PROTECTIVE ANTIBODIES IN THE SERUM OF SYPHILITIC RABBITS*

By THOMAS B. TURNER,** M.D.

(From the Department of Bacteriology of The Johns Hopkins School of Hygiene and Public Health, Baltimore)

PLATE 44

(Received for publication, March 16, 1939)

It has been known for many years that one attack of syphilis confers an increased resistance to a second attack. Persons in whom characteristic generalized lesions of early syphilis develop usually recover, even in the absence of specific treatment, and rarely again develop widespread lesions of the skin or mucous membranes or bones. The presence of a relative immunity in man following infection with *Treponema pallidum* has also been demonstrated by reinoculation. Most of the available information on immunity in syphilis has been gained, however, from a study of the disease in animals.

The work of Neisser and his associates on monkeys and the higher apes, and the studies of Uhlenhuth, Mulzer, Brown, Pearce, Chesney, Kolle, Frei, and Breinl, to mention only a few, on rabbits, have provided a fairly clear picture of the degree of resistance to reinoculation that is developed by animals infected with *T. pallidum*, the distribution of the resistant state among various tissues of the animal's body, and the time relationships concerned in the evolution of this immunity. In brief, these studies show that in rabbits immunity is acquired slowly and does not reach its full development for a number of months after infection; that it is not sufficient completely to rid the body of the infectious agent; that it tends to be strain specific; and that the acquired resistance of the animal may be overcome by conditions which favor the infecting organism as against the host. The experiments bearing on this subject were summarized by Chesney in a comprehensive review published in 1927 (1), while the more recent work has been

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation.

** Staff member of the International Health Division of The Rockefeller Foundation.

reviewed by Harrison (2), Topley and Wilson (3), and Zinsser, Enders, and Fothergill (4), among others.

Included among the studies on the general subject of immunity in syphilis have been many designed to demonstrate the presence of antibodies specific for *T. pallidum* in the blood serum of syphilitic human beings or animals. In general these efforts have met with failure, or, at best, the results have been equivocal. Complement-fixing antibodies and precipitins are commonly present in the serum of syphilitic patients, but the demonstration of these substances rests on tests which superficially at least, are biologically non-specific. A number of workers, including Hoffman and von Prowazek (5), Zabolotny and Maslakowitz (6), Touraine (7), and Blum (8), obtained results suggesting the presence in the serum of man or animals of either agglutinins or treponemicidal substances for virulent *T. pallidum*, but these experiments were far from conclusive and numerous other investigators, including Landsteiner and Mucha (9), Zinsser, Hopkins, and McBurney (10), and Beck (11), have been unable to demonstrate such antibodies. A large number of experiments have also been done with strains of treponemes, supposedly *T. pallidum*, that have been cultivated on artificial media. In general, these studies show that, upon injection of culture treponemes, animals develop agglutinins and treponemicidal substances against these spirochetes, but not against virulent *T. pallidum* derived from man or animals (1). Likewise, the serum of syphilitic persons or animals usually fails to agglutinate or kill culture treponemes, although Zinsser, Hopkins, and McBurney (12) noted that serum from syphilitic rabbits agglutinated these organisms in higher dilution than did the serum from normal rabbits. Moreover, serum from patients with tertiary syphilis showed greater agglutinating power than serum from normal persons. In recent years, however, considerable doubt has been thrown on the identity of culture treponemes and *T. pallidum*, and the failure of syphilitic serum to act upon culture treponemes may not be of great significance.

The studies of two groups of investigators, however, are worthy of special note, since they are not in accord with the majority of studies on the subject of humoral immunity in syphilis. In 1921 Eberson (13) recorded experiments in which serum from persons with late syphilis and from rabbits with syphilitic infection of 6 months' duration or longer, when combined with virulent *T. pallidum* and incubated for 2 hours at 36°C., completely protected rabbits against infection when the mixture was inoculated intratesticularly. Serum from normal persons and persons with early syphilis, and serum from normal rabbits and rabbits infected for less than 6 months exerted no such protective action, for rabbits inoculated with the incubated mixtures developed characteristic evidence of syphilitic infection. Unfortunately, this author failed to give certain important details of these experiments, but apparently no exceptions were noted to the general results as stated. Moreover, no other investigator has succeeded in obtaining such complete protection of normal rabbits with serum from persons with late syphilis and with serum from rabbits infected for 6 months or longer.

More recently, Tani and his coworkers have published a series of papers on the

question of humoral immunity in syphilis. Tani, Saito, and Funada (14) tested the serum of two rabbits with syphilitic infection of long duration (231 and 295 days, respectively) by combining the serum with emulsions of virulent *T. pallidum* in serial dilutions, incubating the mixture for varying periods of time, and inoculating these mixtures intracutaneously in normal rabbits. As controls, serum from normal rabbits, plus spirochete emulsion treated in the same manner, were inoculated intracutaneously in the same rabbits as was the serum from the syphilitic animal. Evidence of the protective action of the syphilitic serum was manifested by a longer incubation period and smaller size of the lesions developing at the site of inoculation of syphilitic serum-spirochete emulsion, compared with those of the controls. In a subsequent paper Tani and Ogiuti (15) reported the results of similar tests made on serum from 6 rabbits infected from 18 to 79 days, and the serum of 7 patients with secondary syphilis and 3 with general paresis. On the whole the serum from the syphilitic persons and animals showed slightly greater protective power than did normal serum, but the differences were not marked. The technique used in these experiments was rather complicated and the results are not altogether clear cut.

Of considerably greater interest, however, are the parabiosis experiments performed by Tani and Aikawa (16). These investigators parabiosed 42 pairs of rabbits in which one of the pair had been infected with syphilis from 99 to 459 days, and the other from 9 to 94 days. None of the former had active syphilitic lesions and all were probably immune; all of the latter had active syphilitic lesions at the time of the parabiosis operation. By tests with trypan blue, and neoarsphenamine, and by other methods it was shown that exchange of body fluids began about the 4th day and free circulation between the parabiosed animals was usually established by the 10th day. Among 35 pairs surviving for 9 days or longer, all but 7 showed definite healing of the active lesions, as manifested by decrease in induration and, frequently, the disappearance of spirochetes from the lesions. Of 14 control pairs in which an animal with active lesions was joined to a normal rabbit and observed for 9 days or longer, evidence of healing was manifest in only 2. The results of these experiments seem to be quite definite and, if confirmed, to prove the existence of humoral antibodies in syphilitic rabbits. Moreover, these antibodies appear to play an important rôle in the defense mechanism of the host.

