Monosaccharides as Specific Precipitinogens of Eel Anti-Human Blood-Group H(O) Antibody*

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ABSTRACT: A novel phenomenon is described: the specific precipitation by monosaccharides of a 7S globulin from eel serum which possesses anti-human blood-group H(O) specificity and many characteristics of an immune antibody. 3-*O*-Methyl-D-fucose and 3-*O*-methyl-D-galactose gave typical precipitin curves with this eel anti-H(O) antibody as did human blood-group H(O) glycoprotein and the highly blood-group H(O)-specific polysaccharides from *Sassafras albidum* and *Taxus cuspidata*. Each precipitinogen removed at equivalence >85% of the precipitin activities of the sera. Inhibitory activities of haptens were of the same order regardless of the precipitinogen employed. Soluble eel anti-H(O) antibody was recovered quantitatively by dialysis of monosaccharide–antibody precipitates. The isolated protein agglutinated human blood-group O erythrocytes and was precipitated equally with each of the antigens and monosaccharides. These monosaccharides are the smallest uncharged, unaggregated precipitinogens yet described. It is difficult to reconcile these findings with the lattice theory of immune precipitation because of the lack of characteristic repeating reactive areas on the monosaccharides. Between 5 and 8 moles of monosaccharide appear to bind per mole of antibody. We have described previously the minimal hapten structure required for complementarity with the eel antibody. While the overall contours of the presumed combining sites of the inhibitors and the precipitinogens are closely similar, there are some additional specific requirements for monosaccharides to function as precipitinogens. They seem to consist, within this contour, of three vicinal oxygens protruding from a C-1 pyranose ring. The oxygen at C-3 carries an apolar group while the two oxygens flanking this group are very well capable of hydrogen bonding. One of these latter oxygens must be equatorial and trans to the oxygen at C-3 and the other axial and cis. Additional apolar groups are not compatible with precipitating activity of the pyranose structure. Chemically the antibody is characterized by predominance of Asp, Gly, Glu, Ala, Ser, and Thr and the scarcity of Met, Trp, and Phe. It possesses equal quantities of NH_2-terminal Ser and Ala and of COOH-terminal Ser and Gly. Only traces of carbohydrates were found.

The structural requirements for activity with the anti-human blood-group H(O) antiserum from the eel have been studied extensively, since it is one of the most potent and specific reagents detecting blood-group H(O)[1] antigens on erythrocytes and in solution (Watkins and Morgan, 1952; *cf.* Springer and Williamson, 1962). Although the stimulus provoking the reactive protein is unknown, it is generally considered to be an antibody.[2] Terminal nonreducing L-fucose residues are a prerequisite for H(O) activity of human and animal blood-group substances with this serum, as determined by hemagglutination inhibition tests, and three necessary preconditions for significant blood-group H(O) activity of fucosides were postulated: the fucose residue must belong to the L series, it must be of pyranosidic structure and any linkage must be α glycosidic (Watkins and Morgan, 1952; Kuhn and Osman, 1956). However, we found that some methyl ethers of L-fucose were more active than L-fucose (Springer *et al.*, 1956) and even some derivatives of the enantiomorphous D-fucose were potent inhibitors of eel anti-H(O) antibody (Springer and Williamson, 1962; Springer *et al.*, 1964). In addition, the unrelated 3-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-D-galactose were as active as the most active methyl ether of L-fucose (Springer *et al.*, 1965). From these studies arose the novel concept that the complementary grouping for an anti carbohydrate antibody may be smaller than a monosaccharide (Springer *et al.*, 1964; Desai and Springer, 1965). For proper spatial arrangements, however, such a grouping needs to be part of a ring structure (Kabat, 1962).

Surprisingly, two of the highly active monosaccharides in the hemagglutination inhibition assay, D-digitalose and 3-*O*-methyl-D-galactose, precipitated the anti-H(O) antibody of most eel sera (Springer *et al.*, 1964, 1965; Kolecki and Springer, 1965).

We report here that both these monosaccharides gave typical precipitin curves with the eel antisera which were closely similar to those obtained with the blood-group H(O)-specific macromolecules. The protein was quantitatively recovered from the precipitates in pure and active form. We also present our findings on the structures required for haptens to act as

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[1] Recent investigations indicate that blood-group H(O) specificity as found in secretions is not the indirect result of the action of a gene allelomorphic to those which express A and B specificities but is rather due to the absence of the expression of these two genes. What is detectable serologically in persons of blood-group O is the H substance, which apparently is the immediate precursor of the A and B substances (*cf.* Watkins, 1966). The situation may be somewhat different for the blood-group substances on cells (Wiener *et al.*, 1966). In order to do justice to historical development and to avoid confusion with a number of unrelated antigens named H, we will use the term H(O) for the activity described.

[2] We likewise will refer to this protein as an antibody although we will point out in this and the following paper that the reactive protein, besides important similarities with human IgG globulin, exhibits some profound physical differences from mammalian antibodies.

precipitinogens rather than as inhibitors and on the mode of interaction between antibody and haptens as well as on the composition and the terminal amino acids of this eel antibody.

Materials and Methods

Haptens, Related Monosaccharides, and Antigens. Sugars used were prepared in this laboratory or obtained from others as described earlier (Springer and Williamson, 1962; Springer *et al.*, 1964, 1965). All sugars were analytically pure. Wherever possible, the more readily available D-digitalose was employed instead of 3-*O*-methyl-D-galactose. Preparations from four different sources, including a crystalline sample kindly given us by Professor T. Reichstein, were employed. Similarly, the crystalline 3-*O*-methyl-D-galactose was not only isolated from *Sassafras* polysaccharide by us but also supplied by Professor T. Reichstein and Professor B. Lindberg.

Isolation, purification, and properties of the blood-group H(O)-specific antigens used have been described previously (Springer *et al.*, 1964, 1965). The highly active, physicochemically and immunochemically homogeneous polysaccharide Ca-606 isolated from *Sassafras albidum* was employed throughout. Since the highly potent human ovarian cyst glycoprotein 277/P1/WS (donated by Professor W. T. J. Morgan) was available only for preliminary experiments, subfractions of human blood-group H(O)-active ovarian cyst glycoprotein $CaII_b$ prepared by us were used although they precipitated only between 30 and 60% as much antibody protein as did glycoprotein 277/P1/WS and were of lower activity in the hemagglutination inhibition assay.

Antisera. Sera, 3–8 ml/fish, were obtained as described previously from live eels (*Anguilla rostrata*) weighing >1.5 lb each (*cf.* Springer and Williamson, 1962). The eels were caught between September and December in fresh or brackish water in eastern Canada, Maine, Maryland, and Virginia.

About one-third of the sera were of high enough anti-H(O) titer (>32 at 22–25°) to be employed in the present experiments. Extensively hemolyzed samples were discarded. Usually sera with a reciprocal preabsorption anti-H(O) titer of 512 or greater were pooled as "h" and those with a titer of 64–256 as "m" and processed in lots of 100–200 ml. The pools were heated at 56° for 35–45 min to inactivate the potent hemolysins and then absorbed with one-quarter to two-thirds volume of fresh, thoroughly washed human blood-group A_1B erythrocytes for 30–40 min at *ca.* 15°. Absorption reduced the anti-H(O) titers by one to two tubes and titers against A_1B erythrocytes were <2. All experiments in this report were performed on pooled, absorbed sera except where indicated otherwise. From two pools, a portion was separated prior to decomplementing. Most sera were stored at 4° after sterilization with 0.25% phenol and 0.015% thimerosal (final concentrations). Thimerosal alone did not interfere with methylpentose determination and was therefore added to those sera and antibody solutions on which these measurements were performed. These latter sera were employed as soon as possible and Seitz-filtered immediately before use.

Anti-eel anti-human blood-group H(O) antiserum was prepared by immunizing three New Zealand white male rabbits (3.6–4.2 kg) with two subcutaneous injections separated by a 4-week interval immediately after an initial bleeding. Each injection consisted of 1.1 mg of alum-precipitated (*cf.* Chase, 1967) isolated antibody in 1.0 ml of buffered saline (see below). An additional rabbit was injected in the same fashion with precipitated alum only. Bleeding was 10 days after the last injection; antibody precipitating the anti-human blood-group H(O) protein had been stimulated in two of the immunized rabbits. Neither the preimmunization sera nor the samples from the control animal contained such precipitins.

Other sera and the *Lotus tetragonolobus* reagent used in this study have been described earlier (Springer and Horton, 1964; Springer *et al.*, 1966). All sera and antibody solutions were centrifuged at *ca.* 1500*g* at 4° for 12–20 hr immediately before use in precipitin assays.

Solutions and Erythrocytes. Aqueous 0.05 M phosphate buffer in 0.10 M NaCl (pH 7.3) (buffered saline) was generally used as diluent and solvent; 0.01% thimerosal (final concentration) was added for dialysis or precipitin assays. Solubility, biological stability, and precipitability of isolated antibody were determined in isoosmotic buffers. Sodium citrate buffer (Gomori, 1955) was employed at pH 4.45. Sodium phosphate buffers adjusted to isoosmolarity with sodium chloride were used between pH 5.50 and 8.00, and Veronal buffers were employed between pH 8.50 and 9.05 (*cf.* Springer *et al.*, 1966).

Human O and A_1B erythrocytes, stored for <2 weeks, were used as described previously (Springer *et al.*, 1964).

