Immunochemical Studies on Blood Groups. XXXI. Destruction of Blood Group A Activity by an Enzyme from *Clostridium tertium* Which Deacetylates *N*-Acetylgalactosamine in Intact Blood Group Substances*

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Received September 25, 1963

An enzyme which destroys the serological activity of blood group A substance has been purified from *Clostridium tertium* culture filtrates by chromatography on DEAE-cellulose and hydroxyapatite columns. The loss of blood-group A activity produced by the purified enzyme is almost completely restored by *N*-acetylation of the enzyme-treated material. A hydrolysate of a dinitrophenyl derivative of an enzyme-treated A substance was analyzed by thin-layer chromatography and the only DNP compound identifiable was DNP-galactosamine.

The use of enzymes which alter the serologic properties of blood group substances has been valuable in elucidating the structure of their antigenic determinants (Kabat, 1956; Watkins, 1962). Culture filtrates of *Clostridium tertium* destroyed the blood group A activity of human saliva (Iseki and Okada, 1951), and of blood group A substances extracted from human erythrocytes or hog gastric mucin (Iseki and Masaki, 1953). Disappearance of blood group A activity was accompanied by an increase in H (group O) reactivity, as measured by hemagglutination inhibition with chicken antisera to human group O erythrocytes (Iseki and Masaki, 1953). Howe *et al.* (1957) found that culture filtrates of the Iseki strain of *Cl. tertium* inactivated the A_1, A_2, M, and N antigens of the intact human erythrocyte, the hemagglutinin receptor for certain strains of influenza virus, and the cross reactivity of A, B, and H (O) substances with type XIV antipneumococcal horse serum. The enzyme-treated A substances did not exhibit an increase in cross reactivity with a bovine anti-H (O) reagent (Howe *et al.*, 1958). Treatment of blood group A substances with culture filtrates of either the Iseki strain of *Cl. tertium* or a strain (McClung 1259) which did not affect blood group A activity, resulted in the liberation of dialyzable sugars amounting to 1–5% of the original weight (Schiffman *et al.*, 1958). In both instances the major components found in the dialysate were galactose and *N*-acetylglucosamine as monosaccharides, with smaller amounts of a disaccharide composed of galactose and *N*-acetylgalactosamine. It was not possible to attribute the loss of serologic activity to release of sugars from the antigenic determinant.

It has been reported recently that a concentrated enzyme preparation from *Cl. tertium* released 31% of the sugar from human gastric mucin A substance (Yamamoto *et al.*, 1962), and 74% of the sugar from an erythrocyte glycolipid with blood group A activity (Fujisawa *et al.*, 1963). Galactose, *N*-acetylgalactosamine, and a disaccharide composed of galactose and *N*-acetylgalactosamine were liberated from both substrates; in addition, *N*-acetylglucosamine was released from the mucin and sialic acid was released from the glycolipid.

The present study describes the partial purification of the A-decomposing enzyme (A enzyme) from *Cl. tertium* culture filtrates and demonstrates that the loss of blood group A activity produced by the purified enzyme is almost completely restored by *N*-acetylation of the enzyme-treated blood group substance.

MATERIALS AND METHODS

Cultivation of Bacteria.—The Iseki strain of *Cl. tertium* was obtained from Dr. C. Howe and the organisms were grown in the medium previously described (Howe *et al.*, 1957) with the following modifications: a final concentration of 0.1% D-glucosamine hydrochloride was used for induction of the A enzyme and 0.075% lactose for induction of β-galactosidase. The procedure used for bulk production of the enzymes has been published (Marcus *et al.*, 1963).

Enzyme Purification.—The *Cl. tertium* culture supernatant was concentrated by dialysis against Carbowax 20000 (Union Carbide Chemicals Co.), dialyzed against a buffer containing 0.01 M Tris, *p*H 7.5, 0.9% NaCl, and 0.001 M $MnCl_2$, and passed through a DEAE-cellulose[1] (Schleicher and Schuell Co.) column to remove β-galactosidase, β-glucosaminidase, and peptidase activities (Marcus *et al.*, 1963). In general, all of the A enzyme was recovered in the initial effluent but on occasion the recovery was as low as 50–66%. No galactosidase or glucosaminidase activity was detectable in these A-enzyme preparations. Between *p*H 7 and 8 the A enzyme is retained by DEAE-cellulose at NaCl concentrations of 0.05 M or lower but the recovery of enzyme activity from the column was low, averaging about 10%.

