

STUDIES ON THE TREPONEMAL IMMOBILIZATION TEST*

BY

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PART I. EXAMINATION OF NORMAL AND SYPHILITIC SERA

Since the introduction of a serological test for syphilis by Wassermann, Neisser, and Bruck (1906), an immense amount of work has been carried out to improve tests for the detection of "reagin" in the sera of patients with syphilis. The relationship of reagin to the *T. pallidum* is still not settled with certainty and, although tests for the detection of this substance have reached a high degree of specificity and sensitivity, the need for a practical test which would demonstrate antibodies clearly directed against the causative organism of syphilis has long been felt. The existence of such antibodies has been known for a long time, as early as 1912 Touraine described a method for demonstrating the agglutination of *T. pallidum* by syphilitic serum (Touraine, 1912). Other methods, such as those described by Turner (1939), have needed the use of animals for the demonstration of protective antibodies against *T. pallidum* and are therefore not suitable for routine laboratory use. The reports by Nelson (1948), and Nelson and Mayer (1949) of a relatively simple technique by which a treponemal immobilizing antibody, clearly distinct from reagin, could be demonstrated in syphilitic sera aroused great interest. The preliminary work suggested that the test was highly specific, reactions only being given by sera from patients with syphilis and other closely allied treponematoses (yaws, bejel, pinta).

In a paper on the techniques of measurement of immobilizing antibody, Nelson and Diesendruck (1951) laid down certain criteria of positivity. Sera which, under test conditions (at a final dilution of 1 in 10, incubation at 35° C. for 18 hrs) gave 0 to 19 per cent. specific immobilization (S.I.) were classed as negative, from 20 to 49 per cent. S.I. as doubtful, and 50 per cent. or more S.I. as positive. These limits were set from practical experience with the test (Nelson, personal communication) and the data on which they were based were not published. They

have been widely accepted by other workers, although Magnuson and Thompson (1949) have used 30 per cent. as the upper limit of the negative zone and 40 per cent. as the lower limit of the positive zone. In the majority of published reports more attention has been paid to the behaviour of the Treponemal Immobilization Test (T.P.I.) in cases of known or presumed syphilis than to its results in normal sera. In the present study an attempt has been made to assess the criteria of negativity by the examination of non-syphilitic sera and to confirm the findings of other workers on the prevalence of immobilizins at various stages of syphilis and thus to examine the utility of the test as a diagnostic aid for the specialist serological laboratory. Some workers (Olansky, Harris, and Hill, 1953; Saurino, 1953) have expressed dissatisfaction with the reproducibility of the T.P.I. Because reproducibility is as important as specificity in the evaluation of any serological test, an attempt has been made to assess it in the present work.

Source and Nature of Sera Examined.—The sera examined came from two sources:

(a) *Department of Venereal Diseases, London Hospital (Clinic Sera).*—These included:

- (i) 359 sera from presumed non-syphilitic patients,
- (ii) 309 sera from patients at various stages of syphilitic infection, both treated and untreated,
- (iii) 17 miscellaneous sera from patients suspected of giving transient non-specific results with serum tests for syphilis (S.T.S.).

(b) *Venereal Disease Reference Laboratory, Medical Research Council (V.D.R.L. Sera).*—These were mainly selected sera sent for examination because of anomalous serum test results in other laboratories or because the S.T.S. results were at variance with the clinical findings. For purposes of discussion they have been divided into four groups and are dealt with in Part II (see p. 149).

- (i) 233 sera giving positive S.T.S. in the absence of any clinical evidence of syphilis, the diagnosis thus lying between latent syphilis and non-specific reactions with the S.T.S. (? Latent syphilis ? N.S.R. group)

* Received for publication May 28, 1954.

- (ii) 99 sera from patients with signs or symptoms which might be attributable to syphilis but in whom the S.T.S. were negative or only weakly positive (Exclusion group).
- (iii) 42 sera from patients with a history or present evidence of syphilis in whom the S.T.S. were positive (Confirmatory group).
- (iv) 30 miscellaneous sera from patients with conditions thought to give chronic non-specific reactions with the S.T.S. (e.g. leprosy, lupus erythematosus, and haemolytic anaemia).

Technique.—Only minor modifications were introduced into the original method.

Glassware.—For distribution of sera and complement, graduated pipettes were replaced by dropping pipettes calibrated so that one drop is equivalent to 0.05 ml. These are easy to operate and far easier to clean than fine-bore graduated pipettes. The test was carried out in tubes measuring 10 × 1 cm., closed by loosely fitting aluminium caps. The extraction flask is similar to that described by Sausse (1951) with the addition of two pairs of glass hooks on the stopper and neck so that these can be held together by elastic bands to avoid accidental loosening of the stopper. Meticulous cleaning of glassware is very important; after use it was left in sulphuric acid-dichromate cleaning fluid overnight and then thoroughly washed with tap water, distilled water, and finally with doubly distilled water from an all-glass still.

Survival Medium.—Reagents (with the exception of inorganic salts) were kept at +4° C. in a vacuum dessicator over calcium chloride and appeared to be stable under these conditions. It was found advantageous to obtain freshly prepared specimens of sodium pyruvate and sodium thioglycollate from the manufacturers* at intervals of 2 to 3 months. The ox serum ultrafiltrate† was stored at +4° C. under an atmosphere of 95 per cent. N₂-5 per cent. CO₂ in 10-ml. amounts in 20-ml. Pyrex tubes with ground-glass stoppers and stopcocks.

Stock solutions of the components were prepared in doubly distilled water at the concentrations described by Nelson. Solutions of sodium thioglycollate and sodium pyruvate were prepared weekly, others at intervals of 2 to 3 weeks; all were kept at -20° C. until required. On the day of the test the requisite amounts were pipetted out and the mixture sterilized by Seitz filtration. Improved survival was obtained by doubling the amounts of

sodium thioglycollate and serum ultrafiltrate and omitting the saline in the original formula.

