Binding studies of a sialic acid-specific lectin from the horseshoe crab
Carcinoscorpius rotunda cauda with various sialoglycoproteins

Somasundaran MOHAN, Devarajan THAMBI DORAI, Subita SRIMAL and Bimal Kumar
BACHHAWAT
Indian Institute of Chemical Biology, Jadavpur, Calcutta-700032, India

(Received 21 September 1981/Accepted 16 December 1981)

Interaction of the sialic acid-specific lectin carcinoscorpin with various sialoglyco-
proteins was studied by using radioiodinated lectin. The binding of carcinoscorpin was
dependent not only on sialic acid content but also on the type of glycosidic linkage and
form (branched or linear) of the carbohydrate chains. Carcinoscorpin has different
classes of binding sites, and binding follows a phenomenon of positive co-operativity.
The effect of Ca$^{2+}$ concentration on the binding was studied, and the optimal
concentration was found to be 0.02M. Effect of pH, temperature and other bivalent
metal ions are also reported. From haemagglutination- and precipitation-inhibition
studies, it was concluded that carcinoscorpin has multispecificity towards acidic sugars,
and its relation to the biological role of the lectin in the horseshoe crab is discussed.

A large number of carbohydrate-specific pro-
teins, called lectins, have been purified from differ-
ent plant and animal sources, and their properties
have been well defined (Lis & Sharon, 1977;
Goldstein & Hayes, 1978). They serve as good
models for study of protein–carbohydrate inter-
actions (Goldstein & Hayes, 1978). These sugar-
specific proteins also serve as a good analytical tool
in the form of immobilized columns with which we
can isolate and characterize various specific glyco-
proteins of cell-surface or cytoplasmic origin. These
have proved to be difficult to purify by classical
methods, owing to their structural diversity and
presence in minute amounts (Gambos et al., 1971;
Dulaney, 1979).

The first report of an agglutinin in an invertebrate
haemolymph was made by Noguchi (1903) when he
showed an agglutinating material in the haemo-
lymph of the marine horseshoe crab, Limulus
polyphemus. A sialic acid-binding lectin, carcino-
scorpin, has been detected and purified to homo-
geneity from the haemolymph of the marine horse-
shoe crab, Carcinoscorpius rotunda cauda
(Bishayee & Dorai, 1980). These horseshoe crabs
are available in the coastal regions of the Bay of
Bengal and are similar to but approximately one-
quarter the size of L. polyphemus, available only off
the North American coast. The haemolymph of L.

polyphemus also contains a sialic acid-specific lectin,
limulin, which has been characterized extensively
(Cohen et al., 1965; Marchalonis & Edelman, 1968;
Roche et al., 1975). Carcinoscorpin has been
partially characterized with regard to its subunit
amino acid composition and immunological cross-
reactivity with limulin (Dorai et al., 1981a). It has
been immobilized and used as an efficient tool to
fractionate sialoglycoproteins such as sheep brain
alkaline phosphatase and rat liver acid phosphatase
(Dorai et al., 1981b; Mohan et al., 1981).

A systematic study was undertaken with a
number of model sialoglycoproteins, each different
with respect to its sialic acid content as well as to
type of oligosaccharide linkage, in order to obtain a
better understanding of the mode and optimal
parameters of binding of carcinoscorpin. The aim
was to widen the applicability of this lectin in serving
as an analytical tool to isolate or resolve the
microheterogeneity of many biologically important
sialoglycoproteins or to use it as a probe in the study of
membrane glycoconjugates.

Materials and methods

Fetuin type III, bovine serum albumin, N-acetyl-
neuraminic acid, N-glycolylneuraminic acid, 3-
deoxy-2-oxo-oxonate, chitobiose, colominic acid,
d-glucuronic acid, d-galacturonic acid, N-acetyl-
galactosamine, N-acetylgalactosamine, phosphochol-
ine (Ca$^{2+}$ salt) and p-nitrophenyl fucoside were
purchased from Sigma Chemical Co., St. Louis,
MO, U.S.A. Human serotransferrin, human lactotransferrin and α-1-acid glycoprotein were given by Dr. H. Debray, Université des Sciences et Techniques de Lille I, Lille, France. Carrier-free $^{125}$I was from Bhaba Atomic Research Centre, Bombay, India. All other chemicals used were of analytical grade.

