not differ appreciably with respect to their physical strength. Indeed, it hardly would be expected that aeration would render cells physically stronger; rather, it might be expected that the cells would be altered in permeability and/or concentration of intracellular metabolites and enzymes. The greater resistance of aerated cells is undoubtedly due to such alterations. It is suggested that the actual cause of death upon freezing and thawing is not due to a mechanical factor but is due to some chemical or physicochemical phenomenon. The effect of solutes and initial cell concentration upon survival, as well as the very shape of the survival curves(9,10) supports this contention.

Summary. There appears to be no correlation between the physical strength of cells and their susceptibility to the lethal effect of freezing. It is therefore unlikely that the lethal factor of freezing-thawing is mechanical.

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Resistance of Specifically-Sensitized *Treponema pallidum* to Methylene Blue Stain.* (22335)

BARBARA J. ROSENAU AND JOHN F. KENT. (With assistance of Robert W. Sanders.)
Department of Serology, Walter Reed Army Institute of Research, Washington, D.C.

During studies of toxoplasmosis Sabin and Feldman(1) developed a serodiagnostic test based on the observation that *Toxoplasma gondii,* which is normally stained by methylene blue, lost affinity for the dye when incubated with homologous antiserum and a thermolabile factor in normal serum. Recent experiments(2) showed that virulent *Treponema pallidum* becomes similarly resistant to methylene blue when incubated with syphilitic serum and complement. The purpose of the present studies was to explore possible applications of the reaction in the diagnosis of syphilis.

Materials and methods. The treponeme stain consisted of 0.10 g methylene blue† and 0.025 g Na_2CO_3 in distilled water to make 100 ml. It was prepared by diluting 1 ml of 2.50% Na_2CO_3 with 89 ml water, then adding rapidly 10 ml of a stock 1% solution of the dye. Both stock and dilute dye solutions were stored at 3-6°C, care being taken however to discard any showing sediment or contamination. New glass slides and coverslips, used in microscopic examination of test mixtures, were cleaned in ethanol (70%) and wiped dry with clean gauze. All other glassware was cleaned scrupulously with sulfuric-chromic acid mixture. The basic test procedure was the treponemal immobilization test(3,4). To 0.05 ml of serum in 13 x 100 mm test tube were added 0.40 ml of suspension containing 7-9 x 10^6 treponemes/ml and 0.05 ml guinea pig complement (C'); 0.05 ml of inactivated guinea pig C' was substituted in otherwise identical serum control. The mixtures were incubated 18 hours at 35°C in an atmosphere of nitrogen (95%) and carbon

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† Methylene blue chloride (dye content, 87%), National Aniline Division, Allied Chemical and Dye Corp., New York City.

dioxide (5%). The treponemes in test and control mixtures were then suspended uniformly by swirling, and samples (about 0.01 ml) transferred with capillary pipettes‡ to opposite ends of a glass slide; delivery was effected by touching capillary tips to the slide. Two such slide preparations were made. The first served for immobilization tests, the droplets being surmounted with coverslips and given to other personnel for independent examination. An equal volume of methylene blue solution was added to the second set of droplets; individual capillaries were used for each addition and the dye and treponemes mixed with the tips. Coverslips were applied and the mixtures allowed to stand for 15 minutes at room temperature; this interval was essential for clearcut differentiation of stained and unstained treponemes. The frequency (%) of stained organisms in test and control mixtures was then determined by dark-field microscopic examination of fifty treponemes (magnification, 430 X). In this connection it should be noted that personnel familiarized with the dye and immobilization tests found the differentiation between stained and unstained organisms simpler than the decision regarding motility which was frequently complicated by sluggish or intermittently motile treponemes. The method adopted in calculating percentage of specifically dye resistant treponemes corresponded with that(4) used in determining percentage specifically immobilized; *i.e.*

$$\text{Specific dye resistance (\%)} = 100\,\frac{(\%\text{ stained in control}) - (\%\text{ stained in test})}{(\%\text{ stained in control})}$$

