THE EFFECT OF COMBINATION WITH DIAZO COMPOUNDS ON THE IMMUNOLOGICAL REACTIVITY OF ANTIBODIES

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Previous investigations into the chemistry of antigen-antibody reactions have dealt largely with antigens. The effect of chemical modification on the immunological reactivity of protein antigens has been extensively studied by Landsteiner and his coworkers; and considerable progress has been made in the chemical identification of the antigenic constituents of bacteria, red blood cells and other complex cellular antigens. In comparison, little is known of the chemistry of antibodies. There is a considerable body of evidence which indicates with a strong degree of probability that antibodies are proteins associated with the globulin fraction of the serum which combine with the homologous antigen to form a surface layer of protein, somehow rendered insoluble by this combination. These conclusions are supported by the chemical properties of the antibody in solution, the surface properties of antigen-antibody compounds, their flocculating tendencies and immunological behavior, and by the N content of antigen-antibody aggregates.[1] Little has been done, however, to identify either the chemical groups in antibodies which endow them with their specific reactivity, or those which are responsible for the characteristic properties of antigen-antibody compounds: susceptibility to aggregation and to phagocytosis, and the extraordinary avidity for complement which results in complement fixation, hemolysis, bacteriolysis and bactericidal action. The

[1] Literature summarized by Marrack; Heidelberger; Mudd, Lucké, McCutcheon and Strumia; Eagle (1930).

present paper, dealing with the effect of various diazo compounds on the reactivity of antibodies, represents an attempt in that direction. The groups in protein which react with diazo compounds are not only the imidazole ring of histidine and the phenyl group of tyrosine (a phenolic derivative) as described by Pauly, but probably include the aliphatic NH_2 groups, the proline and hydroxyproline NH group, and the indole group of tryptophane (Eagle and Vickers). The immunological reactivity of the "coupled" antibody globulin should therefore provide a clue to the extent to which these groups in antibody participate in its combination with antigen, and the extent also to which they are responsible for the characteristic properties of the antigen-antibody complex.

Breinl and Haurowitz have found that antibodies are completely destroyed by diazotized aniline, atoxyl or metanilic acid. Reiner, however, as well as Bronfenbrenner, Hetler and I. O. Eagle, report little or no destruction of horse serum antibodies despite the complete loss of their species specificity.[2] As will appear in the following, the apparent discrepancy is due to differences in the degree of coupling; and particular interest attaches to the partially inactivated antibodies of peculiarly modified reactivity to be here described.

Methods and Materials

The antisera studied were horse antipneumococcus serum and refined globulin; horse diphtheria antitoxin serum and globulin; horse and rabbit antityphoid sera; rabbit antisera *vs.* sheep red blood cells, horse serum, and egg albumen; and the reagin of Wassermann positive human sera.[3] There were thus included representative sera from

[2] That is, the coupled horse antibody preparations no longer reacted immunologically as horse serum protein.

[3] We are indebted to the Mulford Laboratories, Glenolden, Pennsylvania, for their generous cooperation in furnishing large quantities of antipneumococcus serum, diphtheria toxin and antitoxin, and horse antityphoid serum for use in these studies; to the Massachusetts State Antitoxin Laboratory for their kindness in supplying antipneumococcus serum; to Dr. Walther Goebel, of The Rockefeller Institute for Medical Research, for a generous supply of Type I and II pneumococcus carbohydrate, and to the Wassermann laboratories of The Johns Hopkins Hospital, Baltimore, and the Graduate Hospital, Philadelphia, for normal and Wassermann positive human serum.

several animal species, giving agglutination, precipitation and complement fixation, animal protection, hemolysis and hemagglutination with the homologous antigens; and these antigens included bacteria, red cells, carbohydrate, toxin, protein and lipoid.

Coupling of the Antisera with the Diazo Compound.—The experiments were greatly facilitated by a technic which allowed the reaction between the diazo compound and the serum to be terminated after any desired interval. By taking aliquot samples, it was possible to follow the progressive change in the reactivity of the antibody as more and more groups combined with the diazo compound. Essentially the same method was used throughout.

The antiserum and *e.g.* diazotized and neutralized sulfanilic acid were mixed in the proportions indicated in the various tables. Samples were withdrawn at intervals ranging from 15 seconds to 24 hours, and to each sample was added a M/7 solution of Na sulfanilate or metanilate in twice the quantity necessary to combine with the diazo compound originally present in the sample. As shown by tests with α-naphthol, any excess diazo compound combined rapidly and completely (>99.9 per cent)[4] with the sulfanilate; and such a mixture usually remained unchanged as regards antibody activity after 24 hours at room temperature or at 37°C. Occasionally, however, a mixture of Na sulfanilate and diazosulfanilate caused a slight but definite decrease in the antibody activity of dilute antiserum.

Diazotized Sulfanilic Acid.—For 200 cc. of a M/7 solution (the amount usually prepared at one time), 5 gm. of sulfanilic acid were treated with 1.75 gm. of anhydrous Na_2CO_3 dissolved in 75 cc. of water, and with 2.1 gm. of $NaNO_2$ dissolved in 10 cc. of water. The solution was cooled in ice water and diazotized at 3–5°C., using a mechanical stirrer, by the slow addition of 35 cc. of N/1 HCl from a separatory funnel, the tip of which was immersed in the liquid. The pale yellow, somewhat acid diazonium solution was brought to pH 7.2–7.6 with N/1 NaOH. Since the solutions coupled with the pH indicators ordinarily used, it was found necessary to prepare a somewhat yellowish solution of bromthymol blue and note the evanescent color formed on adding a few drops of this to a little of the diazo solution contained in a small tube. 8 cc. of 0.5 M phosphate buffer containing 4 parts of Na_2HPO_4 to one part of KH_2PO_4 were then added, and the whole diluted to 200 cc.

Other Diazo Compounds.—Diazotized arsanilic, *p*-aminobenzoic, 1-amino-2-

[4] The diazo solution originally gave a brilliant red color in a 1:8000 dilution on testing with α-naphthol. Within 1 minute after the addition of the sulfanilate, a 1:10 dilution gave only a trace of color, and within 5 minutes, the reactivity with α-naphthol had almost completely disappeared.

naphthol-4-sulfonic and 4-aminotoluene-2-sulfonic acids were prepared in essentially the same manner.

