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Glycoprotein Catabolism in Rat Liver

LYSOSOMAL DIGESTION OF IODINATED ASIALO-FETUIN

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^{125}I-labelled asialo-fetuin, administered intravenously, rapidly accumulates in rat liver and the radioactivity is subsequently cleared from the liver within 60min. Plasma radioactivity reaches a minimum between 10 and 15 min after injection and rises slightly during the period of liver clearance. Free iodide is the only radioactive compound found in plasma during this latter period. Fractionation of rat liver at 5 and 13min after injection of ^{125}I-labelled asialo-fetuin supports the hypothesis that asialoglycoprotein is taken into liver by pinocytosis after binding to the plasma membrane and is then hydrolysed by lysosomal enzymes. At 5min, radioactivity was concentrated 23-fold in a membrane fraction similarly enriched in phosphodiesterase I, a plasma-membrane marker enzyme, whereas at 13min the radioactivity appeared to be localized within lysosomes. Separation of three liver fractions (heavy mitochondrial, light mitochondrial and microsomal) on sucrose gradients revealed the presence of two populations of radioactive particles. One population banded in a region coincident with a lysosomal marker enzyme. The other, more abundant, population of radioactive particles had a density of 1.13 and contained some phosphodiesterase, but very little lysosomal enzyme. These latter particles appear to be pinocytotic vesicles produced after uptake of the asialo-fetuin bound by the plasma membrane. Lysosomal extracts extensively hydrolyse asialo-fetuin during incubation *in vitro* at pH4.7 and iodotyrosine is completely released from the iodinated glycoprotein. Protein digestion within lysosomes was demonstrated by incubating intact lysosomes containing ^{125}I-labelled asialo-fetuin in iso-osmotic sucrose, pH7.2. The radioactive hydrolysis product, iodotyrosine, readily passed through the lysosomal membrane and was found in the external medium. These results are not sufficient to account for the presence of free iodide in plasma, but this was explained by the observation that iodotyrosines are deiodinated by microsomal enzymes in the presence of NADPH.

The liver from both the rat and rabbit has the specific ability to remove serum glycoproteins from circulation when the penultimate galactose units of their carbohydrate moiety have been exposed by prior removal of the terminal sialic acid residues (see review by Ashwell & Morell, 1974). The uptake mechanism is specific for asialo-proteins, requires intact galactosyl residues (Van Den Hamer *et al.*, 1970) and involves a binding protein present on the liver plasma membrane (Hudgin *et al.*, 1974). The reticuloendothelial system is not involved in this metabolic function, as the asialo-protein enters hepatocytes, not Küpfer cells, and subsequently is processed through the liver lysosomes (Gregoriadis *et al.*, 1970). Blood clearance of other proteins has also been demonstrated. Denatured albumin enters Küpfer cells (Benacerraf, 1958) as does haemoglobin (Goldfischer *et al.*, 1970), whereas ribonuclease is rapidly removed from circulation by mouse kidney (Davidson, 1973). These latter systems do not involve hepatic parenchymal cells nor do they exhibit the great specificity which characterizes the liver uptake of asialo-glycoproteins.

The Ashwell & Morell (1974) system provides a unique opportunity to investigate heterophagy of serum glycoproteins. Gregoriadis *et al.* (1970) followed the liver metabolism of asialo-caeruloplasmin by radioactively labelling the terminal galactose residues and the bound copper moiety of this metal-transport protein. It was found that both radioactive labels accumulated in the lysosomes and subsequently the radioactivity could not be immunoprecipitated. Since these results do not directly determine the digestive fate of the peptide portion of the molecule, we have undertaken a detailed study of asialo-glycoprotein heterophagy to emphasize the individual steps which occur after the initial uptake of the protein by hepatocytes. This report describes experimental data from studies both *in vitro* and *in vivo* on the lysosomal digestion

of ^{125}I-labelled asialo-fetuin, and the results depict a unified process of hepatic clearance and degradation of this glycoprotein.

Experimental

Iodination of fetuin

Fetuin [purified by the method of Spiro (1960)] was purchased from Grand Island Biological Co., Grand Island, N.Y., U.S.A., and was treated with 0.1M-EDTA, pH7, at 37°C for 2h. The EDTA was removed by extensive dialysis against water and the protein was desialylated by mild acid hydrolysis (Tuppy & Gottschalk, 1972).

The iodination procedure of David & Reisfeld (1974) was used to prepare ^{125}I-labelled asialo-fetuin. Generally iodine was incorporated into the reaction product only as monoiodotyrosine residues. In one preparation a higher efficiency of ^{125}I incorporation was obtained by increasing 10-fold the total H_2O_2 and carrier KI used and by adding the reagents in five equal batches over a 2h period. This latter high-specific-radioactivity glycoprotein preparation contained both mono- and di-iodotyrosine, which were found as hydrolysis products during proteolysis.