Gas.—The N₂-CO₂ mixture was passed over heated copper gauze in a silica tube to remove traces of oxygen.

Complement.—Pooled serum from ten or twelve guinea-pigs was sterilized by Seitz filtration and stored at -20° C. It was found satisfactory after storage at this temperature for periods up to a month. The amount used in the test was increased from 0.1 to 0.15 ml., the reaction mixture being :

Serum	0.05 ml.
Complement (fresh or inactivated) ..	0.15 ml.
Treponeme suspension	0.30 ml.

The test for residual complement after incubation and reading of the tests was carried out by adding 0.15 ml. veronal buffer as in Nelson's method, and 0.1 ml. of a 2½ per cent. suspension of sheep cells sensitized with 5 M.H.D. amboceptor. The mixtures were incubated at 37° C. for 10 min. and a further 0.1 ml. sensitized cells was added to those tubes showing sparkling lysis and the degree of lysis read after a further 20 min. at 37° C. Complete lysis of 0.1 but not of 0.2-ml. cells was recorded as ++, and of 0.2-ml. cells as + + + +. In practice + + + + lysis was obtained with nearly every serum and no result was reported as negative unless + + + + lysis was obtained.

Batch Controls.—These included :

- (a) Treponeme suspension alone.
- (b) Negative control using serum ultrafiltrate.
- (c) Positive control with serial dilutions of a pooled rabbit serum stored at -20° C. and used for a series of batches. This control gives an indication of the day-to-day variation in sensitivity, and is thought to be more valuable than (b) as an index of *in vivo* sensitization of treponemes.

Incubation.—18 hrs at 35° C.

Reading.—In the batch controls 50 treponemes were counted; in qualitative tests on sera a count of 25 was considered adequate except where the result fell in the doubtful range. In this case counts of 50 organisms were made on two preparations. Sera showing less than 70 per cent. motility in the control tube were not reported.

Repetition of Tests.—Where the result of the T.P.I. was at variance with the S.T.S. or the clinical findings, the test was repeated on the same specimen of serum. Where the T.P.I. result fell in the doubtful range, two further tests were carried out on the

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same specimen of serum. Sera showing complete immobilization in both tubes were retested after the addition of 0.05 ml. penicillinase per ml. serum.

Preparation of Inoculum.—The method described by Durel and others (1953) was found most satisfactory. The inoculum should contain 25 to 50 million Tp./ml.; 1 ml. is inoculated into each testis, and clinical orchitis usually develops in 6 to 7 days. Animals which did not develop orchitis until the 10th day or later were not used for the test.

Results

Examination of Normal Sera.—These sera came from patients attending the Whitechapel Clinic in whom a diagnosis other than syphilis had been made and whose routine S.T.S. (Wassermann Reaction with crude heart extract antigen, and Price's Precipitation Reaction) were negative. It was realized that the choice of patients of this type was open to certain objections, since many of them had other venereal diseases and might have had unsuspected syphilis in the past. However, since they had all been subjected to a careful examination by venereologists with a high index of suspicion for syphilis, their sera were readily available, and because the machinery for surveillance already existed, it was felt that these advantages more than counterbalanced the disadvantages.

In all, 349 sera were tested. 275 of them came from males (22 of whom were coloured: West Africans, West Indians, Indians, and Chinese), and 74 from females (one coloured). A comparison with the new admissions to the clinic during a 3-month period showed that the group on which the T.P.I. tests were performed was representative of the clinic population as far as the proportion of coloured to white patients was concerned but that the tested group included a larger proportion of males.

The percentage of specific immobilization (S.I.) given by these sera is shown diagrammatically in the Figure. 84.1 per cent. of sera gave an S.I. of 10 per cent. or under, 11.4 per cent. gave an S.I. between 11 and 19 per cent., and fourteen sera (4%) gave an S.I. above the upper limit of negativity. Two sera were toxic to treponemes even after the addition of penicillinase.

When the figures were re-examined according to the stage of the investigation at which the sera were tested, it was found that most of the results in the 11 to 19 per cent. S.I. range were obtained in the early stages. The S.I. readings given by the first 116 and subsequent 233 sera were as set out in Table I. This may only reflect increasing familiarity with the technique of the test; it could not be corre-

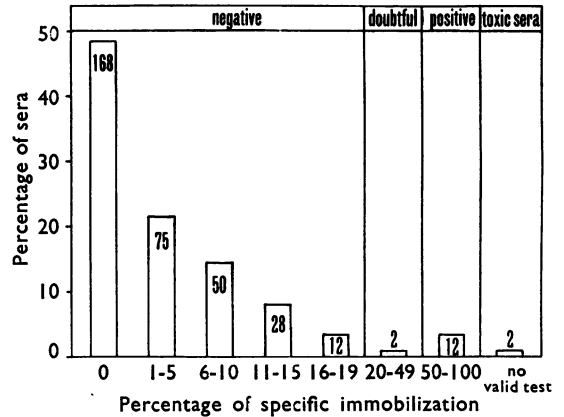


FIGURE.—Percentages of specific immobilization given by 349 supposedly non-syphilitic sera.

TABLE I
SPECIFIC IMMOBILIZATION READINGS OF 349 SERA

Per cent. Specific Immobilization	First 116 "Normal" Sera (per cent.)	Subsequent 233 "Normal" Sera (per cent.)
0	40.5	52
1-5	16.5	24
6-10	14.6	14.1
11-15	13.8	5.2
16-19	9.4	0.4
20 or more	5.1	3.4
Toxic sera	—	0.8

lated with the introduction of any modifications.

Fourteen sera from twelve patients gave a specific immobilization outside the negative range, the results being confirmed by a repetition of the test on the same specimen of serum. Brief details of these patients are given in Table II (opposite); six were Coloured, a far higher proportion than would have been expected by chance in view of the small number of coloured patients in the whole group.

