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A Study of the Effects of Salt and of pH on Precipitation of Antigen–Antibody Compounds

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Summary. Tobacco mosaic virus (TMV) combines with its homologous antibody to much the same extent irrespective of whether or not salt is present, but without salt the complex not only fails to precipitate, but the virus particles do not aggregate. TMV–antibody precipitate formed in the presence of salt, like that formed between human serum albumin (HSA) and its homologous antibody, dissolves when suspended in distilled water to form stable and transparent solutions, although the precipitate may not disaggregate completely.

To dissolve HSA–antibody complex in distilled water, the pH of the water must be raised to about 7·0. At pH near 6·0, HSA–antibody complex precipitates even in the absence of salt, but the precipitate dissolves immediately when the pH is raised to 7·0.

All these facts are incompatible with the theory of precipitation based on the 'lattice hypothesis', and argue strongly in favour of the theory that antigen–antibody complexes are hydrophobic and, as such, flocculate when sufficiently discharged either by salt or by suitably adjusting the pH of the medium.

INTRODUCTION

Any theory of the mechanism of flocculation (precipitation or agglutination) of antigens by antibodies must account for the fact, known since Bordet (1899) demonstrated it for bacterial agglutination, that salt is usually necessary for the flocculation of antigen–antibody complexes.

The theory of precipitation currently most generally accepted is the 'lattice hypothesis', which was put forward by Marrack (1934, 1938) and by Heidelberger and Kendall (1935a, b, c). It is based on the assumption that specific bonding between antigen and antibody alone leads to flocculation by causing aggregates to increase in size, until they are large enough to settle from the solution. For this to happen both antigen and antibody must be multivalent or at least bivalent. Subsequent work has shown that antibody molecules may be bivalent (Pappenheimer, Lundgren and Williams, 1940; Eisen and Karush, 1949; Marrack, Hoch and Johns, 1951). As molecules or particles of various antigens can combine with several molecules of antibody, they obviously can be considered multivalent. Antibody molecules that can combine specifically with antigens but not flocculate them, are usually, on the strength of the 'lattice hypothesis', considered 'monovalent'.

There are various facts that seem to conflict with the 'lattice hypothesis', but its greatest weakness seems to be the difficulty of accounting for the role of salt. Marrack (1938) tried to overcome the difficulty by saying that 'reduction of the charges on the particles

by moderate salt concentrations will assist their aggregation whether this be due to loss of attraction for water or a specific mutual attraction'.

Another theory of flocculation of antigens by antibodies (which can be considered to have been originated by Bordet, 1899) is based on the assumption that, when antigens combine with antibodies, hydrophilic groups on both are blocked, which diminishes attraction to water so that the compound behaves like a hydrophobic colloid that is flocculated by small concentrations of salts. Actually, an alteration only in combined antibody molecules whereby they become hydrophobic and behave as if they were denatured, may often be considered sufficient for an antigen–antibody complex to become precipitable by salt (see Eagle, 1930; Marrack, 1938). The general validity of this theory is made doubtful by the fact that some antigen–antibody complexes can apparently precipitate in salt-free solutions. Aladjem and Lieberman (1952) even obtained more precipitate over a range of antigen/antibody ratios between ovalbumin and dialysed water-soluble globulin fraction of a homologous antiserum, in distilled water than in the presence of NaCl.

In the work described below the effect of salt and of pH on the precipitation of antigen–antibody complexes was investigated in the hope of getting some new evidence concerning the mechanism of the process. Two antigens were used: human serum albumin (HSA) and tobacco mosaic virus (TMV). HSA was chosen as an example of a relatively small molecular weight antigen that forms with antibody, at the ratio of equivalence, a complex consisting mainly of antibody (the weight ratio of antibody to HSA being about 6·0; see Kleczkowski, 1959). TMV was chosen as an example of a large particulate antigen which gives an antigen–antibody complex at the ratio of equivalence consisting mainly of antigen (the weight ratio of antibody to TMV being only about 0·2 (Kleczkowski, 1941, 1961)). This was convenient for the purpose of this work because if the complex did not aggregate, its rate of sedimentation during high speed centrifugation would not be expected to differ appreciably from that of free antigen.

