

THE ADSORPTION OF PROTEINS ON ERYTHROCYTES TREATED
WITH TANNIC ACID AND SUBSEQUENT HEMAGGLUTINATION
BY ANTIPROTEIN SERA

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The adsorption of components of bacteria onto erythrocytes, which are thus rendered agglutinable by antisera directed against these components, has been reported by a number of investigators (1-6). The work of Keogh, North, and Warburton (2) has suggested that, at least in some instances, a bacterial polysaccharide is involved in this adsorption process, and it has been generally assumed that the agglutination by immune sera is due to a reaction between specific antibody and the adsorbed polysaccharide. Purified protein preparations have not been shown to be similarly adsorbed by normal erythrocytes. Interest in the mechanism of these phenomena stimulated investigations which eventually led to the observations reported in this paper.

There seems to be a possibility that, in the case of some sensitizing antigen preparations, the adsorption process might take place in two distinct phases, the first of these consisting of an alteration in the surface properties of the erythrocyte brought about by the action on the cell of some reactive components of the system; and the second phase being the secondary adsorption of other types of molecules by the cells as a result of newly acquired surface properties of the latter.

With this tentative hypothesis in mind, a number of experiments were performed to study the effects on sheep erythrocytes of various preparations of polysaccharides from plants. It was soon observed that treatment of erythrocytes with solutions of some preparations of inulin rendered them capable of adsorbing proteins (*e.g.*, horse serum albumin) from solutions in saline; the cells subsequently washed in saline were found to be agglutinable by the corresponding antiprotein sera. However, not all the preparations of inulin tested were found active in this respect, and it was observed that the preparations capable of promoting protein adsorption by erythrocytes also had the property, in high dilutions, of agglutinating untreated cells. This property was not shared by the preparations inactive in promoting protein adsorption.

As it has long been known that tannic acid agglutinates erythrocytes (7),

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this substance was also tested for ability to promote adsorption of proteins by these cells. It was found that tannic acid was much more active than any of the inulin preparations tested, both in agglutinating red cells and in rendering them capable of adsorbing proteins. The experiments and results now to be described are chiefly concerned with the development of this observation as a serological procedure for titrating sera for antibodies against the proteins thus adsorbed.

Materials and Methods

Sheep Erythrocytes.—Sheep blood was collected aseptically in 1.2 volumes of modified Alsever's solution (8), which had been autoclaved at 10 pounds for 15 minutes. The composition of this solution was as follows:—

Dextrose.....	2.05 gm.
Sodium citrate.....	0.8 gm.
Sodium chloride.....	0.42 gm.
Distilled water.....	100 cc.

Adjust to pH 6.1 with 10 per cent citric acid solution.

The blood was kept for at least 3 days before using and thereafter was used for up to 3 weeks. At the beginning of each day an aliquot was taken and the cells washed in saline three times and then suspended (2.5 per cent) either in saline or buffered saline (pH 7.2) as required.

Buffered Saline.—Buffered saline was prepared by adding 1 volume of saline to 1 volume of a solution (0.15 M) of KH_2PO_4 and Na_2HPO_4 mixed in the proper proportions to produce the desired pH.

Tannic Acid.—Fresh solutions of tannic acid were made in saline at the beginning of each day. Mallinckrodt tannic acid ("reagent grade") was used throughout. Two preparations of Merck and Company ("fluffy" and "reagent") were used in some experiments for comparison and were found effective.

Tuberculin P.P.D. (Weybridge).—This was kindly supplied by Dr. H. H. Green and Mr. A. B. Paterson at the Veterinary Laboratories, Ministry of Agriculture and Fisheries, New Haw, Weybridge, England, where it is prepared for distribution to veterinarians for the tuberculin testing of cattle in the British Isles. The method of preparation is described fully by Green (9). Briefly, it involves precipitation by trichloroacetic acid of mixed filtrates of cultures of three human strains of steam-killed tubercle bacilli. After redissolving, the material is centrifuged in a Sharples centrifuge to clarify the solution. The tuberculin is supplied in the form of a solution in buffered saline (pH 7.0), with 0.5 per cent phenol and 10 per cent glycerol, containing 3.0 mg. of P.P.D. per cc. The dry powder is said to contain 5 per cent polysaccharide, 7 per cent nucleic acid, the remaining 88 per cent being protein.