Titration of the Antibody Content of the Several Sera.—(*a*) The original serum, (*b*) a control containing serum incubated with an inactive mixture of the Na sulfanilate and diazosulfanilate and (*c*) samples of serum allowed to react with the diazo compound for varying periods of time before the addition of Na sulfanilate to terminate the reaction, were all tested for antibody content by the methods to be described in the following pages.

Diphtheria Antitoxin

The biological activity of diphtheria antitoxin can be tested either by observing the flocculation obtained on the addition of toxin (Ramon), in which case the serum: toxin ratio giving the most rapid flocculation is the index of the potency of the antitoxin, or by determining the minimum amount of serum which protects guinea pigs against some arbitrarily chosen multiple of the lethal dose of toxin. Preliminary tests with twenty-three antitoxic sera and ten lots of toxin from seven different laboratories indicated that, as reported by Ramon, Bayne-Jones, and others, and contrary to statements in the literature, the two methods of titration yield essentially similar results. The ratio of the protective titer: flocculation titer in these twenty-three sera was 1.02 ± 0.04, indicating an almost exact correlation.

Despite this parallelism, and despite the probable identity of the serum constituent giving these two reactions, the addition of Na diazosulfanilate caused a complete dissociation between the flocculating and protective properties of diphtheria antitoxin. One of six experiments yielding qualitatively similar results is summarized in Table I. The flocculating activity of the serum was definitely impaired within a short time after the addition of the diazo compound, in some experiments after as little as 30 seconds. This impairment consisted in a marked prolongation of the time required for flocculation. After a few minutes coupling, it no longer gave a definite precipitate, even after overnight incubation with toxin, but became diffusely cloudy; and on longer coupling, the antitoxin no longer reacted in any visible manner with toxin. However, the protective titer of the serum as measured in guinea pigs remained at its original level long after its flocculating activity had thus been completely inhibited. The introduction of a few diazo molecules had completely

inhibited the flocculating tendency of the antitoxin, without affecting its combining affinity for toxin. Similar results were obtained with

TABLE I

The Effect of Diazotized Sulfanilic Acid on the Immunological Reactivity of Horse Diphtheria Antitoxin

2 cc. of the N/7 diazo solution were added to 6 cc. of antiserum. 1 cc. samples were withdrawn at the stated intervals and 0.5 cc. of N/7 Na metanilate were added. The controls consisted of 0.5 cc. serum mixed with 0.5 cc. of either N/7 NaCl or a 2:1 metanilate:diazo mixture.

Duration of coupling	Degree of flocculation* 2 cc. toxin and varying quantities of the coupled serum						Guinea pig protection experiments with 0.2 cc. toxin and varying quantities of the coupled serum					Conclusion
	0.2 cc.	0.15 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.038 cc.	0.2 cc.	0.05 cc.	0.125 cc.	0.0062 cc.	0.0031 cc.	
min.												
0	Cl*	Cl	4	4	4	0	S†	S	S	S	D2	Flocculating activity completely destroyed in 4 to 16 minutes; approximately 50% of protection activity remains after 24 hrs.
1	0	0	Cl	2	2	0	S	S	S	S	D2	
4	0	0	0	Cl	0	0	S	S	S	S	D2	
16	0	0	0	0	0	0	S	S	S	S	D1	
64	0	0	0	0	0	0						
250	0	0	0	0	0	0	S	S	S	D2	D1	
Overnight	0	0	0	0	0	0	S	S	S	D2	D1	
Overnight coupling with twice as much diazo compound	0	0	0	0	0	0	D1	D1	D1	D1	D1	Complete destruction of antibody on sufficient coupling
Control with inactivated diazo solution	Cl	Cl	4	4	4	0	S	S	S	S	D2	Antibody unaffected by inactivated diazo compound

* Cl = cloudy after 24 hours at 37°C.
2,4 = degrees of precipitation after 24 hours at 37°C.
0 = no visible aggregation.
† S = survived > 4 days.
D1 = dead in 1 day, etc.

diazotized preparations of arsanilic acid, *p*-aminobenzoic acid, 1-amino-2-naphthol-4-sulfonic acid and 4-aminotoluene-2-sulfonic acid.

It is noteworthy that the progressive retardation of the flocculating activity of the serum as coupling proceeded was not associated with any significant change in the serum:toxin ratio giving the most rapid flocculation or turbidity. This optimum ratio, which is taken as the index of the serum antitoxin content, remained constant, and only the time required for flocculation or clouding to become apparent was affected. This constitutes strong evidence that all of the antitoxin was being simultaneously affected by the diazo compound (page 637).

It becomes of interest to ascertain whether the partially coupled antibody fails to flocculate on the addition of toxin because it can no longer combine with toxin, or whether, as suggested by the *in vivo* protection experiments, combination proceeds normally, and only the secondary aggregation of the toxin-antitoxin compound is inhibited. The following experiment indicates that the latter is the case. If one adds 1 L + dose of toxin to a neutralizing quantity of partially coupled antibody, and then precipitates the latter with a rabbit antiserum to horse serum, the supernatant fluid is found to be non-toxic, showing that the toxin had been precipitated along with the antitoxin. A similar experiment carried out with normal horse serum instead of partially coupled antitoxin, or with antitoxin so completely coupled that it had lost its ability to neutralize toxin *in vivo*, results in a supernatant fluid of undiminished toxin content (Eagle, 1935 *a*). It would therefore appear that partially coupled antitoxin neutralizes toxin by virtue of the fact that it can still combine with toxin; but the secondary aggregation (Ramon flocculation) is somehow inhibited by the reaction of the diazo compound with the antibody molecule.

The puzzling observation that pseudoglobulin concentrated from antitoxin sera may fail to give the Ramon flocculation reaction, despite the fact that it combines with toxin *in vitro* (Eagle, 1935 *a*) and retains its protective action *in vivo*, may well be due to some analogous chemical modification produced in the course of its salting out and subsequent dialysis. It would appear from the preceding experiments with diazo compound that only a few groups in the antibody molecule need be modified in order to inhibit flocculation completely.

Antipneumococcus Horse Serum

Four immunological reactions given by antipneumococcal sera were available for study: bacterial agglutination, the precipitation given with the type-specific carbohydrate derived from the bacterial capsule, complement fixation with a bacterial suspension and the protection of mice against multiple lethal doses of pneumococcus culture.

The agglutinating titer was determined by the following technic, using heat-killed 24 hour cultures of Type I organisms grown on 0.2 per cent glucose infusion broth, washed and concentrated to 1/5 the original culture volume.

