ENZYMES OF *CLOSTRIDIUM TERTIUM*

EFFECTS ON BLOOD GROUP AND VIRUS RECEPTOR SUBSTANCES[1]

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The enzymatic degradation of purified blood group substances represents an increasingly fruitful approach to the elucidation of their immunochemical specificity, and progress to date has recently been reviewed (Kabat, 1956). A number of enzymes from different microorganisms have been described which split one or more of the blood group substances with resultant changes in serological specificity. Stack and Morgan (1949) have investigated enzymes, first studied by Schiff (1935), of *Clostridium welchii*, which decompose the A, B and O(H) substances. Watkins (1953) described enzymes in cell free extracts of the protozoan *Trichomonas foetus* which inactivated the A, B, O(H), and Lewis blood group substances; and Watkins and Morgan (1954) have shown that partially purified enzyme preparations from the same source destroyed the O(H), M and N substances on intact erythrocytes without affecting the A, B, P or S antigens. Besides inactivating the specific antigens mentioned, the enzymes of *T. foetus* rendered all erythrocytes nonspecifically agglutinable (panagglutinable), rendered D-positive cells agglutinable by incomplete anti-D, and inactivated the receptor sites for the hemagglutinin of influenza virus type A (PR8). Cell free extracts of *Lactobacillus bifidus* (var. *penn*) have been shown by György, *et al.* (1952) to possess enzymatic activity against human and animal blood group A, B, and O(H) substances, being most active against the latter. Iseki and Tsunoda (1952) have reported that cell free extracts of *Bacillus fulminans*, an aerobic spore bearer, show enzymatic activity on O(H) substances, but not on A or B substances. Chase (1938) isolated a gram-negative coccobacillus from leaf mold which was able to grow on media containing blood group A substance, decomposing the A antigen during growth. More recent studies (Gilmore *et al.*, *unpublished data*) have shown that cell free extracts of this organism are capable of degrading A, B, and O(H) substances. Iseki and Okada (1951) have studied a strain of *Clostridium tertium*, isolated from sewage, which split A but not O(H) substance. The present investigation was undertaken with a view toward characterizing more closely the enzymes elaborated by this latter organism.

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MATERIALS AND METHODS

A semidefined medium for propagation of C. tertium. The basal medium consisted of enzymic hydrolyzate of casein (Sheffield N-Z amine, type A), 5 g; K_2HPO_4, 5 g; and NaCl, 1 g/L of water, adjusted to pH 7.2 and autoclaved at 15 lb pressure for 20 min. Two ml each of ferrous sulfate (120 mg in 100 ml N/10 HCl) and a vitamin mixture (20 mg each of calcium pantothenate, nicotinic acid, pimelic acid, pyridoxine, and thiamin, and 2 mg of riboflavin, in 100 ml of water), sterilized by autoclaving, were added for every 100 ml of basal medium. The effect on growth and enzyme production of different sugars was studied as reported below. For bulk enzyme production, D-glucosamine hydrochloride was prepared as a 25 per cent solution in water, sterilized by Seitz filtration, and added to the above medium to a final concentration of 0.5 per cent just before inoculation.

Preparation of blood group substances and inhibitors of viral hemagglutination. Blood group A and O(H) substances were prepared from commercial hog gastric mucin (Wilson) and from single hog stomach linings by phenol extraction and fractional ethanol precipitation (Morgan and King, 1943; Howe and Kabat, 1956). Human

blood group B substance was prepared by the same method from the sputum of a secretor of blood group B. Material from the same donor was used for the preparation of viral hemagglutination inhibitor (sputum, pH 3.6 precipitate, kindly supplied by Dr. B. F. Erlanger) by the method of Marmion *et al.* (1953) and urinary mucoprotein by the procedure of Tamm and Horsfall (1952). Human brain mucolipid was kindly supplied by Dr. A. Rosenberg (Rosenberg *et al.*, 1956).

Preparation of hemagglutinating viruses and hemagglutination tests. Influenza viruses were propagated by chorioallantoic inoculation of embryonated eggs eleven days of age, the chorioallantoic fluid being harvested after 48 hr of incubation at 37 C. The neuro-WS strain of virus had been maintained by intracerebral passage through mice, the chorioallantoic fluid used for hemagglutination tests being the first egg passage from infected mouse brain. All strains were used in the active, infective state, and with the exception of the neuro-WS strain, after heating at 56 C for 2 hr to convert them to "indicator" virus. Tests involving the neuro-WS strain were carried out at 4 C, since this virus could not be converted to an indicator.

Hemagglutination titrations were performed by the standard serial dilution technique, using a 2 per cent suspension of erythrocytes and reading patterns after an hour at room temperature. In hemagglutination-inhibition tests, indicator virus and active neuro-WS were used in a concentration equivalent to 5 hemagglutinating units for 0.1 ml of 2 per cent chicken cells.

A strain of murine encephalomyocarditis virus (Columbia SK), was maintained by intracerebral passage in mice weighing 10 to 15 g. Fresh infected brain, emulsified in saline, was used for hemagglutination studies with the technique of Horvath and Jungeblut (1952). It was found that following refrigeration overnight, light centrifugation of the tubes gave sharper definition of the hemagglutination end point.

Immunochemical methods. Blood group A antigen was estimated by inhibition of agglutination of group A_1 erythrocytes by human anti-A, and was determined quantitatively by precipitation of anti-A from calibrated human antisera, using the precipitin method of Heidelberger and MacPherson (1943) (Kabat and Mayer, 1948). Blood group O(H) activity was measured by inhibition of agglutination of group O cells by bovine anti-O(H) serum absorbed with A_1B erythrocytes. Blood group B activity was estimated by inhibition of B-anti-B hemagglutination. The anti-A serum (Mil) used for quantitative studies and for inhibition of agglutination was obtained from a donor of group O previously injected subcutaneously with 1 mg of purified hog A substance. The anti-A serum (3817) used in agglutination tests was from a donor of group B. Anti-B serum (Stu) was obtained from a donor of group A previously injected subcutaneously with 1 mg of human B substance. Hexosamine was determined by the method of Elson and Morgan (1933).

