# A STUDY OF NON-SPECIFIC COMPLEMENT-FIXATION WITH PARTICULAR REFERENCE TO THE INTERACTION OF NORMAL SERUM AND CERTAIN NON-ANTIGENIC SUBSTANCES.

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(With 10 Charts and 4 Graphs.)

CONTENTS.



## Introduction.

It is now well recognised that *in vitro* effects regarded as characteristic of the reaction between an antigen and its specific antibody, *e.g.* complement-fixation, may also result from the interaction of animal serum and substances which are not antigenic in the true immunological sense. One of the most striking illustrations of this phenomenon is the Wassermann complement-fixation reaction in which the so-called "antigen" consists of lecithin or closely allied lipoids. It has of course been shown that though such lipoid principles are incapable of acting as antigens *in vivo*, they may, when injected along with foreign proteins, stimulate the formation of specific antibodies demonstrable in the serum of the immunised animal by complement-fixation and flocculation reactions along with a suspension of the particular lipoid (see Landsteiner and Simms, 1923). Such reactions might therefore appear to be analogous to the syphilis serum phenomena. Thus, the existence of lipoidophile antibodies has been postulated and an attempt has been made to bring the syphilis serum reactions into line with true immunity phenomena (see Mackie and

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Watson, 1926). It has also been shown that similar *in vitro* effects may be elicited with specific carbohydrate substances derived from bacteria, interacting with the homologous anti-bacterial serum, though presumably these substances, unless in combination with proteins, are not antigenic *in vivo* (Heidelberger and Avery, 1923, and others).

An important feature of the Wassermann reaction is the augmentation produced by cholesterol and by the alcohol of the "antigen" solution. The influence of these agents as auxiliaries to the lipoid "antigen" is quite obscure. Attention has been drawn by Mackie and Watson (1926) to the fact that unheated serum, and particularly the carbonic-acid-insoluble fraction, may, along with diluted ethyl alcohol, substituted for antigen in the complement-fixation test, produce a definite reaction, and that the responsible serum principle varies in its thermolability among different animal species. Thus, no such effect occurs with the normal whole-serum or carbonic-acid-insoluble fraction of certain species (man, guinea-pig, white rat) when heated for half-an-hour at 55° C. On the other hand, the heated carbonic-acid-insoluble fraction and sometimes whole-serum of the rabbit, ox, sheep and horse still retain their activity.

In a study of the occurrence of the natural Wassermann reaction in animals, Mackie and Watson found that this peculiar complement-fixation reaction by heated serum *plus* diluted alcohol showed a parallelism to the complement-fixation effect with the Wassermann "antigen." Some evidence was also elicited that heated syphilitic sera exhibiting a pronounced Wassermann reaction may yield a weak but recognisable fixation effect with alcohol. No further light, however, was thrown on the nature of this phenomenon.

It was shown by Takenomata (1924) that certain substances, varying greatly in chemical constitution, possess the power of interacting with normal serum and producing a complement-fixation effect. Such substances, which he described as "pseudo-antigens," included peptone solutions, bacterial emulsions, cobra-venom, certain bacterial toxins, and inulin suspensions. Normal rabbit serum was used for demonstrating this reaction, which was certainly quite non-specific in nature. Serum heated at 55° C. was active in this respect, but the reacting principle was either completely inactivated or greatly reduced in its effect at 62° C. Takenomata found that when a bacillary emulsion was tested with normal serum, the non-specific complement-fixation which occurred at 37° C. was absent at 0° C. He thus elicited an apparent difference between specific complement-fixation reactions and the analogous non-specific effects, viz. the absence of the latter at 0° C.

Whatever interpretation may be placed on these reactions, it is apparent that non-specific complement-fixation may result from the interaction of animal serum and substances of varying chemical and physical properties, *e.g.* alcohol and the "pseudo-antigens" described by Takenomata, and that these reactions depend on some serum principle which probably varies in thermolability in different species. The question also arises whether the

natural Wassermann reaction of certain animals depends on the thermostability of a similar or analogous normal principle and is parallel to non-specific complement-fixation with other substances.

It was assumed by Mackie and Watson, on the available evidence, that the natural Wassermann effect of animal sera was the homologue of the syphilis reaction and that the active principle of the serum was at the same time analogous to a natural antibody. The further question arises as to the relation of the human Wassermann reaction to these non-specific complement-fixation effects with various agents other than lipoids.

The nature of the reacting substance of the serum in syphilis still remains a matter of uncertainty. It was suggested by Mackie and Watson that the reaction depends on a lipoidophile principle, analogous to a natural antibody, which is present normally in minimal amount and in a masked state even in the heated serum, and is augmented in syphilitic disease, probably in a non-specific manner. It seems possible also that the reaction may depend not only on the augmentation of this principle but also on its increased thermostability. Normal human serum possesses the particular property but is inactivated at 55° C., whereas in syphilis this property of the serum is greatly augmented and relatively thermostable. From analyses of the varying Wassermann effects of different normal animals it would appear that these depend on the varying thermostability of the particular serum principle.

These considerations have therefore led to a systematic analytical study of the phenomenon of non-specific complement-fixation by serum *plus* various agents which for convenience may be called "pseudo-antigens." This has been undertaken both in view of the interest of the phenomenon from the general biological standpoint and also with the object of throwing light on the nature of the natural Wassermann reaction of animals and the syphilis reaction in the human subject.

## Technique.

The method used for carrying out the complement-fixation reaction closely followed the technique described by Mackie and Watson (1926).

In careful comparative tests with the carbonic-acid-insoluble fraction of guinea-pig serum and alcohol (1 : 12 in saline solution) substituted for antigen in the complement-fixation test (*v. supra*), the maximum results were obtained by allowing these reagents to interact for one hour at room temperature before adding complement, and thereafter carrying out the test in the usual way. The addition of complement immediately after mixing the serum and "pseudo-antigen" as in the performance of routine Wassermann tests did not yield such marked effects. The former system was therefore adopted as a routine measure in the course of the investigation.

The Wassermann "antigen" used was the same as that described by Mackie and Watson: the test amount was 0·5 c.c. of a 1 : 12 suspension. The amount of serum used was 0·05 c.c. Preliminary experiments were usually carried out to ascertain the concentration of the "pseudo-antigenic" substance which along with the test amount of serum constituted the optimum for the fixation reaction, *i.e.* giving the maximum effect without being markedly anti-complementary by itself. In some cases varying concentrations of serum and "pseudo-antigen" were tested with a view to determining whether the reaction depended on an

optimal proportion of the reagents as has been described in complement-fixation by certain true antigens and their specific anti-sera (see Dean and Webb, 1926; Goldsworthy, 1928); but no evidence of this was elicited (*v.* p. 184). Guinea-pig serum 18–24 hours after withdrawal of the animal's blood was employed as complement, and the haemolytic system was a 3 per cent. suspension of ox red cells sensitised with a rabbit *v.* ox haemolytic anti-serum, 0·5 c.c. being the quantity used. The reactions were all carried out on a quantitative basis by testing varying amounts of complement (computed in M.H.D.) with fixed amounts of antigen (or its substitute) and serum.

## PRELIMINARY EXPERIMENTS WITH PEPTONE SOLUTION SUBSTITUTED FOR ANTIGEN IN THE COMPLEMENT-FIXATION TEST WITH NORMAL SERUM.

With a view to ascertaining whether the non-specific fixation reaction with peptone recorded by Takenomata showed any parallelism to the natural Wassermann reaction and the analogous effect with alcohol-saline (referred to in the introduction), experiments were carried out with the normal serum of man, rabbit and white rat.

The alcohol was diluted 1 : 10 in normal saline and the test quantity was 0·5 c.c. A 1 per cent. solution of Witte's peptone made up in normal saline was used, the quantity tested being 0·5 c.c. This quantity (and concentration) of the reagent was not specially anti-complementary by itself as will be seen from the various charts.

It has been shown by Mackie and Watson that the rabbit is usually "Wassermann-positive," whereas the white rat is generally "negative," *i.e.* when heated serum is used in the test. For the purposes of analysis of the serum function the protein fractions separated by Liefmann's carbon-dioxide method (1909) were tested in parallel with whole serum, the quantities being those derived from 0·05 c.c. of whole serum. The unheated serum and fractions were also compared with serum and fractions heated at 55° C. for half-an-hour.

*Rabbit Serum.* With the Wassermann "antigen" and alcohol-saline, results similar to those recorded by Mackie and Watson were obtained. In the case of peptone solution, marked fixation effects were elicited with the unheated and heated carbonic-acid-insoluble fraction. It will be noted, however, that heating at 55° C. definitely weakened the effect with peptone, as with the other reagents. As in the reactions with Wassermann "antigen" and alcohol-saline, the carbonic-acid-soluble fraction was more active than whole serum. With peptone the fresh and heated carbonic-acid-soluble fractions were active, though practically inactive with the Wassermann "antigen" and alcohol-saline. Chart 1 illustrates these results.

