

## SPECIFIC AGGLUTINATION OF TREPONEMA PALLIDUM BY SERA FROM RABBITS AND HUMAN BEINGS WITH TREPONEMAL INFECTIONS\* · †

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(Received for publication, November 30, 1954)

For many years it was suspected that a specific immune response develops in the course of infection with *Treponema pallidum*, but despite attempts by many investigators to demonstrate specific antibodies, only recently have such efforts been successful. Work on this problem led to the discovery of the Wassermann reaction which together with its various modifications that employ lipid antigens has been found to have a high degree of accuracy in the detection of syphilitic and related infections. However, it is now generally believed that these so called standard serologic tests for syphilis (STS)<sup>1</sup> do not reveal a specific antibody to *Treponema pallidum*. Moreover, in recent years it has been demonstrated that these tests give positive reactions with sera from a number of individuals who have no history of syphilis or other treponemal disease (1).

The development of specific immunity in treponeme infections was first demonstrated by means of neutralization experiments in rabbits (2, 3) by a method which was too time consuming and expensive to be of practical value. Later, the development of the treponemal immobilization test (4) provided for the first time an *in vitro* method for the detection of specific treponemal antibodies. However, the technical difficulties of this test have limited its application to a few specially equipped laboratories, and there still remains a great practical need for a simple, specific immunological test that can be used in the general diagnostic bacteriological laboratory.

Numerous unsuccessful attempts have been made to develop a diagnostic test that employs treponemes as the antigen for an agglutination reaction. Hoffman (5) and Zabolotny and Maslakowetz (6) were among the first to demonstrate that *T. pallidum* expressed from syphilitic lesions were agglutinated by the sera of syphilitic individuals. Their findings were confirmed by others (7-11) but most investigations of this problem

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\* These studies were supported jointly by grants from the National Institute of Health, The World Health Organization, and the Lederle Laboratories Division, American Cyanamide Co.

† This material was partially presented in preliminary form at the NIH-AVDA Symposium held in Washington, April, 1953.

<sup>1</sup> STS, serological tests for syphilis.

were abandoned because of the marked tendency for suspensions of pathogenic treponemes to agglutinate spontaneously. In an attempt to overcome this difficulty a number of investigators were led to study agglutination reactions with various strains of non-pathogenic cultured treponemes (11-18). They found, however, that agglutination of these organisms by syphilitic sera was frequently weak and no stronger than the agglutination that occurred with a considerable proportion of non-syphilitic human and rabbit sera.

Tani (19), in 1940, reinvestigated the use of pathogenic *T. pallidum* in the agglutination reaction. He found that after treatment with antiformin, suspensions of treponemes did not undergo spontaneous clumping and were still agglutinable by sera from syphilitic animals and human beings, but he also found that these treponemes were agglutinated in low titer by approximately 25 per cent of the non-syphilitic human sera tested (20).

Recently McLeod and Magnuson (21) reported the successful preparation of stable suspensions of *T. pallidum* by heat killing the organisms obtained from early rabbit testicular lesions. They found that such suspensions gave specific agglutination reactions with syphilitic sera when fresh bovine serum was present in the test mixture.

It is the purpose of this paper to report the results of our studies on agglutination of *T. pallidum*. A method will be described for the preparation of suspensions of killed treponemes and evidence will be presented to demonstrate that such suspensions are agglutinated by two distinctly different antibodies, often present together, in the sera of individuals with syphilis and related diseases. Further extension of these studies has resulted in a specific treponeme agglutination test which we believe to be both reliable and practical.

#### *Materials and Methods*

*Treponema Pallidum*.—The Nichols strain of *T. pallidum* was used throughout this study, although stable suspensions of treponemes have been prepared as well with the Chicago strain of *T. pallidum* and Haiti B strain of *Treponema pertenue*. The treponemes were obtained from rabbit testicular syphilomas. During the early part of this study, the animals were infected by direct transfer; later, a standard inoculum pool of infected testicular tissue, suspended in saline that contained 15 per cent glycerin and stored over solid carbon dioxide (22), was used to ensure more uniform infections.

*Rabbits*.—Adult male rabbits of mixed breeds, 2.5 to 4.0 kilos in weight, were employed. They were housed in an air conditioned room at a temperature of 20°C. or less.

Treponeme suspensions obtained from some rabbits were found to agglutinate spontaneously during the course of preparation, or shortly thereafter. This difficulty was eliminated by selection of only those animals whose pre-inoculation sera were negative for treponeme agglutinins.

*Sera*.—The sera were for the most part specimens that had been collected over a period of several years and stored in the frozen state at a temperature of approximately -15°C., although many recently collected specimens were also tested. All sera were heated to 56°C. for 30 minutes prior to use.

