STUDIES IN THE SEROLOGY OF SYPHILIS

VI. The Induction of Antibodies to Tissue Lipoids (a Positive Wassermann Reaction) in Normal Rabbits

By HARRY EAGLE, M.D.*

(From the Syphilis Division of the Medical Clinic of The Johns Hopkins University, Baltimore)

(Received for publication, January 11, 1932)

Contrary to the traditional view that proteins are the only type of substance with antigenic properties, it has been recently found that under certain conditions specific antibodies may be produced in the experimental animal to both lipoids and carbohydrates. Thus, Forssman (1911) found that guinea pig kidneys, injected into rabbits, induce the formation of antibodies to a lipoid present in the kidneys. As Landsteiner and Simms showed (1921, 1923), this lipoid is non-antigenic when injected as such, but when mixed with a foreign protein (*e.g.* pig serum) before injection, it induces the formation of the characteristic antibody. To this type of "incomplete" antigen, which can combine with a specific antibody, but which cannot stimulate its formation without the aid of a foreign protein, Landsteiner gave the name "haptene."

Sachs, Klopstock, and Weil (1925) have obtained similar results with the tissue lipoid used as "antigen" in the Wassermann reaction. Although rabbit lipoid alone is non-antigenic for rabbits, they found that when it is injected together with a foreign protein (pig serum), antibodies are formed against the lipoid; *i.e.*, the rabbit develops a positive Wassermann reaction. Because of this observation, they have suggested that the Wassermann reaction in syphilis is an antibody response to autogenous lipoids liberated at foci of infection and tissue destruction. True, human lipoids as such are non-antigenic; but they draw an analogy to the animal experiment and suggest that

* Aided by a grant from the Committee on Research in Syphilis, Inc.

the spirochetal protein activates the autogenous lipoid into a complete antigen (Weil, 1926).

The question is sufficiently important to warrant an experimental confirmation of the original observation. Moreover, if true, it provides a ready test of the theory proposed in the first paper of this series (Eagle, 1929). Evidence was there adduced to indicate that the precipitation of tissue lipoids by syphilitic serum (Kahn, Kline, Sachs-Georgi, Hinton, Müller, Eagle tests, etc.) is due to the deposition of a specifically reacting protein as a sensitizing film around the lipoid particles. If this theory is correct, the washed precipitate contains lipoid antigens and human protein, in a peculiarly firm combination; and the sensitizing film of human reagin-globulin should function in the same manner as pig serum in the experiment of Sachs, Klopstock, and Weil. Indeed, the precipitate should be even more antigenic than the lipoid-serum mixture they used, for the lipoid and the reagin-globulin are very firmly bound, while in the simple lipoid-serum mixture there is at best only a loose combination between the lipoid and the activating protein.

The purpose of the present paper is therefore twofold: a verification of the Sachs-Klopstock-Weil experiment, and a test of the antigenic properties of the lipoid-reagin precipitate obtained in the precipitation tests for syphilis. An essential preliminary step was to determine whether normal rabbits have a positive Wassermann reaction when tested by the technique employed in this laboratory.

The Wassermann Reaction of Normal Rabbits

The conflicting literature as to the Wassermann reaction of normal rabbits has been summarized by Kolmer and Twist (1916), Kuczynski (1921), Blum (1924), and Thomsen and Christiansen (1930). The incidence of positive findings ranges from less than 1 per cent to greater than 50 per cent.

The discordant results do not necessarily imply mutual contradictions. In my own experience, any desired result can be obtained by appropriate changes in the Wassermann technique used. If fresh normal rabbit serum is tested with a non-cholesterolized beef heart antigen, using a half-hour incubation at 37°C., and a sheep cell system as the hemolytic indicator, a negative Wassermann reaction is obtained

almost invariably; at least, we have yet to encounter a positive result in over 2,000 such tests on more than 300 rabbits. If the serum is tested after inactivating at 56°C. for ½ hour, using a highly sensitized, yet not anticomplementary beef heart antigen, incubating for 4 hours at 0–6°C., followed by ½ hour at 37°C., a positive Wassermann is obtained in one-half to two-thirds of all normal rabbits. Even with this latter procedure, however, the result obtained will often depend upon the amount of rabbit serum used. As illustrated in the following protocol, the presence of native amboceptor for sheep cells in the serum may mask the positive reaction if large quantities of rabbit serum are used.