**Purification of carcinoscorpin**

Carcinoscorpin was purified by the procedure described by Bishayee & Dorai (1980) as modified by Dorai et al. (1981a). The haemagglutinating activity of the lectin was determined as described by Bishayee & Dorai (1980), by using 1% washed rabbit erythrocytes. The purified material, in 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl and 0.01M-CaCl$_2$, was stored at a protein concentration less than 2mg/ml, in small batches at $-20^\circ$C until further use, as reported by Dorai et al. (1981a).

**Iodination of carcinoscorpin**

Carcinoscorpin was iodinated with Na$^{125}$I by the method of Hunter (1967). In a total volume of 30µl, 11.4µg of purified carcinoscorpin (dialysed against 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl to remove Ca$^{2+}$) was treated at room temperature with 0.53mCi of Na$^{125}$I and 6µg of chloramine-T in 0.25M-sodium phosphate buffer, pH 7.5. After 30s, the reaction was terminated by adding 5µl of sodium metabisulphite (10mg/ml). At 1min after the addition of sodium metabisulphite, 5µl of KI (166mg/ml) and 20µl of desialylated bovine serum albumin (20mg/ml) were added. The $^{125}$I-labelled lectin was separated by affinity chromatography. First, $^{125}$I-labelled carcinoscorpin was separated from free Na$^{125}$I by rapid dialysis against 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl, for 2h, followed by dialysis for 1h against 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl and 0.01M-CaCl$_2$. For further separation of $^{125}$I-labelled carcinoscorpin, the non-diffusible material was then loaded on to a fetuin–Sepharose column (1ml) (Dorai et al., 1981b) previously equilibrated with 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl, 0.01M-CaCl$_2$ and 2mg of desialylated albumin/ml (equilibrium buffer). After washing the column with 5 bed vol. of equilibrium buffer, the bound $^{125}$I-labelled carcinoscorpin was eluted with 0.05M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl, 0.04M-sodium citrate and 2mg of desialylated albumin/ml.

The eluate was dialysed thoroughly against two changes of 0.002M-Tris/HCl buffer, pH 8.0, containing 0.1M-NaCl and then once against the same buffer containing 0.01M-CaCl$_2$ also. $^{125}$I-labelled carcinoscorpin had a specific radioactivity of $2 \times 10^4$ c.p.m./ng. The radioactivity was counted in a Packard Autogamma counter 5110 (70% efficiency).

**Iodination of sialoglycoproteins**

The procedure was essentially the same as for carcinoscorpin, except that the iodinated glycoprotein was separated from free Na$^{125}$I by gel filtration on a Sephadex G-50 column (22cm x 0.8cm) previously equilibrated with equilibrium buffer. Fractions (0.5ml) were collected and the radioactivity was measured. The fractions near the void volume (3ml), which contained the first peak of high radioactivity, were pooled. $^{125}$I-labelled fetuin had a specific radioactivity of $1.6 \times 10^4$ c.p.m./ng and that for $^{125}$I-labelled serotransferrin was $1.7 \times 10^4$ c.p.m./ng.

**Preparation of sheep submaxillary mucin**

Sheep submaxillary mucin was isolated from the glands by the method of Murphy & Gottschalk (1961). The carcinoscorpin-specific disaccharide, $O$-($N$-acetyleneuraminyl)-(2→6)-2-acetamido-2-deoxy-d-galactitol, was prepared from sheep submaxillary mucin by reductive cleavage with alkaline borohydride (Murthy & Horowitz, 1968).

**Preparation of fetuin containing only N-glycosidically linked carbohydrate chains**

The procedure followed was the method described by Downs et al. (1973). This alkaline-dimethyl sulphaoxide method consists of incubation of 50mg of fetuin dissolved in 5ml of dimethyl sulphaoxide/water/ethanol (5:4:1, by vol.) containing 0.17M-KOH for 60min at 45°C. The resulting solution was neutralized with 2M-HCl and then dialysed for 15h with three changes against water and then later against 0.002M-Tris/HCl buffer, pH 8.0. The procedure produces no diffusible protein fragments and leaves intact the N-glycosidic linkages since they are resistant to treatment by alkali. The decrease in sialic acid content corresponded to the loss of sialic acid located at the terminal ends of carbohydrate chains, which are O-glycosidically linked to the protein.

