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Distinction between Binding and Endocytosis of Human Asialo-Transferrin by the Rat Liver

By ERWIN REGOECZI, PATRICIA TAYLOR, MARK W. C. HATTON, KWONG-LOI WONG and ALEX KOJ*
Plasma Protein Research Laboratory, McMaster University Health Sciences Centre, Hamilton, Ont., Canada L8S 4J9

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The ability of the rat liver to bind and endocytose human asialo-transferrin was investigated *in vivo*. Asialo-transferrin was separated from incompletely desialylated transferrin and neuraminidase by chromatography before being labelled with ^{125}I. Plasma radioactivity curves and hepatic radioactivity contents measured over a 1270-fold dose range led to the following observation. At the lowest dose (0.4 μg/100g body wt.), the distribution of asialo-transferrin between plasma and liver resembled a reversible reaction reaching equilibrium in approx. 20min. After 35min, 93% of the dose was recovered with the plasma and liver as protein-bound radioactivity. Most of the asialo-transferrin associated with the liver could be displaced by asialo-orosomucoid, indicating that binding of asialo-transferrin to the galactose-specific lectin on the plasma membrane of hepatocytes was not followed by a signal for endocytosis. A range of doses, up to an average of 509.2 μg of asialo-transferrin per 100g body wt., resulted in progressive increments in asialo-transferrin catabolism, as evidenced by lower dose recoveries and increased concentrations of non-protein-associated radioactivity in the liver and plasma volume. These observations indicate that binding and endocytosis of human asialo-transferrin by the rat hepatocyte are distinct phenomena. Individual asialo-transferrin molecules, although readily bound by the hepatic lectin, lack either the quantity or spacing of terminal galactose residues necessary for triggering endocytosis. Although endocytosis is induced by several asialo-transferrin molecules acting synergistically, preliminary experiments with asialo-glycopeptides and other substances have so far failed to provide further insight into the chemical basis of the signal for endocytosis.

The plasma membrane of hepatocytes from several mammalian species contains a lectin (Stockert *et al.*, 1974) that selectively removes glycoproteins from the circulation in which carbohydrate chains terminate in galactose (see review by Ashwell & Morell, 1974). These asialo-glycoproteins are rapidly endocytosed by the hepatocyte and transferred to the lysosomes for degradation (Gregoriadis *et al.*, 1970; Rogers & Kornfeld, 1971; LaBadie *et al.*, 1975). A survey of this pathway in the rat by Morell *et al.* (1971) showed that of ten different asialo-glycoproteins tested nine were promptly removed by the liver, but human asialo-transferrin was apparently not significantly affected.

The exceptional behaviour of asialo-transferrin prompted us a few years ago to undertake further studies with this protein, and our results were different. A comparison of the elimination rate of labelled asialo-transferrin with that of simultaneously injected transferrin showed unequivocally that asialo-transferrin was prematurely catabolized both in rabbits and rats because of preferential hepatic uptake (Regoeczi *et al.*, 1974; Regoeczi & Hatton, 1974).

The aim of the present study was to examine in detail the interaction of human asialo-transferrin with the rat liver and thus to resolve the conflicting views that exist in the literature about this protein.

Materials and Methods

Human asialo-transferrin

Batches of transferrin were isolated from fresh plasma of individual donors and their purity was established by immunoelectrophoresis (Regoeczi *et al.*, 1977). Samples of the protein were desialylated with neuraminidase acylneuraminyl hydrolase (EC 3.2.1.18) (*Clostridium perfringens*; Sigma, St. Louis, MO, U.S.A.) from which proteinases had been removed by a chromatographic technique (Hatton & Regoeczi, 1973). In a typical procedure, 30mg of transferrin was incubated with 1–1.5 units of neuraminidase in 2ml of 0.1M-sodium acetate, pH5.5,

* Present address: Institute of Molecular Biology, Jagiellonian University, Krakow, Poland.

