ON THE IMMUNOLOGICAL NATURE OF THE PRINCIPLE IN SERUM RESPONSIBLE FOR THE WASSERMANN REACTION, WITH REFERENCE ALSO TO THE FLOCCULATION REACTION OF SACHS AND GEORGI.

By T. J. MACKIE and H. FERGUSON WATSON.

(*From the Bacteriology Department, Edinburgh University.*)

(With 14 Charts.)

CONTENTS.

Introduction.

Though the Wassermann reaction and, more recently, the flocculation reaction of Sachs and Georgi have been so extensively investigated and applied, the theoretical explanation of these phenomena is still debatable.

The mechanism of the Wassermann reaction is now well understood in its practical application but there remains a great deal of uncertainty regarding the phenomenon from the strictly immunological standpoint[1]. The antigen function has, of course, been subjected to most careful analysis by many

[1] The whole subject of the theory of the syphilis reactions has been carefully reviewed recently by Browning and his collaborators (1924).

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

workers. It is generally agreed that the antigenic property of the alcoholic extracts of tissue used in the Wassermann test depends on "lecithin" and allied substances, *i.e.* the ether-soluble acetone-insoluble fraction of the extract. The augmentation of the Wassermann reaction by cholesterol (first demonstrated by Browning, Cruickshank and Mackenzie, 1910) and by the alcohol of the solution from which the lipoid suspension is prepared (as originally shown by Sachs and Rondoni, 1908) are important factors in the phenomenon and, together with the recognised influence of the state of dispersion of the lipoid suspension, illustrate the complexity of the associated physical and chemical processes.

The nature of the Wassermann reacting "substance" in the serum and its mode of development present an even more difficult problem. This principle has been regarded as of antibody nature or analogous in many of its properties to antibodies. The work of Taniguchi (1921) has thrown some light on this question. "Heterogenetic" or Forsmann antibody, *i.e.* the haemolytic antibody of the rabbit for sheep's red corpuscles, generated by immunisation with the tissues of the guinea-pig and certain other species ("heterophile" antigen), exhibits complement-fixation and flocculation reactions with the lipoids of heterophile tissues, and an analogy has been drawn between the syphilis serum reactions and that of the Forsmann antibody with the lipoids of heterophile tissue.

It might be postulated, therefore, according to the original theory of Weil and Braun (1907) that the Wassermann reacting substance is a lipoidophile antibody but the actual genesis of the substance remains unexplained. It has been supposed that this antibody is produced in response to some "foreign" tissue product developed as a result of the pathological changes in the disease, which in virtue of its foreign nature is capable of acting as antigen. It is well known, of course, that lipoid substances are not antigenic *in vivo*, though they can act as receptors *in vitro*. It is possible, however, that in combination with protein—a lipoid-protein compound—they may evoke antibody formation *in vivo* (Taniguchi, 1922). Forsmann (1924) supports the idea that the "Wassermann substance" is an antibody and has shown that minute amounts of formaldehyde destroy it in the same way as various other antibodies.

On the other hand the reacting principle may be of the nature of a *natural* antibody present normally in minimal amount and augmented in syphilis as a result of some heterologous stimulus. The occurrence of the Wassermann reaction with the serum of certain normal animals is highly suggestive in this respect (*vide infra*).

The Forsmann antibody certainly represents an augmented production of a natural antibody, as a result of the injection of a heterologous antigen and it has been shown that this natural antibody can be increased by other non-specific stimuli apart from those of antigenic nature (Mackie, 1925). It is of great interest in this connection that in Taniguchi's work (1921) immunisation of rabbits with heterophile tissues, besides increasing the anti-sheep

haemolysin, also caused the increased development of the Wassermann reacting substance in these animals.

The syphilis serum phenomena, depending on colloidal reagents, have also been studied from the physical standpoint and the reactions undoubtedly correspond in some respects to other colloid reactions. Thus the flocculation reaction has been explained as a colloidal precipitation effect on a basis of neutralisation of negatively charged particles in the antigen suspension by positively charged serum globulin, and the fixation of complement has been regarded as an adsorption of complement by the surface action of the precipitated particles (see Schmidt, 1911). While these reactions may be governed, like certain other *in vitro* immunity phenomena, by the laws of colloidal actions, the idea suggested by Sachs (1917) and others that the reacting power of a serum depends on some physical alteration of the globulin affords no satisfactory explanation of all the known data regarding the occurrence and the mechanism of the reactions.

The association of the reacting substances with certain protein components in the serum has been investigated by various workers and different methods of separating out the protein fractions have been applied. The active substances are undoubtedly associated with the globulin. Euglobulin and pseudoglobulin have been separately examined as regards their reactivity in the Wassermann phenomenon. Gilmour (1912), who used careful separation by ammonium sulphate and purified the fractions by repeated precipitation, found that the euglobulin of positive sera usually reacted negatively while in most cases the pseudoglobulin proved active. Other observers have claimed that the active property depends on the euglobulin (Noguchi, 1909; Harold, 1922; Stern, 1924; and others). According to Oeller and Schierge (1923) it is not possible to isolate, by any of the methods commonly used, one fraction which exclusively contains the fixing substance. It is doubtful, of course, whether these separated fractions represent definite protein entities. Results have been variable and this may depend on a variable distribution of the reacting principle in the different protein precipitates.

The carbon-dioxide method of Liefman (1909) which precipitates the euglobulin, usually along with a certain proportion of the pseudoglobulin, separates out in the insoluble fraction a moiety of serum which apparently constitutes the greater part of the Wassermann reacting substance but the soluble fraction which contains most of the pseudoglobulin (along with the albumin) is also active though to a lesser degree. By this method it has been possible in a series of strongly flocculating sera to demonstrate a partial dissociation of the complement-fixing and flocculating properties of the serum (Mackie, 1923) owing to the fact that the latter property is mainly resident in the soluble fraction.

The observations we have to record in this paper have resulted from a further investigation into the nature of the syphilis serum reactions, particularly with regard to the question whether they represent the activity of

a natural antibody present normally in minimal amount but non-specifically reinforced by infection with *Spironema pallidum* and certain other organisms or as a result of the metabolic alterations or chemical changes in the tissues incidental to syphilis and the other conditions in which a positive Wassermann reaction has been noted. The occurrence of similar reactions with the serum of various animal species has been studied with a view to throwing some light on the general question and the variation of the Wassermann reaction of animals under certain influences has also been investigated. While attention has been mainly directed to the complement-fixation phenomenon, the flocculation reaction has been studied to a considerable extent in parallel with the other reaction. A uniform antigen and technique has been used throughout, and though the antigen function has not been specially investigated in the course of this work, the influence on the Wassermann reaction of the alcohol in the antigen suspension and the behaviour of alcohol-free antigen preparations have been considered. The fractioning of positive and negative human and animal sera by carbon-dioxide has been utilised as an aid in the analysis of the serum function.

METHODS.

It is unnecessary to describe in detail the method used for carrying out the Wassermann reaction. The general system of the test is well known and it will be sufficient here to indicate the essential features of the method employed. The antigen was an alcoholic extract of sheep's heart (20 grams extracted with 100 c.c. alcohol) saturated with cholesterol. For the test a 1 : 12 dilution was prepared in saline by slow admixture so as to form a suspension of the maximum turbidity. Fixed amounts of antigen emulsion (0·5 c.c.) and serum (0·05 c.c.) were tested with varying amounts of guinea-pig's complement so that the approximate number of minimum haemolytic doses deviated[1] could be estimated. Where serum fractions were used, an amount of the fraction equal to that derived from 0·05 c.c. of whole serum was tested. The test quantity of haemolytic system (sensitised ox or sheep red corpuscles) was 0·5 c.c. The smallest dose of complement in the series of amounts tested was 2 M.H.D. (for the test quantity of the blood suspension). All the necessary controls were included in any series of tests as in the usual Wassermann reaction and known negative and positive control sera were also tested as standards for comparison with other sera.

The technique of the flocculation reaction was that originally described by Mackie (1921). This method has now been extensively applied by Watson (1925 and unpublished observations) and the original method has been modified by the introduction of mechanical shaking of the mixtures for 5 minutes before incubation. The system of this method consists in preparing 0·3 or 0·4 c.c. of a series of dilutions of the serum, 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64, to which is added a half-volume of a 1 in 6 emulsion of the same antigen as that used in the Wassermann test. The mixtures are incubated in agglutination tubes or in 3 in. × ½ in. tubes for 4 hours and then allowed to stand at room temperature for 36 hours when final readings of the resulting flocculation are made. The flocculation is visible to the naked eye.

Other methods used in the course of the investigation are described later.

[1] The term "deviation" of complement is used in this paper as synonymous with "fixation" of complement.

THE COMPLEMENT-FIXATION AND FLOCCULATION REACTIONS OF ANIMAL SERA WITH THE WASSERMANN LIPOID ANTIGEN.

The fact that the heated (55–56° C.) serum of certain animal species may frequently exhibit the complement-fixation reaction with the lipoid antigen used in the Wassermann reaction is of particular interest and the question has arisen as to whether this reaction is the true homologue of the Wassermann syphilis reaction in man. Lower apes, dogs, goats, sheep, pigs and rabbits have been referred to as exhibiting the reaction (see Browning and Mackenzie, 1924). Reports regarding the reaction in the case of the ox and horse are contradictory. As might appear obvious in view of the use of guinea-pig's serum as complement in the diagnostic test, this serum is characterised by its failure to produce the reaction in the unheated state.

It might be supposed, of course, that the reaction in animals is a function of some common type of infection. Thus in rabbits the frequent occurrence of *Spironema cuniculi* infection or of coccidiosis might be regarded as the exciting cause. Observations by Browning and Mackenzie (1910), Manteufel (1909) and Marcuse (1921) do not support such an explanation. Torres (1924) has also shown that there is no relationship between coccidiosis and the Wassermann reaction, and states the reaction is positive or negative irrespective of the presence of lesions. The striking uniformity in the occurrence of the reaction which we have noted in certain species has led us to regard the property of the serum as a normal or natural one, analogous to that of a natural antibody. Taniguchi (1921) has also shown that when the rabbit haemolysin for sheep's red corpuscles is generated as a result of the injection of heterophile tissues there is also an increase in the power of the serum to react in the Wassermann test.