Serum, *cc.*	0.8	0.4	0.2	0.1	etc.
0.85 per cent NaCl, *cc.*		0.4	0.6	0.7	etc.
Bacterial suspension, *cc.*	0.2	0.2	0.2	0.2	etc.

The precipitating titer of the serum against the deacetylated Type I and Type II capsular carbohydrates was similarly determined, using a 0.01 per cent solution of the carbohydrate instead of the bacterial suspension. In the protection experiments, 0.1 cc. of a 24 hour culture of the organisms in blood broth was injected intraperitoneally, and varying quantities of the serum sample to be tested were injected immediately into the opposite side of the peritoneal cavity. The animals were then observed over a 4 day period.

The diazotized sulfanilic acid caused a complete dissociation of these several properties, this despite the fact that they are reported to be due to the same antibody (Felton). The three experiments summarized in Table II are illustrative of ten others with three different sera and two different globulin preparations, and are diagrammatically summarized in Fig. 1. Qualitatively the same results were obtained with all of the other diazo compounds tested (page 620).

The first effect produced on the antipneumococcus serum (or concentrated globulin) by the Na diazosulfanilate was a disappearance of its ability to give precipitation with the specific soluble substance. This inactivation began to be apparent within a few minutes after the diazo compound was added to an equal volume of serum, and long before there was any change in either the agglutinating, complement fixing or protective titer of the serum.

As coupling proceeded, these also began to be affected. The bacterial aggregates became smaller, the characteristic large coherent

TABLE II

The Effect of Coupling with Diazotized Sulfanilic Acid on the Immunological Reactivity of Antipneumococcus Serum and Globulin

	Duration of treatment	Reactivity of the treated antisera				
		1 Agglutination (4 hrs. at 37°)	2 Centrifuge agglutination*	3 Precipitation with SSSI	4 Complement fixation with bacteria	5 Mouse protection
Experiment 1						
4 cc. of 1:2 serum + 4 cc. of diazo solution. Aliquot samples of 1 cc. were withdrawn at intervals and the reaction terminated by the addition of 1 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 1 ±	4 4 4 4 1	4 4 4 4 1 2	4 4 4 4 4 2	
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 1 ±	4 4 4 4 1	4 4 4 4 1 0	ac† ac 4 4 4 2	
	1 min. coupling	4 4 4 1 ±	4 4 4 4 1	0 1 1 1 0 0	ac ac 4 4 4 2	
	4 " "	4 4 3 1 0	4 4 4 2 0	0 0 1 ± 0 0	ac ac 4 4 2 0	
	8 " "	4 4 2 1 0	4 4 3 3 0	0 0 1 ± 0 0	ac ac 4 4 2 0	
	60 " "	4 1 1 0 0	4 3 2 1 0	0 0 1 0 0 0	ac ac 4 2 0 0	
	Overnight "	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0 0	ac ac ac ac 0 0	
Experiment 2						
15 cc. of 1:2 serum + 5 cc. of diazo solution. Aliquot samples of 3 cc. were withdrawn in intervals and the reaction terminated by the addition of 1.5 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 4 ±	4 4 4 4 ±	4 4 4 4 0 0		
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 4 4	4 4 4 4 4	4 4 4 4 2 0		
	½ hr. coupling	4 3 3 3 1	4 4 4 4 0	4 4 2 0 0 0		
	1½ hrs. "	3 2 1 0 0	4 4 4 4 0	± ± 0 0 0 0		
	2 " "	2 0 0 0 0	2 4 4 4 0	0 0 0 0 0 0		
	4 " "	± 0 0 0 0	4 4 4 ± 0	0 0 0 0 0 0		
	8½ " "	0 0 0 0 0	4 4 4 0 0	0 0 0 0 0 0		
	23 " "	0 0 0 0 0	4 4 3 0 0	0 0 0 0 0 0		

						1.6 cc.	0.4 cc.	0.1 cc.	0.05 cc.	0.25 cc.
Experiment 3										
6 cc. serum + 6 cc. of diazo solution. Aliquot samples of 1.5 cc. were withdrawn at intervals and the reaction terminated by the addition of 1.5 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 4 2	4 4 4 4 2	4 4 1 ± 0 0			S	—	S	D2
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 4 2	4 4 4 4 1	4 4 1 ± 0 0						
	1 min. coupling	4 4 4 4 1	4 4 4 4 1	2 2 ± 0						
	4 " "	4 4 4 4 ±		1 ± 0 0			S	S	D2	D1
	16 " "	4 4 4 4 0		0 0 0 0 0		S	S	S	D3	D1
	64 " "	4 4 4 3 0		0 0 0 0 0		S	S	D2	D1	
	240 " "	4 4 4 2 0	4 4 4 4 0	0 0 0 0 0		S		S	D1	
	Overnight "	4 4 2 0 0	4 4 4 2 0	0 0 0 0 0		S	D2	D2		
	Overnight coupling with twice as much diazo solution	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0		D1	D1	D1		

* *Cf.* text, page 626.

† ac = anticomplementary.

flakes no longer formed and the agglutinating titer as read after 4 hours at 37°C. began to decrease. Generally, however, the precipitating activity with carbohydrate had been completely destroyed at a time when the serum still gave active agglutination, complement fixation and protection to almost the same degree as the original serum. At this intermediate stage, the partially coupled serum therefore resembled that often produced in rabbits by the injection of pneumococci, in so far as it gave all the reactions of a horse antiserum except the precipitation with specific soluble substance. The dissociation between carbohydrate precipitation and bacterial agglutina-

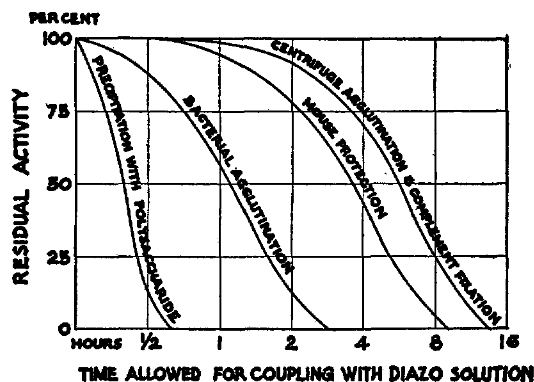

FIG. 1. Diagrammatic representation of the effect of diazo compound on the immunological reactivity of antipneumococcus serum. The order in which protective action, centrifuge agglutination and bacterial complement fixation disappear is not invariable.

tion was even more pronounced than columns 1 and 3 in Table II would indicate. If, after the precipitating activity had been completely destroyed, and the agglutinating titer had been cut to a fraction of its original value, serial dilutions of the serum were incubated with bacteria and the latter then centrifuged, they packed to form a coherent floccule of agglutinated bacteria even in serum dilutions which would otherwise have shown no agglutination. In some experiments (*e.g.* Experiment 2, in Table II) the difference between the apparent agglutinating titer and the centrifuge agglutination titer of the coupled serum was striking. The antibody had apparently retained its affinity for the bacteria, but the deposit of coupled antibody

protein on the surface of the bacteria was not as cohesive as a similar deposit of unaltered antibody. The impacts between bacteria caused by their Brownian movement no longer resulted in cohesion, and it required the intimate pressure packing caused by the centrifuge to produce visible agglutination.