Column chromatography

A column (1.5cm×95cm) of Sephadex G-25 (fine grade) equilibrated with 0.2M-acetic acid was used to separate the low-molecular-weight components in plasma samples and in lysosomal digests of asialo-fetuin. The flow rate was maintained at 15ml/h and fractions (approx. 3ml) were collected. Before application to the column, the sample was mixed with 2mg of KI, 0.5mg of 3′-monoiodotyrosine and where appropriate 0.6mg of 3′,5′-di-iodotyrosine. The sample was acidified to pH2–3 with 6M-acetic acid, and if necessary clarified by low-speed centrifugation. The elution pattern of a lysosomal digest of asialo-fetuin and the elution volumes of a number of standard compounds were determined and are shown in Fig. 1. Appropriate detection of the standard compounds was accomplished by measuring u.v. absorbance at 280nm, radioactivity in a Packard Auto-Gamma scintillation spectrometer, ninhydrin reactivity, or precipitability with $AgNO_3$ (for iodide). The excellent resolution of the three radioactive hydrolysis products (mono- and di-iodotyrosine and I^-) was due to a strong retardation of the iodotyrosines. Incubations of $Na^{125}I$ and 3′-[^{125}I]iodo-L-tyrosine (Amersham/Searle, Arlington Heights, Ill., U.S.A.) with plasma, lysosomal fractions and asialo-fetuin followed by chromatography on Sephadex G-25 established that only negligible amounts of these radioactive compounds complex with the proteins eluted at the void volume.

In one series of experiments, a column of different dimensions (2.5cm×20cm) and flow rate (24ml/h) was used. This column gave satisfactory resolution of the radioactive substrate and the three radioactive products with the advantage that a sample could be processed in less than half the time required for the above column. The elution volumes of other materials were not checked in this system.

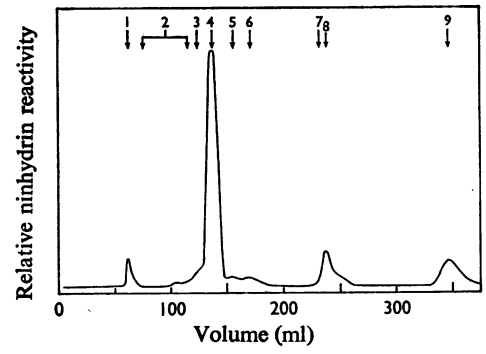

Fig. 1. *Resolution of degradation products of iodinated asialo-fetuin by gel chromatography*

After 23h of incubation at 37°C a sample of a reaction mixture, containing approx. 1mg each of purified lysosomal extract and ^{125}I-labelled asialo-fetuin, was prepared for chromatography as described in the text and fractionated on a column (1.5cm×95cm) of Sephadex G-25 (fine grade). The elution volume of various standard compounds is indicated by arrows and numbered as follows: 1, proteins; 2, oligopeptides; 3, a mixture of eight non-aromatic dipeptides; 4, a mixture of ten non-aromatic amino acids; 5, KI and phenylalanine; 6, tyrosine; 7, tryptophan; 8, monoiodotyrosine; 9, di-iodotyrosine.

Whole-animal experiments

Male rats (150–225g) of the Wistar strain were provided by The Pennsylvania State University Small Animal Facility. Administration of the radioactive protein was accomplished by a 0.2–0.6ml injection of ^{125}I-labelled asialo-fetuin (0.1–0.3mg, 1×10^6–7×10^6c.p.m. in 0.85% NaCl) into a lateral tail vein of a restrained rat. Blood samples after injection were obtained by nicking the tail on the side opposite the injection site and collecting the blood in heparinized capillary tubes. After centrifugation, a measured portion of plasma (10–40μl) was pipetted into a test tube for measurement of radioactivity. Plasma for column chromatography was obtained from blood collected by heart puncture from animals anaesthetized by administration of an intraperitoneal injection of sodium pentobarbital (6.5mg/100g body wt.).

Liver fractionation

The animals were killed by decapitation and the livers quickly removed and perfused with cold 0.25 M-sucrose. Subsequent fractionation followed the differential-centrifugation method of de Duve *et al.* (1955). Highly purified lysosomal extracts for studies *in vitro* were prepared as described by Leighton *et al.* (1968) 4 days after intraperitoneal injection of Triton WR-1339 (Ruger Chemical Co., Irvington, N.J., U.S.A.). The detergent-filled lysosomal fraction was concentrated in an ultrafiltration apparatus and then equilibrated with a buffer solution [10mM-sodium acetate (pH4.7)–1mM-2-mercaptoethanol–0.1mM-EDTA). The extract was stored in frozen batches (3mg of protein/ml) until used. The sucrose solutions used in the isolation and incubation of an intact lysosomal fraction were buffered with 10mM-Tris–acetate, pH7.2, and contained 2mM-2-mercaptoethanol and 1mM-$MgCl_2$. Total radioactivity in the liver was determined by counting a weighed tissue slice or a measured sample of the total homogenate.

