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Specific Purification of Eel Serum and *Cytisus sessilifolius* Anti-H Hemagglutinins by Affinity Chromatography and Their Binding to Human Erythrocytes†

Isamu Matsumoto and Toshiaki Osawa*

ABSTRACT: Eel serum and *Cytisus sessilifolius* anti-H hemagglutinins were purified by affinity chromatography using specific adsorbents prepared by cross-linking the corresponding inhibitory sugar to insoluble starch. The preparations were homogeneous by ultracentrifugal analysis and disc electrophoresis. The sedimentation constants ($s_{20,w}$) and the molecular weights were estimated to be 7.2 S and 140,000 for eel serum anti-H hemagglutinin and 6.8 S and 110,000 for *C. sessilifolius* anti-H hemagglutinin. The binding studies using ^{125}I-labeled eel serum and *C. sessilifolius* hemagglutinins revealed that there are approximately the same number of receptor sites (1.7–1.9 $\times$ 10^6 per cell) for both anti-H hemagglutinins on human group O erythrocytes. Furthermore, the presence of one anti-H hemagglutinin effectively inhibited the binding of the other anti-H hemagglutinin to group O erythrocytes. Although these two anti-H hemagglutinins possess different sugar specificities (Matsumoto, I., and Osawa, T. (1971), *Vox Sang. 21*, 548), the results described above indicate that they bind to different but overlapping portions of the same H-determinant structure on the cell surface. Human peripheral lymphocytes were also found to contain 0.4 $\times$ 10^6 receptor sites per cell for eel serum anti-H hemagglutinin.

Numerous heterologous anti-H hemagglutinins have been found in eel serum and the extracts of certain plant seeds. These anti-H hemagglutinins can be subdivided into two groups on the basis of the inhibition assays using simple sugars as inhibitors: one is a group of eel serum type anti-H hemagglutinins which includes eel serum hemagglutinin, *Lotus tetragonolobus* hemagglutinin, and *Ulex europeus* hemagglutinin I (Matsumoto and Osawa, 1969), and the other is a group of *Cytisus* type anti-H hemagglutinins which includes *Cytisus sessilifolius* hemagglutinin, *Laburnum alpinum* hemagglutinin, and *Ulex europeus* hemagglutinin II (Matsumoto and Osawa, 1970). Since eel serum type anti-H hemagglutinins are inhibited by L-fucose (Springer and Williamson, 1962; Springer *et al.*, 1964; Matsumoto and Osawa, 1969), they are assumed to bind with α-L-fucopyranosyl residue in the H-determinant structure of human erythrocyte surface or soluble blood group substances. On the other hand, *Cytisus* type anti-H hemagglutinins are inhibited by di-*N*-acetylchitobiose and the other oligosaccharides having a terminal nonreducing β-*N*-acetylglucosaminyl residue (Watkins and Moran, 1962; Osawa, 1966), the structure of which is, however, not found in the carbohydrate chain of blood group determinant structure. Thus, the structure which *Cytisus* type anti-H hemagglutinins actually recognize on the human erythrocyte surface has not been clarified, even though β-*N*-acetylglucosaminyl linkage has been found at the internal position in the carbohydrate chains of blood group H glycolipids (Stellner *et al.*, 1973) and soluble blood group substances (Watkins, 1972). In the previous papers (Matsumoto and Osawa, 1970, 1971), we demonstrated that the *Cytisus* type anti-H hemagglutinins could not agglutinate human group O erythrocytes previously treated with purified H-decomposing enzyme (α-L-fucosidase) from *Bacillus fulminans* (Iseki *et al.*, 1962), and they were more effectively inhibited by 2′-fucosyllactose and lacto-*N*-fucopentaose I than by the corresponding fucose-free oligosaccharides, *i.e.*, lactose and lacto-*N*-tetraose. It has been, therefore, assumed that *Cytisus* type anti-H hemagglutinins recognize the *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→3 or 4)-β-D-*N*-acetylglucosamine sugar sequence in the carbohydrate chain of blood group determinant structure. Thus, it can be inferred that eel serum type and

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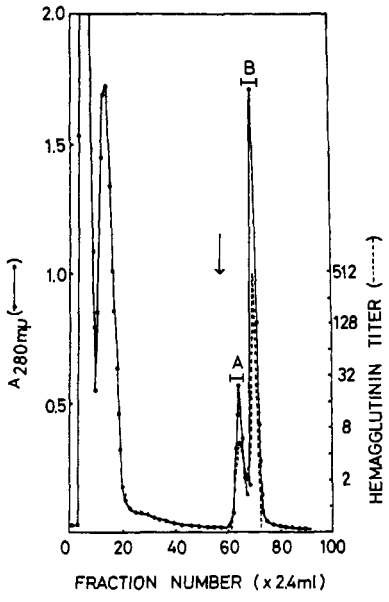

FIGURE 1: Affinity chromatography of eel serum hemagglutinin on an L-fucose–starch column. Five milliliters of eel serum was applied to a column of L-fucose–starch (1.6 × 12.8 cm) equilibrated with 5 mM phosphate-buffered saline (pH 7.0). After washing with the same buffer, elution (arrow) was performed with 0.05 M glycine-HCl buffer (pH 3.0) containing 0.5 M NaCl. Fractions of 2.4 ml were collected at 4°.