This paper will present the results of experiments designed to show the existence of protective antibodies in the serum of rabbits resistant to reinfection, and to describe a technique by which this protective action of the serum may be demonstrated.

Experimental Method

In developing a technique to show the protective power, if any, of syphilitic serum, consideration was given to the idea that, at best,

the antibody titer of serum from rabbits immune to syphilis was probably low, and that any test designed to show the presence of these antibodies must be sufficiently finely adjusted to bring out the small differences that probably exist between the serum of normal and syphilitic animals or human beings. To this end many preliminary experiments were made, and the technique of the test outlined here represents the one which is in use at the present time, although many of the experiments recorded were not performed exactly with this present technique.

In brief, 9 parts of whole serum are combined with 1 part of spirochete emulsion, the mixture is incubated at 37°C. for 6 hours, and then is inoculated intracutaneously on the backs of normal rabbits. The rabbits are observed at daily intervals and the incubation period and size of the resulting syphilitic lesions are noted. The details of the test and the experimental method employed in these studies are given in full below.

Serum Tested.—The serum tested was obtained from rabbits which had been infected with the Nichols strain of *T. pallidum* at least six months prior to bleeding. No antisyphilitic treatment had been given. From the experiments of others, to which reference has already been made, it could be assumed that the rabbits would, upon reinoculation, prove to have at least a chancre immunity and probably to be entirely resistant to reinfection. The presence of chancre immunity was actually proved in a number of these animals by reinoculation of an homologous strain of *T. pallidum.* As controls, serum from 2 or 3 normal rabbits, bled at the same time as the test animals, was pooled and subjected to the same procedures as serum from the test animals. The whole blood was placed in the ice box overnight and the serum pipetted off the following day. It was then tested on that day, or else frozen in solid carbon dioxide and 95 per cent alcohol at −78°C. until ready to be used. The serum was not heated above 37°C. before testing.

Spirochete Emulsion.—Normal rabbits were inoculated in both testes with the Nichols strain of *T. pallidum.* When the resulting orchitis was fully developed the testes were excised, weighed, ground in a mortar to which a small amount of sand had been added, and diluted with ordinary culture broth in an amount in cubic centimeters corresponding to the weight of the organs in grams. The suspension was centrifuged in order to throw down the large tissue particles. The supernatant fluid, which was ordinarily rich in motile *T. pallidum,* was termed a 50 per cent emulsion. The strength of such emulsions as judged by number of spirochetes or its infectivity for rabbits, varies, of course, with each batch, and designation in terms of percentages is meant to carry only an approximate estimate as to its virulence.

Occasionally this emulsion was used in the protection test on the day it was prepared, but the usual procedure followed was to distribute the emulsion in 1 or 2 cc. vials, tightly stopper the vials, and freeze them in solid carbon dioxide and

alcohol at a temperature of −78°C., until needed. As reported in a previous paper, the virulence of *T. pallidum* can thus be preserved essentially unchanged for periods of months or even years (17). Graduated dilutions of this emulsion were then combined with normal rabbit serum in the same proportion as that used in the test and titrated by inoculating the mixture intracutaneously in normal rabbits. It was thereby possible to determine approximately the least amount of spirochete emulsion which would fairly regularly produce a lesion at the point of inoculation within a period of 4 weeks. This may be termed the "minimal chancre dose," and ordinarily varies from a final dilution of 1 to 5 per cent when 0.1 cc. is the amount inoculated, and the period of incubation is 6 hours at 37°C.

Proportion of Spirochete Emulsion to Serum.—Different proportions of spirochete emulsion to serum have been used in the experiments reported here. As the test is now being performed a proportion of 1 part of spirochete emulsion to 9 parts of serum is used, but proportions of 1 to 3 and 1 to 4 have also been employed. It is probable that an excess of serum is being used; this point requires further investigation.

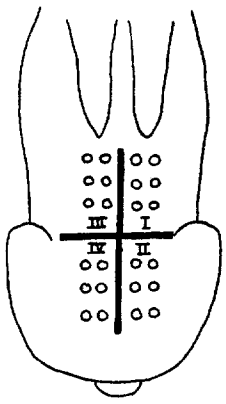
Incubation of Mixture.—The serum-spirochete emulsion is thoroughly mixed and placed in the incubator at 37°C. for 6 hours. Care should be taken to see that the temperature of the incubator does not rise above this point. As will be noted below, in some experiments shorter periods of incubation were used. At the expiration of the period of incubation the serum-spirochete emulsions are again mixed and cultures made on blood agar plates in order to rule out the presence of contaminating bacteria. The mixtures are now ready for inoculation.

Inoculation of Mixtures.—In the preliminary experiments normal rabbits belonging to the ordinary laboratory stock were used. In the latter experiments all test rabbits were male animals of the Dutch Belt variety, all were obtained from one dealer supplying a highly inbred stock, and all animals were young adults of approximately the same age. The backs of the animals were prepared for inoculation by closely clipping the hair with electrically operated clippers.

Inoculations are made with tuberculin syringes and 27 gauge needles. The back of each rabbit is divided into 4 areas, and inoculations are made intracutaneously in 6 sites of each area, 0.1 cc. of the mixture being injected at each site. 4 animals are customarily inoculated with each mixture. If syphilitic lesions develop at each site of an area, a characteristic pattern is noted. This pattern assumes importance when the lesions begin to develop, for it aids materially in distinguishing between syphilitic lesions and non-specific lesions which are not infrequently encountered. The pattern of beginning syphilitic lesions is unmistakable, however, and greatly facilitates accurately establishing the incubation period of the lesions. A diagram of the rabbit's back showing the areas and sites of inoculation is presented in Text-fig. 1.

Reading the Test.—All animals were commonly examined daily during the period of observation. Frequently on the day following inoculation a slight non-specific reaction, characterized by erythema

and slight edema at the sites of inoculation, was noted. This usually subsided by the 2nd or 3rd day, and the inoculated areas remained negative until the beginning of syphilitic lesions in one or another area. As will be noted later, the incubation period of these lesions varied considerably, depending on a number of factors. As the syphilitic lesions develop at the site of inoculation an effort is made to record their relative size for comparative purposes. Characteristically, the lesions begin as small erythematous spots and progress, within a period of a week or 10 days, to typical indurated papules closely resembling the hunterian chancre considered to be so characteristic of primary syphilis in man. Motile *T. pallidum* were demonstrated in many of the lesions. Numerous ways of giving a numerical expression to these lesions have been tried, and the most satisfactory seems to be to record the relative size of the lesions in each animal in terms of 1 plus, 2 plus, 3 plus, or 4 plus (+, ++, +++, ++++). The sum of these numbers in any one area is then used to indicate the size of the lesions in that area. For example, if a well developed chancre is present at each of 6 sites in one area these would be designated 4 plus lesions and the total of these figures would be 24. In another area showing 1 plus lesions the total size would be read as 6. Actual measurements of the lesions have been made, but because the lesions are three dimensional this method often gives a less accurate picture than the one just described.



