SOME EFFECTS OF FORMALDEHYDE ON HORSE ANTIPNEUMOCOCCUS SERUM AND DIPHTHERIA ANTITOXIN, AND THEIR SIGNIFICANCE FOR THE THEORY OF ANTIGEN-ANTIBODY AGGREGATION

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It has been shown in a previous communication (1) that diazo compounds added to antipneumococcus horse serum or to horse diphtheria antitoxin cause a dissociation between the aggregating activity of the antibody *in vitro* and its protective action *in vivo*. When a small amount of sodium diazosulfanilate was added to diphtheria antitoxin, the latter no longer gave the Ramon flocculation reaction with toxin, but its ability to combine with toxin, and its protective action *in vivo* were unaffected. Similarly, when antipneumococcus serum was coupled with a small amount of diazo compound, the azoprotein dye so formed no longer gave the characteristic precipitation with the type specific capsular carbohydrate, but its bacterial agglutinating activity was only slightly affected, and its protective action *in vivo* not at all. A larger amount of diazo compound added to the antipneumococcus serum caused an apparent loss of its agglutinating activity; but if the mixture of treated serum and bacteria was centrifuged, the pressure packing of the sedimented bacteria caused their cohesion to form the characteristic flake of agglutinated pneumococci. At this stage, the serum still protected mice. On further treatment with diazo compound all antibody activity progressively decreased, and eventually disappeared.

It was subsequently shown (2) that the coupling of protein with diazo compounds was a complex reaction in which not only the dye-forming histidine NH and tyrosine OH groups might conceivably be

involved, but also the aliphatic NH_2 groups, and the NH groups of proline and arginine. It remained to ascertain which of these five groups was primarily concerned in the dissociation of antibody activity just described, the reason for this dissociation and its significance for the mechanism of antigen-antibody aggregation.

The simplest experimental attack seemed to be the study of the effect of formaldehyde on the activity of antipneumococcus serum and diphtheria antitoxin. Formaldehyde apparently does not react with the proline or the arginine NH group in protein (3). Of the five groups in protein previously found to react with diazo compounds, it is said that only two, the aliphatic NH_2 (4) and the histidine NH (5), are readily affected by formaldehyde. The present experiments were therefore undertaken to ascertain whether the effects of diazo compound previously described could be duplicated with formaldehyde, and thus, could be reasonably ascribed to modifications in either the aliphatic NH_2 or histidine NH of the antibody molecule.

It is a well known observation that formaldehyde in concentrated solution destroys antibodies (6). Chow and Geobel (7) have recently shown that under certain conditions the inactivation of antipneumococcus globulin by formaldehyde is reversible, presumably due to the hydrolysis of $—N{=}CH_2$ groups formed on the addition of formaldehyde. Several investigators (8) have reported the variable susceptibility of different antisera to the destructive action of formaldehyde. Mudd and Joffe (9), in a study which is particularly germane to the experiments here to be reported, found that agglutinating sera treated with an equal volume of 9 to 37 per cent HCOH lost some of their activity, and showed wide prozones in the agglutination reaction. In the presence of an excess of antiserum there was no obvious agglutination, but cohesion was obtained on centrifugation. That combination with antibody has occurred was further shown by the change in the cataphoretic properties of the organisms. A similar decrease in the agglutinating tendency was observed if the bacteria were first sensitized in untreated antiserum, and if the washed bacteria were then treated with formaldehyde.

As will be shown in the present paper, the bizarre effects of diazo compounds on diphtheria antitoxin and antipneumococcus serum could be reproduced with formaldehyde. A minute amount sufficed

to inhibit the aggregating activity of these sera completely. Although the reaction between HCOH and protein is complex, it seems possible that this inhibiting action on aggregation is primarily due to the modification of a few NH_2 groups in the antibody molecule. The reason for this inhibition, and the implications of these observations with respect to the mechanism of antigen-antibody aggregation are discussed in the text. In contrast to the effect on aggregation, even large quantities of formaldehyde did not affect either the ability of these two antibodies to combine with antigen *in vitro*, or their protective action *in vivo*. It follows that the aliphatic NH_2 groups of diphtheria antitoxin and antipneumococcus serum are not primarily concerned in their combination with the homologous antigens.[1]

EXPERIMENTAL[2]

The Effect of Formaldehyde on Diphtheria Antitoxin

Varying amounts of formaldehyde[3] were added to fixed amounts of diphtheria antitoxin, as indicated in Table I. After 1 hour at room temperature the mixtures were dialyzed in cellophane tubing against running water for 24 hours,[4] made isotonic by the addition of 1/19 volume of 17 per cent NaCl, adjusted to pH 7.0, and tested for antibody activity.

As shown in Table I, 1 part of formaldehyde solution to 2048 parts of serum, acting for 1 hour at room temperature, definitely retarded the Ramon flocculation reaction with toxin, and 1 part to 64 parts of serum prevented flocculation completely. In marked contrast, a 1:8

[1] It should be emphasized that although the formolized antibody might conceivably be reversed to native antibody *in vivo*, such dissociation does not occur under the conditions of the *in vitro* experiment. The formolized antibody itself combines with its antigen in the test tube (*cf.* page 499).