A slurry of 17 g of hydroxyapatite (Tiselius *et al.*, 1956) and 17 g of Celite in 0.001 M sodium phosphate buffer, *p*H 7.0, was packed in a column 3.5 × 16 cm. The charge, in Tris buffer as above, was applied to the column in a volume of about 70 ml, and the column was washed with 0.001 M phosphate buffer. A phosphate gradient was constructed with the use of an external reservoir containing 0.15 M phosphate, *p*H 6.8, and a mixing chamber containing 300 ml of the 0.001 M phosphate buffer. The flow rate was 80–90 ml/hour and 12-ml fractions were collected; all chromatography was carried out at room temperature. The shape of the gradient was determined by measuring the conductivity of the fractions. One experiment was performed with 140–170 mesh hydroxyapatite prepared by the method of Anacker and Stoy (1958),

* Supported by grants from the National Science Foundation (G-18727), the Office of Naval Research, and the General Research Support Grant from the National Institutes of Health, U. S. Public Health Service.

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[1] Abbreviations used in this work: RD, receptor-destroying; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DNP, dinitrophenyl.

using 15 g of hydroxyapatite and 7 g of Celite in a column 2.9 × 14.5 cm. The capacity of different batches of hydroxyapatite to absorb the A enzyme was variable. The quantity of absorbent was kept to a minimum because of the slow flow rate and the necessity of working at room temperature.

The A enzyme could be separated from the receptor-destroying (RD) enzyme[1] by passage through a carboxymethyl-cellulose column (CM-cellulose, Brown and Co.). Five ml of A enzyme, previously fractionated on hydroxyapatite, was applied to a 1.8-g carboxymethyl-cellulose column, 1.2 × 20 cm, in 0.01 M acetate buffer, *p*H 5.5, containing 0.001 M $MnCl_2$. The flow rate was 50 ml/hour and 3-ml fractions were collected. About 50% of the A enzyme was recovered in the initial effluent without detectable RD enzyme activity.

Enzyme Assays—A Enzyme.—The A enzyme was measured by its ability to destroy the hemagglutination-inhibiting activity of soluble blood group A substance. Inhibition of A enzyme by EDTA, disodium salt, noted previously (Howe *et al.*, 1957), was confirmed and the enzyme was found to be activated specifically by Mn^{2+} or Co^{2+} in the range of 5×10^{-5} to 5×10^{-3} M concentration. A enzyme activity is also enhanced severalfold by 0.001–0.01 M mercaptoethanol. The enzyme and substrate, in 0.01 M Tris, *p*H 7.5, 0.9% NaCl, 0.005 M $MnCl_2$, and 0.01 M mercaptoethanol, were incubated overnight at 37° and heated for 5 minutes at 100° to destroy residual A-enzyme activity, and the solution was assayed for blood group A activity by hemagglutination inhibition. The amount of enzyme required to inactive 100 μg of blood group substance under these conditions was defined as one unit.

Treatment of Erythrocytes.—Blood group A erythrocytes were suspended to a concentration of 8% in 2500 units of A-enzyme preparation HA IX, in 0.01 M Tris-saline, 0.001 M $MnCl_2$, 0.001 M mercaptoethanol, incubated for 1 hour at 37°, washed three times, and tested for agglutinability by anti-A reagents.

Receptor-destroying Enzyme.—Assay for RD enzyme was performed (Howe *et al.*, 1957) after enzyme treatment for 1 hour at 37° using type A influenza virus PR 8 as the hemagglutinating agent.

Peptidase, β-galactosidase, and β-glucosaminidase assays were carried out as described (Marcus *et al*, 1963).

Analytical Methods.—Standard analytical methods (Kabat, 1961) were employed with minor modifications as recently described (Marcus *et al.*, 1963). The following materials were measured by the methods given: methylpentose by the method of Dische and Shettles (1948), galactose by the method of Dische (1955), hexosamine by the method of Elson and Morgan (1933), *N*-acetylhexosamine by the method of Reissig *et al.* (1955), and amino nitrogen by a ninhydrin method (Moore and Stein, 1954). Hexosamine determinations were also carried out on *unhydrolyzed* blood group substances by the method of Dische and Borenfreund (1950) on a 1/5 scale.

Immunochemical Methods.—Quantitative precipitin analyses were performed by a modified ninhydrin method (Schiffman *et al.*, 1962b; Kabat and Schiffman, 1962; Schiffman *et al.*, 1964). Anti-A sera were obtained from humans immunized with A substance from hog gastric mucin.

Hemagglutination inhibition assays were performed by a standard method (Kabat, 1961) or with a Takatsy microtitrator (Cooke Engineering Co.) using 0.025 ml each of inhibitor, antibody, and a 2% erythrocyte suspension (Sever, 1962). Five to ten hemagglutinating units of antibody were used. *Dolichos biflorus* and *Ulex europeus* lectins were prepared as 10% extracts (Boyd and Shapleigh, 1954); eel serum was supplied by Dr. G. Springer. Isoantibodies were assayed at room temperature or 37°; lectins and heterologous sera were assayed at room temperature. Results are given as the minimum concentration of substance added to the assay system to give complete inhibition of hemagglutination.

