ISOLATION AND SOME PROPERTIES OF MAMMALIAN HEPATIC MEMBRANE
LECTINS

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Key words: Asialoglycoprotein receptors, Mammalian liver, SDS-PAGE.

Membrane lectins were isolated from sheep, goat and buffalo liver by chromatography on asialofetuin (ASF)-Sepharose 4B column. The lectins moved as single protein band in SDS-PAGE with M of 42, 54 and 50 kDa, respectively, for sheep, goat and buffalo lectins. M remained unchanged in 0.2 M 2-mercaptoethanol. As judged from inhibition of the binding of lectin to ASF-gel, the three lectins were β-galactoside specific. Sheep, goat and buffalo lectins were found to be sialglycoproteins containing 18.6, 27 and 38.8 moles of neutral hexose, respectively; the corresponding values of sialic acid contents being 5.3, 8.7 and 11.8 moles per mole of lectin. Thus, goat and buffalo lectins are physico-chemically different from many mammalian hepatic lectins described so far.

Mammalian hepatic membrane lectins appear to serve as asialoglycoprotein receptors (1,2) in the hepatic uptake of circulatory asialoglycoproteins prior to their catabolism. They have also been implicated in exocytosis of endocyted asialglycoproteins (3) and possibly in specific cellular interaction (4). Available data on membrane hepatic lectins show that the lectins from different animals differ significantly in physico-chemical properties (4-7). Further, hepatic membrane lectins from the same animal may exhibit multiple activities indicating more than one type of lectins (8-10). Studies on hepatic lectins, carried out thus far, are primarily confined to rabbit, rat, and human livers (1, 2) and probable occurrence of lectins in other mammalian livers
and their molecular and functional properties are yet to be investigated. We, therefore, report our results on the isolation and characterization of three uninvestigated hepatic lectins i.e. goat, sheep, and buffalo liver lectins. Strikingly goat and buffalo hepatic lectins were found to be significantly different in molecular morphology from those hitherto described hepatic lectins.

Materials and Methods

Proteins, enzymes, Sepharose 4B, acrylamide, 2-mercaptoethanol, phenylmethanesulfonyl fluoride and saccharides were purchased from Sigma Chemical Co. St. Louis Mo. U.S.A. Other reagents used were of analytical grade. Protein concentration was measured by modified Lowry's method (5). Neutral hexose and sialic acid contents of lectins were determined by the methods of Dubois et al., (11) and Warren (12), respectively, using Sigma D(+) galactose and N-acetyleneuraminic acid as standards. SDS-PAGE was carried out according to Laemmli (13).

Preparation of affinity media:

Sepharose 4B gel slurry (60 ml) activated with 18 g of solid cyanogen bromide according to cuatrescasas and Anfinsen (14) was treated with fetuin (130 mg) in the coupling buffer (0.2 M sodium carbonate buffer pH 9.2 containing 0.15 M NaCl) and the bound fetuin was desialylated with 0.7 mg neuraminidase in 0.1 M sodium acetate buffer pH 5.6 at 37°C for 4 hours. From the amount of sialic acid liberated, it was estimated that about 1.3 mg of fetuin bound per ml settled gel.
Measurement of lectin activity:

The lectin activity was assayed by measuring the interaction of lectin with ASF-gel slurry. Lectin (130 μg in 1 ml) was incubated with 1 ml gel slurry in 10 mM tris-HCl buffer pH 7.5 containing 0.5% triton X-100, 0.5 M NaCl, 20 mM CaCl₂ and 1% BSA for one hour at 4°C and the bound protein was eluted with 50 mM ammonium acetate buffer pH 5.1 containing 0.5 M NaCl and 0.5% triton X-100.