at 37°C under an atmosphere of toluene. [One unit of neuraminidase activity denotes the quantity of enzyme that liberates 1 μmol of sialic acid/min from human α_1-acid glycoprotein (1%, w/v) in 0.1M-sodium acetate buffer, pH5.0 (*Clostridium perfringens* enzyme) or pH5.5 (*Vibrio cholerae* enzyme) at 37°C.] After 48h, the mixture was transferred into Visking dialysis tubing and dialysed overnight against 0.01M-Tris/HCl, pH8.0, at 4°C. The protein was saturated with iron (Charlwood, 1971) and the excess iron removed by further dialysis against the above buffer. A DEAE-cellulose (DE-52, Whatman; W. and R. Balston, Maidstone, Kent, U.K.) column (1.4cm×34cm) was equilibrated with 0.01M-Tris/HCl, pH8.0, before the asialo-transferrin was loaded. The column was developed by a gradient that permitted the separation of transferrin molecules according to their sialic acid contents (Wong & Regoeczi, 1977). The first peak obtained was pooled, concentrated by ultrafiltration and dialysed for 24h against 0.89% (w/v) NaCl. Transferrin concentrations were determined as described by Lane (1971).

Electrophoretic examination of samples of this asialo-transferrin in alkaline polyacrylamide gels (Clarke, 1964) invariably yielded a single band. Analyses of the glycopeptides from two preparations by g.l.c. (Zanetta & Vincendon, 1974) did not show the presence of sialic acid, and neuraminidase activity, assayed by the method of Aminoff (1961), with bovine submaxillary mucin as a substrate, was not detected in the preparations.

Other proteins

Human α_1-acid glycoprotein (orosomucoid) was isolated as described by Charlwood *et al.* (1976) and desialylated as follows. The freeze-dried protein (100–150mg) was dissolved in 5ml of 0.1M-sodium acetate, pH5.5, and mixed with 1ml (0.12 unit) of neuraminidase (*Vibrio cholerae*; GIBCO, Grand Island, NY, U.S.A.). After 20h at 37°C, the incubation mixture was dialysed for 24h against 0.01M-Tris/HCl, pH8.0, and loaded on a DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) column (1.1cm ×20cm) that had been equilibrated with 0.01M-Tris/HCl, pH8.0. After washing of the column with 100ml of the equilibrating buffer, the desialylated protein was step-eluted by 0.2M-Tris/HCl, pH8.0, whereas under these conditions the neuraminidase activity was retained by the column. The product was dialysed against distilled water for 48h at 4°C and freeze-dried. Sialic acid determinations, by the method of Aminoff (1961) after hydrolysis in 0.05M-H_2SO_4 for 1h at 80°C, showed that preparations were 89–92% desialylated by comparison with samples of the starting materials.

Human albumin was obtained from Behringwerke (Marburg, Germany) and bovine fetuin from GIBCO. Chicken α_1-acid glycoprotein was prepared as described elsewhere (Charlwood *et al.*, 1976).

Labelled proteins

Asialo-transferrin (0.5–1mg) was labelled with ^{125}I, and albumin (2–3mg) with ^{131}I, by the method of McFarlane (1958). The reaction was carried out under conditions that limited the number of substituted iodine atoms to an average of less than 2 per molecule of protein.

Animals

Male Sprague–Dawley rats with a mean body wt. of 244 (S.D. ±50)g were used. They received a standard pelleted diet and water *ad lib.* The results are based on observations from 104 animals.

Experimental design

During the prototype metabolic experiment, lasting 30–35min, the rat was restrained but not anaesthetized until 10–15s before being killed. The dose, consisting of a predetermined amount of ^{125}I-labelled asialo-transferrin and approx. 50–100μg of ^{131}I-labelled albumin in a volume of 0.25–0.35ml, was injected into a tail vein and a stop-watch was started. Small blood samples were withdrawn into dry heparin at approx. 5min intervals from another tail vein and their mean time was calculated from the interval that elapsed between the start and the end of the sampling (usually 20–35s). At the end of the experiment, sodium pentobarbital (6mg/100g body wt.) was injected, and the liver was rapidly removed and immediately homogenized in 50ml of 0.89% NaCl containing 16% (v/v) 2-octanol.

Dosage

The quantities of asialo-transferrin injected were related to the body weights and arranged in eight groups spanning a 1270-fold difference in doses. The amount of radioactivity injected in individual experiments was kept relatively constant at approx. 3.9×10^6c.p.m. per animal by adding carrier asialo-transferrin to the ^{125}I-labelled protein as necessary. In addition, each dose contained ^{131}I-labelled albumin (see below). A standard was prepared from each dose stock solution by a gravimetric method (Regoeczi, 1975) and the dose each animal received was determined from the weight of the syringe before and after injection.

