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Purification and Characterization of the Agglutinins from the Sponge *Axinella polypoides* and a Study of Their Combining Sites†

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ABSTRACT: The hemagglutinins from the sponge *Axinella polypoides* were isolated by affinity chromatography using Sepharose 4B as an absorbent and eluting with DGal. Further separation on DEAE-cellulose and preparative disc electrophoresis on polyacrylamide and agarose gave three fractions. The physicochemical properties and binding specificities of the two main agglutinins were studied. Homogeneity was tested by polyacrylamide electrophoresis and immunoelectrophoresis and by sedimentation analysis. In isoelectric focusing, agglutinin I (mol wt 21 000) showed two bands at pH 3.8 and 3.9. Agglutinin II (mol wt 15 000) showed one band at pH 3.9. Both agglutinins have a carbohydrate content of about 0.5%, are immunochemically unrelated, and differ in amino acid composition. Both precipitate A_1, A_2, B, Le^a, and precursor I blood group substances but to different extents. Inhibition experiments revealed that both agglutinins are inhibited best by terminal nonreducing DGal glycosidically linked $\beta 1 \rightarrow 6$ or by *p*-nitrophenyl-βDGal. DGal and DFuc are equally active but about 20 and 12 times less active with agglutinin I and agglutinin II, respectively. DGalNAc and LFuc were inactive even at much higher concentrations. Both agglutinins have similar specificities and react with the immunodominant determinants of blood group B and Le^a but not with A and H substances; in A and H substances, reactivity is with side chains in which β-linked DGal is unsubstituted at the nonreducing terminus. The *Axinella polypoides* lectins are compared with galactose-specific lectins of different origin and with the aggregation factor system in sponges.

The number of lectins which have been isolated from invertebrates is quite small as compared with the great variety of purified lectins of plant origin. Information about invertebrate agglutinins is mostly limited to those from molluscs and crustaceans (Hammarström and Kabat, 1969; Acton et al., 1969; Marchalonis and Edelman, 1968; Jenkin and Rowley, 1970). Agglutinations by these lectins and by those of plant origin are similar.

Since the discovery of hemagglutinins in sponges by Dodd et al. (1968), the serological and immunoelectrophoretic properties of several (*Axinella spec., Cliona celata, Aaptos papillata, and Axinella polypoides*) were investigated in detail by Khalap et al. (1970, 1971) and by Bretting (1973). Hemagglutination of human A, B, O, and AB erythrocytes by crude

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and purified *Axinella polypoides* extracts could be inhibited by galactose and by oligosacharides containing nonreducing terminal α- and β-linked galactosyl residues.

Some of the physicochemical properties of *Axinella spec.* agglutinin were studied by Gold et al. (1974). By ion-exchange chromatography and gel filtration, an agglutinin of molecular weight of 15 000 to 18 000 was isolated. It contained negligible amounts of sugar, had an isoelectric point of 3.9, and bound 15 mol of Ca^{2+} and 12.2 mol of Fe^{3+} ions per mol of protein. Bretting and Renwrantz (1974) showed that at least two distinct agglutinins of similar specificity could be obtained from purified extracts of *Axinella polypoides*. Their molecular weights, 17 000 and 21 000, corresponded closely to the values of Gold et al. (1974). Low molecular weights are not generally characteristic of sponge agglutinins. *Aaptos papillata,* another sponge agglutinin, has a molecular weight of 65 000 (Bretting and Renwrantz, 1974).

Crumpton and co-workers (personal communication) purified *Axinella polypoides* hemagglutinin by affinity chromatography, adsorbing it to Sepharose 6B and eluting with galactose. In the present study *Axinella* hemagglutinin was adsorbed onto Sepharose 4B and eluted with galactose. Further fractionation on DEAE[1]-cellulose and by preparative acrylamide electrophoresis gave three different fractions which were immunologically unrelated as determined with rabbit antiserum to crude *Axinella* extract. Two of these were studied in detail. The third fraction, which is present probably only in low concentrations was purified, but could not be studied due to fragmentation of the material after separation. The two lectins studied differed significantly in molecular weights, isoelectric focusing pattern amino acid compositions, and electrophoretic mobilities. They also differed in mitogenicity (Phillips, Bretting, and Kabat, in preparation): one fraction was almost inactive; the other was comparable to concanavalin A and phytohemagglutinin in blast transformation of human lymphocytes. Both fractions precipitated with A, B, H, Le^a, Le^b, and I blood group substances to different extents. The site on both lectins reacts best with oligosaccharides containing a terminal DGalβ1$\rightarrow$6 residue, the subterminal sugar not being contributory; α-linked terminal DGal was less active than β-linked terminal DGal.

Experimental Section

Materials. The sponge *Axinella polypoides* was collected in the Mediterranean near Banyuls sur Mer (France). The sponge tissue was dried at 35–40 °C after separation from the solid axes which contain almost all of the needles making up the skeleton. The dry material was homogenized in a blender and stored at −20 °C until used. Saline extracts were prepared by stirring 50 g of sponge tissue with 1 l. of 0.9% NaCl for 16 h at 4 °C. The extract was centrifuged at 2000 rpm for 30 min and then for 5 h at 15 000 rpm in the cold. The dark brown supernatant was Millipore filtered and is referred to as crude extract; 0.02% NaN_3 was added as a preservative.

Blood group substances used were the previously described preparations from human ovarian cysts, hog gastric mucins, and horse stomach linings (Kabat, 1956; Schiffman et al., 1964; Lloyd and Kabat, 1968; Vicari and Kabat, 1969). Monosaccharides were obtained commercially (Mann Research Laboratories or Nutritional Biochemicals Corp.). The blood group oligosaccharides used were those isolated and characterized (Lloyd et al., 1966). Streptococcal group A and C polysaccharides were a gift from Dr. R. M. Krause of Rockefeller University (Krause and McCarty, 1961, 1962).

The immunoadsorbent hog A + H blood group substances (PL-hog A + H) were obtained by copolymerization of the purified hog A + H substance (Kabat, 1956) with the *N*-carboxyanhydride of L-leucine, (Pilot Chemicals) by the technique of Tsuyuki et al. (1956), as described by Kaplan and Kabat (1966). Sepharose 4B was purchased from Pharmacia Fine Chemicals.

Analytical Methods. Methylpentose (fucose), hexosamine, *N*-acetylhexosamine, hexose (galactose), and nitrogen were determined by colorimetric methods (Kabat, 1961; Lloyd et al., 1966). Periodate uptake has also been described previously (Kabat, 1961).

