PRECIPITATION PHENOMENA AND THE WASSERMANN REACTION.

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CONTENTS.

Introduction.

In the literature of the diagnosis of syphilis attention has frequently been drawn to the concordance which is shown in the results of two such dissimilar tests as those of Wassermann and Sachs-Georgi. The agreement is certainly of the order of 90 per cent. Indeed, in a recent summary Kahn (1925) shows that with a technique similar to that of Sachs-Georgi the agreement in 156,000 cases tested side by side by the two methods was 99·5 per cent. This indicates, either, that the reacting substances in the serum are identical, or, if not identical, are the products of chemical or physico-chemical action occurring in the syphilitic process which are formed side by side. Epstein and Paul (1921) believe the former alternative to be correct. The common impression that the complement deviation test produces greater agreement with medical findings is not, necessarily, a support to the other view, for it may well be that the Wassermann reaction is more sensitive to the conditions peculiar to a syphilitic serum than simple precipitation tests.

The following study was commenced as an investigation of the principle underlying the precipitation tests of which (*a*) the Sachs-Georgi and the numerous variants employing alcoholic extract of liver or muscle are examples, and (*b*) the class of reactions utilising some electro-negative suspension, of which mastic, benzoin and gold are the best known.

From this we were led to the more intricate problem of the reacting substance in syphilitic serum.

An examination of the various precipitation tests for luetic sera indicates that all "antigens"[1] exhibit somewhat similar phenomena. On their addition

[1] For lack of a better word we use "antigen" to denote the various electro-negative suspensions mentioned in the course of this paper.

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to progressive dilutions of normal and specific sera zones of precipitation take place. These only occur in a definite range. In precipitation tests the only difference to be observed between normal and luetic sera or spinal fluids is usually a broadening of the zone of precipitation in both directions—towards the region of greater concentration of serum and more especially in that of greater dilution. The antigens are prepared in an arbitrary state of dispersion for a particular test and means more or less elaborate and efficient are taken to ensure uniformity.

Since both normal and luetic sera yield precipitation zones differing only in extent, the idea of Wassermann that luetic sera contain a specific substance loses some of its probability. The specificity of the Wassermann test is not opposed to this view, for we know that on occasion it is possible to transform a normal serum into one giving a positive W.R. It is also stated that certain rabbit sera which are generally regarded as normal give a positive test.

In an attempt to throw light on the reacting substances in syphilitic serum, much discussion has naturally centred round the composition of the precipitate which is formed in the precipitation zone of the Sachs-Georgi and related reactions. The points of controversy have mainly been as to whether the constituent in the serum reacting with the antigen is lipoidal in character or whether it is an aggregate containing albumin or globulins.

Many investigators have concluded that the globulins are more closely associated with the specific precipitate than are the albumin fractions. One of the interesting features of the precipitate is its ability on resolution to give a Wassermann test. Indeed, Taoka states that by extracting the Sachs-Georgi precipitate with ether the protein residue, consisting, according to that author, of globulins, gives both the complement deviation test and a renewed Sachs-Georgi reaction. Stern (1923) narrows the active substance down to the euglobulin fraction as distinct from the pseudo-globulin fraction. While this has been called into question by some workers it represents the present consensus of opinion.

A search of the literature in support of or against these views reveals divergent and conflicting statements. It is said that the "rest nitrogen" is higher in syphilitic serum than in normal (Embden and Much, 1914). According to Bachmann (1921) the ninhydrin reaction runs parallel to the Wassermann test, indicating an unusual amount of amino acid nitrogen. These variations are well known to occur in other diseases, but they are of interest in connection with statements that the injection of glycocol into rabbits, or the addition of exceedingly small amounts of some of the amino acids of lower molecular weight, will transform a normal into a positive W.R. serum. We have been unable to confirm these statements. The dispersion of a syphilitic serum is claimed by Peyre to be coarser than a normal one, an observation confirmed by Hirsch and Liebers.

Both the surface tension and viscosity of a specific serum are said to be higher than normal (Holker, 1922). Here again the variations in non-specific

sera are so great that it is difficult to appraise the real significance of these statements. According to Nixon and Saito the globulin fraction from a luetic serum is less easily filtered than that from the normal.

Several explanations have been advanced to account for the precipitation of antigens by specific fluids. The purely chemical explanation of the precipitation, based upon Wassermann's suggestion of a specific substance existing in a luetic serum but not in a normal serum, does not receive much support, because precipitation is found (if the concentration range be wide enough) to form a zone only over a certain concentration range, a phenomenon to be observed in normal, as well as in luetic sera. Again, the material reacts with the most diverse antigens, *e.g.* gold and heart extract and this, in its turn, renders the chemical explanation improbable.

