Biochem. J. (1986) 234, 653–658 (Printed in Great Britain)

Binding of hyaluronate and chondroitin sulphate to liver endothelial cells

Torvard C. LAURENT,*‡ J. Robert E. FRASER,† Håkan PERTOFT* and Bård SMEDSRØD*

*Department of Medical and Physiological Chemistry, University of Uppsala, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden, and †University of Melbourne Department of Medicine, Royal Melbourne Hospital, Vic. 3050, Australia

Hyaluronate is taken up and metabolized in liver endothelial cells by means of a receptor. To characterize the interaction with the receptor, two preparations of ^{3}H-labelled hyaluronate, of M_r 4×10^5 and 6.4×10^6, and a series of hyaluronate oligosaccharides were bound to cultured liver endothelial cells at 7 °C. The dissociation constant varied between 4.6×10^{-6} M for an octasaccharide and 9×10^{-12} M for the largest polymer. The M_r-dependence for the series of oligosaccharides was explained by the increased probability of binding due to the repetitive sequence along the chain. The high affinity of high-M_r hyaluronate for the receptor could also be mainly ascribed to this effect, which rules out any major contribution of co-operative multiple-site attachment to the cell surface. Each liver endothelial cell can bind 10^5 oligosaccharides, about 10^4 molecules with M_r 4×10^5 and about 10^3 molecules with M_r 6.4×10^6. This is explained by mutual exclusion of large molecules from the cell surface. Chondroitin sulphate is also bound to liver endothelial cells. Inhibition studies showed that it binds to the same receptor as hyaluronate and with an affinity that is about 3-fold higher than that of hyaluronate of the same degree of polymerization.

INTRODUCTION

Circulating sodium hyaluronate (HA) is efficiently taken up and metabolized by the endothelial cells in the liver sinusoids (Fraser *et al.*, 1981; Eriksson *et al.*, 1983; Smedsrød *et al.*, 1984; Fraser *et al.*, 1985). Previous investigations (Smedsrød *et al.*, 1984) demonstrated that binding of ^{3}H-labelled HA to liver endothelial cells at 7 °C is saturable and can be inhibited by an excess of unlabelled HA, indicating the presence of a receptor for HA on the cell surface. The binding can also be inhibited by HA oligosaccharides, but less efficiently, showing a preference of the cell surface for high-M_r material. This was tentatively ascribed to multiple-site attachment of large molecules to the surface. The present investigation was carried out to elucidate the mechanism for the M_r-dependence.

Chondroitin sulphate (CSA) is also taken up and metabolized by liver endothelial cells (Smedsrød *et al.*, 1985). The partial inhibition of HA binding by CSA (Smedsrød *et al.*, 1984) and the inhibition of CSA binding by HA (Smedsrød *et al.*, 1985) indicates that the two polysaccharides are recognized by the same receptor. We have now confirmed this hypothesis by the use of oligosaccharides of identical degree of polymerization prepared from the two polymers.

MATERIALS AND METHODS

Unlabelled saccharides

Pure HA (Healon) with a weight-average M_r of several millions was obtained from Pharmacia (Uppsala, Sweden). Unfractionated CSA as well as a sharp fraction with M_r 20000 (fraction no. 3) were kindly donated by Professor Åke Wasteson (see Wasteson, 1971).

Oligosaccharides were prepared from HA by digestion with bovine testicular hyaluronidase and gel chromatography on a 3 cm × 250 cm column of Sephadex G-50 as described previously (Smedsrød *et al.*, 1984). Oligosaccharides were similarly prepared from CSA after the polysaccharide had been freed from traces of HA by precipitation with cetylpyridinium chloride in the presence of 0.125 M-Na_2SO_4 (Scott, 1960). In the following the oligosaccharides are denoted by the number of monosaccharide residues per molecule, e.g. HA-4 stands for a tetrasaccharide prepared from HA.

^{3}H-labelled saccharides

Radioactive HA was synthesized by cultured synovial cells that had been given [^{3}H]acetate, and was purified by centrifugation in CsCl density gradients as described by Fraser *et al.* (1981). The M_r distribution was determined by gel chromatography (Laurent & Granath, 1983). A previously described preparation (Smedsrød *et al.*, 1984) had weight-average (M_w) and number-average (M_n) M_r values of 4×10^5 and 1×10^5 respectively. A new preparation, which was stored in 1% (v/v) dimethyl sulphoxide to prevent radiolysis, had values of 6.4×10^6 and 2.8×10^5 respectively. The specific radioactivities of the two preparations were 5×10^5 and 4×10^5 d.p.m./μg respectively.