P.P.D.-S. from two sources was used. (1) P.P.D.-S. (lot 1112) prepared by Dr. Florence B. Seibert was supplied through her courtesy to Dr. M. W. Chase, who made it available for this work; (2) P.P.D.-S. prepared according to the method of Seibert and Glenn (10) and distributed by Sharp and Dohme in the form of tablets containing lactose.

Old Tuberculin.—Old tuberculin was kindly supplied by Dr. Piersma of the Lederle Laboratories, Division of the American Cyanamid Company, Pearl River, New York. The preparation is described as concentrated tuberculin lot no. 2725-12. 4 X international standard.

Streptococcus Protein and Homologous Rabbit Antiserum.—These were made available through the courtesy of Dr. Rebecca C. Lancefield, who stated that the purified protein was prepared from group A, type 28 hemolytic streptococcus, strain T28. This protein was heretofore considered to be the type-specific substance of this serological type, but recent un-

published data indicate that it is not analogous to the type-specific M protein of other serological types of group A streptococci. Agglutination of streptococci referable to this type 28 protein does, however, occur. The preparation is thought to be free of group-specific C polysaccharide. Analytical results obtained both by electrophoresis and in the ultracentrifuge indicate that the material is almost entirely homogeneous. These findings are in agreement with the serological specificity of the preparation.

Rabbit Anti-H37Rv Serum.—This was kindly supplied by Dr. G. Middlebrook. It had been prepared by repeated intravenous injections over a period of 2 months of tubercle bacilli extracted with ether and acetone and resuspended in 0.5 per cent phenol. The culture used was strain H37Rv, a virulent human strain.

Other Antigens.—The following antigens and their homologous rabbit antisera had originally been prepared in the laboratory of the late Dr. K. Landsteiner. They were kindly supplied by Dr. M. W. Chase.

Ovalbumin—three times recrystallized
Horse serum albumin
Horse serum globulin
Chicken serum globulin

These were all dialyzed for 12 hours against physiological saline in the cold before use to remove the greater part of the preservatives (toluene and 0.25 per cent phenol).

Human Sera.—Dr. S. Rothbard of the Montefiore Hospital, The Bronx, New York City, kindly supplied sera of tuberculous and non-tuberculous individuals and the relevant clinical data.

Treatment of Sera before Use.—All sera were inactivated before use by heating at 56°C. for 30 minutes. They were subsequently absorbed with sheep red cells (1 volume of packed sheep cells per volume of serum) by contact for 10 minutes at room temperature.

Test Tubes.—The hemagglutination tests were carried out in small culture tubes (10 × 75 mm.).

The Hemagglutination Test of Middlebrook and Dubos.—The human sera were titrated by this method with some slight modifications. The test was performed as follows:—

One volume of a 2.5 per cent suspension in saline of sheep red cells was added to 5 volumes of saline containing 0.1 volume of the P.P.D. (Weybridge) preparation. The mixture was incubated for 2 hours at 37°C., then centrifuged, the cells washed once in saline, and finally resuspended in the original volume constituting again a 2.5 per cent suspension. One drop of this suspension was added to each tube of threefold dilutions of the test sera made in 0.5 cc. quantities of saline and to a saline control. As a further control, one drop of untreated sheep cells was added to 0.5 cc. of a 1:5 dilution of the serum. The tubes were shaken 30 minutes after the addition of the cells. Five hours later they were put at 0–4°C. and left overnight. The readings were made the following morning after incubation at 37°C. for 5 minutes by gently shaking the tubes to observe clumping.

