Degradation of Blood Group Antigens in Human Colon Ecosystems

I. IN VITRO PRODUCTION OF ABH BLOOD GROUP-DEGRADING ENZYMES BY ENTERIC BACTERIA

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ABSTRACT Human feces contain enzymes produced by enteric bacteria that degrade the A, B, and H blood group antigens of gut mucin glycoproteins. We have studied their production in fecal cultures to determine if such cultures can be a source for enzyme purification and to explore how blood group antigen-degrading enzymes are adapted in individual human colon ecosystems. They were present in fecal cultures from each of 27 healthy subjects, including ABH nonsecretors. Heat-sensitive obligate anaerobes are their major source. From 39 to 85% of the total enzyme activity produced by growing cultures was extracellular. Commercial hog gastric mucin and salivary glycoproteins, including Le^a saliva which lacks A, B, and H antigens, enhance production of A-, B-, and H-degrading activity in anaerobic fecal cultures irrespective of the glycoprotein's blood group specificity. There is evidence that the host's ABO blood type and secretor status affects the specificity of blood group-degrading enzymes produced by his fecal bacteria in vitro. Thus, fecal inocula from B secretors incubated with hog gastric mucin (A and H specificity) or with Le^a saliva produced greater levels of B-degrading than A- or H-degrading activity, and inocula from A secretors in similar media produced greater levels of A-degrading than B- or H-degrading activity. Blood group-degrading enzymes produced in fecal cultures are glycosidases and not proteases. The B-degrading enzyme cleaves the B antigenic determinant α-D-galactose from the oligosaccharide side chains of mucin glycoproteins with B specificity.

Anaerobic fecal cultures containing blood group substances are a feasible source for purifying blood group antigen-degrading enzymes. Prior adaptation to blood group antigens in the gut mucins of type A and type B secretors affects the specificity of the enzymes produced in vitro.

INTRODUCTION

Enzymes that degrade ABH(O) blood group substances were discovered in human feces over 40 years ago by Schiff and Akune (1). Some of their properties were defined, indicating that they were enzymes (2, 3). Blood group-degrading (BGD)[1] enzymes were absent from meconium and appeared in infants' stools after the first few months of life (4). They were also found in small amounts in saliva (1), in achlorhydric gastric juice but not in normal acidic gastric juice (5), and in feces of several other species (6). But their origin, whether from the host or his indiginous microflora, was unsettled. BGD enzymes have subsequently been found in certain species of bacteria (7-10), in a protozoan (11), in plant and mold sources (12, 13), snail hepatopancreas (14, 15), and in beef liver lysosomes (16). With the exception of a *N*-deacetylase from a strain of *Clostridium tertium* (17), purified preparations appear to be exoglycosidases that cleave the A, B, H, and Le^a antigenic determinant glycosides from their nonreducing terminal positions on the oligosaccharide side chains of mucin

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[1] *Abbreviations used in this paper:* BGD enzyme, blood group-degrading enzyme; HGM, hog gastric mucin.

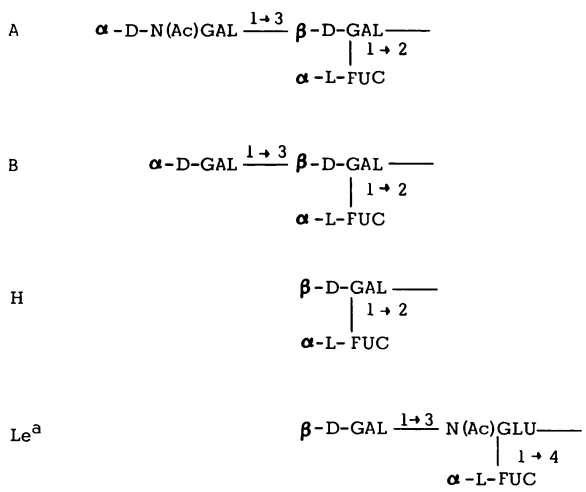

FIGURE 1 Antigenic determinant structures of A, B, H, and Le^a blood group antigens in mucin glycoproteins. Le^a antigen is present in mucous secretions of most ABH nonsecretors. N(Ac)GAL, *N*-acetylgalactosamine; GAL, galactose; FUC, fucose; N(Ac)GLU, *N*-acetylglucosamine.

glycoproteins with blood group antigen specificity (Fig. 1).

There is need for a convenient source of purified, well characterized glycosidases with substrate specificity for the blood group antigenic determinant structures. BGD enzyme preparations from available sources have most frequently been crude and incompletely characterized. Purification has been difficult and yields small (8), or the active pH range has been considerably lower than physiological pH (12-16). For these reasons we have investigated BGD enzymes in human feces to determine if this were a feasible source for their purification and characterization. Fecal BGD enzymes were shown to originate from enteric bacteria and not from the host (18); they presumably had a role in the extensive degradation of the carbohydrate moieties of mucin glycoproteins by enteric bacteria (19). It was shown that BGD enzyme activity in fecal extracts from individual ABH secretors was generally greatest for the host's blood group phenotype antigen (20). This suggests that the enteric microflora adapts degradative enzymes to the blood group antigenic structures in the gut mucins secreted by the host. Therefore, an additional purpose of our investigating fecal BGD enzymes was to study how bacteria in complex microbial ecosystems adapt enzymes to closely related but genetically differing substrates provided by their host.

This report describes studies aimed at defining (*a*) the production of BGD enzymes in fecal cultures, (*b*) the types of enteric bacteria that produce them, (*c*) the effect of mucin glycoproteins and of the host's blood type on the specificity of the enzymes produced in fecal cultures, and (*d*) whether BGD enzymes produced in fecal cultures are glycosidases or proteases. The results indicate that BGD enzymes are glycosidases and that anaerobic fecal cultures are a feasible source. Some of the findings, together with those reported in the accompanying paper (21), suggest how BGD enzymes are adapted in human colon ecosystems.

MATERIALS AND METHODS

Materials

Stool specimens. Stools were obtained from healthy subjects. Each subject's blood type and ABH salivary secretor status was determined as described previously (20).

