

## ENHANCEMENT BY LYSOZYME OF THE SENSITIVITY OF *TREPONEMA PALLIDUM* IMMOBILIZATION TESTS\*

BY

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The addition of lysozyme to the survival medium has been shown (Metzger, Hardy, and Nell, 1961) to increase the rate of immobilization of *Treponema pallidum* by syphilitic serum and complement; this might allow *T. pallidum* immobilization (TPI) tests to be completed in a working day. But, more important from the point of view of diagnosis, was the possibility of increasing the sensitivity of the standard 18-hr TPI test and, perhaps also, of the already highly sensitive 40-hr test (Rosenau and Kent, 1958; Kent and DeWeerd, 1961).

### Materials and Methods

**Treponemal Survival Medium.**—The medium adopted contained the originally described ingredients (Nelson and Diesendruck, 1951) plus streptomycin (Borel, Bentejac, and Durel, 1958), penicillinase, and lysozyme; the sodium thioglycollate concentration was half that used in the initial studies of lysozyme, and bovine plasma fraction V was used instead of gelatin (Metzger and others, 1961). It was composed as follows:

Component	g. (or ml.)/l.
Plasma fraction V (bovine) ..	25·000 g.
Serum ultrafiltrate (bovine) ..	62·500 ml.
NaHCO <sub>3</sub> .. .. .	0·712 g.
Na <sub>2</sub> HPO <sub>4</sub> .. .. .	1·774 g.
KH <sub>2</sub> PO <sub>4</sub> .. .. .	0·643 g.
Streptomycin .. .. .	1·000 g.
Penicillinase (1,000 u./ml.) ..	6·250 ml.
Lysozyme † .. .. .	0·075 g.
Sodium pyruvate .. .. .	0·155 g.
Sodium thioglycollate .. .. .	0·450 g.
Glutathione .. .. .	0·387 g.
Cysteine hydrochloride .. .. .	0·198 g.
Distilled water q.s. .. .. .	1,000·000 ml.

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 † Crystalline (egg white), B grade, California Corp. for Biochemical Research, Los Angeles 63, California.

The pH was adjusted to  $7\cdot0 \pm 0\cdot02$ , and the solution sterilized by filtering through a Millipore membrane of  $0\cdot45\mu$  porosity. Forty ml. volumes were shell frozen in 100-ml. heavy glass ampoules, and desiccated under reduced pressure. The ampoules were sealed hermetically under vacuum and stored at  $-25^{\circ}\text{C}$ . For use they were rehydrated with 40 ml. sterile 0·59 per cent. NaCl solution. The concentration of the rehydrating solution represents the sum of the NaCl amounts used originally (Nelson and Diesendruck, 1951); the salt was not added before desiccation because it lowered the freezing point of the medium enough to cause melting before desiccation was complete.

**TPI Procedure.**—The standard procedure was followed (Nelson and Diesendruck, 1951; Rosenau and Kent, 1958; Kent and DeWeerd, 1961) except that the volumes of serum, antigen, and complement (C') were 0·05, 0·375 and 0·075 ml.; five parts of antigen and one part of C' were mixed and added as 0·45 ml. Human and rabbit sera were heated 30 mins. at  $56^{\circ}\text{C}$ . to inactivate native C'. For titrations they were diluted serially in 0·85 per cent. NaCl solution, and each dilution was tested with antigen and active guinea-pig C'; the lowest dilution was tested as control with antigen and heated C'. Non-syphilitic human sera were included as controls, and provided indices both of treponemal survival and specificity of the reaction. After 18 hrs' anaerobic incubation, a loopful (about 0·01 ml.) was removed from each tube, and the percentage of motile treponemes determined by dark-field microscopic examination. Five hundredths ml. of active and heated C' respectively were then added to tests and controls, and the mixtures re-incubated. At 40 hrs the percentages of motile treponemes were redetermined, and the 18-hr and 40-hr readings converted (Nelson and Diesendruck, 1951) to percentage specific immobilization. Results of qualitative tests were



TABLE I

TITRES OF SYPHILITIC CONTROL SERA WITHOUT AND WITH 0.0075 PER CENT. ADDED LYSOZYME

Serum	Date Tested	Titre*			
		Without		With	
		18 hrs	40 hrs	18 hrs	40 hrs
WHO IV† (Human)	Nov. 20-22, '61 ..	87	240	—	—
	Dec. 26-28, '61 ..	47	178	—	—
	Feb. 14-16, '62 ..	63	135	—	—
	Feb. 27- Mar. 1, '62	44	120	—	—
	Mar. 14-16, '62 ..	—	—	110	214
	May 1-3, '62 ..	—	—	282	575
	May 15-17, '62 ..	—	—	229	631
	May 22-24, '62 ..	—	—	191	363
	Mean .. ..	60	168	203	446
	NYS 100659‡ (Rabbit)	Mar. 6-8, '62 ..	—	—	105
Mar. 21-23, '62 ..		31	162	—	—
Mar. 28-30, '62 ..		—	—	105	550
Apr. 24-26, '62 ..		20	66	—	—
May 8-10, '62 ..		—	—	98	427
Mean .. ..		26	114	103	408

\* Reciprocal of serum dilution producing 50 per cent. immobilization.

† Distributed by the World Health Organization Serological Reference Centre, Statens Seruminstitut, Copenhagen.

‡ Standard of the Division of Laboratories and Research, New York State Department of Health, Albany, N.Y.

To supplement these findings, human sera that had been found negative or weakly reactive in qualitative TPI tests without lysozyme were re-examined with added enzyme. The results, which demonstrate the efficacy of lysozyme in detecting threshold concentrations of treponemal immobilizing antibody, are summarized in Table II.

