Autolysis of Glycoproteins in Rat Kidney Lysosomes *in vitro*

EFFECTS ON THE ISOELECTRIC FOCUSING BEHAVIOUR OF GLYCOPROTEINS, ARYLSULPHATASE AND β -GLUCURONIDASE

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1. Rat kidney lysosomal glycoproteins, prelabelled in the *N*-acetylneuraminic acid and polypeptide portions with *N*-acetyl[^3H]mannosamine and [^{14}C]lysine, or with *N*-acetyl-[^{14}C]glucosamine, were incubated under various conditions. Autolytic cleavage of labelled *N*-acetylneuraminic acid and peptide was maximum at pH 5.0. 2. *N*-Acetylneuraminic acid was released more rapidly than peptide during incubation at 37° or 4°C at pH 5. *p*-Nitrophenyloxamic acid, an inhibitor of bacterial neuraminidase (Edmond *et al.*, 1966), inhibited the cleavage of *N*-acetylneuraminic acid and peptide, and also inhibited cathepsin D activity. 3. Galactono-, mannono-, and glucono-lactone, inhibitors of the corresponding glycosidases, blocked the autolytic cleavage of *N*-acetyl[^{14}C]glucosamine and protein without inhibiting β -*N*-acetylhexosaminidase or cathepsin D activity. These findings suggest that the carbohydrate side chains protect the polypeptide portion of the lysosomal glycoproteins against proteolytic attack by lysosomal cathepsins. 4. In electrofocusing experiments, autolysis was minimized by adding 0.1% *p*-nitrophenyloxamic acid to the media used for extraction and electrofocusing, and by maintaining an alkaline pH (pH 8.8-9) during extraction and dialysis. Arylsulphatase occurred in two forms with pI values of 4.4 and 6.4-6.7, and β -glucuronidase in two forms with pI values of 4.4 and 6.1. When [^{14}C]lysine and *N*-acetyl[^3H]mannosamine were given to rats 1.5 and 1 h before killing, ^{14}C and ^3H were largely restricted to highly acidic glycoprotein species with pI values of 2.1-5.1. 5. When a lysosomal extract was adjusted to pH 5 and incubated at 20°C for 16 h and then at 37°C for 1 h before electrofocusing, 32 and 58% of the labelled peptide and *N*-acetylneuraminic acid was cleaved and the pI values of the labelled glycoproteins were markedly increased. About 80% of the acidic form of arylsulphatase and β -glucuronidase was recovered with the basic form, and the pI of the basic form of both enzymes rose to 7.0. Similar, though less marked changes, were observed when a lysosomal extract was kept at pH 5 for 2 h at 4°C before electrofocusing. 6. When an acidic lysosomal fraction (pI 4.2-4.6) was incubated at pH 5 for 2.5 h and refocused, 80% of the arylsulphatase now occurred in two forms with pI values of 5 and 6.4. When a basic lysosomal fraction (pI 5.8-6.4) was similarly incubated, the pI of arylsulphatase increased from 6.4 to 7.2. The relative increase in pI of arylsulphatases was accompanied by a proportional loss of *N*-acetylneuraminic acid from the glycoprotein associated with these forms. 7. These experiments show that lysosomal glycoproteins and two representative hydrolases, when exposed to a mildly acidic pH, readily undergo autolytic degradation and their pI values increase. These observations may have a bearing on the origin of the molecular heterogeneity of the lysosomal enzymes.

It has been repeatedly observed that lysosomal hydrolases are relatively resistant to inactivation during autolysis (de Duve & Beaufay, 1959; de Duve & Wattiaux, 1966; Aronson & de Duve, 1968). We have shown that these hydrolases in rat liver and kidney lysosomes are glycoprotein enzymes containing *N*-acetylglucosamine, mannose, glucose and *N*-acetyl-

neuraminic acid (Goldstone & Koenig, 1970; Goldstone *et al.*, 1973) and there are indications that galactose (Robineaux *et al.*, 1969; A. Goldstone & H. Koenig, unpublished work) and fucose (Bennett & Leblond, 1971; R. Nayyar & H. Koenig, unpublished work) are also present. The resistance of these hydrolases to autolytic inactivation could be related to their

