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Serologic Properties of Bentonite Particles Coated with Microbial Polysaccharides. (28724)

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The basic procedures for detection and measurement of antibody *in vitro* are relatively few. Some were developed before the turn of the century. Although in recent years variations have been devised for studying the interaction of antigens and antibodies, there is a need for easily prepared, sensitive and dependable serologic reagents for measurement of antibody. The technique of fixing antigen to the surface of normal or of tanned erythrocytes, which came into general use about 10 years ago, has provided a practical and convenient means for measurement of antibodies(1) but despite its utility the coated red cell technique has not proved to be ideal. Disadvantages of the method are: the complex antigenic reactivity native to the red cell surface; lack of uniformity of red cells, and problems related to their lability; and finally, for reasons which are not clear, a number of antigens are not taken up by such cells.

To devise more appropriate surfaces for antigen-antibody reactions various particulates have been investigated including collodion(2), latex(3), barium sulphate(4), bismuth tannate(5), etc. Bozicevich and his coworkers had shown that bentonite (aluminum silicate) particles could be used to advantage for this purpose(6,7,8,9). During studies with various purified polysaccharide antigens fixed on bentonite, certain advantages of these coated particles as serologic reagents became evident. This communication concerns the findings of a systematic exploration of the factors influencing the preparation and performance of polysaccharide-coated bentonite particles.

Materials. Bentonite (Volclay, American Colloid Co., Chicago, Ill.) is a mineral clay, mined in Wyoming, consisting principally of aluminum silicate. The preparation by differential centrifugation of a stock bentonite suspension with appropriate range of particle size has been described(7). The polysaccharides employed as antigens, their distinguishing features, source and reference to their method of preparation are listed in Table I. The immune sera had been prepared previously by immunization of man, rabbit, horse and mouse in connection with other studies. The schedule of injections varied widely for the different situations, but represented intensive immunizations; in some instances the immunizing preparation was the intact bacterium from which the polysaccharide was derived.

Methods and results. The methods employed were those described by Bozicevich and coworkers(7,8,9) with some modifications. Ten ml of stock bentonite is sedimented, the pellet brought up to 1 ml with distilled water, the desired quantity of antigen in 1 ml of saline is added, and the two are mixed and allowed to stand at room temperature for 1 hour. One ml of 1% crystallized bovine serum albumen (BSA) is added, the mixture left at room temperature for an additional 15 minutes, after which 15 ml distilled water and 1 ml of a 0.1% solution of methylene blue are added and the antigen-bentonite particles are washed twice

TABLE I. Polysaccharide Antigens Employed for Bentonite Flocculation.

Microbial source					Reactivity with immune serum	
Organism	Strain	Distinguishing features	Supplied by	Reference to preparation and/or properties	μg antigen for optimal coating of 10 ml stock bentonite	Titer
Salmonella enteritidis	S795	*Endotoxin*—protein free, low in bound lipid; high molecular wt (10S)	E. Ribi	(10)	200	1:8000
" "	"	*Haptene*—derived from endotoxin by acid hydrolysis; low molecular wt (1.4S)	"	(11)	150	1:32,000
Salmonella typhosa	R-2	*Endotoxin*—essentially non-reactive with antibody against somatic polysaccharides	M. Raynaud	(12)	200	1:32,000
Escherichia coli	0127-B8	*Endotoxin*—protein content reduced	T. Haskins	(13)	300	1:2000
Serratia marcescens	724	*Endotoxin*	M. J. Shear	(14)	300	1:2000
Shigella dysenteriae	Type I, strain #K-624	"	A. L. Davies	(15)	200	1:4000
Pasteurella tularense	Schu	Prepared by phenol extraction of acetone dried cells	G. G. Wright	(16)	400	1:4000
Diplococcus pneumoniae	Types I, II, III	*Capsular*—Uronic acid polymers	B. Prescott	(17)	200	I = 1:32,000 II = 1:16,000 III = 1:64,000
Escherichia coli	(Vi) 5396/38	*Capsular*—N-acetyl d-galacto-aminuronic acid polymer	M. Webster	(18)	200	1:4000
Leuconostoc mesenteroides	6025	Native dextran	E. J. Coulson	(19)	250	1:4000
(Non-bacterial) Blood Group A	—	Phenol soluble—derived from pseudomucinous ovarian cyst	H. Baer	(20)	200	1:512