Cytisus type anti-H hemagglutinins may share a common H-determining oligosaccharide chain on the cell surface but they bind to different portions of the H-determinant structure. These assumptions should be verified by the binding studies of labeled anti-H hemagglutinins to the human erythrocytes. In this connection, it is particularly important to obtain pure anti-H hemagglutinins, freed from contaminating proteins which cannot be removed by ordinary purification procedures, for the binding studies. In this paper, we describe the specific purification of eel serum anti-H and *C. sessilifolius* anti-H hemagglutinins by use of sugar–starch specific adsorbents, which we have previously reported (Matsumoto and Osawa, 1972). Also reported in this paper are the results of binding studies of the labeled purified anti-H hemagglutinins to human erythrocytes of various blood groups. Based on these results, the structure which these two kinds of anti-H hemagglutinins actually recognize on the cell surface is discussed.

Experimental Section

Preparation of Specific Affinity Adsorbents. L-Fucose–starch specific adsorbent for the purification of eel serum anti-H hemagglutinin and tri-*N*-acetylchitotriose–starch specific adsorbent for *C. sessilifolius* anti-H hemagglutinin were prepared according to the method previously described (Matsumoto and Osawa, 1972).

Purification of Eel Serum Anti-H Hemagglutinin. Five milliliters of fresh eel serum was directly applied to an affinity column of a L-fucose–starch specific adsorbent. The purified eel serum hemagglutinin was eluted as described in the legend of Figure 1.

Purification of C. sessilifolius Anti-H Hemagglutinin. One-hundred grams of freshly powdered *C. sessilifolius* seeds (purchased from F. W. Schumacher, Sandwich, Mass.) was suspended in 1 l. of 5 mM phosphate-buffered saline (pH 7.0) and allowed to stand overnight at 4° with continuous stirring. To the clear supernatant obtained by centrifugation at 12,000*g* for 20 min, sufficient solid $(NH_4)_2SO_4$ was added to give 0.7 saturation. The precipitate collected by centrifugation at 12,000*g* for 20 min was dialyzed against distilled water until free of NH_4^+ and then lyophilized. Further purification of this fraction was achieved by gel filtration on Sephadex G-200 followed by affinity chromatography on a tri-*N*-acetylchitotriose–starch column as described in the legends of Figures 2 and 3.

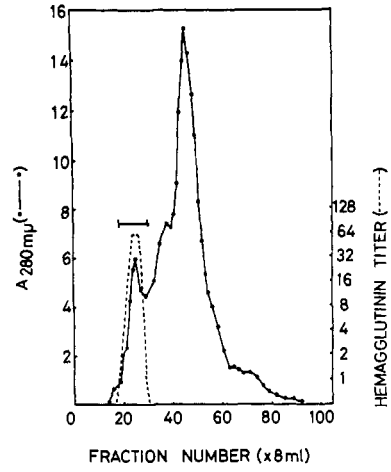

FIGURE 2: Sephadex G-200 gel filtration of crude *C. sessilifolius* hemagglutinin. One gram of the active fraction obtained by $(NH_4)_2SO_4$ fractionation was dissolved in 10 ml of 5 mM phosphate-buffered saline (pH 7.0) and applied to a Sephadex G-200 column (2 × 50 cm) equilibrated against the same buffer. Elution was carried out with the same buffer and fractions of 8 ml were collected at a flow rate of 30 ml/hr at 4°.

