

Three Types of Human Asialo-Transferrin and their Interactions with the Rat Liver

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Three types of asialo-transferrin were obtained from immunologically pure human transferrin by chromatography on DEAE-cellulose, followed by desialylation and affinity chromatography on a column of the immobilized asialo-glycoprotein-binding hepatic lectin from rabbit liver. Of the asialo-transferrins, type 1 was derived from the principal DEAE-cellulose chromatographic component of transferrin, i.e. the one that contains two biantennary glycans. The two other asialo-transferrins (types 2 and 3) were derived from a minor DEAE-chromatographic transferrin component, which is assumed to possess one biantennary and one triantennary glycan. The three asialo-transferrin types were indistinguishable by electrophoretic mobility, but they were readily distinguished on the basis of their binding strengths to the hepatic lectin in intact rats. Glycan structures responsible for the difference in binding strengths between asialo-transferrin types 2 and 3 are not known. Metabolic studies in rats showed that none of the individual asialo-transferrin types was capable of generating a signal for endocytosis at low doses ($<1\ \mu\text{g}/100\ \text{g}$ body wt.) and, consequently, most of the injected protein was recoverable with the plasma and the liver 35 min after injection. However, endocytosis and catabolism of each asialo-transferrin type was readily induced by injecting a larger dose (50–250 $\mu\text{g}/100\ \text{g}$ body wt.) of unlabelled asialo-transferrin of the same type or of a different type a short interval after the labelled dose. These findings support the view that the dose-dependent uptake of human asialo-transferrin by the hepatocyte, as established in an earlier study with asialo-transferrin made from whole transferrin [Regoeczi, Taylor, Hatton, Wong & Koj (1978) *Biochem. J.* 174, 171–178], also holds for these asialo-transferrin subfractions. Furthermore, the present studies indicate that asialo-transferrins of different carbohydrate compositions are capable of synergistically promoting endocytosis of each other.

Catabolism of human asialo-transferrin by the liver of the intact rat is a mass-dependent process. Thus, after the intravenous injection of a small quantity ($<1\ \mu\text{g}/100\ \text{g}$ body wt.), asialo-transferrin attains equilibrium between circulating plasma and the asialo-glycoprotein-binding lectin (Stockert *et al.*, 1974) on the plasma membrane of the hepatocytes with only an insignificant portion of the dose actually being taken up by the cells. After the administration of larger quantities (50–500 $\mu\text{g}/100\ \text{g}$ body wt.), however, significant portions of the asialo-transferrin dose are endocytosed and degraded by the liver. As a possible interpretation of these findings we suggested that the galactose groups of human asialo-transferrin are either quantitatively or qualitatively (e.g. spacing) inadequate to elicit a signal for endocytosis of the individual protein molecules, but two or more

asialo-transferrin molecules acting synergistically can effect endocytosis (Regoeczi *et al.*, 1978).

Our source of asialo-transferrin for the above study was whole human transferrin, which was not further fractionated by any means before desialylation. However, since then it has become clear that such asialo-transferrin preparations consist of a mixture of molecules with markedly different affinities for the immobilized lectin from rabbit liver (Wong *et al.*, 1978). At that time it seemed reasonable to assume the binding heterogeneity to be due to the presence of transferrin molecules containing one bi- and one tri-antennary glycan, instead of two biantennary ones (Spik *et al.*, 1975). This would give rise to an additional terminal galactose group in asialo-transferrin and, consequently, to a firmer lectin binding. However, a subsequent study showed that triantennary transferrin asialo-glycopeptides themselves are heterogeneous with respect to their affinity for the immobilized hepatic lectin (Hatton *et al.*, 1979).

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In view of this rather complex situation, in our search for the signal for endocytosis of asialo-glycoprotein it seemed necessary to isolate these forms of human asialo-transferrin and to investigate their interactions with the rat liver. Here we report our observations with three types of asialo-transferrin.

Materials and Methods

Preparation of the asialo-transferrin types

The principle of the procedure devised for this purpose is as follows. Iron-saturated transferrin was first resolved into its three molecular components on DEAE-cellulose (Regoeczi *et al.*, 1977). The major chromatographic peak [designated 2Fe-TfC-5-6 by Wong *et al.* (1978)], containing molecules with two biantennary glycans, and the following peak (designated 2Fe-TfC-6-6), presumably containing molecules with a combination of one biantennary and one triantennary glycan, were pooled separately and desialylated with neuraminidase. The enzyme and any incompletely desialylated transferrin were removed from the incubation mixture by chromatography on DEAE-cellulose. Asialo-transferrin obtained from the 2Fe-TfC-6-6 peak was then labelled with iodine and resolved into a non-retained and a retained fraction by passage through a Sepharose-hepatic lectin (rabbit) column.

The preparation of transferrin, criteria used to assess its purity, saturation with iron, and resolution of transferrin into its components by DEAE-cellulose chromatography have been described elsewhere (Regoeczi *et al.*, 1977). Desialylation of transferrin with neuraminidase (acylneuraminyl hydrolase; EC 3.2.1.18), separation of completely desialylated transferrin from the enzyme and from partially desialylated products, and iodination of asialo-transferrin were reported in detail in Regoeczi *et al.* (1978).