[2] I am indebted to the Mulford Biological Laboratories, Glenolden; the Eli Lilly Company, Indianapolis; the Lederle Laboratories, Pearl River; and the Health Departments of Massachusetts, New York City and New York State for the antisera, refined globulin and diphtheria toxin used in these and subsequent experiments. The Mulford Biological Laboratories also furnished preparations of acetylated Type I and Type II pneumococcus carbohydrate.

[3] Merck reagent, containing approximately 37 per cent HCOH.

[4] In some of the early experiments, the formaldehyde was almost instantaneously inactivated after the desired interval by the addition of an excess of $NaHSO_3$. The results did not differ from those obtained on dialysis.

TABLE I

The Effect of Formaldehyde Acting for 1 Hour on the Flocculating Activity and Protective Action of Horse Diphtheric Antitoxin

Antitoxin serum	37 per cent HCOH	Ratio of HCOH: protein		N/1 NaOH necessary to neutralize*	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight†	Final volume after dialysis and addition of NaCl	A Ramon flocculation (figures indicate flocculation time) Varying amounts of treated 1:2 serum + 1 cc. toxin						B Protective action in guinea pigs Varying amounts of serum + 1 L+ dose toxin							C Conclusion
		Concentration	Molar				0.2 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.0375 cc.	0.025 cc.	0.02 cc.	0.012 cc.	0.009 cc.	0.006 cc.	0.0045 cc.	0.003 cc.	0.002 cc.	
cc.	*cc.*			*cc.*		*cc.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*								
4	0	0	0	—		8	0	Cl	180	37	**20**	130	S	S	S	S	S, D2	D2	D1	Progressive retardation of Ramon flocculation, with eventual complete disappearance. The protective action is unaffected even by 128 times the quantity which causes a significant retardation of flocculation
4	0.001	1:4096‡	3.75:1§	—		8	0	Cl	180	42	**24**	180								
4	0.002	1:2048	7.5:1	—		8	0	Cl	180	75	**39**	180								
4	0.004	1:1024	15:1	—		8	0	Cl	180	180	**63**	180								
4	0.008	1:512	30:1	—		8	0		Cl	180	180	180								
4	0.016	1:256	60:1	0.03	10	8	0	0	0	180	180	Cl								
4	0.031	1:128	120:1	0.05	15	8	0	0	0	180	180	0	S	S	S	S	D4	D2	D1	
4	0.062	1:64	240:1	0.07	22	8	0	0	0	0	0	0								
4	0.125	1:32	480:1	0.11	35	8				0			S	S	S	S	D4	D1	D1	
4	0.25	1:16	960:1	0.14	43	8				0			S	S	S	S	D2	D3	D1	
4	0.5	1:8	1920:1	0.14	43	8				0			S	S	S	S	D4	D2	D1	Progressive, but incomplete, destruction of protective action
4	1	1:4	3840:1	0.145	46	8				0			S	S	S	S, D5	D2	D1	D1	
4	2	1:2	7680:1	0.15	47	8				0			S	S	S	D4	D1			
4	4	1:1	15,360:1	0.16	50	8				0			S	S	D4	D1	D1			

0 = no flocculation in 24 hours. Cl = cloudy after 24 hours at 37°C. S = survived more than 4 days; D1 = dead in 1 day, etc.

* Back to original serum pH.

† Approximate number of NH_2 groups $= \frac{\text{cc. 1 M NaOH}}{\text{cc. serum}} \times \frac{\text{molecular weight protein}}{\text{gm. serum protein per liter serum}}$.

$$= \frac{\text{cc. 1 M NaOH}}{4} \times \frac{100{,}000}{80} = 312.5 \times \text{cc. 1 M NaOH.}$$

‡ 1 part of 37 per cent HCOH to 4096 parts of serum.

§ 3.75 moles of HCOH to 1 mole of serum protein. See footnote 5, page 499.

ratio had no demonstrable effect on the protective action of the antiserum *in vivo*, as tested with guinea pigs; and even a 1:1 ratio did not wholly destroy the antitoxin. The partially treated antiserum was clearly capable of neutralizing toxin *in vivo*, despite the absence of the usual aggregation.

It is to be noted in Table I (section A, bold-faced column headed by 0.0375 cc.) that as the amount of formaldehyde was increased, the toxin: antitoxin ratio which gave the most rapid flocculation did not significantly vary, despite the progressive retardation of that flocculation. Since this optimum ratio is the index of the "neutral" mixture, in which toxin and antitoxin are combined in "equivalent" proportions, it follows that the ability of the antitoxin to combine with toxin was unaffected by the treatment with formaldehyde. This was further shown by the fact that a rabbit antiserum to horse serum protein, added to a non-flocculating and non-toxic mixture of antitoxin and toxin, precipitated both the antitoxin protein and the toxin with which it had combined, and left a non-toxic supernatant fluid (*cf.* 10). A control mixture of formaldehyde-treated antipneumococcus serum and diphtheria toxin, similarly precipitated by a rabbit antiserum to horse serum, yielded a supernatant fluid of undiminished toxicity.

