TABLE II. Effect of Compounds Structurally Related to Dilantin on Proliferation of Human Fibroblast-Like Cells in Tissue Culture.

	Mean cell count (per ml of medium) $\times 10^5$		
	0	4	p (4 day interval)
Control	.96	2.02	—
Dilantin Na (.0007M)	.97	4.19	<.01
Barbital Na (.0010M)	.98	2.03	N.S.
Hydantoin (.0019M)	.98	2.02	"
Allantoin (.0013M)	.98	2.08	"
Uric acid (.0012M)	.98	2.02	"
Urea (.0033M)	.99	2.03	"
Alloxan (.0014M)	.98	2.02	"

Dilantin Sodium or the other 2 chemical analogues.

None of the structurally related compounds (barbital sodium, urea, uric acid, alloxan, allantoin or hydantoin) produced a stimulatory effect similar to that of Dilantin Sodium (Table II). At the 4-day interval, found adequate through numerous studies in this laboratory for evaluation of cell-stimulation effects, cell counts in all tubes except Dilantin Sodium did not differ significantly from those of control tubes.

Discussion. The studies here obviously confirm the cell stimulatory effect of Dilantin Sodium. However, they fail to clarify that particular portion of the hydantoin molecule or side chain or grouping of side chains responsible for this phenomenon. The phenomenon is apparently unrelated to the basic hydantoin structure, presence of phenyl groups, or presence or absence of various groups at the 1 or 3 position. It is interesting to note that, while analogues B and D and all the structurally related compounds were ineffective in stimulating cell growth, there was no evidence of growth inhibition or cytotoxicity.

Summary. Several chemical analogues and chemically related compounds of Dilantin Sodium have been used to study and clarify the cell stimulatory effects of this compound. Two analogues (1 allyl, 5 phenyl hydantoinate and 5 methyl, 5 phenyl hydantoinate) induce stimulation of fibroblast-like cell proliferation while 2 other analogues (1 propyl, 5 phenyl hydantoinate and 3 methyl, 5,5 diphenyl hydantoinate) were completely ineffective. None of the other structurally related compounds tested (barbital sodium, urea, uric acid, alloxan, allantoin or hydantoin) produced any stimulation of cell proliferation.

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Received November 2, 1960. P.S.E.B.M., 1961, v106.

A Study of Bentonite Flocculation Test for Measurement of Type-Specific Streptococcal Antibodies.* (26288)

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A reliable and sensitive technic for determining type-specific anti-streptococcal antibodies in the blood of patients recovering from Group A streptococcal infections would help greatly in investigating epidemiological and clinical problems associated with such infections and their sequelae. The mouse-protection test(1), which is generally accepted as the ultimate basis for type classification of Group A hemolytic streptococci and for detection and measurement of type-specific protective antibody, has the disadvantage that it requires strains of high mouse virulence (which are infrequent among streptococci freshly isolated from human sources),

* Supported in part by grant from U.S.P.H.S.

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and a high antibody content in the human serum. Furthermore, large amounts of sera are necessary to carry out the tests.

The bacteriostatic test(2,3) is based on the observation that the union of homologous antibody with M antigen on the surface of the bacterial cells increases susceptibility of these cells to phagocytosis by human leukocytes. The inhibitory effect of antibody is most marked with strains containing the largest amount of M antigen. Cross-reactions between some types such as 2, 13, and 48 have been observed(4). This test requires fresh "normal" human whole blood, drawn not more than 2 hours prior to the test proper; blood from laboratory animals cannot replace the "normal" human blood(5).

Stollerman and Ekstedt(6) during their studies on the bactericidal system(2) for detection of type-specific antibody, found a number of strains which grew in extremely long chains (approximately 50 to 100 cocci per unit as compared to chains of 4 to 12 observed in the control), in the presence of homologous antiserum. Their data indicate that formation of long chains in liquid media by the strains of streptococci studied is dependent in part upon the interaction of type-specific antibody and the M protein of the cells.

The complement-fixation test has been employed by some investigators in measurement of type-specific antibody(7), but repeated absorption of the antisera with strains of heterologous types is necessary to minimize cross-reactions.

Boyden(8) showed that treatment of sheep erythrocytes with solutions of tannic acid and insulin rendered them capable of adsorbing proteins, including Group A streptococcal M protein, from solutions in normal saline; the cells subsequently washed in normal saline were found to be agglutinable by the corresponding antiprotein sera. This hemagglutination test, when used to measure type-specific antistreptococcal antibody, also requires absorption of serum with heat-killed Group A streptococci of heterologous types (9).

Since the major difficulty in the latter 2 procedures is the finding that crude antigenic preparations derived from streptococci react not only with homologous type-specific antibodies but also with antibodies stimulated by immunization with heterologous serologic types, and since in any given patient's serum antibodies to multiple Group A streptococcal types may occur, then the specificity of any observed reaction will necessarily depend upon the specificity of the antigen used. It should be noted that preparation of highly purified type-specific antigen is extremely difficult and is regarded by some workers as impossible of achievement with the methods currently used. A second approach to this problem is in the use of absorption to remove heterologous antibody from the patient's serum before testing. While this is possible to achieve, it too is a laborious procedure and one difficult to apply to large numbers of serum specimens.