glycoprotein nature, as the proteolytic degradation of some glycoproteins, e.g. α_1 -acid glycoprotein and bovine submaxillary glycoprotein, by various proteolytic enzymes was facilitated by the removal of *N*-acetylneuraminic acid residues (Schmid *et al.*, 1959; Anantha Samy, 1967; Gottschalk & Fazekas de St. Groth, 1960). The inclusion in the lysosomal enzyme complement of neuraminidase, glycosidases and proteinases that can extensively degrade glycoprotein substrates of non-lysosomal origin (Aronson & de Duve, 1968; Mahadevan *et al.*, 1969) raises the possibility that these and other lysosomal hydrolases may undergo autolytic degradation when incubated at acid pH. In support of this supposition, we recently found that incubation of a soluble extract of rat kidney lysosomes at pH 5 resulted in an autolytic cleavage of *N*-acetylneuraminic acid residues from lysosomal glycoproteins (Goldstone *et al.*, 1971), apparently catalysed by a neuraminidase present in these lysosomes (Mahadevan *et al.*, 1967).

Many of the lysosomal acid hydrolases exist in several molecular forms. For example, we have shown that a number of acid hydrolases in rat kidney and liver lysosomes occur in two or more forms differing in solubility, electrophoretic mobility (Goldstone & Koenig, 1970, 1973a; Goldstone *et al.*, 1971) and pI (Koenig *et al.*, 1972; Needleman & Koenig, 1973). A study of the effect of bacterial neuraminidase on the electrophoretic mobility (Goldstone *et al.*, 1971) and pI (Needleman & Koenig, 1973) of these hydrolases indicated that *N*-acetylneuraminic acid residues are largely responsible for the solubility and electro-negative charge of the lysosomal enzymes.

This communication describes experiments on the autolysis *in vitro* of rat kidney lysosomal glycoproteins, prelabelled in the carbohydrate and peptide moieties with radioisotopic precursors to facilitate following the cleavage of these moieties. Several significant findings have emerged from this investigation. The results indicate that the carbohydrate chains afford the lysosomal glycoproteins limited protection against proteolytic attack by cathepsins. Nevertheless, lysosomal glycoproteins are highly susceptible to autolytic digestion when exposed to an acidic pH, even at 4°C. We have therefore developed procedures for the extraction and processing of lysosomal glycoprotein enzymes that are designed to minimize their autolytic degradation. Isoelectric-focusing experiments showed that newly synthesized macromolecules are packaged in rat kidney lysosomes in the form of highly acidic glycoproteins. When the lysosomal glycoproteins were incubated at pH 5, *N*-acetylneuraminic acid and peptide were split off and the pI values of the partially degraded glycoproteins and several enzymes increased substantially. These observations may have an important bearing on the biodegradation of lysosomal hydrolases and the origin of the molecular heterogeneity of these enzymes.

Materials and Methods

Sprague-Dawley rats weighing 150–200 g were injected intraperitoneally with 5 μ Ci of *N*-acetyl- β -D-[1- 14 C]glucosamine (12 mCi/mmol) at 120 min, or with 40 μ Ci of *N*-acetyl- β -D-[U- 3 H]mannosamine (400 mCi/mmol), a precursor of *N*-acetylneuraminic acid, at 60 min and 10 μ Ci of L-[U- 14 C]lysine (240 mCi/mmol) 90 min before death. Isotopes were supplied by ICN Corp. (Irvine, Calif., U.S.A.). Kidneys from five to ten rats were pooled for each experiment, and heavy lysosomal fractions were isolated by differential and density gradient centrifugation as described previously (Goldstone *et al.*, 1973). Lysosomal fractions showed a 15–27-fold enrichment in acid hydrolase activities over the whole homogenate and contained a maximum of 5–10% contamination by mitochondria, peroxisomes and endoplasmic reticulum, as shown by assay of marker enzymes and electron microscopy (Goldstone *et al.*, 1973). To ascertain the fate of 3 H from *N*-acetyl[3 H]mannosamine, one lysosomal preparation isolated 90 min after injection of label was hydrolysed for 1 h in 0.05 M- H_2SO_4 at 80°C to remove bound *N*-acetylneuraminic acid, the residue further hydrolysed for 3 h in 3 M-HCl at 100°C to release neutral and amino sugars, and the sugars were separated by column and paper chromatography as described earlier (Goldstone & Koenig, 1970). Radioassay of the isolated sugars showed that 90% of the incorporated 3 H label was present in *N*-acetylneuraminic acid, and 10% in *N*-acetylglucosamine.