with distilled water and the final volume is made up to 5 ml. Microscope slides (3″ × 2″) with 9 rings (each ring is 5/8″ in diameter) are prepared for use in this test with a 9 ring template and molten sealing wax. One-tenth ml of serial 2-fold dilutions of the test serum in 0.1% BSA are dispensed into the rings; normal and known positive sera are run simultaneously. A calibrated Pasteur pipette is used to dose each ring with one drop (0.025 ml) of the antigen-bentonite suspension. The slides are rotated for 30 minutes on a Boerner type rotating machine at approximately 110-120 rotations per minute and read under low power magnification. Reactions are scored as 1 to 4 plus according to the extent of flocculation. In a negative test the particles are evenly suspended and there is no discernible flocculation.

Preceding work utilizing the bentonite flocculation test had been concerned with antigenic preparations of complex unknown composition(6), soluble proteins(7) or DNA(8). The present work deals with a different category of antigens, viz., high molecular weight polysaccharides, mostly of microbial origin. It was desirable therefore systematically to determine the conditions for the coating of particles with these antigens so as to provide a test which was sensitive, reproducible and free from non-specific flocculation.

1) *Effect of antigen concentration on reactivity of particles.* All the purified polysaccharides examined coated bentonite particles effectively. The concentration of antigen was a significant factor in the serum titer obtained with the coated particles. For each antigen the concentration providing maximum serum titer was determined by coating standard aliquots of bentonite with doubling dilutions of the antigen. For example, the standard amount of bentonite mixed with 50 μg/ml of Type III pneumococcal polysaccharide yielded coated particles which were flocculated by a concentrated rabbit immune globulin to a titer of 1:16,000; antigen at 4 times the preceding concentration gave a titer of 1:64,000. Particles coated with *Shigella dysenteriae* polysaccharide at 50 μg/ml gave a titer of 1:512 against a mouse anti-shigella serum; 200 μg/ml of this antigen, in the same system, yielded a titer of 1:4096. In the case of *Escherichia coli* 0127, particles coated with 300 μg/ml produced a titer of 1:2048; 100 μg/ml gave no flocculation even with the test antiserum at 1:4 dilution. For the most effective use of a given antigen the quantity was first determined which coated particles so that they were flocculated to maximum titer by antiserum (Table I).

2) *Coating with antigen preparations which fail to fix on erythrocytes.* The surface characteristics of mammalian cells and of an inorganic particulate such as bentonite are likely to have little in common except possibly for certain charge characteristics. It was considered that bentonite might provide a satisfactory surface for adsorption of antigens which are not fixed by erythrocytes. To explore this possibility a group of antigen preparations was assembled which was inoperative in the hemagglutination procedure as generally performed(1), which required treatment with heat or alkali for adsorption by red cells.

The bentonite particles were readily coated by a variety of antigens that were ineffective in modifying red cells (Table II). Following heat or alkali treatment, the *Salmonella enteridis* endotoxin was effective in coating red cells; however, these same products adsorbed to bentonite yielded higher titers. Treatment of the Vi antigen with dilute alkali results in loss of acetyl groups and considerable depolymerization. This partially degraded antigen no longer is capable of coating red cells(21), but still retains much of its antigenic reactivity when attached to bentonite.