^{3}H-labelled oligosaccharides of HA were prepared by reduction with NaB^3H_4 (15.9 Ci/mmol; Amersham International, Amersham, Bucks., U.K.). A 5 mg portion of HA digested by testicular hyaluronidase (Leo, Helsingborg, Sweden) as described by Smedsrød *et al.* (1984) was dissolved in 300 μl of 0.1 M-Tris/HCl buffer, pH 8.5, and mixed with 250 mCi of Na^3HBH_4 and 15 μl of 0.5 M-NaOH. The mixture was left at room temperature overnight. Then 60 μmol of unlabelled $NaBH_4$ was

Abbreviations used: HA, hyaluronate; CSA, chondroitin sulphate.

‡ To whom correspondence should be addressed.

added, and after 4 h the solution was acidified to pH 4 with 4 M-acetic acid. The oligosaccharides were purified from reaction products by chromatography on a Sephadex G-25 PD-10 column (Pharmacia) and subsequently fractionated by chromatography on Sephadex G-50 (see above). After being desalted on Sephadex G-25 columns and freeze-dried each oligosaccharide was dissolved in 1 ml of 0.15 M-NaCl. The concentration of uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962) in four fractions (HA-8, HA-10, HA-14 and HA-24) and the molarities of the oligosaccharides were calculated from the known contents of glucuronic acid in each of them. Subsequent determination of radioactivity showed that their specific radioactivities were 1.78×10^{15}, 1.86×10^{15}, 1.78×10^{15} and 1.80×10^{15} d.p.m./mol respectively. This verifies that the oligosaccharides had been labelled only in the reducing end position. In the following all the oligosaccharides were regarded as having a specific radioactivity of 1.8×10^{15} d.p.m./mol.

Four undefined fractions were collected in the void region of the Sephadex G-50 chromatogram. Analyses of uronic acid and radioactivity and the use of the specific radioactivity obtained for smaller oligosaccharides made it possible to calculate an average of the oligosaccharide size in each of these fractions, namely HA ~ 30, HA ~ 35, HA ~ 47 and HA ~ 51.

The fraction of CSA with M_r 20000 was labelled with NaB^3H_4 as described for the HA oligosaccharides. A specific radioactivity of 1.0×10^{15} d.p.m./mol was obtained.

Cell cultures

Endothelial cells were isolated from rat livers as described in detail by Smedsrød & Pertoft (1985). The cells were suspended in RPMI medium (Flow Laboratories, Irvine, Scotland, U.K.) and seeded on plastic dishes (surface areas 9.6 or 28.2 cm²; Flow Laboratories) coated with fibronectin (Smedsrød *et al.*, 1984; Smedsrød & Pertoft, 1985). After incubation for 0.5–1 h at 37 °C, non-adherent cells were removed by washing. The final number of cells in the cultures was approx. 200000/cm².

Test for receptor-mediated internalization of ^{3}H-HA

Six 9.6 cm² cultures of liver endothelial cells were incubated at 0 °C for 45 min in RPMI medium containing 0.1 μg of ^{3}H-HA (M_w 6.4×10^6)/ml. The medium was removed and the cell layers were washed four times with phosphate-buffered saline (0.13 M-NaCl/0.02 M-sodium phosphate buffer, pH 7.4). Two dishes were used for measuring binding of ^{3}H-HA to the cells (see below). Another two dishes where incubated at 0 °C for 15 min with 5 'turbidity-reducing' units of testicular hyaluronidase/ml of RPMI medium, washed and analysed for bound ^{3}H-labelled material. Finally two dishes were warmed to 37 °C for 10 min, returned to 0 °C and treated with hyaluronidase as above before analysis.

Determination of binding of ^{3}H-saccharides to liver endothelial cells

The procedure adopted previously was followed (Smedsrød *et al.*, 1984). In short, the cell cultures were incubated in RPMI medium containing various concentrations of ^{3}H-oligosaccharides and 1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.). After incubation overnight at 7 °C the medium was removed and the cell layer was washed with phosphate-buffered saline. The cells were dissolved in 1 ml of 0.3 M-NaOH/1% (w/v) SDS and the samples were then neutralized with 2 M-HCl and analysed for radioactivity.