The reaction of TMV with its homologous antibody resembles that of proteins with much smaller molecular weights (such as serum proteins or egg albumin) in that precipitation can be completely inhibited by excess of antigen and that, at a constant concentration of antibody, the ratio of antigen to antibody at which precipitation occurs most rapidly (optimal proportion) corresponds with the ratio of equivalence (when neither antigen nor antibody can be detected in the supernatant fluid after the removal of the precipitate by low speed centrifugation) (Kleczkowski, 1941). With bacterial suspensions it is the most rapid agglutination at a constant concentration of antigen and varying concentration of antibody that corresponds to the ratio of equivalence, i.e. to the maximum amount of absorbed antibody (Duncan, 1934; Kleczkowski, 1941). However, as TMV particles are intermediate in size between antigens with small molecular weights and bacteria, any conclusions concerning flocculation of TMV by its antibody are more likely to apply generally than those obtained with an antigen from either of the extreme ends of the range of sizes.

Electron-microscopy showed that in an extreme antibody excess, precipitation of TMV is not a result of 'lattice' formation. Each virus particle was covered with a continuous layer of antibody molecules and there was no possibility that single antibody molecules had combined with two different virus particles (Kleczkowski, 1961). However, the possibility of this happening at or near the ratio of equivalence still remained, and the work described below is mainly concerned with compounds formed at or near this ratio.

MATERIALS AND METHODS

Human serum albumin (HSA) was prepared by removing globulin from human serum by half-saturation with ammonium sulphate followed by filtration and by complete saturation of the filtrate with ammonium sulphate. The precipitated albumin was dissolved in and dialysed against distilled water. It was stored at 4° with toluene as antiseptic.

When examined electrophoretically in a Perkin-Elmer electrophoresis apparatus after dialysis against M/15 phosphate buffer at pH 7·0, the preparation of HSA appeared almost homogeneous. It formed a pattern identical with that previously obtained with a similarly prepared solution of human serum albumin (Kleczkowski, 1959, Fig. 3B). There was one large peak with the mobility of $-6{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$, which is typical for serum albumin, and there was a very small contamination with globulin which gave two, more slowly moving, very small peaks with the total area of less than 0·5 per cent of the area under the albumin peak. It can be concluded, therefore, that the preparation of HSA consisted almost entirely of albumin. Any further fractionation of the preparation was not considered necessary for the purpose of this work.

Tobacco mosaic virus (TMV), type strain, was prepared from sap of infected tobacco plants by several alternate precipitations by one third saturation with ammonium sulphate and by adjusting the pH to 3·4, followed by differential centrifugation. The final pellet obtained by high speed centrifugation was dissolved in and dialysed against distilled water. The solution was stored at 4°.

Antisera. Solutions containing 5–10 mg. of HSA or TMV in physiological saline were injected intravenously into rabbits twice a week for 3 weeks. After an interval of 4 weeks the animals were again injected three times, and were bled 10 days after the last injection. The sera were kept at 4° with phenol at 0·5 per cent as antiseptic.

The antisera were dialysed at 4° in Cellophane bags for 3 days against frequently changed distilled water whose pH (measured with a glass electrode) was adjusted to about 7·0 with 0·1 N NaOH. Thymol was added to the sera and to water as antiseptic. The precipitates formed during dialysis were removed by centrifugation. This did not cause any appreciable loss of antibody to TMV for most of it was in the water-soluble globulin fraction. The antiserum to HSA lost about half of its antibody during dialysis and the final level was about 1·5 g./l. When 1 mg. of HSA was added to an equivalent amount of antiserum (4 ml.) in the presence of salt and at pH 7·0, about 7·0 mg. of the precipitate was formed; this is the amount of precipitate expected to be formed at equivalence by 1 mg. of albumin (the very small amount of globulin contaminating the preparation of HSA could have produced less than 0·02 mg. of precipitate at equivalence, and less than 0·08 mg. in extreme excess of antibody). In gel diffusion precipitin tests the HSA–antibody system gave only one precipitation band.

The pseudo-globulin fraction of the antiserum to TMV was precipitated by adding one volume of a saturated solution of ammonium sulphate to two volumes of the antiserum. The precipitate was washed on a filter with a one-third saturated solution of ammonium sulphate, dissolved in water and dialysed at 4° for 3 days against frequently changed distilled water with pH adjusted to 7·0. Thymol was added to the water during the first day, but not during the next 2 days, as its presence would have interfered with sedimentation tests using ultraviolet optics. The precipitate that formed during dialysis was removed by centrifugation. The final volume of the pseudo-globulin solution was half of the volume of the original antiserum.