Analytical Procedures. All sugars and the isolated antibody were dried to constant weight at 10^{-1} to 10^{-2} mm over P_2O_5 at room temperature (22–25°) except for elemental analyses and carboxyl-terminus determinations, for which they were dried at 80°. Critical weighings were performed on a Cahn gram electrobalance (Ventron Instrument Corp., Paramount, Calif.). Routine examinations for bacteriological contamination were carried out by standard procedures (*cf.* Springer *et al.*, 1959); incubation of blood-agar plates was at 22–25° and 37°.

The possibility of aggregation of monosaccharides in water was assessed with a Mechrolab vapor-pressure osmometer Model 302 (Hewlett-Packard, Mountain View, Calif.) at 37°. The measurements on each sugar were carried out at four different concentrations ranging from 12.5 to 555 μmoles per ml with sucrose as standard. In addition cryoscopic determinations were performed in deionized water *and* in buffered saline with a Knauer semi-micro osmometer. The freezing point depression afforded by 2.00, 4.00, 8.00, and 16.00 μmoles per ml of the various sugars was compared to the depression given by urea and NaCl of the same normalities. Further cryoscopic determinations were made on 0.1 and 0.2% monosaccharide solutions in 0.15 M aqueous NaCl by Huffman Microanalytical Laboratories Inc., Wheatridge, Colo., with sucrose as standard. These laboratories in addition performed N (Dumas) and wet ash analyses (with H_2SO_4).

Spectrophotometric measurements were carried out in a Beckman DU spectrophotometer to which a Gilford 220 optical density converter, photometer-indicator, and automatic cuvet positioner were attached. All samples, including the reagent blank, were read against water.

All methylpentoses (*cf.* Springer *et al.*, 1965), were determined by the procedure of Dische and Shettles (1948). The difference in optical densities between D-digitalose, L-rhamnose, and L-fucose compared *on a molar basis*, was ±1% at concentrations between 0.03 and 0.3 μmole. When organic substances in addition to methylpentose were present, the unknown sample, to which all reagents had been added except cysteine, was included as an additional blank (Dische and Shettles, 1948); internal standards were also included. Four duplicate levels of the methylpentose investigated were employed as standards.

Methylpentose in the supernatants and in the washes of precipitates was determined on nondeproteinized samples and in case of the supernatants after deproteinization with $Ba(OH)_2$–$ZnSO_4$ according to Somogyi (1945) as well as by ex-

traction of freeze-dried samples with 0.5 ml of 8% trichloroacetic acid as by Heidelberger *et al.* (1955). The trichloroacetic acid as well as dialysis procedures were used in preparation for measurement of the methylpentose content in precipitates; because of the small quantities of D-digitalose it was necessary to combine up to eight washed precipitates. All standards and controls were pretreated in the same way as the samples proper.

Total hexose and heptose were measured spectrophotometrically as D-glucose and D-glycero-D-*gulo*-heptose, respectively, by the primary cysteine–sulfuric acid reaction of Dische (1955). Human γ-globulin (Pentex, fraction II no. 22) was included as reference. Sialic acid was determined by Warren's procedure (1959).

All quantitative amino acid analyses, except Trp were performed on a Beckman 120C amino acid analyzer (Spackman *et al.*, 1958) by Dr. P. Weber. For amino acid determinations 1.0 or 2.0 mg of material was hydrolyzed for 16 hr at 115° under nitrogen with 4.00 ml of 6 N HCl containing 0.28 μmole of norleucine. The material was dried in a rotary evaporator, dissolved in 1.25 ml of 0.2 N sodium citrate buffer, pH 2.20, and 1.00 ml of this solution was added to the column. Cysteic acid was measured after oxidation with performic acid in an ice bath prior to hydrolysis (*cf.* Hirs, 1956). Determination of amino sugars in the analyzer differed from that of amino acids only in that the hydrolysis was with 3 N HCl for 3 hr at 100°. Trp was measured in this laboratory as by Winkler (1934) except that all volumes were reduced by four-fifths and the absorbance determined at 580 nm.

Amino- and carboxyl-terminal amino acids were assessed both qualitatively and quantitatively by standard procedures with the inclusion of appropriate standards and controls.[3] The FDNB[4] (Sanger, 1945), PTC (*cf.* Fraenkel-Conrat *et al.*, 1955), and dansyl methods (*cf.* Gray, 1967) were employed in the qualitative assessment of NH_2-terminal amino acids. Hydrazinolysis (Akabori *et al.*, 1952) followed by dinitrophenylation or dansylation was used for identification of COOH-terminal amino acids. For quantitation of NH_2-terminal amino acids both FDNB and DANS procedures were used directly. Carboxyl-terminal amino acids were quantitated, after prior hydrazinolysis, by the dansylation procedure only.

Hemagglutination and Hemagglutination Inhibition Tests. All tests were done at least twice at 22–25°. Arithmetic averages of the results are reported. The volume of all reagents in a test was constant; either 0.05 or 0.02 ml was used throughout. Mode of titration, incubation, and interpretation were the same as described previously, as were the standards and the positive and negative controls which were included in all titrations (Springer *et al.*, 1959; Springer and Horton, 1969). A different pipet was used for each tube in a titration series. In all inhibition assays four minimum agglutinating doses of serum were employed. Agglutination was read microscopically by three individuals 90 min after the addition of a 0.5% suspension of red cells. Agglutinin titers are expressed as reciprocals and are called "titers." Hapten activities are given on a weight and molar basis in terms of the highest hapten dilution per milliliter giving complete inhibition of agglutination. Activities are given for dilutions before the addition of serum and erythrocyte suspension.

Precipitin and Precipitation Inhibition Tests. These quantitative procedures, based on those of Heidelberger and Kendall (*cf.* Kabat, 1961a), were used with the previously described modifications (Springer *et al.*, 1964, 1965); 0.5-ml portions of all reactants were used. The samples were incubated for 30 min in an ice bath and then for 7–10 days at 4° with daily agitation.

In inhibition studies haptens were added in 0.5-ml volume to 0.5 ml of undiluted serum and incubated in an ice bath for 30 min; thereafter 0.5 ml of precipitinogen solution at a concentration corresponding to the beginning of equivalence was added and the procedure continued as above. The quantity of precipitinogen employed in this area was called "optimal amount." Areas of the precipitin curves are defined as is customary for immunoprecipitation (*cf.* Kabat, 1961a). The standard reference in inhibition assays was eel serum to which only the precipitinogen to be inhibited had been added in the same quantity as in the inhibition tests. Negative controls in all assays consisted of eel serum or isolated antibody alone and antigen or hapten alone each adjusted to appropriate volume. All precipitinogen and hapten concentrations are given as μg or μmoles per 1.5 ml of reaction mixture unless stated otherwise.

Precipitates obtained with antigens were recovered by centrifugation and washed twice with 1.5 ml of ice-cold buffered saline. Because of their somewhat greater solubility the precipitates obtained with monosaccharides were washed only once. The washed precipitates were dissolved in 2.5 ml of water containing 0.01 mequiv of NaOH and their protein content determined colorimetrically (Heidelberger and MacPherson, 1943) against a human γ-globulin standard (Pentex, fraction II no. 22; N, 15.96%; ash, 1.0%) at four different concentrations. All solvents and reagents were reduced by one-half, if the expected protein amounted to <75 μg. The extinction given by the eel antibody was the same as that given by the human γ-globulin between 15 and 200 μg of protein with a deviation of $\pm 1\%$ below and $\pm 3\%$ above 100 μg of protein. All protein values were expressed as μg of antibody precipitated per 0.5 ml of undiluted eel serum, unless indicated otherwise.

Samples, controls, and standards were always assayed in duplicate or triplicate, except in some tests on supernatants. Inhibition was calculated as the percentage decrease in the amount of protein precipitated in the presence of inhibitory hapten as compared to that precipitated in its absence.

Assays for Antibodies and Blood-Group Specific Substances in Supernatants of Precipitin Tests. RESIDUAL ANTIBODIES were determined by agglutination and precipitin assays before and after dialysis. Dialyses were always performed at 1–6° in prewashed Union Carbide cellulose dialyzer tubing on a mechanical shaker and, unless stated otherwise, against four times (changed every 12 hr) 20 volumes of buffered saline.

Precipitin tests for the measurement of antibodies in supernatants were modified from the standard procedure because of the dilution of all reagents; to 1.5 ml of supernatant 0.1 ml of the second precipitinogen was added. One of the two serum control tubes of the original test served again as negative control, while to the other the precipitinogen employed for the test on the supernatant fluids was added in optimally precipitating quantity.

RESIDUAL BLOOD-GROUP H(O)-SPECIFIC SUBSTANCES in the supernatants were determined by hemagglutination inhibition and precipitin assays. Undialyzed supernatants were heated in boiling water for 15 min and insoluble material removed by centrifugation at 12,800*g;* in the precipitin assays 1.0 ml of these boiled supernatants was then added to 0.5 ml of eel

[3] G. F. Springer, and I. Y. Huang to be submitted for publication.

[4] Abbreviations used are: DANS (dansyl), 1-dimethylaminonaphthalene-5-sulfonyl; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; PTC, phenyl thioisocyanate.