The sera were tested quantitatively for the presence of Wassermann antibody by the Eagle and/or VDRL<sup>2</sup> micro-flocculation tests (23, 24). In addition, most of the sera were exam-

<sup>2</sup> VDRL, Venereal Disease Research Laboratory.

ined for the presence of treponemal immobilizing antibodies by the method of Nelson and Mayer (4).

*Anti-VDRL Rabbit Sera*<sup>3</sup>.—Sera that contained Wassermann antibody but no treponemal immobilizing antibody were prepared by immunization of rabbits with washed floccules composed of VDRL antigen and human Wassermann antibody, by 16 intravenous injections over a period of 4 weeks.

*Absorption of Wassermann Antibody from Serum*.—For many of the experiments it was necessary to use sera from which the Wassermann antibody had been removed by absorption with VDRL antigen, as follows: One volume of alcoholic solution of VDRL flocculation antigen was precipitated in 5 volumes of saline (0.85 per cent NaCl solution) and sedimented by centrifugation at 2500 R.P.M. for 30 minutes. The supernate was decanted and the serum to be absorbed was added to the precipitate in a volume equal to the original alcoholic antigen solution. However, with serum samples of less than 1 ml. the precipitated antigen was resuspended to its original volume in saline, and a portion of the resuspended antigen was mixed with an equal volume of serum; with this procedure the absorbed serum was considered to be a 1/2 dilution. The antigen-serum mixtures were incubated for 1 hour in a 37°C. water bath and then placed at 4°C. overnight. On the following day, the precipitates were removed from the sera by centrifugation.

*Technic of Agglutination Tests*.—Agglutination tests were carried out in 10 by 75 mm. test tubes in a total test volume of 0.2 ml.:0.1 ml. of antigen and 0.1 ml. of diluted serum. All sera, except when otherwise indicated, were diluted in saline containing 0.005 M ethylenediamine tetracetate (EDTA),<sup>4</sup> adjusted to pH 7.6.

For routine diagnostic purposes a 1/10 dilution of serum was employed to avoid a slight prozone phenomenon that was occasionally observed in lower dilutions of some sera. Treponeme agglutinin titers were determined by twofold serum dilutions and the titer was taken as the highest dilution which gave definite agglutination. All serum dilutions were recorded as the final dilution in each test mixture.

The reaction mixtures were incubated in a water bath at 37°C. for 18 hours prior to reading.<sup>5</sup> The presence or absence of agglutination was determined by examination of a wet preparation of each mixture under darkfield microscopy. It was found essential to place a large drop on a slide and to examine only those organisms which were floating free between the slide and coverglass because the treponemes that had adhered to either glass surface sometimes gave a false impression of agglutination. The degree of agglutination was read as 0, 1, 2, 3, or 4 plus on the basis of the estimated per cent of treponemes in clumps. In low dilutions of high titer sera the organisms frequently formed large, tightly packed aggregates in which the individual treponeme morphology was completely lost; usually, however, the treponemes agglutinated in loose, lacy clumps.

#### EXPERIMENTAL

*Preparation of Treponeme Antigen*.—After investigation of various procedures the method described below was found most satisfactory for the production of stable suspensions of *T. pallidum* suitable for agglutination reactions.

Rabbits were inoculated into the body of each testis with approximately  $2.5 \times 10^7$  treponemes in a volume of 0.5 ml. On the 2nd or 3rd day after inoculation daily intramuscular injections of cortisone acetate, 6.0 mg./kilo of body weight, were begun and continued until the animals were sacrificed. When a firm orchitis had developed, usually between the 12th and

<sup>3</sup> These sera were kindly provided by Dr. A. G. Osler of this Department.

<sup>4</sup> EDTA, ethylenediamine tetracetate.

<sup>5</sup> Merthiolate in the treponeme suspension prevented bacterial contamination.

16th day after inoculation, the rabbits were anesthetized with 1 to 2 ml. of 6 per cent sodium pentobarbital, injected intravenously, and exsanguinated from the carotid artery. The testes were removed and minced with scissors, placed in 50 ml. of 0.075 M sodium citrate solution and shaken continuously for 3 hours at 4°C. At the end of that time the fluid, which contained a large number of treponemes, was separated from the testicular tissue; an additional 50 ml. of citrate solution was added to the testes, and a second extraction was carried out overnight.

At the end of the extraction each suspension of treponemes was centrifuged at 1000 R.P.M. for 5 minutes in order to remove gross particles. The organisms were then sedimented by centrifugation at 35,000 *G* for 30 minutes, at a temperature of 4°C., in a Spinco model L centrifuge. The supernate was discarded and the organisms were resuspended in saline to a density of approximately  $10^8$  treponemes per ml. The preparation was then heated at a temperature of 65°C. (or occasionally 56°C.) for 2 hours, after which merthiolate, to a final concentration of 0.01 per cent, was added as a preservative. Finally, all antigen preparations, except those used in the study of pH effects on treponeme agglutination, were mixed with 1/20 volume of 0.1 M phosphate buffer, pH 7.6.