**Determination of protein**

Protein was measured by the method of Lowry et al. (1951), with crystalline bovine serum albumin as standard.

**Determination of sialic acid in sialoglycoproteins**

The sialic acid content of sialoglycoproteins was determined by the thiobarbituric acid assay of Warren (1959), as modified by Saifer & Gerstenfeld (1962) by hydrolysis with 0.05M-H$_2$SO$_4$ at 80°C for 1h.
Binding assay

This experiment was based on the observation that carcinoscorpin precipitates as an insoluble complex when it binds with sialoglycoproteins by recognition of the sialic acid moiety. Binding assays were performed in glass tubes (5 cm x 0.5 cm) in a final volume of 50 μl containing 0.05 M-Tris/HCl buffer, pH 8.0, 0.1 M-NaCl, 0.01 M-CaCl₂, and 1 mg of desialylated albumin/ml (incubation buffer). When titration studies were done between various sialoglycoproteins and carcinoscorpin, the incubation system contained 0.05 nmol of unlabelled carcinoscorpin, a fixed amount of 125I-labelled carcinoscorpin corresponding to 2 x 10⁶ c.p.m. (negligible amount of carcinoscorpin compared with unlabelled protein) and various amounts of sialoglycoproteins. Control tubes were used that did not contain the sialoglycoprotein. After 60 min at room temperature, the incubation mixture was filtered rapidly with a Swinnex 13 Millipore filter unit [0.45 μm pore size; presoaked in desialylated albumin (10 mg/ml)]. A small amount of the incubation buffer was used to wash the filter unit to remove any liquid remaining in the dead space of the apparatus. The whole procedure of filtration and washing twice with 0.1 ml of incubation buffer gave reproducible results, and took less than 20 s. The filter paper was carefully folded and counted directly for radioactivity in the gamma counter. In experiments where the bound and free ligands (the sialoglycoproteins) were to be determined for Scatchard (1949) plots, the incubation system contained 0.05 nmol of unlabelled carcinoscorpin, a fixed amount of 125I-labelled sialoglycoprotein and various amounts of unlabelled sialoglycoprotein. The radioactivity obtained from each filter paper corresponds to the amount of precipitate formed when carcinoscorpin binds to the high-molecular-weight ligands and is directly related to the amount of carcinoscorpin (in the titration experiment with various ligands) and ligand (in the Scatchard-plot experiment) present in the complex compared with the controls.

Effect of pH on binding

The binding assay was done essentially by the method described above, with the incubation mixture containing 0.05 nmol of unlabelled carcinoscorpin (that had been dialysed against 0.002 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.01 M-CaCl₂), 125I-labelled carcinoscorpin corresponding to 2 x 10⁶ c.p.m. and 0.4 nmol of fetuin (which was the amount that corresponded to maximal binding of carcinoscorpin as obtained from the titration curve), 0.1 M-NaCl and 0.01 M-CaCl₂ in 0.05 M-Tris/acetate buffer of pH 4.5-7.4 or 0.05 M-Tris/HCl buffer of pH 7.0-9.0.

Effect of metal ions on binding

The incubation mixture used to study the effects of different metal ions was essentially the same as described under 'Effect of pH on binding' and was in 0.05 M-Tris/HCl buffer, pH 7.0, containing various concentrations of each metal ion. In each case, the control tube contained no metal ion. Two different sets of experiments were done to study the effect of metals on binding: one set contained carcinoscorpin that had been dialysed for 15 h with three changes against 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl and the other set contained carcinoscorpin that had been dialysed thoroughly against 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl and 0.01 M-disodium EDTA. All solutions used were prepared in deionized water.

Effect of temperature on binding

The binding assay as described above was performed at different temperatures to study the effect of temperature on the amount of carcinoscorpin that bound to fetuin. In each case, the incubation mixture with fetuin was allowed to attain the required temperature and then carcinoscorpin, which was also allowed to attain the same temperature, was added. After incubation at that temperature for 1 h, the incubation mixtures were passed through the Millipore syringe as described under 'Binding assay' for room temperature (28-30°C). Since the separation procedure took less than 20 s for its completion, any error involved owing to fluctuation of temperature was negligible.