In four of the coloured patients there was other evidence suggestive of the possibility of previous syphilis or yaws. The remaining two failed to attend the clinic so that no final opinion can be given regarding them; as they both came from the West Indies, the possibility of previous infection with yaws cannot be excluded.

Three among the six white patients had findings supporting the possibility of previous syphilis, and a fourth was a prostitute who defaulted after one attendance. The remaining two patients both had venereophobia and no further evidence of syphilis was obtained. One specimen of serum from Patient 11 was examined four times as the results were unstable (48, 19, 33, and 12 per cent. S.I.); the final result has been given as the mean (28 per cent.).

TABLE II
CLINICAL DETAILS OF PATIENTS IN "NORMAL" GROUP WHOSE SERA GAVE SPECIFIC IMMOBILIZATION OUTSIDE THE NEGATIVE RANGE

Patient No.	Nationality	Age	Complaint	Per cent. S.I.	Further Investigation
1	British West Indian	40	Routine test (emigration)	94	—
2	British West Indian	29	Urethral stricture	64	Vague history of yaws in childhood
3	Pakistani	33	Gonorrhoea	60 60*	Treated for penile sore with injections while at sea in 1954
4	British West Indian	28	Gonorrhoea	100	"Cut on penis" in 1940 Treated in Jamaica by tablets and an injection
5	British West Indian	39	Gonorrhoea	45	Defaulted
6	British Guianan	42	Furuncle on penis	100	Cardiolipin Wassermann reaction positive on same specimen of serum
7	White	56	Trichomonas vaginitis	64	History of intravenous injections in a V.D. clinic 20 years ago
8	White	26	Gonorrhoea	100	Cardiolipin Wassermann reaction positive on same specimen of serum
9	White	55	Contact of late syphilis	91	Husband had syphilitic glossitis
10	White	32	Vaginal discharge	100	Prostitute
11	White	25	Venereophobia	28	—
12	White	58	Venereophobia	78 76*	Patient "thought she might have caught something off a lavatory seat." Vague history of injections for boils some years previously

* Tests on two separate specimens of serum.

Such "unstable" sera have been very uncommon in the present series. Thus, among 326 white patients who were presumed to be non-syphilitic, one positive and one doubtful result with the T.P.I. were unsupported by any other evidence suggestive of syphilis.

As the reading of the test depends upon the examination of a relatively small number of treponemes seen consecutively in specimens from the reaction mixtures, the possibility of the influence of random errors in sampling on the result of the test must be considered. The analysis of 300 sera in the "normal" group which gave negative results may be tabulated as follows :

Complement	Inactive	Active
Mean motility (per cent.) ..	92.6	90.2
Standard deviation ..	6.02	7.25

In the inactive complement (control) tubes, motilities of below 2 S.D. less than the mean were found in sixteen sera (5.3 per cent.) and 3 S.D. below the control in one (0.3 per cent.). In the active complement tubes, six sera (3 per cent.) showed motilities of more than 2 S.D. below the mean and none more than 3 S.D. below this value. The probability of a chance variation of 3 S.D. from the mean is 1 in 370 (Dahlberg, 1940). If this occurred in the active complement tube, while the control tube showed the

observed mean motility, the specific immobilization level would apparently be 26 per cent.—*i.e.* just in the doubtful zone. Examination of a second preparation should correct this error, since the probability of the second slide giving a similar low motility due to chance is remote. Erroneous readings due to random sampling can also, of course, be reduced by increasing the number of organisms counted. The increase in accuracy will, however, only vary as the square root of the number counted ; thus by counting 50 instead of 25, the accuracy is only increased in the proportion of 1 : 1.4. Thus from the results obtained above it is felt that the upper limit of negativity of 19 per cent. S.I. is safe, provided that possible errors due to random sampling are guarded against by examination of duplicate specimens where indicated.

Examination of Syphilitic Sera

EARLY SYPHILIS

(a) *Primary*.—Only four sera from untreated patients were tested. Both the T.P.I. and S.T.S. were negative in one, the T.P.I. was negative but the S.T.S. were positive in two, and in the fourth both tests were positive, the T.P.I. showing 62 per cent. S.I.

Forty sera were examined from 36 patients with treated primary syphilis. Two sera were toxic ; the

results of the remaining 38 sera compared with the S.T.S. were these :

S.T.S.	+	+	-	-	-
T.P.I.	+	±	+	±	-
No. of Sera	4	1	2	3	28

Nine patients known to be sero-negative with the S.T.S. at the time of the original diagnosis were all T.P.I. negative ; in thirteen, who were initially sero-positive with the S.T.S. before treatment, the T.P.I. was positive in five and doubtful in two. The original S.T.S. findings at the time of diagnosis of the remaining fourteen patients were not known. Of the group of six patients with a positive T.P.I., in two, whose S.T.S. were negative, the infections dated from 13 and 21 years previously, in two whose S.T.S. were only positive with the more sensitive tests (Cardiolipin Wassermann Reaction, and Kahn), the diagnosis had been made 9 years previously. In the remaining two patients, whose S.T.S. were positive and one of whom had developed neurosyphilis, the original infections were 6 and 11 years old.

(b) *Secondary*.—Three patients with untreated secondary syphilis all had positive T.P.I. tests. Seventeen patients who had been treated gave the following results :

S.T.S.	+	-	-
T.P.I.	+	+	-
Sera	1	11 (1 ±)	5

Sera from eight patients were examined within 5 years after treatment ; the T.P.I. was positive in six, doubtful in one, and negative in one. The other nine sera were examined 6 to 22 years after treatment, and five were positive and four negative with the T.P.I.