Isolation of membrane lectins:

Acetone powder obtained from fresh liver homogenate prepared in 10 mM tris-HCl buffer pH 7.5 containing 0.15 M NaCl, was solubilized in 10 mM tris-HCl buffer pH 7.5 containing 0.5 M NaCl, 0.4 M KCl, 60 mM CaCl₂, 1% triton X-100 and 0.2 mM PMSF. The supernatant (450 ml containing 1 g of protein) was loaded on the affinity column equilibrated with the same buffer. The bound protein was eluted with 50 mM ammonium acetate buffer pH 5.1 containing 0.5 M NaCl and 0.5% triton X-100. The protein fractions showing lactose inhibitable binding to ASF-Sepharose were rechromatographed on the affinity column.

Results and Discussion:

About 1 mg purified lectin was obtained from 25 g acetone powder of goat, sheep, and buffalo liver by chromatography on asialofetuin Sepharose 4B column (see Fig.1). The elution profiles for sheep and buffalo lectins were similar and hence omitted for brevity. Similar yield was reported for hepatic lectins from human (4) and rat liver (15). The three purified lectins were essentially free from extraneous proteins and moved as a single protein band in SDS-PAGE (see inset of Fig.2). The
Elution volume (ml)

Absorbance at 200 nm

Fig. 1: Isolation of goat hepatic lectin by chromatography on asialofetuin Sepharose 4B column. One gram protein in 450 ml of 10 mM tris-HCl buffer pH 7.5 containing 0.5 M NaCl, 0.4 M KCl, 60 mM CaCl$_2$, 1% triton X-100 and 0.2 mM PMSF was applied on ASF-Sepharose 4B column (2.5 x 12 cm) equilibrated with the same buffer and the bound protein was eluted with 50 mM ammonium acetate buffer pH 5.1 containing 0.5 M NaCl and 0.5% triton X-100 in 5 ml fractions at a flow rate of 7 ml per hour. The protein fractions were pooled and the pH was raised to 7.5 with 1 M tris solution. After adding 60 mM CaCl$_2$, the protein solution showing lactose inhibitable binding to ASF-Sepharose were rechromatographed on the affinity column equilibrated with 10 mM tris-HCl buffer pH 7.5 containing 0.5 M NaCl, 0.5% triton X-100 and 60 mM CaCl$_2$. The inset shows the elution profile obtained on rechromatography of hepatic lectin on the same column.

Relative mobilities of sheep, goat, and buffalo lectins were 0.42, 0.33, and 0.34, respectively, which according to the calibration curve, depicted in Fig. 2, would correspond to $M_r$ of 42, 54, and 50 kDa (see Table I). These values remained unaltered in the presence of 0.2 M 2-mercaptoethanol, suggesting absence of interpolypeptide cross-links in SDS denatured lectins. The experimental uncertainty in the measurement of molecular weight by SDS-PAGE was within 10%. Thus, $M_r$ of sheep lectin is significantly lower than those found for goat and buffalo lectins.
Fig. 2: Plot of $R_m$ values of marker proteins versus logarithm of molecular weight. The marker proteins were 1. bovine serum albumin (mol. wt. 68 kDa), 2. heavy chain of IgG (mol.wt. 50 kDa), 3. Ovalbumin (mol.wt. 43 kDa), 4. pepsin (mol.wt. 35 kDa), 5. chymotrypsinogen (mol.wt. 25.5 kDa), and 6. light chain of IgG (mol. wt. 11.7 kDa). Inset shows electrophorograms of hepatic membrane lectins of sheep (A, A'), goat (B, B') and buffalo (C, C'), respectively, under reducing (primed letter) and nonreducing (unprimed letter) conditions. Thirty micrograms each of the three lectins were electrophoresed in 25 mM Tris-192 mM glycine buffer pH 8.3 containing 0.1% SDS for about 3 hours. The slab gel was stained with 1% coomassie brilliant blue R-250 and destained mechanically with 7% acetic acid.

The sialoglycoprotein nature of the three lectins was studied by measuring neutral hexose and sialic acid contents of hepatic lectins and the results are summarized in Table I. The carbohydrate (hexose and sialic acid) contents of goat and buffalo lectins are markedly higher than those of sheep lectin.