Digestions of ^{125}I-labelled asialo-fetuin in vitro

Digestions of ^{125}I-labelled asialo-fetuin *in vitro* were performed in a medium containing 100mM-sodium acetate buffer, pH4.7, 15mM-2-mercaptoethanol, 2.5mM-$MgCl_2$, 2.5mM-$MnCl_2$, 1mM-$CaCl_2$ and 35mM-KCl. Solutions of lysosomal extract and glycoprotein substrate (approx. 2mg/ml each) were mixed just before the start of incubation at 37°C. Samples for ninhydrin and acid-solubility determinations were taken at various times by pipetting 20μl portions of the incubated reaction mixture into 0.5ml of ice-cold water. The diluted samples were then stored frozen for later assay. Acid-soluble radioactivity was obtained by first adding a 20μl portion of sample to a carrier of 1–2mg of fetuin in a total volume of 0.5ml followed by an equal volume of 4% (w/v) phosphotungstic acid solution in 2M-HCl. The resulting suspension was left at 4°C for 15min. After low-speed centrifugation, the supernatant was removed and the pellet washed with 1ml of half-strength phosphotungstic acid solution. The pellet and the combined supernatant were then separately counted for radioactivity.

Lysosomal fractions [fraction L of de Duve *et al.* (1955)] obtained from animals previously injected with ^{125}I-labelled asialo-fetuin were incubated in 0.25 M-sucrose containing 10mM-Tris–acetate, pH7.2, 2mM-2-mercaptoethanol and 1mM-$MgCl_2$, in order to follow digestion of radioactive protein trapped within intact lysosomes. Latency of lysosomal enzymes, a measure of the integrity of the lysosomal membrane, was determined by comparing *N*-acetyl-β-D-hexosaminidase activity during a 3min incubation in the presence and absence of 0.2% Triton X-100. Further characterization of a similarly prepared L fraction was made by density-gradient centrifugation by using a continuous 15–54% (w/w) sucrose gradient in a Spinco 25.2 rotor. The gradients were centrifuged at 75000$g_{av.}$ for 4h and were fractionated with a tube-piercing device. Details of these experiments are provided in the Results section.

Analytical methods

Protein was determined by the method of Miller (1959), with bovine serum albumin as a standard. Ninhydrin reactivity was assayed as described by Rosen (1957), with the results expressed as leucine equivalents. The extent of asialo-fetuin proteolysis was based on a factor of 7.78μmol of ninhydrin reactivity/mg of glycoprotein representing 100% hydrolysis, the value of which was calculated from a published amino acid composition of fetuin (Graham, 1972). Procedures previously described (Touster *et al.*, 1970; LaBadie & Aronson, 1973) were used for the determination of marker enzymes for lysosomes (*N*-acetyl-β-D-hexosaminidase), microsomal fraction (non-specific esterase) and plasma membranes (alkaline phosphodiesterase I).

Results

Plasma clearance, liver uptake and liver clearance of radioactivity from intravenously injected ^{125}I-labelled asialo-fetuin

First-order kinetics of plasma clearance were observed for approximately the first 5min after injection of ^{125}I-labelled asialo-fetuin. The average half-life of radioactivity in the blood was 92s (S.D.$\pm$15, 13 animals tested). Typical clearance of plasma radioactivity (Fig. 2) may be divided into three sections, (1) an initial exponential decrease, (2) a minimum value of approx. 5% of the zero-time value around 10min, and (3) a small but reproducible increase to a relatively constant value, 5–10% of the initially injected radioactivity, for the remainder of the first hour after injection. The radioactivity rapidly accumulated in the liver, so that at 10min 76.5$\pm$4% of the injected radioactivity was found in this organ (five animals tested). However, at 30min only 18% of the total radioactivity remained in the liver and after 45min this decreased to less than 5$\pm$3% of the injected dosage (11 animals). The bile duct was cannulated and bile secretion was collected for a period of 60min during the course of three experiments. Only low radioactivity, amounting to less than 5% of that injected, was detected in the bile fluids. The circulatory system therefore must act as the principal vector for the observed clearance of the radioactivity from the liver.

Identity of the radioactivity in the blood

Samples of blood were taken by heart puncture at various times after administration of 0.2mg of

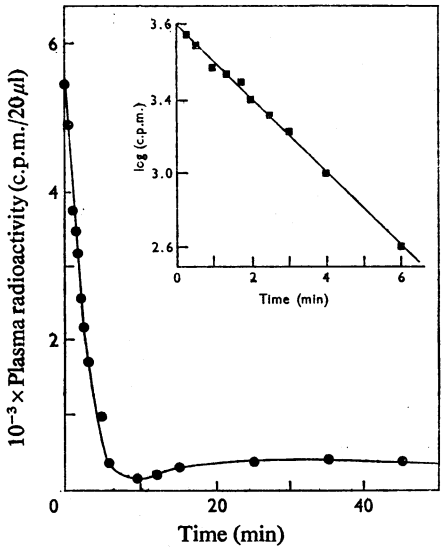

Fig. 2. *Radioactivity in plasma after injection of ^{125}I-labelled asialo-fetuin*

An intravenous injection of 0.26mg of ^{125}I-labelled asialo-fetuin (1.88×10^6c.p.m.) in 0.5ml of 0.85% NaCl was administered to a 170g rat. At the indicated times, blood samples were taken and 20µl of plasma was counted for radioactivity. The inset shows the exponential decrease of plasma radioactivity for the first 6min (ten time-points). The following data were calculated for this experiment: slope of plasma clearance [log (c.p.m.)/min] = −0.198. Half-life of ^{125}I-labelled asialo-fetuin in plasma = 91s. Total radioactivity in blood at zero time (based on 3.4ml of plasma/100g animal weight) = 1.82×10^6c.p.m.

