THE MODIFICATION OF ANTIBODIES BY FORMALDEHYDE

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Interaction of antigen and antibody forms upon the antigen surface[1] a more or less complete film of antibody-globulin held by specific stereochemical forces. The various serological reactions are consequent upon the properties of this surface deposit and the special environing conditions. Thus the antibody-globulin surface deposit under ordinary conditions of test has a surface potential difference below and cohesiveness above the mean critical values for stability, (agglutination occurs (1, 2)); upon the antibody-globulin surface polymorphonuclear and mononuclear phagocytes tend to spread, (phagocytosis occurs (3, 4, 5)); and the antigen-antibody complex is a good adsorbent for the serum components known as complement, (complement-fixation occurs (6, 7)).

Study of the conditions of formation and the physical-chemical attributes of these antibody-globulin films is then clearly of strategic value in gaining further understanding of the serological reactions. The property of the surface film susceptible of most accurate measurement is the electrokinetic P.D. (zeta potential). In undertaking certain studies on this factor we have been aided by possession of strains of bacteria which themselves have only minimal zeta potentials over a wide range of pH, and therefore afford a minimal risk of confusing the zeta potentials of the films with those of the bacterial surfaces (2, 8).

In the present study antibodies before and after combination with antigen have been treated with formaldehyde, and the effects upon

[1] The term "antigen surface" is used without intended implication as to whether the antigen is in molecular dispersion, in micellae, or forms part of a cell surface, and as to whether or not the reaction is stoichiometric.

specific agglutination and upon the isoelectric points of the antibody films have been determined. The data obtained have yielded evidence of the presence of basic groups in the antibody film upon which substitution (or addition) can be effected. Substitution (or addition) upon these groups in the uncombined antibody is possible without greatly interfering with its specific chemical affinity for antigen. It appears, however, that this reaction is not without effect upon the physical properties of the antibody film.

Experimental Methods

Bacteria Used.—In earlier experiments a strain of *Bact. coli* and a strain of *Bact. dysenteriae* (Flexner type) were used; in the experiments graphed or tabulated, *Bact. pullorum* and a non-flagellate typhoid bacillus (Strain 0901). All the bacterial strains were smooth. These strains have only minimal zeta potentials in dilute buffers over a wide range of pH (2, 8). The bacteria were grown in infusion broth, washed, and resuspended in 0.85 per cent NaCl solution.

Chemicals.—Merck's reagent formalin; this was neutral to litmus except where specified as made neutral to phenolphthalein. Walpole's acetate buffers were used in M/50 concentration over the range of pH 3.6 to 5.6. The pH values were determined colorimetrically, or in the later work with a quinhydrone electrode, taking the value for 0.1 N HCl as pH = 1.08.

Glassware.—Glassware was cleaned in concentrated sulfuric acid-potassium dichromate solution, tap water, and distilled water.

Sensitization.—The bacteria were sensitized except when otherwise specified with homologous immune rabbit serum. Given volumes of bacterial suspension in 0.85 per cent NaCl solution were mixed with serial dilutions of antiserum. All dilutions of antiserum were made in 0.85 per cent NaCl solution. The tubes were incubated for 2 hours at 37° and refrigerated overnight. Agglutination readings were made. All tubes were then centrifuged until virtually complete sedimentation had taken place. The supernatant fluids were decanted and the sediments were shaken up in about 3 ml. each of 0.85 per cent NaCl solution. All tubes were again centrifuged and the supernatants decanted. The sediments were again shaken up in a minimal amount of 0.85 per cent NaCl solution and each of the suspensions was divided into two portions. To one portion was added 2 ml. of saline and to the second portion 2 ml. of formalin solution. In Table I, Experiment 5, 37 per cent formaldehyde solution was added; in Experiments 1 to 4, and 6 to 9 inclusive, 18.5 per cent formaldehyde solution was added, and in Experiment 10, approximately 9 per cent. The sensitized bacteria, washed and suspended in 0.85 per cent NaCl, are designated in Table I and in the graphs below as Series A; the sensitized, washed bacteria treated with formalin are designated as Series Ax. Both series were allowed to stand 20 to 30 minutes at room temperature. All tubes were centrifuged and the supernatants decanted. The sediments were shaken up in saline and again centrifuged. The supernatants were decanted.