*Serum of White Rat.* With the Wassermann "antigen" and alcohol-saline, similar effects to those noted by Mackie and Watson were obtained and the results with peptone were found to present a parallelism. Thus, the unheated serum and carbonic-acid-insoluble fraction produced marked reactions, but at 55° C. the property of reacting in this manner was practically absent. The carbonic-acid-soluble fraction was inactive both in the fresh and heated state.

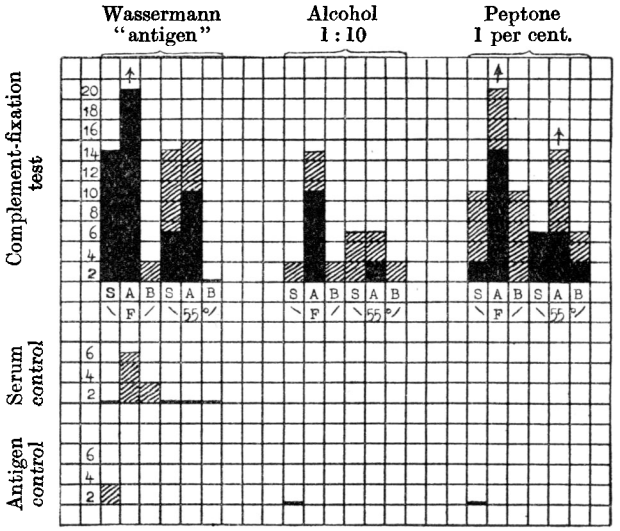


Chart 1. Rabbit serum.

In this and subsequent Charts:

S = whole serum.
A = $CO_2$-insoluble fraction.
B = $CO_2$-soluble fraction.
F = fresh and unheated.
55° C. = heated at this temperature for half-an-hour.
↑ = no end-point reached.
□ = complete lysis.
▨ = partial lysis.
■ = no lysis.

Column of numbers refers to doses of complement tested.

The results are illustrated in Chart 2. In this species as contrasted with the rabbit the active principle or principles of serum concerned in these reactions are labile at 55° C.

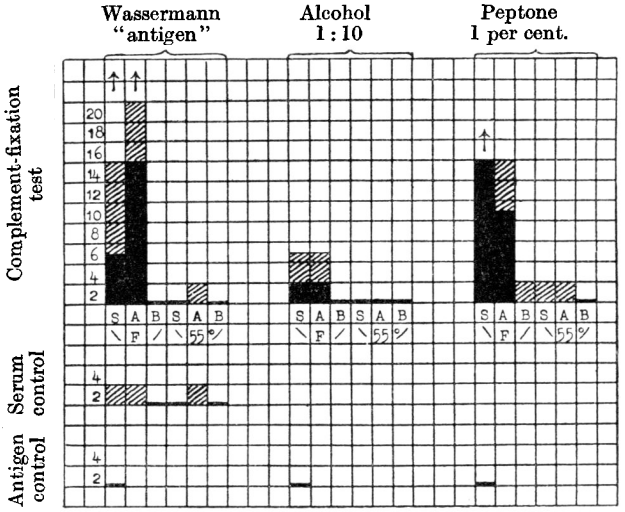


Chart 2. Serum of White Rat.

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*Normal Human Sera.* The results obtained with normal human sera were similar to those with the serum of the white rat. The reactions of the whole-serum with Wassermann "antigen," alcohol-saline and peptone were all annulled at 55° C. In the case of alcohol and peptone the same applied to the carbonic-acid-insoluble fraction, though this fraction was not completely inactivated in the Wassermann reaction (see Chart 3).

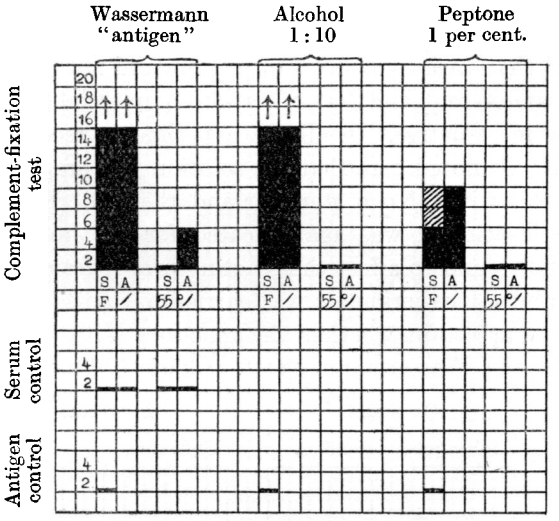


Chart 3. Normal human serum.

COMPARISON OF SERA OF DIFFERENT SPECIES.

It will be shown later how, among various agents tested in the same way as peptone solution and alcohol-saline, a cholesterol suspension acted in a similar fashion. This was prepared by adding one part of a two-thirds-saturated alcoholic solution of cholesterol to ten parts of saline and suspending by slow admixture. The test amount was 0·5 c.c. This strength of suspension was found to yield the most marked reactions without the occurrence of excessive anti-complementary effects in the control tests. Marked anti-complementary effects by the cholesterol suspension *per se* were sometimes encountered, depending probably on the particular specimen of complement used. The reaction with cholesterol was much greater than that produced by the contained alcohol. The filtered suspension was not more active than alcohol-saline (1 : 10). Apparently the activity of the cholesterol suspension was a function of the suspended particles and was not due to any trace of colloidal cholesterol.

It was also found that cholesterolised-peptone constituted one of the most active "pseudo-antigenic" agents tested. Striking quantitative effects were obtained with this agent. It will be seen in the various charts how along with this preparation heated serum may fix at least 12–18 doses of complement while neither has any degree of anti-complementary effect *per se*. The choles-

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terolised-peptone was prepared by adding one part of a two-thirds-saturated solution of cholesterol in alcohol to ten parts of 1 per cent. peptone in normal saline. The test amount was 0·5 c.c. As in the case of cholesterol, marked anti-complementary effects were occasionally elicited with this agent *per se* and certain specimens of guinea-pig complement.

The following agents were therefore selected for all systematic observations of the phenomenon under investigation: alcohol-saline, peptone solution, cholesterol suspension and cholesterolised-peptone.

It was found that various samples of peptone did not possess the reacting property to the same extent, and that, of certain commercial peptones examined, Witte's was the most active. In all the experiments recorded this peptone was used. It may be noted here that the solution was prepared by dissolving the peptone by heat in a Koch steriliser and then filtering, after which for purposes of sterilisation it was again heated for one hour at 100° C. This treatment was not found to affect the reacting properties of the substance.

A large number of experiments was carried out with the sera of various animal species to ascertain whether the reactions with the selected agents were parallel to the Wassermann effect. Chart 4 illustrates the contrast

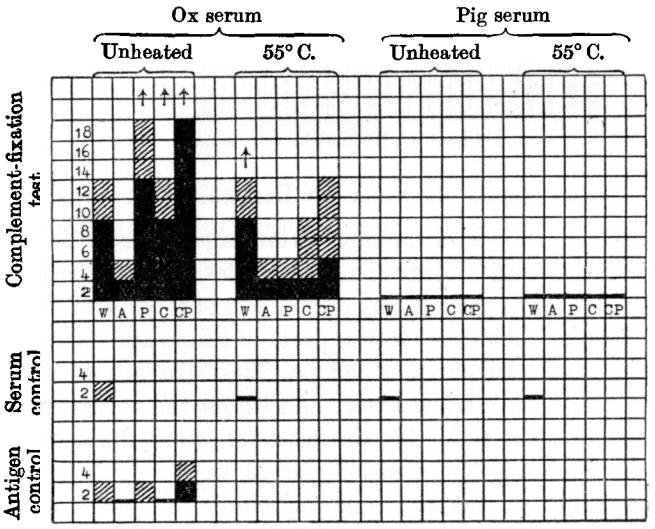


Chart 4.

W = Wassermann "antigen." C = Cholesterol suspension.
A = Alcohol-saline. CP = Cholesterolised-peptone.
P = Peptone solution.

between ox serum (Wassermann-positive) and pig serum (Wassermann-negative). The latter, both in the unheated state and also when heated at 55° C., is negative with the Wassermann "antigen," alcohol-saline, peptone, cholesterol and cholesterolised-peptone. The unheated ox serum shows strong reactions with Wassermann "antigen," peptone, cholesterol and cholesterolised-peptone, but, though the reactions with peptone and cholesterol are

considerably weakened at 55° C., the active principle in the serum is still relatively stable.