Amino Acid Analysis. Salt free samples were hydrolyzed in constant boiling HCl at 110 °C in evacuated, sealed, Pyrex tubes for 24, 48, and 72 h. Quantitative analyses were performed by the technique of Spackman et al. (1958) using a Technicon amino acid analyzer. Methionine and half-cystine values were determined separately on samples hydrolyzed for 24 h after prior oxidation with performic acid and subsequent reduction with hydrobromic acid according to the method of Moore (1963). Tryptophan was estimated by the method of Goodwin and Morton (1946).

Physicochemical Analysis. Sedimentation velocity was measured in a Spinco Model E ultracentrifuge equipped with phase-plate Schlieren optics and automatic temperature control. The sedimentation coefficient was calculated at a lectin concentration of about 12 mg of protein/ml in saline (pH 5.9) from measurements at 50 740 rpm and 8.8 °C.

Disc electrophoresis was performed in polyacrylamide gels (Davis 1964; Reisfeld and Small, 1966; as described by Dorner et al., 1969); 7.5, 10, and 12.5% gels were employed at pH 9.3 and 4.5. Electrophoresis was at an initial constant current of 1.5 mA/tube until the buffer line passed from the spacer gel to the small pore gel. The current was then increased to 2.5 mA/tube and the electrophoresis was stopped shortly before the buffer line reached the end of the gel. The gels were fixed and stained in 0.02% Coomassie blue in 12.5% trichloroacetic acid and destained in methanol–acetic acid–water (75:50:875).

For separation in agarose gels, 30-cm tubes were used with no spacer gel and stained as for polyacrylamide gels. For molecular weight determination, the purified *Axinella* lectins were examined by the method of Weber and Osborn (1969) by electrophoresis in 10% polyacrylamide gels prepared in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate with or without 0.1% β-mercaptoethanol. Protein markers used were: bovine serum albumin (mol wt 68 000), ovalbumin (mol wt 43 000), pepsin (mol wt 35 000), chymotrypsinogen (mol wt 11 000 and 13 700), trypsin (mol wt 23 300), and lysozyme (mol wt 14 300). Approximately 10 μg of N of the purified lectins and of the markers were used. Mobility was measured relative to bromphenol blue. To ensure removal of bound ions, the lectins, dissolved in 0.9% saline, were dialyzed exhaustively at 4 °C against 0.02 M HCl, saturated with EDTA (7 days, two changes per day; Edmundson et al., 1971). Subsequently one part was dialyzed against 0.9% saline, the other against 0.9% saline containing 0.1 mol of Ca^{2+}.

Analytical isoelectric focusing was performed on a thin layer of 5% polyacrylamide with an LKB apparatus according to the instructions of the manufacturer using 2% carrier ampholytes (Ampholine pH 3.5–10).

Separation Procedure. Gel filtration was performed at 4 °C on Bio-Gel P-10. Ion exchange chromatography was on

[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DGal, D-galactose; LFuc, L-fucose; DFuc, D-fucose; LRha, L-rhamnose.

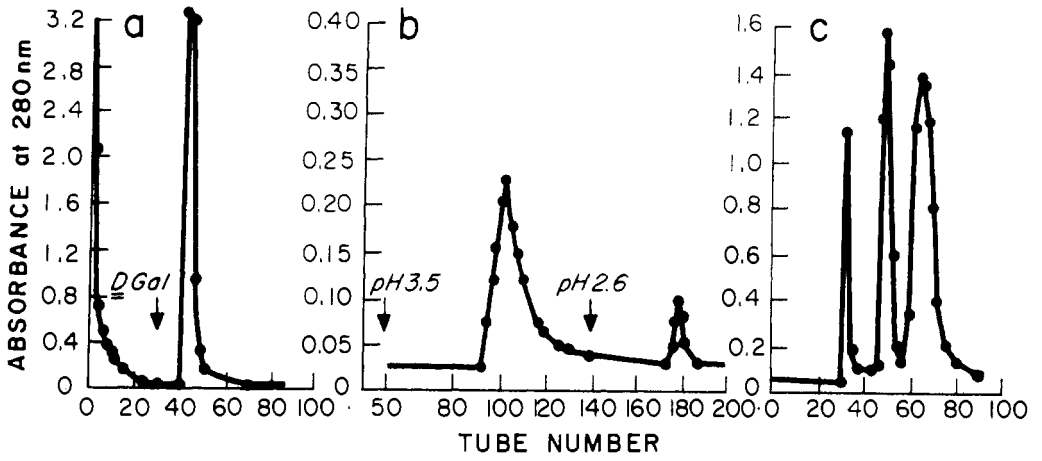

FIGURE 1: Purification of the Sepharose 4B fraction and of agglutinins I and II from *Axinella polypoides*. (a) Elution of the Sepharose 4B fraction from a Sepharose 4B column (2.5 × 1.2 cm) with 0.01 M DGal after the column was saturated with crude extract and washed. (b) Ion exchange chromatography of the Sepharose 4B fraction on a DEAE-cellulose column (15 × 2 cm) equilibrated with 0.07 M barbital buffer (pH 5.0). Elution was carried out stepwise with 0.07 M barbital buffer (pH 3.5 and 2.6). The first peak (tubes 92–130) corresponds to agglutinin I, the second (tubes 174–186) to a mixture of agglutinins I, II, and III. (c) Preparative polyacrylamide gel electrophoresis of the Sepharose 4B fraction (80 mg) on 12.5% acrylamide gel. Peak I (tubes 30–36) represents unpolymerized material, peak II (tubes 46–56) agglutinin II, and peak III (tubes 58–80) a mixture of agglutinins I and III.

DEAE-cellulose equilibrated with 0.07 M barbital-acetate-HCl buffer (pH 6) in a column 25 × 3.2 cm. Stepwise elution was by 0.5 pH units until a pH of 2.5 was reached. Preparative polyacrylamide electrophoresis was performed simultaneously in ten tubes (12 × 0.8 cm), the lower ends of which were closed with dialysis bags. The migrating fractions which had a brownish cast were monitored by eye and, as soon as a fraction had left the gel and entered the dialysis bag, electrophoresis was stopped, the contents of the dialysis bags were pooled and new dialysis bags were attached. The procedure was repeated with every fraction. Instead of tracing the single fraction by eye, which is sometimes not possible, one can calculate the time a fraction needs to migrate out of the tube and change the dialysis bags at the calculated time. In every case, a small part of the pooled fractions was reexamined by analytical polyacrylamide to be sure that complete separation had been achieved. Toward the end of this study preparative disc electrophoresis was performed in a Uniphor 7900 column designed for preparative purposes by LKB. Separations were achieved with a 12.5% acrylamide gel in a Tris-glycine buffer (10 mM) at pH 8.5. To separate nonpolymerized material from the protein, a prerun at 800 V and 10 mA was performed for an hour. After application of the 2-ml sample (80 mg of protein), electrophoresis was run at 200 V and 4 mA. After the protein had entered the gel, the voltage was increased to 800 V. Separations in 1.5% agarose gel containing 10 mg of galactose/ml were obtained in 0.05 ml of barbital buffer, at 240 V and 40 mA. Elution was set to about 16 ml/h and fractions of 1.6 ml were collected. Concentration was by pressure filtration with a Diaflow UM-10 membrane (Amicon Corporation, Lexington, Mass.).