To ensure that only transferrin molecules having biantennary glycans were contained in peak 2Fe-TfC-5-6, the protein was always purified from plasma that was freshly frozen and stored at -70°C . For transferrin containing a bi- and a tri-antennary glycan (peak 2Fe-TfC-6-6), selected samples of pure transferrin, obtained by the courtesy of Dr. N. Heimburger (Behringwerke, Marburg, G.F.R.), were used as an additional starting material (see the Discussion section). The quality of the separation of transferrin molecules of different glycan compositions achieved on the DEAE-cellulose (DE-52, Whatman; W. and R. Balston, Maidstone, Kent, U.K.) column was routinely controlled by analytical electrophoresis in alkaline polyacrylamide gels (Clarke, 1964). According to the results obtained with these gels, pure preparations of the protein in peak 2Fe-TfC-5-6 were usually obtained after a single chromatography. By contrast, isolation of the protein in peak 2Fe-

TfC-6-6 as an electrophoretically homogeneous material depended on rechromatography on DEAE-cellulose at least once, but more often twice. Conditions for rechromatography were identical with those for the first chromatography; the protein peak, pooled from the previous chromatography, was first concentrated by ultrafiltration with an Amicon UM10 membrane to approx. 20ml, followed by concentration to approx. 5ml by pressure dialysis at 4°C in Visking dialysis tubing (20/32) against 0.01 M-Tris/HCl, pH 8.0. After a total dialysis time of approx. 30h, rechromatography was undertaken on a fresh column of DEAE-cellulose.

Sepharose-hepatic lectin columns

The asialo-glycoprotein-binding lectin was isolated from rabbit livers by the technique of Hudgin *et al.* (1974) with minor modifications (Wong *et al.*, 1978), and was coupled to Sepharose 6B as reported by Wong *et al.* (1978). Columns (approx. 10cm \times 0.8cm), containing 1.6–2.6mg of conjugated protein per g of moist gel, were used. For chromatography, small samples (0.5–1mg) of an asialo-transferrin preparation were labelled (McFarlane, 1958) with ^{125}I . Columns were equilibrated before use with 0.05 M-Tris/HCl, pH 7.8, containing 0.15 M-NaCl and 0.05 M-CaCl₂. After up to 100 μg of asialo-transferrin in approx. 0.2ml of 0.15 M-NaCl had been loaded, columns were first eluted with 0.05 M-Tris/HCl, pH 7.8, containing 0.15 M-NaCl and 0.05 M-CaCl₂, and behaviour of the protein was followed by measuring the radioactivity content of the 2ml fractions collected. When the effluent became essentially free of radioactivity, columns were eluted with 0.02 M-sodium acetate, pH 6.4, containing 0.5 M-NaCl and 0.05 M-EDTA (disodium). The whole operation was carried out at room temperature.

Asialo-transferrin preparations from the lectin columns were further studied in animals (see below). For this purpose, fractions were pooled, and concentrated by pressure dialysis against 0.15 M-NaCl as necessary. In initial experiments substantial losses of asialo-transferrin were noted that were due to adsorption on the glassware and dialysis tubing at the prevailing low protein concentrations. Subsequently, these losses were effectively reduced by coating surfaces with unlabelled transferrin. Thus tubes and tubing were filled with 0.05% (w/v) transferrin in 0.15 M-NaCl, kept at 4°C overnight, and emptied just before use.

Sepharose-concanavalin A

Small samples (0.25–0.5mg) of labelled asialo-transferrin preparations were tested for binding to columns (10cm \times 0.8cm) of Sepharose-concanavalin A (Pharmacia, Uppsala, Sweden) under conditions given elsewhere (Wong *et al.*, 1978).

Other proteins

Human α_1 -acid glycoprotein (orosomuroid) was prepared as described by Charlwood *et al.* (1976) and desialylated as reported before (Regoeczi *et al.*, 1978). Human albumin was obtained from Behringwerke. A globulin fraction from goat serum containing antibodies to human transferrin was obtained from Sycco (Sylvana, Millburn, NJ, U.S.A.).

Carbohydrate analyses

Sialic acid contents were determined by the thio-barbituric acid method of Warren (1959) after hydrolysis in 0.05M-H₂SO₄ at 80°C for 1 h. Neutral sugars were identified and quantitatively determined by g.l.c. as their alditol acetates after hydrolysis with Dowex 50 (Lehnhardt & Winzler, 1968). Hexosamines were determined on an amino acid analyser after hydrolysis in 4M-HCl at 100°C for 6 h.

Animals

Male Sprague-Dawley rats with body weights ranging from 230 to 450 g were used. They received a standard pelleted diet and water *ad lib*. A total of 131 animals was studied.

Experiments in vivo

Preparation of dose solutions, blood sampling, processing of samples for counting of radioactivity, determination of protein-bound and non-protein-bound radioactivities in plasma and liver, and correction of the plasma radioactivity curves for diffusion and sampling have been reported in detail earlier (Regoeczi *et al.*, 1978).

