ON THE MECHANISM OF SPECIFIC PRECIPITATION

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It has been pointed out (13, 10, 17) that according to the "alternation" (= "mutual multivalence" = "lattice" = "framework") theory of serological reactions, simple chemical compounds, if they contain as many as two (or at any rate three) groups capable of reacting specifically with antibody, should be able to form precipitates when mixed with the appropriate antisera. It is apparent that such precipitation would not be expected on the basis of the views of Bordet (3) concerning serological reactions, with their emphasis on the covering of the surface of the antigen by a film of antibody.[1] Tests of this prediction of the alternation theory have been made (10, 11, 17) with results which are not in full agreement with the older (or the newer) theory. This raises the question if either point of view can be quite correct, and it is the purpose of the present communication to report the results of experiments designed to throw light on this question.

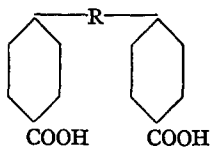
Pauling, Campbell, and Pressman (17) have suggested that the failure of Hooker and Boyd (11) to obtain specific precipitation with their divalent haptens could be attributed in part to the small size of the molecules studied, stating that "Steric repulsion between two antibody molecules attached to such small molecules would be much stronger than for the molecules used by Landsteiner and van der Scheer and by us." It will be seen below that this can hardly be the true explanation, but in view of this suggestion, and in view of the fact that Hooker and Boyd failed to observe precipitation with compound "V" (see below), where R = —N=N—$C_6H_4AsO_3H_2$, but did with "VII," where R = —N=N—C_6H_4=N=N—$C_6H_4AsO_3H_2$, it was considered necessary to make compounds in which the reactive groups were about as far apart as in the latter compound.

In the course of this work 34 different compounds were made and tested. Seven of these had been previously studied (11, 17), the others had not been examined, and apparently the majority of them have been made here for the first time. We may divide the parent compounds on the basis of structure into

[1] Except presumably with rather large molecules such as polysaccharides, or simple molecules which associate in solution to give larger particles.

five classes, I, II, III, IVA, and IVB, which have the composition shown below, where R represents the group which varied.

