ELECTROPHORETIC PURIFICATION OF THE Vi ANTIGEN

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Several investigators have attempted to purify the Vi antigen and have described their products in terms of chemical and biological properties (Topley et al., 1937; Combiesco et al., 1937; Henderson and Morgan, 1938; Boivin and Mesrobeanu, 1938; Ogonuki, 1940; Grabar and Corvazier, 1951; Webster, Landy, and Freeman, 1952; Kozinski et al., 1954; DeBarbieri et al., 1956; Baker et al., 1959). These descriptions vary in kind and extent, but serious contradictions, particularly concerning the chemistry of the antigen, are readily apparent. These discrepancies may generally be ascribed to differences in purity of product, but may also be caused by chemical alteration of the natural antigen during purification. The antigen is known to be altered by relatively mild alkaline conditions (Baker et al., 1959; Ashida, 1949), and the purification and certain of the properties of the altered antigen have been reported by Ashida (1949). Two of the more recent purification methods (Webster et al., 1952; DeBarbieri et al., 1956) employ extended (6 to 24 hr) hydrolyses in hot 1 M acetic acid. Although the antigen is more stable to acid than to alkaline treatment, direct proof that this vigorous treatment causes no alteration of the antigen molecule is lacking. Baker et al. (1959) recently succeeded in purifying the antigen by milder methods and have submitted data which indicate that the hot acetic acid treatment probably causes some degradation of the molecule.

An electrophoretic approach to the problem of purification of the Vi antigen is suggested by its highly acidic character. Although Combiesco and Soru (1939) found that the Vi and O antigens migrated quite differently in an electrophoretic field when tested individually, they failed to separate mixtures of the two by this means. Linton, Smith and Krejci (1944), using a more refined method of electrophoresis, were able to separate crude soluble antigenic preparations from the growth medium of *Salmonella typhosa* into three fractions. The O and Vi antigens appeared to be concentrated in different fractions.

A mild, electrophoretic procedure which leads to a product of considerable apparent purity and some of the properties of that product are described in this paper.

MATERIALS AND METHODS

Organisms and culture conditions. Escherichia (Citrobacter) freundii strain 5396/38 was employed for the major proportion of work. *S. typhosa* strain Ty 2 was used for one study. *E. freundii* was grown on a defined broth medium containing per liter: glucose, 7.5 g; sodium L-glutamate, 0.02 g; calcium pantothenate, 0.0001 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 3.0 g; Na_2HPO_4, 6.0 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; and NaCl, 5.0 g. The experimental basis for the use of this medium and the cultural conditions employed have been reported (Jarvis, Mesenko, and Tibbs, 1960). *S. typhosa* was grown on veal infusion agar (Difco).

Ultracentrifuge. Information on sedimentation properties was obtained with a model E Spinco ultracentrifuge operated at 56,100 rpm and 20 C.

Analytical. Hemagglutination (Ha) titers were obtained in the manner described previously (Jarvis et al., 1960). Precipitin tests for O antigen were conducted in capillary tubes using a 1:2 dilution of O antiserum. The end points were read after storage for two days at 4 C following incubation for 2 hr at 37 C. Vi-positive colonies of *Paracolobactrum ballerup* strain 7851/39 were used to prepare the Vi antiserum; selected Vi-negative colonies of *E. freundii* strain 5396/38 were used to prepare the O antiserum.

Carbon, hydrogen, nitrogen (Dumas) and ash were determined by the microanalytical laboratory, NIAMD, National Institutes of Health, Bethesda, Maryland. A modified Fiske-Subba-Row method (Dryer, Tammes, and Routh, 1957) was used to determine phosphorous; the sulfur content was estimated from the specific radio-

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activities of the input sulfur and the purified antigen.

Viscometry. Viscosity measurements were made with a Zeitfuchs crossarm (constant = 0.01) viscometer (Johnson, LeTourneau, and Matteson, 1952) under accurately controlled conditions. Exactly 1 ml of solution was used for each determination.

