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QUANTITATIVE PRECIPITIN STUDIES ON THE SPECIFICITY OF AN EXTRACT FROM *Tridacna maxima* (RÖDING)*

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ABSTRACT

Haemolymph from the clam *Tridacna maxima* precipitated with purified H-blood-group substances, *Helix pomatia* galactogen, and pneumococcus type XIV polysaccharide. Although gel diffusion, gel electrophoresis, and inhibition experiments indicated that only a single precipitating lectin was present in the haemolymph, quantitative precipitin and haemagglutination results suggested that a second agglutinin with anti-H-like specificity was also present. Evidence obtained from hapten inhibition experiments indicated that the precipitin that reacts with pneumococcus type XIV polysaccharide can be inhibited by a number of simple sugars. Of the compounds tested, 2-acetamido-2-deoxy-D-galactose was the best inhibitor of precipitation with pneumococcus type XIV polysaccharide and of haemagglutination with human erythrocytes, but the inhibition experiments showed that the extract was also markedly inhibited by D-galactosamine hydrochloride, D-galactose, lactose, and *p*-nitrophenyl β-D-galactopyranoside. The latter compound was more active than its parent sugar, which was in turn a more potent inhibitor than *p*-nitrophenyl α-D-galactopyranoside. Melibiose, raffinose, and stachyose, compounds which each contain terminal α-linked D-galactopyranosyl residues, were relatively weak inhibitors. The combining sites of the lectin that reacts with pneumococcus type XIV polysaccharide appear, therefore, to be most complementary to 2-acetamido-2-deoxy-D-galactopyranosyl residues, probably in β-linkage.

INTRODUCTION

Non-antibody agglutinins, or lectins[1], from a variety of plants and invertebrates, although first detected by their ability to agglutinate red blood cells[2,3], are now being increasingly used in many areas of the biological sciences. Lectins are proving valuable in structural investigations of polysaccharides[4–8], for the isolation of polysaccharides

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and glycoproteins[9–11], as reagents for fractionating lymphocyte subpopulations[12,13], in studies with normal and transformed cells[11,14–17], and as mitogens[11,18–20]. In addition, mitogenic stimulation of lymphocytes by some lectins may serve as a model for the study of lymphocyte activation[21,22]. Although the application of lectins to specific research problems in immunology and experimental cell research is being continually expanded[11,23–25], the number of purified lectins with known specificities available for use is, so far, relatively small[7].

While searching amongst the Invertebrata for reagents that react with carbohydrate structures, we found that haemolymph from the elongate clam *Tridacna maxima* (Röding) strongly agglutinated human ABO erythrocytes and the haemagglutinating activity was inhibited by small amounts of purified H-blood-group substances and some invertebrate polysaccharides[26]. Further examinations revealed that the extract precipitated both in gel and in solution with the H-substances, *H. pomatia* galactogen, and pneumococcus type XIV polysaccharide. Before attempting to purify the *T. maxima* precipitin(s) by an affinity-chromatography method, quantitative precipitin and specificity were studied in an effort to find the best inhibitors of the *T. maxima* combining-sites. In this paper, results of the sugar inhibition experiments are reported and the structural features of the most active inhibitors are discussed.

EXPERIMENTAL

Tridacna maxima *extract.* — The shells of clams were opened after subjecting the animals to carbon dioxide-treated sea water. Haemolymph, collected by dissecting the animals with a knife, was dialyzed against distilled water and lyophilized. Solutions for use in haemagglutination and precipitation experiments were prepared by dissolving the required amount of lyophilized extract in phosphate buffered saline, pH 7.3, containing 0.1% of sodium azide.