TEXT-FIG. 1. Schematic representation of rabbit's back showing areas and sites of intracutaneous inoculation of serum-spirochete mixtures.

Schematic drawings of the lesions have been made twice weekly during the period of observation, and these provide the most accurate picture of all. The actual area of the lesion is drawn and the degree of induration or elevation is represented by crossed lines; the closest cross-hatching representing the greatest elevation. Drawings made from four animals are reproduced in Text-fig. 2.

Duration of Observation.—In the experiments reported here, no uniform period of observation was employed, and as a rule animals were observed until the first lesions had apparently reached their

maximum development. In the experiments still in progress, a uniform practice has been adopted of observing the animals 7 days after the first pattern of syphilitic lesions is noted. The size of the lesions recorded in the following experiments represents the readings made on the last day of observation or on the day of maximum development of the lesions, in case they had begun to subside before the animal was discarded.

Factors Affecting the Test.—Among the most important variables affecting the test was the virulence of the spirochete emulsion. In the latter experiments, however, this factor was controlled by using material which had been previously tested and preserved by freezing. Another important variable was the temperature of the room in which the inoculated rabbits were maintained. In general, the higher the temperature the longer the incubation period. This effect of temperature is well known, and is a factor which must always be reckoned with in studies on experimental syphilis. It is possible that protection tests, such as described here, cannot be successfully performed at temperatures usually encountered during the summer months.

Another variable factor is the susceptibility of individual rabbits to syphilis. The vast majority of animals, particularly from so uniform a stock as now in use here, react in much the same manner to the same inoculation. In every large series, however, there will be an occasional animal that seems to be unusually susceptible, as indicated by a much shorter incubation period of the syphilitic lesions and by the larger size of the lesions. Likewise, an occasional rabbit is encountered that seems to be unusually resistant to syphilitic infection, as manifested by a delayed appearance and slow development of the lesions. Rarely an animal will not develop any lesions at all following inoculation of the same mixtures that give rise to characteristic syphilitic lesions in a large number of other rabbits. It is for this reason that normal sera used as controls in these experiments were commonly obtained from two or more rabbits.

EXPERIMENTAL RESULTS

Preliminary Experiments.[1]—As a starting point for the investigation of this problem a number of rabbits that had been infected with

[1] Some of the preliminary experiments were performed in the laboratory of the Syphilis Division of the Johns Hopkins Medical School, which is under the direc-

T. pallidum for from 6 months to 1 year were selected. All had had extensive syphilitic lesions, which at the time of these experiments were apparently inactive. No antisyphilitic treatment had been given. The animals were reinoculated intracutaneously with a homologous strain of treponeme and were shown to be refractory to reinoculation. Serum from these animals was tested numerous times along with serum from normal rabbits. During these preliminary experiments one or another of the various procedures in the test was varied in an effort to find the combination that seemed to yield the best results. Tests were made with varying dilutions of spirochete emulsion, with different amounts of serum, and using varying periods of incubation. In none of these experiments was the serum heated above 37°C. Several other experiments were made to test the effect of heating the serum to 56°C. in order to inactivate the complement but these will be referred to later.

The results of these preliminary experiments will not be given in as much detail as will those of the subsequent experiments, but a summary of the results is shown in Table I. Each separate test with serum from immune rabbits was controlled by inoculating mixtures of normal serum which had been subjected to exactly the same procedures.

The results with immune serum-spirochete mixtures obtained on intracutaneous inoculation of the test rabbits have been designated "definite protection," "questionable protection," or "no protection," in relationship to the results obtained in the same animals with simultaneous inoculation of normal serum-spirochete mixtures. A clearer idea of how these designations were made can be obtained by referring to the more detailed accounts of the experiments presented below.

Without regard to the details of the tests, it is noted that of 56 areas inoculated with immune serum-spirochete mixtures 42 showed evidence of definite protective action on the part of the immune serum, as compared with the areas inoculated with normal serum-spirochete mixtures. In 10 areas questionable evidence of protection was noted, and in only 4 areas was there no evidence of protection.

---

tion of Dr. Alan M. Chesney. During this period the author was assisted by Dr. Abraham Gelperin. Some of the immune animals used in these experiments were kindly supplied by Dr. Chesney.

Of the animals tested, the original inoculation had been intratesticular in rabbits 46-75 and 46-87, and intracutaneous in rabbits 47-70, 47-72, and 47-73. It is of interest that, in each of three series of

TABLE I

Results of Protection Tests Made with Serum from Rabbits Immune to Syphilis
Preliminary Experiments

Source of "immune" serum tested		Details of test			Results of test in inoculated rabbits			
						Number showing		
Rabbit No.	Duration of infection	Dilution of spirochete emulsion	Proportion of spirochete emulsion to serum	Incubation of mixture Temperature and time	Number inoculated	Definite protection	Questionable protection	No protection
	mos.	*per cent*						
46-75	12	10	1:3	37°C. 30 min.	2	2	0	0
"	12	1	"	" " "	2	2	0	0
"	12	0.1	"	" " "	2	2	0	0
"	12	20	"	" " "	3	3	0	0
"	18	50	"	" 3 hrs.	4	3	1	0
46-87	16	"	"	" " "	4	3	0	1
47-70	11	"	"	" " "	4	3	1	0
47-72	11	"	"	" " "	3	0	2	1
47-73	11	"	"	" " "	3	3	0	0
46-75	18	1	"	" " "	3	2	1	0
46-87	16	"	"	" " "	3	3	0	0
47-70	11	"	"	" " "	4	4	0	0
47-72	11	"	"	" " "	3	1	0	2
47-73	11	"	"	" " "	2	1	1	0
46-75	19	10	"	" " "	3	3	0	0
46-87	17	"	"	" " "	3	3	0	0
47-70	12	"	"	" " "	3	2	1	0
47-72	12	"	"	" " "	3	1	2	0
47-73	12	"	"	" " "	2	1	1	0

tests, serum from rabbit 47-72 tended to exhibit less protective power than serum from the other animals.