The numbers of sera from patients with early syphilis are unfortunately much too small to allow more than tentative conclusions to be drawn. The results are in broad agreement with those of other workers and suggest that, unless treatment is given very early in the course of the infection, immobilizins are produced and may persist for long periods of time, despite treatment which is currently accepted as being adequate in amount and intensity as judged by the clinical response and the reversal of the S.T.S. The significance of the residual positive immobilization test is not known and it may well be no more than a "serological scar". It should be emphasized, however, that only qualitative tests have been used. Examination of these patients' sera by periodical quantitative tests is necessary to deter-

mine whether their immobilizin titre remains static, in analogy with "Wassermann-fast" cases, or whether there is a slow decline over the years. This would be, however, a long-term investigation and one which the increasing rarity of early syphilis in this country would render difficult to carry to a satisfactory conclusion.

Late Syphilis (Congenital and Acquired).—This group consisted of 85 sera from 83 patients of whom ten were untreated. They included ten patients with cardiovascular syphilis, thirty with neurosyphilis, 23 with late cutaneous or osseous lesions, and twenty with lesions due to congenital syphilis. The T.P.I. was positive in all but one of the untreated group and for brevity these will be considered with the treated patients. The serological results were as follows :

S.T.S.	+	-	-	+	+	-	+
T.P.I.	+	+	±	±	-	-	Toxic serum
Sera	58	15	4	2	1	4	1

Ten of the eleven sera giving doubtful or negative T.P.I. results were from treated patients. Of the six giving doubtful results, four were from patients who had had interstitial keratitis (aged 18, 19, 53, and 59), the last being the only one of the four who showed positive S.T.S. The remaining two patients giving doubtful results were a woman, aged 71, who had had a gumma of the skin 6 years previously and whose S.T.S. were negative, and a man, aged 67, with "burnt-out" tabes whose S.T.S. were positive at a low titre. Five patients gave a negative T.P.I. One had been diagnosed as a congenital syphilitic 13 years previously and treated on the grounds of a single positive S.T.S.; all his subsequent tests were negative and it seems that there may be some doubt as to the correctness of the original diagnosis. Three other patients had had undoubted congenital syphilis : one, aged 47, had had interstitial keratitis 34 years previously ; a second, aged 45, had Hutchinsonian teeth and tissue-paper scars over the tibiae and had been treated 16 years previously ; the third, aged 4, had been treated at birth. The remaining patient was a woman, aged 56, who was thought on clinical and radiological grounds to have syphilitic aortitis. Her S.T.S. were only weakly positive. She died 2 months after the institution of treatment from a ruptured saccular aneurysm of the aorta.

It would appear from the foregoing that the T.P.I. is positive in the majority of cases of late syphilis and is little influenced by treatment as far as can be judged by qualitative tests. Most of the patients who

gave doubtful or negative results had congenital or acquired infections of long standing. The case of the patient with a negative T.P.I. who died of a ruptured aneurysm raises the question of the possibility of a spontaneous reversal of the T.P.I. with the passage of time in the absence of treatment. The T.P.I. has been advocated as a diagnostic test for syphilis in patients who present lesions which may be due to the disease but whose S.T.S. are negative. While a positive result may be of help, it should be clearly realized that a negative result does not *absolutely* exclude the possibility of past infection, especially in patients where the infection may be congenital in origin or in elderly patients with acquired disease, e.g. in "burnt-out" tabes.

Latent Syphilis.—The sera in this group came from patients who were diagnosed as having latent syphilis on the grounds of positive S.T.S. alone and who gave no previous history of syphilis; 51 sera came from 42 untreated patients, and 94 sera were examined from ninety patients who had been treated in the past.

Untreated Patients.—The results of parallel tests with the S.T.S. and the T.P.I. were as follows:

S.T.S.	+	-	±	Toxic serum
T.P.I.	+	-	±	
Sera	44	3	3	1

Three patients gave negative S.T.S. with a positive T.P.I. One was a woman whose child had congenital syphilis; the second was a coloured woman who had had repeated attacks of gonorrhoea and whose S.T.S. had previously been positive; the third had had latent syphilis in 1945 but defaulted almost immediately after treatment had been started, and is therefore considered in the untreated group. Examination of a second specimen of her serum showed a positive T.P.I. and weakly positive S.T.S. A negative T.P.I. in the face of positive S.T.S. was found in two patients in the untreated group. One was a woman, aged 53, who asked for a blood test because her sister had a skin disease thought to be lupus vulgaris; no other evidence of syphilis was noted and the same pattern of results was found when a second specimen of serum was tested. The second patient was a girl, aged 20, whose S.T.S. had been found positive on routine testing as a blood donor (similar results were found on re-testing two further specimens not included in the present series), but no other evidence of syphilis was found.

Treated Patients.—The comparative serological results were as follows:

S.T.S.	+	-	-	-	-
T.P.I.	+	-	-	-	-
Sera	64	1	20	5	4

It will be seen that in twenty sera the T.P.I. remained positive after the S.T.S. had reversed to negative, while in 65 sera both tests were positive (in one the T.P.I. was doubtful).

Five patients showed a negative T.P.I. with positive S.T.S. All were female, and in four the original diagnosis had been made on the basis of positive S.T.S. found in pregnancy. One patient was subsequently proved to have disseminated lupus erythematosus. In four other patients both the S.T.S. and the T.P.I. were negative; it was noted that all four had rapidly become sero-negative after the institution of treatment, suggesting either that their infections were of recent origin, or that possibly the S.T.S. had originally given transient non-specific reactions.

It was noted that, taking the treated and untreated patients with presumed latent syphilis together, the seven patients who showed negative T.P.I. tests with positive S.T.S. were all women. An analysis of the whole group showed that 65 sera came from males and eighty from females. The preponderance of females is only partly accounted for by the effect of routine antenatal serum tests in revealing presumed latent syphilis; 27 of the eighty females first came under observation through this means.