Bozicevich(10) demonstrated that bentonite‡ suspensions sensitized with an acid-soluble protein fraction extracted from *Trichina* larvae provide 95% or greater accuracy in serological diagnosis of trichinosis. Besides being highly accurate, the test can be completed within 20 minutes in any ordinary laboratory. Another advantage is that the stock bentonite suspension can be kept at room temperature for as long as 4 months without losing its potency, while antigen-coated-bentonite suspension can be stored at 4°C for 2 months(10). He applied this technic successfully to the serological diagnosis of rheumatoid arthritis(11). Both of his tests have withstood intensive evaluations by other investigators(12,13).

There have been no publications on the feasibility of this method for detection of type-specific anti-streptococcal antibodies in sera of infected humans or of immunized laboratory animals; to investigate its possible usefulness the present study was undertaken.

Materials and methods. A. *Bacterial strains and antisera.* The streptococcal strains employed and production of the rabbit antisera have been described(14). B. *Preparation of Group A streptococcal crude M protein.* The crude M protein was ex-

‡ Bentonite is a montmorillonite clay obtained from Fisher Scientific Co., Fair Lawn, N. J.

tracted from each strain with hot hydrochloric acid according to the method of Lancefield(15) with the following modifications: (a) the final extract was precipitated twice with alcohol after it was demonstrated by the capillary precipitin test of Swift, Wilson, and Lancefield(16) that there was no group-specific C substance in the extract; (b) the crude M antigen in buffered normal saline was preserved with 1-5,000 Merthiolate and stored at 4°C. C. *Preparation of Group A streptococcal Somatic Polysaccharide.* Strain S-94 was grown in trypticase-soy broth at 37°C for 18 hours and the group-specific polysaccharide was extracted from the cells by Fuller's formamide method (17). The polysaccharide extract was stored at 4°C. D. *Preparation of stock bentonite suspension.* The stock bentonite suspension was prepared according to the procedure of Bozicevich(10). E. *Preparation of test antigen.* Bentonite suspensions with adsorbed M antigen will hereafter be referred to as "test antigen." Absorption of M antigen onto the bentonite particles was carried out in the following manner:

1) A 2.5 ml portion of M antigen solution was added to 5.0 ml of stock bentonite suspension in a 40.0 ml conical graduated centrifuge tube. 2) The tube was left standing vertically in the refrigerator overnight—approximately 15 hours. 3) The mixture was removed, shaken, and 0.625 ml of 0.1% aqueous methylene blue solution was added to it. 4) It was again shaken gently by hand for 3 to 5 minutes. 5) 10 ml of normal saline were added, and the mixture was shaken thoroughly for one minute. 6) The mixture was centrifuged at 1,600 rpm for 15 minutes, using a size 2 International Centrifuge, with No. 240 head. 7) The supernate was discarded; the sediment was suspended in 15.0 ml of normal saline and centrifuged at 1,600 rpm for 15 minutes. 8) The resultant supernate was discarded; the sediment was resuspended in 4.0 ml of 0.05M phosphate buffer at pH 8.5. 9) Five tenths ml of normal rabbit serum (pre-immunization serum) was added to prevent spontaneous flocculation. 10) The suspension (test antigen) was stored at 4°C until used. F. *Bentonite flocculation test.* The test is a modification of the method used in diagnosis of trichinosis(10). It was carried out as follows: 1) Two-fold serum dilutions were prepared with 0.05M phosphate buffer at pH 8.5. (2) With a clean glass pipette delivering 30 drops per ml, 2 drops of each dilution were put into each ring of a 10-ring glass slide, using a clean pipette for each dilution. 3)The test antigen was removed from storage at 4°C and allowed to reach room temperature before use. It was shaken to obtain a homogeneous suspension, then with a clean glass pipette delivering 30 drops per ml, one drop was added to the center of each ring containing the diluted serums. 4) The slide was then placed on a Clay-Adams horizontal rotator operated at 100-120 rotations per minute for 15 minutes, after which readings were made immediately, to avoid drying, under a microscope (60 ×).

Positive and negative serum controls were included in each series of tests, together with a suspension control consisting of sensitized particles and buffer only.

Results and discussion. Contrary to expectation from the report of Bozicevich(10) it was found that spontaneous flocculation occurred when normal saline buffered at pH 7.2 was used to suspend the antigen-coated bentonite particles. Use of Tween 80 in concentrations even greater than the recommended 1% did not prevent this. However, in presence of 0.05 M acetate buffer, pH 2.6, or 0.05 M phosphate buffer at pH 7.3 or 8.5, spontaneous flocculation did not occur if normal rabbit serum was included, as described in the previous section; the M protein-coated bentonite remained in suspension without loss of potency for as long as 6 weeks.

