Antibodies Detectable by Counterimmunoelectrophoresis Against *Bacteroides* Antigens in Serum of Patients with Inflammatory Bowel Disease

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Heat-extracted antigens from seven species of *Bacteroides* were used in passive hemagglutination and counterimmunoelectrophoretic tests. Sera from 87 normal persons (group I) and 15 patients with ulcerative colitis (group II) were of low and equal reactivity in passive hemagglutination tests; all positive tests were eliminated by 2-mercaptoethanol reduction of the sera. When these same sera were tested by counterimmunoelectrophoresis with six of the *Bacteroides* antigens, no significant difference in the percentage of positive reactions was noted. However, using the chi-square test, the seventh antigen, prepared from *Bacteroides vulgatus*, successfully distinguished the two populations at the 0.025 level. Counterimmunoelectrophoretic tests with the *B. vulgatus* antigen also provided a means to separate the patients in group II with active disease from those in remission at a *P* value of 0.01. All the sera from 12 patients with defined Crohn's disease activity indexes reacted with the *B. vulgatus* antigen in counterimmunoelectrophoretic tests. Reduction and alkylation of patient sera with 2-mercaptoethanol and iodoacetamide removed detectable antibody in 78% of the samples, which suggested a dominant role of immunoglobulin M in the response to *Bacteroides* antigens.

Antibodies to components of the normal human flora are usually present in human sera, though in low quantities, and often go undetected unless sensitive serological techniques such as complement fixation, passive hemagglutination (PHA), or radioimmunoassay are used (5, 18). Such antibodies may be increased in quantity as the result of diseases in which the normal flora serve as opportunistic pathogens or as "bystander" flora which gain a firmer exposure to the host's immune system when its barriers are removed by other pathogens or by physiological disturbances (8, 9).

The etiology of ulcerative colitis and Crohn's disease is unknown (21), but both conditions cause a profound disturbance in the structural integrity of the colon or other portions of the alimentary canal. It is thus surprising that antibody levels to *Escherichia coli* and enterococcus species remain normal or are only modestly elevated in these conditions though increased serum concentrations of anti-*Bacteroides* globulins are detected by radioimmunoassay (5, 6). Fluorescent antibody assays segregate the anti-

bodies formed by patients with ulcerative colitis against normal components of the intestinal flora into an interesting pattern (17). In both the patient and noncolitic control group sera, antibodies were found to react with bacteria of the aerobic gut flora, yet antibodies of the intestinal mucosa of the patient group were directed only against the anaerobic and not the aerobic flora.

Our own study of serum antibodies in the sera of patients with inflammatory bowel disease has been reported in abstract form (C. J. Helphingstine, D. J. Hentges, and J. Butt., Fed. Proc. 36: 1311, 1977). It is described here in additional detail with a study of serum antibodies from another important group of patients with Crohn's disease.

MATERIALS AND METHODS

Cultures. Stock cultures of *Bacteroides fragilis* C1, *B. fragilis* 23745, *Bacteroides distasonis* 8503, *Bacteroides vulgatus* An-1, *Bacteroides ovatus* An-26, *Bacteroides thetaiotaomicron* An-21, and *Bacteroides melaninogenicus* Nf-6 were grown in peptone-yeast broth and perpetuated in an anaerobic glovebox isolator similar to the one described by Aranki and Freter (1). Colonies arising from transfers from stock cultures to prerduced *Brucella* blood agar supple-

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mented with hemin, cysteine, and menadione (12) were periodically examined for Gram-staining reactions, fermentation and other biochemical reactions, and volatile fatty acid production to reconfirm their species identity (12, 22).

Antigens. Bacteria used for the immunization of rabbits were harvested from 48- to 72-h cultures on *Brucella* blood agar plates. The cells were removed from the plates and washed twice in saline before being resuspended in 0.5% Formalinized saline. After 24 h at 4°C the cell density was adjusted to approximately 10^9 cells per ml to use for immunization. To prepare the soluble heat-stable antigen, 0.5 ml of packed cells, rewashed twice in saline, was resuspended in 10 ml of 0.01 M phosphate-buffered saline (pH 7.0) and held in a boiling-water bath for 1 h. The solution was clarified by centrifugation, and the supernatant fluid was stored at -20°C until needed.