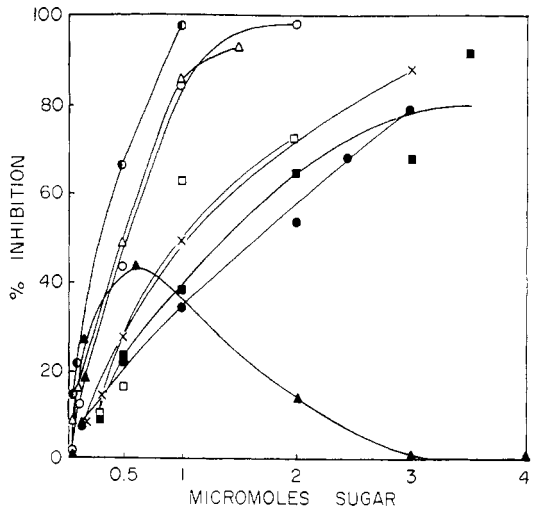

FIGURE 1: Inhibition by fucose derivatives of eel anti-blood-group H(O) antibody precipitation with human ovarian cyst blood-group H(O) glycoprotein. (●) L-Fucose, (○) methyl α-L-fucopyranoside, (△) methyl 3-*O*-methyl-α-D-fucopyranoside, (◐) methyl 3-*O*-methyl-β-D-fucopyranoside, (□) 2,3-di-*O*-methyl-D-fucose, (■) 2-deoxy-3-*O*-methyl-D-fucose, (×) 3-*O*-methyl-L-fucose, and (▲) 3-*O*-methyl-D-fucose (antigen, 150 μg of CaII$_b$2+3PF; antiserum, 0.5 ml of eel h^{-1}).

serum. A control, in addition to the regular ones, consisted of the supernatants of appropriately diluted boiled eel serum to which no blood-group active substance had been added; the reading of this control always was in the same range as that of the serum blank.

Agar Gel Diffusion and Microprecipitin Tests. The two-dimensional double-diffusion procedure (*cf.* Ouchterlony, 1962) was performed as described before (Springer *et al.*, 1965) in 0.8% Oxoid Ionagar II (Colab Laboratories, Chicago Heights, Ill.) containing 3.75% glycine and 0.01% thimerosal in flat-bottom Petri dishes. The reagents were used in 0.1- to 0.2-ml volume and the dishes incubated in a water-saturated atmosphere either at 22–25° or at 4°. Microprecipitin tests were carried out as previously at 4° in capillaries of 0.5- to 0.9-mm diameter and 90 mm length (Springer *et al.*, 1965). Both types of precipitin tests were observed for about 1 month.

Immunoelectrophoresis. The Shandon chamber, slide rack, scraper, and gel punch were purchased from Colab, the power supply was Type 3371C from LKB Instrument Co. (Rockville, Md.) and the high-resolution buffer HR no. 51104 pH 8.8, μ 0.05, was procured from Gelman Instruments Co. (Ann Arbor, Mich.). New microscope slides 10 × 2.5 cm were cleaned and covered with agar and the procedure carried out as described in Colab Operating Instructions No. 11-192-3. Staining was done with Amido-Schwarz 10B.

Results

Blood-Group Specific Inhibition and Precipitation of Eel Anti-Human Blood-Group H(O) Sera by Monosaccharides. Table I shows results obtained with haptens in the semiquantitative hemagglutination inhibition assay. Certain *O*-methyl ethers of L- and D-fucose and D-galactose had high and approximately equal activities. A minor alteration like displacement of the single substituent in mono-O-methylated L-fucose from C-3 to C-4 completely inactivated a highly active compound, while another minor change such as 3-O-methylation of the inactive D-fucose or D-galactose produced high activity *de novo*.

TABLE I: Some Highly Blood-Group H(O)-Active Sugars.

Test Substance	Min Amt Completely Neutralizing 4 Hemagglutinating Doses of Eel Serum	
	mg/ml	μmoles/ml
L-Fucose series[a]		
L-Fucose	0.1	0.6
2-*O*-Methyl-L-fucose	0.05	0.3
3-*O*-Methyl-L-fucose	0.05–0.1	0.3–0.6
2,3-Di-*O*-methyl-L-fucose	0.05	0.3
D-Fucose series[b]		
D-Fucose	>5	>30
3-*O*-Methyl-D-fucose[a]	0.05	0.3
2,3-Di-*O*-methyl-D-fucose	0.05	0.3
D-Galactose series[c]		
D-Galactose	>5	>25
3-*O*-Methyl-D-galactose[d]	0.1	0.5
2,3-Di-*O*-methyl-D-galactose	0.1	0.5

[a] Methyl substituents at C-4 and/or C-5 inactivate. [b] Methyl substituent at C-5 inactivates active methyl ethers. [c] Methyl substituents at C-4, at C-6, and at C-2, C-4, and C-6 do not activate the inactive parent compound D-galactose. [d] Methyl substituent at C-4 inactivates. (For related active and inactive sugars, see Springer *et al.*, 1964, 1965.)

Figure 1 summarizes the results of inhibition in quantitative precipitin assays with ovarian cyst glycoprotein as precipitinogen. The two anomeric methyl 3-*O*-methyl-D-fucopyranosides were the most powerful inhibitors. Inactive fucose derivatives are not depicted. Remarkably, 3-*O*-methyl-D-fucose, which appeared to be an excellent inhibitor up to about 0.5 μmole, decreased its inhibitory effect beyond this level with this serum pool and most individual eel sera; also, besides of the smooth precipitate given by the blood-group H(O) antigen there was some coarse flocculate which was soon recognized as typical for antibody precipitated by this monosaccharide. Inhibition was no longer demonstrable if 3 μmoles or more of D-digitalose were added. In contrast, the enantiomorph, 3-*O*-methyl-L-fucose, was an excellent inhibitor at all levels tested. No other sugar of the L- and D-fucose series was found to precipitate eel antibody, but, as Figure 2 shows, 3-*O*-methyl-D-galactose behaved similar to 3-*O*-methyl-D-fucose in that it reached a peak of inhibitory activity at about 0.5 μmole; thereafter, precipitation dominated. In contrast, 2,3-di-*O*-methyl-D-galactose did not precipitate and was almost 1.7 times as potent an inhibitor as L-fucose at the 1.0-μmole level (Figure 2); 2-*O*-methyl-D-galactose was virtually inactive. In a series of 44 individual sera of medium to high titer two did not precipitate with the two 3-*O*-methyl sugars as determined in microprecipitin assays; they did precipitate with antigens.

These precipitating monosaccharides could be transformed by minor changes into powerful inhibitors of precipitation of the eel antibody by blood-group specific substances. Table II summarizes our findings.

D-Digitalose and 3-*O*-methyl-D-galactose gave typical quantitative precipitin curves with eel serum which rose in the area of antibody excess, formed a plateau in the equivalence zone, and

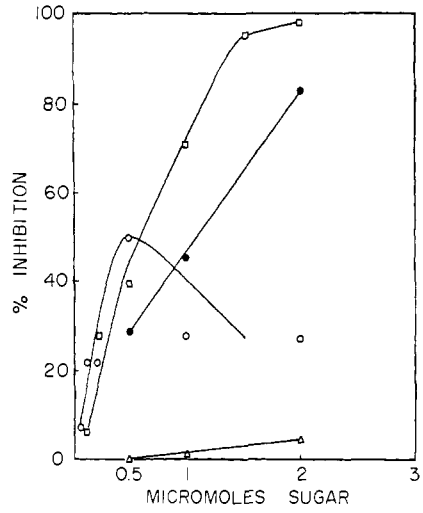

FIGURE 2: Inhibition by galactose derivatives of eel anti-blood-group H(O) antibody precipitation with human ovarian cyst blood-group H(O) glycoprotein. (●) L-Fucose, (□) 2,3-di-*O*-methyl-D-galactose, (○) 3-*O*-methyl-D-galactose, and (△) 2-*O*-methyl-D-galactose (antigen, 110 μg of $CaII_bIII_c$; antiserum, 0.5 ml of eel $m^0/1/63$).

TABLE II: Transformation of Antibody Precipitating Monosaccharides into Inhibitors by Changes on a Single C Atom.

Precipitinogen	Inhibitor	% Inhibn[a] Relative to L-Fucose
3-*O*-Methyl-D-galactose[b]	2,3-Di-*O*-methyl-D-galactose	100–200
3-*O*-Methyl-D-fucose[c]	2,3-Di-*O*-methyl-D-fucose	100–200
	2-Deoxy-3-*O*-methyl-D-fucose	100
	Methyl 3-*O*-methyl-α-D-fucopyranoside	200–500
	Methyl 3-*O*-methyl-β-D-fucopyranoside	500

[a] Hemagglutination inhibition and precipitation inhibition. [b] 3,4-Di-*O*-methyl-D-galactose is inactive. [c] 3-Deoxy-D-fucose is inactive.

depending on the serum pool employed, either declined or remained level in the area of precipitinogen excess. These precipitin curves were closely similar to those obtained with the macromolecular blood-group H(O)-specific precipitinogens (Figure 3) with all serum pools tested except one, where in repeated tests over a 2-year period the *Sassafras* antigen precipitated 271 μg of antibody at equivalence but D-digitalose only half of this amount. Possibly it contained some serum lots not precipitable by monosaccharides. This pool was not used subsequently.

Previous experiments had shown that a *Sassafras* antigen preparation corresponding to that employed here, the H(O)-specific polysaccharide from *Taxus cuspidata* and human ovarian cyst glycoprotein 277/P1/WS precipitated, from a given serum, the same quantity of eel antibody and gave precipitin curves of closely similar shape (Springer *et al.*, 1965).