Immunochemical Methods. Hemagglutination was performed at room temperature with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using 0.025-ml loops and 2% suspension of erythrocytes. Quantitative precipitin analyses were done by a microprecipitin technique (Kabat, 1961) employing a final volume of 250 μl unless otherwise stated. The tubes were incubated for 1 h at 37 °C and kept for 1 week at 4 °C. Nitrogen in washed precipitates was determined by the ninhydrin procedure (Schiffman et al., 1964). Immunodiffusion was by the Ouchterlony (1948) method in 1.5% agar in 0.05 M sodium barbital (pH 8.3). Immunoelectrophoresis was performed at 150 V for 35 min according to the technique of Grabar and Williams (1953) in 1.5% agar containing 10 mg of galactose/ml to avoid binding of the lectin to the agar. Antiserum to a crude extract of *Axinella polypoides* was produced in a rabbit. After obtaining preimmunization sera, the footpads of the rabbit were injected once a week for 3 weeks with an emulsion of about 1 mg of sponge extract in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). The animal was bled on 3 successive days beginning 1 week after the final injection and the three bleedings were pooled. After 1 week the immunization and bleeding schedule was repeated and a second batch of antisera with much higher antibody levels was obtained.

Results

Purification of the Lectin. The clear brown crude extract (980 μg of N/ml) agglutinated human red blood cells of type A_1, A_2, B, and O each to a titer of 32. Quantitative precipitin analysis showed 5% of the total nitrogen to be precipitable by human ovarian cyst blood group A substance (McDon, Cyst A). Purification was carried out according to Crumpton (personal communication) by affinity chromatography on Sepharose 4B which contains terminal DGal residues. In a small scale experiment, a column (1.2 × 2.5 cm) was filled with Sepharose 4B and washed with 0.85% saline overnight until the optical density at 280 nm of the eluate was 0.008. The crude *Axinella* extract was then applied continuously to the column, and 2.9-ml fractions were collected until the titer against A, B, and O erythrocytes of the effluent equaled that of the initial extract. Crude extract (160 ml) was necessary to saturate the column. It was then washed with 0.9% saline until the extinction at 280 nm was less than 0.015.

Specific elution of hemagglutinin was effected by 0.01 M DGal in 0.9% saline (Figure 1a). The peak obtained was pooled and concentrated by ultrafiltration. Further elution attempts with 0.1 and 0.5 M DGal were negative. The DGal was separated from the lectin by gel filtration on a Bio-Gel P-10 column (80 × 1.8 cm). The lectin appeared as a single symmetrical peak in the excluded volume and the DGal was retarded giving a separate peak by periodate uptake. The protein peak was pooled, concentrated by ultrafiltration, and re-chromatographed on the same Bio-Gel P-10 column. A periodate posi-