This method for the preparation of treponeme suspensions while largely empirical was developed through trial and error. The administration of cortisone acetate to the rabbits during the development of infection was found to enhance the final yield of treponemes greatly. Exsanguination of the rabbits prior to removal of the testes markedly decreased the amount of blood which appeared in the extraction medium. The use of isotonic sodium citrate as the extraction medium eliminated the formation of fibrin clots that frequently occurred and enmeshed the treponemes when other extraction media were employed. Immediate heating of the resuspended treponemes appeared to prevent deterioration upon storage.

By this procedure we have obtained good yields of treponeme suspensions that have maintained their specific agglutinability for periods of 6 to 9 months when stored at 4°C.

*Agglutination of T. Pallidum by Wassermann Antibody.*—Evidence accumulated in the course of these studies to suggest rather strongly that Wassermann antibody was responsible to some extent for the agglutination of *T. pallidum*. In an attempt to obtain further data on this point, agglutination tests were performed with anti-VDRL rabbit sera that were known to contain Wassermann antibody but not treponemal immobilizing antibody. The results of a typical experiment are recorded in Table I, in which it will be seen that the serum readily agglutinated the organisms and, as expected, the agglutinins were completely removed by absorption of the serum with VDRL antigen. These findings were substantiated by subsequent experiments with other sera both non-syphilitic and syphilitic.

*Agglutination of T. Pallidum by Specific Treponeme Antibody.*—In addition to agglutination in the presence of Wassermann antibody, it was observed that both rabbit and human syphilitic sera, in which no Wassermann antibody could be demonstrated, produced strong agglutination. These observations suggested that a second treponeme agglutinin develops in response to infection

with *T. pallidum*. This possibility was confirmed by the experiment presented in Table II. The agglutinin titer of a syphilitic rabbit serum repeatedly absorbed with VDRL antigen, remained unchanged after as many as five absorptions whereas the VDRL reaction became negative after the first absorption. For the purpose of identification, the term, specific treponeme agglutinin, will be used throughout this paper to differentiate this antibody from Wassermann antibody.

TABLE I  
*Treponeme Agglutination by Anti-VDRL Rabbit Serum*

Anti-VDRL serum (Ra-110-16)	Dilution of serum*								VDRL titer
	20	40	80	160	320	640	1280	2560	
Unabsorbed	4+	4+	3+	3+	3+	2+	1+	0	16
Absorbed with VDRL	0	0	0	0	0	0	0	0	0

\* Expressed as the reciprocal.

TABLE II  
*Treponeme Agglutination by Syphilitic Rabbit Serum (NIP 14) before and after Absorption with VDRL Antigen*

No. of absorptions	Dilution of serum*										TPI titer	VDRL titer
	20	40	80	160	320	640	1280	2560	5120	10,240		
0	4+	4+	4+	4+	4+	3+	3+	3+	2+	0	300	16
1	4+	4+	4+	4+	4+	3+	3+	3+	2+	0	300	0
3	4+	4+	4+	4+	4+	4+	4+	3+	3+	0	—	—
5	4+	4+	4+	4+	4+	4+	4+	3+	2+	0	—	—

\* Expressed as the reciprocal.

*Effect of Heat upon the Agglutinability of T. Pallidum.*—When the agglutination titers of several standard syphilitic sera were determined with different lots of *T. pallidum* suspensions, it was noted that some lots were agglutinated to a much higher titer than others. In attempting to find an explanation for these inconsistent results, studies were made to determine the effect of heat upon the agglutinability of the treponemes.

Samples of a given suspension were incubated in water baths of different temperatures for varying periods of time. The agglutinability of each sample was then determined in the presence of two sera: (a) a standard syphilitic serum, from which the Wassermann antibody had been removed, and (b) an anti-VDRL serum.

The results obtained with a typical suspension are recorded in Table III. This lot (L<sub>8</sub>) had been previously heated at 56°C. for 2 hours at the time of

preparation in order to kill the organisms. It will be noted that the treponemes became more agglutinable by the syphilitic serum (which contained specific treponeme agglutinin) as both time and temperature of heating were increased, and that they reached their maximum agglutinability after heating at 83°C. for 2 hours or at 100°C. for 1 hour. The effect of heat on the agglutinability of the treponemes by Wassermann antibody was much less marked.