Processing samples

From the plasma, 50μl samples were made up to counting volume (2ml) with 0.89% NaCl. The total

volume of liver homogenates was recorded and 2ml portions were taken for radioactivity counting. All specimens, including the standards, were assayed in duplicate. After counting, quantities of non-protein-associated radioactivity were determined in each sample by recounting deproteinized samples. Plasma samples were deproteinized with 2ml of 20% (w/v) trichloroacetic acid, and homogenates with 3ml of 5% (w/v) phosphotungstic acid in 2M-HCl. Radioactivities were assayed in a Packard model 5986 multi-channel analyser. Protein-bound radioactivities were calculated as the difference between total and non-protein-bound radioactivities.

Evaluation

It was assumed that losses of the injected protein from the plasma volume over 35min would be due to the following mechanisms: (*a*) binding, with or without subsequent degradation, by the liver, (*b*) diffusion into the extravascular space, (*c*) removal with blood samples and (*d*), to a minor extent, regular catabolism (see McFarlane & Koj, 1970). To obviate the combined effect of (*b*)–(*d*), the asialo-transferrin plasma curve was corrected from the data on the co-injected albumin. Thus the disappearance rate constant for albumin-bound ^{131}I radioactivity was calculated from the fifth minute onwards for each sampling interval and was used to correct the corresponding section of the integrated mean asialo-transferrin plasma radioactivity curve.

Albumin was preferred to transferrin as a diffusion marker, because of our earlier finding that storage may affect the sialic acid composition of transferrin (Wong & Regoeczi, 1977). Further encouragement for using albumin was received from experiments with rats in which the plasma curves of simultaneously injected albumin and freshly prepared transferrin paralleled each other closely over the first 40min. The use of albumin also provided a means of measuring residual plasma in the hepatic vascular bed (Regoeczi, 1975).

The magnitude of catabolic response to any asialo-transferrin dose was assessed from the fraction of the dose that could be recovered as protein-bound radioactivity at the end of an experiment. Concentrations of terminal labelled products from asialo-transferrin in the liver and plasma served as an additional semi-quantitative measure of catabolic activity.

Preparation of asialo-glycopeptides from human transferrin and bovine fetuin

The methods have been reported in detail elsewhere (Hatton *et al.*, 1978) and only an outline will be given here. Each protein (final concentration 1%) was digested by a *Streptomyces griseus* proteinase (Sigma) in 0.02M-Tris/HCl, pH8.0, for 48–72h at 37°C. The products were passed through a Sephadex G-50 column in 0.1M-pyridine/acetic acid, pH5.5, and the glycopeptide peaks were identified by the phenol/H_2SO_4 method (Dubois *et al.*, 1956), pooled and freeze-dried. The human transferrin glycopeptide peak was further fractionated by high-voltage electrophoresis, which yielded two major constituents. From amino acid and carbohydrate analyses these two glycopeptides closely resembled glycans I and II as reported by Spik *et al.* (1975) for human transferrin. The glycopeptides of bovine fetuin were eluted from Sephadex G-50 as a sharp major peak followed by a small peak. For the experiments described below only the major peak was taken. On g.l.c. analysis, galactose, mannose, *N*-acetylglucosamine and *N*-acetylneuraminic acid were detected, but not *N*-acetylgalactosamine. It was therefore concluded that only fetuin glycopeptides with *N*-glycosidically linked three-forked heterosaccharides were present (Spiro, 1973).

Chromatographic studies

The chromatographic behaviour of the fraction of ^{125}I-labelled asialo-transferrin that was still in the circulation after 20min was compared with a ^{131}I-labelled sample of the same preparation that had not been injected. A column (2.2cm×36cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05M-sodium phosphate, pH7.4, containing 0.15M-NaCl, and 1ml of heparinized rat plasma, trace-contaminated with the ^{131}I-labelled preparation, was filtered through it at a flow rate of approx. 20ml/h with the equilibrating buffer as eluent. For chromatography on DEAE-cellulose, albumin was first removed from 2ml of rat plasma by passage through a Sepharose–Cibacron Blue column (1cm×8cm), which was prepared and operated as previously described (Koj *et al.*, 1978). A small quantity of ^{131}I-labelled control asialo-transferrin was added to the fraction not adsorbed by this column and the mixture was dialysed against 0.01M-Tris/HCl, pH8.0. Chromatography on a DEAE-cellulose column (1cm×39cm) was undertaken under the same conditions described above for the preparation of transferrin.

