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Isolation and characterization of a mannose/*N*-acetylglucosamine/fucose-binding protein from rat liver

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A rat liver mannan-binding protein was isolated by affinity chromatography on invertase–Sepharose by a modification of the method of Kawasaki, Etoh & Yamashina [(1978) *Biochem. Biophys. Res. Commun.* **81**, 1018–1024] and by a new method involving chromatography on mannose–Sepharose. The binding protein appears as a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent mol.wt. of approx. 30000. Binding of ^{125}I-labelled mannan is saturable and inhibited by mannose, *N*-acetylglucosamine, or L-fucose but not by galactose or mannose 6-phosphate. Neoglycoproteins containing mannose, *N*-acetylglucosamine, or L-fucose, but not galactose, are inhibitory. The neoglycoproteins are 10000-fold more effective (based on moles of sugar) than are free monosaccharides as inhibitors. ^{125}I-labelled mannan binding to the binding protein is calcium-dependent.

Rapid plasma clearance of mannose/*N*-acetylglucosamine-terminated glycoproteins, including certain lysosomal glycosidases, is mediated by receptors associated with the reticuloendothelial system (i.e. Kupffer cells) (Achord *et al.*, 1978; Brown *et al.*, 1978; Schlesinger *et al.*, 1978). Alveolar macrophages possess a pinocytosis receptor whose uptake specificity is quite similar to that which mediates rapid plasma clearance (Stahl *et al.*, 1978). Kawasaki and coworkers have reported the isolation of a mannan-binding protein from rabbit liver that may represent the receptor, or some component thereof, responsible for rapid plasma clearance (Kawasaki *et al.*, 1978).

In the present paper, we wish to report the isolation of the rat liver binding protein by use of invertase–Sepharose and mannose–Sepharose affinity chromatography. The rat liver protein has binding properties similar to those of the mannan-binding protein isolated from rabbit liver by Kawasaki *et al.* (1978). Through the use of neoglycoprotein inhibitors, the binding protein has been demonstrated to express a specificity including mannose, *N*-acetylglucosamine and fucose. A preliminary report of this work has been made (Townsend & Stahl, 1979).

Materials and methods

Materials

Sepharose 4B and 6B, invertase (catalogue no. I-4504), Triton X-100, CNBr, yeast mannan, Tris, and protein standards for electrophoresis were purchased from Sigma. The following gifts are gratefully acknowledged: mutant mannan (MNN2) from Dr. Clinton Ballou (Ballou & Raschke, 1974), neoglycoproteins (monosaccharide–albumin conjugates) from Dr. Y. C. Lee [prepared by the method of Lee *et al.* (1976)], orosomucoid from the American National Red Cross Laboratory, and Emulphogene BC-720 from GAF Corp., New York, NY, U.S.A. $(NH_4)_2SO_4$ (ultra-pure grade) was purchased from Schwartz/Mann, Orangeburg, NY, U.S.A. Whatman GF/C filters were purchased from Fisher Scientific Co., St. Louis, MO, U.S.A. Na ^{125}I (IMS 300) was from Amersham, Arlington Heights, IL, U.S.A. Rat livers were obtained from female Wistar rats purchased from Harlan Industries, Cumberland, IN, U.S.A. Vinyl sulfone was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. All monosaccharides were from Pfanstiehl Laboratories, Waukegan, IL, U.S.A. with the exception of mannose 6-phosphate, which was from Sigma. All other chemicals were of reagent grade and were purchased from local suppliers.

Affinity-resin preparation

Orosomucoid was desialylated by incubation in 0.05 M-H_2SO_4 at 80°C for 1 h (Spiro, 1966). Invertase was further purified by chromatography on DEAE-cellulose (Neumann & Lampen, 1967) before use. Asialo-orosomucoid (100 mg/60 ml of Sepharose) and invertase (150 mg/100 ml of

Abbreviation used: SDS, sodium dodecyl sulphate.