Culture media. The culture medium used in studies comparing BGD enzyme production in aerobic, anaerobic, and heat-treated anaerobic cultures, the earliest work in this series, was thioglycollate broth (Difco Laboratories, Detroit, Mich.) containing 1 mg/ml D-glucose and methylene blue redox indicator. For aerobic cultures the medium was formulated without sodium thioglycollate. In all other studies the following medium, called "anaerobic medium," was used: K_2HPO_4 7.2 g, KH_2PO_4 3.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, Casamino acids (Difco, technical grade) 10 g or Trypticase (Baltimore Biological Laboratories, Baltimore, Md.) 10 g, yeast extract (Baltimore Biological Laboratories) 5 g, sodium thioglycollate 2 g, D-glucose 1 g, hog gastric mucin (HGM) 2 g, resazurin 1 mg, and distilled water to make 1 liter. In the absence of HGM this medium had no detectable A, B, or H hemagglutination inhibiting activity. The final pH was 6.9±0.2. Where specified, crude salivary glycoprotein preparations of known blood group activity were used in place of HGM. These consisted of salivas which were heated 10 min in a 100°C water bath, centrifuged at 700 *g* to remove particulate matter, dialyzed four times against 2 liters of distilled water, and lyophilized. The lyophilized material was dissolved in the culture medium at a final concentration of 2 mg/ml. Le^a saliva used in culture media was obtained from a group O nonsecretor. Its content of Le^a was determined by Dr. Thomas F. Boat of the Department of Pediatrics, using ficin-treated erythrocytes (22). The minimal inhibitory concentration of Le^a antigen in this lyophilized preparation was 2 μg/ml.

Antigen substrates for BGD enzyme assays. The blood group antigen substrates used in assays of BGD enzyme activity were HGM for A and H antigen and boiled, dialyzed, and lyophilized saliva from one blood group B secretor for B antigen. These were dissolved in 0.02 M phosphate buffer, pH 6.4, containing 1 mM $MgCl_2$ and 0.2 mM disodium ethylenediamine tetraacetate ("assay buffer") at final concentrations of 0.2 mg/ml HGM for A antigen, 2 mg/ml HGM for H antigen, and 0.5 mg/ml lyophilized B saliva for B antigen.

The HGM used in culture media and as antigen substrate (lot 4580 Hog Gastric Mucin N.N.R. (Special), ICN Nutritional Biochemical Div., International Chemical & Nuclear Corp., Cleveland, Ohio) possessed strong A, weaker H, and no detectable B antigen activity. The minimum hemagglutination inhibiting concentration of this preparation was 0.08 μg/ml for A antigen and 1.6 μg/ml for H antigen.

Antisera in hemagglutination inhibition assays. Human immune anti-A and anti-B antisera (Ortho Diagnostics, Raritan, N. J.) and an extract of *Ulex europeus* seeds (F. W. Schumacher Co., Sandwich, Mass.) as anti-H lectin

were prestandardized and freshly diluted with saline (0.15 M NaCl) to contain 2 hemagglutination U per 0.05 ml.

Preparation of the L-*fucose-inhibited anti-H lectin from U. europeus.* A 10% wt/vol extract of *U. europeus* seeds in saline was used as the anti-H agglutinin in early phases of this study. But the crude extract tended to produce hemolysis during the microtiter hemagglutination inhibition assays, and it is known to contain two anti-H agglutinins, one inhibited by L-fucose and the other not (23). We purified the fucose-inhibited anti-H lectin from crude *Ulex* extract as follows. A 20% wt/vol saline extract of 50 g ground *Ulex* seeds was filtered. The precipitate that formed upon adding 2 M ammonium sulfate to the filtrate was dissolved in and dialyzed against saline. Ethanol was added to a final concentration of 50% vol/vol and the precipitate was discarded. Ethanol was then added to a final concentration of 70% vol/vol. The resulting precipitate was dissolved in and dialyzed against saline. This fucose-inhibited anti-H lectin solution contained 20–50 mg protein and up to 110 hemagglutinating U per mg protein against O cells. Its anti-H activity remained stable for months at 4°C. Hemagglutination by 2 hemagglutinating U was inhibited by 1.3 mg/ml L-fucose but not by 3.3 mg/ml tri-*N*-acetylchititriose. 2 hemagglutinating U of this purified fraction yielded the same hemagglutination inhibition endpoints as the crude extract but without hemolysis of red cells.

Purified B-degrading enzyme. Preparations of purified B-degrading enzyme were obtained from the cell-free supernates of anaerobic cultures inoculated either with feces from a group B secretor or with a fecal strain of *Ruminococcus* AB that produced B-degrading enzyme activity (see Results). The B-degrading enzyme activity in the culture supernates was purified by ammonium sulfate fractionation, gel filtration on Sephadex G-200, and disk gel electrophoresis.[2] B-degrading activity purified from both sources was devoid of A- and H-degrading activities, sialidase, nitrophenyl-α- and -β-D-galactosidases, and proteolytic activity. The preparation from *Ruminococcus* AB was purified 45-fold to a specific activity of 9,500 U/mg. Disk gel electrophoresis revealed one heavily staining protein band of slow mobility corresponding to B-degrading activity and five faster, weakly staining bands.

Partially purified A- and H-degrading enzymes. A preparation containing both enzyme activities was obtained from the cell-free supernate of an anaerobic culture inoculated with feces from a group A secretor. Partial purification was achieved by ammonium sulfate fractionation, gel filtration of Sephadex G-200, and chromatography of DEAE cellulose.[2] A-degrading activity in this preparation had been purified three-fold to a specific activity of 60 U/mg. Disk gel electrophoresis revealed five protein bands.

Methods

Preparation and storage of prereduced culture medium. Freshly prepared culture medium was boiled in a flask equipped with reflux condenser until the redox indicator became yellow. Aliquots were then transferred into screw-capped tubes or bottles, which were then capped and autoclaved. Air in the flask, tubes, or bottles was displaced by a continuous stream of gas mixture (95% nitrogen, prepurified grade, and 5% carbon dioxide, Coleman Instruments grade, Matheson Gas Products, East Rutherford, N. J.) during all manipulations. Immediately after autoclaving, the tubes or bottles were placed in vacuum desiccator jars, which were then alternately evacuated and flushed four times with 95% N_2-5% CO_2 and stored at slightly negative pressure at room temperature.

Inoculation and incubation of cultures: preparation of cell-free culture supernates. 1 g of fresh feces was suspended in 10 ml anaerobic culture medium and cultures were inoculated with 0.01 vol of this suspension to a final concentration of 10^{-3} g feces per ml. Inoculated cultures were incubated in desiccator jars which had been evacuated and flushed four times with N_2-CO_2. Unless stated otherwise, cultures were incubated for 24 h at 37°C.