The carbohydrate binding specificity of sheep, goat and buffalo hepatic lectins was studied by ascertaining the capacity of simple sugars to inhibit binding of the lectins to ASF-gel. As can be seen in Table II the three lectins appear to be galactose specific.
TABLE - II: Effect of simple sugars on the binding of mammalian hepatic membrane lectins to ASF-Sepharose gel.

<table>
<thead>
<tr>
<th>SUGAR</th>
<th>Relative Inhibitory activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheep</td>
</tr>
<tr>
<td>1. D(+) Lactose</td>
<td>1</td>
</tr>
<tr>
<td>2. D(+) Galactose</td>
<td>0.7</td>
</tr>
<tr>
<td>3. D(+) Fucose</td>
<td>0.03</td>
</tr>
<tr>
<td>4. D(+) Mannose</td>
<td>0.01</td>
</tr>
<tr>
<td>5. D(+) Glucose</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Relative Inhibitory activity is the ratio of $C_M$ for lactose to the $C_M$ for test sugar where $C_M$ is the concentration of the sugar required for 50% inhibition of the binding of lectin to ASF-gel. The values of $C_M$ for lactose were 12, 15 and 15 mM for sheep, goat and buffalo lectins, respectively.

From the results presented in Tables I and II, it is clear that although the three mammalian hepatic lectins are identical, within experimental error, in carbohydrate binding specificity, the goat and buffalo lectins are markedly different from sheep lectin in physico-chemical properties. Interestingly, sheep lectin is similar to human (4) and to major rat (6) liver lectins in molecular weight and in carbohydrate binding properties. Furthermore, carbohydrate content (21.4 moles) of human lectin is similar to that (23.9 moles, see Table I) determined for sheep lectin in this study.

Acknowledgements:

Thanks are due to the Council of Scientific and Industrial Research, New Delhi, for research grant and to Aligarh Muslim University for facilities.
References:


ISOLATION AND CHARACTERIZATION OF SOLUBLE $\beta$-GALACTOSIDE BINDING LECTINS FROM MAMMALLIAN LIVER.

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Key words: $\beta$-galactoside binding lectins, hepatic lectins, characterization

Soluble $\beta$-galactoside binding lectins were isolated by chromatography on asialofetuin-Sepharose 4B column in 10 mM tris-HCl buffer pH 7.5 containing 150 mM NaCl, 5 mM CaCl$_2$ and 1 mM 2-mercaptoethanol. The three lectins moved essentially as single polypeptide band with $M_r$ of 18, 22 and 24 kDa, respectively, for sheep, goat and buffalo hepatic lectins. Sheep and goat lectins each contained 4 moles of hexose whereas the hexose content of the buffalo lectin was 7 moles. The number of sulfhydryl groups in sheep, goat and buffalo lectins were determined to be 3.2, 4.3 and 4.8, respectively. Optical properties of the three lectins were similar to those of tryptophan containing proteins. Lectin mediated hemagglutination of trypsinized rabbit erythrocytes was most effectively inhibited by lactose followed by 0-nitrophenyl-\(\beta\)-galactopyranoside and galactose but remained unaffected by glucose, mannose, fucose and fructose. Calcium ion substantially enhanced their hemagglutinating activity. Goat and buffalo lectins but not sheep lectins, were also stimulated by Mg$^{2+}$, Mn$^{2+}$, Sr$^{2+}$ and Ni$^{2+}$ ions. The lectins lost activity after treatment with parahydroxymercuribenzoate and N-ethyl maleimide. However, iodoacetamide the three lectins are different from the soluble $\beta$-galactoside binding lectins studied thus far.