Hemagglutinins and Antisera. U. europeus hemagglutinins I and II were prepared by the methods previously described (Matsumoto and Osawa, 1969, 1970). *Phaseolus vulgaris* hemagglutinin was a product of Difco (PHA-M). Concanavalin A (Con A) was purified from Jack bean (Sigma) according to the method of Agrawal and Goldstein (1967). *Ricinus communis* hemagglutinin was purified by the method of Tomita *et al.* (1972). *Solanum tuberosum* hemagglutinin was prepared from commercial potatoes according to the method of Marinkovich (1964). Wheat germ agglutinin was purified

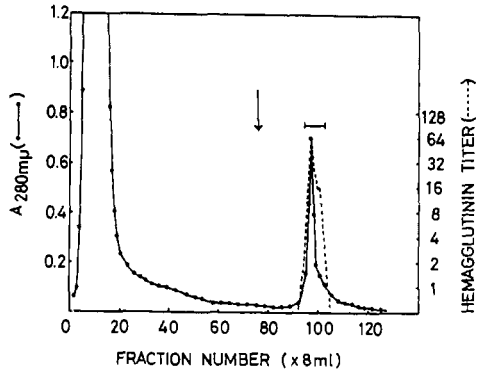

FIGURE 3: Affinity chromatography of *C. sessilifolius* hemagglutinin on a tri-*N*-acetylchitotriose–starch column. The active fractions from Sephadex G-200 gel filtration were pooled, dialyzed against distilled water, lyophilized, and redissolved in 10 ml of 5 mM phosphate-buffered saline (pH 7.0). The solution was then applied to a column of tri-*N*-acetylchitotriose–starch (4.5 × 20 cm) equilibrated with the same buffer. After a large protein peak was eluted out, the column was further eluted with 0.05 M glycine-HCl buffer (pH 2.0) containing 0.5 M NaCl. Fractions of 8 ml were collected at 4°.

TABLE I: Details of Purification of Eel Serum Anti-H Hemagglutinin.

Fraction	Vol (ml)	Yield (mg)	Hemagglutinating Titer for O	A	B	AB	Yield of Act. (%)
Crude serum	5	300	512	128	128	64	100
After affinity chromatography							
Fraction A	14.4	4.0	4	*a*	*a*	*a*	2.4
Fraction B	9.6	7.5	160	40	40	20	60

[a] Not determined.

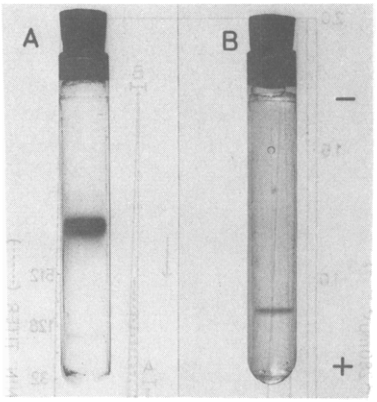

FIGURE 4: Polyacrylamide disc gel electrophoresis of purified anti-H hemagglutinins. Electrophoresis was for 1 hr at 5 mA/tube with 7.5% gels in Tris-HCl buffer (pH 8.9): (A) eel serum anti-H hemagglutinin; (B) *C. sessilifolius* anti-H hemagglutinin.

by the method of Allen *et al.* (1973). Rabbit immune anti-A and anti-B sera were purchased from Tokyo Standard Serum Ltd.

Ultracentrifugation. Measurement of the sedimentation velocity of the hemagglutinins was performed according to the band sedimentation method (Vinograd *et al.*, 1963) in a Spinco Model E ultracentrifuge equipped with an ultraviolet optical system at a speed of 56,100 rpm. The absorbancy at 280 nm of the hemagglutinin solutionst ested was 3.0 in 0.2 M NaCl.

Disc Electrophoresis. Disc electrophoresis in polyacrylamide gel was carried out in 7.5% gels in Tris-HCl buffer at pH 8.9 according to Ornstein (1964) and Davis (1964). Staining was performed with Amido Black in 7% acetic acid, and destaining in an electric field with 7% acetic acid.

Molecular Weight Estimation by Gel Filtration. A column of Sephadex G-200 (1.6 × 90 cm) was equilibrated with 5 mM phosphate-buffered saline (pH 7.0). The relationship between the elution volume and the logarithm of the molecular weights of various proteins was established on this column according to the procedure of Andrews (1964). The following proteins obtained from Mann Research Laboratories were used for this purpose (molecular weights shown in parentheses): myoglobin (17,800), chymotrypsin (25,000), bovine serum albumin (67,000), and human γ-globulin (160,000). Blue dextran (Pharmacia) was used for determining the void volume of the column.

Hemagglutination Assays. The titration and inhibition assays using human erythrocytes freshly obtained from a donor were carried out according to the methods previously described (Matsumoto and Osawa, 1970).

Iodination of Hemagglutinin. Purified anti-H hemagglutinins were iodinated with ^{125}I by the chloramine-T method of Hunter (1967) using a 30-sec exposure to the chloramine-T, and the labeled hemagglutinins were freed from an excess of reagents by passage over Sephadex G-50. This procedure did not affect the agglutinating activity of the hemagglutinins.