If we assign an arbitrary figure of 100,000 as the "average" molecular weight of serum protein, and assume that the antibody protein does not significantly differ in its affinity for formaldehyde from the rest of the serum protein, it follows that an amount of formaldehyde sufficient to combine with approximately 7 to 8[5] NH_2 groups in each molecule of serum protein, and which probably reacted with no more than 2 or 3 groups in the course of 1 hour (*cf.* sixth column of Table I), significantly retarded the flocculating activity of diphtheria antitoxin with toxin. As determined with a glass electrode, this amount of

[5] If we assume an average molecular weight of 100,000 for all the serum proteins, a serum containing 8 per cent protein is 0.0008 M. A 37 per cent solution of HCOH is approximately 12.3 M. 1 part of that solution to 2048 parts of serum is a $\frac{12.3}{0.0008 \times 2048}$, or approximately an 8:1 ratio. The actual number of groups of the antitoxin molecule which might be affected by a given amount of HCOH differs from this calculated value to the extent that the molecular weight of the antitoxin protein itself differs from the arbitrarily chosen average of 100,000.

HCOH had no demonstrable effect on the pH of the serum, further evidence that but few NH_2 groups had been affected. Under the same conditions, the protective action of the antiserum was wholly unaffected even by 250 times that quantity. As shown by the amount of NaOH required to neutralize (*cf.* fifth column of Table I), the latter amount of HCOH sufficed to block practically all the free NH_2 groups of the protein. As was concluded by Mudd and Joffe (9) for antibodies to various bacteria, it seems clear that the aliphatic NH_2

TABLE II

The Effect of Formaldehyde on the Antigenic Activity of Horse Diphtheric Antitoxin, as Determined by Its Reactivity with a Rabbit Antiserum vs. Horse Serum

(Antitoxin treated with formaldehyde as shown in Table I)

Ratio of 37 per cent HCOH to serum	A. Varying amounts of the 1:2 treated serum + 0.4 cc. rabbit antiserum + NaCl up to 0.8 cc. Figures represent degree of precipitation after 4 hrs. at 37°									B. Supernatant fluids from section A + 0.01 cc. fresh horse serum. Figures represent precipitation after 2 hrs. at 37° (test for free antibody)								Conclusion
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0002 cc.	0.0031 cc.	0.0016 cc.									
0	3	4	4	4	4	4	4	3	2	0	±	2	3	4	4	4	4	4	Only slight change in the precipitating activity of partially formolized antitoxin (Table I), acting as antigen with a rabbit antiserum *vs.* horse serum. Marked precipitation prozone
1:32	±	2	4	4	4	4	4	3	2	0	0	2	3	4	4	4	4	4	
1:16	0	1	4	4	4	4	4	3	2	0	0	2	3	4	4	4	4	4	
1:8	0	0	2	4	4	4	4	3	2	0	0	1	2	4	4	4	4	4	
1:4	0	0	1	4	4	4	4	3	2	0	±	1	3	4	–	–	–	–	
1:2	0	0	1	3	4	4	4	3	2	0	±	2	3	4	–	–	–	–	
1:1	0	0	0	±	3	3	3	3	2	0	±	2	3	4	–	–	–	–	

groups play little or no rôle in the combination between diphtheria toxin and horse antitoxin.

It is to be noted (Table II) that the antigenic activity of horse antitoxin, that is, its reactivity as horse serum with a precipitating rabbit antiserum to horse serum, was as little affected by formaldehyde as was its antitoxic activity. An 18 per cent concentration of HCOH acting for 1 hour had little effect on its precipitating activity, save for a wider prozone in the region of antigen excess; and the formaldehyde had even less effect on its combining affinity for the antibody, as

TABLE III

The Effect of Formaldehyde on the Antitoxic and Antigenic Activity of Horse Diphtheric Antitoxin

Time for which equal volumes of 37 per cent HCOH and antitoxin were allowed to react*	pH of reacting mixture (brought to pH 8.85 after 1 hr.)	Activity of treated serum as antibody. Protective action in guinea pigs. Varying amounts of 1:2 treated serum + 1L + dose of toxin											Precipitating activity of treated serum as antigen with rabbit antiserum *vs.* horse serum. A. Varying amounts of treated 1:2 serum + 0.4 cc. antiserum in total volume of 0.8 cc.								B. Supernatant fluids from section A + 0.01 cc. untreated horse serum (test for free antibody)	C. Anaphylaxis experiments with guinea pigs sensitized to native horse serum. Amount of treated 1:2 serum injected		Conclusion
		0.2 cc.	0.15 cc.	0.1 cc.	0.175 cc.	0.05 cc.	0.0375 cc.	0.025 cc.	0.018 cc.	0.125 cc.	0.009 cc.	0.006 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.125 cc.	0.0062 cc.	0.0031 cc.		2 cc.	1 cc.	
hrs.																								
0†								S	S	S	S	D4	4	4	4	4	4	4	3	3	0 0 ± 2 3 4 4 4	D, D	D, D	Progressive slow decrease in the protective action of formolized antitoxin, its precipitating activity with a rabbit antiserum, and its ability to shock guinea pigs sensitized with native horse serum
¾	8.83					S		S	S	D1			0	0	1	2	2	2	2	1	0 1 4 4 4 4 4 4	D, scratch dyspnea, convulsions		
1½	8.81			S				S	S	D2			0	0	1	2	2	2	1	±	± 1 4 4 4 4 4 4		Scratch, dyspnea (2 animals)	
3	8.80			S		S	S	D1					0	0	0	±	1	1	±	0	± 2 4 4 4 4 4 4		0 (slight weakness) (3 animals)	
6	8.80	S				S, D4	D3	D2					0	0	0	0	±	±	±	0	1 3 4 4 4 4 4 4	Scratch, dyspnea, prostration (2 animals)		
12	8.80	S	S	D3		—	D1						0	0	0	0	0	0	0	0	3 4 4 4 4 4 4 4			

S = survived more than 4 days; D1 = dead in 1 day, etc.

