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The role of extra-hepatic tissues in the receptor-mediated plasma clearance of glycoproteins terminated by mannose or *N*-acetylglucosamine

Paul H. SCHLESINGER,* Jane S. RODMAN,* Thomas W. DOEBBER,*
Philip D. STAHL,* Yuan Chuan LEE,† Christopher P. STOWELL†
and Teresa B. KUHLENSCHMIDT†
**Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, MO 63110, U.S.A. and †Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.*

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The mannose- and *N*-acetylglucosamine-specific pathway for the clearance of mammalian glycoproteins has been characterized by using ^{125}I-labelled neoglycoproteins, glycosidase-treated orosomucoid and lysosomal glycosidases (β-glucuronidase and β-*N*-acetylglucosaminidase) as probes. There are two components to this pathway *in vivo*; one liver-dependent and the other extrahepatic or liver-independent. Cells that mediate clearance by the latter component of the pathway are present in spleen, bone and in elements of the reticuloendothelial system, but not in the kidney. Glycoproteins that possess terminal mannose, glucose or *N*-acetylglucosamine residues, including various lysosomal enzymes, are rapidly cleared from plasma via this pathway. Glucose-terminated glycoproteins are recognized by two pathways in the intact animal; the hepatic galatose-specific pathway and the mannose/*N*-acetylglycosamine-specific pathway, which is present in liver and in peripheral tissues. Following removal of the liver by surgical evisceration, glucose-terminated glycoproteins are cleared whereas glycoproteins bearing galactose are not cleared. Uptake of ^{125}I-labelled neoglycoproteins and agalacto-orosomucoid by isolated alveolar macrophages closely mimics clerance *in vivo* by the mannose/*N*-acetylglucosamine pathway. Neoglycoproteins terminated by mannose, glucose or *N*-acetylglucosamine all compete with ^{125}I-labelled agalacto-orosomucoid for uptake by receptor-mediated pinocytosis. The extent of substitution of the neoglycoproteins is a critical determinant of their inhibitory potency. It is proposed that mononuclear phagocytes are an important component of the clearance pathway *in vivo*. The mannose/*N*-acetylglucosamine pathway may be important in the regulation of extracellular levels of various glycosylated macromolecules, including lysosomal hydrolases.

Plasma survival of infused glycoproteins is known to be determined by the nature of the exposed (i.e. terminal) non-reducing sugar residues of the oligosaccharide chains (Ashwell & Morell, 1974). In general, sialylated glycoproteins are not rapidly cleared. Galactose-terminated glycoproteins are cleared rapidly from plasma and are taken up into hepatocytes by a pathway elucidated by Ashwell and his coworkers (Ashwell & Morell, 1974). Glycoproteins having mannose or *N*-acetylglucosamine in the terminal position are cleared rapidly by liver *in vivo* but, by contrast with galactose-terminated glycoproteins, liver sinusoidal cells mediate this clearance (Baynes & Wold, 1976; Winkelhake & Nicholson, 1976; Schlesinger *et al.*, 1976; Stockert *et al.*, 1976; Brown *et al.*, 1978; Schlesinger *et al.*, 1978; Steer *et al.*, 1979). A similar uptake system has been shown to be associated with alveolar macrophages *in vitro* (Stahl *et al.*, 1978*a*). Physiological substrates for the mannose/*N*-acetylglucosamine pathway are the lysosomal glycosidases (Achord *et al.*, 1977*b*; Stahl *et al.*, 1976*a*,*b*,*c*) and perhaps other lysosomal enzymes.

Abbreviations used: The term glycoproteins is used to include neoglycoproteins, modified orosomucoids and lysosomal glycosidases. The neoglycoproteins are indentified by the saccharide residue that has been linked to the protein: mannose–bovine serum albumin (mannose–albumin), glucose–bovine serum albumin (glucose–albumin), galactose–bovine serum albumin (galactose–albumin) and *N*-acetylglucosamine–bovine serum albumin (*N*-acetylglucosamine–albumin).

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To elucidate the specificity and organ distribution of the clearance pathway *in vivo*, we have used a family of synthetic glycoconjugates (i.e. neoglycoproteins) and enzymatically modified orosomucoids as both substrates and inhibitors. The neoglycoproteins are particularly useful because of their uniform physical properties and their homogeneous sugar composition. Naturally occurring glycoproteins frequently have microheterogeneity in their carbohydrate structure which is often amplified during enzymatic modification. Therefore we have employed the neoglycoproteins as primary standards for the structural specificity of clearance and uptake by the alveolar macrophage. This has enabled us to study the specificity in more detail than otherwise would have been possible.

Experimental

Materials

Reagents used in this study were reagent grade and were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and from Fisher Scientific Co., St. Louis, MO, U.S.A.