EXPERIMENTAL RESULTS

Conditions for enzyme production. A variety of sugars, singly and in combination, as well as blood group substance in varying concentration, were incorporated as principal carbon source into the semidefined medium already described. Growth was estimated at 24 and 48 hr, and at 48 hr, cultures were tested for the presence of A-splitting enzyme. Cultures were centrifuged until clear, and equal volumes of culture supernatant and phosphate buffer (M/15; pH 7.4) containing 1 mg of blood group substance (mucin), were mixed. As a control, a second sample of the same culture supernatant, heated to 100 C for 10 min, was similarly treated. After incubation overnight at 37 C under toluene, the mixtures were assayed for residual A antigen by hemagglutination inhibition. Under the conditions of these experiments incubation overnight (16 to 18 hr) was sufficient to detect the presence of any enzyme specific for the A antigen, as judged by complete serological inactivation of the standard amount of blood group substance. The findings can be summarized as follows:

(a) As the principal carbon source in the semidefined basal medium, glucose, lactose, galactose, glucosamine, and N-acetylglucosamine supported growth well in a concentration of 0.5 per cent; fucose, rhamnose, and galactosamine did not. With larger inocula, growth also occurred with N-acetylgalactosamine. With respect to glucose, glucosamine, and lactose, growth occurred at levels down to 0.1 per cent but not below. Intact blood group substance (hog mucin) incorporated in a concentration of 1 per cent into the basal medium without free sugar, did not support growth; but the same substance (0.2 per cent) completely hydrolyzed (2 N HCl, 2 hr and neutralized) allowed good growth. Intact blood group substance (5 per cent to 0.005 per cent) plus

suboptimal glucose (0.1 to 0.01 per cent) also supported good growth.

(b) Glucosamine, N-acetylglucosamine, intact blood group substance plus suboptimal glucose, and completely hydrolyzed blood group substance (2 N HCl, 2 hr and neutralized) as above in (a), in addition to supporting good growth, induced the formation of enzymes inactivating the blood group A antigen. Glucose, galactose, lactose, and N-acetylgalactosamine did not induce the formation of A-splitting enzyme, although, as already noted, each alone in the basal medium supported growth. Enzyme production in the presence of galactose plus glucosamine was variable, although growth was consistently good. The addition of glucose (0.5 to 0.05 per cent) to the basal medium containing 0.5 per cent glucosamine or N-acetylglucosamine resulted in suppression of enzyme production, although growth was undiminished. Lactose above a concentration of 0.1 per cent had a similar effect.

(c) With glucosamine as the principal carbon source, maximum production of A-splitting enzyme occurred at a concentration of 0.5 per cent; at 0.1 per cent growth was much reduced and no enzyme could be detected. At a concentration of 0.05 per cent no growth occurred.

The rate of production of A-splitting enzyme. The rate of production of A-splitting enzyme in the semidefined liquid medium containing 0.5 per cent glucosamine was investigated. Assays for enzyme were performed at regular intervals following inoculation. Aliquots of culture supernatant were centrifuged, and added to a standard solution of blood group substance in phosphate buffer. Samples of each enzyme-blood group substance mixture were then taken at intervals, heated to 100 C for 5 min to inactivate enzyme, and assayed for residual A antigen. The speed with which a given sample of culture supernatant inactivated the A antigen was taken as an estimate of the amount of enzyme present. It was found that the most rapid evolution of enzyme took place during the first 24 hr of growth, followed by a slower rise to a maximum level reached after about 48 hr of incubation at 37 C. The level of glucosamine dropped to less than 20 per cent of its original concentration within 30 hr after inoculation of the medium, indicating active utilization of this carbohydrate during growth and enzyme production (figure 1). Pure glucosamine was not altered with respect to its ability to give the Elson-Morgan color reaction after 30 hr of incubation with cell free culture supernatant possessing A-splitting activity.

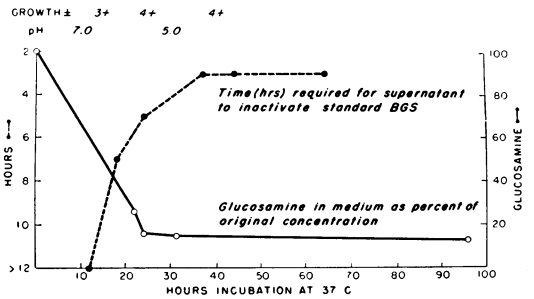

Figure 1. Utilization of glucosamine and production of blood group A-splitting enzyme during growth of *Clostridium tertium* in semidefined medium.

Comparison of the Iseki strain with other strains of C. tertium. Twenty-nine additional strains of *C. tertium* were examined for their capacity to elaborate the blood group A-splitting enzyme. All showed typical morphological and growth characteristics. Transfers were made from stock meat broth cultures to NZ-amine glucosamine broth, assays for enzyme being carried out at each successive transfer. On the first transfer through glucosamine broth, 6 strains showed blood group A-splitting activity; 12 strains required 2 transfers and 1 strain 3 transfers before they produced detectable enzyme. Eleven strains failed to produce enzyme for blood group A antigen after at least 2 transfers and in some cases up to 4 transfers through glucosamine broth. Culture supernatants from 8 strains when tested simultaneously for blood group A and blood group B splitting activity were active only against the A antigen.