Syphilis of Unknown Status.—Sera were examined from fourteen patients in whom the original stage of the disease at which treatment had been begun was unknown. Both S.T.S. and T.P.I. were positive in two and negative in seven. The S.T.S. were negative in four patients, three of whom had a positive and one a doubtful T.P.I. In the remaining case, which has been reported elsewhere (Wilkinson, 1954), the T.P.I. was negative while the S.T.S. showed strongly positive but anomalous reactions.

Patients with Transiently Positive Reagin Tests.—Seventeen sera were examined from thirteen patients whose S.T.S. had shown transient weak positive reactions on routine tests. In all save one (a coloured patient) the T.P.I. was negative.

PART II. EXAMINATION OF "PROBLEM" SERA

In this section the results of immobilization tests on sera sent to the Venereal Diseases Reference Laboratory (Medical Research Council) are reported. The great majority were "problem" sera, in the sense that tests at other laboratories had given results at variance with the clinical findings and

confirmatory tests were requested; they therefore form a highly selected group. Upon receipt in the laboratory, each specimen was split into two parts. On one, an immobilization test (T.P.I.) was carried out by the methods outlined in Part I of this paper; on the other a battery of four serum tests for syphilis (S.T.S.) in routine use in the laboratory were performed. These consisted of:

- (a) Wassermann reaction with a crude heart extract antigen at its optimum titre.
- (b) Wassermann reaction with cardiolipin antigen.
- (c) Standard Kahn reaction.
- (d) Price's Precipitation Test (Price, 1948).

Group 1, ? Latent Syphilis, ? Non-Specific Reaction.—This comprised 233 sera from 209 patients whose positive reagin tests were the only presumptive evidence of syphilis. Of these, sera from pregnant women (97) and from blood donors (27) formed the largest single groups.

A comparison of the results obtained with the T.P.I. and the battery of reagin tests viewed as a whole is shown in Table III. There was agreement

TABLE III

COMPARISON OF RESULTS OF S.T.S. AND T.P.I. IN SERA OF THE ? LATENT SYPHILIS, ? N.S.R. GROUP

S.T.S.	All +	All +	Partial +	Partial +	All Neg.*	All Neg.	
T.P.I.†	+	—	+	—	—	+	N.V.T.
Sera	91	22	36	47	15	2	20
Per cent.‡	42.5	10.2	16.8	22	7.4	0.9	—

The S.T.S. are recorded as "Partial +" when some tests gave positive and others negative results.

*These sera had given a positive S.T.S. on previous specimens.

†Five doubtful T.P.I. results included among the positives.

‡Percentage calculated from the 213 sera in which a valid comparison would be made.

between the T.P.I. and one or more of the S.T.S. in 142 sera (66.6 per cent.), and disagreement in 71 sera (33.3 per cent.). If the T.P.I. is taken as a yardstick, this suggests that one or more of the S.T.S. had given non-specific results in 69 sera, or in 32.2 per cent. of the 213 sera in which valid comparisons could be made (in two sera the T.P.I. was positive but the S.T.S. were negative on the specimens tested, although they had given positive results on previous specimens from these patients). The figure of 32.2 per cent. is slightly exaggerated, since repeat tests were carried out on sera from eight patients in whom the S.T.S. were positive and the T.P.I. negative; in each case the T.P.I. was again negative. Allowing for this, the incidence of presumed non-specific results with the S.T.S. is 29.7 per cent.

For comparison, the results of a similar analysis of the sera from pregnant women and blood donors are shown in Table IV.

TABLE IV

COMPARISON OF RESULTS OF S.T.S. AND T.P.I. IN SERA FROM PREGNANT WOMEN AND BLOOD DONORS

S.T.S.	All +	Partial +	All +	Partial +	Neg.	Neg.
T.P.I.	+	+	—	—	—	—
Pregnant Women	47	10	10	23	6	1
Blood Donors	8	4	3	12	—	—

The remainder of the sera came from patients with a diversity of clinical conditions but the numbers were too small for analysis to be profitable.

It should be emphasized again that the sera in this group were selected, in the sense that the results of the S.T.S. were at variance with the clinical findings and the sera were submitted for the T.P.I. test for this reason. The figure of 29.7 per cent. presumed non-specific results with the S.T.S. is considerably higher than that found in the latent syphilis group of clinic patients (4.8 per cent. in untreated and 5.5 per cent. in treated patients). The latter figures, however, are much lower than that of 45.3 per cent. reported by Moore and Mohr (1952a) among treated and untreated patients in private practice, and that of 42.5 per cent. reported by Nelson (1953) in 496 untreated service personnel. Gaté and others (1953), in an analysis of collected material from French clinics, found 134 out of 493 patients (27 per cent.) with treated latent syphilis to have negative T.P.I. tests.

The results obtained in this group of sera have also been analysed to compare the T.P.I. results with those given by the four S.T.S. of the battery. The comparison is shown in Table V (opposite).

If the T.P.I. results are again taken as a yardstick for the comparison, Table V shows that the order of specificity of the four S.T.S. is P.P.R. > * Standard W.R. > Kahn > Cardiolipin W.R. A comparison of sensitivity can also be made from those sera in which the individual S.T.S. and the T.P.I. were both positive. The relative order of sensitivity of the four tests is then Cardiolipin W.R. > Standard W.R. > Kahn > P.P.R. These relative orders of specificity and sensitivity of the four tests are those which had been arrived at on the basis of long practical experience with them with sera of known provenance.

It had long been suspected that the Cardiolipin Wassermann Reaction, while an extremely sensitive

* > = greater than.