Agar Diffusion.—Double diffusion in agar was performed by the method of Ouchterlony (1949). Rabbit antiserum to unfractionated *Cl. tertium* culture supernatant (Howe *et al.*, 1957) was supplied by Dr. C. Howe.

Blood-Group Substances.—Blood group A substances were prepared from hog gastric mucin and human pseudomucinous ovarian cyst fluid by peptic digestion, phenol extraction, and precipitation with ethanol or isopropyl alcohol. Details of the preparations have been published; hog mucin fraction 2 (Carsten and Kabat, 1956); hog 50 (Howe and Kabat, 1956); McDon. (Schiffman *et al.*, 1962a); MSS and MSM (Schiffman *et al.*, 1964). Three of the substances, hog 50, McDon., and MSM were *N*-acetylated before enzyme treatment.

N-Acetylation.—*N*-acetylation of blood group substances was performed by a slight modification of the method of Roseman and Daffner (1956); one volume each of saturated $NaHCO_3$ and 5% acetic anhydride were added to four volumes of blood group substance (3–40 mg). After standing for 10 minutes at room temperature and 2 hours at 37° the substances were dialyzed against water and lyophilized. When acetylation was performed with acetic-1-^{14}C anhydride (10^4 dps/mg, New England Nuclear Corp.), the anhydride was used undiluted rather than at 5% concentration and the reaction mixture was held at 4° until all the reagents were added. Radioactivity measurements were made with a Baird Atomic or Nuclear-Chicago gas-flow counter. The activity of the sample of the anhydride used was determined in this laboratory to be 1.5×10^4 dps/mg.

Enzyme Treatment of Blood Group A Substances.—The A-enzyme preparations employed were all purified by chromatography on DEAE-cellulose and hydroxyapatite columns, and the preparation used to treat hog mucin fraction 2 was passed through a carboxymethyl-cellulose column to remove RD enzyme. The enzyme and substrate, in 0.01 M Tris, *p*H 7.5, 0.9% NaCl, 0.005 M $MnCl_2$, and 0.01 M mercaptoethanol, were incubated for 2–9 days at 37° (Table I). The course of the reaction was followed by hemagglutination-inhibition assay of the A substances, and additional portions of enzyme were added if necessary to abolish totally the serological activity of the substrate. As a control, substrate was incubated with enzyme heated for 5 minutes at 100°. The reaction was terminated by heating to destroy residual A enzyme and the preparation was dialyzed exhaustively against water. The total recovery of nondialyzable weight ranged from 90% to over 100%, and the nondialyzable material did not differ significantly from the starting material in percentage N, hexosamine, galactose, or methylpentose. When additional weight was acquired in handling, activity measurements were calculated on the assumption of 100% recovery of the starting material. None of the dialysates contained measurable amounts of nitrogen or sugar components. During the course of treatment of hog mucin fraction 2, 45% of the nondialyzable material became insoluble. The composition of the soluble nondialyzable material did not differ from the untreated material, and none of the other enzyme-treated substances exhibited more than a trace of insoluble material.

TABLE I
THE EFFECT OF ENZYME TREATMENT AND REACETYLATION ON THE SEROLOGICAL ACTIVITY OF BLOOD GROUP A SUBSTANCES

Blood Group Substance	mg	Enzyme Preparation	Protein (mg)	Treatment	Blood Group Activity (μg inhibiting) Anti-A[a]	Anti-H(O) *Ulex europeus*		Anti-H(O) Eel Serum	
Hog mucin fr. 2 (A and H(O))	100	CM III fr. 7–9	0.39	No enzyme treatment	3.1	5	8	8	
				Enzyme-treated	>990	5	8	8	
				Enzyme-treated reacetylated	6.3				
				Enzyme-treated + HNO_2			6	8	
				Enzyme-treated + HNO_2 reacetylated	>130				
MSS	20	HA VI fr. 42–45	0.35	No enzyme treatment	1.6	104	>100	>100	
				Enzyme-treated	>1440	>101	330	>985	
				Enzyme-treated reacetylated	1.4	>107		>178	
				Enzyme-treated + HNO_2			12	23	
McDon	21	HA VII fr. 46–49	0.68	No enzyme treatment	1.3	23		183	
				Enzyme-treated	>2100	8		128	
				Enzyme-treated reacetylated	1.6	13		410	
Hog 50	19	HA VII fr. 46–49	0.68	No enzyme treatment	1.2	83		670	
				Enzyme-treated	>1800	56		224	
				Enzyme-treated reacetylated	1.4	89			
MSM	109	HA VII fr. 51–57	0.87	No enzyme treatment	3	400	540	>1600	1600
				Enzyme-treated	>1700	6.8	6	13.5	34
				Enzyme-treated reacetylated	25	3.9		15.5	
				Enzyme treatment + HNO_2			6		27
				Enzyme-treated + HNO_2 reacetylated	>500				

[a] Serum I, ten hemagglutinating units.

