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

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Interaction of the sialic acid-specific lectin carcinoscorpin with various sialoglycoproteins 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 0.02 M. 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 proteins, called lectins, have been purified from different 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 interactions (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 glycoproteins 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 haemolymph of the marine horseshoe crab, *Limulus polyphemus*. A sialic acid-binding lectin, carcinoscorpin, has been detected and purified to homogeneity from the haemolymph of the marine horseshoe 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*-acetylneuraminic acid, *N*-glycolylneuraminic acid, 3-deoxy-2-oxo-octonate, chitobiose, colominic acid, D-glucuronic acid, D-galacturonic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine, phosphocholine (Ca^{2+} salt) and *p*-nitrophenyl fucoside were purchased from Sigma Chemical Co., St. Louis,

Abbreviations used: GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid.

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 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.01 M- CaCl_2 , was stored at a protein concentration less than 2 mg/ml, in small batches at -20°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 30 μl , 11.4 μg of purified carcinoscorpin (dialysed against 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl to remove Ca^{2+}) was treated at room temperature with 0.53 mCi of Na^{125}I and 6 μg of chloramine-T in 0.25 M-sodium phosphate buffer, pH 7.5. After 30 s, the reaction was terminated by adding 5 μl of sodium metabisulphite (10 mg/ml). At 1 min after the addition of sodium metabisulphite, 5 μl of KI (166 mg/ml) and 20 μl of desialylated bovine serum albumin (20 mg/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 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, for 2 h, followed by dialysis for 1 h against 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.01 M- CaCl_2 . For further separation of ^{125}I -labelled carcinoscorpin, the non-diffusible material was then loaded on to a fetuin-Sepharose column (1 ml) (Dorai *et al.*, 1981b) previously equilibrated with 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, 0.01 M- CaCl_2 and 2 mg 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 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, 0.04 M-sodium citrate and 2 mg of desialylated albumin/ml.

The eluate was dialysed thoroughly against two changes of 0.002 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, and then once against the same buffer containing 0.01 M- CaCl_2 also. ^{125}I -labelled carcinoscorpin had a specific radioactivity of 2×10^4 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 (22 cm $\times$ 0.8 cm) previously equilibrated with equilibrium buffer. Fractions (0.5 ml) were collected and the radioactivity was measured. The fractions near the void volume (3 ml), which contained the first peak of high radioactivity, were pooled. ^{125}I -labelled fetuin had a specific radioactivity of 1.6×10^4 c.p.m./ng and that for ^{125}I -labelled serotransferrin was 1.7×10^4 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*-acetylneuraminy)-(2 $\rightarrow$ 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 sulphoxide method consists of incubation of 50 mg of fetuin dissolved in 5 ml of dimethyl sulphoxide/water/ethanol (5:4:1, by vol.) containing 0.17 M-KOH for 60 min at 45°C . The resulting solution was neutralized with 2 M-HCl and then dialysed for 15 h with three changes against water and then later against 0.002 M-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 0.05 M- H_2SO_4 at 80°C for 1 h.

Binding assay

This experiment is based on the observation that carcinoscorpion 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 × 0.5 cm) in a final volume of 50 μ l 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 carcinoscorpion, the incubation system contained 0.05 nmol of unlabelled carcinoscorpion, a fixed amount of ¹²⁵I-labelled carcinoscorpion corresponding to 2 × 10⁴ c.p.m. (negligible amount of carcinoscorpion 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 μ m 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 carcinoscorpion, 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 carcinoscorpion binds to the high-molecular-weight ligands and is directly related to the amount of carcinoscorpion (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 carcinoscorpion (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 carcinoscorpion corresponding to 2 × 10⁴ c.p.m. and 0.4 nmol of fetuin (which was the amount that corresponded to maximal binding of carcinoscorpion 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 carcinoscorpion 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 carcinoscorpion 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 carcinoscorpion that bound to fetuin. In each case, the incubation mixture with fetuin was allowed to attain the required temperature and then carcinoscorpion, 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 carcinoscorpion, a fixed amount of ¹²⁵I-labelled carcinoscorpion corresponding to 2 × 10⁴ 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 carcinoscorpion.