TABLE V

COMPARISON OF T.P.I. RESULTS WITH INDIVIDUAL S.T.S. IN THE ? LATENT SYPHILIS ? NON-SPECIFIC REACTION GROUP

S.T.S.		Agreement						Disagreement					
		TPI +		TPI -		Total		TPI +		TPI -		Total	
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Cardiolipin Wassermann Reaction	+	124	(58)	—	—	150	(70)	—	—	58	(27)	63	(30)
	-	—	—	26	(12)			5	(2.3)	—	—		
Standard Wassermann Reaction	+	110	(52)	—	—	154	(72)	—	—	40	(18.8)	59	(28)
	-	—	—	44	(20)			19	(8.9)	—	—		
Kahn	+	107	(50.2)	—	—	144	(67.5)	—	—	46	(21.5)	69	(32.3)
	-	—	—	37	(17.3)			23	(10.8)	—	—		
P.P.R.	+	99	(46.5)	—	—	155	(73)	—	—	29	(13.6)	58	(27.2)
	-	—	—	56	(26.5)			29	(13.6)	—	—		

test, was giving an undue proportion of non-specific results. The P.P.R., on the other hand, while considerably less sensitive, was thought to be very specific. It should be understood that the finding of low specificity of the Cardiolipin Wassermann Reaction applies to the antigenic formula used (cardiolipin 1, lecithin 1, cholesterol 10; Price, 1953) and may well not be applicable to cardiolipin antigens of different composition.

It has usually been thought that non-specific results with the S.T.S. are generally low titred, and that a serum giving a high titre is most probably syphilitic. A comparison of P.P.R. titres with T.P.I. results on 213 sera in the group under discussion gave the results shown in Table VI.

TABLE VI

COMPARISON OF P.P.R. TITRE WITH T.P.I. RESULT IN SERA OF THE ? LATENT SYPHILIS ? N.S.R. GROUP

T.P.I.	P.P.R. Titre							
	0	Neat*	2	4	8	16	32	32+
Positive	29	52	16	12	9	4	2	4
Negative	56	18	4	4	2	1	—	—

*Sera giving a negative result by the routine test but a positive P.P.R. on centrifuging have been included in the "neat" group.

If the T.P.I. result is taken as an index of the specific nature of the reaction, it will be seen that the generalization cited above is true in the majority of cases. There is, however, a sufficiently large proportion of strongly reacting sera giving P.P.R. titres of 4 or higher, which in view of the negative T.P.I. are thought to be non-specific, to make undue reliance on the titre unwise. There have not been any reports of the prozone phenomenon in the T.P.I. test. A few sera which gave a negative T.P.I. in the face of a P.P.R. of high titre have been ex-

amined by a quantitative technique to detect possible zoning; no evidence of this was found.

Group 2, "Exclusion" Sera.—This group consisted of 98 sera from 91 patients who had signs or symptoms which might be attributable to syphilis but whose S.T.S. gave negative or anomalous results. The comparative results are shown in Table VII which shows that there was agreement between the T.P.I. and S.T.S. in 59 sera and disagreement in thirty where comparisons could be made. In seventeen the T.P.I. was positive or doubtful while the S.T.S. were negative; three of these patients were old congenital syphilitics, three were the mothers of siblings of known congenital syphilitics, three were cases of clinical tabes, and two were coloured patients who gave a definite history of yaws in childhood.

TABLE VII

COMPARISON OF T.P.I. AND S.T.S. IN THE "EXCLUSION" GROUP OF SERA

S.T.S.	All +			Partial +			Neg.			A.C.
	All +	Partial +	Neg.	All +	Partial +	Neg.	All +	Partial +	Neg.	
T.P.I.	+	+	+	-	-	-	-	-	-	N.V.T. (Toxic serum)
Sera	1	10*	17†	1	12	48	2	7		

*Includes one doubtful T.P.I.

†Includes five doubtful T.P.I. results.

Group 3, "Confirmatory" Sera.—This was a small group of 41 sera from 34 patients who either showed clinical evidence of syphilis or in whom the diagnosis had been made in the past and treatment given. Six sera were toxic, but in the 35 where comparison could be made between the S.T.S. and T.P.I., there was agreement in thirty and disagreement in five. The T.P.I. was negative in six sera; two from a patient with aortic incompetence who

had been treated for syphilis, one from a patient with treated secondary syphilis whose S.T.S. were still weakly positive, one from a treated contact whose marital partner had latent syphilis, and the remaining two from old treated cases. It will be seen that the T.P.I. was of little value in these cases, although, if positive, it may be of use as a means of retrospective confirmation of diagnosis in patients who have been treated for latent syphilis on the basis of positive S.T.S. alone.

Group 4, Conditions thought to give Non-Specific Reactions with the S.T.S.

Leprosy.—Twelve sera were examined from nine patients. The S.T.S. were positive in all (in one the complement-fixation reactions were anticomplementary). The T.P.I. was negative in seven patients and positive in two; one of the two was a coloured man who was known to have neurosyphilis, the other was a white man who showed no clinical evidence of syphilis.

Disseminated Lupus Erythematosus.—Nine sera were examined from seven patients. The S.T.S. were positive in six and negative in one while the T.P.I. was negative in all.

Acquired Haemolytic Anaemia.—Four sera were examined from three patients. The T.P.I. was negative in all and the S.T.S. positive in two, six previous specimens of this last patient's serum had given positive S.T.S. results.

Miscellaneous.—The T.P.I. was negative and the S.T.S. positive in one patient with sarcoidosis; one with anaemia, thrombocytopaenia, and splenic enlargement; one with leukaemia; and one with anaemia, splenomegaly, and achlorhydria. One patient with thrombocytopaenic purpura gave a positive T.P.I. and S.T.S.

Reproducibility of the Test

Tests were carried out on second specimens of sera from 46 patients to confirm the original T.P.I. result. In 41 cases the second test was in complete agreement with the first, in five there were minor discrepancies. The percentages of specific immobilization found on the two occasions were: 32 and 51 per cent., 40 and 76 per cent., 62 and 33 per cent., 40 and 20 per cent., and 44 and 12 per cent. In the last instance treatment had been given before the submission of the second specimen. It will be seen that these discrepancies were of only minor degree and in no case was there a reversal from a frank positive to negative or *vice versa*.