* It is obvious that all these times are in error to the extent that the formaldehyde was not removed instantaneously on dialysis. However, the concentration probably fell to less than ½ its original level within an hour.

† Original serum, diluted with equal volume NaCl.

shown by the subsequent addition of untreated horse serum (Table II, section B). Like the antitoxic activity, the species specificity of horse serum protein apparently does not depend primarily on its free NH_2 groups.

It is true that this concentration of formaldehyde, acting over a 24 hour period, eventually almost completely destroyed the protective action of the antitoxin serum, as well as its reactivity with an antibody to horse serum (Table III). However, this destruction cannot be ascribed to the simple addition of HCOH to the NH_2 groups of protein. The latter reaction proceeded very rapidly in the presence of so large an excess of HCOH, as evidenced by the approximately constant pH of the reacting mixture after the first hour. The destruction of antibody, on the other hand, was incomplete even after 12 hours. Some reaction other than the blockade of the NH_2 groups is apparently responsible for this slow destruction.

The loss of flocculating activity caused by small amounts of formaldehyde is probably due to its addition to a few aliphatic amino groups.[6] The minute amounts which suffice (too small even to affect the pH of the serum), and the speed with which the inactivation may proceed,[7] both suggest that this is the case. Nevertheless, in view of the complexity of the reaction between HCOH and protein the possibility of some other reaction must be considered.

It seems possible that the loss of Ramon flocculating activity frequently observed in the course of concentrating and refining diphtheric antitoxin globulin, may be due to a similar modification of relatively few groups, perhaps the NH_2 groups. Thus, as is seen in Table IV, when antitoxin serum of pH 9.4 to 10.0 was kept at 56°C. for 1 to 4 hours there was a significant retardation or even loss of Ramon flocculation, without any change either in the optimum

[6] The total number of amino acid NH_2 groups incorporated in globulin far outnumbers those of histidine NH, which constitutes only 2.8 per cent of the serum protein (11 *a*). If we assume that the number of free NH_2 groups in protein bears a similar relationship to the number of histidine NH groups capable of reacting with formaldehyde, and if we assume an equal reactivity with formaldehyde, it follows that the first few groups in the antibody to react with HCOH are the NH_2 rather than the NH.

[7] Almost instantaneous with *e.g.*, a 1:20 ratio of formaldehyde:serum.

toxin:antitoxin ratio, or in the protective action of the preparation *in vivo*. Similar heating at pH 5.8 to 6.8 had only a slight effect on the flocculation reaction. It is of interest that a similar loss of precipitation and agglutination, with no impairment of protective action. was noted by Felton and Bailey (11 *b*) on heating antipneumococcic serum for ½ hour at 56°C.

TABLE IV

The Effect of Heating at 56°C. on Ramon Flocculation Time of Diphtheric Antitoxin Serum

Antiserum No.	pH before heating	Heating at 56°C.				pH after heating at 4 hrs. at 56°C.
		0	1 hr.	2 hrs.	4 hrs.	
		Optimum flocculation time with toxin				
		min.	*min.*	*min.*	*min.*	
1	9.36	75	95	100	210	—
	5.8 to 6.1	67		80	80	
2	9.57	70	180	360	600	9.3
	5.8 to 6.1	65		80	95	
3	10.1	95	180	95	105	9.35
	5.8 to 6.1	65	—			
4	9.58	65	180	420	1440	—
	5.8 to 6.1	65	—	80	—	
5	9.4	60	140	420	—	9.0
	5.8 to 6.1	55	—	100	—	
6	10.05	75	420	1440		—
	5.8 to 6.1	65	—	100	—	
7	9.78	70	420	420	1440	9.4
	5.8 to 6.1	65		125	120	

The Effect of Formaldehyde on Antipneumococcus Serum

In the case of a mixed Type I and II antipneumococcus serum, 1 part of 37 per cent formaldehyde to 2048 parts of serum, acting for 24 hours at room temperature, largely inhibited its precipitating activity with the type specific capsular carbohydrates; and a 1:1024 ratio

TABL

The Effect of Formaldehyde Acting for 24 Hours on the Type I Precipitating, Agglutina

