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Immunochemical Properties of Vi Antigen from *Salmonella typhi* Ty2: Presence of Two Antigenic Determinants

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Antigen Vi of *Salmonella typhi* was found to have at least two antigenic determinants. In one of them, *O*-acetyl moiety played a dominant role. The second antigenic determinant did not involve *O*-acetyl residues, but both carboxyl and *N*-acetyl groups were necessary for the antigen-antibody reaction. These results were obtained by performing serological tests with antigen Vi, with its structural analog, polygalacturonic acid, and with the derivatives of both polysaccharides.

The Vi antigen is a highly acidic polysaccharide produced by some members of *Enterobacteriaceae*. The Vi antigen of *Escherichia freundii* 5396/38 is composed of *O*- and *N*-acetylated galactosaminouronic acid units connected through α (1-4) linkages (1). The structure of this antigen produced by *Salmonella typhi* Ty2 is essentially the same (B. Lindberg, P. E. Jansson, B. Szewczyk, and A. Taylor, manuscript in preparation), the only difference being the lower *O*-acetylation degree. Though the structure of Vi antigen is established, the data concerning its immunochemical properties are not complete. Whiteside and Baker (14) compared Vi antigen preparations from different bacterial species and found that purified Vi antigen preparations from *Citrobacter ballerup, Salmonella typhi,* and *E. freundii* were serologically identical, though their acetyl content was not the same. However, after the *O*-deacetylation of Vi antigen, a serological difference between the antigen of *S. typhi* and *C. ballerup* was observed, indicating the possibility of minor structural differences in these antigens (15). Landy et al. (4) observed that *O*-deacetylated Vi antigen loses the serological properties of the native material, as it precipitates only about 20% of Vi antibodies and is 400 times less active than Vi antigen in hemagglutination inhibition tests with Vi-coated erythrocytes.

This paper presents evidence of the presence of at least two antibody types directed against different parts of the Vi antigen molecule. These antibodies were present in variable ratios in rabbit Vi antisera.

MATERIALS AND METHODS

Bacteria. *S. typhi* Ty2, obtained from the National Reference Laboratory for Enteric Phage Typing in Gdańsk, Poland, was grown on 1% agar (Oxoid) supplemented with 1% peptone and 0.35% NaCl.

Isolation and purification of antigen Vi. The procedure employed for the isolation and purification of Vi antigen was essentially the same as that described by A. Taylor (11) with the following modifications: (i) the step where acids were used was omitted; and (ii) the chromatography of the Vi antigen on diethylaminoethyl (DEAE)-Sepharose CL-6B was added as the final step of purification (elution with 0 to 2 M NaCl gradient in 0.05 M phosphate buffer, pH 6.8).

Chemicals. The polygalacturonic acid was from Serva Feinbiochemica, Heidelberg, West Germany. The *N*-acetyl D-galactosamine was from Sigma Chemical Co., St. Louis, Mo. The D-glucuronic acid and D-galacturonic acid were from Phanstiehl Laboratories Inc., Waukeegan, Ill.

Chemical modifications of antigen Vi. *O*-deacetylated antigen Vi (derivative I) was obtained by the overnight treatment of the native antigen with 0.25 N NaOH at room temperature. *O*- and *N*-deacetylated antigen Vi (derivative II) was obtained by using the procedure of Nilsson and Svensson (7). Carboxyl-reduced antigen Vi (derivative III) was prepared by the procedure of Taylor and Conrad (12). The procedure was repeated three times. To obtain reacetylated derivative I (derivative IV of antigen Vi), we dissolved 10 mg in formamide (20 ml), and a mixture (5 ml) of equal volumes of acetic anhydride and pyridine was added. The mixture was stirred for 3 h at room temperature and dialyzed, and the product was lyophilized (yield about 10 mg). The reacetylated derivative III (derivative V) was obtained by the same procedure.

Chemical modifications of polygalacturonic acid. Polygalacturonic acid was dialyzed extensively against distilled water to remove the material of lower molecular weight. Lyophilized polygalacturonic acid was dissolved in formamide (1-mg/ml solution). This solution was acylated either with a mixture of acetic acid anhydride-pyridine or with propionic acid anhydride-pyridine as described above for the acetylation of antigen Vi derivatives. The acetyl or propionyl derivatives of polygalacturonic acid were purified on a DEAE-Sephadex A-25 column (1.6 by 60 cm) using a gradient of 0 to 2 M NaCl (in 0.05 M phosphate buffer, pH 6.8) as an eluant.