The pH values of small amounts of distilled water and of salt-free protein solutions were measured with a glass electrode and adjusted with 0·01 N HCl or NaOH.

Routine precipitin tests to titrate antigen or antibody, or to determine optimal proportions, were done with solutions in M/15 phosphate buffer at pH 7·0. Antigen (1 ml.) at different dilutions was added to a series of tubes all containing 1 ml. of antiserum, or the pseudo-globulin fraction of antiserum, at a constant dilution, or *vice versa*. The tubes were placed in a water-bath at 45° and readings were made at intervals during 3 hours. The antiserum to TMV, or its pseudo-globulin fraction, was used at a constant dilution of 1/50, and that of the antiserum to HSA at 1/10; when the concentration of TMV was kept constant, it was 0·03 g./l.

The rates of sedimentation of TMV and of TMV–antibody compounds were measured with a Spinco Model E Analytical Ultracentrifuge. Sedimentation coefficients were obtained by the graphic method described by Markham (1960).

RESULTS

PRECIPITATION OF HSA–ANTIBODY COMPOUNDS

Table 1 shows that, whereas at pH 7·0 HSA–antibody mixtures precipitated only in the presence of salt, at pH 5·9 they precipitated from salt-free solutions. The amounts of precipitates were not measured, but they were obviously greater, and formed much more rapidly, in the salt-free solutions at pH 5·9 than at pH 7·0 in the presence of salt. At pH 5·9 precipitation also occurred in salt-free solutions at levels of antigen excess which inhibited precipitation at pH 7·0 in the presence of salt.

TABLE 1

THE EFFECT OF pH AND OF NaCl ON PRECIPITATION OF HSA BY ITS ANTISERUM

Test No.	*pH**	*Dilution†*							
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
1. No salt added	5·9	++++	++++	++++	++++	++++	+++	++	–
2. No salt added	7·0	–	–	–	–	–	–	–	–
3. Salt added‡	7·0	–	+	++	+++	+++	++	+	–

The mixtures were placed in a water-bath at 45° and inspected at intervals; the final readings were made after 3 hours.

* The pH of the diluted antiserum was 7·0 in tests Nos. 2 and 3, and was adjusted to 5·9 for test No. 1. A slight opalescence developed but was not followed by flocculation unless antigen was added.

† 1 ml. of dialysed antiserum diluted 1/10 in distilled water + 1 ml. of 0·02 per cent salt-free solution of HSA diluted in distilled water as indicated.

‡ 0·1 ml. of 17 per cent NaCl added to each tube.

+ Indicates the presence and degree of precipitation; – Indicates the absence of precipitation.

The fact that, over the whole range of antigen/antibody ratios, there was more precipitate at pH 5·9 in the absence of salt than at pH 7·0 in the presence of salt, suggests that at pH 5·9 some non-antibody globulin precipitated with the antigen–antibody complex. This probably occurred because of low solubility of serum globulins at pH 5·9; the dialysed antiserum showed a slight opalescence when it was diluted 1/10 in distilled water and adjusted to pH 5·9. However, no flocculation occurred in the antiserum after 3 hours' incubation at 45° in the absence of HSA or after addition of horse serum albumin, whereas precipitation occurred rapidly when HSA was added.

To test for reversibility of precipitation at pH 5·9 from a salt-free solution, 0·5 ml. of the

dialysed antiserum was added to 4·5 ml. of distilled water, the pH of the mixture was adjusted to 5·9, and 5·0 ml. of 0·0025 per cent solution of HSA in distilled water was added. A precipitate soon formed. The mixture was incubated for 3 hours at 37°, and then the pH was raised to 7·0. The precipitate dissolved immediately, and the fluid became water-clear. It was divided into two portions. One was left untreated, and 1/20 volume of 17 per cent NaCl was added to the other. The fluids were then incubated for 3 hours at 37°. The salt-free portion remained water-clear, whereas the other became opalescent almost immediately and a precipitate soon formed. The amount of NaCl formed by first adjusting the pH to 5·9 with HCl and then raising it to 7·0 with NaOH, was insufficient to precipitate the HSA–antibody mixture at pH 7·0, but precipitation rapidly occurred with 0·85 per cent NaCl.

The following experiment showed that precipitation at pH 7·0 in the presence of salt can also be easily reversed.