In brief, a calculated quantity of ¹²⁵I-labelled asialo-transferrin, in the iron-saturated form, was mixed with ¹³¹I-labelled human albumin and injected in rats in a volume of 0.2–0.3 ml. Blood samples were collected at approx 5 min intervals for 35 min, then the liver was removed under anaesthesia and homogenized. Plasma and homogenate samples were assayed in duplicate for protein-bound and non-protein-bound radioactivities. Plasma volume was calculated from the dilution of the dose of labelled

albumin in 5 min, and non-hepatic losses of asialo-transferrin (i.e. those due to diffusion and sampling) from the disappearance rate of labelled albumin from the plasma volume. Quantities of asialo-transferrin in the residual plasma in the hepatic vascular bed were calculated from the data on labelled albumin (Regoeczi, 1975).

Results

The three types of human asialo-transferrin

An example for the isolation of transferrin components 2Fe-TfC-5-6 and 2Fe-TfC-6-6 from whole transferrin by chromatography on DEAE-cellulose is shown in Fig. 1, and data pertinent to the carbohy-

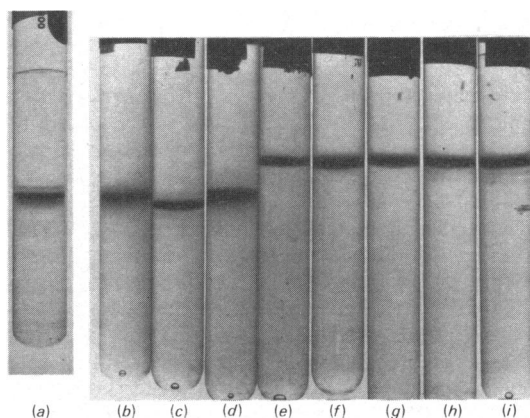


Fig. 1. Polyacrylamide-gel electrophoresis of various forms of human transferrin and asialo-transferrin (a) Whole human transferrin; (b) DEAE-cellulose peak 2Fe-TfC-5-6; (c) DEAE-cellulose peak 2Fe-TfC-6-6; (d) mixture of (b) and (c); (e) asialo-transferrin from (b); (f) asialo-transferrin from (c); (g) mixture of (e) and (f); (h) mixture of (e) with asialo-transferrin from (a); (i) mixture of (f) with asialo-transferrin from (a). Each gel was loaded with 30–40 μ g of protein and run for 1 h at 4 mA/gel at pH 8.2. Migration was from top to bottom, with the anode at the bottom. All samples were in the 2Fe form.

Table 1. Carbohydrate analyses of human transferrin fractions prepared by chromatography on DEAE-cellulose. Monosaccharides are expressed as number of residues per molecule of protein. Values were calculated by using an $A_{1\text{cm}}^{1\%}$, $_{278}$ value of 13.0 (Lane, 1971). Analyses were performed in duplicate for two individual samples (a and b) of each fraction. Glucosamine is probably present as N-acetylglucosamine. For the sialic acid value of 4.23 see the text.

Monosaccharide	Peak 2Fe-TfC-5-6		Peak 2Fe-TfC-6-6	
	a	b	a	b
N-Acetylneuraminic acid	3.81	3.83	4.80	4.23
Galactose	3.68	3.56	4.75	4.82
Mannose	5.95	5.71	6.03	6.12
Glucosamine	8.13	7.96	9.31	8.85

drate compositions of these proteins are summarized in Table 1. It is evident that the composition of the transferrin species that electrophoresed as the major band (2Fe-TfC-5-6; Fig. 1b) was consistent with the presence of two biantennary glycans, each consisting of four *N*-acetylglucosamine, three mannose, two galactose and two sialic acid residues. By comparison, the composition of the more anodic transferrin species (2Fe-TfC-6-6; Fig. 1c) was consistent with the presence of a biantennary glycan and of a tri-antennary glycan, the latter composed of five *N*-acetylglucosamine residues and three residues each of mannose, galactose and sialic acid. The sialic acid content of 4.23 residues/molecule, measured for one of the preparations in Table 1, was less than the expected value of approx. 5. However, on electrophoresis, this protein fraction was shown to have selectively lost some sialic acid during preparation (Wong & Regoeczi, 1977).

The protein obtained by desialylating transferrin from peak 2Fe-TfC-5-6 (Fig. 1b) is designated asialo-transferrin type 1. It electrophoresed as a single band (Fig. 1e) and it was not retained by Sepharose–hepatic lectin, but it was quantitatively retained by Sepharose–concanavalin A in the presence of 0.1 M-NaCl.

Desialylation of transferrin from peak 2Fe-TfC-6-6 (Fig. 1c) yielded an electrophoretically homogeneous protein (Fig. 1f) with the same mobility as asialo-transferrin type 1 or desialylated whole transferrin (Figs. 1g and 1i). Chromatography of this material on Sepharose–hepatic lectin resulted in a non-retained (designated asialo-transferrin type 2) and a retained (designated asialo-transferrin type 3) fraction, their relative proportions being approx. 40–50% and 60–50%. After asialo-transferrin made from whole transferrin had been loaded on Sepharose concanavalin A, asialo-transferrin types 2 and 3 passed through the column on washing with the equilibrating buffer supplemented with 0.1 M-NaCl.

All three asialo-transferrin types were quantitatively precipitated with an anti-globulin fraction to human transferrin by the technique of Farr (1958).