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 carcinoscorpion 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 carcinoscorpion

The results are plotted in Fig. 1 as percentage of carcinoscorpion 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 carcinoscorpion (0.05 nmol) and time of incubation (60 min) were fixed on the basis of our previous studies on binding of carcinoscorpion 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 & Bhoyroo, 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 carcinoscorpion bound was proportional to the sialic acid content. Each glycoprotein differed in the amount of carcinoscorpion that was bound maximally at the equivalence point. Modified fetuin, which has 40% less sialic acid content than fetuin, precipitated 40% less carcinoscorpion than fetuin. Sheep submaxillary mucin, even with its high content of sialic acid, had a different profile of binding to carcinoscorpion (Fig. 1b). The titration data are summarized in Table 1. Ratios of sialic acid content to the percentages of carcinoscorpion 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

Fig. 1. *Titration of various sialoglycoproteins with carcinoscorpion*

Binding was performed at room temperature in a total volume of 50 μ l containing 0.05 M-Tris/HCl buffer, pH 8.0, 0.1 M-NaCl, 0.01 M-CaCl₂, desialylated albumin (1 mg/ml), 0.05 nmol of unlabelled carcinoscorpion, ¹²⁵I-labelled carcinoscorpion corresponding to 2×10^4 c.p.m. and different amounts of each of the sialoglycoproteins. Other conditions were as described in the text. Proportion of carcinoscorpion bound (%) = $100 \times [\text{radioactivity in the precipitate (c.p.m.)} / \text{total radioactivity added (c.p.m.)}]$. (a) Symbols: ■, α_1 -acid glycoprotein; □, fetuin; △, lactotransferrin; ●, modified fetuin; ○, serotransferrin. (b) shows the results for sheep submaxillary mucin.

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 carcinoscorpion 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 carcinoscorpion 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-

Table 1. *Structural features of the carbohydrate portions of the sialoglycoproteins and their relation to carcinoscorpin 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.

Glycoprotein	Type of linkage of the oligosaccharide chains	No. and form of oligosaccharide chains per molecule	Amount of carcinoscorpin bound (nmol)	Ratio of sialic acid content to percentage of carcinoscorpin bound	Ratio of sialic acid content per oligosaccharide chain to percentage of carcinoscorpin bound
Fetuin	<i>N</i> - and <i>O</i> -glycosidic	6; tri- and bi-antennary	0.04	8	2
α_1 -Acid glycoprotein	<i>N</i> -Glycosidic	5; bi-, tri- or tetra-antennary	0.044	9	2
Modified fetuin*	<i>N</i> -Glycosidic	3; tri-antennary	0.023	9	3
Serotransferrin	<i>N</i> -Glycosidic	2; bi-antennary	0.025	11	6
Lactotransferrin	<i>N</i> -Glycosidic	2; bi-antennary	0.048	7	3
Sheep submaxillary mucin	<i>O</i> -Glycosidic	>800; linear	0.042	68	0.1

* 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 carcinoscorpin with fetuin and modified fetuin might differentiate carcinoscorpin 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 carcinoscorpin bound.

Scatchard plots

Figs. 2 and 3 give the binding profiles of radioiodinated sialoglycoproteins with unlabelled carcinoscorpin 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 carcinoscorpin. The binding of fetuin at room temperature is represented by the unusual curvilinear plot (Fig. 2a) consisting of a concave-down initial region followed by linear regions with different slopes. Fig. 3 shows the Scatchard plot obtained for the binding of carcinoscorpin 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 carcinoscorpin follows a phenomenon of

Fig. 2. *Scatchard plot of the binding of fetuin to carcinoscorpin*

The binding system contained ^{125}I -labelled fetuin (2×10^4 c.p.m.) with various amounts of unlabelled fetuin in a total volume of $50 \mu\text{l}$ of incubation system similar to that given in the legend to Fig. 1. Other conditions were as described in the text. $\bar{N} = [\text{bound fetuin}] (\mu\text{M})/[\text{carcinoscorpin}] (\mu\text{M})$; $L = [\text{free fetuin}] (\mu\text{M})$. (a) Incubation at room temperature; (b) incubation at 4°C .