Analytical methods. Determinations of *O*-acyl content were performed by the method of Snyder and Stephens (10). Acidic polysaccharides in eluates from columns were determined by the method of Webster et al. (13).

Antisera. Antisera against *S. typhi* 21802 and *C. ballerup* 107 (Vi antiserum) were obtained from the National Reference Laboratory for Enteric Phage Typing, Gdańsk, Poland.

Serological techniques. Quantitative precipitin assays were performed by adding increasing quantities of the antigen to a series of centrifuge tubes, each tube containing 0.1 ml of antiserum (2). The antibody protein in the precipitate was determined by the method of Lowry et al. (5). Inhibition of the immune precipitation was carried out by the method of Hungerer et al. (2).

Precipitin reactions in agarose gels were performed by the Ouchterlony technique (8). The dried gels were stained with a 0.5% solution of Coomassie brilliant blue in acetic acid-methanol-water (1:5:5, vol/vol) for 20 min at room temperature. The excess of the stain was removed by washing the gels with the same solution without Coomassie brilliant blue.

Indirect hemagglutination (HA) was performed with formolized sheep erythrocytes (SRBC). To sensitize SRBC with antigens, we added 50 μg of antigenic material to 10 ml of 1% washed SRBC suspension in saline. After incubation at 37°C for 30 min, the excess antigen was washed off and the sensitized SRBC were resuspended in saline to give a 1% suspension. The SRBC suspension was added to series of antiserum dilutions in microtiter plates as described previously (6).

To measure the inhibiting capacity of a substance, we also used the HA inhibition (HAI) test. Serial dilutions (0.2 ml) of the inhibitor were incubated at 37°C for 1 h with 0.2 ml of a serum dilution containing 2 HA units. Then 0.2 ml of the sensitized SRBC was added, and the plates were again incubated for the same time. The lowest inhibitor concentration giving a total inhibition of HA was recorded after 2 h at room temperature.

RESULTS

Characterization of rabbit antisera. The results of quantitative precipitation tests of native Vi antigen and *O*-deacetylated Vi antigen in various Vi antisera showed that the ratio of antibodies precipitated by these compounds was not constant. In each case Vi antigen precipitated more antibodies than *O*-deacetylated Vi antigen. The amount of antibodies precipitated by *O*-deacetylated antigen in relation to the native Vi antigen ranged from 0 to 65%. This observation led us to the assumption that Vi antisera contain at least two types of antibodies recognizing different parts of the Vi antigen molecule. For further studies of immunochemical properties of Vi antigen, we chose two antisera: unabsorbed *S. typhi* 21802 antiserum and *C. ballerup* 107 antiserum repeatedly absorbed with killed bacteria of the Vi^- strain of *C. ballerup* until the agglutination of these bacteria was no longer observed. The first of the antisera did not contain any antibodies against *O*-deacetylated Vi antigen (antiserum A), while in the second one (antiserum B), *O*-deacetylated Vi antigen precipitated about 65% of the total Vi antibodies.

Synthesis and physicochemical properties of derivatives of polygalacturonic acid. Polygalacturonic acid is a vegetal polysaccharide built up of galacturonic acid residues connected through α (1–4) linkages (9). Its molecular weight ranges from 10,000 to 50,000. When the structures of antigen Vi and polygalacturonic acid were compared, the following differences were observed: (i) antigen Vi is of a much higher molecular weight (ca. 10^6); (ii) antigen Vi has an *O*-acetyl residue on carbon-3 of the hexose ring; and (iii) antigen Vi has an *N*-acetyl residue on carbon-2 of the hexose ring instead of the OH group. Nevertheless, the similarity is significant, as it was found that *O*-acetylated polygalacturonic acid is a substrate for the enzyme of Vi phages, which specifically deacetylates antigen Vi (3).

O-acylated polygalacturonic acid was eluted from the DEAE-Sephadex column at a salt concentration equal to about 1 M. The degree of acylation of polygalacturonic acid and antigen Vi derivatives is given in Table 1.

Indirect HA test. Antigen Vi and polygalacturonic acid, as well as their derivatives, were tested in passive HA. It appeared that only native antigen Vi and 2,3-di-*O*-acetyl polygalacturonic acid were active in coating formolized SRBC. Their reciprocal titers with antiserum A were comparable—2,560 for the native antigen Vi and 1,280 for 2,3-di-*O*-acetyl polygalacturonic acid.