Blood-group substances, purified polysaccharides, and sugars. — Purified human H-substance (MH), isolated from ovarian cyst fluid, and purified hog H-substance were provided by Prof. Winifred M. Watkins, Lister Institute, London. Galactogen from *Helix pomatia* was a gift from Prof. Helene Weinland, Institute of Biological Chemistry, University of Erlangen (FRG). Purified pneumococcus type XIV polysaccharide was a gift from Dr. Grace I. Pardoe, Medical School, Birmingham University, England. Sugars were obtained from Sigma Chemical Co. and British Drug Houses. In all cases, compounds of the highest purity available were used. *p*-Nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside were purchased from Koch–Light Laboratories, England.

Immunological methods. — The preparation of erythrocyte suspensions and the haemagglutination (HA) and haemagglutination inhibition (HAI) techniques used have been previously described[27,28]. Solutions of sugars, blood-group substances, and polysaccharides used in HAI tests were prepared by techniques applicable to quantitative analytical procedures. Substances that did not inhibit at least 8 full HA

doses of *T. maxima* extract, at concentrations of 10 mg/ml, were considered to be inactive.

Immunodiffusion experiments were carried out in Petri dishes. The gels consisted[29] of 1% of Special Noble Agar (Difco Laboratories, Detroit, Michigan) containing 0.85% of sodium chloride, 0.05% of sodium azide in mM phosphate buffer, pH 7.3. Precipitates were stained with Amido Black.

Quantitative precipitin assays, based on the pioneering studies of Heidelberger *et al.*[30,31], were performed on a microscale with Lang–Levy pipettes and according to the procedures laid down by Kabat[32], and Schiffman, Kabat, and Thompson[33]. A solution of dialyzed and lyophilized *T. maxima* extract (25 µl) containing approximately 350–400 µg of N per ml was mixed with varying amounts of polysaccharide in a total volume of 135 µl, in 3-ml tapered centrifuge tubes. *T. maxima* controls without polysaccharide were also set up. After 1 h at room temperature and 72 h at 4°, tubes were centrifuged and the precipitates washed twice with 140 µl of cold saline solution. The nitrogen content of the washed precipitates was determined by the ninhydrin method after digestion with sulphuric acid[33].

Quantitative inhibition experiments, based on the above precipitation methods and adapted for inhibition studies by Kabat[32], were also performed on a microscale. Known quantities of the compounds to be tested for inhibition, each in a total volume of 100 µl, were mixed with 25 µl of *T. maxima* extract (350–400 µg of N per ml). After 1 h at room temperature, an amount of polysaccharide, known to give maximum precipitation with 25 µl of the *T. maxima* solution used, was added and the contents of the tubes were mixed. After a further 1 h at room temperature, tubes were placed at 4° and mixed once or twice daily over a period of 72 h. Controls of *T. maxima* extract alone, *T. maxima* extract with polysaccharide but no inhibitor, and *T. maxima* extract with inhibitor but no polysaccharide were also set up. Precipitates were centrifuged off, washed, and analysed for total N as just described[33]. Percentage inhibition was calculated from the amounts of precipitated N in the control (*T. maxima* extract plus polysaccharide) and test (*T. maxima* extract plus inhibitor plus polysaccharide) tubes after subtracting the *T. maxima* extract-saline blank[32].

RESULTS

Precipitation studies. — *T. maxima* extract precipitated in gel with the purified H-substances, pneumococcus type XIV polysaccharide, and the galactogen from *H. pomatia* albumin gland, forming a single and continuous precipitin line with no sign of spurring (Fig. 1). This precipitation was confirmed by quantitative studies using *T. maxima* extract together with microgram quantities of the different polysaccharides. Fig. 2A shows that of the four preparations tested, *H. pomatia* galactogen precipitated most N. The other three preparations, each precipitated approximately the same amount of N, but the shape of the pneumococcus type XIV polysaccharide precipitin-curve was different from the other three curves. With this preparation, less than half the amount of antigen was needed at equivalence than was needed to produce maximum precipitation with H-substance and *Helix* galactogen.