Neoglycoproteins were prepared from bovine serum albumin as previously described (Lee *et al.*, 1976). The proteins contained 25–31 mol of sugar/mol of protein. Orosomucoid was a generous donation of the American Red Cross. Asialo-orosomucoid, agalacto-orosomucoid and ahexosamino-orosomucoid (kindly donated by Dr. Gilbert Ashwell, N.I.H., Bethesda, MD, U.S.A.), were prepared by sequential enzymic removal of terminal sugars with exoglycosidases. β-Glucuronidase (E.C. 3.2.1.31) was prepared from rat preputial glands essentially as described by Himeno *et al.* (1975) to a specific activity of 2400 units/mg. Lysosomal β-*N*-acetylglucosaminidase (E.C. 3.2.1.30) was purified by affinity chromatography by the method of Doebber & Stahl (1978) to a specific activity of greater than 10000 units/mg. Units are expressed as μmol of substrate hydrolysed/h. Both enzyme preparations were homogeneous as tested by gel electrophoresis under alkaline conditions (Davis, 1964). Protein was estimated by the Miller (1959) method.

Animal preparations and clearance studies

Female Wistar rats (200g) were obtained from Harlan Industries (Cumberland, IN, U.S.A.) or National Laboratory Animals (St. Louis, MO, U.S.A.). Animals were anaesthetized with sodium pentobarbital (30mg/kg, intraperitoneal) and the femoral artery and vein were cannulated as described by Stahl *et al.* (1976*b*). Animals were nephrectomized or eviscerated as described by Schlesinger *et al.* (1976). Samples were administered intravenously in 0.5ml of saline (0.9% NaCl) over a period of 70s by using a Harvard constant-infusion pump. ^{125}I-labelled neoglycoproteins were diluted with unlabelled carrier to a specific radioactivity of 2.5×10^5 c.p.m./μg of protein; 20μg of protein was routinely infused. When purified lysosomal enzymes were studied 20 units of activity were infused in 0.5ml of saline as just described. Arterial blood samples were taken in heparinized haematocrit tubes beginning at 20s after termination of the infusion and continuing at the indicated times for up to 60min. Plasma was either used directly for the enzyme assays as previously described (Stahl *et al.*, 1976*b*) or was precipitated with 0.5ml of 5% phosphotungstic acid in 2M-HCl. For radioisotope counting, the precipitate was dissolved in 1M-NaOH.

Macrophage assays

Alveolar macrophages were isolated from anaesthetized female Wistar rats as described by Stahl *et al.* (1978*a*). The isolated cells had a viability of greater than 95% as indicated by Trypan Blue exclusion. A sample of 5×10^5 cells suspended in 0.1ml of minimal essential medium as described by Stahl *et al.* (1978*a*) was dispensed into microcentrifuge (Microfuge, Beckman) tubes (capacity 0.4ml) over a layer of 0.15ml of oil. The oil phase was a mixture of 4vol. of silicone fluid (no. 550; *d* 1.07; Accumetric Inc., Elizabethtown, KY, U.S.A.) and 1vol. of mineral oil (*d* 0.88; Taylor Chemical Co., St. Louis, MO, U.S.A.). An air space separated the cells from the oil. After appropriate incubation, ligand was rapidly separated from cells by centrifugation in a Microfuge for 30s. The tips of the tubes were cut off with a sharp scalpel and assayed for radioactivity, as was the medium remaining above the oil. In the absence of cells, very little ligand was detected in the cut tips. Ligand and inhibitors were added to the cells simultaneously.

Radiolabelling

Glycoproteins were iodinated with Na^{125}I by using the Chloramine-T method (Greenwood *et al.*, 1963) followed by extensive dialysis against Tris-buffered saline. Proteins with specific activities of 0.5×10^6–2.0×10^6 c.p.m./μg were employed as ligands.

Results

Plasma clearance of ^{125}I-labelled neoglycoproteins in the intact rat

The plasma clearance rates of infused radiolabelled mannose–albumin, glucose–albumin, *N*-acetylglucosamine–albumin and galctose–albumin were measured in the anaesthetized rat as described in the legend to Fig. 1. All of the neoglycoproteins were rapidly cleared from plasma with half-survival times of less than 5min (Fig. 1*a*). Infused ^{125}I-

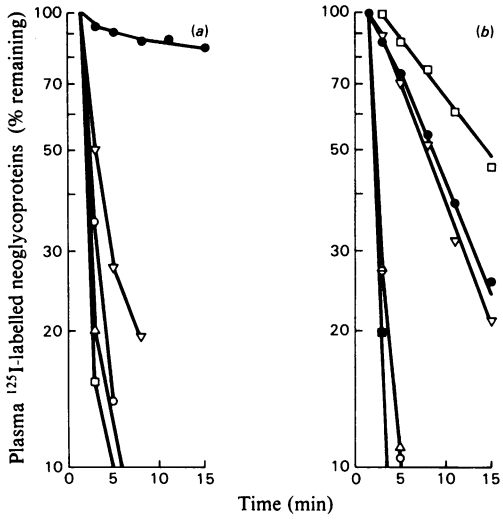

Fig. 1. *Clearance of ^{125}I-labelled neoglycoproteins in the intact rat*

Female Wistar rats were prepared and infusions were performed as described in the Experimental section. (*a*) The following ^{125}I-labelled neoglycoproteins were infused: galactose–albumin (□), glucose–albumin (△), mannose–albumin (○), and *N*-acetylglucosamine–albumin (▽) as well as bovine serum albumin alone (●). (*b*) The effect of blocking doses of neoglycoproteins on the clearance of radiolabelled glucose–albumin (■) was tested by including in the infusate 3.0mg of galactose–albumin (△), mannose–albumin (○), or glucose–albumin (▽), or combinations of galactose–albumin + mannose–albumin (●, 1.5mg each; □, 3.0mg each). Data are expressed as a percentage of the radioactivity (c.p.m.) remaining/ml when compared with the first time point. Each line represents the results from three experiments.