Partition of small doses (approx. 1 µg/100 g body wt.) of each asialo-transferrin type between plasma and liver was tested in groups of rats. The results (Table 2) showed that binding to the liver increased in the order: asialo-transferrin type 1 < type 2 < type 3. Furthermore the asialo-transferrin types were displaced from the liver surface by 2.5 mg of asialo-orosomucoid per 100 g body wt. in the reverse order. By injecting asialo-orosomucoid 5 min before asialo-transferrin, the association of each asialo-transferrin type with the liver was reduced to insignificant values (1–3% of the dose). In this case, disappearance of ¹²⁵I-labelled asialo-transferrin from the plasma closely paralleled that of the simultaneously injected ¹³¹I-labelled albumin. This is shown for asialo-transferrin type 3 in Fig. 2. Similar curves were obtained also with types 1 and 2 in other animals.

The different affinities of the three asialo-transferrin types for the hepatic lectin *in vivo* were also evident from the quantities of unbound asialo-transferrin that remained detectable in the plasma under quasi-equilibrium conditions after the injection of small comparable doses of these proteins (Fig. 3).

Mass-action and synergism in the endocytosis and catabolism of the asialo-transferrin types by the rat liver

Metabolic-balance studies, lasting 35 min, were conducted with each type of asialo-transferrin in groups of rats to determine (a) if any of the types is taken up by the liver at low plasma concentrations, (b) the effect of increasing the plasma concentration on the catabolism of each asialo-transferrin type and (c) whether asialo-transferrins of different types can act synergistically in promoting endocytosis and catabolism.

Table 2. Partition of the radioactivity between plasma and liver 5 min after the injection of different types of ¹²⁵I-labelled asialo-transferrin

The dose per animal was 0.97 (±0.05 S.D.) µg/100 g body wt. and each value is the mean ±S.E.M. for five experiments. Asialo-transferrin types 1–3 are defined in the text. Treatment signifies the intravenous injection of 2.5 mg of human asialo-orosomucoid/100 g body wt. at 5 min after the asialo-transferrin followed by an additional 5 min before death to allow displacement of asialo-transferrin from the liver surface. All liver values have been corrected for the plasma asialo-transferrin trapped in the hepatic circulation at the time of death.

Asialo-transferrin type	Treatment	Radioactivity (% of dose)		
		In plasma volume	In liver	Recovery
1	–	47.3 ± 1.4	49.6 ± 1.8	96.9 ± 0.6
1	+	91.6 ± 1.1	8.7 ± 0.4	100.3 ± 0.9
2	–	24.4 ± 1.8	71.2 ± 1.7	95.6 ± 0.5
2	+	84.4 ± 1.8	11.3 ± 1.2	95.7 ± 0.8
3	–	5.0 ± 1.9	93.8 ± 1.9	98.8 ± 0.4
3	+	72.1 ± 2.2	28.5 ± 1.5	100.6 ± 1.4

In studies on mass action, a small dose (approx. 1 µg/100g body wt.) of ¹²⁵I-labelled asialo-transferrin (type 1, 2 or 3) was injected, followed 4min later by a larger quantity (50, 100 or 250 µg/100g body wt.) of unlabelled asialo-transferrin of the same or of a different type. The quantities of asialo-transferrin types 2 and 3, which are prepared by affinity chromatography on Sepharose-hepatic lectin, required for the second injection were too large for the use of purified proteins. Therefore these types were administered as a mixture obtained by desialylating transferrin from peak 2Fe-TfC-6-6. As already pointed out, the mixture contains asialo-transferrin types 2 and 3 in approx. equal proportion.

Results with the three asialo-transferrin types are summarized in Tables 3, 4 and 5, and the following prominent features should be pointed out. At the

lowest doses (0.6–1 µg/100g body wt.), large portions of the dose (85–90%) of all asialo-transferrin types were recovered as protein-bound radioactivity; protein degradation, as judged from quantities of non-protein-bound activity in liver and plasma, was minimal, though values increased in the order of type 1 < type 2 < type 3. Injection of a larger quantity of unlabelled asialo-transferrin 4min later resulted in reductions in dose recoveries and increases in the concentrations of labelled products of proteolysis.

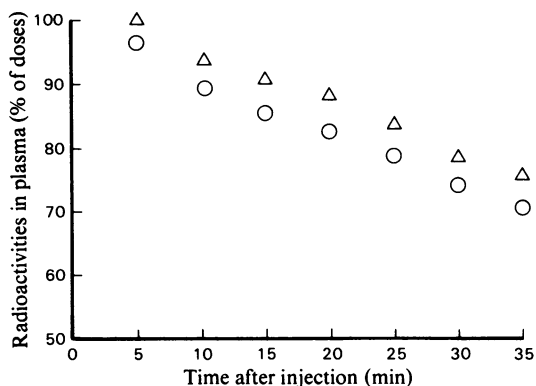


Fig. 2. Disappearance of ¹³¹I-labelled human albumin (Δ) and ¹²⁵I-labelled asialo-transferrin type 3 (○) in a rat pre-injected with asialo-orosomuroid

Unlabelled asialo-orosomuroid (2.5 mg/100g body wt.) was given 5 min before the mixed dose of albumin (approx. 2 µg/100g body wt.), and asialo-transferrin (0.95 µg/100g body wt.) was injected at zero time.