Production of enzyme in large quantity. In order to derive sufficient material for further study, two methods for enzyme production were employed. In the first, lots of 1 or 2 L of NZ-amine glucosamine broth were inoculated with 20 ml of a 24 hr anaerobic culture in the same medium. After incubation for 48 hr at 37 C and removal of bacterial cells by centrifugation, the supernatant was dialyzed against water and lyophilized. The total yield of material per liter of culture was 1 to 2 g. This type of preparation is referred to subsequently as dialyzed culture supernatant (DCS). In the second method, batches of 7 L of the same medium were inoculated with 100 ml of a 24 hr anaerobic culture. After incubation for 48 hr, the organisms were removed by centrifugation; and, to the supernatant, ammonium sulfate was

added in solid form to full saturation. Following refrigeration overnight, the resultant precipitate was collected by centrifugation, taken up in a small amount of water, and dialyzed against water. This material was centrifuged in the cold until clear and lyophilized. The yield of water soluble material thus obtained was about 0.1 g/L of culture. Enzyme so prepared is referred to subsequently as full saturation precipitate (FSP). Anaerobic incubation of these bulk cultures was not required, since *C. tertium* is a facultative aerobe and actively multiplying cultures were used as inocula.

Characteristics of the enzyme specifically inactivating blood group A antigen. (a) pH optimum: —Blood group substance (mucin) and enzyme (FSP) were mixed in a proportion of approx 3:1 in veronal-acetate buffers (Michaelis, 1931), 0.03 M, from pH 2.3 to 9.0. Samples of each mixture were taken at zero time, and after various intervals of incubation at 37 C, heated to stop enzymic action, and then assayed for residual A antigen. Figure 2 shows that the most rapid destruction of the A antigen took place between pH 7 and 8, and that there was relatively little activity above pH 8.5 or below pH 6.0. All other enzyme experiments dependent on maximum A-splitting enzyme activity were therefore carried out in phosphate or veronal buffer at pH 7.4 to 7.6.

(b) Inhibition of blood group A-splitting enzyme by divalent cations:—Enzyme (DCS) and blood group substance (mucin) were incubated overnight at pH 7.5 (veronal) in the presence of several metal salts in a concentration of 10^{-3} M, and then assayed for residual A antigen. Under these conditions, nickel acetate, zinc acetate, and copper sulfate inhibited the action of the enzyme; manganese chloride, calcium chloride, cobalt acetate, magnesium sulfate and ferrous sulfate did not.

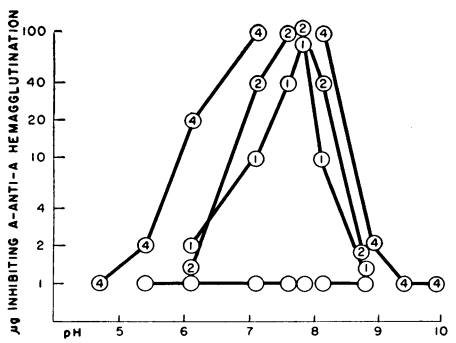

Figure 2. pH optimum for blood group A-splitting activity by full saturation (ammonium sulfate) precipitate (FSP) from *Clostridium tertium* culture supernatant. Circled figures indicate hours of incubation of enzyme-substrate mixtures at 37 C before assay for residual A antigen.

Quantitative measurement of enzymatic degradation of blood group A substance. 13.5 mg of purified hog A substance [Hog 55, fraction 10(2)] (Howe and Kabat, 1956) were mixed with enzyme (DCS) in M/15 phosphate at optimum pH. Samples were removed at zero time, and after various periods of incubation at 37 C up to 48 hr, heated, and tested for residual A antigen by quantitative precipitation of anti-A from a calibrated human antiserum. Figure 3 shows that with increasing duration of enzyme treatment, there was marked decrease in the amount of blood group substance capable of precipitating anti-A, virtually none remaining at the end of 48 hr.

Effect of C. tertium enzymes on other blood group substances, on soluble inhibitors of viral hemagglutination, and on intact erythrocytes. Various soluble blood group substances with Group A, B, or O(H) specificity, from several different species, were treated with enzyme (DCS) and were then tested for residual serological activity. From table 1, it will be seen that only the blood group A antigen, when present, was inactivated and that blood group B and O(H) antigens were unaffected.

Several soluble inhibitors of influenza virus hemagglutination were similarly treated with *C. tertium* enzyme (FSP). It was found that the inhibitory capacity of these substances was markedly reduced or abolished for the strains of virus which they were able to inhibit to high titer before

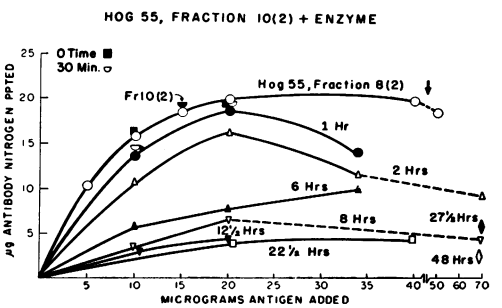

Figure 3. Inactivation of purified blood group A substance (hog) by *Clostridium tertium* culture supernatant. Antigen added to duplicate 0.5 ml samples of serum Mil_4.

