Interaction of Vi Antigen with Proteins

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ABSTRACT

WHITESIDE, ROBERTA E. (Boston University School of Medicine, Boston, Mass.), AND EDGAR E. BAKER. Interaction of Vi antigen with proteins. J. Bacteriol. 92:1597-1603. 1966.—Purified Vi antigen (Vi) mixed in equal amounts with bovine serum albumin (BSA) or human γ globulin (HGG) at pH values above 4.7 formed a complex which was not precipitated by trichloroacetic acid or tungstic acid. At pH values below 4.7, the interaction between Vi and either BSA or HGG produced insoluble complexes except when excess Vi antigen was present. When sufficient Vi was present at the lower pH values, the soluble complex was not precipitated by trichloroacetic acid. Other acid polysaccharides tested did not form trichloroacetic acid-soluble complexes with BSA. When subjected to immunoelectrophoresis, the Vi-BSA complex migrated in agar at a rate different from that of either BSA or Vi alone. The complex reacted with both Vi and BSA antiserum. The addition of either BSA or Vi antiserum to a Vi-BSA complex resulted in dissociation of the complex and precipitation of either Vi or BSA, depending upon the antisera used. Vi antigen mixed with purified O antigen from Salmonella typhosa formed a complex which migrated in agar at a rate different from that of either component alone when subjected to immunoelectrophoresis.

Webster, Sagin, and Freeman (13) showed that turbidity developed upon the addition of albumin to Vi antigen at pH 4.0 and that this phenomenon could be used as an assay method for Vi antigen. Baker and Whiteside (2) found that albumin or other proteins were necessary for the activity of a Vi antigen-degrading enzyme produced by Bacillus sphaericus. In this study, it was noted that albumin was not precipitable with certain protein-denaturing agents when Vi antigen was present at neutral pH values. These observations suggested that a protein-polysaccharide complex is formed in which the protein is less easily denatured. Recently, Olitzki and Godinger (10) found that, in the peritoneal cavity of mice infected with Salmonella typhosa, Vi antigen formed trichloroacetic acid-soluble complexes with globulins, but not with other proteins. The present communication is concerned with further studies of the interaction of Vi antigen and albumin or the O antigen of S. typhosa.

MATERIALS AND METHODS

Vi antigen (Vi) was prepared from S. barkeri by the method of Baker et al. (1). Deacetylated Vi was obtained by treating Vi with 0.1 N NaOH at room temperature (15). Enzymatically degraded Vi was made by the procedure previously described (2). O antigen was prepared from water extracts of acetone-killed S. typhosa 0-901. The method, to be published elsewhere, involved treatment of the water extract with picric acid to remove material with a high nitrogen content. The picric acid-soluble material was neutralized, dialyzed, and then fractionated on a column of Sephadex G-200. The first material excluded from the column after recovery of the void volume was serologically pure O antigen with the following chemical analyses: nitrogen, 1.95%; phosphate, 1.45%; total hexose, 35.87%; glucose, 25.5%; rhamnose, 13.45%; and lipid, 7.90%. Bovine serum albumin (BSA), polygalacturonic acid, and hyaluronic acid were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Colaminic acid was obtained through the courtesy of W. C. Boyd, Boston University School of Medicine. Human γ globulin (HGG) was obtained from the Institute of Laboratories, Massachusetts Department of Public Health. Pneumococcus polysaccharides were prepared according to the method described by Kabat and Mayer (8). Deacetylated type I pneumococcus polysaccharide was prepared by the same technique used for deacetylating Vi. Klebsiella pneumoniae type B polysaccharide was prepared in a manner similar to that used for pneumococcus polysaccharides.

Various protein precipitants were used as follows. (i) A 0.1-ml amount of a saturated solution of trichloroacetic acid (approximately 55% by weight) was added to 1 ml of a solution to give a final concentration of approximately 5%. (ii) Tungstic acid was used according to the method described by Folin and Wu (6). (iii) Heat was used to precipitate proteins by immersion of the sample in a boiling-water bath for 10 min.
Antiserum was prepared against Vi and O antigens by administration of suspensions of acetone-killed S. marcescens and S. typhosa, respectively, to rabbits, according to the procedure described previously (14). BSA antiserum was prepared by injection of 25 mg of BSA in Freund's complete adjuvant (Difco) into the toe pads of each rabbit, followed by an intravenous booster injection of 100 mg of BSA 1 month later. At 1 week after the booster injection, the rabbits were exsanguinated and the sera were collected.

Serological tests employed were the classical tube precipitin and agglutination tests, and a modification of the Ouchterlony agar-gel precipitin technique (16). Immunoelctrophoresis was carried out according to the method of Scheidegger (11), with 1% Noble agar (Difco) in 0.05 M Veronal buffer (pH 8.4) for 2 hr with a constant current of 2.5 ma per slide.