The dialysed antiserum (0·5 ml.) was mixed with 2·0 ml. of M/15 pH 7·0 phosphate buffer and with 0·5 ml. of 0·025 per cent HSA in the buffer. This corresponded with the ratio of equivalence. Opalescence developed immediately and a precipitate soon separated. After 3 hours incubation at 37° the precipitate was centrifuged down (10 minutes at 8000 rev./min.), washed in the buffer and suspended in 3 ml. of distilled water previously adjusted to pH 7·0. The precipitate immediately dissolved completely. When 1/20 volume of 17 per cent NaCl was added to the solution, it became opalescent immediately and precipitation soon followed. After removing the precipitate by centrifugation, neither antigen nor antibody could be detected in the supernatant fluid.

To dissolve the HSA–antibody precipitate in distilled water, the pH of the water (originally about 5·4) had to be raised to 7·0. When the pH of the distilled water was not raised, the pH of the suspended precipitate was about 6·0, and the precipitate did not dissolve.

COMBINATION BETWEEN TMV AND ANTIBODY

Salt-free solutions of TMV and serum precipitate unspecifically at pH 6·0 or below: the precipitate is crystalline and is produced by the virus combining with serum globulin, probably because the two are oppositely charged (Kleczkowski, 1946). In all serological tests with salt-free fluids, therefore, the pH was kept well above 6·0.

After confirming that at pH 7·0 TMV precipitates with its antiserum only when salt is present (at least 0·005 M NaCl), the effect of salt on the extent of combination between TMV and its antibody was investigated. This was done by making two identical series of salt-free mixtures of various amounts of antigen with a constant amount of antiserum, and then adding salt to one of the series. The antigen was then removed from the salt-free mixtures (which remained clear) by high speed centrifugation, which would leave any free antibody in the supernatant fluid. The precipitates formed in the mixtures containing salt were removed by a low speed centrifugation. All the supernatant fluids were then assayed for antibody and tested for the presence of antigen.

Table 2, which gives the details of the experiment, shows that antibody combined with the antigen to much the same extent whether or not salt was present. In particular, all antibody was combined at the same ratio of equivalence in the presence of salt (test No. 6) or in its absence (test No. 2). Thus, whatever the mechanism responsible for antigen–antibody precipitation when salt is present, it is not based on the effect of salt upon the extent of specific combination between antigen and antibody.

TABLE 2

COMBINATION BETWEEN TMV AND ANTIBODY IN THE PRESENCE AND ABSENCE OF SALT

Test No.	*TMV (mg.) added to 4 ml. antiserum dilution* 1/16	*Tests of the supernatant fluids*						
		1 ml. of TMV solution, at 0·03 g./l. + 1 ml. of the supernatant fluid at a dilution of 1 *in:*						*1 ml. of the supernatant fluid + 1 ml. of antiserum dilution* 1/50
		1	2	4	8	16	32	
Combination in the absence of salt								
1	0·5	−	−	−	−	−	−	−
2	0·25	−	−	−	−	−	−	−
3	0·125	++	++	+	−	−	−	−
4	0·0625	+++	++	++	+	−	−	−
Combination in the presence of salt								
5	0·5	−	−	−	−	−	−	++
6	0·25	−	−	−	−	−	−	−
7	0·125	++	++	+	−	−	−	−
8	0·0625	+++	++	++	+	−	−	−
Untreated antiserum (control)								
9	0	+++	+++	+++	++	++	+	

One ml. of a salt-free water solution of TMV at different concentrations was added to a series of tubes each containing 4 ml. of the dialysed antiserum diluted 1/16 in water. (The amounts of added TMV are shown on the left-hand side of the Table.) The fluids were then incubated for 3 hours at 37°. They all remained clear.

Tests Nos. 1–4. The fluids were centrifuged for 1 hour at 40,000 rev./min. The supernatant fluids were decanted from above the pellets: 0·5 ml. of M/1·5, pH 7·0, phosphate buffer was added to each supernatant fluid.

Tests Nos. 5–8. 0·5 ml. of M/1·5, pH 7.0, phosphate buffer was added to each fluid. Precipitates formed rapidly in all the fluids. After 1 hour's incubation at room temperature the precipitates were sedimented by centrifugation for 10 minutes at 8000 rev./min. to obtain the supernatant fluids.