Attention has been drawn to certain differences as regards the reaction between rabbit's serum (which has been most studied) and positive human sera. As is well known, heating at 55° or 56° C. apparently reduces the activity of syphilitic sera. It has been stated by Browning and Mackenzie (1910) that in the case of the rabbit, the unheated serum may react negatively while heated serum gives a positive result. This, however, is not a uniform occurrence as indicated by Hessberg (1909). Browning and Mackenzie have shown that this peculiarity is not due to the fact that the presence of the rabbit's serum in the unheated state increases the amount of haemolytic complement in the mixtures and therefore obscures the fixation of the test complement. Further, the result is not apparently due to non-deviable complement in the rabbit's serum. Griffith and Scott (1920) have also stated that the human and rabbit reactions differ in the thermostability of the reacting principle in the serum. Sachs and Georgi (1923) have claimed that treatment of rabbit's serum with dilute hydrochloric acid removes the reacting substance along with the resulting globulin precipitate, while in rabbits infected with *Spironema pallidum* this procedure only produces a weakening of the reaction. This is confirmed by Blum (1924).

Table I. *Showing results of a survey of the complement-fixation and flocculation reactions of animal sera with the Wassermann antigen.*

Exceptions	General results Wassermann reaction Flocculation ,,		Animal	Rabbit	Ox	Sheep	Horse	Mouse	White rat	Guinea-pig	Pig	Cat	Dog	*Macacus rhesus*	Pigeon	Fowl	Duck	Frog
			Total No. examined	120	22	21	12	20	32	54	58	12	7	1	10	14	1	6
			Both reactions	67	15	13	12	20	32	54	58	12	7	1	10	14	1	6
			Wassermann reaction only	53	7	8	—	—	—	—	—	—	—	—	—	—	—	—
Wassermann reaction positive Flocculation ,, negative – 6 Wassermann ,, ,, Flocculation ,, positive – 2	Positive																	
Wassermann reaction negative – 4 Flocculation reaction not tested)	Positive																	
Wassermann reaction negative – 1 Flocculation reaction not tested)	Positive																	
—	Positive																	
Wassermann reaction positive Flocculation ,, negative – 9 Wassermann ,, ,, Flocculation ,, ,, – 1	Positive but both reactions weak	10																
In 4 cases Wassermann reaction not frankly negative—only partial lysis with 2 and 4 doses of complement while negative control sera showed complete lysis with 2 doses	Negative																	
—	Negative																	
Wassermann reaction negative Flocculation ,, ,, – 1 Wassermann ,, positive Flocculation ,, ,, – 11	Wassermann reaction negative Flocculation ,, positive																	
Wassermann reaction positive Flocculation ,, ,, – 1 Wassermann ,, negative Flocculation ,, ,, – 6	Wassermann reaction negative Flocc. reaction weakly positive	– 5																
Wassermann reaction positive Flocculation ,, ,, – 2 Wassermann ,, negative Flocculation ,, ,, – 1	Wassermann reaction positive Flocculation ,, negative	– 4																
—	Positive																	
—	Wassermann reaction negative Flocculation ,, positive																	
Wass. reaction weak positive Flocculation reaction positive – 1	Wassermann reaction negative Flocculation ,, positive																	
—	Wassermann reaction negative Flocculation ,, positive																	
	Negative																	

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

In the course of this investigation, a survey of the occurrence of the complement-fixation and flocculation reactions has been carried out in animals, specimens of whose blood were easily obtainable for the necessary tests.

The table shows the general results of this survey, the species and the numbers of animals examined. The results refer to adult animals. The sera were heated at 55° C. for the systematic tests.

It will be noted that the lipoidophile reacting substances in the serum are widely distributed among animals, occurring not only in mammalian species but also in birds. Certain species including man, however, are characterised by negative reactions. In some species there is great uniformity in the occurrence of both reactions; in others the general result has been that the flocculation reaction is positive (often strongly positive) while the Wassermann reaction is negative. In certain species there has been considerable diversity among individuals in the results of the two reactions.

Reactions typical of the different species are illustrated in Table II.

Rabbits. While both reactions have been positive in the majority of the adult individuals tested, in a certain proportion the flocculation test was negative while the complement-fixation reaction was positive. In two animals the reverse effect was noted—fixation test negative, flocculation test positive. Thus every rabbit tested showed the presence of reacting substances—evidenced in the vast majority by both reactions. The fixation reaction varied greatly in degree in different individuals. Of course variation in the deviability of different specimens of complement accounted for some of these differences but variation was noted quite distinctly among different animals tested with the same complement. Though in all cases the end-point was not ascertained, in the majority the fixation did not exceed 12 doses of complement allowing for any inhibitory effects of serum and antigen noted in the controls. Where careful titrations were made, the maximum deviation noted was 16 doses. These results contrast with the high degree of fixation usually elicited with sera from cases of active syphilis where over 30 doses of complement are fixed in the method we have used.

The degree of the flocculation reaction also varied considerably, the highest titre being in most cases from 1 in 4 to 1 in 16; occasionally it reached 1 in 32 or 1 in 64.

There was some tendency to correspondence in strength between the two reactions with the same sera—strongly positive Wassermann effects being associated with strong flocculation reactions, but frequently there was no parallelism. As compared with the Wassermann results the flocculation effects were relatively weak. This was in contrast with the behaviour of sera from certain other animals (*e.g.* ox, sheep, *vide infra*).

Comparisons were made with a series of specimens between the complement-fixing power of the serum in the fresh and heated states respectively. In the case of positively reacting human sera, the unheated serum reacts

more strongly than when heated at 55–56° C. The results have usually been the same with rabbit's serum. In some cases both heated and fresh serum showed an equal complement deviation. In two instances the fresh serum gave a weaker but still definitely positive effect as compared with the heated specimen and in one instance the peculiar result recorded by Browning and Mackenzie (1910) was noted: fresh serum reacted negatively while the heated serum was positive. This result has never been observed in the case of any other animal and as it has been so rarely noted, it is impossible to offer any explanation of its occurrence.

Browning and Mackenzie also drew attention to the fact that in the case of tests with fresh serum, when the tubes were allowed to stand overnight, there was progressive lysis even if the readings after a shorter period, from the time of adding the haemolytic system, indicated a positive result. This has been investigated but the lysis progressing overnight has not been more marked than that noted in the diagnostic Wassermann tests with heated positive sera.

Ox and sheep. The results have been uniformly positive with the exception of four specimens of ox serum and one specimen of sheep serum that reacted negatively in the Wassermann test—the flocculation test had not been carried out in these cases. The strength of the complement-fixation effect varied considerably as in the case of the rabbit. In most cases the serum did not fix more than 12 doses of complement. One specimen of ox serum deviated 20 doses of complement. The flocculation reaction was uniformly marked, the end-titre being 1 in 32 or 1 in 64 in most cases.

Horse. The sera tested were uniformly positive in both reactions. The complement deviation was usually under 10 doses. The flocculation results were in all cases marked, the usual end-point being not less than 1 in 32.

Mouse. With one exception, all the specimens tested showed positive Wassermann effects but the results were usually weak—represented by a deviation of only 3–6 doses of complement. A considerable proportion reacted quite negatively in the flocculation test and even those which were positive exhibited a weak reaction.

Among the various species that may be classified as positive reactors, mice showed the reacting property of the serum to a very limited degree.

White rats. The tests with the serum of this species have elicited interesting results. In general the heated serum reacted quite negatively in both tests but in a few instances the Wassermann reaction was not frankly negative and partial inhibition of lysis occurred with 2 and 4 doses of complement though negative control sera showed complete lysis with 2 doses. A number of sera (15) were tested in the flocculation reaction, both in the fresh and heated state, with the result that the fresh sera showed a weak reaction (end-titre 1 in 4 or 1 in 8) while the heated serum proved quite inactive. This result is, of course, contrary to what has been noted with human positive sera, viz. that heating at 54–56° C. tends to increase the activity (Mackie,

1923; Watson, 1925) possibly by annulling some inhibitory principle in the serum. Such results with rat's serum suggest that this species is not devoid of lipoidophile reacting substances though a definite Wassermann reaction is uniformly absent. It is interesting to note, however, that the active principle is completely thermolabile at 55° C.

Guinea-pig. As we have noted above, it must be apparent that the serum of this animal in the fresh condition lacks the power of reacting with the Wassermann antigen. A large number of specimens of heated guinea-pig's serum have been tested in the Wassermann and flocculation reactions with negative results in all cases. Fresh serum, unlike the serum of the white rat, also failed to yield a flocculation effect.

Pig. The majority of specimens from these animals exhibited a striking dissociation of the Wassermann and flocculation reactions, the former reaction being quite negative while the latter was strongly positive (end-titre 1 in 32 or 1 in 64). This result is of interest in showing how the flocculation reaction may occur independently of the Wassermann reaction. In certain specimens, however, the Wassermann reaction was also positive, the deviation varying from 5 to over 12 doses of complement. In one animal both reactions were negative. According to Friedemann (1910) pig's serum fails to react in the Wassermann test.

The blood specimens were obtained at an abattoir and the animals were about 6 to 7 months old, the usual age at which these animals are slaughtered. Older animals have not been examined.

Cat. Only one of the twelve sera examined showed a positive result in both tests. In five cases the flocculation reaction was positive but the Wassermann effect was quite absent. Six specimens reacted negatively in both reactions but one of these tested for the flocculation reaction in the unheated state gave a weakly positive result.

Dog. Four of the seven sera examined yielded a positive complement-fixation reaction with a negative result in the flocculation test. Two specimens were positive and one negative in both reactions. The positive reactions were well marked.

In the cat and dog, dissociation of the two reactions has been a distinct feature of the results.

Monkey. The serum of a *Macacus rhesus* was strongly positive in both reactions.

Pigeons and fowls. The results obtained were generally similar to the typical reaction of pig's serum: the Wassermann effect was absent while the flocculation reaction was strongly positive (end-titre 1 in 32 to 1 in 64).

The serum of a duck gave a similar result.

In one instance (fowl) both reactions were positive.

Frog. Six specimens were examined with quite negative results in both tests.

Table II.

Showing typical results with animal sera in the Wassermann and flocculation tests.