Electrophoresis. This operation was conducted in a Spinco model. CF continuous flow cell at 4 C. Two types of curtain material were used, the usual paper provided by Spinco and a ceramic paper obtained from Hurlbut Paper Company (type 970FH). The cellulose curtain contains soluble, nondialyzable impurities which are difficult to remove; the ceramic curtain shows such a high endosmotic flow toward the anode that it is impossible to avoid contaminating the buffer with electrically neutral materials (in the crude extracts) and still collect the Vi antigen with good resolution. Both difficulties were circumvented by using the paper curtain for a first electrophoretic step and the ceramic curtain for a second. The ceramic curtains were pretreated with hot concentrated H_2SO_4 (with a few drops of concentrated HNO_3) to remove occasional spots of organic impurities. Buffer flow to the top of the ceramic wicks was increased by introducing siphons with larger bore than those supplied with the instrument.

The buffer used (0.05 M borate at pH 8.8) provides freedom from bacterial contamination, causes no appreciable loss of activity, and is transparent to ultraviolet light, which makes direct spectrophotometric analyses of the curtain fractions possible. Bromphenol blue was added to the input solution as a visible reference point. The Vi antigen is the only major component which leaves the curtain between the dye and the cathode. The antigen foams at very low concentrations and may be located tentatively by this property. It may be located more specifically and quantitatively by hemagglutination titer or by optical activity. The buffer does not interfere with either of these tests.

PURIFICATION

Procedure. The centrifuged and saline-washed cells from each liter of medium were resuspended in about 50 ml of physiological saline and shaken or stirred vigorously with an equal volume of diethyl ether for 10 min at room temperature. The original ether and one ether wash were separated and discarded. Any remaining ether was eliminated under reduced pressure and the cells removed by centrifugation. The clear supernatant was adjusted to pH 4.0, cooled, and four volumes of cold acetone added to precipitate the antigen. After allowing the precipitate to flocculate, it was collected by centrifugation and the acetone removed by decantation and evaporation under reduced pressure. The sediment was dissolved in about 25 ml of pH 8.8, 0.05 M borate buffer and the Vi antigen separated from O antigen and other impurities by continuous flow, paper curtain electrophoresis. The Vi antigen fractions were collected and treated with a few micrograms of ribonuclease at pH 7.5 for ½ hr at about 30 C. The antigen was precipitated with acetone at pH 4.0, dissolved in borate buffer and the electrophoretic step repeated using the ceramic type of curtain described above. To remove the buffer and ribonucleic acid residues not separated by electrophoresis, the Vi antigen fraction was dialyzed first against 2 M NaCl (Markham and Smith, 1952) and then against water. The antigen may be obtained in the acid form by passing the dialyzed solution through a sulfonic acid (as Amberlite IR-120) exchange column, or in the salt form by neutralizing (pH 7.0) the dialyzed solution. Lyophilized preparations of the free acid may not be completely soluble in distilled water or in acidic buffers, but dissolve readily in buffers which neutralize the acidity of the antigen. Lyophilized preparations of the sodium salt of the antigen dissolve readily in water, in neutral buffers, and in pH 4.7 acetate buffer. Dilute preparations may be sterilized without loss of antigen by filtration through ultrafine sintered glass.

Experimental. The degree of separation of the major carbon constituents of the crude preparation by the first electrophoretic step is shown in figure 1. Radiocarbon (C^{14}) was introduced into the growth medium before inoculation in the form of randomly labeled invert sugar. The excellent correlation between optical rotation values and the Vi antigen content even of crude preparations has led to the use of this measurement as a valuable supplementary means for assay. The additional excellent correlation among radiocarbon content, hemagglutination titer, and optical activity peaks shown in figure 1