To avoid the difficulties inherent in a chemical mechanism, explanations based upon properties of the serum and antigen in virtue of their colloidal states are now generally advanced. Of these the simplest, but by no means the earliest in point of historical development, is one which we, for a time, believed to be correct. We were forced to reject it although during the course of this work it received support from a similar suggestion made by Wright and Kermack (1923). As this view is one which we feel must be definitely disposed of we give it in some detail. It is as follows: the antigens are negatively charged colloids and electrical precipitation might well result if discharge could be effected by some electropositive material in the serum. As will be noted in the experimental part of this paper the determination of the hydrogen ion concentration of the mixture of serum and gum benzoin antigen indicates that neither the albumins nor globulins are necessarily on the acid side of their isoelectric points. Furthermore, the precipitation zones, as well as the differentiation between normal and syphilitic sera, persist from *p*H 3·8 to alkalinities as high as *p*H 8·5. The electrical precipitant, if present, must therefore be a strongly adsorbed and highly basic material. Contrary experiments with highly basic materials occurring in tissues or resulting from protein degradation, as well as some observations on the effect of neutral salt concentration on the precipitating zones, which will be referred to later, led us to reject this hypothesis.

Epstein and Paul (1921) believe that luetic sera differ from normal sera in that they possess a larger number of positively charged colloidal aggregates and are less disperse and that the lipoid particles undergo coalescence among themselves and are fixed to the negative lipoid of the added antigen. The protein phase of the serum is claimed to act as an electrically indifferent insulating layer whilst the negative lipoid of the antigen is discharged both by the positively charged particles in the luetic serum and by the sodium ions present in the solution. It will be noted that Epstein and Paul ascribe a very important rôle to the sodium ions in specific precipitation and indeed in other reactions, such as that of Wassermann. In this they are undoubtedly correct.

From precipitation experiments in salt solutions Epstein and Paul (1921) concluded that the proteins in the specific serum are not responsible for the reaction with the antigens. To this is opposed the large body of evidence concerning the globulin and especially the euglobulins, already referred to.

Nathan (1918) attributes the peculiar action of syphilitic serum to a specific combination of two independent variables: (*a*) the change in the constitution of the globulins and (*b*) a change in the lipoids. He deduces this from the difference in the behaviour of a true syphilitic serum and of a normal serum made artificially Wassermann positive. The latter is said to lose its specific quality by heating to 56° C. This change is due to the fact that the artificial specific serum derives its quality from changes in the globulin. This W.R. + reaction is thermolabile and therefore disappears on heating the artificial W.R. + serum to 56° C. If the lipoid constituents are changed, as in truly luetic serum, this confers a thermostability on the globulin and hence no change takes place in the reactivity of a syphilitic serum on heating it to 56° C.

We have tried to repeat some of Nathan's experiments on which the above hypothesis is based and have been unsuccessful. There is evidence in the papers of this author that the methods he employs do not invariably transform a normal serum into one giving a positive Wassermann test.

These speculations as well as the conclusions of Epstein and Paul (1921) lose some of their validity from the observation of Taoka (1922) and also from our own experiments, where it is shown that a delipoided specific euglobulin still yields a precipitation zone as well as a positive Wassermann reaction.

The Preparation of Standard Antigens.

From the above review of the more salient points concerning precipitation phenomena in the diagnosis of syphilis, it appeared to us that the so-called precipitation tests were essentially purely colloidal reactions and that the various extracts of heart or liver employed, together with the methods devised for mixing, were in reality attempts to produce an electro-negative suspension of the required degree of sensitivity. The sensitivity of the suspension may be determined, as Jacobsthal and Kafka (1916) have done, by ascertaining the concentration of some salt which will be effective in producing precipitation. Whilst the liminal values of salts for colloids vary with the nature both of the cations and anions, it appeared likely, for this purpose, that a comparison of several antigens with one particular salt over definite precipitation ranges might indicate the limits of precipitation. With this information one would be in a position to determine the availability with regard to sensitiveness of any synthetic antigen.

It is well known that the emulsions are less sensitive to salt precipitation than suspensions. By forming a complex colloid, consisting of a suspensoid with an emulsoid coating, the sensitivity can be decreased. Cholesterin for example, which is very coarse and highly suspensoid when mixed with water, becomes extremely fine when the dispersion medium contains saponin. These

coated suspensions of cholesterin are among the most stable with which we have dealt. The gums are variable in their behaviour, since they are composites of lyophilic and lyophobic groups. It is owing to the predominance of the latter in the Siamese gum, that the Sumatra product was selected by Guillain, Laroche and Lechelle (1922). Gum myrrh is so emulsoid in character that it is practically impossible under the conditions of the present experiments to precipitate it. Balsam of Tolu occupies an intermediate place.

Some antigens are suitable for cerebro-spinal fluid, but cannot be used without modification for serum. This is due partly to the great increase in protein concentration in the latter. It is, therefore, necessary to protect the antigen by an added emulsoid coating, for example, of saponin, in order to render it available. Guillain, Laroche and Kudelski's (1922) objection to Arnaud's (1922) statement that gum benzoin was suitable to use as a reagent for blood serum in the diagnosis of syphilis is certainly not well founded if the gum benzoin is properly coated with saponin and the blood serum is employed in the proper concentration.