TABLE 1

Effect of Clostridium tertium enzymes on blood group antigens and on viral hemagglutination inhibitors in soluble mucoids

Preparation	Species, Source	Blood Group Antigens			Strains of "Indicator" Influenza Virus for which Hemagglutination Inhibitors were Inactivated
		Present	Inactivated	Not inactivated	
Blood group substances*					
6A	Hog mucin	A, O(H)	A	O(H)	
7A	Hog mucin	A, O(H)	A	O(H)	
52, fraction 4(2)	Single hog stomach	A	A		
55, fraction 8(2)	Single hog stomach	A	A		
38, fraction 8(2)	Single hog stomach	O(H)		O(H)	
43, fraction 10(2)	Single hog stomach	O(H)		O(H)	
54, fraction 4(2)	Single hog stomach	O(H)		O(H)	
54, phenol insoluble	Single hog stomach	O(H)		O(H)	
H-2, 15%	Single horse stomach	B		B	
H-2, 25%	Single horse stomach	B		B	
Phenolin soluble†	Human bronchial secretion	B		B	
Viral hemagglutination inhibitors‡					
pH 3.6 precipitate†	Human bronchial secretion	B		B	PR8, Lee, Great Lakes, FM1, Neuro-WS¶
Urinary mucoprotein†	Human urine	None§			PR8, Lee, Great Lakes, FM1; PR301
Mucolipid	Human brain	None§			PR8, PR301

* Treated with DCS. Numbers refer to preparations described in Howe and Kabat (1956) (hog) and Baer *et al.*, (1950) (horse).

† Prepared from the same donor, a secretor of group B.

‡ Treated with FSP.

§ No detectable A, B, or O(H) antigen.

¶ Active virus.

enzyme treatment. These virus strains are listed in table 1.

The effect of *C. tertium* enzymes on the antigens and receptors on the surface of the intact erythrocyte was determined. Human erythrocytes, washed 3 times, were grouped for ABO, Rh, and M, N, Duffy^a (Fy^a), and Kell factors. They were then treated with enzyme (DCS) in phosphate saline for several hours or overnight at 37 C. As controls, the same donors' cells, suspended in phosphate saline, without enzyme, were incubated under similar conditions. The enzyme preparation used in each case was also tested against soluble blood group A substance to verify the presence at least of the A-splitting activity. At the end of the incubation period, both enzyme treated and control cells were washed to remove the original suspending medium, and then tested in equivalent concentration with various reagents in concurrent agglutination titrations or plate tests. The results are given in tables 2 and 3. In those instances in which titrations were performed, the difference between the reciprocals of the titers with normal (N) and enzyme treated (E) cells with a given reagent is expressed as a ratio. Thus if the ratio is greater than 1, the antigen or receptor in question was not significantly altered. With respect to the A antigen, on A cells as well as on A_1B and A_2B cells, there was regularly a significant difference between the agglutinability of cells treated with enzyme and the same donor's normal cells as shown by the high ratios (column 3). A regular finding was the appearance of panagglutinability after treatment with enzyme, the cells becoming agglutinable, often to quite high titers, in the same donor's

TABLE 2

Effect of Clostridium tertium enzymes on reactivity of intact erythrocytes*

Donor	Grouping	Reciprocal of Titer with Normal Cells/Reciprocal of Titer with Enzyme Treated Cells in:				Agglutination of Normal (N) and Enzyme Treated (E) Cells by:									
		Anti-A†	Anti-B‡	Same donor's plasma or serum (panagglutinin)	Columbia SK virus	Anti-M		Anti-N		Anti-D§		Anti-K§		Anti-Fy^a§	
						N	E	N	E	N	E	N	E	N	E
(1)	(2)	(3)	(4)	(5)	(6)	(7)		(8)		(9)		(10)		(11)	
22409	$A_1MNDFy^{a+}K^-$	6¶		0.025	8	+	−	+	−	+	+	−		+	+
22299	$A_1MNDFy^{a+}K^-$	32¶		0.2	32	+	−	+	−	+	+	−		+	+
21878	$OMNDFy^{a+}K^+$			0.1	32	+	−	+	−	+	+	+	+	+	+
21955	$OMDFy^{a+}K^-$			0.05	16	+	−	−	−	+	+	−		+	+
Ho	$OMNDFy^{a-}K^-$			0.05	128	+	−	+	−	+	+	−		−	−
2 donors	$ORh(D)^+$			0.5–0.025						+	+				
5 donors	A_1B	>256‖	1	0.5–0.025											
24110	A_2BN	>4‖		0.025		−	−	+	−						
23945	A_2B	>32‖	1												
13628	A_1	64¶	0.25**	0.025											
22600	$ARh(D)^-$	128¶		0.06						−	−				
22183	$BRh(D)^-$		1	0.5	16					−	−				
22033	$BRh(D)^-$		1	0.25	16					−	−				
14063	B	0.05**	1	0.5											
Gil	A_1	160††		0.01											

* DCS used throughout except for cells Gil, which were treated with FSP.

† Serum 3817, a donor of group B, used with all cells except Gil; titer not enhanced by antiglobulin.

‡ Serum Stu_2, a donor of group A, immunized with human B substance; titer not enhanced by antiglobulin.

§ Tested with antiglobulin serum.

¶ Low titer of panagglutinin for enzyme treated cells.

‖ Panagglutinin absorbed from anti-A serum with enzyme treated O cells; enzyme treated cells not agglutinated.

** Panagglutinin titer not extended by antiglobulin; normal cells not agglutinated.

†† Using serum Go_1 (table I, McDuffie and Kabat, 1956), low panagglutinin titer with enzyme treated cells not enhanced, anti-A titer with normal cells enhanced 4- to 8-fold with antiglobulin serum.

plasma or serum. In column 5, the ratios of less than 1, represent the titers of the enzyme treated cells in the same donor's plasma or serum, since the corresponding normal cells of course were never agglutinable. The cells of 5 individual donors of blood group A_1B were treated with enzyme as described. Their agglutinability was then measured by titrating with anti-A absorbed with enzyme treated O cells, this absorption having been carried out in order to remove the panagglutinin present. The enzyme treated A_1B cells were found to have been rendered wholly inagglutinable in undiluted absorbed anti-A serum, while retaining their full agglutinbility with anti-B. This is shown by the ratio of 1 in column 4. A_1 cells (donor 13628) treated with enzyme lost their agglutinability in anti-A, and showed low titers of agglutination with anti-B; and B cells (donor 14063), while retaining their full titer with anti-B after enzyme treatment, became agglutinable to low titer with anti-A. In both cases, this panagglutinin could be removed by absorption of the sera with enzyme treated cells of group O. It was found, using normal A_1 cells, that the titer of anti-A in serum 3817 could not be extended by antiglobulin serum. In order to determine whether the low titer of agglutinins regularly found with enzyme treated A cells might be due to residual A antigen rather than to panagglutination, a sample of A_1 cells (Gil) after enzyme (FSP) treatment was tested with a serum (Go_1) the anti-A titer of which had been shown to be capable of 4- to 8-fold enhancement by Coombs serum (Table I, McDuffie and Kabat, 1956).