Protocol 1. Effect of Native Amboceptor in Masking the Wassermann Reaction of Normal Rabbits.—

1. Rabbit serum: Inactivated at 56°C. ½ hour.

2. Antigen: Powdered beef heart was extracted three times with ether (5 cc. per gm.) at 37°C., each extraction lasting 10 minutes. The ether extracts were discarded, and the dry residue extracted for 3 days with 95 per cent alcohol (5 cc. per gm.). The alcoholic extract was concentrated to two-thirds its original volume, and fortified with 0.6 per cent cholesterol. It was used in 1:40 dilution (not anticomplementary in 1:2 dilution).

3. Complement: Guinea pig serum, bled 24 hours before using and kept on the clot at 0–4°C. overnight. It was used diluted 1:10 with NaCl N/7.

4. Sheep cells: Citrated blood, washed with NaCl N/7 and resuspended in NaCl N/7 to form a 2½ per cent suspension. This was sensitized with 4 units of amboceptor in an equal volume of saline. 0.4 cc. was used in the test, hemolysis being read after ½ hour.

Rabbit serum, *cc*	0.2	0.1	0.05	0.025	0.0125	0.0062	0.0031
NaCl N/7, *cc*	0	0.1	0.15	0.175	0.2	0.2	0.2
Antigen 1:40, *cc*	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Complement 1:10, *cc*	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Reading of fixation after 4 hrs. at 0–6°C., ½ hr. at 37°C., and ½ hr. incubation with sensitized cells	0	0	+	+	+	±	0
Reading of fixation after native amboceptor had been removed from the rabbit serum before testing	+	+	+	+	+	±	0

That such a positive Wassermann reaction is not an artifact is shown by the fact that normal rabbit sera which are Wassermann

positive frequently give a positive precipitation test with tissue lipoid antigens; more significant, upon the removal of this normal antibody to lipoid by absorption of the serum with a beef heart antigen, the Wassermann and precipitation tests both become completely negative (Protocol 2).

Protocol 2.—To 2 cc. of Wassermann positive normal rabbit serum was added 0.4 cc. of beef heart flocculation-antigen suspension.[1] After 4 hours at 37°C., 3.6 cc. of NaCl N/7 were added, the mixture (*a*) centrifuged, (*b*) passed through a Berkefeld filter, and the clear supernatant fluid (1:3 serum) tested for its reagin content as outlined in the following tabulation, using the technique described in Protocol 1.

Serum, *cc*.....	0.2	0.1	0.05	0.025	0.0125	0.0062
(*a*) Complement fixation by supernatant fluid obtained after centrifugation.....	+	±	0	0	0	0 (anticomplementary in first two tubes)
(*b*) Complement fixation by Berkefeld filtrate of absorbed serum.....	±	0	0	0	0	0
Complement fixation by untreated serum, 1:3.....	+	+	+	±	0	0
Precipitation test, absorbed serum.....	0	0	0	0	0	0
Precipitation test, untreated serum, 1:3.....	+	+	±	0	0	0

As already stated, we believe the discordant findings in the literature can be reconciled on the basis of the differences in the Wassermann techniques employed. The reagin of normal rabbit serum, present in one-half to two-thirds of 100 animals tested by the technique of Protocol 1 can be masked in whole or in part (1) by using fresh serum, (2) by using a non-cholesterolized antigen, (3) by incubating for only ½ hour at 37°C., or (4) by using an excess of serum containing native amboceptor.

[1] The method of preparation of this antigen is to be described in a forthcoming paper. The same result is obtained with the suspension formed in diluting a cholesterolized (0.6 per cent) beef heart antigen with an equal volume of NaCl N/7.

To be sure, this Wassermann reaction of normal rabbits differs materially from that observed in human syphilis. The reactions are not as clear-cut, there being frequently incomplete hemolysis and fixation; the aggregates in the precipitation test are difficult to see; the two types of test do not parallel each other as consistently as they do in human syphilis; finally, rabbit sera give this same type of weak complement fixation with many lipoid suspensions which do not possess any reactivity with syphilitic serum. Nevertheless, in using the rabbit as an experimental animal for the study of the Wassermann reaction, this pseudo-fixation given by normal rabbit sera, however unlike the fixation as given by syphilitic sera, must be constantly kept in mind as a potential source of error. In testing the effect of any particular procedure upon the Wassermann reaction of rabbits, it is essential to titrate the sera quantitatively, and to include some normal rabbit sera in every Wassermann series.