The heated sera of ox, sheep, horse, and rabbit have generally given definite effects with peptone and cholesterol and particularly with cholesterolised-peptone, whereas pig, guinea-pig, white rat, and selected normal human sera have been negative (see Charts 5 and 6). Unheated guinea-pig serum,

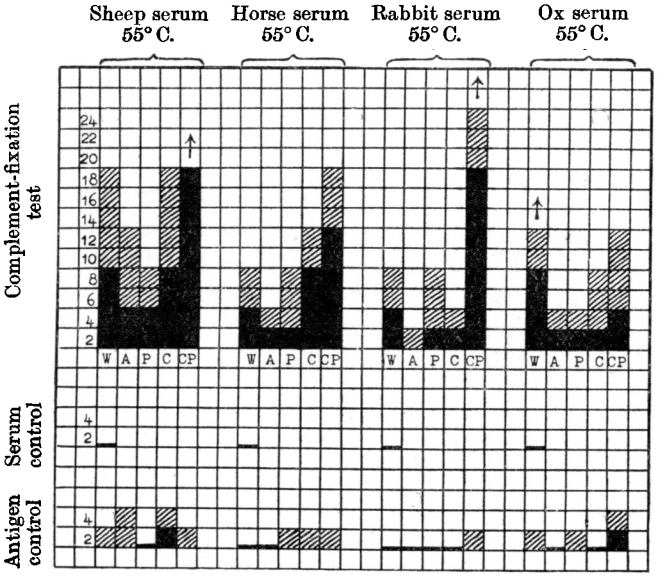


Chart 5.

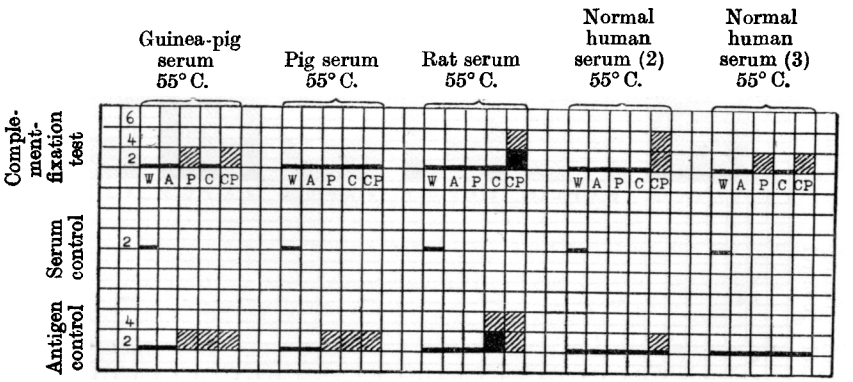


Chart 6.

under the conditions in which it has been used as complement in these tests, does not as a rule show the complement-fixing property elicited with the fresh serum of other animals. Dunlop (see Browning, 1927) has described a non-specific complement-fixation effect produced by guinea-pig serum interacting in certain concentrations with bacterial suspensions, and thus characterised by a "zone phenomenon" when varying amounts of the serum

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(containing both the fixing agent and complement) are tested. We have found that occasional specimens of guinea-pig serum, especially from recently withdrawn blood, have given zone phenomena with various "pseudo-antigens," independently of any other serum tested for fixing properties. This effect has been exceptional but has been most obvious with cholesterol and cholesterolised peptone. It is apparently analogous to the phenomenon described by Dunlop.

*Thus among animal species the reaction with peptone has only occurred* with sera that were Wassermann-positive and has been negative with Wassermann-negative sera. The reactions with Wassermann "antigen," alcohol, peptone, cholesterol, and cholesterolised-peptone have been parallel in this respect.

It is to be noted that the cholesterolised-peptone reaction was frequently much greater than the mere summation of the separate effects of peptone and cholesterol. This is well illustrated in Chart 5 (rabbit serum). When the cholesterolised-peptone was filtered to remove the suspended cholesterol, the filtrate did not yield a reaction quantitatively greater than that produced by a peptone solution containing alcohol in the same proportion as the cholesterolised-peptone.

Though the reacting properties of ox and sheep sera were relatively stable at 55° C., greater thermolability was noted generally with these sera than with

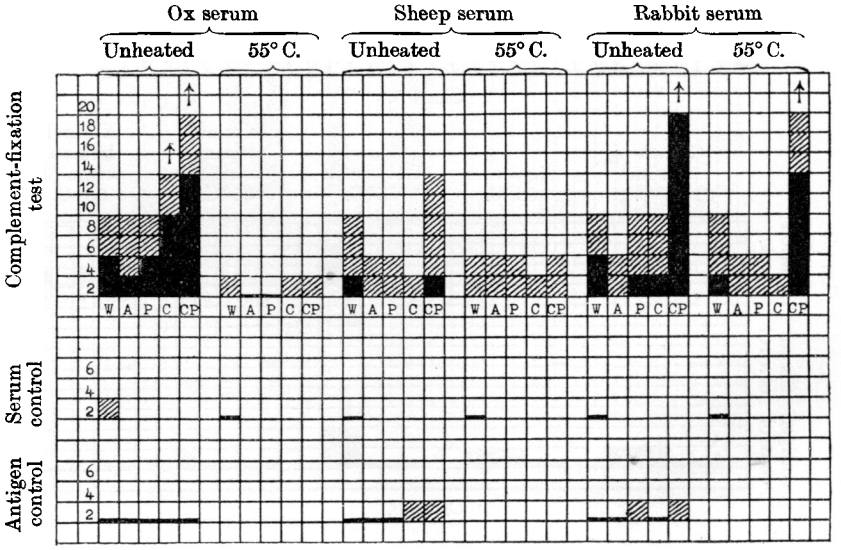


Chart 7.

rabbit serum, and in a few instances there was almost complete inactivation at 55° C. as in the case of rat serum. It was noteworthy also that in individual animals in which the Wassermann effect was weak, the alcohol, peptone, cholesterol and cholesterolised-peptone effects were correspondingly weak (see Chart 7).

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The results show the close correspondence between these various non-specific reactions and the Wassermann effect.

## THE INFLUENCE OF TEMPERATURE.

Takenomata (1924) has stressed the differentiation of such non-specific reactions from specific complement fixation by the absence of the former when the "pseudo-antigen," serum and complement are allowed to interact at 0° C., the specific reactions developing even at such low temperature.

We have found, however, that non-specific reactions definitely occurred at 0° C. (3 hours), though less pronounced at this temperature than after incubation at 37° C. (1½ hours)—see Chart 8.

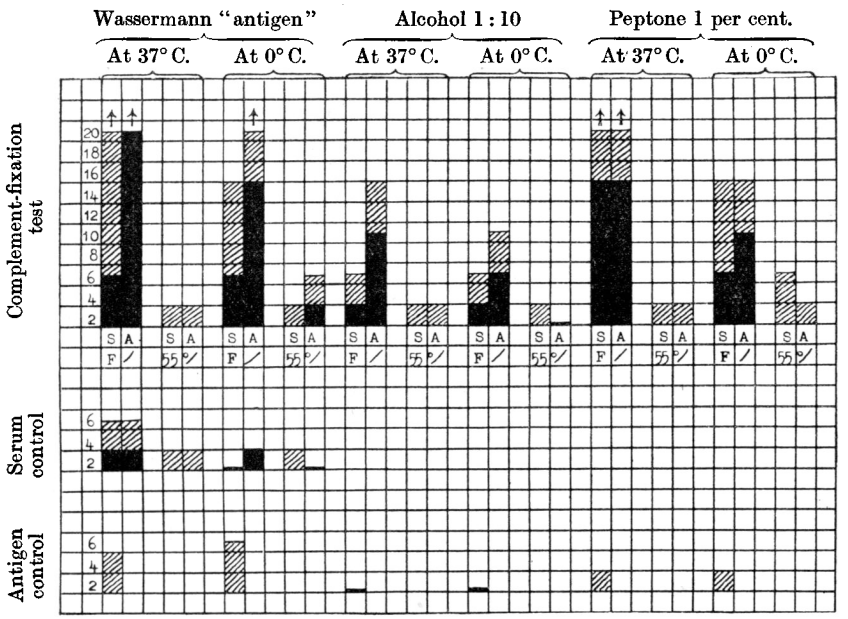


Chart 8. Rat serum.

In comparative tests of a quantitatively marked specific reaction and a reaction of less pronounced degree, as in the case of the non-specific effects investigated, any influence that weakens the effect, for example, lowering the temperature to 0° C., may elicit a difference which though merely quantitative appears to be qualitative. This may explain the difference emphasised by Takenomata.