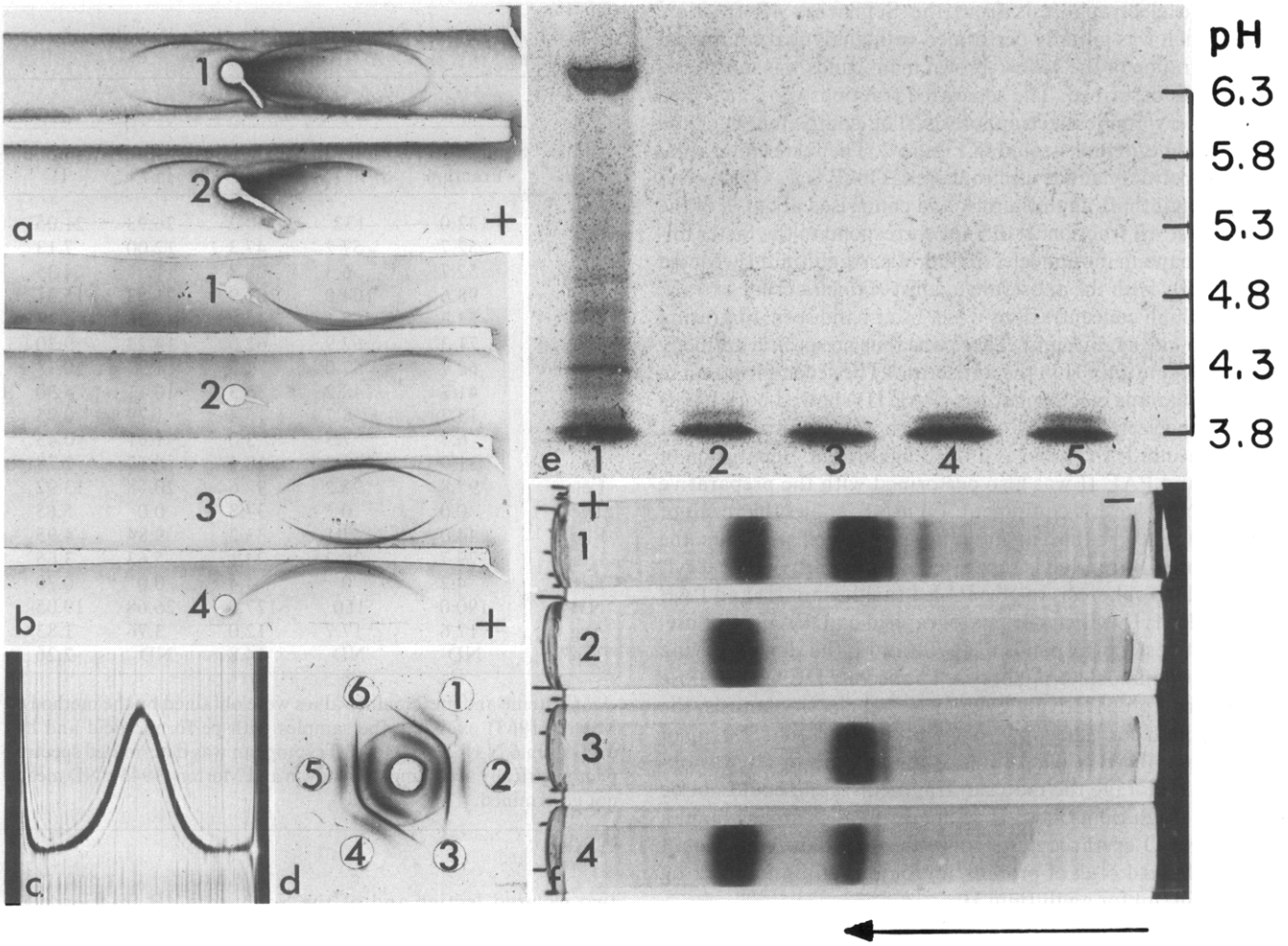

FIGURE 2: Immunoelectrophoretic, immunodiffusion, ultracentrifugal, isoelectric focusing, and disc electrophoresis patterns of the *Axinella polypoides* lectins. (a) Immunoelectrophoresis of crude extract (well 1), 980 μg of N/ml, and of crude extract after absorption on Sepharose 4B (well 2), 850 μg of N/ml. (b) Immunoelectrophoresis of the Sepharose 4B fraction (well 1), 61 μg of N/ml; agglutinin III (well 2), 5 μg of N/ml; separated agglutinins I plus III (well 3), 60 μg of N/ml; and agglutinin I (well 4), 61 μg of N/ml. The troughs contain rabbit antiserum to the crude extract of *Axinella*. (c) Schlieren pattern of agglutinin I (12.5 mg/ml in saline) photograph after 304 min at 50 740 rpm. (d) Immunodiffusion of crude extract (wells 1 and 4) of crude extract after absorption on Sepharose 4B (wells 2 and 5) and of purified lectin (wells 3 and 6), 61 μg of N/ml; center well, rabbit antiserum to crude extract. (e) Isoelectric focusing pattern of: (1) crude extract, 9.8 μg of N; (2) Sepharose 4B fraction, 3.7 μg of N; (3) agglutinin II, 4.0 μg of N; (4) fraction II from preparative polyacrylamide electrophoresis, 4.5 μg of N; (5) agglutinin I, 3.7 μg of N. (f) Disc electrophoresis at pH 9.2: (1) purified lectin 15.8 μg of N; (2) agglutinin I, 11.5 μg of N; (3) fraction II from preparative polyacrylamide electrophoresis, 9 μg of N; (4) agglutinin I, 5.5 μg of N, plus fraction II from preparative polyacrylamide electrophoresis, 4.5 μg of N.

tive peak was not found. Lectin (11.5 mg) was recovered. The isolated lectin agglutinated a 2% suspension of erythrocytes (A, B, and O) at a minimum protein concentration of 2.5 μg/ml.

PL-hog A + H was also used for affinity chromatography. A small column (2.5 × 0.5 cm) containing 1 part PL-hog A + H plus 5 parts of washed Celite (Pereira and Kabat, 1974) was saturated with lectin (270 ml). After extensive washing (OD at 280 nm less than 0.030), the lectin was eluted with 0.01 M DGal as a single symmetrical peak and chromatographed twice on Bio-Gel P-10 to separate the lectin from galactose. Twenty-five milligrams of protein of purified lectin was recovered from a column containing 300 mg of PL-hog A + H.

The material eluted from Sepharose 4B and from PL-hog A + H was tested for purity by double diffusion and immuno- and polyacrylamide electrophoresis. As seen in Figure 2, purified hemagglutinin from Sepharose 4B gave two weak bands in the Ouchterlony test, two bands moving toward the anode in immunoelectrophoresis, and two major and three minor bands in 12.5% polyacrylamide. Separations were poor in gels with lower acrylamide concentrations or at acid pH (4.5). The material eluted from PL-hog A + H behaved identically.

In all further purification the starting material was the Sepharose 4B fraction because it was easier to wash the saturated Sepharose 4B than the PL-hog A + H.

By ion exchange chromatography on DEAE-cellulose the Sepharose 4B fraction gave two fractions. A typical separation was achieved with a column (15 × 2 cm) equilibrated with 0.07 M barbital-acetate-HCl (pH 5.0). All of the applied protein (650 μg of N) was bound under these conditions. As seen in Figure 1b, stepwise elution with buffer at different pH gave two fractions. The first, DEAE I, agglutinin I, eluted at pH 3.5; the second, DEAE II, eluted at pH 2.6. Analysis showed that DEAE I gave only the slower migrating band in immunoelectrophoresis and only the upper main band and two minor bands in polyacrylamide gel. The second fraction, DEAE II, was a mixture of at least two agglutinins, giving two bands in immuno- and in polyacrylamide electrophoresis.

A second agglutinin was purified by preparative polyac-

rylamide electrophoresis from the Sepharose 4B fraction. Separation was initially performed with analytical equipment and migration of the yellow-brown main bands was monitored by eye, as described. The separated fractions were analyzed in polyacrylamide electrophoresis. The completeness of the separation is demonstrated in Figure 2. The faster migrating band in polyacrylamide electrophoresis (PAE I) was the second major agglutinin, agglutinin II, and comprised about ⅓ of the Sepharose 4B fraction. It did not correspond to the faster migrating band in immunoelectrophoresis. Agglutinin II reacted very poorly with the antiserum against *Axinella* crude extract. Only at high concentrations was a faint band seen, migrating anodally like agglutinin I. There was thus no specific antibody against agglutinin II in the antiserum. The second fraction in polyacrylamide electrophoresis (PAE II) showed both bands in immunoelectrophoresis, one of which was identical with that of agglutinin I; the other is a third agglutinin. Separation of PAE I and PAE II was also performed with the preparative disc electrophoresis equipment. Figure 1c shows the result of a typical run. The third agglutinin was obtained as follows: the Sepharose 4B fraction is separated by preparative polyacrylamide electrophoresis to give PAE I (agglutinin II) and PAE II. The PAE II is then chromatographed on DEAE-cellulose. The first peak corresponds to agglutinin I, the slower moving band in immunoelectrophoresis. The second DEAE-cellulose peak is run preparatively by agarose gel electrophoresis, the first fraction corresponding to agglutinin III. From 800 mg of Sepharose 4B fraction, 18 mg of the agglutinin III was obtained; on storage the material fragmented and could not be studied. Agglutinins I and II do not distinguish among human A, B, and O erythrocytes. However, the minimum amount required was 1.5 μg of protein/ml for agglutinin I and 11 μg of protein/ml for agglutinin II.