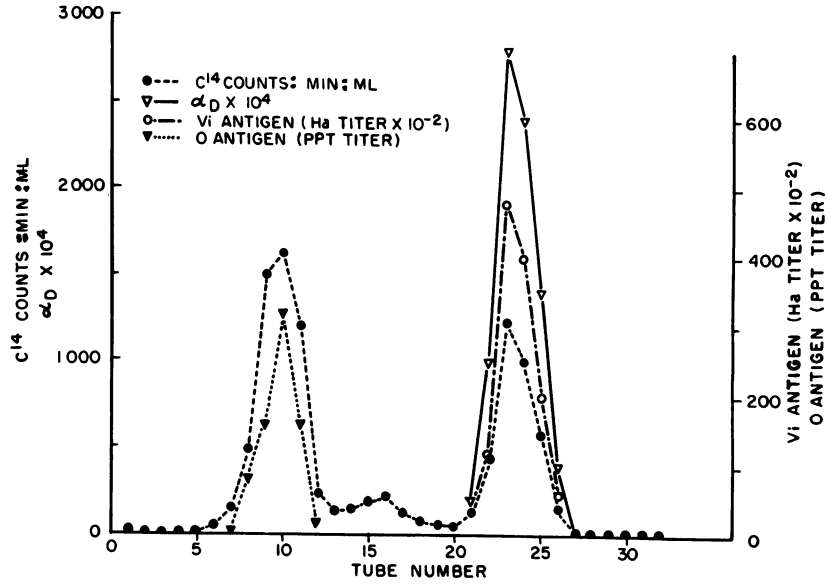

Figure 1. The distribution of material eluted from paper curtain during first electrophoretic run. Optical rotation, Vi antigen titers and O antigen titers not shown were essentially zero.

TABLE 1

Data on purification of Vi antigen

Stage of Purification	Antigen Concn,* Hemagglutination Titer: mg:ml	Total Vi Antigen† Present	Total C^{14}‡
		mg	*(counts/min)*
Cells	4,000	80	12.6×10^6
Ether extract	40,000	80	12.3×10^5
Acetone precipitate	50,000	80	5.8×10^5
Preparation after first electrophoresis	160,000	70	2.1×10^5
Final product	200,000	60	1.8×10^5

* With the exception of the value shown for the cells (*see* text), these data were obtained from dialyzed samples.

† Total antigen values were estimated from optical activity and hemagglutination titer data from a typical 4 liter run.

‡ These data were obtained from undialyzed samples.

suggests that only minor quantities of impurities are present after the first electrophoretic separation.

Typical recoveries and the extent of purification achieved by each major step in the procedure described above are shown in table 1. The amount of Vi antigen originally present on the bacterial cells was assumed to be equal to the amount present in the ether extract, as further ether treatment removed very little additional antigen and the ether-treated cells no longer agglutinated with Vi antiserum. About 5 per cent of the antigen from the anode side of the Vi antigen peak from each electrophoretic run was discarded to facilitate purification. The antigen activity data (hemagglutination titer: mg:ml) were obtained from dialyzed samples; the C^{14} counts were obtained from samples which were not dialyzed. One may conclude from these data that the acetone precipitation step serves largely to remove dialyzable organic material from the antigen preparation. It also serves as a convenient means for concentration. Somewhat higher yields could have been obtained by washing the residual antigen in the delivery vessel onto the curtains with buffer.

The addition of ribonuclease to preparations which had been purified by acetone precipitation, electrophoresis, and dialysis caused the appearance of appreciable quantities of dialyzable material with an absorption peak at about 260 mμ. The addition of deoxyribonuclease to the same preparations had no such effect. Ultraviolet absorption analysis of elution fractions from either electrophoretic run shows that material of

similar absorption characteristics and a slightly lower migration rate is not completely separated from the Vi antigen. A small amount of this material fails to dialyze and appears in the final product.

The introduction of radioactive sulfur (as $Na_2S^{35}O_4$) into the growth medium reveals a minor sulfur-containing impurity not completely separated from the antigen by our procedure. This material migrates at about the same rate as the 260 mμ light-absorbing material during electrophoresis.

The purification schedule outlined above has also been used successfully to purify Vi antigen from *S. typhosa* (grown on veal infusion agar) and from the growth medium of *E. freundii* which was given a preliminary dialysis but was not ether treated. Although comprehensive studies of these preparations have not been made, they are free of O antigen activity, and comparative infrared spectra show no appreciable differences from the antigen obtained from *E. freundii*.

PROPERTIES

The purified antigen (free acid or sodium salt) is a white, amorphous solid, soluble in water, formamide, and ethylene glycol and very slightly soluble in methanol. It is insoluble in ethanol and the usual fat solvents. Lyophilization in the free acid form may cause a fraction of the preparation to become insoluble except in neutralizing solutions.