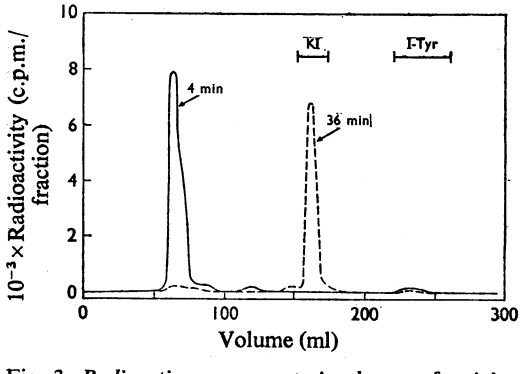

Fig. 3. *Radioactive components in plasma after injection of ^{125}I-labelled asialo-fetuin*

Blood was collected by heart puncture at intervals after injection of ^{125}I-labelled asialo-fetuin. The plasma was separated from the cellular components and fractionated on Sephadex G-25 as described in the Experimental section. Bars indicate the elution volumes of added KI and mono-iodotyrosine. ——, 4min after injection; ----, 36min after injection.

^{125}I-labelled asialo-fetuin. Sephadex chromatography of plasma at 3 min after injection showed no significant radioactivity (less than 5% of the total) in any fractions except those eluted with the high-molecular-weight materials (Fig. 3). In contrast, the radioactive components of plasma obtained 35min after injection consisted of 94% iodide, 1% iodotyrosine and 4% excluded radioactivity. Up to 1h after glycoprotein injection the only significant concentration of radioactivity to be found in other tissue was in the stomach walls and its contents, which accumulated approx. 10% of the injected label. On injection, free iodide is known to behave in a similar manner (Rhodes, 1968). The radioactive product leaving the liver thus is either free iodide or a transient precursor which, on entering the blood, is rapidly assimilated and deiodinated in another tissue before being released into the circulation as iodide. As shown below, the liver is the likely site of deiodination.

Deiodination of [^{125}I]iodotyrosine in vivo

An injection of a tracer amount of [^{125}I]iodo-L-tyrosine was followed for blood clearance, organ location and identity of the labelled compound. Clearance of the label from the blood was so rapid that a meaningful half-life could not be calculated (less than 1min). The radioactivity in plasma from blood collected 3min after injection was principally iodide (>80%). No organ was found to have accumulated a high proportion of the radioactivity (all less than 7% of the injected dose). The organs tested were liver, kidneys, spleen, stomach, intestines, contents of the gastrointestinal tract and the thyroid. Incubation of [^{125}I]iodotyrosine with whole heparinized blood did not result in the production of free iodide.

Subcellular localization of ^{125}I-labelled asialo-fetuin in the liver

Fractionation of the liver 5min after injection of ^{125}I-labelled asialo-fetuin (Table 1) revealed a 23-fold concentration of radioactivity in P_2, a subfraction purified from the microsomal fraction which is rich in plasma-membrane fragments (Touster *et al.*, 1970), most likely coming from the blood-sinusoidal surfaces (Wisher & Evans, 1975). Gregoriadis *et al.* (1970) observed a similar concentration of labelled asialo-caeruloplasmin in the liver microsomal fraction early after injection, but their work was not further extended to correlate this observation with the plasma-membrane location of the radioactive protein. Studies *in vitro* have clearly shown that asialo-glycoprotein uptake by hepatocytes is initiated by a specific binding to a plasma-membrane glycoprotein (Hudgin *et al.*, 1974). When tissue fractionation was performed 13min after injection, ^{125}I-labelled asialo-fetuin radioactivity was most

Table 1. *Distribution of radioactivity in liver fractions 5 min after injection of ^{125}I-labelled asialo-fetuin*

A rat was killed 5 min after injection of 0.3 mg of ^{125}I-labelled asialo-fetuin (9.3×10^5 c.p.m.) and the liver fractionated as described by Touster *et al.* (1970). The fractions obtained were: (H) total homogenate (E+N), (E) postnuclear supernatant, (N) nuclear, (ML) heavy plus light mitochondrial, (P) microsomal, (S) supernatant and (P_2) a fraction enriched in plasma membranes isolated by density-gradient flotation of fraction P. Relative specific activity is defined as the specific activity of the fraction divided by the specific activity of the homogenate. Specific radioactivity is defined as c.p.m./mg of protein. Enzyme units are μmol of substrate hydrolysed/min.