After incubation the cultures were centrifuged at 10,000 *g* for 30 min. The cell-free culture supernatant fraction was then assayed for BGD enzyme activity. A measured aliquot was dialyzed four times in 100 vol or more of distilled water at 4°C and assayed for protein by the method of Warburg and Christian (24). Since BGD enzyme activity produced in thioglycollate broth was weak compared to that produced in "anaerobic medium," enzyme activity in the culture supernates of thioglycollate broth was concentrated by lyophilizing the supernates after preliminary dialysis in distilled water. BGD enzyme assays were then performed on solutions of 5 mg/ml culture lyophilate in assay buffer.

Assay of BGD enzyme activity. BGD enzyme activity was estimated from the decrease in titer of A, B, or H blood group antigen substrate during incubation with samples containing enzyme activity. Aliquots of each culture supernate were incubated separately with A, B, or H antigen substrate solutions at 37°C. Equal volumes of culture supernate and antigen substrate solution were mixed, and aliquots were removed at intervals including 0, 10, 30 min and 4 h, heated 2 min in a boiling water bath to inactivate the enzyme, and the titer of antigen remaining in each aliquot was determined by hemagglutination inhibition. Titers are expressed as the exponent, n, of serial $1/2^n$ dilutions, and the decrease in antigen titer as $\Delta n = n_0 - n_t$, representing a decrease from the initial value of $1/2^{n_0}$ to $1/2^{n_t}$ at time t. "1 U" of enzyme activity is defined as that amount of enzyme which will cause the antigen substrate titer to decrease 1 twofold dilution from $1/2^n$ to $1/2^{n-1}$ in 30 min at pH 6.4 and 37°C. Strong enzyme activity in the culture supernate resulted in $\Delta n \geq 1$ after 10 min incubation whereas weak activity resulted in $\Delta n \geq 1$ only after 2 or 4 h incubation. The amount of enzyme activity in the incubation mixture was estimated from the decrease in antigen substrate titer, Δn, occurring in the earliest incubation period, Δt. This was converted to units of enzyme activity per milliliter of incubation mixture from the equation: units/milliliter $= (\Delta n/\Delta t) \times 30$ min. Enzyme activity in the culture supernate is expressed as units per milliliter culture supernate or as specific activity, units per milligram protein.

The variability of the assay was tested in five replicate cultures from a single stool specimen and did not exceed $\Delta n \pm 1$ at each of three different incubation periods. The coefficient of variation of the protein concentration in the same five cultures was ±6%

Measurement of antigen titers by hemagglutination inhibition. The titers of A, B, and H antigens in each aliquot were determined in triplicate hemagglutination inhibition tests using microtiter plates, loops, and pipettes (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). Each 0.05-ml sample was serially diluted in wells containing 0.05 ml saline, then 2 hemagglutinating U of antiserum in 0.05 ml saline was added followed by 0.05 ml of a 1% saline suspension of washed A_1, B, or O erythrocytes. Appropriate red cell and antiserum controls

[2] Details are available upon request.

were included. The plates were shaken to suspend the mixtures and were stored at 4°C for at least 4 h before the initial reading of hemagglutination inhibition titers. Clear-cut hemagglutination inhibition endpoints required suspending the cells and allowing them to settle at least twice before making a final reading. The highest dilution of antigen that completely inhibited hemagglutination was defined as the antigen titer. The titer endpoints of the triplicates were averaged to the nearest whole integer to give the antigen titer of the sample. The endpoints generally were either identical or were identical in two while the third differed by one serial dilution.

Comparison of extracellular and intracellular BGD enzyme activity. Anaerobic cultures inoculated with feces from two subjects were centrifuged at 10,000 *g* while in the active growth phase at 16 h. The cells were washed twice in pH 6.4 phosphate-buffered saline and 5-ml aliquots were disrupted by ultrasonication with a Branson model S-75 Sonifier (Heat Systems-Ultrasonics, Great Neck, N. Y.) and 12 4-s bursts of 2.0 A interspersed with 15 s cooling periods. Preliminary studies showed that this sequence gave maximal release of protein and lactic dehydrogenase, the latter being used as an intracellular enzyme marker. The suspension of sonicated cells was centrifuged, and the supernate of the sonicated cells and the cell-free culture supernate were dialyzed in pH 6.4 phosphate-buffered saline and were analyzed for A-, B-, and H-degrading activities and lactic dehydrogenase. Lactic dehydrogenase was measured by the method of Kornberg (25) and is expressed in international units. The supernate of the sonicated cells contained no inhibitors of BGD enzyme activity.

Measurement of galactose released during treatment of B saliva with B-degrading enzyme. 2 ml of a solution containing 2 mg/ml lyophilized B saliva in assay buffer and 0.5 ml of a solution containing B-degrading enzyme purified from the culture supernate of a fecal strain of *Ruminococcus* AB were mixed and incubated at 37°C. Aliquots were removed at various times and were assayed for B-degrading activity and for free galactose released during incubation. An identical mixture containing heat-inactivated enzyme served as a control. Free galactose was measured by the method of Meisler (26), with galactose dehydrogenase and nicotinamide adenine dinucleotide obtained from Boehringer Mannheim Corp., New York.

Identification of the products released from HGM by a partially purified preparation containing A- and H-degrading activity. HGM was precipitated twice from solution with 60% vol/vol ethanol, dissolved in 0.1 M NaCl, dialyzed against distilled water, and lyophilized. 20 mg of the lyophilized material in 2 ml assay buffer was incubated with 0.1 ml of the enzyme preparation for 27 h at 37°C under toluene. Residual HGM was removed by precipitation with 70% vol/vol ethanol. The supernate was flash evaporated to dryness, dissolved in distilled water, desalted by passage through Dowex 50W-x2 and Dowex 1x-8 ion exchange resins (27), flash evaporated to dryness, and redissolved in 0.2 ml distilled water. Diluted aliquots were assayed for hexoses by the anthrone method and L-fucose by the cysteine-sulfuric acid method (27). Galactose was determined from the hexose assay by correcting for fucose content. For identification of neutral sugars descending chromatograms were run on Whatman 3 MM paper in two different solvent systems: *n*-butanol-pyridine-water (6:4:3) and ethyl acetate-pyridine-water (24:10:8). Neutral sugars were detected with silver nitrate spray reagent (28). For identification of hexosamines chromatograms were run in the same solvent systems on Whatman 3 MM paper previously impregnated with 0.2 M borate buffer, pH 8.0. They were detected with the Elson-Morgan spray reagent (28). The sugars were identified by comparing their migration in the two solvent systems with that of standard sugars applied to each chromatogram.

