THE ACTION OF ENZYMES FROM CLOSTRIDIUM TERTIUM ON THE I ANTIGENIC DETERMINANT OF HUMAN ERYTHROCYTES*

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High titer erythrocyte cold agglutinins occur most frequently in association with atypical pneumonia (1, 2), leukemia, lymphoma, and in patients with hemolytic anemia without obvious underlying disease, the so-called "cold-hemagglutinin syndrome" (3; 4, p. 342). These antibodies have been thought to be non-specific in nature in that they usually react with all human erythrocytes tested, although it has been recognized that red cells from normal individuals vary widely in their sensitivity to agglutination by a given antibody (5). In addition, there are several reports of cold agglutinins reacting preferentially with group O cells (2, 6, 7; 8, p. 333), and one report of their acting like a "pseudo anti-A_1" (9).

Knowledge of the anti-I specificity of some cold agglutinins started with the observation of Wiener *et al.* (10) that the red cells of five individuals, of over 22,000 tested, were not agglutinated at 24°C by a high titer cold agglutinin. These cells were agglutinated at 4°C, but to much lower serum dilutions than were other test cells, and the agglutinability of both usual and exceptional cells was increased proportionally by treatment with ficin. The antibody was termed anti-I, usual cells were considered to be I-positive, and the cells of the five exceptional individuals were designated as i, or I-negative. The sera of three of the five i individuals were tested for anti-I agglutinins, and they possessed low titer cold agglutinins of "average normal titer."

In 1960, six additional healthy individuals with type i cells were reported (11, 9), three of whom were siblings. Their cells were agglutinated weakly, or not at all, at 20°C by two high titer cold agglutinins, one of which was the antibody employed previously by Wiener (10). The individual whose cells were weakest in I antigen possessed a powerful anti-I cold agglutinin; the other five sera also contained anti-I agglutinins, but it is not clear whether their titers exceeded the normal cold agglutinin range. The extremely weak reaction of umbilical cord erythrocytes with anti-I was also noted (11).

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Marsh (12) later reported two sera with cold agglutinins which reacted strongly with adult i cells and weakly with normal I cells and designated this antibody as anti-i. Erythrocytes from newborn infants reacted strongly with the anti-i agglutinins; this reactivity gradually declined in strength during the first 18 months of life, during which time I reactivity appeared.

The limited genetic data currently available indicate a recessive pattern of inheritance for the adult i phenotype (9; 8, pp. 330–332).

Although all of the cold agglutinins studied in the early observations on the Ii system appeared to be specific for either the I or i determinants, Dacie has reported five sera and van Loghem *et al.* fourteen of thirty-nine sera which do not discriminate between I and i cells (4, pp. 497, 532; 7).

During the course of purification from *Clostridium tertium* culture filtrates of an enzyme which acts on blood group A substance (13–16), it was found that a fraction containing partially purified beta galactosidase and beta glucosaminidase activities reduced the I activity of human erythrocytes. Both enzymes seemed to be needed to effect maximum reduction in activity. Treatment of red cell stroma with these enzymes resulted in a loss of ability of the stroma to absorb anti-I with the liberation of galactose and *N*-acetylglucosamine as monosaccharides, suggesting that these two sugars are involved in the I antigenic determinant.

Materials and Methods

Cultivation of Bacteria.—The strain of *Clostridium tertium* isolated originally by Iseki (13) was obtained from Dr. Calderon Howe. The organisms were cultivated in the medium described by Howe *et al.* (14) with the following modifications: for induction of both beta galactosidase and beta glucosaminidase activities, a final concentration of 0.05 per cent lactose and 0.1 per cent D-glucosamine–HCl was used; to obtain beta galactosidase with much less glucosaminidase activity, a final concentration of 0.075 per cent lactose was employed without added glucosamine. For initiation of growth and serial passage 10 ml cultures were grown in an anaerobic jar at 37°C and transferred at 48 hour intervals. For bulk production of enzymes, 6 liter lots of culture medium were inoculated with 100 ml of a 24 hour anaerobic culture and incubated aerobically for 48 hours. The bacteria were sedimented by centrifugation at 2000 RPM for 1 hour in an International refrigerated centrifuge model PR-2.

Fractionation of Enzymes.—Six liters of centrifuged culture supernatant were concentrated to about 200 ml by dialysis against carbowax[1] 20000 and then dialyzed against the chromatography starting buffer, 0.01 M tris, tris(hydroxymethyl)aminomethane, pH 7.5, 0.9 per cent NaCl, and 0.001 M Mn^{++}. The dialyzed supernatant was applied to a 20 gm DEAE cellulose[2] column, 3.4 × 20 cm, and the column washed with starting buffer until absorption at 2800 A of the effluent was negligible. An NaCl gradient was then imposed, using an external reservoir containing the limit buffer, 0.01 M tris, pH 7.5, 1.0 M NaCl, and 0.001 M Mn^{++}, and two mixing chambers in series, each containing 400 ml of starting buffer. In general, the flow rate was 200 ml/hour, and 20 ml fractions were collected. When the column effluent no longer exhibited either absorption at 2800 A or enzyme activity, the column was

[1] Obtained from Union Carbide Chemicals Company, New York.

[2] DEAE selectacel standard, Schleicher and Schüll Company, Keene, New Hampshire.

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flushed with the limit buffer. The shape of the gradient was determined by measuring the conductivity of the fractions with a serfass conductivity bridge, model RCM 15 B_1.[3]

Manganese was used in the chromatography buffer to stabilize the enzyme which acts on the blood group A substance. It serves no useful function in the preparation of either beta galactosidase or beta glucosaminidase and interferes with the reducing sugar, hexosamine, and acetylhexosamine assays.

Enzyme Assays.—Beta galactosidase activity was determined by measuring the hydrolysis of *o*-nitrophenyl beta-D-galactopyranoside[4] (17). The reaction mixture consisted of enzyme, buffer, and saline in a total volume of 0.5 ml, plus 1.0 mg of substrate in a volume of 0.5 ml. After 30 minutes at 37°C, 0.5 ml of 0.5 M Na_2CO_3 was added and the optical density was read at 4050 A. With this substrate, the pH activity curve showed a broad optimum between pH 6.0–7.5; assays were performed in a 0.05 M phosphate buffer, pH 7.5, in the early studies, and in a 0.02 M phosphate buffer, pH 6.5, in the later work.