Coupling with the diazo compound beyond this stage caused a complete destruction of antibody; the protective action, complement fixing activity and the centrifuge agglutination test eventually disappeared completely. Usually, but not invariably, these reactions disappeared in the order named.

There are several possible explanations for these progressive changes in the reactivity of antipneumococcal sera.

1. The failure to precipitate might conceivably be due to the fact that partially coupled antibody loses its combining affinity for carbohydrate, or, equally likely, the coupled antibody may combine with carbohydrate, but fail to precipitate, just as partially coupled diphtheria antitoxin combines with toxin but fails to flocculate. However, the experiments described in Protocol 1 and illustrated in Table III indicate that the partially coupled antibody actually fails to combine with the carbohydrate, despite the fact that it can still cause bacterial agglutination.

Protocol 1

Experiments indicating that the failure of the partially coupled antibody to give precipitation with Type I and Type II pneumococcus polysaccharide is due to the absence of combination.

Varying quantities of diazotized sulfanilic acid were added to antipneumococcus serum, and the mixtures allowed to interact overnight in the ice box. The excess diazo compound was then neutralized by the addition of sulfanilic acid and the reactivity of the several solutions tested by adding Type I and Type II carbohydrate to varying quantities of the serum as indicated in Table III. Precipitation was read after 4 hours at 37°C. followed by overnight in the ice box. The supernatant fluid was then tested for excess antibody and excess carbohydrate by the addition of fresh carbohydrate and serum respectively. The quantities of serum and carbohydrate in the original mixtures were so chosen that if combination occurred, it would become apparent in the disappearance of free carbohydrate in some of the tubes of each series. A single experiment with Type I carbohydrate is given in Table IV and is illustrative of four similar experiments with both Type I and Type II carbohydrates. The results can be briefly sum-

TABLE III

Showing That a Coupled Serum Which Has Lost Its Precipitating Reactivity with Type I Deacetylated Carbohydrate (SSSI) and Still Agglutinates Bacteria, Actually Fails to Combine with the Carbohydrate

Serum	Diazotized sulfanilic acid, 2 N/7	Agglutinating activity of treated serum: Cc. serum + N/7 NaCl up to 1 cc. + 0.4 cc. bacterial suspension						Precipitating activity of treated serum: 1. Cc. serum + N/7 NaCl up to 1.6 cc. + 0.1 cc. 1:4000 SSSI						2. 0.8 cc. of each supernatant from (1) + 0.1 cc. 1:4000 SSSI (test for residual free antibody)						3. 0.8 cc. of each supernatant from (1) + 0.2 cc. fresh serum (test for residual free carbohydrate)						Conclusion
		1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.													
cc.	cc.																									
8	0	4	4	4	4	4−	2	4	4	4	4	3	3	+	+	+	+	±	0	0	0	0	0	+	+	No free carbohydrate in first 4 tubes, combination
8	1	4	4	4	4	4	1	4	4	3	3	2	1	+	+	±	0	0	0	0	0	±	+	+	+	Carbohydrate combined only in first 3 tubes, decreased affinity of coupled antibody for carbohydrate
8	2	4	4	4	4	4	1	3	3	3	2	1	±	0	0	0	0	0	0	±	+	+	+	∓	+	Free carbohydrate in all tubes, no combination between carbohydrate and the coupled antibody, despite persistence of bacterial agglutination
8	4	4	4	4	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	
8	8	4	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	
8	8 cc. of inactivated diazo compound as control	4	4	4	4	4	2	4	4	4	4	3	2	+	+	+	±	0	0	0	0	0	±	+	+	

marized as follows: Whenever a coupled serum loses its ability to give precipitation with Type I or Type II carbohydrate that serum no longer combines with the carbohydrate. Even minimal quantities of the latter added to a large excess of the treated serum remain free and uncombined, as shown by the heavy precipitation obtained on the subsequent addition of fresh, untreated antiserum.

Despite the experiment of Protocol 1, it is conceivable that the partially inactivated antibody does combine loosely with carbohydrate to form a non-precipitating compound, and that the altered antibody is displaced from the compound by normal antibody, with the formation of the usual precipitate. This theory, although tenable, involves two unproved assumptions. In the following discussion, the experiment of Protocol 1 will be considered to prove that the partially coupled antibody actually fails to combine with capsular carbohydrate; nevertheless, the possibility of the alternative explanation must be borne in mind.

2. If we accept the validity of the conclusion that the partially coupled serum actually fails to combine with polysaccharide, although it can still agglutinate bacteria, several possible explanations of the apparent paradox suggest themselves. The carbohydrate used in these experiments was not the acetyl polysaccharide actually present in the bacterial capsule (Avery and Goebel), but the deacetylated form. Conceivably, the partially coupled antibody may lose its affinity for the degraded antigen while retaining its affinity for the acetyl polysaccharide, and thus, for the bacterial surface.

In the second place, the antiserum may well contain antibodies to cell constituents other than the type-specific carbohydrate, antibodies capable of combining with the cell surface and thus causing agglutination long after the peculiarly susceptible antibody to carbohydrate has been completely inactivated by the diazo compound.

Either of these theories would account for the experimental findings; and further work with the acetyl polysaccharide and other cell constituents will be necessary to decide which represents the true picture.

3. The fact that the antiserum on further coupling loses its ability to cause agglutination unless the bacteria are thrown into violent and intimate contact, as by centrifugation, is difficult to explain. Clearly, the antibody can still combine with the organisms, but the

antibody deposit seems to be less "sticky" than normally. We must leave as an open question whether the addition of the diazo compound to the antibody molecule makes the antibody deposit on the surface of the bacteria more water-soluble, and correspondingly less susceptible to aggregation by electrolyte (Eagle, 1930); or, whether the diazo compound inhibits aggregation by blocking some of the specifically reacting groups in the antibody molecule, which would impair that combination between the antibody deposit and other unsensitized bacteria suggested by Marrack[5] as the cause of bacterial agglutination.