Deamination of Blood Group Substances.—Deamination was performed by addition of 0.5 ml of 5% $NaNO_2$ and 0.5 ml of 33% acetic acid to 2.0 ml of blood group substance (4–6 mg). After 15–30 minutes at room temperature the preparations were dialyzed against water and lyophilized.

DNP–Blood Group Substance.—Samples of human cyst substance MSM untreated, enzyme-treated, and enzyme-treated reacetylated were allowed to react with dinitrofluorobenzene by the method of Sanger (1945) and dialyzed against water. About one-third of the enzyme-treated MSM preparation became insoluble shortly after addition of the dinitrofluorobenzene and this material was washed with water, alcohol, and ether and dried *in vacuo*. Separate samples of the DNP–blood group substances were hydrolyzed for 2 hours at 100° in 2 N HCl and in sealed evacuated tubes for 18 hours at 100° in 6 N HCl, lyophilized, and dissolved in water for chromatography. DNP-glucosamine and DNP-galactosamine were prepared by Leskowitz and Kabat (1954), and DNP–amino acids by Dr. Helen Van Vunakis.

Thin-Layer Chromatography.—Thin-layer chromatography was performed with apparatus and materials obtained from the Brinkmann Instrument Co. For analytical work a slurry of 30 g of silica gel G in 60 ml of 0.1 M $K_2B_4O_7$, *p*H 10.8, was used to coat five plates with a layer 250 μ thick, and the plates were heated for 30 minutes at 100–105°. For preparative purposes a layer 1 mm thick was employed. A good separation of 5–10 μg of DNP-glucosamine and 5–10 μg of DNP-galactosamine could be obtained with a 60-to 90-minute development (10–12 cm migration) at 21° using several solvent systems: 1-propanol–ethyl acetate–water (7:1:2), butanol-pyridine-water (7:3:3), and ethanol-chloroform-water (4:6:1, organic phase). Using the first solvent system average R_F values for DNP-glucosamine and DNP-galactosamine were 0.46 and 0.33, respectively; bands were 2–3 mm in width. The presence in the chromatography tank of a paper lining saturated with buffer aided in maintaining an even solvent front and sharp bands.

RESULTS

Enzyme Purification.—The A-enzyme preparation which had been passed through a DEAE-cellulose column, as described above, was chromatographed on a hydroxyapatite column (Fig. 1). The A enzyme was consistently eluted in two peaks which were similar in specific activity, sensitivity to EDTA inhibition, and activation by Mn^{2+} and mercaptoethanol. About 34% of the enzyme activity was recovered in this experiment and the specific activity of the peak fractions (fr. 47, 52–56) represented 15- and 10-fold purifications over the starting material. In another experiment, using hydroxyapatite prepared by a different method (Anacker and Stoy, 1958), all the activity was recovered and a 125- to 160-fold purification was achieved. The specific activities of the purified enzyme preparations varied within a factor of ± 2, which was the limit of error of the assay.

The active fractions, which also contained RD enzyme, were pooled, concentrated, and dialyzed against Tris buffer for use in treatment of blood-group substances or erythrocytes. The RD enzyme could be removed from this preparation by passage through a carboxymethyl-cellulose column but in most experiments the A enzyme was used without further purification.

Agar Diffusion.—Pooled and concentrated A enzyme from the two peaks was examined by double diffusion in agar with a rabbit antiserum to an unfractionated *Cl. tertium* enzyme preparation. Each peak gave one