Each lot of heat-stable antigen was analyzed for its carbohydrate content by the orcinol procedure (24) and for protein content by the Folin phenol method (15).

Serological tests. PHA tests utilized human blood group O, Rh-negative erythrocytes, which were washed three times in 0.1 M phosphate-buffered saline (pH 7.0) by centrifugation for 10 min at $2,500 \times g$ and resuspension. A mixture of 1.2 ml of heat-stable antigen, at a concentration of 0.1 mg of carbohydrate per ml, and 2.0 ml of the erythrocytes in phosphate-buffered saline at a concentration of 20% was held for 1 h at 37°C with occasional mixing. Thereafter, the cells were washed three times in 5 ml of phosphate-buffered saline and adjusted to a 0.25% suspension. These cells, in 1% normal rabbit serum in phosphate-buffered saline, were used in hemagglutination assays conducted with patient sera and using microtiter equipment (Cooke Engineering Co., Alexandria, Va.) essentially as described by Takatsky (23).

Sera were tested for their content of precipitating antibodies by the counterimmunoelectrophoresis (CIEP) procedure (19) using prepared gel plates and

the REC-300-C electrophoresis unit (Cordis Corp., Miami, Fla.), with 40 μ l of serum and 20 μ l of antigen. All antigens were adjusted to contain 0.1 mg of carbohydrate per ml, except for the *B. thetaiotaomicron* preparation, which contained 0.05 mg/ml. After the reservoirs were filled, the plates were wicked to a pH 8.2 barbitol buffer, ionic strength 0.05, and were exposed to a direct current of 15 mA per plate for 1 h. Each plate was examined immediately and after one day at 4°C for precipitation bands with the aid of a dark-background viewer. Positive controls consisting of antigen and hyperimmune rabbit serum were included with each experiment (Fig. 1).

The concentrations of immunoglobulin G (IgG), IgA, and IgM in the sera of 13 patients with inflammatory bowel disease were determined by single radial immunodiffusion (16). Reagents from three separate commercial firms were used according to the manufacturer's directions.

Sera. The 93 normal human serum samples used in this study, referred to as group I, were collected from persons who fell within the normal limits on 12 separate blood chemistry determinations. The 15 patients from the University of Missouri Medical Center diagnosed to have either Crohn's disease or ulcerative colitis comprised group II. This group contributed 27 samples. Twenty-five sera from 12 patients with Crohn's disease were obtained from the patient pool of the National Cooperative Crohn's Disease Study, Denver, Colo.

Equal volumes of selected sera and a 0.2 M solution of 2-mercaptoethanol in 0.2 M phosphate buffer at pH 7.2 were held for 1 h at room temperature to dissociate IgM in the serum samples (2). Thereafter, 1 volume of 0.22 M iodoacetamide in the phosphate buffer was added. Since this resulted in a threefold dilution of the serum sample, it was applied only to those sera with serological titers greater than 1:3.

RESULTS

The carbohydrate and protein contents of the

FIG. 1. A portion of a counterimmunoelectropherogram showing (from left to right) a negative test, a positive test with a patient's serum, and a strong positive test with a hyperimmune rabbit serum against the *B. vulgatus* antigen.

heat-stable antigen preparations from the several *Bacteroides* species are shown in Table 1. There was a remarkable constancy in the carbohydrate content of these preparations, most of which ranged from 0.110 to 0.240 mg/ml. Unexpected was the finding that these preparations, though boiled for 1 h, had protein contents ranging from 0.120 to 0.530 mg/ml.

The PHA titers presented in Table 2 were obtained by calculating the geometric mean titers and then expressing them as arithmetic titers. Four antigens from three *Bacteroides* species were tested against the sera from the 86 normal persons in group I and 27 serum samples from the 15 patients in group II. This serological test did not clearly distinguish the normal population from the population with inflammatory bowel disease (group II).