Amboceptor Serum

A rabbit antiserum against sheep red blood cells added to a washed suspension of the same cells causes (*a*) hemagglutination, and, in the presence of complement (*b*) hemolysis. The serum also causes (*c*) the flocculation of lipoids extracted from the cells, and (*d*) gives a positive complement fixation reaction with the lipoid suspension. The titer of the serum with respect to all four reactions was followed quantitatively as amboceptor serum coupled with the various diazo compounds. A single experiment with diazosulfanilic acid is described in Protocol 2 and Fig. 2; similar results were obtained with the other diazo compounds tested.

Protocol 2

10 cc. of a rabbit anti-sheep cell serum were mixed with 5 cc. of N/7 diazotized Na sulfanilate. 3 cc. samples were withdrawn after ½, 2, 4 and 16 hours at room temperature, and the reactions terminated by the addition of 2 cc. N/7 Na sulfanilate. The control consisted of 2 cc. serum plus 3 cc. of a 1:2 diazosulfanilate:sulfanilate mixture, tested after 16 hours at room temperature. The following technic was used to determine the several reaction titers.

Hemolysis.—(*a*) Serially decreasing quantities of treated serum, (*b*) 0.2 cc. of a 3 per cent sheep cell suspension, (*c*) NaCl N/7 up to 0.8 cc. and (*d*) 0.2 cc. of 1:10 guinea pig complement were mixed in the order named. The degree of hemolysis was read after ½ hour at 37°C.

Hemagglutination.—(*a*) Serially decreasing quantities of treated serum, (*b*) NaCl N/7 up to 0.4 cc. and (*c*) 0.05 cc. of a 3 per cent sheep cell suspension were mixed in the order named. Agglutination was read after 2 hours at room temperature and checked after 18 hours in the refrigerator.

Lipoid Flocculation.—(*a*) Serially decreasing quantities of treated serum,

[5] Marrack, page 115.

(*b*) NaCl N/7 up to 0.4 cc. and (*c*) 0.02 cc. of a sheep cell lipoid suspension fortified with sterols (Eagle, 1935*b*) were mixed, shaken and incubated for 4 hours at 37°C. The tubes were then centrifuged and aggregation read after the addition of 1 cc. of N/7 NaCl.

Lipoid Complement Fixation.—(*a*) Serially decreasing quantities of treated serum, (*b*) NaCl N/7 up to 0.2 cc., (*c*) 0.2 cc. of the lipoid suspension diluted 1:100 with NaCl and (*d*) 0.2 cc. of a 1:10 guinea pig complement were mixed and placed in the ice box for 4 hours, followed by ½ hour at 37°C. 0.4 cc. of a 1½ per cent suspension of sensitized cells was then added, and the results read after ½ hour at 37°C.

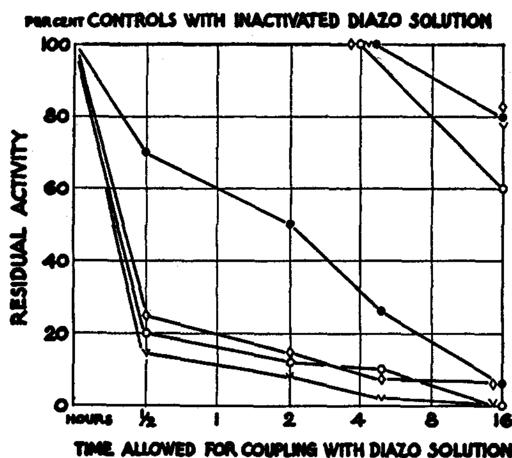

FIG. 2. The effect of diazotized sulfanilic acid on the hemolytic (○ — ○), hemagglutinating (● — ●), lipoid flocculating (◇ — ◇) and lipoid complement fixing (v — v) activities of an anti-sheep red blood cell serum.

As is seen in Fig. 2, the hemolytic, lipoid-flocculating and lipoid complement-fixing activity of the antiserum fell off exactly in parallel. The hemagglutinating activity, however, seemed somewhat more resistant to the diazo inactivation. The difference in susceptibility was not nearly as pronounced as that found between the carbohydrate-precipitating and bacteria-agglutinating activity of antipneumococcus serum, or between the toxin-combining and toxin-precipitating activity of antidiphtheria serum; nevertheless, it suggests either the presence of several antibodies in an amboceptor serum, or a dissociation between the hemagglutinating and other manifestations of a single antibody.

Rabbit Antisera vs. Egg Albumen

As with every antibody studied in this paper, coupling this antiserum with the diazo compound resulted in a progressive and eventually complete disappearance of antibody activity, as manifested both by precipitation and complement fixation. The two reactions fell off in parallel.

Rabbit Antiserum vs. Horse Serum

It became of interest to ascertain whether the progressive decrease in the reactivity of precipitating rabbit antisera as they coupled with diazotized sulfanilic acid was due to a loss in combining affinity for antigen, as in the case of horse antipneumococcus sera, or whether combination proceeded normally, and only the secondary aggregation was affected, as with diphtheria antitoxin. The inhibition experiment with rabbit antiserum *vs.* horse serum, summarized in Table IV, seemed to show that the former was the case: the gradual disappearance of precipitating activity was apparently due to the fact that the coupled antibody was losing its affinity for the homologous antigen (*cf.* page 629).

Serum from Syphilitic Patients

The results summarized in Table V are self explanatory. The diazo compound, added to human syphilitic serum in the quantities indicated in the table, caused an extraordinarily rapid and complete disappearance of the characteristic reactivity with alcoholic extracts of beef heart. Both the flocculation and Wassermann reactions, originally positive up to 1:32 dilutions of serum, became completely negative within 1 minute after the addition of 1 volume of N/7 diazo compound to 3 volumes of serum.

Horse and Rabbit Antityphoid Serum

The effect of diazotized sulfanilic acid on the agglutinating activity of horse antityphoid serum is illustrated in Table VI and Fig. 3, in which one of four similar experiments is summarized. There was the usual progressive decrease in antibody activity; but the amount of coupling required to produce this inactivation seemed significantly greater than in the case of the sera previously described. Although only the results with the horse serum are given in Fig. 3, rabbit antiserum was similarly inactivated.