Physical and Chemical Analyses. In the analytical ultracentrifuge using Schlieren optics, the purified Sepharose 4B fraction, agglutinin I, and agglutinin II at concentrations of 10, 12.5, and 10.3 mg of protein/ml, respectively, sedimented as single peaks with a $s_{20,w}$ of 2.3, 2.6, and 2.8.

On polyacrylamide gels in sodium dodecyl sulfate, the unfractionated purified *Axinella* lectin migrates as two bands, one corresponding to a molecular weight of 15 000, the other of 21 000. Agglutinin I showed two bands, a stronger one at 21 000 and smaller one at 15 000, while agglutinin II gave only one band of molecular weight 15 000.

In polyacrylamide gels in sodium dodecyl sulfate with β-mercaptoethanol, all fractions showed only one band of molecular weight of 15 000. Since agglutinin I contains cysteine, it is possible that the molecule is completely reduced only in sodium dodecyl sulfate-polyacrylamide gels with β-mercaptoethanol and split into a larger component of 15 000 and a smaller nondetected fragment.

Analytical Isoelectric Focusing. Figure 2 shows the results of the analytical isoelectric focusing of the different fractions. The Sepharose 4B fraction shows two bands, a major band at pH 3.8 and a minor band at pH 3.9. Agglutinin II, the first fraction in preparative polyacrylamide electrophoresis, shows only one band at pH 3.8, while the second fraction from the preparative polyacrylamide electrophoresis gave the same pattern as the Sepharose 4B fraction. Agglutinin I, the first fraction from DEAE-cellulose, also has the two bands seen in the Sepharose 4B fraction.

Carbohydrate Analysis. Agglutinins I and II contained about 0.5% hexoses by the orcinol method. Hexosamine, methylpentose, and *N*-acetylhexosamine were absent.

Amino Acid Analysis. The amino acid compositions of the two isolated lectins and of the Sepharose 4B fraction were studied and compared in Table I. Since the amino acid composition of the Sepharose 4B fraction revealed only minor differences between 24, 48, and 72 h hydrolysates, the amino acid analysis of the two fractions was carried out only for 24 h. Both lectins resemble each other, but differ in three amino acids. Agglutinin I contains no histidine and tyrosine and has significantly higher serine. Both contain cysteine and methionine and are high in aspartic acid, glutamic acid, and leucine. The Sepharose 4B fraction is not entirely comparable to these two fractions since the proportions of agglutinins I and II vary from one preparation to another.

TABLE I: Amino Acid Composition of the *Axinella polypoides* Agglutinins.

Amino Acid	Residues/100 000 g			mol/mol	
	Sepharose 4B Fraction	Agglutinin I	Agglutinin II	Agglutinin I	Agglutinin II
Asp	132.0	132	140.2	26.93	21.05
Thr	52.7	57.2	47.3	12.00	7.13
Ser	52.7	56.3	28.8	11.84	4.35
Glu	98.6	104.0	101.9	21.83	15.31
Pro	53.3	58.1	40.9	12.26	6.17
Gly	71.3	67.9	62.0	14.23	9.30
Ala	62.7	57.0	71.6	11.9	10.78
Val	41.7	48.2	35.3	10.11	5.30
Cys[a]	13.7	8.7	6.4	1.79	0.95
Met[a]	6.3	7.5	3.0	1.61	0.43
Ile	21.9	29.5	18.4	6.17	2.78
Leu	97.8	95.2	92.6	20.58	13.92
Tyr	0.0	0	37.8	0.0	5.65
Phe	44.0	47.1	33.2	9.84	4.95
Lys	29.7	26.2	24.1	5.63	3.65
His	4.2	0	4.4	0.0	0.70
NH_3	190.0	150	127.0	26.68	19.05
Arg	17.6	17.7	12.0	3.76	1.83
Trp[b]	ND	ND	14.8	ND	2.26

[a] Cysteine and methionine values were obtained by the method of Moore (1963) oxidizing the samples with performic acid and hydrolysis in 6 N HCl for 24 h. [b] Tryptophan was determined spectrophotometrically according to Goodwin and Morton (1946). ND means not determined.

Immunochemical Specificity. Horse 4 25% and Beach phenol insoluble (two B substances), Cow 21P1 obtained by mild acid hydrolysis, OG 20% from 10% (a precursor human ovarian cyst blood group substance), N-1 (an Le^a-active substance), and Tighe (an H-active material) all precipitated close to 6 μg of N with the Sepharose 4B fraction, which is essentially all of the lectin added (Figure 3). For 50% precipitation 5 to 8 μg of blood group substance was required; A substances MSM 10%, MSS 10% 2x, and cyst 14 reached the same maximum (about 6 μg of N) but 10 to 34 μg was required for 50% precipitation. Cyst 9, an A-active substance, and cyst JS phenol insoluble, an H substance, precipitated about ⅔ of the total N, JS 1st IO_4^-/BH_4^-, obtained by periodate oxidation and Smith degradation, precipitated only 26% of the lectin. However, after a second periodate oxidation and Smith degradation, 50% of the lectin was precipitated by 8 μg of JS 2nd IO_4^-/BH_4^-; inhibition by excess was very rapid, however.

Inhibition experiments on the Sepharose 4B fraction (Figure 4) were carried out with monosaccharides, methyl and *p*-nitrophenyl glycosides, and some di- and oligosaccharides. DGal and DFuc were equal on a molar basis and were the best mo-