CIEP (Fig. 1) testing of the human serum samples produced characteristic precipitin bands that were much fainter than the reaction produced by hyperimmunized rabbit serum. Efforts to improve the density of these precipitates or to visualize additional lines of precipitations by alcohol precipitation of immune complexes were not successful. This method was applied to all sera in group II previously examined by PHA testing and to most of the sera from the normal population. The results (Table 3) indicated that there was a slight increase in the percentage of positive tests with the two *B. fragilis* strains when comparing the patient group with the

norms, but this difference was statistically insignificant. The difference noted with the *B. vulgatus* antigen, however, was significant at the level $P \leq 0.025$. If the sera of the patients in group II that were taken during periods of inactive disease were not used in the comparative analysis, the difference was more significant and could be distinguished from the norms at the level $P = 0.005$ (chi-square analysis).

The reaction of each serum sample from each individual in group II is recorded in Table 4 with the notation of the disease status of the patient. It can be noted that there is little correlation of positive results between the *B. vulgatus* antigen and *B. fragilis* and *B. distasonis* antigens. One serum (T.B., A) produced a negative reaction with *B. vulgatus* antigen and a positive reaction with *B. distasonis* antigen. Four of the six positive *B. distasonis* reactions were negative with *B. fragilis* antigen, and two were positive. Patients with active disease could be distinguished from those in remission on the basis of the reaction of their serum with *B. vulgatus* antigen ($P \leq 0.01$).

When the first serum samples available from the patients in group II were evaluated for a positive or negative result with the *B. vulgatus* antigen, 11 of the 15 patients (73.3%) produced a positive test (Table 4). When all serum samples were analyzed, 16 of 27 sera (59%) were positive (Table 3).

Quantitation of IgG, IgM, and IgA in the sera of 13 of the 15 patients with inflammatory bowel disease in group II revealed (Table 5) a striking elevation of serum IgA levels. Seven of the 13 sera were noted to exceed the normal serum level of 170 to 280 mg/ml for this immunoglobulin. One patient (M.F.) was hypergammaglob-

TABLE 1. Polysaccharide and protein content of heat-stable antigens

Organism	Poly-saccharide (mg/ml)	Protein (mg/ml)
<i>B. fragilis</i> C1	0.110	0.120
<i>B. fragilis</i> 23745	0.150	0.215
<i>B. vulgatus</i> An-1	0.135	0.300
<i>B. distasonis</i> 8503	0.240	0.530
<i>B. ovatus</i> An-26	0.130	0.260
<i>B. melaninogenicus</i> Nf-6	0.145	0.200
<i>B. thetaiotaomicron</i> An-21	0.050	0.315

TABLE 2. PHA titers of sera from a normal population (group I) and patients with inflammatory bowel disease (group II)

Organism	Normal: group I (86) ^a	Patients: group II (27) ^a
<i>B. fragilis</i> C1	1:14	1:20
<i>B. fragilis</i> 23745	1:6	1:6
<i>B. vulgatus</i> An-1	<1:4	1:8
<i>B. distasonis</i> 8503	1:9	1:8

^a Parentheses indicate number of sera tested.

TABLE 3. CIEP tests with sera from a normal population (group I) and patients with inflammatory bowel disease (group II)

Organism	Normal: group I, positive/ tested (%)	Patients: group II, positive/ tested (%)
<i>B. fragilis</i> C1	2/93 (2.1)	2/27 (7.4)
<i>B. fragilis</i> 23745	3/93 (3.2)	2/27 (7.4)
<i>B. vulgatus</i> An-1	29/93 (32)	16/27 (59) ^a
<i>B. distasonis</i> 8503	21/93 (22.6)	6/27 (22.2)
<i>B. ovatus</i> An-26	0/25 (0)	0/27 (0)
<i>B. melaninogenicus</i> Nf-6	0/26 (0)	0/27 (0)
<i>B. thetaiotaomicron</i> An-21	0/25 (0)	0/27 (0)

^a Statistically significant increase of positive results in the patient group ($P \leq 0.025$ to 0.01) above the normal population. If only patients with active disease in group II are considered, $P \leq 0.005$.

ulinemic in regard to all three immunoglobulins. One additional patient (R.We.) had an increase in IgG, and a different individual (L.S.) had an increase in IgM.