2 drops of saline from a 1 ml. pipette were added to each tube. The tubes were arranged in a row in a rack with the control tubes of bacteria plus saline without serum, in the center. The rack was shaken uniformly until the control tubes showed even suspensions. The sensitized bacteria resuspended in aggregates, the size and resistance to dispersion of which affords a rough estimate of the cohesiveness of the bacteria. Isoelectric point estimations were then made.

In order to determine whether specific combination between antigen and antibody occurred if the immune serum had been previously treated with formalin, the following procedure was followed in addition to the above. The serum dilutions were made up to a given volume, usually 0.5 ml. To each tube the same volume of neutral formaldehyde solution was added, and allowed to stand 20 to 30 minutes at room temperature. In Experiments 1 to 5 the concentration of HCHO was 37 per cent, in Experiments 6 to 9, 18.5 per cent, and in Experiment 10, 9 per cent. To these tubes were added the bacteria suspended in neutral formaldehyde solution of 18.5 per cent concentration (Series B) or in saline (Series D). (In Experiment 5, Table I, the bacteria were suspended in 37 per cent formaldehyde, and in Experiment 10 in 9 per cent formaldehyde. In Experiment 1 the HCHO was neutral to phenolphthalein.) The tubes were incubated for 2 hours in a 37° water bath and refrigerated overnight. Agglutination readings were made, the tubes were centrifuged, the sediments washed in saline solution, and "resuspension" readings were made as described above. Isoelectric points were estimated.

In a few experiments also a set of serial serum dilutions in saline were made and a suspension of bacteria in formalin solution was added. Such series are designated Series C.

The reversibility of the formaldehyde antibody combination was tested in two special experiments. The isoelectric point of the sensitized HCHO-treated bacteria was estimated as explained below. The bacteria were then washed three times and the isoelectric points estimated after each washing. Under these conditions the washings did not cause appreciable reversion of the isoelectric points toward those of the untreated antibody films. This result does not imply, however, that longer contact of the sensitized HCHO-treated bacteria with saline would not have brought out evidence of slow reversibility.

Cataphoresis and Estimation of Isoelectric Points.—The Kunitz modification of the Northrop-Kunitz microcataphoresis cell (9) was used with dark-field condensor and Bausch and Lomb 8 mm., 0.50 n.a., 21 × objective. Three readings were taken for each suspension at the two stationary levels; *i.e.*, 0.21 and 0.79 of the inside depth of the cell. Radio B batteries were used; the applied potential of 125 volts gave a gradient through the cell of about 6.5 volts per cm.

The buffer series were made up so that successive members differed by 0.4 of a pH unit. The cell was first washed with the buffer to be used and then a drop of bacterial suspension in about 1 ml. of buffer was washed into the cell with about 2 ml. of buffer. Two successive buffers were found in one of which the particles migrated to the anode and in the other to the cathode; the isoelectric point was then estimated from the relative mean velocities of the two pH values.

TABLE I

No. of experiment	Name of organism	Serum	Interval since serum drawn	Agglutination titre of serum	Agglutination titre of serum treated with HCHO	Tube No.	pH of isoelectric point				
							A	Ax	B	C	D
1	*Bact. pullorum* (smooth)	Rabbit 18-84 homologous	6 days	1:256	None or slight	1	5.0	4.3	4.5	4.3–4.2	4.4
						2	5.0	4.3	4.45	4.3–4.2	4.4
						3	5.0	4.3	4.45	4.4	4.4
						4	4.9+	4.2			
2	*Bact. pullorum* (smooth)	Horse typhoid	6½ yrs.	1:256	None or slight	1	4.95	4.1	4.15–2		
						2	4.9	4.1	3.7–3.8		
						3	4.5	3.6?	3.7–3.8		
						4	4.4	3.6?			
3	*Bact. pullorum* (smooth)	Cow triple typhoid	6½ yrs.	1:256	None or slight	1	4.9–5.0	4.3	4.25	4.2–4.3	4.25
						2	5.0–5.1	4.3	4.3	4.2–4.3	4.25
						3	5.1	4.35	4.35		4.35
						4	5.05	4.35			
4	*Bact. pullorum* (smooth)	Rabbit 18-87 homologous	2 wks.	1:4096	1:1024 Prozone	1	5.1	4.35	4.6	4.5	4.5
						2	5.0	4.3	4.45	4.45	4.5–4.4
						3	4.9	4.3	4.4	4.4	4.4–4.35
						4	4.7	4.3–4.2	4.3–4.4	4.4–4.3	
						5	4.8	4.4	4.3	4.35	
5	*Bact. pullorum* (smooth)	Rabbit 18-84 homologous	4 wks.	1:4096	1:256	1	5.2–5.1	4.4–4.5	4.5	4.4–4.5	
						2	5.0–5.1	4.4–4.5	4.4	4.4	
						3	5.0	4.4	4.4	4.4–4.3	
					Prozone	4	4.75	4.35	4.2	4.4	
						5	4.7	4.3			