positive co-operativity (Scatchard, 1949). The linear profiles with different slopes again suggest different classes of binding sites of carcinoscorpin; one class

Fig. 3. Scatchard plot of the binding of serotransferrin to concanavalin A

The binding system contained ^{125}I -labelled serotransferrin (2×10^4 c.p.m.) with various amounts of unlabelled serotransferrin in a total volume of $50 \mu\text{l}$ of incubation system similar to that given in the legend to Fig. 1. Other conditions were as given in the text. $\bar{N} = [\text{bound serotransferrin}]$ (μM)/[concanavalin A] (μM); $L = [\text{free serotransferrin}]$ (μM). (a) Incubation at room temperature; (b) incubation at 4°C .

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 concanavalin A 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 concanavalin A 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 concanavalin A with fetuin at pH 4.5–9.0 was studied. There was a slow increase in the amount of concanavalin A 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 concanavalin A bound was only 8–10% higher than that at pH 8.0. The same pH profile was also obtained when serotransferrin–concanavalin A 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 concanavalin A with various sialoglycoproteins and also the extent of concanavalin A 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 concanavalin A is not temperature-dependent.

Effect of metal ions

(a) Ca^{2+} . There is an absolute requirement of Ca^{2+} for the binding activity of limulin (Marchalonis & Edelman, 1968; Nowak & Barondes, 1975). It is known from circular-dichroism studies that Ca^{2+} induces a partial conformation change in limulin, which seems to be a prerequisite for its binding activity (Roche *et al.*, 1978). Concanavalin A is also dependent on Ca^{2+} for its activity (Dorai *et al.*, 1981b). Our studies on the concentration of Ca^{2+} (0.005–0.030 M) required for maximum binding activity of concanavalin A with fetuin showed an optimal value of 0.02 M, although there was no appreciable difference in binding activity at 0.01 M.

(b) *Other metal ions*. To date, the metal-binding sites of limulin and concanavalin A 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 Mg^{2+} , Mn^{2+} , Ba^{2+} and Sr^{2+} were used in place of Ca^{2+} . Concanavalin A dialysed against neutral 0.01 M-EDTA solution in buffer and salt had no binding activity. The original activity was restored with 0.02 M- CaCl_2 . Since EDTA is known to be a metal-chelating agent, the observation that the original binding activity was regained by the addition of 0.02 M- Ca^{2+} suggests the possibility that concanavalin A depends solely on Ca^{2+} for its

activity. The normal binding activity could not be restored in the EDTA-dialysed carcinoscorpion by addition (0–0.02 M each) of Mg^{2+} , Mn^{2+} , Ba^{2+} and Sr^{2+} , demonstrating the incapability of these bivalent cations to substitute for Ca^{2+} . We also observed that carcinoscorpion dialysed against Ca^{2+} -free buffer possessed binding activity, but less by 40% of that of the original carcinoscorpion (undialysed), and this was restored by 0.01 M- Ca^{2+} . Although Mg^{2+} and Ba^{2+} did not affect this activity even at 0.02 M, Mn^{2+} and Sr^{2+} abolished it, implicating the presence of endogenous metal sites that were deleteriously affected by Mn^{2+} and Sr^{2+} . More investigations are required to arrive at the exact role of bivalent metal ions in the binding activity of carcinoscorpion.

Inhibition of binding

Table 2 summarizes the inhibition studies on both the binding of carcinoscorpion to fetuin and haemagglutination by carcinoscorpion. The concentration of each inhibitor required for inhibition of carcinoscorpion–fetuin complex-formation or haemagglutination reflects the affinity of carcinoscorpion

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 carcinoscorpion–fetuin complex-formation. The specificity of carcinoscorpion towards sialic acid was concluded from its inability to bind and precipitate asialofetuin. Also, asialofetuin, even at a high concentration of 350 μ g/150 μ l, did not inhibit the haemagglutination. The non-inhibitory effects of *N*-acetylhexosamines differentiate carcinoscorpion 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–carcinoscorpion interaction and a low inhibitor of agglutination by carcinoscorpion. 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–carcinoscorpion interaction was studied

Table 2. *Inhibitory effect of simple and complex saccharides and glycoproteins on binding activity of carcinoscorpion*
The values for '50% inhibition' were obtained from studies on inhibition of carcinoscorpion–fetuin interaction. The values for inhibition of one haemagglutination dose were obtained from studies on inhibition of agglutination of rabbit erythrocytes by carcinoscorpion. Abbreviations used: KDO, 3-deoxy-2-oxo-octonate; disaccharide, *O*-(*N*-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxy-D-galactitol; trisaccharide, *N*-acetylneuraminyl(2→3)- or (2→6)- β -D-galactopyranosyl(1→4)-D-glucopyranose; ND, not determined.