Measurement of proteolytic and BGD enzyme activity in fecal extracts and anaerobic fecal cultures. Fresh feces was inoculated into an anaerobic culture and incubated as described above. A 10% wt/vol suspension of the same feces was prepared in saline and centrifuged at 700 *g* and 20,000 *g* to obtain a clear supernatant fraction. The culture supernate and the fecal supernate were dialyzed in saline. Portions of the dialyzed supernates were adjusted to pH 6.4 and 9.5 with 0.1 M phosphate and 0.1 M glycine-NaOH buffer, respectively, and were assayed for proteolytic and BGD enzyme activity. Proteolytic enzyme activity was estimated by the method of Tomarelli et al. (29), with sulfanilic acid-azoalbumin (Sigma Chemical Co., St. Louis, Mo.) as substrate. Activity is expressed as the increase of trichloroacetic acid-soluble products absorbing light at 440 nm during 4 h incubation at 37°C.

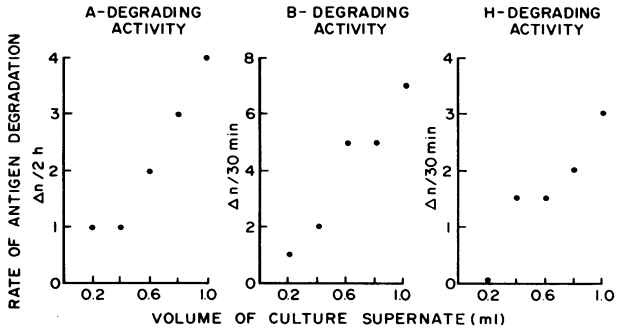

FIGURE 2 Relationship between the rate of antigen degradation and BGD enzyme concentration in the incubation mixture. Total volume of each incubation mixture was 2.0 ml.

RESULTS

Estimation of BGD enzyme activity in fecal culture supernates. It is not possible to assay BGD enzyme activity with simple synthetic glycoside substrates since BGD enzymes do not cleave them (references 8 and 30 and unpublished observations), and glycosidases that do cleave such substrates are also present in the cultures. Therefore, we estimated BGD enzyme activity by the rate of decrease of blood group antigen substrate titer, using hemagglutination inhibition titration to measure antigen levels. Despite the crudeness of this procedure it appears to be adequate to detect the differences described herein. As was found with fecal extracts (20), the rate of decline of antigen titer, $\Delta n/\Delta t$, during incubation with fecal culture supernate is directly related to enzyme concentration (Fig. 2).

A typical BGD enzyme assay is shown in Fig. 3, where the fecal culture supernate from a group A secretor was incubated separately with A, B, and H antigen substrates. The culture shows strong A-degrading,

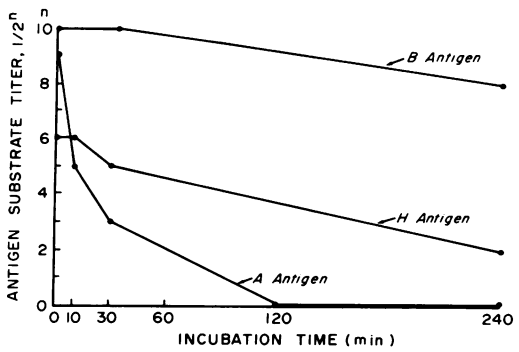

FIGURE 3 BGD enzyme activity in the supernatant fraction of an anaerobic fecal culture from a group A secretor.

intermediate H-degrading, and weak B-degrading activity amounting to 24, 2, and 0.5 U/ml culture supernate, respectively.

Bacterial sources of fecal BGD enzymes. BGD enzymes were produced in fecal cultures from each of 27 healthy subjects. Heat-sensitive obligate anaerobes are their major source. This is shown in Table I by the levels of enzyme activity obtained when identical fecal inocula from each of 11 subjects were incubated anaerobically, anaerobically after heating 20 min at 70°C to select for sporulated organisms, and aerobically. Strong BGD enzyme activity was produced in every unheated anaerobic culture. In contrast, BGD enzyme activity was either weak or absent in the preheated anaerobic and aerobic cultures. Facultative anaerobes present in the aerobic cultures did not produce BGD enzyme activity when subcultured under anaerobic conditions.

The capacity to produce BGD enzymes is not shared by many anaerobic fecal bacteria. Isolates of anaerobes were obtained from feces of a blood group B secretor whose fecal cultures consistently produced B-degrading activity. No B-degrading activity was detected in the culture supernates and ultrasonically disrupted cells of one or more strains of *Bacteroides* sp., *B. melaninogenicus,* bifidobacteria, *Clostridium perfringens,* and an anaerobic streptococcus, but a gram-positive nonsporulating anaerobe was isolated that constitutively produced extracellular B-degrading enzyme activity.[3] This was tentatively identified as a strain of a newly described species, *Ruminococcus* AB (31), by the staff of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.

Production of BGD enzyme activity in anaerobic fecal cultures. Production of BGD enzyme activity parallels bacterial growth in anaerobic fecal cultures containing HGM. This is shown in Fig. 4. Fresh feces from a blood group B secretor was inoculated into the medium at 0 time. Bacterial growth, measured by increase in optical turbidity, became detectable at 6 h and proceeded rapidly until 24 h when it ceased. Production of B-degrading enzyme activity paralled bacterial growth and likewise stabilized at 24 h. The A and H antigen titers imparted to the medium by HGM decreased rapidly from high levels at the time of inoculation to zero at 12 h, indicating early degradation of the antigenic determinant structures.

[3] Hoskins, L. C., and M. Agustines. In preparation.

TABLE I

BGD Enzyme Production in Fecal Cultures Incubated Anaerobically, Anaerobically after Heating 20 min at 70°C, and Aerobically from Each of 11 Subjects

Culture conditions	BGD enzyme activity*		
	Strong	Weak	Absent
Anaerobic	7	4	0
Anaerobic preheated 20 min at 70°C	0	6	5
Aerobic	0	6	5

* Strong activity, ≥1.6 U/mg lyophilized culture supernate; weak activity, ≤0.4 U/mg lyophilized culture supernate.

Comparison of extracellular and intracellular BGD enzyme activity. Significant amounts of BGD enzymes produced by living fecal bacteria are elaborated into the medium. This is shown in Table II, where from 39 to 85% of the total activity was in the cell-free supernatant fraction of fecal cultures. Insofar as the cultures were actively growing and none of the intracellular marker enzyme, lactic dehydrogenase, was detected extracellularly, it is likely that BGD enzymes are elaborated as extracellular enzymes by living bacteria and not as intracellular enzymes released by cell autolysis.