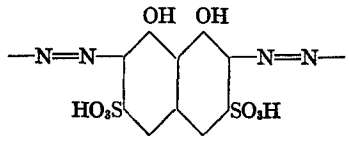
I



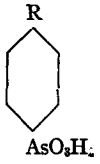
(C_2) R = —

(ZC_2) R = —N=N—

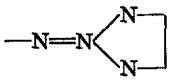
(NC_2) R =



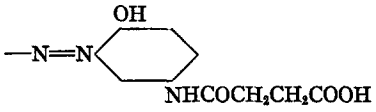
II



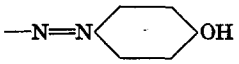
(IA) R =



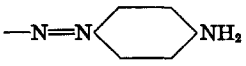
(SA) R =



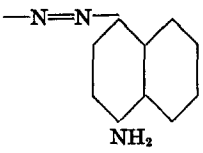
(HA) R =



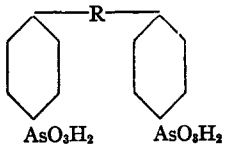
(AA) R =



(NA) R =

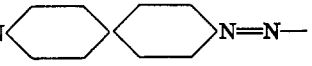


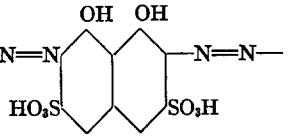
III



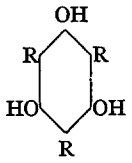
(ZA_2) R = —N=N—

(SA_2) R = —NHCOCH$_2$CH$_2$CONH—

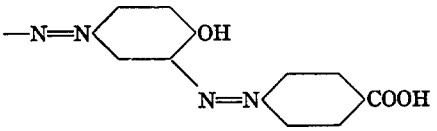
(DA_2) R = 

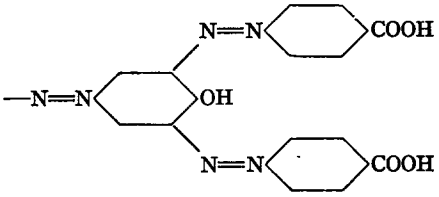
(NA_2) R = 

IV



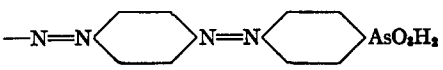
A

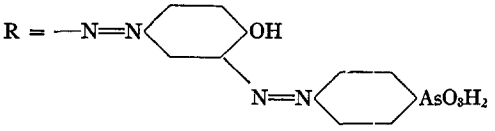
(PC_3) R = 

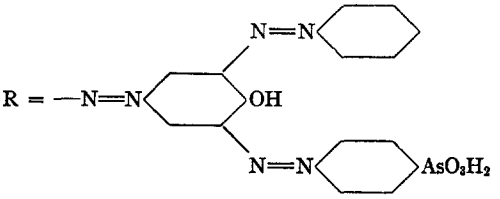
(PC_6) R = 

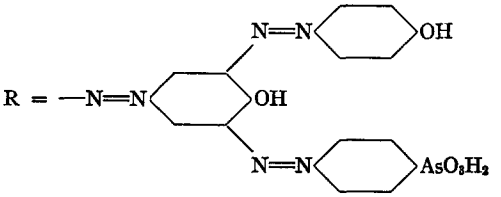
B

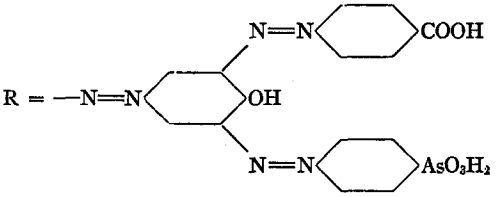
(V) R = 

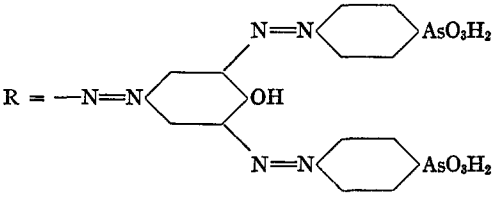
(VII) R = 

(PA_3) 

(PP_3A_3) 

(PH_3A_3) 

(PC_3A_3) 

(PA_6) 

The combinations of letters in parentheses are shorthand designations for the various compounds, for convenience in reference. It will be noted that A represents the arsonic acid group, C the carboxylic acid group, Z the azo linkage, P phloroglucinol, etc. The compounds designated by the Roman numerals V and VII are the compounds thus numbered in the paper of Pauling, Campbell, and Pressman (17).

In addition to these compounds, the following were made by acetylating or benzoylating certain of the above (a represents acetyl, b, benzoyl): NAb, NA_2b, PC_3a, PC_3b, PC_6a, PC_6b, PA_3a, PA_3b, PC_3A_3a, PC_3A_3b, PA_6a, PA_6b.

All the haptens were brought into solution as the sodium salt, by addition of the minimum of NaOH. The concentration of the stock solutions was in each case 1 mg./cc.

Tests with a few of the haptens brought to definite pH indicated that the exact pH of the solutions was not important, doubtless because the pH of the serum-hapten mixtures is in all cases controlled almost completely by the relatively large buffer power of the serum.

Materials and Methods

The preparation of compounds of class I has already been described (11). The compounds of class II were all made by coupling diazotized arsanilic acid with the appropriate substance. Of the compounds of class III, NA_2 has been described (11), SA_2 was made as described by Morgan and Walton (14), DA_2 was made by the Bart reaction from

Of the compounds of class IV, V was made by coupling three equivalents of diazotized arsanilic acid with phloroglucinol (17), VII by coupling three equivalents of diazotized *p*-amino-azobenzene-arsonic acid (AA) with phloroglucinol. In addition to my own preparations of V and VII, small samples were available which Drs. Pauling, Campbell, and Pressman kindly sent me, prepared as described by them (17); no differences in behavior were observed. The other compounds were made by coupling three equivalents of diazotized *p*-amino phenol with phloroglucinol, purifying, then coupling to this in alkaline solution the appropriate amounts of other diazotized amines, in the presence of pyridine (18). These compounds were purified first by repeatedly dissolving in alkali and precipitating with acid, then by crystallization from alcohol-water mixtures.

The constitution of several of the arsenic-containing compounds was checked by drying and analyzing them for arsenic.