A further examination was then undertaken of sera from 12 patients with Crohn's disease, who had been regularly monitored for the status of their disease and assigned a Crohn's disease

TABLE 4. CIEP testing of sera from patients in group II with inflammatory bowel disease

Patient	Status ^a	<i>B. vul-</i> <i>gatus</i> ^b An-1	<i>B. fra-</i> <i>gilis</i> C1	<i>B. fra-</i> <i>gilis</i> 23745	<i>B. dis-</i> <i>tasonis</i> 8503
T.B.	A	—	—	—	+
	R	—	—	—	—
B.B.	R	+	—	—	+
	R	—	—	—	—
M.F.	R	+	—	+	—
	R	—	—	—	—
J.H.	A	+	—	—	—
	R	—	—	—	—
P.H.	A	+	—	—	+
	R	—	—	—	—
P.K.	R	—	—	—	—
	R	—	—	—	—
M.L.	A	+	—	—	+
N.O.	A	+	—	—	—
	A	+	—	—	—
M.Ri.	A	—	—	—	—
	A	—	—	—	—
M.Ro.	A	+	—	—	—
J.S.	A	+	—	—	—
	A	+	—	—	—
L.S.	R	—	—	—	—
	R	+	—	—	—
D.S.	A	+	—	+	+
	A	+	—	—	—
R.We.	R	+	—	—	—
	A	+	+	—	—
R.Wi.	A	+	+	—	+
	A	+	—	—	—
Summary ^c		16/27 ^b	2/27	2/27	6/27

^a Active disease (A) or in remission (R).

^b A statistically significant difference between sera from patients with active and inactive disease exists for this antigen at a level of $P \leq 0.01$.

^c Sera positive/sera tested.

TABLE 5. Serum immunoglobulin levels in patients in group II with inflammatory bowel disease

Patient	IgG (mg/100 ml)	IgM (mg/100 ml)	IgA (mg/100 ml)
M.R.	1,100	75	170
B.B.	800	40	110
D.S.	800	75	>400 ^a
R.We.	2,000 ^a	160	170
P.H.	1,100	75	170
R.Wi.	1,350	75	>400 ^a
N.O.	1,100	75	370 ^a
T.B.	800	50	90
M.F.	1,700 ^a	240	>400 ^a
P.K.	900	110	>400 ^a
M.L.	1,350	160	>400 ^a
J.H.	110	160	370 ^a
L.S.	1,100	240 ^a	200

^a Denotes elevated value.

activity index. This is a numerical index based on objective criteria established (4) for classifying the status of Crohn's disease patients. The results of the CIEP tests with the *B. vulgatus* antigen and the sera from these patients are recorded in Table 6. When tested before dilution, all sera gave positive results with this antigen. Of the 25 sera, 17 were positive at a 1:2 dilution and 14 at a 1:4 dilution, and the latter could withstand the dilution necessary for reductive alkylation with 2-mercaptoethanol. Only three remained positive after this treatment, indicating a dominance of IgM in the remaining 11 serum samples. A similar reduction of the sera from the patients with inflammatory bowel disease (Table 4) removed the precipitating antibody from 6 of the 11 sera tested.

DISCUSSION

These results indicate that heat extracts of whole *Bacteroides* cells contain antigens suitable for conducting CIEP and PHA tests with human sera; however, the PHA test was not capable of distinguishing a normal population from one that had inflammatory bowel disease. This is reminiscent of the recent description of the inability of PHA tests to detect a rise in antibody level in persons colonized with *E. coli*, although a radioimmunoassay was effective for this purpose (13).

CIEP testing with the *B. vulgatus* antigen distinguished between the normal population (group I) and the patients with inflammatory bowel disease (group II) at the highly significant level of $P \leq 0.025$ and, more importantly, separated patients with active disease from the norms at a level of $P \leq 0.005$. Group II patients with active disease could be distinguished from those in a state of remission at a level of $P \leq$