Eighteen eel serum pools were employed and all precipitated with the four different precipitinogens. Turbidity occurred within 5 min to 24 hr after addition of 0.5 μmole of D-digitalose or 3-*O*-methyl-D-galactose, depending on the serum pool. Over 120 complete precipitin curves were obtained. More than 80 of these assays were with undiluted, decomplemented, and absorbed eel sera, and 23 of these were with D-digitalose. Five different lots of D-digitalose and ten different eel serum pools were used. Maximal precipitation varied with the sera and occurred between 2.0 and 6.0 μmoles of D-digitalose. The arithmetic average of antibody protein maximally precipitated per 0.5 ml of eel serum in these 23 assays was 458 μg (range 148–1360 μg). For 7 of the 10 serum pools the range of antibody protein precipitated at equivalence was between 222 and 588 μg.

Figure 1 shows that 0.5 μmole of D-digitalose inhibited precipitation of eel antibody with blood-group H(O) antigen by >40%. Nevertheless, even 0.1 μmole of D-digitalose added to three different eel sera by itself precipitated some antibody. The average quantity of protein precipitated at 0.1–0.3 μmole was not more than 10% of that in the equivalence zone.

A quantitative precipitin test was performed at 37° and compared to a control carried out at 4°. At 37° precipitates were first recognized after overnight incubation with 2.0 μmoles or more of D-digitalose. The experiment was interrupted after 26-hr incubation; no bacteria were demonstrable by routine culturing procedures. The collection of the precipitates was standard except that the series incubated at 37° was centrifuged at 30°. The supernatants of both sets were reincubated at 4° for 9 more days and additional precipitates were recovered as usual. It can be seen from Table III, which lists the results of the first part of the experiment, that during the incubation at 37° precipitation resulted at all D-digitalose concentrations tested, and that after 26 hr 6.0 μmoles of D-digitalose precipitated nearly 25% of the antibody precipitated during the same time at 4°; this corresponded to about one-fifth of the total anti-H(O) antibody precipitable. This table also demonstrates that during the first day of incubation at 4° about 80% of the total antibody precipitable by a given

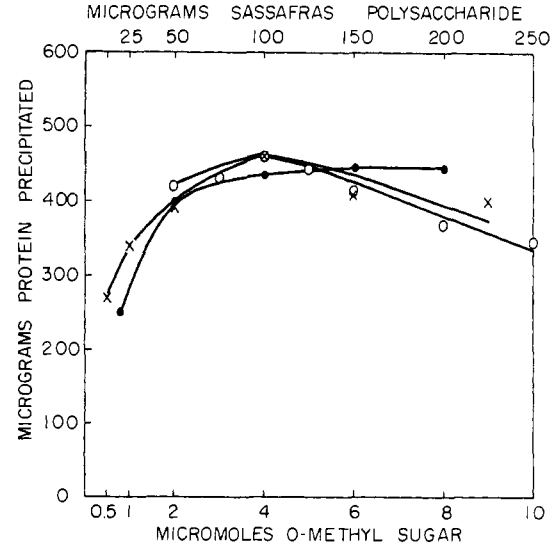

FIGURE 3: Precipitation of eel anti-human blood-group H(O) serum by various precipitinogens. (×) 3-*O*-Methyl-D-fucose, (●) 3-*O*-methyl-D-galactose, and (○) *Sassafras* polysaccharide (Ca-606).

TABLE III: Effect of Temperature on Precipitation of Anti-Human Blood-Group H(O) Eel Antibody by 3-*O*-Methyl-D-fucose.

D-Digitalose Added (μmoles)	Antibody Precipitated during First 26 hr (% of Antibody Precipitable[a]) 37°	4°
2.0	1.2	79.2
4.0	2.6	86.6
6.0	18.7	83.0

[a] On 10-days incubation at 4°.

D-digitalose concentration at 4° was obtained. The sum of antibody precipitated during the first and second incubation was the same for the experimental (37°) and the control (4°) sets.

Precipitin tests were also performed with modifications: the samples were incubated during the first 90 min at 22–25° instead of in an ice bath; or the sera were exhaustively dialyzed against buffered saline prior to use, freeze-dried, and reconstituted with distilled water; or monosaccharides recovered after dialysis were employed. None of these manipulations influenced the results. Also, the presence of a H(O)-inactive carbohydrate such as 3-*O*-methyl-D-glucose did not influence the precipitability of eel antibody by D-digitalose. Serological activities were completely destroyed within 15 min when sera diluted 1:3 were heated in a boiling-water bath.

The effect of dilution of eel serum on its precipitability is shown in Figure 4. The amount of protein precipitated decreased in parallel with increasing serum dilution at all precipitinogen levels. The steepness of the slope of the curves in the area of large antibody excess decreased also, indicating diminished avidity of the diluted sera. A shift of the maximum to the left on dilution was noted with the cyst glycoprotein ($CaII_b2+3PF$). Planimetry of the areas under the curves gave

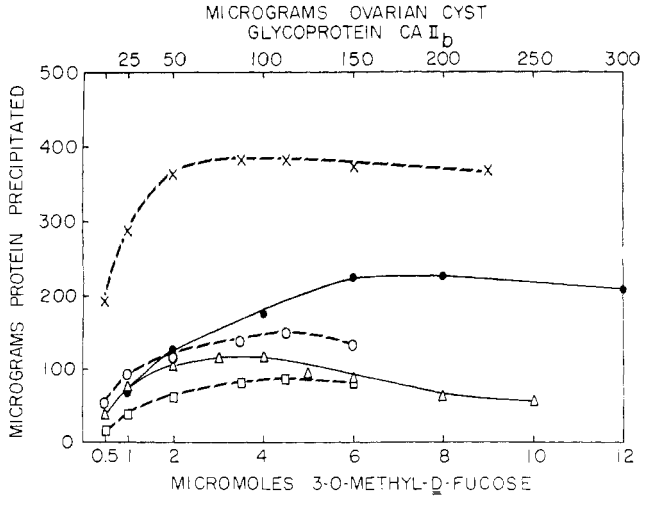

FIGURE 4: Precipitin curves obtained with diluted eel anti-blood-group H(O) sera. Precipitinogen $CaII_b2+3PF$ and antiserum, 0.5 ml of h/1/63: (●) undiluted antiserum and (△) antiserum diluted 1:2. Precipitinogen, 3-*O*-methyl-D-fucose, and antiserum, 0.5 ml of m/11/65: (×) undiluted antiserum, (○) antiserum diluted 1:2, and (□) antiserum diluted 1:3.

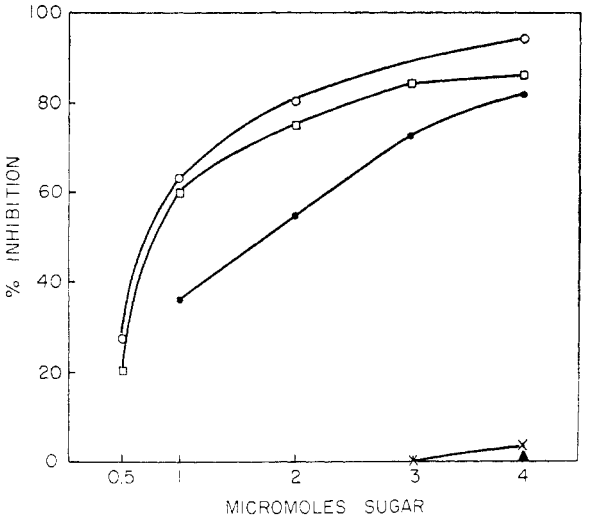

FIGURE 5: Inhibition by haptens of eel anti-blood-group H(O) antibody precipitation with 3-*O*-methyl-D-fucose. (●) L-Fucose, (○) methyl α-L-fucopyranoside, (□) 2,3-di-*O*-methyl-D-galactose, (×) D-glucose, and (▲) 3-*O*-methyl-D-glucose (precipitinogen, 2.0 μmoles of 3-*O*-methyl-D-fucose; antiserum, 0.5 ml of eel m/1/67).

proportions of undiluted to twofold and threefold diluted serum of 1.0:0.36:0.19 when D-digitalose was used as precipitinogen. The proportion was 1.0:0.50 for undiluted to twofold diluted serum with the cyst glycoprotein as precipitinogen. With a different eel serum and D-digitalose the proportion of protein precipitated in the equivalence zone from undiluted compared to 1:2 diluted serum was 1.0:0.49.

To aliquots of two <3-weeks-old serum pools which had neither been decomplemented nor absorbed with A_1B erythrocytes and which contained powerful hemolysins, guinea pig complement (double strength, Markham Laboratories, Chicago, Ill.) was nevertheless added. They were run in parallel with the same decomplemented sera both before and after absorption. As in the agglutination assays, decomplementing had no influence on the amount of antibody precipitated, whereas absorption with A_1B erythrocytes led to a 15–20% decrease of protein precipitated.

Human anti-blood-group A, B, and H(O) sera, rabbit anti-*Escherichia coli* O_{128} serum, which reacts specifically with human blood-group H(O) antigen (Springer *et al.*, 1961), rabbit anti-*E. coli* O_{86} serum, horse anti-pneumococcus type XIV serum, and the anti-human blood-group H(O) *Lotus tetragonolobus* reagent were not precipitated by D-digitalose.

Inhibition of D-Digitalose Precipitation of Anti-Human Blood-Group H(O) Antibody by Blood-Group Specific Haptens. Figure 5 shows that precipitation of eel antibody by D-digitalose was inhibited by blood-group H(O)-specific haptens such as L-fucose, methyl α-L-fucopyranoside, and 2,3-di-*O*-methyl-D-galactose. Similar results were obtained with the four different serum pools tested. All three haptens, at 4 μmoles, gave >80% inhibition of precipitation by D-digitalose; methyl α-L-fucopyranoside and 2,3-di-*O*-methyl-D-galactose were more active than L-fucose. Blood-group inactive sugars had no inhibitory effect even at concentrations thrice the optimal amount of inhibitors.