The enzyme treated cells and the corresponding normal cells were titered simultaneously in this serum by the technique of McDuffie and Kabat (1956). With the enzyme treated cells, the low titer in saline was identical to that in antiglobulin serum; whereas with the normal cells, an enhancing factor of 4 to 8 was demonstrable with antiglobulin. These findings give further support to the view that the low titer of agglutinins in anti-A sera for enzyme treated cells which originally had the A antigen is not due specifically to anti-A.

Of the other blood group antigens readily measured, only the M and N antigens were altered by *C. tertium* enzymes, as shown in columns 7 and 8, table 2. Six samples from different donors of $Rh(D)^+$ cells exhibited no difference in reactivity with anti-D following enzyme treatment when tested in saline and with anti-globulin serum (column 9); nor did four samples from individual donors of Fy^a positive cells, nor one example of Kell (K) positive cells (columns 11 and 10). There was no change in the negative reaction of 3 examples of $Rh(D)^-$ cells following enzyme treatment.

The effect of *C. tertium* enzymes on certain virus hemagglutination receptors was also determined. Human erythrocytes, treated with enzyme as already described, were used in titrations with active influenza virus and with 1 strain of murine encephalomyocarditis virus (Columbia SK), parallel titrations being run at the same time with the corresponding normal cells. It will be seen from table 2, column 6, that there was a regular and significant drop in titer with Columbia SK virus, as shown by the high ratios. Table 3 shows the results with various strains of active influenza virus. Receptors for type A (PR8) and for one type A′ strain (Conley) were regularly inactivated by *C. tertium* enzymes (FSP); those for the two other A′ strains (PR301 and FM1), for type B (Lee), and B′ (Great Lakes), and for NWS being unaffected. Chicken erythrocytes (samples from 4 birds) showed a similar pattern after *C. tertium* enzyme treatment, in that receptors for the PR8, FM1, Conley and PR301 strains were rendered inactive and those for the other strains were unaffected.

Neutralization of A-splitting enzyme by specific antibody. Soluble enzyme (FSP) was precipitated on alum for the immunization of rabbits. Two rabbits received a series of 16 intravenous injections over a 4 week period for a total of 69 mg of bacterial protein each. Both rabbits were bled before immunization and 10 days after the final injection. For the measurement of anti-enzyme, dilutions of serum in a volume of 0.2 ml were mixed with soluble enzyme in two amounts, namely 200 μg and 40 μg, each amount contained in 0.2 ml. After mixing and incubation at room temperature for 1 hr, 400 μg of blood group substance (mucin) in a volume of 0.4 ml were added to the serum-enzyme mixtures, which were then incubated at 37 C. Samples of each were taken at 4 hr and 20 hr, respectively, and assayed for residual blood group A antigen. Appropriate controls were included. The results are shown in table 4, dilutions of serum being given as the final dilution after the addition of enzyme and substrate. The preimmunization serum from both

TABLE 3

Effect of Clostridium tertium (Iseki strain) enzymes (FSP) on influenza virus receptors of human and chicken erythrocytes

Sample		Ratio of Highest Dilution Agglutinating Normal Cells to that of Agglutinating Enzyme Treated Cells of 7 Strains of Influenza Virus*						
		Strain						
		PR8	Conley	PR-301	FM1	Lee	Great Lakes	Neuro-WS
		Serotype						
Human	Blood Group	A	A′	A′	A′	B	B′	A
1†	B	>64		1	1	1	1	1
2	B	512	128	4	1			
3	O	512	128	2	1			
4†	O	>64		1	1	1	1	1
5	A_1	>64		1		1		
6	B	>64		1		1		
7‡	A_2	320	160		1			
Chicken								
1		128		>64	>64	1	1	1
2		128	64	>64	>64			
3		>64		>64		1		
4		320	40	320	160	1		

* Ratios are based on reciprocals of dilutions. Ratios preceded by > represent titers with enzyme treated cells of less than 1:8.

† Two separate samples from each of these donors, treated with enzyme, gave identical results.

‡ Cells from the same donor after treatment with material (FSP) from *C. tertium* strain 1259 were still agglutinable to full titer with A and A′ strains of influenza virus.

TABLE 4

Inhibition of Clostridium tertium enzyme action by specific antibody

Rabbit Serum Number	Final Serum Dilution*	Enzyme	Blood Group Substance	Loss of Blood Group A Activity after Incubation at 37 C for:	
				4 Hr	20 Hr
		μg	μg	%	%
Pre-immunization 1,2	1:4	200	400	100	
	1:4	40	400	100	
	1:4	—	400	0	0
Post-immunization 1,2	1:4	200	400	0	0
	1:40–1:640	200	400	100	
	1:4–1:80	40	400	0	0
	1:160	40	400	80–90	100
	1:320–1:640	40	400	100	
	1:4	—	400	0	0
Enzyme controls	—	200	400	100	
	—	40	400	100	
Substrate control	—	—	400	0	0

* Neither rabbit serum agglutinated A_1 cells in a dilution higher than 1:2; the first tube in assays for residual A antigen contained rabbit serum in a dilution of 1:8.

animals failed to neutralize the A-splitting enzyme. Both immune sera neutralized 200 μg of enzyme completely in a dilution of 1:4 but not at higher dilution. With the smaller amount of enzyme, definite neutralization was apparent through a serum dilution of 1:160 using the 4 hr end point, and 1:80 using the 20 hr end point. None of the rabbit sera showed agglutinins for Group A erythrocytes above a final dilution of 1:2. All of the enzyme controls remained fully active.