Inhibition of binding was measured by incubating cultures with ^{3}H-labelled saccharide in the absence and in the presence of an unlabelled inhibitor of known concentration.

Unspecific binding of ^{3}H-labelled saccharides was determined on cultures to which also had been added a large excess (usually 100-fold) of unlabelled but otherwise identical saccharide. Non-specific binding was subtracted from total binding to obtain specific binding.

Table 1. Internalization of ^{3}H-HA bound to the cell surface

Endothelial cells were seeded on 9.6 cm² dishes and incubated with 0.1 μg of ^{3}H-HA/ml for 45 min at 0 °C as described in the Materials and methods section. The radioactivity associated with the cell layer was determined after the treatments described below. Each value is an average of two experiments. The background binding in the presence of 10 μg of unlabelled HA/ml was 95 d.p.m. and has been subtracted from the total binding.

Treatment of the cells	Cell-associated radioactivity (d.p.m.)	(%)
Incubation with ^{3}H-HA at 0 °C	1470	100
Incubation with ^{3}H-HA at 0 °C followed by hyaluronidase treatment	170	12
Incubation with ^{3}H-HA at 0 °C followed by incubation at 37 °C and hyaluronidase treatment	705	48

Determination of radioactivity

Radioactivity was measured by liquid-scintillation counting as previously described (Smedsrød *et al.*, 1984).

RESULTS

Receptor-mediated internalization of ^{3}H-HA

An experiment was carried out to show that the specific binding of HA to liver endothelial cells mediates the internalization of the polysaccharide. The result is displayed in Table 1. HA bound to the cell surface in the cold can be removed to an extent of approx. 90% by hyaluronidase. After 10 min at 37 °C about half of the material becomes insensitive to extracellular hyaluronidase, indicative of an internalization.

Binding of high-M_r HA to liver endothelial cells

The binding of a ^{3}H-HA preparation with M_w 4×10^5 was reported in a previous publication (Smedsrød *et al.*, 1984; see also Table 2). The binding of ^{3}H-HA with M_w 6.4×10^6 is reported in Fig. 1 and Table 2.

Binding of HA oligosaccharides to liver endothelial cells

Binding curves were obtained for a few selected oligosaccharides (Fig. 2). Owing to scarcity of material (both cells and oligosaccharides) only three or four points

Table 2. Binding of HA saccharides of increasing size to liver endothelial cells

The dissociation constant, K_d, is the concentration at half-maximal binding in the binding curves displayed in Figs. 1 and 2. The inhibitor constant, K_i, was calculated from the inhibition of the binding of HA~35 to the cells. The values for HA of M_w 4×10^5 are from Smedsrød *et al.* (1984). For explanation of the three last columns see the Discussion section.

Saccharide	No. of molecules bound/cell	K_d (μM)	K_i (μM)	No. of octasaccharide sequences (n)	$n \cdot K_d$	$n \cdot K_i$
HA-4			75			
HA-6			13			
HA-8	(130000)*	(1.4)*	4.6	1		4.6
HA-10			2.0	2		4.0
HA-12	200000	1.0	1.9	3	3.0	5.7
HA-14			1.3	4		5.2
HA-16			0.9	5		4.5
HA-18	120000	0.50	0.8	6	3.0	4.8
HA-20			0.6	7		4.2
HA-22			0.4	8		3.2
HA-24	140000	0.36	0.4	9	3.2	3.6
HA~35	120000	0.20		14	2.8	
HA (M_w 4×10^5)	8100	6×10^{-5}		10^3	0.06	
HA (M_w 6.4×10^6)	900	9×10^{-6}		16×10^3	0.14	

* The background binding was in the order of 50% of the total binding in this experiment.

could be obtained on each curve. This was, however, sufficient to show an increasing affinity with increasing chain length. An uncertainty was introduced by the high background binding observed for the smallest saccharides (for HA-8 between 48 and 59% of the total binding, for HA-12 between 18 and 44% and for HA-18 between 10 and 27%; the background then continually declined with increasing chain length).

As is apparent in Fig. 2 and Table 2 the level of maximal binding varies in the different experimental series. This was also observed previously in experiments on HA with M_w 4×10^5 and is part of the biological and experimental variations inherent in cell biological work. The maximal binding per cell and the concentration of free saccharide at half-maximal binding are tabulated in Table 2.