All the supernatant fluids were tested for antibody and for antigen. All the reagents used in these tests were in M/15, pH 7·0, phosphate buffer. The dilutions of *untreated antiserum* (Test No. 9) corresponded to antiserum dilutions in Tests No. 1–8.

+ Indicates the presence and degree of precipitation; − Indicates the absence of precipitation.

THE BEHAVIOUR OF TMV–ANTIBODY COMPOUNDS FORMED IN SALT-FREE MEDIA

The TMV–antibody complex formed at equivalence in salt-free medium was investigated in the analytical ultracentrifuge. The amount of UV-absorbing inert protein was reduced by using the dialysed, water-soluble pseudo-globulin fraction of the antiserum; this was compared with a similarly prepared pseudo-globulin fraction from a normal rabbit serum.

Salt-free mixtures were made at equivalent ratios by mixing 0·4 ml. of 0·07 per cent solution of antiserum pseudo-globulin or normal serum pseudo-globulin with 1·6 ml. H_2O and 0·2 ml. of 0·06 per cent solution of TMV. The pH of all solutions was adjusted to 7·0. After incubation for 3 hours at 37°, the mixtures (which remained clear) were centrifuged at 21,740 rev./min. in the analytical ultracentrifuge. Fig. 1 shows that when a Schlieren optical system was used, the two mixtures (of TMV with normal serum pseudo-globulin and with antiserum pseudo-globulin) produced similar peaks which moved with similar speeds, the sedimentation coefficients, S_{20}, being 192s and 194s, respectively. When a UV-optical system was used, very similar concentration gradients were found for the boundary of sedimenting TMV in the two mixtures (Fig. 2).

To check that the centrifuged mixture of TMV with antiserum pseudo-globulin corresponded to the ratio of equivalence, the mixture was withdrawn from the centrifuge cell

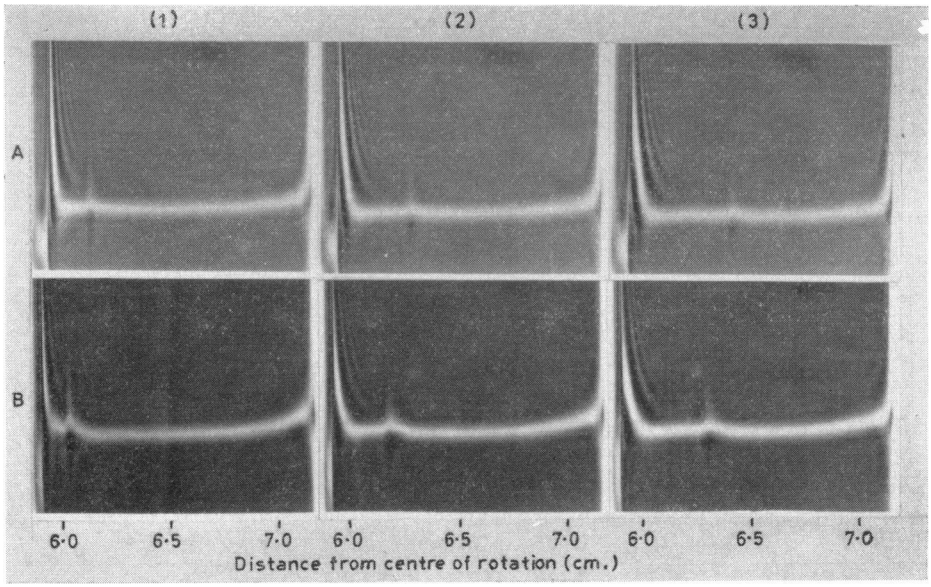

FIG. 1. Sedimentation at 21,740 rev./min. of mixtures of salt-free solutions of TMV with euglobulin (A) from normal rabbit serum and (B) from an antiserum to TMV. Positions (1), (2) and (3) are photographs taken at 4 minute intervals using Schlieren optics.