Beta glucosaminidase activity was measured by following the hydrolysis of beta methyl-*N*-acetylglucosaminide.[5] The reaction mixture contained enzyme, 0.01 M phosphate buffer, pH 6.5, and 10.0 μg of substrate in a total volume of 0.25 ml. After incubation for 1 hour at 37°C an analysis for free *N*-acetylhexosamine was performed as described below.

A semiquantitative estimation of peptidase activity was obtained by following the hydrolysis of L-leucylglycylglycine,[6] using the ninhydrin reaction (18, 16) to measure the increase in free amino nitrogen. The reaction mixture contained enzyme, 0.017 M tris buffer, pH 7.5, 0.004 M Mn^{++}, and 15.7 μg of substrate in a volume of 0.3 ml. After 1 hour at 37°C, 0.1 ml of 4.0 M acetate buffer, pH 5.5, was added, the solutions were mixed, and 0.1 ml of 5.0 per cent ninhydrin in methyl cellosolve added. The ninhydrin was activated just before use by the addition of 0.05 ml of 0.01 M KCN per 2.0 ml of 5 per cent ninhydrin. The tubes were heated in a water bath at 95°C for 20 minutes, cooled, and 4.5 ml of 50 per cent ethanol added; readings were made at 5700 A.

Zone Electrophoresis.—Electrophoresis was performed using pevikon C-870[7] as a supporting medium (19) in a block of dimensions 7.0 × 1.2 × 29.0 cm using the E-C electrophoresis apparatus. The buffer employed was 0.07 M phosphate, pH 6.5. Electrophoresis was carried out for 17 hours at 200 v, 16 to 24 ma at room temperature, with the block enclosed in a jacket through which cold water was circulated. The block was cut into 1.0 cm segments which were placed on sintered glass filters of medium porosity, and the enzymes eluted with two 2 ml portions of saline, using gentle positive pressure. Protein concentration in the eluates was estimated by reading the absorption at 2800 A.

Hemagglutination.—Blood was preserved at 4°C in Alsever's solution (20). Hemagglutination and hemagglutination inhibition tests were performed as previously described (20, chapter 3), using light centrifugation. Most of the assays were carried out on a microscale in a total volume of three to four drops, including one drop of 2 per cent or 4 per cent erythrocytes. When group A cells were used, the anti-A isoagglutinin was neutralized by addition of blood group A substance from hog gastric mucin to the anti-I serum.

Sera.—Sera were obtained from patients with hemolytic anemia associated with cold agglutinins. Patient Bu. had a reticulum cell lymphosarcoma; patient Ch.[8] had a lympho-

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[3] Obtained from Arthur H. Thomas Company, Philadelphia.

[4] Obtained from the California Corporation for Biochemical Research, Los Angeles.

[5] Alpha and beta methyl-*N*-acetylglucosaminide were generously supplied by Professor Richard Kuhn.

[6] Obtained from Mann Research Labs, Inc., New York.

[7] Obtained from Stockholms Superfosfat Fabriks A.-B., Stockholm, Sweden.

[8] Serum generously supplied by Dr. Peter Miescher.

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sarcoma; patient N.P.[9] had no obvious associated disease. The anti-I specificity of serum Bu. was confirmed by Dr. R. Race; the anti-I specificity of serum N. P. was documented in reference 21, page 391; the anti-I specificity of serum Ch. was established by tests with various red cells including adults of blood types Bi, A_1i, Oi, and numerous samples of cord blood cells. Type XIV antipneumococcal horse serum, H 615, bleeding of 4/15/39, was obtained from the New York State Department of Health through the courtesy of Dr. Jesse L. Hendry. Hemagglutination tests with serum Bu. were carried out at 20°C; all other sera were tested at 4°C.

Preparation and Treatment of Stroma.—The erythrocytes from an outdated unit of whole blood were washed with saline and the buffy coat removed by aspiration. The cells were lysed in 2 liters of saline containing 0.5 gm of digitonin[10] and the stroma washed with saline until the supernatant contained traces of hemoglobin. Stroma preparations varied considerably in their content of residual hemoglobin and in the ease with which they could be sedimented; the basis of this variability is not known. The washed stroma preparation was divided into two portions, one of which was suspended in a DEAE column fraction containing beta galactosidase and beta glucosaminidase, in 0.02 M phosphate, 0.15 M saline, pH 6.5, and the other portion was suspended in buffer. The total volume of the suspensions varied from 45 to 70 ml. The stroma suspensions were placed on a mechanical rotating device and incubated at 37°C for 17 to 19 hours, packed by centrifugation in the cold for 1 hour at 2000 RPM, washed three times with water, and the pooled supernatants from each preparation desalted by passage through amberlite[11] MB 3, 125 gm in a column 4.5 × 14 cm, and concentrated by evaporation under reduced pressure.

For further purification and characterization of the sugars present in the stroma supernatants, chromatography was performed on columns composed of equal weights of darco G-60 charcoal and celite No. 535 (22, 16). The blood group A and O stroma supernatants were chromatographed on 8 gm columns (4 gm charcoal + 4 gm celite), 2 × 9 cm, at a flow rate of 40 ml/hour; the B stroma supernatants were chromatographed on a 3 gm column, 1.2 × 8.5 cm, at a flow rate of 20 ml/hour.

The effect of enzyme treatment on the I determinant of erythrocyte stroma was evaluated by comparing the ability of control and enzyme-treated stroma to absorb cold agglutinins. Approximately equal volumes of packed control and enzyme-treated stroma were suspended in a 1:5 dilution of serum Ch., held at 4°C for 1 hour, and centrifuged at 2000 RPM for 1 hour, at 4°C. The supernatant sera were aspirated and aliquot portions were subjected to a second absorption.

If the stroma retained appreciable I activity following the initial overnight enzyme treatment, an aliquot portion was retreated for 72 hours at twice the initial enzyme:substrate ratio.

Analytical Methods.—Reducing sugars were measured by a modification of the procedure of Park and Johnson (23, 16). Methylpentose was measured by the method of Dische and Shettles (24), and galactose by the method of Dische (25), both on a one-fifth scale. Hexosamine was determined by a modification of the method of Elson and Morgan and others (26, 27, 16) in which the hydrolysis is carried out in a 5.0 ml volumetric flask. *N*-Acetylhexosamine was measured by the procedure of Reissig *et al.* (28). Nitrogen was measured by a ninhydrin method described recently (29, 30). The ethanol concentration in the charcoal column fractions was determined by a modification of a procedure employing Anstie's reagent (31, 16). The methods used are also given in reference 20.

