# THE CHEMICAL ALTERATION OF A BACTERIAL SURFACE, WITH SPECIAL REFERENCE TO THE AGGLUTINATION OF B. PROTEUS OX-19

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The complex antigenic structure of bacteria raises many practical difficulties in the use of intact bacteria as diagnostic serological reagents, since the presence of several reactive antigenic groupings may confuse the significance of positive reactions. One such problem and the manner in which it has been partially overcome is exemplified by the classification of organisms in the *Salmonella* group. Infection with an organism of this group will give rise to the production of flagellar and somatic agglutinins. The classification of the organism responsible for the particular infection, according to the Kaufmann-White scheme (1), depends in part on the differentiation of these agglutinins by the use of chemically treated bacterial suspensions. Formolized suspensions enable detection of flagellar agglutinins, whereas alcohol-extracted organisms, devoid of flagellar antigens permit the detection of somatic agglutinins.

A typhus infection will result in the production of agglutinins for *B. proteus* OX-19, utilized in the Weil-Felix reaction. The sera of a considerable number of individuals, having no previous history of typhus, agglutinate this latter organism. It has been shown that *B. proteus* OX-19 possesses at least two antigenic groupings reactive in intact organisms. One of these is reactive with agglutinins elaborated as a result of a typhus infection, another with agglutinins elaborated as a result of immunization or infection with *B. proteus* organisms which may not be of the OX-19 type (2, 3). Thus a positive serological reaction with *B. proteus* OX-19 is not necessarily characteristic of typhus or even an infection with *B. proteus* OX-19.

Recent methods for the serological diagnosis of typhus employ rickettsial antigens (4–6). Even with these antigens, the differential diagnosis of epidemic or murine typhus is again complicated by the complexity of the diagnostic reagent.

It was considered that an increase in the specificity of a serological reaction might be accomplished by means of chemical substitution of some antigenic

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groupings on the bacterial or viral surface. This might possibly eliminate the reaction of the organism with antibodies not specifically characteristic of the infection for which the test was made. The exploratory experiments described in this paper were performed with the organism employed in the diagnosis of typhus by the Weil-Felix reaction, namely *B. proteus* OX-19.

The polar groups of a bacterial surface may be expected to contribute to the electrophoretic mobility of the organism as a function of their acid and base-binding capacities (7). A study of the electrophoretic mobilities of native and altered organisms under the proper conditions of pH, ionic strength, etc. should yield information approximating the titration curve of the surface complex. Coupled with data on the serological behavior of native and altered organisms, indications of the nature of antigenic groups responsible for specific serological reactions might become available.

The only reagent used in the studies to be described below was benzene sulfonyl chloride at a weakly alkaline pH. Under these conditions, this substance had been found to react with amino groups at a greater rate than with the phenolic hydroxyl of tyrosine (8). The treatment of *B. proteus* OX-19 with benzene sulfonyl chloride ($\phi SO_2Cl$) promptly resulted in considerable changes in the pH-mobility curve of the organism. The shape of the curves indicated the substitution of both amino and imidazole groups. The ability of the reagent to affect the latter under the conditions employed was confirmed by experiments with gelatin, but was not observed with free histidine or $\beta$-alanyl histidine. Hirayama had noted the elimination of the Pauly diazo reaction in clupein and sturine after treatment with aromatic sulfochlorides (9).

Treatment with $\phi SO_2Cl$ resulted in a marked reduction of the agglutinability of the organisms in rabbit antisera to *B. proteus* OX-19. The antibody-combining capacity of these substituted organisms, as determined by quantitative methods (10), was unaltered however. This apparent contradiction led to a comparison of the agglutinating properties of native and benzene-sulfonated organisms.

## *Materials and Methods*

*Bacterial Preparation.*—48 hour cultures of *B. proteus* OX-19 were grown on nutrient agar or in nutrient broth. The former were washed off with saline and sedimented; the latter were sedimented. A mixed pool of both types was employed in the experiments, in 0.1 M borate buffer at pH 7.8 containing 0.01 per cent merthiolate. The organisms were repeatedly sedimented and resuspended in the borate-merthiolate buffer. The stock suspension contained 1.14 mg. nitrogen per cc.