Animal (Unless where otherwise indicated, result refers to serum heated at 55° C.)	Wassermann reaction Doses of complement deviated*	Flocculation test. Dilution of serum (Number of + marks denotes degree of flocculation) Readings after 36 hours					
		1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64
Rabbit 425	10	+ + + +	+ + +	+	−	−	−
,, 442	6	−	−	−	−	−	−
,, 436	12	+ + + +	+ + +	−	−	−	−
,, I unheated	18	+ + +	+ + +	+ + +	+ +	−	−
,, ,, 55°	11	+ + + +	+ + +	+ +	+	−	−
,, II unheated	11	+ + +	+ + +	+ +	−	−	−
,, ,, 55°	11	+ + +	+ + +	+ +	−	−	−
Ox 25/11	11	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
,, 11/11	11	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+
Sheep 25/11	11	+ + + +	+ + + +	+ + + +	+ + +	+ +	−
,, 30/1	8	+ + + +	+ + +	+ + +	+ +	+ +	−
Horse 15/1	10	+ + + +	+ + +	+ + +	+ +	+	−
,, 5/2	6	+ + + +	+ + + +	+ + +	+ + +	+ +	+ +
Mouse 7/11	3	−	−	−	−	−	−
,, 24/11	6	+ + +	+ +	+	−	−	−
White rat 1 unheated	.	+ + + +	+ + +	+ +	−	−	−
,, 55°	Nil	−	−	−	−	−	−
,, 3 unheated	.	+ + +	+ +	+	−	−	−
,, 55°	Nil	−	−	−	−	−	−
,, 5 unheated	.	+ +	+ +	−	−	−	−
,, 55°	Nil	−	−	−	−	−	−
Pig 1	,,	+ + +	+ + +	+ + +	+ +	+	−
,, 5	,,	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ +
,, 12/9 IV	11	+ + + +	+ + + +	+ + +	+ +	+	−
Cat 11/9	Nil	+ +	+	−	−	−	−
,, 17/11	,,	+	+	−	−	−	−
,, 13/12	7	+ +	+ + +	+ + +	+ + +	+ + +	†
Dog 2/7	10	−	−	−	−	.	†
,, 19/12	11	+ + +	+ + +	+ + +	+ + +	+	†
Macacus rhesus	12	+ + + +	+ + +	+ + +	+ +	+	−
Pigeon 17/11	Nil	+ + + +	+ + +	+ +	+ +	+	−
Fowl 3/6	,,	+ + + +	+ + + +	+ + +	+ +	+ +	+
Duck	,,	+ + + +	+ + + +	+ + +	+ +	+	†

* Allowing for any deviation produced by the serum or antigen alone in the respective control tests.

† Not tested.

The absence of the reactions in young animals.

It was observed that, while the serum of adult cattle, sheep and rabbits were, with few exceptions, positive in both reactions, the serum from calves (about 6–8 weeks old), lambs (about 3–4 weeks) and rabbits (3–4 weeks) generally reacted negatively. Among the young animals tested (10 calves, 9 lambs, 18 rabbits), however, one specimen of calf's serum showed a weakly positive flocculation reaction associated with a negative Wassermann reaction. It has also been shown by Wendt (1925) that while the majority of adult cattle yield positive reactions, the majority of calves are negative.

In the case of the young rabbits examined, the mothers' sera yielded strongly positive Wassermann reactions.

With a view to tracing the development of the reacting substances, litters of rabbits were examined at intervals. In litter 1, the first appearance of the

reacting principle was noted when the sera were examined on the 55th day. The reactions were well developed on the 69th day though more marked still by the 105th day (Table III).

The question as to whether this development of lipoidophile substance was parallel with that of the natural antibody for sheep's red corpuscles was investigated in a second litter (Table IV). The technique of titrating the natural haemolysin was that described by Mackie (1925).

Table III.

Litter 1.

Age in days	Wassermann reaction Complement doses deviated				Flocculation test End-titre of reaction			
	25	55	69	105	25	55	69	105
Rabbit 1	–	3	10	>10	–	1 in 2	1 in 32	1 in 64
2	–	–	6	>10	–	–	1 in 8	1 in 32
3	–	–	10	>10	–	–	1 in 16	1 in 64
4	–	–	10	>10	–	–	1 in 16	1 in 64
Mother	>10				1 in 64			

Table IV.

Litter 2.

Age in days	Wassermann reaction Complement doses deviated					Flocculation test End-titre of reaction				Natural I.B. *v.* sheep's R.C. M.H.D. for 0·5 c.c. 1·5 % R.Cs suspension. (c.c.)			
	27	73	88	96	101	27	73	88	96	27	73	88	96
Rabbit 1	–	>10	.	.	9	–	1 in 8	.	1 in 8	No lysis with 0·5 c.c. serum	>0·5	.	0·3
2	–	>10	.	.	16	–	1 in 16	.	1 in 16		0·1	.	0·025
3	–	8	.	12	.	–	1 in 8	.	1 in 16		0·5	.	0·1
4	–	>10	15	.	.	–	1 in 8	1 in 16	.		0·5	0·3	.
Mother	10					–				0·05			

The natural lipoidophile principle observed in the serum of adult animals is therefore apparently absent in very young animals, but, as evidenced in the case of the rabbit, appears at an early stage and is progressive in development. In rabbits, absent at 3–4 weeks, it appears in 8–10 weeks. The same holds also for the natural antibody for sheep's red corpuscles, and this is highly suggestive of the analogy between these lipoidophile reacting substances and natural antibodies.

Thermostability of the reacting substances in animal sera as compared with human syphilitic serum.

It has been stated that the Wassermann substance of rabbit's serum is more thermolabile than in the case of human syphilitic serum (Griffith and Scott, 1920).

For testing thermostability the sera were diluted 1 in 4 with saline to obviate coagulation above 60° C. and specimens heated at varying temperatures were examined simultaneously.

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

In the first specimen of rabbit's serum tested, a distinct weakening of the Wassermann reaction occurred at 60° C. and the effect was completely annulled at 70° C.

Rabbit's serum	Wassermann reaction Doses of complement deviated
55° C. 30 minutes	>10
60 ,,	7
70 ,,	0

A similar result was obtained with a specimen of human syphilitic serum:

Syphilitic serum	Wassermann reaction Doses of complement deviated
56° C. 30 minutes	>10
60 ,,	10
65 ,,	6
70 ,,	0

In another specimen of rabbit's serum the Wassermann reaction was completely annulled at 60° C. though the flocculation reaction was only abolished between 60° C. and 70° C. The flocculating property in rabbit's serum seemed to be more resistant to heat than the Wassermann substance.

Rabbit's serum (462)	Wassermann reaction Doses of complement deviated	Flocculation reaction End-titre
55° C. 30 minutes	10	1 in 16
60 ,,	0	1 in 8
70 ,,	0	—

In a further specimen subjected to a series of increasing temperatures above 55° C., weakening of both reactions occurred at 57·5° C., was more marked at 60° C. and the Wassermann effect was annulled at 62·5° C. The flocculating principle was more stable and was only completely destroyed at 65° C.

Rabbit's serum (453)	Wassermann reaction Doses of complement deviated	Flocculation reaction End-titre
55 °C. 30 minutes	>10	1 in 16
57·5 ,,	> 8	1 in 16 (but degree of reaction less)
60 ,,	8	1 in 8
62·5 ,,	0	1 in 4
65 ,,	0	—

A specimen of horse serum and another positive human serum were tested in the same way *simultaneously* with this sample of rabbit's serum. The horse serum showed marked thermolability of the Wassermann principle which was completely inactivated at 57·5° C. though the flocculation reaction was only annulled at 62·5° C.

Horse serum (28/3)	Wassermann reaction Doses of complement deviated	Flocculation reaction End-titre
55 °C. 30 minutes	8	1 in 32
57·5 ,,	0	1 in 16
60 ,,	0	1 in 8
62·5 ,,	0	—

The syphilitic serum showed inactivation of the Wassermann substance at 60° C. and of the flocculating principle at 62·5° C.

Syphilitic serum	Wassermann reaction Doses of complement deviated	Flocculation reaction End-titre
55 ° C. 30 minutes	8	1 in 16
57·5 „	4	1 in 4
60 „	0	1 in 4
62·5 „	0	—
65 „	0	—

As in the other sera, the Wassermann reaction was annulled at a lower temperature than the flocculation reaction. In these experiments the rabbit's serum was more stable than the human syphilitic serum; on the other hand the horse serum was most labile. It seems doubtful whether stress can be laid on such differences. It is probable that slight variations in thermostability occur with different specimens of serum. A striking result of these tests has been the difference of the two reacting properties in their degree of thermostability.

Persistence of Wassermann reaction in animals and the influences that affect the reaction.

It has been stated by Fürst (1914) that rabbit's serum may react positively and negatively at different times though Dohi (1911) indicates that variations in the reaction are slight. In testing for the occurrence of fluctuation where specimens of serum are examined at different times with different complements, variation in the deviability of complement may give an appearance of fluctuation.

We have investigated this question by bleeding rabbits at weekly intervals during 3 or 4 weeks, separating the serum and, after heating for 1 hour at 55° C., storing in sealed quill-tubes in a refrigerator and finally, after again heating at 55° C. for 30 minutes, testing all the specimens simultaneously with the same complement. The samples of blood taken were about 2·5 c.c. in amount. Under these conditions the Wassermann substance is remarkably stable and like immune bodies retains its activity for a considerable time in stored serum. There has been no instance of a serum reacting positively at one time and negatively at another. In fact in normal rabbits the results have been remarkably uniform in the degree of deviation produced by the serum tested at intervals, though some slight variations have been noted (Table V).

The flocculation reaction tested at weekly intervals (without storage of the serum) also proved exceedingly uniform, the end-titre remaining constant.

As referred to above, Taniguchi has shown that when the natural antibody of the rabbit for sheep's red corpuscles is increased by injection of heterophile antigen, the Wassermann reacting substance may also be augmented, *i.e.* fixation of complement with lipoids from non-heterophile tissues is increased. This is illustrated from our own experiments in Table VI.

Table V.

Day on which blood sample was taken	*Rabbit* 501. Wassermann reaction Doses of complement deviated	*Rabbit* 502. Wassermann reaction Doses of complement deviated	*Rabbit* 414. Wassermann reaction Doses of complement deviated
1	6	5	14
7	6	4	14
15	6	5	16
21	6	5	16

Table VI.

Day of experiment		Concentration of natural haemolysin*	Wassermann reaction Doses of complement deviated
	Rabbit 517.		
1	Injected intraperitoneally on 3 occasions at weekly intervals with emulsified kidney tissue of guinea-pig	3·3	7
28	—	200	>18
	Rabbit 518.		
1	Treated as in case of Rabbit 517	10	9
28	—	500	>18

* Figure denoting concentration of natural haemolysin is the reciprocal of the M.H.D. (See Mackie, 1925.)