Vertebrate tissues contain soluble lectins that can be isolated by affinity chromatography on asialofetuin (ASF)- or lactosyl-Sepharose-4B column (1,2). They are solubilized from tissues with aqueous buffer and are $\beta$-galactoside specific (1,3). The major $\beta$-galactoside binding lectin occurring in vertebrate tissues is a dimer with subunit $M_r$ of about 14 kDa and requires thiol but not divalent metal ions for its hemagglutinating activity (1). The dimeric lectins, from as distinct species as the electric eel, the chicken, rat and humans possess similar properties and exhibit considerable sequence homology especially in the putative saccharide binding site region comprising residues 70-76 which, among others, include one Trp, one Arg and two Glu residues (4,5).
Besides 14 kDa lectin, other β-galactoside binding lectins have been found in mammalian tissues especially human (HL) and rat (RL) lungs (1,2,6,7). These include prominent monomeric lectins (2,7) with molecular weight of 29 kDa, 18 kDa (in rat lung) and 22 kDa (in human lung). Sparrow et al., (2) have shown that HL-29 and RL-29 as well as HL-22 and RL-18 differ significantly in carbohydrate binding specificity or immunological reactivity. Further, affinity purified HL-14 and HL-29 could be resolved into at least 5-6 acidic forms (2). Thus available data (1-3) show that multiple forms of β-galactoside binding lectins may occur in mammalian tissues. It would be interesting to find out their tissues and species specificity. Thus far studies have been focused primarily on the soluble lectins from lung tissues and very little information exists on such lectins from other mammalian tissues.

This paper describes, for the first time, isolation and characterization of soluble hepatic β-galactoside binding lectins from sheep, goat and buffalo. Our results show that these soluble lectins differ significantly from hitherto reported β-galactoside binding lectins from mammalian tissues.

Materials and Methods.

Proteins, sugar substrates, phenylmethylsulfonyl-fluoride (PMSF), parachloromercuribenzoate (which on solubilization with dilute alkali is converted into its hydroxy form, i.e. parahydroxymercuribenzoate (pHMB)); trypsin type II (lot No. T-8128), neuraminidase type V (lot No. 96C-8125), N-ethylmaleimide (NEM), Sepharose-4B were purchased from Sigma Chemical Company, St. Louis, Mo. U.S.A. One hundred microgram of trypsin when incubated with 10 mg casein in 2 ml of 10 mM sodium phosphate buffer pH 7.5 for 30 min at 37°C produced a change in absorbance (due to hydrolysis) at 700 nm of 0.52 optical density unit so that the specific activity of the enzyme was 10.4 OD unit/hour/mg;
the hydrolysed products were measured by the method of Lowry et al., (8). One milligram of neuraminidase caused release of 2.25 micromoles of sialic acid from 1 mg of fetuin in 100 mM sodium acetate buffer pH 5.6 in one hour and at 37°C. Reagents used in sodium dodecyl sulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) were the same as reported earlier (9). Other chemicals were of reagent grade and were used without further purification. Alpha forms of lactose and galactose were converted into their β-anomers as recommended by Hudson and Yonovsky (10). Trypsinized rabbit erythrocytes were obtained by treating cells with trypsin (10 mg per ml packed cells) for 10 min. at 37°C. Cells were washed ten times by suspending them in 150 mM NaCl and pelleting down by centrifugation at 3000 rpm for 5 min at a field of 700 RCF. Pelleted cells were then suspended in 2 ml of PBS pH 7.5 (10 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl). Fresh animal liver was obtained from Allgarh slaughter house.

Light absorption measurements in the ultra-violet region were made on Cecil UV Beam Spectrophotometer model CE 202 in conjunction with a recorder. Fluorescence measurements were made on Shimadzu spectrofluorometer model R 450 using a slit of 10 nm.

Protein concentration was determined either by the method of Lowry et al., (8) or by Bradford's method (11). Occasionally protein concentration was estimated spectrophotometrically by measuring optical densities at 260 nm \(A_{260}\) and 280 nm \(A_{280}\). The protein concentration in mg/ml was \((1.55 \times A_{280} - 0.77 \times A_{260})\). The hexose contents of the three lectins were determined according to Dubois et al., (12). SDS-PAGE was carried out in 12.5% gel as recommended by Laemmli (13). In order to prepare ASF-Sepharose, 60 ml of Sepharose-4B was activated according to Cuatrucasas and Anfinson (14) by addition of 20 g of solid cyanogen bromide, the pH was maintained to 10 with 4 M NaOH. The activated
gel slurry was then treated with 120 mg of fetuin in the coupling buffer (200 mM sodium carbonate buffer pH 9.2 containing 150 mM NaCl). After washing the fetuin-Sepharose-gel, the bound fetuin was desialylated with 0.7 mg of neuraminidase in 100 mM sodium acetate buffer pH 5.6 at 37°C for 4 hours.