Lysosomal fractions prelabelled with *N*-acetyl-[14 C]glucosamine alone were suspended in 5 ml of 0.1 M-sodium acetate buffer, pH 5.7, lysed by sonication (Goldstone *et al.*, 1971) and clarified by centrifuging for 1 h at 37 000g, all at 4°C. These extracts were adjusted to pH 5 and incubated at 37°C for various time-intervals in the absence and presence of 0.4% D-galactono-1,4-lactone (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 0.4% D-glucono-1,5-lactone and 0.4% L-mannono-1,4-lactone (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), inhibitors of β -galactosidase, β -glucosidase (Robinson *et al.*, 1967) and α -mannosidase (Conchie & Hay, 1959) respectively. Autolysis was stopped by the addition of trichloroacetic acid to a final concentration of 7.5% (w/v) at 4°C. The acid-soluble and acid-insoluble fractions were separated by centrifugation and assayed for protein with bovine serum albumin as a standard (Lowry *et al.*, 1951) and for 14 C in an automatic gas-flow counter (Nuclear-Chicago model 183B).

Lysosomal pellets doubly labelled with *N*-acetyl-[3 H]mannosamine and [14 C]lysine were cooled to –20°C immediately after isolation and covered with 3 ml of 0.2% Triton X-100 in 0.1 M-glycine-NaOH buffer, pH 9.0. After 30 min the pellet was dispersed,

disrupted by sonication (Goldstone *et al.*, 1971), and the sonicate clarified by centrifugation at 100000g for 1 h at 4°C. Extracts were incubated under various conditions in the absence and presence of 0.1% *p*-nitrophenyloxamic acid (K & K Laboratories, Plainview, N.Y., U.S.A.), an inhibitor of bacterial neuraminidase (Edmond *et al.*, 1966), as described in the Results section. After completion of the incubations, proteins were precipitated with 7.5% trichloroacetic acid at 4°C and samples of acid-soluble and acid-insoluble fractions mixed with 10ml of Aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.) for counting of ^3H and ^{14}C in a Beckman model LS-250 liquid-scintillation system. In several experiments the effects of the inhibitors on cathepsin D and β -*N*-acetylhexosaminidase were examined in rat kidney lysosomes. Cathepsin D (EC 3.4.23.5) was assayed against a denatured haemoglobin substrate (Serra *et al.*, 1972), and β -*N*-acetylhexosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) against a 4-methylumbelliferyl β -D-glucosaminide substrate (Goldstone *et al.*, 1973).

Extracts of doubly labelled lysosomal pellets, prepared as described above, were used for electrofocusing experiments. The experimental lysosomal extract was adjusted to pH 5 with 2M-acetic acid and divided into two portions. One portion, hereafter designated Expt. A, was kept at pH 5 for 2 h at 4°C, and a second, designated Expt. B, at pH 5 at 20°C for 16 h followed by 1 h at 37°C. The experimental extracts were fractionated immediately after incubation by isoelectric focusing in a pH 10–3 Ampholine gradient in an LKB model 7100 column at 4°C for 16 h (Vesterberg & Svensson, 1966). Fractions (3 ml) were collected and portions dialysed overnight at 4°C against several changes of water to remove the Ampholines, which interfere with the protein determination. To minimize autolysis in the control lysosomal sample, 0.1% *p*-nitrophenyloxamic acid was included in the extraction medium and the Ampholine gradient and portions of fractions were dialysed against 0.005M-glycine-NaOH buffer, pH 8.8. Dialysed proteins were precipitated with cold 7.5% trichloroacetic acid, taken up in 0.5M-NaOH, measured for protein (Lowry *et al.*, 1951) and assayed for ^3H and ^{14}C in a Beckman model LS-250 liquid-scintillation system as described above. The pH determinations and enzyme assays were done on undialysed samples. Arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1) and β -glucuronidase (β -D-glucuronide glucuronosohydrolase, EC 3.2.1.31) were assayed as described previously (Goldstone *et al.*, 1973). For measurement of β -glucuronidase, 0.2ml of sample and 0.1ml of 0.2M-sodium acetate buffer, pH 5, were incubated for 1 h at 37°C. The reaction was terminated by addition of 2ml of 0.2M-glycine-0.2M-NaOH, pH 10.4, and the E_{540} measured. For assay of arylsulphatase, 0.2ml of

sample, 0.1ml of 0.1M-*p*-nitrocatechol sulphate and 0.7ml of 0.2M-sodium acetate buffer, pH 5.5, was incubated at 37°C for 15 min. The reaction was terminated by adding 2ml of 1M-NaOH and the E_{515} measured.