Table VII.

Day of experiment		Concentration of natural haemolysin	Wassermann reaction Doses of complement deviated
	Rabbit 430.		
1	Intravenous injection 0·25 c.c. *M*/1000 $BeCl_2$, $4H_2O$	2	6
2	—	20	10
	Rabbit 450.		
1	Intravenous injection 1·0 c.c. Colloidal manganese solution	2	8
7	,,	5	8
13	,,	10	5
17	—	20	8
24	—	40	12
	Rabbit 426.		
1	Intravenous injection of *M*/1000 $MnCl_2$, $4H_2O$	10	7
3	—	20	>9
	Rabbit 477.		
1	Intravenous injection 1 c.c. 5 % Sodium nucleinate	100	13
3	—	200	13
6	Intravenous injection 1 c.c. 5 % Sodium nucleinate	—	—
8	—	200	>17
13	Intravenous injection 1 c.c. 5 % Sodium nucleinate	40	>17
17	—	40	>17

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

A considerable number of experiments have been carried out in which rabbits were subjected to various non-specific agents and the effect on the Wassermann reaction and natural antibody for sheep's red corpuscles noted in parallel tests. In experiments in which manganese chloride, beryllium chloride, colloidal manganese, and sodium nucleinate were used and in which an increase of the natural haemolysin occurred (see Mackie, 1925), there was apparently an associated parallel increase in the Wassermann reagent, but in some instances there was no such correspondence. The apparent augmentation in the Wassermann substance was not so proportionately great as in the case of the natural antibody and certain of the results do not entirely exclude the possibility of the variation being due to natural fluctuation (Table VII).

A marked increase in the Wassermann reaction has been noted in animals during the course of an experimental infection with the bovine tubercle bacillus and the augmentation was still present when the animals were moribund (Table VIII). The flocculation reaction also underwent a parallel increase though to a much lesser degree. That these effects were not due to the presence of heterophile antigen in the organism is shown by the fact that the anti-sheep haemolysin was not increased.

Table VIII.

Day of experiment		Concentration of natural haemolysin	Wassermann reaction Doses of complement deviated	Flocculation reaction End-titre
	Rabbit 498.			
1	Injected intravenously with culture of *B. tuberculosis*, bovine type	10	5	Not tested
7	—	10	4	,,
13	—	10	>14	,,
20 (moribund)	—	10	>14	,,
	Rabbit 524.			
1	Injected with *B. tuberculosis* as in case of Rabbit 498	20	6	1 in 8
8	—	10	8	1 in 8
14	—	<5	12	1 in 8
19 (moribund)	—	20	>16	1 in 16
	Rabbit 525.			
1	Injected with *B. tuberculosis* as in case of Rabbit 498	Not tested	6	1 in 4
8	—	,,	12	1 in 8
14	—	,,	14	1 in 16
18 (moribund)	—	,,	8	1 in 2

It has not been possible to augment the Wassermann substance by any of the non-specific agents we have used to a degree comparable with the marked reactions in active syphilis.

According to Dohi (1911), starvation may annul the Wassermann property in animals and subcutaneous injections of nucleic acid exert a similar

effect. It has been noted in our own investigation of the Wassermann reaction in animals, that the reacting power of the serum may be markedly reduced and even abolished by subjecting animals to certain chemical influences. Such effects have not, however, been systematically studied and in the experiments quoted the chemical substances used were not specially selected with a view to producing the result which they illustrate.

In the case of a rabbit injected subcutaneously with turpentine with the object of producing aseptic inflammation, there was a progressive decrease in the Wassermann substance paralleled by a decrease (after a preliminary augmentation) in the natural antibody for sheep's red corpuscles.

Rabbit 445.

Day of experiment		Concentration of natural haemolysin	Wassermann reaction Doses of complement deviated
1	Subcutaneous injection of 8 c.c. 2 % turpentine in olive oil	3·3	9
3	—	5	5
11	—	2	5
19	—	2	3

It has been found possible in a *series* of experiments to abolish the reaction in rabbits by successive subcutaneous injections of alcohol, and the natural haemolytic antibody also underwent a corresponding decrease.

Rabbit 487.

Day of experiment		Concentration of natural haemolysin	Wassermann reaction Doses of complement deviated
1	Subcutaneous injection of 6 c.c. alcohol dil. with 2 vols. of saline	5	10
2	—	.	.
3	—	.	.
4	—	.	.
7	—	<1	0

The general health of these animals was not markedly affected and the experiments have all yielded striking results such as that in Rabbit 487.

β-tetra-hydro-naphthylamine injected subcutaneously with the object of producing a pyrexial reaction also brought about a marked weakening of both the Wassermann substance and the natural haemolytic antibody.

Rabbit 473.

Day of experiment		Concentration of natural haemolysin	Wassermann reaction Doses of complement deviated
1	1 c.c. 5 % β-tetra-hydro-naphthylamine subcut.	40	>11
3	—	5	2

Intravenous injection of glucose (2 c.c. of a 10 per cent. solution) effected a parallel reduction in the Wassermann substance and the anti-sheep haemolysin. Salvarsan had no influence on the Wassermann reaction though it

apparently increased the concentration of the natural antibody (see Mackie, 1925). Anaesthesia by chloroform or ether did not alter the strength of the Wassermann and flocculation reactions in rabbits.

These results were apparently due to some effect on the antibody producing function. It seems possible that various poisonous chemicals even in sublethal doses might produce similar effects. The parallelism between the effect on the Wassermann reacting substance and the natural antibody is further evidence of the analogy between the former principle of serum and natural antibodies.

The analysis of the Wassermann reaction by carbon-dioxide fractioning of the serum.

This method, which was originally used by Liefman (1909) for the "splitting" of serum-complement, has previously been applied by one of us in the analysis of the Wassermann and flocculation reactions of human syphilitic serum (Mackie, 1923). It depends on the precipitation of part of the globulin by passing carbon-dioxide through serum diluted 1 in 10 with ice-cold distilled water. The technique followed was in accordance with Liefman's original method. Though it cannot be claimed that this method separates different protein entities, the subdivision of the complementing principle that results, even though it is variable and frequently only a partial separation, has been a striking serological phenomenon. It was shown by Browning and Mackie (1914), in studies of guinea-pig's serum, that the fractioning of complement depends not only on a precipitation (by carbon-dioxide) of the euglobulin but also on the "splitting" of the pseudoglobulin into two moieties—a carbonic-acid-insoluble and -soluble respectively. It was noted by Mackie in a series of syphilitic sera that the Wassermann substance is contained in both fractions of serum separated by the carbon-dioxide method but to a lesser extent in the soluble than the insoluble fraction. As shown by salting out with ammonium sulphate, the whole activity of the serum was resident in the globulins (both euglobulin and pseudoglobulin) and the albumin was inactive.

A further study of syphilitic sera fractioned by the carbon-dioxide method was carried out for purposes of comparison with animal sera.

For convenience of description, the carbonic-acid-insoluble and -soluble fractions are designated the *A* and *B* fractions respectively.

It was found that in syphilitic sera the *A* fraction is more active quantitatively than the *B* fraction. In fact in a weakly reacting serum the *A* fraction may represent the whole activity of the serum, the *B* fraction being practically inactive. On the other hand, if a strongly reacting serum is fractioned, the *B* fraction shows marked activity though to a lesser degree than the other. Apparently as the serum increases in the strength of its reaction, the "distribution" of the active principle in the serum proteins is extended to the carbonic-acid-soluble pseudoglobulin. This is illustrated in Chart 1. The

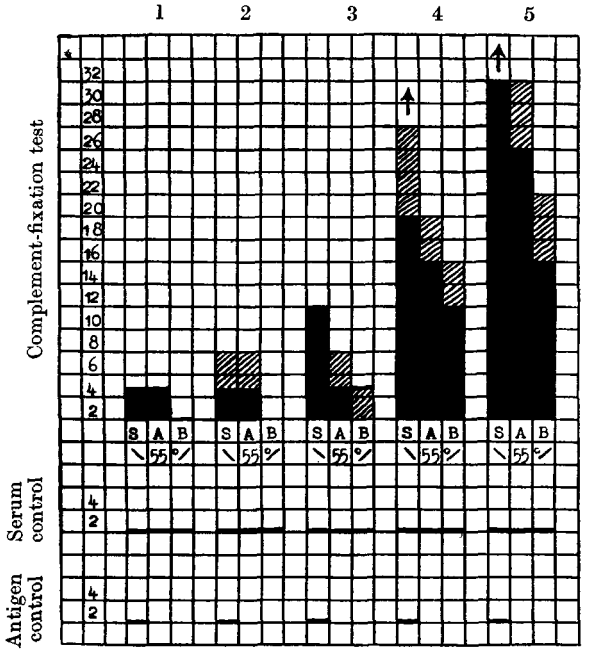

Chart 1. Syphilitic sera.

In this and subsequent Charts:—

S = whole serum.
A = CO_2 – insoluble fraction.
B = CO_2 – soluble fraction.
Column of numbers refers to doses of complement tested.

↑ = no end-point reached.
□ = complete lysis.
▨ = partial lysis.
■ = no lysis.

Note.—Though not shown in the Charts, known negative sera were included in the sets of tests from which the illustrations are drawn, the negative sera showing complete or almost complete lysis with 2 M.H.D. in the fixation test.

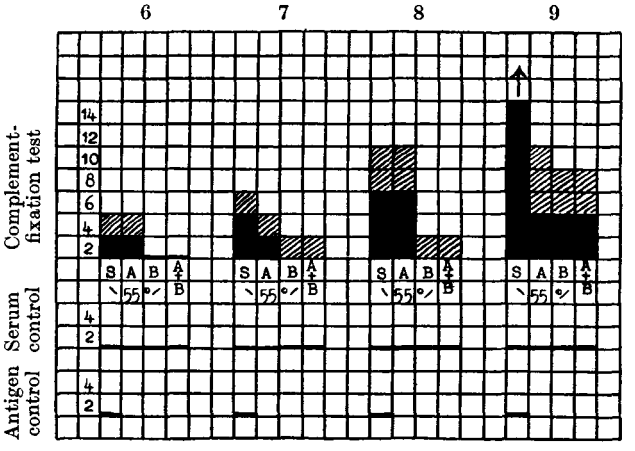

Chart 2. Syphilitic sera.