## THE ABSENCE OF REACTING PROPERTIES FROM THE SERUM OF YOUNG ANIMALS.

It was shown by Mackie and Watson (1926) that the Wassermann reaction usually obtained with rabbit serum was invariably absent in young animals of 3 to 4 weeks. The reacting property had developed by the 8th to 10th week

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after birth and at this time the appearance of the natural haemolytic antibody for sheep's blood (also previously absent) was observed in the serum. The active principle or principles capable of reacting with the "pseudo-antigens" were similarly absent at an early stage of life and developed coincidently with the Wassermann "substance" and the natural haemolytic antibody (Table I).

Table I. *Rabbits—Litter A. Doses of complement fixed.*

| Rabbit | Wassermann "antigen" | Alcohol-saline | Peptone | Cholesterol | Cholesterolised-peptone | Natural haemolytic antibody *v.* sheep red cells |
|---|---|---|---|---|---|---|
| Age 22 days | | | | | | |
| I | 0 | 0 | 0 | 0 | 0 | No lysis of 0·25 c.c. 3 % suspension of red cells by 0·5 c.c. serum |
| II | 0 | 0 | 0 | 0 | 0 | |
| III | 0 | 0 | 0 | 0 | 0 | |
| IV | 0 | 0 | 0 | 0 | 0 | |
| Mother | 4 | 2 | 4 | 6 | 8 | Complete lysis by 0·1 c.c. serum |
| 37 days | | | | | | |
| I | 2 | 2 | 8 | 8 | >18 | Complete lysis by 0·5 c.c. serum |
| II | 8 | 4 | 8 | 8 | >18 | ,, ,, 0·1 ,, |
| III | 4 | 4 | 8 | 4 | >18 | ,, ,, 0·5 ,, |
| IV | 0 | 0 | 8 | 2 | 12 | Partial lysis by 0·5 c.c. serum |
| 65 days | | | | | | |
| I | >12 | 2 | >12 | 10 | >18 | Complete lysis by 0·5 c.c. serum |
| II | 12 | 6 | >12 | 10 | >18 | ,, ,, 0·025 ,, |
| III | >12 | 2 | >10 | 6 | >18 | ,, ,, 0·3 ,, |
| IV | 2 | 0 | 10 | 2 | 10 | ,, ,, 0·3 ,, |

The figures stated are the number of doses fixed by serum *plus* "antigen" or "pseudo-antigen," less the sum of the doses of complement fixed by each separately in the control tests.

Unheated sera of young animals 16 days after birth (Table II) were also tested and were found practically inactive like the heated sera. This clearly

Table II. *Rabbits—Litter B. Doses of complement fixed.*

| Rabbit | Serum | Wassermann "antigen" | Alcohol-saline | Peptone | Cholesterol | Cholesterolised-peptone |
|---|---|---|---|---|---|---|
| Age 16 days | | | | | | |
| I | Unheated | 0 | 0 | 2 | 0 | 2 |
| | 55° C. | 0 | 0 | 0 | 0 | 0 |
| II | Unheated | 0 | 0 | 0 | 0 | 2 |
| | 55° C. | 0 | 0 | 0 | 0 | 0 |
| III | Unheated | 0 | 0 | 0 | 2 | 2 |
| | 55° C. | 0 | 0 | 0 | 0 | 0 |
| IV | Unheated | 0 | 0 | 2 | 0 | 0 |
| | 55° C. | 0 | 0 | 0 | 0 | 0 |
| V | Unheated | 0 | 0 | 0 | 0 | 0 |
| | 55° C. | 0 | 0 | 0 | 0 | 0 |
| 58 days | | | | | | |
| I | Unheated | >16 | 0 | >14 | 0 | >14 |
| | 55° C. | >10 | 0 | >14 | >8 | >14 |
| II | Unheated | 2 | 0 | 4 | 0 | 14 |
| | 55° C. | 0 | 2 | 4 | 0 | 14 |
| III | Unheated | >16 | 4 | 8 | 0 | >14 |
| | 55° C. | 6 | 2 | 8 | 8 | >14 |
| 102 days | | | | | | |
| I | 55° C. | 12 | 2 | 18 | 4 | >14 |
| II | 55° C. | 12 | 6 | >18 | >8 | >18 |

The figures stated are the number of doses fixed by serum *plus* "antigen" or "pseudo-antigen," less the sum of the doses of complement fixed by each separately in the control tests.

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shows that the negative results with heated sera were due to absence of the active principle and not to any thermolability effect.

It will be noted that in the case of Rabbit IV of Litter A the reaction with peptone and cholesterolised-peptone appeared before the reaction with the Wassermann "antigen" and alcohol-saline, and at this stage the cholesterol effect was exceedingly weak. Even on the 65th day the Wassermann and cholesterol reactions were slight and no reaction occurred with alcohol. It is of interest that the heated serum of Rabbits I and III of Litter B (58 days) gave a reaction with cholesterol though inactive in the unheated state. This has sometimes been observed with the Wassermann "antigen" and was first described by Browning and Mackenzie (1910).

### ABSORPTION EXPERIMENTS.

Attempts were made to absorb the non-specific reacting substances from rabbit, ox and sheep sera by means of a 1 : 10 cholesterol suspension. The technique adopted was as follows: 0·5 c.c. of serum heated to 55° C. was mixed with 4·5 c.c. of a 1 : 10 cholesterol suspension (as used in the complement-fixation experiments), left in contact with the cholesterol at 37° C. for two hours and then separated by centrifuging. The supernatant fluid now represented a 1 in 10 dilution of the original serum and 0·5 c.c. was used for a fixation test along with the cholesterol suspension. Untreated serum was also tested in the same way. No quantitative change in the reaction of the various treated sera was noted.

It has already been shown how in rabbits the development of natural haemolysin runs parallel with the development of non-specific complement-fixing properties. It has been found that this natural antibody is contained in the carbonic-acid-insoluble fraction of the serum, corresponding in this respect with the complement-fixing principle. Absorption of the natural haemolytic antibody from rabbit serum was carried out by treatment with the homologous red corpuscles for 1 hour at 0° C. and the serum was then tested with the various "pseudo-antigens." It was found that although the haemolysin was removed from the serum, the reactions with Wassermann "antigen," peptone and cholesterolised-peptone were quite unchanged quantitatively. The same result was obtained when both fresh and heated serum were used.

These experiments would appear to show that the serum principles which cause the non-specific reactions although presenting an analogy to a natural haemolytic antibody are quite independent of it.

### FURTHER EXPERIMENTS WITH PEPTONE SOLUTION.

In regard to the action of commercial peptone as a non-specific complement-fixing agent, the possibility of lipoid-like substances being present was considered. A purified peptone was prepared by taking a 10 per cent. solution of peptone in water and pouring it into five volumes of ice-cold alcohol. This

was allowed to stand at 0° C. for 24 hours when the precipitate was filtered and extracted with several times its bulk of ether. The resultant product was dried at room temperature and dissolved in saline. This purified peptone was tested in parallel with the untreated peptone and the substances extracted by alcohol and ether, heated rabbit serum being used for the reaction. The alcohol- and ether-soluble substances were found to be inactive, while the purified peptone was found to retain the full activity of the original sample.

Comparative tests were carried out with heated ox serum and peptone solutions of varying pH values from 5·5 to 8·0: no quantitative differences were noted.

The question also arose whether the complement-fixation was due to the formation of a precipitate; no obvious precipitation effect was noted, however, in experiments in which varying dilutions of serum were tested with varying concentrations of peptone.

In detailed tests involving variation in the concentration of both serum and peptone solution no evidence was elicited that the reaction depended on optimal proportions. The maximum results were obtained with the maximum amounts of the reagents which were practicable for testing purposes, *i.e.* were not excessively anti-complementary *per se*.

### Substances Capable of Producing Complement-fixation with Normal Serum.

A large number of varied agents have been tested mainly with a view to ascertaining whether such "pseudo-antigen" action pertained to any particular chemical groups. The selection of the substances tested was based generally on a consideration of the chemical constitution (or composition) of the active agents referred to above: ethyl alcohol, commercial peptone, cholesterol, and the cholesterolised alcoholic tissue extract used in the Wassermann reaction. Thus various alcohols, proteins, amino-acids and fatty acids were tested and also a non-cholesterolised alcoholic extract of sheep's heart muscle, ox heart and ox liver lecithins, sodium oleate, sodium nucleate, glycero-phosphoric acid, and guanidine hydrochloride. Other substances examined were cobra-venom, agar and gelatin solutions, inulin, starch, a suspension of kaolin, colloidal benzoin and a suspension of paraffin.