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Sepharose) were coupled to Sepharose 4B that had been activated with 100mg of CNBr/ml of gel (Cuatrecasas & Anfinsen, 1971). Mannose–Sepharose 6B was prepared by using vinyl sulphone by the method of Fornstedt & Porath (1975).

Isolation of binding protein

All purification steps were done at 4°C unless otherwise noted. All centrifugations were done at 21500*g* in a GSA rotor (Sorvall) for 25min. Two different methods were used to isolate the rat liver binding protein. The first method employed rat liver acetone-dried powder and was similar to that used by Kawasaki *et al.* (1978) for rabbit liver except that the affinity column used was invertase–Sepharose 4B instead of mannan–Sepharose 4B. A 2% (v/v) Triton X-100 crude extract, prepared as described by Kawasaki *et al.* (1978), was adsorbed to the invertase–Sepharose 4B (100ml) by stirring the extract (800ml) with the affinity resin for 2h with a mechanical stirrer. After allowing the resin to settle and siphoning off the supernatant, the invertase–Sepharose was loaded into a glass column, washed with Ca^{2+}-containing buffer and eluted with EDTA as described by Kawasaki *et al.* (1978). Chromatography on asialo-orosomucoid–Sepharose 4B (60ml) and a second invertase–Sepharose column (2–5ml) was then carried out as described by Kawasaki *et al.* (1978). Protein was measured by the method of Miller (1959), scaled down to one-tenth volume, with bovine serum albumin (Armour Pharmaceutical Co., Phoenix, AZ, U.S.A.) as standard.

The second method of binding-protein purification used frozen rat liver as starting material. Frozen rat liver (300g) was processed into flakes with a Cuisinart food processor. The flakes were suspended in 1000ml of buffer A (0.2M-NaCl/10mM-Tris/HCl, pH7.5) with a Polytron homogenizer (Brinkman) at low speed. The suspension was centrifuged and the supernatant was discarded. The pellet was suspended in 1000ml of buffer A as before and centrifuged. The resulting pellet was suspended in 750ml of buffer B [0.4M-KCl/2% (v/v) Emulphogene/10mM-Tris/HCl, pH7.8] with gentle use of the Polytron and centrifuged. The supernatant was saved and the pellet was re-extracted with 750ml of buffer B and centrifuged. The two 2% (v/v) Emulphogene extracts were pooled and stirred while solid $(NH_4)_2SO_4$ was added over 1–2h to achieve 30% saturation (170g/l of extract). The solution was stirred for 2h to allow complete precipitation and was then centrifuged. The pellet was discarded and the supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ (120g/l of solution). After stirring for 2h, the suspension was centrifuged and the pellets were dissolved in a minimum volume (200–300ml) of buffer B and dialysed for at least 12h against 7 litres of buffer C (1.25M-NaCl/15mM-$CaCl_2$/10mM-Tris/HCl, pH7.8) containing 1% (v/v) Emulphogene. A flocculent precipitate that formed during dialysis was removed by centrifugation and the supernatant was loaded onto a mannose–Sepharose column (50ml) that had been pre-equilibrated with buffer C containing 1% (v/v) Emulphogene. The column was then washed with 400ml of this buffer followed by 100ml of buffer C containing only 0.2% (v/v) Emulphogene. The binding protein was eluted with buffer D (1.25M-NaCl/2mM-EDTA/10mM-Tris/HCl, pH7.8) containing 0.2% (v/v) Emulphogene and fractions containing mannan-binding activity (see below) were pooled. $CaCl_2$ was added to a final concentration of 15mM and the solution was loaded onto a mannose–Sepharose column (1ml) that had been equilibrated with buffer C containing 0.2% Emulphogene. The column was washed with buffer C without detergent and eluted with buffer D without detergent. The isolated protein was subjected to SDS/polyacrylamide-gel electrophoresis [10% (w/v) acrylamide] under reducing conditions by the method of Laemmli (1970), with bovine serum albumin, ovalbumin, trypsinogen, β-lactoglobulin and lysozyme as molecular weight standards.