Inhibition of binding of HA~35 to liver endothelial cells

Owing to insufficient supply of radioactive oligosaccharides for proper binding curves another technique was adopted in which unlabelled oligosaccharides were used to inhibit the binding of a labelled saccharide with known dissociation constant. As labelled compound we chose HA~35, which had a reasonably high affinity (K_d 0.20 μM; Table 2), had a relatively low background binding (10–20%) and was available in the required amounts.

In a system with added radioactive ligand but without an inhibitor the binding can be described by:

$$\frac{C_* \cdot C_{R'}}{C_{*-R'}} = K_d \qquad (1)$$

where C_* is the concentration of free radioactive ligand, $C_{R'}$ the concentration of free receptor and $C_{*-R'}$ the concentration of receptor–ligand complex. K_d is the dissociation constant.

If we make an identical experiment but also add an inhibitor with the concentration C_i and the inhibitor

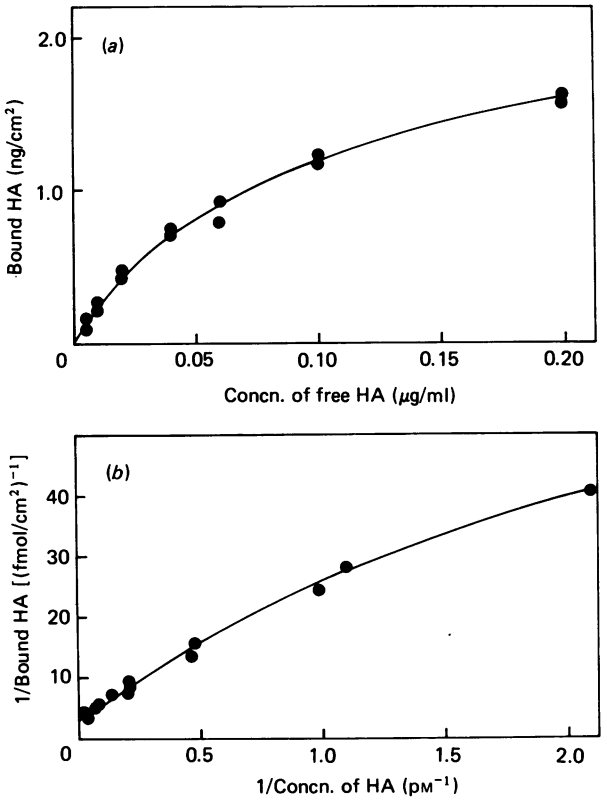

Fig. 1. Binding of HA (M_w 6.4×10^6) to cultured liver endothelial cells at 7 °C

(*a*) The binding is plotted as a function of the concentration of free HA in the system outlined in the Materials and methods section. Background binding amounted to less than 3% of the total binding. (*b*) Double-reciprocal plot of the binding curve. Extrapolation to infinite concentration gives a maximal binding of 0.29 fmol/cm² of culture or approx. 900 molecules/cell. Half-maximal binding is obtained at a concentration of free HA of approx. 9 pM.

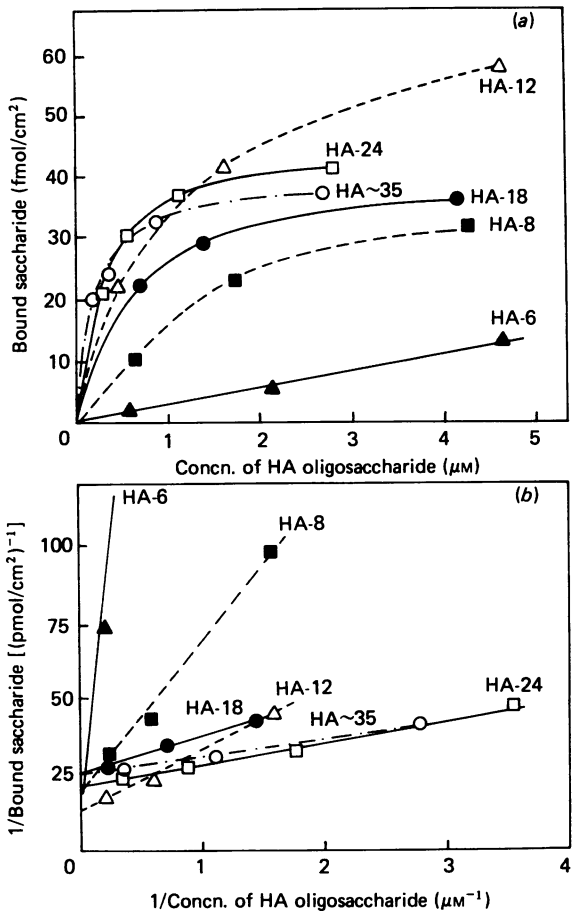

Fig. 2. Binding to cultured liver endothelial cells of oligosaccharides derived from HA