TABLE IV

Showing That Coupling a Rabbit Antiserum vs. Horse Serum with Diazosulfanilic Acid Destroys the Affinity of the Antibody for the Corresponding Antigen. (All Coupling Overnight at Room Temperature)

Serum	N/7 diazo solution	N/7Na sulfanilate added after overnight coupling	Precipitating activity of the treated serum																					Conclusion
			1. Cc. serum + N/7 NaCl up to 1.6 cc. + 0.1 cc. of 1:50 horse serum							2. 0.8 cc. of supernatant fluids of (1) + 0.1 cc. of 1:50 horse serum. (Test for residual free antibody)							3. 0.4 cc. of supernatant fluids of (1) + 0.2 cc. of fresh antiserum. (Test for residual free antigen)							
										Tube							Tube							
			1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
cc.	*cc.*	*cc.*																						
5	0	0	4	4	4	4	4	3	2	+	+	+	+	±	0	0	0	0	0	0	±	+	+	The decreasing precipitation in section 1 is due to the decreased ability of the antibody to combine with antigen, as shown by the presence of free antigen
5	0.25	0.375	4	4	4	4	3	2	1	+	+	+	+	0	0	0	0	0	0	±	+	+	+	
5	0.5	0.75	4	4	4	3	2	1	1	+	+	±	0	0	0	0	0	0	±	+	+	+	+	
5	1.0	1.5	4	3	2	1	1	1	±	0	0	0	0	0	0	0	±	+	+	+	+	+	+	
5	2.0	3.0	1	1	1	±	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	4.0	6.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	8.0	14.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	10 cc. of a 1:1.5 diazosulfanilate: sulfanilate mixture (comparable to row 6)		4	4	4	4	3	2	1	+	+	+	±	0	0	0	0	0	±	+	+	+	+	Control antiserum, treated with inactivated diazo compound, can still combine with antigen, as shown by the absence of free antigen in the first few tubes

Of particular interest was the effect of the diazo compound on the isoelectric point of antibody to *Bact. typhosum*. Mudd and Joffe observed an acid shift of 0.6–0.8 pH units in the isoelectric point of sensitized typhoid bacteria treated with formaldehyde, probably due to a reaction between basic groups in the antibody film and the formaldehyde. *A priori*, one would expect that the addition of a sulfanilate radical in place of or adjacent to an NH, NH_2 or OH group in a molecule of antibody protein would also result in a more acidic

TABLE V

The Effect of Sodium Diazosulfanilate on the Reactivity of Syphilitic Serum

To 9 cc. serum were added 3 cc. N/7 Na diazosulfanilate. At the stated intervals, 1.6 cc. were withdrawn and the reaction terminated by the addition of 0.8 cc. N/7 Na sulfanilate. The usual controls were set up by diluting 2 cc. samples of serum with 2 cc. of salt solution and with 2 cc. of diazosulfanilate solution inactivated by the addition of sulfanilate.

Time allowed for coupling	Flocculation with beef heart lipoid					Wassermann reaction					
	Cc. serum + 0.85% NaCl to 0.4 cc. + 0.03 cc. flocculation antigen (Eagle, 1932)					Result with varying amounts of serum					
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.001 cc.
Control with N/7 NaCl after 60 min.	+	+	+	+	±	+	+	+	+	±	0
Control with inactivated diazo solution after 60 min.	+	+	+	+	±	+	+	+	+	±	0
¼ min. coupling	+	±	0	0	0	0	0	0	0	0	0
½ " "	±	0	0	0	0	0	0	0	0	0	0
1 " "	0	0	0	0	0	0	0	0	0	0	0
2 " "	0	0	0	0	0	0	0	0	0	0	0

antibody, and that the greater the number of sulfanilate groups so introduced, the more acid would be the isoelectric point of the coupled protein. The strain of *Bact. typhosum* described by Mudd and Joffe was peculiarly well suited to test this surmise. Most other bacteria and suspended particles have a more or less pronounced cataphoretic potential. In consequence, their isoelectric point after sensitization with antiserum is intermediate between that of the antibody deposit and that of the uncovered bacterial surface, the

TABLE VI

The Effect of Diazotized Sulfanilic Acid on the Immunological Reactivity of Antityphoid Serum

To 20 cc. of 1:10 serum were added 20 cc. of N/7 sodium diazosulfanilate. At the intervals indicated in the table, 7 cc. samples were withdrawn and the reaction terminated by the addition of 7 cc. N/7 sodium metanilate. The usual controls were set up by adding 2 cc. of 1:10 serum to 6 cc. of (*a*) 0.85 per cent NaCl; (*b*) a 2:1 diazosulfanilate: sulfanilate mixture.

Treatment of serum	Agglutination								Relative cataphoretic mobility of sensitized bacteria at pH						Isoelectric points* of sensitized bacteria
	0.8 cc.	0.2 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0.0031 cc.	0.0016 cc.	4.7	4.4	4.1	3.8	3.5	3.2	*pH*
Overnight control with N/7 NaCl	4	4	4	4	4	4	2	0	−2.9	+1.5	+3.1				4.5
Overnight control with inactivated diazo solution	4	4	4	4	4	4	2	0	−2.5	0	+2.9				4.4
1 min. coupling	4	4	4	4	4	3	±	0	−3.0	+1.5	+3.3				4.5
4 " "	4	4	4	4	1	0	0	0	−3.3	−0.7	+3.2				4.35
16 " "	4	4	4	4	2	0	0	0	−4.3	−1.8	Barely +	+3.2			4.15
60 " "	4	4	4	3	0	0	0	0			−4.2	−2.6	+2.1	+4.1	3.65
240 " "	4	4	2	2	0	0	0	0				−2.8	−1.0	+1.2	3.35
Overnight "	0	0	0	0	0	0	0	0	No significant cataphoretic mobility						—

* These are the isoelectric points of bacteria sensitized with a fixed amount of coupled antiserum, and therefore with decreasing quantities of effective antibody. However, a preceding experiment had shown that over a 64-fold range in antibody concentration the isoelectric point of these bacteria was practically independent of the degree of sensitization. The values given for the isoelectric point were obtained from the cataphoretic mobilities by graphic interpolation.

