A reassessment of the assay for the asialoglycoprotein receptor and its use in the quantification of receptor distribution in hepatocytes

David A. W. GRANT and Naheed KADERBHAI*

Department of Surgery, St. George's Hospital Medical School, London SW17 0RE, U.K.

The assay for the fucose-binding protein described by Lehrman & Hill [(1983) *Methods Enzymol.* **98**, 309–319] was adapted for the measurement of the asialoglycoprotein receptor in rat liver. The amount of ligand bound to the plasma-membrane-associated or affinity-purified receptor was acutely sensitive to the concentrations of Triton X-100 and NaCl in the assay: 0.02% (v/v) Triton X-100 increased ligand binding to the two preparations by 100% and 40% respectively. Higher concentrations of detergent progressively decreased binding, and in 0.32% Triton X-100 it was about 30% of the value obtained in detergent-free buffer. The addition of increasing concentrations of NaCl to the assay progressively inhibited ligand binding to the membrane-associated receptor, whereas there was a 60% increase in binding to the pure receptor in the presence of 0.1–0.2 M-NaCl. These effects could not be identified in the original assay procedure described by Hudgin, Pricer, Ashwell, Stockert & Morell [(1974) *J. Biol. Chem.* **249**, 5536–5543]. Using optimal assay conditions the binding of ^{125}I - β -D-galactosyl-bovine serum albumin to both the membrane-associated and purified receptor was inhibited by 50% by 1 nM- β -D-galactosyl-bovine serum albumin and -asialo-orosomucoid and by approx. 100 μM - β -L-fucosyl-bovine serum albumin, whereas β -D-galactose, lactose and β -L-fucose had no effect on ligand binding up to concentrations of 1 mM, 500 μM and 5 mM respectively. K_D values of 0.94 and 1.25 nM and B_{max} values of 40 and 1660 pmol of D-galactosyl-bovine serum albumin bound/mg of receptor were obtained for the membrane-bound and purified receptor respectively. Hill-plot analysis of the same data gave slopes of 0.96 and 1.01. Scatchard analysis of saturation-binding studies with other subcellular fractions indicated that the receptor was distributed in the proportions 72:23:2.5:2.5 between total microsomal fractions, plasma membrane, Golgi and canalicular membrane respectively. The receptor was about 1% of the total protein in each compartment and was estimated to be about 0.3% of the total liver protein.

INTRODUCTION

The asialoglycoprotein receptor is expressed on the sinusoidal plasma membrane of hepatocytes, and its sole function appears to be the clearance and endocytosis of serum-derived glycoproteins which have either peripheral galactose or *N*-acetylgalactosamine residues in their oligosaccharide structures (Ashwell & Harford, 1982). We have shown that intact, catalytically active, enterokinase is also bound to this receptor before internalization, although fucose, and not galactose, appears to be the predominant terminal sugar on this heavily glycosylated enzyme (Grant & Hermon-Taylor, 1976; Grant, 1985). Once internalized, enterokinase appears to be processed in a manner analogous to asialoglycoproteins, in that most of it is directed to lysosomes but a small amount (1–2% of the internalized enzyme) is secreted, catalytically active, into bile (Grant *et al.*, 1981).

During the course of our studies on the transcellular transport of enterokinase from blood to bile, we have

compared the subcellular distribution of enterokinase with that of its receptor (Grant *et al.*, 1984). The assay of choice for the asialoglycoprotein receptor has been 'assay A' described by Hudgin *et al.* (1974), which involves precipitation of the receptor- ^{125}I -labelled ligand complex with $(\text{NH}_4)_2\text{SO}_4$ and harvesting on glass-fibre discs. More recently, an assay for a hepatic fucose-binding protein has been described which substitutes polyethylene glycol 6000 (PEG 6000) for $(\text{NH}_4)_2\text{SO}_4$ to decrease non-specific binding (Lehrman & Hill, 1983). We have adapted this assay for the measurement of the asialoglycoprotein receptor from rat hepatocytes and have found that the amount of ligand bound to both membrane-associated and purified receptor is acutely sensitive to the concentrations of detergent and salt in the assay. Scatchard analysis of saturation-binding studies with various subcellular fractions has shown a distinct compartmentalization of the receptor within the hepatocyte.