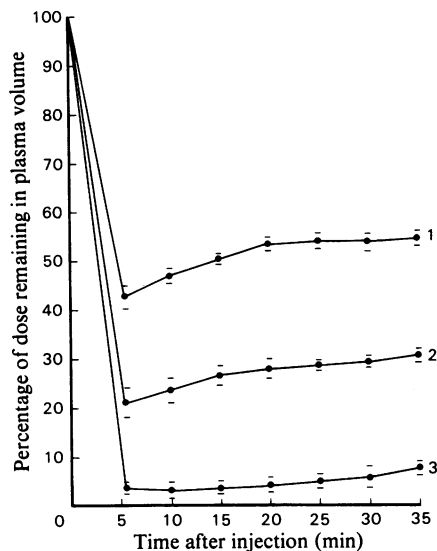


Fig. 3. Protein-bound radioactivities in the plasma of rats after the injection of ¹²⁵I-labelled asialo-transferrin types 1, 2 or 3

Curves are numbered in accordance with the asialo-transferrin types. Each value has been corrected for diffusion and sampling and is the mean of six to eight experiments. Bars denote s.e.m. The doses were 0.9–1.1 µg/100g body wt.

Table 3. Metabolic balance at 35 min after the injection of ¹²⁵I-labelled asialo-transferrin type 1

Each group consisted of four to six rats, and the values listed are their means ±s.e.m. The dose for groups A, B, C, E and F was 0.98 (±0.04s.d.) µg/100g body wt., and for group D it was 241 (±2.1s.e.m.) µg/100g body wt. At 4min after the injection of the dose, the following types and quantities of unlabelled asialo-transferrin were injected per 100g body wt.: group A, none; B, type 1, 100 µg; C, type 1, 250 µg; D, none; E, mixture of types 2 and 3, 100 µg; F, mixture of types 2 and 3, 250 µg.

Group	Protein-bound ¹²⁵ I (% of dose)		Non-protein-bound ¹²⁵ I (% of dose)		Recovery of dose as protein-bound ¹²⁵ I (%)
	In plasma volume	In the liver	In plasma volume	In the liver	
A	54.5 ± 1.5	30.5 ± 1.6	0.58 ± 0.11	0.48 ± 0.04	85.0 ± 1.5
B	47.6 ± 1.2	21.4 ± 2.4	1.72 ± 0.24	1.52 ± 0.42	69.0 ± 2.1
C	38.1 ± 2.0	25.4 ± 1.2	3.12 ± 0.44	3.32 ± 0.42	63.5 ± 2.9
D	41.5 ± 0.9	17.6 ± 1.2	2.90 ± 0.32	2.46 ± 0.28	59.1 ± 1.4
E	56.8 ± 2.4	18.6 ± 1.4	1.58 ± 0.22	2.42 ± 0.47	75.4 ± 1.8
F	52.3 ± 1.5	21.3 ± 2.8	1.58 ± 0.24	2.82 ± 0.41	73.6 ± 1.3

Table 4. *Metabolic balance at 35 min after the injection of ¹²⁵I-labelled asialo-transferrin type 2*

Each group consisted of four to six rats, and the values listed are their means \pm s.e.m. The dose was $0.85 (\pm 0.1 \text{ s.d.}) \mu\text{g}/100\text{g}$ body wt. At 4 min after the injection of the dose, the following types and quantities of unlabelled asialo-transferrin were injected per 100g body wt.: group A, none; B, type 1, $100 \mu\text{g}$; C, type 1, $250 \mu\text{g}$; D, mixture of types 2 and 3, $100 \mu\text{g}$; E, mixture of types 2 and 3, $250 \mu\text{g}$.

Group	Protein-bound ¹²⁵ I (% of dose)		Non-protein-bound ¹²⁵ I (% of dose)		Recovery of dose as protein-bound ¹²⁵ I (%)
	In plasma volume	In the liver	In plasma volume	In the liver	
A	31.4 ± 1.2	53.2 ± 1.2	0.47 ± 0.17	0.90 ± 0.08	84.6 ± 1.7
B	26.7 ± 1.7	38.0 ± 2.7	2.51 ± 0.24	4.38 ± 0.54	64.7 ± 3.8
C	22.4 ± 0.9	27.6 ± 1.7	4.26 ± 0.27	6.17 ± 0.35	50.0 ± 2.0
D	26.1 ± 2.1	32.9 ± 1.2	3.53 ± 0.27	5.21 ± 0.21	59.0 ± 1.8
E	37.3 ± 2.3	23.4 ± 1.2	2.86 ± 0.25	3.67 ± 0.34	60.7 ± 3.2

Table 5. *Metabolic balance at 35 min after the injection of ¹²⁵I-labelled asialo-transferrin type 3*

Each group consisted of four to eight rats, and the values listed are their means \pm s.e.m. The dose was $0.62 (\pm 0.3 \text{ s.d.}) \mu\text{g}/100\text{g}$ body wt. At 4 min after the injection of the dose, the following types and quantities of unlabelled asialo-transferrin were injected per 100g body wt.: group A, none; B, type 1, $100 \mu\text{g}$; C, type 1, $250 \mu\text{g}$; D, mixture of types 2 and 3, $100 \mu\text{g}$; E, mixture of types 2 and 3, $250 \mu\text{g}$; F, desialylated whole transferrin, $50 \mu\text{g}$; G, desialylated whole transferrin, $100 \mu\text{g}$; H, desialylated whole transferrin, $250 \mu\text{g}$.