Compounds	Concn. needed for 50% inhibition (mM)	Minimum concn. needed for inhibition of one haemagglutination dose (mM)
NeuAc	>20	>1
NeuGc	0.4	>0.5
KDO	>10	0.84
<i>O</i> -Phosphoethanolamine	>50	>8
Phosphocholine	>50	>10
D-Glucose	>100	>9
α -L-Fucose	>70	>8
<i>p</i> -Nitrophenyl fucoside	>30	2.5
GalNAc	>80	8
GlcNAc	>50	8
D-Glucuronic acid	11	0.8
D-Galacturonic acid	>80	>8
Disaccharide	0.035	0.002
Trisaccharide	1.1	0.12
Chitobiose	>80	>6.5
Colominic acid	>70*	>0.34†
Asialofetuin	ND	>0.012
Fetuin	ND	0.0002
α_1 -Acid glycoprotein	ND	0.0002
Sheep submaxillary mucin	ND	0.00002
Serotransferrin	ND	0.0003
Lactotransferrin	ND	0.0005

* In μ g/0.05 ml.

† In mg/0.15 ml.

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 G_M₂ ganglioside containing NeuGc [NeuGc α (2 $\rightarrow$ 3)-Gal β (1 $\rightarrow$ 4)-Glc β (1 $\rightarrow$ 1)ceramide], as the major sialoglycoconjugate. Similar results were obtained by others with limulin also (Maget-Dana *et al.*, 1981).

The trisaccharide neuraminyl-lactose [NeuAc α (2 $\rightarrow$ 3)- and NeuAc α (2 $\rightarrow$ 6)-Gal β (1 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 6) rather than α (2 $\rightarrow$ 3) linkage is possible because of the high rotational freedom of α (2 $\rightarrow$ 6) linkages (Montreuil & Vliegthart, 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 $\rightarrow$ 3)-linked NeuAc isomer, the rest being α (2 $\rightarrow$ 6) isomer. Since it was observed that carcinoscorpin has greater affinity for NeuGc than for NeuAc, the higher binding capacity to α (2 $\rightarrow$ 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 colominic acid, a linear polysaccharide of sialic acid linked α (2 $\rightarrow$ 8), was unable to inhibit precipitation or agglutination by carcinoscorpin even at a concentration higher than 1.4 mg/150 μ l. 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 $\rightarrow$ 3)-NeuAc in modified fetuin. However, detailed experiments of inhibition with a specific disaccharide containing α (2 $\rightarrow$ 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-octonate 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 & Steinhansen, 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-operative and post-co-operative binding of car-

cinoscorpin 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 carcinoscorpin (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 carcinoscorpin, suggests a prominent biological role for this lectin in the horseshoe crab.