*Preparation of Antiserum.*—A single antiserum was employed in the experiments described below. Similar experiments on other antisera similarly prepared have yielded the same results. A rabbit was immunized by eight subcutaneous inoculations of 0.5 cc. amounts of the stock bacterial suspension over a 2½ week period. Two months later, six injections were again given over a 2 week interval. The animal was bled 7 days after the last injection.

*Preparation of Substituted Organisms.*—To 10 cc. of the stock suspension of organisms was

added 90 cc. of 0.2 M phosphate buffer at pH 8.4. After removal of 25 cc., the remainder was chilled to 4°. One-tenth of a cubic centimeter of benzene sulfonyl chloride was added to the constantly stirred suspension. Aliquots were removed at suitable intervals, sedimented in an angle centrifuge at 4000 R.P.M. for 30 minutes, and the sediment was finely resuspended in distilled water or saline containing 0.01 per cent merthiolate. Organisms exposed to treatment in excess of 3 hours gave coarser suspensions than the original organisms.

*Quantitative Agglutinations.*—The native and altered organisms were sedimented and resuspended in merthiolate saline until suspensions containing 1 mg. of nitrogen per cc. yielded 0.01 mg. of unsedimented nitrogen or less in the supernatant solution. These washed suspensions did not give off soluble nitrogen after storage for several days. To 1 cc. aliquots of the rabbit antiserum to *B. proteus* OX-19, and a normal rabbit serum were added 1 cc. aliquots of suspensions of native or altered organisms at various concentrations of total nitrogen. After incubation with frequent stirring at 38°, the tubes were permitted to stand at 4° overnight. The tubes were sedimented while immersed in ice chips at 2500 R.P.M. for 1 hour and the supernates were decanted. The sediments were thoroughly mixed in 1.5 cc. of cold merthiolate saline and similarly resedimented. This procedure was repeated. The sediments were transferred to Kjeldahl flasks and their nitrogen contents were estimated by the micro-Kjeldahl method. The antibody-combining capacities of the organisms were taken as the difference between total nitrogen sedimented in antiserum and in normal serum, after slight corrections were made for the nitrogen sedimented in these sera without antigen added.

*Electrophoretic Examination.*—The Northrop-Kunitz microelectrophoresis apparatus (11, 12) was employed. It was established that, when the organisms were examined at different levels within the cell, a parabolic curve was obtained when mobility was plotted against cell depth. Velocities were measured for ten particles in each direction, generally at only one level in the cell at which the movement of the fluid was zero. The twenty readings were averaged and the mobilities were calculated from the formula, mobility $= dcKq/t \cdot I$, where $d$ represents the distance in centimeters which the particle moves; $c$, the conductivity of the buffer at room temperature; $K$, the constant of the conductivity cell; $q$, the cross-sectional area of the cell; $t$, the observed time in seconds; $I$, the current in amperes.

Aliquots of the suspensions of organisms in water were added to buffer within 2 minutes of beginning electrophoretic examination. This was done to minimize the possibility of chemical changes in the organisms. The final ionic strength of the buffers was usually 0.02, uni-univalent buffers being used throughout the entire pH range studied. The buffers used were veronal buffers at pH 6.8—9.2, acetate buffers at pH 4.5–5.5, and glycine-HCl buffers at pH 2–3.

*Colorimetric Detection of Histidine.*—The Pauly diazo reaction was employed. To 1 cc. aliquots of the solution to be tested were added 1 cc. of 12.5 per cent sodium carbonate and 1 cc. of 0.2 per cent diazobenzene sulfonic acid in 0.7 per cent hydrochloric acid. The mixture was diluted to 10 cc. and was read, after 1 minute, in the Klett-Summerson photoelectric colorimeter with a 540 filter.