Lipoids and Lipoid-Protein Compounds as Antigens

A. The Effect of Lipoids Alone.—

1. Rabbit, beef, and human hearts were extracted by the method described in Protocol 1, Paragraph 2. The yellow residues obtained by evaporating the alcohol extracts to dryness were resuspended in a minimum volume of NaCl N/7. Amounts corresponding to 12 cc. of extract were injected intravenously into rabbits three times a week for 3 to 4 weeks. Three rabbits were injected with each type of lipoid. Of the nine rabbits, four died in the course of the injections; the remaining five showed no significant changes in their Wassermann titre at any time during or after the course of injections.

2. Nine rabbits were similarly injected with suspensions of dried cholesterolized (0.6 per cent) antigens, again with negative results.

3. Each of the three alcoholic heart extracts were concentrated to one-fifth their original volume, and each extract dropped slowly, with shaking, into four volumes of NaCl N/7. 10 cc. of the milky stable suspensions formed were slowly injected intravenously. Five rabbits were used for each type of antigen, a total of 15 rabbits in all. As before, injections were also carried out with cholesterolized (0.6 per cent) extracts. The results observed in the surviving animals are given in Table I.

The results can be briefly summarized as follows: Rabbit lipoid, no matter how dispersed, does not produce antibodies when injected into rabbits intravenously. Beef or human lipoid is also non-antigenic

TABLE I

Response of Rabbits to Intravenous Injections of Colloidally Dispersed Lipoid

Source of lipoid	Type of extract	Wassermann titre* before injections	Wassermann titre after 3 wks. injections
Rabbit heart	Plain alcoholic extract (ether-insoluble)	32	24
		0	0
		24	8
		24	32
	Cholesterolized alcoholic extract	8	8
		32	32
		32	24
Beef heart	Plain alcoholic extract	0	32
		16	16
		6	16
		24	24
		32	96
	Cholesterolized alcoholic extract	64	96
		8	4
Human heart	Plain alcoholic extract	24	16
		24	96
		32	16
	Cholesterolized alcoholic extract	16	128
		24	96
		0	48
		32	24

* The method used in estimating the reagin titre is illustrated by the following example:

Serum, *cc*	0.2	0.1	0.05	0.025	0.0125	0.0062
Wassermann result	+	+	+	+	+	±
Dilution of serum	1:1	1:2	1:4	1:8	1:16	1:32

0.0062 cc., *i.e.* 0.2 cc. of a 1:32 dilution, gave a ± result; 0.2 cc. of a 1:16 dilution gave a + result. The maximum dilution of which 0.2 cc. gave a positive result was therefore approximately 1:24, a reagin titre of 24. The sources of error inherent in the Wassermann reaction do not justify any finer approximation. Any change of < 200 per cent is to discarded as being within the limits of experimental error.

when the dry material is crudely suspended in NaCl N/7; but a colloidally dispersed suspension does seem to have some slight antigenic activity. In the light of the subsequent experiments, it is possible that this antigenicity rests upon the presence in the extracts of traces of beef and human protein. Too much stress should not be laid on the positive results of Table I, as the serum of normal rabbits may contain from 0 to 64 reagin units, while the highest titre induced by colloidally dispersed lipoid was 128 units.

B. Lipoid-Human Serum Mixtures as Antigens.—

The cholesterolized (0.6 per cent) alcoholic extracts of rabbit, beef, and human hearts were evaporated down to one-third their original volume, and dropped slowly with shaking into ten volumes of 1:10 normal Wassermann negative human serum. After standing for 1 hour at 37°C., 15 cc. of each mixture were injected intravenously into each of three rabbits. Injections were repeated three times a week for 3 weeks, the Wassermann reaction being tested each week for 1 month. The results are given in Table II. As rabbits died, they were replaced.