Group	Protein-bound ¹²⁵ I (% of dose)		Non-protein-bound ¹²⁵ I (% of dose)		Recovery of dose as protein-bound ¹²⁵ I (%)
	In plasma volume	In the liver	In plasma volume	In the liver	
A	6.3 ± 0.4	84.4 ± 2.2	0.89 ± 0.16	1.65 ± 0.24	90.7 ± 2.0
B	4.3 ± 0.5	60.9 ± 3.7	3.36 ± 0.15	2.85 ± 0.34	65.2 ± 3.8
C	6.2 ± 0.5	46.5 ± 4.7	4.57 ± 0.70	3.48 ± 0.59	52.7 ± 5.2
D	4.7 ± 1.8	30.4 ± 5.7	4.55 ± 0.64	7.32 ± 1.76	35.1 ± 6.1
E	5.3 ± 0.6	22.9 ± 2.0	7.04 ± 0.39	5.25 ± 0.68	28.2 ± 1.6
F	7.9 ± 1.1	65.7 ± 3.1	1.99 ± 0.30	3.79 ± 0.58	73.6 ± 3.7
G	9.3 ± 1.0	49.1 ± 3.6	3.62 ± 0.27	6.06 ± 0.51	58.4 ± 3.5
H	6.8 ± 0.7	28.8 ± 2.8	5.72 ± 0.39	8.81 ± 1.20	35.6 ± 2.8

The magnitude of this effect depended on the dose and the type of asialo-transferrin. Catabolism of one type of asialo-transferrin was also induced by the administration of appropriate amounts of another asialo-transferrin type or a mixture of several types. However, quantification of the catabolic response induced by non-identical asialo-transferrin types was complicated by the prevailing differences in affinities for the hepatic lectin; thus any second injection containing significant amounts of unlabelled asialo-transferrin type 3 displaced a portion of the pre-injected labelled type-1 or type-2 asialo-transferrin from the liver surface, with the consequence that the catabolic response was reduced (see group F in Table 3 and group E in Table 4).

Displacement of asialo-transferrin type 1 from the liver surface by an asialo-transferrin of a higher affinity type was deduced from the plasma radioactivity curves (Fig. 4, curves E and F). A portion of labelled asialo-transferrin type 1 was also released by the hepatic lectin when a larger dose of unlabelled asialo-transferrin type 1 was injected (Fig. 4, curves

B and C). However, the latter phenomenon is thought to be a result of the dynamic equilibrium between free and bound forms of asialo-transferrin of the same type. In support of this explanation, plasma radioactivity concentrations became virtually identical by the 10th minute irrespective of whether the large unlabelled dose ($240\text{--}250 \mu\text{g}/100\text{g}$ body wt.) was added to the radioactive dose at zero time or injected separately 4 min later (Fig. 4, curves C and D). Asialo-transferrin type 2, despite its comparatively higher affinity for the rat hepatic lectin, was displaced to some extent by large injections of asialo-transferrin type 1 (Fig. 5, curves B and C). Injection of a large dose of mixed types 2 and 3 had a more prominent effect on the plasma curve of asialo-transferrin type 2 (Fig. 5, curve E), which is probably attributable partly to displacement of type 2 by the higher-affinity type 3 and partly to the dynamic equilibrium of type 2 itself. In contrast with the situation with asialo-transferrin types 1 and 2, return of the radioactivity from the bound to the free form was only observed with asialo-transferrin type 3 if a

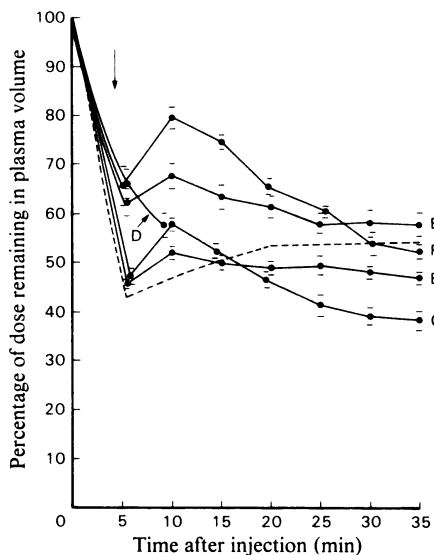


Fig. 4. Effect of a large dose of an unlabelled asialo-transferrin given at 4 min on the plasma protein-bound radioactivity curve of a small dose of ¹²⁵I-labelled asialo-transferrin type 1 injected at zero time

Groups of animals shown here are identical with those listed in Table 3 and curves are lettered accordingly. Bars denote ±S.E.M. The arrow indicates injection of the unlabelled asialo-transferrin in quantities as specified in Table 3. To avoid congestion, curve D is only drawn in for 9 min after which it closely paralleled curve C. The broken curve is a reproduction of curve 1 from Fig. 3 as the control. Each curve has been corrected for losses due to diffusion and sampling from the data on ¹³¹I-labelled albumin contained in the labelled asialo-transferrin dose.