	Fraction							Recovery
Assay	H (units/mg)	E	N	ML	P	S	P_2	(%)
Protein (mg)	—	1090	68	315	212	445	13	90
		Relative specific activity						
Radioactivity	480	1.0	0.9	0.5	2.5	0.5	22.6	85
Esterase	3.4	1.0	0.7	0.7	3.9	0.1	3.1	98
Hexosaminidase	0.01	1.0	1.0	2.4	0.3	0.4	1.2	95
Phosphodiesterase I	0.04	0.8	3.2	0.4	2.8	0.1	22.0	86

Table 2. *Distribution of radioactivity in liver fractions 13 min after injection of ^{125}I-labelled asialo-fetuin*

The liver of a rat killed 13 min after injection of 0.25 mg of ^{125}I-labelled asialo-fetuin (4.51×10^6 c.p.m.) was fractionated by the method of de Duve *et al.* (1955). The sucrose solution used in the fractionation was buffered as described in the Experimental section. Data are presented as described in the legend for Table 1.

	Fraction						Recovery
Assay	H (units/mg)	E	N	M	L	P	S	(%)
Protein (mg)	—	1060	306	199	58	176	460	88
		Relative specific activity						
Radioactivity	1911	1.2	0.2	0.8	5.6	2.1	0.5	87
Hexosaminidase	0.02	1.2	0.3	2.1	7.6	0.9	0.1	84
Esterase	2.9	1.1	0.7	0.6	2.3	4.3	0.1	96

enriched in the L fraction (Table 2), indicating its shift from the plasma membrane to the lysosomes.

Degradation of ^{125}I-labelled asialo-fetuin in vitro by lysosomal extracts and within intact lysosomes

Extracts of purified Triton-filled lysosomes are capable of extensively digesting the peptide portion of fetuin and asialo-fetuin (Aronson & de Duve, 1968). When ^{125}I-labelled asialo-fetuin (containing 2.2 mg of non-labelled asialo-fetuin and 0.02 mg of ^{125}I-labelled asialo-fetuin) was incubated for 20 h with such a lysosomal preparation, peptide cleavage was 80 and 85% of the theoretical, based respectively on the increase in ninhydrin colour and on the acid-soluble radioactivity produced. On Sephadex chromatography of this digest mixture, 95% of the radioactivity co-chromatographed with a mono-iodotyrosine standard, whereas the remaining 5% appeared as free iodide (Fig. 4).

At 13 min after injection of ^{125}I-labelled asialo-fetuin into a rat, the liver was fractionated (see Table

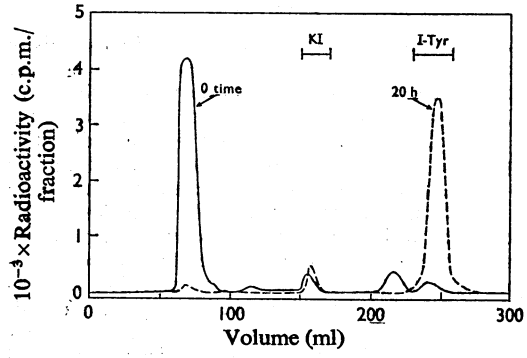

Fig. 4. *Digestion of ^{125}I-labelled asialo-fetuin by extracts of purified lysosomes*

^{125}I-labelled asialo-fetuin (0.02 mg) plus a carrier of 2.2 mg of asialo-fetuin was incubated with 2.4 mg of an extract of purified lysosomes at 37°C in a pH 4.7 buffer solution as described in the Experimental section. Samples were analysed by column chromatography on Sephadex G-25: ——, zero-time incubation; – – – –, 20 h incubation.

2) and the isolated L fraction, including intact lysosomes containing radioactive material, was incubated in iso-osmotic buffer, pH7.2 (see the Experimental Section). The lysosomes were very stable during this incubation at 30°C, since there was only a 5% decrease in the initial latency (88–90%) of *N*-acetyl-β-D-hexosaminidase during the 4h of the experiment. The intactness of the lysosomes was also indicated by the fact that at 2h 97% of the total hexosaminidase activity was pelleted by centrifugation at 30000*g* for 15min. Column chromatography was used to measure the distribution of degradation products in a sample of the total reaction mixture which was obtained by adding Triton X-100 (Fig. 5*a*). This pattern of products was then compared with that found in the non-sedimentable portion of the incubation which was obtained after centrifugation of a reaction sample at 30000*g* for 15min (Fig. 5*b*). At zero time the detergent-solubilized total sample contained small amounts of mono- and di-iodotyrosine, with most of the radioactive material being high-molecular-weight (Fig. 5*a*). After 4h of incubation the content of iodotyrosines increased and additionally a small amount of radioactive iodide appeared. The radioactivity found collectively as these three degradation products increased from 5% of the total radioactivity in the L fraction at the beginning of the experiment to 17% after 4h of incubation.