Binding Studies. Binding reactions were carried out in silicon coated tubes presoaked overnight with 0.25% bovine serum albumin solution in 5 mM phosphate-buffered saline (pH 7.0). The reaction mixture contained 2–4 × 10^6 erythrocytes or 1.4 × 10^6 lymphocytes and 1–10 μg of ^{125}I-labeled hemagglutinin in a final volume of 0.3 ml of 5 mM phosphate-buffered saline–0.25% bovine serum albumin. After 30 min of incubation at room temperature with occasional mixing, the cells were washed twice with each 3 ml of the cold phosphate-buffered saline–0.25% bovine serum albumin, and the amount of bound ^{125}I was determined in an Alloka autogamma counter with appropriate corrections for the nonspecific binding to the tube. The data were plotted by the method of Steck and Wallach (1965) according to the equation

$$\frac{C}{[HA_b]} = \frac{1}{Kn}\frac{1}{[HA_f]} + \frac{1}{n}$$

where [HA] = concentration of hemagglutinin (M) (HA_b, bound; HA_f, free), n = number of hemagglutinin binding sites per cell (expressed as micrograms of hemagglutinin bound per cell), C = number of cells, and K = the affinity constant of hemagglutinin.

Trypsin Treatment of Human Erythrocytes. To a 10% erythrocyte suspension in 0.05 M phosphate-buffered saline (pH 7.7) was added 0.25 mg of crystalline trypsin per 10^9 cells and the suspension was shaken gently for 3 hr at 37°. The cells were then collected and washed several times with chilled 5 mM phosphate-buffered saline (pH 7.0).

Purification of Lymphocytes. Human group O venous blood was withdrawn into syringes previously treated with heparin. The heparinized blood was transferred to glass cylinders, and the erythrocytes were allowed to sediment by gravity. The leukocyte-rich plasma was withdrawn, and layered over the same volume of the following solution in a glass centrifuge tube: 9.1 g of Ficoll (Pharmacia Fine Chemical, Sweden) and 25 ml of Urografin solution (60%, Schering, Germany) in 125

TABLE II: Details of Purification of *C. sessilifolius* Anti-H Hemagglutinin.

Fraction	Yield from 20 g of Seeds (mg)	Hemagglutinating Act.[a] (μg/ml) O	A	B	AB
Crude extract	1139	313	625	625	1250
$(NH_4)_2SO_4$ fraction 0–0.7 saturation	600	156	313	313	625
After Sephadex G-200 gel filtration	75	39	78	78	156
After affinity chromatography	4.2	4.9	9.8	9.8	20

[a] Minimum hemagglutinating dose against human O, A, B, or AB cells.