The estimation of the 50 per cent. immobilization titre of the positive control serum affords a means of

assessing the reproducibility of the test from day to day. A positive control serum was supplied to laboratories undertaking the T.P.I. test by the World Health Organization. Serial dilutions of this serum were prepared in saline, kept at -20°C ., and used as the positive control for about nine successive batches per ampoule of undiluted serum. The results of tests on the first three ampoules of serum are shown in Table VIII. The ampoules of undiluted serum were stored at $+4^{\circ}\text{C}$., in accordance with the suggestion of the W.H.O. The results with dilutions prepared from one ampoule were fairly consistent, but there was a drop in the mean titre as the period of storage increased.

TABLE VIII
SERIAL ESTIMATIONS OF THE 50 PER CENT. IMMOBILIZATION TITRE ON THE SAME SERUM

Ampoule	No. of Tests	50 per cent. Immobilization Titre	Range	S.D.
1	10	334	210-447	64
2	8	266	160-850*	222
3	9	175	135-260	41

**Treponemata sensitized in vivo.*

About 8 months elapsed between the first estimation on Ampoule 1 and the last on Ampoule 3; at one test on Ampoule 2 the *treponemata* were sensitized *in vivo* and the test had to be abandoned. If this value is omitted, the mean titre for Ampoule 2 becomes 183 (with a range of 160-250 and an S.D. of 29), and the results with all three ampoules fall within ± 2 S.D. of the means. These results are comparable with those reported by Khan and others (1951) and Chorpenning (1953), and suggest that the test can give reproducible results with a quantitative technique.

Adequacy of Criteria of Positivity

It is very desirable in any serological test that the demarcation between positivity and negativity should be as sharply defined as possible. The results obtained in the present series of sera from very varied sources have shown that, in the great majority of cases, the result fell clearly into a positive or negative category. Table IX (opposite) shows that only 26 of 991 sera fell into the doubtful range and these were mainly from patients with treated or old congenital syphilis.

Discussion

In the evaluation of a serological test for syphilis most stress should be laid on its specificity. The T.P.I. has, from its inception, been acclaimed as highly specific for *treponemal* infection. Examination

TABLE IX

OCCURRENCE OF DOUBTFUL T.P.I. REACTIONS IN THE VARIOUS TYPES OF SERA TESTED

Serum Group		T.P.I. Result		
		Positive	Doubtful	Negative
" Normal " Patients		12	2	323
Primary Syphilis (treated)		6	4	28
Secondary Syphilis (treated)		11	1	5
Latent Syphilis	Untreated	47	0	3
	Treated	85	1	9
Late Syphilis	Untreated	9	0	1
	Treated	63	6	4
V.D.R.L.	? Latent syphilis, ? N.S.R.	126	5	84
	Exclusion	23	7	62
	Confirmation	30	0	6
	Miscellaneous	4	0	24
Totals out of 991 Sera		416	26	549

of published reports shows, however, that only relatively small numbers of tests on sera from presumed normal persons or from patients with diseases other than syphilis have been reported by individual workers. Thus, Nelson and others (1950) found negative results in 101 normal persons and in 107 with diseases other than syphilis; Magnuson and Thompson (1949) found that 73 normal patients and 81 with diseases other than syphilis all gave negative results; Miller and others (1952) reported negative results in thirteen normal persons and in seventy with diseases other than syphilis; Chacko (1953) found no positive results among 241 non-syphilitic sera; Sequeira (1954) examined 256 sera from pregnant women and found two which gave positive T.P.I. tests but negative S.T.S.

In the present series, one doubtful and two positive results were found in 326 sera from white patients attending a venereal diseases clinic with diseases other than syphilis.

The serum giving a doubtful result was examined early in the series and four tests of the specific immobilization varied from 12 to 48 per cent.; it is felt that errors in technique may account for the divergence in results.

Technical error can be excluded in the second patient as positive results were obtained on two separate specimens of serum; there was no clinical evidence or history of infection, but it was felt that the patient was not being entirely frank and she gave a vague history of having received "injections" for boils some years previously. If these were injections of penicillin, they might well account for the serological findings.

A third patient also showed no evidence or history of syphilis, but was a prostitute.

Positive T.P.I. results were obtained in three further patients in this group, but further investigations revealed evidence suggestive of syphilis.

It is felt that the results in this group of presumed non-syphilitic individuals are in agreement with those of other workers and support the reputation of the T.P.I. as a highly specific test.

The results obtained from tests on sera from patients at various stages of syphilis are in broad agreement with those previously reported by other workers. Comparison of the various clinical groups according to the stage of the disease at the time of the original diagnosis showed that the proportion of treated patients who were found to be T.P.I. negative progressively fell with increasing duration of the disease. The achievement of negativity with the T.P.I. has been suggested by some workers as a criterion of cure in early (primary and secondary) syphilis.

The persistence of a positive T.P.I. in patients with early syphilis, who, after treatment which is currently considered adequate, show negative S.T.S. and C.S.F. findings, raises a problem of interpretation. It is not yet known whether the persistence of immobilizins in such patients is a harbinger of trouble to come, but in the present state of our knowledge it would seem prudent to keep such patients under observation and perform serial quantitative T.P.I. tests to determine the trend of the titre.

In late symptomatic syphilis the T.P.I. is almost invariably positive and, as far as can be judged from qualitative tests, treatment has little effect on the test. It must be remembered, however, that a small proportion of these patients are T.P.I. negative; the finding of a negative T.P.I. in an untreated patient who has signs or symptoms which might be attributable to late syphilis does not absolutely rule out the diagnosis, although it makes it improbable.

Thus, among thirty patients with clinical evidence of congenital syphilis, four had a negative T.P.I. test. The ages of three of these were 33, 47, and 55, that of the fourth being unknown. All four had been treated many years previously. A further instance is that of a woman with signs and symptoms of aortic disease which were thought on clinical grounds to be syphilitic in origin, despite very weakly positive S.T.S. and a negative T.P.I. test. She died suddenly soon after treatment had been begun and autopsy showed a ruptured saccular aortic aneurysm.