In our early experiments a dispersion was effected by using a 10 per cent. solution of Sumatra gum benzoin in alcohol made according to the directions of Guillain, Laroche and Lechelle (1922). Three-tenths of a c.c. of this solution was dispersed in 20 c.c. of medium. The aqueous solution was contained in a small beaker and agitated by a rapidly rotating glass stirrer. The alcoholic solution of the gum was admitted slowly from a pipette, the tip of which was below the surface of the medium. The temperature of the medium was 55° C. When the dispersion is made in this way there are no particles of clotted gum and it is unnecessary to filter the suspension before using it in a series of tests. Dispersions of gum benzoin in simple aqueous solution are extremely sensitive to electrolytes. Experiments in the direction of protection with typical colloids such as oleic acid, potassium stearate and castor oil were not particularly successful. Saponin was finally employed in order to render the suspension more emulsoid in character. The stock saponin used contained 0·05 per cent. of saponin. Magnesium sulphate was chosen as the precipitating electrolyte.

The following results were obtained when 0·3 c.c. of the electrolyte solutions were added to 1·0 c.c. of emulsion protected with saponin[1]:

Saponin solution	$MgSO_4$ %					
Per 20 c.c.	10	5	2·5	1·25	0·625	0·31
0·25 c.c.	4	4	4	2	1	0
0·5 ,,	4	4	3	1	0	0
0·75 ,,	4	4	3	1	0	0
1·0 ,,	4	2	0	0	0	0
1·25 ,,	3	1	0	1	0	0

[1] 4 indicates complete precipitation, 0 indicates no change in the dispersion when viewed against a dark background by indirect illumination, 3, 2 and 1 indicate various stages in coarsening. A + sign over a number indicates that in the judgment of the observer the coarsening has proceeded further than the number indicates. With practice it is not difficult to divide flocculation into 9 grades. Duplicate readings are very satisfactory.

Heart extract cholesterin dispersion prepared according to Dreyer's directions gave the following results:

	$MgSO_4$ %					
Dreyer antigen	2·5	2·25	2·0	1·75	1·5	1·25
Dilute	2	0	0	0	0	0
Concentrated	4	4	3	2	0	0

The concentrated antigen is thus more sensitive to precipitation by the divalent salt. This we have also found in working with suspensions of benzoin unprotected with saponin, as is shown in the following table where a suspension has been diluted from 50 per cent. to 12·5 per cent. of its original concentration, 0·3 c.c. of electrolyte solution added to 1 c.c. of the antigen.

	$MgSO_4$											
	M/14	*M*/16	*M*/18	*M*/20	*M*/24	*M*/28	*M*/32	*M*/36	*M*/40	*M*/44	*M*/48	*M*/52
100 %	4	4	4	4	4	4	4	4	4	4	4	4
50 %	4	4	4	4	4	4	4	4	4	4	4	4
33·3 %	2	4	4	1	0	1	4	4	4	0	0	1
12·5 %	3	1	0	1	1	0	4	3	2	0	0	0

In this series are noted the double zones of precipitation caused by the polyvalent ion which were observed by Kermack and Voge (1925) in precipitating benzoin with ferric chloride. The zones were found to be unaffected whether the different concentrations of benzoin dispersion were made by simple dilution of a more concentrated suspension or by direct dispersion.

Attempts were made to substitute the Bordet Ruelens heart extract for saponin in decreasing the sensitivity of the benzoin dispersion; in this we were not successful, although since the experiments were performed Sachs, Klopstock and Ohashi have shown that this is possible.

An antigen prepared by dispersing 0·9 c.c. of 10 per cent. Sumatra gum benzoin in alcohol in 100 c.c. of water containing 0·75 c.c. of 0·05 per cent. saponin proved to have about the same range of sensitivity to magnesium sulphate as the more concentrated antigen of Dreyer. Accordingly a series of dilutions of normal and luetic sera in 0·9 per cent. sodium chloride were prepared starting with an initial dilution of serum of 1/100 and ending with a dilution of 1/204,800 by successive dilution of 1–1. The following were the results:

Normal	123,344,421,000
Luetic	344,444,432,000

It is obvious that such an antigen is quite capable of distinguishing between a normal and a specific serum. One characteristic is to be noted here which recurs in many other experiments and which is noted in the curves obtained with cerebrospinal fluid by Guillain and his co-workers. The specific serum differs from the normal in exhibiting less protection in the more concentrated ranges than does the normal serum. There is, therefore, a widening of the zone of precipitation in both directions.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION.

From the foregoing experiments it was clear that artificial antigens could be prepared from gum benzoin and saponin of suitable sensitivity to react selectively with specific sera and the use of gum benzoin was not restricted to cerebrospinal fluid, as insisted upon by Guillain, Laroche and Lechelle. It seemed to us that the effect of the addition of an antigen of one hydrogen ion concentration to different concentrations of serum of another hydrogen ion concentration might have an important effect on the precipitation and reaction by reason of a progressive change in acidity. This was confirmed by the work of Kermack and his colleagues which appeared while this was being investigated. They found as did we that the reaction of the mixture played an important part in the precipitation of cholesterol, benzoin and gold sols by electrolytes.