Inhibition studies

The procedure was similar to the above experiments except that the assay mixture contained, in addition to 0.05 nmol of carcinoscorpin, a fixed amount of 125I-labelled carcinoscorpin corresponding to 2 x 10⁶ c.p.m., 0.4 nmol of fetuin and various amounts of the inhibitor. Fetuin was added after 1 h of incubation of the inhibitor with carcinoscorpin.

Haemagglutination tests

The inhibition of haemagglutination by various simple sugars or glycoproteins was performed by the method described by Matsumoto & Osawa (1970). The incubation was performed in microtitre U-plates (Laxbro, Pune, India) by a 2-fold serial dilution. To each 0.05 ml of 2-fold serially diluted sugar or glycoprotein inhibitor solution in haemagglutination buffer (0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.1 M-CaCl₂) was added an equal volume of carcinoscorpin solution diluted to contain four minimum haemagglutination doses. After incubation for 1 h at room temperature, 0.05 ml of 3% normal rabbit erythrocyte suspension in haemagglutination buffer was added. The mixture was kept for 1 h at room temperature and then examined
for agglutination. Results were expressed as the minimum concentration (mm) required to inhibit completely one haemagglutination dose after taking into account the 3-fold dilution caused by addition of lectin and erythrocytes.

**Results and discussion**

*Titration of sialoglycoproteins with carcinoscorpin*

The results are plotted in Fig. 1 as percentage of carcinoscorpin bound against sialic acid content (nmol) of the glycoproteins. All points represent the average of duplicate experiments. Appropriate corrections were made for non-specific binding, which was less than 4%, and were obtained from the control tubes of each titration. The albumin added in the incubation mixture was desialylated because of the previous observation that the commercial sample of albumin contains sialic acid (4 nmol of sialic acid/mg of protein; Dorai et al., 1981b). The amount of carcinoscorpin (0.05 nmol) and time of incubation (60 min) were fixed on the basis of our previous studies on binding of carcinoscorpin to fetuin and rat liver acid phosphatase (S. Mohan, S. Bishayee & B. K. Bachhawat, unpublished work).

The amount of sialoglycoproteins taken for titration was 0—3 nmol of protein, with sialic acid content ranging from 0 to 300 nmol. The model glycoproteins were chosen so as to have a wide variety of sialoglycoproteins that differ not only in their sialic acid content but also with respect to the distribution of the carbohydrate chains and their linkage to protein (see Table 1). In other words, the topology of sialic acid differed in all of these glycoproteins. The sequence of the carbohydrate chains in each of these glycoproteins has been well established (Bertolini & Pigman, 1970; Spiro & Bhyroo, 1974; Baenziger & Fiete, 1979; Debray et al., 1981).

The general pattern of all the curves was similar to that of antigen—antibody precipitin curves that follow a pseudo-first-order reaction. The plots showed that the amount of carcinoscorpin bound was proportional to the sialic acid content. Each glycoprotein differed in the amount of carcinoscorpin that was bound maximally at the equivalence point. Modified fetuin, which has 40% less sialic acid content than fetuin, precipitated 40% less carcinoscorpin than fetuin. Sheep submaxillary mucin, even with its high content of sialic acid, had a different profile of binding to carcinoscorpin (Fig. 1b). The titration data are summarized in Table 1. Ratios of sialic acid content to the percentages of carcinoscorpin bound at equivalence point were constant for globular proteins with large numbers of antennary-type carbohydrate chains. The different value for serotransferrin compared with fetuin may be attributed to the smaller number of carbohydrate chains, which are spaced out farther from each other on a molecule larger (mol.wt. 80000) than fetuin (mol.wt. 48000). Lactotransferrin, which is similar in structure to serotransferrin except for its fucosylated carbohydrate chains, surprisingly bound more carcinoscorpin than did serotransferrin. This anomalous binding behaviour was also reflected in the ratio of sialic acid content per oligosaccharide chain to that of percentage of carcinoscorpin bound. If the abnormal binding of lactotransferrin may be attributed to its fucose residues and the unusual type of binding by mucin to its linear arrangement of sialic acid, our studies then suggest that carcino-
Carcinoscorpin binding to sialoglycoproteins