Determination of hemagglutinating activity.

Hemagglutinating activity of hepatic lectin with trypsinized erythrocytes was measured by light scattering method at 610 nm. About 150-250 ug of a lectin in 2 ml of 10 mM tris-HCl buffer pH 7.5 containing 150 mM NaCl, 5 mM CaCl₂ and 1 mM 2-mercaptoethanol (assay buffer) was incubated with 3 ml of trypsinized erythrocytes (1x10⁶ cells) suspension in PBS pH 7.5 for 15 min. The clump thus formed was vigorously shaken in a tube and its absorbance at 610 nm was recorded with time; absorbance decreased with time. The absorbance at 5 min was routinely taken to measure the extent of hemagglutination reaction. A similar procedure was used in this laboratory to follow antigen-antibody precipitin reaction (15). The percent hemagglutinating activity of lectin was (A-A')/A X 100, where A and A' were the optical densities at 610 nm in the absence and presence of a lectin.

Isolation of soluble hepatic lectins.

Acetone powder of liver (25 g) prepared from 100 g of fresh liver of sheep, goat and buffalo according to Hudgin et al., (16) was solubilized in aqueous assay buffer pH 7.5 (see above) containing 0.2 mM PMSF. The extract was fractionated on asialofetuin-Sepharose-4B column (2.5x12 cm) equilibrated with the assay buffer without PMSF. The bound protein was specifically eluted with 0.5 M lactose. The final yield of soluble β-galactoside binding hepatic lectins was found to be in the range 2-3 mg from 100 g of fresh liver of the three species. The lectin was
rechromatographed on the affinity column and was used throughout this study.

Results and Discussion.

About 2-3 mg of soluble lectin was obtained from 100 g of sheep, goat and buffalo livers by affinity chromatography on ASF-Sepharose-4B column equilibrated with 10 mM tris-HCl buffer pH 7.5 containing, among others, 5 mM CaCl₂ and 1 mM 2-mercaptoethanol. The bound lectins were specifically eluted with 0.5 M lactose. The eluted soluble lectins were completely bound to the affinity column; their size homogeneity was tested by SDS-PAGE. The electrophoretogram of goat lectin is given in Fig.1; the SDS-PAGE patterns of sheep and buffalo lectins were similar although the relative mobilities were different. Results showed that the goat as well as the other two lectins were essentially homogeneous with respect to size. The values of relative mobilities, \( R_m \), of goat, sheep and buffalo lectins were found to be 0.64, 0.71 and 0.63,

<table>
<thead>
<tr>
<th>Property</th>
<th>Sheep</th>
<th>Goat</th>
<th>Buffalo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight, ( M ), kDa</td>
<td>18</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Neutral hexose contents, moles/mole of protein</td>
<td>4</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Absorption maximum in 10 mM tris-HCl buffer pH 7.5 containing 0.15 M NaCl and 1 mM 2-mercaptoethanol, nm</td>
<td>276</td>
<td>278</td>
<td>278</td>
</tr>
<tr>
<td>Emission maximum in 10 mM tris-HCl buffer pH 7.5 containing 0.15 M NaCl and 1 mM 2-mercaptoethanol, nm</td>
<td>340</td>
<td>340</td>
<td>338</td>
</tr>
</tbody>
</table>