particular value obtained varying to a large extent with the degree of sensitization. This strain has no significant cataphoretic potential against water. When the organism combines with antibody, it does develop a cataphoretic mobility and a cataphoretic isoelectric point which are presumably determined solely by the bound antibody and are largely independent of the degree of sensitization. It was thus possible to follow the progressive change in the isoelectric point of the antibody to *Bact. typhosum* as it coupled with the diazo compound, and to correlate that change with the progressive decrease in its im-

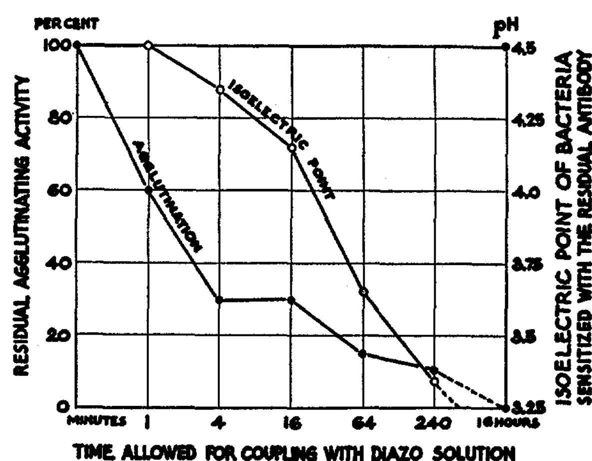

FIG. 3. The effect of coupling with diazotized sulfanilic acid on the agglutinating activity (● — ●) of a horse antityphoid serum, and on the isoelectric point (○ — ○) of the residual antibody.

munological reactivity. Aliquot samples were periodically withdrawn from a serum:diazo mixture, and the reaction terminated by the addition of sulfanilic acid. The residual agglutinating titer was obtained in the usual manner; and the cataphoretic isoelectric point of bacteria sensitized with that sample and washed free of serum was then measured, using acetate buffers for pH values between 5 and 3.5 and dilute HCl for the more acid ranges. The results are summarized in Fig. 3, and are self explanatory. As the antibody coupled with the diazosulfanilate, there was a progressive acid shift in the isoelectric point of the antibody as measured on the surface of bacteria, paralleling the progressive decrease in the antibody titer of the serum.

In this particular experiment, the most acid isoelectric point observed was pH 3.35; in another experiment, an antibody was obtained with an isoelectric point at pH 2.7; and, as is indicated by the dotted line in Fig. 3, there is reason to believe that on further coupling, just before the antiserum loses all of its reactivity, the residual traces of antibody may have an isoelectric point even more acidic than pH 2.7.

DISCUSSION

1. Every antibody studied in this paper lost all reactivity with the corresponding antigen on sufficient coupling with the diazo compounds. The susceptibility of the several antibodies to this inactivation varied widely, from the almost instantaneous destruction of syphilitic reagin and the rapid inactivation of a rabbit antiserum against egg albumen, to the slow decrease in the agglutinating activity on an antityphoid serum.

2. The gradual and eventually complete disappearance of antibody activity as the sera coupled with the diazosulfanilic acid might be due to the inactivation of an increasing proportion of antibody molecules; however, it might also reflect a progressive decrease in the reactivity of each antibody molecule. To take a specific example: if the agglutinating titer of a treated serum was found to be only 25 per cent of its original value, this could be interpreted on the basis that 75 per cent of the antibody molecules had been inactivated, leaving 25 per cent with normal activity, or, it might mean that every antibody molecule had lost approximately 75 per cent of its agglutinating activity.

On *a priori* statistical grounds, it seems improbable that at any one moment, some molecules of antibody protein would have reacted with *e.g.* 50 molecules of diazo compound and be completely inactivated, while others would have combined with *e.g.* only 5 molecules of the diazo substance. The experiment with antityphoid serum provided a clear answer to this question as regards this particular antibody. As coupling proceeded, the isoelectric point of the residual active antibody, determined by measuring the cataphoretic mobility of bacteria sensitized with that antibody, became progressively more acidic. This indicates that the residual reactivity was not due to residual normal antibody, but to a chemically altered antibody which

became progressively more acidic and less reactive as it added on more and more of the diazotized sulfanilate. The possibility of non-specific adsorption of coupled acidic protein by the bacteria was excluded by the fact that when the serum had lost all agglutinating activity it had no effect on the isoelectric point of the bacteria.

There is no reason to doubt that a similar situation obtains in the case of all the antibodies studied in this paper: that each molecule combines with progressively increasing quantities of the diazo compound, gradually losing its immunological reactivity as it does so. Indeed, the experiments with antipneumococcus serum and with diphtheria antitoxin furnish further evidence that such is the case. Partial coupling of the diphtheria antitoxin molecule completely inhibited the flocculation reaction with toxin without impairing its ability to combine with and neutralize the toxin. Moreover, the gradual disappearance of flocculating activity was not associated with a corresponding decrease in the flocculating titer of the serum: the optimum proportion of serum and toxin for flocculation remained the same, only the degree and velocity of the flocculation being affected. This strongly suggests that all the antibody molecules were being simultaneously affected. In the case of antipneumococcus serum, the first change noted was a disappearance of ability to react with carbohydrate, and the serum became almost wholly inactive in this respect before there was any decrease in its agglutinating action. It is difficult to explain these several observations with antityphoid, antipneumococcus and antitoxin sera on any basis other than the progressive addition of more and more diazo molecules to each molecule of antibody, the immunological reactivity being progressively modified as coupling proceeded.

3. The fact that four other diazo compounds as different as diazotized arsanilic, *p*-aminobenzoic, *p*-amino-2-naphthol-4-sulfonic and 4-amino-2-sulfonic acids had qualitatively the same effect as diazosulfanilic acid on antipneumococcus serum, amboceptor serum and diphtheria antitoxin indicates that the observed effects were largely independent of the type of substance added on to the antibody molecule.

4. As was shown in a preceding paper, it is probable that diazo compounds react with at least six groups in proteins: free aliphatic

NH_2 groups, the imidazole ring of histidine, the NH of proline and hydroxyproline, the indole group of tryptophane and the phenyl group of tyrosine. The fact that every antibody studied in this paper was eventually completely destroyed by the diazo compound would seem to imply that one or all of these groups are essential for the reaction with antigen.[6] The present series of experiments do not allow of a more accurate localization, which must await the development of a technic whereby individual groups on protein can be blocked or modified.[7]

5. It was shown for two antibodies, rabbit antiserum *vs.* horse serum, and horse antiserum *vs.* type-specific pneumococcus polysaccharide, that the complement-fixing and precipitating titer of the serum after coupling with diazosulfanilic acid apparently depended solely on the ability of the serum to combine with antigen; these manifestations of antibody activity fell off together, and in parallel with the decreased combining affinity of the antibody for antigen. This suggests, with the reservation noted on page 629, that so long as these two antibodies remained capable of combining with antigen, the secondary objective manifestations of this combination followed as a matter of course, unaffected by the coupling with the diazo compound; and that the groups affected by the latter are not primarily concerned in the secondary aggregation or complement fixation.