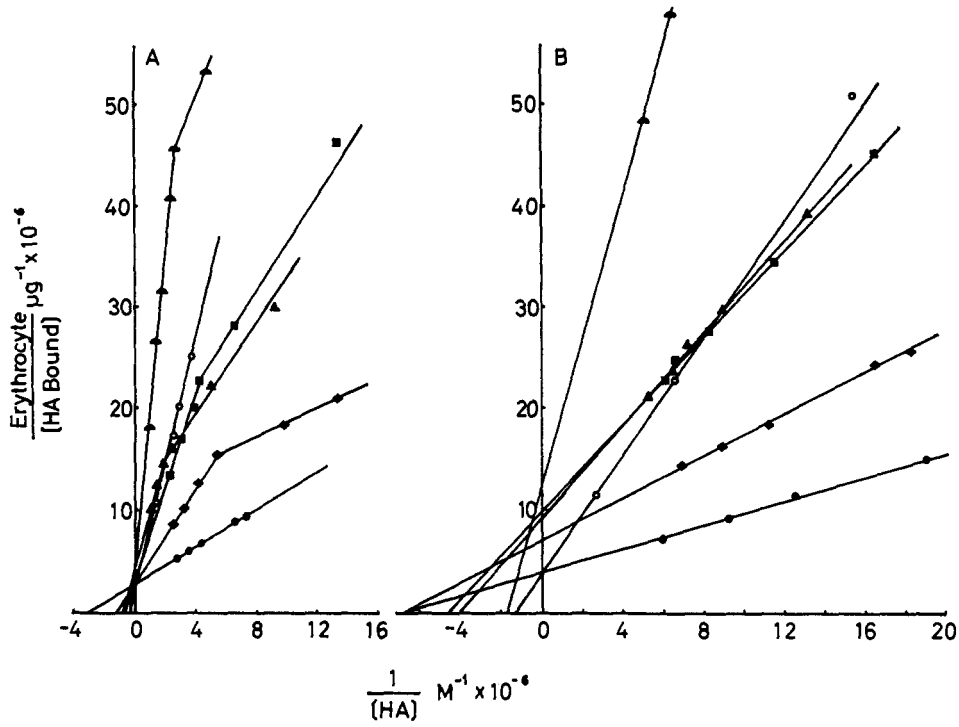

FIGURE 5: Binding of ESH and CSH to human erythrocytes of various blood groups. The binding reactions of [^{125}I]ESH (in A) and [^{125}I]CSH (in B) were performed as described in the text. The data were plotted by the method of Steck and Wallach (1965): (●) O erythrocytes (2.3 × 10^6 cells); (○) O erythrocytes (2.3 × 10^6 cells) with inhibitory sugars (100 μg of L-fucose in A; 500 μg of di-*N*-acetylchitobiose in B); (■) A_1 erythrocytes (3.1 × 10^6 cells); (◆) A_2 erythrocytes (2.6 × 10^6 cells); (▲) B erythrocytes (3.2 × 10^6 cells); (◓) AB erythrocytes (3.1 × 10^6 cells).

ml of twice distilled water. The centrifugation was performed at 400*g* for 20 min. The white fluffy interphase was removed by aspiration. The average proportion of lymphocytes in this layer was higher than 98%, and the viability was found to be 98–100% by a trypan blue exclusion test. The cells were then washed with 5 mM phosphate-buffered saline–0.25% bovine serum albumin and used for the binding studies.

Results

Purification of Hemagglutinins. Table I summarizes data pertaining to the purification of eel serum anti-H hemagglutinin. Of particular note is the fact that a single-step specific purification could be effectively achieved by affinity chromatography on an L-fucose–starch column as shown in Figure 1. The hemagglutinating activity was recovered in two protein peaks (fractions A and B) by lowering the pH of the buffer. Fraction B, which represented a major active protein, was used as such for the further experiments. The minimum hemagglutinating dose of this purified hemagglutinin (ESH)[1] was 4.1 μg/ml against human group O erythrocytes.

The results of the purification of *C. sessilifolius* anti-H hemagglutinin are summarized in Table II. Considerable enrichment of the hemagglutinating activity was achieved by ammonium sulfate fractionation of the crude extract. In order to remove inert low molecular weight substances which were found to be difficult to separate in the following affinity chromatography, the active ammonium sulfate fraction was further purified by Sephadex G-200 gel filtration as shown in Figure 2. Strong hemagglutinating fractions were pooled and then applied to a specific affinity column of tri-*N*-acetylchitotriose–starch adsorbent (Figure 3). *C. sessilifolius* anti-H hemagglutinin was clearly retained by the column and could be eluted by lowering the pH of the buffer. The minimum hemagglutinating dose of the purified hemagglutinin thus obtained (CSH) was 4.9 μg/ml against human group O erythrocytes.

The anti-H specificity of these purified hemagglutinins was confirmed by inhibition experiments with human salivas. Both hemagglutinins were inhibited by salivas from secretor individuals, but not those from nonsecretors.

Further tests for the homogeneity of the purified hemagglutinins were carried out by ultracentrifugation and disc electrophoresis.

Homogeneity and Molecular Weight Estimation. Ultracentrifugation of ESH and CSH yielded a single peak in each case during the whole of the run in the densitometer chart obtained from the experiment. The sedimentation coefficients ($s_{20,w}$) calculated from the sedimentation velocity data were 7.2 S for ESH and 6.8 S for CSH.

The electrophoretic homogeneity of these two purified hemagglutinins was confirmed by disc electrophoreses on polyacrylamide gel. A single band was observed in both instances as shown in Figure 4.

Approximate molecular weights of 140,000 and 110,000 were calculated for ESH and CSH, respectively, from the curve relating elution volume to logarithm of molecular weights of various standard proteins on a Sephadex G-200 column.

Binding Studies of Purified Hemagglutinins. The binding experiments of ^{125}I-labeled ESH (sp radioact., 13,000 cpm/μg) and CSH (sp radioact., 40,000 cpm/μg) to human erythrocytes of various blood groups were carried out, and the data obtained were plotted according to the method of Steck and Wallach (1965) as shown in Figure 5. The apparent associa-

[1] Abbreviations used are: ESH, eel serum hemagglutinin; CSH, *Cytisus sessilifolius* hemagglutinin.

TABLE III: Bindings of [^{125}I]ESH and [^{125}I]CSH to Human Erythrocytes and Lymphocytes.