Paper Chromatography.—Chromatograms were run on Whatman No. 1 or Schleicher and

[9] Serum generously supplied by Dr. Scott Swisher.

[10] Obtained from Mann Research Labs.

[11] Obtained from Mallinckrodt Chemical Works, New York.

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Schüll No. 589 green label paper, using a 1-propanol–ethyl acetate–water (7:1:2) solvent (32), and developed with alkaline silver nitrate (33, 34).

RESULTS

Enzyme Fractionation.—The fractionation of a concentrated *Cl. tertium* culture supernatant on a DEAE cellulose column is illustrated by the chromatogram reproduced in Fig. 1. Under the conditions specified, beta galactosidase, beta glucosaminidase, and peptidase activities were retained on the column, neuraminidase and peptidase activities, and the enzyme which acts on blood group A substance appear in the breakthrough volume. The A enzyme is retained on the column at NaCl concentrations below 0.9 per cent; 1 to 5 per cent of the total activity may be held on the column at 0.9 per cent NaCl. The recovery of galactosidase and glucosaminidase activities ranged from 75 to 100 per cent.

As seen in Fig. 1, the peak of galactosidase activity is only one tube (20 ml) ahead of that for glucosaminidase. This degree of separation could be improved somewhat by rechromatography on DEAE with the collection of smaller fractions, and by using stepwise elution with closely spaced NaCl concentrations. In this manner fractions with beta glactosidase activity with no detectable beta glucosaminidase activity were obtained from the front of the peak, but the beta galactosidase peak always overlapped the glucosaminidase region and it was not possible to obtain glucosaminidase without appreciable galactosidase activity. It should be noted that the method of assay for galactosidase is much more sensitive than that for glucosaminidase.

A beta glucosaminidase preparation with low beta galactosidase activity was obtained in the following manner. The fractions containing the peak galactosidase and glucosaminidase activities (fractions 89 to 94, Fig. 1) were pooled and rechromatographed on a DEAE cellulose column, using stepwise elution with NaCl. Both the glucosaminidase and galactosidase were eluted at 0.30 M NaCl and were separated from the peptidase, which was eluted at 0.25 M NaCl. The glucosaminidase-containing fractions were pooled, excluding the earlier beta galactosidase peak fractions, concentrated by dialysis against carbowax 20000 and subjected to electrophoresis on a pevikon block. As seen in Fig. 2, the material eluted from segment +7 had a considerably enhanced ratio of beta glucosaminidase/beta galactosidase activities.

Enzymatic Treatment of Erythrocytes.—The enzyme activity in unconcentrated *Cl. tertium* culture supernatants was too low for use in treatment of red cells. The unfractionated, concentrated supernatant could be employed but long treatment is required, several hours to overnight, because of the relatively low glucosaminidase activity. In addition, as noted previously (14), after treatment with the unfractionated supernatant the cells became agglutinable in autologous serum. In general, the DEAE cellulose column fractions with peak

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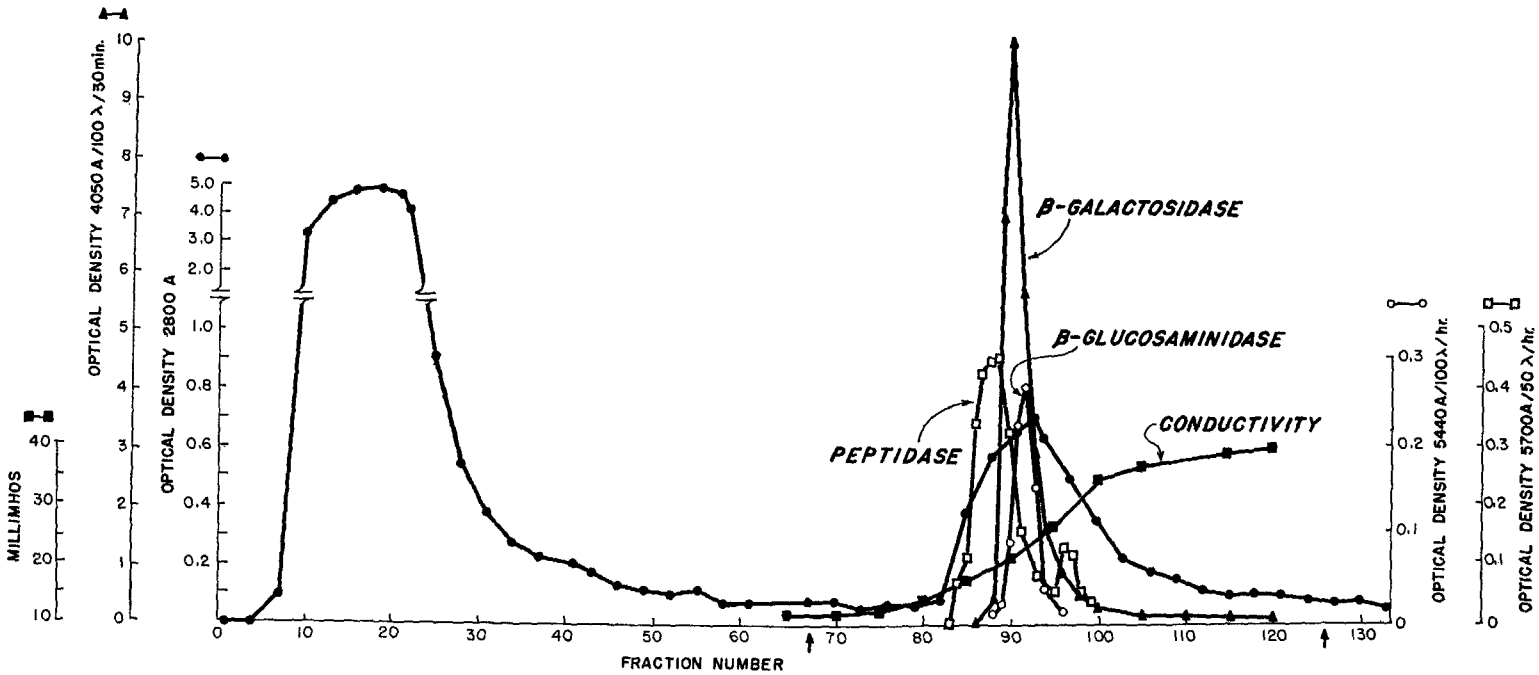

FIG. 1. Fractionation of a concentrated supernatant from 6 liters of a *Cl. tertium* culture on a 20 gm DEAE cellulose column, 3.4 × 20 cm. The starting buffer was 0.01 M tris, pH 7.5, 0.9 per cent NaCl, 0.001 M Mn^{++}; at the first arrow a gradient was imposed with a buffer containing 0.01 M tris, pH 7.5, 5.8 per cent NaCl, and 0.001 M Mn^{++}. This limit buffer was then applied directly to the column at the second arrow. Chromatography was carried out at room temperature with a flow rate of 200 ml/hour; 20 ml fractions were collected.