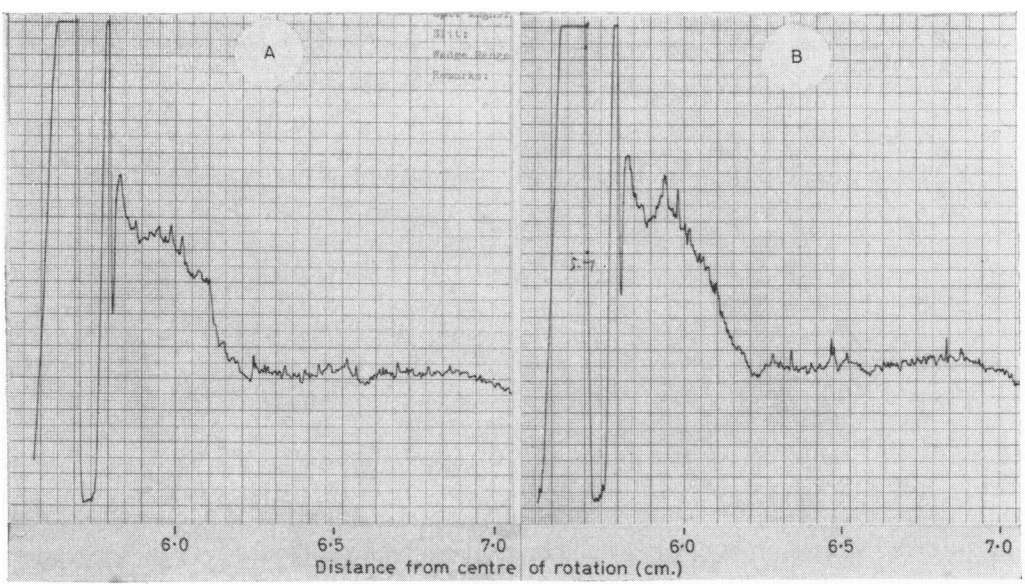

FIG. 2. Sedimentation at 21,740 rev./min. of mixtures of salt-free solutions of TMV with euglobulin (A) from normal rabbit serum, and (B) from antiserum to TMV. The graphs are densitometer tracings of photographs taken using a UV-optical system. They correspond approximately to the position (2) shown in Fig. 1.

and 1/20 volume of 20 per cent solution of NaCl was added to it. The fluid became opalescent almost immediately and a precipitate soon formed. It was removed by centrifugation for 10 minutes at 8000 rev./min. and neither antigen nor antibody was detected in the supernatant fluid (by the procedure shown in Table 1).

It is concluded that sedimentation of TMV during high speed centrifugation is little affected by combination with antibody at the ratio of equivalence in the absence of salt. This can be interpreted to mean: (1) that the amount of antibody combined with TMV at the ratio of equivalence (about 0·2 g. of antibody per 1·0 g. of TMV) is too small to affect appreciably the rate of sedimentation of TMV; (2) that TMV combined with antibody in salt-free medium does not aggregate.

TMV–ANTIBODY PRECIPITATE FORMED IN THE PRESENCE OF SALT AND THEN DISPERSED IN WATER

The precipitate formed by TMV and its antiserum in the presence of salt dissolves when suspended in distilled water. To find the extent to which the precipitate is dispersed, equivalent ratios of dialysed antiserum and TMV were mixed in the presence of phosphate buffer at pH 7·0. A precipitate formed rapidly. After incubation for 3 hours at 37° the precipitate was centrifuged down (10 minutes at 8000 rev./min.), washed in the buffer and suspended in 2·0 ml. of distilled water, when it formed a clear solution from which only a trace of material was sedimented by centrifugation for 10 minutes at 8000 rev./min. The fluid was centrifuged at 21,740 rev./min. in the analytical ultracentrifuge, simultaneously with a mixture of 1·5 ml. H_2O + 0·5 ml. of 0·2 per cent solution of TMV in H_2O.