6	*Bact. pullorum* (smooth)	Rabbit 18-84 homologous	9 wks.	1:1024	1:256	1	5.2+	4.45	4.5		
						2	5.2	4.45	4.5–4.4		
						3	5.2–5.1	4.4	4.45		
					Prozone	4	4.9	4.3	4.35		
7	*Bact. pullorum* (smooth)	Rabbit 42 homologous	7 wks.	1:1024	1:256	1	5.4	4.5	4.2	4.2–4.3	4.2
					Prozone	2	5.3	4.4–4.5	4.3	4.3	4.3
8	*Bact. typhosum* 0 901 (smooth)	Horse 9391B, Rawlings typhoid	5 wks.	1:4096	1:4096	1	4.6	4.0			4.1
					(Longer	2		4.1			4.1–4.2
				(Prozone)	prozone)	3	4.6	4.0			4.1
						4	4.5	4.0?			
9	*Bact. typhosum* 0 901 (smooth)	Rabbit 7	3 days	1:4096	1:1024	1	5.0				4.5
						2	4.9				
					Prozone	3	4.9				4.4
10	*Bact. pullorum* (smooth)	Rabbit 18-84	7 wks.	1:4096	1:1024	1	5.1–5.0	4.8	4.7		
						2	5.1–5.0	4.85	4.7		
					Prozone	3	5.0	4.75	4.7		

RESULTS

The essential results are shown in Figs. 1 to 4[2] and in Table I. The isoelectric points of these bacteria when maximally sensitized with rabbit antisera fell in the range pH 5.0 to 5.2. These values are about 0.6 pH unit lower than those found for the sensitizing films formed by antibodies against certain other antigens (3, 11). It appears that the sensitizing antibody films formed upon various antigens may have isoelectric points as in this case similar to, or more alkaline (10, 3, 11), or more acid (8) than those of the normal globulins of the species from which the serum is obtained. This point will be developed more fully in a later paper.

Treatment with formaldehyde under the conditions of these experiments has shifted the isoelectric points of the sensitizing films by about 0.6 to 0.8 pH unit toward the acid side. A shift of this approximate magnitude occurred whether the formaldehyde was allowed to react with the antibody film after its formation on the antigen surface, (Figs. 1 to 4, Series Ax), or whether the antisera were mixed with formaldehyde before combination with antigen, (Figs. 1 to 4, Series B, C, and D).

When the antisera were treated with formaldehyde before combination with antigen, agglutination was consistently inhibited in the highest antiserum concentrations, *i.e.* agglutination prozones appeared; see Fig. 1, Series B, C, and D, Fig. 2, Series B and C, and Table I. Cataphoresis and resuspension both showed that these agglutination prozones were due to changes in the physical properties of the sensitizing films rather than to failure of antigen-antibody combination. For the bacteria so sensitized showed appreciable migration velocities in the cataphoresis cell on both sides of their isoelectric points, and the bacteria cohered in the resuspension reaction, indicating that the sensitizing films were present on the bacterial surfaces and merely altered in their properties by the treatment with formaldehyde. Further analysis of the mechanism by which combination with HCHO affects agglutination, and inquiry as to its possible effect on phagocytosis we have not yet attempted.

The end-titre of agglutination was regularly somewhat reduced

[2] The points in Figs. 1 to 4 are joined by lines merely to aid in reading.