Of twenty specimens that had been negative at 18 hrs, five remained negative at 18 hrs, but six became weakly reactive, and nine were reactive; of thirteen originally found weakly reactive in the same circumstances, three remained weakly reactive (but with percentage treponemal immobilization increased in the given range), and ten became reactive. Sensitivity at 40 hrs was correspondingly increased. Of six sera initially found weakly reactive after this incubation period, one remained weakly reactive (but

TABLE II

RELATIVE SENSITIVITY OF QUALITATIVE 18-HR and 40-HR TREPONEMAL IMMOBILIZATION TESTS CONDUCTED WITHOUT AND WITH ADDED LYSOZYME

Result	Numbers of Sera yielding Given Result under Given Conditions			
	18 hrs		40 hrs	
	Without	With	Without	With
Negative .. ..	20	5	4	—
Weakly Reactive	13	9	6	1
Reactive .. ..	—	19	23	32

with percentage immobilization increased), and five became unequivocally reactive. Unfortunately, the number (33) of specimens available for comparative tests was limited because sera of threshold antibody content occur infrequently in diagnostic practice. However, there was no exception to the rule that reactivity was increased in the presence of added enzyme.

Discussion

Preliminary trials with the suggested medium (Metzger and others, 1961), containing twice the original concentration (Nelson and Diesendruck, 1951) of sodium thioglycollate, gelatin instead of plasma fraction V, and 0.015 per cent. added lysozyme, were only partially successful. At 18 hrs, specific treponemal immobilization was significantly increased and treponemal survival was satisfactory, but at 40 hrs survival was less than the accepted 70 per cent. (Nelson and Diesendruck, 1951), making it impossible to estimate percentage specific immobilization. Since it was important to realize, if possible, the increase in sensitivity afforded by prolonging incubation, the same amount of lysozyme (0.015 per cent.) was incorporated in a medium (Nelson and Diesendruck, 1951; Borel and others, 1958) known to yield satisfactory 40-hr results (Rosenau and Kent, 1958; Kent and DeWeerd, 1961). This somewhat improved treponemal survival, but 40-hr motility remained less than 70 per cent. in about one-third of trials. Consistently satisfactory results were not achieved until the lysozyme concentration was halved (0.0075 per cent.). This concentration was adopted; it appeared unnecessarily time-consuming and expensive to determine the optimum more precisely. The resultant 2- to 4-fold increase in sensitivity for treponemal antibody, superimposed on that realized by prolonging incubation to 40 hrs, made the mean overall titre increase 7- to 8-fold in the case of an international control serum (human), and 15- to 16-fold in that of a state control serum (rabbit) (see Table I). A corresponding increase in sensitivity was achieved also in qualitative diagnostic TPI tests (Table II).

Some side-effects of added lysozyme are worth noting. The elution of treponemes from syphilitous testes was greatly accelerated, and the yield of organisms markedly increased. Infectivity for rabbits, however, was impaired, animals injected intratesticularly with the usual number of treponemes (10<sup>8</sup>/testis) failing to develop orchitis within the interval (7 to 9 days) accepted for TPI tests; apparently organisms treated with lysozyme are more susceptible to the normal defence mechanisms of the host. It was

also noted that, in qualitative TPI tests, the treponemidal (immobilizing) effect occasionally progressed to treponemolysis (Metzger and others, 1961). A similar lysozyme-activated progression has been reported (Amano, Inai, Seki, Kashiba, Fujikawa, and Nishimura, 1954; Inoue, Tanigawa, Takubo, Satani, and Amano, 1959) in the case of *Escherichia coli*. The effect was ordinarily restricted to qualitative tests of undiluted, strongly reactive sera; it was not encountered in titrations beginning with relatively higher serum dilutions (WHO and NYS controls). While it reduced the number of organisms available for microscopic enumeration, it was an occasional difficulty, not considered serious enough to contraindicate the use of lysozyme.

A similar study (Fribourg-Blanc, 1962) has been published while this report was being prepared. Lysozyme was found to increase sensitivity 3-fold, not only over short incubation periods but over the standard 20- to 22-hr period for TPI tests. This finding, like ours, was at variance with that of the original investigators (Metzger and others, 1961) and was recognized as having obvious applications in serodiagnosis. The observed maximally-enhancing concentration, 100 µg./ml. medium, and toxic concentration, 400 µg./ml., were in close agreement with those originally established (Metzger and others, 1961). However, a concentration of 200 µg./ml. medium was adopted for diagnostic TPI tests. As indicated by our experience, concentrations of this order are toxic when incubation is extended beyond 18 hrs, and this may have discouraged attempts to achieve a more sensitive 40-hr test.

#### Summary

The incorporation of 0.0075 per cent. lysozyme in treponemal survival medium effects a 3- to 4-fold increase in the sensitivity of tests for specific *T.*

*pallidum* immobilizing (TPI) antibody. This increase, superimposed on that achieved by incubating for 40 hrs instead of the usual 18 hrs, resulted respectively in 7- to 8-fold and 15- to 16-fold mean increases in the titres of syphilitic human and rabbit sera. Practical applications in the sero-diagnosis of syphilis are demonstrated.

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#### Augmentation par lysozyme de la sensibilité du test d'immobilization des tréponèmes (T.P.I.)

#### RÉSUMÉ

L'addition de 0,0075% lysozyme au milieu de survie des tréponèmes augmente de trois ou quatre fois la sensibilité des tests spécifiques de l'anticorps immobilisant les tréponèmes.

Cette augmentation, ajoutée à celle obtenue par incubation de 40 heures au lieu de 18 heures, fait monter le titre moyen des sérums syphilitiques de 7 à 8 fois chez l'homme et de 15 à 16 fois chez le lapin.

Où décrit l'application pratique de cette méthode dans le diagnostic de la syphilis.