TABLE II

Effect of Lipoid-Human Serum Mixtures upon Reagin Titre of Rabbits

Antigen injected	Animal No.	Original reagin titre	Reagin titre after wk. No.				Maximum reagin titre / Original titre	Average increase
			1	2	3	4		
								per cent
Cholesterolized beef heart lipoid + normal human serum	1	32	24	48	128	64	128/48	
	2	24	32	24	96	24	96/24	300
	3	0	0	0	48	48	48/0	
Cholesterolized human heart lipoids + normal human serum	1	16	16	16	96	128	128/16	
	2	48	24	32	128	64	128/24	400
	3	24	32	48	192	48	192/24	
Cholesterolized rabbit heart lipoid + normal human serum	1	8	8	8	48	24	48/8	
	2	32	32	24	128	128	128/32	400
	3	0	0	0	0	0	0/0	
Controls with injections of NaCl N/7	1	48	32	48	48	32	48/48	
	2	16	24	12	16	24	24/16	50
	3	24	24	16	48	32	32/24	

C. The Lipoid-Reagin Precipitate as Antigen.—We have thus verified the finding of Sachs, Klopstock, and Weil that a mixture of foreign protein (*i.e.* foreign to the rabbit) and tissue lipoids, injected into rabbits, produces antibodies against the lipoids. If, as we maintain, the precipitate formed by adding these lipoids to syphilitic serum consists of a firm combination of the lipoid and the reagin-globulin, such a precipitate should be antigenic for rabbits. That such is the case is shown in the following experiment.

The cholesterolized heart extracts (beef, rabbit, and human),[2] prepared as described in Protocol 1, were diluted with an equal volume of NaCl N/7. The milky suspension thus formed was added to ten volumes of strongly Wassermann positive syphilitic serum, previously inactivated at 56°C. for ½ hour. After 24 hours at room temperature, the mixture was diluted with two-third volumes of NaCl N/7, and centrifuged at high speed. The white precipitate was washed repeatedly in NaCl N/7 until the supernatant fluid was protein-free, and finally resuspended in NaCl N/7 to form a heavy milky suspension of approximately half the volume of the antigen used in its preparation. The average solid content of this suspension was 8 to 12 mg. per cc.

2 to 4 cc. of the three suspensions were injected intravenously three times a week for 3 weeks into a total of twenty-five rabbits, quantitative Wassermann and precipitation tests being performed on each rabbit at intervals in the course of 4 weeks after the beginning of injections.

The results are summarized in Table III.

The results were clear-cut; not only did every rabbit develop antibodies to lipoids, but the titres obtained were several times higher than the strongest Wassermann reaction we have ever observed in syphilitic serum, whether of human beings or of rabbits, and many times higher than the titres induced by the injection of lipoid-serum mixtures.

Exception might be taken to the validity of our interpretation of this experiment, on the ground that the lipoid-reagin compound may conceivably dissociate into its component parts when injected into the rabbit. The rabbit's blood might in such a case contain circulating human syphilitic reagin, and not an antibody produced as a response

[2] In keeping with the suggestions embodied in the 1928 report of the League of Nations Conference on the Serodiagnosis of Syphilis, throughout this paper completely positive results, whether fixation or precipitation, are reported +, completely negative results are reported 0, and doubtful or incomplete results are reported ±.

to the antigenic stimulus of the lipoid-reagin mixture. True, in our experience such dissociation cannot be accomplished *in vitro*; furthermore, there is no evidence that it takes place *in vivo*. Nevertheless,

TABLE III

Effect of the Lipoid-Reagin Precipitate When Injected Intravenously into Rabbits

Antigen injected	Animal No.	Original Wassermann titre*	Wassermann titre after wk. No.				Maximum titre* / Original titre	Increase
			1	2	3	4		
								per cent
Beef heart lipoid-human reagin precipitate	1	30	—	—	480	320	480/30	1,600
	2	20	20	—	320	—	320/20	1,600
	3	10	20	40	80	160	160/10	1,600
	4	30	16	48	—	320	320/30	1,000
	5	60	32	—	1,024	158	1,024/60	1,500
	6	0	0	48	512	512	511/0	
	7	8	—	—	256	1,024	1,024/8	12,000
	8	16	—	—	512	128	512/16	3,000
	9	10	—	—	1,600	400	1,600/10	16,000
	10	20	20	40	1,600	1,200	1,600/20	8,000
	11	64	32	96	1,024	1,024	1,024/64	1,500
	12	32	16	16	128	64	128/32	400
Rabbit heart lipoid-human reagin precipitate	1	10	10	40	320	160	320/10	3,200
	2	0	0	0	320	320	320/0	
	3	0	0	16	512	256	512/0	
	4	8	16	8	256	128	256/8	3,000
	5	16	8	64	512	512	512/16	3,000
	6	10	20	80	1,600	640	1,600/10	16,000
	7	24	16	16	1,600	356	1,600/24	7,000
Human heart lipoid-human reagin precipitate	1	16	0	32	1,024	256	1,024/16	6,000
	2	0	0	64	512	128	512/6	8,000
	3	32	32	32	480	480	480/32	1,600
	4	0	0	0	320	160	320/0	
	5	0	0	32	1,600	128	1,600/0	
	6	0	0	0	1,600	100	1,600/0	

