CIV. COLLOIDAL PROPERTIES OF WASSERMANN ANTIGENS. II.

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INTRODUCTION.

IN certain serological tests and in particular in the Wassermann reaction, use is made of a colloidal suspension prepared by the addition of an alcoholic extract of an animal organ, such as heart or liver, to a 0.85 % solution of sodium chloride. Some of the colloidal properties of such antigens have been described in the first paper of this series [Kermack and MacCallum, 1924]. It was found that such antigens resemble ordinary colloidal suspensions, such as gold or gum benzoin, in being precipitated by small quantities of positively charged protein (*e.g.* gelatin), but differ from them by not being precipitated by salts of univalent cations however high in concentration these may be and in not showing precipitation in presence of large quantities of calcium chloride but complete precipitation in presence of moderate quantities of this salt.

It is the purpose of the present paper to describe more completely these peculiar abnormalities with a view to elucidating their cause. It is of especial interest to inquire to what extent the zone phenomenon observed when calcium chloride is used to precipitate the antigen is related to the phenomenon known as ion antagonism, such as may be observed with Selmi's sulphur sol. This colloidal solution requires less sodium chloride for precipitation in the absence than it does in the presence of a small quantity of magnesium chloride, the concentration of the latter being of course so chosen as to be insufficient to effect precipitation alone. A similar effect is observed to some extent with almost any combination of a monovalent along with a bivalent or trivalent cation, and it appears whether the multivalent cation is added first or whether a mixture of these two cations is added to the sol. In some respects the effect observed using calcium chloride with the Wassermann antigen resembles the phenomenon of ion antagonism, inasmuch as in both cases increase in the total cationic concentration results actually in a decrease in the precipitation observed.

A systematic study has therefore been made of the action of univalent and bivalent cations on the antigen. The salts used have been chiefly lithium chloride, sodium chloride, calcium chloride and magnesium chloride; and systematic observations have been made on the precipitation of antigen by these salts separately and by mixtures of any two of them.

The experimental work contained in this paper is divided into four sections. The first contains a description of the method of preparing the antigen, some of its properties and the effect on these of variations in the method of preparation. The second describes the effect of the addition of antigens prepared under definite conditions to solutions of inorganic salts covering a wide range of concentration. The third contains experiments on the protecting power of these antigen solutions on gold, cholesterol, gum mastic and gum benzoïn sols; and in particular, the effect on this of modifications in the methods of preparation of the antigen and of the presence of the cations and anions; whilst in the fourth section, certain results relating to lecithin sols are given, which may be compared with those obtained with the Wassermann antigen.

It is convenient to record these in this paper, in order that a comparison may be made of the two somewhat similar colloidal systems. It is not claimed that the observations recorded are exactly reproducible, as different preparations of antigen appear to give slightly differing results and the degree of precipitation observed may alter in a quantitative sense with certain factors such as the age of the antigen used in the experiment. The general type of result, however, can be reproduced without difficulty.

I. PROPERTIES OF THE ANTIGEN.

In the previous paper [Kermack and MacCallum, 1924], several antigens were used but, as the same general results were obtained with all of them, that of Bordet and Reuben was selected for further work, as this antigen appeared to be the most uniform and highly active in the Wassermann reaction, in proportion to its content of solid material. The alcoholic solution was prepared as described by Kermack and MacCallum [1924]. On evaporation it left 0.3 % of a yellow waxy solid which readily dispersed on mixing with water. No cholesterol could be detected by the Liebermann-Burchard or Salkowski tests and it left no ash on ignition.

It is well known that the physical appearance of such antigens depends on the exact conditions of mixing with water or sodium chloride solution. With Bordet's antigen this phenomenon is quite well marked. When water or saline is added to it slowly the mixture becomes opalescent, but if the alcoholic extract is added drop by drop to the water, or if the two are mixed suddenly, less turbidity is observed and the resulting mixture may appear quite clear. It has been observed that the activity of such sols in the Wassermann reaction depends on their opacity, and it was thought therefore of some interest to ascertain to what extent their colloidal properties were influenced by the method of preparation. Certain of the experiments have therefore been carried out with sols prepared so as to be opalescent, while others have been carried out with sols which appeared almost water-clear. The first type (A) was prepared by the addition of 90 cc. of water drop by drop, with constant stirring during 20–25 minutes, to 10 cc. of the alcoholic extract. The second

type (*B*) was prepared by adding 10 cc. of the alcoholic extract drop by drop, to 90 cc. of water.

The ultramicroscopic observations of these antigens are of interest. The opaque antigen (*A*) shows the presence of a large number of particles about the same size as the particles in gum benzoin sol with a marked Brownian movement. When high concentrations (2 *N*) of calcium chloride are added the particles become very much larger with a proportionate decrease in movement. The fine antigen (*B*) shows exceedingly small particles and a similar effect is observed when calcium chloride in high concentration is added.

It appears from these observations that the coarse antigen consists of moderately small particles which, in the presence of salt, tend to aggregate, but that this aggregation is arrested at a certain point if sufficient salt is present. In the fine antigen, the particles, much finer to begin with, likewise aggregate, under the action of salt solutions, but here the arrest in aggregation takes place with quantities of salt insufficient to effect flocculation.