Results

Effect of pH on autolysis

Lysosomal glycoprotein extracts from rat kidney, prelabelled in the *N*-acetylneuraminic acid and peptide moieties with *N*-acetyl[^3H]mannosamine and [^{14}C]lysine, were incubated at various pH values. Fig. 1 shows that, under the conditions chosen (4 h, 37°C), the autolytic cleavage of *N*-acetylneuraminic acid and protein, as measured by the release of acid-soluble ^3H and ^{14}C , was maximum at pH 5 and declined sharply with increasing and decreasing pH. However, appreciable cleavage of these residues, 25–75% of the maximum, occurred over a rather wide range of pH (3–8).

Fig. 1. Effect of pH on the autolytic cleavage of *N*-acetylneuraminic acid and peptide in lysosomal glycoproteins

Rat kidney lysosomal glycoproteins, prelabelled in the *N*-acetylneuraminic acid and peptide portions with *N*-acetyl[^3H]mannosamine and [^{14}C]lysine respectively, were incubated at 37°C for 4 h in the following buffers: pH 3 and 4, 0.2M-sodium citrate; pH 5, 0.2M-sodium acetate; pH 6 and 7, 0.2M-sodium phosphate; pH 8, 0.2M-Tris-HCl; pH 9, 0.2M-glycine-0.2M-NaOH. Cleavage is expressed as percentages of the total trichloroacetic acid-precipitable radioactivity released into solution during incubation. The results are the means and standard errors of three experiments. ●, ^3H from *N*-acetyl[^3H]mannosamine; ○, ^{14}C from [^{14}C]lysine.

Effect of time, temperature and *p*-nitrophenyloxamic acid on autolysis

Figs. 2 and 3 show the results of experiments in which prelabelled lysosomal glycoprotein extracts were autoincubated at pH 5 for several time-periods at 37° and 4°C. At 37°C the autolytic cleavage of *N*-acetylneuraminic acid residues began at once at its maximum rate and then slowed down, but was still progressing after 24 h, at which time 38% of the total ³H had been released. Peptide cleavage commenced at a slower rate, but was still progressing at 24 h when 34% of the incorporated ¹⁴C had been released (Fig. 2). When the incubation temperature was lowered to 4°C, *N*-acetylneuraminic acid cleavage declined only 23–42% from the corresponding values at 37°C, whereas proteolysis diminished by 74% after 1 h, and by 50 and 43% after 4 and 24 h of incubation respectively (Fig. 3). *p*-Nitrophenyloxamic acid (0.1%) inhibited the autolytic cleavage of *N*-acetylneuraminic acid and peptide by about 32–50% during the first hour of incubation, but this inhibition declined after longer incubations. However, *p*-nitrophenyloxamic acid (0.1%) also inhibited cathepsin D in rat kidney lysosomes by 44±1%.

Fig. 2. Autolytic cleavage of *N*-acetylneuraminic acid and peptide in lysosomal glycoprotein during incubation at 37°C and the effect of *p*-nitrophenyloxamic acid

Rat kidney lysosomal glycoproteins, prelabelled in the *N*-acetylneuraminic acid and peptide portions as in Fig. 1, were incubated in 0.1 M-sodium acetate buffer, pH 5, at 37°C in the presence and absence of 0.1% *p*-nitrophenyloxamic acid for various periods of time. The results are the means and standard errors of three experiments. (a) ³H released: ●, control; ○, +0.1% *p*-nitrophenyloxamic acid. (b) ¹⁴C released: ■, control; □, +0.1% *p*-nitrophenyloxamic acid.

Effect of lactones on autolysis

When lysosomal glycoproteins, prelabelled with *N*-acetyl[1-¹⁴C]glucosamine, were incubated at pH 5 and 37°C, incorporated ¹⁴C was released rapidly at

Fig. 3. Autolytic cleavage of *N*-acetylneuraminic acid and peptide in lysosomal glycoproteins during incubation at 4°C and the effect of *p*-nitrophenyloxamic acid

The conditions were as in Fig. 2. The results are the means and standard errors of three experiments. (a) ³H released: ●, control; ○, +0.1% *p*-nitrophenyloxamic acid. (b) ¹⁴C released: ■, control; □, +0.1% *p*-nitrophenyloxamic acid.