EXPERIMENTAL

The Effect of Tannic Acid on the Adsorption of Tuberculin P.P.D. (Weybridge) by Erythrocytes

In general, the preliminary adsorption and hemagglutination experiments were carried out as follows: Erythrocytes were treated with dilute solutions of tannic acid, then centrifuged, and the cells washed once in saline. They were then exposed to solutions of Tuberculin P.P.D. (Weybridge) for a few minutes,

after which they were washed again a single time, and then added to dilutions of rabbit anti-H37Rv serum and to dilutions of normal rabbit serum. These serum dilutions were made in normal rabbit serum (hereafter designated as N.R.S.) diluted 1:100 in saline. Hemagglutination was observed in the higher concentrations of anti-H37Rv serum, but not in the lower concentrations of this serum, nor in any of the tubes containing only N.R.S.

Details are given below of such an experiment that was carried out after the introduction of various modifications of the original simple technique for reasons to be discussed in a later section.

It should be mentioned that the dilutions of the test sera are made in N.R.S. 1:100 because erythrocytes which have been treated with tannic acid and P.P.D. and washed, agglutinate when suspended in saline. However, in N.R.S. 1:100 the suspension is more stable, and the cells settle to the bottom in the form of a small central "button." This subject will be discussed again in another section.

Two cc. of a 2.5 per cent suspension of sheep erythrocytes in buffered saline (pH 7.2) was added to 2 cc. of 1:5,000, 1:20,000, 1:80,000, and 1:320,000 tannic acid solutions in saline. The mixtures were incubated at 37°C. for 10 minutes and then centrifuged and the cells washed in buffered saline at the same pH. After washing they were resuspended in 2 cc. saline and 0.5 cc. of each suspension added to 2 cc. buffered saline (pH 6.4), containing 0.05 cc., 0.0125 cc., and 0.003 cc. of the tuberculin P.P.D. (Weybridge) preparation. After 10 minutes at room temperature, the cells were centrifuged and washed in 1:250 N.R.S. and then resuspended in 0.5 cc. of the same medium. One drop (0.05 cc.) of each suspension was added to each tube of dilutions of rabbit anti-H37Rv serum. These dilutions were made in fourfold steps in N.R.S. (1:100), beginning at 1:1,000 of the test serum. One drop of each suspension was also added to a control tube containing only N.R.S. (1:100). As further controls, 1 cc. of cells was treated under the same conditions with tannic acid only, and another 1 cc. with P.P.D. only, and these cells were added to the highest concentration (*i.e.*, 1:1,000) of the rabbit antiserum in N.R.S. (1:100). The tubes were shaken immediately and again in 30 minutes and a reading made 2 hours after the second shaking. The results are shown in Table I.

In the presence of adequate amounts of the anti-H37Rv serum, the cells which had been treated with the higher concentrations of tannic acid and P.P.D. were agglutinated. The control tubes all failed to show any hemagglutination.

This agglutination occurred in the form of a typical pattern, covering the whole of the curved bottom of the tube, forming a uniformly thin carpet of red cells, as seen in influenza virus agglutination (11) and in agglutination by antimallein sera of cells which have been sensitized by exposure to antigens of *Pfeifferella mallei* in mallein (5). On gentle shaking the cells were completely redispersed.

This type of agglutination differs from that caused by antisera to the tubercle bacillus in the hemagglutination test of Middlebrook and Dubos (3), in which sheep erythrocytes are

exposed for 2 hours at 37°C. to saline extracts of phenol-extracted tubercle bacilli, or to certain tuberculin preparations (O.T. (Lederle), P.P.D. (Weybridge)). After washing, the cells are added to the serum dilutions and allowed to stand overnight. The test is read the following morning by gently shaking the tubes and watching the manner in which the cells re-suspend; in the presence of the lower dilutions of antisera they rise from the bottom in large clumps which do not easily break up, while in normal sera they rise evenly dispersed as a cloud. In this test the typical virus agglutination pattern referred to above is seldom observed, except sometimes in the highest dilutions of the serum causing agglutination.