Other materials

Cytochalasin B (grade B) was obtained from Calbiochem, San Diego, CA, U.S.A.

Results

Radioactivity in plasma and liver after injection of small quantities of ^{125}I-labelled asialo-transferrin

Injections of ^{125}I-labelled asialo-transferrin in the lowest dose range shown in Table 1 resulted in

Table 1. *Metabolic balance at 35 min after the injection of different quantities of ^{125}I-labelled asialo-transferrin in rats*
Experimental details are provided in the text. Values are means ± S.E.M. —, Not determined.

Dose (μg/100g)	No. of experiments	Protein-bound ^{125}I in plasma volume (μg/100g)	Protein-bound ^{125}I in plasma volume (% of dose)	Protein-bound ^{125}I in the liver (μg/100g)	Protein-bound ^{125}I in the liver (% of dose)	Non-protein-bound ^{125}I in plasma volume (% of dose)	Non-protein-bound ^{125}I in liver (% of dose)	Recovery of dose as protein-bound ^{125}I (%)
0.4 ± 0.02	4	0.24 ± 0.01	55.3 ± 1.0	0.16 ± 0.002	37.5 ± 1.4	0.49 ± 0.06	—	92.8 ± 1.4
5.3 ± 0.02	4	3.0 ± 0.1	56.5 ± 1.2	1.7 ± 0.1	32.6 ± 1.7	0.52 ± 0.1	0.43 ± 0.07	89.2 ± 1.2
50.2 ± 1.2	5	23.8 ± 1.4	47.4 ± 1.9	12.3 ± 0.7	24.6 ± 1.4	1.8 ± 0.3	1.1 ± 0.1	72.0 ± 3.0
102.9 ± 2.2	5	47.0 ± 1.8	45.8 ± 2.1	22.2 ± 1.7	21.7 ± 1.4	2.2 ± 0.5	1.4 ± 0.2	67.5 ± 2.8
185.7 ± 9.1	4	75.3 ± 4.3	40.4 ± 2.1	42.2 ± 1.7	22.2 ± 0.2	3.0 ± 0.1	1.6 ± 0.1	63.2 ± 1.2
243.7 ± 3.2	5	85.8 ± 3.7	35.1 ± 1.2	42.5 ± 1.5	17.4 ± 0.5	3.4 ± 0.1	2.2 ± 0.1	52.6 ± 1.5
337.1 ± 18.0	4	124.7 ± 9.6	34.5 ± 3.3	66.8 ± 9.0	19.6 ± 1.3	2.8 ± 0.2	2.4 ± 0.1	56.7 ± 2.1
509.2 ± 9.3	5	166.2 ± 14.3	32.5 ± 2.4	86.7 ± 4.9	17.0 ± 0.8	4.8 ± 0.5	5.4 ± 0.1	50.3 ± 2.6

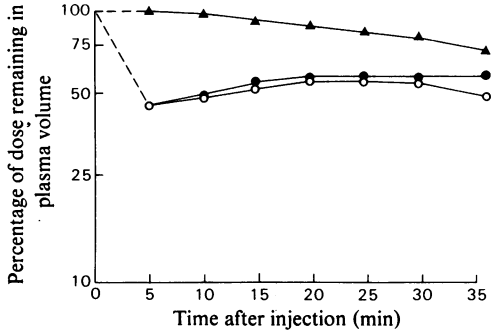

Fig. 1. *Protein-bound radioactivities in the plasma of a rat after the mixed injection of ^{125}I-labelled asialo-transferrin (0.43 μg/100g body wt.) and ^{131}I-labelled albumin (25 μg/100g body wt.)*
In the plot it is assumed that at 5 min the intravascular distribution of albumin (▲) was complete and that the volume in which the albumin dose was diluted at this time corresponded closely to the plasma volume of the animal. It is also assumed that the same volume of plasma was available for the distribution of asialo-transferrin (○) as that for albumin. ●, Asialo-transferrin curve after correction for diffusion and sampling as described in the Materials and Methods section. For further details see the text.

