CLXXXVI. SOME COLLOIDAL PROPERTIES OF WASSERMANN ANTIGENS.

BY WILLIAM OGILVIE KERMACK AND PETER MACCALLUM.

*From the Research Laboratory of the Royal College of Physicians, Edinburgh,
and the Pathological Department of the Royal Infirmary of Edinburgh.*

(Received October 23rd, 1924.)

A VERY large amount of work has been done on the more direct and obvious properties of the colloidal lyophobic sols known as "antigens," which are used, for example, in carrying out the Wassermann, Sachs-Georgi or Sigma reactions. So far as we are aware no general investigation of the colloidal properties of these antigens has been made from a fundamental point of view.

Such antigens are prepared by mincing the tissue of an organ, preferably bullock or human heart, the material in a dry or moist state being then, with or without preliminary extraction with acetone, extracted with alcohol under given conditions. On adding the alcoholic extract to normal saline solution an opalescent sol results. This sol added to serum which has been first heated to 55° (inactivated) causes under certain conditions the formation of a precipitate, and under certain other conditions it can be shown that if in addition some fresh serum (complement) is added, the mixture when incubated at 37°, loses its power of haemolysing red blood corpuscles sensitised by specific immune body (the Wassermann reaction). This is hypothetically regarded as being due to some kind of incipient precipitate formation which results in the binding of the hypothetical "complement" present in the fresh serum added and necessary for the haemolysis of the cells. When the complement is "bound" it cannot act in producing haemolysis. It appears to be fairly well established that such precipitation (Sigma test), or fixation of complement (Wassermann test), by antigen plus serum, occurs with a very high percentage of sera from patients suffering from syphilis, and is rare, if it exists at all, in the absence of such syphilitic infection, but the physical or chemical mechanism at the basis of these reactions is very obscure.

It was considered that an investigation of the physical properties of these Wassermann antigens on the lines adopted in the paper on Colloidal "Gum Benzoin" [Wright and Kermack, 1923], would be of much interest. However, it soon became apparent that it was desirable to investigate more closely such questions as the magnitude of the potential difference between the particles of the sol and the medium. We therefore decided to investigate quantitatively the simple case of a colloidal solution of cholesterol, an important constituent of Wassermann "antigens." The results of this investigation will be described in a later paper. Meanwhile the experimental results obtained with the Wassermann "antigens" themselves are here recorded.

A reference may be made to the very interesting results obtained by Holker [1922] on the effect of increasing concentrations of sodium chloride on the antigen. Using the antigen of Bordet and Ruelens (cholesterolised), with progressive variation of sodium chloride concentration, he found rhythmic alternations in the opacity of the antigen, an effect particularly obvious where his special opacimeter is used, but also visible to the naked eye. We have been concerned rather with the gross alteration in dispersion of the nature of total precipitation. It is of interest to note that even very great concentrations of sodium chloride do not cause the complete precipitation which is effected quite easily by calcium chloride.

I. PREPARATION OF ANTIGEN.

The method of preparation of the "antigen," is that described in the Medical Research Committee's Report on the Wassermann test [1918]. It is a modification of that of McIntosh and Fildes [1916]. The muscle tissue of human heart, obtained as fresh as possible and freed carefully from fat with scissors, was minced finely in a mincing machine. The material was weighed, placed in a Winchester bottle with a closely fitting stopper, and for each gram 10 cc. of absolute alcohol were added. The bottle was stoppered, and sealed over with plasticine, and, after its contents had been thoroughly shaken, was placed in an incubator at 37° for 21 days. The mixture was well shaken up daily, and at the end of the period was filtered, a clear colourless fluid being obtained. This extract, without the addition of cholesterol, was the antigen, McIntosh and Fildes I. The antigen McIntosh and Fildes II was prepared in a similar manner from the muscle tissue of bullock's heart.