## EXPERIMENTAL

*Electrophoretic Effects of Treatment with Benzene Sulfonyl Chloride.*—In initial experiments, it was observed that maximal effects were obtained after intervals of 120 minutes of treatment. The first preparation was examined in 0.05 N buffers. At pH 1.5, a reversal of charge occurred after 1 hour of treatment. At the 60 minute interval, a slight mobility increase (indicative of imidazole ionization) was still observable between pH 4 and 8.4 although the slope of the curve

in this region was less than that of the original organisms. By 120 minutes, however, the mobility of the organisms was constant in this range, at a level equivalent to the mobility of "60 minute" organisms at pH 8.4. These data are summarized in Table I.

TABLE I

*The Electrophoretic Mobilities of Native and Benzene-Sulfonated B. proteus OX-19*

| Time of treatment with $\phi SO_2Cl$ | Mobility × $10^{-4}$cm.²/volt sec. at various pH levels | | | |
|---|---|---|---|---|
| | pH 1.51 | pH 2.07 | pH 3.98 | pH 8.40 |
| *min.* | | | | |
| 0 | +0.65 | +0.29 | −0.41 | −1.33 |
| 30 | 0 | −0.58 | −1.60 | −2.54 |
| 60 | −0.39 | −0.84 | −2.48 | −3.12 |
| 120 | −0.87 | −0.95 | −2.93 | −2.92 |

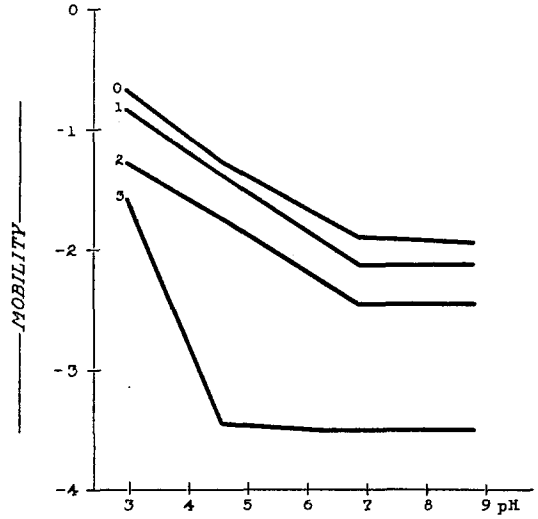


FIG. 1. The electrophoretic mobilities of native and benzene-sulfonated *B. proteus* OX-19. The numbers on the individual curves, 0, 1, 2, 3, represent hours of treatment with $\phi SO_2Cl$. The mobilities are given in $10^{-4}$ cm.² per volt sec.

In subsequent preparations, however, it was observed that for some unknown reason, the substitution proceeded at a slower rate. Effects to be noted in the region of imidazole ionization now required periods of 3 hours, and intermediate hourly periods did not result in mobility changes as marked as those described in Table I. A typical series of curves is given in Fig. 1. From the shape and quantitative relations of these curves, it can be seen that those groups which tend to diminish the mobility of this organism in the range studied have been

eliminated in large measure, if not completely. The ionization of imidazole and amino groups was markedly repressed, presumably as a result of the addition of the benzene sulfonyl group.

Although the data demonstrate that the microelectrophoretic method is applicable to the observation of chemical changes on *B. proteus* OX-19, it is clear that the fullest utilization of the method would be facilitated by a particle whose cationic ionization is more readily determinable. This criterion is fulfilled by an organism such as *R. prowazeki* (13). With that virus it has been observed that the decrease in cationic mobility from 1.19 to 0 at pH 2.5 after substitution almost exactly corresponds to the increase in anionic mobility at pH 9.[1]

*Substitution of Imidazole groups.*—To 25 cc. of solutions of histidine derivatives in 0.2 M phosphate buffer at pH 8.4 was added 0.1 cc. of benzene sulfonyl chloride with stirring at 4°. The intensity of the Pauly diazo reaction was followed. The materials employed were histidine, β-alanyl histidine, and gelatin. The latter protein was selected because of the absence of tyrosine which reacts with diazobenzene sulfonic acid. After 180 minutes, neither histidine nor β-alanyl histidine showed significant reduction of the diazo reaction. Gelatin, however, fell to 24 per cent of the original color intensity in that time. At 25°, the substitution was even more rapid, color development falling to 15 per cent of the original in 30 minutes and to 6 per cent in 90 minutes.