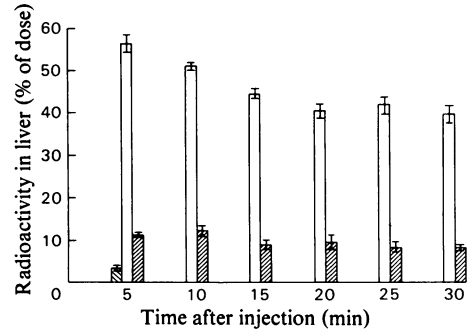

Fig. 2. *Recovery of ^{125}I-labelled asialo-transferrin in the rat liver at various intervals after intravenous administration*
Each value is the mean of four experiments; the vertical bars are S.E.M. The dose was 1.4 (±1.0 S.D.) μg/100g. Groups of animals shown by open columns received no treatment. Those shown by hatched columns received, at the time at which they appear in the graph, 2.51 (±0.51 S.D.) mg of asialo-orosomucoid per 100g body wt., and an additional 5 min was allowed before death for the displacement of asialo-transferrin from the liver surface. The small additional hatched column on the left at 5 min denotes results from a group of rats that received the asialo-orosomucoid 1 min before the labelled asialo-transferrin. All values have been corrected for the plasma asialo-transferrin trapped in the hepatic circulation at the time of death.

characteristic plasma radioactivity curves, as evident from an example shown in Fig. 1. Three kinetic phases were distinguishable after correction for diffusion and sampling: 1, a rapid initial fall to 40–50% of the dose at 5 min; 2, a slow reversal between 5 and 20 min, resulting in the return of some 10–15% of the dose to the circulation as protein-bound radioactivity; 3, a steady state over the rest of the observation period with plasma asialo-transferrin concentrations either remaining constant or rising only insignificantly (1–3%) with time.

Observations on the quantities of asialo-transferrin associated with the liver after a low dose are summarized in Fig. 2. At each time, the hepatic asialo-transferrin radioactivity closely complemented the corrected plasma radioactivity curve in Fig. 1. However, most of the radioactivity associated with the liver was readily displaced by asialo-orosomucoid, indicating that the asialo-transferrin bound by the lectin did not enter the cell. The presence of a small fraction of the dose (8–10%) not displaced by

asialo-orosomucoid is explained by the heterogeneity of asialo-transferrin with regard to galactose content (Spik *et al.*, 1975).

Chromatography of plasma samples obtained from three animals at 20min on Sephadex G-200 showed that the injected asialo-transferrin was eluted in the same position as the control ^{131}I-labelled asialo-transferrin sample. Furthermore rechromatography of plasma samples obtained from two rats at 20min, on DEAE-cellulose under conditions that would permit resolution of transferrin fractions of different sialic acid content, provided no evidence for a partial resialylation of the asialo-transferrin still in circulation.

Induction of asialo-transferrin catabolism by larger doses of asialo-transferrin

Data from the small-dose experiments presented above clearly indicate that the quantity of asialo-transferrin endocytosed and degraded by the rat liver was negligible. However, as shown by the analyses in Table 1, the situation changed considerably as the dose was increased. The effect of a dose increase from 0.4 to 5μg/100g body wt. was comparatively small, resulting in a slight decrease in dose recovery (from 93% to 89%) due to a decreased amount of protein-bound radioactivity associated with the liver. Further increases in the dose resulted in the following characteristic changes: fractions of the dose recoverable as protein-bound radioactivity progressively decreased in both plasma and liver, and these decreases were paralleled by increasing amounts of non-protein radioactivity.

In contrast, quantities of non-protein radioactivity derived from the simultaneously injected ^{131}I-labelled albumin exhibited no significant changes in relation to time or asialo-transferrin dose. Thus, in 20 rats drawn from the various groups, the non-protein-bound ^{131}I radioactivity of the total liver amounts to 0.13 (±0.01 S.E.M.)% of the ^{131}I dose at 35min, the corresponding value in seven rats at 5min being 0.15 (±0.04 S.E.M.)%. Serial determinations of the ^{131}I non-protein-bound activity in the circulating plasma volumes of 25 rats yielded steady values averaging at approx. 0.4% of the dose.