Antiserum	37 per cent* HCOH	Molar ratio of HCOH: protein†	M/1 NaOH necessary to neutralize	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight‡	A Precipitation of type specific acetyl polysaccharide: Varying amounts of treated serum + 0.2 cc. 1:400,000 acetylated SSSI								A: Supernatant of previous section + 0.2 cc. untreated serum (test for free carbohydrate)							B Agglutination: Varying amounts of treated serum + 0.2 cc. bacterial suspension: 4 hrs. at 37°C.						
					0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.								0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0
cc.	*cc.*		*cc.*																							
12.8	0	0	0	—	4	4	4	3	2	1	±	0	0	0	0	4	4	4	4	4	4	4	0	0	0
12.8	0.0062	7.5:1	0.03	3	Cl	Cl	Cl	0	0	0	0	0	Cl	Cl	Cl	4	4	4	4	3	4–	4–	±	0	0	0
12.8	0.0125	15:1	0.09	8	0	0	0	0	0	0	0	0	Cl	Cl	Cl	2	4	4	4	1	1	1	0	0	0	0
12.8	0.025	30:1	0.14	14									0	0	0	Cl	2	4	4	±	±	±	0	0	0	0
12.8	0.05	60:1	0.21	21									0	0	0	0	Cl	2	2	0	0	0	0	0	0	0
12.8	0.1	120:1	0.28	27	No precipitation in any								0	0	0	2	4	4	4	0	0	0	0	0	0	0
12.8	0.2	240:1	0.37	36									0	0	2	4	4	4	4							
12.8	0.4	480:1	0.42	41									±	2	4	4	4	4	4							
12.8	0.8	960:1	0.44	43																No agglutination						
12.8	1.6	1920:1	0.46	45									1	2	4	4	4	4	4							
12.8	3.2	3840:1	0.49	48									2	3	4	4	4	4	4							
12.8	6.4	7680:1	0.5	49									2	4	4	4	4	4	4							
12.8	12.8	15,360:1	0.51	50									2	4	4	4	4	4	4							

Cl = cloudy; numbers 1 to 4 represent increasing degrees of precipitation after 4 hours at 37°C., S = survived; 1 = dead in 1 day, etc.

* Allowed to act overnight at room temperature.

† Assuming a molecular weight of 100,000 for serum protein, serum containing 8 per cent prote

‡ See †, Table I and footnote 5, page 499.

E V

ting and Protective Action of a Mixed Type I and Type II Antipneumococcus Serum

of bacteria	C Protection of mice								
Readings after mild centrifugation	Varying amounts of treated 1:2 serum + 0.1 cc. pneumococcus culture								Conclusions
	1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0	
4 4 4 4 4 0 0 4 4 4 4 4 0 0	2SSSS		1SSSS	SSSSS	4SSSS	223SS	22345	11111	Carbohydrate-precipitating activity of antiserum inhibited; combining affinity unaffected
4 4 4 4 4 0 0 4 4 4 4 4 0 0 ~~4 4 4 4 4~~ 0 0	134SS	11123	11233		22333				Agglutination inhibited; but antiserum can still combine with organisms, as shown by centrifuge agglutination, and can still combine with carbohydrate. Marked decrease in protective action
~~4 4 4 4~~ 0 0 0 2 ± 0 0 0 0 0 0 0 0 No agglutination	1112	11111		12222					Progressive decrease, and eventual disappearance of combining affinity for carbohydrate, centrifuge agglutination and protective action. Activity of antiserum acting as antigen in guinea pigs sensitized to horse serum also impaired (*cf.* Table VI)

followed by 18 hours at 2°C.

in is 0.0008 M; commercial formaldehyde is approximately 12 M.

prevented precipitation entirely. However, as is shown in Tables V and VI, the treated antibody could still combine with the carbohydrates.[8] On the addition of normal antibody to a non-precipitating mixture, no precipitation was observed; the carbohydrate had apparently been found by the treated antibody, but the secondary aggregation had been somehow prevented. The rough measure of combining affinity illustrated in section A of Tables V and VI revealed no demonstrable decrease.

At this stage the treated serum could still agglutinate bacteria. Larger amounts of 37 per cent formaldehyde (1 part to 64–256 parts serum) caused an apparent loss of agglutinating activity. On centrifugation, however, the bacteria cohered to form the characteristic flake. The treated antibody could apparently still combine with the bacteria, and its activity in this respect was not significantly less than that of the original serum, as shown by centrifuge agglutination. However, the surface deposit of antibody protein was apparently less conducive to aggregation than normally, and it required the pressure packing of the centrifuge to produce cohesion.

With larger amounts of formaldehyde, there was a progressive decrease and eventual disappearance of both centrifuge agglutination and protective action. As long as the antibody could cause spontaneous agglutination, it was capable of protecting mice; but when the protein had been so altered that centrifugation was required in order to produce aggregation, its protective action was definitely impaired.

These effects of formaldehyde on pneumococcus antiserum, as well as those discussed in the following section, have been qualitatively reproduced with acetaldehyde, benzaldehyde and butyraldehyde. The first was almost as active as formaldehyde; benzaldehyde was only a fraction as active, and butyraldehyde was almost inert.

[8] Heidelberger and Kabat (15) have recently shown that the diazo-treated pneumococcus antibody also combines with carbohydrate. This we have been able to confirm. In the original paper of Eagle, Smith and Vickers (1) on the effect of diazo compounds, some evidence was presented against such combination; but as was there stated (page 629), the possibility of combination could not be excluded. The experiments of Heidelberger and Kabat clearly show that it does occur with diazo-treated antipneumococcus serum; and the present experiments further show that it occurs with formaldehyde-treated serum.