labelled bovine serum albumin was not appreciably cleared from plasma during this period. As a test for the structural specificity of neoglycoprotein clearance *in vivo*, two of the synthetic ligands, galactose–albumin and mannose–albumin, were tested in separate experiments by cross-competition with yeast mannan (5mg) and asialo-orosomucoid (5mg) as blocking reagents. These two inhibitors have been shown to block the plasma clearance of mannose-terminated (Achord *et al.*, 1978) and galactose-terminated (Ashwell & Morell, 1974) glycoproteins, respectively. The results (not shown) indicated that mannan impaired the clearance of mannose–albumin but not that of galactose–albumin, whereas asialo-orosomucoid retarded the clearance of galactose–albumin but not that of mannose–albumin.

Despite the lack of cross-competition between mannose- and galactose-terminal glycoconjugates, recovery of radioactivity after infusion and clearance of ^{125}I-labelled neoglycoproteins in the intact animal indicates that the liver is the major organ responsible for clearance of both neoglycoproteins (Table 1). However, it is noteworthy that considerable radioactivity accumulated in spleen and bone after infusion of ^{125}I-labelled mannose–albumin or ^{125}I-labelled glucose–albumin.

Earlier work has shown that glucose–albumin (i) competes with galactose–albumin for binding to the isolated hepatocyte galactose receptor (Krantz *et al.*, 1976; Stowell & Lee, 1978) and (ii) is taken up by alveolar macrophages *in vitro* (Stahl *et al.*, 1978*a*). Because of these observations *in vitro*, one might predict that glucose–albumin would show dual recognition *in vivo*. To test this possibility, the inhibitory effect of galactose–albumin and mannose–albumin on ^{125}I-labelled glucose–albumin clearance

Table 1. *Tissue distribution of ^{125}I-labelled neoglycoproteins after plasma clearance in the intact rat*

Neoglycoproteins (5×10^6c.p.m.; 20μg) in 0.5ml were infused intravenously as described in the Experimental section. Animals were killed by decapitation 10min after completion of infusion. Tissues were removed, blotted and counted for radioactivity. Ribs were removed, cleaned of extraneous tissue, and used as an indicator of bone tissue in general. Fat samples were taken from the peritoneal cavity. Muscle was derived principally from the tibialis anterior and also, partially, from the extensor digitorum longus. Bone, muscle, fat and plasma were assumed to represent 10, 40, 15 and 5% of body weight, respectively. Results are the average of two animals.

		Proportion of injected dose (%)		
Tissue	Glycoprotein ...	Gal–albumin	Glc–albumin	Man–albumin
Liver		81.5	88.2	76.5
Lung		0.2	0.2	0.2
Kidney		1.9	0.6	0.5
Bone		0.6	2.0	3.4
Fat		0.4	0.7	1.2
Spleen		0.1	2.6	5.0
Muscle		0.9	2.3	0.8
Plasma		0.9	1.0	1.3
Total recovery		86.2	98.1	89.0

was measured in the intact rat. Blocking doses of galactose–albumin, mannose–albumin, glucose–albumin or a combination of mannose–albumin and galactose–albumin were employed. Inhibitors were infused simultaneously with ligand. The results (Fig. 1*b*) show that large doses (3 mg) of galactose–albumin and mannose–albumin, when administered alone, were ineffective in retarding clearance of ^{125}I-labelled glucose–albumin. However, a combination of the two antagonists, even in smaller doses, significantly shifted the glucose–albumin clearance curve, resulting in a much slower clearance rate. Glucose–albumin (3 mg) significantly decreased the clearance rate of ^{125}I-labelled glucose–albumin.

Clearance of ^{125}I-labelled neoglycoproteins in the eviscerated and eviscerated–nephrectomized rat preparation

Clearance of the neoglycoproteins was tested in an animal preparation where liver and associated intraperitoneal organs were surgically removed from the circulation (i.e. evisceration). In some experiments, the animal preparations were also acutely nephrectomized to avoid artifacts arising from glomerular filtration of injected ligands. Nephrectomy alone had no selective effect on the clearance of any of the ligands under study.