TABLE III  
*Agglutination of Treponemes after Heating at Various Temperatures*

Serum	Heat treatment of treponemes*		Dilution of serum								
	Temperature	Time	20	40	80	160	320	640	1280	2560	5120
	°C.	min.									
Absorbed syphilitic rabbit serum	0	0	1+	1+	1+	±	0	0	0	0	0
	65	60	2+	2+	1+	±	0	0	0	0	0
	65	120	3+	3+	3+	2+	±	±	0	0	0
	83	30	3+	3+	3+	3+	2+	2+	±	0	0
	83	60	4+	3+	3+	3+	3+	1+	1+	0	0
	83	120	4+	4+	4+	4+	3+	3+	2+	1+	0
	100	60	4+	4+	4+	4+	4+	4+	3+	1+	0
	100	120	4+	4+	4+	4+	4+	4+	3+	0	0
Anti-VDRL rabbit serum	0	0	3+	3+	3+	3+	2+	1+	0	0	0
	65	60	4+	4+	3+	3+	3+	2+	1+	0	0
	65	120	4+	4+	3+	3+	3+	2+	±	0	0
	83	30	4+	4+	4+	4+	3+	2+	1+	0	0
	83	60	4+	4+	4+	4+	3+	2+	±	0	0
	83	120	4+	4+	4+	4+	4+	3+	2+	0	0
	100	60	4+	4+	4+	4+	4+	3+	2+	0	0
	100	120	4+	4+	4+	4+	3+	3+	1+	0	0

\* Suspension L<sub>4</sub> 1 month after preparation.

When the effect of heat upon the agglutinability of treponemes by the specific treponeme agglutinin was determined with a number of different lots markedly variable results were obtained, as shown in Table IV. These findings could not be explained until it was realized that the agglutinability of a given treponeme suspension, both before and after heating, depends upon the age of the suspension at the time of testing. It will be noted in Table IV that treponeme lots L<sub>2</sub> and M reacted in essentially the same manner to heating as L<sub>3</sub> when they were tested approximately 1 month after preparation. Another portion of suspension L<sub>3</sub>, when subjected to heat treatment 5 months after preparation, reached maximum agglutinability with much less heating than was required at 1 month of age. Suspensions K and J, 7 to 8 months old, were both completely aggluti-

nable before heat treatment, and did not change with moderate heating, but they rapidly lost their capacity to be agglutinated when both the time and temperature of heating were increased. Slight loss of agglutinability had been noted with the younger suspensions also after excessive heating (100°C. for 2 hours), but the changes were not sufficiently great to be significant by themselves. Suspensions F and D, which had been readily agglutinable at 8 to 12 months of age, rapidly lost their agglutinability, or agglutinated spontaneously, upon heat treatment 16 months after preparation. These suspensions were

TABLE IV  
*Agglutination Titers of a VDRL-Absorbed Syphilitic Rabbit Serum Obtained with Different Lots of Treponemes Heated at Various Temperatures and Times*

Heat treatment		N	M	L <sub>1</sub>	L <sub>2</sub>	L <sub>3</sub>	K	J	F	D
		Storage time before heat treatment								
		2 wks.	1 mo.	1 mo.	1 mo.	5 mos.	7½ mos.	8 mos.	15½ mos.	16 mos.
°C.	min.									
—	—	80	320	320	80	80	5120	2560	2560	320
65	60	320	—	320	80	1280	5120	2560	1280	320
65	120	320	—	1280	160	2560	5120	2560	320	80
83	30	—	—	—	640	2560	2560	2560	320	80
83	60	—	—	—	1280	2560	2560	2560	320	640*
83	120	—	—	—	2560	1280	1280	320	640	640*
90	60	640	—	1280	—	—	—	—	—	—
90	120	640	—	2560	—	—	—	—	—	—
100	30	—	—	—	—	2560	1280	1280	‡	‡
100	60	1280	2560	2560	2560	1280	640	320	‡	‡
100	120	1280	—	1280	1280	1280	320	320	‡	‡

\* Agglutination 1+ at all dilutions of serum.

‡ Spontaneous agglutination.

obviously undergoing deterioration, as the treponemes were distorted in appearance and few in number. Lot N, tested at 2 weeks of age, did not attain the same agglutinability as other suspensions but this could possibly be explained by the fact that it had purposely not been subjected to the initial heating that the other treponeme lots received in the course of preparation.

Although the agglutinability of young suspensions of treponemes by Wassermann antibody was much less affected by heat than their agglutinability by specific treponeme agglutinin, the loss in the reactivity of the older suspensions after heating was observed with both antibodies.

It appears from these results that treponeme suspensions, upon storage at 4°C., probably undergo a gradual change, reflected in their increased agglutinability of specific treponeme agglutinin, and eventually reach maximal aggluti-

nability. After this, deterioration sets in with eventual loss of ability to be agglutinated. Furthermore, it seems that subjection of the suspensions to heat at any time greatly accelerates both reactions (*i.e.*, increase then decrease in reactivity) and accomplishes in several hours the changes that require several months at 4°C. Our findings to date give no knowledge of the nature of the changes that take place in the treponemes upon storage or heating, or of the mechanisms by which they take place. Further study of these phenomena is therefore necessary.