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beta galactosidase and beta glucosaminidase activities were pooled to provide a preparation for treatment of red cells or stroma. These pooled preparations contained variable amounts of peptidase activity, but this did not interfere with the determination of erythrocyte reactivity with anti-I agglutinins; fractions with high peptidase activity but no galactosidase or glucosaminidase

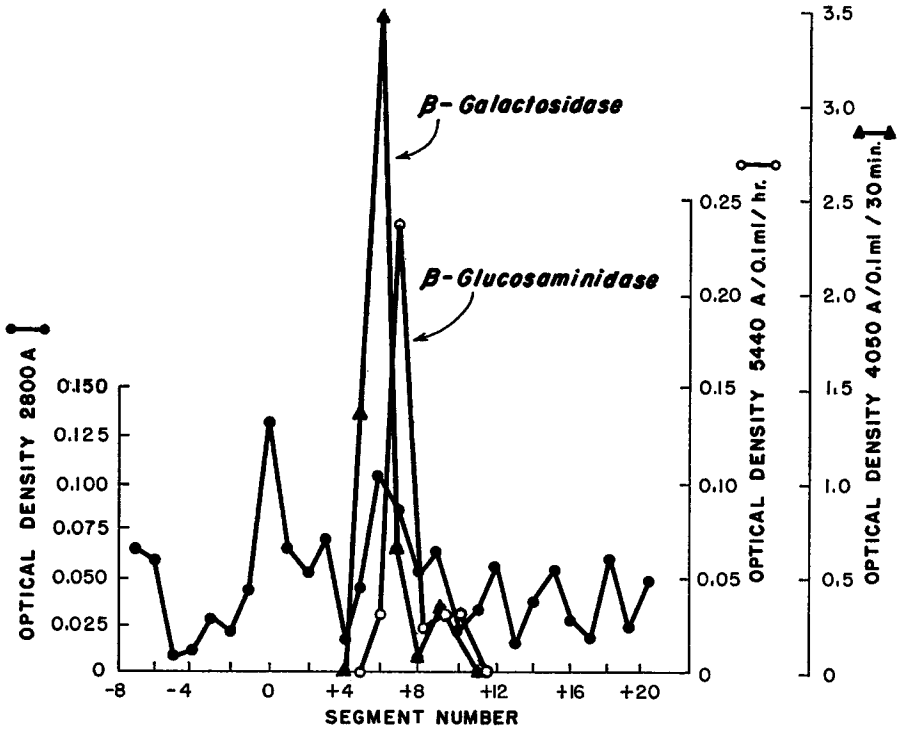

FIG. 2. Separation of beta galactosidase and beta glucosaminidase from *Cl. tertium* by zone electrophoresis in a pevikon block 7 × 1.2 × 29 cm. Electrophoresis was carried out for 17 hours at 200 v, 16 to 24 ma, in 0.07 M phosphate buffer, pH 6.5. The block was cut into 1 cm segments.

activity; *e.g.*, fraction 88, Fig. 1, did not alter the I activity of red cells, nor did any of the fractions from the earlier part of the chromatogram.

The data in Table I illustrate the decrease in reactivity with anti-I of groups A_1 and O erythrocytes treated with a preparation containing beta galactosidase and beta glucosaminidase. Two important features of this data should be noted: the differences among the three anti-I sera in the extent of decrease in titer against treated cells; and the effect of the ABO group of the cells on their susceptibility to enzymatic modification. Thus, when titrated simultaneously against A_1 cells from a single treated sample, sera Bu., Ch., and

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TABLE I

The Effect of Treatment with Cl. tertium Beta Galactosidase and Beta Glucosaminidase on the Agglutinability of Group A_1 and O Erythrocytes by Anti-I Cold Agglutinins and Type XIV Antipneumococcal Horse Serum*

Serum Bu.—reciprocal of dilution

		2	4	8	16	32	64	128	Saline
A_1 cells	Untreated	++++‡	++++	++++	++++	+++±	+++	+±	0
	Enzyme-treated	0	0	0	0	0	0	0	0
O cells	Untreated	++++	++++	++++	++++	++++	++++	+++±	0
	Enzyme-treated	+++±	+++	++	0	0	0	0	0

Serum Ch.—reciprocal of dilution

		4	8	16	32	64	128	256	512	1024	2048	4096	Saline
A_1 cells	Untreated	++++	++++	++++	++++	++++	+++±	+++±	++	+±	0	0	0
	Enzyme-treated	++	++	++	+±	+	0	0	0	0	0	0	0
O cells	Untreated	++++	++++	++++	++++	++++	++++	+++±	++	+±	+	0	0
	Enzyme-treated	++++	++++	++++	++++	++++	++++	+++	+	0	0	0	0

Serum N. P.—reciprocal of dilution

		4	8	16	32	64	128	256	512	1024	2048	4096	Saline
A_1 cells	Untreated	++++	++++	++++	++++	++++	++++	++±	++±	+±	±	0	0
	Enzyme-treated	+++	++	++	++	+±	+±	+±	0	0	0	0	0
O cells	Untreated	++++	++++	++++	++++	++++	++++	+++±	++±	+±	±	0	0
	Enzyme-treated	++++	++++	++++	++++	++++	++++	+++±	++±	++	+	0	0

Serum Bu.—reciprocal of dilution

		2	4	8	16	32	64	128	256	512	Saline
A_1 cells	Untreated	++++	++++	++++	+++±	++±	++	+	0	0	0
	Enzyme-treated	0	0	0	0	0	0	0	0	0	0
O cells	Untreated	++++	++++	++++	++++	+++±	++±	+±	+	0	0
	Enzyme-treated	++	++	±	0	0	0	0	0	0	0

Horse 615 Type XIV antipneumococcal specific polysaccharide (SXIV)—reciprocal of dilution

		2	4	8	16	32	64	Saline
A_1 cells	Untreated	++++	+++±	++	+	0	0	0
	Enzyme-treated	0	0	0	0	0	0	0
O cells	Untreated	++++	++++	++++	++++	++±	0	0
	Enzyme-treated	++	++±	+±	±	0	0	0

* A 4 per cent suspension of the erythrocytes was incubated in the enzyme preparation for 2 hours at 37°C.