Previous work reported from our laboratory has shown that ^{125}I-labelled ahexosamino- (i.e. mannose-terminated) orosomucoid and ^{125}I-labelled agalacto- (i.e. *N*-acetylglucosamine-terminated) orosomucoid were appreciably cleared in the eviscerated–nephrectomized rat, whereas ^{125}I-labelled asialo- (i.e. galactose-terminated) orosomucoid was indistinguishable from ^{125}I-labelled orosomucoid (Stahl *et al.*, 1978*b*). These results suggested the presence of an extra-hepatic recognition system that displays specificity for ligands having mannose or *N*-acetyl-

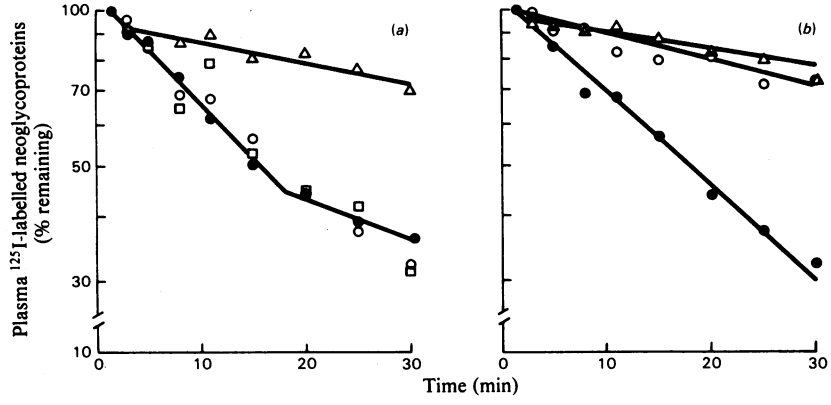

Fig. 2. *Clearance of neoglycoproteins in the eviscerated–nephrectomized rat*

Animals were prepared as described in the legend to Table 2. The infusion protocol and sampling procedure are described in the Experimental section. (*a*) The clearance of 20 μg (10^6 c.p.m.) of each of the following neoglycoproteins was tested: galactose–albumin (△), glucose–albumin (□), mannose–albumin (●), or *N*-acetylglucosamine–albumin (○). Plasma samples were acid precipitated and counted. (*b*) ^{125}I-labelled glucose–albumin (●) was employed as ligand (20 μg; 10^6 c.p.m.). Blocking doses (3 mg) of mannose–albumin (△) or glucose–albumin (○) were administered simultaneously.

Table 2. *Relative tissue distribution of ^{125}I-labelled neoglycoproteins after clearance in the eviscerated–nephrectomized rat*

Animals were anaesthetized and eviscerated as described in the Experimental section. ^{125}I-labelled neoglycoproteins (1×10^6 c.p.m.; 20 μg) were infused as described and the animals were killed by decapitation 30 min post-infusion. Tissue samples were taken as described in Table 1. Results are the average for two to three animals per group. Results are expressed as specific activity relative to muscle radioactivity · (g of tissue)$^{-1}$/radioactivity · (g of muscle)$^{-1}$.

		Relative specific radioactivity		
Tissue	Glycoprotein ...	Gal–albumin	Glc–albumin	Man–albumin
Lung		16.4	9.3	8.5
Bone		2.7	8.6	7.8
Fat		1.6	0.9	1.1
Muscle		1.0	1.0	1.0

glucosamine in the terminal position on the oligosaccharide chain. Recovery of radiolabelled asialo-orosomucoid, agalacto-orosomucoid and ahexosamino-orosomucoid after clearance in the intact rat demonstrated that liver was largely responsible for uptake. After clearance in the eviscerated–nephrectomized preparation, radiolabelled ahexosamino-orosomucoid showed a diffuse distribution with some enrichment in bone.

The notion that terminal mannose and/or *N*-acetylglucosamine serve as recognition markers for systemic clearance in the absence of the liver, as suggested by the orosomucoid study, was further established by plasma clearance experiments with injected neoglycoproteins in the eviscerated rat preparation. Previously we reported the clearance of radiolabelled galactose–albumin, glucose–albumin, mannose–albumin and *N*-acetylglucosamine–albumin in the eviscerated–nephrectomized rat. These results were repeated (see Fig. 2*a*) to serve as a control for the blockade and tissue distribution experiments (Stahl *et al.*, 1978*b*). Glucose–albumin, mannose–albumin and *N*-acetylglucosamine–albumin were promptly cleared ($t_{\frac{1}{2}}$ less than 15 min) whereas galactose–albumin had a much longer plasma survival time ($t_{\frac{1}{2}}$ greater than 30 min). Tissue recovery of radiolabelled neoglycoproteins after clearance is summarized in Table 2. The results in Table 2 reflect tissue distributions of labelled ligands 30 min after infusion. Since evisceration can remove up to 30% of the animal's plasma volume and ligands are cleared at very different rates in the eviscerated animal, the absolute radioactivity/g of tissue in these animals can be a function of the plasma content of the tissue and the extent of clearance by that tissue of a particular ligand. Therefore, the tissue distributions in Table 2 are expressed as a ratio of the specific radioactivity/g of tissue to the specific radioactivity/g of muscle for each ligand. This allows one to compare the relative recovery of different ligands in a particular tissue. The results show substantial enrichment of radiolabelled mannose–albumin and glucose–albumin in bone. Similar results were observed with *N*-acetylglucosamine–albumin. Enrichment of all neoglycoproteins in lung tissue was observed.