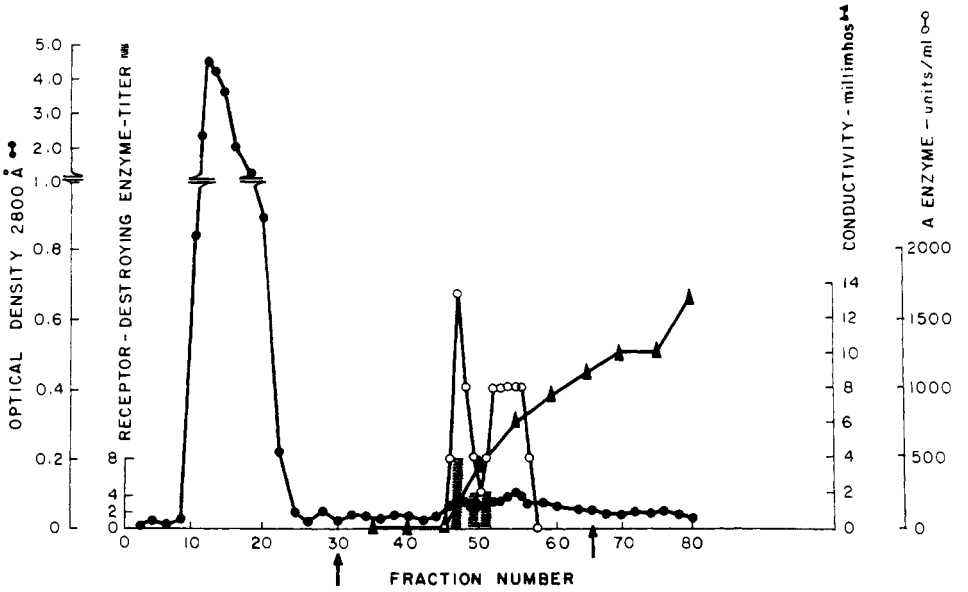

FIG. 1.—Fractionation of RD enzyme and A enzyme from *Cl. tertium* on a hydroxyapatite-Celite column, 3.5 × 16 cm. At the first arrow a phosphate gradient was imposed, as described in the text, and at the second arrow the column was flushed with 0.15 M phosphate buffer, *p*H 6.8.

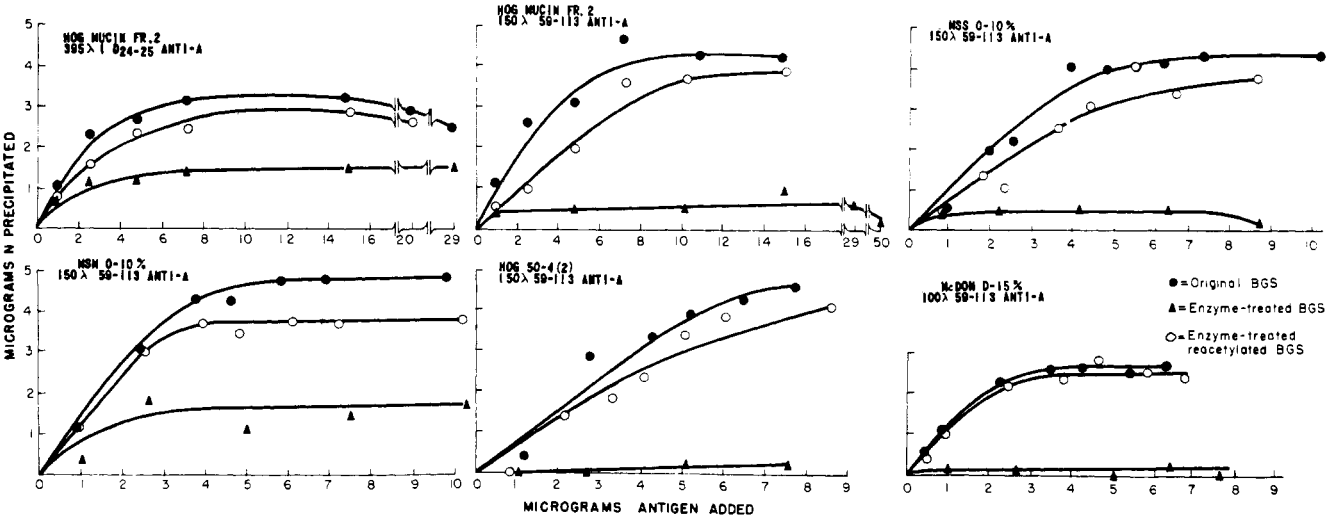

FIG. 2.—The decrease in capacity of enzyme-treated human and hog blood group A substances to precipitate anti-A, and restoration of blood group A activity by *N*-acetylation.

major precipitin line, and the lines fused completely. The first peak also gave another faint line which appeared to represent RD enzyme.

Enzyme Treatment of Blood Group A Substances.—The decrease in precipitability of blood group A substances produced by enzyme treatment is illustrated in Figure 2. The percentage of total antibody precipitated by an enzyme-treated substance varies with the antiserum employed: enzyme-treated hog mucin fraction 2 precipitated 50% of the antibody from antiserum 1 but only 15% of the antibody in antiserum 59-113. With the former serum the other 50% of the original antibody was precipitable from the supernatant by untreated mucin fraction 2, indicating that the two substances were reacting with the same population of antibodies. Enzyme-treated A substances exhibited no change in capacity to precipitate with type XIV antipneumococcal horse serum.

The enzyme-treated A substances were completely inactive in inhibiting hemagglutination by anti-A sera (Table I). An unequivocal increase in ability to inhibit anti-H (O) reagents following enzyme treatment was found only with ovarian cyst substance MSM. Although enzyme treatment alone did not produce an increase in H (O) activity of MSS, subsequent exposure to nitrous acid caused a marked increase in H (O) activity as assayed with both Ulex and eel anti-H (O) (Table I).

Enzyme Treatment of Erythrocytes.—Treatment of group A_1 erythrocytes with purified A enzyme decreased their agglutinability by anti-A reagents and consistently increased their reactivity with the two anti-H (O) reagents employed (Table II). Similar results were obtained using unfractionated *Cl. tertium* extracts or the effluent from a DEAE-cellulose column as a source of A enzyme.