* With beef heart lipoid as antigen. Approximately the same results are obtained if either human or rabbit heart lipoid is used in the test.

even granting the possibility that it may take place, the experiment itself affords data which indicate that the antibody demonstrated in the rabbits' sera is not human reagin liberated from its combination

with lipoid, but is an antibody formed *de novo* in response to an antigenic stimulus.[3] These data are as follows:

1. The chronological curve of reagin titre during a series of injections shows the latent period characteristic of antibody production. For 1 to 2 weeks there is no significant change in titre, and then, almost explosively, it multiplies 10-, 20-, and even 50-fold within a few days. If this were merely reagin liberated from the injected precipitate, the curve of its appearance should be roughly linear.

2. The amount of reagin present in the injected rabbits is many times the total amount of reagin potentially present in the lipoid-reagin precipitate. The 18 to 36 cc. of precipitate suspension injected into each rabbit, correspond to a maximum of 1,600 cc. of syphilitic serum, with an average titre of 25 to 50 reagin units per cc. Assuming that this is recovered in its entirety by dissociation of the precipitate *in vivo*, a total of 40,000 to 80,000 reagin units have been injected. The maximum titre we have observed after nine injections is approximately 1,600 units. Assuming that blood constitutes 7 per cent of the body weight, this rabbit (weighing 3.0 kilos) may be said to have 210 cc. blood; *i.e.*, about 120 cc. of serum. The total reagin content of this blood was therefore approximately 200,000 units. Remembering that the tissue juices contain antibody and reagin in high concentration, it is clear that even a 100 per cent dissociation of reagin from the precipitate injected and a 100 per cent retention in the animal body would account for only a small fraction of that present in this rabbit.

3. If we assume that the dissociation of reagin takes place with a precipitate derived from a foreign serum, it should occur as readily with antibody derived from homologous serum. Actually, however, 90 cc. of a lipoid-reagin precipitate suspension, obtained from a rabbit antiserum and representing approximately 200,000 reagin units, failed to cause any significant increase in the Wassermann titre of another rabbit when injected intravenously over a period of 3 weeks (see Protocol 4).

[3] It should be noted that no matter what type of lipoid was used in the preparation of the precipitate, whether human, rabbit, or beef, the antibody formed reacts equally well with all three. There is no evidence of species specificity. In this respect the antibody is like the reagin of syphilitic serum.

4. Finally, the antibody which appears as the result of the injections differs materially from the reagin of human syphilitic serum. Although the latter reacts equally well with a human, beef, or rabbit lipoid, it fails to react with alcoholic extracts of milk, of human or sheep red cells, or with solutions of lecithin; the antibody induced in rabbits, however, is characterized by a surprising lack of specificity. It gives complement fixation with every one of the above antigens, as well as with finely divided suspensions of cholesterol and sitosterol (Protocol 3, Table IV).

Protocol 3. The Antibody Response to Tissue Lipoids Is Not Specific.—(1) Dried sheep cells, (2) human cells, and (3) skimmed milk powder were extracted three times with ether (4 cc. per gm.), and the dried residue extracted for 2 weeks with 95 per cent alcohol. The alcoholic extract was concentrated to one-third its original volume, and fortified with 0.6 per cent cholesterol. (4) Lecithin (Merck) was dissolved in 95 per cent alcohol to form a 2 per cent solution, which was then fortified with 0.6 per cent cholesterol. (5) Cholesterol and (6) sitosterol were dissolved in 95 per cent alcohol to form a 1 per cent solution.