The antisera used in these experiments were of two kinds, designated as anti-C and anti-A. The former were made by injecting rabbits with *Limulus* hemocyanin coupled with diazotized *p*-amino-benzoic acid, the latter by injecting *Limulus* hemocyanin coupled with diazotized arsanilic acid. Four different anti-C sera, each represented by two bleedings at different stages of immunization, were available, *viz.*, 161, 162, 163, 165. The corresponding anti-A sera were 239, 241, 243, 244, 271. Two different bleedings from each of these rabbits were also available. The earlier bleedings seemed somewhat superior. The anti-C sera contained about 0.2 mg. of antibody N per cc., the anti-A sera about 0.3.

The tests for precipitability of the various haptens were made by mixing in small tubes 0.3 cc. of antiserum with 0.3 cc. of an appropriate dilution of the hapten, allowing to stand overnight in the ice box, then examining for precipitate. Controls consisting of hapten alone, hapten plus saline, and hapten plus normal rabbit serum were always included, and were consistently negative.

RESULTS

None of these haptens were precipitated by any of the anti-C sera, in any of a large number of dilutions tested. The results with compounds of classes I and IVA were thus consistently negative.

None of the univalent arsenic-containing haptens (class II) were precipitated. It will therefore be unnecessary to present details of tests with compounds of any of these three classes.

TABLE I

Tests for Specific Precipitation of Anti-A Sera Mixed with Haptens of Class III

Serum No.	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1.6
271-I	ZA_2	−	−	++	+	−
239-I	ZA_2	−	+w	++	+	−
271-I	SA_2	−	−	−	−	−
239-I	SA_2	−	−	−	−	−
271-I	DA_2	−	−	−	−	−
239-I	DA_2	−	−	−	−	−

− means no precipitation; +w means very slight precipitation; + means definite precipitation; ++ means marked precipitate formation.

TABLE II

Tests for Specific Precipitation of Anti-A Sera Mixed with Haptens of Class IV B

Serum No.	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1.6
243-I	PA_3	−	−	−	−	−
271-I	PA_3	−	−	−	−	−
239-I	PC_3A_3	−	−	−	−	−
271-I	PC_3A_3	−	−	−	−	−
271-I	PH_3A_3	−	−	±	−	−
271-I	PP_3A_3	−	±	+±	+w	−
243-I	PA_6	−	−	−	±	−
271-I	PA_6	−	−	−	+	−

± means doubtful formation of precipitate. Other symbols as in Table I.

The results of tests with compounds of class III are shown in Table I. It may be seen that only one (ZA_2) of these divalent haptens gave any precipitate.

Of compounds of class IVB, a number were specifically precipitable by anti-A sera, in a way which was seen to depend definitely on their constitution, but not in the way demanded by the "alternation" theory.

The results obtained with compounds of class IVB are shown in Table II. (It will be recalled that class IVA (carboxy compounds) was entirely negative). Of the haptens shown in Table II, two were negative, one gave a very weak reaction, another a slight positive reaction, and only one precipitated well. All the compounds which failed to precipitate were retested with other anti-A sera, with the same results, and compounds which did precipitate were tested with other anti-A sera and with unrelated antisera to control the specificity of the reactions. These results are not shown here.

DISCUSSION

It is apparent from Tables I and II, and the results obtained with compounds of classes I and IVA, that predictions based on the alternation theory are by no means regularly fulfilled. No carboxy compound was ever observed to precipitate, and many of the arsonic haptens, even those containing three reactive groups, adequately separated, failed to precipitate. In all, six divalent haptens, four trivalent, and one hexavalent, failed to precipitate. These compounds nevertheless reacted with the appropriate antibodies, as was shown by inhibition experiments. It seems clear that the possibility of "lattice" (framework) formation is by no means sufficient to insure that a hapten will precipitate. As a matter of fact, a careful examination of Table II discloses a different sort of correlation between constitution and precipitability which probably provides a much more valid basis on which to predict the behavior of haptens.

It may be assumed that all the arsonic acid groups in the first four haptens shown in Table II are capable of combining with molecules of antibody, and calculations of the size of antibody molecules and the relative distances in these hapten molecules support this idea. This combination blocks off the solubilizing action which the arsonic acid groups are known to exert, and at the same time at least one, possibly several, polar groups of the antibody are combined with. The result is a complex of one hapten and three antibody molecules, which being larger than one antibody molecule, demands at least three times, perhaps more, the number of solubilizing polar groups which would just suffice to keep an antibody molecule in solution. From what is known of protein chemistry, we may surmise that this latter number probably lies somewhere between 5 and 40. In hapten PA_3, three outer[2] hydroxyl groups still remain uncombined with, and in PC_3A_3 there are in addition three carboxyl groups. This, in addition to the polar groups remaining free on the antibody molecules, evidently is sufficient to keep the complex in solution, for no precipitate is formed. The importance of solubilizing groups in immune reactions has been commented on by Eagle (6) who observed that the introduction of a

[2] The inner hydroxyls, *i.e.*, those on the phloroglucinol residue, are probably too completely blocked off mechanically (by steric hindrance) to have their full solubilizing effect.

few soluble groups into the antitoxin molecule rendered it incapable of flocculating toxin, although it could still combine.