Stimulating effect of mucin glycoproteins on BGD enzyme production. Commercial HGM added to the

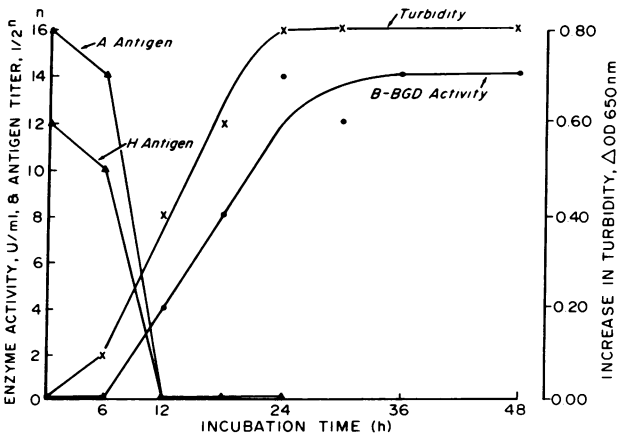

FIGURE 4 Bacterial growth and production of B-degrading enzyme activity in an anaerobic culture containing HGM inoculated at 0 time with feces from a group B secretor.

TABLE II

Extracellular Release of BGD Enzymes by Bacteria in 16-h-Old Anaerobic Fecal Cultures

Subject	A-degrading activity		B-degrading activity		H-degrading activity		Lactic dehydrogenase	
	Total	% extra cellular	Total	% extra cellular	Total	% extra cellular	Total	% extra cellular
	U		*U*		*U*		*U*	
A secretor	1,200	49	680	85	250	39	1.8	0
B secretor	700	79	1,200	48	330	55	7.9	0

culture medium stimulates BGD enzyme production in anaerobic cultures of fecal bacteria. This is illustrated in Table III, where a fecal suspension from a blood group B secretor was inoculated into anaerobic medium containing 1 mg/ml D-glucose and HGM at the concentrations indicated. In the absence of HGM, BGD enzyme activity was not detected in the 2-h incubation period of the enzyme assay. With increasing concentrations of HGM, BGD enzyme production increased, reaching a maximum at a concentration of 2 mg/ml. This stimulating effect of HGM on BGD enzyme production by fecal bacteria has been consistent; in studies of cultures from more than 20 subjects, irrespective of their blood group or secretor status, 2 mg/ml HGM in the culture medium enhanced A-, B-, and H-degrading enzyme activity from 2- to 20-fold or greater over that produced in parallel cultures lacking HGM. It is also noteworthy that B-degrading activity produced in the fecal culture with 2 mg/ml HGM in Table III exceeded the levels of A- and H-degrading activity even though the medium lacks B antigen substrate. As noted below this is related to fecal donor's being a blood group B secretor.

The stimulating effect of HGM on BGD enzyme production is also a property of salivary glycoproteins, irrespective of their ABH antigen specificity. This is shown in Table IV by the levels of BGD enzyme activity produced in cultures containing glucose alone or with 2 mg/ml HGM or boiled, dialyzed, and lyophilized salivas. Two studies are shown, one using feces from a group A secretor and the other feces from a group B secretor. The results are similar in both studies: compared to the control cultures containing glucose alone, A- or B-degrading enzyme activity was clearly enhanced in all the cultures containing blood group substances including those that lack A or B antigenic determinant structures. Similarly, H-degrading activity was enhanced in cultures containing Le^a saliva which lacks the H antigenic determinant structure. It is unlikely that the stimulating effect of these glycoproteins is due solely to their being an additional source of carbohydrate since the glucose concentration in the control culture, 3 mg/ml, exceeds the amount of carbohydrate available from 2 mg/ml glycoprotein and 1 mg/ml glucose in the other media. Although glucose is known to inhibit induced synthesis of β-galactosidase by *Escherichia coli* (32), it is unlikely that the low levels of BGD enzyme activity in the cultures containing only glucose represent a similar inhibition since we also found low levels in media con-

TABLE III

Anaerobic Fecal Cultures from a Blood Group B Secretor, Showing Enhanced Production of BGD Enzymes in the Presence of HGM

Concn of HGM	BGD enzyme activity		
	A	B	H
mg/ml	*U/mg protein*		
0	0*	0*	0*
0.02	0	0	0
0.20	0	5	5
2.0	26	105	55
10.0	10	140	40

* No decrease in antigen substrate titer during 2 h incubation with culture supernate.

TABLE IV

Enhanced Production of Blood Group-Degrading Enzymes in 24-h Cultures Containing HGM or Salivary Glycoproteins

Culture*	BGD enzyme activity			
	Fecal inoculum from a group A secretor		Fecal inoculum from a group B secretor	
	A	H	B	H
	U/mg protein			
Glucose	0.8	0	1.0	0
HGM	26	4	28	2
A saliva	12	2	55	3
B saliva	19	6	15	1
O saliva	13	7	29	1
Le^a saliva	13	4	21	2

* Glucose medium contained 3 mg/ml D-glucose; all others contained 1 mg/ml D-glucose and 2 mg/ml HGM or saliva.

TABLE V

Specific Activities of BGD Enzymes Produced in Anaerobic Fecal Cultures Containing HGM

Blood group	Subject	BGD enzyme activity A	B	H
		U/mg protein		
A secretors	1	5	0*	1
	2	12	0	1
	3	48	1	4
	4	20	2	15
	5	37	0	11
B secretors	6	4	22	11
	7	7	21	1
	8	5	54	5
	9	12	24	8
	10	14	86	14
O secretors	11	14	0	2
	12	4	0	4
	13	5	5	15
	14	7	4	4
	15	27	2	5
Nonsecretors	16 (A)	8	4	0.3
	17 (A)	75	1	3
	18 (B)	33	6	4
	19 (O)	9	0.4	3
	20 (O)	4	2	0.5

* No decrease in antigen substrate titer during 4 h incubation with culture supernate.

taining glycerol instead of glucose. Other sugars that did not stimulate BGD enzyme production were D-galactose, L-fucose, *N*-acetyl-D-galactosamine, melibiose, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-α-L-fucopyranoside. Soluble starch, a different type of polysaccharide molecule, likewise did not stimulate BGD enzyme production at a concentration of 2 mg/ml.

It is unlikely that the apparent stimulating effect of mucin glycoproteins is actually due to their binding and protecting the enzymes from degradation by bacteria, for BGD enzymes appear to be resistant to bacterial degradation. Thus, when a crude preparation of BGD enzymes was added to anaerobic medium lacking HGM and inoculated with feces, there was no decrease of A-, B-, or H-degrading activities from their initial levels during bacterial growth over 24 hours' anaerobic incubation.