RESULTS
Effect of pH, concentration of reactants, and trichloroacetic acid on Vi-protein complexes. Preliminary experiments showed that, at neutral pH values, the addition of approximately equal amounts of Vi to BSA rendered the BSA non-precipitable by trichloroacetic acid, tungstic acid, or heat. In the following experiment, the effect of pH and relative concentration of Vi was studied in detail. Mixtures of BSA and various amounts of Vi were made in water, and the pH was adjusted with the addition of an equal amount of an appropriate buffer, 0.2 ionic strength. Final concentrations of the components were 1 mg/ml of BSA or HGG and increasing twofold concentrations of Vi from 0.125 mg/ml to 2.0 mg/ml of buffer (ionic strength, 0.1). Buffers used were acetate, for pH 4.0 to 5.0; cacodylate, for pH 6.0 to 7.0; and tris(hydroxymethyl)aminomethane (Tris), for pH 8.4. Immediately upon addition of buffer, each sample was mixed and, within 10 sec, the development of turbidity was determined with a Bausch & Lomb colorimeter at 600 nm. The pH of the mixture was determined with a Radiometer pH meter. An additional turbidity measurement was made after 5 min. Then trichloroacetic acid was added to each sample, and another turbidity measurement was made immediately after mixing (within 10 sec). The results are recorded in Table 1 as per cent transmission.

The addition of Vi to protein solutions and subsequent adjustment of pH to 4.7 or lower resulted in the spontaneous development of turbidity even before trichloroacetic acid was added. This phenomenon was dependent upon the concentration of Vi. When Vi was present in the protein mixtures in a concentration less than 1 mg/ml at pH 4.0, 4.5, and 4.7, spontaneous turbidity developed immediately, and the quantity of precipitate increased with decreasing ratios of polysaccharide to protein. As the ratio of Vi in the mixture was decreased below certain levels (for example, 0.125 mg/ml of Vi to 1 mg of BSA at pH 4.0), the turbidity again decreased. The addition of larger amounts of Vi (1 or 2 mg/ml) to BSA or HGG at pH values of 4.7 or lower did not result in precipitation. When any amount of Vi was mixed with BSA or HGG at pH 5.0, 6.0, 7.0, or 8.4, turbidity did not develop spontaneously. When the mixture of Vi and protein did not result in spontaneous precipitation, the subsequent addition of trichloroacetic acid rarely resulted in precipitation of protein. Only when small amounts of Vi were present (less than 1 mg/ml) did the addition of trichloroacetic acid result in visible precipitation. The quantity of precipitate was approximately inversely proportional to the amount of Vi in the mixture.

Effect of trichloroacetic acid on mixtures of various polysaccharides and BSA. To determine whether the ability of Vi to interact with protein and to render the protein nonprecipitable was a unique property of this substance, several other acid polysaccharides were examined in a similar fashion. Solutions containing 1 mg/ml of BSA and 1 mg/ml of any one of the following were prepared in 0.033 M Tris buffer (pH 8.4): polygalacturonic acid, hyaluronic acid, type I pneumococcus polysaccharide, type I d-eacetylated pneumococcus polysaccharide, type II pneumococcus polysaccharide, colominic acid, or K. pneumoniae type B polysaccharide. In every case, BSA was precipitated upon the addition of trichloroacetic acid. Likewise, decetylated Vi or enzymatically degraded Vi did not interact with BSA in such a way as to inhibit its precipitation by the reagents used. Thus, the ability to form trichloroacetic acid-soluble complexes appeared to be unique to Vi antigen.

Immunoelectrophoretic studies of Vi-BSA complexes. The following experiments were conducted to obtain additional evidence of complex formation. Varying concentrations of Vi and BSA were mixed in 0.05 M Veronal buffer (pH 8.4) and were submitted to immunoelectrophoresis. After electrophoresis, the mixtures were tested with both Vi and BSA antisera. Vi alone produced a line which extended from the origin nearly to the end of the slide (Fig. 1). BSA migrated as a fairly well-defined zone (Fig. 2). However, when mixtures of the two substances were subjected to immunoelectrophoresis, a component formed which migrated at a rate different from that of either BSA or Vi alone. Figures 3 illustrates the results of immunoelectrophoresis of a mixture of Vi in a concentration of 1 mg/ml and BSA in a concentration of 2 mg/ml of buffer (a ratio of 1:2). It may be noted that the zone produced by
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Fig. 1. Immunoelectrophoresis pattern of Vi tested with Vi antiserum. (A) Vi (0.5 mg/ml of buffer). (B) Vi antiserum. (C) Vi (2.5 mg/ml of buffer).