It may also be stated here that observations have been made on the sign of the electric charge carried by the colloidal particles. Ultramicroscopic observations on this point have been made on both antigens by a method described in a previous communication by Wright and Kermack [1923]. Confirmatory observations have also been made by the use of the inverted U-tube method, the U-tube used being similar to that described by Northrop and Cullen [1922]. As was to be expected both antigens were found to have a negative charge.

The effect of adding these antigens to a series of dilutions of calcium chloride is shown in Table I; 1 cc. of the antigen was added to 1 cc. of the calcium chloride solution contained in a small test-tube and the results were read after 24 hours. (In this and in other tables 4 denotes complete precipitation, 3 almost complete, 2 partial, 1 slight and 0 no change. Where the antigen became more opaque "op." is entered. The final concentrations after addition of the antigen are given.)

Table I. *Precipitation of antigens A and B by calcium chloride.*

		Concentration of calcium chloride									
		1 <i>N</i>	0.5 <i>N</i>	0.25 <i>N</i>	0.125 <i>N</i>	0.062 <i>N</i>	0.031 <i>N</i>	0.016 <i>N</i>	0.008 <i>N</i>	0.004 <i>N</i>	0.002 <i>N</i>
Antigen <i>A</i>	op.	op.	1	4	4	4	4	0	0	0	0
„ <i>B</i>	0	0	0	0	0	0	0	0	0	0	0

It appears from these results, which have been confirmed on many occasions, that the antigen (*A*) is precipitated much more easily than the antigen (*B*), and so, at least with calcium chloride, it is only antigen (*A*) which shows the zone effect, as antigen (*B*) is not precipitated in any tube.

It is difficult to observe the presence or absence of precipitation with the fine antigen (*B*); but a strong beam of light from an arc lamp was used with

advantage as the antigen shows a marked Tyndall cone which becomes more marked with the opaque suspensions produced by the high concentrations of salt, but which would practically disappear if the colloid was precipitated.

The following observations show that the colloidal properties of the antigen are fairly stable and that various treatments of a mild nature do not materially alter the sol. Antigen (*A*) was treated in the following ways.

- (1) Heated on water-bath at 100° for several hours.
- (2) Aerated at room temperature for several hours.
- (3) Aerated at 100° for several hours by a current of air which had previously passed through water at 100°.
- (4) Treated similarly to (3) but with the addition finally of a quantity of alcohol equal to that removed in (2) and (3).
- (5) Hydrogen peroxide (2 cc. 90–100 vols.) added to 50 cc. antigen, the mixture being then treated as in (3) and neutralised with dilute sodium hydroxide solution before use.

These experiments were carried out to ascertain whether heating and mild oxidation rendered the antigen incapable of giving the peculiar zone effect. It was found, however, that after such treatment the same type of result was obtained. Similar experiments were also carried out with the fine antigen (*B*) but in no case was any material difference effected in its colloidal properties.

II. PRECIPITATION BY DIFFERENT SALTS.

In this section we shall detail the results of experiments in which the actions of various salts and of mixtures of different salts were investigated. In most experiments antigen *A* was used. For this purpose a series of dilutions of various salts was prepared in distilled water, and to 1 cc. of each dilution of the solution 1 cc. of antigen was added, and the mixtures were allowed to stand for about 24 hours. The results indicated in Table II were obtained.

Table II. *Precipitation of antigen A by salts.*

	Concentration of salt									
	1 <i>N</i>	0.5 <i>N</i>	0.25 <i>N</i>	0.125 <i>N</i>	0.062 <i>N</i>	0.031 <i>N</i>	0.016 <i>N</i>	0.008 <i>N</i>	0.004 <i>N</i>	0.002 <i>N</i>
CaCl ₂	op.	op.	1	4	4	4	4	0	0	0
MgCl ₂	op.	1	2	4	4	4	op.	0	0	0
SrCl ₂	op.	op.	4	4	4	op.	0	0	0	0
BaCl ₂	op.	op.	4	4	4	3	op.	0	0	0
NaCl	op.	op.	0	0	0	0	0	0	0	0
KCl	1	op.	0	0	0	0	0	0	0	0
LiCl	2	1	1	0	0	0	0	0	0	0
NH ₄ Cl	1	op.	0	0	0	0	0	0	0	0

It will be seen that magnesium, calcium, strontium and barium chlorides all show the same peculiar abnormal effect and that in the case of each salt less precipitation occurs at concentrations of 0.5 *N* than at 0.016 *N*. In this table are also included the results obtained with certain univalent cations and it will be seen that these resemble sodium in their action.

It is well known that in most cases of ion antagonism it is necessary to use ions of different valency in order that the phenomenon may be evident and that it is particularly well marked with lithium and magnesium.

In a certain sense the results obtained with the bivalent cations may be considered as a particular type of ion antagonism inasmuch as the presence of a larger number of ions antagonises the action of a smaller number. For this reason it appeared of interest to investigate systematically the result of having present simultaneously two types of cations. Four salts were therefore chosen, calcium, sodium, magnesium and lithium chlorides, and six experiments were carried out in each of which a particular pair of these salts was used. A series of dilutions of each of the salts was prepared and into a test-tube was placed 0.5 cc. of a particular dilution of one salt and 0.5 cc. of a particular dilution of another. To the mixture was added 1 cc. of the antigen. The results are given in Tables III-VIII in which the concentrations given are the final concentrations of salt present after dilution with the antigen.