Hemagglutinin	Cell	Blood Group	No. of Receptor Sites[a] ($\times 10^6$)	App Affinity Constant[a] ($\times 10^6$ M^{-1})
ESH	Erythrocytes	O	1.7	2.7
		O[b]	2.2	5.1
		A_1	1.0	1.3
		A_2	1.2	1.9
		A_2[c]	0.9	3.1
		B	0.9	1.0
		B[d]	0.6	1.4
		AB	0.9	0.5
	Lymphocytes[e]		0.4	1.0
CSH	Erythrocytes	O	1.9	5.5
		O[b]	2.0	6.0
		A_1	0.7	5.0
		A_2	1.1	6.9
		B	0.9	3.0
		AB	0.6	1.9

[a] Average values of duplicate experiments. [b] Trypsin-treated cells. [c] Anti-A was added. [d] Anti-B was added. [e] From a blood group O donor.

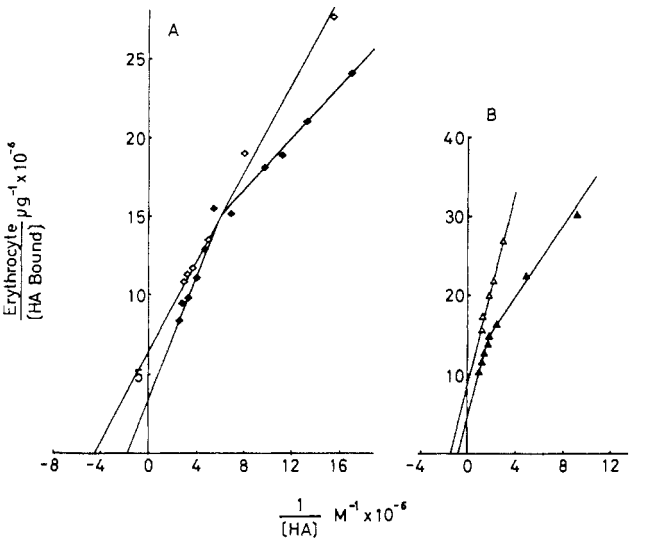

FIGURE 6: Effect of antiserum on ESH binding to human erythrocytes. The binding reactions of [^{125}I]ESH to human A_2 erythrocytes (in A) and to human B erythrocytes (in B) were carried out in the absence and presence of antiserum (titer: X256). Experimental details are described in the text. The data were plotted by the method of Steck and Wallach (1965): (◆) A_2 erythrocytes (2.6×10^6 cells); (◇) A_2 erythrocytes (2.6×10^6 cells) + 0.1 ml of anti-A serum; (▲) B erythrocytes (3.2×10^6 cells); (△) B erythrocytes (2.5×10^6 cells) + 0.1 ml of anti-B serum.

tion constants and the average number of receptor sites per cell were calculated using the molecular weights estimated by gel filtration, and these values are listed in Table III. There are approximately the same number of receptor sites for both ESH and CSH on group O erythrocytes and the lower number of receptor sites was observed on the erythrocytes of other blood groups in the decreasing order of O > A_2 > A_1 = B > AB. Furthermore, it can be seen from Table III that, in general, CSH binds to the erythrocytes with a greater affinity than ESH, and the trypsin treatment of erythrocytes increases their affinity to the hemagglutinins, especially to ESH. Of particular interest is the fact that human peripheral lymphocytes from a group O donor specifically bind significant amounts of [^{125}I]ESH. It is also interesting to note that the binding of [^{125}I]ESH to the erythrocytes other than group O gives two-phasic lines when the data are plotted according to Steck and Wallach (1965) as shown in Figure 5A. Since ESH is homogeneous at least from the criteria of ultracentrifugation and electrophoresis, these results seem to suggest that there exist two independent classes of receptor sites on these cells. In order to verify this assumption, anti-A or anti-B serum was included in the binding reaction of [^{125}I]ESH to the erythrocytes. This blockage of blood group A or B determinants on the cell surface made the plots linear as shown in Figure 6 with a decrease of the number of receptor sites and an increase of the apparent affinity constants (Table III).

Effect of Simple Sugars on Binding to Human Group O Erythrocytes. In parallel with the facts that eel serum anti-H hemagglutinin is most effectively inhibited by L-fucose and *C. sessilifolius* anti-H hemagglutinin is best inhibited by di-*N*-acetylchitobiose, these sugars are strongly inhibitory against the bindings of ^{125}I-labeled ESH and CSH to human group O erythrocytes. At the [^{125}I]ESH concentration of 5 μg/ml, the concentration of L-fucose which caused a 50% inhibition of the binding was 34 μg/ml and, at the [^{125}I]CSH concentration of 5 μg/ml, 230 μg/ml of di-*N*-acetylchitobiose caused a 50% inhibition of the binding. From standard competitive inhibition curves (Figure 5), these sugars were found to be competitive inhibitors.