*Effect of Ethylenediamine Tetracetate upon Treponeme Agglutination.*—In the course of studies to establish the most satisfactory environmental conditions for treponeme agglutination, experiments were designed to determine the effect of certain divalent cations,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , upon the reaction. The introduc-

TABLE V  
*Effect of Ethylenediamine Tetracetate (EDTA) upon Agglutination of Treponemes by Syphilitic Rabbit Serum*

Serum—NIP 14		Dilutions of serum									
Absorption	Diluent	20	40	80	160	320	640	1280	2560	5120	10,240
Unabsorbed	Saline	4+	4+	4+	4+	3+	2+	2+	1+	±	0
	Saline-EDTA	4+	4+	4+	4+	4+	4+	4+	3+	1+	0
Absorbed with VDRL antigen	Saline	1+	1+	1+	1+	1+	2+	2+	3+	1+	0
	Saline-EDTA	4+	4+	4+	4+	4+	4+	3+	3+	1+	0

tion of additional  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  ions in concentrations up to four times those normally present in serum did not influence agglutination with either Wassermann antibody or specific treponeme agglutinin. However, the latter type of agglutination was profoundly affected by the binding of these cations with a strong chelating agent, ethylenediamine tetracetate (EDTA).

The results of treponeme agglutination by a standard syphilitic rabbit serum, diluted in saline alone and in saline containing 0.005 M EDTA, are recorded in Table V. It will be noted that the presence or absence of EDTA had very little effect upon the agglutination of the treponemes by the unabsorbed serum. On the other hand, after Wassermann antibody had been removed from the serum, agglutination was enhanced markedly when EDTA was incorporated in the reaction system. Except in very high serum dilutions, little agglutination occurred if free divalent cations were present.

Additional experiments were performed in an effort to determine whether the enhanced reactivity of specific treponeme agglutinin in the presence of EDTA was indeed due to the binding of divalent cations. It was found that

agglutination was inhibited by the addition of  $\text{CaCl}_2$  in concentrations above the binding capacity of the EDTA, but that the addition of  $\text{MgCl}_2$  to the same extent had no effect. In order to interpret these findings it should be recognized that EDTA has a greater binding affinity for  $\text{Ca}^{++}$  than for  $\text{Mg}^{++}$ , and therefore, in a mixture of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and EDTA,  $\text{Ca}^{++}$  is bound preferentially. It follows from this that  $\text{Ca}^{++}$  is probably responsible for the interference with agglutination.

*Effect of pH upon Treponeme Agglutination.*—In addition to other environmental conditions that might influence the agglutination reaction it was of interest to determine the effect of pH upon treponeme agglutination by the two antibodies. An anti-VDRL rabbit serum and a VDRL-absorbed syphilitic rabbit serum were titered in the usual manner but at various pH levels by addition to the reaction mixtures of 1/10 volume of 1 M phosphate buffer at the desired pH.

TABLE VI  
*Treponeme Agglutination Titers Obtained at Different pH Levels*

Serum	pH of reactions			
	5	6	7	8
Absorbed syphilitic rabbit serum	320	640	1280	2560
Anti-VDRL rabbit serum	2560	2560	1280	1280

The results are presented in Table VI. It will be noted that the agglutination titer of the anti-VDRL serum was essentially unaffected through a pH range of 5 to 8, whereas the VDRL-absorbed syphilitic serum titer decreased as the pH dropped. Below pH 5 spontaneous agglutination of the treponemes occurred and at pH levels above 9 the organisms began to lyse. Since the ability of EDTA to bind divalent cations decreases markedly at levels below pH 7 it was not surprising to find pH 8.0 the optimal level for the demonstration of specific treponeme agglutinin.

*Effect of NaCl Concentration on Treponeme Agglutination.*—Agglutination titrations were carried out in various NaCl concentrations in order to determine the concentration most satisfactory for the reaction.

The results that were obtained are summarized in Table VII in which it will be seen that the highest titers occurred in 0.42 to 0.85 per cent saline.

*Incubation Conditions for Treponeme Agglutination.*—Agglutination titers of both Wassermann antibody and specific treponeme agglutinin were determined after various incubation periods at different temperatures. No differences in incubation requirements were observed between the two antibodies.

When reaction mixtures were incubated at various temperatures between 4 and 56°C. the rate of agglutination increased somewhat as the temperature

of incubation was raised from 4 to 37°C. but all reactions eventually reached full titer if sufficient time was allowed. Temperatures above 37°C. did not exert sufficient additional effect to be of practical value. The time of incubation was found to be a factor of great influence upon the reaction as agglutination proceeded very slowly under conditions of low antibody concentration, and reached completion only after 15 hours at 37°C. Agglutinations, therefore, were incubated in a routine manner for 18 hours at 37°C.