Cobra-venom and inulin have been referred to as "pseudo-antigens" by Takenomata (1924). Colloidal benzoin has been extensively employed as a reagent in a precipitation reaction with cerebro-spinal fluid and serum in syphilis. Silber and Friese (1925) have stated that an alcoholic solution of paraffin added to saline solution forms a stable colloid and can be substituted for the Wassermann "antigen." Their tests were not apparently controlled with alcohol-saline mixtures. They also stated that there was a parallelism between the Wassermann reaction of rabbit serum and a complement-fixation effect obtained when a gelatin solution was substituted for the lipoid antigen.

Sodium oleate was stated by Sachs and Altmann (1909) to be active as an "antigen" in the Wassermann reaction with syphilitic serum, and Levaditi and Yamanouchi (1907) found sodium taurocholate similarly active.

For testing purposes a serum or the carbonic-acid-insoluble fraction known to be active along with the Wassermann "antigen" was used. Generally, heated rabbit serum was employed; in a few instances the unheated carbonic-acid-insoluble fraction of rat serum was tested and occasionally the heated serum of the ox, sheep or horse. All the substances found to be active were also tested with Wassermann-negative animal sera, *e.g.* pig serum, and found to react negatively.

In the case of many of the substances quoted the tests were repeated several times with different sera. The concentrations of the various substances used are indicated in Table III, and 0·5 c.c. was in all cases the test amount.

Table III.

*Alcohols.*

| | | |
|---|---|---|
| Ethyl alcohol | Diluted 1 : 10 with normal saline | Active (*v. supra*) |
| Allyl alcohol<br>Isopropyl alcohol | Diluted 1 : 10 with normal saline | Inactive |
| Methyl alcohol | Diluted 1 : 10 with normal saline | Weakly active but results irregular; generally less active than ethyl alcohol |
| Glycerol | Diluted 1 : 10 with normal saline | Weakly active |
| Cholesterol (two-thirds-sat. soln. in ethyl alcohol) | Diluted 1 : 10 with normal saline | Strongly active (*v. supra*) |
| Phenol | 0·5 % in saline | Inactive |

*Amino-acids.*

(Made up in 1 % solution in normal saline when the solubility of the substance permitted; if not soluble to this degree, a saturated solution in normal saline was used.)

| | | |
|---|---|---|
| Tryptophan<br>Arginine | Saturated solution (less than 1 %) | Weakly active |
| Leucine | Saturated solution (less than 1 %) | Inactive |
| Alanine<br>Phenyl-alanine<br>Histidine | 1 % fully dissolved | Weakly active |
| Sarcosine<br>Valine | 1 % fully dissolved | Inactive |
| Glycine | Saturated solution (less than 1 %) | Strongly active |

*Proteins.*

| | | |
|---|---|---|
| Haemoglobin | Saturated solution in saline (less than 1 %) | Inactive |
| Egg albumin | 1 % solution in saline | Inactive |
| Proteins precipitated by alcohol from a saline extract of ox liver | Extract obtained by grinding up 20 grm. ox liver with sand and mixing with 100 c.c. saline; the extract was filtered and 2 volumes of absolute alcohol added; the precipitate was separated by centrifuging and re-dissolved in 100 c.c. saline | Strongly active |
| Proteins precipitated by alcohol from a saline extract of sheep's heart | Prepared as above | Active |
| Gelatin | 1 % solution in saline | Inactive |

Table III—*continued.*

*Other substances.*

| | | |
|---|---|---|
| Agar | 0·1 % solution in saline | Inactive |
| Paraffin suspension | Prepared by adding 1 part saturated solution of liquid paraffin in alcohol to 5 parts of saline | Effect not greater than that of contained alcohol |
| Sodium oleate | 0·06 % alcoholic solution of sodium oleate added to 10 parts saline and made up like Wassermann "antigen" | Active |
| Starch | 1 % in saline | Inactive |
| Colloidal benzoin | 0·3 c.c. of a 10 % alcoholic solution of Sumatra benzoin to 10 c.c. saline | Effect not greater than that of contained alcohol |
| Guanidine hydrochloride | 1 % in saline | Inactive |
| Kaolin suspension | 1 % in saline | Inactive |
| Inulin | 1 % in saline | Weakly active |
| Sodium nucleate | 1 % in saline | Strongly active |
| Alcoholic extract of sheep's heart | Prepared in same way as Wassermann "antigen" but not cholesterolised; a 1:10 suspension in saline used | Active |
| Purified lecithin from ox heart and ox liver | 1 % in alcohol prepared by method de-described by Browning (1924); a 1 : 10 suspension in saline used | Active |

The solutions were rendered as far as possible isotonic, and any anti-complementary effect or haemolytic action was ascertained by the usual controls. The results are summarised in Table III. The fixation effects elicited with certain of these substances are illustrated in Table IV.

Table IV. *Doses of complement fixed.*

| Heated serum of | Wassermann "antigen" | Alcohol-saline | Peptone | Cholesterol | Glycine | Proteins pptd. from aq. ext. of liver | Sodium oleate | Sodium nucleate | Tryptophan | Ox heart lecithin | Ox liver lecithin |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit | 18 | 0 | 4 | — | 4 | — | — | — | — | — | — |
| Ox | 12 | 8 | 8 | 8 | 8 | — | — | — | 4 | — | — |
| Rabbit | 2 | 2 | 8 | 4 | — | 10 | — | — | — | — | — |
| Rabbit | 2 | 0 | 4 | — | — | 4 | — | — | — | — | — |
| Rabbit | 4 | 4 | 10 | 14 | — | — | 12 | — | — | — | — |
| Rabbit | 4 | 2 | 12 | 2 | — | — | 8 | — | — | — | — |
| Rabbit | 10 | 4 | 16 | 8 | — | — | — | 14 | — | — | — |
| Rabbit | 6 | 4 | 16 | 8 | — | — | — | 6 | — | — | — |
| Ox | >12 | 0 | >12 | >12 | — | — | — | — | — | >12 | >14 |
| Rabbit | 4 | 0 | >12 | 4 | — | — | — | — | — | 4 | 6 |

The figures stated are the number of doses fixed by serum *plus* "antigen" or "pseudo-antigen," less the sum of the doses of complement fixed by each separately in the control tests.

Various substances tested were found unsuitable for complement-fixation reactions either in virtue of their anti-complementary action or lytic effect, *e.g.* butyl and propyl alcohol, hippuric acid, a glycyl-glycine preparation, glutaminic acid, glycero-phosphoric acid, stearic, oleic and linoleic acids, sodium taurocholate and cobra-venom.

The complicated monohydric alcohol cholesterol was the most active alcohol tested. As shown elsewhere, this effect appears to depend on the suspended particles. Of the remaining alcohols the most active was ethyl alcohol; methyl alcohol and glycerol were only slightly active. No apparent relation-

ship has been elicited between the complement-fixation effect and the chemical or physical properties of the alcohols tested.

Of the amino-acids, glycine yielded the most pronounced reactions; tryptophan, alanine, phenyl-alanine, arginine and histidine also showed the same property but to a lesser degree. These constitute a group of amino-acids which appear to have little or no relationship to each other as contrasted with other amino-acids.

The activity of the proteins precipitated by alcohol from saline extracts of tissue is noteworthy, when considered with the analogous effect produced by alcoholic extracts of similar tissues, *e.g.* the lipoid "antigens" used in the Wassermann reaction.

Of the other substances examined, sodium oleate and sodium nucleate were most active. Both exhibit some relationship in chemical structure to the lecithin-like lipoids, the one in respect of the fatty acid grouping and the other the phosphoric acid group.

Reference has been made above to the observations by Takenomata (1924) that bacterial suspensions are capable of yielding non-specific complement-fixation with normal serum. A preliminary investigation of this property of serum was also made in the course of the present inquiry. In most of the tests a strain of *B. coli communis* was used and prepared in the form of saline suspensions from agar-slope cultures. When unheated bacterial suspensions were used, the fixation effect showing the zone phenomenon described by Dunlop (see Browning, 1927) frequently occurred with the guinea-pig serum *per se* used as complement in the test and thus introduced a complicating factor. Under these conditions it was difficult to estimate the fixation produced by the serum under investigation as apart from that produced by the complement-containing serum itself. Apparently fresh guinea-pig serum possesses an active principle capable of effecting complement-fixation under certain conditions with various bacteria. This property has recently been discussed by Browning (1927). This effect with fresh guinea-pig serum can be reduced to a minimum by incubating the serum at 37° C. for several hours before using it for the test, and in this way it has been possible to carry out with other sera fixation tests in which guinea-pig complement is used. Specimens of unheated ox, sheep, pig and rabbit sera have thus been found to yield pronounced reactions (*e.g.* fixation of over 18 doses of complement), but the property has proved markedly thermolabile, being usually annulled at 55° C. It is noteworthy that the activity of the bacteria in this respect is also reduced or annulled by heating at temperatures above 55° C. Similar effects have been elicited with various other organisms. These non-specific reactions with bacteria are still under investigation and require further study before any statement can be made regarding their relationship to the reactions with the "pseudo-antigenic" substances described.