Peptidases of C. tertium. Two strains of *C. tertium* were investigated for peptidase activity, namely the Iseki strain and strain 1259 (McClung), the latter having failed to produce blood group A-splitting enzyme after at least 3 passages through glucosamine-NZA broth. Material (FSP) derived from both strains was tested against di- and tripeptides. Aliquots of enzyme solution were incubated with known di- and tripeptides at pH 7.2 to 7.5. The mixtures were then chromatographed on Whatman no. 1 paper, using phenol-ammonia or butanol-acetic acid-water as solvents. The presence of free amino acids was subsequently determined on the dried papers by spraying with ninhydrin. Known amino acids, di- and tripeptides without enzyme, and enzyme solutions alone were incubated and chromatographed as controls. The peptides found to be split by the material from the Iseki strain were glycyl-L-leucine, L-leucyl-leucine, DL-leucyl-glycine, DL-isoleucyl-glycine, DL-valyl-glycine, glycyl-glycyl-glycine, DL-alanyl-glycyl-glycine, and L-leucyl-glycyl-glycine. Material from strain 1259 hydrolyzed DL-leucyl-glycine and L-leucyl-glycyl-glycine—the only peptides tested with this material. The strain 1259 product failed to inactivate blood group A antigen in hog gastric mucin in the standard assay at pH 7.5, did not inactivate the A, M, and N antigens and influenza virus receptors on intact erythrocytes, and did not render the cells panagglutinable to any appreciable extent. Influenza virus receptors on chicken erythrocytes were likewise unaffected by the strain 1259 material.

Enzymes from both strains were then tested for sensitivity to EDTA (ethylenediaminetetraacetic acid) inhibition. Samples of 2 peptides (leucyl-glycine and leucyl-glycyl-glycine) and blood group A substance (mucin) were each mixed with equal amounts of each of the 2 enzyme preparations. Aliquots of these enzyme-substrate mixtures were then mixed with the trisodium salt of EDTA to make 3 final ratios (W:W) of enzyme:EDTA, namely 12, 2.4 and 1.2. The mixtures with peptides were chromatographed as before and those with blood group substance were assayed for residual A antigen, after appropriate incubation periods. The peptidases of strain 1259, the Iseki strain enzyme hydrolyzing leucyl-glycyl-glycine, and the blood group A splitting enzyme were inhibited at enzyme: EDTA ratios of 1.2 and 2.4, but not of 12. The Iseki strain peptidase hydrolyzing leucyl-glycine was not inhibited at any of the 3 ratios used. These findings suggest that both peptidase and blood group A splitting activities are metal activated. Under the conditions of these experiments, they could therefore not be separated by differential sensitivity to versene.

The material from the Iseki strain was also tested against azocoll (Oakley *et al.*, 1946) for total proteolytic activity. None was detectable when as much as 2 mg of the enzyme preparation (FSP) was used in the standard assay (Mandl *et al.*, 1953).

Fractionation of culture supernatants. In an attempt to concentrate and purify the enzyme responsible for inactivation of blood group A

TABLE 5

Fractionation of Clostridium tertium enzymes (FSP)

Fraction Number	Concentration of Ammonium Sulfate		Preparation					
			1			2		
	%	% of Saturation	Yield	Total Yield	Activity*	Yield	Total Yield	Activity*
			g	%		g	%	
I	0–18	0–33	0.099		Inactive	0.071		Inactive
II	18–23	33–42	0.050		16 hr	0.050		1 hr
III	23–28	42–53	0.120	26	15–30 min	0.083†	21	15 min
IV	28–35	53–65	0.155		Inactive	0.120		90 min
V	35+	65–full	0.032		Inactive	0.063		Inactive

* Time necessary to inactivate blood group A antigen (mucin) in standard assay.

† Single electrophoretic component at pH 8.6, 7.4, and 5.5.

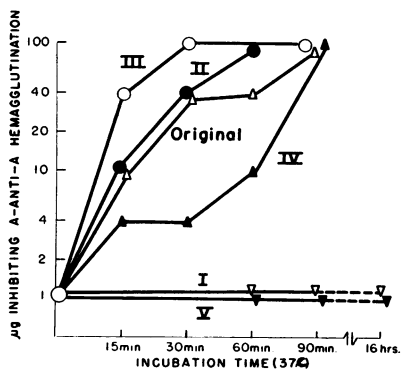

Figure 4. Refractionation (ammonium sulfate) of blood group A-splitting enzyme from full saturation precipitate (FSP).