Radioiodination

Mannan (MNN 2) was iodinated by using chloramine-T as previously described (Stahl *et al.*, 1980). Specific radioactivities of 1.1–4.5μCi/μg were achieved and the radiolabelled ligands were diluted with unlabelled ligand to known specific activities for use in assays.

Binding assay

The standard binding assay is a modification of the $(NH_4)_2SO_4$-precipitation assay described by Hudgin *et al.* (1974). The assays were carried out in 250mM-NaCl/40mM-$CaCl_2$/0.1% (v/v) Triton X-100/6mg of bovine serum albumin/ml/50mM-Tris/HCl, pH7.8, in a total volume of 0.5ml containing 0.5–2μg of binding protein. Binding reactions were initiated by adding 0.3μg of ^{125}I-labelled mannan (MNN-2) and tubes were incubated at room temperature for 10min. The reaction was stopped by addition of an equal volume of cold saturated $(NH_4)_2SO_4$ (buffered to pH7.8 with solid Tris and containing 10mM-$CaCl_2$) and the mixture was kept on ice for 10min. The mixture was then filtered on a GF/C filter and the filter was washed with a total of 6ml of 50%-satd. $(NH_4)_2SO_4$ containing 10mM-$CaCl_2$, 3mg of bovine serum albumin/ml and 10mM-Tris/HCl, pH7.8. Filters were counted in a Packard Auto-Gamma scintillation spectrometer. Non-specific binding was estimated by adding excess unlabelled mannan to the incubation mixture. Specific binding was estimated by subtracting non-specific binding from total radioactivity bound.

Results

Binding protein isolation and ^{125}I-labelled mannan binding

The mannan-binding protein of rat liver was isolated by two methods. Both methods take advantage of the sensitivity to Ca^{2+} of ligand binding to the solubilized protein (see below), facilitating adsorption to affinity resins in the presence of Ca^{2+} and elution with EDTA. The first method involved invertase–Sepharose affinity chromatography of a Triton X-100 extract of rat liver acetone-dried powder. Chromatography on asialo-orosomucoid–Sepharose was included to remove any contaminating galactose-binding protein (which also bound to the invertase–Sepharose). This procedure yielded 200–250 μg of mannan-binding protein from 150 g of rat liver (1.3–1.7 μg/g of liver).

The second method gave higher yields of mannan-binding protein with fewer chromatographic steps. The mannose–Sepharose affinity column used in this procedure did not bind the galactose-binding protein, so the use of an asialo-orosomucoid–Sepharose column was not required. $(NH_4)_2SO_4$ fractionation of the detergent extract of frozen rat liver decreased the protein concentration sufficiently to allow direct loading of this material onto the mannose–Sepharose column, instead of the stirring procedure used in the first method to adsorb the binding protein to the affinity gel. The second method yielded approx. 600 μg of mannan-binding protein from 300 g of rat liver (2 μg/g of liver).

The isolated rat liver binding protein appears as a single band on SDS/polyacrylamide-gel electrophoresis in the presence of β-mercaptoethanol with an apparent mol.wt. of approx. 30000 (Fig. 1). The properties of the protein purified by either of the two methods appear to be identical.

The ligand used for measuring the binding activity of the soluble binding protein was ^{125}I-labelled MNN2, a mannan from a mutant yeast with an unsubstituted α 1→6 linked backbone (Ballou & Raschke, 1974). The binding of various concentrations of this ^{125}I-labelled mannan to a constant amount of binding protein is shown in Fig. 2. Specific binding was determined by subtracting radioactivity obtained with excess unlabelled yeast mannan in the assay mixture (non-specific binding) from radioactivity obtained from otherwise identical tubes (total binding). Binding is seen to saturate at approx. 100 ng of ^{125}I-labelled mannan bound/μg of binding protein. The standard binding assay used 300 ng of ^{125}I-labelled mannan since the ratio of radioactivity specifically bound to total radioactivity precipitated was highest at this ligand concentration.