Fig. 2. Immunoelectrophoresis pattern of BSA tested with BSA and Vi antiserum. (A) Vi antiserum. (B) BSA (2 mg/ml of buffer). (C) BSA antiserum.

Fig. 3. Immunoelectrophoresis patterns of a mixture of Vi and BSA tested with Vi and BSA antisera. (A) Vi antiserum. (B) Vi (1 mg/ml of buffer) plus albumin (2 mg/ml of buffer). (C) BSA antiserum.

Fig. 4. Immunoelectrophoresis patterns of a mixture of Vi and BSA tested with Vi and albumin antisera. (A) Vi antiserum. (B) Vi (2 mg/ml of buffer) plus albumin (0.25 mg/ml of buffer). (C) BSA antiserum.

testing with anti-BSA serum shows the presence of two components. The slowest of these has about the same position as does BSA alone (Fig. 2) and presumably represents free BSA. Material in the more rapidly moving zone also reacts with anti-Vi serum and presumably represents the complex. Whenever BSA was present in the mixture in an amount of at least one-half the amount of Vi present in the mixture, free BSA could be detected, as well as complexed Vi and BSA.

Figure 4 illustrates the results of immunoelectrophoresis of a mixture of 2 mg of Vi per ml and 0.25 mg of BSA per ml of buffer (a ratio of 8:1). The Vi-BSA complex was again represented by material which migrated more rapidly than BSA alone and which reacted with both anti-albumin and anti-Vi sera. With this excess of Vi, no free BSA was detected serologically. However, the excess Vi reacted with Vi antiserum to form a zone of precipitate which extended almost to the antigen origin, as was the case with Vi alone (Fig. 1). When the amount of Vi present in the mixture was increased to more than twice the amount of BSA present in the mixture, all of the BSA was complexed with Vi, and free Vi could be detected serologically.

Serological studies of Vi-BSA complexes. To determine whether a Vi-BSA complex could be isolated by the addition of either anti-Vi or anti-BSA serum, the following experiments were carried out. Vi was mixed with albumin in a 4:1 ratio which, according to the immunoelectrophoretic experiments outlined above, left no free BSA. Upon addition of an amount of anti-Vi serum sufficient to precipitate all of the Vi in solution, the supernatant fluid still showed the
presence of BSA when tested with an anti-BSA serum. Similarly, when Vi was mixed with BSA in a 2:1 ratio, which should result in complete complexing of Vi with free BSA, the addition of an equivalent amount of anti-BSA serum did not remove Vi from the supernatant fluid. Supernatant fluids were tested by both tube precipitin and gel precipitin tests. The persistence of soluble Vi or BSA after addition of sufficient antibody to effect complete precipitation of the complex suggested that addition of specific antiserum to either Vi or BSA resulted in dissociation of the complex.

Examination of the specific precipitate resulting from precipitation of Vi-BSA complexes by either anti-Vi serum or anti-BSA serum confirmed this suggestion. A mixture of Vi and BSA (2:1 ratio) was precipitated with anti-BSA serum at equivalence for the amount of BSA present. The precipitate was collected by centrifugation, washed five times, and finally resuspended in saline. The washed precipitate was digested with trypsin for 18 hr at pH 8.5 and at 37 C, with a trace of chloroform present as a preservative. No serologically active Vi was detected after degradation of the BSA. Simultaneous control tests showed that, under the identical conditions used, serological activity of BSA, but not of Vi, was destroyed by the tryptic digestion.

Rabbits were immunized with washed specific precipitates obtained by precipitation of a Vi-BSA mixture (ratio, 2:1) with either anti-Vi serum or anti-BSA serum at equivalence. The specific precipitates were incorporated in Freund's complete adjuvant. After immunization, the rabbit sera were examined for Vi and BSA antibodies by tube precipitin and by agar-gel precipitin tests. Rabbits immunized with specific precipitates obtained by precipitation with Vi antiserum of a Vi-BSA mixture and of Vi alone did not produce detectable antibodies against either Vi or BSA. Whiteside and Baker (14) showed that Vi in the purified state essentially was nonantigenic in the rabbit. However, rabbits immunized with specific precipitates obtained by precipitation with a BSA antiserum of a Vi-BSA mixture or BSA alone did produce antibodies against BSA, but no Vi antibody. Thus, it appeared that reaction of the components of a Vi-BSA mixture with specific Vi or BSA antisera resulted in dissociation rather than precipitation of the intact complex.