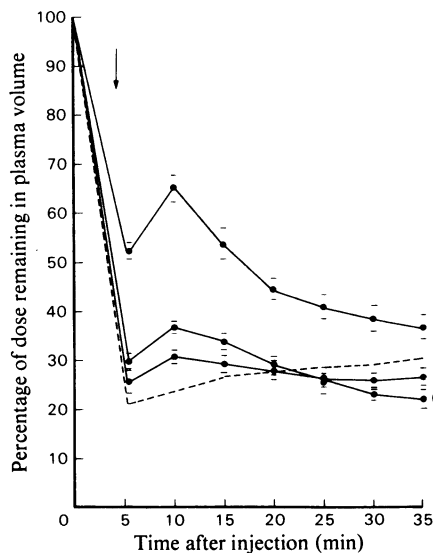


Fig. 5. Effect of injecting a large dose of unlabelled asialo-transferrin at 4 min on the plasma protein-bound radioactivity curve of a small dose of ¹²⁵I-labelled asialo-transferrin type 2 given at zero time

Groups of animals shown here are identical with those listed in Table 4 and curves are lettered accordingly. Bars denote ±S.E.M. The arrow indicates injection of the unlabelled asialo-transferrin in quantities as specified in Table 4. The broken curve is a reproduction of curve 2 from Fig. 3 as the control. Each curve has been corrected for losses due to diffusion and sampling from the data on ¹³¹I-labelled albumin contained in the labelled asialo-transferrin dose.

high dose (250 µg/100 g body wt.) of mixed types 2 and 3 was given (Fig. 6).

After successful induction of endocytosis on a mass-action basis, degradation of asialo-transferrin became evident within a few minutes from the rising values for non-protein-bound radioactivity in the plasma samples (Fig. 7). Values for non-protein-bound radioactivity in the livers at 35 min correlated very well ($r = +0.81$) with the corresponding values in plasma. Furthermore, excellent inverse correlations existed between values for recovery of protein-bound radioactivity and hepatic non-protein-bound radioactivity ($r = -0.86$), as well as between those for recovery and plasma non-protein-bound radioactivity ($r = -0.96$). Hepatic non-protein-bound radioactivity arising from the labelled albumin averaged at 0.18% of the albumin dose for all animals. Group means for this type of radioactivity did not correlate with hepatic non-protein-bound radioactivity from asialo-transferrin ($r = +0.02$).

Discussion

Not long ago, human transferrin type C was thought to be a homogeneous glycoprotein (Putnam, 1975) containing two identical biantennary glycans (Jamieson *et al.*, 1971). The presence of three electrophoretic components in purified transferrin was first reported by Heide & Haupt (1964), but their finding failed to gain broad recognition, perhaps because their preparation originated from pooled starting material. However, a subsequent study of the electrophoretic morphology of transferrins from 11 individual donors did confirm the heterogeneity of transferrin as observed by Heide & Haupt (Regoeczi *et al.*, 1977). A possible explanation for the electrophoretic heterogeneity of transferrin came from the work of Spik *et al.* (1974), who demonstrated the presence of an additional *N*-acetylneuraminyl- α (2-6)-*N*-acetyl-lactosamine sequence in a fraction of the transferrin glycopeptides. Using Sepharose-concanavalin A, a lectin capable of distinguishing

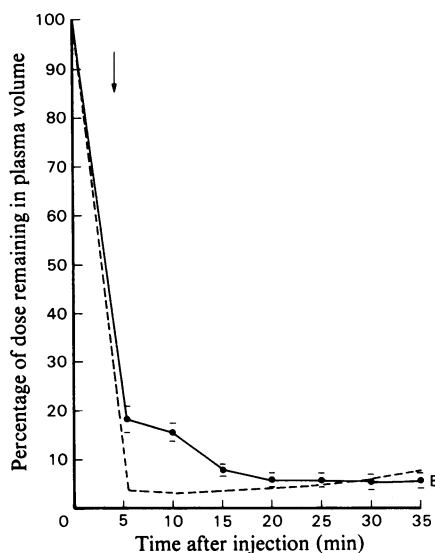


Fig. 6. Effect of injecting a mixture of unlabelled asialo-transferrin types 2 and 3 ($250 \mu\text{g}/100\text{g}$ body wt.) at 4 min on the plasma protein-bound radioactivity curve of a small dose of ^{125}I -labelled asialo-transferrin type 3 given at zero time

Curve E represents group E in Table 5. Bars denote \pm S.E.M. The arrow denotes time of the injection of unlabelled asialo-transferrin. The broken curve is curve 3 from Fig. 3 for comparison. Each value has been corrected for losses due to diffusion and sampling from the data on ^{131}I -labelled albumin contained in the labelled asialo-transferrin dose.