The actual catabolic performance of the liver under different loading conditions is shown in Fig. 3. Two points that should be noted are (*a*), when doses less than 100μg/100g body wt. were used, the quantities of asialo-transferrin catabolized were disproportionately smaller than those observed from doses greater than 100μg/100g body wt., and, (*b*), within the dose range examined, complete catabolism was not achieved at any concentration of asialo-transferrin. Incomplete elimination of asialo-transferrin by the liver was not due to termination of the experiments at 35min, as is evident from Fig. 4, in which five plasma protein-bound radioactivity curves, each from a different dose group, are depicted. The striking feature from Fig. 4 is that the net transfer of protein

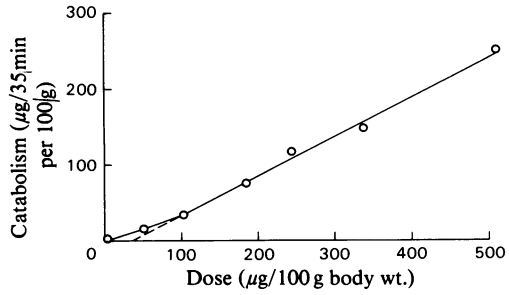

Fig. 3. *Relationship between dose and catabolism of asialo-transferrin in the rat*

Values were calculated from the data of the dose range 5–509μg/100g body wt. in Table 1. The plot indicates that during the linear section of the curve an increment of 1μg/100g body wt. in dose was associated with the catabolism of an additional 0.53μg of asialo-transferrin per 100g body wt.

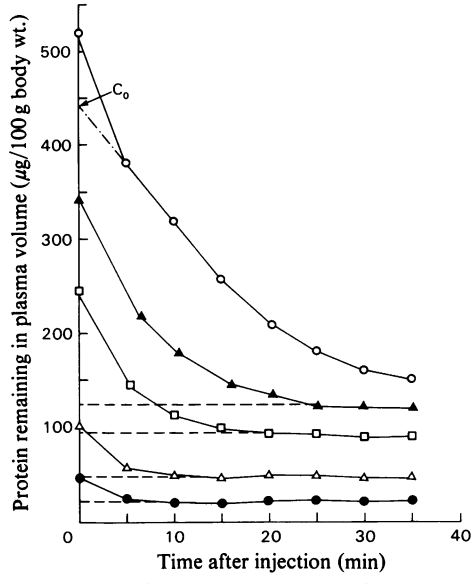

Fig. 4. *Corrected plasma protein-bound radioactivity curves from five rats, each of which received a different dose of ^{125}I-labelled asialo-transferrin*

The first point of each curve at 0 time is theoretical, i.e. obtained by dividing the dose by the plasma volume. Plasma volumes were calculated from the initial distribution of simultaneously injected ^{131}I-labelled albumin. The doses per 100g body wt. were: 519μg (○); 343μg (▲); 244μg (□); 100μg (△); 47μg (●). C_0 is the extrapolated value of the linear section of the curve at 0 time. For further explanations see the text.

ceased between plasma and liver at different residual amounts of asialo-transferrin in the plasma volume, depending on the dose administered. This was not conclusively shown for the highest dose (519 μg/100g body wt.) over the observation period of 35min, but from the shape of the curve it is predictable that equilibrium would have been achieved at a value of approx. 135–140 μg/100g body wt. between 43 and 45min.

Analyses of the plasma curves from the group that received the highest dose yielded information that could not be obtained from animals injected with lower doses. Thus quantities of asialo-transferrin in the plasma volume decreased linearly from the fifth minute until a value of 220–250 μg/100g body wt. was reached, then hepatic transfer rates became continuously smaller. During the linear phase of the curves, hepatic transfer rates averaged at 10.3 (±0.7 S.E.M.) μg of asialo-transferrin/min per 100g body wt. The values obtained by extrapolation of the linear portion of the curves to zero time (C_0 in Fig. 4) fell significantly short of the expected values, the difference averaging at 73.3 (±9.0 S.E.M.) μg of asialo-transferrin per 100g body wt.