HAI tests. HAI tests were performed with antiserum A and antiserum B. Antisera were used in dilutions corresponding to 2 HA units.

TABLE 1. *Acylation degree of derivatives of antigen Vi and polygalacturonic acid*

Polysaccharide	Degree of polymerization	No. of acyl groups per monosaccharide unit
2,3-di-*O*-acetyl polygalacturonic acid	50–250	1.8
2,3-di-*O*-propionyl polygalacturonic acid	50–250	1.7
Native antigen Vi	5,000	0.4
Derivative IV of antigen Vi	ND[a]	0.2
Derivative V of antigen Vi	ND	0.2

[a] ND, Not determined.

Antigen Vi, polygalacturonic acid, and their derivatives were tested for their capacity to inhibit the hemagglutination of Vi-coated SRBC. The data of Table 2 clearly indicate that the inhibition is distinct only for *O*-acylated compounds irrespective of whether it is the derivative of antigen Vi or polygalacturonic acid.

Quantitative precipitation tests. As mentioned earlier, the choice of antisera was made on the basis of the ratio of antibodies precipitated by *O*-deacetylated antigen Vi and the native antigen Vi. Both of the antisera (antiserum A and antiserum B) were tested in quantitative precipitation tests with native antigen Vi, polygalacturonic acid, and their derivatives (Fig. 1 and 2). The following conclusions can be drawn from this set of experiments: (i) 2,3-di-*O*-acetyl polygalacturonic acid precipitates over 80% of Vi antibodies from antiserum A, but only about 30% from antiserum B; (ii) about 15 times more 2,3-di-*O*-acetyl polygalacturonic acid is necessary to precipitate the same amount of antibodies from antiserum B as from antiserum A; (iii) the precipitation of antibodies in antiserum A is observed only when a compound is *O*-acetylated; (iv) the exchange of *O*-acetyl to *O*-propionyl in the derivatives of polygalacturonic acid destroys the property to precipitate; and (v) *O*- and *N*-deacetylation of antigen Vi (derivative II), as well as *O*-deacetylation and carboxyl reduction of antigen Vi (derivative III), leads to about a 80% decrease of the amount of precipitated antibodies from antiserum B; *O*-reacetylation of the carboxyl reduced antigen Vi restores the serological reactivity in antiserum A (to about 60% of that of the native antigen Vi).

TABLE 2. *Serological activity of antigen Vi, polygalacturonic acid, and their derivatives in the HAI*[a]

Polysaccharide	Inhibitory amt in HAI (μg/ml)		Ratio of inhibitory amt in HAI of the given polysaccharide/antigen Vi	
	Antiserum A	Antiserum B	Antiserum A	Antiserum B
Native Vi	0.2	0.1		
Derivative I of Vi	—[b]	—		
Derivative II of Vi	—	—		
Derivative III of Vi	—	—		
Derivative IV of Vi	6.4	6.4	32	64
Derivative V of Vi	6.4	102.4	32	1,024
Polygalacturonic acid	—	—		
2,3-di-*O*-acetyl polygalacturonic acid	0.4	3.2	2	32
2,3-di-*O*-propionyl-polygalacturonic acid	25.6	25.6	128	256

[a] The maximum concentration of antigen in HAI was 1 mg/ml.

[b] —, No inhibition.

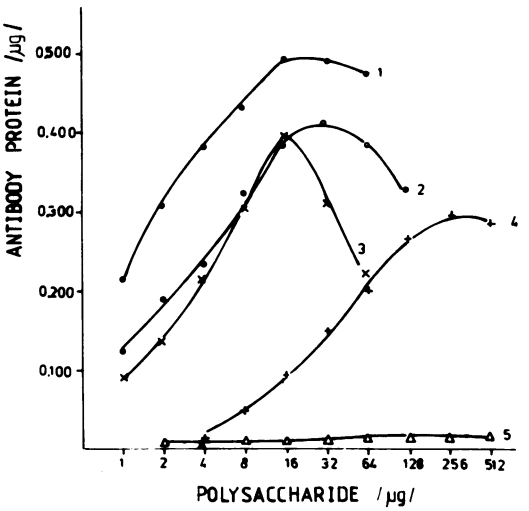

FIG. 1. *Quantitative precipitation curves for the native Vi antigen, 2,3-di-O-acetyl polygalacturonic acid, and their derivatives reacting in antiserum A (100 μl). (1) Native Vi antigen; (2) 2,3-di-O-acetyl polygalacturonic acid; (3) Vi antigen that was first O-deacetylated and next reacetylated (derivative IV); (4) Vi antigen that was first carboxyl reduced and next reacetylated (derivative V); (5) O-deacetylated Vi antigen (derivative I) and O- and N-deacetylated Vi antigen (derivative II) that were carboxyl reduced and O-deacetylated Vi antigen (derivative III), 2,3-di-O-propionyl polygalacturonic acid, and polygalacturonic acid.*