‡ Agglutination is graded from 0 to ++++; + to ++ represent agglutination, as read with a hand lens, and ++± to ++++ represent macroscopic agglutination.

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N. P. showed a decrease in titer of 7, 4, and 2 tubes, respectively; with O cells the decreases were only 4, 2, and 0 tubes, respectively. The decrease in reactivity of A_1 cells with sera Ch. and N. P. was also manifested by the weakened agglutination produced by the lower serum dilutions. A_1 cells were found to be most susceptible to enzymatic attack and O cells least susceptible; A_2, B, and A_1B cells were intermediate in susceptibility to alteration. Treatment of O erythrocytes for periods up to 18 hours at 37°C produced a progressive loss in agglutinability, but prolonged incubation at this temperature was limited by increasing red cell fragility.

The cross-reaction of Type XIV antipneumococcal horse serum with human blood group substances is known to involve non-reducing beta galactosyl end groups (35, 36). It has also been demonstrated that treatment of human and hog blood group substances with a crude unfractionated enzyme preparation from *Cl. tertium* virtually abolished this cross-reaction (15). As seen in Table I, treatment of human erythrocytes with partially purified beta galactosidase and beta glucosaminidase reduces their agglutinability by Type XIV antipneumococcal horse serum. A_1 cells were more susceptible to the enzyme than were O cells.

Specificity of the Erythrocyte Alteration.—Enzyme-treated A_1 erythrocytes, which were not agglutinated by a 1:2 dilution of serum Bu. at 20°C, displayed no detectable alteration in their reactivity with the following reagents: anti-A_1 (*Dolichos biflorus* extract), anti-B, anti-H (*Ulex europeus* extract), anti-P (anti-Tj^a) and anti-P_1, anti-Rh (1, 2, 5, and 7 [D, C, e, Ce]), anti-M (rabbit), anti-N (*Vicia graminea* extract), anti-S, anti-s, anti-Le^a, anti-Le^b, anti-K2 (Cellano), anti-Jk^a, anti-Fy^a, and anti-Ge. A sample of anti-i[12] revealed a slight reduction in reactivity: adult cells scored 9 *versus* 21 for the untreated control, and cord blood scored 53 *versus* 60 for the untreated control.

Requirement for Both Beta Galactosidase and Beta Glucosaminidase Activities.—Preparations which contained beta galactosidase with little or no beta glucosaminidase were much less effective in decreasing the I activity of red cells than were those containing both enzymes. Table II presents the results of treatment of erythrocytes with DEAE cellulose column fractions of a *Cl. tertium* culture which had been grown on a medium containing lactose but no glucosamine. Fractions 102 and 104, which were low in beta glucosaminidase activity, were unable to make the cells inagglutinable by a 1:16 dilution of serum Bu., despite their high beta galactosidase activity relative to fraction 114.

The effect of glucosaminidase alone could not be tested, inasmuch as no preparation without beta galactosidase activity was obtained. A glucosaminidase preparation with low galactosidase activity, eluted from pevikon segment

[12] Kindly supplied by Dr. W. J. Jenkins, Northeast Metropolitan Regional Blood Transfusion Centre, London, England.

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+7, Fig. 2, produced a slight decrease in the agglutinability of A_1 cells by sera Bu. and Ch. A bacterial beta glucosaminidase which acts on the streptococcal Group A carbohydrate[13] (37) was without effect on erythrocyte I activity.

It is difficult to provide an exact estimate of the amount of enzyme activity required to alter the erythrocyte I determinant because of several factors: differences in susceptibility to enzymatic modification among cells of different ABO groups; differences among anti-I sera; and the limited precision of the conventional hemagglutination system employing serial twofold dilutions. As an approximation, A_1 cells became completely inagglutinable by a 1:16 dilu-

TABLE II

The Effect of Treatment with Enzyme-Containing Fractions from a DEAE Cellulose Column on the Agglutinability of A_1 Erythrocytes by Anti-I*

Fraction No.	Beta galactosidase activity OD 4050 A/0.1 ml/30 min.	Beta glucosaminidase activity OD 5440 A/0.1 ml/14 hrs.	Serum Bu. 1:16	Saline
No treatment	—	—	++++	0
90	0	0	++++	0
100	3.7	0.024	+++	0
102	23.0	0.025	++±	0
104	28.0	0.049	+±	0
108	11.5	0.111	0	0
111	6.8	0.254	0	0
114	4.5	0.204	0	0

* A 4 per cent suspension of erythrocytes was suspended in the enzyme preparation for 30 minutes at 36°C.

tion of serum Bu. after incubation of cells at a concentration of 4 per cent at 37°C for 30 minutes in an enzyme preparation with beta galactosidase activity of 3.000 optical density units at 4050 A/0.1 ml/30 minutes and beta glucosaminidase activity of 0.075 to 0.100 optical density units at 5440 A/0.1 ml/hour.

Treatment of Erythrocyte Stroma.—Since intact red cells could be incubated at 37°C for only limited periods of time, stromata were employed as substrate, and the ability of enzyme-treated and untreated stroma to absorb anti-I agglutinins was determined. As can be seen in Table III, stroma derived from A_1 cells lost all ability to absorb anti-I agglutinins after overnight incubation with an enzyme preparation containing beta galactosidase and beta glucosaminidase. Treatment of equivalent amounts of B and O stromata with the same enzyme preparation resulted in only limited loss of absorptive capacity for anti-I. Retreatment of a stroma aliquot for 72 hours at twice the original

[13] Generously provided by Dr. Maclyn McCarty.