N-Acetylation of Enzyme-treated A Substances.—The observation that the action of the purified A enzyme on A substances was not accompanied by the release of dialyzable sugars, and the evidence that *N*-acetylgalactosamine is the terminal nonreducing sugar in the A determinant (Morgan and Watkins, 1953; Kabat and Leskowitz, 1955; Watkins and Morgan, 1955; Côté and Morgan, 1956; Cheese and Morgan, 1961; Schiff-

TABLE II
THE EFFECT OF ENZYME TREATMENT OF GROUP A_1 ERYTHROCYTES ON THEIR AGGLUTINABILITY BY ANTI-A AND ANTI-H(O) REAGENTS

Anti-A Reagent	Titer: Before Enzyme Treatment	Titer: After Enzyme Treatment	Anti-H(O) Reagent	Titer: Before Enzyme Treatment	Titer: After Enzyme Treatment
Serum No. 1	32	2	*Ulex europeus*	4	64
Serum 59-113	256	4	Eel serum	0	128
Dolichos biflorus	128	0			

man *et al.*, 1962a) suggested that the action of the A enzyme was a deacetylation of this *N*-acetylgalactosamine residue. *N*-Acetylation of the enzyme-treated A substances restored almost completely their ability to precipitate with (Fig. 2) and to inhibit hemagglutination (Table I) with anti-A. Treatment with nitrous acid prior to reacetylation prevented restoration of activity in hemagglutination (Table I). The precipitating power of the substances after exposure to A enzyme and nitrous acid was comparable to that of the enzyme-treated material. Enzyme-treated hog mucin fraction 2, which showed residual precipitation with serum $1D_{24-25}$ (Fig. 2), precipitated comparable amounts of antibody after treatment with nitrous acid; the minimal activity of other enzyme-treated samples studied was not affected by nitrous acid. Acetylation of untreated A substances produced no change in specific immunological antibody.

Estimation of the Extent of Deacetylation.—Hexosamine determinations were performed by the method of Dische and Borenfreund (1950); the values obtained with unhydrolyzed enzyme-treated substances are presented in Table III. Unhydrolyzed and untreated or enzyme-treated reacetylated substances gave no measurable hexosamine except for untreated hog mucin fraction 2 which had 0.9 μg hexosamine/100 μg mucin. Intensive treatment of two ovarian cyst B substances and two O substances with A enzyme produced no measurable hexosamine and no loss in serological activity.

The number of amino groups exposed by the action of the A enzyme was also estimated by determining the radioactivity of blood group substances reacetylated with acetic-1-^{14}C anhydride (Table IV). These data are also presented in terms of μg hexosamine reacetylated/100 μg of blood group substance in Table III to facilitate comparison with the results obtained by the Dische hexosamine method.

TABLE III
ESTIMATION OF THE NUMBER OF AMINO GROUPS EXPOSED ON BLOOD GROUP A SUBSTANCES BY THE ACTION OF THE A ENZYME OF *Cl. tertium*[a]

	μg Hexosamine (Dische)/ 100 μg Blood Group Substance	μg Hexosamine, Acetylated by Acetic-1-^{14}C Anhydride/ 100 μg Blood Group Substance[a]
Hog mucin fr. 2	4.7	
MSS	19	
McDon	7.4	3.4
Hog 50	16	10
MSM	9.8	5.2

[a] Calculated on the assumption that all of the acetyl-^{14}C had *N*-acetylated hexosamine.

TABLE IV
SPECIFIC ACTIVITY OF BLOOD GROUP A SUBSTANCES ACETYLATED WITH ACETIC-1-^{14}C ANHYDRIDE[a]

	Net cpm/100 μg of Blood group Substance: Untreated	Net cpm/100 μg of Blood group Substance: Enzyme-treated	Enzyme-treated First then Reacetylated with Unlabeled Acetic Anhydride
McDon	10	137	14
Hog 50	22	265	17
MSM	3	138	19

[a] Samples containing 80–200 μg of blood group substances in volumes from 20 to 100 μl were placed on planchets and dried in air, and radioactivity was determined.

Dinitrophenyl derivatives of cyst substance MSM untreated, enzyme-treated, and enzyme-treated reacetylated were prepared. The optical density at 3600 A of the untreated and enzyme-treated reacetylated preparations amounted to 3–5% of the optical density of the enzyme-treated material and they were not further characterized. Samples of DNP-enzyme-treated MSM were hydrolyzed for 2 hours in 2 N HCl and for 18 hours in 6 N HCl at 100° and the hydrolysates were analyzed by thin-layer chromatography. One band with the mobility of DNP-galactosamine was found in both hydrolysates but in the 2-hour hydrolysate some yellow color was visible at the origin and also as a smear between the origin and the DNP-galactosamine band; less yellow color was visible at the origin in the chromatogram of the 18-hour hydrolysate but a rapidly migrating band with an R_F approximately equal to that of dinitroaniline was observed. The main band had the R_F of DNP-galactosamine in three solvents. Identical results were obtained with that portion of DNP-enzyme-treated MSM which became insoluble during the dinitrophenylation procedure. A 2-hour hydrolysate of a mixture of hog mucin fraction 2, DNP-galactosamine, and DNP-glucosamine gave two bands on thin-layer chromatography, plus a smear as described above; addition of the two DNP-amino sugars to mucin fraction 2 after hydrolysis and lyophilization produced two sharp bands on chromatography without smearing.