EFFECT OF CHOLESTEROLISATION.

This has already been referred to in the case of peptone. It was found that certain substances which were practically inactive, or only weakly active by themselves, along with cholesterol, yielded marked results in excess of the separate reactions.

Thus, cholesterolised-glycine and -tryptophan gave reactions which were greater than the summation of effects produced by cholesterol and the particular substance separately. Gelatin by itself in 1 per cent. solution was inactive, but cholesterolised-gelatin yielded a reaction quantitatively greater than that due to the ordinary cholesterol suspension (Chart 9).

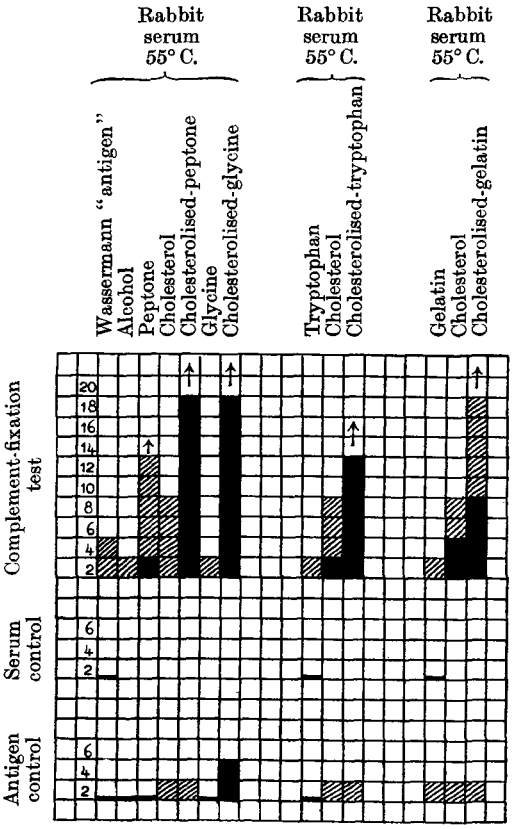


Chart 9.

Cholesterolisation of substances in saline solution was effected by adding two-thirds-saturated cholesterol in the proportions of 1 : 10 and mixing as in the preparation of the Wassermann "antigen" emulsion.

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## COMPLEMENT-FIXATION EFFECTS OBTAINED WITH WASSERMANN-POSITIVE AND -NEGATIVE HUMAN SERA ALONG WITH VARIOUS "PSEUDO-ANTIGENS."

The close parallelism between the Wassermann reaction of certain normal animals and the complement-fixation effects with various "pseudo-antigens" raised the question whether a similar relationship might be demonstrated in the case of human syphilitic serum. It has also been shown how normal sera (obtained from selected healthy persons) exhibited in the heated state no reaction with alcohol-saline, peptone solution, cholesterol and cholesterolised-peptone. Mackie and Watson (1926) found that heated syphilitic sera and particularly the carbonic-acid-insoluble fraction frequently showed a weak complement-fixation reaction with alcohol-saline, whereas normal sera were negative in this respect. A number of sera from a Venereal Diseases Clinic and a Mental Hospital were therefore tested simultaneously with the Wassermann "antigen" and the other reacting substances referred to. Many of the Wassermann-negative sera were from treated syphilitic cases. One hundred and twenty-five sera were examined and the general results are shown in Table V.

Table V.

*Wassermann-positive sera* (54).

| Alcohol-saline | | Peptone | | Cholesterol | | Cholesterolised-peptone | |
|---|---|---|---|---|---|---|---|
| Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. |
| 8 | 46 | 17 | 37 | 28 | 26 | 33 | 21 |

*Wassermann-negative sera* (71).

| | Alcohol-saline | Peptone | Cholesterol | Cholesterolised-peptone | Clinical condition |
|---|---|---|---|---|---|
| 64 sera | Neg. | Neg. | Neg. | Neg. | |
| 1 serum | Neg. | Pos. | Pos. | Pos. | Treated syphilitic case |
| 2 sera | Neg. | Neg. | Neg. | Pos. | (1) Treated syphilitic case<br>(2) Case under observation—no clinical signs of syphilis |
| 2 sera | Neg. | Pos. | Neg. | Pos. | (1) Gonorrhoea<br>(2) Treated syphilitic case |
| 1 serum | Pos. | Pos. | Neg. | Pos. | Balanitis |
| 1 serum | Neg. | Neg. | Pos. | Pos. | Treated syphilitic case |

Negative control sera were included in the various sets of tests, and in determining positive and negative results the criteria generally adopted in reading Wassermann reactions were also applied to the other tests. Where positive results are recorded, the fixation produced was quite definite; any doubtful or border-line results have been recorded as negative.

It will be noted in the first place that there was no definite parallelism between the reactions with any of the "pseudo-antigens" tested and the Wassermann effect. The results are nevertheless of considerable interest. Only a small proportion of Wassermann-positive sera yielded a reaction with

alcohol-saline; a higher proportion gave reactions with peptone and more than half showed definite complement-fixation with cholesterol suspension; the proportion of positive reactions was highest with cholesterolised-peptone (61 per cent.), but still a considerable proportion of Wassermann-positive sera failed to react with the last named agent. There has been no definite evidence from previous work (see Browning, 1924) that cholesterol *per se* is capable of acting as a Wassermann "antigen."

It will be seen how a small minority of Wassermann-negative sera reacted with alcohol-saline, peptone and cholesterol. In the case of cholesterolised-peptone about 10 per cent. of Wassermann-negative sera yielded positive results. All these Wassermann-negative sera which gave positive reactions with the other reagents were from patients in a Venereal Diseases Clinic and certain of them were cases of treated syphilis. It is possible that in these cases the Wassermann effect had been abolished by treatment, though the other reactions were more persistent. Chart 10 illustrates the results recorded: definite parallelism among the various reactions is shown in the case of sera (1) to (4).

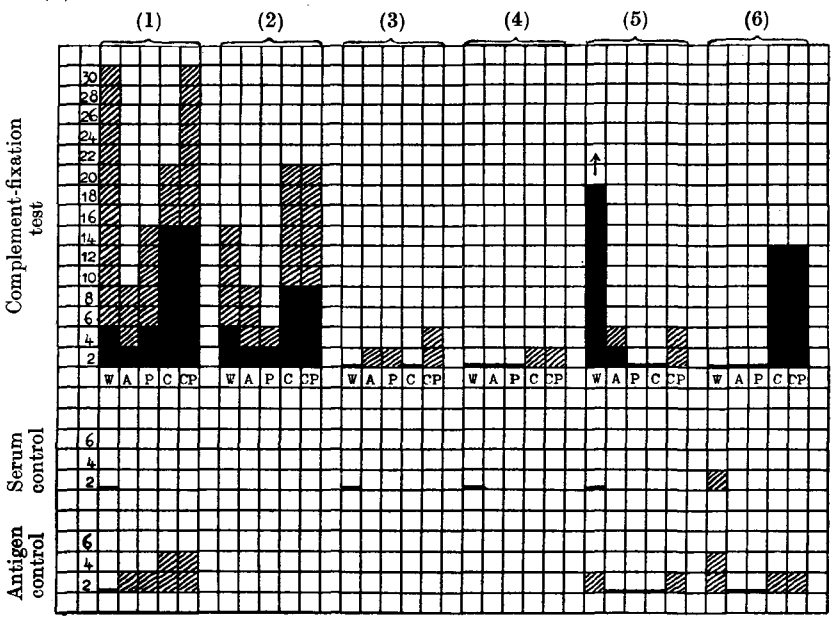


Chart 10. Human sera. Heated 55° C.