In bentonite flocculation tests involving 3 types of Group A streptococci (types 12, 14, 36) (Tables I, II, and III), the titers were considerably lower than those obtained by the hemagglutination technic(9). The results thus suggest that a fairly high antibody level in the test serum may be required for bentonite flocculation, which would somewhat lessen the value of the test for diagnostic use. It is of interest to note that the

TABLE I. Flocculation Reactions: Test Antigen M (Lot #8) from Type 14 Strain S-23 *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256
	Buffer	0	—	—	—	—	—	—	—
73	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
16	Homologous	4+*	4+	4+	4+	3+	2+	0	0
54	"	4+	4+	4+	2–3+	2+	1+	0	0
72	"	4+	4+	4+	2+	0	0	0	0
16	"	4+	4+	4+	4+	3+	2+	1+	0
76	Anti-type 36	3+	1+	0	0	0	0	0	0
77	*Idem*	4+	0	0	0	0	0	0	0
51	Anti-type 12	0	0	0	0	0	0	0	0
9	*Idem*	0	0	0	0	0	0	0	0

* 4+ indicates agglutination of all particles.

TABLE II. Flocculation Reactions: Test Antigen M (Lot #10) from Type 36 Strain S-94 *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256
	Buffer	0	—	—	—	—	—	—	—
77	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
77	Homologous	4+	4+	4+	3+	1+	0	0	0
76	"	4+	4+	4+	3+	1+	0	0	0
74	"	4+	4+	4+	3+	2+	0	0	0
73	"	3+	3+	2–3+	1+	0	0	0	0
9	Anti-type 12	0	0	0	0	0	0		
51	*Idem*	0	0	0	0	0	0	—	—
52	"	0	0	0	0	0	0	—	—
16	Anti-type 14	0	0	0	0	0	0	—	—
54	*Idem*	0	0	0	0	0	0	—	—

TABLE III. Flocculation Reactions: Test Antigen M (Lot #1) from Type 12 Strain Bailey *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256
	Buffer	0	—	—	—	—	—	—	—
9	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
9	Homologous	4+	4+	3+	2+	0	0	0	0
51	"	4+	4+	4+	1+	0	0	0	0
52	"	4+	4+	4+	2+	0	0	0	0
16	Anti-type 14	0	0	0	0	0	0	0	—
72	*Idem*	0	0	0	0	0	0	0	—
73	Anti-type 36	0	0	0	0	0	0	0	—
74	*Idem*	0	0	0	0	0	0	0	—

cross-reaction between types 36 and 14 M antigens was not reciprocal. M antigen extracted from strain S-94 (type 36) failed repeatedly to react with heterologous anti-S-23 (type 14) antisera. However, in these cross-reactions, even though they were either 3+ or 4+ in degree, the clumps were small as compared to the large, compact clumps seen in the homologous reactions at low dilutions.

A mixture of C substance and M antigen was obtained by hot acid extraction (18) from a culture of strain S-23 (type 14). Capillary precipitin tests using both group-specific and type-specific antisera were performed. A 4+ reaction was obtained in each case. Sensitization of stock bentonite was carried out in the usual way but with substitution of this mixed antigen for the ethyl alcohol-precipi-

tated M antigen. Flocculation tests using both type-specific and group-specific antisera were negative with all of the test serum dilutions. Why no flocculation occurred, especially in presence of the type-specific antisera, is not known. Partially purified M protein obtained from such a preparation was normally reactive with homologous antisera when adsorbed to bentonite particles.

There appear to have been no publications in the North American literature on adsorption of polysaccharide antigens by bentonite. C substance, obtained by the hot formamide extraction method of Fuller(17) was used in an attempt to sensitize bentonite particles by the standard procedure. In the presence of group-specific antiserum there was no flocculation.

Summary. A flocculation test for detection and measurement of type-specific antibody to Group A streptococci has been evaluated. A single strain of each of 3 serological types of streptococcus was employed. When a suspension of bentonite particles between 1.0 and 1.5 μ in size was exposed to an unadsorbed homologous rabbit antiserum after being coated with partially purified M protein antigen, flocculation was obtained. The highest homologous flocculation titer encountered was 1-32. A weak non-reciprocal cross-reaction between types 36 and 14 M antigens was found: while M antigen of strain S-94 (type 36) did not react with anti-S-23 (type 14) antisera, M protein of strain S-23 exhibited some cross-reactivity with anti-S-94 antisera. The test antigen could be stored at 4°C for at least 6 weeks without loss of potency. No group-specific or type-specific flocculation occurred when the test antigen was prepared by exposing the bentonite particles to a crude antigen mixture, obtained by hot HCl extraction of streptococcal cells, which was shown by precipitin tests to contain large amounts of both group-specific and type-specific antigens. Partially purified M protein obtained from such a mixture, however, could be used to sensitize particles, which were then reactive with antisera. No flocculation was observed when bentonite particles treated with group-specific polysaccharide solution were exposed to homologous group-specific antiserum.

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