Fig. 4. Autolytic cleavage of *N*-acetylglucosamine and protein in lysosomal glycoproteins during incubation at 37°C and the effect of lactones

Rat kidney lysosomal glycoproteins, prelabelled with *N*-acetyl[1-¹⁴C]glucosamine, were incubated alone and in the presence of 0.4% mannitolactone, 0.4% gluconolactone and 0.4% galactonolactone in 0.1 M-sodium acetate buffer, pH 5, at 37°C for various time-periods. The results are the means and standard errors of three experiments. ¹⁴C released: ●, control; ○, + lactones. Protein released: ■, control; □, + lactones.

first and then more slowly, reaching 27 and 38% of the total after 3 and 24 h of incubation (Fig. 4). The lactones inhibited the cleavage of *N*-acetylglucosamine and peptide by 37–60% and 44–53% at 0.5–3 h, but only 17 and 19% after 24 h of incubation. The lactones had no effect on the cathepsin D and β -*N*-acetylhexosaminidase activities in rat kidney lysosomes.

Isoelectric-focusing experiments

Radioactivity profiles. Nearly 100% of the ^{14}C and 76% of the ^3H in the control glycoprotein extract that was applied to the gradient was recovered in the dialysed fractions. This suggests that some autolytic cleavage of *N*-acetylneuraminic acid still occurred during the electrofocusing and/or dialysis procedure.

About 90% of both labels occurred in glycoprotein fractions with pI values between 2.1 and 5.1. However, only 4% of the ^{14}C and no ^3H was present in fractions with pI values of 6.0 and higher (Figs. 5 and 6).

In Expt. A (pH 5, 2 h at 4°C) about 14 and 31% of the labelled peptide and *N*-acetylneuraminic acid respectively were cleaved (Table 1) and the ^{14}C and ^3H radioactivity profiles were displaced from glycoprotein fractions of low pI into fractions of higher pI (Figs. 5 and 6). In Expt. B (pH 5 at 20°C for 16 h and 37°C for 1 h) peptide and *N*-acetylneuraminic acid cleavage increased to 32 and 58% respectively (Table 1), and the ^{14}C and ^3H radioactivity profile showed a more marked shift into glycoprotein fractions of higher pI (Figs. 5 and 6).

Table 1 presents the average specific ^{14}C and ^3H radioactivities (c.p.m./mg of protein) of an acidic

Fig. 5. Effect of autolysis on the isoelectric-focusing behaviour of [^{14}C]lysine-labelled peptide in lysosomal extracts

Rats were killed 1.5 h after receiving [^{14}C]lysine and 1 h after *N*-acetyl[^3H]mannosamine, a precursor of *N*-acetylneuraminic acid, by intraperitoneal injection and lysosomes were isolated from pooled kidneys. One lysosomal extract (Expt. A) was adjusted to pH 5 and kept for 2 h at 4°C, and a second (Expt. B) at pH 5 for 16 h at 20°C and 1 h at 37°C. Extracts were fractionated immediately after incubation by electrofocusing in a pH 10–3 Ampholine gradient. In the control extract, autolysis was minimized by adding 0.1% *p*-nitrophenyloxamic acid, a neuraminidase inhibitor, to the media used for extraction and electrofocusing, and by maintaining an alkaline pH (pH 8.8–9) during extraction and dialysis. Experimental details are given in the text. The specific ^{14}C radioactivity is shown on the ordinate and the pH (pI) of the fractions at equilibrium is noted on the abscissa. (a) Control; (b) Expt. A; (c) Expt. B.

Fig. 6. Effect of autolysis on the isoelectric-focusing behaviour of *N*-acetylneuraminic acid labelled by *N*-acetyl- ^3H mannosamine in lysosomal glycoproteins

For details, see legend to Fig. 5. The specific ^3H radioactivity is shown on the ordinate. ●, Control; ▲, Expt. A; ○, Expt. B.

(pI 3.0–4.9) and a basic (pI 5.8–8.0) glycoprotein fraction containing the acidic and basic forms, respectively, of arylsulphatase, β -glucuronidase and several other hydrolases (Goldstone & Koenig, 1974), and

an intermediate fraction (pI 5.0–5.7). In Expt. B the specific ^{14}C and ^3H radioactivities of the acidic glycoprotein fraction declined 2- and 3-fold respectively, whereas those of the intermediate glycoprotein fraction increased 5.6- and 4.2-fold and those of the basic glycoprotein fraction increased 4.6- and 3.2-fold. In Expt. A these changes were intermediate in degree.