A 1.2-mg sample of the 2-hour hydrolysate of DNP-enzyme-treated MSM was run on a preparative scale on a thick plate. After development the gel was divided into three areas, the leading band, the origin, and the intermediate area, and these were scraped off the plate and extracted with water. On rechromatography the material eluted from the leading band (about 40% of the original sample) migrated with the mobility of DNP-galactosamine and gave a faint smear extending back to the origin; most of the material eluted from the intermediate area remained on the origin and the rest gave a smear up to the position of the DNP-galactosamine band.

The spectra of DNP-galactosamine, DNP-glucosamine, and DNP-enzyme-treated MSM were identical between 3200 and 4000 A, with a maximum at 3600 A. Assuming that all the dinitrophenyl groups were on galactosamine residues, and that the molar extinction of DNP-galactosamine in the blood group substance was unchanged, one can calculate that 100 μg of the DNP–blood group substance contained 7 μg of DNP-galactosamine, a value in good agreement with the other data (Table III) on the extent of deacetylation of MSM.

Discussion

Evidence that the purified A enzyme deacetylates *N*-acetylamino groups in blood group A substance has been obtained by three independent methods; labeling of enzyme-treated material by acetic-1-^{14}C anhydride and dinitrofluorobenzene, and its ability to react in the hexosamine method of Dische. Presumably, the nitrous acid converted the hexosamine into a 2,5-anhydrosugar which was then hydrolyzed from the blood group substance. This interpretation is supported by the observation that enzyme-treated MSM after treatment with nitrous acid and dialysis gave no hexosamine value and the dialysate contained material which formed a chromogen on heating with indole and hydrochloric acid (as done in the final step of the Dische hexosamine procedure). The deamination and subsequent rapid hydrolysis of glucosaminides by nitrous acid is well documented (Foster *et al.*, 1953; Baddiley *et al.*, 1962). Deamination with nitrous acid of enzyme-treated A substance provides a convenient method for obtaining A substances from which the terminal *N*-acetylgalactosamine residues have been removed.

Although the partial purification of the A enzyme(s) which has been achieved has been valuable in elucidating the nature of the alteration produced in the blood group molecule, several questions remain which require further enzyme purification and characterization. The separation of A enzyme activity into two peaks on hydroxyapatite chromatography may result from the presence of genetically distinct enzymes or from an alteration acquired during the 48-hour growth period or during fractionation. If the enzymes are genetically distinct they may differ in specificity, cofactor requirements, etc. Many aspects of the subject of multiple molecular forms of enzymes have recently been reviewed (Kaplan, 1963). In the limited studies carried out, the A enzyme failed to deacetylate monosaccharide *N*-acetylhexosamines or to decrease the inhibitory activity of partially purified fragments derived from blood group A substance by alkaline hydrolysis (A_1 and A_3, Schiffman *et al.*, 1964). This may reflect an extremely limited substrate specificity or a failure to achieve proper conditions for the enzyme to act on small substrates. There has been little study of purified *N*-acetylhexosamine deacetylases; a partially purified *N*-acetylglucosamine deacetylase obtained from *E. coli* strain K 12 was also found to deacetylate *N*-acetylgalactosamine at a slow rate, but was inactive against phenyl-*N*-acetylglucosaminides (Roseman, 1957).

The data obtained in this study cannot be compared directly with the work of Yamamoto *et al.*, (1962) or Fujisawa *et al.* (1963) since their A-enzyme preparation apparently contained galactosidase, *N*-acetylglucosaminidase, *N*-acetylgalactosaminidase, and neuraminidase activities. However, the release of large amounts of dialyzable sugar from human gastric mucin and erythrocyte blood group substances noted in their studies contrasts with the release of only 1–5% of the sugar from hog gastric mucin and ovarian cyst A substances by the unfractionated *Cl. tertium* enzyme preparation used by Schiffman *et al.* (1958). This apparent discrepancy may result in part from the use of different substrates and conditions, but a mutation may have occurred in the strain of *Cl. tertium* used by one of the two laboratories. The disaccharide obtained by Yamamoto *et al.* (1962), which they assumed to have the structure of α-*N*-acetylgalactosaminoyl-(1 → 3)-galactose, gave a positive Morgan-Elson reaction, suggesting that the *N*-acetylgalactosamine may be on the reducing end of the molecule, and that it may be the same disaccharide α-galactosyl-*N*-acetylgalactosamine isolated from enzyme hydrolysates[2] by Schiffman *et al.* (1958); Côté and Morgan (unpublished data cited by Morgan 1959c) identified α-galactosyl-(1 → 3)-*N*-acetylgalactosamine from an acid hydrolysate.