Table III. *Precipitation of antigen A by NaCl and CaCl₂.*

Concentration of calcium chloride	Concentration of sodium chloride									H ₂ O
	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	
H ₂ O	op.	op.	0 (7.0)	0	0	0	0	0	0 (6.8)	0 (6.4)
0.004 N	op.	op.	0	0	0	0	0	0	0	0
0.008 N	op.	op.	0 (7.2)	0	0	0	0	0	0	op.
0.016 N	op.	op.	op.	0	0	op.	3	4	4	4
0.031 N	op.	op.	1	3	3	4	4	4	4	4
0.062 N	op. (7.4)	1	3	3	4	4	4	4	4	4 (7.4)
0.125 N	op.	1	3	4	4	4	4	4	4	4
0.25 N	op.	1	2	3	3	3	3	3	2	2
0.5 N	op.	1	1	1	1	1	1	1	1	op.
1 N	op. (7.6)	op.	op.	0	0	0	0	op.	op.	op. (8.0)

Table IV. *Precipitation of antigen A by LiCl and CaCl₂.*

Concentration of calcium chloride	Concentration of lithium chloride									H ₂ O
	2 N	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	
H ₂ O	3 (6.4)	2	2	0	0	0	0	0	0	0 (6.4)
0.004 N	3	2	2	0	0	0	0	0	0	0
0.008 N	3	2	2	op.	0	0	0	0	0	0
0.016 N	3	2	1	1	0 (6.4)	0	0	op.	3	4
0.031 N	3	2	2	2	2	3	4	4	4	4
0.062 N	3 (6.7)	2	2	2	3	3	4	4	4	4
0.125 N	3	2	2	2	2	3	3	3	3	4
0.25 N	3	2	2	2	2	2	2	1	1	1
0.5 N	3	2	2	2	1	op.	op.	op.	op.	op.
1 N	2 (7.0)	op.	op.	op.	0	0	0	op.	op.	op. (7.4)

It will be seen from Table III that the action of sodium chloride is usually protective, for instance 0.015 N calcium chloride produces precipitation but if sodium chloride is also present in concentration greater than 0.031 N precipitation is inhibited. Also 0.12 N calcium chloride just causes complete precipitation but if sodium chloride in concentration of 1 N be present no

precipitation takes place. A very similar phenomenon is observed when both magnesium chloride and sodium chloride are present or when lithium chloride and calcium chloride or lithium chloride and magnesium chloride are present, although in some of these cases the effects are somewhat more complicated and can be readily seen from the table. When both calcium and magnesium chloride are present it is found that if a concentration of calcium is taken which itself effects precipitation, that precipitation may be inhibited provided

Table V. *Precipitation of antigen A by NaCl and MgCl₂.*

Concentration of magnesium chloride	Concentration of sodium chloride										H ₂ O
	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N		
H ₂ O	1 (5.9)	1	1	op.	op.	0	0	0	0	0	0 (6.4)
0.004 N	1	1	1	op.	op.	0	0	0	0	0	0
0.008 N	1	1	1	op.	op.	0	0	0	0	0	0
0.016 N	1	2	2	1	op.	op.	0	op.	op.	4	4
0.031 N	1	2	3	3	3	3 (5.8)	4	4	4	4	4
0.062 N	1	3	3	4	4	4	4	4	4	4	4
0.125 N	2	3	3	4	4	4	4	3	3	3	4
0.25 N	3	3	3	3	3	3	3	3	3	3	3
0.5 N	op.	4	4	3	3	3	3	2	2	2	2
1 N	2 (5.1)	3	3	3	2	1	1	1	1 (6.0)	1	1

Table VI. *Precipitation of antigen A by NaCl and LiCl.*

Concentration of sodium chloride	Concentration of lithium chloride										H ₂ O
	2.5 N	1.25 N	0.62 N	0.31 N	0.16 N	0.08 N	0.04 N	0.02 N	0.01 N		
H ₂ O	2 (6.2)	2	2	op.	0	0	0	0	0	0	0 (6.4)
0.004 N	2	2	2	op.	0	0	0	0	0	0	0
0.008 N	2	2	2	op.	0	0	0	0	0	0	0
0.016 N	2	3	3	op.	0	0	0	0	0	0	0
0.031 N	3	3	3	op.	0	0	0	0	0	0	0
0.062 N	3	3	3	1 (6.4)	op.	0	0	0	0	0	0
0.125 N	3	3	3	2	2	op.	0	0	0	0	0
0.25 N	1	2	3	3	3	3	3	3 (6.5)	3	3	2
0.5 N	0	2	2	3	3	3	3	3	3	3	2
1 N	0 (6.3)	1	2	2	2	2	2	2	2	1 (6.6)	1