Influence of host blood type and secretor status on the specificity of BGD enzymes produced in anaerobic fecal cultures. The ABO blood type and secretor status of the host appear to affect the specificity of A-, B-, and H-degrading enzyme activities produced by his fecal bacteria in anaerobic cultures. Evidence for this is clearly seen in anaerobic fecal cultures from B secretors grown in the presence of HGM (Table V). Despite the lack of B antigenic structures in this medium, fecal cultures from B secretors produced 2- to 20-fold greater B-degrading than A- or H-degrading activities. In contrast, fecal cultures from A secretors consistently produced greater A-degrading than B- or H-degrading activities. Fecal cultures from O secretors and nonsecretors produced BGD enzyme specificities that were generally in the order A-degrading > H-degrading > B-degrading. Since the A and H antigenic structures in HGM might have induced A- and H-degrading activity in vitro, a more suitable medium for observing effects of the host's blood type on the specificity of BGD enzymes produced in vitro would be one that lacks A, B, and H antigenic structures. Such a medium would be one containing glycoproteins with Le^a specificity. BGD enzyme specificity was therefore ascertained in cultures containing Le^a saliva inoculated with feces from four subjects. The results (Table VI) show that in three out of four cultures the specificity of BGD enzyme activity was greatest for the subjects' blood group phenotype antigen. The exception was the culture from an O secretor where A-degrading activity equaled H-degrading activity.

Evidence that BGD enzymes in anaerobic fecal cultures are glycosidases. BGD enzymes produced in anaerobic fecal cultures appear to be glycosidases and not proteases. Thus, galactose was released during degradation of salivary B antigen by a B-degrading enzyme purified from the culture supernate of a fecal strain of *Ruminococcus* AB (Fig. 5*a*). During the degradation of B antigen by purified B-degrading enzymes obtained from anaerobic fecal cultures and from cultures of *Ruminococcus* AB the titer of H antigen increases (Fig. 5*b*). These results indicate that B-degrading enzymes are α-galactosidases that cleave the terminal α-D-galactose from the underlying H antigenic structure.

A preparation of A- and H-degrading activity, partially purified from the anaerobic fecal culture supernate of a group A secretor, was devoid of B-degrading activity but also contained hydrolases active against *o*-ni-

TABLE VI

Specific Activities of BGD Enzymes Produced in Anaerobic Fecal Cultures Containing Le^a Antigen

Host	BGD enzyme activity A	B	H
	U/mg protein		
A secretor	13	0.6	4
B secretor	1	21	2
O secretor	7	3	7
O nonsecretor	0	0	3

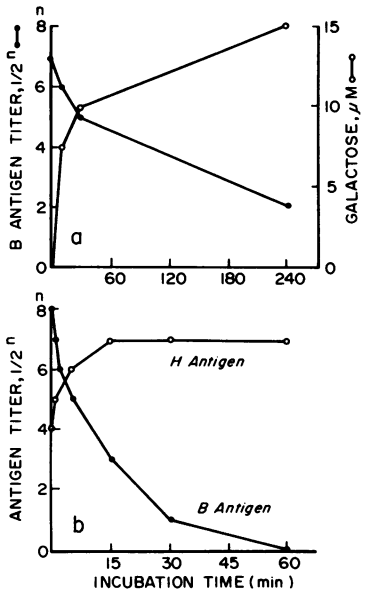

FIGURE 5 (*a*) Release of galactose (micromoles/liter) during degradation of B saliva antigen by a B-degrading enzyme from a fecal strain of *Ruminococcus* AB. (*b*) Increase in H antigen titer during degradation of B saliva antigen by B-degrading enzyme purified from a fecal anaerobic culture.

trophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-*N*-acetylglucosamine. After incubation of this preparation with HGM the split products were separated from the residual glycoprotein and desalted. Paper chromatography revealed four spots that corresponded to galactose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. None of these were detected on chromatograms from HGM incubated identically with heat-inactivated enzyme preparation. No unacetylated hexosamines were detected in the 2 N HCl eluate from the Dowex 50W column used in the desalting procedure. This suggests that A-degrading activity in the enzyme preparation was not a *N*-deacetylase (17). Analysis of the amounts of fucose and galactose in the split products indicated that 47% of the total fucose and 7.5% of the total galactose in the HGM had been liberated by enzyme action.

Fecal BGD enzyme activity appears to be distinct from fecal proteolytic activity. Thus, the pH optima of fecal BGD enzymes from two subjects was in the range pH 5.5–6.5 whereas the pH optimum of fecal proteolytic activity from six subjects was in the range pH 8.0–9.5. Assays were performed of both activities at pH 6.4 and pH 9.5 in fecal extracts from six subjects and in anaerobic cultures of the same fecal samples. The results were consistent and are shown for one subject in Table VII. Fecal proteolytic activity was greater at pH 9.5, but at pH 9.5 BGD activity was inactive. Of greater significance, despite its presence in each fecal extract, proteolytic activity was either not produced in the anaerobic cultures from the fecal samples or it was present in trace amounts that could be accounted for by the amount of feces in the inoculum. No proteolytic activity was detected in the purified B-degrading enzyme produced by *Ruminococcus* AB or in the partially purified A- and H-degrading preparation.

DISCUSSION

Blood group A-, B-, and H-degrading enzymes were present in the supernates of fecal cultures from each of 27 healthy subjects; their production is therefore a normal feature of the human enteric microflora. Production was greatest and was uniformly consistent in cultures incubated anaerobically; therefore, obligate anaerobic species are their most likely source. As noted here and by others previously (2, 33, 34), many species of fecal bacteria do not produce BGD enzymes. Bacteria with BGD activity described in the literature have generally been isolated from soil or sewage. Most are gram-positive and members of the family Bacillaceae, primarily *Clostridia* and to a lesser extent *Bacillus* species (7–10, 33). Two have been isolated from human feces. These were a strain of *Cl. perfringens* (type A (34) and a strain of bifidobacteria (*L. bifidus var. Penn*) that produced intracellular blood group-degrading activity (9). The capacity of *Clostridia* to produce BGD enzymes is not a species characteristic but varies among strains (33–35). Our isolation of a strain of *Rumino-*

TABLE VII

Proteolytic and BGD Enzyme Activities in an Extract of Feces and in an Anaerobic Culture of the Same Fecal Specimen

	Proteolytic activity		BGD enzyme activity					
			A		B		H	
	At pH		At pH		At pH		At pH	
	6.4	9.5	6.4	9.5	6.4	9.5	6.4	9.5
	ΔOD/4 h				Δn/4 h			
Fecal extract (100 mg/ml)	0.219	0.496	3	1	4	0	3	0
Fecal culture (1 mg/ml)	0.000	0.005	3	0	4	0	4	0

coccus AB that produces extracellular B-degrading activity suggests that this and strains of other gram-positive nonsporulating anaerobic species produce extracellular BGD enzymes in human colon ecosystems.