Abbreviations used: BSA, bovine serum albumin; D-Gal-BSA, β -D-galactosyl-bovine serum albumin; D-Glc-BSA, β -D-glucosyl-bovine serum albumin; L-Fuc-BSA, β -L-fucosyl-bovine serum albumin; ASOR, asialo-orosomucoid; PEG 6000, polyethylene glycol 6000.

* Present address: Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth SY23 3DD, Dyfed, Wales, U.K.

MATERIALS AND METHODS

Materials

Cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-galactopyranoside and cyanomethyl-2,3,4-tri-*O*-acetyl-1-thio- β -L-fucopyranoside were prepared by Peninsula Laboratories Europe Ltd., St. Helens, Merseyside WA9 3AJ, U.K. The neoglycoproteins D-Gal-BSA and L-Fuc-BSA were prepared by the method of Stowell & Lee (1980). A 25-molar excess of each sugar intermediate to protein amino groups was mixed with 250 mg of BSA (fraction V; Sigma Chemical Co., Poole, Dorset, U.K.) to give a final molar ratio in the freeze-dried product of 4 mol of sugar/mol of albumin (kindly determined by Professor John Clamp, University of Bristol). For Gal-BSA a value of 14 mol of sugar/mol of protein was determined by colorimetric analysis (François *et al.*, 1962). ASOR was prepared by digesting 200 mg of orosomucoid at 37 °C with 1 unit of immobilized neuraminidase (Sigma Chemical Co.) in 0.2 M-sodium acetate buffer, pH 5, for 48 h.

125 I-labelled D-Gal-BSA and ASOR were prepared by using Na 125 I (Amersham International) and Iodobeads (Pierce Chemical Co., Rockford, IL, U.S.A.): 25 μ g of each ligand was mixed with 2 mCi of Na 125 I and one Iodobead in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7.6, and frequently agitated for 10 min at room temperature. The radiolabelled ligand was separated from Na 125 I by passage through a 10 ml bed of Sephadex G-15 equilibrated in 10 mM-sodium phosphate (pH 7.6)/0.15 M-NaCl. The specific radioactivity of each ligand prepared in this way was approx. 1 Ci/ μ mol; this was diluted to an appropriate radioactivity with unlabelled ligand before use in the binding assays.

Peroxide-free Triton X-100 was prepared by the procedure of Lehrman & Hill (1983) and stored as a 10% (v/v) solution. The asialoglycoprotein receptor was purified by affinity chromatography on immobilized D-Gal-BSA and ASOR-Sepharose 4B as previously described (Grant, 1985). Smooth and rough microsomal fractions ('microsomes') were prepared from liver homogenates by the method of Bergstrand & Dallner (1969), sinusoidal membrane was prepared by the method of Touster *et al.* (1970), and Golgi membrane was prepared by the method of Fleischer & Fleischer (1970). The bile-canalicular membrane was prepared by the procedure of Wisher & Evans (1975).

Measurement of receptor-binding activity

A portion (2.5 μ g) of smooth-microsomal or sinusoidal-plasma-membrane protein or 0.1 μ g of the purified receptor was mixed at 0 °C with 200 fmol of 125 I-D-Gal-BSA (1 μ Ci/10 pmol) in 0.25 ml of 25 mM-Tris/HCl, pH 7.8, containing 20 mM-CaCl₂ and 1 mg of BSA/ml (buffer A). The concentrations of Triton X-100 and NaCl were adjusted from 0 to 1% and 0 to 1.6 M respectively. Non-specific binding was assessed in the presence of a 10³-molar excess of unlabelled D-Gal-BSA. After standing on ice for at least 4 h, the receptor-ligand complex was harvested as described for the rat liver fucose-binding protein (Lehrman & Hill, 1983). Briefly, 50 μ l of fibrinogen (5 mg/ml) in 20 mM-Tris/HCl, pH 7.8, containing BSA (0.5 mg/ml), 10 mM-CaCl₂ and 0.2 M-NaCl (buffer B) was added to the assay mixture, followed by 2.5 ml of ice-cold 5% (w/v) PEG 6000 prepared in buffer B except that BSA was omitted. After

10 min on ice, the complex was harvested by vacuum filtration on Whatman GF/C discs presoaked in buffer B. The assay tubes and discs were washed with 2 $\times$ 5 ml of 5% PEG 6000 before counting the filters for radioactivity in an LKB Compugamma instrument. When appropriate, the assay was compared with 'assay A' of Hudgin *et al.* (1974) by using identical amounts of receptor protein and 125 I-labelled ligand.