(*a*) The binding at 7 °C is plotted as a function of the concentration of the oligosaccharides in the system outlined in the Materials and methods section. Each curve represents the mean of two separate experiments. (*b*) Double-reciprocal plots of the binding curves. The maximal binding of each oligosaccharide and the concentration when half-maximal binding is obtained are tabulated in Table 2 (with the exception of HA-6, for which reliable values could not be obtained). ▲, HA-6; ■, HA-8; △, HA-12; ●, HA-18; □, HA-24; ○, HA~35.

constant K_i we obtain the following equilibrium situation:

$$\frac{C_* \cdot C_{R''}}{C_{*-R''}} = K_d$$

$$\frac{C_i \cdot C_{R''}}{C_{i-R}} = K_i \qquad (2)$$

where $C_{R''}$ is the concentration of free receptor under these conditions, $C_{*-R''}$ the concentration of receptor–ligand complex and C_{i-R} the concentration of receptor–inhibitor complex.

By combining eqns. (1) and (2), assuming a constant total amount of receptor and keeping the concentration of free ligand so high that it is the same in both experiments, we obtain:

$$\frac{C_{*-R'}}{C_{*-R''}} = \frac{K_d + C_* + K_d \cdot C_i / K_i}{K_d + C_*} \qquad (3)$$

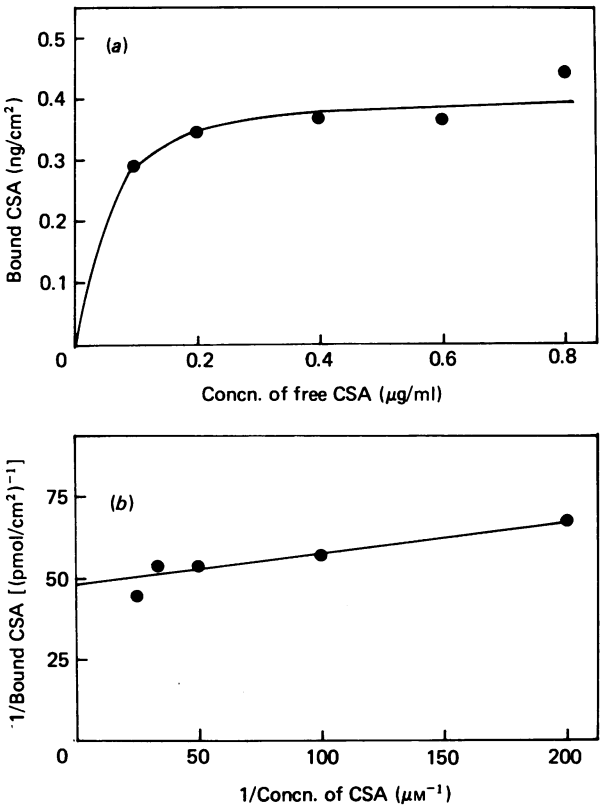

Fig. 3. Binding of CSA (M_w 2×10^4) to cultured liver endothelial cells at 7 °C

(*a*) The binding is plotted as a function of the concentration of free CSA in the system outlined in the Materials and methods section. Background binding varied between 14 and 50% of the total binding. (*b*) Double-reciprocal plot of the binding curve. Extrapolation to infinite concentration gives a maximal binding of approx. 21 fmol/cm² or 63000 molecules/cell. Half-maximal binding is obtained at a concentration of 2.5 nM.

Table 3. Competition of oligosaccharides prepared from HA or CSA with the binding of HA-30 or HA-35 to liver endothelial cells

The inhibitor constant, K_i, was determined as described in the text by using a determined value of K_d for HA~35 of 0.2 μM and an interpolated value for K_d of HA~30 of 0.3 μM. Values are given as means ±S.E.M., or as means with individual values where only two or three determinations were made.