During the early part of the study reaction mixtures were centrifuged or shaken constantly for part of the incubation period but both of these procedures were discontinued because they did not appear to materially accelerate agglutination except in the presence of high antibody concentrations.

*Treponeme Agglutination with Human Sera.*—The evidence presented in the preceding experiments showed the presence of two antibodies reactive with *T. pallidum* in syphilitic serum and established the conditions necessary for

TABLE VII  
*Treponeme Agglutination Titers Obtained at Different NaCl Concentrations*

Serum	NaCl concentration—per cent				
	0.42	0.85	1.7	3.4	6.8
Absorbed syphilitic rabbit serum	5120	5120	2560	1280	640
Anti-VDRL rabbit serum	1280	1280	1280	640	80

the demonstration of the specific treponeme agglutinin. These findings made it possible to devise a test for the detection of the specific agglutinin in human serum. In order to determine the practical value of this test, sera from patients in various stages of syphilitic infection and from individuals with no history of syphilis were examined for the presence of this antibody.

The sera employed in this survey as non-syphilitic controls were obtained from medical students, student nurses, or hospital patients whose medical history and examination revealed no evidence of past or present syphilitic infection. The sera from patients with syphilis and the sera from patients with a diagnosis of biologic false positive reaction were obtained from thoroughly studied patients of the Syphilis Clinic (Medicine I) of the Johns Hopkins Hospital or the private practice of Dr. J. E. Moore of Baltimore.<sup>6</sup>

The sera were absorbed with VDRL antigen prior to examination for treponeme agglutination in order to remove Wassermann antibody if present. Absorptions were carried out with 0.2 ml. of serum and 0.2 ml. of resuspended VDRL antigen as described. All sera were tested for agglutination at a 1/10 dilution in saline-EDTA.

The results obtained with the sera from 430 individuals are summarized in Table VIII. 116 sera were from normal individuals or from patients with dis-

<sup>6</sup> The sera and clinical information from these patients were kindly supplied by Dr. Moore.

eases other than syphilis; all gave negative agglutination reactions, and in all of 76 tested the treponemal immobilization test was negative. In addition, negative tests were obtained with all but 2 sera from 58 patients diagnosed as biologic false positive reactors on the basis of positive standard serologic tests and negative treponemal immobilization tests. 1 of the 2 patients in this group whose serum showed a positive agglutination reaction gave a history of possible exposure to syphilitic infection; this patient was known to have had a husband with syphilis but the infectiousness of his disease during the period of exposure was not known.

In contrast were the results obtained with the sera from 256 patients with a diagnosis of syphilitic infection. 14 of 19 sera from patients with dark-field-positive primary syphilis and 15 of 16 sera from patients with untreated sec-

TABLE VIII  
*Summary of Results of Treponeme Agglutination Tests with Human Sera after Absorption with VDRL Antigen*

Diagnosis	No. positive	No. negative	Total sera tested	Per cent positive
Normal	0	53	53	0
Diseases other than syphilis	0	63	63	0
Biologic false positive reactors	2	56	58	3.5
Primary syphilis	14	5	19	73.7
Secondary syphilis	15	1	16	93.8
Latent syphilis	89	6	95	93.7
Asymptomatic CNS syphilis	31	1	32	97.0
Late symptomatic syphilis	76	2	78	97.4
Congenital syphilis	16	0	16	100.0

ondary syphilis gave positive agglutination tests. In addition, 89 of the 95 sera from individuals with a diagnosis of latent syphilis, and 123 of 126 sera from patients with various types of late syphilitic infection, gave positive tests, although approximately two-thirds of the patients in each of these groups had had antisyphilitic therapy.

Successful treponemal immobilization tests were completed on 244 of the 256 sera from patients with syphilis, so that it was possible with those sera and the 58 sera from patients with a diagnosis of biologic false positive reactions to compare the results obtained by the three tests: the agglutination test, the immobilization test, and the standard serological test. The comparative results are summarized in Table IX in which it will be noted that the three tests were in agreement in 201 of the sera from the patients with syphilis, 199 of which were positive and 2 negative. 25 of the remaining sera from the syphilitic patients gave positive results with both the agglutination and immobilization tests but negative standard serological tests. In addition, the results of the

agglutination and immobilization tests were negative in 56 of the 58 sera from patients with a biologic false positive reaction. These sera, by definition, all gave positive standard serologic tests.