In addition to the above antigen (McIntosh and Fildes I) which was the one used in general, two other antigens were also prepared and used for comparison. Their modes of preparation are briefly as follows:

Antigen of Keim and Wile [1922] was prepared by drying by means of a fan a thinly spread layer of finely minced fresh bullock's heart muscle tissue and grinding up the dried product. This was extracted for 24 hours in the ice chest with fresh ether on four successive days. After filtration the tissue was dried at room temperature till odourless, and extracted with 100 cc. of absolute alcohol for each 20 g. for nine days in the ice chest and one day at room temperature. The alcoholic filtrate (cholesterolised) was used in preparing the antigen.

Antigen of Bordet and Ruelens [1919] was prepared by first extracting 100 g. of finely minced calf's heart muscle tissue with 125 cc. of 95 % alcohol for five days at room temperature. The residue from filtration was spread in a thin layer and dried at 37° till dark in colour and just brittle, and then extracted with 200 cc. of pure acetone for ten days at 21°, shaking daily. The acetone was poured off and replaced with 100 cc. of fresh acetone for six hours. The residue from filtration was dried for two hours, and after grinding to a coarse

COLLOIDAL PROPERTIES OF WASSERMANN ANTIGENS 1383

powder, was extracted for 20 days (with daily shakings) at 21° with 200 cc. of 95 % alcohol. The filtrate (non-cholesterolised) was used in preparing the antigen.

For use, 10 cc. of the antigen of McIntosh and Fildes was placed in the bottom of a dry 100 cc. measuring cylinder, and 90 cc. of distilled water were poured in rapidly and the mixture shaken vigorously. The antigen thus prepared differs from that used in the Wassermann reaction in that it contains no sodium chloride. It was considered that this variation was advisable as sodium chloride could be added as required and it seemed important to investigate the antigen first of all in the absence of that salt. In the experiments described below Keim and Wile's extract was similarly treated. The antigen of Bordet and Ruelens, however, was prepared by taking 1 cc. of the extract and evaporating to dryness in a porcelain basin. The yellowish residue was dispersed in 2 cc. of distilled water by triturating the material with a glass rod and the result was diluted to 10 cc. with distilled water. The determination of the hydrogen ion concentration in the following experiments was carried out colorimetrically with the Clark-Lubs sulphonephthalein series of indicators [Clark, 1920].

II. PRECIPITATION BY ACIDS AND SALTS.

(a) *Precipitation by Acid.*

Compared with ordinary suspensoid sols, a very high concentration of acid is required to precipitate Wassermann antigens. A comparison of the action of various acids has been made on four antigens. The results are shown in the following table:

Table I. *Effect of Acids on Antigens.*

Antigen	Concentrations of sulphuric acid					Concentrations of hydrochloric acid				
	N/20	N/40	N/80	N/160	N/320	N/20	N/40	N/80	N/160	N/320
Keim and Wile (1 in 10)	4	4 (p _H 1·8)	1 (p _H 2·2)	0	0	4 (p _H 1·6)	2	0 (p _H 2·4)	0	0
McIntosh and Fildes I (1 in 10)	3	3 (p _H 1·8)	1 (p _H 2·0)	0	0	4	3 (p _H 1·9)	2	0 (p _H 2·8)	0
McIntosh and Fildes II (1 in 10)	4 (p _H 1·6)	2	1 (p _H 2·1)	0	0	4 (p _H 1·6)	2	0 (p _H 2·4)	0	0
Bordet (1 in 10)	4 (p _H 1·4)	2 (p _H 1·6)	0 (p _H 2·0)	0	0	op. (p _H 1·6)	0 (p _H 2·1)	0	0	0
Bordet (1 in 40)	op. (p _H 1·6)	0 (p _H 1·8)	0	0	0					
Antigen	Concentrations of acetic acid									
	N/20	N/40	N/80	N/160	N/320					
Keim and Wile (1 in 10)	4 (p _H 1·4)	0 (p _H 2·0)	0	0	0					
McIntosh and Fildes I (1 in 10)	4 (p _H 1·4)	2	0	0	0					
McIntosh and Fildes II (1 in 10)	3 (p _H 1·4)	3 (p _H 2·0)	0 (p _H 2·4)	0	0					
Bordet (1 in 40)	op. (p _H 1·4)	0 (p _H 1·8)	0 (p _H 2·3)	0	0					

The antigen of Bordet and Ruelens as prepared contains little solid material and an opalescence (op.) rather than a precipitation was obtained. In the other cases 4 denotes complete precipitation, 3 almost complete precipitation, 2 partial precipitation, but supernatant fluid not nearly clear, 1 very slight precipitation, and 0 no precipitation.