Table 1. Structural features of the carbohydrate portions of the sialoglycoproteins and their relation to carcoscorpin binding

All glycoproteins have antennae-type carbohydrate chains, except for sheep submaxillary mucin, which has a linear arrangement. Values for binding and ratios are those obtained at equivalence point.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Type of linkage of the oligosaccharide chains</th>
<th>No. and form of oligosaccharide chains per molecule</th>
<th>Amount of carcoscorpin bound (nmol)</th>
<th>Ratio of sialic acid content to percentage of carcoscorpin bound</th>
<th>Ratio of sialic acid content per oligosaccharide chain to percentage of carcoscorpin bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>N- and O-glycosidic</td>
<td>6: tri- and bi-antennary</td>
<td>0.04</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>α2-Acid glycoprotein</td>
<td>N-Glycosidic</td>
<td>5: bi-, tri- or tetra-antennary</td>
<td>0.044</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Modified fetuin*</td>
<td>N-Glycosidic</td>
<td>3: tri-antennary</td>
<td>0.023</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Serotransferrin</td>
<td>N-Glycosidic</td>
<td>2: bi-antennary</td>
<td>0.025</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>N-Glycosidic</td>
<td>2: bi-antennary</td>
<td>0.048</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Sheep submaxillary mucin</td>
<td>O-Glycosidic</td>
<td>&gt;800; linear</td>
<td>0.042</td>
<td>68</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Fetuin with its O-glycosidically linked carbohydrate chains removed.

scorpin interaction with bound sialic acid depended not only on the total number of residues of sialic acid available but also on the 'local density' of these residues on the molecule, i.e. on the topography of these residues on the protein. Our observations on the binding of carcoscorpin with fetuin and modified fetuin might differentiate carcoscorpin from limulin, which was reported to have a higher affinity for NeuAc-GalNAc residues (Roche & Monsigny, 1979), since modified fetuin has its O-glycosidically linked carbohydrate units, consisting of NeuAc-GalNAc removed, and still showed no marked difference in the amount of carcoscorpin bound.

Scatchard plots

Figs. 2 and 3 give the binding profiles of radioiodinated sialoglycoproteins with unlabelled carcoscorpin in the form of Scatchard plots. All points represent the average of duplicate experiments. The amount of fetuin used for this titration varied from zero through one-hundredth to ten times the concentration of carcoscorpin. The binding of fetuin at room temperature is represented by the unusual curvilinear plot (Fig. 2a) consisting of a concave-down initial portion followed by linear regions with different slopes. Fig. 3 shows the Scatchard plot obtained for the binding of carcoscorpin to serotransferrin, a glycoprotein of simpler structure and lower content of sialic acid. The concave-down pattern was observed in this case also at room temperature (Fig. 3a). The similar curvilinear Scatchard plots suggest that the binding of ligands to carcoscorpin follows a phenomenon of positive co-operativity (Scatchard, 1949). The linear profiles with different slopes again suggest different classes of binding sites of carcoscorpin; one class
of high affinity/low capacity and the other of low affinity/high capacity. The binding of both fetuin and serotransferrin at lower temperature (4°C) gave similar curvilinear Scatchard plots (Figs. 2b and 3b), eliminating the functional heterogeneity in binding owing to the competition of sugar residues for the binding sites as reported for hepatic lectin (Debanne et al., 1980). These studies give only the effective valency of the lectin and not its true valency. From the plot for fetuin at room temperature, it was observed that two molecules of fetuin bound to a molecule of carcinoscorpin with high affinity, whereas six other molecules of fetuin bound with low affinity. At lower temperature, although the Scatchard plot was similar, it was observed that the total number of molecules that bound to carcinoscorpin was half of that bound at room temperature (two molecules of fetuin at the high-affinity sites and only two more molecules at the low-affinity sites). Although this could be due to the possible dissociation of the protomers, similar to the tetramer–dimer dissociation reported for concanavalin A (Ochoa et al., 1979), further work is required to prove this point conclusively.