If the size of the hapten, and at the same time its non-polar character, are increased by the addition of three phenyl groups, as in hapten PP_3A_3, the result is so insoluble that the three free hydroxyl groups are no longer sufficient, and the compound is precipitable. If the size is similarly increased, but the polar character kept approximately the same by the introduction of three new hydroxyl groups, as in hapten PH_3A_3, the resulting hapten does not precipitate, or precipitates only slightly.

From spatial considerations, it is doubtful if all six of the reactive groups in hapten PA_6 can simultaneously combine with antibody molecules, but it is

TABLE III

Tests for Specific Precipitation of Acetylated (a) and Benzoylated (b) Derivatives of Compounds of Class IV B

Serum No.	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1.6
271-I	PA_3a	−?	+	±	−	−
271-I	PA_3b	−	−	±	+	−
271-I	PC_3A_3a	−	−	+w	−	−
271-II	PC_3A_3a	−	−	+w	−	−
271-I	PC_3A_3b	−	−	−	±	−
271-I	PA_6a	−	−	++	+	±
271-I	PA_6b	−	−	+w	++	+

Symbols as in Tables I and II.

probable that more than three are capable of doing so, leaving less than three solubilizing groups in addition to the hydroxyls. It is therefore not surprising that this compound precipitates, although it will be noted that its precipitability is much inferior to that of PP_3A_3 (or of ZA_2 or VII). It is probable that a mechanism proposed by Hooker and Boyd (11), namely, mechanical occlusion of polar solubilizing groups of neighboring antibody molecules, also comes into play in this instance, and perhaps in the above cases too.

If the above explanation were correct, we should expect that acetylating or benzoylating the unprecipitable class IV haptens, so as to block off the solubilizing hydroxyls, would render them specifically precipitable. That this is so is shown by Table III, which gives the results of tests on acetylated and benzoylated derivatives of some of the compounds of Table II.

It is of considerable interest that the precipitability of the acetylated or benzoylated PC_3A_3 is very slight, which may doubtless be attributed to the

three carboxyl groups still remaining. In fact the precipitability of PC_3A_3a and PC_3A_3b is quite comparable with that of the unmodified PA_6 (Table II). Similarly benzoylation of hapten NA_2, which in addition to the two hydroxyls has two sulfonic acid groups, did not render it precipitable.

The relation of structure to precipitability in these compounds seems quite clear, and the possibility of "lattice" formation is evidently quite irrelevant, even if we grant the unproven assumption that the anti-A antibody is divalent. How may we explain the results obtained with divalent haptens of class III, shown in Table I, where one compound precipitated, whereas two did not, although few if any additional solubilizing groups are present?

It seems probable that the explanation of these results again rests upon the mechanical occlusion of polar groups of the neighboring antibody molecules, which in hapten ZA_2 would be brought quite close together, whereas in haptens SA_2 and DA_2 they would be farther separated, so that all their polar groups, with the exception of those actually concerned in combining with the arsonic acid groups, would remain free to keep the complex in solution. If this suggestion is correct, the idea of Pauling, Campbell, and Pressman, that the smaller hapten molecules cannot precipitate, is erroneous. Instead, the converse would seem to be true, namely, the reason haptens SA_2 and DA_2 do not precipitate is that they are too large (*i.e.*, their combining groups are too widely separated).

The above interpretation is strengthened by the observation that univalent haptens (class II) never precipitate, even in the case of a biggish molecule (NAb) having no free polar groups in addition to the arsonic acid group through which combination is effected. Evidently the reduction in free polar groups of the antibody molecule which follws this combination is by itself insufficient to reduce the solubility significantly, in the absence of mechanical hindrance due to the near-by presence of another molecule of antibody.

