of the acidic forms of two putative lysosomal enzymes in human tissues, prostatic acid phosphatase (Smith & Whitby, 1968) and splenic β -*N*-acetylhexosaminidase (Robinson & Stirling, 1968). Neuraminidase also raises the isoelectric point of human prostatic acid phosphatase I (Dziembor *et al.*, 1970), a glycoprotein enzyme with an isoelectric point of 4.9 which contains 6 residues of *N*-acetylneuraminic acid/molecule (Derechin *et al.*, 1971). The acidic (anionic) forms of seven hydrolases in rat

kidney lysosomes, acid phosphatase, β -*N*-acetylhexosaminidase, β -galactosidase, β -glucuronidase, arylsulphatase, acid ribonuclease and acid deoxyribonuclease, are converted partly or wholly into more basic (cationic) forms by incubation with neuraminidase (Goldstone *et al.*, 1971a). Neuraminidase treatment also raises the isoelectric points of the acid forms of these hydrolases, as measured by the technique of isoelectric focusing (S. Needleman & H. Koenig, unpublished work), and renders a variable portion of these soluble enzymes sedimentable when mixed with particulate material (A. Goldstone & H. Koenig, unpublished work). These findings indicate that the electronegative charge and solubility of the acidic forms of these various lysosomal hydrolases are due to the presence of *N*-acetylneuraminic acid residues and provide further evidence that they are glycoprotein enzymes. Kinetic studies of isotope uptake in rat kidney indicate that sugar residues are incorporated into nascent lysosomal glycoprotein in the rough endoplasmic reticulum and in the Golgi apparatus just before packaging of labelled glycoproteins in lysosomes (Goldstone *et al.*, 1971b; Goldstone & Koenig, 1972; Nayyar & Koenig, 1972). Therefore it would be expected that the nascent lysosomal hydrolases would acquire negative charge and augmented solubility in the Golgi apparatus as a consequence of the attachment of *N*-acetylneuraminic acid residues in this site.

This proposition has now been tested by investigating the solubility and electrophoretic mobility of five lysosomal hydrolases in rat kidney subcellular fractions enriched in structural elements that participate in the synthesis, transport and storage of these enzymes. This investigation was facilitated by the recent discovery that a special population of rough microsomal material (microsomes) could be separated from the bulk of rough microsomes in rat kidney as a discrete fraction which is enriched 10- to 30-fold in lysosomal hydrolases over an ordinary rough microsomal fraction (Goldstone *et al.*, 1973). These special rough microsomes are the site of synthesis of lysosomal glycoproteins (Goldstone *et al.*, 1971b; Goldstone & Koenig, 1972). In addition, a lysosomal, standard rough microsomal, smooth microsomal and Golgi membrane fraction, the latter not previously isolated from kidney tissue, were prepared and characterized by ultrastructural, biochemical and enzymic criteria. The results show that the solubility and electrophoretic mobility of acid phosphatase, β -galactosidase, β -*N*-acetylhexosaminidase, β -glucuronidase and arylsulphatase vary in an orderly manner according to their subcellular location in rat kidney. Each of these hydrolases is evidently synthesized in the rough endoplasmic reticulum as a single bound basic glycoprotein enzyme. The soluble, anionic forms of these hydrolases are generated mainly in fractions containing Golgi membranes. A

brief report of a portion of this work has appeared in abstract form (Goldstone & Koenig, 1971).

Materials and Methods

Fractionation of tissues

Young adult Sprague-Dawley rats (Holtzman Co., Madison, Wis., U.S.A.) weighing about 200g were killed by cervical dislocation. Kidneys were removed and homogenized in 0.3M-sucrose (4ml per g) with a Potter-Elvehjem-type homogenizer. All operations were at 4°C. A heavy lysosomal (L), standard rough microsomal (RM₁) and special rough microsomal (RM₂) fraction were prepared according to Goldstone *et al.* (1973). A smooth microsomal (SM) and a Golgi membrane (GM) fraction were prepared according to the method devised for rat liver by Leelavathi *et al.* (1970). Protein, phospholipid and RNA were determined in fractions as described by Goldstone *et al.* (1973).

Enzyme assays

The following total acid hydrolase activities were measured in fractions as described by Goldstone *et al.* (1973): acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2); β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23); β -*N*-acetylglucosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucosylhydrolase, EC 3.2.1.30); β -glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31); arylsulphatase (aryl sulphate sulphohydrolase, EC 3.1.6.1). The specific activity of acid phosphatase, β -glucuronidase and arylsulphatase is expressed as μ mol, and of β -galactosidase and β -*N*-acetylhexosaminidase as nmol \times 10, of substrate hydrolysed/h per mg of protein.

N-Acetyl-lactosamine synthetase (UDP-galactose-*N*-acetylglucosamine galactosyltransferase), a marker enzyme for Golgi membranes (Morré *et al.*, 1969; Fleischer *et al.*, 1969; Leelavathi *et al.*, 1970), was assayed by the method of Morré *et al.* (1969). The reaction mixture contained the enzyme sample, 0.02ml of Tris-HCl buffer, pH 7.5, 0.01ml of 0.10M-MgCl₂, 0.01ml of 0.05M-MnCl₂, 0.05ml of 0.1M-mercaptoethanol, 0.05ml of 0.01M-*N*-acetyl- β -D-glucosamine, 0.1 μ Ci of UDP-[¹⁴C]galactose and 0.05ml of 5% Triton X-100 in a final volume of 0.5ml. Control tubes contained all of these ingredients except *N*-acetylglucosamine. After incubating at 37°C for 10min the reaction was stopped by pouring incubation mixtures through anion-exchange columns containing about 1ml of packed Bio-Gel AG₁-X₂ resin (Cl⁻ form) (Bio-Rad, Richmond, Calif., U.S.A.). *N*-Acetyl-lactosamine and galactose were eluted from columns with 3 \times 0.4ml

washes of water. Washes were placed on stainless-steel planchets, dried and radioassayed in an automatic gas-flow counter (Nuclear-Chicago model 183B). The radioactivity of the control reaction mixture was subtracted to correct for non-specific hydrolysis of UDP-[¹⁴C]galactose. Specific enzyme activity was expressed as μmol of [¹⁴C]galactose transferred to *N*-acetyl-lactosamine/h per mg of protein.

Galactosyltransferase (UDP-galactose-endogenous glycoprotein acceptor galactosyltransferase), also a reference enzyme for Golgi membranes (Wagner & Cynkin, 1971), was assayed in fractions without exogenous acceptor protein by the method of Fleischer *et al.* (1969). The reaction mixture, containing 0.25 ml of 0.2M-Tris-HCl buffer, pH 6.85, 0.1 ml of 0.1M-MnCl₂, 0.1 ml of 0.5M-mercaptoethanol, 0.1 μCi of UDP-[¹⁴C]galactose and enzyme sample in a total volume of 1 ml, was incubated at 37°C for 1 h. The reaction was terminated by adding 0.5 ml of 30% (w/v) trichloroacetic acid. A control reaction to which the same amount of trichloroacetic acid had been added at zero time was similarly incubated. Acid-insoluble material was deposited by centrifugation, the pellets were washed twice with cold 10% (w/v) trichloroacetic acid, dissolved in 0.5 ml of 0.5M-NaOH and radioassayed in a gas-flow counter as described above. The acid-insoluble radioactivity of the control incubation mixture was subtracted from that of the enzyme reaction mixture. Specific enzyme activity was expressed as μmol of [¹⁴C]galactose transferred to acid-insoluble protein/h per mg of protein.

Sialyltransferase (CMP-*N*-acetylneuraminic acid-glycoprotein sialyltransferase), a Golgi enzyme marker in rat liver (Schachter *et al.*, 1970; Wagner & Cynkin, 1971), was assayed with a neuraminidase-treated α_1 -acid glycoprotein as described by Schachter *et al.* (1970). α_1 -Acid glycoprotein (50 mg) was incubated with 2 mg of *Clostridium perfringens* neuraminidase in 0.1M-sodium acetate buffer, pH 5.2, at 37°C for 3 h, heated for 3 min at 100°C to stop the reaction and dialysed overnight against water at 4°C. The reaction mixture, containing 0.5 ml of 0.1M-sodium acetate buffer, pH 5.7, 1 mg of neuraminidase-treated α_1 -acid glycoprotein, 0.1 ml of 0.05 mM-CMP-*N*-acetyl[¹⁴C]neuraminic acid, 0.1 ml of 5% Triton X-100 and enzyme sample in a total volume of 1.0 ml, was incubated at 37°C for 2 h along with a control reaction mixture without the α_1 -acid glycoprotein acceptor. The reaction was stopped by adding 0.5 ml of 30% (w/v) trichloroacetic acid, the precipitate centrifuged at 5000g for 15 min and the pellet washed twice with cold 10% trichloroacetic acid. Pellets were dissolved in 0.5 ml of 0.5M-NaOH and samples plated on stainless-steel planchets for counting in a gas-flow counter. The radioactivity of the control was subtracted from that of the complete

reaction mixture. Specific enzyme activity was expressed as μmol of *N*-acetyl[¹⁴C]neuraminic acid transferred to acid-insoluble protein/h per mg of protein.