Although not yet fully characterized, BGD enzymes produced in anaerobic fecal cultures appear to be glycosidases that act on the oligosaccharide side chains of mucin glycoproteins and not proteases that act on the polypeptide core. We have shown that B-degrading enzyme catalyzes cleavage of the B antigenic determinant α-D-galactose from B saliva with a concomitant uncovering of the underlying H antigenic structure. We have not yet purified A- and H-degrading enzymes sufficiently to define their site of action. Our evidence suggests that they are also exoglycosidases that split their respective antigenic determinant terminal glycosides from glycoproteins with A and H specificity. First, L-fucose (the antigenic determinant of H antigen) was identified as a product when HGM was incubated with a preparation containing several glycosidases including A- and H-degrading activity. This saccharide has been found only in nonreducing terminal positions on oligosaccharide side chains of blood group substances (36). Second, the presence of D-galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine as other monosaccharides in the split products indicates that the preparation contained exoglycosidases that cleaved these from HGM. Finally, recovery of *N*-acetylgalactosamine and no unacetylated hexosamines in the split products suggests that the A-degrading enzyme was a *N*-acetylhexosaminidase and not a deacetylase that cleaves the acetyl group from the A antigenic determinant α-*N*-acetylgalactosamine (17).

It is likely that BGD enzyme production by certain strains of fecal bacteria is related to bacterial degradation of the host's gastrointestinal mucins. Gut mucins with ABH blood group activity are resistant to their host's digestive tract glycosidases (18, 37), but their carbohydrate moieties are degraded extensively by intestinal bacteria (19). The saccharides in gut mucin glycoproteins are a potential source of nutrition for enteric bacteria providing that the latter can produce the specific glycosidases required for hydrolytic cleavage of the various glycoside linkages in the oligosaccharide side chains. The A-, B-, and H-BGD enzymes presumably are essential for the initial step in the degradation of oligosaccharide side chains with A, B, or H antigen specificity. Their elaboration as extracellular enzymes into the bacterial microenvironment should provide other bacteria lacking this enzymatic capability with nutrient monosaccharides from mucin glycoproteins.

We earlier reported (20) that the host's blood type and secretor status influenced the specificity of A-, B-, and H-degrading activities in feces. The present studies indicate that these genetic traits also influence the specificity of BGD enzymes produced in anaerobic fecal cultures. These effects presumably represent prior adaptation of the host's microflora to the blood group antigens secreted in his gut mucins. In the case of blood type A and type B secretors this adaptation is apparently transferred to anaerobic fecal cultures.

The stimulating effect of commercial HGM on BGD enzyme production provides a means of obtaining enhanced levels of these enzymes in anaerobic fecal cultures. With fecal inocula selected from a group A or group B secretor, culture supernates with high levels of either A- or B-degrading enzyme activities may be obtained and can be used as a starting point for enzyme purification.

The mechanism by which HGM and salivary glycoproteins enhance BGD enzyme production is unclear and requires further study. Reports in the literature differ concerning whether or not blood group substances enhance BGD enzyme production by pure cultures of bacteria. Stack and Morgan (33) found that A- and H-degrading activities produced by several strains of *Cl. perfringens* type B were not enhanced by the addition of HGM to a simplified culture medium. On the other hand, Howe et al. (38) found that the A-degrading activity produced by the Iseki strain of *Cl. tertium,* subsequently shown to be a deacetylase (17), was enhanced by the addition of either HGM, glucosamine, or *N*-acetylglucosamine to the culture medium. Glycoprotein substrates are capable of inducing glycosidase synthesis in certain bacteria. Barker et al. (39-41) showed that glycosidases were sequentially induced in a strain of *Klebsiella aerogenes* during its degradation of the oligosaccharide side chains of urinary orosomucoid and pneumococcal type XIV polysaccharide. But our observation that HGM and salivary glycoproteins enhance production of A-, B-, and H-degrading enzymes when the enzyme's antigenic determinant substrate is not present in the glycoprotein is inconsistent with the concept that substrate-induced enzyme synthesis is the principal mode of BGD enzyme adaptation by communities of fecal bacteria in vitro. In addition, the capacity to produce BGD enzymes appears to be restricted largely to certain anaerobic bacteria among many others in feces. These observations suggest an alternative mode of BGD enzyme adaptation by bacterial communities in the human colon: a populational adaptation of constitutive mutant strains that, by virtue of producing BGD enzymes specific for their host's blood group phenotype antigen, possess a selective advantage in their host habitat. In the following paper we present evidence supporting this mechanism in blood group B secretors.