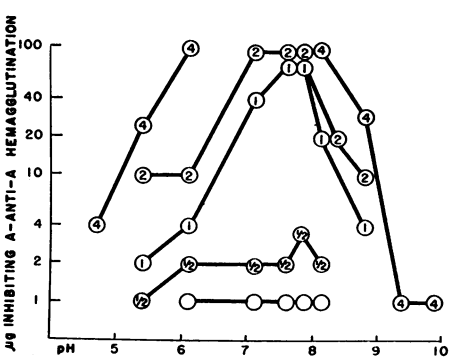

Figure 5. pH optimum for blood group A-splitting activity in fraction III (42 to 53% of saturation). Circled figures indicate hours of incubation of enzyme-substrate mixture at 37 C before assay for residual A antigen.

antigen, material (FSP) derived from the Iseki strain was fractionated further with ammonium sulfate, all manipulations being carried out in the cold. Saturated ammonium sulfate, neutralized with ammonium hydroxide, was added to a neutral solution of the crude material (FSP) to 18 per cent. After standing about 8 hr, the precipitate was removed by centrifugation and the supernatant brought to 23 per cent ammonium sulfate concentration. Three more successive cuts were taken in a similar manner at the final ammonium sulfate concentrations indicated in table 5. The precipitates were taken up in water, dialyzed against water in the cold, centrifuged clear, and lyophilized. The results of 2 preparations, each starting from 1 g of crude material (FSP), are given in table 5. In both preparations, the third fraction, between 42 per cent and 53 per cent of saturation representing ¼ to ⅕ of the total yield, was the most active, and in both was more active per unit weight than the original crude material. Peptidase activity was present in all fractions. Figure 4 shows the comparative rate at which equal amounts of the 5 fractions of preparation 2 and the original material inactivated blood group A antigen. It will be seen that fraction III inactivated the substrate completely after 30 min, being more active than either the original or any other fraction. The pH optimum for inactivation of the A antigen by fraction III in the second preparation, determined as previously described, and shown in figure 5, was similar to that in the crude starting material.

Fraction III in the second preparation was found to have a single component on free electrophoresis in the Tiselius apparatus at pH 8.6 (veronal), 7.4 (phosphate) and 5.5 (acetate).

Limited agar diffusion studies, however, with the rabbit antisera described above showed the presence of several antigenic components in all fractions.

DISCUSSION

It has been readily established that *C. tertium* elaborates an enzyme, or enzymes, directed against blood group A antigen in the presence of glucosamine, N-acetylglucosamine, intact blood group substance with suboptimal glucose, or completely hydrolyzed blood group substance. With strains which can produce the A-splitting enzyme, its elaboration in chopped meat as described by Iseki can be explained by the undoubted presence in this medium of intact blood group A substance and glucose, a combination shown in the present study to allow enzyme formation. Although culture supernatants with A-splitting activity showed no detectable action on glucosamine itself, this carbohydrate was shown to be utilized by the organism during growth and enzyme production. The capacity for the formation of adaptive enzymes specific for blood group A antigen appears to be a fairly readily elicited characteristic of *C. tertium* as evidenced by a study of 30 different strains. The only other blood group antigen so far found to be affected by these enzymes are the M and N factors.

The pH optimum found for the enzyme splitting the A substance, both in the crude material (FSP) and in the partially purified fraction (Fraction III), corresponds with that noted by Iseki, being somewhat higher than that of the A enzyme of *C. welchii* (Stack and Morgan, 1949). The pH optimum for the *T. foetus* enzymes (Watkins and Morgan, 1954) has not been stated.

The blood group A-splitting activity of *C. tertium* enzymes was inhibited by copper, zinc, and nickel ions, but not by other divalent cations. Although this effect was not studied further, its pattern resembles that found with certain peptidases obtained from other members of the genus *Clostridium*, notably *C. histolyticum* (Mandl *et al.*, 1957). Peptidases from the latter organism are completely inhibited in the presence of relatively small amounts of chelating agent (EDTA) and can be reactivated by subsequent addition of metal ions in the proper concentration. Limited studies with enzymes of *C. tertium* have shown that the blood group A-splitting activity and at least one of the peptidases elaborated by this organism are inhibited by much larger amounts of EDTA. Peptidases elaborated by a strain of *C. tertium* (1259) which shows no blood group A-splitting activity were completely inhibited by EDTA. These results suggest that peptidases of both strains, as well as the blood group A-splitting enzyme, may be activated by metal ions. From the way in which certain tripeptides were split (e. g., leucyl-glycyl-glycine to leucine and glycyl-glycine) it may be inferred that *C. tertium* elaborates aminopeptidases as well as dipeptidases. Watkins and Morgan (1954) ascribed the changes in reactivity of whole cells treated with *T. foetus* enzymes to trypsin-like activity as distinct from the O(H) splitting action. The similar activities of *C. tertium* enzymes, apart from the A-splitting enzyme, can perhaps be ascribed to the peptidases elaborated by this organism since it produces no proteolytic enzymes. On the other hand, the strain not producing the A enzyme (1259) also failed to inactivate the M and N antigens and the influenza virus receptors, although it produced potent peptidases. This finding would tend to identify the enzymes inactivating M and N antigen and influenza receptors more with the A enzyme than with the peptidases. Progress on the problem will depend to some extent on the separation of peptidase activity from that directed against the blood group antigens, either by sharper fractionation methods or by the determination of the effect of various metal ions on the different enzymes. The immunochemical changes effected by *C. tertium* enzymes on soluble A and O(H) substances, and the nature of the split products of enzymic action on these substrates, are currently under investigation and will be the subject of a forthcoming communication.

It is of interest that several viral hemagglutinin receptors on the human erythrocyte surface can be altered by enzymes from the strain of *C. tertium* which also elaborates blood group A-splitting enzyme, but not from material from a strain lacking the latter activity. The susceptible receptors are those for murine encephalomyocarditis virus and for type A and certain type A′ strains of influenza virus. The changes brought about by the *C. tertium* enzymes on chicken erythrocytes were similar with respect to the influenza viruses, but involved a broader range of receptors for A and A′ strains. Certain mucoids have been studied extensively because of their capacity to

inhibit viral hemagglutination, being therefore regarded as "receptor analogues." Definite differences in their reactivity with influenza virus are known to exist, as reflected in the different types of virus which they are able to inhibit. Several of these inhibitors, namely urinary mucoprotein, sputum mucoid (pH 3.6 precipitate), and human brain mucolipid were rendered inactive as inhibitors of viral hemagglutination by the action of *C. tertium* enzymes. Such pronounced effects by these enzymes on virus receptor substances both on intact cells and in soluble form offer a possible basis for more precise characterization of the viral enzyme substrates. Further studies of this aspect of the problem are in progress and will be reported elsewhere.