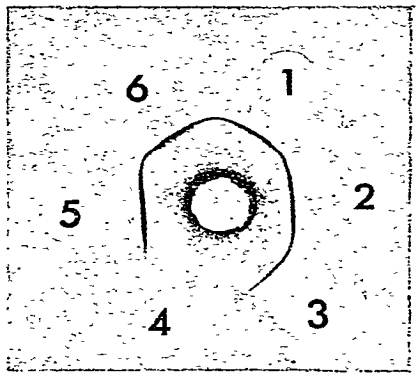

Fig. 1. Agar gel-diffusion pattern formed from the reaction of *Tridacna maxima* extract (744 μg N/ml) with pneumococcus type XIV polysaccharide, H-substances, and *Helix pomatia* galactogen. Peripheral wells: 1, Human H-substance, 1 mg/ml; 2, *H. pomatia* galactogen, 2 mg/ml; 3 and 6, hog H-substance, 1 mg/ml; 4, saline solution; and 5, pneumococcus type XIV polysaccharide, 1 mg/ml. Capacity of wells: 15 μl.

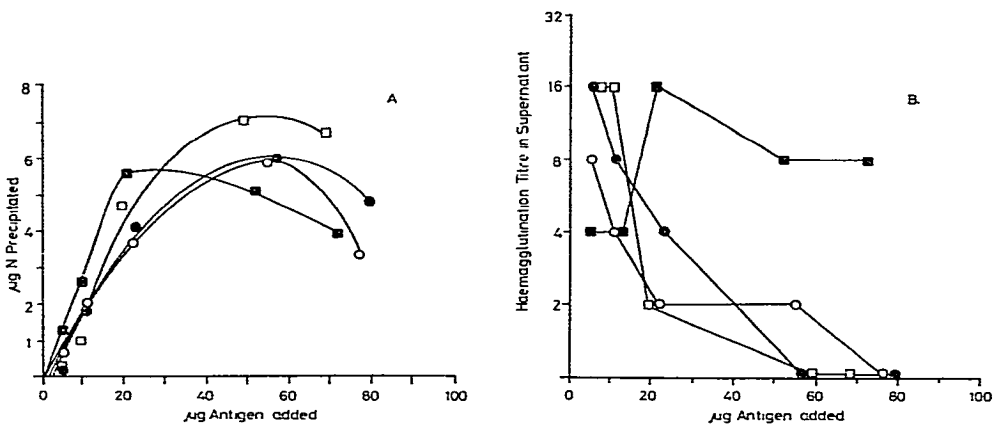

Fig. 2. A. Precipitation of human H-substance (●), hog H-substance (○), pneumococcus type XIV polysaccharide (■), and *Helix pomatia* galactogen (□) by *Tridacna maxima* extract.*T. maxima* extract (25 μl, 9.3 μg of N) was added to each tube; total volume: 135 μl. B. Haemagglutination titres against human group O erythrocytes found in supernatants after precipitation of human H substance (●), hog H substance (○), pneumococcus type XIV polysaccharide (■), and *H. pomatia* galactogen (□) by *T. maxima* extract (see A). Haemagglutination titre in control tubes (no antigen), 32.

Supernatants from each of the precipitin tubes were examined in HA experiments by use of human group O erythrocytes (Fig. 2B). With the supernatants from tubes containing H-substances or *H. pomatia* galactogen, HA activity was reduced as the amount of precipitated N increased, and all of the haemagglutinin could be removed. With pneumococcus type XIV polysaccharide, however, reduction of HA

activity was much less evident. Although a 4-fold reduction was obtained in the supernatants from tubes containing 5.2 μg and 10.3 μg of pneumococcus type XIV polysaccharide, even at the maxima, appreciable titres of anti-O agglutinins remained in the supernatants. The supernatant from tube No.3, for example (20.7 μg polysaccharide), showed only a 2-fold reduction in HA titre, as compared to the control. This effect was noticed in three separate experiments with pneumococcus type XIV polysaccharide and two different *T. maxima* extracts.