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REFERENCES

1. Schiff, F., and M. Akune. 1931. Blutgruppen und Physiologie. *Muench. Med. Wochenschr.* **78**: 657–660.
2. Schiff, F., and G. Weiler. 1931. Fermente und Blutgruppen. I. *Biochem. Z.* **235**: 454–465.
3. Schiff, F., and G. Weiler. 1931. Fermente und Blutgruppen. II. *Biochem. Z.* **239**: 489–492.
4. Witebsky, E., and T. Satoh. 1933. Zur Frage des Blutgruppen-fermentes und der Ausscheidung von Blutgruppensubstanz. *Klin. Wochenschr.* **12**: 948–949.
5. Schiff, F., and F. A. Burón. 1935. Zur Kenntnis der sogenannten Blutgruppenfermente. *Klin. Wochenschr.* **14**: 710–712.
6. Eisler, M. 1932. Weitere Versuche über das Verhalten von Antigenen gegenüber verschiedenen Stuhlextrakten. *Z. Immunitaetsforsch.* **77**: 472–476.
7. Iseki, S. 1970. Glycosidases and serological changes in blood group substances. *In* Blood and Tissue Antigens. D. Aminoff, editor. Academic Press, Inc., New York. 379–394.
8. Aminoff, D., and K. Furukawa. 1970. Enzymes that destroy blood group specificity. I. Purification and properties of a α-L-fucosidase from *Clostridium perfringens*. *J. Biol. Chem.* **245**: 1659–1669.
9. György, P., C. S. Rose, and G. F. Springer. 1954. Enzymatic inactivation of Bifidus Factor and blood group substances. *J. Lab. Clin. Med.* **43**: 543–552.
10. Kabat, E. A. 1956. Blood group substances. Their chemistry and immunochemistry. Academic Press, Inc., New York. 88.
11. Watkins, W. M. 1959. Enzymes of *Trichomonas foetus*. The action of cell-free extracts on blood-group substances and low-molecular-weight glycosides. *Biochem. J.* **71**: 261–274.
12. Zarnitz, M. L., and E. A. Kabat. 1960. Immunochemical studies of blood groups. XXV. The action of coffee bean α-galactosidase on blood group B and BP1 substances. *J. Am. Chem. Soc.* **82**: 3953–3957.
13. Bahl, O. P. 1970. Glysosidases of *Aspergillus niger*. II. Purification and general properties of 1,2-α-L-fucosidase. *J. Biol. Chem.* **245**: 299–304.
14. Howe, C., and E. A. Kabat. 1953. Immunochemical studies on blood groups. XIII. Action of enzyme from snail (Busycon) liver on blood group A and O(H) substances (hog). *J. Am. Chem. Soc.* **75**: 5542–5547.
15. Tuppy, H., and W. L. Staudenbauer. 1966. The action on soluble blood group A substances of an α-*N*-acetylgalactosaminidase from *Helix pomatia*. *Biochemistry.* **5**: 1742–1747.
16. Weissmann, B., and D. F. Hinrichsen. 1969. Mammalian α-acetylgalactosaminidase. Occurrence, partial purification, and action on linkages in submaxillary mucins. *Biochemistry.* **8**: 2034–2043.
17. Marcus, D. M., E. A. Kabat, and G. Schiffman. 1964. 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. *Biochemistry.* **3**: 437–443.
18. Hoskins, L. C. 1968. Bacterial degradation of gastrointestinal mucins. II. Bacterial origin of fecal ABH (O) blood group antigen-destroying enzymes. *Gastroenterology.* **54**: 218–224.
19. Hoskins, L. C., and N. Zamcheck. 1968. Bacterial degradation of gastrointestinal mucins. I. Comparison of mucus constituents in the stools of germ-free and convential rats. *Gastroenterology.* **54**: 210–217.
20. Hoskins, L. C. 1969. Ecological studies of intestinal bacteria. Relation between the specificity of fecal ABO blood group antigen-degrading enzymes from enteric bacteria and the ABO blood group of the human host. *J. Clin. Invest.* **48**: 664–673.
21. Hoskins, L. C., and E. T. Boulding. 1976. Degradation of blood group antigens in human colon ecosystems. II. A gene interaction in man that affects the fecal population density of certain bacteria. *J. Clin. Invest.* **57**: 74–82.
22. Marcus, D. M., and A. P. Grollman. 1966. Studies of blood group substances. I. Caprine precipitating antisera to human Le^a and Le^b blood group substances. *J. Immunol.* **97**: 867–875.
23. Matsumoto, I., and T. Osawa. 1969. Purification and characterization of an anti-H(O) phytohemagglutinin of *Ulex europeus*. *Biochim. Biophys. Acta.* **194**: 180.
24. Layne, E. 1957. Spectrophotometric and turbidometric methods for measuring proteins. *Methods Enzymol.* **3**: 447–454.
25. Kornberg, A. 1955. Lactic dehydrogenase of muscle. *Methods Enzymol.* **1**: 441–443.
26. Meisler, M. 1972. β-Galactosidase from human liver. *Methods Enzymol.* **28**: 820–824.
27. Spiro, R. G. 1966. Analysis of sugars found in glycoproteins. *Methods Enzymol.* **8**: 3–26.
28. Wheat, R. W. 1966. Analysis of hexosamines in bacterial polysaccharides by chromatographic procedures. *Methods Enzymol.* **8**: 60–85.
29. Tomarelli, R. M., J. Charney, and M. L. Harding. 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. *J. Lab. Clin. Med.* **34**: 428–433.
30. Aminoff, D., and K. Furukawa. 1970. Stereo-specificity of glycosidases that act on blood group substances. *In* Blood and Tissue Antigens. D. Aminoff, editor. Academic Press, Inc., New York. 395–413.
31. Moore, W. E. C., and L. V. Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl. Microbiol.* **27**: 961–979.
32. Cohn, M., and K. Horibata. 1959. Physiology of the inhibition by glucose of the induced synthesis of the β-galactosidase-enzyme system of *Escherichia coli*. *J. Bacteriol.* **78**: 624–635.
33. Stack, M. V., and W. T. J. Morgan. 1949. The preparation and properties of enzymes from *Cl. welchii* (type B) filtrates which destroy blood-group substances. *Br. J. Exp. Pathol.* **30**: 470–483.
34. Schiff, F. 1935. Über den Abbau gruppenspezifischer Substanzen durch Bakterien. *Klin. Wochenschr.* **14**: 750–751.
35. Kostuch, Z. 1938. Ferments anti-groupaux du *Bacillus perfringens*. *C. R. Seances Soc. Biol.* **129**: 352–354.
36. Watkins, W. M. 1972. Blood-group specific substances. *In* Glycoproteins, Their Composition, Structure and Function. A. Gottschalk, editor. Elsevier Scientific Publishing Company, Amsterdam. 830–891.

37. Hsu, L., and Tappel, A. L. 1965. Lysosomal enzymes and mucopolysaccharides in the gastrointestinal tract of the rat and pig. *Biochem. Biophys. Acta.* **101**: 83–89.

38. Howe, C., J. D. MacLennan, I. Mandl, and E. A. Kabat. 1957. Enzymes of *Clostridium tertium*. Effects on blood group and virus receptor substances. *J. Bacteriol.* **74**: 365–379.

39. Barker, S. A., G. I. Pardoe, M. Stacey, and J. W. Hopton. 1963. Sequential enzyme induction: a new approach to the structure of complex mucoproteins. *Nature* (*Lond.*). **197**: 231–233.

40. Barker, S. A., G. I. Pardoe, M. Stacey, and J. W. Hopton. 1964. The fine structure of the carbohydrate moiety of urinary orosomucoid. *Protides Biol. Fluids Proc. Colloq. Bruges.* **11**: 284–287.

41. Barker, S. A., G. I. Pardoe, M. Stacey, and J. W. Hopton. 1964. Degradation of type XIV *Pneumococcus* polysaccharide by induced enzymes. *Nature* (*Lond.*) **204**: 938–939.