Protein profiles. In the control, protein was widely distributed in the gradient in the range of pI 2.1–7.7 with a maximum at pI 5.3. In Expt. A there was a small decrease in protein in the most acidic portion of the gradient, and a relative increase in the more basic region with maxima at pI 5.3 and 6.2. In Expt. B new protein maxima appeared at pI 7.4 and 8.6 at the expense of protein with pI values of 2.1–3.7 and 5.1–6.1. The protein remaining in the region of pI 3.7–5.0 would be mainly lysosomal lipoglycoprotein without enzymic activity (Koenig, 1974; A. Goldstone & H. Koenig, unpublished work).

β -Glucuronidase activity in the gradient. About 13% of the β -glucuronidase activity in the control lysosomal extract was present in a discrete acidic peak with a pI of 4.4, and 87% in a basic peak with a pI of 6.1 (Fig. 7). In Expt. A the acidic peak was decreased to 4% and the basic peak increased to 96% of the total β -glucuronidase activity. In Expt. B the acidic peak was decreased to 2% of the total activity and its pI increased to 4.5, whereas the basic form increased to 98% of the total and its pI rose to 7.0. There was no change in the total β -glucuronidase activity during incubation.

Arylsulphatase activity in the gradient. About 41% of the arylsulphatase activity in the control occurred in an acidic form with a pI of 4.4 and 59% in a basic form with a pI of 6.6 (Fig. 8). In Expt. A the acidic fraction was decreased to 21% and the basic fraction increased to 79% of the total enzyme activity. In Expt. B the acidic form was decreased to 8% of the total activity and its pI rose to 4.7, whereas the basic form increased to 92% of the total activity and its pI rose to 7.0 without any change in the total arylsulphatase activity.

In another experiment, lysosomal glycoproteins prelabelled in the *N*-acetylneuraminic acid moiety with *N*-acetyl ^3H mannosamine were fractionated by electrofocusing. The fractions containing the acidic form (pI 4.2–4.6) and the basic form (pI 5.8–6.4) of arylsulphatase were pooled. The acidic fraction contained 37% of the total arylsulphatase and had a specific ^3H radioactivity of 2200 c.p.m./mg of protein. The basic fraction contained 63% of the arylsulphatase activity and had a specific ^3H radioactivity of 312 c.p.m./mg of protein. These fractions were incubated separately at pH 5 and 37°C for 2.5 h and again fractionated by electrofocusing. The incubated acidic fraction yielded three peaks of arylsulphatase with pI 4.2, 5.0 and 6.8, containing 19, 60 and 21% respectively of the total enzyme activity. The specific ^3H

Table 1. *Specific radioactivities of glycoprotein fractions from rat kidney lysosomes: effects of incubation at pH5*

Rats received [¹⁴C]lysine and *N*-acetyl[³H]mannosamine by intraperitoneal injection 1.5 and 1 h before death. In Expt. A, a lysosomal extract was kept at pH5 for 2 h at 4°C. In Expt. B, a lysosomal extract was kept at pH5 for 16 h at 20°C and for 1 h at 37°C. Extracts were fractionated directly after incubation by isoelectric focusing in a pH10–3 Ampholine gradient. In the control extract, autolysis was minimized by adding 0.1% *p*-nitrophenyloxamic acid to the extraction medium and Ampholine gradient, and by maintaining an alkaline pH (pH8.8–9.0) during extraction and dialysis. Protein, enzymes, pH, ¹⁴C and ³H were measured in the whole extracts and in all fractions as described in the Materials and Methods section. To compute mean specific radioactivities of the fractions given in this table, the sum of the radioactivities recovered in fractions from the gradient in the pH ranges shown was divided by the sum of the protein recovered in these fractions. Values are also expressed (in parentheses) as a percentage of the corresponding control value.