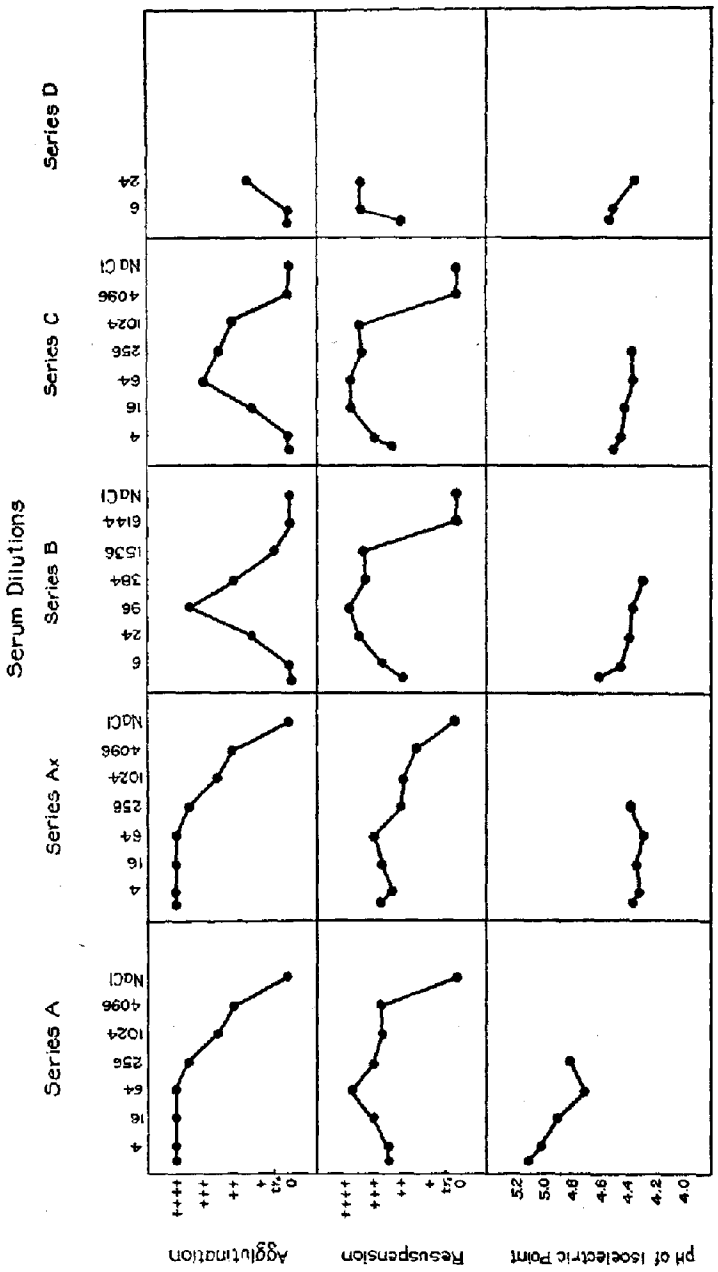

FIG. 1. The effects of formaldehyde treatment on the agglutination, resuspension, and the isoelectric points of sensitized bacteria. Other data relevant to this experiment are given under Experiment 4 in Table I.