The sera of (1) normal rabbits, of (2) rabbits injected with the lipoid-reagin precipitate, and of (3) syphilitic human beings were tested for complement fixation with each of these antigens, care being taken to use the antigen in a dilution sufficiently removed ($< 1/8$) from the anticomplementary concentration to ensure against technical false positive reactions. The technique was otherwise the same as that described in Protocol 1. Only one experiment is here given in detail. Qualitatively similar results were obtained in eight similar comparative tests.

As is seen in Table IV the injections cause an increase in the complement-fixing titre of the rabbit serum against all the lipoids listed; the antibody formation could therefore not be due to the liberation *in vivo* of human reagin which reacts only with the beef heart lipoid.

It follows from these lines of evidence that the large quantities of antibody to tissue lipoids present in the rabbit serum after the injection of a (beef, rabbit, human) lipoid-reagin precipitate are formed *de novo* in the rabbit, and are not due to dissociation in the animal of the human reagin present in the precipitate. This result furnishes an additional verification of our original thesis that the precipitation of lipoids by syphilitic human serum is due to the deposition of a sensitizing globulin film around the individual lipoid particles. As in the experiments of Sachs and of Landsteiner, this foreign (human) protein confers antigenicity upon the lipoid; but the reagin-globulin film,

TABLE IV

The Antibody Response in Rabbits to Tissue Lipoids Differs from the Reagin of Syphilitic Human Serum

Type of serum	Dilution of serum	Complement fixation with							Anticomplementary control
		Beef heart extract 1:40	Cholesterol 1:100	Sitosterol 1:100	Lecithin 1:8,000	Milk 1:200	Sheep cell 1:800	Human cell extract 1:100	
Normal rabbit serum	1	+	±	+	+	+	+	+	0
	1:2	+	±	±	+	+	+	+	0
	1:4	+	±	±	+	+	+	+	0
	1:8	+	0	0	±	+	+	+	0
	1:16	0	0	0	0	+	+	0	0
	1:32	0	0	0	0	+	+	0	0
	1:64	0	0	0	0	±	0	0	0
	1:128	0	0	0	0	0	0	0	0
	1:256	0	0	0	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0
	1:1,024	0	0	0	0	0	0	0	0
Serum titre.....		8	4	4	6	48	32	8	0
Rabbit serum after 9 injections with rabbit heart lipoid-human reagin precipitate	1	+	+	+	+	+	+	+	+
	1:2	+	+	+	+	+	+	+	+
	1:4	+	+	+	+	+	+	+	+
	1:8	+	+	+	+	+	+	+	+
	1:16	+	+	+	+	+	+	+	+
	1:32	+	+	+	+	+	+	+	+
	1:64	+	+	+	+	+	+	+	0
	1:128	+	+	+	+	+	+	+	0
	1:256	+	+	+	+	+	+	+	0
	1:512	+	0	0	+	+	+	0	0
	1:1,024	0	0	0	0	0	±	0	0
Serum titre.....		500	250	250	500	500	750	250	32
Syphilitic human serum	1	+	0	0	0	0	+	0	0
	1:2	+	0	0	0	0	+	0	0
	1:4	+	0	0	0	0	±	0	0
	1:8	+	0	0	0	0	0	0	0
	1:16	+	0	0	0	0	0	0	0
	1:32	+	0	0	0	0	0	0	0
	1:64	+	0	0	0	0	0	0	0
	1:128	0	0	0	0	0	0	0	0
	1:256	0	0	0	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0
	1:1,024	0	0	0	0	0	0	0	0
Serum titre.....		64	0	0	0	0	2-4	0	0

firmly anchored to each lipoid particle, is apparently more efficient in this respect than the loosely adsorbed protein of the simple serum-lipoid mixture which they used.

That the foreign protein (human reagin-globulin) plays an essential rôle in conferring antigenicity upon the lipoid with which it has combined is shown by the fact that a similar precipitate obtained from rabbit sera instead of human sera and thus containing rabbit protein instead of human protein is completely non-antigenic for rabbits, even when injected in massive quantities (Protocol 4). Moreover, if the lipoid-human reagin precipitate is heated at 100°C. for 5 minutes, it loses its antigenicity entirely, despite the fact that the lipoid as such is not affected. This is considered to be due to the heat coagulation of the reagin-globulin film.