Fractions	Specific radioactivity of fractions (c.p.m./mg of protein)							
	Whole extract		pI3.0–4.9		pI5.0–5.7		pI5.8–8.0	
	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C
Control	800 (100%)	300 (100%)	1725 (100%)	480 (100%)	159 (100%)	55 (100%)	70 (100%)	45 (100%)
Expt. A	553 (69%)	256 (86%)	1267 (74%)	427 (89%)	181 (114%)	169 (307%)	42 (60%)	115 (255%)
Expt. B	337 (42%)	204 (68%)	550 (32%)	245 (51%)	667 (419%)	308 (560%)	225 (322%)	206 (460%)

Fig. 7. *Effect of autolysis on the isoelectric-focusing behaviour of β -glucuronidase in lysosomal extracts*

For details, see legend to Fig. 5. The absorbance (540nm) is shown on the ordinate.

Fig. 8. *Effect of autolysis in the isoelectric-focusing behaviour of arylsulphatase in lysosomal extracts*

For details, see legend to Fig. 5. The absorbance (515nm) is shown on the ordinate.

radioactivity of the separated pI4.2, 5.0 and 6.0 fractions was 1910, 1260 and 270 c.p.m./mg of protein respectively. The incubated basic fraction yielded a single peak of arylsulphatase activity with pI7.2. The specific ^3H radioactivity of the pI7.2 fraction was 240 c.p.m./mg of protein.

Discussion

Our findings clearly demonstrate that the lysosomal glycoproteins are highly susceptible to autolytic degradation. Autolytic cleavage of *N*-acetylneuraminic acid and peptide was greatest at pH5 (Fig. 1). However, substantial cleavage still occurred over a rather wide pH range. Cathepsin D seems to be responsible for the autolytic digestion of lysosomal protein, as it is prevented by pepstatin (A. Goldstone & H. Koenig, unpublished work), a potent inhibitor of this enzyme (Barrett & Dingle, 1972; Serra *et al.*, 1972). *N*-Acetylneuraminic acid was cleaved more rapidly than the peptide during short incubations at pH5 (Figs. 2 and 3). These kinetic differences suggest that removal of *N*-acetylneuraminic acid may expedite the autolytic digestion of the protein component of the lysosomal glycoproteins. In keeping with this inference *p*-nitrophenyloxamic acid protected *N*-acetylneuraminic acid from autolytic cleavage and also retarded the digestion of protein during a short incubation (Figs. 2 and 3). However, the latter effect could also be due partly to a direct inhibition of cathepsin D by *p*-nitrophenyloxamic acid.

Galactonolactone, gluconolactone and mannonolactone, in combination, inhibited the release of *N*-acetylglucosamine and retarded peptide cleavage during a short incubation (Fig. 4). These lactones did not inhibit β -*N*-acetylhexosaminidase activity in rat kidney lysosomes when measured against a synthetic substrate. Inasmuch as β -*N*-acetylhexosaminidase would be expected to remove only terminal amino sugars, we conclude that the lactones blocked the cleavage of *N*-acetylglucosamine by interfering with enzymic removal of the corresponding monosaccharides located externally to the latter in the carbohydrate chains of the lysosomal glycoproteins. Further, the lactones did not inhibit the activity of rat kidney cathepsin D when measured with denatured haemoglobin as substrate. Therefore they probably retarded the autolytic digestion of peptide through the same mechanism, namely by interfering with the autolytic removal of monosaccharides. These findings provide additional support for the view that the carbohydrate side chains protect the protein core of the lysosomal hydrolases from catheptic attack. Accordingly, the glycoprotein nature of the lysosomal hydrolases may account for their resistance to inactivation during autolysis.

The present isoelectric-focusing experiments showed that only the highly acidic glycoproteins,

which include the acidic form of several hydrolases in rat kidney lysosomes, were extensively labelled by radioisotopic precursors during a short uptake period, whereas the more basic glycoproteins, which contain the basic form of these hydrolases, were essentially unlabelled (Figs. 5 and 6 and Table 1). This pattern of incorporation was clearly demonstrable only when special precautions were taken to keep autolytic degradation of the lysosomal constituents to a minimum, namely rigorous avoidance of acidic conditions during extraction and processing, and the inclusion of the neuraminidase inhibitor, *p*-nitrophenyloxamic acid (0.1%), in the extraction medium and Ampholine gradient.