TABLE 6. CIEP testing of sera from patients with Crohn's disease

Patient	Date of serum sample	Duration of disease (months)	CDAI ^a	CIEP test			
				Undiluted	1:2	1:4	2-ME ^b
P.B.	3/73	12	213	+	+	+	—
	3/75		95	+	+	+	—
R.A.	9/74	72	193	+	—	—	
	2/75		—47	+	—	—	
D.H.	3/74	1	236	+	+	+	—
	4/75		26	+	+	+	—
F.M.	2/74	3	256	+	—	—	
	6/74		140	+	—	—	
C.A.	10/72	1	279	+	—	—	
	1/73		497	+	+	—	+
M.O.	8/73	1	185	+	+	—	
	8/75		80	+	+	+	—
J.P.	10/73	22	181	+	+	+	—
	10/75		12	+	+	+	—
J.D.	2/74	8	228	+	—	—	
	2/76		94	+	+	+	—
R.S.	1/75	1	264	+	+	+	—
	2/75		486	+	+	+	+
J.W.	5/75	2	324	+	+	+	—
	6/75		439	+	—	—	
W.S.	5/74	190	126	+	+	+	—
	5/74		333	+	+	+	+
V.M.	9/74	14	213	+	+	—	
	1/74		50	+	+	+	—
	6/75		179	+	—	—	

^a Crohn's disease activity index.^b CIEP test result after reduction of sera with 2-mercaptoethanol (2-ME).

0.01. Approximately half of the group II patients (7/13) had elevated serum IgA levels.

All sera from 12 patients with Crohn's disease were positive in CIEP tests with the *B. vulgatus* antigen. This further substantiates the value of CIEP testing to distinguish a normal population from one with inflammatory bowel disease. Since sera from patients with both high and low Crohn's disease activity index values produced positive tests with the *B. vulgatus* antigen, it was not possible to correlate these values with positive CIEP tests. Retesting the 14 positive sera that could withstand the dilution necessary for 2-mercaptoethanol reduction revealed that 78% of the samples became negative.

The patients studied tended to have increased serum levels of IgA, but the ease by which 2-mercaptoethanol eliminated the precipitating

activity of the sera indicated a strong reliance of positive tests on immunoglobulins of the IgM class. It is well known that polysaccharide antigens tend to favor the IgM response. Although the antigen used contained proteins or peptides as determined by the Folin phenol method, polyacrylamide gel electrophoresis (C. Helphingsstine, Ph.D. thesis, University of Missouri, Columbia, 1977) indicated a relative paucity of proteins with discrete electrophoretic mobility. The antigen preparation also contained components which adsorbed directly to erythrocytes, a characteristic of polysaccharides, lipids, and conjugates with these biopolymers (14).

Since the diagnosis of ulcerative colitis and Crohn's disease represents a synthesis of several subjective and objective criteria (4), a simple diagnostic acid would be welcome. Further ex-

perience with the CIEP test using a more purified *B. vulgatus* antigen preparation may improve the diagnostic and prognostic utility of CIEP testing for inflammatory bowel disease. Sera from patients with other forms of bowel disease, such as colonic cancer or diverticulitis, should also be examined with the CIEP test to determine if the test would be useful in the differential diagnosis of these conditions.

None of the evidence presented here should be used as proof that *B. vulgatus* has an etiological role in ulcerative colitis or Crohn's disease. Cooke (7) found both quantitative and qualitative similarities in the bacterial flora of patients with ulcerative colitis and healthy individuals, although Seneca and Henderson (20) noted that patients with colitis tended to have higher total fecal bacterial counts. An overpopulation of the small bowel in Crohn's disease has been reported (3, 11), but these studies did not incriminate *Bacteroides* species as causative agents. From these experiments, the most logical explanation of the elevated antibody levels to *B. vulgatus* is that the disruption of the cellular integrity of the gut provides a more potent and continuous exposure to these bacteria than exists in the healthy bowel. The question why other ubiquitous members of the intestinal flora are not equally proficient in stimulating the immune response of their host is still perplexing. It may be answerable when further information on the chemistry of the antigens is available and when the immune status of these unique patients is more sharply defined.