Effect of ligand specific radioactivity on the binding assay

A sample (2.5 μ g) of plasma-membrane protein in 0.25 ml of ice-cold buffer A containing optimal concentrations of Triton X-100 and NaCl was mixed with 10 μ l batches of buffer A containing 0.02 μ Ci of 125 I-D-Gal-BSA which had been serially diluted from 1 to 0.0625 Ci/ μ mol with unlabelled ligand so that the final concentration in the assay was between 0.08 and 1.28 nM. Non-specific binding was assessed as described above, and after standing on ice for over 4 h, the receptor-ligand complex was harvested by using PEG 6000 as above.

Displacement studies

Portions (10 μ l) of serially diluted stock solutions of unlabelled ligands were added to 0.25 ml of ice-cold buffer A containing 200 fmol of 125 I-D-Gal-BSA and optimal concentrations of Triton X-100 and NaCl for assay of either the plasma-membrane-associated or purified receptor respectively to give the following final assay concentrations: D-Gal-BSA (0–80 nM), ASOR (0–80 nM), L-Fuc-BSA (0–250 μ M), lactose (D-galactosyl- β -D-glucose) (0–5 mM), β -D-galactose (0–10 mM), β -L-fucose (0–5 mM). After standing on ice for over 4 h, the receptor- 125 I-ligand complex was harvested and counted for radioactivity as described above.

Saturation binding of D-Gal-BSA to hepatocyte subcellular fractions

For the determination of the apparent equilibrium binding constant, K_D , and the apparent maximum number of binding sites, B_{max} , the specific radioactivity of the 125 I-D-Gal-BSA was diluted in five equal steps from 1 Ci/4 μ mol to 1 Ci/64 μ mol by the addition of unlabelled ligand. Samples (0.02 μ Ci) of each were added to 0.25 ml of ice-cold buffer A containing the optimal concentrations of Triton X-100 and NaCl and either 0.1 μ g of purified receptor or 2.5 μ g of protein from appropriate subcellular fractions. The final concentration of D-Gal-BSA in the assay was therefore between 0.32 and 5.12 nM. Samples were prepared in duplicate together with a control sample containing a 10³-molar excess of unlabelled ligand. The remainder of the assay procedure was exactly as described above, and Scatchard and Hill plots were constructed from the binding data by linear regression analysis. As before, appropriate assays were repeated with the 'assay A' procedure of Hudgin *et al.* (1974), by using identical ligands and preparations of purified and plasma-membrane-associated receptor.

RESULTS

Preliminary studies with the plasma-membrane-associated receptor indicated that, at 0 °C in buffer A, and in the absence of Triton X-100 and NaCl, the binding of 125 I-D-Gal-BSA reached equilibrium after 2 h. Subsequent assays were left for at least 4 h, and it was often convenient to leave samples on ice overnight. In the

Fig. 1. Effect of Triton X-100 on the binding of ^{125}I -D-Gal-BSA to the asialoglycoprotein receptor

Assays were performed with (a) affinity-purified receptor (O) or (b) sinusoidal membrane (▲) either as described in the text (see the Materials and methods section) or by the 'assay A' procedure of Hudgin *et al.* (1974) (●).