In direct contrast, diphtheria antitoxin completely lost its ability to give flocculation with antigen on moderate coupling with diazo compound, while it still retained almost its original combining af-

[6] It is conceivable, although improbable, that the observed loss in immunological reactivity might be due to steric hindrance: the diazo molecules, added on to the antibody protein, might block some specifically reacting group in the latter adjacent to, but not identical with, the group which binds the diazo compound.

[7] Although not germane to the present paper, it is to be noted that diazo compounds inactivate Type I pneumococcus polysaccharide, but have no effect on the Type II polysaccharide. The difference may well be due to the fact that the Type I polysaccharide contains NH_2 groups capable of reacting with the diazo compound; while the Type II derivative, lacking NH_2 groups, may not react with the diazo compound. If this proves to be correct, it indicates that not only the COOH groups (Chow and Goebel), but the NH_2 groups as well may be essential for the immunological reactivity of the Type I polysaccharide.

finity for the antigen. In the case of this antibody, the first groups to be attacked by the diazo compound (and there is as yet no indication as to which of the six groups are most reactive) apparently play no part in the combination of its antitoxin with toxin, but are essential for the flocculation of the formed compound.

Similarly, the first few groups in the proteins of an antipneumococcus serum to react with diazo compound seem essential for its precipitating reactivity with the type-specific capsular carbohydrate, but play little or no part in its reaction with the whole bacterial cell. On further coupling with the diazo compound, antibody groups are inactivated which seem essential for the agglutination of bacteria but which are not the actual combining groups. The chemical identification and differentiation of the groups responsible for precipitation with polysaccharide, for bacterial agglutination and for combination with bacteria await the development of an appropriate chemical technic.[7]

The recent work of Chow and Goebel, who found that formaldehyde caused a reversible inactivation of the precipitin to Type I pneumococcus polysaccharide, suggests that free amino groups are essential for the precipitating reactivity of this particular antibody. It will be of interest to ascertain whether formaldehyde causes a dissociation between the agglutinating and precipitating activity of a pneumococcus antiserum analogous to that produced by diazo compounds.

6. It has been suggested (Heidelberger; Marrack) that the precipitation of antigen-antibody compounds is not due to an altered solubility of the combined antibody as suggested by Eagle (1930), but results from the combination of antigen-antibody aggregates with similar aggregates by virtue of specifically reacting groups on the surface of each, until the complex becomes sufficiently large to become macroscopically visible as sedimenting clumps. The observation that diphtheria antitoxin can be chemically altered so that it remains capable of combining with toxin, but no longer gives the flocculation reaction is difficult to reconcile with the latter hypothesis.

7. The extraordinary difference in the rates of inactivation of the various antibodies under comparable conditions of volume, diazo

concentration, etc., exemplified by the data summarized in Table VII, suggests that antibodies differ in the number and type of specifically reacting groups. It should be pointed out that the relative times indicated in Table VII are not a direct measure of the vulnerability of the antibody, in view of the progressive spontaneous deterioration of the diazo compound.

TABLE VII

The Varying Susceptibility of Different Antibodies to Inactivation by M/7 Na Diazosulfanilate

Antiserum	Type of reaction	Approximate time (in hours) required for 75 per cent inactivation				
		Diazo: serum = 1:6	Diazo: serum = 1:4	Diazo: serum = 1:2	Diazo: serum = 1:1	Diazo: serum = 10:1
Syphilis reagin	Wassermann and flocculation			0.01		
Diphtheria antitoxin	Ramon flocculation		0.05–0.3	0.02		
	Animal protection		4–24	0.25		
Antipneumococcus horse serum	Carbohydrate precipitation	1.5		0.2 –0.07	0.02–0.06	
	Agglutination	24		0.24–1	4	
	Mouse protection			0.5 –2	1	
Rabbit antiserum *vs.* horse serum	Precipitation				0.25	
Horse antityphoid serum	Agglutination			24	8–24	0.5–4

SUMMARY

Sufficient coupling with any of five different diazo compounds eventually destroyed the reactivity of all the antisera here studied. The rates of inactivation varied considerably among the several antisera. By stopping the reaction at intervals, it was possible to prepare partially inactivated antibodies of peculiarly modified reactivity.

Thus, the flocculating activity of diphtheria antitoxin with toxin was completely destroyed long before there was any demonstrable impairment of its protective titer *in vivo*. The first change induced in

antipneumococcus horse sera was the apparently complete loss of reactivity with the capsular carbohydrate at a time when the agglutinating, animal-protecting and complement-fixing activity of the sera were only slightly affected. On further coupling, the sera no longer caused visible agglutination; but aggregation of the serum-treated bacteria could be induced by centrifugation. Still further coupling destroyed all antibody activity.

Rabbit antisera to egg albumen and horse serum no longer precipitated the homologous antigen after treatment with diazo compounds, probably due to their failure to combine with the antigen. The hemolytic, complement-fixing and lipoid-flocculating activity of coupled rabbit antisera to sheep red blood cells fell off in parallel; the hemagglutinin seemed somewhat more resistant. The reagin of syphilitic serum was destroyed almost instantaneously by comparatively small amounts of diazo compounds. Finally, in the case of antityphoid agglutinin, the isoelectric point of the coupled antibody, measured on the surface of specifically sensitized bacteria, was found to shift from an original value of pH 4.7 to one of less than pH 2.7 as progressively more sulfanilic acid radicals added on to the antibody molecule.

The groups in protein which participate in its reaction with diazo compounds probably include aliphatic amines, the imidazole ring of histidine, the indole group of tryptophane, the NH of proline and hydroxyproline and the phenyl group of tyrosine. Although it has been possible to modify antibodies chemically so that they combine with the corresponding antigens without causing their aggregation, the experiments here described furnish no indication as to which of these groups in antibody protein are primarily concerned in the antigen-antibody reaction, and which are responsible for the secondary flocculation. Such localization awaits the development of a technic for attacking individual groups in the protein molecule.

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