Fig. 3 shows the increase in ^{125}I-labelled mannan bound as the concentration of binding protein

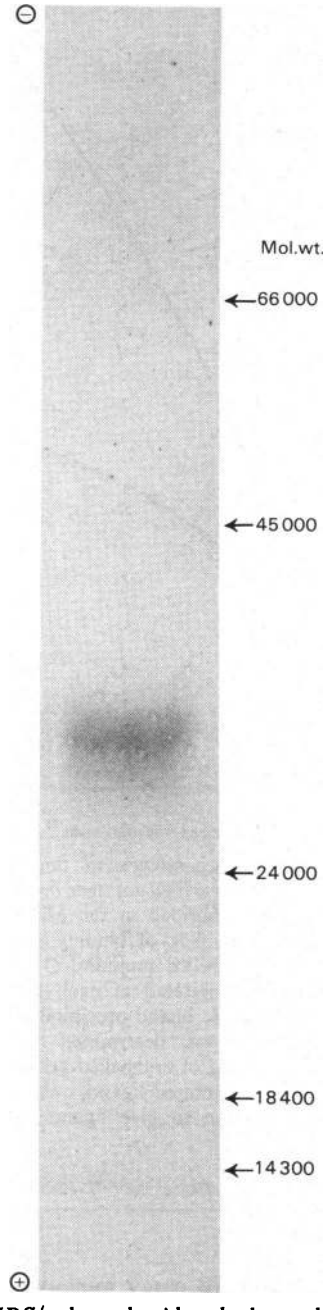

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of isolated binding protein*

Purified binding protein (20 μg) was electrophoresed in a 1.5 mm thick 10% (w/v) acrylamide-slab gel under reducing conditions in the presence of SDS by the procedure of Laemmli (1970). The gel was fixed in acetic acid and stained with Coomassie Brilliant Blue R. Protein standards in adjacent lanes of the gel (not shown) were bovine serum albumin, ovalbumin, trypsinogen, β-lactoglobulin, and lysozyme. The binding protein migrated with an apparent mol.wt. of approx. 30000.

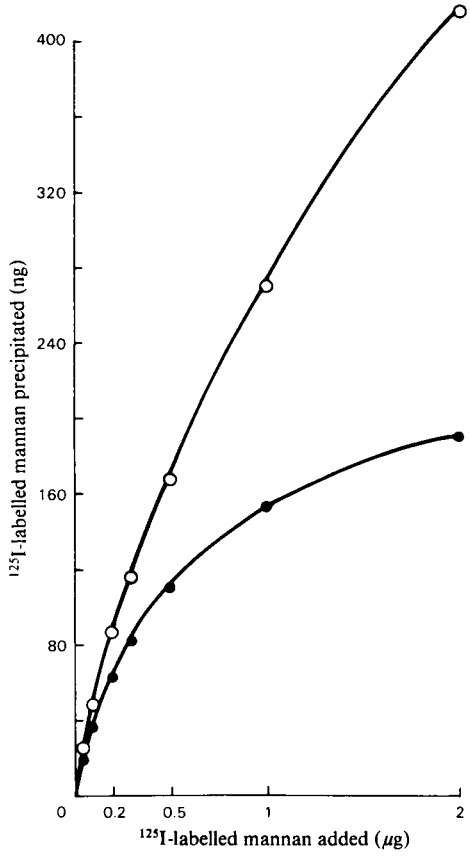

Fig. 2. *Concentration-dependence of binding of ^{125}I-labelled mannan to the purified rat liver binding protein*
The assay was as described in the Materials and methods section with 2 μg of binding protein and 50–2000 ng of ^{125}I-labelled mannan. O, total ^{125}I-labelled mannan precipitated at each ligand concentration. Non-specific ligand precipitation at each ligand concentration was determined in duplicate assays containing 2 mg of unlabelled yeast mannan. Non-specifically precipitated ligand was subtracted from each total value to give ligand specifically bound (●).