TABLE VI

The Effect of Formaldehyde Acting for 24 Hours on the Type II Precipitating, Agglutinating and Protective Action of a Mixed Type I and Type II Antipneumococcus Serum

(Serum treated as in first few columns of Table V)

Molar ratio of HCOH: protein	Approximate number of formolized NH_2 groups in antibody molecule per 100,000 molecular weight*	A. Precipitation of type specific polysaccharide: Varying amounts of treated serum + 0.2 cc. 1:400,000 acetylated SSS II	A. Precipitation of type specific polysaccharide: Supernatant + 0.2 cc. untreated serum (test for free carbohydrate)	B. Agglutination of bacteria (Type II): Varying amounts of treated serum + 0.2 cc. bacterial suspension: 4 hrs. at 37°C.	B. Agglutination of bacteria (Type II): Readings after mild centrifugation	C. Protection of mice	Anaphylaxis experiments with guinea pigs sensitized to native horse serum. Amount of treated 1:2 serum injected: 2 cc.	1 cc.	½ cc.	Conclusions
0	—	4 4 3 2 1 ± 0	0 0 0 4 4 4 4	4 4 4 4 4 2 0	4 4 4 4 4 2 0	4SSSS 14SSS 234SS 234SS	D	D, D	D, D	Carbohydrate-precipitating activity of antiserum inhibited; combining affinity unaffected
7.5:1	3	± ± ± 0 0 0	Cl Cl Cl 4 4 4 4	3 4 4 4 0 0 0	4 4 4 4 4 0 0					
15:1	8	0 0 0 0 0 0	0 0 0 3 4 4 4	2 2 ± 0 0 0 0	4 4 4 4 4 0 0					
30:1	14		0 0 0 2 4 4 4	1 1 ± 0 0 0 0	4 4 4 4 2 0 0					Agglutination inhibited; but antiserum can still combine with organisms, as shown by centrifuge agglutination, and can still combine with carbohydrate. Marked decrease in protective action
60:1	21		0 0 0 0 0 Cl 2	0 0 0 0 0 0 0	4 4 4 4 4 0 0	34SSS 12234 2223S 12234			D, D	
120:1	27		—	0 0 0 0 0 0 0	4 4 4 4 2 0 0					
240:1	36		—	—	4 4 4 4 3 0 0	224SS 11124 11234				
480:1	41		—		4 4 4 3 1 0 0					Progressive decrease, and eventual disappearance of combining affinity for carbohydrate, centrifuge agglutination and protective action.
960:1	43		—		3 3 3 ± 0 0 0	12344 11111	Prostrated	Scratch		
1920:1	45		0 0 0 4 4 4 4		2 2 2 ± 0 0 0		0, 0	0		
3840:1	48		Cl 2 4 4 4 4 4		± ± ± ± 0 0 0		D, 0			
7680:1	49		2 4 4 4 4 4 4		0 0 0 0 0 0 0					
15,360:1	50		4 4 4 4 4 4 4		0 0 0 0 0 0 0		0, scratch	Scratch	0	Activity of antiserum acting as antigen in guinea pigs sensitized to horse serum also impaired

Cl = cloudy; numbers 1 to 4 represent increasing degrees of precipitation after 4 hours at 37°C., followed by 18 hours at 2°C.

* See †, Table I, and footnote 5, page 499.

In order to make a rough approximation of the number of groups in the antibody molecule affected by the HCOH, we may assume that serum protein has an average molecular weight of 100,000. If the molecular weight of the antibody is several times that quantity, as recent measurements by Heidelberger, Pedersen and Tiselius (12 *a*) indicate, the number of antibody groups affected is the corresponding multiple of the calculated number. It follows from the data of Tables V and VI that an amount of formaldehyde which could combine with at most 7 to 8 NH_2 groups in the antibody molecule for each 100,000 molecular weight (1 part 37 per cent HCOH to 2048 parts serum), which probably blocked no more than 3 to 4 such groups, and which did not demonstrably change the pH of the serum, nevertheless sufficed to destroy its precipitating activity with carbohydrate almost completely, without affecting its combining power with either the carbohydrate or the bacterial cell. An amount of HCOH which could combine with 15 NH_2 groups per 100,000 molecular weight, and which did combine with 9, inhibited spontaneous agglutinating activity, but again did not affect the combining power with bacteria, as shown by centrifuge agglutination. Eight to 32 times that quantity of HCOH was necessary before the combining power with either carbohydrate or bacteria began to be significantly impaired. This represents a concentration of 0.3 to 1.2 per cent HCOH, enough to block most of the NH_2 groups in the antibody molecule (sixth column of Table V). As in the case of other agglutinating antibodies (9), and of diphtheria antitoxin, it would therefore appear that free NH_2 groups are not primarily concerned in the combination between horse antipneumococcus serum and either the bacterial cell or the free carbohydrate. Paradoxically, concentrated refined antipneumococcus globulin was not affected by HCOH in concentrations which were found to destroy the aggregating activity of the native antiserum. This decreased susceptibility to HCOH of the isolated antibody is being further investigated.