Table VII. *Precipitation of antigen A by LiCl and MgCl₂.*

Concentration of magnesium chloride	Concentration of lithium chloride										H ₂ O
	2.5 N	1.25 N	0.62 N	0.31 N	0.16 N	0.08 N	0.04 N	0.02 N	0.01 N		
H ₂ O	1 (5.8)	1	1	op.	0	0	0	0	0	0	0 (6.4)
0.004 N	1	1	1	op.	op.	0	0	0	0	0	0
0.008 N	1	1	1	2	2	3	3	4	4	4	4
0.016 N	1	1	2	2	2	3	4	4	4	4	4
0.031 N	2	2	2	2	3 (5.9)	3	4	4	4	4	4
0.062 N	2	2	2	2	3	4	4	4	4	4	4
0.125 N	3	3	2	3	3	4	4	4	4	4	4
0.25 N	3	3	3	3	3	4	4	4	4	4	4
0.5 N	3	3	4	4	2	2	2	2	2	2	2
1 N	2 (5.2)	2	2	2	op.	op.	op.	op.	op.	op.	op. (6.0)

Table VIII. *Precipitation of antigen A by CaCl_2 and MgCl_2 .*

		Concentration of calcium chloride										H_2O
		1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N		
Concentration of magnesium chloride	H_2O	op (7.6)	op.	3	4	4	4	4	op.	0	0 (6.4)	
	0.004 N	4	4	4	4	4	4	4	4	0	0	
	0.008 N	4	4	4	4	4	4	4	4	op.	0	
	0.016 N	4	4	4	4	4	4	4	4	3	op.	
	0.031 N	4	4	4	4	4 (7.0)	4	4	4	4	4	
	0.062 N	4	4	4	4	4	4	4	4	4	4	
	0.125 N	4	4	3	3	3	3	4	4	4	4	
	0.25 N	4	4	3	3	3	2	2	2	2	2	
	0.5 N	4	4	3	2	2	1	1	1	1	1	
	1 N	4 (7.8)	4	2	2	2 (6.8)	1	1	1	1	op. (6.0)	

sufficient magnesium is also present. On the other hand, calcium, in any amount within the range used, does not appreciably inhibit precipitation by magnesium. It would appear then that this sol shows to a very marked extent the phenomenon of ion antagonism and that this is exhibited not only with mixtures of univalent and bivalent ions, but even to some extent in the case of two bivalent ions. In the case of two univalent ions, lithium and sodium, the effects are somewhat obscured because of the very weak action of the univalent ions on this sol; but, if we take a series of tubes containing lithium chloride in concentration of 2.5 N, the effect of increasing amounts of sodium chloride is first of all to cause increased precipitation and in higher concentrations decreased precipitation.

It will be observed that the ion antagonism here described is peculiar in that it is exhibited by ions of the same valency and even in the case of a single type of ion. Further, as mentioned above, with many bivalent ions, a zone effect is observed and so it would appear that an excess of ions antagonises the precipitating reaction of a smaller quantity. This inhibiting action of large concentrations of bivalent ions does not seem to be due to their effect in reversing the electric charge. Cataphoretic experiments were made in a U-tube in order to ascertain the magnitude and the sign of the cataphoretic potential difference between the particles and the medium in presence of a concentration of calcium chloride greater than that effecting precipitation. No cataphoretic movement, however, was obtained and, although any conclusion from this experiment must be drawn with reserve, in view of the difficulties introduced by the presence of the high concentrations of electrolyte, yet this result makes it appear probable that the failure of precipitation under these conditions is to be found not in a reversal of electric charge on the particle but in some other direction.

With the above results, obtained with salts of sodium, lithium, magnesium and calcium, may be compared Table IX which represents experiments in which for one of the salts there has been substituted hydrochloric acid. It will be seen that the hydrogen ion acts like a bivalent rather than a univalent ion in as far as complete precipitation occurs in comparatively low concentrations

Table IX. *Precipitation of antigen A by CaCl_2 and HCl .*

Concentration of calcium chloride	Concentration of hydrochloric acid										H_2O
	H_2O	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	
	4 > (1.2)	4	op.	0	0	4 (1.4)	4 (1.7)	0	0	0	0
0.004 N	4	4	1	0	0	4	4	4	4	4	0
0.008 N	4	4	1	0	0	4	4	4	4	4	4
0.016 N	4	4	1	0	0	3	4	4	4	4	4
0.031 N	4	4	1	op.	0	0	4	4	4	4	4
0.062 N	4	4	1	0	0	0	0	4 (2.4)	4	4	4 (7.4)
0.125 N	4	4	4	4	0	0	0	0	4	2	2 (7.6)
0.25 N	4	4	2	1	0	0	op.	op.	op.	op.	op.
0.5 N	4	4	2	op.	0	0	0	0	0	0	op.
1 N	4 > (1.2)	4	2	0	0	0	0	0	0	0	op.

and absence of precipitation at high concentrations of acid. It is to be observed, however, that with still higher concentrations (0.5 N) of hydrochloric acid precipitation again occurs and this precipitation is not very much altered by the presence of calcium ions in addition to the hydrogen ions. Reference may here be made to Fig. 1 of the previous paper. In this it was shown that the zone obtained with calcium was not affected even although the hydrogen ion concentration was varied over a wide range (p_{H} 3.5–10.0). The results now given extend these previous observations which were not carried beyond a p_{H} of 3.5 and show that the zone of precipitation gradually widens as the p_{H} decreases until at p_{H} 1.2 complete precipitation of the antigen takes place with all concentrations of calcium chloride until a concentration of 0.06 N is reached. It appears that the acid does not act in any specific way but that it is similar in its effect to an electrolyte containing a bivalent cation.