The hydrogen ion concentrations of a number of antigens, as determined with the hydrogen electrode, are given in the following tables:

	*p*H
Cholesterin 5 c.c. of a 1 % alcoholic solution dispersed in 100 c.c. of water with a trace of KCl	4·5
Gum benzoin 0·9 c.c. of a 10 % alcoholic solution in 100 c.c. of water	3·9
Dreyer's antigen (concentrated)	4·4
Guillain's antigen	3·9

On the addition of sera to these antigens, the solution became more alkaline, with the result that there is a very marked change in the *p*H of the mixtures as dilution takes place. To eliminate this variable, important in dealing with amphoteric materials, small quantities of phosphate or phthalate, or for the more alkaline ranges, borate buffer mixtures, were used. These effectually maintained the *p*H of the dilution constant.

How sensitive the precipitation of benzoin is to changes in hydrogen ion concentration is shown by the following table. Dispersions of benzoin were made at *p*H 5·2, 7·1 and 8·5 and the precipitation effected by dilution of serum in normal saline from 1/100 to 1/204,800.

*p*H				
5·2	123	444	432	100
7·1	002	344	321	000
8·5	000	000	000	000

That the range in the region of the isoelectric point of the globulins is critical is shown by the precipitation conditions where these are effected at closer spacing.

*p*H				
4·5	444	444	432	110
5·0	112	333	321	000
5·5	001	333	210	000

The characteristic differences in the precipitation of normal and luetic sera are still observable when the hydrogen ion concentration is maintained constant by means of buffer mixtures and the alkalinity of these mixtures is carried far above the neutral point. This phenomenon at first led us to the view which, during the course of this work, was also suggested by Kermack

and his co-workers, viz., that the material in the serum causing the precipitation was a positively charged substance strongly adsorbed, even at great dilutions. In addition to this material there exists the albumin and globulin. On the alkaline side of their isoelectric points the antigen is further protected by adsorption of the anions of these proteins, the protective power decreasing, however, as the dilution increases until, at a certain point, the positive material present is sufficient to precipitate the negatively charged protein antigen.

On the acid side of the isoelectric point both the albumin and globulins would convert the negative suspension into a protected positive suspension in high concentration but would precipitate it in diluted solutions and render it more sensitive to precipitation by electrolytes in solutions still more dilute. An increase in the positive material would extend the range of precipitation towards the range of greater dilution.

The following considerations have shown that this cannot be the mechanism of the precipitation process.

In the first place, it will be noted that the difference in the concentration of the hypothetically positively charged material in the serum, *i.e.* as is imagined to occur in a normal or a luetic serum, would affect the zones of precipitation in a different manner according as we operated on the acid or basic side of the isoelectric points of the albumins or globulins.

This fact led to a critical test of the hypothesis by examining how far into the alkaline range the antigen would still be able to differentiate between normal and luetic sera. We succeeded in obtaining satisfactory precipitation zones in a region as alkaline as pH = 9·0. The following data were obtained at this pH:

Non-heated serum normal	000	$\overset{+}{0}14$	$33\overset{+}{1}$	$\overset{+}{0}00$
„ „ specific	000	$\overset{+}{0}13$	$\overset{+++}{3}11$	$\overset{+}{1}00$
Serum heated to 56° C. normal	000	134	$43\overset{+}{2}$	100
„ „ „ specific	000	$\overset{+}{0}44$	$\overset{++}{4}21$	$\overset{+}{0}00$

The difference between a normal and a luetic serum is quite marked at pH 8·5, as is shown by the following experiment. The serum had not been heated.

Normal	$00\overset{+}{0}$	$\overset{+}{0}14$	$44\overset{+}{3}$	$\overset{++}{3}10$
Specific	$0\overset{++}{0}0$	$\overset{+}{0}13$	$\overset{++}{3}34$	$\overset{+}{2}21$

As we proceed towards the isoelectric points of the albumin and globulin, and thus decrease the concentration of albuminate and globulinate ions, the diagnostic selectivity of the precipitation test increases, the antigen becoming more sensitive.

The Effect of Basic Substances on the Precipitation.

It would appear that the hypothetical positive precipitating material would be an extremely strong base since its isoelectric point would have to be on the alkaline side of pH 9·0 and even pH 9·5. A critical examination of the zones

in solution of this alkalinity failed to reveal any definite difference between the two classes of sera.

A search of the literature for bases of this character which, at the same time, would be strongly adsorbed, failed to elicit any with the requisite alkalinity and power of adsorption.

That the presence of strongly adsorbable basic materials would not, in fact, duplicate the observed precipitation zones was shown by experiments on the effect of such substances as choline, histone from calves' thymus, clupein sulphate, acetate and carbonate from herring sperm and spermins from human and bovine sperms (for these last compounds we are greatly indebted to Dr Otto Rosenheim).

In all cases no extension of the zone towards diluter concentrations was observed. What was noted, however, was an extension of the prezone in the region of greater concentration. This effect was clearly shown when small quantities of clupein sulphate were added to a globulin solution in normal saline. The washed euglobulin from horse serum was dispersed in normal saline of half the volume of the original serum from which it was precipitated.

1. To 9 c.c. of 0·85 per cent. sodium chloride solution was added 1 c.c. of the globulin solution.

2. To 8 c.c. of 0·9 per cent. NaCl solution was added 1 c.c. of globulin solution and 1 c.c. of a solution of clupein sulphate containing 0·001 grm. of the protamin salt. Precipitation was effected with a benzoin suspension of *p*H 5·0.