The reverse problem of interpretation is presented by patients showing a positive T.P.I. with negative standard tests who have clinical findings suggesting syphilis. In these cases the positive T.P.I. results give strong supporting evidence of past infection. Where there is no clinical evidence to support the positive T.P.I., a problem of interpretation arises

which will become more frequent with the wider adoption of the test. If technical error is excluded by confirmation of the result with a second specimen of serum, it is felt that previous treponemal infection is indicated. Several examples have been encountered in the present series. Two instances were of women whose children had developed signs of congenital syphilis. The mothers had not been treated but their infections had presumably become latent by the time of the test. In a third case, a brother 2 years older than the patient was known to have juvenile general paresis, and in a further case the patient's husband was known to have late syphilis. The concept of a possible type of silent latent syphilis which is revealed only by the presence of a positive immobilization test raises the question of its significance for the patient and whether treatment is indicated. In the present state of our knowledge this must be left as an open question.

The use of the T.P.I. as a verification test in cases of presumed latent syphilis has given divergent results in the hands of various workers. Moore and Mohr (1952a), in a study of treated and untreated patients in private practice, found 45.3 per cent. of 300 patients to have negative immobilization tests. In general the result of the T.P.I. test agreed fairly well with the authors' clinical assessment of patients as probable latent syphilitics or probable "chronic biological false positive reactors". Durel and others (1952) found 89 (27 per cent.) patients with treated latent syphilis to have negative immobilization tests. Nelson (1953), in a study of 496 service personnel who had positive S.T.S. (mainly standard Kahn tests) but no other evidence of syphilis, found 42.5 per cent. to have negative immobilization tests.

Gate and others (1953), in a combined study from several clinics in France, found 134 (27 per cent.) of 493 treated latent syphilitics with negative immobilization tests.

Other workers have found a much lower incidence: thus Magnuson and Thompson (1949) found only one negative T.P.I. in 106 patients with early latent syphilis and none in twenty cases of late latent syphilis; Miller and others (1952) found no T.P.I.-negative cases among 37 patients with early latent and only nine in 213 patients with late latent syphilis (these nine patients all had positive or weakly positive S.T.S.); Chacko (1953) found nine patients out of 101 with treated latent syphilis to have negative immobilization tests.

In the present series of clinic patients with presumed latent syphilis, two out of 42 untreated and five out of ninety treated patients were T.P.I. negative. In contrast to this, 29.7 per cent. of 209 sera sent to the V.D. Reference Laboratory from

patients in whom the diagnosis lay between latent syphilis and non-specific S.T.S. reactions had negative immobilization tests. This last group of sera was selected, and hence the results cannot be taken as representative.

The disparity between the results in various laboratories calls for some comment. It is not known what proportion, if any, of patients with early latent syphilis become T.P.I. negative as a result of treatment or spontaneously, nor whether such a reversal, if it occurs, can precede that of the S.T.S. If the proportion is significant it may well influence the results in different series containing varying proportions of early and late latent cases. The incidence of syphilis in the population from which the patients are drawn will have a profound effect on the results; this is well illustrated in figures quoted by Magnuson (1953). Where the incidence of syphilis is high, most of the cases of "latent syphilis" will, in fact, have the disease, while in populations where the incidence is low, a higher proportion of non-specific reactions with the S.T.S. may be expected. The levels of specificity and sensitivity of the S.T.S. on which the original diagnosis was founded are obviously of paramount importance.

The work reported by Moore and Mohr (1952 a, b), showing that the occurrence of non-specific reactions with the S.T.S. may be indicative of serious disease in some patients, has aroused wide interest. It has been established that diseases of the collagen group may be associated with persistent non-specific reactions with the S.T.S. in some patients; Haserick and Long (1952) have reported cases of disseminated lupus erythematosus in which such reactions preceded the manifestations of the disease by as much as 7 years. It would therefore seem desirable that special attention should be directed to patients showing such non-specific reactions, and that they should be investigated along the lines suggested by Moore and Mohr (1952a) and not discharged from observation once the clinician is satisfied that they are not suffering from syphilis. In the majority of patients no cause for the non-specific S.T.S. can be found and these patients still offer a challenging field for research.

Conclusions

On the basis of the sera personally studied and the reports of other workers, the field of use of the immobilization test in routine diagnostic serology can be summed up as follows:

(i) In early (primary and secondary) syphilis it offers no advantages over existing diagnostic methods.

(ii) In late (symptomatic) syphilis where the S.T.S. are positive the T.P.I. may be expected to be positive; in these circumstances the test has only confirmatory value and is not indicated for routine use.

(iii) In the diagnosis of suspected latent syphilis it is a most valuable confirmatory test. In such cases, if the T.P.I. is negative and this finding is confirmed by examination of a second specimen of serum, the positive S.T.S. should be strongly suspected of being non-specific in nature.

(iv) In patients who present signs or symptoms which may be attributable to late syphilis but in whom the S.T.S. are negative, a positive T.P.I. is strong confirmatory evidence of the disease. A negative T.P.I. does not absolutely exclude syphilis, but renders the diagnosis improbable.

(v) Although the immobilization test is highly specific, it does not distinguish between syphilis and the other treponematoses.

(vi) In the routine serology of syphilis it does not replace the S.T.S. but should be used as an ancillary to them.

Many thanks are due to Dr. I. N. Orpwood Price for providing facilities for carrying out the test and to Mr. A. J. King, F.R.C.S., Senior Physician, Department for Venereal Diseases, London Hospital, for allowing access to clinical records. I am also greatly indebted to Mr. C. J. Storey and Dr. B. Boxall for their help in carrying out the tests.

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