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

amounts of the serum fractions tested were those derived from the test quantity of whole serum, *i.e.* 0·05 c.c.

A somewhat anomalous result obtained was that the combination of the *A* and *B* fractions usually failed to restore the full activity of the serum and the effect of the two fractions acting together was equal quantitatively only to that of the *B* fraction (Chart 2). In some cases also the sum of the deviation produced by the two fractions acting independently was apparently greater than that of the whole serum.

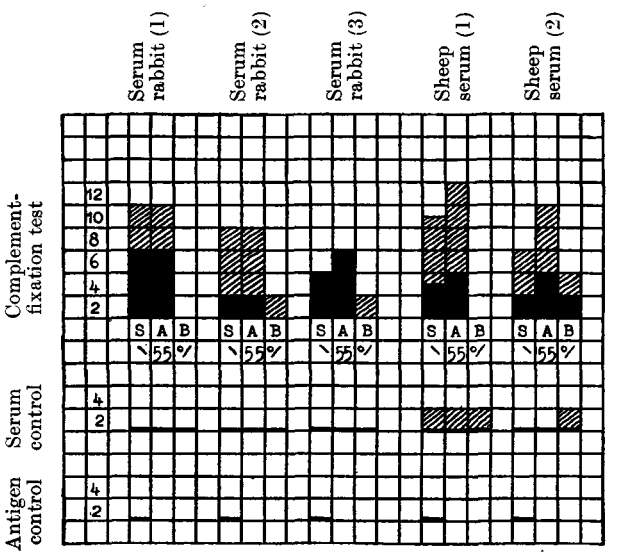

Chart 3 A.

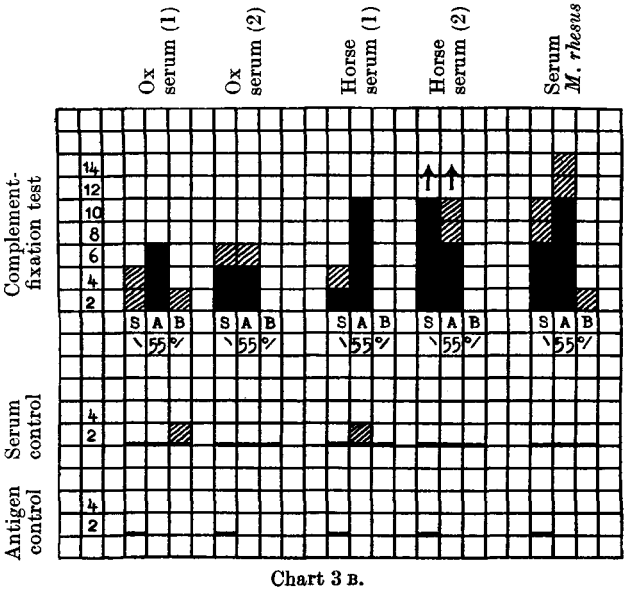

Chart 3 B.

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

In the case of the animal Wassermann reaction (rabbit, ox, sheep, horse, *M. rhesus*), the *A* moiety contains practically all the reacting substance and in the majority of instances is more active than the whole serum (Charts 3 A and 3 B). This peculiar augmented effect of the *A* fraction has never been observed in testing *heated* syphilitic serum. In animals it does not depend on any greater anti-complementary effect of the fraction as compared with whole serum.

According to Sachs and Georgi (1923), treatment of rabbit's serum with dilute hydrochloric acid and the removal of the precipitated globulin abolishes the normal Wassermann effect but only weakens the reaction associated with experimental syphilis. By this method a portion of the globulin is removed as in carbon-dioxide precipitation. Since the reacting power of normal rabbit's serum resides in this fraction of the serum, its removal therefore annuls the reaction. We have used Sachs and Georgi's method with normal rabbit's serum and have noted that the fluid, after removal of the globulin precipitate, is inactive while in the case of strongly reacting human sera the fluid is still active though less so than the whole serum owing to the fact that in this case the precipitate does not contain all the active substance. Though we have not investigated the serum of syphilitic rabbits, the results of Sachs and Georgi's method applied comparatively to normal and syphilitic rabbits might thus be explained on the assumption that in infected animals the Wassermann substance is increased and corresponds in its distribution in the serum proteins to that observed in human syphilitic serum. It seems doubtful if the method serves to demonstrate that the syphilis reaction and the Wassermann effect in normal animals are different properties.

It was noted that if normal human serum and the serum of animals that reacted negatively in the Wassermann test, such as the white rat and guinea-pig, were fractioned and tested (the serum and fractions being heated at 55° C. for 30 minutes), the *A* fraction usually gave a weak fixation reaction, varying in degree from 2 to 6 doses of complement, though the whole serum reacted quite negatively (Chart 4). The addition of the *B* to the *A* fraction abolished this effect. There appeared to be some reacting principle present in the carbonic-acid-insoluble globulin which was masked in the serum by the other proteins.

Even by adding to whole serum the insoluble fraction so as to increase its concentration three-fold in the serum and then testing the product, its activity was still masked.

This effect was not due to any anti-complementary action of the fraction by itself. It was abolished when the fraction was heated at temperatures above 60° C. for 30 minutes and was most marked when the mixtures of antigen and the fraction were allowed to stand at room temperature for half-an-hour before the addition of complement. This result was not invariable but occurred in the vast majority of tests. It appeared to vary with the deviability of the complement used.

In view of the abolition of this effect by heating above 60° C., it seemed possible that it might be more marked in the case of the fresh unheated fraction. Unheated sera and their fractions (normal human, rat, guinea-pig) were therefore tested in the same way. The deviation produced by the *A* fraction was often very marked but some unheated whole sera also exhibited

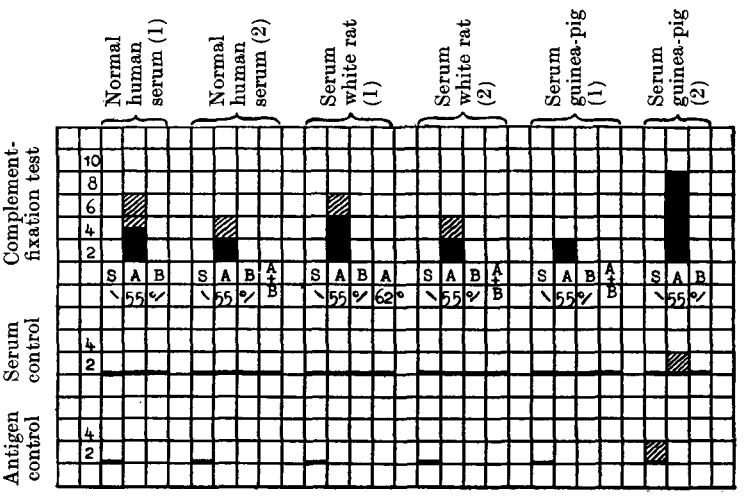

Chart 4.

a deviation reaction though invariably to a lesser degree than the *A* fraction (Charts 5 and 6). The *B* fraction was usually inactive. Again it was found that the *B* fraction exerted a partial or complete masking effect when the two fractions were combined. In the case of guinea-pig's serum, the *A* fraction was frequently anti-complementary by itself but even then the deviation with antigen considerably exceeded this anti-complementary action (Chart 6).

The question arose as to whether the apparent masking effect in the case of the fresh serum might be due to the presence of the complement of the whole serum or of the combined *A* and *B* fractions. It seemed possible that the absence of deviation or the lesser degree of deviation with whole serum as compared with the *A* fraction might be due to the complement in the test mixtures derived from the fresh reacting serum additional to that introduced as the test amount of complement. The *A* fraction would not by itself act as complement representing only the so-called "mid-piece" fraction. To ascertain this, the complement was removed from the serum to be tested by treatment with the sensitised stromata of sheep's corpuscles according to the method described by Browning and Mackie (1913), but sera after absorption of the complement still remained negative and did not assume the activity of the *A* fraction. This was ascertained in the case of normal human, rat and guinea-pig serum.

It might be argued from such results that the Wassermann principle is present normally in low content in the serum of man and those animals which

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

give an apparent negative reaction but in a masked state; that it is present in the *A* fraction but masked by the other proteins; that the partial lability at 55° C. (which is a feature of this substance in syphilitic serum) explains the difference between the heated and unheated *A* fraction. It has been

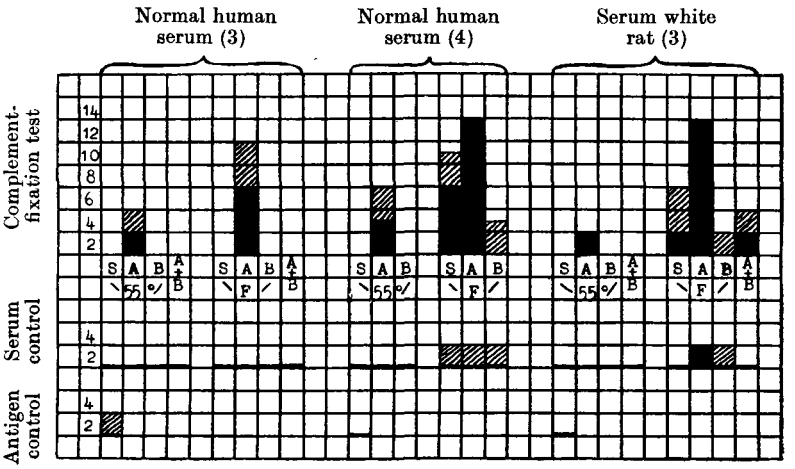

Chart 5.

F = fresh and unheated.

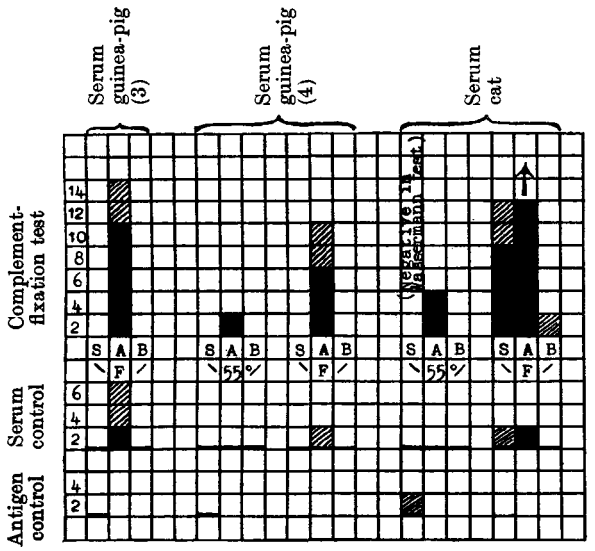

Chart 6.

shown, however, by Browning, Dunlop and Kennaway (1922) that when the concentration of the alcohol in the antigen emulsion exceeds a certain degree, unheated normal human serum may fix complement, often markedly, and thus appear to give a Wassermann effect. The antigen suspension we have used for the tests was a 1 : 12 dilution of the cholesterolised alcoholic extract

(*vide supra*) and the alcohol factor in these results noted with the unheated *A* fraction demanded further investigation.