Globulin	000	$\overset{++}{0}34$	$\overset{+}{3}32$	$\overset{+}{1}00$
Globulin and clupein	430	144	$43\overset{+}{1}$	$\overset{+}{1}00$

Similar experiments in the presence of sera show that the electro-negative protected antigen is readily precipitated by strongly adsorbed positive ions such as are present in protamin salts. The precipitating power falls off rapidly with dilution and much more rapidly than the protective power of the albuminates and globulinates decreases with increasing dilution. This powerful effect of the protamin salts results in a prezone of precipitation but not in an extension of the zone usually found with sera or with globulins. Obviously, therefore, the mechanism of the precipitation of a benzoin antigen with a protamin is of a different class from the interaction of protein and antigen.

The Influence of Sodium Chloride.

Since it is clear that the zone of precipitation is not caused by a variation in the hydrogen ion concentration consequent on dilution nor is it due to basic materials present in the serum and since, at the same time, it appeared likely that the albumins and globulins, in strong concentration, at least, were apparently adsorbed by the antigen and altered it in sensitivity to precipitation, we came to the conclusion that the precipitating agent was the salt itself present in the system. The importance of salt concentration in the precipitation test has recently been emphasised by Brandt (1922) and Georgi and Lebenstein (1919).

This was confirmed by experiments showing the extension of the zone of precipitation of a globulin-benzoin system on increase of the salt content. One c.c. of a globulin solution prepared as in previous experiments was added to 9 c.c. of sodium chloride solutions of the following strengths: (*a*) 0·1 per cent., (*b*) 0·9 per cent., (*c*) 1·8 per cent., and (*d*) 3·6 per cent. Dilutions of these four solutions were made, using the corresponding salt concentration for the diluting fluid. These four series were then tested with a benzoin suspension containing 1 c.c. of 0·05 per cent. saponin per 100 c.c. and buffered to *p*H 8·5. The results were the following:

(*a*)	001	343	100	000
(*b*)	000	344	332	000
(*c*)	000	244	443	320
(*d*)	000	244	444	430

That albumin is adsorbed by gum benzoin can be demonstrated by the alteration of the surface tension produced by the addition of an emulsion of gum benzoin to a diluted solution of egg albumin and also by the marked change in the sensitivity of the suspension. By adding egg albumin, diluted 1 : 100, with a surface tension of 72·0 dynes per cm. to a benzoin suspension, having a surface tension of 64·3 dynes per cm., the surface tensions of the resulting mixtures were as follows:

Dilution	Surface tension
1 : 50	60·57
1 : 25	61·45
1 : 12·5	62·30

The Mechanism of the Precipitation Process.

The process of precipitation clearly involves a sensitisation of the antigen to sodium chloride. Two such processes have already been definitely established in simple colloidal systems. In one partial neutralisation of the charge on the electro-negative suspension is effected by the addition of protein on the acid side of its isoelectric point. If sufficient protein be added, precipitation will ensue in the absence of electrolytes. If an excess be added, repeptisation will take place. This is due to the formation of a protected colloid opposite in sign to that of the original suspension.

Since sensitisation in the class of precipitation at present under discussion takes place irrespective of the hydrogen ion concentration of the medium, the zone of precipitation is not caused in this way.

In the second type of sensitisation, we assume that the antigen suspension adsorbs the sensitising agent, and this complex suspension has different adsorbing powers for sodium and chlorine ions than the unsensitised suspension.

Thus, gum benzoin adsorbs sodium ions more strongly than chlorine ions. If the adsorption of these latter ions by the suspension is cut down on sensitisation, *i.e.* on adsorption of the protein, and at the same time the capacity of the colloid to adsorb sodium ions is little affected, the sensitised antigen will now be more sensitive to the precipitating effect of sodium chloride.

Excess of the sensitising agent will, of course, cut down the capacity for adsorbing both ions. Under these circumstances the antigen will be rendered less sensitive (Rideal, Weiser). This explanation fits in closely with the observed reaction between antigen and serum.

Both albumin and globulin in stronger concentrations undoubtedly protect the antigen. In higher dilutions they sensitise the antigen to precipitation with sodium chloride. The sensitisation is not affected so much by change in hydrogen ion concentration as is the protective power, we may conclude therefore that the protective power of albumin and globulin is determined to a greater extent by the concentration of albumin and globulin ions than is the sensitising power.

The Sensitising Material.

The three constituents in serum which must be considered as potentially important in the process of sensitisation are the globulins, albumins and the lipoids. There is no valid reason why an amphoteric substance should not effect sensitisation on either side of its isoelectric point. In support of this is evidence to show that not only the ions but also the neutral protein molecules (Zwitterionen) may exert these effects. This is shown in the following experiment, where the sensitising effect of a horse euglobulin on a benzoin suspension at varying hydrogen ion concentrations is quite clearly demonstrated.