The question of whether clearance of mannose–albumin or glucose–albumin is mediated by a specific uptake system was investigated by infusing ^{125}I-labelled glucose–albumin into the eviscerated–nephrectomized rat in the absence or presence of blocking doses (3 mg) of mannose–albumin or glucose–albumin (see Fig. 2*b*). Contrary to the finding in the intact rat, where mannose–albumin was a poor antagonist of clearance of ^{125}I-labelled glucose–albumin, mannose–albumin and glucose–albumin were equally effective in antagonizing clearance of glucose–albumin.

Clearance of lysosomal glycosidases in the intact rat and its inhibition by neoglycoproteins

Lysosomal glycosidases are rapidly cleared from

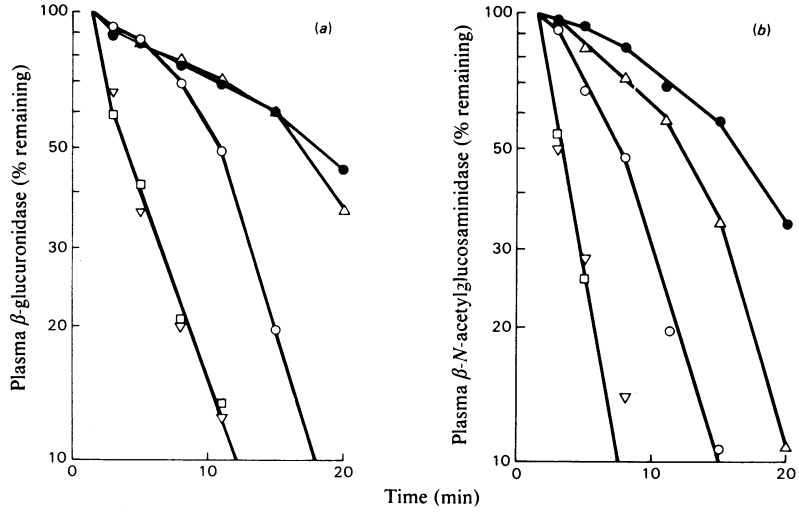

Fig. 3. *Clearance of lysosomal hydrolases in the intact rat*

Female Wistar rats were prepared for infusion, enzyme or enzyme plus inhibitor were infused, and plasma samples were prepared as described in the Experimental section. (*a*) Preputial β-glucuronidase was infused; (*b*) lysosomal β-*N*-acetylglucosaminidase was infused. The plasma enzyme concentrations were measured with 4-methylumbelliferyl β-glucuronide (*a*) or 4-methylumbelliferyl β-*N*-acetylglucosaminide (*b*). The enzymes were either infused alone (□) or with blocking doses (1 mg) of the following neoglycoproteins: galactose–albumin (▽), glucose–albumin (○), mannose–albumin (●), or galactose–albumin + glucose–albumin (△).

plasma by a sugar-specific receptor-mediated mechanism (Achord *et al.*, 1977*b*; Stahl *et al.*, 1976*a*,*b*,*c*). In the present study, the clearance of two highly purified lysosomal glycosidases, rat preputial β-glucuronidase and rat liver lysosomal β-*N*-acetylglucosaminidase, was examined. Both enzymes were rapidly cleared (Figs. 3*a* and 3*b*) from plasma after intravenous injection. Blocking doses (1 mg) of galactose–albumin, administered simultaneously, had no effect on the clearance of β-glucuronidase or of β-*N*-acetylglucosaminidase, whereas mannose–albumin (1 mg) was an excellent antagonist. On the contrary, glucose–albumin (1 mg), when administered with the lysosomal hydrolases, was only partially inhibitory to enzyme clearance and produced a transient blocking effect. The results in Fig. 3 show that glucose–albumin produced a more persistent blocking effect when administered with galactose–albumin than when administered alone. Galactose–albumin thus potentiates the antagonistic effects of glucose–albumin (Figs. 3*a* and 3*b*) but is ineffective when administered alone.

Uptake of ^{125}I-labelled agalacto-orosomucoid by alveolar macrophages

Concentration dependence and time course. The uptake of ^{125}I-labelled agalacto-orosomucoid by isolated macrophages was followed by incubating cells (5×10^5) with ligand under standard conditions. Fig. 4 shows the concentration-dependence of ligand uptake at 10 min when ligand concentration was varied from 0–10 μg/ml in the assay