It will be seen that, as far as the experiments in the above table go, it is the hydrogen ion concentration which is the determining factor in precipitation by acids. Precipitation is always obtained at p_H 1.8 or 1.9 in the case of the antigens of Keim and Wile or McIntosh and Fildes II (bullock's heart), whilst the human heart antigen (McIntosh and Fildes I) appears to be slightly more difficult to precipitate, requiring a p_H of 1.6, and Bordet's antigen, which is so dilute that it gives only an opalescence, behaves similarly.

(b) *Behaviour in the presence of Sodium Chloride.*

No precipitation of antigen (human heart) was obtained on adding sodium chloride in concentrations from 10 % to 0.0045 % with variations in the p_H of from 6.4 to 3.5.

(c) *Precipitation by Calcium Chloride.*

It was found that calcium chloride in comparatively low concentrations produced precipitation of the antigen, but that such precipitation did not occur in the presence of larger amounts of the salt. At first sight this appeared rather similar to the zone effect observed, for example, with ferric chloride and colloidal "gum benzoin," but that the phenomena are different would appear from the fact that precipitation did not again occur in the presence even of very great concentration of calcium chloride. It was thought to be of interest to investigate the effect of variation of p_H on this action of calcium chloride on the antigen, and the results are shown in Fig. 1. It will be noticed that the highest concentration of calcium chloride at which precipitation was obtained is 0.625 %, and is little if at all influenced by the hydrogen ion concentration, and in the same way, within wide limits, the minimum amount required to produce precipitation is also independent of the reaction.

III. PRECIPITATION BY GELATIN.

In view of the comparatively great stability of the antigen, it was of particular interest to ascertain whether its behaviour towards gelatin was similar to that of normal lyophobic sols such as "gum benzoin" [Wright and Kermack, 1923], or cholesterol. The following experiment shows that it is. The isoelectric gelatin was prepared as described by Loeb [1922], and contained a negligible amount of ash. A series of solutions of decreasing concentration was prepared and also a similar series of dilutions of acid. Test tubes were arranged in rows and columns so that each row corresponded to a certain concentration of gelatin and each column to a certain concentration of acid (or of distilled water). Into each test tube was put 0.5 cc. of the proper dilution of gelatin, 0.5 cc. of the appropriate solution of acid (or distilled water), and 1 cc. of the antigen. The contents of the tubes were thoroughly mixed, the tubes corked, and allowed to stand overnight. The results are shown in Fig. 2. The nature of the charge on the particles determined by observation

COLLOIDAL PROPERTIES OF WASSERMANN ANTIGENS 1385

of their behaviour in an electric field under the ultramicroscope is also indicated. It will be noted from the figure that the zone of precipitation, as in the case of gum benzoin, separates the positively from the negatively charged region and that this zone does not extend beyond a p_H of 4.7.

Fig. 1. Effect of p_H on the precipitation of "Antigen" by calcium chloride.

It may be noted that gelatin without the addition of acid does not produce a precipitate with the antigen as it does with the "benzoin," since the resultant p_H without the addition of acid is about 5.0, and therefore on the alkaline side of the isoelectric point of gelatin. All the gelatin exists as negatively charged anion and so does not cause precipitation.

DISCUSSION.