*p*H				
5·2	444	444	444	444
5·9	$0\overset{+}{0}2$	344	444	$3\overset{++}{3}3$
7·0	$0\overset{+}{0}2$	444	$4\overset{+}{3}2$	$2\overset{+}{1}0$
8·0	$0\overset{+}{0}2$	444	321	000

Attention has already been drawn to the importance of the globulin and especially the euglobulin fraction by many workers in this field. We decided to investigate to what extent the variation in the sensitising power of luetic and normal sera could be associated with the globulin fractions and finally, whether the difference in the sensitising power of a luetic euglobulin from a normal serum euglobulin was quantitative or really qualitative in essence.

In the precipitation of the euglobulin fraction of a serum by means of carbon dioxide a variable amount of lipoid material is constantly brought down with the precipitate. So much so is this the case that it has been said that globulins are compounds of lipoid and protein.

According to Hartley, specific sera, when treated by Hardy and Gardiner's method, lose their Wassermann reacting power, and this quality cannot be restored by mixing the removed lipoid with the redispersed protein of the serum. He naturally ascribes considerable importance to these lipoids in the Wassermann test.

We decided to examine the euglobulin from normal and luetic sera from which the lipoids had been removed as completely as possible with respect to their behaviour in sensitising suspensions and to their action with the Wassermann test.

That a change in sensitising power of a CO_2 globulin does occur when treated according to this method is clear from the following experiment. The euglobulins from horse and sheep sera, precipitated by carbon dioxide and removed by centrifuging after washing with distilled water, were dissolved in sodium chloride of a volume equal to that of the serum from which they were precipitated. Portions of these globulin solutions were treated by Hardy and Gardiner's method. The desiccated globulins were redispersed in volumes of sodium chloride solution giving the same apparent concentration as in the original serum. The solutions were then tested with benzoin at *p*H 7·0. The following data were obtained:

Sheep globulin	Precipitated	024	$44\overset{+}{2}$	$\overset{+}{2}1\overset{+}{0}$	$\overset{+}{0}00$
	Delipoided	444	$\overset{++}{3}22$	$\overset{+}{1}11$	$\overset{+}{1}00$
Horse globulin	Precipitated	$03\overset{+}{3}$	$\overset{++}{3}31$	$\overset{++}{0}00$	000
	Delipoided	$44\overset{+}{3}$	$\overset{+}{3}01$	000	000

A syphilitic serum globulin made in the same way yielded the following results:

Luetic euglobulin	$\overset{++}{0}13$	$\overset{+}{3}44$	$32\overset{+}{0}$	$\overset{+}{0}00$
Luetic euglobulin delipoided	344	$43\overset{+}{2}$	$\overset{+}{0}00$	000

What is of considerable interest in view of Hartley's results with delipoided serum is that the delipoided luetic euglobulin is still capable of giving a positive Wassermann reaction.

The delipoided globulin was made up to approximately the same strength as the untreated euglobulin. The Wassermann test was as follows:

Untreated euglobulin	$0/+ +\underset{\cdot\cdot}{0}\underset{\cdot\cdot}{0}$
Delipoided globulin	$0/\pm$ 000

When the delipoided globulin was made up to practically double the strength of the original globulin we obtained a Wassermann reaction as follows:

Delipoided globulin (double strength) $\pm/+ +\underset{\cdot\cdot}{0}\underset{\cdot\cdot}{0}$

The anti-complementary control was carried out at all dilutions and inhibition without antigen only occurred with the highest concentration of globulin. It can therefore be stated definitely that a euglobulin from a luetic serum treated by Hardy and Gardiner's method still yields a positive Wassermann reaction. This experiment has been repeated a number of times, always with the same result.

It cannot be affirmed that the change in the sensitising properties of a euglobulin on removal of the lipoids is a change due entirely to the removal of these compounds from the protein. There is little doubt that proteins themselves, when treated with alcohol and ether, do suffer some change in lyophilic characteristics.

It seems probable, therefore, that the euglobulin fraction of a serum minus any lipoids contained therein is the seat of some material, a variation in which in normal and luetic sera is responsible both for the Wassermann and pre-

cipitation tests. Since, as has already been shown, alteration in globulin concentration shifts the zone of precipitation, it might be anticipated that an increase in the concentration of globulin would give a positive Wassermann reaction. To this idea is, of course, opposed the statements which have been made to the effect that there is no increase in the concentration of globulins in luetic sera above the normal.

In experiments with euglobulin, however, it can be shown that normal euglobulins deviate complement not only in concentrations with antigen where the absence of antigen produces no inhibition of haemolysis. The following experiment will show to what extent this occurs.

The euglobulins from 58 c.c. of horse serum diluted to 600 c.c. were precipitated by carbon dioxide. They were separated off by centrifuging and washed four times with distilled water, the water being removed by rapid spinning each time. The proteins were dissolved in 8 c.c. of saline, so that the concentration of euglobulin was about seven times what it was in the original serum. Seven c.c. of this euglobulin solution were delipoided by Hardy and Gardiner's method. The residue was washed on a hardened filter ten times with ice cold dry ether, and after drying in an evacuated desiccator over sulphuric acid was dissolved in 3·5 c.c. of saline, so that the euglobulin concentration was now apparently 14 times that of the original serum.