Electron microscopy

Fractions were processed for electron microscopy according to Goldstone *et al.* (1973). For negative staining, samples of glutaraldehyde-fixed fractions were taken on grids, dried and stained with 2% phosphotungstic acid, pH 6.8. Sections and negatively stained preparations were examined and photographed in an RCA EMU3F electron microscope.

Solubilization of enzymes

Fractions from about 10 g of kidney tissue were suspended in 40 ml of 0.1M-sodium acetate buffer, pH 5.2, and soluble enzymes were released into solution with 10 cycles of rapid freezing and thawing. Insoluble material was removed by ultracentrifugation at 100000g for 60 min. Bound enzymes were extracted from the residue by suspending the latter in 5 ml of 0.2% Triton X-100 in 0.1M-sodium acetate buffer, pH 5.2. The suspension was sonicated for 2 min at 4°C in a Branson model W185D Sonifier (in four 30 s periods separated by 15 s intervals to avoid overheating) and centrifuged at 100000g for 60 min to remove the residue. Protein and acid hydrolase activities were assayed in each fraction.

Polyacrylamide-gel electrophoresis

Enzymes were extracted from pelleted fractions by sonication in 2 ml of 0.2% Triton X-100 containing 0.1M-sodium acetate buffer, pH 5.2, as described above. Because of the extreme lability of the acidic forms of the lysosomal enzymes even at -20°C (Patel & Koenig, 1972) soluble extracts were subjected to electrophoresis directly after preparation. All procedures were done at 4°C. Electrophoresis of samples containing about 0.1-0.3 mg of protein was done in 5% (w/v) polyacrylamide gels at pH 8.8 or 4.4 for about 45 min at 4 mA/tube (Davis, 1964). Gels were fixed in 2% formaldehyde, pH 5.0, at 4°C for 15 min, washed in cold 0.2M-sodium acetate buffer, pH 5.0, and incubated for enzyme activities as described previously (Goldstone & Koenig, 1970; Goldstone *et al.*, 1971a). Gels were incubated in appropriate substrates at 37°C for 0.5-1 h to demonstrate the following acid hydrolase activities: acid phosphatase (α -naphthol phosphate as substrate) (Barka & Anderson, 1962); β -galactosidase (4-methylumbelliferyl β -D-galactopyranoside as substrate) (Robinson *et al.*, 1967); β -*N*-acetylhexosaminidase (4-methylumbelliferyl β -D-glucosaminide as substrate) (Robinson *et al.*, 1967); β -glucuronidase

with the β -glucuronide of naphthol AS-BI (7-bromo-3-hydroxy-2-naphth-*O*-anisidide) as substrate (Hayashi, 1964); arylsulphatase (*p*-nitrocatechol sulphate as substrate) (Roy, 1958). Enzyme reaction product was measured by densitometry in gels at appropriate wavelengths with a Gilford 2410 linear-transport and a Gilford 2000 absorbance recorder. The fluorescent bands of 4-methylumbelliferone released by the action of the glycosidases were made visible in gels under near-u.v. illumination and recorded photographically. The fluorescence was intensified with NH_3 (Robinson *et al.*, 1967).

Chemicals

UDP-[^{14}C]galactose (specific radioactivity 28.5 mCi/mmol) and CMP-*N*-acetyl[^{14}C]neuraminic acid (specific radioactivity 196 mCi/mmol) were obtained from New England Nuclear Co., Boston, Mass., U.S.A. Sodium β -glycerophosphate, phenolphthalein glucuronic acid, *p*-nitrocatechol sulphate (dipotassium salt), α -naphthyl phosphate (sodium salt), naphthol AS-BI β -D-glucuronic acid and neuraminidase (from *Clostridium perfringens*, type VI, specific activity 1.1 units/mg) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 4-Methylumbelliferyl β -D-galactopyranoside and β -*N*-acetyl- β -D-glucosaminide were bought from Pierce Biochemical Co., Rockford, Ill., U.S.A. Triton X-100 was obtained from Rohm and Haas, Philadelphia, Pa., U.S.A. α_1 -Acid glycoprotein was purchased from Calbiochem, San Diego, Calif., U.S.A. Reagents for polyacrylamide-gel electrophoresis were supplied by Canalco Co., Rockville, Md., U.S.A. Other chemicals were of the best obtainable analytical grade.

Results

Ultrastructure of fractions

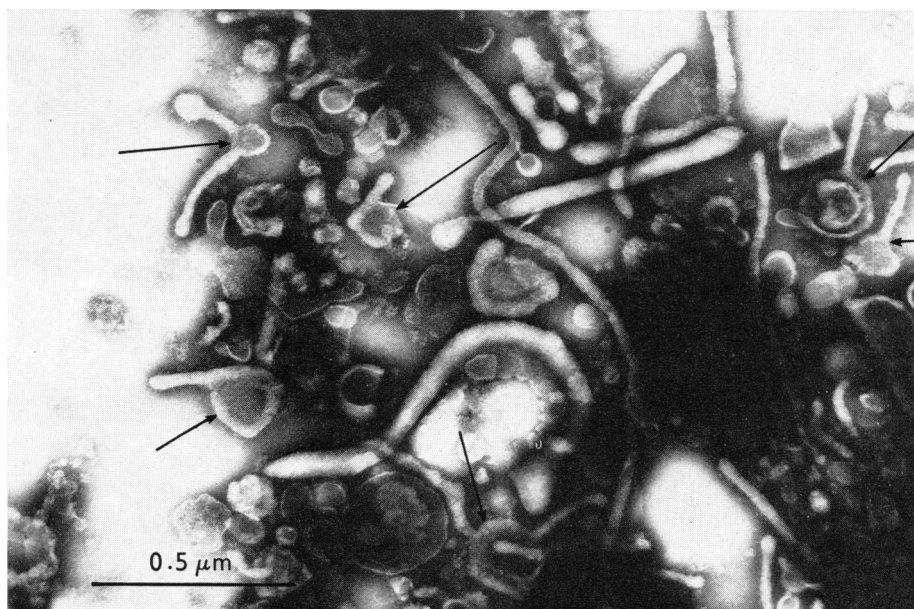
The GM fraction contained numerous recognizable Golgi sacs and tubules. In negatively stained preparations, isolated tubules (some with bulging ends) and flattened sacs were the predominant elements (Plate 1). Tubules frequently formed simple anastomotic arrays. Intact Golgi cisternae consisting of a central plate-like sac continuous with a simple array of peripheral anastomotic tubules were also present. These Golgi cisternae differed from their counterpart in liver (Morré *et al.*, 1970) in that they were considerably less intricate and without associated peripheral secretory vesicles. It is not clear whether the relative simplicity of the Golgi cisternae isolated from rat kidney reflects their real structure in this tissue or is a consequence of partial fragmentation of more elaborate Golgi cisternae during the isolation procedure. In stained sections, the GM fraction consisted predominantly of smooth membranous sacs and tubules (Plate 2). Occasional

dictyosomes or stacks of Golgi cisternae were seen in cross section. These appeared as parallel arrays of flattened cisternae, some with bulging ends, surrounded by small vesicular profiles representing transverse sections of the anastomotic tubular network. In sections cut tangentially to the surface of the dictyosomes, simple plate-like arrays of anastomotic tubules were continuous with a central or eccentric sac. Many tubular and saccular profiles considered to be of Golgi origin appeared to be isolated and without obvious relationship to each other. The cisternal content of these tubules and saccules varied considerably in degree and character, but generally it tended to be sparse, amorphous and of moderate electron density. Microvilli, mitochondria, autophagic vacuoles, atypical dense bodies and other structures were occasionally seen in this fraction. Except for the differences already noted, the membranous components in the GM fraction generally resembled those described in Golgi fractions from ox and rat liver (Fleischer *et al.*, 1969; Morré *et al.*, 1970; Schachter *et al.*, 1970; Wagner & Cynkin, 1971).

The SM fraction contained many smaller smooth vesicles. Simple arrays of anastomotic tubules continuous with a plate-like sac similar to those in the GM fraction were fairly common. Some rough and transitional microsomes, free ribosomes, microvilli and infrequent mitochondria and dense bodies also were present. The RM_1 fraction consisted largely of rounded, ribosome-studded sacs with an amorphous content of moderate electron density, together with numerous free ribosomes, some smooth vesicles, microvilli and glycogen granules and infrequent small mitochondria and dense bodies. Occasional anastomosing tubules and sacs resembling those in the GM and SM fraction were also present. The cisternal content of these tubular elements tended to be more electron-dense than that of tubules in the GM fraction. The RM_2 fraction contained a distinctive kind of rough-surfaced microsome, as described elsewhere (Goldstone *et al.*, 1973). These special rough microsomes, comprising about one-half of the RM_2 fraction, presented ovoid or elongate, rather than spherical, profiles and were filled with an osmiophilic electron-dense content. Some smooth vesicles, small mitochondria, dense bodies, free ribosomes and glycogen particles also were present together with infrequent anastomosing tubules and sacs of the Golgi system. The relative numbers of Golgi elements in these fractions, as judged from their frequency in electron micrographs, increased in the order: $\text{RM}_2 < \text{RM}_1 < \text{SM} < \text{GM}$.