Parallel tests were therefore carried out in which sera and their fractions were tested, both in the fresh and heated state with (*a*) the usual antigen suspension, (*b*) a suspension of the antigen from which the alcohol had been removed by evaporation, (*c*) a 1 : 12 mixture, of absolute alcohol and normal saline. The "alcohol-free" antigen was prepared in the first experiments by evaporation over a water bath to about two-thirds of the volume and then restoration to the original by the addition of distilled water. In subsequent experiments, to ensure complete removal of the alcohol, evaporation was continued till the volume was reduced to 20 per cent. The results obtained were similar with both preparations. As a result of heating, the lipoids underwent some degree of flocculation but the uniformity of the suspension could be restored by thorough shaking. It was anticipated that the heated emulsion would be inferior in reacting power to the original, owing to the physical alteration produced. Thus the heated emulsion, after restoration to the original volume, showed a markedly diminished turbidity. It is well known that the turbidity of the lipoid suspension is a factor in its antigenic activity.

Heated normal human serum never showed any complement-deviation effect with alcohol substituted for antigen. The *A* fraction after heating also reacted negatively with alcohol except in one experiment in which a weak deviation (2 doses) was noted. On the other hand, fresh normal human serum frequently yielded a definite deviation effect with alcohol which was even more marked and quite constant in the case of the *A* fraction. This effect of both whole serum and the *A* fraction was, however, less than that with the antigen emulsion.

The same results were obtained with the sera of white rats and guinea-pigs.

Thus the apparent Wassermann effects previously noted with the unheated *A* fraction were due in part to complement deviation along with the alcohol of the antigen suspension and not entirely to an interaction with the lipoids. Such complement-fixation by fresh serum and the unheated *A* fraction along with alcohol, however, was less than the corresponding reaction with the antigen suspension. Further, the unheated *A* fraction still reacted with the alcohol-free antigen and its effect was masked in the whole serum.

Chart 7 shows how the total effect of the unheated *A* fraction with the antigen emulsion is contributed to mainly by the alcohol but depends also on the antigen proper. The action of the fresh whole serum with alcohol is less than that with the lipoid antigen, showing that the latter effect is not entirely due to the alcohol in the suspension though the serum is quite inactive with the heated suspension. The effect of the *heated A* fraction with the usual antigen is not paralleled with the alcohol-free suspension. In Chart 8 a similar result is illustrated: the *A* fraction possesses a distinct activity with

alcohol-free antigen, though markedly active with alcohol alone. The same effect in lesser degree is also shown in Chart 9.

Corresponding results have been obtained in a considerable series of similar tests and demonstrate that the carbonic-acid-insoluble fraction of

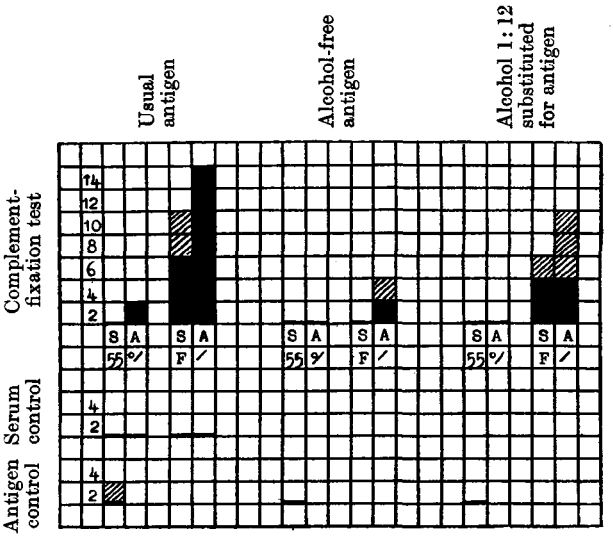

Chart 7. Normal human serum.

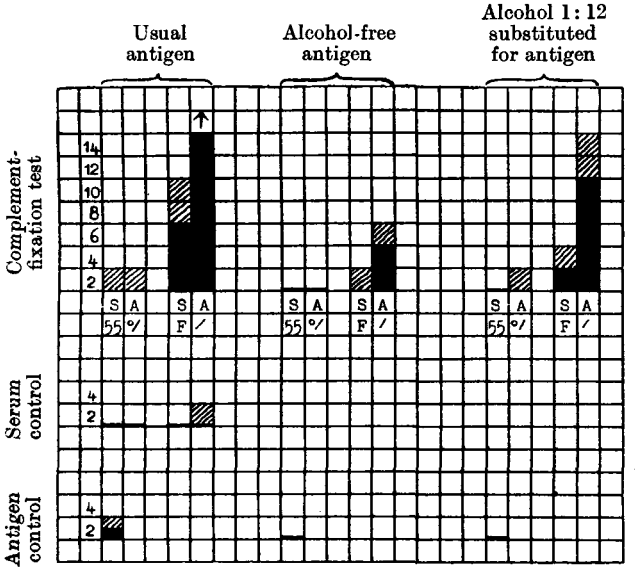

Chart 8. Normal human serum.

normal serum in the fresh state deviates complement along with the lipoid antigen proper, apart from any effect it may have with the alcohol present in the usual antigen suspensions. Further, the deviation produced by fresh

serum along with the usual antigen, when well developed, is greater than that observed with alcohol substituted for the antigen.

It is a phenomenon of some interest, however, from the serological standpoint that normal serum should react with alcohol and produce deviation of complement simulating the specific interaction of antigen and antibody. This property of serum is apparently labile at 55° C. evidenced by its absence in heated normal serum (man, white rat, guinea-pig), and like the principle of normal serum which reacts with the lipoid antigen, is most marked in the carbonic-acid-insoluble globulin and is partially masked by the carbonic-acid-soluble fraction of the serum.

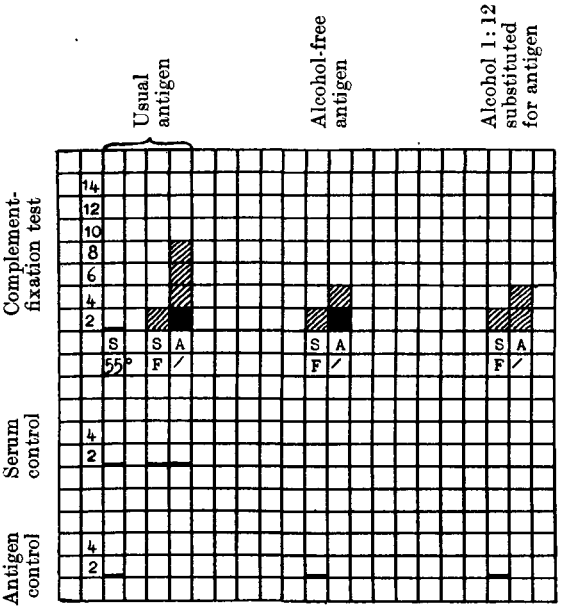

Chart 9. Normal human serum.

The effect is variable in degree, depending on the serum and the complement. In our tests the deviation has varied in the case of whole serum (with a 1 : 12 concentration of alcohol) from nil to 6 doses of complement and in the case of the *A* fraction from 4 to 14 doses. A 1 : 12 concentration of alcohol is not usually anti-complementary by itself. The effect diminishes on decreasing the concentration of the alcohol and while definitely noticeable in the case of whole serum and the *A* fraction along with an alcohol dilution of 1 : 25, it is usually absent with a 1 : 50 dilution. It can be intensified by increasing the concentration of alcohol above that corresponding to the antigen (1 : 12) but the higher concentrations tend to be markedly anti-complementary by themselves.

While heated normal human serum did not produce complement deviation when alcohol 1 : 12 was substituted for antigen and the same generally applied to the heated *A* fraction (*vide supra*), it was found that the heated *A* fraction

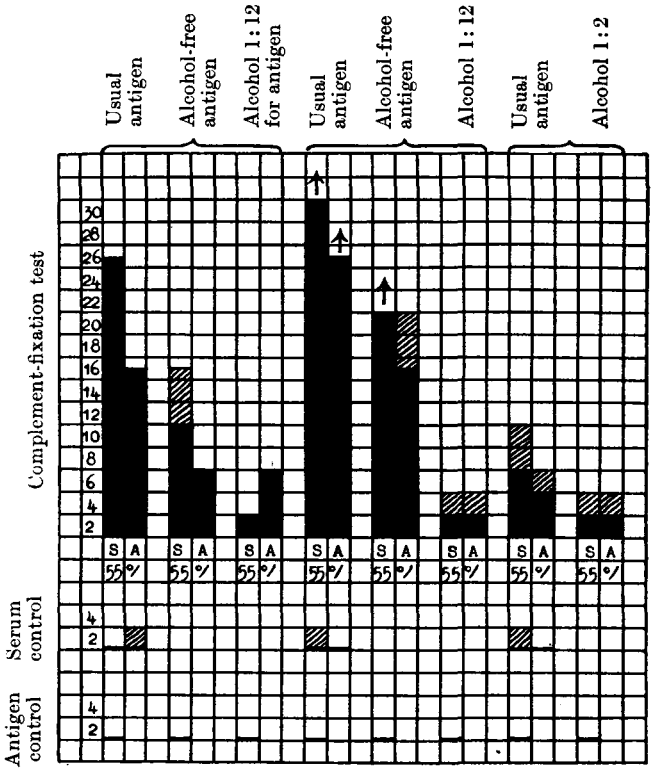

Chart 10. Syphilitic sera.