Effect of Other Hemagglutinins on Binding to Human Group O Erythrocytes. The facts that L-fucose-containing oligosaccharides, *i.e.*, 2′-fucosyllactose and lacto-*N*-fucopentaose I, were potent inhibitors for both ESH and CSH, and that there exist approximately the same number of receptor sites for both hemagglutinins on group O erythrocytes strongly suggest that these hemagglutinins bind to different portions of the same oligosaccharide chain on the cell surface. If this were the case, the binding of one anti-H hemagglutinin could be expected to interfere with the binding of the other anti-H hemagglutinin. To test this assumption and to determine the relationship between the receptor sites of these anti-H hemagglutinins and those of other blood group nonspecific hemagglutinins, competitive inhibition studies were performed and the results are listed in Table IV. In these experiments, the amount of each competing hemagglutinin added was more than ten times the amount which was sufficient to saturate its receptor sites on the cell surface. As shown in Table IV, the [^{125}I]ESH binding to human group O erythrocytes was strongly inhibited not only by ESH itself and *U. europeus* hemagglutinin I (eel serum type) but also by CSH and *U. europeus* hemagglutinin II (*Cytisus* type) and the [^{125}]CSH binding was, in turn, inhibited by ESH and *U. europeus* hemagglutinin I. Among the nonspecific hemagglutinins tested, *Solanum tuberosum* hemagglutinin is a potent inhibitor against the [^{125}I]CSH binding but is not an inhibitor against [^{125}I]ESH binding, and wheat germ agglutinin has remarkable inhibitory activity against the bindings of both [^{125}I]ESH and [^{125}I]CSH. On the other hand, Con A, PHA-M, and *R. communis* hemagglutinin were found to be virtually devoid of inhibitory activity against the binding of both anti-H hemagglutinins.

Discussion

The specific purification of eel serum anti-H hemagglutinin has been previously reported by Springer and Desai (1971) by means of the precipitation reaction with digitarose. In the present work, we have purified eel serum and *C. sessilifolius* anti-H hemagglutinins by affinity chromatography using immobilized haptenic inhibitors as specific adsorbents. These specific adsorbents, sugar–starch adsorbents, are very easy to prepare and very effective for the purification of the hemagglutinins which can be inhibited by simple sugars and oliosaccharides. The hemagglutinins thus purified were found to be homogeneous by ultracentrifugal analysis and electrophoresis on polyacrylamide gel. Particularly, the purification of eel serum anti-H hemagglutinin could be achieved by a single passage over an L-fucose–starch specific affinity column. The sedimentation constant and the molecular weight of our purified eel serum hemagglutinin (estimated to be 7.2 S and 140,000) are in good agreement with the values (7.2 S and 123,000) reported by Bezkorovainy *et al.* (1971).

Fujisawa *et al.* (1963) estimated the number of H determinants on human group O erythrocytes to be 7.0–9.3 $\times$ 10^6 per cell from the amount of liberated L-fucose residue when the cells were treated with an H-decomposing enzyme from *Bacillus fulminans* (Iseki *et al.*, 1962). However, this value is considerably larger than the number of H determinants (1.7–1.9 $\times$ 10^6 per cell) determined in this paper by the binding studies of ^{125}I-labeled anti-H hemagglutinins. The latter value is, in turn, in close agreement with the number of A or B determinants (0.6–1.2 $\times$ 10^6 per cell) on human group A_1 or B erythrocytes reported by Greenbury *et al.* (1963) and Economidou *et al.* (1967). In view of the proposed biosynthetic pathway of ABH blood group determinants, the number of H determinants on group O erythrocytes should be either equal to or somewhat larger than the number of A or B determinants on group A_1 or B erythrocytes. The presence of H determinants on group A or B erythrocytes as incomplete carbohydrate chains was demonstrated by the fact that the Steck and Wallach plots of the binding of [^{125}I]ESH to group A_1, A_2, B, or AB erythrocytes gave a two-phasic line (Figure 5). It is known that, if there are two or more independent classes of receptor sites or binding proteins, the Steck and Wallach plots will no longer give a straight line (Klotz and Hunston, 1971). Since ESH is homogeneous in the criteria of ultracentrifugation and electrophoresis, it is reasonable to assume that there exist more than two kinds of receptor sites on these cells for ESH. In the previous paper (Matsumoto and Osawa, 1971), we reported that eel serum type anti-H hemagglutinins are more strongly affected by the steric hinderance caused by the α-galactosaminyl or α-galactosyl unit joined (1$\rightarrow$3) to the terminal D-galactose in the carbohydrate chain of blood group determinant structure. Therefore, eel serum type anti-H hemagglutinins act more strongly on group O erythrocytes than group A and B erythrocytes, whereas no significant difference of reactivity between blood groups is observed in the reaction with *Cytisus* type anti-H hemagglutinins. These facts explain why ESH gives two-phasic lines in the Steck and Wallach plots differentiating A and B determinants from H determinants which remain on these cells as incomplete carbohydrate chains. This explanation was further confirmed by the experimental evidence that, in the [^{125}I]ESH binding to group A_2 or B erythrocytes, the blockage of A or B determinants with anti-A or anti-B serum made the Steck and Wallach plots linear (Figure 6) and, furthermore, this treatment resulted in the decrease of the number of binding sites and the simultaneous increase of the apparent affinity constants.