## Thermolability of Serum Properties.

It has been shown how in certain species the non-specific reacting property of the serum is annulled at 55° C., whereas in others it is relatively stable at this temperature. Even in the latter, however, a distinct weakening in activity is usually noted at 55° C. as compared with the fresh unheated serum. A study of the relative lability of the reacting properties of rabbit serum at various

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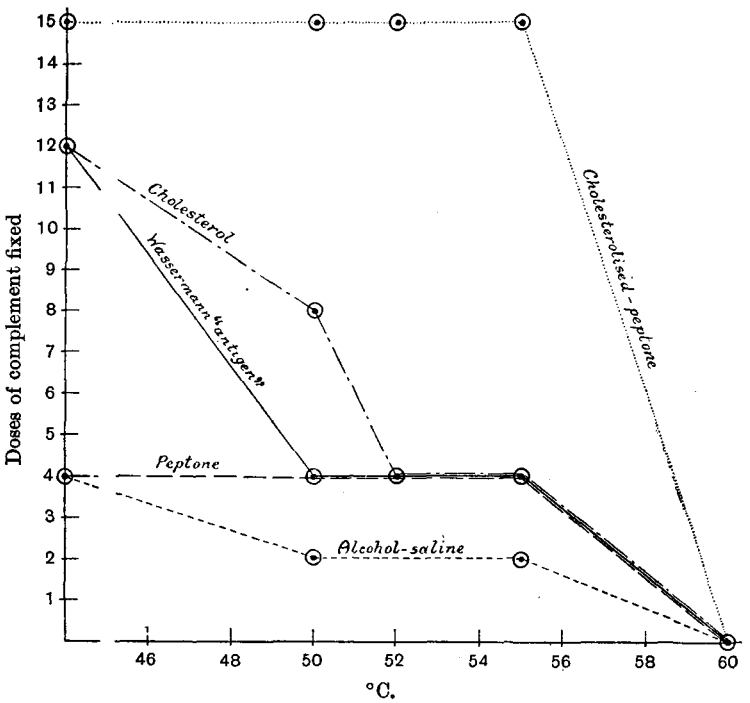
temperatures and also of Wassermann-positive and -negative human sera was carried out with a view to ascertaining whether the degree of lability as regards the reaction with different "pseudo-antigens" was equal, and whether different lability curves could be demonstrated with positive and negative human sera respectively.

The sera were heated at varying temperatures (*e.g.* 46°, 50°, 52°, 54°, 56°, 58°, 60°) for half-an-hour in a constant-temperature water-bath and then tested in parallel with the various substances as in the previous experiments. A standardised thermometer was used. When serum was exposed to temperatures over 58° C. it was first diluted 1 in 4 with normal saline to obviate inspissation or coagulation, and the test amount of the diluted serum was 0·2 c.c.

*Rabbit.* Tests were carried out with a number of specimens of serum from individual animals and two types of thermolability curve were met with.

In certain animals heating produced a progressive weakening of the reacting power, inactivation occurring at 60° C. (Graph 1).

In other animals at 50° C. to 52° C. there was almost complete inactivation




Graph 1. Rabbit serum.

In this and subsequent graphs the horizontal axis represents temperature and the vertical axis the degree of complement-fixation. For convenience the unheated state (of the serum) is taken as zero and the actual curves are contracted between this and 46° or 50° C. as the case may be.

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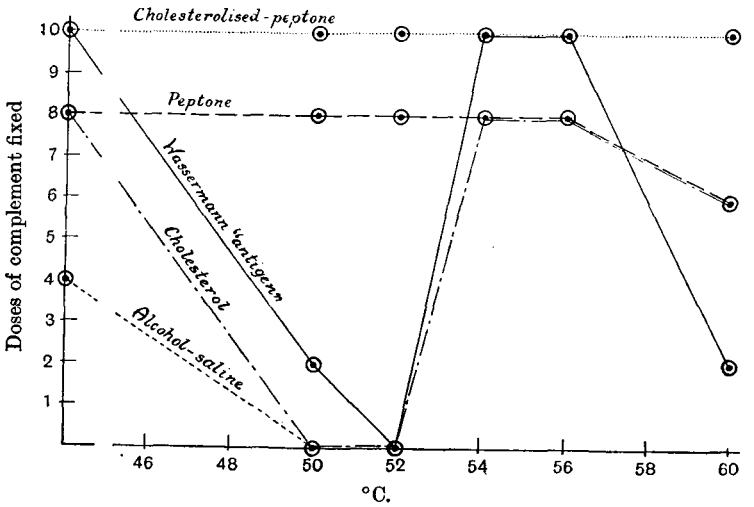
as regards the reactions with certain substances, *e.g.* Wassermann "antigen" and cholesterol, but at 54°–56° C. the effects were again almost equal to those of unheated serum; above 60° C. inactivation occurred (Graph 2). This result represents a striking phenomenon illustrative of the remarkable variation in reacting power after heating at different temperatures. Analogous effects produced by heating syphilitic sera at varying temperatures have been described by Watson (1925) in the Sachs-Georgi reaction, *i.e.* inactivation occurred at 50°–52° C. and at 58° C., the serum being fully active at 54°–56° C. It will be noted in Graph 2 that, while the curves for the Wassermann "antigen" and cholesterol correspond, they differ from those for peptone and cholesterolised-peptone which did not elicit the double optimum temperature zone.




Graph 2. Rabbit serum.

An analogy has already been drawn between these serum properties in the rabbit and the natural haemolytic effect for sheep blood. It is interesting to note that the latter property also shows the same degree of thermolability, being completely inactivated at 60° C. (see Table VI), and contrasting in this respect with the stability of an acquired haemolytic antibody for the same cells, which is unaltered quantitatively even at 70° C. Jones (1927) has

Table VI. *Rabbit serum. Doses of complement deviated.*

| Temp. | Wassermann "antigen" | Alcohol-saline | Peptone | Cholesterol | Cholesterolised-peptone | M.H.D. of natural antibody *v.* sheep red cells (c.c.) |
|---|---|---|---|---|---|---|
| Unheated | 12 | 4 | 4 | 12 | 18 | 0·025 |
| 50° C. | 4 | 2 | 4 | 8 | 18 | 0·05 |
| 51° C. | 4 | 2 | 4 | 4 | 18 | 0·05 |
| 55° C. | 4 | 2 | 4 | 4 | 18 | 0·1 |
| 60° C. | — | — | — | — | — | No lysis by 0·5 |

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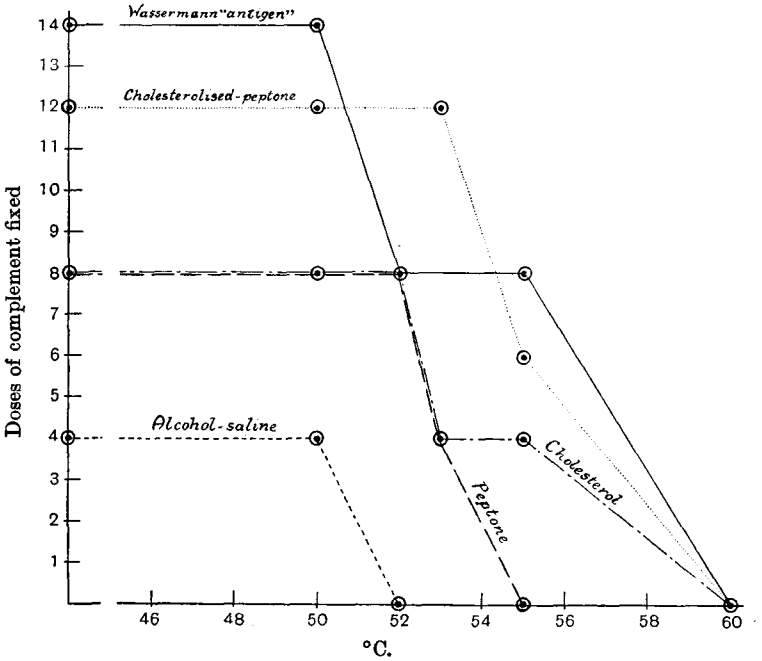
recently emphasised the degree of stability of a specific haemolysin which he states is only inactivated at 85° C.

In some cases a progressive decrease in the activity of the natural haemolysin has been noted on heating, which showed some parallelism to the inactivation of the serum in its reactions with the "pseudo-antigens" (Table VI). This is further evidence of the analogy between the two phenomena.




Graph 3. Human serum. Wassermann-positive.

Graphs 3 and 4 show the comparative curves presented by selected Wassermann-positive and -negative human sera. The occurrence of the characteristic weak reactions with the unheated negative sera are illustrated and also their disappearance between 50° C. and 54° C. In the case of the positive serum the reactions with the Wassermann "antigen," cholesterol and cholesterolised-peptone are maintained over a higher temperature range, though all are annulled at 60° C.