With reference to the details of the technique of the test, the most definite finding drawn from the preliminary experiments was that the virulence of the spirochete emulsion was probably the most important

feature. Emulsions which were too virulent tended to obliterate differences in the incubation period, while if the spirochete emulsion was of relatively low infectivity even the normal serum-spirochete emulsions failed to produce lesions at the site of inoculation. Because of these findings a spirochete emulsion which had been previously tested for virulence and preserved by freezing was employed in subsequent experiments.

Effect of Inactivating Complement.—During the course of the preliminary experiments samples of sera from the same animals were tested after having been heated for 30 minutes at 56°C. in order to inactivate the complement. Altogether, 11 sera from 5 immune rabbits were tested by the same technique as used in the foregoing experiments. All animals had been infected by intratesticular inoculation from 7 to 12 months prior to bleeding. Of a total of 42 areas injected with immune serum-spirochete mixtures, 13 showed definite evidence of the protective action of the serum as compared with the areas in which normal serum had been injected, 12 showed questionable evidence of protection, and in 17 there was no evidence of protection. These results are distinctly poorer than when sera from the same animals were tested without heating above 37°C., and they suggest that the presence of active complement is favorable to the demonstration of the protective action of immune serum. To what extent active complement was present in the testicular emulsion is not known, but it is possible that enough was available to satisfy the requirements of the test. This point needs further study.

Experiment 1.—The four sera used in this experiment were obtained from 2 immune animals, 46-75 and 46-87, and 2 normal rabbits, normal A and normal B. One part of a 25 per cent testicular emulsion containing numerous treponemes was added to 4 parts of serum and the mixture incubated for 6 hours at 37°C. At the end of 6 hours 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area on each of 6 normal rabbits. The incubation period and the approximate size of the resulting lesions in these inoculated animals are shown in Table II.

In general the incubation period of the syphilitic lesions which developed in the test rabbits was about the same for the 2 "normal" areas in the same animal, but in different animals the incubation period in these areas ranged from 12 to 24 days. It is evident that

rabbits, even from the same stock and of approximately the same age vary considerably in their reaction to the same inoculum. In the

TABLE II

Results of Protection Tests with Serum from Normal and Syphilitic Rabbits
Experiment 1

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Duration of observation	Interpretation of test Degree of protection
		days		*days*	
1-79	Normal A	12	19	51	Control
	Normal B	12	18	"	"
	46-75	Neg.	0	"	Definite
	46-87	26†	3	"	"
1-80	Normal A	24	17	"	Control
	Normal B	24	19	"	"
	46-75	33	8	"	Definite
	46-87	Neg.	0	"	"
1-81	Normal A	19	24	41	Control
	Normal B	21	24	"	"
	46-75	24†	2	"	Definite
	46-87	Neg.	0	"	"
1-82	Normal A	18	18	"	Control
	Normal B	18	18	"	"
	46-75	28†	3	"	Definite
	46-87	24	6	"	"
1-83	Normal A	23	24	51	Control
	Normal B	22	24	"	"
	46-75	Neg.	0	"	Definite
	46-87	"	0	"	"
1-84	Normal A	24	9	50	Control
	Normal B	24	8	"	"
	46-75	33	4	"	Definite
	46-87	Neg.	0	"	"

* See page 872.

† Pattern of lesions not complete.

12 areas inoculated with immune serum-spirochete mixtures either no lesions at all developed during the period of observation or, when

lesions did develop, the incubation period was significantly longer than for those in the normal areas. Likewise, the size of such lesions was uniformly smaller than the size of the lesions in the normal areas. In 3 areas inoculated with immune serum mixtures, even though lesions did develop, they did not appear at each inoculated site and the typical pattern was not observed. Photographs of the inoculated areas in rabbits 1-79 and 1-83 are shown in Fig. 1.

It seems evident, therefore, that the serum of these two untreated immune syphilitic rabbits (46-75 and 46-87) possessed some power to inhibit the development of syphilitic lesions in previously uninfected rabbits, when compared under identical experimental conditions with serum from normal rabbits.

Experiment 2.—In this experiment sera from 10 immune syphilitic rabbits were tested for protective power in comparison with two lots of pooled serum from normal rabbits. The duration of infection in the immune rabbits at the time of bleeding is shown in Table IV. Rabbits 47-70, 47-72, and 47-73 had been infected by intracutaneous inoculation and all the others by intratesticular inoculation. No antisyphilitic treatment had been given. Rabbits 46-75, 46-87, 47-70, 47-72, 47-73, and 8-9 were shown to be resistant upon reinoculation of virulent *T. pallidum.* Rabbits 1-3, 1-4, 1-11, and 1-12 were not tested by reinoculation, but it is assumed that they would have shown a chancre immunity. Both normal A and normal B serum consisted of pooled serum from 3 normal rabbits. All animals were bled the day before the test was made, the serum meanwhile being kept in the refrigerator. The inoculum consisted of 1 part of a 25 per cent testicular emulsion, containing numerous *T. pallidum,* to 4 parts of serum. The spirochete emulsion had been frozen prior to inoculation. The serum-spirochete mixtures were incubated at 37°C. for 6 hours. Following incubation, 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area in each of 5 normal rabbits.

The results of these inoculations are shown in Table III. Of the animals inoculated, one (2-19) died prematurely and was omitted from the protocol; another (2-07) failed to develop lesions at the site of the control inoculations and has been classified as unsatisfactory; and in rabbit 2–17 two inoculated areas, obscured by pigmentation and irregular thickening of the skin, were classified as unsatisfactory. Altogether there were 90 satisfactory inoculated areas in 23 animals. Of these areas, 45 were inoculated with one of the normal serum-spirochete mixtures and an equal number with one or another of the

immune serum-spirochete mixtures. Considering all the immune areas as a group, 37 areas inoculated with immune serum mixtures showed definite evidence of the protective power of the immune serum as compared with the normal serum; in 5 areas the evidence of protection was only questionable; and in 3 areas no protective power was manifest.

Again, it is noted that the incubation period of the syphilitic lesions developing in the normal areas was about the same, in most animals, for the two normal serum mixtures, although in one animal (2-02) there was a difference of 5 days. In those animals in which lesions developed at the site of inoculation of immune serum mixtures the incubation period of these lesions was usually significantly longer than in the control areas. As a rule the relative size of the lesions in the different areas showed a direct correlation with the incubation period, the shorter the incubation period, the larger the lesions. Exceptions to this rule are noted, however. Drawings made from representative test animals (2-05, 2-11, 2-16, and 2-21) are shown in Text-fig. 2, and a photograph of the lesions in rabbit 2-21 is shown in Fig. 1.