One can only speculate as to whether the loss of flocculating activity with carbohydrate or bacteria caused by small concentrations of HCOH is due to the blocking of a few amino groups, or whether there is some more complicated reaction between the antibody protein and the formaldehyde. The successful reversal of the inactivated anti-

body by Chow and Geobel would indicate that the formation of a few —N=CH_2 groups is primarily responsible for the loss of flocculating activity (*cf.* page 502).

Some Observations on the Mechanism of Antigen-Antibody Aggregation

It was suggested in a previous communication (12 *b*) that the specific combining groups of antibody may be strongly hydrophilic, and that their elimination in the course of the antigen-antibody combination may result in a relatively insoluble compound. Antigen-antibody flocculation would simply reflect this decreased solubility. On this theory, only the combination of antigen and antibody is due to specific forces of attraction, and the secondary aggregation is non-specific. An alternative explanation of antigen-antibody aggregation has been suggested by Marrack (13) and Heidelberger (14). An elementary antigen-antibody compound would combine with similar compounds by virtue of residual specific linkages to form aggregates of increasing size, which eventually reach the limits of visibility. The antigen-antibody aggregate would accordingly be a lattice-like structure in which each molecule of antigen is bound to several molecules of antibody, and each molecule of antibody is similarly bound to several molecules of antigen. On this theory, both the first stage of combination and the second stage of aggregation are due to the same specific forces of attraction between antigen and antibody.

As shown in the present paper, an amount of HCOH sufficient to couple with only 7 or 8 groups of antibody for each 100,000 molecular weight, and which probably blocked no more than 3 to 4 groups, did not affect its combining affinity for the corresponding antigen, but completely inhibited the flocculating activity of antitoxin with toxin, and of antipneumococcus serum with carbohydrate. This finding is difficult to reconcile with the Marrack-Heidelberger theory of antigen-antibody aggregation. If aggregation were due to the same specific linkages which make for combination, as long as the antibody remains capable of combining with antigen, aggregation should follow as a matter of course; and the addition of a few molecules of formaldehyde should have no effect. Formaldehyde-treated (or diazo-treated) diphtheria antitoxin which combines with toxin should precipitate

at the unchanged optimum toxin : antitoxin ratio; and similarly treated pneumococcus antibody, which combines with carbohydrate,[8] should cause its precipitation. In both cases, the observed absence of visible aggregation is clearly not due to a loss of combining affinity, and cannot be explained on the Marrack-Heidelberger theory that formation of visible antigen-antibody compounds (agglutination and precipitation) is due solely to the specific combining groups.

Similarly, the fact that pneumococcus antibody adequately treated with either formaldehyde or diazo compounds fails to agglutinate pneumococci, despite the fact that combination has occurred (page 506), seems inconsistent with the mechanism of specific agglutination postulated by the investigators.[9]

The present observations are, however, consistent with the hypothesis that the specifically reactive groups of antibody protein contribute to its solubility, and that their elimination in the course of antigen-antibody combination results in a relatively insoluble antibody protein, and thus, in the precipitation of the antigen-antibody compound. One need only assume that formaldehyde (or diazo compounds) added on to antibody protein, most probably to the free NH_2 groups, increases its solubility. The following experiments were carried out to test that assumption.

Antipneumococcus antibody is normally water-insoluble and is precipitated from the antiserum on dilution with water. After treating serum for 24 hours at room temperature with as little as 1 part of 37 per cent HCOH to 2048 parts of serum there was a significant increase in the solubility of the antibody, as shown by a marked increase in the amount of water necessary to cause its immediate precipitation, and by a decreased amount of precipitate on dilution with ten volumes of cold water. This decrease was reflected both by the decreased agglutinating titer of the redissolved precipitate and by the actual amount of protein precipitated. Higher concentrations of HCOH resulted in an antibody which could no longer be precipitated

[9] Hooker (16) has recently presented evidence from an entirely different point of view which seems equally inconsistent with the theory that the secondary aggregation of antigen-antibody compounds is due to the same specific forces of attraction which bring about the original combination.

TABLE VII

The Effect of Formaldehyde, Acting for 24 Hours at 26°C., on the Water Solubility of Pneumococcus Antibody

Antiserum	37 per cent HCOH	Approximate molar ratio of HCOH: protein*	Reactivity of whole treated antiserum (Same technic as indicated in Tables V and VI)								Amount of water necessary to cause beginning precipitation of treated antibody	Amount of antibody N precipitated after 2 hrs. at 2°C. on dilution with 20 cc. cold water	Conclusions
			Carbohydrate precipitation SSS II						Agglutination (Type I) Same amounts as previous section	Centrifuge agglutination (Tubes of preceding section)			
			0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.01 25 cc.	0.0062 cc.					
cc.	*cc.*										*cc.*	*mg.*	
1.6	0	0	4	4	4	4	Cl†	0	444400	4444?0	3.3	2.5	Progressive increase in water solubility as antibody is treated with formaldehyde. Point at which antibody is no longer precipitable by water coincides with disappearance of precipitability by specific carbohydrate: Combining power with carbohydrate or bacteria unaffected
1.6	0.0006	6:1	4	4	4	3	Cl	0	444400	444400	10.0	1.0	
1.6	0.0008	8:1	4	4	4	Cl	Cl	0	444400	444400	20.0	0.9	
1.6	0.0012	12:1	3	3	1	Cl	Cl	0	444400	444400	No immediate precipitation on dilution with water in any amount	0.4	
1.6	0.0016	16:1	Cl	Cl	Cl	Cl	±	0	444300	444400		No significant amount of precipitate	
1.6	0.0024	24:1	Cl	Cl	±	0	0	0	443200	444400			
1.6	0.0032	32:1	0	0	0	0	0	0	333200	444400			
1.6	0.0048	48:1	0	0	0	0	0	0	332100	444200			