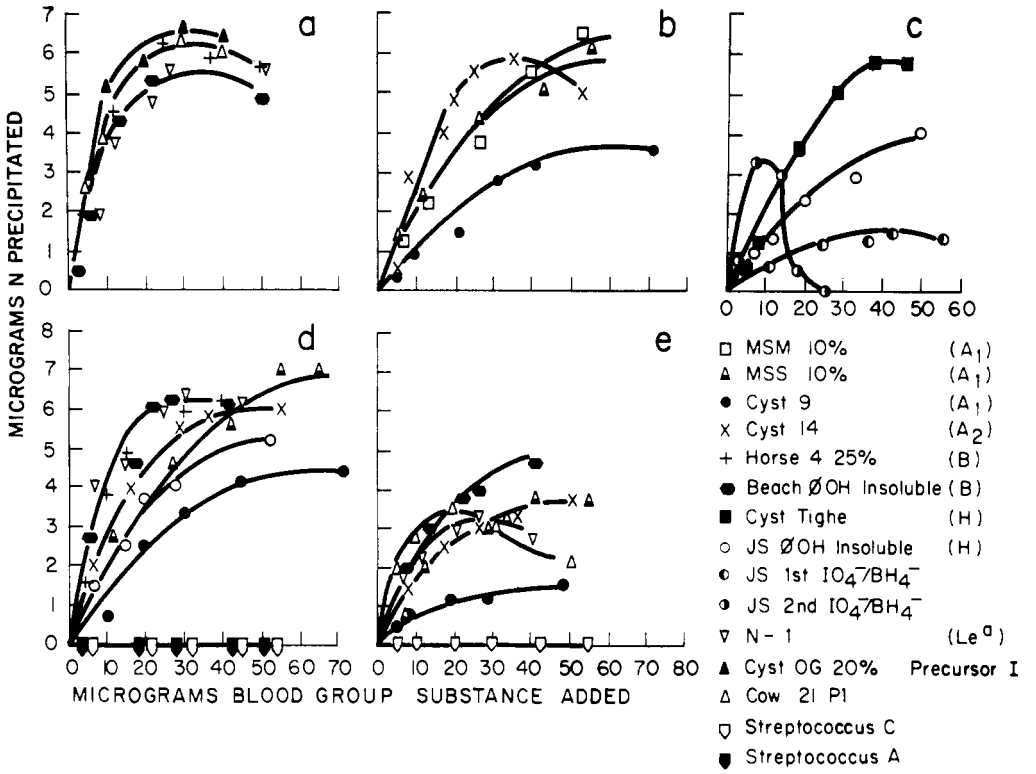

FIGURE 3: Quantitative precipitin curves of (a–c) the Sepharose 4B fraction, 6.1 μg of N; (d) agglutinin I, 6.1 μg of N, and (e) agglutinin II, 5.75 μg of N, with blood group substances and other polysaccharides. Total volume in all cases was 250 μl.

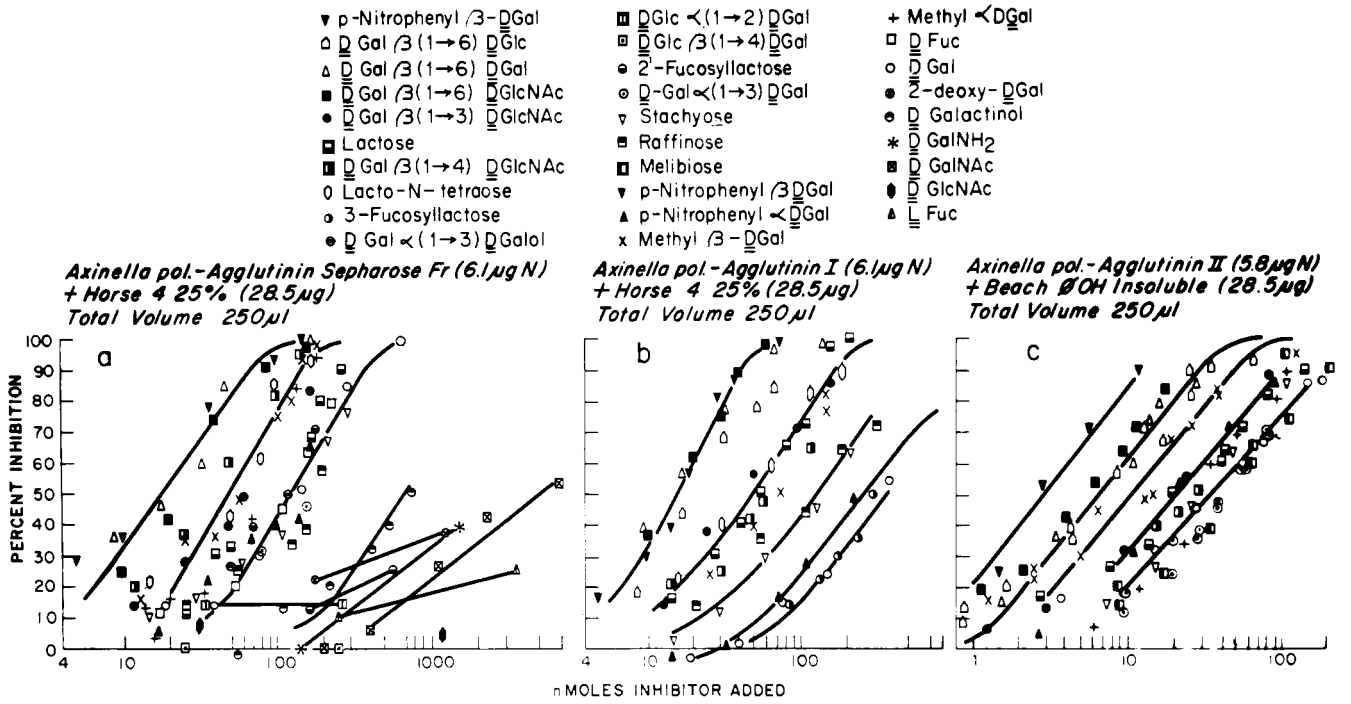

FIGURE 4: Inhibition by monosaccharides and various oligosaccharides of precipitation of horse 4 blood group B substance with the Sepharose 4B fraction (a) and agglutinin I (b) and of human blood group B substance Beach ϕOH insoluble with agglutinin II (c).

nosaccharide inhibitors; 2-deoxy-DGal was about one-tenth as active. LFuc and DGlcNAc were inactive up to 1500 nmol, $DGalNH_2$ showed 40% inhibition at this concentration, and DGalNAc inhibited 50% at 5000 nmol, being 35 times less active than DGal. Thus the site is specific for a terminal nonreducing DGal with the OH at C2 being important and that at C6 not being essential. Methyl-βDGal was two times and *p*-nitrophenyl-βDGal was ten times more potent than DGal, while methyl-αDGal, as well as *p*-nitrophenyl-αDGal, was as potent as DGal, indicating specificity for β-linked DGal. Ethyl-βDGalNAc was inactive up to 400 nmol. Of the disaccharides, those with terminal nonreducing β-linked DGal were more active than those with terminal nonreducing αDGal. DGalα1→3DGal, melibiose, raffinose, stachyose, and 3-fu-

cosyllactose were about as active or slightly less active than DGal and galactinol (αDGal-1→1-*myo*-inositol) was one-fifth as active. DGal linked β1→3 or β1→4 was almost as active as methyl-βDGal as seen with DGalβ1→3DGlcNAc, DGalβ1→4DGlcNAc, lactose, and lacto-*N*-tetraose. All sugars tested with DGal linked β1→6 at the nonreducing end, such as DGalβ1→6DGal and DGalβ1→6DGlcNAc were the most potent inhibitors and equal to *p*-nitrophenyl-βDGal. Thus the linkage rather than the subterminal sugar seemed to have the predominant influence on inhibitory activity. The requirement for a terminal nonreducing DGal is seen by the failure of DGlcβ1→4DGal, DGlcα1→2DGal, and LFucα1→2DGalβ1→4DGlc to give any inhibition.