*Serological behavior of Native and Altered Organisms.*—Preliminary observations were made with the organisms defined in Table I. By the method described previously, it was determined that the available antiserum had an agglutinin content of 0.23 mg. antibody nitrogen per cc., which was completely absorbed by the addition of a suspension of *B. proteus* OX-19 containing 0.8 mg. nitrogen per cc. At the concentration employed in the substitution experiments, namely 0.1 mg. nitrogen per cc., saline suspensions of organisms treated for 30, 60, and 120 minutes were observed to have markedly changed their agglutinability in this antiserum. While control suspensions rapidly agglutinated on incubation at 38° with antiserum, organisms treated for 120 minutes did not agglutinate in antiserum in 2 hours under these conditions. Materials treated for 30 and 60 minutes agglutinated at rates which were much slower than the control. The addition of free histidine to antiserum plus native organisms did not appreciably inhibit agglutination.

The antibody-combining capacities of the organisms in various stages of substitution were determined quantitatively at this same region, namely antibody excess. Despite the progressive loss of agglutinability, the amounts of antibody bound by all four of the control and substituted products were identical; *e.g.*, 0.10 mg. of antigen nitrogen bound 0.05 mg. of antibody nitrogen when 1 cc. aliquots of bacterial suspension were incubated with 1 cc. aliquots of antiserum.

[1] Cohen, S. S., unpublished data.

The antibody-combining capacities of native and altered organisms were then determined in the entire range from antibody excess to antigen excess. Using the four types of organisms defined in Fig. 1, it was found that despite the marked differences in pH-mobility curves and in the qualitative aspects of agglutination, the curves of antibody N bound plotted against antigen N bound were identical in regions of antibody excess, equivalence, and antigen excess. This common curve is presented in Fig. 2. Furthermore, in region of antigen excess, slightly past the equivalence zone, organisms treated for 3 hours absorbed all agglutinins capable of reacting with native organisms.

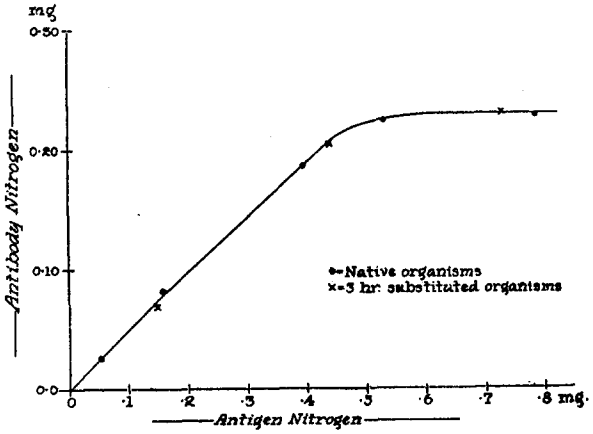


FIG. 2. The antibody-combining curve of the four types of organisms described in Fig. 1. Experimental values are given only for 0 and 3 hour organisms.

It was considered that an explanation for the marked difference in agglutinability noted in antibody excess might be derived from the hypothesis that two types of chemical groups or regions on the organism were essential for cross-linkage by agglutinating antibody. For purposes of simplicity, let us assume that these groups are amino and carboxyl groups, as in Fig. 3(*a*). Thus antibody might still bind according to the same quantitative relations with one unaltered type of group but could not bind at both ends to provide a lattice since the other essential grouping was blocked. This is represented in Fig. 3(*b*). The following experiment, represented in Fig. 3(*c*), was considered pertinent to the validity of this hypothesis:—

A suspension of non-agglutinating substituted organisms at 0.10 mg. nitrogen per cc. was added to an equivalent amount of antiserum. After incubation for 30 minutes at 38°, the non-agglutinating complex of antigen plus antibody was sedimented in the centrifuge and washed several times with saline until agglutinins were not detectable in the supernatant fluids. The complex was smoothly resuspended in saline and small amounts of a suspen-

sion of unaltered organisms were added. Within 30 minutes, agglutination took place in which all the particulate elements participated. When similar amounts of substituted organisms were added to the non-agglutinating complex of substituted organisms and antibody, agglutination did not take place. Agglutination did not occur when substituted and unsubstituted organisms were mixed in the absence of antiserum. On addition of antiserum to this latter mixture, total agglutination took place.