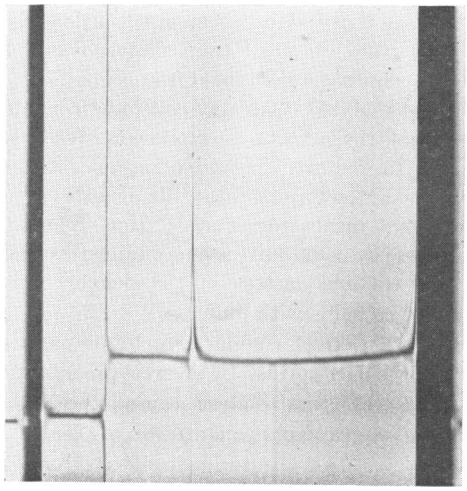

Figure 2. Sedimentation of 0.1 per cent Vi antigen solution in 0.10 M acetate buffer at pH 4.7 and 20 C. Photograph taken after 64 min at 56,100 rpm.

Analysis. Calculated for $C_{10}H_{14}NO_7$: C, 46.15; H, 5.42; N, 5.38. Found (after drying in vacuo at 100 C): C, 45.96; H, 5.42; N, 5.51.

Additional analyses include (all values based on dry, free acid form of the antigen): ash, 2.70 per cent; P, 0.25 per cent; S, 0.015 per cent; neutral equivalent, 280; $[\alpha]_D^{23}$, 300 (sodium salt in H_2O, c = 0.1); and specific viscosity, 1.10 (0.1 per cent in 0.1 ionic strength, pH 4.7 acetate buffer at 20 C).

The preparation sediments as a single peak in the ultracentrifuge at both pH 4.7 (0.1 M acetate) and pH 7.2 (0.1 M phosphate). The ultracentrifuge pattern of a 0.1 per cent solution in 0.1 M acetate buffer at pH 4.7 is shown in figure 2. A preliminary account of detailed studies of sedimentation rate, viscosity, and infrared absorption behavior has been reported (Jarvis, Mesenko, and Perrine, 1960). The final product absorbs ultraviolet light with a peak at about 260 mμ. The extinction coefficient ($E_{1cm}^{1\%}$) at 260 mμ has varied from about 3 to 6 in different preparations.

A 1 mg/ml solution of the antigen gives a hemagglutination titer of about 200,000, which corresponds to an end point concentration of 0.005 μg/ml. It protects mice against challenge with *S. typhosa*, and is antigenic for mice, slightly antigenic for rabbits and essentially nontoxic for either. It is free of O antigen activity as judged by precipitin tests using homologous O antiserum (Vi antibody free). Details of the biological properties of our purified antigen have not been published.

DISCUSSION

Although the treatment with ribonuclease leads to a final product considerably more transparent to 260 mμ light, this product still has an absorption peak at this wave length. Some of this activity is probably caused by ribonucleic acid residues that failed to dialyze even against 2 M salt solutions, but some may be caused by the Vi antigen molecule. At least part of the ultraviolet absorption activity and the antigen itself sediment at identical rates at pH 4.7 in 0.1 M acetate buffer. We could discern no other sedimenting peak with either schlieren or ultraviolet optics. If all the ultraviolet activity repre-

sents extraneous ribonucleic acid residues, the extent of contamination would vary from 1.5 to 3 per cent.

The second electrophoretic step removes very little extraneous material; but that it does remove some is shown by increased hemagglutination activity per mg, and by the elimination of a residual toxicity for rabbits always present after only one electrophoretic step.

Although the analytical values found correspond rather closely to the ratio $C_{10}H_{14}NO_7$, we do not wish to imply that this necessarily represents an accurate formulation of a simple repeating unit of the antigen molecule. It is evident, however, that unless the isolated antigen is much less pure than our data suggest, it cannot be described as a simple, partially acetylated polymer of an aminohexuronic acid, as might be construed from previously published data (Baker et al., 1959; Clark, McLaughlin, and Webster, 1958).

The high specific viscosity of our preparation as compared with that found by Webster et al. (1954) under essentially identical conditions indicates a less degraded product. The specific viscosity of water solutions of the sodium salt of the antigen (1.5 to 1.7 at 1 mg/ml) is higher than the value obtained in buffer as would be expected of a polyelectrolyte. The absence of information pertaining to concentration and solvent conditions employed by Baker et al. (1959) makes comparison with their viscosity data impossible.

SUMMARY

A method for the purification of the Vi antigen by continuous flow, paper curtain electrophoresis is described. Evidence of the degree of purity achieved by the procedure is presented, and the product is partially described in terms of chemical, physical, and biological properties.

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