Solubility of Anti-Blood-Group H(O) Eel Serum Precipitates. SOLUBILIZATION BY INHIBITORY HAPTENS. Methyl α-L-fucopyranoside or 2,3-di-*O*-methyl-L-fucose at three times the equivalent amount of D-digitalose served as inhibiting haptens and D-glucose as inactive control. When 0.1 ml of either of

the inhibitory haptens was added to the precipitin tubes containing both supernatants and precipitates after standard incubation, there was >92% dissolution of the precipitates obtained with the macromolecules from ovarian cyst and *Sassafras* on reincubation at 4° for 4 days or during 150 min at 37°. In contrast, the precipitates obtained with D-digitalose dissolved from 13.5 to 17.5% only.

When the supernatants were removed and either of the two inhibitory haptens added to the washed precipitates, which had been reconstituted to the original volume, >98% of the precipitates obtained with any of the precipitinogens dissolved at either of the incubation temperatures. D-Glucose, used at the same conditions as the inhibitory haptens, did not solubilize (<1.5%).

SOLUBILIZATION WITHOUT INHIBITORY HAPTENS. Precipitates obtained with any of the precipitinogens did not dissolve (<3.5%) on incubation at 37° for up to 3 hr when they were left with their supernatants. Similarly, washed precipitates, obtained with any of the precipitinogens, showed little solubilization (1.5–7.0%) upon reincubation for 96 hr at 4° with buffered saline adjusted to the original volume. At 37°, however, >99% of the resuspended precipitates obtained with D-digitalose dissolved within 10 min while those obtained with macromolecular blood-group H(O)-active substances showed a solubility from 15.5 to 39% after 150 min at 37° in buffered saline.

Addition of equivalent amounts of D-digitalose suppressed solubilization of precipitates, but if D-digitalose was added in thrice the equivalent quantity, it solubilized the D-digitalose precipitate by about 20% and increased the solubility of those obtained with the macromolecules by about 30%.

Precipitates obtained with D-digitalose and suspended in their original supernatants dissolved by >98% upon dialysis. The precipitates with *Sassafras* polysaccharide were solubilized from 3.6 to 11.5% and those with ovarian cyst glycoprotein $CaII_bIII_A$ were solubilized from 8.5 to 22.5%. The solubilization was slightly increased if the precipitates had been washed before dialysis.

Studies on Supernatants of Precipitin Tests. RESIDUAL HEMAGGLUTININS. These were determined with human blood-group O erythrocytes on the supernatants of 63 different precipitin tests and inhibition assays of 18 different serum pools; supernatants of 49 tests from far antibody excess to antigen excess were assayed.

UNDIALYZED SUPERNATANTS. Zero to nine per cent of the original agglutinins was demonstrable in the supernatants of precipitates obtained with any of the four precipitinogens in the equivalence area, except that two of the 32 supernatants of monosaccharide precipitates contained 15–25% of the original agglutinins. At twice the equivalent precipitinogen concentration the same quantity of agglutinins was found in the supernatants.

The hemagglutinin titers of the supernatants at one-fourth the precipitinogen concentration of the equivalence point possessed 4–32 times the agglutinin titer of the equivalence zone supernatants.

The nonprecipitating monosaccharides methyl α-L-fucopyranoside, L-fucose, 2-*O*-methyl-L-fucose, and 2,3-di-*O*-methyl-L-fucose incubated under the same conditions as the precipitin assays decreased at equivalent concentrations the agglutinin titers to the same extent as did the precipitating monosaccharides. However, dialysis completely restored the original agglutinating power of the sera.

DIALYZED SUPERNATANTS. The anti-H(O) agglutinin titers of the supernatants from precipitates with either of the two antigens were not changed by dialysis. In contrast, if monosaccharides had been the precipitinogen, dialysis significantly increased the titers of nearly three-quarters of the 21 equivalence zone supernatants investigated to as much as 25% of the agglutinins originally demonstrable. The same results were obtained with dialyzed supernatants of monosaccharide precipitates at twice the equivalent concentration. About one-quarter of the supernatants of monosaccharide precipitation in the antibody excess area (one-fourth equivalence) had a 4- to 32-fold titer increase.

AGGLUTININS IN SUPERNATANTS AFTER SUCCESSIVE ADDITION OF TWO PRECIPITINOGENS. To undialyzed supernatants obtained with one precipitinogen another precipitinogen was added in concentration equivalent for the untreated serum. There was no change in agglutinin titer of the supernatants regardless of whether the two precipitinogens were monosaccharides or macromolecules unless supernatants obtained with less than one-third the equivalent concentration of the first precipitinogen were used; here a reduction of the residual titers by 75 to >90% occurred.

RESIDUAL HEMAGGLUTINATION INHIBITORS. Boiled supernatants of 24 complete precipitin assays, including 14 with monosaccharides, were investigated. The monosaccharides were always demonstrable at approximately their original concentrations (see below) due to the insensitivity of the procedure employed. Macromolecular precipitinogens were never demonstrable in supernatants of the antibody excess area and only occasionally at equivalence, but regularly in the antigen excess area, where they amounted for the *Sassafras* antigen to from 10 to 20% of the polysaccharide originally added.

PRECIPITINS IN UNDIALYZED SUPERNATANTS. Only antibody in supernatants of precipitates with D-digitalose will be described since virtually identical results were obtained on supernatants of precipitates with 3-*O*-methyl-D-galactose or *Sassafras* polysaccharide.

Upon addition of optimal amounts of either 3-*O*-methyl-D-galactose, *Sassafras* polysaccharide Ca-606 or ovarian cyst glycoprotein $CaII_bIII_c$ the quantity of protein precipitated from the supernatant of the equivalence zone, or that of 2- to 3-fold precipitinogen excess of the original precipitin test, was <0.5–7% of that maximally obtainable from eel serum to which no precipitinogen had previously been added. In the area of one-eighth to one-twelfth equivalence concentration, 20–25% of the protein maximally precipitable was obtained from supernatants with all precipitinogens except ovarian cyst glycoprotein which precipitated only 7.5%. The sum of protein precipitated by the first and second precipitinogen was nearly equal (within ±7%) to that maximally precipitable, by the first or the second precipitinogen alone from untreated serum, throughout the entire range of the curve. Simultaneous addition of optimal quantities of D-digitalose and 3-*O*-methyl-D-galactose to an eel serum pool did not yield a larger precipitate than addition of either of these precipitinogens alone.

PRECIPITINS IN DIALYZED SUPERNATANTS. Dialysis had no influence on the quantities of precipitins demonstrable from 2-fold antibody excess to 2.5-fold precipitinogen excess; depending on the shape of the precipitin curve, dialysis increased demonstrable precipitins in the area of 3-fold antibody excess up to four times when monosaccharides were the precipitinogen.

RESIDUAL PRECIPITINOGENS. Less than 0.5% of the protein precipitable by the original concentration of *Sassafras* antigen resulted from the polysaccharide remaining in the supernatants of the antibody excess area and the equivalence zone; supernatants from twice and four-times precipitinogen excess pre-

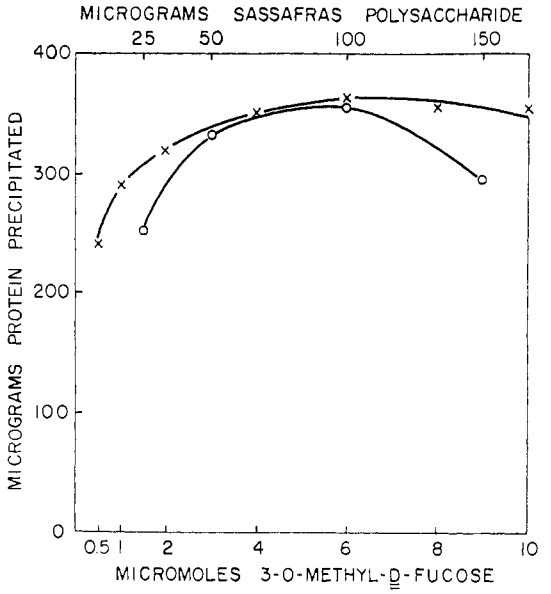

FIGURE 6: Precipitin curves obtained with isolated eel anti-blood-group H(O) antibody and various precipitinogens. (×) 3-*O*-Methyl-D-fucose and (○) *Sassafras* polysaccharide (Ca-606).

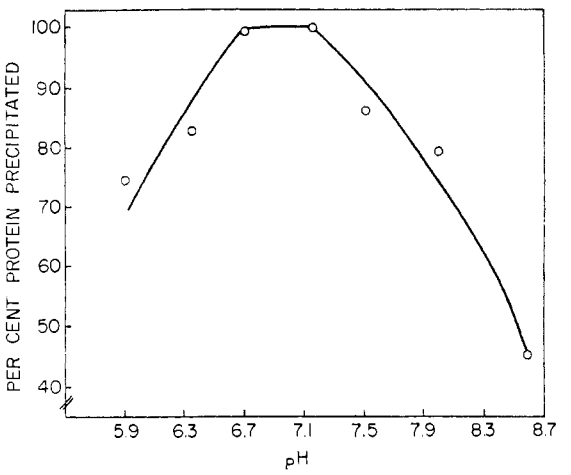

FIGURE 7: Effect of pH on precipitation of isolated eel anti-blood-group H(O) antibody with 3-*O*-methyl-D-fucose.

cipitated between 54 and 99% of the protein precipitated in the equivalence zone of an ordinary precipitin test. In contrast, supernatants from the equivalence zone of tests with monosaccharide precipitinogens precipitated virtually the same quantity (on the average 99.1%) of protein as did the original concentrations of these precipitinogens.