Inhibition studies. — Quantitative precipitin-inhibition experiments were performed with a number of monosaccharides, oligosaccharides, and glycosides of D-galactose, together with *T. maxima* extract and pneumococcus type XIV polysaccharide. This polysaccharide was selected since, unlike the H-substances and *Helix* galactogen, it was available in sufficient quantities to be used with all of the compounds examined in inhibition experiments (Fig. 3). Of the compounds tested, 2-acetamido-2-deoxy-D-galactose proved to be the best inhibitor, only a concentration of 17 nM being required to produce 50% inhibition of precipitation. This sugar was approximately 10 times as active as the next best inhibitors, *p*-nitrophenyl β-D-galactopyranoside and galactosamine hydrochloride, and approximately 25 times as effective as D-galactose and β-lactose. *p*-Nitrophenyl α-D-galactopyranoside was much less active than the β-anomer, producing only 33% inhibition at a concentration of 1.03μM. Two oligosaccharides, melibiose and raffinose, each containing terminal α-linked D-galactose groups, produced 50% inhibition of precipitation at concen-

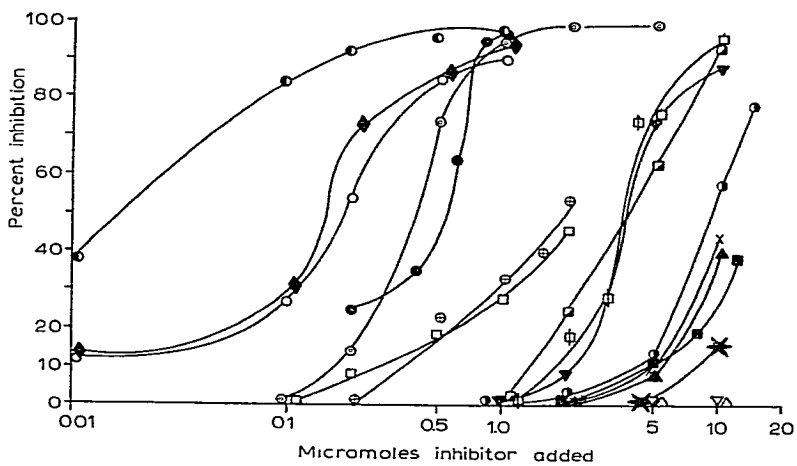

Fig. 3. Inhibition by monosaccharides, galactosides, and oligosaccharides of precipitation of pneumococcus type XIV polysaccharide by *Tridacna maxima* extract. *T. maxima* extract (9.3 μg of N) used with 20.7 μg of pneumococcus type XIV polysaccharide in a total volume of 135 μl. Key: ◐, 2-Acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine); ◆, *p*-nitrophenyl β-D-galactopyranoside; ○, 2-amino-2-deoxy-D-galactose (D-galactosamine) hydrochloride; ◎, lactose; ●, D-galactose; ⊕, *p*-nitrophenyl α-D-galactopyranoside; □, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine); □, raffinose; ▼, D-fucose; ⧈, melibiose, ◑; stachyose; ×, L-rhamnose; ▲, D-mannose; ■, D-glucose; ★, methyl β-D-glucopyranoside; ▽, L-fucose; and △, 2-acetamido-2-deoxy-D mannose (*N*-acetyl-D-mannosamine).

trations of 3.7 and 3.8μM, respectively. The tetrasaccharide stachyose, however, which also contains a terminal α-D-galactosyl group was significantly less active than melibiose and raffinose. Of the remaining sugars tested, only D-fucose showed any significant activity when used at a concentration of less than 5μM. Two sugars, L-fucose and 2-acetamido-2-deoxy-D-mannose, proved completely inactive when tested over the range of 1–10μM (Fig. 3).