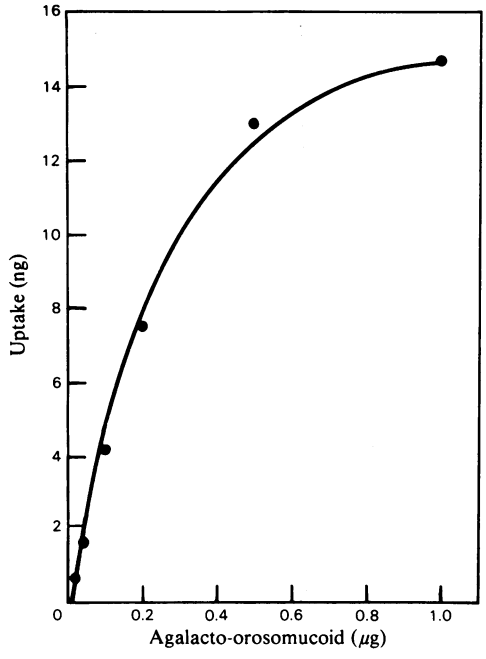

Fig. 4. *Concentration dependence of ^{125}I-labelled agalacto-orosomucoid uptake by alveolar macrophages*
Cells (5×10^5 in 0.1 ml) were incubated with increasing concentrations of ^{125}I-labelled agalacto-orosomucoid for 10 min at 37°C over oil as described in the Experimental section. Uptake was terminated by centrifugation. Non-specific uptake (corresponding to less than 15% of the total uptake) was estimated by adding an excess (100 μg) of yeast mannan to parallel assays at each ligand concentration.

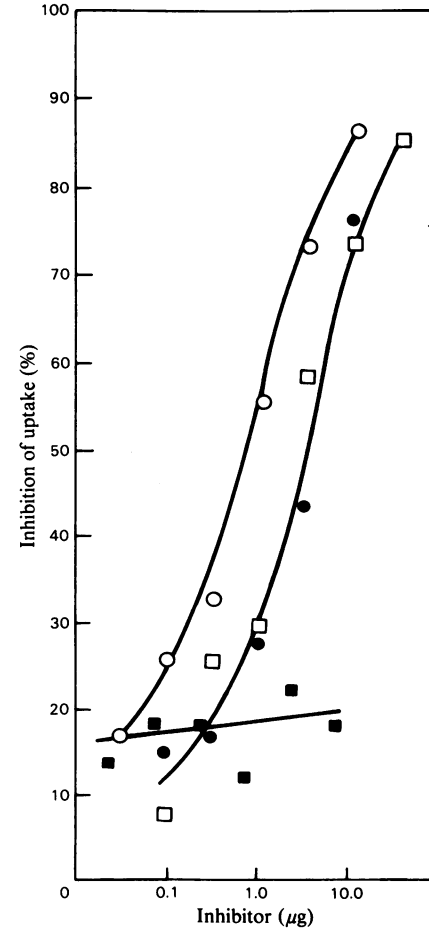

Fig. 5. *Effect of neoglycoproteins on the uptake of ^{125}I-labelled agalacto-orosomucoid by alveolar macrophages*
Cells (5×10^5 in 0.1 ml) were incubated with 1 μg of ^{125}I-labelled agalacto-orosomucoid (10 μg/ml) under standard assay conditions for 10 min at 37°C together with increasing concentrations of mannose–albumin (○), glucose–albumin (□), galactose–albumin (■) or *N*-acetylglucosamine–albumin (●). Uptake was terminated by centrifugation as described in the Experimental section.

Table 3. *Inhibition of uptake of ^{125}I-labelled agalacto-orosomucoid by macrophages by mannose–albumin with increasing sugar substitution*

Cells (5×10^5 in 0.1 ml) were incubated with 1 μg of ^{125}I-labelled agalacto-orosomucoid for 10 min at 37°C in the absence or presence of 1 μg of mannose–albumin in which sugar substitution was varied from 5 to 37 mol of mannose/mol of albumin. Inhibition was calculated by comparing total uptake (c.p.m.) without inhibitor with total uptake in the presence of inhibitor. Results are the average of two experiments.

Substitution of mannose–albumin (mol of sugar/mol of protein)	Uptake (c.p.m.)	Inhibition of uptake (%)
None added	4475	0
5	3716	17
8	3080	32
12	2802	38
28	1058	77
33	1236	73
37	778	83

mixture. The Microfuge oil-assay was used as described in the Experimental section. Non-specific uptake was determined by including a large excess of yeast mannan in the assay. Specific uptake was calculated by subtracting non-specific uptake from total uptake. The results show that uptake is saturable and specific. In separate experiments, the time course for uptake was followed with a ligand concentration of 1.0 μg/assay. The results indicated that uptake is linear over the 10 min assay period employed in the studies.

Inhibition of agalacto-orosomucoid uptake by neoglycoproteins. To study the specificity of uptake, neoglycoproteins were employed as antagonists to the uptake of ^{125}I-labelled agalacto-orosomucoid by alveolar macrophages. Cells were incubated at 37°C with ^{125}I-labelled agalacto-orosomucoid in the absence or presence of increasing concentrations of inhibitors. Fig. 5 shows the inhibition of agalacto-orosomucoid uptake as a function of the amount of inhibitor added. Three neoglycoproteins (mannose–albumin, glucose–albumin and *N*-acetylglucosamine–albumin) are potent inhibitors of ^{125}I-labelled agalacto-orosomucoid uptake; galactose–albumin has no concentration-dependent effect on uptake. The inhibition curves in Fig. 5 have not been corrected by subtracting non-specific uptake. If one considers only the specific component, the active neoglycoproteins completely block uptake. Mannose–albumin was the best inhibitor of the three, followed by *N*-acetylglucosamine–albumin and glucose–albumin.