Inhibition of immune precipitation. Studies of the inhibition of the antigen Vi precipitation in antiserum A and the inhibition of *O*-deacetylated antigen Vi precipitation in antiserum B further confirmed the results of quantitative precipitation tests and HAI tests. As concluded from Fig. 3 and 4, the *O*-propionyl derivative of polygalacturonic acid is the inhibitor of precipitation in both antisera, while polygalacturonic acid itself inhibits only the precipitation of the native antigen Vi in antiserum B. *O*-deacetylated antigen Vi, which precipitates antibodies in antiserum B, is not an inhibitor of the native antigen Vi precipitation in antiserum A. Not only galacturonic acid but also glucuronic acid inhibit the precipitation of the antibodies against *O*-deacetylated antigen Vi in antiserum B, which suggests that the binding of *O*-deacetylated Vi antigen to the antibody molecule is via the carboxyl group. This is further supported by the lack of inhibition in the case of *N*-acetyl galactosamine.

Absorption of antisera with 2,3-di-*O*-acetyl polygalacturonic acid. To show that *O*-acetylated polygalacturonic acid precipitates Vi antibodies from antiserum A and antiserum B, we absorbed both antisera 2,3-di-*O*-acetyl poly-

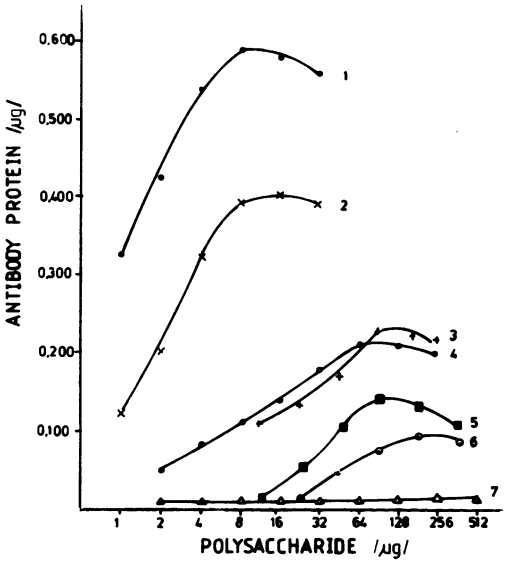

FIG. 2. *Quantitative precipitation curves for the native Vi antigen, 2,3-di-O-acetyl polygalacturonic acid, and their derivatives reacting in antiserum B (100 µl). (1) Native Vi antigen; (2) O-deacetylated Vi antigen (derivative I); (3) Vi antigen first carboxyl reduced and next reacetylated (derivative V); (4) 2,3-di-O-acetyl polygalacturonic acid; (5) carboxyl reduced and O-deacetylated Vi antigen (derivative III); (6) O- and N-deacetylated Vi antigen (derivative II); (7) 2,3-di-O-propionyl polygalacturonic acid.*

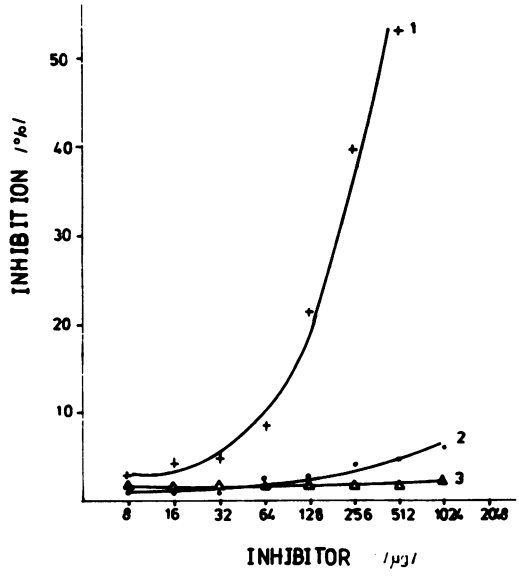

FIG. 3. *Inhibition of precipitation of the native Vi antigen in antiserum A by various concentrations of (1) 2,3-di-O-propionyl polygalacturonic acid; (2) O-deacetylated Vi antigen (derivative I); (3) polygalacturonic acid and galacturonic acid.*

glacturonic acid. Figure 5 represents the immunodiffusion pattern of precipitation of the native antigen Vi and 2,3-di-*O*-acetyl polygalacturonic acid with antiserum A and the same antiserum absorbed with the latter polysaccharide. Both compounds appeared to be serologically identi-