Comparison of dye and immobilization tests was further simplified by adopting the same convention in interpretation; *i.e.*, more than 50% specific dye resistance was considered a reaction, 21-50% a weak reaction, and less than 21% no reaction. Tests for residual C′ in each serum-treponeme C′ mixture were conducted shortly after removal of samples for dye and immobilization tests; each tube containing test mixture received 0.25 ml of a suspension containing 2 x 10^8 optimally-sensitized(5) erythrocytes/ml, and was incubated 30 minutes at 37°C. Fifty to 100% hemolysis was considered an acceptable result(4); less than 50% hemolysis however indicated a degree of C′ deterioration warranting repetition of test. *Syphilitic rabbit sera* were obtained from animals infected experimentally with Nichol's strain of *T. pallidum*. Samples of representative specimens were absorbed with cardiolipin antigen floccules(6); others were absorbed with virulent treponemes, the serum-treponeme-C′ mixtures being incubated anaerobically for 18 hours at 35°C and the organisms separated by centrifugation. Human sera were obtained from patients with syphilis and from individuals without clinical or anamnestic evidence of treponematosis. All were stored at −20°C. Just prior to testing, the required volume of thawed serum was heated for 30 minutes in water bath at 56°C.

Results. The results obtained in parallel methylene blue (TPMB), immobilization (TPI), and cardiolipin flocculation (CF) tests of a syphilitic rabbit serum appear in Table I A; aliquants of the same serum dilutions were used in the 3 titrations. Essentially the same degrees of reaction were observed in the parallel TPMB and TPI tests, and complement (C′) was required in both reactions (*cf* tests and C′-free controls). Attempts to absorb the serum components responsible for dye resistance or immobilization were unsuccessful, sera treated repeatedly with 6 x 10^7 *T. pallidum* per ml remaining unchanged in reactivity. Absorption with cardiolipin antigen floccules, however, reduced the titer of lipid flocculating (CF) antibody without affecting reactivity in the TPMB or TPI tests; the results obtained in titrations of a lipid absorbed rabbit serum are given in Table I B.

The relative sensitivity and specificity of the dye test were evaluated in a serologic study of 169 selected individuals; the TPI test was performed concurrently as a control. Table II summarizes the findings. Sixty patients presenting clinical or anamnestic evidence of syphilis showed almost identical TPMB and

‡ Melting Point Capillary Tubes, 1.5-2.0 x 100 mm, No. 34500, Kimble Glass Division, Owens-Illinois Glass Co., Toledo, O.

TABLE I. Treponemal Immobilization (TPI), Methylene Blue Resistance (TPMB), and Cardiolipin Flocculation (CF) Tests of a Syphilitic Rabbit Serum.

Serum dilution	TPI test: Treponemes motile, % Test	TPI test: Treponemes motile, % Control	TPI test: Specific immobilization, %*	TPMB test: Treponemes stained, % Test	TPMB test: Treponemes stained, % Control	TPMB test: Specific dye resistance, %*	CF test: Degree of reaction
A. Reactions of original serum							
1: 8	10	100	90	6	100	94	4+
1: 16	36	98	63	26	98	73	3+
1: 32	68	96	29	74	94	21	1+
1: 64	90	100	10	84	98	14	—
1:128	98	100	2	98	96	—	—
B. Reactions after absorption with cardiolipin antigen floccules							
1: 8	8	100	92	10	96	90	—†
1: 16	32	98	67	22	100	78	—
1: 32	70	94	26	74	98	25	—
1: 64	90	100	10	86	98	12	—
1:128	92	100	8	92	98	6	—

* Calculated as $100 \frac{b-a}{b}$, where a is % of stained (or motile) treponemes in the test with complement (C'), and b is that in the C'-free serum control.

† A trace (±) reaction persisted in the test with undiluted (1:1) serum.