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References

- Baenziger, J. U. & Fiete, D. (1979) *J. Biol. Chem.* **254**, 789–795
- Bertolini, M. & Pigman, W. (1970) *Carbohydr. Res.* **14**, 53–63
- Bishayee, S. & Dorai, D. T. (1980) *Biochim. Biophys. Acta* **623**, 89–97
- Cohen, E., Rowe, A. W. & Wissler, F. C. (1965) *Life Sci.* **4**, 2009–2016
- Debanne, M. T., Regoeczi, E. & Halton, M. W. C. (1980) *Can. J. Biochem.* **58**, 1414–1420
- Debray, H., Decout, D., Strecker, G., Spik, G. & Montreuil, J. (1981) *Eur. J. Biochem.* **117**, 41–55
- Dorai, D. T., Bachhawat, B. K., Bishayee, S., Kannan, K. & Rao, D. R. (1981a) *Arch. Biochem. Biophys.* **209**, 325–333
- Dorai, D. T., Bachhawat, B. K. & Bishayee, S. (1981b) *Anal. Biochem.* **115**, 130–137
- Dorai, D. T., Srimal, S., Mohan, S., Bachhawat, B. K. & Balganes, T. S. (1981c) *Biochem. Biophys. Res. Commun.* in the press
- Downs, F., Herp, A., Moschera, J. & Pigman, W. (1973) *Biochem. Biophys. Acta* **328**, 182–192
- Dulaney, J. T. (1979) *Mol. Cell. Biochem.* **21**, 43–63
- Gambos, G., Morgan, I. G., Waenholdt, T. V., Vincendon, G. & Breckenridge, W. C. (1971) *Adv. Exp. Med. Biol.* **25**, 101–113
- Goldstein, I. J. & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 127–340
- Hammarström, S. (1974) *Ann. N.Y. Acad. Sci.* **234**, 183–197
- Hunter, W. M. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 608–642, F. A. Davis Co., Philadelphia
- Lis, H. & Sharon, N. (1977) in *The Antigens* (Sela, M., ed.), vol. 4, pp. 429–529, Academic Press, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maget-Dana, R., Veh, W. R., Sander, M., Roche, A.-C., Schauer, R. & Monsigny, M. (1981) *Eur. J. Biochem.* **114**, 11–16
- Mangum, C. P. & Schick, J. M. (1972) *Comp. Biochem. Physiol.* **42A**, 693–697
- Marchalonis, J. J. & Edelman, G. M. (1968) *J. Mol. Biol.* **32**, 453–465
- Matsumoto, I. & Osawa, T. (1970) *Arch. Biochem. Biophys.* **140**, 484–491
- Mohan, S., Bishayee, S. & Bachhawat, B. K. (1981) *Indian J. Biochem. Biophys.* **18**, 177–181
- Montreuil, J. & Vliengenthart, J. F. G. (1979) in *Proceedings of the 4th International Symposium on Glycoconjugates* (Gregory, J. D. & Jeanloz, R. W., eds.), pp. 35–78, Academic Press, New York
- Murphy, W. H. & Gottschalk, A. (1961) *Biochim. Biophys. Acta* **52**, 349–360
- Murthy, V. L. N. & Horowitz, M. I. (1968) *Carbohydr. Res.* **6**, 266–275
- Noguchi, H. (1903) *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Abt. 1* **34**, 286–288
- Nowak, T. P. & Barondes, S. H. (1975) *Biochim. Biophys. Acta* **393**, 115–123
- Ochoa, J. L., Kristiansen, T. & Pahlman, S. (1979) *Biochim. Biophys. Acta* **577**, 102–109
- Robey, F. A. & Liu, T.-Y. (1981) *J. Biol. Chem.* **256**, 969–975
- Roche, A.-C. & Monsigny, M. (1979) in *Biomedical Applications of the Horseshoe Crab (Limulidae)* (Cohen, E., ed.), pp. 603–616, Alan R. Liss, New York
- Roche, A.-C., Schauer, R. & Monsigny, M. (1975) *FEBS Lett.* **57**, 245–249
- Roche, A.-C., Maurizot, J. C. & Monsigny, M. (1978) *FEBS Lett.* **91**, 233–236
- Saifer, A. & Gerstenfeld, S. (1962) *Clin. Chim. Acta* **7**, 467–475
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–667
- Shimizu, S., Ito, M. & Niwa, M. (1977) *Biochim. Biophys. Acta* **500**, 71–79
- Spiro, R. G. & Bhoyroo, V. D. (1974) *J. Biol. Chem.* **249**, 5704–5717
- Thom, D., Cox, S. D., Safford, R. & Rees, A. D. (1979) *J. Cell Sci.* **39**, 117–136
- Uhlenbruck, G. & Steinhansen, G. (1972) *Blut* **25**, 335–337
- Vaith, P., Uhlenbruck, G. & Holz, G. (1979) *Protides Biol. Fluids Proc. Colloq.* **27**, 455–458
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975