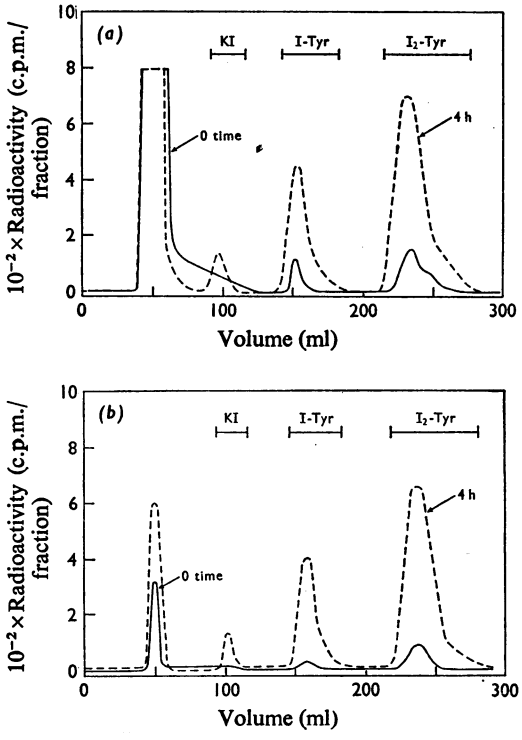

Fig. 5. *Digestion of ^{125}I-labelled asialo-fetuin within intact lysosomes*

A liver fraction, enriched in lysosomes and radioactivity (fraction L, Table 2), containing 58mg of protein and 6.2×10^5c.p.m. in 8ml total volume was incubated at 30°C in a pH7.2 sucrose solution as described in the Experimental section. The identity of the radioactive components of the reaction was determined by chromatographing 1ml portions of the incubation mixture on a column (2.5cm×20cm) of Sephadex G-25. Intactness of the lysosomal membrane was determined by measuring the latency and sedimentability of *N*-acetyl-β-D-hexosaminidase (see the text). The digestion of ^{125}I-labelled asialo-fetuin was followed by measuring acid-soluble and non-sedimentable radioactivity. (*a*) Composition of the total reaction mixture which was obtained by solubilizing a sample with Triton X-100. (*b*) Composition of the non-sedimentable supernatant portion of the reaction mixture which was obtained by centrifuging for 16min at 30000*g* a sample diluted 3-fold with ice-cold 0.25M-sucrose. Bars above the chromatogram indicate the elution volumes of carrier compounds added to the samples before chromatography. ——, Zero-time incubation; ----, 4h incubation.

Most of the degradation products arising from the hydrolysis of ^{125}I-labelled asialo-fetuin were released to the external medium (Fig. 5*b*), as evidenced by the increased radioactivity found in the supernatant fraction at 4h as iodide and iodotyrosine. The monomeric products must easily have crossed the lysosomal membrane, since at this time almost all (82%) of the iodide and iodotyrosine radioactivity in the total sample was present in the external medium in a non-sedimentable form. The hydrolysis of ^{125}I-labelled asialo-fetuin in intact lysosomes appeared to have ceased within 1h after the start of incubation. Determinations made at hourly intervals showed that the fraction of total radioactivity which was non-sedimentable increased from 6% initially to 16% at 1h. During the remaining 3h of incubation, only a 4% further increase in extralysosomal radioactivity was noted. Rapid initial but incomplete total hydrolysis of included protein has also been observed in similar experiments by Mego & McQueen (1965) and by Davidson (1973). Initially the non-sedimented samples (Fig. 5*b*) contained only 2% of the corresponding amount of radioactivity in the high-molecular-weight component of the total sample (Fig. 5*a*), and after 4h this value increased to 3%. Since this material probably leaked out of damaged organelles, this again indicates the overall intact nature of the lysosomes during the experiment.

The iodide produced during lysosomal digestion of the iodinated asialo-fetuin (Fig. 5) may have been due to a deiodination activity of the microsomal component in fraction L (see Table 2). Only 11% of the low-molecular-weight radioactivity (iodide, mono- and di-iodotyrosine) in a 2h incubation of intact

Table 3. *Deiodination of iodotyrosines by lysosomal and microsomal fractions*

A rat liver was fractionated in pH 7.2 buffered sucrose (see the Experimental section) 10min after injection of ^{125}I-labelled asialo-fetuin (0.25mg, 5.74×10^6c.p.m.). Portions of the intact L and P fractions (4.9 and 3.7mg respectively) were incubated in 2ml of this iso-osmotic sucrose solution at 30°C. NADH and NADPH, 3mg each, were added as indicated. After 2h, the extent of deiodination of the lysosomal hydrolysis products was determined by Sephadex G-25 chromatography.

Fractions	Addition of NADH and NADPH	Content (% of low-mol.-wt. radioactivity)		
		Iodide	Monoiodo-tyrosine	Di-iodo-tyrosine
L	(–)	11	24	65
L	(+)	94	1	5
L+P	(+)	97	1	2
P+[^{125}I]iodo-tyrosine	(+)	100	0	0

lysosomes is free iodide (Table 3). Addition of NADH and NADPH without or with added microsomal protein resulted in 94 and 97% deiodination of the iodotyrosine. Similar incubation of the microsomal fraction (P) alone with added [^{125}I]-iodotyrosine results in the complete deiodination of the substrate.