In the interpretation of the results of these tests, both the incubation period and the size of the lesions are considered. Doubtless, in the case of some areas, all observers might not make the same interpretation of the results in the test animal, but there can be no question that, on the whole,the immune serum exerted an inhibitory effect on the development of syphilitic lesions in those areas when compared with the results in the areas injected with normal serum mixtures. Even in those areas designated as showing questionable protection, either the incubation period of the lesions was slightly longer than that of the controls or else the lesions did not reach the size of the control lesions.

In Table IV is given a summary of the results obtained with the serum from each immune rabbit. Serum from rabbits 47-72 and 47-73 tended to show somewhat less protective power than did serum from the other animals; it may be significant that these animals were originally inoculated intracutaneously. It should be noted, also, that rabbit 1-11, at the time of bleeding had an active syphilitic testicu-

TABLE III

Results of Protection Tests with Serum from Normal and Syphilitic Rabbits

Experiment 2

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection	Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*				*days*		*days*	
2-01	Normal A	20	12	35	Control	2-11	Normal A	20	12	43	Control
	Normal B	22	12	"	"		Normal B	20	12	"	"
	46-75	Neg.	0	"	Definite		47-73	27	4	"	Definite
	46-87	Neg.	0	"	"		1-3	Neg.	0	"	"
2-02	Normal A	16	24	"	Control	2-12	Normal A	22	24	36	Control
	Normal B	21	22	"	"		Normal B	26	16	"	"
	46-75	29†	3	"	Definite		47-73	30	9	"	Definite
	46-87	34	8	"	"		1-3	30	13	"	"
2-03	Normal A	31	12	41	Control	2-13	Normal A	27	9	41	Control
	Normal B	31	9	"	"		Normal B	27	8	"	"
	46-75	34	4	"	Definite		47-73	27	6	"	None
	46-87	34	5	"	"		1-3	27	5	"	Quest.
2-04	Normal A	27	12	"	Control	2-14	Normal A	16	12	36	Control
	Normal B	27	12	"	"		Normal B	16	18	"	"
	46-75	Neg.	0	"	Definite		47-73	15	12	"	None
	46-87	36	6	"	"		1-3	27†	3	"	Definite
2-05	Normal A	21	18	35	Control	2-15	Normal A	23	6	41	Control
	Normal B	18	23	"	"		Normal B	22	12	"	"
	46-75	Neg.	0	"	Definite		47-73	Neg.	0	"	Definite
	46-87	31	5	"	"		1-3	"	0	"	"
2-06	Normal A	29	7	41	Control	2-16	Normal A	10	19	35	Control
	Normal B	27	10	"	"		Normal B	13	20	"	"
	47-70	Neg.	0	"	Definite		1-4	29	4	"	Definite
	47-72	29†	2	"	Quest.		8-9	29	7	"	"
2-07	Normal A	Neg.	0	"	Unsatis.	2-17	Normal A	18	13	41	Control
	Normal B	"	0	"	"		Normal B	Area obscured			Unsatis.
	47-70	"	0	"	"		1-4	Neg.	0		Definite
	47-72	"	0	"	"		8-9	Area obscured			Unsatis.
2-08	Normal A	13	12	"	None	2-18	Normal A	15	10	37	Control
	Normal B	13	12	"	"		Normal B	13	18	"	"
	47-70	15	9	"	Quest.		1-4	29	3	"	Definite
	47-72	13	12	"	None		8-9	27	6	"	"

* See page 872.

† Pattern of lesions not complete.

TABLE III—*Concluded*

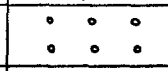
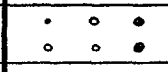
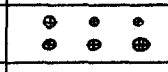
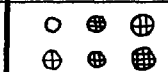
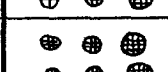
Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection	Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*				*days*		*days*	
2-09	Normal A	16	12	43	None	2-20	Normal A	24	5	43	Control
	Normal B	16	8	"	"		Normal B	20	12	"	"
	47-70	27	6	"	Definite		1-4	Neg.	0	"	Definite
	47-72	34	5	"	"		8-9	"	0	"	"
2-10	Normal A	15	18	36	None	2-23	Normal A	15	12	41	Control
	Normal B	16	18	"	"		Normal B	15	10	"	"
	47-70	18	4	"	Definite		1-11	16	6	"	Quest.
	47-72	18	4	"	"		1-12	16†	1	"	Definite
2-21	Normal A	16	24	35	Control	2-24	Normal A	18	17	35	Control
	Normal B	16	24	"	"		Normal B	18	17	"	"
	1-11	27	10	"	Definite		1-11	22	17	"	Quest.
	1-12	34†	5	"	"		1-12	Neg.	0	"	Definite
2-22	Normal A	18	11	41	Control	2-25	Normal A	18	18	37	Control
	Normal B	18	9	"	"		Normal B	18	18	"	"
	1-11	27	9	"	Definite		1-11	27†	3	"	Definite
	1-12	29†	2	"	"		1-12	Neg.	0	"	"

lar lesion. While it seems likely that this animal would have exhibited a chancre immunity on reinoculation at that time, it is probable that its resistance was not as great as that of some of the other animals.

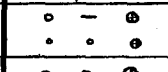
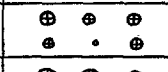
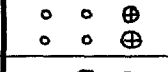
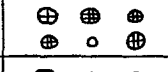
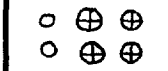
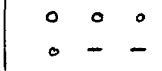
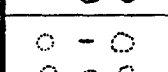
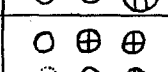
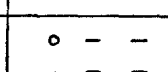
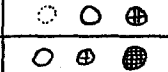
Experiment 3.—In the preceding experiments tests with serum from immune syphilitic animals were controlled with serum from normal rabbits which, as a rule, had been in the laboratory for a much shorter period of time than had the immune animals. Perhaps, too, animals that were the source of the control serum were, on the whole, younger than the immune animals. It is not known whether either of these factors, *i.e.*, duration of time in the laboratory or age of the rabbit, has an appreciable effect on the power of serum to protect against the development of syphilitic lesions, but Experiment 3 was designed to test this point.