III. PROTECTIVE ACTION OF ANTIGEN.

It was thought that some insight might be obtained as to the mechanism whereby the peculiar results detailed above are produced by means of experiments on the protective action of these antigens. Most of the work described in this section has been carried out with colloidal gold sol, but some experiments have been carried out with gum benzoin, gum mastic and cholesterol sols.

The following experiments have been carried out with gold sol, to which antigens had been added. The mixtures were prepared in two ways; in the first (C), the alcoholic extract was added to the gold sol, drop by drop with stirring, so that the colloidal condition of the antigen would correspond to that in the clear sol (B) described above. In the second method (D), the gold sol was added, drop by drop to the antigen with constant stirring, so that in this sol the antigen is in the turbid condition (A); 1 cc. of one of these sols was added to an equal amount of the salt solution at various concentrations. In the following experiment the salt used was calcium chloride, and gold sol containing various quantities of antigen was added, the antigen being in the fine condition C. The results are given in Table X.

Table X. *Precipitation of antigen-gold sol C by CaCl₂.*

Concentration of calcium chloride										
1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	
0	0	0	0	0	0	0	0	0	0	5 % C
0	0	0	0	0	0	0	0	0	0	0.5 % C
0	0	0	1	2	3	3	3	3	0	0.1 % C
1	1	2	4	4	4	4	4	4	0	0.075 % C
4	4	4	4	4	4	4	4	4	0	0.05 % C

It will be seen that no protection takes place until the concentration of the antigen is equal to 0.1 %. At this concentration antigen precipitation does not occur with 0.25 N salt, but occurs with rather smaller concentrations of salt, and continues to 0.004 N. If the concentration of antigen is increased, precipitation fails to occur over a larger number of tubes until with 0.5 % antigen no precipitation occurs in any tube.

A series of experiments was carried out with the coarse antigen-gold sol (D), similar to those with the fine antigen-gold sol (C). The results are shown in Table XI.

Table XI. *Precipitation of antigen-gold sol D by CaCl₂.*

Concentration of calcium chloride										
CaCl ₂ 1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	
0	0	0	0	0	0	0	0	0	0	1 % D
0	0	0	0	0	0	1	0	0	0	0.6 % D
0	0	0	0	0	3	3	3	2	0	0.5 % D
1	1	1	1	3	4	4	3	2	0	0.4 % D
4	4	4	4	4	4	4	4	4	0	0.1 % D

On comparing these results with the fine antigen-gold sol (C), it will be seen that qualitatively they correspond, but that quantitatively there is the difference that the fine antigen is more effective in preventing precipitation than is the coarse antigen. It has more marked protective power, a result which fits in with the fact mentioned above, that the fine antigen itself (B) is much more difficult to precipitate with the salts than the coarse antigen (A). Table XII shows the results when sodium chloride is used instead of calcium

Table XII. *Precipitation of antigen-gold sols C and D by NaCl.*

Concentration of sodium chloride										
1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	
4	4	4	4	4	4	0	0	0	0	0.5 % antigen-gold D
2	4	4	4	4	4	0	0	0	0	1 % " "
1	3	4	4	4	4	0	0	0	0	2 % " "
1	2	4	4	4	4	2	0	0	0	5 % " "
4	4	4	4	4	4	0	0	0	0	Gold sol alone
4	4	4	4	4	4	0	0	0	0	0.5 % antigen-gold C
4	4	4	4	4	4	0	0	0	0	1 % " "
3	4	4	4	4	4	0	0	0	0	2 % " "
3	4	4	4	4	4	0	0	0	0	5 % " "

chloride. The very striking fact is revealed by these experiments that, in presence of a suitable quantity of antigen, gold sol is more readily precipitated by sodium chloride than by calcium chloride.

The conclusion is clearly suggested, that in presence of salts some protective property of the antigen is produced or enhanced, and that salts containing bivalent cations are much more effective in causing this than salts containing univalent cations. It will be recalled that in respect to its action on the antigen the hydrogen ion behaves like the bivalent ions and that a zone effect is obtained with the hydrogen ion just as with the bivalent ions. It is therefore of interest to determine the protective power of the antigen when hydrochloric acid is used as precipitant. The results of the experiments are shown in Table XIII. When these results are compared with those in Tables X, XI and XII it is clear that hydrogen chloride is markedly different from sodium chloride and is very similar to calcium chloride. The hydrogen ion therefore behaves anomalously in the protective, as well as in the precipitation, experiments. In order to obtain information on the dependence of this effect on the specific chemical nature of the ions, and also on the influence of the anions, similar experiments were carried out with a series of salts and the results are shown in Tables XIII and XIV.