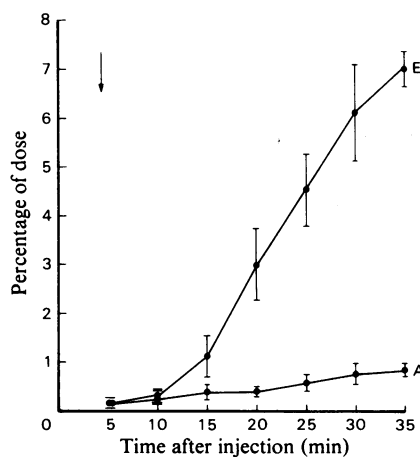


Fig. 7. Non-protein-bound radioactivity from ^{125}I -labelled asialo-transferrin type 3 in the plasma volume in two groups of rats

Both groups are from Table 5 and they are lettered correspondingly. Bars denote \pm S.E.M. Doses are given in Table 5. The arrow denotes the injection of a mixture of unlabelled asialo-transferrin types 2 and 3 ($250 \mu\text{g}/100\text{g}$ body wt.) in group E.

between bi- and tri-antennary glycans (Krusius *et al.*, 1976), we were able to localize transferrin molecules having the additional carbohydrate in the most anodic electrophoretic transferrin band (Wong *et al.*, 1978). More recently, we have found evidence of heterogeneity within the triantennary glycan population of transferrin; after desialylation, only a portion of the triantennary glycan fraction binds to the Sepharose-hepatic lectin, whereas the remainder is retarded by the conjugate (Hatton *et al.*, 1979). The effect of minor variations in the structure of transferrin asialo-glycopeptides on the binding properties of the protein is illustrated by the present work.

Successful separation of transferrin molecules possessing two biantennary glycans from those having mixed (one two- and one three-forked) glycans on DEAE-cellulose relies on the use of fully sialylated protein. However, during prolonged (and not sterile) fractionation procedures, transferrin can lose sialic acid residues (Wong & Regoeczi, 1977). Consequently, a transferrin molecule that originally had five sialic acids (mixed glycan) and now is missing one, is co-eluted from the DEAE-cellulose column with transferrin molecules that have four sialic acid residues associated with two biantennary glycans. To obtain homogeneous preparations of asialo-transferrin type 1, it is therefore essential to use as a starting material transferrin that exhibits no signs of carbohydrate decomposition by polyacrylamide-gel electrophoresis.

Separation of asialo-transferrin types 2 and 3 requires affinity chromatography on the immobilized lectin from rabbit liver. This lectin does not separate asialo-transferrin type 1 from type 2, hence the need for the elimination of asialo-transferrin type 1 before desialylation by chromatography on DEAE-cellulose. Asialo-transferrin type 1 exhibits low affinity and type 3 exhibits high affinity both for the immobilized rabbit lectin and the rat lectin *in situ*. However, the strength of binding of asialo-transferrin type 2 to the two lectins is markedly different. Whether this is due to a species-specific difference between rat and rabbit lectins, or an artificial performance of the rabbit lectin in the conjugated state (restricted subunit mobility?), is unknown. Nevertheless, the observed difference is highly reproducible and is the proof for the existence of asialo-transferrin type 2.

The chemical nature of the difference between asialo-transferrin types 2 and 3 remains to be established. From our previous work (Hatton *et al.*, 1979) and the present data (Table 1) a structural difference between the triantennary glycans from both types, which could result in different spatial arrangements of the three galactose groups, seems a probability. This could be brought about, for example, by variations in the site of attachment of the additional *N*-acetyl-lactosamine sequence (Spik *et al.*, 1974) to the trimannosido-*N*-acetylchitobiose core.

In view of the marked differences that exist among the three asialotransferrin types with regard to their binding to the rat hepatic lectin *in vivo*, it was interesting to investigate whether differences existed among them with respect to their uptake by the rat liver. From the results of the metabolic studies (Tables 3-5) this is clearly not the case, thus confirming that our earlier conclusion of mass-dependent endocytosis of human asialo-transferrin by the rat hepatocyte, as established in our earlier study (Regoeczi *et al.*, 1978) with asialo-transferrin from whole transferrin, is also valid for the individual asialo-transferrin types. Furthermore the present data also show that asialo-transferrin molecules of different carbohydrate structures can act synergistically in eliciting a signal for endocytosis.

In 5 min, the liver of the intact rat removes from the circulation 93-94% of the injected asialo-transferrin type 3 (Table 2), which is a value similar to that observed with comparable quantities of asialo-fetuin or asialo-orosomucoid (Charlwood *et al.*, 1979). Yet, whereas most of the asialo-transferrin is held by the plasma membrane, asialo-fetuin and asialo-orosomucoid are qualitatively taken up and promptly degraded. This astonishing difference illustrates the need to reappraise the critical number of galactose groups that an asialo-glycoprotein is required to possess for its effective elimination and degradation via the hepatic lectin pathway. From studies with partially desialylated caeruloplasmin, this minimal number was originally thought to be two (Van den Hamer *et al.*, 1970). However, as shown by asialo-transferrin type 3 in the present study, five galactose groups, arranged into one bi- and one tri-antennary glycan, are clearly inadequate to effect endocytosis when administered at a low dose ($< 1 \mu\text{g}/100\text{g}$ body wt.). A possible explanation is that the experiments by Van den Hamer *et al.* (1970) did not distinguish between binding and cellular uptake and, consequently, the number of galactose groups calculated from their results may simply be a threshold for binding.

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