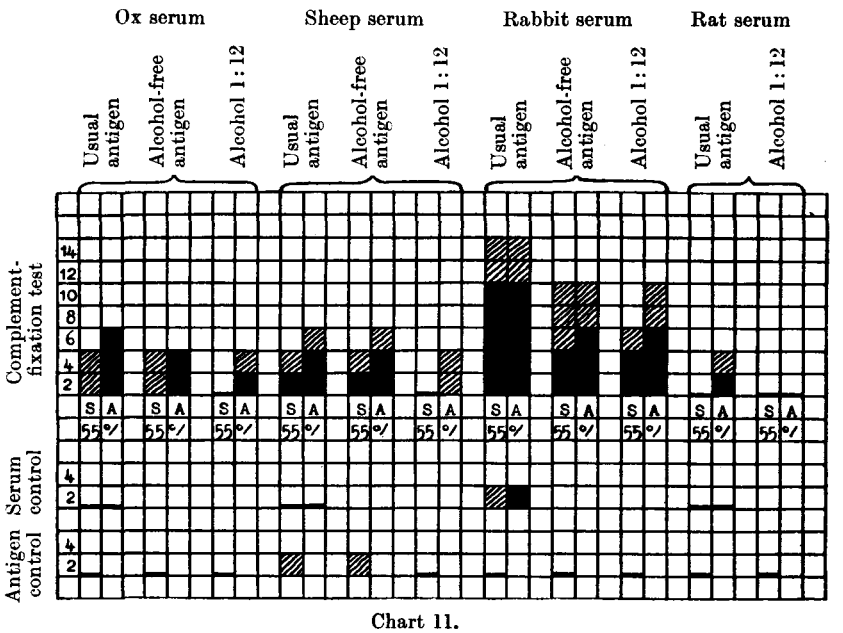

Chart 11.

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

of syphilitic sera reacted with alcohol and in many cases the whole serum also reacted similarly though usually to a lesser degree (Chart 10). This was also true for the *A* fraction and in some cases the whole serum of animals that yielded a positive result in the usual Wassermann test (Chart 11).

Positive human sera showed a much weaker Wassermann effect with the alcohol-free antigen preparations than with the usual antigen. The weakening of the antigen was more than could be accounted for by the withdrawal of the alcohol: the reacting power of the suspension was probably reduced to some extent as a result of heating (*vide supra*).

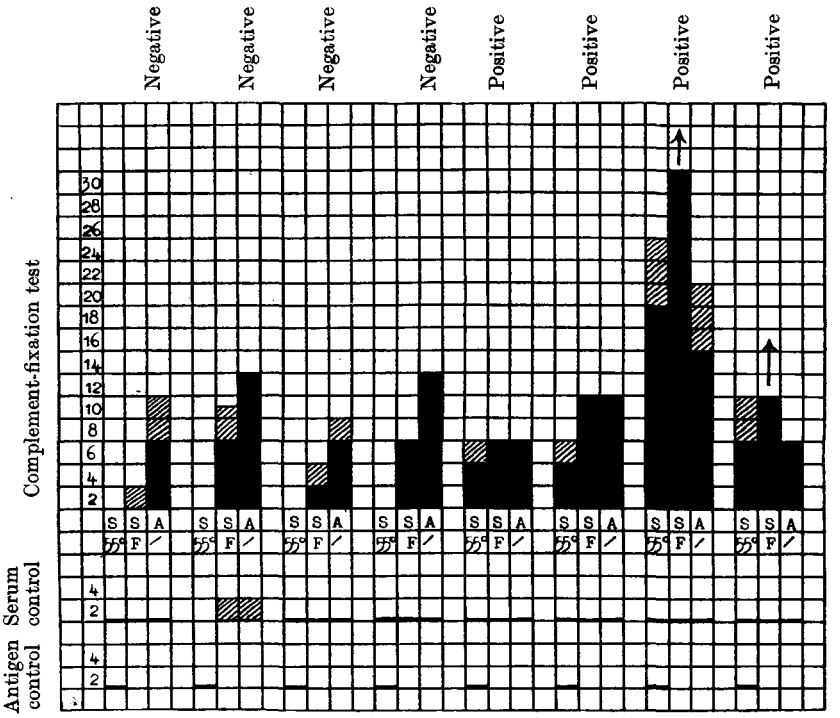

Chart 12. Human sera.

Positive animal sera also showed a weaker effect with the alcohol-free antigen. It was found that the heated *A* fraction of these sera yielded a relatively marked deviation reaction when alcohol was substituted for antigen and that in some cases (particularly in rabbits) the heated whole serum acted in the same way though usually to a lesser extent than the fraction (Chart 11). Thus the alcohol factor may apparently play a considerable part in the Wassermann reaction of normal animals (even when heated serum is used). In rabbits the deviation of the heated serum and of the *A* fraction with alcohol may be almost equal to that with the alcohol-free antigen (Chart 11).

An interesting difference was elicited between Wassermann-positive and -negative human sera when analysed by the carbon-dioxide method, the

whole serum and the *A* fraction being tested in the unheated state. In the negative serum the *A* fraction is more active than the whole serum (*i.e.* there is a partial masking of the effect of the *A* fraction by the other proteins) whereas in the positive serum this masking is absent and the *A* fraction is less active than the whole serum or at the most equal to the whole serum. This difference, illustrated in Chart 12, depends apparently on the absence of any masking of the reacting property in positive sera and on the relative predominance in the negative sera of the reaction with the alcohol of the antigen suspension, which is specially marked in the *A* fraction and partially masked in the whole serum (see Charts 7 and 8). This is obscured in positive sera by the reaction of the whole serum with the lipoid reagent proper. The augmented effect of the heated *A* fraction of positive animal sera as compared with the whole serum (*vide supra*) might also be similarly explained—the alcohol factor being relatively marked in this case even with the heated fraction.

THE ANALYSIS OF THE FLOCCULATION REACTION OF ANIMAL SERA BY CARBON-DIOXIDE FRACTIONING.

It was shown by this method that the principle responsible for the flocculation reaction in strongly reacting syphilitic sera is associated mainly with the carbonic-acid-soluble globulins and that in sera after some days from the withdrawal of the blood the insoluble fraction may exert an inhibitory effect on the activity of the other (Mackie, 1923).

The dissociation of the two reactions has been strikingly demonstrated in certain of the tests, already recorded, with animal sera. Thus in the pig, pigeon and fowl the flocculation reaction is usually well marked while the Wassermann effect is entirely absent. Examples have also been quoted where the flocculation effect is absent though the Wassermann reaction is positive (*vide* Table II). Though the two reactions are so frequently parallel, even in animals that usually react positively in both tests, there may be complete dissociation and one reaction is positive while the other is negative.

Parallel flocculation and Wassermann tests have been carried out with the carbonic-acid fractions of animal sera in a number of experiments. The general result has been that the flocculating principle (like the Wassermann substance) resides almost entirely in the *A* fraction (rabbit, ox, horse, pig—Chart 13). Exceptions have been noted in single specimens of horse and sheep serum and a result similar to that recorded in human serum was observed, *i.e.* the carbonic-acid-soluble fraction (*B*) contained practically all the flocculating property though devoid of action in the Wassermann test.

It should be noted that the complement-fixation produced by the fresh and heated *A* fractions of "negative" sera (human and animal) was not paralleled by a corresponding flocculating effect, unless in two specimens of rat's serum and one specimen of cat's serum which in the heated state reacted

negatively in the usual Wassermann test. In these exceptional instances the *A* fraction (heated) gave a definite but weak flocculation reaction though the whole serum was inactive.

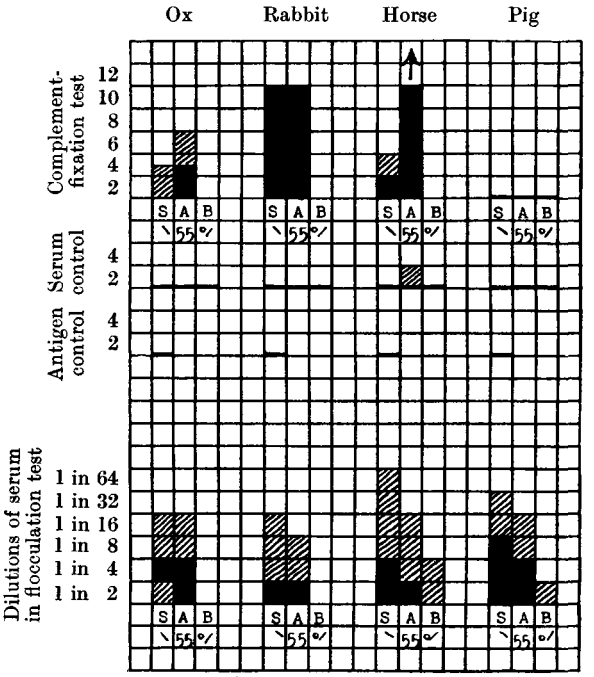

Chart 13.

No flocculation.
Partial flocculation.
Complete flocculation.

Note.—The flocculation effects have been charted in this manner only for convenience in illustrating the action of the serum and fractions along with the Wassermann effects.

THE BEHAVIOUR OF THE CARBON-DIOXIDE FRACTIONS OF HUMAN SYPHILITIC SERA IN THE FLOCCULATION TEST.

A certain amount of variation has been encountered, though the general result has been that the soluble fraction (*B*) is the more active in the flocculation reaction; the insoluble moiety (*A*) is more active in the Wassermann test. In some cases the whole flocculating activity of the serum is resident in the *B* fraction and the *A* fraction is inactive. On the other hand the separation may be less apparent, the insoluble fraction showing a certain amount of activity and in exceptional cases being even more active than the *B* fraction. Analogous variations have been noted by Browning and Mackie in the "splitting" of serum complement by the same method and it was shown that they were independent of technique (Browning and Mackie, 1925). Such variations depend either on a variable distribution of the reacting

https://doi.org/10.1017/S0022172400017319 Published online by Cambridge University Press

substances in the serum proteins, variable adsorption of these substances by the globulin precipitate or a variable splitting of the proteins. These results, in spite of their variability, show how in syphilitic human serum (as occasionally noted in animal serum) the Wassermann and flocculating properties can be partially dissociated (Chart 14).