Exposure of a lysosomal extract to acidic conditions (pH5), even for a brief period in the cold, resulted in an autolytic cleavage of *N*-acetylneuraminic acid and peptide and a concomitant increase in the pI of labelled glycoproteins. Moreover, this treatment profoundly modified the isoelectric focusing behaviour of β -glucuronidase and arylsulphatase [and also β -galactosidase, β -*N*-acetylhexosaminidase and acid phosphatase (Needleman & Koenig, 1973; A. Goldstone & H. Koenig, unpublished work)] in the same direction (Figs. 7 and 8). The possibility that the loss of the acidic form of these hydrolases was due to a selective inactivation of the latter, rather than its conversion into a more basic form, is made unlikely by the finding that the basic form of these hydrolases was increased and the total hydrolase activities unaltered during autolysis. This inference was verified as follows. When an acidic fraction isolated from a lysosomal extract by isoelectric focusing was incubated at pH5, the acidic form of arylsulphatase (pI4.4) was largely converted into two more basic forms with a pI of 5.0 and 6.4 and the *N*-acetylneuraminic acid content of the glycoprotein fractions containing these forms declined by 43 and 87% respectively. When an isolated fraction containing the basic form of arylsulphatase was similarly incubated, the pI of the enzyme increased from 6.4 to 7.2 and the *N*-acetylneuraminic acid content of the glycoprotein in this fraction decreased by 23%. Therefore these results confirm the earlier findings, based on polyacrylamide-gel electrophoresis (Goldstone *et al.*, 1971), showing that the anionic forms of arylsulphatase, β -glucuronidase and five other hydrolases were converted, partially or wholly, into cationic forms during incubation at pH5 owing to the autolytic removal of *N*-acetylneuraminic acid.

The present results provide the first direct evidence for the proposition that each newly synthesized hydrolase is packaged in lysosomes in a highly acidic form. The finding that the less acidic glycoprotein fractions, which contain the basic forms of a number of hydrolases, are essentially unlabelled shortly after administration of radioisotope argues against the view that the basic forms are synthesized and packaged as

separate enzymes. These findings support the scheme for the biosynthesis, intracellular transport and storage of the lysosomal enzymes which is based on an analysis of the solubility and electrophoretic mobility of five acid hydrolases in rat kidney subcellular fractions (Goldstone & Koenig, 1973a; Goldstone *et al.*, 1973) and ultrastructural radioautographic (Nayyar & Koenig, 1972; R. Nayyar & H. Koenig, unpublished work) and radiochemical (Goldstone & Koenig, 1972) findings of radioisotope incorporation into lysosomal glycoproteins. According to this scheme, the various hydrolases are synthesized as bound, basic glycoprotein enzymes in a restricted portion of the rough endoplasmic reticulum. The soluble, acidic forms are generated from the latter by attachment of sugar sequences containing *N*-acetylneuraminic acid as the nascent glycoprotein enzymes migrate through the Golgi apparatus *en route* to the lysosomes (Goldstone & Koenig, 1973a).

Evidence that the basic forms of the lysosomal enzymes are derived from the corresponding acidic forms during biodegradation has been obtained in a radioisotopic turnover study in rat kidney lysosomes (Goldstone & Koenig, 1973b, 1974). This study showed that initially *N*-acetylneuraminic acid turned over twice as rapidly as peptide and the labelled lysosomal glycoproteins became progressively more basic with the passage of time. In addition to a loss of electronegative charge resulting from a removal of *N*-acetylneuraminic acid, lysosomal glycoproteins undergo a cleavage of neutral and amino sugars and peptides during incubation at a mildly acidic pH. Therefore it is unlikely that the basic forms of the lysosomal hydrolases are merely asialo derivatives of the corresponding acidic forms, as suggested earlier (Goldstone *et al.*, 1971). Moreover, in studies involving the treatment of purified arylsulphatase A (Graham & Roy, 1973) and α -galactosidase (Beutler & Kuhl, 1972) with bacterial neuraminidase, the desialylated enzymes demonstrated the expected change in electrical charge, but retained the other properties of the native enzymes, e.g. substrate specificity, heat stability, sedimentation coefficient. On the other hand, a sequential cleavage of neutral and amino sugars catalysed by the concerted action of the appropriate lysosomal glycosidases would be expected to modify the molecular conformation and physicochemical and enzymic properties of partially degraded lysosomal hydrolases. Further, a limited cleavage of peptide moieties, possibly in the form of glycopeptides, may also alter the molecular conformation and properties of incompletely degraded enzymes. Therefore as a working hypothesis we propose that the basic forms of the various hydrolases present in lysosomes are produced during intracellular digestion *in vivo* through a partial autolytic degradation of the corresponding acidic forms.

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