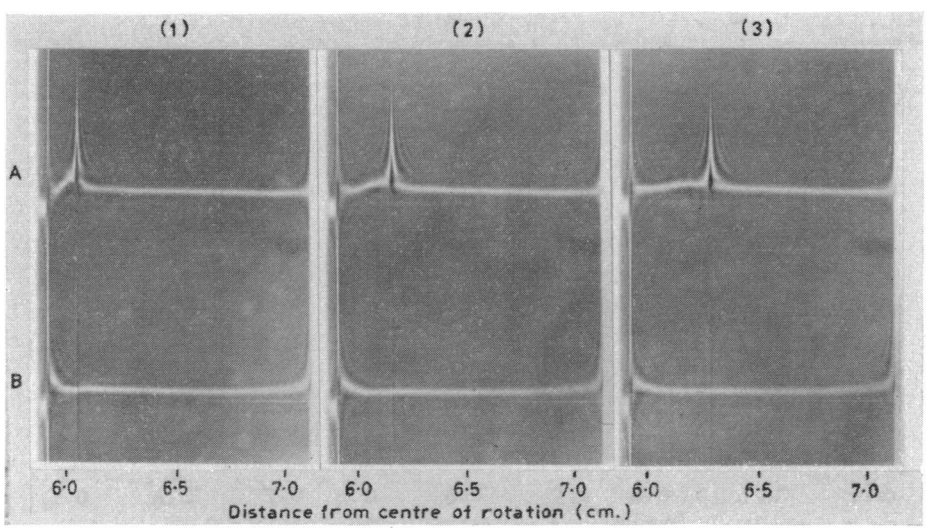

FIG. 3. Sedimentation at 21,740 rev./min. of (A) 0·05 per cent salt-free solution of TMV, and (B) the precipitate obtained by mixing TMV with antiserum in the presence of salt, and dissolved by suspending in distilled water. Positions (1), (2) and (3) are photographs taken at 4 minute intervals using Schlieren optics.

Fig. 3 shows that, using the Schlieren optical system, the control solution of TMV formed a peak which moved at the usual rate (S_{20} = 180s), whereas no peak was formed

by the dissolved precipitate. A densitometer tracing of a photograph obtained using UV-optics showed that the dissolved precipitate behaved like a typical polydisperse material with a concentration gradient spread uniformly almost over the whole length of the centrifugation cell (Fig. 4). This can be contrasted with the concentration drop observed in the salt-free mixture of TMV and antiserum euglobulin (see Fig. 2B).

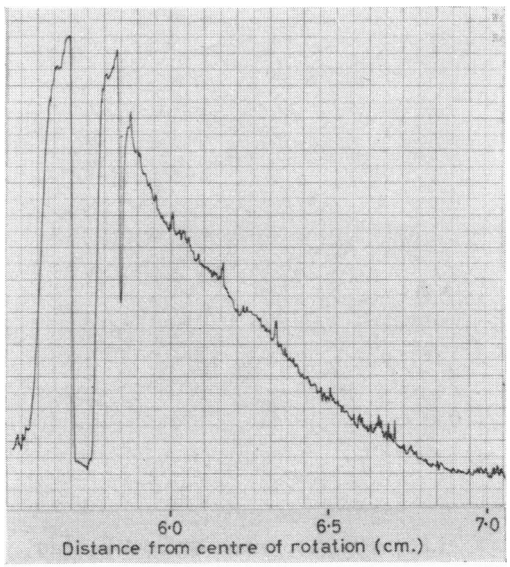

FIG. 4. Sedimentation at 21,740 rev./min. of the precipitate obtained by mixing TMV with antiserum in the presence of salt and dissolved by suspending in distilled water. The graph is a densitometer tracing of a photograph taken using a UV-optical system, and it corresponds approximately to position (2) in Fig. 3.

It is concluded that when the TMV–antibody complex, which had precipitated in the presence of salt, was suspended in water, a large proportion of the virus particles did not separate from each other completely, but remained in aggregates of various sizes that sedimented at different rates in the ultracentrifuge. Nevertheless, the precipitated material was sufficiently dispersed in water to form a stable and transparent solution without any tendency to flocculate. Therefore, parts of the precipitated material separated when salt was removed, which shows that their cohesion depends on the presence of salt.

When 1/20 volume of 17 per cent NaCl was added to the solution, precipitation occurred rapidly, and when the precipitate was removed by centrifugation for 10 minutes at 8000 rev./min., neither antigen nor antibody was detected in the supernatant fluid.

To dissolve the TMV–antibody precipitate in distilled water, the pH of the water did not have to be raised to 7·0 (as it had to be to dissolve HSA–antibody precipitate). This was probably because the TMV–antibody complex, formed at the ratio of equivalence, consists mainly of TMV, and therefore the solubility of the compound may largely depend on the solubility of TMV. As TMV is isoelectric at pH 3·4 in the presence of salt (Eriksson-Quensel and Svedberg, 1936; Best, 1936), and at 4·2 when salt-free (Bawden and Pirie, 1937), the pH of the suspended precipitate was far from the isoelectric point even when the pH of distilled water was not raised.

DISCUSSION

The use of two different antigens led to results that can be considered in some respects as complementary. For some types of experiments described above only one of the antigens appeared suitable, and so only one was used, but there was no indication that the mechanism of precipitation of the two antigens was not basically the same.