The two solutions were tested by the Wassermann reaction and gave the following results:

	Natural euglobulin × 7		Delipoided euglobulin × 14
Without antigen	+ 0 0 0	Without antigen	± 0 0 0
With antigen	+ + + +	With antigen	+ + + ±

These experiments indicate clearly that the euglobulin fraction of a non-specific—a horse—serum can yield a positive Wassermann reaction, even when deprived of the lipoids coming down with the precipitate.

It certainly cannot be concluded that the only difference between a luetic and a normal serum is one due to the relative concentration of euglobulin to other proteins, although this would effect a broadening of the precipitation zones. It is more probable that the difference is one of dispersity or of nature of the globulins rather than a marked increase in quantity.

We have compared two solutions of euglobulins of equal nitrogen content one of which was prepared from a syphilitic serum. The two solutions were so diluted that the nitrogen content in each case was 0·014 per cent. The precipitation ranges were as follows:

Normal	000	$00\overset{+}{2}$	$\overset{+}{3}44$	$\overset{+}{3}32$	$\overset{+}{2}11$	100
Luetic	000	$0\overset{+}{1}3$	$\overset{+++}{3}33$	$330\overset{+}{}$	000	000

The difference between the two solutions is that which we believe to be the main characteristic of a specific protein—the decrease in protective power in more concentrated solutions.

The Change of Sera in Specific Reaction.

Friedberger and Scimone (1922) among others have shown that the exposure of a luetic serum to ultra-violet light rendered it Wassermann negative. Among the many methods which have been suggested and which we have tried with a view to changing the sign of a specific serum this is undoubtedly the most efficient. No visible change takes place in the dispersion of the serum during the process. It appeared to us to afford an opportunity to compare the Wassermann reaction and the sensitising effect of a single serum when giving a Wassermann reaction and when deprived of this quality. At the same time a normal horse serum served as a control.

In the first experiment the sera were radiated undiluted. They were exposed in stoppered quartz vessels to the radiation of a mercury vapour lamp (200 v. 2·5 amps.) at a distance of 3–4 cm. There was but little rise of temperature. The flasks were gently shaken from time to time.

The Wassermann reaction receded from 0/+++0 to 0/++00. The effect on the sensitising action was as follows:

Horse serum	Non-radiated	000	012	344	444	443	$\overset{+}{1}$
	Radiated	000	$0\overset{+}{0}2$	444	444	442	2
Luetic serum	Non-radiated	000	$0\overset{++}{1}3$	$\overset{+}{3}44$	444	$\overset{+}{3}32$	$\overset{+}{1}$
	Radiated	000	013	444	444	$3\overset{+}{1}1$	1

One sees here a slight decrease in the protective power coincident with the action of ultra-violet light.

The euglobulins from 3 c.c. of a specific serum were dispersed in 6 c.c. of normal saline and exposed to ultra-violet light under similar conditions to the previous experiment. The Wassermann test of the globulin solution before radiation was 0/++00, after radiation 0/0000. The sensitising action of the euglobulin solution on benzoin at *p*H 7·0 was as follows:

Before radiation	144	443	$\overset{++}{2}0\overset{+}{0}$	$\overset{+}{0}00$
After radiation	444	443	$\overset{++}{1}00$	000

It is clear that the albumin and other constituents of the serum serve as a shield to the globulin when exposed to the ultra-violet radiation. A decrease in the Wassermann reaction is accompanied by a decrease in the sensitising action of the globulins.

In order to ascertain whether some other not very drastic method might serve to change the physical condition of a Wassermann + globulin we resorted to freezing.

A globulin solution from a specific serum was frozen in a mixture of salt and ice four times, complete thawing being carried out between each freezing. The globulin solution, which before treatment gave a Wassermann reaction of 0/+++ 0, receded to 0/++ – 0, at the same time another portion of the solution was radiated, this treatment changing the Wassermann reaction to

0/+ 000. The sensitising power of the three globulin solutions was then tested on benzoin.

Globulin	134	444	432	110	000
Globulin frozen	034	444	331	100	000
Globulin radiated	034	443	321	000	000

It will be observed that the decrease in Wassermann reactivity is accompanied by a decrease in sensitising action at both ends of the precipitating zone. The effect of a given treatment of a serum is much more evident in the Wassermann test than it is in precipitation tests.

The cerebro-spinal fluid of a patient whose blood gave a Wassermann test of 0/+ + + + was radiated and tested with benzoin with the following result:

Spinal fluid	333	333	321	000
Spinal fluid radiated	333	333	100	000

In this case, also, the parallelism between the two tests is maintained. The experiment also confirms the view that luetic sera may differ from normal sera in the composition, or more likely the dispersity of the globulins, rather than alteration in quantity or relation to other proteins of the serum.

That other slight changes, such as the heating of a serum to 56° C. during the process of inactivation, may alter the sensitising power is exemplified in the following table. The sensitising action was tested in the acid range near the isoelectric point of the proteins where we have already seen sensitising is very sharp. A normal serum was used for the tests.