Precipitin studies with the fractionated agglutinin I showed only minor differences from the Sepharose 4B fraction. With agglutinin II quantitative precipitin curves showed some differences. Only Beach phenol insoluble was able to precipitate 80% of the total lectin N, the other blood group substances precipitating only $\frac{2}{3}$ of the total lectin N; Cyst 9 only precipitated about 25% of the lectin N (Figure 3).

Agglutinin I and the Sepharose 4B fraction showed the same behavior in inhibition assays, except that in the case of agglutinin I oligosaccharides with terminal DGal linked α1→6 were about twice as active as DGal; *p*-nitrophenyl-βDGal, DGalβ1→6DGlc, DGalβ1→6DGlcNAc and DGalβ1→6DGal were the most active and all were of equal potency on a molar basis. When inhibition findings on agglutinin I and II are compared, DGal and terminal β-linked nonreducing DGal are good inhibitors with agglutinin II showing that both have similar specificities. However, some differences in inhibitory activity were observed with some disaccharides. With agglutinin II *p*-nitrophenyl-βDGal is also the best inhibitor but allolactose, DGalβ1→6DGal, and DGalβ1→6DGlcNAc are only about $\frac{1}{2}$ as active as found with agglutinin I. DGal linked β1→3 or β1→4 is about six times better than DGal with agglutinin I but only twice as good with agglutinin II. With agglutinin II methyl-βDGal is twice as potent as disaccharides with terminal DGalβ1→3 or β1→4 but with agglutinin I methyl-βDGal is slightly less active than these disaccharides. Moreover, disaccharides with terminal nonreducing α-linked DGal are equal to DGal in inhibitory power with agglutinin II, but are more than twice as active with agglutinin I.

Inhibition studies with the Sepharose 4B fraction and agglutinin I were carried out using Horse 4 25% for precipitation, while those with agglutinin II were performed with Beach phenol insoluble. Figure 4 shows that the inhibition curves with agglutinin II are displaced so that smaller amounts of material are needed. However when agglutinin I was studied with Beach the curves were similarly displaced; inhibition with Beach phenol insoluble was more favorable perhaps because of differences in number of determinants or in their binding affinity.

The influence of bivalent metal ions on the precipitin reaction was evaluated with agglutinin I. It was dialyzed against saturated EDTA solution in 0.02 M HCl. After 7 days, a portion was dialyzed against a 0.9% NaCl and another against 0.9% saline containing 0.1 M $CaCl_2$. Precipitin assays with both fractions and Horse 4 25% showed no differences in the amounts of blood group substance needed to precipitate 50% of the total nitrogen. The maximum amount of precipitable N was slightly higher with the material dialyzed against $CaCl_2$.

Discussion

The hemagglutinins of *Axinella polypoides* were purified by affinity chromatography on Sepharose 4B using 0.01 M galactose for elution. Three fractions were then isolated by preparative polyacrylamide gel electrophoresis, ion-exchange chromatography on DEAE-cellulose, and preparative agarose gel electrophoresis. Two major hemagglutinating fractions, agglutinin I, eluting first from DEAE-cellulose, and agglutinin II, the fastest component in preparative polyacrylamide electrophoresis, were obtained in amounts adequate for study. Agglutinin I gave one major and two faint bands and agglutinin II only one band in polyacrylamide electrophoresis. In immunoelectrophoresis with rabbit antiserum to crude extract of *Axinella polypoides*, one precipitin line is observed with agglutinin I and none with agglutinin II. In analytical isoelectric focusing agglutinin I displays two bands, a major band at 3.8 and a minor at 3.9; agglutinin II shows one band at pH 3.8. From electrophoresis in sodium dodecyl sulfate-polyacrylamide, the molecular weights of agglutinins I and II are about 21 000 and 15 000, respectively. Agglutinin I is split by β-mercaptoethanol to a component of 15 000 and a fragment which could not be detected; agglutinin II is not affected by β-mercaptoethanol. These results are in agreement with ultracentrifugation experiments, which show an $s^0_{20,w}$ of 2.6 for agglutinin I and a $s^0_{20,w}$ of 2.8 for agglutinin II. But since electrophoresis was in sodium dodecyl sulfate, association by noncovalent bonds remains a possibility. Bretting and Renwrantz (1974) using gel filtration on Sephadex G-75 showed a mixture of agglutinins I and II to have a molecular weight between 17 000 and 21 000. Gold et al. demonstrated (1974) that a lectin of *Axinella spec.* had a molecular weight of 15 000 to 18 000 and an isoelectric point of 3.8 to 3.9. The two *Axinella* agglutinins, and the hemagglutinin from *Streptomyces spec.* (mol wt 11 000, Fujita et al., 1975), are among the smallest found. The molecular weight of wheat germ agglutinin has been revised upward to 36 200 and is a dimer (Nagata and Burger, 1974; Rice and Etzler, 1974). Both agglutinins from *Axinella polypoides* are high in glutamic and aspartic acids and low in cysteine and methionine; histidine and tyrosine are absent in agglutinin I, but agglutinin II contains 0.75 and 5.65 residues per mol, respectively. There is about twice as much serine in agglutinin I as in II. Lectins of plant and animal origin are usually high in acidic amino acids and low in cysteine and methionine (cf. Sharon and Lis, 1972; Lotan et al., 1974; Kawaguchi et al., 1974). Carbohydrate contents below 1% as seen in agglutinins I and II are rare but occur in *Dictyostelium discoideum* (Simpson et al., 1974), in the small seed lentil (Fialova et al., 1975) and in concanavalin A (Agrawal and Goldstein, 1968).