absence of NaCl, the addition of increasing amounts of Triton X-100 to the assay had a biphasic effect on the binding of ^{125}I -D-Gal-BSA to both the plasma-membrane-associated and purified receptor species. The addition of 0.02% Triton X-100 to the assay mixture increased ligand binding to the two preparations by 100% and 40% respectively (Fig. 1). Thereafter, ligand binding progressively declined and in 0.32% Triton X-100 was about 30% of the value obtained in Triton X-100-free buffer. The 'assay A' procedure of Hudgin *et al.* (1974) was insensitive to similar changes in Triton X-100 concentration (Fig. 1). In the presence of 0.02% Triton X-100, the addition of increasing concentrations of NaCl to the assay progressively inhibited ligand binding to the plasma-membrane-associated receptor (60% of NaCl-free value in 1 M-NaCl), whereas there was a 60% increase in binding to the pure receptor in the presence of 0.1–0.2 M-NaCl (Fig. 2). This latter effect was abolished as the NaCl concentration was increased to 1 M. The binding of ^{125}I -ASOR to the two forms of the receptor showed a dependence identical with that of ^{125}I -D-Gal-BSA

Fig. 2. Effect of NaCl in the presence of 0.02% Triton X-100 on the binding of ^{125}I -D-Gal-BSA to the asialoglycoprotein receptor

(a) Affinity-purified receptor (O); (b) sinusoidal membrane-bound receptor (▲).

Fig. 3. Effect of ligand concentration on the binding of ^{125}I -D-Gal-BSA to sinusoidal-membrane-bound asialoglycoprotein receptor in the presence of 0–0.08% Triton X-100

^{125}I -D-Gal-BSA (0.02 μCi) which had been serially diluted from 1 to 0.0625 Ci/ μmol with unlabelled ligand, was added to the assay mixture to give final concentrations of: ■, 0.08 nM; △, 0.16 nM; ▲, 0.32 nM; ○, 0.64 nM; ●, 1.28 nM.

on the concentrations of Triton X-100 and NaCl in the assay mixture (results not shown). When 0.02 μCi of ^{125}I -D-Gal-BSA (sp. radioactivity 1 Ci/ μmol) was used in the assay, the stimulatory effect of low concentrations of Triton X-100 was not seen (Fig. 3). This effect was gradually restored as the ligand was serially diluted with unlabelled D-Gal-BSA, until a 100% increase in binding was recorded with 0.02 μCi of ligand with a specific radioactivity of either 0.125 or 0.0625 Ci/ μmol .

Fig. 4. Displacement of ^{125}I -D-Gal-BSA from the asialoglycoprotein receptor by unlabelled D-Gal-BSA

(a) Sinusoidal-membrane-bound receptor in (●) the absence and (▲) the presence of 0.02% Triton X-100; (b) affinity-purified receptor (○).

Fig. 5. Scatchard plots for the binding of ^{125}I -D-Gal-BSA to the asialoglycoprotein receptor

(a) Sinusoidal-membrane-bound receptor; (b) affinity-purified receptor. The assay conditions were either those described in the text (▲) or the 'assay A' procedure of Hudgin *et al.* (1974) (●). Inserts show concentration-dependent binding of ligand to the receptor.

By using optimal conditions for the plasma-membrane-associated receptor (buffer A containing 0.02% Triton X-100) and for the purified receptor (buffer A containing 0.02% Triton X-100 and 0.15 M-NaCl), the displacement of ^{125}I -D-Gal-BSA by unlabelled D-Gal-BSA and other ligands could be assessed in order to give a first approximation of K_D . The binding of ^{125}I -D-Gal-BSA was decreased by 50% by both unlabelled D-Gal-BSA and ASOR at a concentration of approx. 1 nM (Fig. 4). The neoglycoprotein L-Fuc-BSA decreased the binding of ^{125}I -D-Gal-BSA to the plasma-membrane-associated receptor by 50% at approx. 100 μM . Under the same conditions, β -D-galactose, lactose and β -L-fucose had no effect on ^{125}I -D-Gal-BSA binding to the receptor up to concentrations of 1 mM, 500 μM and 5 mM respectively.