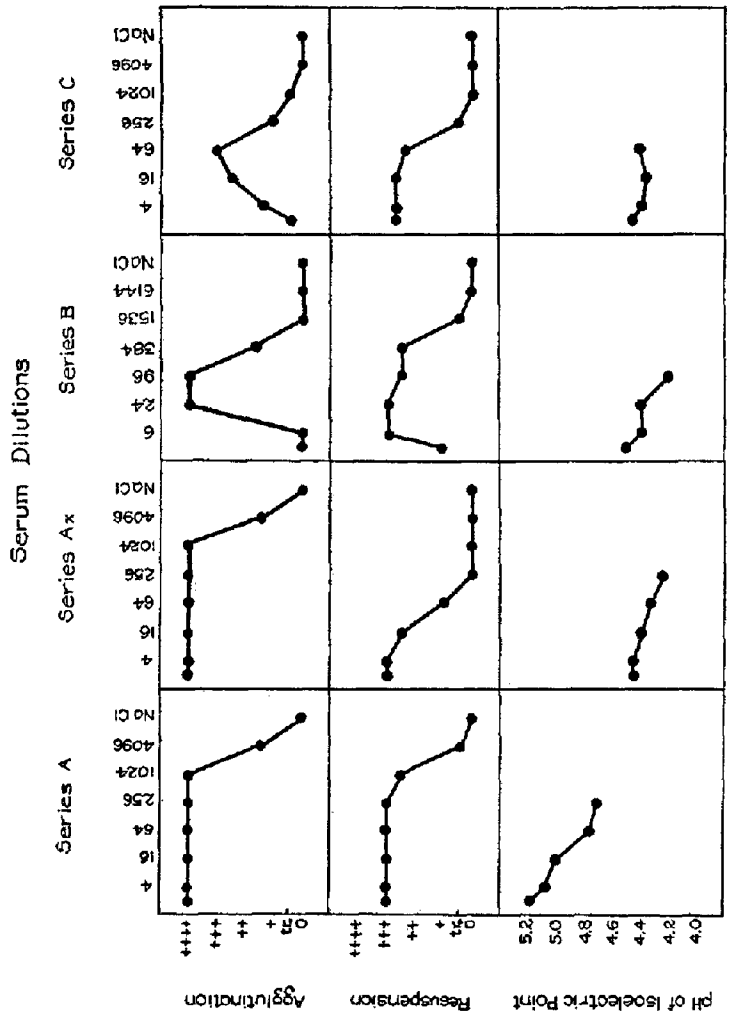

FIG. 2. The effects of formaldehyde treatment on the agglutination, resuspension, and the isoelectric points of sensitized bacteria. Other data relevant to this experiment are given under Experiment 5 in Table I.

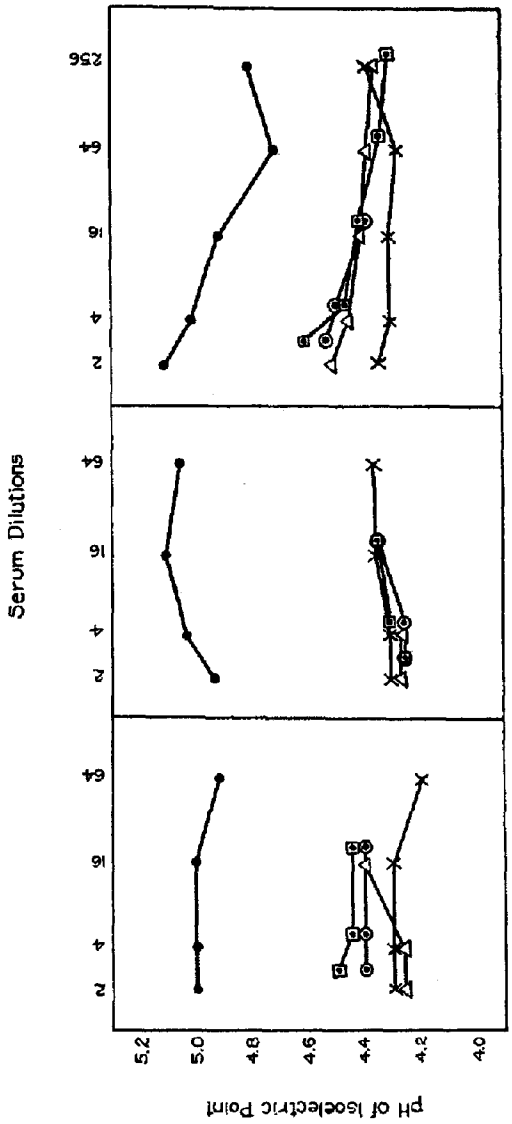

FIG. 3. Effect of formaldehyde treatment on the isoelectric points of sensitized bacteria. The basicity of the sensitizing antibody-protein is diminished in each case by HCHO treatment, as indicated by the shift of the isoelectric point by 0.6 to 0.8 pH unit toward the acid side.

●, Series A, bacteria sensitized with immune serum.
×, Series Ax, bacteria sensitized, then treated with HCHO.
⊡, Series B, bacteria suspended in HCHO, and sensitized with immune serum treated with formaldehyde.
△, Series C, bacteria suspended in HCHO, and sensitized with immune serum.
⊙, Series D, bacteria suspended in saline, and sensitized with immune serum treated with HCHO.
Other data relevant to these experiments are given under Experiments 1, 3, and 4, respectively, in Table I.

when the antisera were treated with HCHO before combination with antigen; see Figs. 1 and 2 and Table I. Whether this reduction was due to a slight loss of combining power of antibody for antigen due to HCHO treatment, or merely to the alterations in physical properties is not clear.