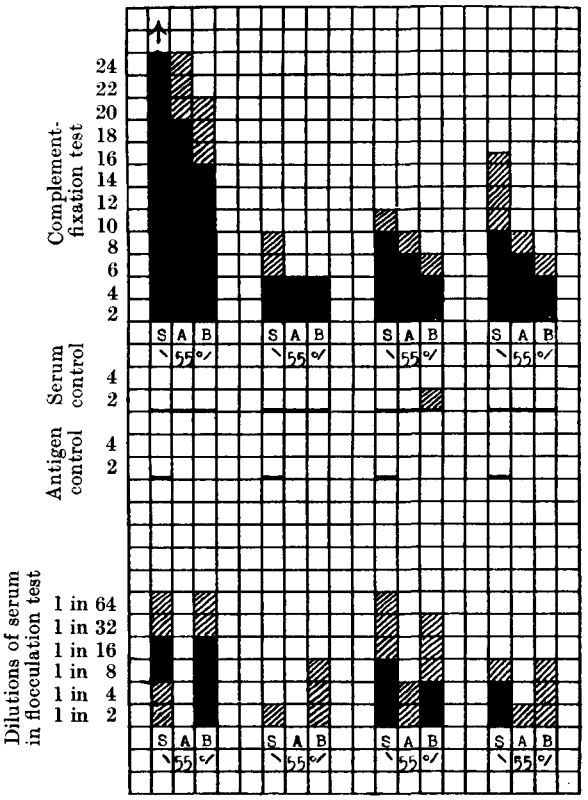

Chart 14. Syphilitic sera.

SUMMARY AND CONCLUSIONS.

The following summarises the findings elicited from the investigation, the significance we attach to the results and the conclusions we have drawn:

A careful study has been made of the reputed Wassermann reaction exhibited by the serum of certain normal animals with a view to throwing light on the nature of the reacting substance or principle in syphilitic serum.

The heated (55° C.) serum of various normal adult animals may fix complement along with the antigen used in the Wassermann test, *e.g.* rabbit, ox, sheep, horse, mouse, cat, dog, macacus, pig.

The complement-fixation reaction may be associated with the flocculation effect similar to that of syphilitic serum.

These reactions apparently represent a natural or normal property of the serum and do not appear to depend on any pathological condition.

Certain species, including man, are characterised by negative reactions, *e.g.* white rat, guinea-pig, frog.

The serum of the white rat in the unheated state, however, yields a definite but weakly positive flocculation reaction which is annulled on heating the serum to 55° C., showing that this species is not devoid of the particular principle. In some species there is great uniformity in the occurrence of both reactions (*e.g.* rabbit, ox, sheep, horse). Such uniformity has led us to regard the reaction as a natural one. In other species there is less regularity and both reactions in certain individuals may be quite negative. In certain animals there may be dissociation of the two reactions: the Wassermann reaction may be negative while the flocculation effect is positive (*e.g.* pig, pigeon, fowl, cat): the reverse may occur, the Wassermann reaction being positive, the flocculation test negative (*e.g.* dog, mouse, rabbit).

Certain positively reacting species are characterised by uniformly weak reactions, *e.g.* mouse.

The Wassermann reaction in normal animals is always limited in degree, contrasting in this respect with the very marked effects obtained with the serum from cases of active syphilis.

In species in which adult animals exhibit positive reactions, very young animals, *e.g.* up to 3–8 weeks of age, react negatively. The reacting power (observed in rabbits) is thereafter progressive in development and is parallel with the development of a natural antibody (anti-sheep haemolysin). This parallelism supports the view that these reactions are due to antibody-like principles in the serum.

No constant difference in thermostability of the reacting substances can be established between normal animal serum and the serum in syphilis. The degree of thermostability seems to vary slightly among individuals. The flocculating property is more stable than the complement-fixing principle both in animals and in syphilis.

These reactions in normal animals seem to be homologous with the corresponding reactions in syphilis. Our observations suggest that the syphilis serum reactions are due to antibody-like substances homologous with antibodies natural to certain species and widely distributed among animals.

The Wassermann and flocculation reactions in individual rabbits tested at intervals are relatively constant.

Marked augmentation of the Wassermann reaction has been produced experimentally in rabbits: (*a*) by immunisation with heterophile antigen, as originally shown by Taniguchi; (*b*) by experimental infection with *B. tuberculosis*. The effects of other non-specific agents have also been studied but marked alterations have not been observed.

Repeated subcutaneous injection of alcohol in rabbits abolishes the Wassermann property. The injection of certain other chemical substances

also produces a weakening influence. Parallel effects are observed on the anti-sheep haemolysin.

An analytical study has been made of the Wassermann and flocculating substances in the serum of normal animals and human syphilitic serum. For this purpose carbon-dioxide fractioning of the serum has been used. The results have been recorded and differences have been elicited in the behaviour of the serum fractions between normal animals and human syphilitics. These differences probably depend on the total content of the reacting substances in the serum and their distribution in the serum fractions.

The carbonic-acid-insoluble fraction of normal human serum and the serum of negatively reacting animals even after heating at 55° C. may yield a weak complement-fixation effect with the Wassermann antigen. This property is "masked" in whole serum. It is lost on heating above 60° C. and is more marked in the unheated fraction.

The deviation effect produced by the unheated fraction is contributed to by an independent complement-deviation reaction with the alcohol present in the antigen suspension but is still manifest with alcohol-free antigen preparations, showing it is partly a true reaction with the lipoids proper. This effect of the carbonic-acid-insoluble fraction has not been paralleled in the flocculation test unless in certain exceptional instances.

These observations seem to indicate that the principle in serum responsible for the Wassermann reaction is present normally in minimal amount and in a masked state even in those animals which are negative reactors when tested in the usual way. It might be supposed, therefore, that the diagnostic reaction is due to the non-specific augmentation of this natural antibody-like substance.

The influence of various non-specific agents and different infective conditions on the content of the reacting substances in the serum of normal animals requires further investigation.

The complement-deviation reaction of fresh serum with diluted alcohol (substituted for antigen) has been studied in animals and man. The fact has been elicited that the heated carbonic-acid-insoluble fraction of the serum of animals (positive in the usual Wassermann test) and of syphilitic sera yields, along with diluted alcohol, a definite though weak complement-deviation reaction. This may also occur with heated whole serum. This effect is absent from heated normal human serum and the heated serum of "Wassermann-negative" animals. This reaction shows some analogy with the Wassermann reaction and the alcohol of the Wassermann antigen suspensions seems to be a contributory factor to the total complement-fixation in the Wassermann test. If heated serum is used, no fallacy is introduced in the diagnostic test even when the concentration of the alcohol is 1: 12.

The dissociation of the Wassermann and flocculating properties in syphilitic sera is evidenced as a result of carbon-dioxide fractioning—the soluble fraction being more active in the flocculation test, the insoluble moiety

being more active in the Wassermann test, though in some cases the dissociation is less obvious and may be quite absent.

It is suggested from the observations recorded that the Wassermann syphilis reaction represents an increase of a "lipoidophile" antibody naturally present in the serum in minimal amount and in a masked state, which in syphilis is non-specifically augmented in an analogous manner to the augmentation of the natural anti-sheep haemolysin by heterologous stimuli.

We have to thank Mr David Lees of the V.D. Department, the Edinburgh Royal Infirmary, for certain of the sera used in this investigation.

REFERENCES.

BLUM, K. (1924). *Zeitschr. f. Immunitätsforsch.* XL. 195.
BROWNING, C. H., CRUICKSHANK, J. and MACKENZIE, I. (1910). *J. Path. and Bact.* XIV. 484.
BROWNING, C. H., DUNLOP, E. M. and KENNAWAY, E. L. (1922). *Ibid.* XXV. 36.
BROWNING, C. H. in BROWNING and MACKENZIE (1924). *Recent Methods in the Diagnosis and Treatment of Syphilis*, 2nd ed. London.
BROWNING, C. H. and MACKENZIE, I. (1910). *J. Path. and Bact.* XV. 182.
BROWNING, C. H. and MACKIE, T. J. (1913). *Zeitschr. f. Immunitätsforsch.* XVII. 1.
—— —— (1914). *Ibid.* XXI. 422.
—— —— (1925) in Browning's *Immunochemical Studies*, London, p. 188.
DOHI, S. H. (1911). *Arb. a. d. Kais. Ges. Amt.* XXXVII. 514.
FORSMANN, C. R. (1924). *C.R. Soc. de Biol.* XCI. 471.
FRIEDEMANN, U. (1910). *Zeitschr. f. Hyg.* LXVII. 278.
FÜRST, S. (1914). *Zeitschr. f. Immunitätsforsch.* XXIII. 358.
GILMOUR, W. (1924). See Browning and Mackenzie: *Recent Methods in the Diagnosis and Treatment of Syphilis*, 2nd ed. London, p. 346.
GRIFFITH, F. and SCOTT, W. M. (1920). *Ministry of Health Reports on Public Health and Medical Subjects*, I, London.
HAROLD, C. H. H. (1922). *J. Roy. Army Med. Corps*, XXXIX. 83.
HESSBERG, P. (1909). *Biochem. Zeitschr.* XX. 349.
LIEFMAN, H. (1909). *München. med. Wochenschr.* LVI. 2097.
MACKIE, T. J. (1921). *S. African Med. Record*, XIX. 363.
—— (1923). *Journ. of Hyg.* XXI. 386.
—— (1925). *Ibid.* XXIV. 176.
MANTEUFEL (1909). *Centralbl. f. Bakt.* I, Ref. XLII, Beiheft, p. 95*.
MARCUSE, K. (1921). *Ibid.* I Orig., LXXXVII. 355.
NOGUCHI, H. (1909). *J. Exper. Med.* XI. 84.
OELLER, H. and SCHIERGE, M. (1923). *Zeitschr. f. Immunitätsforsch.* XXXVI. 59–67.
SACHS, H. (1917). *Ibid.* XXVI. 451.
SACHS, H. and GEORGI, F. (1923). *Berlin. klin. Wochenschr.* II. 880.
SACHS, H. and RONDONI, P. (1908). *Ibid.* p. 1968.
SCHMIDT, P. (1911). *Zeitschr. f. Hyg.* LXIX. 513.
STERN, R. (1924). *Zeitschr. f. Immunitätsforsch.* XXXIX. 293–300.
TANIGUCHI, T. (1921). *J. Path. and Bact.* XXIV. 122 and 241.
—— (1922). *Ibid.* XXV. 77.
TORRES, C. M. (1924). *C.R. Soc. de Biol.* XCI. 986–987.
WATSON, H. F. (1925). *J. Path. and Bact.* XXVIII. 171.
WEIL, E. and BRAUN, H. (1907). *Berlin. klin. Wochenschr.* p. 1570.
WENDT, H. (1925). *Centralbl. f. Bakt.* XCIV. 26.

(*MS. received for publication* 3. II. 1926.—Ed.)