The somatic antigens (endotoxins) of Gram-negative bacteria are polysaccharide complexes (containing associated protein and lipid) whose molecular weight is in excess of 1×10^6. When subjected to acid hydrolysis by refluxing with 0.1 N acetic acid these complexes are dissociated into haptenic polysaccharides (molecular weight approximately 20,000-30,000) which can no longer be adsorbed on erythrocytes(22). It is especially noteworthy that these haptenic polysac-

TABLE II. Coating with Polysaccharides Which Fail to Fix on Normal Erythrocytes.

Source	Treatment prior to test	Hemagglutination	Bentonite flocculation
Salmonella enteritidis (endotoxin)	None	Negative at 1:10	1:16,000
	100°C, 1 hr	1:640	1:16,000
	0.02 N NaOH for 18 hr at 37°C	1:1280	1:8000
Salmonella enteritidis (haptenic polysaccharide)	Obtained from endotoxin by acid hydrolysis	Negative at 1:10	1:32,000
Pneumococcus Type I	None	*Idem*	1:32,000
" Type II	"	"	1:16,000
" Type III	"	"	1:64,000
Human Blood Group A substance	"	*	1:512
		Negative at 1:10	
Escherichia coli Vi strain 5396/38	"	1:3820	1:4000
(capsular strain)	Deacetylation by alkali	Negative at 1:2	1:512
Leuconostoc mesenteroides (native dextran)	None	Negative at 1:10	1:4000

* 'A' substance was adsorbed on sheep erythrocytes. Normal human Group A cells were agglutinated to a titer of 1:1024 by this serum.

charides readily coat bentonite particles and that the resultant serum titers compare favorably with those obtained with the parent endotoxin.

That most of the purified pneumococcal type specific polysaccharides cannot be adsorbed on erythrocytes has largely restricted their use as serological reagents. The data in Table II attest to their capacity to be fixed on bentonite particles.

3) *Avoidance of spontaneous flocculation.* In previous reports concerned with bentonite as the carrier particle, a variety of surface-active agents (e.g. 'Tween-80, polyvinylpyrolidone, PVP) were used to avoid spontaneous clumping. In the DNA-bentonite test, BSA was used; there too, it was found that saturated potassium chloride (KCl) had to be added to the mixture. However, KCl was found to be without significant effect on flocculation of polysaccharide-treated bentonite particles.

Previous use of Tween-80 and PVP was designed to prevent spontaneous flocculation particularly at higher serum dilutions. In the present work it was found that, while useful, these agents did not act consistently. However, tests with BSA (in various concentrations) added to serial dilutions of serum showed that 0.1% sufficed to prevent spontaneous flocculation. One ml of 1% BSA added to the mixture of polysaccharide and bentonite was also helpful in preventing spontaneous flocculation. Accordingly the use of BSA was adopted as routine.

4) *Removal of unadsorbed antigen.* Previously it was considered desirable that following exposure to antigen the bentonite should be washed 3 times to remove unadsorbed antigen. However it was found that a single washing or as many as 3 washings resulted in the same titer. Successive washings diminish the intensity of the methylene blue on the particles and make for ease in reading of the reaction. Therefore the preparation is washed only twice.

5) *Conditions for interaction of antigen and particles.* Since a 1% solution of BSA is routinely added to the antigen-bentonite mixture, parallel tests were made on aliquots of the antigen-coated bentonite after they had been subjected to BSA for intervals ranging from 5 to 60 minutes. After 15 minutes of contact the titer remained unchanged. Likewise the importance of the time allowed for adsorption of the antigen to bentonite was investigated; the antigen was exposed to bentonite for ¼, ½, 1, 2, 4 and 24 hours. As little as 5 minutes sufficed for maximal adsorption of the antigen to the bentonite. Adsorption of antigen at 4, 25 or 37°C yielded coated particles of similar serologic reactivity. The tests reported here were carried out at or near neutrality. Since strongly acidic poly-

TABLE III. Serum Antibody Determinations by Several Procedures.