Isopycnic centrifugation of an intact L fraction containing ^{125}I-labelled asialo-fetuin

The liver of a rat injected with ^{125}I-labelled asialo-fetuin 12min before being killed was fractionated as described for Fig. 5. Samples of the M, L and P fractions were further subdivided by centrifugation through a sucrose gradient. The recovered fractions were assayed for hexosaminidase, phosphodiesterase and radioactivity. The results for the L fraction are depicted in Fig. 6. Hexosaminidase, a lysosomal marker, bands in the dense region of the gradient, whereas phosphodiesterase, a marker for plasma membranes, forms a wider band which is less dense. The distribution of these two enzymes was essentially the same in the gradients for the M and P fractions (not shown).

The distribution of radioactivity in the gradient was bimodal. One component was coincident with hexosaminidase activity and is presumably lysosomal. The highest ^{125}I radioactivity was found in fractions of very low density (fraction 11, $\rho = 1.13$) and this did not correspond to the distribution of either marker enzyme assayed, although these fractions did contain considerable phosphodiesterase. The proportion of radioactivity in the upper part of the gradient varied with the liver fraction

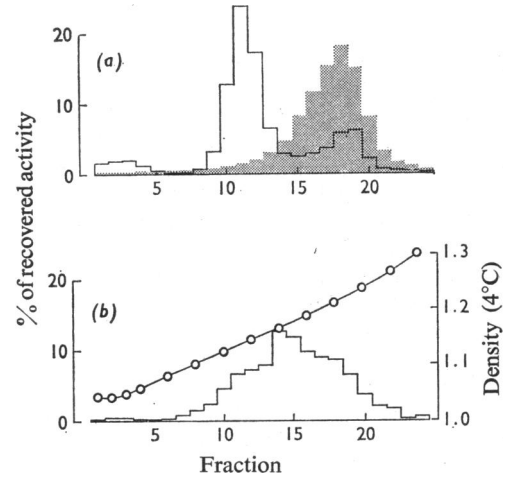

Fig. 6. *Isopycnic centrifugation of a lysosomal fraction obtained 12min after injection of ^{125}I-labelled asialo-fetuin*

A rat was treated with an injection of ^{125}I-labelled asialo-fetuin (0.3mg, 3.84×10^6c.p.m.) and at 12min the liver was removed, homogenized and fractionated in buffered sucrose (see the Experimental section). Portions (8ml) of fractions M (70mg of protein), L (56mg) and P (171mg) were layered on top of separate sucrose gradients made by mixing equal weights of 15 and 54% (w/w) sucrose solutions. The samples were centrifuged for 4h at 25000rev./min in a Spinco SW 25.2 rotor at 3°C. Fractions of each gradient were collected with a tube-piercing device and were analysed for radioactivity, *N*-acetyl-β-D-hexosaminidase and phosphodiesterase. Only the results obtained for the L-fraction gradient are shown. The height of each bar represents the percentage of the total activity recovered from the gradient. (*a*) Upper curves: solid line, radioactivity; stippled area, hexosaminidase. (*b*) Lower curves: solid line, phosphodiesterase; ○, density based on sucrose concentrations measured with an Abbe 3L refractometer (Bausch and Lomb).

analysed. For fractions M, L and P the radioactivity in the low-density region was respectively 1.3-, 3.6- and 12.5-fold as great as the radioactivity which was located at high density. Some 88–93% of the radioactivity in the light fractions could be sedimented at 100000*g* after dilution with 0.25M-sucrose, indicating the particulate nature of this material.

Discussion

Previous studies of hepatic uptake of asialo-glycoproteins have concentrated on characterizing this process in terms of the interaction of the protein with the plasma membrane (Ashwell & Morell, 1974). Some evidence supports the assumption that, after entering hepatic lysosomes, the asialo-glycoprotein is degraded to free amino acids

and carbohydrates. The evidence includes the progressive loss of radioactive label from the liver after uptake, a progressive decrease of immunoprecipitable ^{64}Cu-labelled caeruloplasmin, and a progressive change in the ratio of radioactive galactose to ^{64}Cu of dual-labelled asialo-caeruloplasmin after its clearance from the circulatory system (Gregoriadis *et al.*, 1970). In addition, a number of investigators have shown the release of radioactive degradation products from intact lysosomes isolated from other tissues, containing iodinated ribonuclease or albumin (Davidson, 1973; Mego & McQueen, 1965). Our data provide direct evidence as well as additional indirect evidence for the heterophagic processing of asialo-glycoproteins by hepatocytes. Each stage of heterophagy was observed in our experiments, including protein uptake in phagosomes, transfer of substrate from phagosomes to lysosomes, peptide digestion to free amino acids, release of these products from the lysosomes and their reutilization by other metabolic compartments.