Serum was obtained from 2 immune syphilitic rabbits, Nos. 8-9 and 2-54. Both animals had been inoculated intratesticularly, the former 17 months previously and the latter 7 months previously. Typical syphilitic orchitis developed in each, but at the time of bleeding there was no evidence of active syphilitic lesions. No

antisyphilitic treatment had been given. After bleeding, both animals were reinoculated intracutaneously with a homologous strain of *T. pallidum* and both

Days After Inoculation	Inoculated Areas			
	I. Normal A	II. Normal B	III. 46-75	IV. 46-87
15	Negative	Negative	Negative	Negative
21			Negative	Negative
24			Negative	Negative
28			Negative	Negative
33			Negative	Negative

TEXT-FIG. 2*a*

Days After Inoculation	Inoculated Areas			
	I. Normal A	II. Normal B	III. 47-73	IV. 1-3
18	Negative	Negative	Negative	Negative
22			Negative	Negative
25			Negative	Negative
29				Negative
34				Negative
41			Negative	Negative

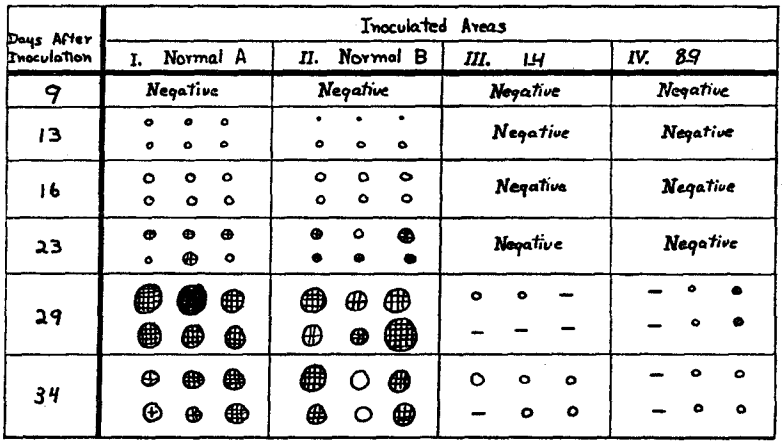
TEXT-FIG. 2*b*

TEXT-FIG. 2. Schematic drawing of lesions in inoculated areas in 4 rabbits belonging in Experiment 2. The square area of each lesion is reproduced in the drawing. The crossed lines represent degrees of elevation or induration of the lesion. The sera in the serum-spirochete mixtures inoculated into the different areas were as follows:

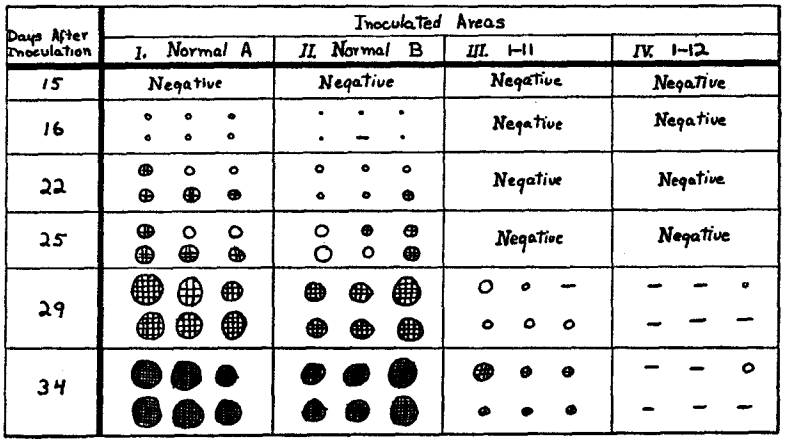
(*a*) Rabbit 2-05. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 46-75, immune. IV, rabbit 46-87, immune.

(*b*) Rabbit 2-11. I, normal A (3 pooled). II, normal B (3 pooled). III rabbit 47-73, immune. IV, rabbit 1-3, immune.

remained negative for a period of 60 days. Controls similarly inoculated developed typical syphilitic lesions within 30 days.



TEXT-FIG. 2*c*



TEXT-FIG. 2*d*

(*c*) Rabbit 2-16. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-4, immune. IV, rabbit 8-9, immune.

(*d*) Rabbit 2-21. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-11, immune. IV, rabbit 1-12, immune.

While in the 4 rabbits represented here, the two normal serum-spirochete mixtures were inoculated in areas I and II, respectively, in other rabbits of this experiment these mixtures were inoculated in different areas.

TABLE IV

Summary of Results of Protection Tests Performed in Experiment 2 with Serum from Immune Syphilitic Rabbits

Source of "immune" serum tested			Results of test in inoculated rabbits			
Rabbit No.	Site of original inoculation	Duration of infection	Number inoculated	Number showing		
				Definite protection	Questionable protection	No protection
		mos.				
46-75	Testis	24	5	5	0	0
46-87	"	22	5	5	0	0
47-70	Skin	17	4	3	1	0
47-72	"	17	4	2	1	1
47-73	"	17	5	3	0	2
1-3	Testis	10	5	4	1	0
1-4	"	10	4	4	0	0
8-9	"	10	3	3	0	0
1-11	"	6*	5	3	2	0
1-12	"	6	5	5	0	0

* Active testicular lesions at time of bleeding.

TABLE V

Results of Protection Tests with Serum from Immune Syphilitic Rabbits and with Serum from Normal Rabbits Which Had Been Maintained under Laboratory Conditions over a Period of Months

Experiment 3

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*	
4-02	1-94 (normal)	30	8	34	Control
	3-13 (normal)	30	10	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-03	1-94	18	10	22	Control
	3-13	18	11	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-04	1-94	21	6	29	Control
	3-13	20	18	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-05	1-94	23	7	"	Control
	3-13	19	16	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"

* See page 872.

As controls in this experiment serum was obtained from 2 normal rabbits, Nos. 1-94 and 3-13. The former animal had been in the laboratory 8 months at the time of bleeding and the latter animal 7 months. One part of a 50 per cent testicular emulsion, containing numerous active *T. pallidum*, was added to 9 parts of serum. The mixture was incubated at 37°C. for 6 hours and at the end of this period 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area in each of 4 normal rabbits.

The results of these inoculations are shown in Table V. In each of the test rabbits a typical pattern of syphilitic lesions developed in the areas inoculated with normal serum-spirochete mixtures, while not a single lesion developed during the period of observation in any area inoculated with immune serum-spirochete mixtures. (See photograph of rabbit 4-03, Fig. 1.) The conclusion must be drawn that the serum from the immune animals inhibited the development of syphilitic lesions under the conditions of this experiment. While the results are not conclusive, the experiment indicates that simply maintaining normal rabbits under laboratory conditions for a period of months does not serve appreciably to increase the titer of protective antibodies against *T. pallidum*.