Effect of various treatments on the binding and catabolism of asialo-transferrin by the rat liver

Four rats were given an intravenous injection of cytochalasin B (80 μg/100g body wt., dissolved in 0.15ml of dimethyl sulphoxide) 15min before 2.7 (±0.7 S.E.M.) μg of ^{125}I-labelled asialo-transferrin per 100g body wt. and the amount of radioactivity associated with the liver in 5min was determined. The value found corresponded to 53.9 (±1.2 S.E.M.)% of the dose, which is not significantly different from the value determined for untreated rats (see Fig. 2).

A limited series of experiments was conducted with various substances to examine whether they were capable of influencing the equilibrium and catabolism of asialo-transferrin *in vivo*. As seen in Table 2, each substance, except for sucrose, was administered at 4.25min after asialo-transferrin, when asialo-transferrin bound to the liver was close to maximum (Fig. 1). Comparison of the data in Table 2 with those from control rats receiving a corresponding asialo-transferrin dose (see Table 1) shows that none of the treatments had any dramatic effect. Nonetheless, some minor changes were observed, which, if reproducible, could be of statistical significance in a larger group of animals. Thus, after the larger dose of asialo-glycopeptides from transferrin, less asialo-transferrin appears to have been associated with the liver and the overall recovery of protein-bound radioactivity was slightly decreased. In contrast, after sucrose administration, more asialo-transferrin was associated with the liver and the dose recovery was higher than expected from the data in control animals. Injection of chicken α_1-acid glycoprotein seems to have slightly increased the proportion of liver-bound asialo-transferrin with no effect on the dose recovery. The action of chicken α_1-acid glycoprotein was probably due to redistribution of asialo-transferrin: according to the plasma radioactivity curves, the chicken protein temporarily displaced approximately one-third of the bound asialo-transferrin from the liver without delaying or changing ultimate equilibrium.

Discussion

The plasma curve of asialo-transferrin with its three phases (Fig. 1) is very different from the rapid straight disappearance curves observed by LaBadie *et al.* (1975) and Moore *et al.* (1977) after the injection of labelled asialo-fetuin in rats. The first question to be considered is therefore whether our results could have suffered from artifacts, such as substrate heterogeneity. However, this seems unlikely for the following reasons. Each transferrin preparation was

Table 2. *Effect of various substances on the distribution and catabolism of ^{125}I-labelled asialo-transferrin in rats*

Substances A–D were injected 4.25min after the asialo-transferrin dose. The substances and the quantities injected were: A, asialo-glycopeptides from bovine fetuin, 12.2 μg/100g body wt.; B, asialo-glycopeptides from human transferrin, 7 μg/100g body wt.; C, the same as B, but 21 μg/100g body wt.; D, chicken α_1-acid glycoprotein, 100 μg/100g body wt.; E, sucrose, 10mg/100g body wt., mixed with the asialo-transferrin dose. Values are means± S.E.M. where appropriate. Experiments were terminated at 35min after the injection of asialo-transferrin.

Substance	No. of experiments	Asialo-transferrin (μg/100g)	Protein-bound ^{125}I in plasma volume (μg/100g)	Protein-bound ^{125}I in plasma volume (% of dose)	Protein-bound ^{125}I in liver (μg/100g)	Protein-bound ^{125}I in liver (% of dose)	Non-protein-bound ^{125}I in plasma volume (% of dose)	Non-protein-bound ^{125}I in liver (% of dose)	Recovery of dose as protein-bound ^{125}I (%)
A	2	5.5	2.9	53.4	2.0	36.4	0.60	0.62	89.8
B	3	5.1 ±0.1	2.7 ±0.08	54.5±1.7	1.7 ±0.1	33.2±2.6	0.50±0.05	0.49±0.09	87.7±1.1
C	2	0.52	0.30	56.7	0.16	31.5	0.76	0.43	88.2
D	2	5.1	2.6	51.0	1.9	37.4	0.85	0.60	87.9
E	3	54.0 ±2.9	25.2 ±3.4	46.3±3.9	17.5±0.7	32.7±2.9	1.3 ±0.1	0.90±0.05	79.7±1.5

checked by immunoelectrophoresis and each batch of asialo-transferrin was separated from incompletely desialylated transferrin molecules and the neuraminidase by chromatography on DEAE-cellulose. The apparently homogeneous asialo-protein could have subsequently been rendered heterogeneous either *in vitro* by the labelling procedure or *in vivo* by some molecular transformation. Iodination, within the limits given in the methods, does not noticeably alter the affinity of asialo-transferrin for the hepatic lectin, evidence for this being obtained from comparisons of the chromatographic behaviour of ^{125}I-labelled asialo-transferrin with that of ^{59}Fe-labelled asialo-transferrin on Sepharose–hepatic lectin columns (Wong *et al.*, 1978). As to the possibility of molecular changes *in vivo*, examination of the asialo-transferrin still in the circulation at 20min by gel filtration and DEAE-cellulose chromatographies showed no evidence for macromolecular complex-formation or partial resialylation.