* *Cf.* footnote 5, page 499; and Table I.

† Contents cloudy: no immediate precipitate.

by dilution with water or by dialysis (Table VII).[10] It is significant that the same amount of treatment which rendered the antibody water-soluble, also largely inhibited its precipitating activity with capsular carbohydrate (*cf.* Tables V, VI and VII). Similar experiments with diazo compounds have yielded qualitatively similar results. Wholly analogous to the increased solubility caused by formaldehyde and diazo compounds is the observation by Felton and Bailey (11 *b*) that horse antipneumococcus sera heated at 56°C. for 30 minutes in large measure lost their precipitating, agglutinating and complement fixing activity, but that their protective action *in vivo* was unaffected; and that such heated sera no longer yielded a precipitate on dilution with water.

These several observations with antipneumococcus and antitoxin serum strongly support the theory that antigen-antibody aggregation is primarily determined by the insolubility of the bound antibody. The formaldehyde-treated, diazo-treated or heated antibody can still combine with antigen, and specifically reactive water-soluble groups are thus eliminated. Normally, this would suffice to make the antibody protein sufficiently insoluble to cause visible flocculation of the antigen-antibody compound. In the treated antibody, however, the highly soluble groups formed by the addition of a few molecules of formaldehyde or of diazo compound to the antibody, groups which are not involved in its combination with antigen, apparently suffice to keep the compound in solution, and there is no aggregation.[11]

SUMMARY

Small amounts of formaldehyde inhibited the precipitating activity of horse diphtheria antitoxin with toxin and of horse antipneumococcus

[10] This amount of treatment with formaldehyde did not significantly affect either the pH of the solution, or the isoelectric point of the serum protein as determined by the optimum pH for precipitation. At that isoelectric point, however, there was a copious precipitate, no less than that obtained from untreated serum; indeed, strongly formolized serum yielded even more precipitate than the control, untreated serum.

[11] The fact that the antibody content of some antipneumococcus sera is lower when tested by carbohydrate precipitation than it is when tested by mouse protection or carbohydrate combination suggests that in these sera the antibody may be normally water-soluble to a greater extent than is usually the case (*cf.* 17).

serum with the homologous capsular carbohydrate. Approximately 1 part of commercial formaldehyde to 1000 parts of serum, acting for 24 hours, inhibited the flocculating activity completely. In both cases, the combining affinity of the treated antibody for the corresponding antigen was not demonstrably affected, as determined both by *in vitro* experiments and by animal protection. More intensive treatment of the antipneumococcus serum caused an apparent loss of its bacterial agglutinating activity, but on centrifugation the organisms cohered: combination had occurred, and only the spontaneous aggregation was prevented. These effects are the same as those previously described for diazo compounds, and have been qualitatively reproduced with acetaldehyde, benzaldehyde and butyraldehyde.

The quantitative relationships suggest that only a few groups in the antibody molecule need be modified by formaldehyde in order to prevent aggregation; and it is probable that these are some of the free NH_2 groups of the antibody protein. In marked contrast, the combining affinity of both antipneumococcus antibody and diphtheria antitoxin for the corresponding antigens was only slightly affected by amounts of formaldehyde which sufficed to block the free NH_2 groups rapidly and almost completely. Similarly, this amount of treatment did not affect the reactivity of these two antisera acting as antigen with a rabbit antiserum *versus* horse serum. The integrity of the NH_2 groups is apparently not essential for the activity of these sera acting either as antigen or as antibody; and the slow disappearance of their activity in concentrated HCOH is apparently to be ascribed to some secondary reaction other than the simple addition of HCOH to free NH_2 groups.

The present experiments do not support the theory that antigen-antibody aggregates are lattice-like structures built up from elementary antigen-antibody compounds because of residual specific combining groups. The aggregating activity of both antipneumococcus serum and diphtheria antitoxin was completely inhibited by procedures which did not demonstrably affect their combining power with antigen. This suggests that the aggregation of antigen-antibody compounds is a secondary, non-specific reaction. It is perhaps significant that the amount of formaldehyde which just sufficed to prevent aggregation also caused a marked increase in the solubility of the pneumococcus antibody, which could then no longer be precipitated

at serum pH by dilution with water or by dialysis. This strongly suggests that the loss of precipitating activity is actually due to the increased solubility of the antibody and supports the hypothesis that the primary cause of specific antigen-antibody aggregation is the relative insolubility of the bound antibody.

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