Possibility of Aggregation of Precipitating Monosaccharides in Solution. Our cryoscopic determinations indicated no aggregation of D-digitalose. The values for D-digitalose and L-fucose in deionized water were the same and within $<\pm 2.5\%$ of those expected for the pure monomeric form at all levels investigated; in buffered saline they also reached this level with $\pm 3.5\%$ deviation from the theoretical value. The cryoscopic determinations carried out by Huffman Laboratories in 0.85% aqueous NaCl at two concentrations indicated no aggregation of D-digitalose. Similarly, the molecular weights found by vapor pressure osmometry at 37° for 3-*O*-methyl-D-fucose, 2-*O*-methyl-L-fucose, and D-glucose were in good agreement with the theoretical values for these sugars in monomeric form; for the precipitating D-digitalose and the nonprecipitating 2-*O*-methyl-L-fucose they were within $\pm 0.25\%$ of the theoretical values.

Isolation of Pure, Homogeneous Anti-H(O)-Specific Antibody from Eel Serum. The ready solubilization of the D-digitalose–eel serum precipitates upon dialysis suggested a unique way to isolate this antibody in pure form. The anti-human blood-group H(O) antibody was precipitated under standard conditions in large series of tubes with an equivalent amount of D-digitalose. The protein content of the precipitates of two tubes from each experiment was determined. The washed precipitates were either worked up immediately or stored at −20° in the freeze-dried state. The precipitates were suspended in about one-half their original volume of buffered saline and dialyzed on a mechanical shaker at 4° against 20 volumes of buffered saline which was gradually replaced by deionized distilled water until no more Cl^- was demonstrable with $AgNO_3$. This stepwise electrolyte decrease minimized antibody precipitation. Insoluble matter amounting on the average to 3.8% of the total recovered was removed by centrifugation. The materials were freeze-dried and then dried at 22–25° over P_2O_5 at 10^{-1} mm. The average recovery, by weight, of soluble antibody in ten experiments was 94.1%. Prior to chemical analyses the antibody was electrodialyzed at 4° through a dialysis membrane at 200 V (*cf.* Springer *et al.*, 1965).

Immunochemical Properties of the Isolated Eel Antibody. The preparations were white, fluffy powders which gave clear or slightly opalescent solutions up to at least twice their concentration in the eel serum. The average wet ash content was 3.7%; maximal weight loss on drying was 8%. A typical electrodialyzed preparation contained <1% ash and 15.74% N (corrected). Hemagglutinating activity of the antibody recovered after dialysis or electrodialysis was the same as that of the original eel serum; that of the insoluble residue after dispersion with a Vortex mixer amounted to <4%.

Antibody solutions in buffered saline or deionized water were stable for at least 1 month at 4°. Repeated freezing and thawing severely damaged the antibody activity. The effect of different hydrogen ion concentrations on the agglutinating activity of 0.1% antibody was determined at intervals of about 0.5 pH unit; solubility was complete between pH 9.00 and 5.50; some protein precipitated at lower pH. The antibody changed the pH of the solutions by $<\pm 0.05$ unit during a 12-day incubation at 4°. Thereafter, all samples were adjusted to pH 7.3 by dialysis and the agglutinin titers of all of the samples were fully recovered.

The isolated eel antibody gave typical precipitin curves, and in the same proportions as those when it was still in serum, with the blood-group H(O)-specific precipitinogens; Figure 6 lists D-digitalose and *Sassafras* polysaccharide as examples; results with 3-*O*-methyl-D-galactose were virtually identical. Protein precipitated by these blood-group active substances in the equivalence zone ranged from 68.7 to 95.9% of the total antibody protein, although the latter figure was exceptional; the average recovery in ten experiments with antibody from three different pools was 74.7%. An average of 2.3% protein was found in the washes while 22.9% remained in the supernatants, whose agglutinin titers paralleled their protein content. After adjustment of the protein content of dialyzed supernatants of monosaccharide precipitin tests to its pre-precipitation concentration, the same proportion of protein was precipitable by the precipitinogens as from the original antibody solution. These results were also obtained if eel serum was used instead of the isolated antibody solution.

TABLE IV: Composition of Eel Anti-Human Blood-Group H(O) Antibody.[a]

Components	%	Moles/Mole of Antibody
Lysine	3.67	31
Histidine	4.85	38
Arginine	7.48	53
Aspartic acid	11.92	110
Threonine	7.16	74
Serine	6.34	74
Glutamic acid	10.77	90
Proline	3.97	42
Glycine	5.59	92
Alanine	6.44	89
Valine	6.28	66
Methionine[b]	0.77	6
Isoleucine	6.22	58
Leucine	5.83	55
Phenylalanine	2.39	18
Tyrosine	4.29	29
Cysteic acid[c]	6.36	46
Tryptophan	1.58	10
Glucosamine	0.39	3
Galactosamine	0.06	
Sialic acid	0.10	
Methylpentose	<0.2	
Hexose	<0.6	
Heptose	<0.2	
NH_3	1.80	130

[a] Values corrected for ash and moisture. [b] Includes value derived from methionine sulfoxide. [c] A second determination showed only 2.53%.

A slight but reproducible shift of the beginning of the equivalence zone to the right was generally noted with precipitinogens when precipitin curves obtained with isolated antibody were compared to those given by the serum pools from which the antibody originated.

No precipitation of electrodialyzed eel antibody by D-digitalose was observed in deionized water, isotonic glucose, and isotonic sucrose. When electrodialyzed *Sassafras* polysaccharide, which contains *ca.* 6% galacturonic acid (Springer *et al.*, 1965) was used instead of D-digitalose, 28.7 and 14.8% of the protein maximally precipitable resulted at equivalence with deionized water and isotonic sucrose as solvents, respectively.

Figure 7 shows the dependence of the precipitation of eel antibody with D-digitalose on the hydrogen ion concentration at equivalence. The precipitation at optimal pH is listed as 100%. The precipitation optimum was between pH 6.70 and 7.15. There was a steady decline in precipitation toward both the acid and alkaline ranges. The same proportions in precipitation depending on pH were found at D-digitalose concentrations one-third as well as twice that of the equivalence point.

Immunochemical homogeneity of the eel antibody was indicated by the shape of the precipitin curves and by the single symmetric arc obtained by immunoelectrophoresis (Figure 8). Similarly, agar gel diffusion showed only one sharp band with the H(O)-specific human ovarian cyst, *Taxus* and *Sassafras* antigens as well as with the blood-group H(O)-specific antigen from *E. coli* O_{128}; all these bands showed complete fusion with one another.

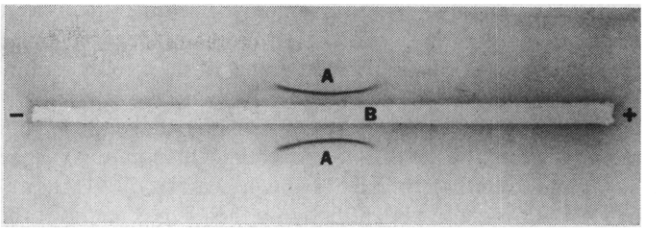

FIGURE 8: Immunoelectrophoresis pattern of recovered anti-human blood-group H(O) eel antibody (REAB). (A) REAB Ca-1214, 1–2 μg, (B) rabbit 10 anti-REAB, 25-30 μl.

Distribution of Monosaccharide Precipitinogen in Precipitin Tests. The isolated eel antibody solution used contained <0.001 μmole of methylpentose per ml. Precipitates and supernatants with isolated eel antibody were investigated not only in the equivalence zone but also at twice and one-fourteenth the equivalent amount of D-digitalose. Protein recovery ranged from 99.6% to 100.7%; in the precipitates were found: 32.0% protein in the antibody excess area, 70.9% at the beginning of the equivalence area, and 67.9% protein was recovered from the precipitate at double the equivalent concentration. Recovery of added D-digitalose amounted to 100% (±1%); >97.8% of the D-digitalose remained in the supernatant at all levels of the precipitin curve investigated. The results were closely similar whether the determinations were made on supernatants as such or after deproteinization. Between 0.11% and 0.61% of the total D-digitalose added was recovered from the precipitates. The methylpentose content of the washes varied from 1.97 to 1.52%; it showed a small but steady percentage decrease from antibody excess to precipitinogen excess.

The molar ratio of methylpentose/antibody in the precipitates ranged from 3.15 in the antibody excess area to 5.37 in the equivalence zone to 7.78 in the area of precipitinogen excess. These results were obtained with trichloroacetic acid extracts. Methylpentose determinations on dialysates of equivalence zone precipitates gave a molar ratio of 5.51 for D-digitalose/antibody. This suggests that 3, 5, and 8 moles of D-digitalose may combine with 1 mole of antibody, depending on the area of the precipitin curve. Additional D-digitalose distribution experiments gave closely similar results.

Unspecific adsorption of methylpentose onto the precipitated protein was assessed by addition of 9.8 μmoles of L-rhamnose to 0.5 ml of eel serum immediately prior to addition of an optimal quantity of *Sassafras* polysaccharide. The precipitate caused by the polysaccharide after standard incubation contained <0.03 mole of free methylpentose/mole of antibody.