Table XIII. *Precipitation of antigen-gold sol C by HCl.*

Concentration of hydrochloric acid										
1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	
0	0	0	0	0	0	0	0	0	0	5 % C
0	0	0	0	0	0	0	0	0	0	1 % C
4	3	3	3	3	2	0	0	0	0	0.5 % C

Table XIV. *Effect of anions on precipitation of antigen-gold sol C.*

Concentration of salt										
	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	0.001 N
NaCl	4	4	4	4	4	0	0	0	0	0
NaBr	1	1	3	3	4	0	0	0	0	0
NaF	2	3	4	4	0	0	0	0	0	0
NaI	1	1	2	0	0	0	0	0	0	0
NaClO ₃	1	1	2	3	0	0	0	0	0	0
Na ₂ SO ₄	3	4	4	4	0	0	0	0	0	0
NaCl	4	4	4	4	4	0	0	0	0	0
NaF	4	4	4	4	0	0	0	0	0	0
NaBr	1	1	3	4	3	1	0	0	0	0
NaI	4	4	4	1	0	0	0	0	0	0
KCl	4	4	4	4	4	2	0	0	0	0
KF	4	4	4	4	0	0	0	0	0	0
KBr	4	4	4	4	4	1	0	0	0	0
KI	4	4	4	3	0	0	0	0	0	0

It is at once clear that the most important factor in determining the protective action of the salt is the valency of the cations; the anion, however,

seems to be of some importance. The nature of the cation, apart from its valency, does not appear to exert much influence; rather curiously, the nature of the anion appears to have a more marked influence. This is shown by the different effects of sodium chloride, bromide, iodide and fluoride on sols containing 5 % and 0.5 % antigen. In order to make certain of this somewhat remarkable result, the above experiment was repeated using the corresponding salts of potassium, instead of those of sodium. These results are also included in Table XIV. As before, the protection is more marked with the iodide than with the others; but the differences are not quite so great as when the sodium salts are used. Sodium iodide protects better than does potassium iodide, although in the precipitation of a gold sol containing no antigen these salts are approximately equal in effectiveness.

It has been ascertained by ultramicroscopic observations that the protective effect is not brought about as the result of the acquisition by the particles of a positive charge. Neither in the case of a coarse antigen alone nor in the case of the gold sols containing antigen was any movement of the particles observed, when so large a quantity of salt was present that precipitation did not occur.

It is clearly important to ascertain whether this protective action of the antigen is exerted on gold sol alone or whether it is as characteristic as the protective power exercised by a solution of gelatin which, as is well known, protects almost every lyophobic colloid. Sols of gum benzoin, gum mastic and cholesterol were therefore prepared and to 90 cc. of the sol 10 cc. of the alcoholic antigen were added with constant stirring, the antigen being in the finely dispersed condition (*B*); 1 cc. of each of these sols was added to 1 cc. of varying concentrations of calcium chloride solution and the whole left for 24 hours. The results are given in Table XV.

Table XV. *Precipitation by CaCl_2 of various sols with antigen B.*

Concentration of calcium chloride												
1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	0.001 N	0.0005 N	
4	4	4	4	4	4	3	0	0	0	0	0	Cholesterol + antigen
4	4	4	4	4	4	4	4	4	2	0	0	„ alone
4	4	4	4	4	4	3	0	0	0	0	0	Gum mastic + antigen
4	4	4	4	4	3	0	0	0	0	0	0	„ alone
4	4	4	4	4	4	2	0	0	0	0	0	Gum benzoin + antigen
4	4	4	4	4	4	2	0	0	0	0	0	„ alone

It will be seen that whereas a very marked protective action is exerted by antigen upon gold sol the effect is practically non-existent on gum benzoin, very slightly marked on gum mastic and slightly more marked on cholesterol. It is clear that the exact conditions existing at the surface of the colloidal particles are of great importance. In the case of cholesterol either a definite protective film is not formed or, because of the disturbing effects of the

cholesterol surface, the protective properties of the film are interfered with. This might occur as the result of an unusual orientation of the molecules constituting the film.

The general trend of these experiments is to emphasise the importance of the constitution of the surface layers surrounding the colloidal particles. In the case of the coarse antigen it was found that the material precipitated by small quantities of calcium chloride is readily redispersed when stirred up with distilled water, and the resulting suspension, when made up to a volume equal to that of the antigen from which the material was originally precipitated, is considerably coarser than was the antigen. It is now found that this coarse suspension, when added to a series of dilutions of calcium chloride, exhibits a zone of precipitation which is even more sharply defined than in the case of the original coarse antigen. This experiment at first sight seems to indicate that a film of protective material must have surrounded the particles when first precipitated by calcium chloride. It is, however, possible that the larger particles which are precipitated contain enclosed within them quantities of protective material and that this diffuses out when the precipitate is suspended in water.

It is evident that this question can be settled only when further work has been carried out and indeed the explanation of the phenomena described in this paper does not at present appear to be at all simple. The existence of protection in presence of sufficient quantities of electrolytes and its absence when the amount of electrolytes is less but still sufficient to neutralise the charge on the colloidal particles has been definitely demonstrated. It is also clear that the physical nature of the colloidal antigen is of considerable importance in determining the results in a quantitative sense. Though it seems clear that the zone phenomenon observed with the antigen alone is closely related to the protective effects upon gold sol and other colloids, there are certain peculiar results such as the fact that the antigen itself is practically unaffected by sodium chloride, although sodium chloride is but slightly active in calling forth the protective action of the antigen upon gold sol. Such results indicate the very complex behaviour of the materials commonly used in routine serological tests.