**pH and temperature effect**

The binding of carcinoscorpin with fetuin at pH 4.5–9.0 was studied. There was a slow increase in the amount of carcinoscorpin bound to fetuin with pH up to pH 7.0, and then a decrease with a further increase in pH. The optimal pH was around pH 7.0, where the proportion of carcinoscorpin bound was only 8–10% higher than that at pH 8.0. The same pH profile was also obtained when serotransferrin–carcinoscorpin interaction was studied. This is in agreement with the physiological pH of the horse-shoe-crab haemolymph, which was reported as 7.29 (Mangum & Schick, 1972). The binding profile of carcinoscorpin with various sialglycoproteins and also the extent of carcinoscorpin bound were identical for incubations at temperatures from 0 to 37°C. Also, the haemagglutination titre with rabbit erythrocytes was the same for incubation at 37°C and 4°C. The temperature profile suggests that the binding activity of carcinoscorpin is not temperature-dependent.

**Effect of metal ions**

(a) **Ca²⁺**. There is an absolute requirement of Ca²⁺ for the binding activity of limulin (Marchalonis & Edelman, 1968; Nowak & Barondes, 1975). It is known from circular-dichroism studies that Ca²⁺ induces a partial conformation change in limulin, which seems to be a prerequisite for its binding activity (Roche et al., 1978). Carcinoscorpin is also dependent on Ca²⁺ for its activity (Dorai et al., 1981). Our studies on the concentration of Ca²⁺ (0.005–0.030M) required for maximum binding activity of carcinoscorpin with fetuin showed an optimal value of 0.02M, although there was no appreciable difference in binding activity at 0.01M.

(b) **Other metal ions**. To date, the metal-binding sites of limulin and carcinoscorpin have not been studied like that of concanavalin A. Hence, we undertook metal-substitution studies to obtain an idea of the metal requirements. Chlorides of Mg²⁺, Mn²⁺, Ba²⁺ and Sr²⁺ were used in place of Ca²⁺. Carcinoscorpin dialysed against neutral 0.01M-EDTA solution in buffer and salt had no binding activity. The original activity was restored with 0.02M-CaCl₂. Since EDTA is known to be a metal-chelating agent, the observation that the original binding activity was regained by the addition of 0.02M-CaCl₂ suggests the possibility that carcinoscorpin depends solely on Ca²⁺ for its
activity. The normal binding activity could not be restored in the EDTA-dialysed carcinoscorpin by addition (0–0.02 M each) of Mg²⁺, Mn²⁺, Ba²⁺ and Sr²⁺, demonstrating the incapability of these bivalent cations to substitute for Ca²⁺. We also observed that carcinoscorpin dialysed against Ca²⁺-free buffer possessed binding activity, but less by 40% of that of the original carcinoscorpin (undialysed), and this was restored by 0.01 M-Ca²⁺. Although Mg²⁺ and Ba²⁺ did not affect this activity even at 0.02 M, Mn²⁺ and Sr²⁺ abolished it, implicating the presence of endogenous metal sites that were deleteriously affected by Mn²⁺ and Sr²⁺. More investigations are required to arrive at the exact role of bivalent metal ions in the binding activity of carcinoscorpin.

Inhibition of binding

Table 2 summarizes the inhibition studies on both the binding of carcinoscorpin to fetuin and haemagglutination by carcinoscorpin. The concentration of each inhibitor required for inhibition of carcinoscorpin–fetuin complex-formation or haemagglutination reflects the affinity of carcinoscorpin for these inhibitors. The concentration of ligand required to inhibit one haemagglutination dose in the haemagglutination-inhibition assays (although the value is known to be empirical) agree with the value of concentration required for 50% inhibition of carcinoscorpin–fetuin complex-formation. The specificity of carcinoscorpin towards sialic acid was concluded from its inability to bind and precipitate asialofetuin. Also, asialofetuin, even at a high concentration of 350 μg/150 μl, did not inhibit the haemagglutination. The non-inhibitory effects of N-acetylhexosamines differentiate carcinoscorpin from the lectin isolated from the Japanese horseshoe crab, Tachypleus tridentatus, which has been reported to be specific for N-acetylhexosamines (Shimizu et al., 1977). The monosaccharide NeuAc, when compared with the disaccharide and other sialoglycoproteins, was a weak competitor of fetuin–carcinoscorpin interaction and a low inhibitor of agglutination by carcinoscorpin. This can be explained by the avidity phenomenon reported for limulin and other lectins (Roche & Monsigny, 1979). The higher inhibitory capacity of NeuGc (50-fold) when fetuin–carcinoscorpin interaction was studied