It will be noticed that in several respects a Wassermann antigen of the type investigated differs from an ordinary sol. The differences are particularly obvious in connection with the precipitation by salts. Sodium chloride, which

in comparatively low concentrations precipitates colloidal gold or colloidal "gum benzoin," does not precipitate the antigen even in very high concentration. In the same way calcium chloride does not precipitate the antigen till a concentration of about 0.07 % is reached, and, when a concentration of 1.25 % or more is used there is no precipitation but rather a protective effect. When acid alone is used it will be observed that whereas colloidal gold and colloidal "gum benzoin" are precipitated at about p_H 2.8 or 3.0, the Wassermann antigen is not precipitated till the p_H is as low as 1.9. Notwithstanding the great stability of the antigen, it is of interest to note that the precipitation by protein appears to be as marked as ever and apparently as normal.

Fig. 2. Illustrating the effect of gelatin on the stability of "Antigen" at various p_H .
x indicates actual observations.

It appears probable that the stability of a sol depends on two factors: the particles, aggregates, micelles or whatever units compose the discrete phase may have an electric charge which prevents close contact and coalescence of these units; on the other hand, these units may themselves contain, or they may be associated with, molecules containing groups of atoms whose affinity for water is great enough to overcome the tendency of the particles to aggregate. This tendency to aggregate is of course presumably occasioned by the decrease of surface energy which accompanies such aggregation. Such views have been advanced by Loeb [1923] and confirmatory evidence is given elsewhere.

Precipitation by gelatin is apparently the result of a neutralisation of the electric charge on the particles of the antigen, the gelatin used being too small

in amount to enable its hydrophile groups to resist the tendency of the sol towards aggregation. As noted above, the precipitation of antigen particles by gelatin does take place. The fact that sodium chloride does not effect precipitation would appear then to be due to its being unable to reduce the charge on the particles sufficiently. Measurements of the actual cataphoretic potential difference (P.D.) between the particles and the medium are being made.

The presence on the surface of the particles of molecules of a fairly strong acid which can form an insoluble calcium salt might explain the phenomena observed. Sodium chloride even in high concentrations would not be able to diminish the degree of ionisation of the acid and thus decrease the cataphoretic P.D. between particles and medium caused by the negatively charged ions of the acid, nor, if sufficient acid ions are present, will the Donnan equilibrium effect of sodium chloride (or of monovalent salts in general) on layers of indiffusible ions result in a sufficiently large decrease of the charge. In the same way to obtain precipitation by acid, a relatively high concentration will be required. On the other hand, a small quantity of gelatin, owing to its tendency to be concentrated at interfaces, will be sufficient to neutralise the charge. Also, because of the hypothetical insolubility of the calcium salt of the acid, calcium salts will precipitate the sol in comparatively low concentrations. The exact mechanism of these phenomena can, of course, only be elucidated by further experimental investigation.

CONCLUSIONS.

1. An alcoholic extract of heart muscle when added to water forms an opalescent sol which is not precipitated by even high concentrations of sodium chloride, and by acid only when a p_H as low as (approx.) 1.9 is reached. It is precipitated by an equal volume of 0.15 % calcium chloride solution, but not by an equal volume of calcium chloride solution of 2.5 % or more. This action of calcium chloride is little influenced by the p_H .

2. Precipitation of the antigen sol by solutions of gelatin of the requisite (low) concentration occurs at p_H values below 4.7. In this respect the sol resembles the "benzoin" sol previously investigated.

REFERENCES.

- Bordet and Ruelens (1919). *Compt. Rend. Soc. Biol.* **82**, 880.
 Clark (1920). The Determination of Hydrogen Ions. (*Williams and Wilkins Company, Baltimore.*)
 Holker (1922). *J. Path. Bact.* **25** 522.
 Keim and Wile (1922). *J. Amer. Med. Assoc.* **79**, 870.
 Loeb (1922, 1). *Proteins and the Theory of Colloidal Behaviour.* (*McGraw Hill, Book Company, Inc. New York*).
 — (1923). *J. Gen. Phys.* **6**, 105.
 McIntosh and Fildes (1916). *Lancet*, ii, 751.
 Medical Research Committee (1918). *Special Report Series*, **14**, 15.
 Wright and Kermack (1923). *Biochem. J.* **17**, 635.