Results obtained from HAI experiments support the findings of the precipitation and just described inhibition studies (Table I). High dilutions of human and hog H-substances inhibited agglutination of human group O erythrocytes. Only a little more than 4 μg/ml of each preparation was needed for complete inhibition. Approximately four times as much *H. pomatia* galactogen as H-substance was needed to inhibit HA by the *Tridacna* reagent, but 125 μg/ml of pneumococcus type XIV polysaccharide was necessary for complete inhibition. The inhibition observed with pneumococcus polysaccharide, however, was transient and observable only up to 15 min after addition of the red cells.

TABLE I

RESULTS OF HAEMAGGLUTINATION INHIBITION EXPERIMENTS WITH *Tridacna maxima* EXTRACT IN CONJUNCTION WITH H-BLOOD-GROUP SUBSTANCES, POLYSACCHARIDES, AND SUGARS

Test substance	*Minimum concentration of substance*[a,b]	
	mg/ml	*μmole/ml*
Human H-substance	0.004	
Hog H-substance	0.004	
H. pomatia galactogen	0.015	
Pneumococcus type XIV polysaccharide	0.125[c]	
D-Galactose	5.0	27.7
D-Galactosamine hydrochloride	5.0	23.2
Lactose	2.5	7.3
2-Acetamido-2-deoxy-D-galactose	0.3	1.4
p-Nitrophenyl β-D-galactopyranoside	2.5	8.3
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside	0.4–0.8	1.2–2.4

[a]Minimum concentration completely inhibiting the agglutination of human group O erythrocytes by 8 HA doses of *T. maxima* extract. Human secretor salivas (O, A, and B) inhibited at dilutions of 1:128–1:256. Nonsecretor salivas inhibited at a dilution of 1:8 after 15 min; no inhibition after 1 h. Galactan (Calbiochem), L-galactono-1,5-lactone (Calbiochem), 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, cellobiose, D-glucose, D-fucose, L-fucose, D-mannose, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, melibiose, raffinose, L-rhamnose, and stachyose did not inhibit up to a concentration of 10 mg/ml. *p*-Nitrophenyl α-D-galactopyranoside inhibited at 13 mg/ml. [b]Results were read 1 h after addition of erythrocytes. [c]Inhibition observed 15 min after addition of erythrocytes. No inhibition after 1 h.

Of the sugars tested (Table I), 2-acetamido-2-deoxy-D-galactose proved to be the best inhibitor of agglutination, but *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside proved almost as active as this sugar. Weaker inhibitory activity

was seen with D-galactose, D-galactosamine hydrochloride, and *p*-nitrophenyl β-D-galactopyranoside and lactose, but none of the other compounds tested except *p*-nitrophenyl α-D-galactopyranoside inhibited the agglutination of group O erythrocytes. The latter compound was approximately 1/5 as active as the β-glycoside.

DISCUSSION

Results obtained with pneumococcus type XIV polysaccharide and the behaviour of H-substances, *H. pomatia* galactogen, and human secretor and nonsecretor salivas in HAI experiments (Table I) suggest that more than one specificity may be present in the *T. maxima* extract. The H-substances and the *H. pomatia* polysaccharide, a preparation known to have human blood-group O (H) cross-reactivity[34], inhibited HA in high dilution, and human secretor saliva was a much stronger inhibitor than human nonsecretor saliva. These results, and the finding of residual HA activity in the supernatants of the tests with pneumococcus type XIV polysaccharide, suggest that the *T. maxima* extract may contain an anti-H-like specificity as well as the specificity responsible for the precipitation with pneumococcus type XIV polysaccharide. When *T. maxima* extract was examined in gel diffusion experiments with pneumococcus type XIV polysaccharide, H-substances, and *H. pomatia* galactogen, a single and continuous line of precipitation formed in front of each of the antigen wells (Fig. 1). After electrophoresis of *T. maxima* haemolymph in 1% agar gel, pH 8.6, and subsequent diffusion against H-substances, *H. pomatia* galactogen, or pneumococcus type XIV polysaccharide, a single precipitin arc was observed in the α-region[35]. These findings, and the complete inhibition of the *T. maxima*–pneumococcus polysaccharide precipitation reaction by some simple sugars, suggested that a single lectin is involved in the reaction of the whole extract with pneumococcus type XIV polysaccharide. It is emphasized, however, that the number of lectins in the extract can be clearly established only by purification experiments and more specificity studies.