Oligosaccharide	K_i (μM)
HA-10	2.0±0.28 (n = 7)
CSA-10	0.63 (0.8 and 0.46)
HA-16	0.89±0.17 (n = 7)
CSA-16	0.18 (0.14, 0.15 and 0.24)
HA-22	0.38±0.06 (n = 7)
CSA > 20	0.13±0.05 (n = 4)

Thus, by knowing the concentration of free radioactive ligand, C_*, the value of its dissociation constant, K_d, and the concentration of inhibitor, C_i, and measuring the ratio of specifically bound radioactive ligand without and

with inhibitor present, one can calculate the inhibitor constant, K_i.

The experiments were designed so that the concentration of free ligand, C_*, was high and essentially the same in both experiments. Whenever possible the concentration of inhibitor was chosen to give half-maximal binding of radioactive ligand. The values of the inhibitor constants so obtained are tabulated in Table 2.

Binding of CSA to liver endothelial cells

The binding curve for CSA with M_r 20000 is shown in Fig. 3. Owing to the low specific radioactivity of the polysaccharide experiments could not be performed at lower concentrations. The dissociation constant (2.5×10^{-9} M) must therefore be regarded as tentative.

Comparison between HA oligosaccharides and CSA oligosaccharides with regard to affinity for the HA receptor

Three HA oligosaccharides, HA-10, HA-16 and HA-22, were compared with corresponding CSA oligosaccharides. As the fractionation of CSA oligosaccharides was less efficient than that of the HA oligosaccharides, a less well-defined fraction (CSA > 20) eluted just before CSA-20 was compared with HA-22.

The oligosaccharide fractions were used as inhibitors of binding to the cells of HA ~ 35 or in a few experiments HA ~ 30. The inhibitor constants are given in Table 3. Both types of oligosaccharides inhibit the binding of HA, i.e. HA and CSA compete for the same receptor. In each of the three sizes of oligosaccharides CSA has an inhibitor constant of about one-third or less than that of HA, i.e. a higher affinity for the receptor. As is apparent in Table 3, the errors in the determinations were relatively large, but the difference between HA and CSA seems significant.

DISCUSSION

Our previous investigation (Smedsrød *et al.*, 1984) demonstrated that both HA oligosaccharides and CSA only partly inhibited the binding of HA to liver endothelial cells, which indicated a higher affinity of high-M_r HA for the cell surface. Underhill and Toole (Underhill, 1982; Underhill & Toole, 1979, 1980, 1981; Underhill *et al.*, 1983) likewise described an M_r-dependent binding of HA to the surface of 3T3 and BHK cells. The higher affinity for the larger polymers was in both cases explained in terms of multiple-site attachment of the large molecules to the cell surface. Our present data are not in accordance with the multiple-site attachment hypothesis.

The experiment described in Table 1 confirms that HA

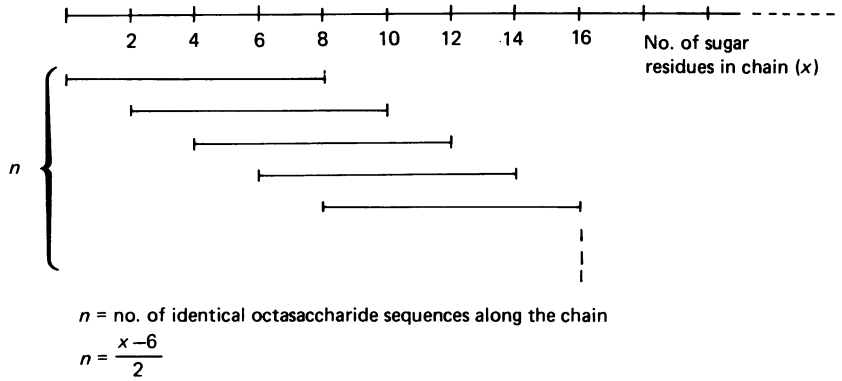

Fig. 4. Illustration of the number of identical octasaccharide sequences in an HA molecule

HA is a linear-chain molecule built from repeating disaccharide units containing glucuronic acid and *N*-acetylglucosamine.