Chemical composition of fractions

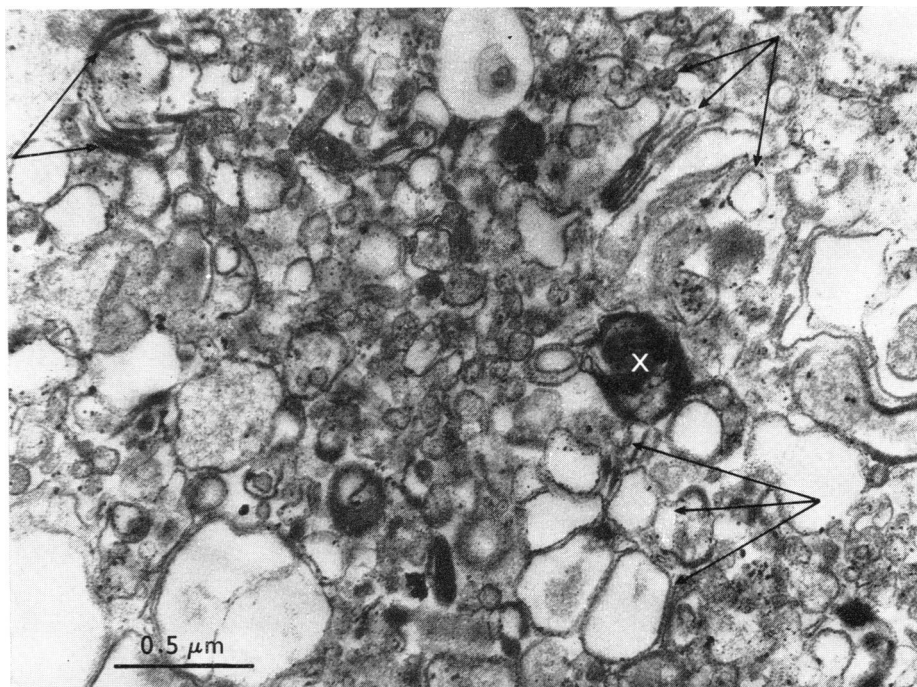
The protein, RNA and phospholipid contents of rat kidney subcellular fractions are given in Table 1.



EXPLANATION OF PLATE I

Electron micrograph of the GM fraction

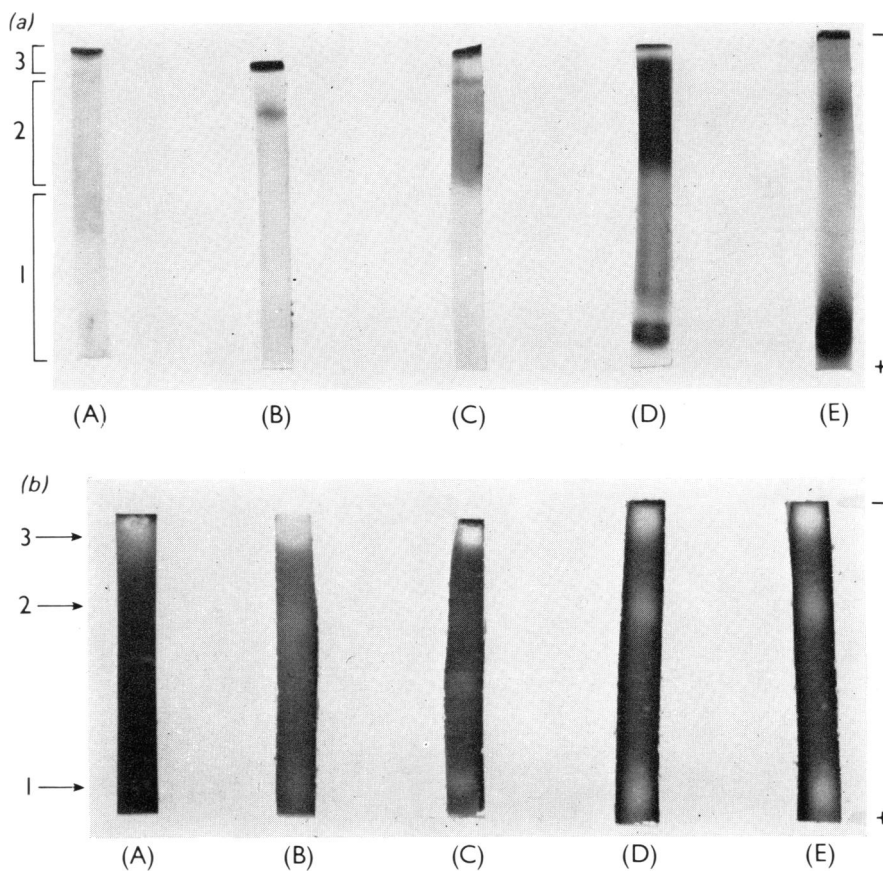
Isolated sacs and tubules are the predominant elements. Some simple cisternae consisting of flattened sacs and one to several attached tubules (→) are also present. The preparation was negatively stained with 2% phosphotungstic acid, pH 6.8 (see the text). Magnification $\times 52800$.



EXPLANATION OF PLATE 2

Electron micrograph of the GM fraction

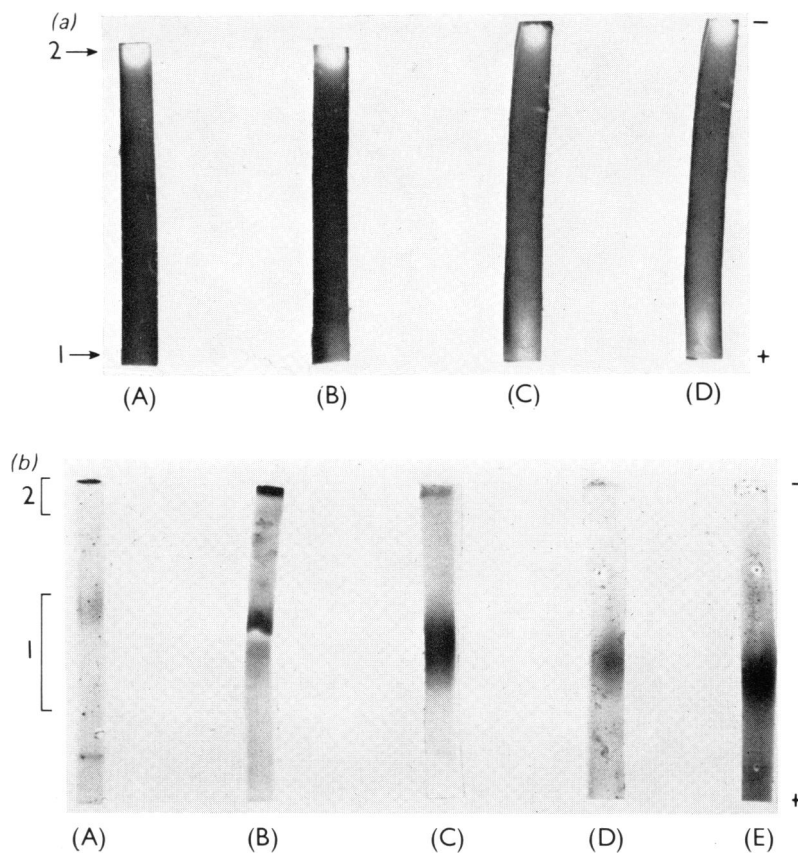
Saccular and tubular profiles predominate in this field. Some stacks of Golgi cisternae ('dictyosomes') (→) are seen in cross-section. Several electron-dense structures (X), possibly of a lysosomal nature, are present. The uranyl acetate-lead citrate stain was used. Magnification $\times 36480$.



EXPLANATION OF PLATE 3

Gel electrophoretograms of 0.2% Triton X-100 extracts of subcellular fractions from rat kidney stained for (a) acid phosphatase and (b) β -galactosidase activity

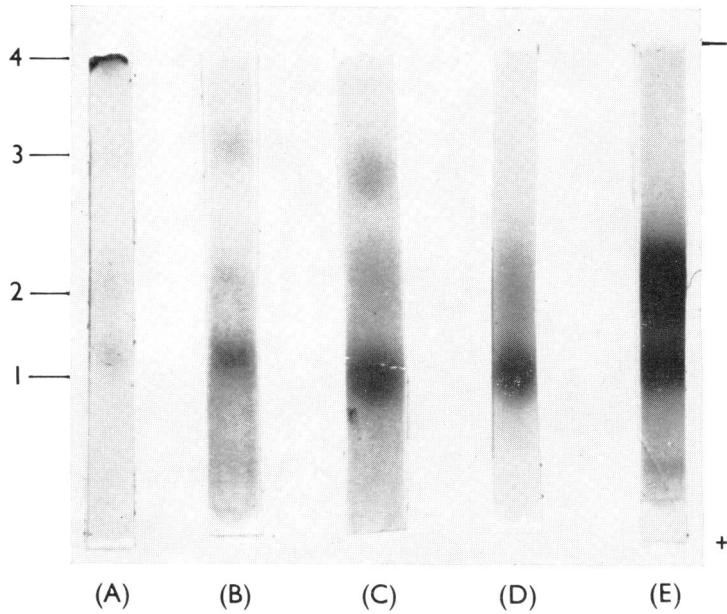
For details see the text. Migration was towards the anode (+). (A) RM_2 fraction. (B) RM_1 fraction. (C) SM fraction. (D) GM fraction. (E) L fraction. In (b) the gels were photographed in near-u.v. light to show the fluorescent enzyme reaction product.