DISCUSSION

Most writers reviewing the large amount of experimental work of the past 30 years on the nature of immunity in syphilis have concluded, and rightly so, on the basis of the available evidence, that this acquired resistance is a property primarily of the fixed tissue cells. Despite the fact that both man and animals, under certain conditions, exhibit a high degree of resistance to reinoculation, only an occasional investigator has been able to obtain evidence of a humoral expression of this immunity. In reviewing the experiments bearing directly on this point, however, it is evident that in many instances the methods of testing for the presence of humoral antibodies specific for *T. pallidum* were relatively crude compared with the techniques now available. Much more is now known concerning the general course of experimental syphilis and, in particular, much more is known of the various factors that influence the development of the immune state.

In the experiments reported in this paper liberal use has been made of techniques developed in the study of filtrable viruses and other

infectious agents. Likewise, improved technical methods in experimental syphilis have made it possible to control certain variable elements which materially affect the reaction between host and parasite. For example, a large amount of infectious material can be prepared at once, its relative infectivity for rabbits can be determined, and the whole lot of material can be preserved essentially unaltered over long periods of time by freezing at low temperatures. Thus, it is practicable fairly regularly to employ in the protection test an inoculum which closely approaches the minimal chancre dose of *T. pallidum*. Perhaps this feature is the most important element in the protection tests described above. It is probable that the inocula ordinarily used in experimental syphilis vary widely in their degree of infectivity for normal animals, which may in large measure explain the negative or equivocal results obtained by other investigators in their attempt to demonstrate protective antibodies in syphilis.

From the foregoing experiments it seems clear that the serum of rabbits which had had syphilis for 6 months or longer and had not been treated exerted a treponemicidal or treponemistatic effect on virulent *T. pallidum* belonging to a homologous strain, as compared with the effect of serum from non-syphilitic rabbits. It is not known whether this effect was exerted *in vitro* or only after injection of the mixtures into a living host. Nor is the mechanism of this action known. Because prolongation of the incubation period of the serum-spirochete mixtures seemed to accentuate the differences between normal and immune serum, it is assumed that some change occurs *in vitro*. Other evidence is also available which suggests that this may be a direct treponemicidal effect. Since, however, under conditions of these experiments spirochetes are present in the mixtures in only relatively small numbers, the point is difficult to determine. Experiments bearing on this question are in progress.

It seems likely that active complement must be present in serum-spirochete mixtures in order to demonstrate this protective action. In experiments in which the complement in the serum was inactivated, much poorer and much more variable results were obtained than when unheated serum was tested. It is probable, however, that some complement is usually present in the testicular tissue emulsion and

this amount may be sufficient in many instances to fulfill the requirements of the reaction.

In these experiments no effort has been made to determine the titer of the protective antibodies. Probably an excess of serum was used in the test. It may be possible to reduce the amount of serum to the point where smaller differences in antibody content can be detected.

These experiments and those of several other workers mentioned above indicate that specific humoral antibodies are produced during the course of syphilitic infection in rabbits. If this is true, it would serve to remove syphilis from the rather unique position among infectious diseases which it has occupied in the past, and place it among the ever growing group of diseases in which resistance to reinfection is associated at some period during the course of the infection with the presence of humoral antibodies specific for the causative agent of that disease. This phenomenon is observed more characteristically in the acute infections. The mere fact that a disease is chronic and the infection ordinarily of long duration in itself suggests that the resistance to the causative organisms, whether to those already within the body, or to those that reach it from without, is not highly developed. By the same reasoning, it could be hypothesized that the humoral expression of this immunity would, likewise, probably be only imperfectly developed. This seems to be the case in syphilis.

SUMMARY

1. When an emulsion containing virulent *Treponema pallidum* is added to serum from normal rabbits and from untreated immune syphilitic rabbits that have been infected with a homologous strain of *T. pallidum*, the mixture incubated at 37°C., and injected intracutaneously into normal rabbits, typical syphilitic lesions commonly develop at the sites of inoculation of the normal serum-spirochete mixture, while at the sites of inoculation of immune serum-spirochete mixtures usually either no lesion develops or else the incubation period of the resulting lesions is shorter and the lesions remain smaller than those produced by normal serum-spirochete mixtures.

2. In a series of preliminary experiments, of 56 areas inoculated with serum-spirochete mixtures, in 42 the suppressive action of the

syphilitic serum was manifest, in 10 areas questionable evidence of protection was noted, and in 4 areas there was no evidence that the syphilitic serum had exerted a suppressive or protective action.

3. The protective action of syphilitic serum seems to have been lessened by heating to 56°C.

4. The results of the protection test in three other series of experiments were as follows: (*a*) Of 12 areas in 6 rabbits inoculated with normal serum-spirochete mixtures typical syphilitic lesions developed, while in the same number of areas inoculated with immune serum-spirochete mixtures there was complete or partial suppression of lesions in all. (*b*) Of 45 areas inoculated with serum from 10 different immune syphilitic rabbits, definite evidence of protection was observed in 37, questionable evidence in 5, and no evidence of protection in 3. (*c*) Of 8 areas in 4 rabbits inoculated with immune serum-spirochete mixtures no lesions developed during the period of observation, while of 8 areas in the same rabbits inoculated with one of two normal serum-spirochete mixtures typical syphilitic lesions developed in each.

CONCLUSION

During the course of syphilitic infection rabbits develop specific humoral antibodies which can be demonstrated by an appropriate "protection test." The presence of these antibodies is associated with a high degree of acquired immunity to the disease.

BIBLIOGRAPHY

1. Chesney, A. M., Immunity in syphilis, Baltimore, Williams & Wilkins Co., 1927.
2. Harrison, L. W., Syphilis, in A system of bacteriology in relation to medicine, Great Britain Medical Research Council, London, His Majesty's Stationery Office, 1931, **8**, Chapter VII, 185.
3. Topley, W. W. C., and Wilson, G. S., Principles of bacteriology and immunity, Baltimore, William Wood & Co., 1937, Chapter 77, 1430.
4. Zinsser, H., Enders, J. F., and Fothergill, L. D., Immunity: principles and application in medicine and public health, New York, Macmillan, 1939.
5. Hoffman, E., *Dermat. Z.*, 1906, **13**, 561.
6. Zabolotny, D., and Maslakowitz, *Centr. Bakt., 1. Abt., Orig.*, 1907, **44**, 532.
7. Touraine, A., Les anticorps syphilitiques, Paris, Thèse-Steinheil, 1912, cited by Blum (8).