15-PAGE of goat hepatic \( \beta \)-galactoside binding lectin. Lectin electrophoresed in 25 mM tris-192 mM glycine buffer pH 9 0.1% SDS with or without (a b) 0.2 M 2-mercaptoethanol gel ([13]).
respectively which when compared with the \( R_m \) values of marker proteins gave \( M_r \) values of 18, 22 and 24 kDa, respectively, for sheep, goat and buffalo lectins (see Table I). The marker proteins with their molecular weights in parentheses were; bovine serum albumin \( (68 \text{ kDa}) \), heavy chain of goat IgG \( (50 \text{ kDa}) \), ovalbumin \( (43 \text{ kDa}) \), pepsin \( (35 \text{ kDa}) \), chymotrypsinogen A \( (25.5 \text{ kDa}) \), light chain of goat IgG \( (25 \text{ kDa}) \) and cytochrome c \( (11.7 \text{ kDa}) \). A plot of \( \log M \) versus \( R_m \) was found to be linear with slope and intercept of 1.27 and 5.15, respectively. The experimental uncertainty with which \( M_r \) could be measured by SDS-PAGE was within 10%. As we shall see below the three lectins are glycoproteins and hence our molecular weight estimate by SDS-PAGE should be treated with caution (17). Clearly, goat and buffalo \( \beta \)-galactoside binding lectins possess different \( M_r \) from that of the major 14 kDa lectin extensively studied in vertebrate tissues (1-3).

Neutral hexose contents of sheep and goat lectins were identical and were substantially lower than that estimated for buffalo lectin (see Table I). The three lectins absorbed maximally in the ultraviolet region near 276 and 278 nm in 10 mM tris-HCl buffer pH 7.5. In the same buffer the emission maximum of sheep, goat and buffalo lectins were found at 339 nm and 338 nm, respectively, indicating that the three lectins contained tryptophan residue(s).

The effect of eleven saccharides on lectin mediated agglutination of trypsinized rabbit erythrocytes \( (1 \times 10^6 \text{ cells}) \) was investigated in 10 mM tris-HCl buffer pH 7.5 containing 150 mM NaCl, 5 mM CaCl\(_2\) and 1 mM 2-mercaptoethanol. Increasing concentration of specific saccharide caused substantial decrease in hemagglutination. A curve was drawn between the extent of hemagglutination and the concentration of a specific sugar. The saccharide concentration \( (C_M) \) corresponding to the middle point of the curve where the hemagglutination reaction was reduced to half, was
determined for all of the eleven sugars used in this study. The results are summarized in Table II where it can be seen that glucose, mannose, fucose and fructose had virtually no specific effect on the lectin mediated hemagglutination. Further, the two anomeric forms of lactose as well as galactose were indistinguishable within experimental error in their inhibitory potential. However, lactose was more potent inhibitor than galactose. As mutarotation of the two anomeric forms is possible, we have studied the inhibitory effect of methyl-\(\alpha\)-galactopyranoside and methyl-\(\beta\)-galactopyranoside. Clearly, \(\beta\)-form of the saccharide is more effective inhibitor than its \(\alpha\)-form. Strikingly, substitution of hydroxyl hydrogen atom at C-1 position in galactose by nitrophenyl group had