EXPLANATION OF PLATE 4

Gel electrophoretogram of 0.2% Triton X-100 extracts stained for (a) β -N-acetylhexosaminidase and (b) β -glucuronidase activity

For details see the text. Migration was towards the anode (+). For (a): (A) RM₂ fraction. (B) SM fraction. (C) GM fraction. (D) L fraction. The electrophoretogram of the RM₁ fraction, not shown here, was identical with that of the RM₂ fraction. The gels were photographed in near-u.v. light. For (b): (A) RM₂ fraction. (B) RM₁ fraction. (C) SM fraction. (D) GM fraction. (E) L fraction.



EXPLANATION OF PLATE 5

Gel electrophoretograms of 0.2% Triton X-100 extracts stained for arylsulphatase activity

For details see the text. Migration was towards the anode (+). (A) RM₂ fraction. (B) RM₁ fraction. (C) SM fraction. (D) GM fraction. (E) L fraction.

Table 1. *Protein, RNA and phospholipid content of rat kidney subcellular fractions*

Subcellular fractions were prepared and analysed for protein, RNA and phospholipid as described in the text. The values for mouse kidney are from Kato *et al.* (1972). The values for rat liver RNA and phospholipid are from Leelavathi *et al.* (1970) and Dallner (1963) respectively.

Tissue and fraction	Protein (mg/g of tissue)	RNA (mg/mg of protein)	Phospholipid (mg/mg of protein)
Rat kidney			
Whole homogenate	47.00		
RM ₂	0.08	0.192	0.44
RM ₁	3.40	0.293	0.44
SM	0.55	0.057	0.31
GM	0.32	0.032	0.21
L	0.70	0.007	0.105
Mouse kidney			
Rough microsomal		0.164	0.287
Smooth microsomal		0.022	0.420
Rat liver			
Rough microsomal		0.304	0.16
Smooth microsomal		0.045	0.37
Golgi		0.034	

The RM₂ fraction represented about 2% of the protein in the RM₁ fraction and had the same phospholipid content but a slightly lower RNA content than the latter. The RNA content of the other fractions declined sharply in the direction of the SM₁, GM and L fractions, whereas the phospholipid content declined more gradually in the same direction. It can be seen in Table 1 that the chemical composition of the RM₁, SM and GM fractions from rat kidney approximated that of comparable fractions from mouse kidney (Kato *et al.*, 1972) and rat liver (Dallner, 1963; Leelavathi *et al.*, 1970).

Acid hydrolases in fractions

The distribution and specific activities of the lysosomal acid hydrolases in these subcellular fractions are shown in Table 2. All five lysosomal marker enzymes displayed a similar subcellular distribution. A substantial portion of the acid hydrolase activities (20–29% of the total) was recovered in the L fraction, whereas the other fractions altogether contained only 8–10% of the total in the homogenate. The RM₂ fraction was enriched 11- to 24-fold in acid hydrolases over the RM₁ fraction, as already noted (Goldstone *et al.*, 1973). The relative specific hydrolase activities (homogenate = 1) of the RM₂ fraction were 7–9, compared with 15–19 for the L fraction, and 0.4–0.6 for the RM₁ fraction. It is noteworthy that the specific hydrolase activities of the SM and GM fractions were enriched about twofold and fourfold respectively over the RM₁ fraction.

Glycosyl transferases in fractions

The Golgi marker enzymes, *N*-acetyl-lactosamine synthetase, galactosyltransferase and sialyltransferase, were distributed in a more-or-less parallel manner in rat kidney subcellular fractions (Table 3). The GM fraction contained the greatest portion of these transferases (7–13% of the total activity), but the SM and RM₁ fractions also contained substantial activities (3% and 8–11% of the total homogenate respectively). The relative specific activities (homogenate = 1) of *N*-acetyl-lactosamine synthetase and galactosyltransferase in the GM fraction were 16–19, compared with 2.5–3.0 in the SM fraction, 1.5 in the RM₁ fraction and 0.5 in the RM₂ fraction. The relative specific activity of sialyltransferase in these fractions was 10.1, 2.0, 1.1 and 0.7 respectively. Thus the concentration of Golgi membranes in these fractions, as judged by the specific glycosyltransferase activities of fractions, increased in the order: RM₂ < RM₁ < SM < GM. These findings are in fair agreement with the relative number of Golgi structures found in these fractions by electron microscopy.

Solubility of acid hydrolases in fractions

The solubility of acid phosphatase, β -galactosidase, β -*N*-acetylhexosaminidase, β -glucuronidase and arylsulphatase was determined in the various subcellular fractions. This was accomplished by measuring the percentage of the enzyme activity released into solution by freeze-thawing fractions

Table 2. Acid hydrolase activities of rat kidney subcellular fractions

Subcellular fractions were prepared and protein and total acid hydrolase activities were determined as described in the text. The specific activity of acid phosphatase, β -glucuronidase and arylsulphatase is expressed as μ mol and of β -galactosidase and β -N-acetylhexosamine as nmol \times 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.

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	100.0	12.4	1.0	100.0	6.4	1.0	100.0	5.7	1.0	100.0	10.0	1.0	100.0	1.5	1.0
RM ₂	1.2	83.7	6.7	1.4	48.0	7.5	1.2	40.5	7.0	1.1	94.0	9.4	1.1	13.0	8.7
RM ₁	4.6	7.6	0.6	5.0	4.1	0.6	3.7	3.1	0.5	5.0	8.0	0.8	4.4	0.9	0.8
SM	1.7	17.9	1.4	1.7	9.1	1.4	1.2	3.9	1.0	1.2	18.0	1.8	0.9	1.2	0.6
GM	1.7	30.5	2.5	1.7	15.4	2.4	1.3	11.5	2.0	1.5	36.0	3.6	1.3	3.2	2.1
L	28.2	230.0	18.5	28.6	120.0	18.8	22.2	87.0	15.3	20.0	160.0	16.0	21.0	23.5	15.7

suspended in 0.1M-sodium acetate buffer, pH5.2. The results are presented in Tables 4 and 5. The solubility of all five hydrolases was least in the RM₂ fraction and increased in the direction of the RM₂, SM, GM and L fractions. In the RM₂ fraction only 5–10% of the hydrolases was soluble, whereas 66–84% of the hydrolases in the L fraction was soluble. The solubility of the protein paralleled that of the hydrolases in the RM₂ and L fractions, but this was less evident in the SM and GM fractions where the lysosomal hydrolases were only minor constituents.

A major portion of the bound, i.e. sedimentable, hydrolases was extracted by sonicating the pelleted residues from freeze-thawing in 0.2% Triton X-100 buffered to pH5.2. However, a significant portion of the hydrolases, 9–30% of the total, was still sedimentable. The latter activities were not extracted by raising the concentration of Triton X-100 to 5%, but could be quantitatively solubilized by sonicating pellets in alkaline buffers, pH8.5–10.0 (A. Goldstone & H. Koenig, unpublished work). On polyacrylamide-gel electrophoresis, the soluble hydrolases in rat kidney (Goldstone *et al.*, 1971a; A. Goldstone & H. Koenig, unpublished work) and brain (Patel & Koenig, 1972) are primarily anionic isoenzymes, whereas the bound hydrolases are mainly cationic isoenzymes. These observations suggest that electrostatic, as well as hydrophobic, forces are involved in the binding of the lysosomal hydrolases to cell particulates.

Electrophoretic mobility of acid hydrolases in fractions

The electrophoretic heterogeneity and mobility of these hydrolases were investigated by subjecting 0.2% Triton X-100 extracts of subcellular fractions to electrophoresis in 5% polyacrylamide gels. Bands of acid phosphatase, β -galactosidase, β -N-acetylhexosaminidase, β -glucuronidase and arylsulphatase activities were satisfactorily demonstrated by incubating gels in appropriate chromogenic or fluorogenic substrates. The distribution of coloured enzyme product in gels was quantitatively determined by recording densitometry. The isoenzyme patterns of these hydrolases varied markedly according to their subcellular location.

The results for acid phosphatase are shown in Plate 3(a) and Fig. 1. In gel electrophoretograms of the RM₂ fraction, acid phosphatase activity was confined to a single cathodic band in zone 3. In the L fraction, 75% of the total acid phosphatase activity in the gel occurred in two overlapping fast-moving anionic bands in zone 1, 18% in the cationic band in zone 3, and 7% in one or two slow-moving anionic bands in zone 2. The intervening fractions disclosed intermediate isoenzyme patterns, the cationic component diminishing and the anionic components increasing progressively in the direction of the RM₁,

Table 3. *Sugar transferase activities of rat kidney subcellular fraction*

Subcellular fractions were prepared and protein, *N*-acetyl-lactosamine synthetase, galactosyltransferase and sialyltransferase activities determined as described in the text. The specific activity of *N*-acetyl-lactosamine synthetase and galactosyltransferase is expressed as nmol of [¹⁴C]galactose transferred from UDP-[¹⁴C]-galactose to *N*-acetyl-lactosamine and acid-insoluble protein respectively/h per mg of protein. The specific activity of sialyltransferase is expressed as nmol of *N*-acetyl[¹⁴C]neuraminic acid transferred from CMP-*N*-acetyl[¹⁴C]neuraminic acid to α_1 -acid glycoprotein/h per mg of protein.