Antigen	Antibody content	Titer of antisera		
	Quantitative precipitation, μg Ab N/ml	Bacterial agglutination	Conditioned hemagglutination	Bentonite flocculation
Blood Group A	23*	†	†‡	1:512
Esch. coli Vi (capsular strain)	270	1:640	1:3820	1:4000
Pneumococcus type III	2600	†	†	1:64,000
Sal. enteritidis (somatic endotoxin)	260	>1:5120	>1:5120	1:4000

* Kindly done by Dr. H. Baer.
† The antigen cannot be used in this procedure.
‡ Normal human Group A cells were agglutinated by the immune serum to a titer of 1:1024.

saccharides, their sodium salts, and neutral polysaccharides all developed good reactivity it is rather unlikely that pH greatly affects the reaction.

6) *Stability of the coated particles.* It was observed that bentonite particles coated with either the Vi antigen or *S. enteritidis* endotoxin, and stored at 4°C, retained their serologic reactivity for long periods. Controlled experiments designed to determine stability have not been made. Nevertheless, considerable information has been obtained indicating that in the case of the Vi antigen, for example, coated particles stored for 5 months flocculated to titer with specific antisera just as they did on the day they were prepared. Findings such as these indicate that whatever its nature, the bonding between antigen and bentonite is very firm. They also attest to the stability of these complex polysaccharides.

7) *Conditions for flocculation of antigen-coated particles by antibody.* Following the addition of antigen-coated bentonite particles to the serum dilutions, the slides were rotated for varying periods. After 30 minutes of shaking there was no increase in titer.

Previous methods for bentonite tests have been carried out on sera inactivated by heating for 30 minutes at 56°C. Parallel tests, using fresh and inactivated sera yielded essentially the same titers. Inactivated sera, however, produced a more clear-cut flocculation.

8) *Comparison with other serological procedures.* Several situations were examined in which the same antigens and antisera were used to compare the bentonite test and other standard serological procedures (Table III). The bentonite test and conditioned hemagglutination gave similar titers. Human group A cells and bentonite particles coated with blood group A polysaccharide did not differ greatly in their capacity to be agglutinated by rabbit anti-A serum. On the basis of the 4 immunochemically standardized sera examined (Table III), it appears that in terms of weight units the minimum amount of antibody nitrogen which the bentonite procedure can measure with reasonable assurance is in the range of 0.1–1 μg/ml. In several other instances rabbit and human antityphoid sera were also examined. The titers by the bentonite procedure and conditioned hemagglutination were in the same range, while those obtained by bacterial agglutination were usually considerably lower.

9) *Reproducibility of the polysaccharide bentonite flocculation test.* On frequent occasions different lots of bentonite particles were coated with *S. enteritidis* endotoxin and then set up in a standard dilution scheme against the same immune serum. The serum flocculation titers obtained did not differ significantly, *i.e.*, they were within one doubling dilution. Employing the same preparations of polysaccharide, bentonite particles and specific antiserum, the test has been performed under various circumstances by different operators. The serum titers as determined by various individuals were remarkably uniform. Also in numerous instances flocculation tests were read as unknowns by several individuals; invariably the readings were in good agreement.

Discussion. The various serologic techniques now in use represent a balance of advantages and limitations. The principal factors which determine their acceptance are specificity, sensitivity, reproducibility, sim-

plicity and speed of performance. While the bentonite test with an array of polysaccharide antigens is no more sensitive than other tests in general use such as conditioned hemagglutination, it offers other important advantages. The antigen-particle mixture is stable thus facilitating serial tests over extended periods with the same antigen-particle suspension. The usual variables and the time expended on daily preparation of the carrier-antigen reagent are thus obviated.