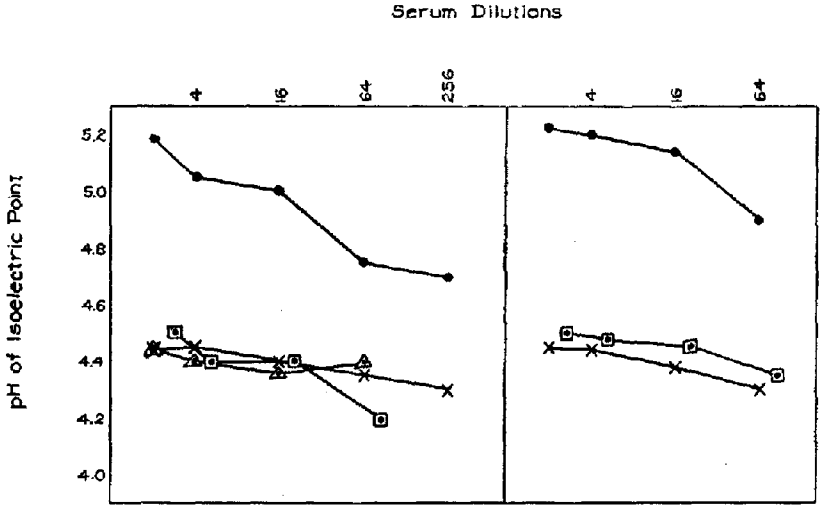

FIG. 4. Effect of formaldehyde treatment on the isoelectric points of sensitized bacteria. Symbols and interpretation as in Fig. 3. Other data relevant to these experiments are given under Experiments 5 and 6, respectively, in Table I.

DISCUSSION

Chemical combination of formaldehyde with proteins, involving alterations in the physical properties of the proteins, was demonstrated by Blum (12) in 1896. Blum formalinized a mixture of ovoalbumin and ovomucoid; the resulting proteins were not coagulated by heat and preserved their solubility in water after precipitation by alcohol or acetone. Benedicenti (13) extended these observations in several directions. He allowed gelatin, fibrin, casein, blood serum, and egg albumin to stand in contact with formol and determined titrimetrically a decrease in formaldehyde content of the solution. The "formaldehyde proteins" were in important respects different from the original proteins. Thus gelatin became hardened and insoluble, blood serum underwent gelation, fibrin and casein lost their ability to swell and to be digested. Schwarz (14) performed elementary analyses upon such compounds of proteins with formaldehyde.

Compounds of formaldehyde with amino acids were studied by Schiff (15).

He interpreted the formation of these compounds as a substitution of $= CH_2$ for $= H_2$ on the free amino groups. On the basis of Schiff's work, Sørensen (16) elaborated the well known formol titration. More recent investigators have found evidence that the combination of HCHO with amino groups is more complex than the simple substitution of a methylene group for hydrogen.[8] However, there is general agreement in the literature that the principal chemical effect of HCHO on proteins, protein decomposition products, and amino acids is a combination with free amino groups which weakens their basicity.

Kossel (21) and Iodidi (22) have sought to identify the basic groups in proteins which react with HCHO. According to both investigators the $-NH_2$ group in the ω-position in lysine combines with formaldehyde, but the amino group in the guanidine nucleus in arginine is inert. Both agree that HCHO combines with the imino group in the imidazole-ring of histidine; according to Kossel and Gawrilow the imino nitrogen in proline when built into protein is not reactive to formaldehyde.

Pirie and Pinhey (23) and Harris (19) have found evidence of a combination at alkaline reactions between HCHO and the $-SH$ group of cysteine which results in a perceptible weakening of the acidity of the $-SH$ group and a shift of the pK to increased basicity.

The rate of gelation of blood serum in contact with HCHO and the rate of change in solubility in ammonium sulfate of the blood proteins in formaldehyde solutions has been studied by Henley (24). Both changes occur slowly. With a given undiluted serum the rate increases with the HCHO concentration.

The action of 0.5 per cent formaldehyde solution at 37°C. on certain proteins, proteoses, and tryptic digestion products has been investigated by Freeman (25). A decrease in amino nitrogen content as determined by the Van Slyke method was observed after formaldehyde treatment in all the solutions investigated; in no case was loss of amino nitrogen complete, however. Holden and Freeman (26) demonstrated progressive loss of amino nitrogen when amino acids were incubated with 0.5 per cent formaldehyde solutions at 37°C. They showed the combination of meta-protein with HCHO to be to some extent reversible.