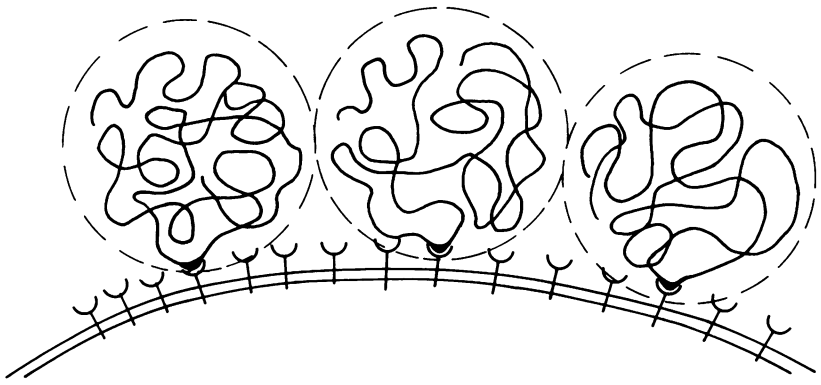

Fig. 5. Representation of the binding of HA molecules to a liver endothelial cell

The surface of the endothelial cell is occupied completely with coiled HA molecules. Each coil is bound to a receptor, but only a small fraction of the total number of receptors is utilized for the binding.

binds specifically to a cell-surface receptor that mediates internalization of the polysaccharide. The binding of HA oligosaccharides to this receptor is strongly M_r-dependent (Table 2), and this M_r-dependence cannot be explained by multiple-site attachment since the oligosaccharides are too small for binding to two or more receptors. The explanation is rather that with increasing length of the oligosaccharide the number of sequences along the chain that can be recognized by the receptor will increase. This is illustrated in Fig. 4. Provided that the recognizable sequence is an octasaccharide (which conforms best with the experimentally obtained data), then the number of sites, n, at which the receptor can bind to the chain is $(x-6)/2$ where x is the number of sugar residues in the molecule. The probability for an oligosaccharide to bind to the receptor increases proportionally to the number of binding sequences; thus when the dissociation constant, K_d, is multiplied by n we should obtain a constant value. The product is tabulated in Table 2, and it is apparent that it attains an approximately constant value. By trial and error one can show that the most nearly constant value is obtained if one assumes a binding sequence of the size of an octasaccharide. However, hexa- and tetrasaccharides at high concentrations also inhibit the binding of HA ~ 35 (Table 2) and can presumably be recognized by part of the receptor.

Table 2 also contains K_d for high-M_r HA and the product $n \cdot K_d$. In the calculation of n we have used the weight-average M_r value. The rationale for this choice is the fact that the polysaccharides are uniformly labelled with radioisotope and that the radioactivity measurements thus will give weight averages. The $n \cdot K_d$ values for the two polysaccharides deviate from the corresponding product for oligosaccharides by a factor of 20–50-fold, which is much less than one would expect from co-operative binding of two equal receptors to the same ligand. Therefore the main reason for the high affinity of high-M_r HA to the liver endothelial cells is essentially the large size of the chain rather than multiple-site attachment.

The dramatic fall in the number of HA molecules bound per cell with increasing M_r can be explained by steric exclusion at the cell surface (Fig. 5). If we assume that a cultured liver endothelial cell is flat and circular with a diameter of 20 μm, then the cell will cover a surface of 314 μm^2. An HA molecule of M_r 6.4×10^6 (Table 2) has a radius of gyration in the order of 300 nm (Laurent & Gergely, 1955). Assuming that 900 of these molecules are packed as closely as the radius of gyration allows, then they will fill 254 μm^2, which corresponds to the area exposed by one cell. An HA molecule with M_r 4×10^5 (Table 2) has a radius of gyration of 65 nm (Laurent, 1955). Then 8100 of these molecules will fill an area of 108 μm^2, which is also of the same order as the available area. We may thus conclude that there are at least 10^5 receptors for HA on the surface of a liver endothelial cell that can bind oligosaccharides, but that for steric reasons only a small fraction of these are utilized in the binding of high-M_r HA.

CSA also binds to the surface of liver endothelial cells, as demonstrated in Fig. 3, and the number of receptors available for molecules of M_r 20000 is of the same order as those available for HA oligosaccharides. Moreover, data in Table 3 clearly demonstrate that HA and CSA are bound to the same receptor species. Interestingly the data also show that CSA is bound with a higher affinity than is HA. The low power of CSA to compete with HA binding (Smedsrød *et al.*, 1984) is actually due to the much lower M_r of CSA.

The skilful technical assistance by Ms. Sigbritt Eriksson is gratefully acknowledged. This project was supported by grants from the Swedish Medical Research Council (3X-4), The Swedish Board for Technical Development (84–4706), Gustaf V:s 80-årsfond, AB Pharmacia, The National Health and Medical Research Council of Australia and the Utah Foundation of Australia.

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Received 16 August 1985; accepted 11 November 1985