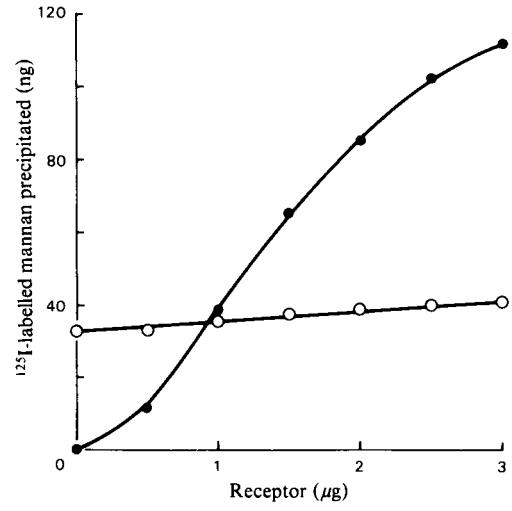

Fig. 3. *Effect of varying the concentration of binding protein on ^{125}I-labelled mannan bound*
The assay was as described in the Materials and methods section with 0–3 μg of purified binding protein and 0.3 μg of ^{125}I-labelled mannan. Non-specific ligand precipitation in the presence of 2 mg of unlabelled yeast mannan (O) was subtracted from each value to obtain ligand specifically bound to binding protein (●).

present in the standard assay mixture (containing 300 ng of ^{125}I-labelled mannan) is increased. Over the restricted range of binding protein concentrations used in the standard binding assay (0.5–2 μg of binding protein/0.5 ml) the amount of ligand bound increases linearly with the amount of binding protein added. The specific binding activity of the purified binding protein under these conditions is approx. 40 ng of ^{125}I-labelled mannan bound/μg of binding protein. Radiolabelled mannan precipitated in the presence of excess unlabelled yeast mannan (Figs. 2 and 3) represents some precipitation of free ligand rather than nonspecific adsorption to the binding protein. This is concluded from the observation that, under all assay conditions, the radiolabel precipitated in binding protein-free control tubes was equal to the radiolabel precipitated in tubes containing the same amount of ^{125}I-labelled mannan and binding protein plus excess unlabelled yeast mannan.

Effect of neoglycoproteins and monosaccharides on ^{125}I-labelled mannan binding to the isolated liver binding protein

The specificity of the binding of ^{125}I-labelled mannan to the binding protein preparation was studied by using neoglycoproteins and monosaccharides as inhibitors. Mannose$_{28}$–albumin (28 mol of mannose/mol of albumin), L-fucose$_{28}$–albumin and *N*-acetylglucosamine$_{31}$–albumin were effective inhibitors of mannan binding; glucose$_{28}$–albumin was intermediate, and galactose$_{33}$–albumin was ineffective (Fig. 4). Table 1 shows the effects of monosaccharides on the binding of ^{125}I-labelled mannan to purified binding protein in the standard binding assay. Although monosaccharides in general were poor inhibitors of binding, D-mannose, L-fucose, *N*-acetylmannosamine, and *N*-acetylglucosamine were more potent than D-galactose and

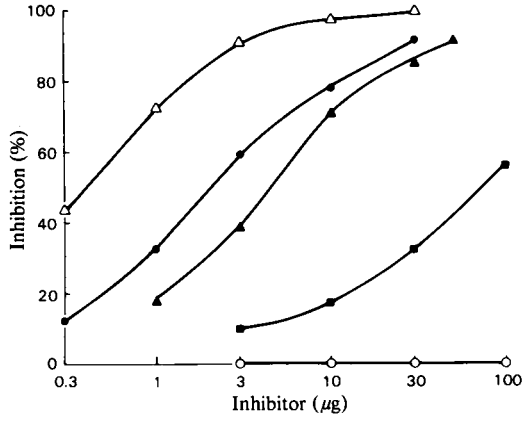

Fig. 4. *Effect of neoglycoproteins on binding of ^{125}I-labelled mannan to isolated binding protein*