Zeiger (27) studied the adsorption of dyes by histological sections of tissue after fixation with formalin as compared with those fixed with alcohol. The reaction at which the tissue elements reversed the sign of their charge as indicated by dye adsorption was shifted toward the acid side by formalin fixation.

Landsteiner and his associates (28) have studied the action of HCHO on the antigenic properties of serum proteins. Rabbit serum was mixed with an equal volume of commercial formalin and allowed to stand at room temperature for 20 hours; the proteins were precipitated with alcohol, washed, triturated and reinjected into rabbits. Antisera were thus produced; these fixed complement specifically in the presence of formalinized rabbit protein but not with formalinized pro-

[8] See Reiner and Marton (17), Bergmann (18), Harris (19), and Levy (20).

tein from the horse, cattle, or fowl. In later work (29) horse sera were formalinized and injected into rabbits. The sera of rabbits injected either with native or formalinized horse serum precipitated and fixed complement with either native or formalinized horse serum but not with the sera of other species.

An extensive investigation of formalinized antisera was made by von Eisler and Löwenstein (30). The power of such antisera to neutralize specific exotoxins was unaffected or suffered only slight loss. The amboceptor function (bacteriolytic and hemolytic), and the precipitating function of agglutinins and precipitins were greatly reduced; specific combination of agglutinin and agglutinogen occurred, however. Baivy (31) and recently Braun (32) have inhibited the complement fixing, agglutinating, and precipitating action of various antisera by treatment with formaldehyde.

Electrophoresis affords a method of studying proteins adsorbed on surfaces and thus of obtaining data not yielded by ordinary titrimetric procedures. This method has proven peculiarly useful in studying the protein deposited upon bacterial surfaces by interaction with normal and immune serum.

A major difficulty in such studies arises from the fact that the surfaces of many or most bacteria contain materials which are themselves sources of measurable zeta potentials. The isoelectric points of such bacteria sensitized with increasing concentrations of serum fall along ascending curves which rise from the values for the unsensitized bacteria toward those of the sensitizing proteins (2, 11). When strong homologous antisera are used for sensitization such isoelectric point curves may reach levels such that further increase in concentration of serum does not cause further rise in the pH of the isoelectric points (3, 11); whether or not such plateaus represent conditions in which the isoelectric points are due solely to the film-forming substances we do not at present know. At all events, however, normal or heterologous sera rarely sensitize sufficiently to yield such plateau values, and the resulting isoelectric points represent indeterminate values intermediate between those of the substances in the bacterial surface and those of the film-forming substances. Discovery of these bacterial strains with minimal zeta potentials over a wide range of pH therefore affords means of studying adsorbed substances under the many conditions in which the adsorbed film is incomplete.

Electrophoresis has not previously been used, so far as we are aware,

to study the reaction of proteins with formaldehyde. The results here recorded are in essential accord with those obtained by other methods; the protein shows decreased basicity, here evidenced by a shift in the isoelectric point; concomitantly there occur changes in physical properties evidenced in this study by decreased tendency to agglutinate. It is noteworthy that the effects of formaldehyde upon isoelectric points and agglutination were similar whether HCHO treatment preceded or followed combination of antibody with antigen. This result, together with those of von Eisler and Löwenstein, and of Braun, would seem to warrant the conclusion that the HCHO-combining basic groups of antibodies are not involved, or are involved only in a minor degree, in the specific chemical union of antigen and antibody.

SUMMARY

Certain strains of bacteria which have only minimal zeta potentials over a wide range of pH, and upon which surface deposits can be formed, afford a favorable means of studying certain chemical and physical properties of the surface deposits.

Films of specific antibody-globulin upon these bacteria possess basic groups which can combine with formaldehyde. Combination of these groups with HCHO under the conditions of the present experiments shifts the isoelectric point of the sensitizing film toward the acid side by about 0.6 to 0.8 pH unit, and reduces the agglutinating tendency of the sensitizing film.

Antibodies may be formalinized before combination with antigen without marked change in their specific combining affinities. The properties of the sensitizing films are similar whether formol treatment occurs before or after the antigen-antibody combination.

The nature of the basic groups has been discussed.

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