Serum	*p*H				
Unheated	5·0	001	344	320	000
Heated	5·0	011	344	421	100
Unheated	5·5	001	123	211	000
Heated	5·5	000	144	421	000

Heating to 56° C. renders a serum somewhat more powerful as a protecting and sensitising agent when maintained on the acid side of the isoelectric point of the globulin but rather less effective on the alkaline side.

As we have already noted, the region between *p*H 5·0 and 6·0 appears to be the most suitable for differentiation between luetic and normal sera by the benzoin and similar methods. In order to show this, dispersion of the antigen should be made in a buffer solution so that the change in reaction due to the introduction of the alakaline serum may be compensated. It is within the regions of *p*H 5·0–6·0 that the isoelectric points of most of the euglobulins lie. This is a matter of possible considerable significance in all reactions of the character under discussion.

With a view to converting a normal serum with one giving a Wassermann reaction we carried out a very large number of experiments. These were all made with the definite purpose of avoiding, if possible, any change in globulin concentration. We have attempted to confirm statements appearing in the literature concerning the action of tannin, hydrolysis by dilute acids, contact

with *Bacillus prodigiosus*, with inulin, etc. It is possible on occasion to produce a serum which inhibits haemolysis but as a rule these sera are anticomplementary. The most satisfactory experiments were with inulin. On the whole, however, the experiments can only be looked upon as unsatisfactory. We have not been able to control the conditions determining the transformation of a normal serum into one giving a proper positive Wassermann reaction.

Finally a series of experiments was carried out employing adsorbent gels of definite electric sign, but used in varying concentrations and also in varying states of dispersion. Attempts were made at fractional adsorption. The alumina gels and silica gels were purified by long dialysis against distilled water.

In one set of experiments 1 part of serum diluted 1–50 was mixed with 1 part of the colloidal gel and 1 part of saline and allowed to stand at room temperature for 47 hours. The mixture was then centrifuged, the supernatant fluid carefully removed and this was again centrifuged in order to remove any of the colloidal reagent. Tests of the sensitising power were made with a benzoin suspension at *p*H 7·5.

The following results are typical for this class of experiment:

Serum	Adsorbent				
Normal	$Al(OH)_3$	$4\overset{++}{1}1$	000	000	
Luetic	$Al(OH)_3$	421	200	000	
Normal	SiO_2	000	000	000	
Luetic	SiO_2	$1\overset{+}{0}0$	000	000	

It would appear that the specific serum is less affected than normal by treatment with alumina. The sensitising components of a normal serum are completely removed by treatment with silica gel, but a similar treatment of a syphilitic serum did not remove all sensitising power. The use of freshly precipitated tungstic acid was effectual in removing all trace of sensitising substance from serum.

Gradation in the adsorptive power of a gel will produce a certain amount of selective action. An alumina gel evaporated to dryness and heated at 160° C. for 1 hour in contact with diluted sera as in the previous experiments gave the following results:

Serum	Adsorbent	*p*H				
Normal	Dry $Al(OH)_3$	7·5	$34\overset{+}{3}$	$2\overset{+}{1}0$	000	000
Luetic	,,	7·5	$\overset{+}{2}44$	311	$1\overset{++}{0}0$	$\overset{+}{0}00$
Normal	,,	8·5	$\overset{++}{3}20$	$\overset{++}{0}00$	000	000
Luetic	,,	8·5	$21\overset{+}{0}$	$\overset{++}{0}10$	000	000

From these experiments it would seem that if there exists in the globulin fraction a specific material which is not globulin-like in character, it does not differ from the globulin in its adsorption on either acidic or basic colloidal gels. This fact again lends support to the hypothesis that the globulin fractions of normal and luetic sera differ only in their dispersity or structure and that

the sera do not differ because of a varying content of total globulins, or of a specific non-globulin material.

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Summary.

1. "Antigens" of varying sensitivity may be prepared by coating a dispersion of gum benzoin with saponin.

2. Using suitable dispersions so prepared a zone of precipitation is observed which is more extended for specific than for normal sera. Differentiation is more pronounced as the region of the isoelectric point of the globulin is approached.

3. Precipitation is effected by the ions of salts present in the solution discharging the negative antigen suspension which has been sensitised by the protein of the serum.

4. The mechanism of sensitisation is described.

5. Both albumins and globulins can protect or sensitise gum benzoin suspensions, the effect depending on an appropriate dilution. This effect occurs qualitatively irrespective of the hydrogen ion concentration of the medium. Quantitatively the position of the zone of precipitation and its extent is contingent on the reaction of the medium.

6. The euglobulin fractions of normal and of luetic sera deprived as far as possible of their lipoids can be differentiated both by the Wassermann test, and by precipitation tests.

7. Delipoided euglobulins from horse serum will yield a Wassermann positive reaction when used in certain concentrations. In more concentrated solutions an anticomplementary effect is exhibited.

8. Evidence is presented for the view that the euglobulins of specific sera differ rather in their composition or state of aggregation than in quantity from the euglobulin of non-specific sera.

9. The changes in sensitising power and in the Wassermann reactivity of euglobulin caused by repeated freezing or exposure to ultra-violet light run a parallel course.

10. Attempts to change the Wassermann properties of normal sera are briefly described.

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