There is little information on the nature of the attachment of antigens to erythrocytes; however in the case of bentonite it is difficult to visualize this as involving other than a charge effect. Some polysaccharide antigens appear to be readily fixed by both erythrocytes and bentonite particles, while others range from products which coat erythrocytes only after appropriate treatment of the antigen, to those which consistently fail to adsorb (Table II). Despite their widely differing properties in the passive hemagglutination procedure all of these products are effective in coating bentonite. It is interesting that products of much lower molecular weight derived from some of these complex polysaccharides by procedures leading to depolymerization or dissociation and incapable of being fixed to erythrocytes are still effective in attaching to bentonite. Since the antigenic attributes of such preparations have been greatly diminished in that they no longer fix complement and they precipitate less antibody(23), bentonite serology should facilitate their study.

There is little doubt that in speed, ease of performance, reproducibility and stability of the serologic reagent, the procedure has much to commend it. However, the sensitivity of the method, particularly in comparison with passive hemagglutination is more difficult to evaluate. The impression was gained that the sensitivity varied considerably with different polysaccharides. Moreover the various immune sera employed differed in their content of antibody; this also made comparison more difficult. Differences in sensitivity may be related to presently unidentified characteristics of individual polysaccharide preparations. On the whole the findings suggest that sensitivity is of the same order as that of conditioned hemagglutination.

These procedures and findings are referable to a major category of complex polysaccharides. Previous work indicates that the conditions for coating and stabilizing are rather different for other antigens. The use of bentonite as a carrier of antigenic material for serologic tests was first reported 12 years ago. Its relatively limited use has probably been influenced by the kind of information obtainable with the heterogenous antigen preparations employed. The earliest application of the technique was with a complex mixture of antigens obtained by an extract of parasites. More recently, the method has been applied to serologic study of "auto-immune diseases"; particles were coated with Cohn Fraction II for detection of rheumatoid factor, and calf thymus DNA preparations for measurement of anti-DNA antibodies in systemic lupus erythematosus. The present work has shown that a considerable number of complex polysaccharides, adsorbed on bentonite provide useful serologic reagents. These coated particles are stable, produce well defined flocculates, and yield reproducible results in a variety of test situations. The present findings further attest to the remarkable surface characteristics of bentonite particles in their capacity to adsorb a diverse array of antigens, to react with antibody and consistently to form aggregates which readily serve as indicators of antigen-antibody reactions.

Summary. A flocculation test utilizing polysaccharide-coated bentonite particles is described. The conditions for coating the particles and performance of the test are reported. The method offers advantages such as simplicity, reproducibility, stability and specificity. A number of polysaccharide antigens, which fail to coat erythrocytes, readily adsorb onto bentonite, permitting serologic tests with these antigens.

Addendum: Since this manuscript was prepared, a report has appeared (Diena, B. B., Wallace, R., Greenberg, L., *Canad. J. Microbiol.*, 1963, v9, 221) describing the serological properties of bentonite particles coated

with crude extracts of Salmonella.

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"Rebound" Recovery from Deprivation of Paradoxical Sleep in the Rabbit.* (28725)

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"Paradoxical sleep" (PS) in mammals has been described as a distinct phase of sleep, apparently profound, characterized by a total loss of postural tone and by rapid eye movements. It has an abrupt onset in the course of ordinary "spindle sleep," and its electroencephalographic (EEG) correlate consists of low voltage fast cortical activity similar to that of arousal(1-3). In man the PS phase has been associated with dreaming, and deprivation of PS by awakening the subject at the onset of each PS episode led to psychological disturbances and an increased incidence of PS when sleep was again undisturbed(4).

In rabbits a pattern now recognized as PS was described(5) as a phase in the "EEG afterreaction" to coitus in the female with "chronic" electrodes implanted in cortical and subcortical regions of the brain. The EEG record was characterized by a fast (8c/sec) high amplitude theta rhythm in the hippocampus and related regions (called the phase of "hippocampal hyperactivity") and a depression of intrinsic activity in the olfactory bulb. During this phase the rabbit's head was on the floor with her ears depressed and the only notable motor activity was a twitching of the eyelids and jerky movements of the jaws. The reaction could be induced by

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