Effect of extent of substitution on the inhibition by neoglycoproteins of agalacto-orosomucoid uptake. The extent of substitution of bovine serum albumin by sugars is an important factor in determining the potency of the neoglycoprotein inhibitor. To study the effect of substitution, preparations of mannose–albumin with increasing numbers of mannosyl residues attached were employed as inhibitors. With a constant amount of inhibitor under the assay conditions described in Table 3, the extent of substitution of albumin by sugar was varied from 5 mol/mol to 33 mol/mol. The results in Table 3 show the relationship between substitution and inhibition and suggest an enhancement by multivalency of ligand–receptor interaction. In this respect, the results are in general agreement with the binding of glucose- and galactose-neoglycoproteins to rabbit liver membranes (Krantz *et al.*, 1976).

Discussion

Glycoproteins that possess *N*-acetylglucosamine or mannose in the terminal non-reducing position are rapidly cleared *in vivo* via a clearance pathway that operates independently of the well-characterized hepatocyte galactosyl-recognition system (Baynes & Wold, 1976; Stahl *et al.*, 1975; Stahl *et al.*, 1976*a*; Steer *et al.*, 1979; Stockert *et al.*, 1976; Winkelhake & Nicholson, 1976; Achord *et al.*, 1977*a*). The present evidence indicates that mannose- and *N*-acetylglucosamine-terminal glycoproteins compete for a common clearance pathway (Achord *et al.*, 1977*a*; Brown *et al.*, 1978; Schlesinger *et al.*, 1978). A systematic study of the factors that influence glycoprotein clearance *in vivo* has been hitherto difficult because of the unavailability of glycoprotein ligands that possess homogeneous non-reducing terminal sugar residues and yet have uniform molecular dimensions. Gel-filtration studies in our laboratory (results not shown) with orosomucoid, asialo-orosomucoid, agalacto-orosomucoid and ahexosamino-orosomucoid have demonstrated that the molecular dimensions of a glycoprotein such as orosomucoid are appreciably decreased by sequential removal of the terminal saccharides. Since dilution, by distribution into spaces greater than the vascular volume, represents some component of the clearance curve, the modified orosomucoids are

not suitable ligands for comparative studies *in vivo*. The neoglycoproteins provide an opportunity to study sugar-specific glycoprotein recognition in the eviscerated–nephrectomized preparation where differences caused by variations in the volume of distribution of the injected ligand can be ignored. The molecular dimensions of the neoglycoproteins employed are similar when studied by gel filtration and by electrophoresis under denaturing conditions (results not shown).

Our results confirm, that in the intact rat, glycoproteins terminating in mannose, *N*-acetylglucosamine or galactose are cleared from the plasma primarily by the liver. Minor sites of mannose–albumin clearance are indicated by the increased recovery of radiolabelled glycoproteins in bone and spleen when compared with galactose–albumin recovery in the same tissue. When the peritoneal contents (including liver, spleen and intestine) are removed from the circulation by surgical evisceration, the behaviour of mannose–glycoproteins differs more strikingly from that of galactose–glycoproteins. Asialo-orosomucoid and galactose–albumin are very poorly cleared in the eviscerated preparation. This result is consistent with the notion that the galactose-specific pathway is confined to the liver. The mannose/*N*-acetylglucosamine–glycoproteins have a much shorter circulating lifetime in the eviscerated rat, indicating the existence of extrahepatic sites for their clearance. Evisceration enhances the clearance of radiolabelled mannose–albumin by extrahepatic sites (e.g. bone) because these sites are no longer in competition with the liver for infused ligand. Tissue distribution of ligands in the eviscerated preparations shows that bone is especially enriched in ^{125}I-labelled mannose–albumin when compared with muscle, whereas both radiolabelled mannose–albumin and galactose–albumin are concentrated in lung. No enrichment of either glycoprotein in pulmonary tissue was observed in the intact animal, which suggests that the latter finding may be artifactual. It is possible that the pooling of blood that occurs in this organ after decapitation is responsible for the observed enrichment. It appears that pulmonary enrichment is more dependent upon circulating concentrations at the time of decapitation than upon the terminal saccharide of the glycoprotein (see Fig. 2 and Table 2). Alternatively, galactose–albumin may be cleared by a galactose-specific lectin such as the one that has been isolated from lung (Teichberg *et al.*, 1975; Waard *et al.*, 1976). The cellular disposition of this lectin is primarily cytoplasmic (Briles *et al.*, 1979) and it is unclear whether the results observed here reflect its functional activity. The mannose/*N*-acetylglucosamine-specific lectin of alveolar macrophages is a potential mechanism for mannose–albumin clearance, but once again there is no evidence to support its role in the clearance of circulating glycoproteins.