The washed non-agglutinating complex of antibody and substituted organism was incubated in saline at 38° for 2 hours. The supernatant fluid after centrifugation was tested

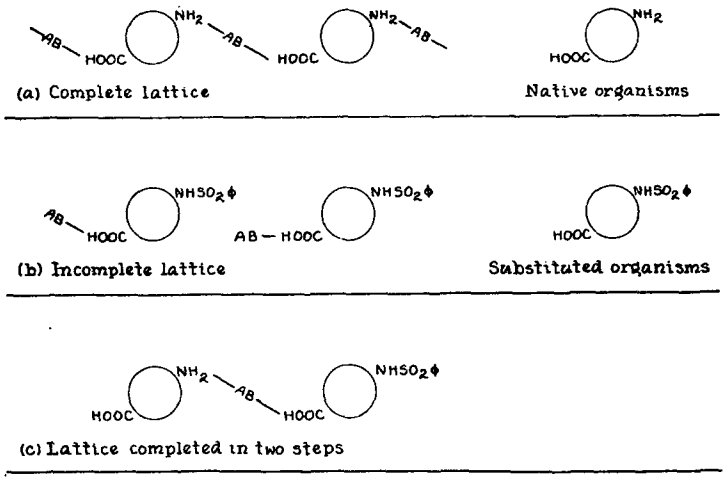


FIG. 3. A schematic representation of a hypothesis of the reaction of antigen and antibody (AB). The two ends of antibody, A and B, are so differentiated that A can combine with basic groups only, and B with carboxyl groups only. In Figs. 3(*a*) and 3(*c*), both types of groups are provided. In Fig. 3(*b*), the substitution of basic groups prevents the A portion of antibody from fulfilling cross-linkage and lattice completion as in Fig. 3(*c*). It is assumed that at least twenty pairs of groups exist in native organisms.

for agglutinins. Since they were not present, it was concluded that dissociation of antibody did not occur under these conditions.

In the course of the estimations of the antibody-combining capacities of the native and altered organisms throughout the entire range from antibody excess to antigen excess, it was noted that substituted organisms did agglutinate readily at very high concentrations of antigen, while native organisms failed to agglutinate at the same high concentration. When these experiments were repeated with diluted antiserum, it was observed that the agglutination of both types of organisms occurred in zones. The optimal agglutination region of altered organisms was at a concentration approximately 9 times greater than that of unaltered organisms. The altered organisms referred to are character-

ized by the disappearance of the imidazole ionization in their pH-mobility curve.

The following experiment was devised to test whether the agglutination of a complex of substituted organisms plus antibody would show similar zone phenomena on addition of substituted or unsubstituted organisms:—

To 2 cc. of undiluted anti-serum was added 0.4 cc. of substituted organisms, at a concentration of 0.35 mg. nitrogen per cc. After incubation for 30 minutes at 38°, the complex was sedimented, washed with saline several times, and finally smoothly resuspended in saline to 2 cc. Serial dilutions of the native or altered organisms were added at the same nitrogen concentration in 0.1 cc. amounts to 0.1 cc. aliquots of the non-agglutinating complex. The tubes were incubated at 38° and read in 60 minutes for the intensity of agglutination.

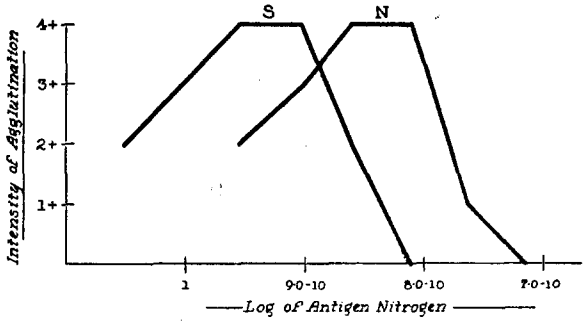


FIG. 4. The zones of agglutination when native or substituted organisms are added to a complex of substituted organisms plus antibody, S represents substituted, N native organisms.