There were 20 sera in the total of 302 that gave discrepant results between the agglutination and the immobilization tests. Analysis of these revealed that 6 were from patients with primary syphilis, 6 from patients with late symptomatic or asymptomatic neurosyphilis, 6 from patients with a diagnosis of latent syphilis, and 2 from individuals with supposedly biologic false positive reactions. In the first two groups of patients whose diagnoses were based primarily on clinical findings, 2 of the 12 sera were positive in the agglutination

TABLE IX  
*Comparison of Results of Agglutination, Treponemal Immobilization, and Standard Serological Tests in Sera from 302 Patients with a Diagnosis of Syphilis or Biologic False Positive Reactor*

Agglutination test	Treponemal immobilization test	Standard serological test	Primary syphilis	Secondary syphilis	Latent syphilis	Asymptomatic CNS syphilis	Late symptomatic syphilis	Congenital syphilis	Biologic false positive reactor	Total
+	+	+	9	13	75	27	59	16	0	199
+	+	-	0	0	9	2	14	0	0	25
+	-	+	3	0	0	1	1	0	2	7
+	-	-	1	0	0	0	1	0	0	2
-	+	+	1	0	6	0	2	0	0	9
-	+	-	1	0	0	1	0	0	0	2
-	-	+	0	0	0	0	0	0	56	56
-	-	-	2	0	0	0	0	0	0	2
Total .....			17	13	90	31	77	16	58	302

test alone, 5 were positive in both the agglutination and standard serological tests, 2 were positive in the immobilization test only, and the remaining 3 were positive in the immobilization and standard serological tests. In the last two groups of patients, however, in which the diagnoses were based largely upon the results of the immobilization and standard serological tests the agglutination test was in opposition to the accepted diagnosis in all 8 sera, negative in the 6 sera from patients with latent syphilis, and positive, together with the standard serological tests, in the 2 sera from biologic false positive reactors.

It appears from the results of this small survey that the treponeme agglutination test, as employed here, is quite specific for the detection of treponemal infections in man.

Although the agglutination tests reported above were performed in sera after absorption with VDRL antigen agglutination reactions were also determined in these sera before absorption. These pre-absorption tests were done

in an effort to prove that removal of Wassermann antibody was necessary before the agglutination test could be utilized for the differentiation of patients with treponemal infections from individuals with other conditions that stimulated Wassermann antibody production.

As was expected, the differences between the results of agglutination tests before and after absorption of the sera were found only in sera that gave negative tests in the absorbed state. 2 of the 116 sera from the non-syphilitic control groups of patients gave weakly positive agglutination tests before absorption. These 2 sera were from patients with gonorrheal infection and were both negative with the standard serological tests. 7 of the 15 sera from syphilitic patients that give negative agglutination tests after absorption were positive when the unabsorbed sera were tested; they were also positive with the standard serological tests. 1 of these was from a patient with primary syphilis, 1 from a patient with secondary syphilis, 3 from patients with latent syphilis, and 2 from patients with late symptomatic syphilis. 4 sera from patients with primary syphilis and 1 serum from a patient with treated asymptomatic neurosyphilis were negative for agglutination both before and after absorption; these sera were also negative in the standard serological tests. The remaining 3 sera were from patients with a diagnosis of latent syphilis and they gave negative agglutination tests before absorption as well as after although the standard serological tests were positive to a low titer in all 3.

The most marked differences in the results of the agglutination tests before and after absorption were found in the sera from patients with a diagnosis of biologic false positive reactions. 17 of the 56 sera in this group that were negative after absorption gave positive agglutination tests before absorption.

Results of this type had been expected but it was rather surprising to find that the incidence of positive tests before absorption was not higher in this group since all these sera had given positive standard serological tests. The failure of 39 of the sera to agglutinate treponemes before absorption cannot be readily explained on the basis of lower Wassermann antibody content as the range of antibody titers of these sera in the standard serological test was the same as that of the 17 sera that were positive, and so it is assumed, for the present, that the Wassermann antibody in the sera with negative pre-absorption agglutination tests is probably qualitatively different from that in the sera with positive pre-absorption tests. Nevertheless, it is evident from these results that the treponeme agglutination test attains greater specificity in the recognition of treponemal infections when absorbed sera are employed.

#### DISCUSSION

The treponemal immobilization reaction demonstrated that there exists in the serum of syphilitic animals as well as man a specific treponeme antibody, in addition to the long recognized "non-specific" Wassermann antibody. The

recognition of this specific antibody has stimulated a renewed interest in the serology that involves the treponeme proper as distinguished from the numerous investigations of the Wassermann antigen-antibody system during the past 50 years. Since it has been possible with many microorganisms to perform serological studies with a variety of immunological tools, such as precipitin tests, complement-fixations, bactericidal reactions, and agglutination, it seemed reasonable to believe that this might also be true for *Treponema pallidum*. Furthermore, the technical difficulties inherent in the treponemal immobilization test gave added impetus to a further investigation of this possibility and led to the present study.