Fraction	<i>N</i> -Acetyl-lactosamine synthetase			Galactosyltransferase			Sialyltransferase		
	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity	(% of total)	Specific activity	Relative specific activity
Homogenate	100.0	92.3	1.0	100.0	3.30	1.0	100.0	0.91	1.0
RM ₂	0.1	44.5	0.5	0.1	1.56	0.5	0.1	0.59	0.7
RM ₁	8.8	111.0	1.2	10.6	4.50	1.5	8.0	0.96	1.1
SM	3.2	249.1	2.7	3.3	9.10	3.0	2.6	1.88	2.1
GM	10.8	1460.0	15.8	13.1	58.20	19.1	7.4	9.12	10.1
L	0.6	35.0	0.4	0.5	1.62	0.5	0.8	0.48	0.5

SM and GM fractions. In the RM₁ fraction the bulk of the enzyme activity, 83% of the total, occurred in the cationic form in zone 3 with a minor slow-moving anionic component in zone 2. In the SM fraction the cationic form declined to 23% of the total activity, 74% appearing as a broad slow-moving anionic band in zone 2, and 3% as a fast-moving anionic band in zone 1. In the GM fraction the cationic band comprised only 8% of the total activity; 66% of the activity appeared in zone 2 as a wide band of heavy activity attributable to multiple overlapping forms, whereas three fast-moving anionic bands totalling 26% of the activity were present in zone 1.

β -Galactosidase and β -*N*-acetylhexosaminidase activities were assessed qualitatively by visual inspection and photography in near-u.v. light as the fluorescent reaction product, 4-methylumbelliferone, diffuses rapidly in polyacrylamide gels. In gel electrophoretograms of the RM₂ fraction, β -galactosidase was confined to a single cationic band in zone 3, whereas those prepared from the L fraction revealed three bands of β -galactosidase activity, a heavy fast-moving anionic band in zone 1, a weak band in zone 2 and another weak band in zone 3 (Plate 3b). The intermediate fractions revealed transitional isoenzyme patterns, the fast-moving anionic form in zone 1 increasing progressively at the expense of the other two forms in the direction of the RM₁, SM and GM fractions.

β -*N*-Acetylhexosaminidase activity in gel electrophoretograms prepared from both the RM₂ and RM₁ fractions occurred as a single cationic band in zone 3 (Plate 4a). In the SM fraction a feeble fast-moving

anionic component appeared in zone 1 together with the major cationic band, whereas in the GM and L fractions the fast-moving anionic band equalled or surpassed the cationic component of β -*N*-acetylhexosaminidase activity in staining intensity.

The bulk of the β -glucuronidase activity, 84% of the total, in the RM₂ fraction was present as a single cationic band in zone 2, and 16% in a slow-moving component in zone 1 (Plate 4b and Fig. 2). In the RM₁ fraction, 75% of the activity occurred in the cationic band, and 25% in the anionic band. In the SM fraction, the cationic component declined to 14% of the total, 86% appearing in a somewhat faster-moving anionic band in zone 1. In the GM fraction the cationic component represented only 3% of the total β -glucuronidase activity, 97% appearing in the fast-moving anionic form in zone 1. In the L fraction, 10% of the activity was in the cationic band, and 90% in the fast-moving anionic band.

Four bands of arylsulphatase activity were detected in polyacrylamide-gel electrophoretograms of these subcellular fractions (Plate 5 and Fig. 3). In the RM₂ fraction, 59% of the arylsulphatase activity was present in the cathodic region in band 4, 22% in band 2, and 19% in band 1. In the RM₁ fraction, only 8% of the arylsulphatase activity remained in band 4, 34% appearing in band 3, 10% in band 2, and 48% in band 1. In the SM fraction, no enzyme activity occurred in band 4; 26% of the activity was present in band 3, 14% in band 2, and 60% in band 1. In the GM fraction, bands 4 and 3 were missing; 15% of the activity appeared in band 2, and 85% in band 1. In the L fraction, bands 4 and 3 also were lacking; 60% of the activity appeared in band 2, and 40% in band 1.

Table 4. Solubilization of protein and acid hydrolase activities in rat kidney subcellular fractions by freeze-thawing and Triton X-100

Fractions were suspended in 0.1M-sodium acetate buffer, pH 5.2, and freeze-thawed 10 times to release soluble protein and enzymes. The particulate material was sedimented, sonicated in 0.2% Triton X-100 and 0.1M-acetate buffer, pH 5.2, to release bound enzymes, and centrifuged to deposit the insoluble residue. Additional experimental details are given in the legend to Table 2 and the text. N.D., not determined.

Fraction	Acid phosphatase			β -Galactosidase			β -N-Acetylhexosaminidase			β -Glucuronidase			Arylsulphatase			
	Protein (% of total)	(% 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
RM₂																
Freeze-thaw extract	8.2	8.6	181	7.7	4.9	15.9	3.5	5.4	28.0	4.9	7.1	76.0	6.9	10.3	20.2	10.1
Triton X-100 extract	36.6	73.0	347	13.9	70.1	68.8	14.9	68.0	79.0	14.9	71.5	168.0	15.3	69.0	30.0	15.0
Residue (homogenate)	55.2	18.4	58	2.3	25.0	16.8	3.6	26.6	20.7	3.6	21.4	33.0	3.0	20.7	6.0	3.0
			(25)	(1.0)		(4.6)	(1.0)		(5.7)	(1.0)		(11.0)	(1.0)		(2.0)	(1.0)
SM																
Freeze-thaw extract	7.4	21.0	48.5	3.2	N.D.	N.D.		10.0	11.2	2.2	14.3	14.3	1.3	13.5	2.2	1.1
Triton X-100 extract	22.2	31.5	40.0	2.6				60.5	24.5	4.8	61.0	19.8	1.8	58.5	3.0	1.5
Residue (homogenate)	70.4	27.5	6.7	0.4				29.5	3.8	0.7	24.7	2.2	0.2	28.0	0.6	0.3
			(15.2)	(1.0)					(5.1)	(1.0)		(11.0)	(1.0)		(2.0)	(1.0)
GM																
Freeze-thaw extract	31.6	64.5	108.0	7.1				48.0	23.5	4.6	63.0	44.0	4.0	53.0	8.0	4.0
Triton X-100 extract	20.1	15.4	40.0	2.6				22.6	17.2	3.4	20.0	24.1	2.2	21.4	5.0	2.5
Residue (homogenate)	48.3	20.1	22.0	1.5				29.4	9.4	1.7	17.0	6.6	0.6	25.6	2.6	1.3
			(15.2)	(1.0)					(5.1)	(1.0)		(11.0)	(1.0)		(2.0)	(1.0)
L																
Freeze-thaw extract	53.5	79.5	333	18.4	77.0	65.0	16.2	66.0	88.0	18.4	78.8	183.0	20.3	84.1	29.9	18.7
Triton X-100 extract	14.0	6.3	95.4	5.3	7.2	24.0	6.0	6.0	31.4	6.5	4.0	55.0	6.1	5.3	7.1	4.4
Residue (homogenate)	32.5	14.2	78.5	4.3	15.8	22.0	5.5	28.0	60.0	12.5	17.2	57.0	6.3	10.6	5.4	3.4
			(18.1)	(1.0)		(4.0)	(1.0)		(4.8)	(1.0)		(9.0)	(1.0)		(1.6)	(1.0)

Table 5. Solubilization of protein and acid hydrolases in rat kidney subcellular fractions by freeze-thawing

Fractions in 0.1 M-sodium acetate buffer, pH 5.2, were freeze-thawed to release soluble acid hydrolases and centrifuged to deposit the bound enzymes and particles. Results are based on results given in Table 4. Protein and enzyme activities are expressed as % of the total recovered in the supernatant and sediment. N.D., not determined.

Fraction	Protein		Acid phosphatase		β-Galactosidase		β-N-Acetyl-hexosaminidase		β-Glucuronidase		Arylsulphatase	
	% in supernatant	% in sediment	% in supernatant	% in sediment	% in supernatant	% in sediment	% in supernatant	% in sediment	% in supernatant	% in sediment	% in supernatant	% in sediment
RM ₂	8.2	91.8	8.6	91.4	4.9	95.1	5.4	94.6	7.1	92.9	10.3	89.7
SM	7.4	92.6	21.0	79.0	N.D.	N.D.	10.0	90.0	14.3	85.7	13.3	86.7
GM	31.6	68.4	64.5	35.5	N.D.	N.D.	48.0	52.0	63.0	37.0	53.0	47.0
L	53.5	46.5	79.5	20.5	77.0	23.0	66.0	34.0	78.8	21.2	84.1	15.9

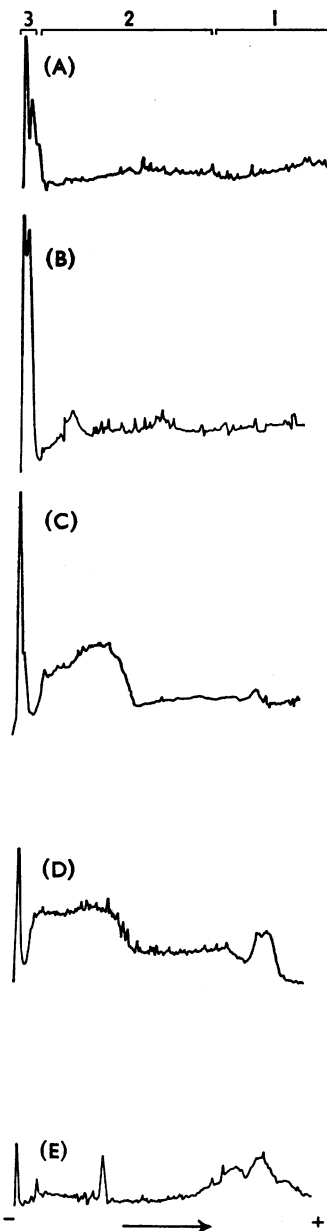


Fig. 1. Densitometric scans of gel electrophoretograms shown in Plate 3(a)

For details see the text and Plate 3(a). Migration was towards the anode (+).