The standard binding assay was used in the presence of various amounts of mannose$_{28}$–albumin (●), fucose$_{28}$–albumin (△), *N*-acetylglucosamine$_{31}$–albumin (▲), glucose$_{28}$–albumin (■) or galactose$_{33}$–albumin (○). All data are expressed as percentage inhibition of control (no inhibitor) specific binding.

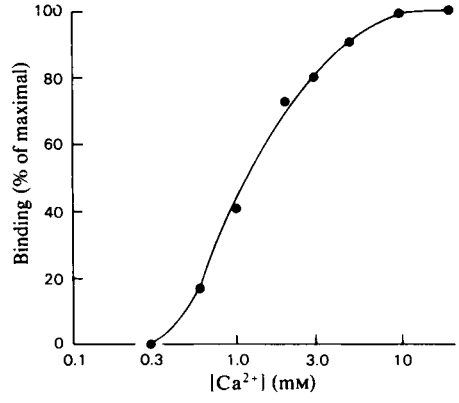

Fig. 5. *Effect of $[Ca^{2+}]$ on binding of ^{125}I-labelled mannan to the isolated rat liver binding protein*

Samples of isolated binding protein in 0.5 ml of solution containing Tris, albumin, Triton X-100 and NaCl at the concentrations used in the standard binding assay were dialysed overnight against 2 litres of 250 mM-NaCl/0.1% (v/v) Triton X-100/40 mM-Tris/HCl, pH 7.8, containing 0–40 mM-$CaCl_2$. ^{125}I-labelled mannan binding was then determined (see the Materials and methods section) and the results are expressed as percentage of maximum (10 mM-$CaCl_2$) specific binding.

Table 1. *Inhibition of ^{125}I-labelled mannan binding to purified binding protein by monosaccharides*

The standard binding assay, with 1.5 μg of binding protein and 0.3 μg of ^{125}I-labelled mannan was performed in the presence of 30 mM of each of the sugars indicated. Results are expressed as percentage inhibition of binding-protein-specific binding (see the legend to Fig. 2).

Sugar	Inhibition (%)
N-Acetylmannosamine	84
α-Methyl mannoside	74
N-Acetylglucosamine	59
D-Mannose	56
L-Fucose	42
α-Methyl glucoside	34
D-Glucose	33
D-Mannose 6-phosphate	18
D-Galactose	14
N-Acetylgalactosamine	0

mannose 6-phosphate. On the basis of moles of sugar present, the neoglycoproteins were more effective than monosaccharides as inhibitors by several orders of magnitude. Free mannose, for example, inhibits binding by 50% at approx. 2×10^{-2} M whereas mannose$_{28}$–albumin gives 50% inhibition at approx. 1.4×10^{-7} M-protein or 4×10^{-6} M-mannose.

Effect of Ca^{2+} on ^{125}I-labelled mannan binding to soluble binding protein

The Ca^{2+}-concentration dependence of mannan binding to the liver binding protein was studied by varying the $[Ca^{2+}]$ in the assay mixtures. The results in Fig. 5 show that binding to the soluble binding protein requires Ca^{2+}, with 50% of maximal binding at approx. 1 mM-Ca^{2+}. This is similar to the Ca^{2+} concentration in extracellular fluids.