Protocol 4. A Lipoid-Reagin Precipitate Derived from Rabbit Serum Is Non-Antigenic for Rabbits.—A cholesterolized rabbit heart antigen was diluted with an equal volume of NaCl N/7, and the milky suspension added to five volumes of the rabbit antisera formed as described in Protocol 3. As before, the precipitate was washed and injected in quantities of 1, 2, 5, and 10 cc. three times a week for 3 weeks. No significant changes in Wassermann titre (<50 per cent) were observed in any of the four rabbits.

The same lipoid-reagin precipitate suspension which is so highly antigenic for rabbits is not in the slightest antigenic for guinea pigs; at least, repeated intracardial injections of comparatively enormous quantities (1 to 2 cc. three times a week for 4 weeks) failed to cause any of the four guinea pigs to develop a positive Wassermann reaction. The intravenous route is the only one which is successful even in rabbits; at least, we find that intraperitoneal and subcutaneous injections give uniformly negative results, none of eight rabbits so injected showing any change in Wassermann titre. Although two out of six rabbits injected intramuscularly did develop a slightly increased Wassermann titre, the increase was not sufficiently marked to be considered significant.

It is interesting also to note that rabbits with this high artificially induced Wassermann titre are just as susceptible to intratesticular inoculations with pathogenic *Sp. pallida* as are control animals, judged by the duration of the incubation period and by the size of the lesion produced.

SUMMARY AND CONCLUSIONS

More than one-half of normal rabbits contain complement-fixing or precipitating antibodies against Wassermann antigens (the alcohol-soluble lipoids of beef, rabbit, and human hearts) by a sufficiently sensitive technique. Normal human sera tested by the same technique are uniformly negative. The intravenous injection of colloidal suspensions of beef and human heart lipoids into rabbits occasionally causes a significant increase in this normal Wassermann (antilipoid) titre. This may indicate a certain degree of antibody response to the lipoids as such; it may be due to the presence in such extracts of traces of foreign protein, which would activate the lipoid haptene into a complete antigen; or it may be a non-specific increase in a normal antibody, not due to a specific antigenic stimulus.

Confirming the results of Sachs, Klopstock, and Weil, the addition of normal foreign (human) serum to rabbit, beef, and human heart lipoids makes them antigenic for rabbits. The intravenous injection of such lipoid-serum mixtures usually causes a significant increase in the titre of the complement-fixing and precipitating antibody against tissue lipoids.

The precipitate which forms upon the addition of tissue lipoids to human syphilitic serum is by far the most efficient antigen for the production, in rabbits, of antibodies to tissue lipoids which we have as yet encountered. Rabbits injected intravenously with such a precipitate regularly develop a Wassermann titre which is many times higher than either the titre observed in human syphilis, or that induced by the injection of a normal serum-lipoid mixture. The very marked antigenic property of the precipitate as compared with that of a normal serum-lipoid mixture is considered to be due to the fact that it contains a foreign protein firmly bound to the lipoid particles, namely, the human reagin-globulin with which they have combined. This interpretation is supported by the observations (1) that heating at 100°C., which does not affect the lipoid constituent of the precipitate, destroys its antigenic power for rabbits, and (2) that a similar precipitate derived from Wassermann positive rabbit serum instead of syphilitic human serum, and therefore containing tissue lipoid in combination with homologous (rabbit) protein, is completely non-antigenic for rabbits.

BIBLIOGRAPHY

Blum, K., *Z. Immunitätsforsch.*, 1924, **40**, 195.
Eagle, H., *J. Exp. Med.*, 1929, **52**, 717.
Forssman, J., *Biochem. Z.*, 1911, **37**, 78.
Kolmer, J. A., and Twist, M. E., *J. Infect. Dis.*, 1916, **18**, 20.
Kuczynski, M. H., *Berl. klin. Woch.*, 1921, **58**, 125.
Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, **38**, 127.
Landsteiner, K., *Biochem. Z.*, 1921, **119**, 294.
Sachs, H., Klopstock, A., and Weil, A. J., *Deutsch. med. Woch.*, 1925, **51**, 589.
Thomsen, O, and Christiansen, T., *Z. Immunitätsforsch.*, 1930, **69**, 31.
Weil, A. J., *Z. Immunitätsforsch.*, 1926, **46**, 81.