It is not easy to say precisely how these considerations apply to the precipitation of the divalent haptens studied by Landsteiner and van der Scheer (12), such as resorcinol-disazo-*p*-suberanilic acid. At first sight, it might seem that in this hapten the two reactive groups are quite separated. However, the antisera used in these experiments were rather specific for the various anilic acids made from fatty acids of various lengths, which may indicate that the antibody when reacting with this hapten combined not only with the carboxy group but with the whole side chain right up to the resorcinol residue, which would bring the two molecules of antibody in rather close apposition. Only two solubilizing groups, the two hydroxyls of the resorcinol, then remain, and they may be somewhat hindered by the presence of the antibody molecules. Our present knowledge of the numbers of polar groups required to keep such complexes in solution does not seem sufficient to enable us to say whether this would account for the precipitability. That it may is suggested by the ex-

planation offered by Landsteiner and van der Scheer themselves, which was in terms of peculiarities in constitution such as the long aliphatic chains, which would be fairly insoluble. The observation that these compounds precipitated better after their solutions had been allowed to stand, however, unlike the compounds studied by Pauling, Campbell, and Pressman, and by myself, suggests, as Landsteiner and van der Scheer pointed out, the possibility that this hapten might be somewhat aggregated in solution, giving particles possessing several combining groups, and large enough to combine simultaneously with several molecules of antibody.

The failure of any of the carboxy compounds to precipitate with anti-C sera rests undoubtedly upon the poor flocculating quality of these sera. Although they gave good reactions by the interfacial ("ring") technique, these sera, even when fresh, flocculated only slowly with casein coupled with diazotized *p*-aminobenzoic acid. (I have previously observed that the carboxy group does not have nearly the antigenic power of the arsonic acid group.) The precipitation of the arsenic-containing haptens was observed to be relatively slow compared to the flocculation of casein-arsanilic compounds by the same sera, in which case it was practically instantaneous. It is therefore not surprising that the much less "avid" anti-C antibodies could not effect precipitation of the carboxy haptens, all of which would be much less precipitable than conjugated protein antigens. Failure to take account of such facts seems to be another way in which the alternation theory is an oversimplification of the true mechanism.[3]

Finally we must discuss the failure of compound V to precipitate, when hapten VII, apparently so similar, precipitated readily. We can hardly avoid mention of this fact on the basis of the report of Pauling, Campbell, and Pressman that V did precipitate, for the discrepancy may possibly depend upon the differences in the antibodies in their sera and in mine, in which case it is unknown how the compounds studied here would have reacted with their sera. There is no doubt that V did not precipitate in my hands; it was tested against both bleedings of each of the five anti-A sera prepared, while these sera were still fresh and capable of precipitating hapten VII powerfully. In not a single one of these mixtures, no matter what the concentration of V, was the faintest trace of precipitation or clouding observed.

It seems likely that the failure of V to precipitate is due to the fact that the

[3] The test of the theory carried out by Hooker and Boyd (11) therefore seems to have been a fair test, within the framework of the hypothesis itself. It is rather interesting to consider, however, that if their sera had been sufficiently "avid," comparable to the anti-A sera studied here (assuming such anti-C sera can be made), Hooker and Boyd might have observed precipitate formation with the shorter of their haptens. Since the interpretation which they would have put on this result would probably have been the erroneous one that the alternation theory is perfectly correct, it is perhaps fortunate that this did not happen.

combining groups are insufficiently separated for more than two molecules of antibody to be able to combine simultaneously with the molecule, so that there are always free three hydroxyls and one arsonic acid group, which are sufficient to keep the complex soluble. In addition, the polar groups of the antibody molecules not concerned in the combination, might remain relatively free, as the antibody molecules would not be forced into intimate enough contact. In VII, the combining groups, being more widely separated, are probably all three able to combine simultaneously with antibody molecules. This leaves no arsonic acid groups free, and probably results in a good deal of steric hindrance of polar groups on the antibody molecules.

It is thus seen that the *possibility* of "framework" formation is by no means sufficient to insure specific precipitation, for although this possibility does not exist with hapten V if the above interpretation of the behavior of V is correct, it does exist in the case of haptens PA_3 and PC_3A_3 which did not precipitate, and hapten PH_3A_3, which gave only a trace of precipitate. Conversely, the possibility of framework formation does not seem to be necessary for precipitation, for hapten ZA_2 precipitated readily, which it could not do by framework formation unless the valence of antibody is more than two, which there is some reason to doubt, both on experimental grounds, and on the basis of theories of antibody formation (15, 2, 5, 16). It is true that Pauling has described a possible mechanism for the formation of trivalent antibody, but this seems to the present author to have been introduced chiefly for the purpose of accounting for the precipitation of divalent haptens, and to have no great plausibility, even from the point of view of Pauling's own theory. There is no experimental evidence for it.