### Table II

**EFFECT OF SACCHARIDES ON HEMAGGLUTINATING ACTIVITY OF MAMMALIAN LIVER \(\beta\)-GALACTOSIDE BINDING LECTINS.**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sugar</th>
<th>Relative inhibitory activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sheep</td>
</tr>
<tr>
<td>1</td>
<td>(\alpha)-Lactose</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>(\beta)-Lactose</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>(\alpha)-Galactose</td>
<td>0.033</td>
</tr>
<tr>
<td>4</td>
<td>(\beta)-Galactose</td>
<td>0.033</td>
</tr>
<tr>
<td>5</td>
<td>Methyl-(\beta)-galactopyranoside</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>Methyl-(\alpha)-galactopyranoside</td>
<td>0.018</td>
</tr>
<tr>
<td>7</td>
<td>D-nitrophenyl-(\beta)-D-galactopyranoside</td>
<td>0.160</td>
</tr>
<tr>
<td>8</td>
<td>D (+) Glucose</td>
<td>0.003</td>
</tr>
<tr>
<td>9</td>
<td>D (+) Mannose</td>
<td>0.003</td>
</tr>
<tr>
<td>10</td>
<td>D (+) Fucose</td>
<td>0.003</td>
</tr>
<tr>
<td>11</td>
<td>D (+) Fructose</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*The relative inhibitory activity was obtained by dividing \(C_M\) for lactose by \(C_M\) for the test sugar as described earlier (2). The values of \(C_M\) for lactose were 0.7, 1.1, and 0.9 mM for sheep, goat and buffalo lectins, respectively.
marked inhibitory effect on the hemagglutination. Lactose and 0-nitrophenyl-\(\beta\)-D-galactopyranoside had comparable inhibitory effects on the hemagglutinating activity of goat lectin but the two sugars differed significantly in their inhibitory effects on the activity of sheep lectin; sheep lectin evidently prefers lactose. The fact that nitrophenylated galactose is far more effective inhibitor than galactose or methyl-\(\beta\)-D-galactopyranoside, suggests the participation of hydrophobic interaction in saccharide binding by hepatic soluble lectins. Here it is instructive to recall earlier plausible speculation about the carbohydrate binding site in \(\beta\)-galactoside binding lectins \((4,5)\). In chicken, eel and human \(\beta\)-galactoside binding lectins, residues from 70-76 have been assigned as part of \(\beta\)-galactoside binding site \((4)\). This particular segment contains, among others, a tryptophan residue. The observation that the three soluble hepatic lectins possess higher affinity for lactose than galactose, suggest that the carbohydrate binding site in the three lectins is extended one which presumably remains unfilled completely by small and specific monosaccharide.

**Cofactor requirements.**

Vertebrate soluble \(\beta\)-galactoside binding lectins, unlike membrane lectins, do not require metal ion such as \(\text{Ca}^{2+}\) for their carbohydrate binding activity \((1,3)\). In contrast to major soluble \(\beta\)-galactoside specific lectins from vertebrate tissues, the three soluble hepatic lectins described here were found to show hemagglutinating activity against trypsinized rabbit red blood cells only in the presence of \(\text{Ca}^{2+}\). No detectable activity was observed in tris-HCl buffer pH 7.5 containing NaCl and 2-mercaptoethanol but no \(\text{Ca}^{2+}\). It should be pointed out that the effect of metal ions on the activity of sheep, goat and buffalo liver lectins was studied with lectin preparation freed from metal ion by extensive dialysis against the buffer containing 10 mM EDTA.
The effect of increasing concentration of Ca\(^{2+}\) on hemagglutinating activity of goat liver lectin is depicted in Fig.2, where it can be seen that the metal ion concentration corresponding to the middle point of the curve (i.e. \(C_M\)) is 20 mM; the maximum attainable activity was about 87% (see Fig.2). Similar curves were obtained for other metal ions e.g. Mg\(^{2+}\), Mn\(^{2+}\), Sr\(^{2+}\) and Ni\(^{2+}\). The values of \(C_M\) and maximum attainable activity (given in parentheses) are listed in Table III. Strikingly, the hemagglutinating activity of sheep liver lectin was stimulated only by Ca\(^{2+}\), the remaining four metal ions had no effect on the lectin activity. Another notable observation is that sheep lectin showed absolute requirements of thiol for its activity whereas the other two mammalian soluble lectins were active even in the absence of 2-mercaptoethanol. In fact, the latter was omitted in all of the experiments which were performed to investigate the effect of metal ions (excluding Ca\(^{2+}\)) on the activity of goat and buffalo lectins (see Table III). Regardless of

<table>
<thead>
<tr>
<th>Metal Salt</th>
<th>(C_M) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{CaCl}_2)</td>
<td>49(85) 20(87) 20(86)</td>
</tr>
<tr>
<td>(\text{MgCl}_2)</td>
<td>- 32(79) 34(75)</td>
</tr>
<tr>
<td>(\text{MnCl}_2)</td>
<td>- 28(80) 32(75)</td>
</tr>
<tr>
<td>(\text{Sr(CH}_3\text{COO)}_2)</td>
<td>- 42(78) 40(75)</td>
</tr>
<tr>
<td>(\text{NiCl}_2)</td>
<td>- 30(78) 40(75)</td>
</tr>
</tbody>
</table>