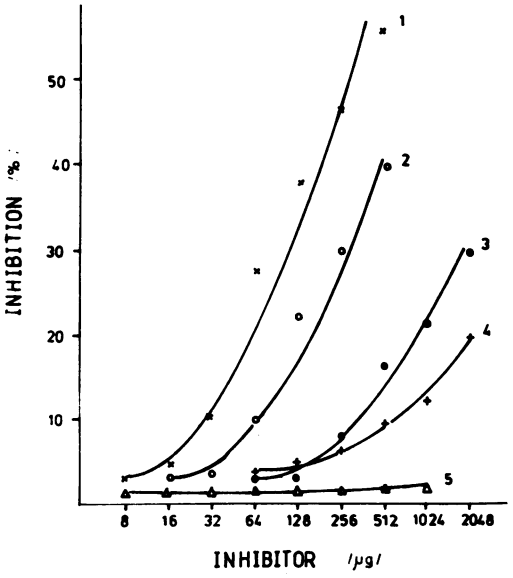

FIG. 4. *Inhibition of precipitation of O-deacetylated Vi antigen (derivative I) in antiserum B by various concentrations of (1) 2,3-di-O-propionyl polygalacturonic acid; (2) polygalacturonic acid; (3) galacturonic acid; (4) glucuronic acid; (5) N-acetyl galactosamine.*

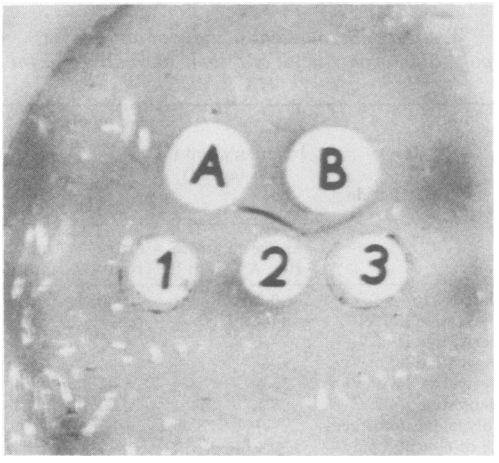

FIG. 5. *Immunodiffusion in gel showing precipitin bands of Vi antigen (2) and 2,3-di-O-acetyl polygalacturonic acid (1, 3). Well A contains antiserum A absorbed with 2,3-di-O-acetyl polygalacturonic acid; well B contains original antiserum A.*

cal in antiserum A. A more precise quantitative precipitation test showed that only about 10% of Vi antibodies were left in the antiserum after the absorption with *O*-acetylated polygalacturonic acid. The tests of the inhibition of immune precipitation indicated that 2,3-di-*O*-propionyl polygalacturonic acid, as well as the native polygalacturonic acid, blocks the antibodies reacting with *O*-deacetylated antigen Vi. It was reasonable to suppose that *O*-acetylated polygalacturonic acid also inhibits this reaction. In fact, instead of two intersecting lines in the immunodiffusion pattern for the precipitation of 2,3-di-*O*-acetyl polygalacturonic acid and *O*-deacetylated antigen Vi in antiserum B, we observed (Fig. 6) partial fusion of lines, indicating the binding of acetylated polygalacturonic acid to the *O*-deacetylated Vi antibodies. The absorption of this antiserum with 2,3-di-*O*-acetyl polygalacturonic acid yielded the antiserum which reacted with neither *O*-deacetylated antigen Vi nor the native antigen Vi.

DISCUSSION

The data presented in this paper show that at least two types of antibodies reacted with the molecule of antigen Vi. Two serological determinants of antigen Vi were characterized by various serological techniques using the native and modified antigen Vi, the structural analog of antigen Vi, 2,3-di-*O*-acetyl polygalacturonic acid, the native polygalacturonic acid, and 2,3-*O*-propionyl polygalacturonic acid.