IV. SOME PROPERTIES OF LECITHIN SOLS.

From the method of preparation of the antigen it is likely to contain much of the lecithin present in the original heart tissue. It is therefore of interest to ascertain to what extent the properties of the sols described above are similar to those of a lecithin sol prepared in a similar way. Some of the properties of lecithin sols have already been described by Kakiuchi [1922] and by Rona and Deutsch [1926]. Kakiuchi finds that lecithin suspensions are not precipitated by salts with univalent cations and that with salts with bi- or ter-valent cations they show a zone effect.

Rona and Deutsch find that the antagonistic effects of the various cations are well marked although Kakiuchi considers that the inhibiting action of the anions has more effect than the antagonising effect of the cations. We have carried out a number of experiments with a commercial lecithin preparation under conditions parallel to the experiments described above with the Wassermann antigen so that a direct comparison could be made between the two sols. Some of these are described below.

It may be stated here that in some respects, *e.g.* the precipitation of the colloidal solutions by salts, there is very considerable similarity but that the lecithin sol appears to have less protective action than the antigen.

In these experiments Merck's lecithin was used. An alcoholic solution was prepared by dissolving 0.5 g. in 100 cc. alcohol. It was first of all ascertained that, as in the case of the antigen, the properties of the sol made by mixing 10 cc. of the alcoholic solution with 90 cc. water depend very much on the method of mixing. A coarse or fine sol could be prepared at will by adding the alcoholic solution to water or by adding the water slowly to the alcoholic solution. The results given in Table XVI indicate the general effects of these sols.

Table XVI. *Precipitation of lecithin by salts.*

	Concentration of salt									
	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N
CaCl ₂	4	2	2	0	0	4	4	4	4	0
MgCl ₂	2	3	4	4	4	4	4	4	0	0
NaCl	1	2	2	0	0	0	0	0	0	0

The result of a typical experiment showing the action of mixtures of salts on lecithin is given in Table XVII.

Table XVII. *Precipitation of lecithin by LiCl and CaCl₂.*

	Concentration of lithium chloride									
	2.5 N	1.25 N	0.62 N	0.31 N	0.16 N	0.08 N	0.04 N	0.02 N	0.01 N	H ₂ O
H ₂ O	1	1	2	3	3	0	0	0	0	0
0.004 N	1	2	2	3	3	3	1	3	1	4
0.008 N	1	2	2	4	4	4	4	4	4	4
0.016 N	1	2	3	4	4	4	4	4	4	4
0.031 N	1	2	3	4	4	4	3	0	4	4
0.062 N	1	2	3	4	3	1	0	0	0	0
0.125 N	1	2	3	3	1	1	1	0	0	0
0.25 N	1	2	3	3	3	3	2	2	2	2
0.5 N	0	1	3	3	3	3	3	3	3	2
1 N	0	0	2	2	2	2	2	2	2	4

These tables may be compared with Tables I–VIII and it will be seen that although the similarity is marked, the appearance of the extra zone of precipitation with calcium chloride alone may be noted. This effect seems to be a real one: it has been obtained several times but the factors causing it have

not so far been analysed, but it may be recalled that a similar phenomenon was observed with antigen when hydrogen chloride was used. It may be said, however, in general terms that the tendency towards complete precipitation is more marked in the case of the lecithin sol than in the case of the antigen in the sense that precipitation takes place over a wider range of concentration; but on the other hand it should be mentioned that in many tubes in which precipitation of the lecithin occurs, sedimentation takes place slowly, so that satisfactory readings cannot be obtained at the end of 24 hours. The above readings were all made after the tubes had been left for more than 48 hours.

Table XVIII. *Precipitation of lecithin-gold sol by salts.*

		Concentration of salt													
	1 N	0.5 N	0.25 N	0.125 N	0.062 N	0.031 N	0.016 N	0.008 N	0.004 N	0.002 N	0.001 N	0.0005 N			
CaCl ₂	4	4	4	4	4	4	4	4	4	4	3	0	Fine lecithin	5 %	in gold sol
NaCl	4	4	4	4	0	0	0	0	0	0	0	0	"	"	5 %
CaCl ₂	3	1	1	4	4	4	4	4	4	4	0	0	"	"	10 %
NaCl	4	4	4	4	0	0	0	0	0	0	0	0	"	"	10 %
CaCl ₂	3	4†	4†	4	4	4	4†	4†	4†	4	0	0	Coarse lecithin	10 %	
NaCl	4	4	4	4	0	0	0	0	0	0	0	0	"	"	10 %

Note. In 5th row the lecithin is only precipitated in tubes marked †.