## DISCUSSION.

The results recorded are of great interest in illustrating how complement-fixation may result from the interaction of normal serum and various substances that are not true antigens, *e.g.* alcoholic extracts of tissue, lecithin, sodium oleate, ethyl alcohol, cholesterol, commercial peptone, certain amino-acids, sodium nucleate. A similar non-specific complement-fixation reaction is produced by normal serum *plus* the proteins precipitated by alcohol from a watery extract of tissue. In certain respects the results fall into line with

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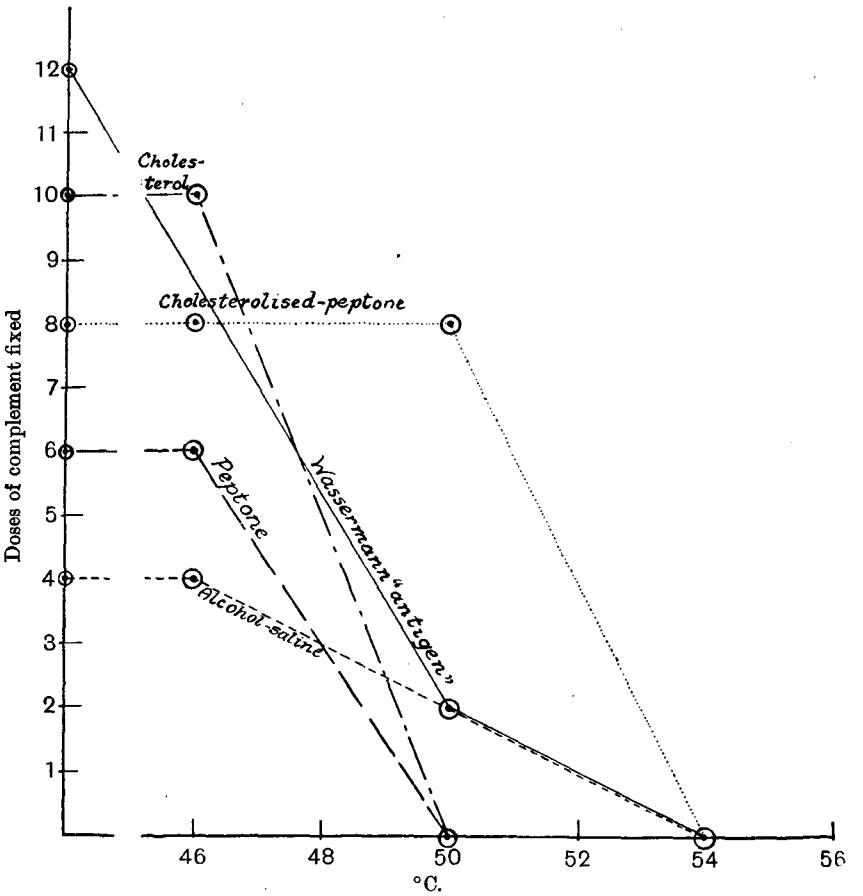
those of Takenomata (1924), who described a similar non-specific reaction by normal serum *plus* various agents including peptone, bacterial suspensions, inulin, etc. He designated these agents "pseudo-antigens"; as a convenient term to signify how, in the complement-fixation test, they may simulate the effect of a true antigen, this designation may in the meantime be adopted. The fact, however, that the specificity of certain bacterial antigens depends




Graph 4. Human serum. Wassermann-negative.

on components of non-protein nature, *e.g.* carbohydrate substances, which though not antigenic *in vivo* are still capable of reacting like antigens *in vitro*, has tended to broaden the general chemical conception of antigens (Heidelberger and Avery, 1923). The occurrence of these reactions with normal serum does not of course exclude their being due to antibody-like principles as in true immunity reactions; the presence of natural antibodies for various types of antigens is well recognised.

The observations are of special interest in that they serve to classify the Wassermann reaction of normal animals in a category of serological pheno-

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mena, *viz.* complement-fixation by normal serum *plus* certain substances varying greatly in chemical and physical properties and not necessarily capable of acting as antigens *in vivo*. The results all tend to demonstrate the close parallelism between complement-fixation reactions with these "pseudo-antigens" and the similar effect with the Wassermann "antigen." There exists therefore a type of non-specific serum reaction natural to various animals and dependent on some serum principle (or principles) varying in thermostability in different species, but more labile than immune bodies formed in the process of immunisation. In certain species the lability of this principle corresponds to the "Wassermann substance" of human syphilitic serum. The absence of this type of serum principle from young animals and its later development is of particular interest and brings it into relationship with a natural antibody, *e.g.* the haemolysin of rabbits for sheep blood. In thermolability this principle also resembles the natural haemolysin. These analogies with a recognised antibody-like substance are of undoubted significance. Browning (1927) has put forward the suggestion that a common prototype of "immune" antibodies may exist normally in blood serum in an undifferentiated state. It seems possible that the active principle of these non-specific reactions may be of this nature, but any conclusive interpretation of the phenomenon seems impossible in the present state of our knowledge regarding the physical and chemical basis of serological reactions.

Among the various chemical agents examined, it has not been possible to establish any definite relationship between their activity or non-activity in this reaction with normal serum and their chemical and physical properties, but this aspect of the subject still requires further study and the present investigation has been more concerned with the analysis of the serum function.

No uniform parallelism between the reaction with any of the "pseudo-antigens" tested and the Wassermann effect of syphilitic serum has been elicited, but there is a sufficient degree of correspondence to point to some relationship. In view of the thermolability curves of rabbit serum it seems probable that more than one serum principle is concerned in these non-specific reactions, and it is possible that in syphilis a type of serum principle of the category referred to is quantitatively increased and exalted in thermostability and particularly a principle capable of reacting with lecithin—a lipoidophile antibody-like substance.

## SUMMARY AND CONCLUSIONS.

1. When a solution of commercial peptone is substituted for antigen in a complement-fixation test with the unheated normal serum of certain species (man, ox, sheep, horse, rabbit, white rat), a definite fixation reaction occurs both at 37° C. and at 0° C. In the ox, sheep, horse and rabbit this property of serum is partially stable at 55° C., but normal human serum and the serum of the white rat are inactive after heating at this temperature. The property is resident mainly in the carbonic-acid-insoluble globulins of the serum.

2. The same results are obtained when ethyl alcohol diluted with several volumes of normal saline solution is substituted for antigen in a complement-fixation test with normal serum.

3. Analysis of these reactions shows a close correspondence with complement-fixation by the sera of normal animals *plus* the Wassermann "antigen" —the Wassermann reaction of normal animals.

4. Marked complement-fixation effects are also obtained with heated normal serum of the rabbit, ox, sheep, horse *plus* cholesterol suspension, and particularly cholesterolised-peptone, these effects occurring in parallel with those produced by serum *plus* alcohol-saline, peptone solutions and the Wassermann "antigen." The heated normal serum of the pig, white rat and guinea-pig do not exhibit these reactions, and the same applies to heated normal human serum. Unheated pig serum fails to react. Such results also elicit a close relationship between these non-specific reactions and the Wassermann reactions of normal animals.

5. The reacting property is absent from the serum (heated and unheated) of young rabbits during the first 2 to 3 weeks of life, but appears soon after this (*e.g.* by the 37th day) and is progressive in development. Its development in early life runs parallel to that of the natural haemolytic property of the serum for sheep's blood (due to a natural antibody-like substance). The two properties are, however, independent as illustrated by absorption tests.

6. Besides the agents referred to above as capable of fixing complement along with normal sera, other substances possess a similar property, *e.g.* certain alcohols, sodium oleate, tissue proteins, certain amino-acids and sodium nucleate. Commercial peptone purified by precipitation with alcohol is equally active with the original material. Cholesterolisation of these agents may yield a product whose activity is greater than that due to summation of effects.

7. Wassermann-positive and -negative human sera have been tested in the complement-fixation reaction with certain of these "pseudo-antigens," viz. alcohol-saline, peptone, cholesterol, and cholesterolised-peptone, but a uniform parallelism has not been demonstrated between the reactions with these agents and the Wassermann effect. Some Wassermann-positive sera react also with alcohol-saline, peptone, cholesterol and cholesterolised-peptone, while sera from selected normal persons are quite inactive. A considerable proportion of Wassermann-positive sera yields definite complement-fixation with cholesterol and cholesterolised-peptone; a small proportion of Wassermann-negative sera reacts with these agents.

8. The thermolability of the serum principles acting with various "pseudo-antigens" has been studied by testing unheated serum and serum heated at temperatures ranging from 46° to 60° C. Two types of thermolability curve have been demonstrated with different specimens of rabbit serum: (1) a more or less progressive weakening of the various reactions with inactivation at 60° C.; (2) inactivation of the effects with Wassermann "antigen," alcohol-

saline and cholesterol at 50–52° C., activation of the effects with the Wassermann "antigen" and cholesterol at 54–56° C. and inactivation again above 60° C.; in this case the curves for peptone and cholesterolised-peptone do not show such double inactivation. Unheated normal human serum yields reactions with the various agents (including the Wassermann "antigen") but inactivation occurs at 50° to 54° C. whereas certain syphilitic sera yield thermolability curves somewhat similar to type (1) of rabbit serum, with inactivation at 60° C. or over.

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