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Conc. needed for 50% inhibition (mM)</th>
<th>Minimum concn. needed for inhibition of one haemagglutination dose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuAc</td>
<td>&gt;20</td>
<td>&gt;1</td>
</tr>
<tr>
<td>NeuGc</td>
<td>0.4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>KDO</td>
<td>&gt;10</td>
<td>0.84</td>
</tr>
<tr>
<td>O-Phosphoethanolamine</td>
<td>&gt;50</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Phosphocholine</td>
<td>&gt;50</td>
<td>&gt;10</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>&gt;100</td>
<td>&gt;9</td>
</tr>
<tr>
<td>α-L-Fucose</td>
<td>&gt;70</td>
<td>&gt;8</td>
</tr>
<tr>
<td>p-Nitrophenyl fucoside</td>
<td>&gt;30</td>
<td>2.5</td>
</tr>
<tr>
<td>GalNAc</td>
<td>&gt;80</td>
<td>8</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>&gt;50</td>
<td>8</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>11</td>
<td>0.8</td>
</tr>
<tr>
<td>D-Galacturonic acid</td>
<td>&gt;80</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Disaccharide</td>
<td>0.035</td>
<td>0.002</td>
</tr>
<tr>
<td>Trisaccharide</td>
<td>1.1</td>
<td>0.12</td>
</tr>
<tr>
<td>Chitobiose</td>
<td>&gt;80</td>
<td>&gt;6.5</td>
</tr>
<tr>
<td>Colominic acid</td>
<td>&gt;70*</td>
<td>&gt;0.34†</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>ND</td>
<td>&gt;0.012</td>
</tr>
<tr>
<td>Fetuin</td>
<td>ND</td>
<td>0.0002</td>
</tr>
<tr>
<td>α₁-Acid glycoprotein</td>
<td>ND</td>
<td>0.0002</td>
</tr>
<tr>
<td>Sheep submaxillary mucin</td>
<td>ND</td>
<td>0.00002</td>
</tr>
<tr>
<td>Serotransferrin</td>
<td>ND</td>
<td>0.0003</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>ND</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

* In μg/0.05 ml.
† In mg/0.15 ml.

Vol. 203
showed the higher affinity that carcinoscorpin has towards NeuGc, which was also supported by the report that the minimum concentration of carcinoscorpin (0.12 ng/ml) to agglutinate horse erythrocytes, was 250 times less than that required to agglutinate rabbit erythrocytes (Dorai et al., 1981a). It is well established that horse erythrocytes have haematoside, a derivative of GM₁, ganglioside containing NeuGc [NeuGca(2→3)-Galβ(1→4)-Glcβ(1→1)ceramide], as the major sialoglycoconjugate. Similar results were obtained by others with limulin also (Maget-Dana et al., 1981).

The trisaccharide neuraminyl-lactose [NeuAcα(2→3) and NeuAcα(2→6)-Galβ(1→4)-Glc] was 20-fold more effective than NeuAc in inhibiting the binding interaction, but had 3-fold less inhibitory capacity when compared with NeuGc. Nevertheless, it was a very weak competitor (35-fold less) when compared with the disaccharide O-(N-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxygalactitol. This was reported previously with respect to its inability to quench intrinsic fluorescence of carcinoscorpin, unlike the disaccharide (Dorai et al., 1981a). Although one can presume this to be due to a higher affinity for NeuAc-GalNAc residues, as reported for limulin (Roche & Monsigny, 1979), it should be noted that the spatial configuration of the glycan moiety of glycoproteins can also play a role in determining the efficient binding of lectins. Greater affinity for sialic acid bound in the α(2→6) rather than α(2→3) linkage is possible because of the high rotational freedom of α(2→6) linkages (Montreuil & Vliegenthart, 1979). This might be one of the possible explanations for the low affinity of the commercial sample of the trisaccharide, which contains 85–90% of α(2→3)-linked NeuAc isomer, the rest being α(2→6) isomer. Since it was observed that carcinoscorpin has greater affinity for NeuGc than for NeuAc, the higher binding capacity to α(2→3)-linked NeuGc of the haematoside cannot eliminate the possible role of linkages in determining the binding capacity. This gains importance from the observation that colonic acid, a linear polysaccharide of sialic acid linked α(2→8), was unable to inhibit precipitation or agglutination by carcinoscorpin even at a concentration higher than 1.4 mg/150 μl. In this regard, our observation that modified fetuin, with a sialic acid content twice that of serotransferrin, could only bind less than that of serotransferrin at its equivalence point may be related to the high content of terminal α(2→3)-NeuAc in modified fetuin. However, detailed experiments of inhibition with a specific disaccharide containing α(2→3)-NeuAc will have to be carried out to clarify their role.