The absence of any gross error from the above sources encourages us to think that the interaction of human asialo-transferrin with the rat liver may be envisaged as a consecutive chemical reaction with a reversible first reaction according to the scheme:

$$P + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PR \xrightarrow{k_{+2}} P' + R$$

where P denotes asialo-transferrin, P′ endocytosed asialo-transferrin and R the hepatic lectin. After the injection of a small quantity of asialo-transferrin, the second reaction is negligible and the first reaction exhibits three phases. The initial condition, [PR] = 0, strongly favours the forward reaction (k_{+1}), hence the rapid decrease in plasma asialo-transferrin concentrations during phase 1. As P is injected in a volume that is small relative to the final mixing volume (plasma volume), concentration of P in the blood perfusing the liver is initially too high, resulting in the formation of excess PR. Correction of this situation takes place in phase 2 through the predominance of the reverse reaction (k_{-1}), yielding more R and P until final equilibrium conditions are reached in phase 3.

The comparatively weak binding of asialo-transferrin by rat liver plasma-membrane preparations (Van Lenten & Ashwell, 1972), together with the data summarized in Figs. 2 and 4, support the view that, in the living animal, free and bound forms of asialo-transferrin tend to attain equilibrium in accordance with the law of mass action.

Hitherto it has been widely assumed that binding of an asialo-glycoprotein molecule by the hepatic lectin is automatically followed by the uptake of the bound protein into the hepatocyte and its subsequent lysosomal catabolism. The present studies with low doses of asialo-transferrin show, however, that binding to the hepatic lectin on the one hand, and endocytosis of the bound protein on the other, are two distinguishable events, and therefore, binding itself may not necessarily be a signal for endocytosis.

The chemical background of this distinction awaits further elucidation. It is possible that the galactose groups of a single asialo-transferrin molecule are either insufficient in number, or their spacing is unsuitable for lectin activation and subsequent endocytosis. However, as implied by the data in Table 1, the galactose residues of two or more asialo-transferrin molecules together can induce endocytosis. This suggests that, for endocytosis to take place, multiple sites of attachments must exist between an asialo-glycoprotein and the hepatic lectin, or perhaps several adjacent lectins. The fact that the lectin consists of a number of subunits (Kawasaki & Ashwell, 1976) may be important if endocytosis is the result of their concerted action. Alternatively, endocytosis may depend on the simultaneous occupancy of adjacent lectins through an asialo-glycoprotein bridge, in distant analogy to pinocytosis of capped membrane receptors (Taylor *et al.*, 1971).

Experiments summarized in Table 2 illustrate the difficulties in finding the portion of the signal that is missing from asialo-transferrin by complementation with asialo-glycopeptides. The fetuin asialo-glycopeptides administered corresponded to an asialofetuin dose of approx. 60μg/100g body wt., and the two doses of transferrin asialo-glycopeptides to 146μg and 439μg of asialo-protein per 100g body wt. Three-forked heterosaccharide chains are found in the fetuin glycopeptides (Spiro, 1973), whereas transferrin glycopeptides are predominantly two-forked (Montreuil, 1975), and both types of asialo-glycopeptides failed to induce endocytosis by the hepatocyte of asialo-transferrin already bound. Chicken α_1-acid glycoprotein, possessing affinity for the lectin because of its exposed galactose groups in the native state (Regoeczi *et al.*, 1975), proved equally ineffective in this respect. Sucrose, which is thought to be taken up by the liver by simple pinocytosis (Jacques, 1973), was used as a control of specificity. These preliminary observations provide no clue to the chemical basis of the signal for endocytosis, and clearly much remains to be accomplished before the mechanism by which human asialo-transferrin traverses the rat hepatocyte plasma membrane is fully understood.

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