It is to be expected from the above results that lecithin sol will exert a protective action in a considerably weaker degree than an antigen. The results of experiments on this point are given in Table XVIII. It will be seen that even in the presence of 10 % of the fine lecithin only a very slight protective action shows itself in the presence of considerable concentrations of calcium chloride. With sodium chloride no protection takes place. It appears, then, that the constituents of the lecithin sol are unable to form a protective film round the gold, or at most can do so only with great difficulty. This is emphasised by the experiments in which the lecithin-gold sol was prepared so that the lecithin was in the coarse condition. Both the gold and lecithin are precipitated in certain tubes but the gold is precipitated in the blue condition.

It may be concluded from the above experiments that in commercial lecithin there is a substance which possesses, in a small degree, protective power in presence of electrolytes but either is present in small amounts or shows very much less tendency to be adsorbed on the surface of colloidal particles than does the corresponding substance in the antigen.

Whether pure lecithin is quite devoid of protective power or whether sols of pure lecithin will not show a zone phenomenon under any conditions it is hoped to investigate in a continuation of the present researches.

DISCUSSION.

The above results seem to be unique in demonstrating, in the extracts of heart used for the preparation of Wassermann antigens, the existence of a substance, the protective effect of which cannot be detected in the absence of salts but which exerts a marked protective effect in the presence of considerable quantities of bivalent cations. We have not so far examined the action of trivalent cations on the colloidal solutions employed above, since, as is well known, anomalous precipitation effects are observed with trivalent cations even with simple colloids such as gold sol, and it seems desirable at this stage to limit the investigation to salts of uni- and bi-valent cations which behave normally with simple colloidal solutions. The results now obtained make less peculiar the observations recorded in the previous paper that gelatin cations (p_H below 4.7) in very small concentration are able to effect precipitation of the antigen whereas sodium chloride is quite unable to do so. This at first sight appears a remarkable result, but gelatin is normally a colloid with high protective properties whereas sodium chloride does not effect protection in the case of ordinary colloidal solutions. This result is now seen to depend on the fact that the protective action of the antigen is exhibited in the presence of sodium ions at a concentration insufficiently great to cause precipitation, whereas gelatin is able to effect precipitation at extraordinarily small concentrations and so neither the gelatin nor the antigen restrains the flocculation of the particles. Another curious result is that a gold sol containing antigen may be prepared which is readily precipitated by sodium chloride over a wide range of concentrations but shows very little or no precipitation by calcium chloride. We are so far unable to offer any explanation of the mechanism whereby this protection in presence of salts is effected, nor have we any evidence as to the chemical nature of the substance responsible except that it is stable to heat and mild oxidation and is probably not lecithin. Experiments on these points are being continued.

The question of course suggests itself whether this protective substance plays a part in the Wassermann reaction. It is clear that its presence has a marked influence on the properties at the surface of the colloidal particles at which complement is presumably fixed when the Wassermann reaction is positive. It is well known however that the efficiency of a colloidal solution such as a Wassermann antigen does not depend solely on its chemical constitution but to a very marked degree on its physical properties. It has been frequently observed that coarse antigens tend to be more effective than fine ones. This fact is exemplified in a striking manner by antigens *A* and *B* described above which are identical chemically but of which *A* is a strong Wassermann antigen whilst *B* is quite inactive. We are therefore elaborating a method whereby active substances can be detached in spite of the fact that they do not give rise to the necessary physical conditions and it is hoped that

it will then be possible to discover the relation between the antigenic properties on the one hand, and the protective powers on the other.

Finally it may be pointed out that these substances are obtained from animal tissue and that fuller understanding of the properties may help in some measure to elucidate the physico-chemical equilibrium existing in the animal cells. It is for this reason as well as because of high practical importance that it seems justifiable to attempt to explore the colloidal properties of mixtures so complex as Wassermann antigens which are the subject of this paper.

SUMMARY.

1. The effect of variations in the method of preparation of a Wassermann antigen on its properties have been investigated and standard methods of preparing (1) turbid, and (2) clear antigens have been evolved.

2. The observation previously made that a Wassermann antigen is precipitated by small quantities of calcium chloride but not by large quantities has been extended and it is shown that a similar effect with salts of other bivalent cations is obtained whilst the salts of univalent cations, with the exception of the hydrogen ion, do not cause complete precipitation at any concentration. The hydrogen ion however acts like the bivalent cations.

3. Many observations have been recorded of the action of mixtures of two of the four salts, lithium chloride, sodium chloride, magnesium chloride and calcium chloride on the precipitation of the antigen *A*. In general the presence of a sufficient quantity of another salt, whether of a univalent or bivalent cation, to a salt (with a divalent cation) present in concentration sufficient to cause precipitation if it were alone tends to prevent precipitation. Similar experiments in which hydrogen chloride was used as one of the salts, demonstrate the similarity of the hydrogen ion to the bivalent cation.

4. The protective effect of antigens both clear and turbid has been investigated, using gold sol. It is found that the protective effect is excited by the presence of salts but especially by salts with bivalent cations or hydrogen ions much more than with salts of the other univalent cations. The hydrogen ion behaves like a bivalent cation. The fine antigen *B* exhibits rather greater protective power than the turbid antigen *A*. The protective power of the antigen has also been determined using cholesterol, gum mastic or gum benzoin sol; it is most marked in the first and least in the last.

5. Analogous experiments have been made with sols of commercial lecithin and it was found that these possess, only in small degree, the peculiar properties of the Wassermann antigens.

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