Estimates of K_D and B_{max} for the binding of ^{125}I -D-Gal-BSA to both the plasma-membrane-associated and purified receptor were made over a concentration range of approx. $\frac{1}{5}$ to 5 times the approximate K_D estimated from the displacement study. K_D values of 0.94 and 1.25 nM and B_{max} values of 40 and 1660 pmol of D-Gal-BSA bound/mg of receptor were obtained for the plasma-membrane-associated and purified receptor respectively (Fig. 5). Hill-plot analysis of the same data gave slopes of 0.96 and 1.01 respectively. Similar results were obtained with ^{125}I -ASOR (results not shown). By using the 'assay A' procedure of Hudgin *et al.* (1974), the equivalent K_D and B_{max} values were found to be 20 nM and 134 pmol/mg and 1.2 nM and 240 pmol/mg for the plasma-membrane-bound and purified receptor respectively (Fig. 5). Under these conditions the binding of ^{125}I -D-Gal-BSA to the membrane-bound receptor was not saturable.

For the other subcellular fractions, the apparent K_D was within the range 0.9–1.6 nM (mean value 1.3 ± 0.235 nM; 1 S.D.); the mean of four determinations for the pure receptor was 1.28 ± 0.13 nM (1 S.D.). The B_{max} values for the various fractions are listed in Table 1.

The net recovery of active receptor from the various subcellular fractions was estimated to be about 43% of the total homogenate content. From this value, the percentage distribution of receptor in various membranous compartments could be amended from Table 1 to give the proportions 72:23:2.5:2.5 for microsomes

Table 1. Subcellular distribution of the asialoglycoprotein receptor from rat hepatocytes

	Total protein (mg)	$B_{\max.}$ (pmol/mg)	Total receptor units (mg of protein $\times B_{\max.}$)	Receptor	
				(mg)*	(% of total)
Homogenate	1650	5	8250	4.97	100
Endoplasmic reticulum					
Smooth	70.9	20	1419	0.86	17
Rough	97	12	1164	0.70	14
Golgi	6	15	90	0.05	1
Plasma-membrane fractions†					
I	4.7	22	102	0.06	1
II	6.2	40	248	0.15	3
III	9.75	10	98	0.06	1
IV	33.75	15	506	0.3	6

* Total receptor units

 $B_{\max.}$ for pure receptor

† Plasma-membrane fractions were assigned as follows (enrichment in 5'-nucleotidase over homogenate given in parentheses); I, canalicular membrane (75-fold); II, sinusoidal membrane (24-fold); III, plasma membrane (25-fold); IV, sinusoidal plasma membrane/smooth microsomes (5-fold).

(smooth and rough):plasma membrane:Golgi:canalicular membrane respectively. From these calculations, the receptor represented about 1% of the total protein in each of these compartments and was estimated to be about 0.3% of the total liver protein.

DISCUSSION

The principal disadvantage of 'assay A' described by Hudgin *et al.* (1974) for the detection and quantification of the asialoglycoprotein receptor is the use of saturated $(\text{NH}_4)_2\text{SO}_4$ to precipitate the receptor-ligand complex. In our hands non-specific binding of ^{125}I -labelled D-Gal-BSA and ASOR to the plasma-membrane-bound receptor was often 30% or more of the total ligand bound even in the presence of a 10^3 -molar excess of unlabelled ligand and substantially lower concentrations of Triton X-100, NaCl and BSA than those described by Hudgin *et al.* (1974) (results not shown). Similar observations have been reported by Blomhoff *et al.* (1982), who suggested that the inconsistency may have been due to the precipitation of Ca^{2+} , which is essential for binding of the ligand to the receptor. The same authors substituted polyethylene glycol with an average M_r of 6×10^6 for harvesting the receptor-ligand complex. Preliminary experiments using the procedure of Lehrman & Hill (1983) showed that precipitation of the receptor-ligand complex with PEG 6000 decreased non-specific binding of both ligands to < 5%. This latter assay, which had been developed for the detection of a fucose-binding protein, contained 5-fold and 6-fold lower concentrations of NaCl and BSA respectively but 5-fold more Triton X-100 than that described for 'assay A'. The procedure for harvesting the receptor-ligand complex appeared, therefore, to be the main parameter affecting the selectivity and reproducibility of the assay, but it was not clear what contribution other factors such as the protein/detergent ratio, ionic strength and specific radioactivity of the ligand made to its overall sensitivity. A detergent-induced biphasic effect on the activity of the membrane-bound asialoglycoprotein receptor was apparently analogous to