Discussion

Rapid plasma clearance of lysosomal enzymes and certain glycoconjugates is mediated by a binding protein associated with reticuloendothelial cells, especially in the liver (Achord *et al.*, 1978; Brown *et al.*, 1978; Schlesinger *et al.*, 1978). Alveolar macrophages possess a binding protein that binds and internalizes mannose- or *N*-acetylglucosamine-terminal glycoconjugates (Stahl *et al.*, 1980). Moreover, a binding protein has been isolated from rabbit liver that binds mannan and is inhibited by certain glycoproteins containing mannose or *N*-acetylglucosamine (Kawasaki *et al.*, 1978).

In the present paper we describe the purification of a mannan-binding protein from rat liver. The binding protein was isolated by detergent extraction of a rat liver homogenate followed by $(NH_4)_2SO_4$

fractionation and affinity chromatography on invertase–Sepharose or mannose–Sepharose. This scheme eliminated the necessity of preparing an acetone-dried powder of the liver as starting material, as done by Kawasaki *et al.* (1978) with rabbit liver. The use of mannose–Sepharose as the affinity absorbent eliminated the need for an asialo-orosomucoid–Sepharose column in the procedure. The isolated binding protein migrated as a single band on SDS/polyacrylamide-gel electrophoresis with an apparent mol.wt. of approx. 30000. The maximum yield of isolated binding protein obtained from rat liver was about 2 μg/g of liver. It is unclear how this compares with the actual amount present in the liver, since the yield at each of the steps in our isolation procedure is unknown. This is because we have been unable to measure binding activity in crude extracts, which clog the GF/C filters, resulting in high background levels of ligand adsorbing to the filters. This problem is presently being addressed by development of a radioimmunoassay for the binding protein.

Binding of ^{125}I-labelled mannan to the binding protein was inhibited by mannose–albumin, L-fucose–albumin and *N*-acetylglucosamine–albumin and, to a lesser extent, by glucose–albumin. Galactose–albumin had no effect. Monosaccharide-inhibition data, although much less sensitive indicators of specificity, agree with this specificity. The inclusion of L-fucose in the specificity for the liver binding protein has not previously been noted. The recognition of L-fucose-terminal structures by the alveolar macrophage binding protein has also been demonstrated (V. L. Shepherd, Y. C. Lee, P. H. Schlesinger & P. D. Stahl, unpublished results).

The liver mannan-binding proteins from rat and from rabbit (Kawasaki *et al.*, 1978) display similar properties. Both display apparent mol.wts. of approx. 30000 as measured by SDS/polyacrylamide-gel electrophoresis. The specific binding activities for mannan binding by the two proteins are also similar. Furthermore, the sugar specificities of the two proteins, as indicated by the monosaccharide-inhibition data, are nearly identical.

The rat liver binding protein exhibits a binding specificity very similar to that observed for rat alveolar macrophages with neoglycoprotein inhibitors of agalacto-orosomucoid and β-glucuronidase uptake (Stahl *et al.*, 1978). Binding of ligand by the isolated liver binding protein and uptake of ligand via the macrophage binding protein require Ca^{2+} in similar concentration. Since hepatic clearance of mannose-terminal glycoproteins occurs via non-parenchymal cells (including Kupfer cells, the resident macrophages of the liver) it is interesting to speculate that the binding protein mediating uptake of mannose/*N*-acetylglucosamine/fucose-terminal glycoproteins by alveolar macrophages is identical with the binding protein isolated from liver. Anti-(rat liver binding protein) immunoglobulin G (from rabbit) inhibits mannan binding to the purified liver binding protein but has no effect on mannose–albumin binding to alveolar macrophages (R. Townsend & P. Stahl, unpublished results). This raises the possibility that either (1) the liver binding protein and the binding protein on the surface of the alveolar macrophage are antigenically different or (2) the antibody binds to some site on the molecule other than the ligand-binding site and this site is not exposed on the surface of the alveolar macrophage. Further studies to compare the mannose/*N*-acetylglucosamine/fucose-specific lectins of liver and alveolar macrophage are necessary.

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