In considering the bearing of these experiments on the "alternation" theory, we should keep clearly separted in our minds two aspects of this theory. The first is the clearly implied claim of its proponents that the possibility of framework ("lattice") formation is necessary and sufficient for the initiation of a serological reaction ("... aggregation would occur regardless of the affinity of the groupings for water" (7)). The second is the claim that larger aggregates are formed solely by the specific linkage of antibody groups with antigen groups. It seems to the present author that the experiments reported here completely disprove the first claim of the alternation theory. On the other hand, it is clear that if antibody is always divalent, these experiments do not bear particularly on the second claim.[4] I do not wish to be understood as denying that the alternation theory, in making this second claim, may be entirely justified. Indeed, evidence indicating that in certain cases the formation of larger aggregates is a phenomenon of a certain degree of specificity (19, 20) is difficult to explain, on the basis of present knowledge, unless we assume some such mechanism.

[4] The precipitability of the divalent hapten ZA_2 would appear to demand trivalent antibody.

The question would seem to depend ultimately on whether antibody molecules have in general more than one combining group, and the evidence now available is not sufficient to settle this point.

As an explanation of the *cause* of precipitation, it would seem that neither the alternation nor the Bordet theory is adequate. The alternation theory seems to be simply incorrect, and the Bordet theory too vague to account for the very definite facts presented here. For the theory suggested above (which is probably not original with me[5]), namely, that precipitation is due to lowering of solubility by neutralization of polar groups of antibody and hapten (or antigen) and concommitant steric hindrance of other polar groups of neighboring antibody molecules in the complex, I wish to propose the name, occlusion theory.

It will be noted that the occlusion theory simply attempts to explain why combination of antibody and antigen or hapten produces, in certain cases, a compound having too low a solubility to remain in solution. Nothing is said about the mechanism by which these primary aggregates unite with each other to build up the larger aggregates which are observed to form during serological precipitation. I shall for the present make no attempt to provide a detailed hypothetical mechanism for this, any more than I propose to explain how the aggregates are formed which result when a protein is salted out of solution by ammonium sulfate. *If* antibody has more than one combining group, it may well be that in specific precipitation the primary aggregates unite in a way very similar to that demanded by the alternation theory; indeed, it is hard to see how such unions could fail to play a prominent rôle in the formation of larger aggregates. It must be remembered, however, that "apparently decisive evidence" (8) has been offered that under some circumstances aggregates can be built up by a non-specific mechanism not involving "framework" formation (1, 9, 4).

If antibody were trivalent, it would certainly seem that all the divalent and trivalent haptens studied here should have been able to form frameworks, and this would be true with the trivalent haptens even if antibody were only divalent. Also divalent antibody ought to be able to build up with divalent haptens long chain-like aggregates showing pronounced birefringence of flow (Pauling), but this has not been observed (11). Until more experimental evidence is available, the multivalency (including divalency) of antibody remains almost purely a postulate. In any case, the present communication attempts simply to present evidence which seems to throw light on the reason for the tendency of primary aggregates not to remain in solution, a reason which seems to have been overlooked by the proponents of the alternation theory.

SUMMARY

A study of the precipitability by the appropriate antisera of 34 different haptens, containing from one to six reactive groups, leads to the conclusion that

[5] Compare Marrack (13), p. 150.

the possibility of framework ("lattice") formation is neither necessary nor sufficient for specific precipitation, but that instead precipitation depends upon the reduction, by mutual neutralization of polar groups of antibody and antigen (or hapten) and mechanical blocking off of polar groups of closely neighboring molecules of antibody, of the solubility of the complex below the point at which it can remain in solution. The decisive factors appear to be the number of polar groups of the antigen (hapten) left free, and the distance separating the different reactive groups, which determines the amount of steric hindrance exerted by one antibody molecule on another. No hypothesis is offered as to how these primary insoluble aggregates unite with each other to produce the larger aggregates which are finally observed.

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