A bacterial suspension suitable for agglutination studies must be sufficiently dense to permit the occurrence of agglutination, and yet free from spontaneous clumping. This has been difficult to attain with *T. pallidum* because of the necessity to employ syphilitic animal tissues as the sole source of organisms. The present satisfactory method for the preparation of treponeme suspensions has been developed for the most part by trial and error, but its successful accomplishment has depended largely upon the greatly increased knowledge concerning experimental syphilis and other treponemal infections in animals that has been acquired in recent years. The effect of cortisone upon rabbit syphilomas (25) made available greater yields of treponemes in the overwhelming infections that develop through depression of the inflammatory response or inhibition of antibody formation. The recognition of naturally occurring treponemal infections in rabbits (26) and the close immunological relationship of these infections to syphilis (27) provided an explanation for the spontaneous clumping of *T. pallidum* suspensions and a clue to the means for prevention of this difficulty.

The effect of heat to render freshly prepared suspensions of *T. pallidum* more readily agglutinable by one of its antibodies is interesting in its similarity to the condition that has been found with some of the enteric bacteria (28), in which a surface antigen must be partially or completely removed by heating before the organisms can be agglutinated by antibody to a more deeply placed somatic antigen. This suggests the possibility that the antigen of the treponeme reactive with Wassermann antibody may be more superficially located than the antigen that reacts with specific treponeme agglutinin, in which case it eventually may be possible to strip the superficial antigen from the treponeme and leave only the antigen that appears to be of more diagnostic value.

The demonstration of the specific reactivity of Wassermann antibody with *T. pallidum* explains Tani's observations of a close correlation between positive standard serological tests and treponeme agglutination with sera from syphilitic rabbits (19). It also provides an explanation for the positive reactions that have been observed in unabsorbed sera from some individuals with no history of treponeme infections. On the other hand, the failure of many sera from biologic false positive reactors to agglutinate treponemes in spite of

their ability to flocculate lipoidal antigens offers additional evidence for the lack of specificity of the standard serological tests and the complexity of the Wassermann antibody.

Although other investigators failed to recognize two antibodies in syphilitic sera that agglutinate *T. pallidum* the demonstration of specific treponeme agglutinin was not surprising in light of studies of the treponemal immobilization reaction. The recognition of this second agglutinin immediately raised the possibility that it might be identical with immobilizing antibody, but conclusive evidence for or against this possibility must come from further studies. The failure to show with human sera a complete correlation between these two antibodies does not necessarily preclude their possible identity. The agglutination test, in general, gives titers approximately tenfold higher than the immobilization test and, therefore, is considerably more sensitive than the latter test as presently conducted. On the other hand, agglutination probably does not take place under conditions in which the antibody is "univalently reactive" as the immobilization reaction well may do.

It is perhaps sound in the present state of our knowledge to accept the treponemal immobilization test as a reference in the evaluation of any new test for the recognition of treponemal diseases, and, therefore, to judge the results of the agglutination test against those of the immobilization test. It must be borne in mind, however, that the immobilization test may not be infallible and an occasional discrepant result may not necessarily indicate that the agglutination test is in error (29). In any case, the limited data presented in this paper demonstrate that the agglutination reaction is a very sensitive test for treponeme antibodies and that under the proper conditions it has a specificity which compares favorably with that of the immobilization test. Moreover, in spite of the necessity to absorb sera for removal of Wassermann antibody, the technic of the test is simple and it can be carried out under conditions in which the immobilization test is impossible to perform. The final evaluation of the agglutination test, however, must come, as in the case of the immobilization test, from the critical analysis of the results obtained with sera from a large number of patients who have been subjected to the most searching clinical and epidemiological study.

#### SUMMARY

A method has been described for the preparation of *Treponema pallidum* suspensions that are suitable for specific agglutination studies and can be stored at 4°C. for months without loss of agglutinability. Such suspensions have been shown to react with two distinct antibodies in the serum of syphilitic animals and man: Wassermann antibody and a specific treponeme agglutinin.

It has been demonstrated that the agglutination of treponemes by specific treponeme agglutinin is enhanced by heat treatment or aging of the suspension, and inhibited by a divalent cation, probably  $\text{Ca}^{++}$ , normally present in serum.

This inhibition has been overcome by the use of a chelating agent, ethylenediamine tetracetate.

These findings have been utilized to devise a simple agglutination test for the diagnosis of treponeme infections that is very sensitive and highly specific. This test has been carried out with 430 human sera, and a comparison has been made of the results of the agglutination, treponemal immobilization, and standard serological tests on these sera. The agglutination test appears to have a specificity comparable to the treponemal immobilization test and considerably greater than the standard serological tests.

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