Digestion of proteins *in vitro* by lysosomal extracts has been demonstrated by a number of workers (Aronson & de Duve, 1968; Mahadevan *et al.*, 1969). The extent of hydrolysis of asialo-fetuin attained in the present study is somewhat higher than was reported by Aronson & de Duve (1968) and may reflect the addition of known cathepsin activators in our incubation medium. In longer incubations (48h) over 90% of the peptide bonds are hydrolysed. This rate is not sufficient to account for the rapid clearance of the iodine label of ^{125}I-labelled asialo-fetuin from rat liver (30–50min after uptake) but may be explained by a higher dilution of lysosomal cathepsins in the digestion *in vitro*. Alternatively, it may be that the release of iodotyrosine in particular and aromatic residues in general are not representative of the overall rate of digestion. Kussendrager *et al.* (1972) reported that tyrosine and phenylalanine are released as free amino acids from the B chain of insulin by lysosomal extracts at a much higher rate than the remaining amino acids are released. This difference was attributed to the initial action of an endopeptidase (cathepsin D) preferentially acting at aromatic residues and exposing those residues to lysosomal exopeptidases sooner than the bulk of the protein is exposed. In our experiments the rate of release of acid-soluble radioactivity from ^{125}I-labelled asialo-fetuin was somewhat higher than the appearance of ninhydrin-reactive groups, a result that corroborates this proposal.

The digestions *in vitro* described above indicate that lysosomal proteinases are capable of extensive or complete hydrolysis of asialo-fetuin to free amino acids, including iodotyrosine. Cell-fractionation studies 10–15min after injection of ^{125}I-labelled asialo-fetuin (Table 2) demonstrate that the protein does enter the lysosomes. However, the distribution of radioactivity does not exactly correspond to the lysosomal distribution and suggests a heterogeneity of particles containing radioactivity. This was also observed by Davidson (1973) in mouse kidney and Mego & McQueen (1965) in mouse liver. Both of these investigations showed that slower-sedimenting particles were less digestively active when incubated in iso-osmotic medium.

This hypothesis is confirmed herein by showing that two populations of radioactive particles, differing in their density and enzyme content, exist in rat liver at short times after injection of asialo-fetuin. Digestion of the administered glycoprotein is presumably occurring in those particles accumulating in the lysosomal region of the gradient. The remainder of the radioactivity (81.6% of the total radioactivity found in the M, L and P fractions at 12min after injection) is in a particle of very low density containing almost no *N*-acetyl-β-D-hexosaminidase, which we consider is the pinocytotic vesicle or phagosome.

The phagosome would not be digestively active, since it has not fused with lysosomes, and this accounts for the high content of unhydrolysed protein observed in the experiments described in Fig. 5, a result which is contrary to the rapid digestion observed *in vivo*. Considering the bimodal distribution of radioactivity in the L fraction, a recalculation of the extent of hydrolysis obtained in the intact lysosome experiments can be made. The radioactivity released in non-sedimentable form during a 1h incubation amounts to 74% of the radioactivity in the phagolysosomes, although this represents only 16% of the total L fraction radioactivity. Davidson (1973) established that although intact mouse kidney lysosomes did not completely degrade included ribonuclease, the initial rate of proteolysis was high enough to account for the rapid degradation that was observed *in vivo*. This result plus our data suggest that the intact lysosome system does approach the rate of digestion *in vivo* for included proteins.

The permeability of the lysosomal membrane to iodotyrosine is evident from the experiment in which intact lysosomes containing ^{125}I-labelled asialo-fetuin were incubated in iso-osmotic sucrose. At the time-points measured, although only 15–25% of the total radioactivity was not associated with the sedimentable fraction, 80–90% of the iodinated hydrolysis products were found in the non-sedimentable fraction and thus were released to the medium. In the cell, it is likely that after release from the lysosome, the iodotyrosine is deiodinated by microsomal enzymes in a reaction requiring NAD(P)H (Dumas *et al.*, 1973). The free iodide would then be released directly to the blood and enter the iodide cycle described by Rhodes (1968).

However, the extremely rapid deiodination of intravenously injected monoiodotyrosine *in vivo* does not permit us to distinguish unequivocally between liver deiodination and its release as the iodinated amino acid before deiodination elsewhere, as either sequence would account for our observation *in vivo* that the form of plasma radioactivity is essentially all iodide. On using a perfused rat liver which has been supplied with ^{125}I-labelled asialo-fetuin, we have recently found that the only radioactive metabolite which accumulates in the perfusate is iodide (W. A. Dunn & N. N. Aronson, Jr., unpublished work). This is strong support for the liver deiodination of the iodotyrosine *in vivo*.

It is not known whether the behaviour of iodotyrosine, especially the rapidity of passage through the lysosomal membrane, mimics the behaviour of the natural amino acids. Only one other amino acid derivative has been studied in this system. Gregoriadis & Ryman (1973) reported that 90% of the radioactivity from ε-*N*-[*Me*-^{3}H]methyl-lysine is retained in rat liver 60min after injection of asialo-fetuin containing this modified amino acid. Digestion of methylated asialo-fetuin *in vitro* by a lysosomal extract results in the complete release of free methyl-lysines, and the rate of lysosomal proteolysis of asialo-fetuin with this peptide alteration is not decreased (LaBadie *et al.*, 1975). Methyl-lysine possibly either is released from the lysosome only slowly or is retained in the liver by a metabolic pathway which reutilizes this amino acid. The differences observed in the metabolic behaviour of iodinated and methylated asialo-fetuin are not currently understood.

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