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enzyme: stroma ratio reduced, but did not eliminate, the reactivity of B and O stromata with anti-I.

Data on the composition of the supernatant fluids from the original overnight treatment of the stroma are given in Table IV. The low nitrogen value for the O stroma supernatant resulted from the passage of this material through a charcoal column in 20 per cent ethanol, a step omitted in the workup of the

TABLE III

Absorption of Anti-I Cold Agglutinin (Ch. Serum) by Control and Enzyme-Treated Stromata

	Reciprocal of serum dilution*											
	5	10	20	40	80	160	320	640	1280	2560	5120	Saline
Unabsorbed	++++	++++	++++	++++	++++	++++	+++±	+++	++±	+	0	0
Absorbed with			20	40	80	160	320	640	1280	2560	5120	Saline
Untreated A_1 stroma			0	0	0	0	0	0	0	0	0	0
Enzyme-treated A_1 stroma 17 hrs.			++++	++++	++++	++++	++++	+++	++±	+	0	0
Absorbed with			18	36	72	144	288	576	1152	2304		Saline
Untreated B stroma			0	0	0	0	0	0	0	0		0
Enzyme treated B stroma 19 + 72 hrs.			++++	++++	++++	+++	++	++	+	0		0
Absorbed with			15	30	60	120	240	480	960	1920		Saline
Untreated O stroma			0	0	0	0	0	0	0	0		0
Enzyme-treated O stroma 18 + 72 hrs.			++++	++++	++++	++++	+++	++±	0	0		0

* When the serum was absorbed with an equal volume of packed stroma it was assumed to have undergone a 1:2 dilution. All titrations were performed with group O cells.

A and B stromata. Paper chromatography of these solutions demonstrated that they were extremely complex in composition, and in view of the relative non-specificity of the colorimetric analytical methods employed, the solutions were chromatographed on charcoal-celite columns.

As seen in Fig. 3, two peaks appeared in the chromatograms of the supernatants from the enzyme-treated stroma, the first was eluted with water, and the second was eluted with 2 to 5 per cent ethanol. The fractions in the peak areas were pooled and concentrated, and the analytical data on these solutions are presented in Table V. With all three stroma samples, paper chromatog-

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raphy showed a single major component with the mobility of galactose in the peak eluted with water. Traces of two more slowly moving compounds were seen in the B stroma supernatant. Galactose has a reducing sugar equivalent as glucose of 79 per cent, and comparison of columns 3 and 4 in Table V shows good agreement between galactose determined directly and the value calculated from the reducing sugar except for the B stroma supernatant which contained very small amounts of sugar.

The *N*-acetylhexosamine was tentatively identified as *N*-acetylglucosamine by differential colorimetric analysis (38), since *N*-acetylgalactosamine and *N*-acetylmannosamine yield 31 per cent (38, 16) and 55 per cent (39) respectively of the color value of *N*-acetylglucosamine. The agreement between *N*-acetylhexosamine values obtained by multiplying the hexosamine value by

TABLE IV

Composition of the Supernatant Fluid from the Untreated and Enzyme-Treated Stromata

	Reducing sugar (glucose equivalents)	Galactose	*N*-Acetylhexosamine	Nitrogen
	μg/ml	μg/ml	μg/ml	μg/ml
A untreated	78	5	5	549
A enzyme-treated	285	124	131	643
B untreated	10	15	0	32
B enzyme-treated	54	55	14	33
O untreated	127	10	5	34
O enzyme-treated	263	92	57	27

1.23, and the direct *N*-acetylhexosamine determination using an *N*-acetylglucosamine standard (Table V, columns 6 and 7), indicates absence of appreciable amounts of *N*-acetylgalactosamine or *N*-acetylmannosamine.

Hemagglutination Inhibition with Anti-I Sera.—Hemagglutination inhibition tests were performed by adding one drop of inhibitor to a drop of diluted serum, incubating the mixture at 20°C or 4°C for 30 minutes, and then adding one drop of 2 per cent group O cells. No inhibition of the three anti-I sera tested was found with any of the following compounds: galactose, glucose, mannose, *N*-acetylglucosamine, lactose, melibiose, or maltose, all at an initial concentration of 50.0 mg/ml; galactinol 9.4 mg/ml; alpha methyl-*N*-acetylglucosaminide 38 mg/ml; beta methyl-*N*-acetylglucosaminide 40 mg/ml; and the following polysaccharides at initial concentrations of approximately 1 to 4 mg/ml,—Type XIV pneumococcal polysaccharide, Group A streptococcal polysaccharide,[13] type A *Cryptococcus neoformans* capsular polysaccharide,[14]

[14] Generously provided by Dr. Irving Abrahams.