8. Blum, K., *Z. Immunitätsforsch.*, 1924, **40**, 491.
9. Landsteiner, K., and Mucha, *Centr. Bakt., 1. Abt., Ref.*, 1907, **39**, 540.
10. Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, **23**, 341.
11. Beck, A., *J. Path. and Bact.*, 1937, **44**, 399.
12. Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, **24**, 561.
13. Eberson, F., *Arch. Dermatol. and Syphilol.*, 1921, **4**, 490.
14. Tani, T., Saito, K., and Funada, H., *Centr. Bakt., 1. Abt., Orig.*, 1935, **134**, 232.
15. Tani, T., and Ogiuti, K., *Japan. J. Exp. Med.*, 1936, **14**, 457.
16. Tani, T., and Aikawa, S., *Japan. J. Exp. Med.*, 1936, **14**, 465.
17. Turner, T. B., *J. Exp. Med.*, 1938, **67**, 61.

EXPLANATION OF PLATE 44

FIG. 1. Photographs of the backs of rabbits inoculated with serum-spirochete mixtures.

(*a*) Rabbit 1-79. Experiment 1. Excised skin of back, 51 days after inoculation. Areas inoculated with the following sera: I, rabbit 46-75, immune. II, normal A (2 pooled). III, rabbit 46-87, immune. IV, normal B (2 pooled). Note pattern of large syphilitic lesions in areas II and IV. There are no lesions in areas I and III, although these areas are partially obscured by pigment.

(*b*) Rabbit 1-83. Experiment 1. Excised skin of back, 51 days after inoculation. Areas inoculated with the following sera: I, rabbit 46-75, immune. II, normal A (2 pooled). III, rabbit 46-87, immune. IV, normal B (2 pooled). Note pattern of large syphilitic lesions in areas II and IV and absence of lesions in areas I and III.

(*c*) Rabbit 2-21. Experiment 2. Excised skin of back, 35 days after inoculation. Areas inoculated with the following sera: I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-11, immune. IV, rabbit 1-12, immune. Note pattern of syphilitic lesions in areas I and II, and absence of lesions in areas III and IV.

(*d*) Rabbit 4-03. Experiment 3. Excised skin of back 22 days after inoculation. Areas inoculated with the following sera: I, rabbit 1-94, normal. II, rabbit 2-54, immune. III, rabbit 3-13, normal. IV, rabbit 8-9, immune. Note pattern of small lesions in areas I and III, and absence of lesions in areas II and IV.

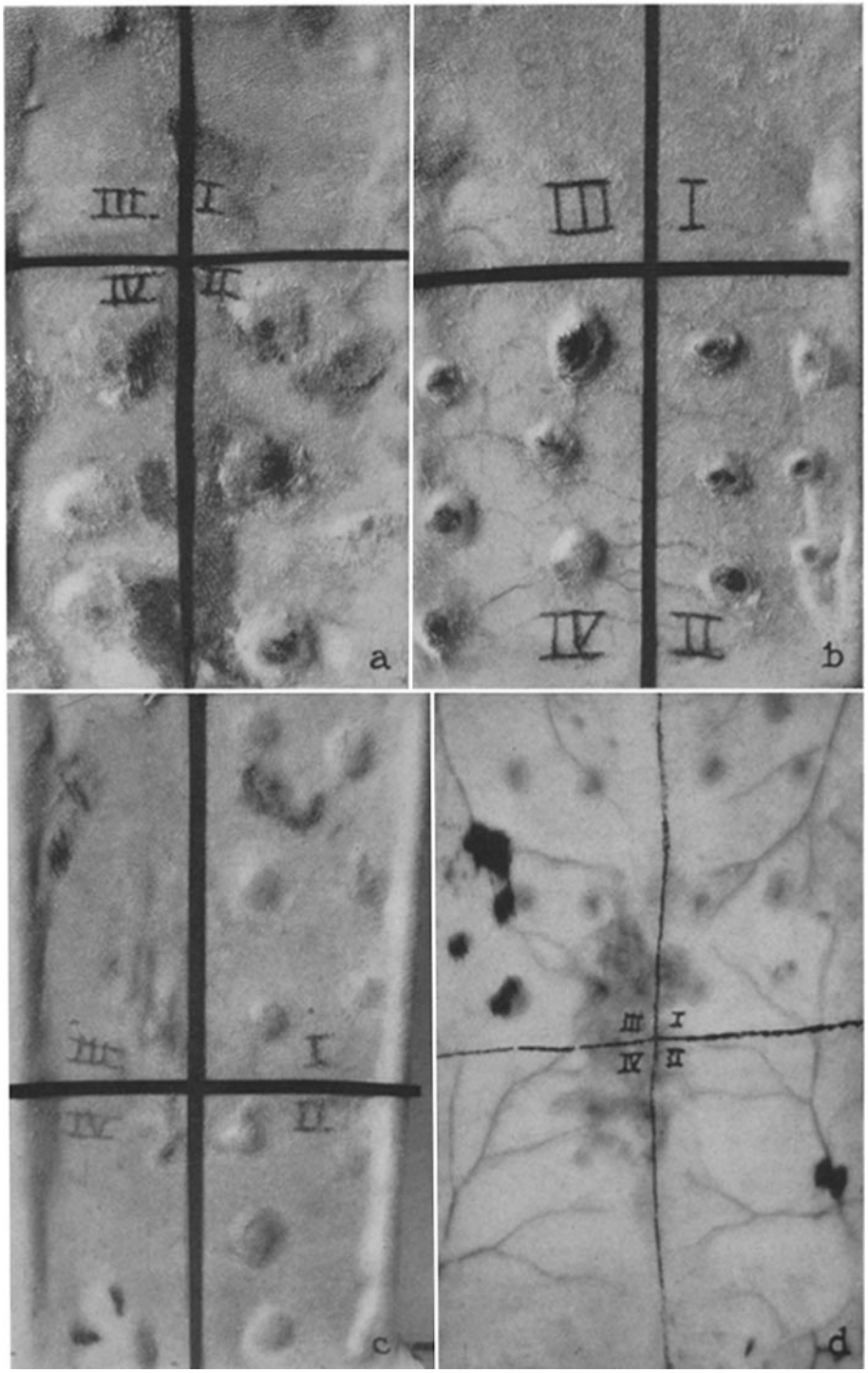


FIG. 1

(Turner: Protective antibodies in syphilitic serum)