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SUMMARY

Thirty strains of *Clostridium tertium* were examined for their capacity to elaborate adaptive enzymes specifically inactivating blood group A antigen. Enzymes produced by 2 strains (Iseki; McClung no. 1259) were obtained in quantity from cultures in a semidefined medium containing glucosamine. Material from the Iseki strain inactivated soluble blood group A antigen and certain inhibitors of influenza virus hemagglutination and acted upon human erythrocytes to destroy the A, M, and N antigens, the influenza virus (strains PR8 and PR301) and Columbia SK virus hemagglutinin receptors, rendering the cells panagglutinable. Chicken erythrocyte receptors for influenza virus (strains PR8, PR301, FM1 and Conley) were also inactivated. Strain 1259 did not produce these enzymes. Both strains were shown to elaborate peptidases, which along with the A-splitting activity of the Iseki strain, were inhibited by ethylenediaminetetraacetic acid, suggesting their activation by metal ions. The A enzyme activity was inhibited by specific antibody.

REFERENCES

BAER, H., KABAT, E. A., AND KNAUB, V. 1950 Immunochemical studies on blood groups. X. The preparation of blood group A and B substances and an inactive substance from individual horse stomachs and of blood group B substance from human saliva. J. Exptl. Med., **91**, 105–114.

CHASE, M. W. 1938 A microorganism decomposing group-specific A substances. J. Bacteriol., **36**, 383–390.

ELSON, L. A. AND MORGAN, W. T. J. 1933 A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. (London), **33**, 1824–1828.

GYÖRGY, P., KUHN, R., NORRIS, R. F., ROSE, C. S., AND ZILLIKEN, F. 1952 A hitherto unrecognized biochemical difference between human milk and cows' milk. Am. J. Diseases Children, **84**, 482–484.

HEIDELBERGER, M. AND MACPHERSON, C. F. C. 1943 Quantitative micro-estimation of antibodies in the sera of man and other animals. Science, **97**, 405–406; **98**, 63.

HORVATH, B. AND JUNGEBLUT, C. W. 1952 Studies on hemagglutination by Columbia-SK virus. J. Immunol., **68**, 627–643.

HOWE, C. AND KABAT, E. A. 1956 Immunochemical studies on blood groups. XVIII. Fractionation of hog gastric mucin and individual hog stomach linings. Arch. Biochem. and Biophys., **60**, 244–254.

ISEKI, S. AND OKADA, S. 1951 On a specific enzyme which decomposes group A substance. Proc. Japan. Acad., **27**, 455–458.

ISEKI, S. AND TSUNODA, S. 1952 On a bacterial enzyme which specifically decomposes O substance. Proc. Japan. Acad., **28**, 370–373.

KABAT, E. A. AND MAYER, M. M. 1948 *Experimental immunochemistry.* Charles C Thomas, Springfield, Illinois.

KABAT, E. A. 1956 *Blood group substances.* Academic Press, New York.

MANDL, I., MACLENNAN, J. D., AND HOWES, E. L. 1953 Isolation and characterization of proteinase and collagenase from *Clostridium histolyticum.* J. Clin. Invest., **32**, 1322–1329.

MANDL, I., FERGUSON, T. T., AND ZAFFUTO, S. F. 1957 Exopeptidases of *Clostridium histolyticum.* Arch. Biochem. and Biophys., *accepted for publication.*

MARMION, B. P., CURTAIN, C. C. AND PYE, J. 1953 The effect of human bronchial secretions (sputum) on the haemagglutinin and

infectivity of influenza virus. Australian J. Exptl. Biol. Med. Sci., **31**, 505–518.

McDUFFIE, F. C. AND KABAT, E. A. 1956 The behavior in the Coombs test of anti-A and anti-B produced by immunization with various blood group A and B substances and by heterospecific pregnancy. J. Immunol., **77**, 61–71.

MICHAELIS, L. 1931 Der acetat-veronal Puffer. Biochem. Z., **234**, 139–141.

MORGAN, W. T. J. AND KING, H. K. 1943 The isolation from hog gastric mucin of the polysaccharide-amino acid complex possessing blood group specificity. Biochem. J. (London), **37**, 640–651.

OAKLEY, C. L., WARRACK, G. H., AND VAN HEYNINGEN, W. E. 1946 The collagenase of *Clostridium welchii* type A. J. Pathol. Bacteriol., **58**, 229–235.

ROSENBERG, A., HOWE, C., AND CHARGAFF, E. 1956 Inhibition of influenza virus haemagglutination by a brain lipid fraction. Nature, **177**, 234–235.

SCHIFF, F. 1935 Über den Abbau Gruppenspezifischer Substanzen durch Bakterien. Klin. Wochenschr., **14**, 750–751.

STACK, M. V. AND MORGAN, W. T. J. 1949 The preparation and properties of enzymes from *Clostridium welchii* (type B) filtrates which destroy blood group substances. Brit. J. Exptl. Pathol., **30**, 470–483.

TAMM, I. AND HORSFALL, F. L., JR. 1952 A mucoprotein derived from human urine which reacts with influenza, mumps, and Newcastle disease viruses. J. Exptl. Med., **95**, 71–97.

WATKINS, W. M. 1953 The serological inactivation of the human blood group substances by an enzyme preparation obtained from *Trichomonas foetus*. Biochem. J. (London), **54**, xxxiii.

WATKINS, W. M. AND MORGAN, W. T. J. 1954 Inactivation of the H receptors on human erythrocytes by an enzyme obtained from *Trichomonas foetus*. Brit. J. Exptl. Pathol., **35**, 181–190.