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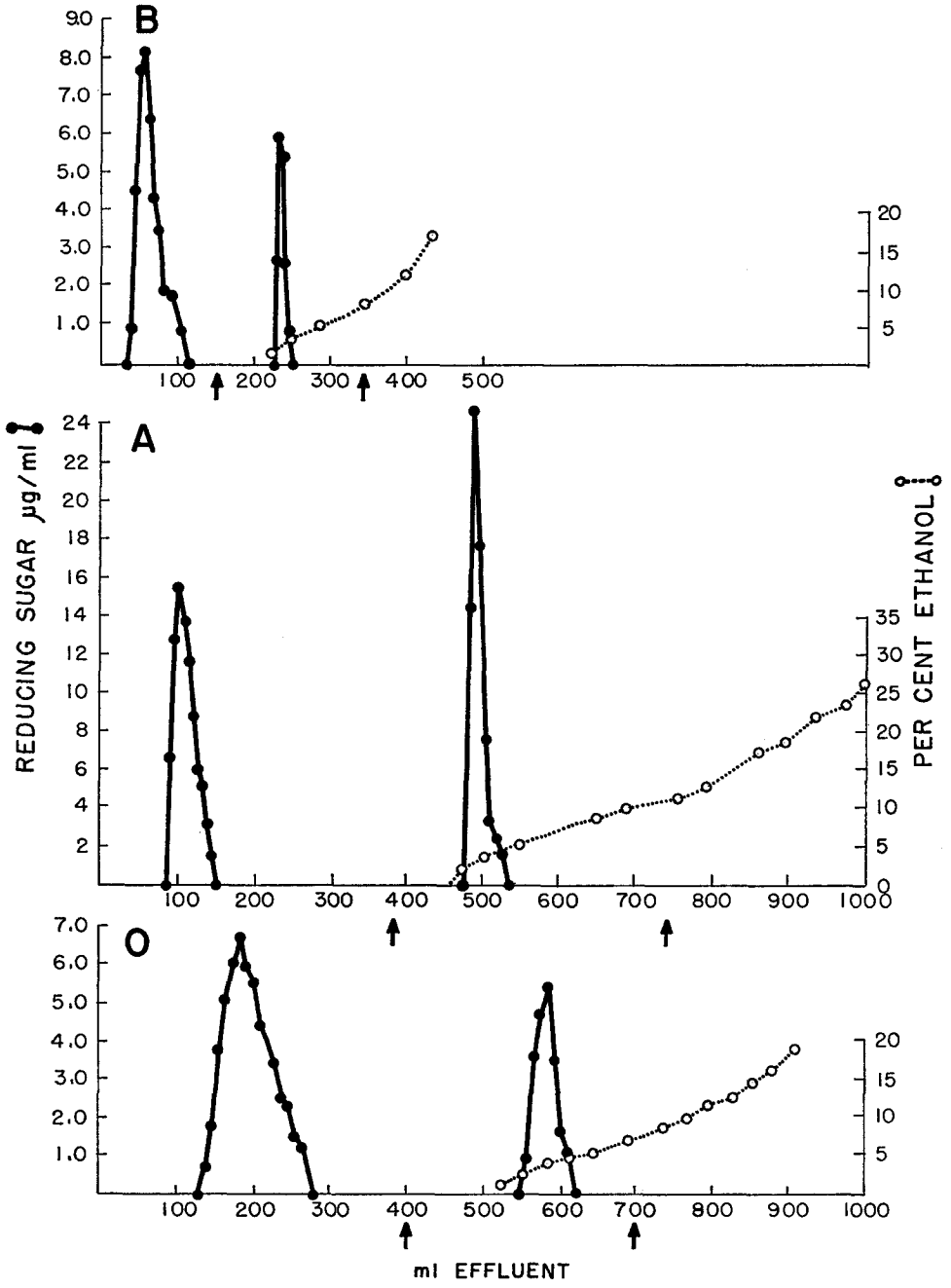

FIG. 3. Chromatography on charcoal-celite columns of sugars released from group A, B, and O erythrocyte stromata by the action of *Cl. tertium* enzymes. The sugars were applied to the column in water; at the first arrow an ethanol gradient was imposed with 25 per cent ethanol, and at the second arrow 50 per cent ethanol was used for the gradient. The supernatants from the A and O stromata were fractionated on 8 gm columns at a flow rate of 40 ml/hour; 3 ml fractions were collected. The B stroma supernatant was fractionated on a 3 gm column at a flow rate of 20 ml/hour; 3 ml/fractions were collected.

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and human and hog blood group substances and their P1 fractions (40). In addition, serum Bu. was not inhibited by any of the following compounds: α-galactosyl-1 → 3-galactose 23.7 mg/ml; α-galactosyl-1 → 6-*N*-acetylglucosamine 9.2 mg/ml; β-galactosyl-1 → 3-*N*-acetylglucosamine 18.2 mg/ml; β-galactosyl-1 → 4-*N*-acetylglucosamine 23.5 mg/ml; β-galactosyl-1 → 6-*N*-acetylglucosamine 26.3 mg/ml; and lacto-N-tetraose 8.7 mg/ml.[5]

The enzymatic alteration of erythrocytes could be inhibited partially with lactose, and to a lesser extent with galactose, melibiose, and *N*-acetylglucosamine in concentrations of about 12 mg/ml. At this concentration only minimal inhibition was produced by glucose, mannose, and maltose.

TABLE V

Analytical Data on Components Obtained by Charcoal Column Chromatography of Supernatants From Enzyme-Treated Stroma

	Peak I			Peak II		
	Reducing sugar (glucose equivalents)	Reducing sugar × 1.25	Galactose	Hexosamine	Hexosamine × 1.23	*N*-acetyl-hexosamine
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
A stroma	125	156	150	100	123	125
B stroma	48	60	44	25	31	33
O stroma	95	119	106	32	39	37

DISCUSSION

The ability of *Cl. tertium* enzymes to decrease the reactivity of anti-I sera with erythrocytes and stroma is clearly demonstrated by the data in Tables I and III. However, interpretation of the data is limited by the semiquantitative nature of the assay system, and the apparently heterogeneous nature of both the I antigen and anti-I.

Since one cannot at present determine directly the number of active I sites on the erythrocyte surface, it is necessary to measure the effect of enzyme treatment by assaying erythrocyte agglutinability, or the ability of cells or stromata to absorb antibody. Moreover, the fraction of the original I receptors which must be altered to produce a decrease in agglutinability is not known, so that this type of assay serves as a very crude estimate of the number of sites affected. The weak reaction of treated A_1 cells with a wide range of dilutions of serum N. P. (Table I) illustrates the difficulty of attempting to correlate the extent of enzyme action with change in titer. The use of stroma as a substrate allows a more protracted period of enzymatic treatment, and the ability of stroma to absorb anti-I provides a more direct estimate of the number of active antigenic sites.

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The heterogeneous nature of the I antigen was noted by Wiener (10), who classified I cells into subgroups I_1, I_2, etc., based on their reactivity with anti-I at room temperature. This notation has not been generally accepted since cells from random donors appear to fall on a continuous normal distribution curve with respect to antigen strength (5, 11). The use of the designation "i" for all cells which react weakly with high titer antisera is now felt to be inadequate, and a classification into subgroups i_1, i_2, and $i_{(coord)}$ is used (42, 12; 8, p. 328), in which the cells are listed in order of increasing I strength.

The difference between group A_1 and O erythrocytes in susceptibility to enzymatic action on the I receptor(s) is another manifestation of the heterogeneous nature of this blood group system. Factors which conceivably could be involved in this differential susceptibility include: differences in the total number of receptor sites present on cells of the various ABO phenotypes; heterogeneity in structure of the I determinants and differences in the accessibility of the I receptors to the enzymes. A large difference in the number of I receptors on A_1 and O cells seems unlikely in view of the grossly similar titers obtained with the I antisera employed. With regard to heterogeneity of I determinants, the ability of stroma of any ABO group to absorb anti-I completely makes it unlikely that a fraction of the I antibodies reacts exclusively with O cells. However, it is possible that determinant groups of similar, but not identical, structure could cross-react extensively, but differ appreciably in their susceptibility to enzymatic hydrolysis. The last possibility, as well as differences in the accessibility to enzyme action of the I determinants on erythrocytes of different blood groups, provide the most likely explanation on the basis of the existing data.