Glucose–albumin is recognized by two clearance pathways in the intact rat, a galactose-specific pathway localized in liver hepatocytes and a mannose/*N*-acetylglucosamine-specific pathway both in liver sinusoidal cells and in peripheral tissues (e.g. bone marrow). Only when inhibitors of both pathways were employed simultaneously was the clearance of tracer doses of ^{125}I-labelled glucose–albumin impaired. Glucose–albumin alone is at best a transient inhibitor of lysosomal enzyme clearance, but, when injected simultaneously with galactose–albumin, glucose–albumin becomes a more potent inhibitor. Since galactose–albumin alone does not inhibit lysosomal enzyme clearance, we have formulated the following hypothesis. At small injected doses of ^{125}I-labelled glucose–albumin (subsaturating), the limiting step in clearance is probably the rate of circulation and distribution of the radiolabelled ligand. Therefore blocking either the mannose/*N*-acetylglucosamine-specific pathway or the galactose-specific pathway will have no effect upon the rate of clearance of glucose–albumin. Blockade of glucose–albumin clearance can be achieved by simultaneous blockade of both pathways. When using glucose–albumin to block the clearance of lysosomal enzymes, the effect will be transient because the blocking agent is being cleared from the circulation by two pathways simultaneously. Apparently the amount of glucose–albumin necessary to saturate, and therefore block, both clearance systems for 20–30 min was greater than we injected in these experiments. However, by simultaneously administering blocking doses of galactose–albumin one can saturate the galactose-specific clearance. As a consequence, glucose–albumin is cleared by the mannose/*N*-acetylglucosamine pathway alone. Under these conditions, the plasma half-life of glucose–albumin is extended, making it a more potent inhibitor of lysosomal enzyme clearance.

In the eviscerated–nephrectomized animal ^{125}I-labelled glucose–albumin clearance is blocked by mannose–albumin alone. This observation supports the conclusion that mannose/*N*-acetylglucosamine-specific clearance occurs in extra-hepatic tissues but galactose-specific clearance is confined to the liver.

The kidney seems to play no role in the clearance of the test ligands other than filtration. Moreover, nephrectomy alone had no selective effect on any of the ligands under study. This report is contrary to earlier studies (Stockert *et al.*, 1976; Winkelhake & Nicholson, 1976) suggesting a role for the kidney in the uptake of mannose-terminated glycoproteins. An alternative explanation of the earlier data is that, after degradation of the injected ligands, radiolabelled fragments are filtered and concentrated by

the kidney, thereby accounting for apparent accumulation.

Experiments with isolated alveolar macrophages confirm and extend the results *in vivo*, showing that mannose, glucose and *N*-acetylglucosamine glycoconjugates all compete for the macrophage uptake mechanism. Agalacto-orosomucoid was selected as a ligand because it is rapidly taken up by macrophages, and uptake is saturable (Fig. 5) and linear over the time course employed in the inhibition experiments. As one might expect, the number of sugars associated with the glycoconjugates is a critical factor in the potency of the inhibitor. The data do not indicate a minimal number of sugar residues required for inhibition, but rather than inhibitory potency increases with the extent of substitution from 5 to 33 mol of sugar/mol of protein. This is consistent with the observation that α-mannosidase treatment of ribonuclease B enhances its plasma survival.

In conclusion, macromolecules having terminal mannose, glucose or *N*-acetylglucosamine residues, and certain lysosomal glycosidases, are rapidly cleared from plasma in the intact or eviscerated rat, whereas rapid clearance of galactose-terminal macromolecules is liver-dependent. Uptake of glycoconjugates by alveolar macrophages closely mimics the specificity of clearance in the eviscerated–nephrectomized rat. Glucose, when covalently bound to protein, is recognized by both the hepatocyte galactose receptor (Stowell & Lee, 1978) and by the macrophage mannose/*N*-acetylglucosamine receptor (Stahl *et al.*, 1978*a*). Glucose–albumin *in vivo* behaves as if it were recognized by both clearance pathways. However, in the eviscerated rat and with isolated macrophages, glucose–albumin behaves like a mannose/*N*-acetylglucosamine glycoconjugate. Finally, some caution should be exercised in the interpretation of experiments with glycoconjugates prepared from thiosugars. However, preliminary experiments with isomaltose–albumin glycoconjugates (J. Smith and P. D. Stahl, unpublished work) have confirmed the recognition of glucose-terminal glycoconjugates by alveolar macrophages. By using a procedure similar to that reported by Kawasaki *et al.* (1978), a binding protein has been isolated from rat liver which has the same specificity and ion requirements as alveolar macrophage uptake (Townsend & Stahl, 1979). Moreover, antibody to the rat liver receptor inhibits ligand binding by rat alveolar macrophages. In view of these findings, it is likely that the binding protein isolated from liver is very similar, if not identical, to that found in alveolar macrophages.

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