the release of latent enzyme activity from membrane vesicles. However, the 2-fold stimulation of binding of ^{125}I -D-Gal-BSA to the receptor was observed at a concentration of Triton X-100 (0.02%) which was too low to cause membrane solubilization (Helenius & Simons, 1975). Furthermore, we established by electron microscopy that the structural integrity of individual membranes or whole hepatocytes was maintained at this concentration of detergent (D. A. W. Grant, S. Siddiqui & R. J. Sharma, unpublished work). A doubling in the number of receptor sites in the presence of such a low concentration of Triton X-100, without lipid disassembly, was unexpected, since both microsomal and plasma-membrane-derived vesicles adopt a 'right-side out' orientation during preparation, i.e. exoplasmic-facing receptors retain their topology. The stimulatory effect of 0.02% Triton X-100 may have been due to the removal of competing peripheral proteins, to the creation of sufficiently large pores for the diffusion of ligand to internal receptor sites, or to an alteration in some physical parameter(s) of the membrane (Klausner *et al.*, 1980). An identical phenomenon has been reported for the binding of insulin to the partially purified receptor from adipocytes (Marshall *et al.*, 1985). The transfer of the asialoglycoprotein receptor into mixed micelles of Triton X-100, lipid and protein was associated with a progressive inhibition of ligand binding as the concentration of Triton X-100 was increased. This may have been due to the shielding of binding sites within the micellar structure or the disruption of a putative oligomeric structure for the functional receptor (Schwartz, 1984). The concentration of Triton X-100 in the assay procedure of Hudgin *et al.* (1974) was 0.1%, but in the present study the amount of ligand bound to the membrane-bound or purified receptor at the same concentration was less than that recorded in detergent-free buffer. In fact, the 'assay A' procedure was completely insensitive to changes in Triton X-100 concentration (Fig. 1). This was almost certainly due to the high background generated by saturated $(\text{NH}_4)_2\text{SO}_4$ obscuring any direct effect of detergent. In the present study, the Triton X-100-mediated increase in ligand binding was not seen

when 0.02 μCi of ^{125}I -D-Gal-BSA at a specific radioactivity of 1 Ci/ μmol was used in the assay (Fig. 3). This represents a final concentration of ligand of 0.08 nM, which was 10-fold below the expected K_D . The detergent effect became progressively more apparent as the specific radioactivity of the ligand was lowered and its concentration in the assay approached the approximate K_D value, i.e. around 1 nM. Many of the assays described in the literature have used ^{125}I -labelled ligand (usually ASOR) with a specific radioactivity of around either 2 or 0.02 Ci/ μmol . As a result the final concentration of ligand can vary by as much as 100-fold in apparently identical assays.

Most groups, including ourselves, have successfully purified the asialoglycoprotein receptor by absorption to immobilized D-Gal-BSA or ASOR in the presence of up to 1% Triton X-100. At this detergent concentration ligand binding in our modified assay was only 10% of the value in detergent-free buffer. The reason for this anomaly is not clear, but the high concentration of immobilized ligand used for successful chromatography (typically 2 mg/ml of Sepharose 4B, equivalent to approx. 30 μM -D-Gal-BSA and 50 μM -ASOR respectively), and an undefined, but presumably fortuitous, ratio of detergent to protein in most crude receptor preparations would favour absorption of the receptor (Grant, 1985). For the same reasons, the apparently inhibitory effect of NaCl on ligand binding to the membrane-bound receptor was unexpected, since most purification protocols have included 0.2–1.0 M-NaCl in their chromatography buffer and the 'assay A' buffer also contains 1.0 M-NaCl. In the present study, 1.0 M-NaCl decreased ligand binding to 50% of the NaCl-free value. By contrast NaCl, like Triton X-100, had a biphasic effect on the binding of ^{125}I -D-Gal-BSA to the purified receptor. The 60% increase in ligand binding at physiological concentrations of NaCl (0.1–0.2 M) may have been indicative of additional changes in receptor conformation or organization.