TABLE IV: Inhibition of Bindings of [^{125}I]ESH and [^{125}I]CSH by Other Hemagglutinins.

Competing Unlabeled Hemagglutinin	Binding of Labeled Hemagglutinin (% of Control)[a]	
	[^{125}I]ESH	[^{125}I]CSH
None	100	100
ESH	8	7
Ulex I[b]	27	6
CSH	34	8
Ulex II[c]	35	7
S. tuberosum	100	11
Wheat germ agglutinin	29	27
PHA-M	100	86
R. communis	60	100
Con A	100	100

[a] Average value of duplicate experiments. [b] *Ulex europeus* hemagglutinin I (eel serum type). [c] *Ulex auropeus* hemagglutinin II (*Cytisus* type).

The direct evidence for the assumption that ESH and CSH share a common H determinant on the cell surface was obtained by competitive inhibition studies in which CSH was found to effectively inhibit the binding of [^{125}I]ESH and *vice versa*. These facts in addition to the data from the inhibition studies with simple sugars and oligosaccharides indicate that these two kinds of anti-H hemagglutinins bind to different but overlapping portions of the same H determinant structure on the cell surface as shown in Figure 7. In the competitive inhibition studies with blood group nonspecific hemagglutinins, *S. tuberosum* hemagglutinin was a potent inhibitor against the [^{125}I]CSH binding and wheat germ agglutinin was an effective inhibitor against the bindings of both [^{125}I]ESH and [^{125}I]CSH to group O erythrocytes. Since it has been previously proposed that *S. tuberosum* hemagglutinin interacts with a reducing or inner-linked *N*-acetylglucosamine residue in the carbohydrate chain on the cell surface (Pardoe *et al.*, 1969; Matsumoto and Osawa, 1971), and CSH possibly interacts with the Fuc-Gal-GlcNAc sugar sequence in the H-determinant chain on the cell surface (Matsumoto and Osawa, 1970), the interaction of *S. tuberosum* hemagglutinin with the inner-linked *N*-acetylglucosamine residue in the H-determinant chain may strongly affect the binding of [^{125}I]CSH. On the other hand, ESH which probably interacts with the Fuc-Gal sugar sequence is not affected by the binding of *S. tuberosum* hemagglutinin. These relationships between the receptor sites of the hemagglutinins are also illustrated in

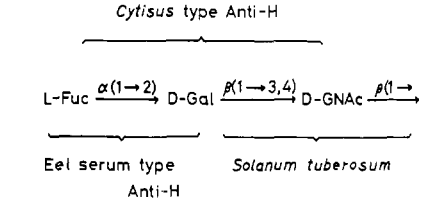

FIGURE 7: Relationship between receptor sites of various hemagglutinins.

Figure 7. A possible explanation of the inhibitory effect of wheat germ agglutinin may lie in the fact that this agglutinin binds to sialic acid residue on the cell surface (Greenaway and Levine, 1973). That is, this binding may somehow make the receptor sites inaccessible to ESH and CSH.

Flory (1967) reported that blood group H antigens could not be detected on human leucocytes by the mixed agglutination test using *U. europeus* and *C. sessilifolius* anti-H hemagglutinins. In contrast, several investigators clearly demonstrated the presence of blood group A antigens on human leucocytes using the mixed agglutination test (Gurner and Coombs, 1958) and the binding studies with ^{131}I-labeled anti-A serum (Anderson and Walford, 1963). The presence of the A antigen and the absence of the H antigen on leucocytes are surprising because the proposed biosynthetic pathway for blood group antigens on human erythrocytes suggests that the A and B antigens are synthesized from H antigen, and that the H antigens must therefore be present first. In the present study, we observed the binding of [^{125}I]ESH to human lymphocytes from group O donors and this binding was found to be of specific nature because it was strongly inhibited by the addition of L-fucose. However, it remains unresolved at the present time why these H antigens on the lymphocyte cell surface are ineffective for the agglutination and the mixed agglutination. In this respect, further investigations are now in progress in this laboratory.

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