The results obtained seem incompatible with the theory of precipitation based on the 'lattice hypothesis'. If precipitation occurred only because the specific combining sites of antibody molecules join more and more antigen particles to each other, such joining would be expected also in salt-free solutions, because TMV and antibody combine to about the same extent whether or not salt is present. Yet, in salt-free solutions and at pH about 7·0, not only was there no precipitation, but most or all antigen particles remained apart from each other. There is evidence that TMV–antibody complexes did not aggregate at all in the absence of salt.

The other phenomenon incompatible with the 'lattice hypothesis' is the dissolution of the precipitate in distilled water. It is true that TMV–antibody precipitate, formed in the presence of salt, did not disaggregate completely when taken up in distilled water, but it disaggregated sufficiently to form a stable and transparent solution. The precipitate formed by HSA and its antiserum in the presence of salt dissolved in distilled water adjusted to pH 7·0. When the pH was near 6·0, HSA precipitated with its antiserum even in the absence of salt, although the precipitate dissolved when the pH was raised to about 7·0.

The isoelectric point of rabbit antibody was found by Tiselius and Kabat (1939) to be about pH 5·8. Alberty (1949) later found that the isoelectric point of γ-globulin depends on ionic strength of the medium. For example, the isoelectric point of human γ-globulin is about pH 5·9 when the ionic strength is 0·1, and about pH 6·5 when the ionic strength is 0·01. Therefore the solubility of antibody seems to be a factor determining the solubility of HSA–antibody complex in salt-free solutions; the complex can precipitate in the absence of salt when the pH is near the isoelectric point of antibody, probably because antibody forms a large proportion of the compound. The fact that the precipitates formed without salt at about pH 6·0 were greater than those formed with salt at pH 7·0, suggests that the small solubility of globulin near pH 6·0 led to some non-antibody globulin combining with the antigen–antibody complex. The slight solubility of the complex also prevented the inhibition of precipitation by antigen excess. An ovalbumin–antibody complex may also have precipitated without salt (Aladjem and Lieberman, 1952) because the pH of the dialysed solutions used was about 6·5, and so not far from the isoelectric point of antibody. Whether raising the pH to 7·0 would have prevented precipitation was not tested.

If precipitation at pH 7·0 were caused only by the specific combination of antibody with antigen molecules or particles to form aggregates, then the dissolution of the precipitate in distilled water at pH 7·0 would indicate a reduction in the size of aggregates which would imply that some specific bonds were broken. Therefore the presence of salt would appear to be needed not only to 'assist aggregation' when a precipitate is forming (as suggested by Marrack, 1938), but also to prevent the specific bonds, already formed in the presence of salt, from breaking. This is clearly not compatible with the fact that the extent of combination between TMV and antibody does not depend to any appreciable degree on the presence of salt. Similarly, if precipitation of HSA by its antibody at pH 6·0 from salt-free solutions depends only upon specific combining sites of antibody joining

more and more antigen molecules together, then adjusting the pH to 7·0 must be assumed to break the specific bonds, which is obviously absurd.

All the results described above are compatible with the view that antigen–antibody complexes are hydrophobic and flocculate when sufficiently discharged either by adding salt or by suitably adjusting the pH. This theory, but not the 'lattice hypothesis', can also explain the behaviour of complexes formed between either antibodies or antigens, and other proteins such as serum albumins (Kleczkowski, 1943, 1949).

The validity of the 'lattice hypothesis', has been tested with cellular antigens, such as bacteria and red blood cells; microscopic examination reveals whether the aggregates formed during agglutination of mixtures of such antigens by mixtures of corresponding antibodies, consist of separate antigens or of mixtures. The results, reviewed by Wilson and Miles (1955) differ, but the fact that in some cases mixed aggregates were obtained when conditions were suitably adjusted can be used as an argument against the 'lattice hypothesis'.

The 'lattice hypothesis' may, however, apply in some special circumstances or in a modified form. This is suggested, for example, by the results obtained by Nisonoff and Rivers (1961) with artificially produced 'hybrid antibodies' which could precipitate with mixtures of corresponding antigens but not with single antigens, or by the results of Fudenberg, Drews and Nisonoff (1964) who found that such 'hybrid antibodies' could aggregate together antigenically distinct red blood cells. However, any definite conclusions from these results must be postponed until phenomena of this kind are investigated more extensively.

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