Chemical Characteristics of the Eel Antibody. As shown in the accompanying paper (Bezkorovainy *et al.*, 1971), the isolated anti-H(O) protein is homogeneous by physicochemical criteria; its molecular weight is 123,000; the molecule is nearly spherical and consists of 12 subunits of equal size. Conformational studies indicated virtual identity of the anti-human blood-group H(O) antibodies from all eels investigated (Jirgensons *et al.*, 1970).

The averaged results of two amino acid and carbohydrate analyses are listed in Table IV. Asp, Gly, Glu, Ala, Ser, and Thr were the predominant amino acids while there was a scarcity of Met, Trp, and Phe. No significant amounts of carbohydrate were demonstrable besides some GlcN.

About equal quantities of NH_2-terminal Ser and Ala were

found by the three methods employed. While there were traces of other amino acids these were never detected by all three procedures. Qualitative assessment of the COOH termini by two methods showed Ser and Gly to be the major components. There were also traces of Asp and Ala by the FDNB method but not by the DANS procedure. Quantitation yielded 5.60 moles of NH_2-terminal Ser and 4.99 moles of NH_2-terminal Ala per mole of eel antibody by the FDNB method; the corresponding figures by the DANS method were 4.89 and 4.79. The dansylation procedure revealed 4.71 moles of COOH-terminal Ser and 4.49 moles of COOH-terminal Gly. These results indicate polypeptide chains with alternating amino as well as carboxyl termini.

Discussion

We have investigated a novel and surprising phenomenon: the precipitation with uncharged monosaccharides of an eel serum protein with the properties of an antibody. The pure, homogeneous antibody was readily isolated from the precipitate by dialysis of the monosaccharide precipitinogen. The isolated antibody was equally precipitable by 3-*O*-methyl-D-galactose, 3-*O*-methyl-D-fucose, or the blood-group H(O)-active macromolecules, and it agglutinated human blood-group O red cells to the same extent as the original serum. That the same antibody was responsible for all these interactions was proven by analyses of the supernatants of the precipitin assays. Furthermore, the same haptens, in the same order of activity, inhibited precipitation of eel antibody by the monosaccharides as well as by the H(O)-active antigens. When monosaccharides were the precipitinogens, the quantities of inhibitors needed to obtain optimal inhibition were approximately twice those required in assays with macromolecules, possibly due to the large concentration of the precipitating monosaccharides.

The failure of any of the numerous sera and a plant extract which interact with blood-group O erythrocytes to precipitate with these monosaccharides attests to the specificity of the reaction.

Precipitation of antisera by relatively small molecules has been described before; however, all of these were strongly charged and contained at least two haptenic groups; related monohaptenic compounds did not precipitate (*e.g.*, Landsteiner and Van der Scheer, 1932; Pauling *et al.*, 1942). The precipitating monosaccharides do not possess any characteristic repeating groups and these would not be necessary according to the old hypothesis of Bordet (1899) although they are a prerequisite for precipitation according to the now universally accepted lattice theory (Marrack, 1934; Heidelberger and Kendall, 1935).

It has been held since the beginning of interpretation attempts of immune precipitin and agglutination reactions that combination of antigen with antibody results in the formation of particles whose surfaces, predominantly furnished by antibody, are more hydrophobic than those of the uncombined reactants (*cf.* Bordet, 1899; Reiner and Fischer, 1929; Eagle, 1930); the presence of sufficient electrolytes further reduces the surface charge of the particles and this results in precipitation. Landsteiner stated "... the presence of two or more binding groups (on the precipitinogen) alone appears not to be sufficient for precipitability" (Landsteiner, 1945), and others accepted the lattice theory, but adduced evidence that nonspecific factors in addition to specific framework formation must be considered (*e.g.*, Duncan, 1938; Boyd, 1942).

The use of dialyzed monosaccharides clearly showed that the precipitation was not due to a macromolecular contaminant. Both the precipitating monosaccharides gave precipitin curves typical for polysaccharides (*e.g.*, Heidelberger and Kendall, 1937) which were indistinguishable from those resulting with blood-group H(O)-specific antigens. The precipitability of the antibody with the O-methylated monosaccharides at different hydrogen ion concentrations was in accord with those reported for ordinary antigen–antibody precipitin reactions (Kleinschmidt and Boyer, 1952), as was the effect of dilution (Kabat, 1961b).

Closer investigation did reveal differences between precipitin tests with macromolecules and monosaccharides. Precipitates obtained with monosaccharides were granular flocculates instead of the smooth button-like precipitates given by antigens. Also, while the solubility of precipitates obtained with the monosaccharides upon washing was approximately equal to that resulting from macromolecules in the equivalence and precipitinogen excess areas, the former were considerably more soluble in the antibody excess region.

The distribution of the precipitinogens in the supernatants was profoundly different for macromolecules and monosaccharides; <0.5% of the *Sassafras* antigen originally added remained in the supernatants of the equivalence area. In contrast, supernatants from monosaccharide precipitates showed no significant diminution of precipitinogen content by either serological or colorimetric methods even in the area of antibody excess. This is explained by comparing the quantitative aspects of the precipitability by monosaccharides to that by the *Sassafras* polysaccharide, whose determinant hapten is 3-*O*-methyl-D-galactose, with that by free 3-*O*-methyl-D-galactose. Approximately 100 μg of polysaccharide gave optimal precipitation (Figures 3 and 6), while for the same effect about 800–1200 μg of 3-*O*-methyl-D-galactose was required. The differences become even more evident by comparison on a molar basis. The molecular weight of the *Sassafras* polysaccharide is 250,000 (Springer *et al.*, 1965) and that of 3-*O*-methyl-D-galactose is 194; therefore 0.4 nmole of *Sassafras* polysaccharide optimally precipitated the eel antibody while 4×10^3 to 6×10^3 nmoles of monosaccharide were required for the same effect. This calculation may be modified by considering that 1 mole of *Sassafras* antigen contains 355 moles of 3-*O*-methyl-D-galactose residues (Springer *et al.*, 1965). Thus the 1.42×10^2 nmoles of this sugar of the *Sassafras* antigen precipitate optimally as do 4×10^3 to 6×10^3 nmoles of free monosaccharide. Based on the quantity of covalently linked hapten the antigen would then be only about 35 times as active. However, it is certain that not all antigen-bound 3-*O*-methyl-D-galactose residues are free to interact (Springer *et al.*, 1965).

Approximately 3–4 nmoles of antibody protein was precipitated in the equivalence zone (Figures 3 and 6); since no antigen remained in the supernatant about 8–10 moles of antibody combined with 1 mole of *Sassafras* antigen. Haurowitz *et al.* (1957) found 7–15 moles of homologous antibody to combine in the equivalence zone with 1 mole of azoprotein precipitinogen. We calculated that Kabat *et al.* (1950) had a ratio of *ca.* 10–15 for human anti-blood-group A antibody to human A substance if the antibody is assumed to be IgG. Our methylpentose measurements on precipitates indicate that between 5 and 8 moles of monosaccharide may bind per mole of antibody. As mentioned, the surface of the blood-group active macromolecules contains many combining sites and thus our monosaccharide findings agree with those obtained with antigens.

Even though 50–100 times as many moles of free monosaccharide as antigen combine with the antibody in the equiva-

lence zone, only about 20 nmoles of monosaccharide of a total of 4000–6000 combine with the total antibody precipitated (Figures 3 and 6). While this finding readily explains the undiminished demonstrability of monosaccharide precipitinogens in the supernatants it raises the important question (assuming a reasonable accuracy of the determination of the precipitinogen in the precipitate) as to why a several-hundredfold excess of monosaccharide precipitinogen over that actually bound is required for precipitation. No wholly satisfactory explanation can be offered. The large quantity of *O*-methyl sugars in solution may change the character of the immediate environment of the antibody molecules and may decrease their solvation and alter their conformation. This may enable antibody molecules to interact with one another; all these changes promote precipitation. Evidence has been adduced by Karush and his colleagues (Warner *et al.*, 1970) that interaction of an anti lactose antibody with univalent hapten makes the antibody more compact.

It is remarkable that the optimally precipitating quantity of D-digitalose or 3-*O*-methyl-D-galactose is of precisely the same order of magnitude, on a molar basis, as that which is needed for inhibiting haptens to afford maximal inhibition of precipitation of eel antibody (Springer *et al.*, 1965) even though the latter reaction is thought to be a competing one between antigens and haptens for the antibody combining sites. Even in different systems such as the human blood-group B anti-B precipitating system the quantity of immunodeterminant disaccharide required for optimal inhibition is of the same order; we calculated 12,600 moles based on the experiments of Schiffman *et al.* (1964). The question therefore arises as to whether the tremendous excess of hapten required for an optimal inhibitory effect in antigen–antibody reactions really is needed only for equilibrium competition or whether these haptens have another function in addition, such as the possibilities discussed above. Similarly in equilibrium dialysis the inability to reach experimentally the binding of 2 moles of hapten/mole of antibody (*e.g.*, Karush, 1956) may need explanations in addition to the varying association constants of a heterogeneous antibody population.