When gels of the L fraction were incubated in the *p*-nitro catechol substrate modified to demonstrate arylsulphatase A activity (Percy & Brady, 1968), 50% of the activity appeared in band 1 and 50% in band 2. On

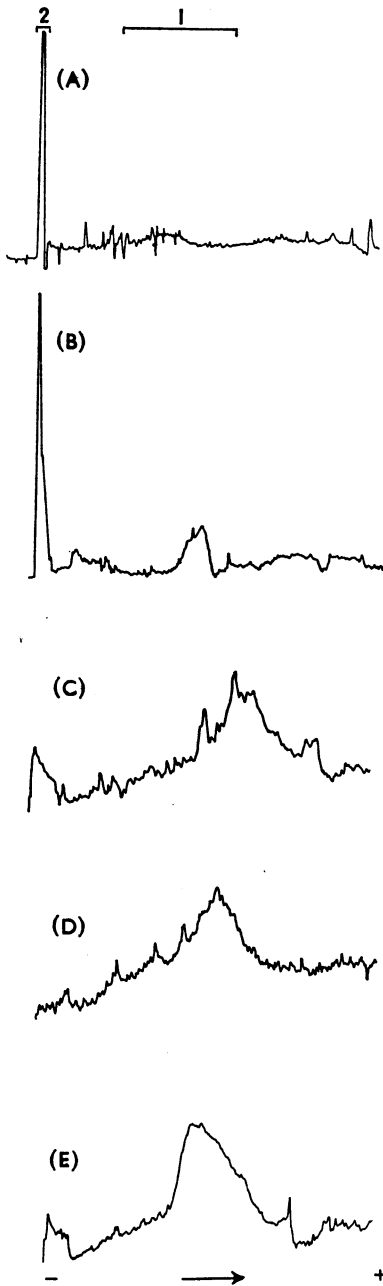


Fig. 2. Densitometric scans of gel electrophoretograms shown in Plate 4(b)

For details see the text and Plate 4(b). Migration was towards the anode (+).

incubation of gels in a *p*-nitrocatechol substrate designed to demonstrate arylsulphatase B activity (Baum *et al.*, 1959), 19% of the activity appeared in

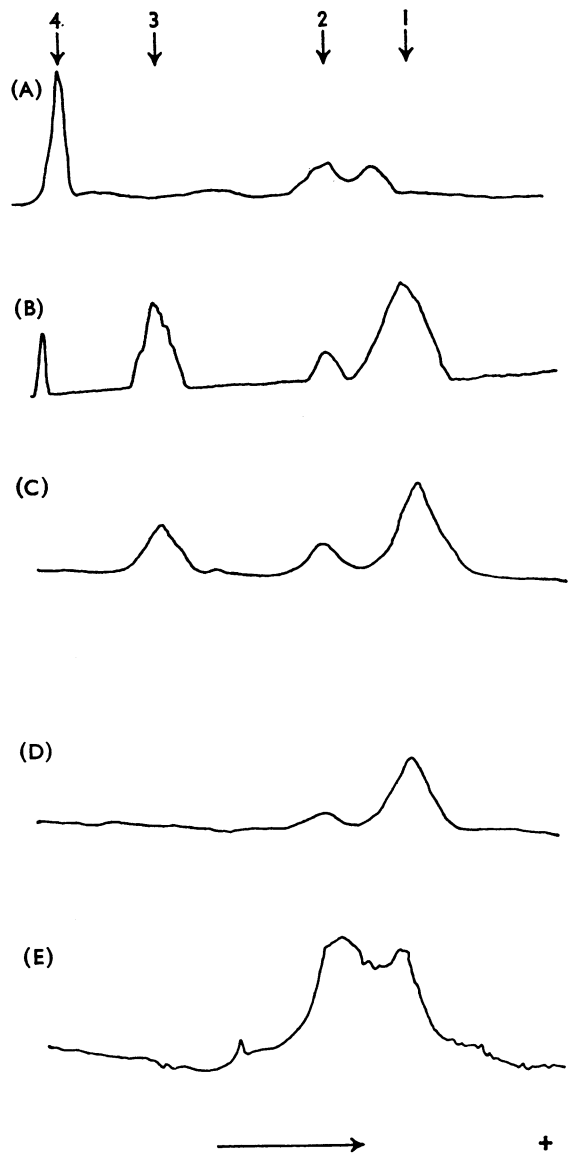


Fig. 3. Densitometric scans of gel electrophoretograms shown in Plate 5

For details see the text and Plate 5. Migration was towards the anode (+).

band 1, and 81% in band 2. Incubation of gels of the RM₁ fraction in this sequence of substrates revealed a similar inhibition of bands 1 and 2 by the substrates for arylsulphatase B and A respectively. However, bands 3 and 4 were essentially unaffected. These findings indicate that band 1 corresponds to arylsulphatase A, and band 2 to arylsulphatase B.

Discussion

Characterization of subcellular fractions

To investigate the physicochemical properties of lysosomal hydrolases during the various stages of synthesis, intracellular transport and packaging, it is necessary to isolate the organelles responsible for these functions. The subcellular fractions which were prepared were characterized by ultrastructural, biochemical and enzymic criteria and proved to be reasonably adequate for this purpose.

The RM₂ fraction consisted largely of a special population of rough microsomes with an electron-dense cisternal content that gives electron-cytochemical reactions for acid phosphatase and glycoprotein (Goldstone & Koenig, 1972; Goldstone *et al.*, 1973). The RM₂ fraction resembled the RM₁ fraction, consisting mainly of ordinary rough microsomes, in its RNA and phospholipid content (Table 1) and its glucose 6-phosphatase activity (Goldstone *et al.*, 1973), but was enriched 10- to 30-fold over the latter in acid hydrolase activities (Table 2; Goldstone *et al.*, 1973). Kinetic studies of isotope incorporation have shown that amino acids, *N*-acetylglucosamine, mannose and glucose are incorporated into lysosomal glycoprotein in these special rough microsomes (Goldstone *et al.*, 1971*b*; Goldstone & Koenig, 1972; R. Nayyar, H. Koenig & C. Hughes, unpublished work). The RM₂ fraction was contaminated by small numbers of Golgi elements and dense bodies containing acid phosphatase, and by inference other acid hydrolases (Goldstone *et al.*, 1973), but the enzymes associated with these structures would make a relatively small contribution to the total acid hydrolase content of this fraction. Therefore, except for this minor contamination, the lysosomal hydrolases in the RM₂ fraction may be taken as the newly synthesized glycoprotein enzymes present in the cisternae of the rough endoplasmic reticulum. The sugar residues probably are added to the peptide chains after their release from the ribosomal site and entry into the cisternal space (Priestly *et al.*, 1969). It is not known whether the completed enzyme polypeptides acquire catalytic properties at the polyribosomal site or subsequent to glycosidation in the cisternae of the endoplasmic reticulum.

The GM fraction consisted predominantly of smooth membranous elements which originated from Golgi apparatuses, as documented by electron microscopy (Plates 1 and 2), and by a 10-, 15- and 19-fold enrichment in sialyltransferase, *N*-acetyl-lactosamine synthetase and galactosyltransferase activities respectively (Table 3). These findings indicate that the GM fraction from rat kidney homogenates resembles Golgi fractions isolated from liver of rat (Cheetham *et al.*, 1970; Leelavathi *et al.*, 1970; Fleischer & Fleischer, 1970; Morré *et al.*, 1969, 1970; Wagner & Cynkin, 1971) and cow (Fleischer *et al.*,

1969) with respect to sedimentation properties, ultrastructure, RNA and phospholipid content and glycosyl transferase activities.

The GM fraction also contained substantial acid hydrolase activities (Table 2). Indeed, these enzymes account for about 10–15% of the protein in the GM fraction, if the specific activities of the L fraction are taken as the standard of purity. The GM fraction contained a few autophagic vacuoles and dense bodies, but the acid hydrolases in these contaminating structures would constitute only a minor portion of the total in this fraction. The bulk of acid phosphatase in the GM fraction has been shown by ultrastructural cytochemistry to reside in the cisternal spaces of the Golgi sacs and tubules (R. Nayyar, H. Koenig, A. Goldstone & C. Hughes, unpublished work). Golgi fractions from rat liver reportedly contain increased amounts of acid phosphatase activity (Cheetham *et al.*, 1970; Fleischer & Fleischer, 1970) and arylsulphatase A and B activities (Nyquist, 1971). A smooth microsomal fraction containing Golgi membranes exhibited a higher acid phosphatase and β -glucuronidase activity than its rough counterpart from kidney of rat (Goldstone & Koenig, 1972) and mouse (Kato *et al.*, 1972). These biochemical and cytochemical findings on Golgi fractions are consistent with previous electron-cytochemical observations in a wide variety of cell types showing the presence of acid phosphatase and arylsulphatase *in situ* in portions of the Golgi apparatus (Ericksson, 1969; Smith, 1969; Nichols *et al.*, 1971).