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LITERATURE CITED

- Aranki, A., and R. Freter. 1972. Use of anaerobic gloveboxes for the cultivation of strictly anaerobic bacteria. *Am. J. Clin. Nutr.* 25:1329-1334.
- Banatvala, J. E., J. M. Best, E. A. Kennedy, E. E. Smith, and M. E. Spence. 1967. A serologic method for demonstrating recent infection by rubella virus. *Br. Med. J.* 3:285-286.
- Beeken, W. L., and R. E. Kanich. 1973. Microbial flora of the upper small bowel in Crohn's disease. *Gastroenterology* 65:390-397.
- Best, W. R., J. B. Beckett, J. W. Singleton, and F. Kern. 1976. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 70:439-444.
- Brown, W. R., and E. M. Lee. 1973. Radioimmunologic measurement of naturally occurring bacterial antibodies. I. Human serum antibodies reactive with *Escherichia coli* in gastrointestinal and immunological disorders. *J. Lab. Clin. Med.* 82:125-136.
- Brown, W. R., and E. Lee. 1974. Radioimmunological measurement of bacterial antibodies. II. Human serum antibodies reactive with *Bacteroides fragilis* and *Enterococcus* in gastrointestinal and immunological disorders. *Gastroenterology* 66:1145-1153.
- Cooke, E. M. 1967. A quantitative comparison of the faecal flora of patients with ulcerative colitis and that of normal persons. *J. Pathol. Bacteriol.* 94:439-444.
- Danielsson, D., D. W. Lambe, Jr., and S. Persson. 1972. The immune response in a patient to an infection with *Bacteroides fragilis* ss. *fragilis* and *Clostridium difficile*. *Acta Pathol. Microbiol. Scand. Sect. B* 80:709-712.
- Danielsson, D., D. W. Lambe, Jr., and S. Persson. 1974. Immune response to anaerobic infections, p. 173-191. In A. Balows, R. M. DeHaan, and L. B. Guze (ed.), *Anaerobic bacteria: role in disease*. International Conference on Anaerobic Bacteria, Center for Disease Control. Charles C Thomas Publisher, Springfield, Ill.
- Finegold, S. M. 1977. *Anaerobic bacteria in human disease*. Academic Press, New York.
- Gorbach, S. L., L. Nahas, A. G. Plaut, L. Weinstein, J. Patterson, and R. Levitan. 1968. Studies of intestinal microflora. V. Fecal microbial ecology in ulcerative colitis and regional enteritis: relationship to severity of disease and chemotherapy. *Gastroenterology* 54:575-587.
- Holdeman, L. V., and W. E. C. Moore (ed.). 1975. *Anaerobe laboratory manual*, 3rd ed. Virginia Polytechnic Institute and State University, Blacksburg.
- Jodal, U., S. Ahlstedt, L. A. Hanson, G. Lidin-Janson, and A. S. Åkerlund. 1977. Intestinal stimulation of the serum antibody response against *Escherichia coli* O83 antigen in healthy adults. *Int. Arch. Allergy Appl. Immunol.* 53:481-489.
- Keogh, E. V., E. A. North, and M. F. Warburton. 1948. Adsorption of bacterial polysaccharides to erythrocytes. *Nature (London)* 161:687-688.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2:253-254.
- Monteiro, E., J. Fossey, M. Shiner, B. S. Drasar, and A. C. Allison. 1971. Antibacterial antibodies in rectal and colonic mucosa in ulcerative colitis. *Lancet* i:249-251.
- Quick, J. D., H. S. Goldberg, and A. C. Sonnenwirth. 1972. Human antibody to *Bacteroidaceae*. *Am. J. Clin. Nutr.* 25:1351-1356.
- Rose, N. R., and P. E. Bigazzi. 1973. Methods in immunodiagnosis, p. 19-23. John Wiley and Sons, New York.
- Seneca, H., and E. Henderson. 1950. Normal intestinal bacteria in ulcerative colitis. *Gastroenterology* 15:34-39.
- Shorter, R. G., T. B. Tomasi, K. A. Huizenga, R. J. Spencer, and J. D. Stobo. 1976. The immunology of chronic ulcerative colitis and Crohn's disease. *Ann. N.Y. Acad. Sci.* 278:586-591.
- Sutter, V., H. Attebury, J. Rosenblatt, K. Bricknell, and S. Finegold. 1975. *Anaerobic bacteriology manual*. Anaerobic Bacteriology Laboratory, Wadsworth Hospital Center, Los Angeles.
- Takatsky, G. 1956. The use of spiral loops in serological and virological micromethods. *Acta Microbiol. Acad. Sci. Hung.* 3:191-202.
- Winzler, R. J. 1955. Determination of serum glycoproteins, p. 279-283. In D. Glick (ed.), *Methods of biochemical analysis*, vol. 2. Interscience Publishers, Inc., New York.