Both agglutinins precipitate with human blood group A_1, A_2, B, H, Le^a, and precursor I substances from ovarian cysts and with H substances after first and second Smith degradations. The precipitin data do not by themselves provide any insight into the groupings with which the lectins are reacting except that they are not blood group specific. Inhibition studies reveal both agglutinins to have similar specificities with *p*-nitrophenyl-βDGal being the best inhibitor. Terminal DGal linked β1→6 was as potent as *p*-nitrophenyl-βDGal with agglutinin I and half as active with agglutinin II. With agglutinins I and II DGal is respectively $\frac{1}{20}$ and $\frac{1}{12}$ as active as disaccharides with a terminal DGal linked β1→6 and βDGal residues linked 1→3 or 1→4 are four times and twice as active as DGal. Terminal DGal residues linked α are generally less effective than those linked β, but oligosaccharides with DGal linked α1→6 were twice as active as DGal with agglutinin I but of equal potency with agglutinin II. Thus both agglutinins show minor differences in specificity. They react best with oligosaccharides with β-linked DGal but are also capable of

reacting with α-linked DGal.

Terminal α- and β-linked DGal are characteristic of the immunodominant portions of B, Le^a, and precursor I substances. In the H-specific determinant, the βDGal at the nonreducing terminus is substituted by an LFucα-1→2 linked residue. Because LFucα1→2DGalβ1→4DGlc is a very poor inhibitor, it seems unlikely that agglutinin I or II can react with the H-specific determinant. This inference is supported by the finding that JS phenol insoluble, an H-specific blood group substance, after two cycles of Smith degradation to expose terminal β-linked unsubstituted DGal, precipitates the Sepharose 4B fraction much better than does the original material or the first stage of Smith degradation. Only 8 μg of JS 2nd IO_4^- gives 3 μg of specific precipitate N while 35 μg of JS phenol insoluble is required to reach the same level.

Since DGalNAc is very poor inhibitor, it is likely that the α-linked DGalNAc is not the reactive group in A substances and this is supported by the failure of the lectin to react with the streptococcal group C specific polysaccharide with immunodominant DGalNAcα1→3DGalNAcα1→3LRha (Coligan et al., 1975, and personal communication).

It would therefore appear that both agglutinins are precipitating A and H substances by interacting with side chains possessing β-linked unsubstituted DGal at nonreducing terminal ends. Oligosaccharides with terminal nonreducing DGal in β1→3 or β1→4 linkage were isolated from the HLe^b substance JS phenol insoluble after alkaline borohydride degradation (Rovis et al., 1973).

Though many lectins are inhibited by DGal their exact specificities have not been extensively studied. Only lectins which are inhibited better by DGal than by DGalNAc may be considered DGal specific. Among these are *Bandeiraea simplicifolia* (Hayes and Goldstein, 1974), *Streptomyces spec.* (Fujita et al., 1975), *Abrus precatorius* and *Ricinus communis* (Olsnes et al., 1974; Nicolson et al., 1974), and *Salmon salar* and *Salmon gairdeneri* (Voak et al., 1974). As expected from its blood group B specificity, the precipitation of Bandeiraea lectin by guar gum is inhibited best by α-linked DGal which is about four times better than DGal while β-linked DGal is inactive. The salmon lectins are unstable (Voak et al., 1974) and have not been isolated but they also appear to be specific for α-linked DGal, accounting for its B, P, and P^k specificity (Voak et al., 1974). The *Streptomyces spec.* lectin is B specific; however, by hemagglutination inhibition, methyl-α- and -β-glycosides of DGal are of equal potency and twice as active as DGal, and *p*-nitrophenyl-α- and -βDGal are equal but four times active as DGal. The data do not provide insight into the basis for its B specificity.

Ricinus communis as well as *Abrus precatorius* seeds each contain two different lectins. Their specificities seem to be similar. By hemagglutination inhibition with all four lectins, DGal is active. Precipitin inhibition studies with both *Ricinus* agglutinins show that lactose inhibits best and methyl-βDGal is more potent than DGal and methyl-αDGal, indicating specificity for terminal β-linked DGal; the effect of linkage of the DGal to subterminal oligosaccharides has not been studied. The relative discriminating ability of *Axinella* agglutinins I and II for DGal linked β1→6 will make them a useful tool in carbohydrate research.

Since the number of binding sites per molecule in *Axinella* agglutinins (mol wt 15 000–17 000) is not known, it has been considered to be a monomer and hemagglutination hypothesized to result from polymerization. In our studies agglutinins I and II reacted with blood group substances like other lectins previously studied (Etzler and Kabat, 1970; Hammarström and Kabat, 1969). Since each agglutinin by itself precipitates with blood group subtances, any polymerization would not have to involve agglutinins I and II. Aged solutions of agglutinin I and II did not show additional bands in polyacrylamide electrophoresis as compared with those freshly prepared, as might be expected if aggregation had occurred. Until the valence of the *Axinella* agglutinins is established, the basis for agglutination will be unresolved.

Bivalent cations which were thought to be involved in the polymerization of *Axinella spec.* (Gold et al., 1974) are either so strongly bound that they cannot be removed by EDTA or are not required for the precipitin reaction. *Axinella polypoides* agglutinin I precipitated with Horse 4 25% as well after EDTA treatment as did the untreated material. EDTA-treated material, dialyzed against solutions containing Ca^{2+}, did not affect the quantitative precipitin curve.

The biological functions of these and other lectins are unknown. One may attempt to compare them to the aggregation factor system in sponges which mediates cell contact (Turner and Burger, 1973; Weinbaum and Burger, 1973; Kuhns et al., 1974). These workers propose that two proteins must cooperate for cell contact to occur. One protein, the baseplate, is attached to the cell surface and interacts with the carbohydrate moiety of another protein termed aggregation factor. The interaction between baseplate and aggregation factor can be inhibited specifically by sugars. In analogy to the aggregation factor system, the sponge lectins might correspond to the baseplate protein because of their reaction with carbohydrates and their specific inhibition by oligosaccharides. They are species specific as are the aggregation factors. Rabbit antiserum against crude extracts of *Axinella polypoides* neither cross-reacts with *Axinella darmicormis,* a closely related species, nor with the more distant *Aaptos papillata.* However, too little is known about the structure of the baseplates for further comparison.

The aggregation factor in *Microciona parthena* is a large molecule with a molecular weight of several million, containing 50% carbohydrate and requiring Ca^{2+} for interaction (Henkart et al., 1973). These features do not accord with the properties of the agglutinin. However, Margoliash et al. (1965) showed it to break into subunits with a molecular weight of 15 000 to 22 000 on dialysis against Ca^{2+}-free medium. It is not known whether these subunits contain carbohydrate. Further data are needed to clarify whether sponge agglutinins are analogous to baseplate-aggregation factors.

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