D-Glucuronic acid, but not D-galacturonic acid, was a good inhibitor of both the precipitation and agglutination by carcinoscorpin, which was also reported previously in terms of its capacity for eluting bound ligands from a column of immobilized carcinoscorpin (Dorai et al., 1981b). Similar binding ability for limulin has been reported (Vaith et al., 1979; Nowak & Barondes, 1975), but the mechanism of the specific inhibition is unknown. The defence mechanism of invertebrates such as this horseshoe crab is directed against various microorganisms, including Gram-positive and Gram-negative bacteria, which contain acidic sugars such as glucuronic acid and 3-deoxy-2-oxo-octanate in their lipopolysaccharides. Hence the observations of specific inhibition by 3-deoxy-2-oxo-octonate, which is 2-fold greater in comparison with free NeuAc, suggests a multispecific binding nature of carcinoscorpin, which could be equated with the biological necessity for carcinoscorpin in the haemolymph of the horseshoe crab. Detailed studies regarding interaction of carcinoscorpin and lipopolysaccharides have been worked out in our laboratory (Dorai et al., 1981c). The possibility that carcinoscorpin has any binding sites for phosphocholine, as previously reported for limulin (Robey & Liu, 1981), should also be investigated, although preliminary studies reported in the present paper on the effect of phosphocholine on the binding of fetuin to carcinoscorpin suggests that the binding site may be different from that for sialic acid.

Although information about the interaction of carcinoscorpin with sialoglycoproteins and the use of immobilized carcinoscorpin for resolution of glycoproteins with respect to their heterogeneity in sialic acid content has been published (Dorai et al., 1981b; Mohan et al., 1981), a detailed investigation of the interaction of carcinoscorpin with various sialoglycoproteins has not yet been carried out. From such studies, information about its exact specificity and binding parameters could be obtained. This was important, since, unlike other carbohydrate-specific proteins of plant origin, this lectin is from an invertebrate species and might have important biological implications in the haemolymph. This gains importance also in the light of various reports about the broad-spectrum agglutinins of snails and some proposed biological functions for limulin (Uhlenbruck & Steinhausen, 1972; Hammarström, 1974; Robey & Liu, 1981).

Our studies with model systems emphasize the fact that not only the sialic acid content but also its topography on glycoproteins determine the mode and extent of interaction of carcinoscorpin with its ligands. The phenomenon of "positive co-operativity", in which the binding of one ligand molecule to one site increases the affinity for the ligand of the neighbouring unoccupied sites, in the early phase of binding of a number of multivalent lectins to the cell surface has been reported (Thom et al., 1979). The co-operotive and post-co-operative binding of car-
Carcinoscorpin to a total of eight residues of ligand (hitherto unreported for any lectin) is possibly accompanied by an alteration in the structural architecture with respect to the monomers of a big molecule such as carcoscorpin (mol.wt. 420000), leading to a heterogeneous type of interaction with its ligands, with no single dissociation-constant value. This, as well as multispecific binding of carcoscorpin, suggests a prominent biological role for this lectin in the horseshoe crab.

We are very grateful to Dr. H. Debray for his generous gift of human serotransferrin, lactotransferrin and α,-acid glycoprotein. This work was partly supported by the Department of Science and Technology, India. We thank Prasanta Chakraborty for his skilful technical assistance. S. M. is a Senior Research Fellow, S. S. is a Junior Research Fellow and D. T. D. is a Research Associate of the Council of Scientific and Industrial Research, India.

References