The selective displacement of ^{125}I -D-Gal-BSA from both the membrane-bound and purified asialoglycoprotein receptor by unlabelled D-Gal-BSA and ASOR [IC_{50} (concn. of unlabelled ligand at which the maximum binding of labelled ligand is displaced by 50%) ~ 1 nM] confirmed the specificity of this receptor for ligands containing terminal galactose and/or *N*-acetylgalactosamine residues (Fig. 4). The likely multivalent interaction between the asialoglycoprotein receptor and the multi-antennary oligosaccharide structures of appropriate ligands (Baenziger & Fiete, 1980; Connolly *et al.*, 1982) was demonstrated by the failure of up to a 10^6 -fold molar excess of the sugars β -D-galactose or lactose, over D-Gal-BSA or ASOR, to inhibit binding of ^{125}I -D-Gal-BSA. Not unexpectedly, β -L-fucose was also without effect and, although the neoglycoprotein L-Fuc-BSA was weakly inhibitory (IC_{50} 100 μM), it was unlikely that this reflected a dual specificity of the receptor for both galactose- and fucose-containing ligands. By contrast with these results, the asialoglycoprotein receptor has been reported to bind L-Fuc-BSA more avidly than D-Gal-BSA (Lehrman *et al.*, 1980). β -D-Galactose, lactose and β -L-fucose (at 0.1 M) have been reported to inhibit binding of ^{125}I -labelled Glc-BSA and ASOR to rabbit liver plasma membrane by 90%, 100% and 70% respectively (Stowell & Lee, 1978), but these concentrations of sugar represent a 10^8 -fold molar excess over

the apparent K_D values for D-Gal-BSA and ASOR binding to the receptor and are unlikely to be of physiological significance. In the present study, the binding of ^{125}I -D-Gal-BSA to both the purified and membrane-bound asialoglycoprotein receptor was saturable with the optimal concentrations of Triton X-100 and NaCl in the modified assay. In both cases, a single class of binding sites was identified, with very similar K_D values (1.25 and 0.94 nM respectively). Hill-plot analysis of the saturation-binding data gave slopes of 0.96 and 1.01 for the membrane-bound and purified receptor respectively, indicating that binding between receptor and ligand was equimolar. An arbitrary unit of specific activity (ng of ligand/ μg of protein) has been used by several groups to monitor the purification of the asialoglycoprotein receptor, and final values of 45–70 ng of ASOR bound/ μg of receptor protein have been reported from non-saturable binding studies using 'assay A'. These estimates, re-expressed in molar quantities as 1125–1725 pmol/mg, compare with a B_{max} value for D-Gal-BSA binding to the receptor under the modified conditions described in this paper of 1660 pmol/mg (equivalent to 113 ng of ligand/ μg of receptor protein). When it is recognized that the modified assay has been developed to overcome some of the limitations inherent in 'assay A', it is remarkable that these values should be in such close agreement.

We have confirmed by Scatchard analysis of saturation-binding studies of ^{125}I -D-Gal-BSA binding to various subcellular membranous fractions that the asialoglycoprotein receptor is almost exclusively distributed between the microsomal fraction (72%) and the plasma membrane (23%) (Table 1). The remainder was evenly distributed between the Golgi complex and the canalicular membrane. In every case the apparent K_D was within the range 0.9–1.6 nM, suggesting that the same receptor species was present in each membrane. An immunological homogeneity between similar receptor populations has been demonstrated previously (Pricer & Ashwell, 1976). It may be calculated from the estimates of B_{max} for the pure receptor (1660 pmol/mg) and the purest plasma-membrane fraction (40 pmol/mg) that the receptor constituted about 2.5% of the total protein in this membrane. The absence of a major Golgi-associated receptor pool is in complete contrast with previous biochemical and immunocytochemical studies, which have reported that 20–40% of the receptor is localized to the Golgi (Pricer & Ashwell, 1976; Gueze *et al.*, 1983). Overall the data in Table 1 show that the receptor constitutes about 1% of the total protein in each of the four subcellular compartments tested (microsomes, plasma membrane, Golgi, canalicular), and represents about 0.3% in total of the total liver homogenate.

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