**Figure 2.** Stimulation of goat hepatic \(\beta\)-galactoside binding lectin (250 \(\mu\)g/ml) freed from metal ion was determined by light scattering method in the presence of increasing concentration of \(\text{CaCl}_2\) (2-40 mM) in 10 mM tris-HCl buffer pH 7.5 containing 150 mM NaCl and 1 mM 2-mercaptoethanol.
metal ions, maximum hemagglutinating activity that could be obtained by increasing metal ion concentration lies in the range 74-87 per cent for all the three lectins (see Table III).

Major soluble β-galactoside binding mammalian lectins require thiol but no metal ion for their carbohydrate binding activity (1-3, 5). Of the three β-galactoside binding hepatic lectins, only sheep lectin required 2-mercaptoethanol for its hemagglutinating activity; in addition it also required Ca\(^{2+}\) (see Table III). Therefore, our results show that the three soluble β-galactoside binding hepatic lectins differ significantly in cofactor requirements from the known major mammalian β-galactoside specific lectins (1-7). The effect of thiol blocking reagents on hemagglutinating activity of the sheep, goat and buffalo lectins was studied by measuring the time course of inactivation by 5 mM each of iodo-

![Figure 3. Effect of thiol blocking reagents on hemagglutinating activity of sheep hepatic β-galactoside binding lectin. Lectin (250 μg/ml) was incubated with 5 mM of pHMB (○), NEM (■) and iodoacetamide (△) for different time intervals as indicated and the residual hemagglutinating activity was measured by light scattering method.](image)
acetamide, NEM and pHMB. As can be seen in Fig. 3, iodoacetamide had no detectable effect on goat lectin activity upto 60 min but the other two reagents caused substantial inactivation; inactivation with pHMB was rather rapid. Results obtained with goat and buffalo lectins were similar and therefore omitted. As pHMB is substantially more hydrophobic than NEM, the rather rapid inactivation of lectins by pHMB appears to suggest that one (or more) functionally relevant thiol group in the lectin is partially shielded from the solvent so that it is not readily accessible to NEM but can react with more hydrophobic pHMB. Our observations are in agreement with those of Hirabayashi et al.,(18) who reported inactivation of β-galactoside binding lectin from human placenta by NEM and pHMB. Curiously, two cysteine residues out of the six in the dimeric rat lung lectin react rapidly with iodoacetamide and the modified lectin showed significantly higher activity (5, 19). Reaction with iodoacetate was slow but it resulted in the loss of lectin activity (5). The loss of lectin activity has been attributed to a possible conformational change that occurred during modification of cysteine residues (5). The loss of activity does not appear to be directly due to the modification of functionally important cysteine residues in the carbohydrate binding site but due to conformational change accompanied by reaction of cysteine residues with the reagent (19). The β-galactoside binding lectins from sheep, goat and buffalo were found to be different from dimeric rat lung lectins (5,19), because the activity of the three lectins were not stimulated by iodoacetamide.

The thiol group in goat lectin was titrated against increasing concentration of pHMB (1.8 - 18x10^{-3} mM) in 10 mM tris-HCl buffer pH 7.0. The results are graphically shown in Fig. 4 where the inflection point can be seen to occur at a molar ration of 4.3, suggesting the
Figure 4. Titration of thiol group of goat hepatic β-galactoside binding lectin. Following Neuman et al., (20), lectin (1.8 μM) was incubated with increasing concentration of pHMB in 10 mM tris-HCl buffer pH 7.0 for 4 hours in the dark and increase in absorbance at 250 nm was recorded.

presence of 4.3 free sulphhydryl groups in the lectin. Similar pHMB titration with the sheep and buffalo lectins indicated the presence of 3.2 and 4.8 free sulphhydryl groups, respectively, in sheep and buffalo lectins.

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