Studies of Vi-O antigen complexes. Since Vi complexed readily with BSA and other proteins, it was thought that Vi might exist in complex with O antigen in the bacterial cell. The following experiments were designed to determine whether Vi could interact with isolated S. typhosa O antigen. Mixtures of pure O antigen and Vi were subjected to immunoelectrophoresis and tested with anti-Vi and anti-O sera. Vi alone behaved as stated above (Fig. 1). O antigen subjected to electrophoresis alone at concentrations of either 0.5 mg/ml or 2.5 mg/ml migrated toward the negative electrode, and, when tested with S. typhosa antiserum, formed a single zone (Fig. 5). A mixture containing 2.5 mg/ml of O antigen and 0.5 mg/ml of Vi was subjected to immunoelectrophoresis. When tested with antisera against O and Vi antigens, it was found that the O antigen migrated toward the positive pole (Fig. 6). Lower concentrations of O antigen behaved in a similar fashion.

Mixtures of Vi and pure O antigen injected intravenously into two rabbits stimulate O, but not Vi, antibody production. Rabbits received a total of 4.5 mg of Vi and 0.9 mg of O antigen in

![Fig. 5. Immunelectrophoresis pattern of Salmonella typhosa O antigen tested with O antiserum. (A) O antigen (0.5 mg/ml of buffer). (B) O antiserum. (C) O antigen (2.5 mg/ml of buffer).](image1)

![Fig. 6. Immunelectrophoresis patterns of a mixture of Salmonella typhosa O antigen and Vi tested with O antiserum and Vi antiserum. (A) Vi antiserum. (B) Vi (0.5 mg/ml of buffer) plus O antigen (2.5 mg/ml of buffer). (C) O antiserum.](image2)
six injections. Two control rabbits, which received the same amount of Vi alone, produced no detectable antibody. Two rabbits that received the same amount of O antigen intravenously did produce specific O antisera (agglutination titer, 5,120). Thus, either Vi complexed with O antigen is not antigenic, or the complex dissociates in vivo.

**Discussion**

At low pH values, purified Vi can combine with proteins in certain relative concentrations to form a precipitate, a property used by Webster, Sagin, and Freeman (13) as a method of assaying Vi. At higher pH values, the complex remains soluble even in the presence of protein precipitants such as trichloroacetic or tungstic acid. The complex is probably in equilibrium with free protein (e.g., BSA) and Vi, since the addition of antibody specific for either component results in precipitation of that component only and the dissociation of the complex. A variety of polysaccharides, both acidic and neutral, have been shown to interact with proteins. In most instances, the interaction results in the formation of an insoluble complex. This interaction may be highly specific, as in the case of antigen-antibody reactions (8) and the blood group polysaccharides and lectins (3). On the other hand, the interaction may be relatively nonspecific. For example, hyaluronic acid or chondroitin sulfuric acid interacts nonspecifically with a variety of proteins (4). Luderitz et al. (9) reported that the O antigens of a variety of gram-negative bacteria will combine with various proteins. This property was increased by treatment of the O antigen with alkali.

The interaction of Vi with proteins appears to be unique in that it will combine in such a way that the precipitation with protein precipitants is prevented. Whether the failure to precipitate means preservation of native structure is not determined by the present studies. This polysaccharide differs from other acidic polysaccharides in that it is a polymer of N-acetyl-galactosamine (5, 7). In addition, each residue in the native substance contains one or more O-acetyl groups (1). However, since the Vi is hydrolyzed only with great difficulty by acid (1, 5, 12) and incompletely by enzyme (2), its exact composition and structure is not known at present. Until more information is available, it is not possible to compare Vi with other acid polysaccharides in their interaction with proteins.

Olitzki and Godinger (10) reported that Vi combined only with globulins in the peritoneal cavity of mice infected with *S. typhosa*. Their observations are in contrast to the present data showing that Vi combines with many proteins. In the present experiments, relatively large amounts of Vi were allowed to interact with proteins. In the experiments of Olitzki and Godinger, it is probable that, in vivo, only very small amounts of Vi were present, in comparison with very large amounts of proteins. It may be that, under these conditions, Vi has a greater affinity for globulin than for other serum proteins.

Vi in crude water extracts or in intact bacterial cells is antigenic in the rabbit. However, after extraction and purification by the method described by Baker et al. (1), the antigen is no longer capable of stimulating antibody production in the rabbit. Since many acidic polysaccharides complexed with proteins exist in nature, it was thought that Vi might exist complexed with O antigen in the bacterial cell. Although Vi can complex with O antigen, attempts to stimulate Vi antibody production in rabbits by injection of mixtures of Vi and O antigens failed. It is possible that either the complex was broken in vivo or that the purification process altered the Vi molecule, even though the procedure used employed very mild methods. The failure of the Vi-O complex to stimulate Vi antibody production also may be explained by assuming that the immunogenic Vi present in intact bacterial cells is linked to O antigen in a manner different from the linkage formed in the artificial mixtures studied.

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**Literature Cited**


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