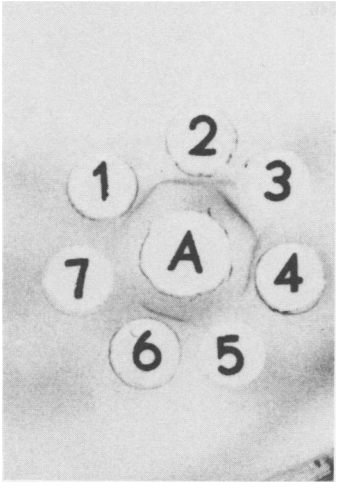

FIG. 6. *Immunodiffusion in gel showing precipitin bands of Vi antigen (1, 2), O-deacetylated Vi antigen (3), and 2,3-di-O-acetyl polygalacturonic acid (4, 6). Wells 5 and 7 contain 2,3-di-O-propionyl polygalacturonic acid; center well contains antiserum B.*

Vi antisera contain these two types of antibodies in different proportions. The first of the antisera chosen for our studies (antiserum A) contained antibodies against only one of the determinants. This determinant has *O*-acetyl as the main component of its structure. The removal of *O*-acetyl from antigen Vi renders it inactive in this antiserum, though the rest of the antigen Vi molecule seems to be relatively less important. This is evidenced by the reaction of 2,3-di-*O*-acetyl polygalacturonic acid (lack of *N*-acetyl on carbon-2 as compared to antigen Vi) in this antiserum, as well as by the reactivity, though diminished, of *O*-acetylated, carboxyl-reduced antigen Vi. The change of *O*-acetyl to *O*-propionyl in the molecule of acetylated polygalacturonic acid eliminates the property of the precipitation of Vi antibodies but, as the results of the inhibition of precipitation and HA show, the compound is still a good inhibitor of the immune reaction. Taking into account that *O*-deacetylated antigen Vi inhibits neither precipitation nor HA, it is reasonable to think that in this case the antibody recognizes only a small fraction of the antigen Vi molecule.

Another antiserum taken for our studies (antiserum B) possesses, as do most other Vi antisera, antibodies of the same type as antiserum I, but also contains antibodies against *O*-deacetylated antigen Vi. These antibodies are much more specific. The elimination of the *N*-acetyl group or the reduction of the carboxyl group of antigen Vi renders the derivatives capable of precipitating only less than 30% of the antibodies against *O*-deacetylated antigen Vi. Inhibition of the immune reaction by polygalacturonic acid, its *O*-propionyl derivative, and simple monosaccharides, like galacturonic acid and glucuronic acid, indicates that inhibitors and the antigen molecule are bound to the antibody via the carboxyl group, but to precipitate from the solution, the molecule must possess the *N*-acetyl group. As might be expected, 2,3-di-*O*-acetyl polygalacturonic acid is also an inhibitor of this immune reaction. Comparison of the precipitation curves in the two antisera shows that about 15 times more 2,3-di-*O*-acetyl polygalacturonic acid is needed to precipitate the same amount of antibody from antiserum B than from antiserum A. This fact may be well-explained if we assume that two reactions take place in antiserum B, the first being the precipitation of one type of antibody and the second being the inhibition of the other type of antibody. This conclusion is well supported by HAI of Vi-coated SRBC where 2,3-di-*O*-acetyl polygalacturonic acid is a much better inhibitor in antiserum A than in antiserum B. HAI results also confirm

that 2,3-di-*O*-propionyl polygalacturonic acid is an inhibitor of both types of antibodies.

The passive HA tests throw some light on the nature of the binding of antigen Vi to erythrocytes. Coating of erythrocytes with 2,3-di-*O*-acetyl polygalacturonic acid, but not *O*-deacetylated antigen Vi, clearly shows that *O*-acetyl is an indispensible group in this process. Somewhat surprising is the lack of binding of *O*-reacetylated antigen Vi (derivative IV). This fact suggests that *O*-acetyl, though indispensable, is not the exclusive factor in binding to erythrocytes. It may be that the fairly low acetylation degree (Table 1) of this compound is responsible for the lack of erythrocyte coating.

Failure to obtain the monovalent antiserum against *O*-deacetylated antigen Vi due to the inhibitory action of 2,3-di-*O*-acetyl polygalacturonic acid inclined us to attempt to obtain such an antiserum by immunizing rabbits with protein-conjugated *O*-deacetylated antigen Vi. The results of these studies will be reported later.

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