A number of observations have led to the consideration of an "association" between the I and ABO systems (9; 8, p. 334). These include the preferential reactivity of some anti-I sera with specific ABO phenotypes (11, 9); the weak reaction of cells from one i_1 and one i_2 individual with anti-H and anti-"O" reagents (11, 9); and the presence of "naturally occurring" anti-I in most of the i individuals reported (11, 9, 12). Wiener (43) has suggested that cold auto-antibodies might be directed at the "nucleus" of the ABO complex. At present, there is no evidence of genetic linkage between the genes of the ABO and I loci (8). The occurrence of the erythrocyte I determinants as discrete structures apart from the ABO determinants is suggested by the absence of abnormalities in the ABO phenotype of most i cells, and the normal I strength of "Bombay" cells (11, 9, 21), a rare type of erythrocyte which is not agglutinated by anti-A, anti-B, or anti-H (44). It is possible that the "association" of the ABO and I groups is spatial in nature, rather than genetic, and that the influence of ABO phenotype on agglutinability by anti-I, or susceptibility of the I determinant to enzymatic attack, is a manifestation of steric interaction of adjacent I and ABO structures on the erythrocyte membrane.

There is a gross parallelism in the behavior of enzymatically-treated A_1 and O erythrocytes with anti-I and Type XIV antipneumococcal horse sera (Table I). In addition, Levine *et al.* noted that horse Type XIV antipneumococcal sera agglutinated O and A_2 cells more strongly than A_1 cells, and that these sera could be partially absorbed with A_1 cells in such a manner that they agglutinated O and A_2 cells but not A_1 cells (45). These observations raise the question of whether these anti-I and anti-XIV sera are reacting in part with the same determinants. The Type XIV

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antipneumococcal horse serum used in this study agglutinated i_i cells,[15] indicating that the two types of antisera are not reacting extensively with the same determinants.

Even if the I determinant should also prove to have a terminal beta galactosyl residue, it has already been demonstrated that two antigenic determinants may have the same terminal non-reducing sugar, in the same anomeric form, and show little or no cross-reactivity (40, 41, 46).

Anti-I sera also appear to differ in specificity. A division of these sera into subgroups anti-I^a and anti-I^b has been proposed (47), based on their differential reactivity with umbilical cord cells. Anti-I sera also vary widely in the extent to which they are inhibited by sheep liver hydatid cyst fluid (9, 12). The differences among the anti-I sera used in this study in the extent to which they reveal a decrease in titer with a single sample of enzyme-treated cells, may reflect differences in specificity and/or heterogeneity in size of combining sites (*cf.* 20, p. 342, for general discussion of heterogeneity of antibodies).

The data in this report do not establish a causal relationship between the release of galactose and *N*-acetylglucosamine from the erythrocyte membrane and the decrease in I antigen activity. The beta galactosidase and beta glucosaminidase were only partially purified, and the possibility exists that unknown contaminating enzymes are responsible for, or participate in, the alteration of the I determinant.

Assuming, for the sake of discussion, a causal relationship between the release of both galactose and *N*-acetylglucosamine and the loss of I activity, the role of these two sugars in the I determinant should be considered. The partial loss of I activity produced by treatment with beta galactosidase preparations without appreciable beta glucosaminidase activity indicates a terminal, non-reducing location for at least a portion of the galactose residues. No inferences can be drawn from the available information as to whether *N*-acetylglucosamine occurs in a subterminal position, in glycosidic linkage with galactose, or in a terminal position, presenting a branched determinant, analogous to that postulated by Watkins and Morgan for the Le^a determinant (48).

The significance of the differences in the amounts of galactose and *N*-acetylglucosamine liberated from the A, B, and O stromata (Table IV) is also not clear, since a very limited number of stroma preparations were used, and the fraction of the released sugar actually related to the I determinant is unknown.

A variety of low molecular weight compounds containing galactose and *N*-acetylglucosamine failed to inhibit hemagglutination by the three I antisera examined. Although these data provide no support for the suggested occurrence of these sugars in the I determinant, they do not militate against this possibility. It is extremely difficult to inhibit with oligosaccharides the hemagglutinating activity of many sera. For example, 0.17 to 0.34 mg of lactose produced 50 per cent inhibition of the precipitation of a variety of blood group substances by 0.5 ml of the Type XIV horse antipneumococcal serum used

[15] Generously provided by Dr. R. R. Race.

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in this study (36). Agglutination of O erythrocytes by 0.1 ml of a 1:20 dilution of the same antiserum could not be inhibited by 3 mg of lactose. The failure of polysaccharides with non-reducing beta galactosyl and beta-*N*-acetylglucosaminoyl groups to cross-react with the anti-I sera is similarly uninformative. As noted above, two antigenic determinants with the same terminal sugar residue may show no appreciable cross-reactivity.

A more direct approach to the elucidation of the structure of the I determinant would be to extract and characterize material from the erythrocyte cell membrane, and work along these lines is planned. Delineation of the structure of the I and i determinants would be of interest in several respects: it would add to our knowledge of the relationship between chemical structure and antigenic specificity (49), investigation of the biochemical lesion which leads to the adult i phenotype would be facilitated; and a knowledge of the specificity of this autoantibody would aid in studying the pathogenesis of hemolytic anemia associated with cold agglutinins.

SUMMARY

A method was described for the partial purification of beta galactosidase and beta glucosaminidase from *Clostridium tertium* culture supernatants. Treatment of erythrocytes with preparations containing both enzymes decreases their ability to react with anti-I cold agglutinins, and with Type XIV antipneumococcal horse serum. Erythrocytes of blood group A_1 are altered more rapidly and extensively than are group O cells. The enzymatic treatment of stroma results in a decrease in ability to absorb anti-I agglutinins and the release of galactose and *N*-acetylglucosamine as monosaccharides. The data suggest that these two sugars may be structural units of the erythrocyte I determinant, but no direct evidence is available.

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