The data plotted in Fig. 4 show that similar zone phenomena were obtained with this system as well, and that the quantitative relations of the concentration optima were unchanged, being approximately 9:1.

## DISCUSSION

The chemical modification of *B. proteus* OX-19 has resulted in intermediate products and end products whose qualitative behavior in agglutination phenomena is grossly different from that of native organisms. Nevertheless it has proven impossible to distinguish these materials by means of quantitative determinations of antibody-combining capacity. This apparently unusual behavior suggested a hypothesis and an experiment which might seem pertinent to its proof, namely the stepwise agglutination of the complex of substituted organisms plus antibody by native organisms.

An analogous stepwise agglutination has been observed by Heidelberger and Kabat (14). A resuspended complex of pneumococci plus antibody, prepared in antibody excess, was rapidly agglutinated by the addition of new organisms. The essential difference between their experiments and those presented above

resides in the inefficacy of substituted organisms in agglutinating the complex in contrast to the action of native organisms.

A hypothesis accounting for this situation is outlined in Fig. 3 in a simplified form. According to this hypothesis, an antigenic region or group (possibly a basic group), which can be blocked by benzene sulfonyl chloride, is essential for the full reactivity of the *B. proteus* OX-19 agglutinin in effecting agglutination. Other groups or regions unaffected by this reagent permit the binding of one end of the antibody. A supply of unsubstituted bacterial groups now enables binding to the other reactive site of antibody and larger clumps are formed. Thus it is suggested that at least two types of reactive regions, both of which are essential for agglutination, exist in *B. proteus* OX-19 and its agglutinin. This is a possibility raised by the lattice theory of agglutination, quite apart from these experiments.

An alternative hypothesis to account for these phenomena stresses the fact that, at pH 7, the negative charge of the substituted organisms is approximately twice that of native organisms. This might repress the continuing interaction of sensitized substituted cells as a function of the increase in electrostatic repulsive forces of the reaction participants. Thus, although antibody could still be bound, the net effect might be the formation of smaller clumps than is necessary for visible agglutination. It may be pointed out, however, that many organisms of appreciably higher negative charge than substituted organisms agglutinate readily.

Objections may be marshalled for both hypotheses and it is not expected that a definitive choice can be made in this complex system. Also other hypotheses may adequately explain the phenomena. The hypothesis of two essentially different chemical groups both of which are necessary for lattice formation is undoubtedly more easily tested in crucial experiments on simpler antigen-antibody systems. The system in which these observations were made was chosen initially solely because of its practical significance.

If one proceeds on the assumption that the observed effects are specific, *i.e.* that the first hypothesis holds, several additional comments are in order. The quantitative relations observed in the zones of optimal agglutination suggest, as one explanation, that 90 per cent of an essential grouping have been substituted. Whether this represents a residual 10 per cent of unsubstituted cells or a residual 10 per cent of unsubstituted groups per cell may be tested by a statistical analysis of cellular behavior in an electric field. Qualitatively, at least, the latter appears to be true, since in the isoelectric region, the most sensitive region, the initial and final products appeared to be markedly homogeneous with respect to their mobilities. The intermediate products were not as homogeneous, indicating differing degrees of substitution per particle. This is of interest in view of the experiments of Miller and Stanley on substituted tobacco mosaic virus (8, 15) in which electrophoretic homogeneity was observed in all stages of substitution.

It is apparent that the microelectrophoretic technique provides a sensitive quantitative method for the examination of particular groups on a bacterial surface and for following the course of their substitution. This substitition would not be subject to analysis by any other method known at present and hence the combined techniques will probably be of assistance in the clarification of serological behavior.

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## SUMMARY

The chemical substitution of a bacterial surface has been effected by the addition of benzene sulfonyl chloride to *B. proteus* OX-19. The substitution of imidazole and amino groups on the bacteria has been followed by the microelectrophoretic method. Concomitant changes in the agglutinability of substituted organisms have been observed, without appreciable change in the antibody-combining capacities of the organisms. The significance of these observations has been discussed.

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