The RM₁ fraction contained mainly ordinary rough microsomes and free ribosomes whereas the SM contained mainly smooth and transitional microsomes. In accord with these ultrastructural findings, the RM₁ fraction exhibited a fivefold greater content of RNA than the SM fraction (Table 1). In addition, these fractions contained occasional microvilli, mitochondria, dense bodies and Golgi elements. Indeed, Golgi elements were distributed throughout all the submicrosomal fractions. As judged by ultrastructural criteria and glycosyl transferase activities, the relative numbers of Golgi elements in fractions increased in the order: RM₂ < RM₁ < SM < GM. Electron-cytochemical observations indicate that acid phosphatase present in the RM₁, SM and GM fractions resides mainly in cisternae of Golgi sacs and tubules (Goldstone *et al.*, 1973; R. Nayyar, H. Koenig, A. Goldstone & C. Hughes, unpublished work).

Characterization of acid hydrolases in subcellular fractions

The physicochemical properties of several lysosomal enzymes in mammalian tissues were reported to vary according to their subcellular distribution. The microsomal acid phosphatase in mouse kidney

(Ide & Fishman, 1969) and rat liver (Walkinshaw *et al.*, 1964; Sloat & Allen, 1969) differs from the lysosomal enzyme in that a large proportion is in the bound form. The soluble form of acid phosphatase in rat liver is more anionic than the bound form on polyacrylamide-gel electrophoresis (Barka, 1961; Sloat & Allen, 1969) and more acidic on ion-exchange chromatography (Anderson, 1965). The microsomal β -glucuronidase in mouse kidney (Ide & Fishman, 1969) and rat liver (Walkinshaw *et al.*, 1964; Ganschow & Bunker, 1970) also differs from the lysosomal β -glucuronidase in that a larger proportion occurs in the bound state. Moreover, it is more cationic than the lysosomal β -glucuronidase on polyacrylamide-gel electrophoresis (Ganschow & Bunker, 1970; Mameli *et al.*, 1972) and more basic than the latter on ion-exchange chromatography (Sadahiro *et al.*, 1965). The soluble β -*N*-acetylhexosaminidase in human spleen is exclusively in the acidic form, whereas the lysosomal and microsomal enzymes occur in both the basic and acidic form (Robinson & Stirling, 1968). Price & Dance (1967) found that the acidic form of β -*N*-acetylhexosaminidase in rat kidney predominated in the soluble and microsomal fractions, whereas the basic form was predominant in the heavy and light mitochondrial fractions. Only the acidic form of β -galactosidase was present in the soluble fraction, and the basic form in particulate fractions of rat kidney. Our findings are at variance with those of Price & Dance (1967). We found that the acidic forms of β -*N*-acetylhexosaminidase and β -galactosidase predominate over the basic form in purified rat kidney lysosomes. A selective loss of the acidic form of these glycosidases during extraction and storage of enzyme extracts may account for the results of Price & Dance (1967). The acidic forms of these, and other, lysosomal hydrolases are quite unstable in tissue extracts and are converted into the corresponding basic forms by lysosomal neuraminidase even at -20°C (Goldstone *et al.*, 1971a; A. Goldstone & H. Koenig, unpublished work; Patel & Koenig, 1972).

The present investigation has revealed striking differences in solubility, electrophoretic heterogeneity and anodic mobility of five lysosomal hydrolases in subcellular fractions of rat kidney. In the RM_2 fraction these enzymes occurred largely or exclusively in a single insoluble, cationic form which coincided with the cationic glycoprotein bands in these gels (Goldstone *et al.*, 1973). Only the cationic form of acid phosphatase, β -galactosidase and β -*N*-acetylhexosaminidase was identified in gel electrophoretograms (Plates 3a, 3b, 4a and Fig. 1), whereas one minor anionic form of β -glucuronidase (Plate 4b and Fig. 2) and two of arylsulphatase (Plate 5 and Fig. 3) were demonstrated in addition to the major cationic form in the RM_2 fraction. The minor anionic forms of the latter hydrolases probably originated from the

lysosomal dense bodies and Golgi elements present in the RM_2 fraction, as these forms increased in prominence in subcellular fractions which were enriched in these organelles. In the L fraction these hydrolases occurred mainly as soluble, anionic isoenzymes. In the intervening subcellular fractions the bound, cationic forms were replaced by forms whose anodic mobility and solubility increased progressively in the order of the RM_2 , RM_1 , SM, GM and L fractions.

Physicochemical modifications of acid hydrolases during synthesis and intracellular transport

It is generally believed that the lysosomal hydrolases are synthesized on the ribosomes of the rough endoplasmic reticulum and transported via the smooth endoplasmic reticulum to the Golgi apparatus for packaging into primary lysosomes (de Duve & Wattiaux, 1966; Cohn & Fedorko, 1969) in a somewhat similar manner to proteins manufactured for extracellular secretion, e.g. pancreatic enzymes (Caro & Palade, 1964; Jamieson & Palade, 1967). This view rests largely on two lines of evidence. (1) Ultrastructural cytochemical observations in a variety of cell types have demonstrated the presence of acid phosphatase (Novikoff *et al.*, 1964; Erickson, 1969; Smith, 1969; Nichols *et al.*, 1971) and arylsulphatase (Nichols *et al.*, 1971) in portions of the Golgi apparatus and infrequently in the smooth and rough endoplasmic reticulum. (2) Radioautographic observations with the electron microscope in differentiating mononuclear phagocytes (Cohn *et al.*, 1966) and neutrophilic leucocytes (Fedorko & Hirsch, 1966) have revealed a flow of newly synthesized protein, assumed to be mainly acid hydrolases, from the site of synthesis in the rough endoplasmic reticulum through the Golgi apparatus before incorporation into lysosomes. Novikoff and associates have advanced an alternative theory based on ultrastructural cytochemical evidence in which lysosomal enzymes are concentrated and packaged in the GERL (abbreviated from Golgi, Endoplasmic Reticulum and Lysosomes), a membranous organelle distinct from the Golgi apparatus which consists of small cisternal portions and anastomosing tubules of smooth endoplasmic reticulum that are frequently continuous with the rough endoplasmic reticulum in which these enzymes are synthesized (Holtzman *et al.*, 1967; Novikoff, 1967; Novikoff *et al.*, 1971). According to the GERL hypothesis, primary lysosomes arise from the tubular portions, i.e. the smooth endoplasmic reticulum, as well as the cisternal portions of the GERL.

Biochemical studies on the synthesis, migration and packaging of lysosomal enzymes have been limited to date. Evidence for the synthesis of lysosomal hydrolases in the microsomal fraction and their

subsequent transfer to the lysosomal fraction has been presented for acid phosphatase and β -glucuronidase in rat liver (Walkinshaw & van Lanckner, 1964; van Lanckner & Lentz, 1970) and for β -glucuronidase in mouse kidney (Ide & Fishman, 1969; Kato *et al.*, 1970, 1972). Fishman and associates (Kato *et al.*, 1972) have reported findings consistent with the view that β -glucuronidase synthesized in gonadotrophin-stimulated mouse kidney is transported from the rough endoplasmic reticulum to the lysosomes via the smooth endoplasmic reticulum.

A kinetic study of isotope incorporation into rat kidney subcellular fractions showed that labelled lysine, *N*-acetylglucosamine and mannose are incorporated into lysosomal peptides in a special rough microsomal fraction, whereas *N*-acetylneuraminic acid residues are incorporated into nascent glycoproteins in a smooth microsomal fraction enriched in Golgi membranes; labelled glycoproteins subsequently appear in lysosomes (Goldstone *et al.*, 1971*b*; Goldstone & Koenig, 1972). These findings have been confirmed and extended by an ultrastructural radioautographic study of isotope incorporation *in vivo* into these fractions (Goldstone *et al.*, 1971*b*; R. Nayyar, H. Koenig & A. Goldstone, unpublished work) and into epithelial cells in proximal convoluted tubule in rat kidney. The latter study revealed that [^{14}C]lysine, [^3H]mannose, [^{14}C]glucose and *N*-acetyl[^3H]glucosamine were incorporated initially in the rough endoplasmic reticulum. Labelled macromolecules arrived in the Golgi apparatus by 15–30 min, and shortly thereafter appeared in lysosomes. By 1 h approximately 90% of all the lysosomes showed uptake of labelled glycoproteins. *N*-Acetylneuraminic acid originating from *N*-acetyl[^3H]mannosamine was taken up in the Golgi apparatus and rapidly appeared in lysosomes, about 90% of the latter becoming labelled by 5 min (Nayyar & Koenig, 1972).