It has been suggested by Professor E. A. Kabat that characteristic repeating groups on the precipitating monosaccharides may result from their aggregation (personal communication). For this we found no evidence by vapor pressure osmometry and cryoscopy in either water or buffered saline over a wide concentration range. Dr. K. O. Lloyd proposed (personal communication) that a Schiff base or *N*-glycosylamine type of bond between the eel anti-H(O) protein and the precipitating 3-*O*-methyl sugars may afford repeating haptenic groups on the antibody itself. However, no other sugars gave such a precipitation including the enantiomorph of D-digitalose, 3-*O*-methyl-L-fucose. 3-*O*-Methyl-D-glucose in equivalent and even larger quantities did not increase precipitation by small amounts of D-digitalose and did not interact with the antibody at any concentration. The absence of any precipitate with D-digitalose in an electrolyte-free environment and the prompt occurrence of a precipitate upon salt addition indicates a requirement for a decrease of polarity of the antibody molecules for precipitation to occur. Precipitates obtained with monosaccharides dissolved completely in deionized water and those obtained with macromolecular precipitinogens partially. These findings are in agreement with observations of Bordet (1899) and Kleczkowski (1965).

The minimum requirements for a monosaccharide to function as a precipitinogen of the eel antibody and the mode of action of the precipitating monosaccharides will now be discussed.

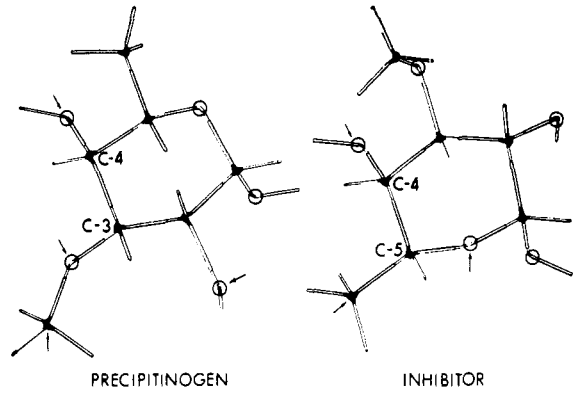

FIGURE 9: Dreiding models of 3-*O*-methyl-D-fucose a precipitinogen and 3-*O*-methyl-L-fucose an inhibitory hapten for eel anti-H(O) antibody. →, reactive areas and O, location of oxygen atoms. The inhibitor model is inverted by 180°.

The energetically most favored conformation for the fucose derivative D-digitalose is C-1 while that for the L enantiomorph is 1-C. We have previously shown that certain fucose derivatives of both the L and the D series possess about equal inhibitory activity for the eel antibody (Springer and Williamson, 1962); these findings seemed to disagree with the proven concept of the strict stereospecificity of serological reactions. However, Professor Kabat has explained this equal activity, based on the overall symmetry of these sugars and the similar profile of their hydrophobic areas as seen in molecular models provided one enantiomorph is inverted by 180° as in Figure 9. The CH_3 at C-5 of 3-*O*-methyl-L-fucose was thought to correspond to the OCH_3 at C-3 of D-fucose, and in turn the C-5 methyl substituent of the 3-*O*-methyl-D-fucose was postulated to be equivalent to OCH_3 of the L enantiomorph. These areas were presumed to interact most significantly with the antibody combining site (Kabat, 1962, 1968).

Our subsequent finding of the high activity of some O-substituted D-galactoses necessitated the then novel assumption that the minimal inhibitory structure complementary to the combining site of an antibody may be smaller than a monosaccharide (Springer *et al.*, 1964; Desai and Springer, 1965). For complementarity to the eel anti-H(O) antibody the hapten must contain an equatorial CH_3 attached to a pyranose ring with an ether oxygen adjoining this CH_3 group either as part of a methoxyl group or as the pyranose ring oxygen and on a contiguous C atom there must be an axial, hydroxyl group cis to the equatorial methyl group. Such a conformation (lower left of both models in Figure 9) was found in all H(O)-active precipitinogens and inhibitors of eel serum (Springer *et al.*, 1964, 1965).

It is evident that the just described regions of the haptens have areas capable of both apolar interaction and hydrogen bonding; in addition to hydrogen bonds, apolar interactions have been shown to be important for carbohydrate–antibody interactions by Karush (1957). The CH_3 group permits apolar interaction between the ligand and the binding site of the antibody; this in turn facilitates the formation of hydrogen bonds by extrusion of interstitial water (Eisen and Siskind, 1964; see also Duncan, 1938, and Boyd, 1942).

The present observation that monosaccharide precipitinogens of the eel antibody are confined to 3-*O*-methyl sugars of the D series, while inhibition is given by L and D sugars

which fulfill the requirements discussed above (Springer and Williamson, 1962; Springer *et al.*, 1964), indicates a greater specificity of precipitation when compared to inhibition. Our studies on models revealed that there are profound differences between the presumed active sites of precipitinogens and inhibitors in spite of their similar contours. These differences are clearly recognizable in the examples of the enantiomorphs of 3-*O*-methylfucose shown in Figure 1. The C-3 region of the precipitating D enantiomorph differs from the C-6 area of the inhibiting L enantiomorph in several pronounced aspects: the shortest distance from C-3 of 3-*O*-methyl-D-fucose to its CH_3 substituent is much larger (2.32 Å), because of the interposed oxygen, than that from C-5 of 3-*O*-methyl-L-fucose to its CH_3 substituent (1.54 Å), which has no interposed ether oxygen but instead a ring oxygen adjoins C-5. The C-3 of 3-*O*-methyl-D-fucose is flanked by an equatorial trans hydroxyl group at C-2 and by an axial cis hydroxyl group at C-4; only the latter half of this configuration and conformation exists around C-5 of 3-*O*-methyl-L-fucose (Figure 9).

Both 3-*O*-methyl-D-fucose and 3-*O*-methyl-D-galactose possess three oxygens protruding from the pyranose ring in the above described combining area, of which two are very well capable of hydrogen bonding whereas 3-*O*-methyl-L-fucose possesses only one protruding oxygen, that on C-4, in this region. We have shown earlier that the only hydrogen-bonding OH group required for inhibitory activity of a hapten in this system is that at C-4 (Springer and Williamson, 1962). Both the additional equatorial hydrogen-bonding region at C-2 and the pronounced protrusion of all three oxygens may thus be decisive for the precipitating ability of the sugar molecule. In any case such a configuration and conformation would facilitate the binding of these relatively hydrophobic ligands (*cf.* Eisen and Siskind, 1964).

Additional apolar groups are not compatible with precipitating ability of a pyranose structure. For example, a minor change such as methyl glycosidation at C-1 and/or O-methylation or reduction at C-2 transforms the precipitating molecule into an inhibitor (Table II) even though this change makes the molecule more hydrophobic.

Eels (*Anguilla anguilla* and *Anguilla rostrata*) are teleostean fish and belong to the order Apodes (Berg, 1947). Eels are thus higher in vertebrate order than the cyclostomes, of which the lamprey (*Petromyzon marinus*) has already been shown unequivocally to produce antibodies (*cf.* Good and Papermaster, 1964). The anti-human blood-group H(O) protein of the eel has been uniformly considered to be an antibody (Jonsson, 1944; Watkins and Morgan, 1952) although the nature of the immunogenic stimulus provoking it has not been demonstrated; it has been suggested to be seaweed-bound L-fucose (Heidelberger, 1956). The eel anti-H(O) protein resembles the human IgG globulin in that it is a 7S globulin, apparently made up of two different kinds of polypeptide chains and has Ser and Gly as carboxyl-terminal amino acids. In contrast to the immunoglobulins of higher animals it contains chains of only one size and there exist only noncovalent interactions between the tetrameric subunits which constitute the antibody. However, the polypeptide chains in the tetramers are linked by disulfide bridges. These requirements for the structural integrity of the eel antibody are reminiscent of the findings with the anti-human O erythrocyte antibodies from the lamprey (Litman *et al.*, 1970).

The anti-H(O) protein from the eel may thus represent a kind of primordial antibody. At present it may even be argued that it is not an antibody. Sequence studies of the eel serum protein will help establish its position in the phylogenetic development of antibodies and whether or not it lies in the straight line of immunoglobulin evolution. However, this problem is irrelevant to the immunochemical aspects of the present study, since the phenomena observed are due to forces which may also be of consequence, perhaps in a more subtle way, in various immune-precipitating systems.

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Physicochemical Properties of the Eel Anti-Human Blood-Group H(O) Antibody*

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ABSTRACT: The eel anti-human blood-group H(O) antibody isolated as described in the preceding paper was homogeneous by ultracentrifugal and electrophoretic criteria. It is a FS globulin, has a molecular weight of 123,000, and is of nearly spherical shape. It consists of three physically bonded subunits of apparently identical molecular weight of 40,000. Each subunit consists of four polypeptide chains of identical size; the chains, whose molecular weight is near 10,000, are joined by disulfide linkages.

The previous paper (Springer and Desai, 1971) described the immunochemical properties, isolation, and composition of the eel anti-human blood-group H(O) antibody. The present communication is concerned with some physical properties of this protein, and its apparent tertiary and quaternary structures. These studies were deemed especially worthwhile in view of the novel phenomenon of specific precipitation of the eel protein with blood-group active monosaccharides, and because of the profound difference in the conformation of the eel and mammalian antibodies (Jirgensons *et al.*, 1970). The quantity of the eel anti-human blood-group H(O) antibody available for this study was limited, and extensive repetition of the operations performed was impossible. Some experiments done with the eel protein were therefore accompanied by identical procedures on a previously characterized protein, the bovine colostral IgG, in order to test the reliability of our methodology. In contrast to the eel antibody, the colostral IgG (Kickhöfen *et al.*, 1968) was shown not to be split into subunits either in urea or by succinylation, but was dissociable into two heavy and two light chains by reduction–alkylation (Bezkorovainy *et al.*, 1970).

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