Inhibition experiments with unfractionated *T. maxima* extract yielded clear-cut results. Inhibition of precipitation between the extract and pneumococcus type XIV polysaccharide, and inhibition of the HA of human erythrocytes by *Tridacna* showed that 2-acetamido-2-deoxy-D-galactose was the best inhibitor of the compounds tested. It should be remembered that, as only a limited number of compounds were tested, it is possible that none of the sugars examined represent the true complementary structure to the *Tridacna* combining sites. However, from the results obtained, it is clear that the carbohydrate-binding sites of the *T. maxima* precipitin are capable of combining with compounds having a D-*galacto* configuration. The finding that 2-acetamido-2-deoxy-D-galactose and *p*-nitrophenyl β-D-galactopyranoside are both significantly better inhibitors than D-galactose and *p*-nitrophenyl α-D-galactopyranoside indicates that the *Tridacna* lectin is probably specific for β-linked 2-acetamido-2-deoxy-D-galactose residues. The precipitin is not absolutely specific for β-linked structures, however, since some inhibition was also obtained with *p*-nitrophenyl α-D-galactopyranoside. These conclusions are reinforced by the results obtained with lactose,

where D-galactose is β-linked, and with the three oligosaccharides, melibiose, raffinose, and stachyose where, in each case, D-galactose is terminal and α-linked. On a molar basis, lactose was approximately 9 times as effective in producing inhibition as melibiose and raffinose, and approximately 20 times as effective as stachyose.

The importance of the groups and the stereochemical arrangement of the groups at positions 2, 4, and 6 on the pyranose ring warrants some discussion. The inhibition obtained with 2-acetamido-2-deoxy-D-galactose, the weak inhibition seen with 2-acetamido-2-deoxy-D-glucose, and the lack of inhibition seen with a concentration as high as 10μM of 2-acetamido-2-deoxy-D-mannose indicates that the orientation of the acetamido group at C-2 is important. The presence of an acetamido group in the correct orientation at C-2 is not absolutely essential for binding, however, since both D-galactose and D-galactosamine reacted with the lectin combining site. A hydroxyl group above the ring at C-4 in the Haworth's projection is preferred since 2-acetamido 2-deoxy-D-galactose and D-galactose were each markedly better inhibitors than 2-acetamido-2-deoxy-D-glucose. The importance of the substituent at C-6 and a further demonstration of the importance of the stereochemical arrangement of the hydroxyl groups at C-2 and C-4 was demonstrated by the results obtained with D-fucose (6-deoxy-D-galactose). This monosaccharide differs from D-galactose only at C-6, but it proved a much poorer inhibitor than D-galactose. By contrast, D-fucose was significantly more active than D-glucose, which differs from D-galactose only in the orientation of the hydroxyl group at C-4.

Explanations for the transient inhibition observed when pneumococcus type XIV polysaccharide was examined in HAI experiments, and the residual HA activity found in the supernatants from the quantitative precipitin tests are, at this stage, speculative. These findings may be due to the presence of anti-H-like agglutinins in the *T. maxima* extract, but purification studies are necessary before this explanation can be accepted. Use of a pure lectin preparation is also necessary to check the just mentioned sugar inhibition results obtained with unfractionated *T. maxima* extract. Based on the specificity data obtained so far, affinity chromatography experiments are being undertaken in an attempt to purify the lectin(s) present in *T. maxima* haemolymph.

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