The present experiments do not clarify the role of the smooth endoplasmic reticulum in the formation of soluble, anionic lysosomal isoenzymes. The slow-moving anionic isoenzymes that were present in the RM_1 and SM fractions could be associated with smooth microsomes arising from the smooth endoplasmic reticulum and/or the Golgi membranes present in these fractions. A few dense bodies resembling lysosomes were present in all of these fractions, but their contribution to the isoenzyme patterns is not considered to be significant as the slow-moving anionic forms are only minor components of the L fraction. Thus two interpretations can be advanced to explain our results: (1) the production of soluble, acidic forms is initiated in elements of the smooth endoplasmic reticulum contained within the RM_1 and SM fractions and completed in the Golgi apparatus present in the RM_1 , SM and GM fractions; (2) the production of the

soluble acidic forms is initiated and completed entirely within various portions of the Golgi apparatus (or GERL) which are retrieved in the RM_2 , RM_1 , SM and GM fractions. In either event the electro-negativity and solubility of these enzymes would be related to the attachment of *N*-acetylneuraminic acid residues.

The following findings strongly favour the latter hypothesis. (a) The Golgi elements in these fractions, as demonstrated ultrastructurally and by the Golgi marker enzymes, increased in the direction of the RM_2 , RM_1 , SM, GM and L fractions. (b) The uptake of *N*-acetylneuraminic acid *in vivo* in rat kidney subcellular fractions during brief incorporation periods roughly paralleled the specific activities of Golgi marker enzymes in these fractions (Goldstone & Koenig, 1972; A. Goldstone, K. Strohl & H. Koenig, unpublished observations). (c) *N*-Acetylneuraminic acid uptake, as demonstrated by electron-microscope radioautography of rat kidney proximal convoluted tubule, occurred mainly in the Golgi apparatus just before its appearance in lysosomes (Nayyar & Koenig, 1972). (d) Acid phosphatase and glycoprotein, as revealed by electron-microscope cytochemistry, were localized in the cisternal spaces of the Golgi tubules and sacs, rather than ordinary smooth microsomes, in the RM_2 , RM_1 and SM fractions (Goldstone *et al.*, 1973; R. Nayyar, H. Koenig, A. Goldstone & C. Hughes, unpublished work). (e) The relative content of the soluble, acidic forms of all five lysosomal enzymes increased in the order of the RM_2 , RM_1 , GM, SM and L fractions. These findings support the hypothesis that the soluble acidic forms of these enzymes are generated in the Golgi apparatus through the attachment of sugar sequences containing *N*-acetylneuraminic acid residues.

Physicochemical heterogeneity of the Golgi apparatus

The buoyant density of the Golgi elements in sub-microsomal fractions diminished in the order: $\text{RM}_2 > \text{RM}_1 > \text{SM} > \text{GM}$. Inasmuch as the relative content of the soluble anionic isoenzymes increased progressively in this order, we may assume the physicochemical nature of the contained hydrolases, or of an associated constituent, somehow influences the equilibrium density of Golgi elements during isopycnic centrifugation in a sucrose-density gradient. This supposition in turn implies that the structural components comprising the Golgi apparatus manifest an orderly physicochemical heterogeneity with respect to the migration pathway of the nascent enzymes through the apparatus. This supposition is compatible with electron-cytochemical observations showing a concentration gradient of osmiophilic material (Mollenhauer & Whaley, 1963), glycoprotein (Rambourg *et al.*, 1969), acidic macromolecules

(Berlin, 1967), thiamin pyrophosphatase (Novikoff, 1967), acid phosphatase (Novikoff, 1967; Ericksson, 1969; Smith, 1969) and arylsulphatase (Nichols *et al.*, 1971) in the cisternae of Golgi saccules, which increases gradually on moving from the saccules in the 'forming' or 'immature' face to the saccules in the 'mature' face on the concave surface of the Golgi stacks. The fact that the special rough microsomes in the RM₂ fraction are substantially denser than the bulk of rough microsomes (Goldstone *et al.*, 1973) suggests that the high density of the former is due to its glycoprotein content. In the light of this finding it is plausible that the denser Golgi tubules sedimenting in the RM₂, RM₁ and SM fractions may have a similar glycoprotein content.

The Golgi elements retrieved in the GM fraction revealed the greatest content of acidic lysosomal isoenzymes. The low density of these Golgi elements could be due to the accumulation of soluble acidic lipoglycoprotein, a major constituent of lysosomes (Koenig, 1969*a,b*; Goldstone *et al.*, 1970). An anionic glycoprotein component in the RM₂ fraction, which has the same electrophoretic mobility as the lysosomal lipoglycoprotein but lacks its lipid constituents, is believed to be the precursor, i.e. the apoglycoprotein, of the latter (Goldstone *et al.*, 1973). The lipid content of this lipoglycoprotein, as detected in appropriately stained gel electrophoretograms of rat kidney submicrosomal fractions, increased in the direction of the RM₂, RM₁, SM and GM fractions (A. Goldstone & H. Koenig, unpublished work). This finding suggests that lipid may be attached to the lysosomal apoglycoprotein in the Golgi apparatus coincident with glycosidation of the nascent enzymes. Serum lipoproteins are present in Golgi fractions from rat liver (Fleischer & Fleischer, 1970) and rat small intestinal epithelium (Mahley *et al.*, 1971) and are thought to be assembled or otherwise processed in the Golgi apparatus (Stein & Stein, 1967; Claude, 1970).

On the basis of these experiments and other observations mentioned in this paper, we propose the following scheme for the biosynthesis of lysosomal acid phosphatase, β -*N*-acetylglucosaminidase, β -galactosidase, β -glucuronidase and arylsulphatase. These hydrolases are each synthesized in a restricted portion of the rough endoplasmic reticulum as a single bound basic glycoprotein enzyme with oligosaccharide side chains containing *N*-acetylglucosamine, mannose and glucose. The soluble, acidic isoenzymes are generated from the bound basic forms through the stepwise attachment of linear sequences of sugar residues containing *N*-acetylneuraminic acid as the nascent glycoprotein enzymes migrate through the Golgi apparatus. These hydrolases are probably packaged as acidic isoenzymes in lysosomes. Preliminary experiments suggest that the basic isoenzymes present in secondary lysosomes may arise

during intracellular digestion through the autolytic cleavage of *N*-acetylneuraminic acid residues by lysosomal neuraminidase (H. Koenig, P. Sanghavi & A. Patel, unpublished work). According to this scheme, the molecular heterogeneity of these enzymes is introduced after translation as a result of glycosidation reactions during intracellular transport, and may be augmented by catabolic reactions during physiological autolysis.

This hypothesis is consistent with the finding that the anodic mobility, solubility and isoelectric point of the acidic lysosomal isoenzymes in rat kidney is dependent on their *N*-acetylneuraminic acid content (Goldstone *et al.*, 1971*a*; S. Needleman & H. Koenig, unpublished work), and with isotope uptake studies in rat kidney already cited, which show that labelled leucine, lysine, *N*-acetylglucosamine, mannose and glucose are initially incorporated into lysosomal polypeptide in the special rough microsomes, whereas *N*-acetylneuraminic acid is incorporated in the Golgi apparatus (Goldstone *et al.*, 1971*b*; Goldstone & Koenig, 1972; Nayyar & Koenig, 1972). Some of the *N*-acetylneuraminic acid residues in these glycoprotein enzymes apparently are located in an internal, i.e. non-terminal, position in the carbohydrate side chains, as they are not cleaved by neuraminidase (Goldstone *et al.*, 1971*a*; Patel & Koenig, 1971). Carroll & Robinson (1972) reported that the acidic and basic form of human liver β -*N*-acetylhexosaminidase are immunologically identical. Moreover, the basic form (isoenzyme III) and the acidic form (isoenzyme I) of β -glucuronidase are completely precipitated by antiserum to β -glucuronidase I (Okochi *et al.*, 1968). These findings support the view that the acidic and basic isoenzymes of these two hydrolases possess the same polypeptide backbone and differ only in their carbohydrate side chains.

The acidic form of a lysosomal enzyme is lacking in two inherited lipid-storage diseases, namely, β -*N*-acetylhexosaminidase A in Tay-Sachs disease (Sandhoff, 1969; Okada & O'Brien, 1969), and arylsulphatase A in metachromatic leucodystrophy (Austin *et al.*, 1965; Mehl & Jatzkewitz, 1965), and the corresponding basic form may be present in normal or elevated amounts in these disorders. If the acidic lysosomal isoenzymes are formed after translation, as proposed here, then these enzyme deficiencies cannot be due to a defect in synthesis alone. We suggest that the following mechanisms, operating jointly, may account for this type of enzyme deficiency. 1. A genetic deficiency in synthesis of the basic glycoprotein in the rough endoplasmic reticulum limits the rate of formation of the acidic isoenzyme in the Golgi apparatus and its segregation in lysosomes. 2. The limited stores of the acidic isoenzyme may be further depleted during intracellular digestion through autolytic cleavage of its *N*-acetylneuraminic acid residues by lysosomal neuraminidase with the

resultant formation of a less soluble, more basic isoenzyme. 3. Inactivation of the basic isoenzyme through catabolic degradation or other means may be retarded by substrate stabilization, i.e. binding of the enzyme to the undegraded acidic sphingolipids, GM₂ ganglioside and cerebroside sulphate, which accumulate in Tay-Sachs disease and metachromatic leucodystrophy respectively (Brady & Kolodny, 1972).

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