TPI reactions, the single exception being an individual with secondary syphilis whose serum was negative in the dye test but reacted weakly in the test for immobilizing antibody. Of special interest were 12 persons whose sera were known to yield non-specific immobilization (NSI) reactions; *i.e.*, immobilization of treponemes in C'-free controls; they were retested to determine whether factors invalidating the TPI test would interfere in the test for methylene blue resistance. All 12 sera yielded non-specific (NSMB) reactions in the dye test. In the group of non-syphilitic individuals there were 19 patients with non-treponemal infections or disorders, 7 of whom reacted serologically in tests(6,7) using cardiolipin antigens. Eighteen of the 19 proved negative in both TPMB and TPI tests. One, a patient whose serum reacted with cardiolipin antigens, was negative in the TPMB but reacted weakly in the TPI test; the diagnosis in this instance was erythema nodosum. The

TABLE II. Treponemal Immobilization (TPI) and Methylene Blue Resistance (TPMB) Tests of Sera from Syphilitic and Non-Syphilitic Persons.

Clinical diagnosis	Persons tested	Persons showing given result* in the TPI test: R	wr	—	NSI	TPMB test: R	wr	—	NSMB
Syphilis									
Primary	5			2	3			2	3
Secondary	6	2	1		3	2		1	3
Early treated	5	1		4		1		4	
Congenital	3	3				3			
Neurological	24	17	1	2	4	17	1	2	4
Cardiovascular	4	2			2	2			2
Latent (probable)	13	11	2			11	2		
Diseases other than syphilis									
Reactive in STS†	7		1	6				7	
Non-reactive in STS	12			12				12	
Healthy	90			89	1			89	1

* Designated as reaction (R), weak reaction (wr), or no reaction (—); the symbols NSI and NSMB indicate that non-specific immobilization or dye resistance was encountered both in the test with complement (C'), and in the C'-free serum control.

† Standard flocculation and/or complement fixation tests(6,7) using cardiolipin antigens.

90 healthy persons who served as controls were young men undergoing comprehensive physical and laboratory tests as candidates for a service academy. One of this group yielded non-specific reactions (NSMB and NSI); the remainder were sero-negative.

Attempts to substitute killed *T. pallidum* in the TPMB test were generally unsuccessful. A variety of treponemacidal agents were tested. Among the chemicals found unacceptable were aqueous ethanol (5%), formalin (1%), phenol (0.1%), benzalkonium chloride (0.1%), sodium hypochlorite (0.1%), sodium ethyl mercuri thiosalicylate (0.1%), sodium citrate (2%), and a polyethylene derivative of sorbitan monooleate (Tween 80, 10%). The possibility of utilizing the treponemacidal effect of methylene blue was also explored; pre-stained organisms however proved resistant to decoloration despite prolonged incubation (18 hrs at 35°C) with syphilitic serum and complement. Heating the treponemal suspension for 10 min. at 56°C frequently immobilized the organisms without altering their capacity for specific dye resistance, but the effect was not a predictable one.

Discussion. The component of syphilitic serum conferring specific resistance to methylene blue appeared to be associated if not identical with treponemal immobilizing antibody. This conclusion was suggested by the close agreement observed in quantitative dye and immobilization tests and by the common requirement for complement (Table I). It was supported by almost identical distribution of reactions in qualitative TPMB and TPI tests of patients in the various stages of syphilis (Table II). The relationship of the responsible serum component to *T. pallidum* was not demonstrable by specific absorption, an experience which has an interesting parallel in the recent failure to neutralize treponemal immobilizing antibody with soluble antigen(8). Whatever its nature, however, the essential factor in syphilitic serum was distinct from the antibody concerned in reactions with cardiolipin antigens (Table I). As such it provides an additional basis for differentiating the patient with latent syphilis from the uninfected individual whose serum reacts with lipid antigens; evidence of the usefulness of the TPMB test in this respect was obtained in the present study (Table II). Further evaluation of the test is essential, however, and continuing studies should emphasize particularly the development of a killed treponemal suspension exhibiting the specific dye resistance characteristic of live *T. pallidum.* Being based on specific alterations in the treponemal staining reaction, the TPMB test has certain advantages over one(3,4) relying on changes in motility, and the development of a stable, killed antigen would mark an important advance in standardization.

Summary. Virulent *T. pallida,* which are normally stained by methylene blue, become resistant to the dye following incubation with syphilitic serum and complement. The responsible factor in syphilitic serum is associated if not identical with treponemal immobilizing antibody. The reaction provides the basis for a specific diagnostic test for syphilis.

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