The only compound identifiable in the hydrolysate of DNP-enzyme-treated MSM was DNP-galactosamine, but inasmuch as some DNP compounds could not be identified because of failure to migrate or smearing, the presence of DNP-glucosamine or DNP–amino acids cannot be excluded. If all of the galactosamine originated from the nonreducing end of blood group A determinants, the figure of 7 μg of DNP-galactosamine/100 μg MSM would yield a value of about 110 determinant groups/mole of MSM, assuming a molecular weight of 300,000; a similar estimate was obtained for B substance from the amount of galactose isolated after treatment with coffee bean α-galactosidase (Zarnitz and Kabat, 1960). Since only 40% of the DNP-labeled material could be identified by chromatography as DNP-galactosamine, a minimum figure would be about 47 determinant groups/mole. DNP derivatives of the other enzyme-treated substances were not studied but the high Dische hexosamine value given by preparations MSS and hog 50, and the high uptake of radioactive acetyl by the latter, raises the possibility that *N*-acetylglucosamine may also be deacetylated in some preparations. The blood group A substances contain 33% hexosamine, of which 40–50% is estimated to be galactosamine (Leskowitz and Kabat, 1954), so that the amount of hexosamine deacetylated in these two preparations, estimated by Dische hexosamine, would exceed the galactosamine content of the blood group substances.

Enzyme-treated MSM differed from the other enzyme-treated materials in that it acquired the ability to inhibit H (O) reagents. The persistence of H (O) activity after r acetylation, and the relatively incomplete restoration of A activity by acetylation indicates that an irreversible alteration had occurred, possibly the hydrolysis of some terminal galactosamine residues.

The finding that enzyme-treated MSS developed H (O) activity only following treatment with nitrous acid, which causes removal of the terminal amino sugar, may result from differences in the enzyme preparation employed, or may indicate a distinct difference between the blood group A substances obtained from serous and mucinous cysts removed from an ovary of a single individual. Two different trisaccharides with A activity have been isolated from a single mucinous ovarian cyst (Cheese and Morgan, 1961).

That the enzyme-treated A erythrocytes consistently develop H (O) activity may possibly indicate antigenic determinants more comparable in structure to that of the substance from the mucinous rather than to that

[2] This disaccharide retained 80% of its galactose after reduction with sodium borohydride while 95% of the *N*-acetylhexosamine was destroyed (unpublished data).

from the serous fluid. Interpretation must be guarded because of the heterologous nature of the anti-H (O) reagents (Watkins and Morgan, 1962; Iseki and Furukawa, 1962; Springer and Williamson, 1963).

The loss of blood group A activity which accompanies deacetylation of *N*-acetylgalactosamine adds to the evidence cited above that it is the terminal nonreducing sugar in the A determinant.

In earlier studies, *N*-acetylgalactosamine inhibited A-anti-A precipitation with 2 pools of antiserum to hog A substance and one sample of human anti-A, while galactosamine inhibited only w th the human anti-A but was less potent than *N*-acetylgalactosamine (Kabat and Leskowitz, 1955).

It is of interest to note an earlier report that the activity of blood group A substance from equine urine was destroyed by alkaline deacetylation and restored by acetylation (Freudenberg and Westphal, 1938). The decreased affinity of anti-A for the deacetylated antigen may result both from partial loss of the normal configuration presented by the acetylated hexosamine, and from the appearance of a positive charge at this previously uncharged site. Preliminary studies indicate that the A activity of DNP-enzyme-treated MSM is slightly greater than that of enzyme-treated MSM, in support of the latter point.

The extent to which enzyme treatment diminishes A activity, and the degree of restoration by reacetylation, is a function of the method of assay employed, the antiserum, the blood group substance, and possibly the enzyme preparation. It has been noted previously that blood group substances altered by enzymatic (Morgan, 1959a; Zarnitz and Kabat, 1960) and physical means (Morgan, 1959b) appeared to be totally inactive in inhibiting hemagglutination but were capable of precipitating substantial amounts of antibody from the same serum. One factor responsible for this apparent discrepancy is that in hemagglutination inhibition the antigen must compete with unaltered erythrocyte determinants for the antibody, whereas in precipitin analyses the altered antigen may retain capacity to form insoluble complexes with antibody. The exclusive use of either system to assay an antigen for blood group activity provides limited, and possibly misleading, information.

The modification of antigenic determinants by removal of terminal residues, or substitution on these residues, may provide additional information about the size of antigenic determinants and the basis of antigenic specificity.

Acknowledgment

The authors with to acknowledge the technical assistance of Mr. John Holland.

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