TABLE I

Amount of Tannic Acid and Tuberculin P.P.D. (Weybridge) Necessary to Sensitize Red Cells

0.5 cc. of 2.5 per cent red cell suspension (in buffered saline (pH 7.2) for treatment with tannic acid)		Antiserum dilutions in N.R.S. 1:100						1:100 N.R.S. control
Tannic acid 0.5 cc. of:	P.P.D. in 2 cc. buffered saline (pH 6.4)	1:1,000	1:4,000	1:16,000	1:64,000	1:256,000	1:1,024,000	
1:5,000	cc.							
	0.05	++++	++++	+++	++	+	-	-
	0.0125	++++	++++	+++	+	-	-	-
1:20,000	0.003	++++	-	-	-	-	-	-
	0.05	++++	++++	+++	+	-	-	-
	0.0125	++++	++++	++++	+	-	-	-
1:80,000	0.003	++++	-	-	-	-	-	-
	0.05	+++	++	-	-	-	-	-
	0.0125	++	-	-	-	-	-	-
1:320,000	0.003	-	-	-	-	-	-	-
	0.05	-	-	-	-	-	-	-
	0.0125	-	-	-	-	-	-	-
	0.003	-	-	-	-	-	-	-

As a result of this experiment, the addition of 1 volume of 1:20,000 tannic acid to 1 volume of 2.5 per cent erythrocyte suspension was adopted as a routine procedure, as was the exposure of the cells so treated to 0.1 volume of the P.P.D. preparation in 4 volumes of saline per volume of red cell suspension.

The Influence of Variations in the Conditions of the Test

Following the observation that red cells treated with tannic acid adsorb P.P.D. onto their surfaces and thereby become agglutinable by antiserum to *Mycobacterium tuberculosis*, experiments were designed to determine the best conditions under which to carry out the various steps in the test. These steps may be classified for convenience as follows: (1) preparation of the erythrocytes

with tannic acid; (2) adsorption of tuberculin P.P.D. onto the cell surfaces; (3) exposure of the cells to the test serum.

Effect of pH on the Preparation of Erythrocytes with Tannic Acid.—It was found that slight variation of the pH at which the saline was buffered during treatment of the erythrocytes with tannic acid had no effect on the final outcome of the test.

However, it was observed that when this step was carried out in a slightly acid medium, for instance, in unbuffered saline or in saline buffered at pH 6.4, there was considerable clumping of the cells, which were particularly difficult to resuspend after centrifugation. Accordingly, in subsequent experiments the cells were suspended in saline buffered at pH 7.2 for treatment with tannic acid. Under these conditions clumping did not occur and resuspension was not difficult.

Effect of pH on the Adsorption of Tuberculin P.P.D. by Cells Treated with Tannic Acid.—As mentioned above, a certain amount of hemolysis associated with difficulty in resuspending the cells had been observed on occasion during the washing after treatment with tuberculin, and even sometimes to such a degree as to necessitate the preparation and sensitization of another aliquot of cells. It was established that this was not due to the preservatives (phenol and glycerol) in the tuberculin preparation. Accordingly, experiments were performed to determine whether the pH at which the exposure of the cells to P.P.D. was carried out had any effect on the degree of lysis.

The treatment with tannic acid was carried out as described above, with the cells suspended in buffered saline (pH 7.2). The P.P.D. preparation was diluted in saline buffered at pH 6.4, pH 7.2, pH 8, and in unbuffered saline. Following exposure to P.P.D., each set of cells was divided into two fractions; in one the cells were washed and resuspended in saline buffered at the same pH as that at which they had been treated; and in the other, in unbuffered saline. As in the last experiment, a slight degree of hemolysis occurred during washing. They were then added as before to dilutions of rabbit anti-H37Rv serum prepared in N.R.S. (1:100).

The cells which had been exposed to P.P.D. at pH 7.2 and pH 8 showed some agglutination in all tubes, including the normal serum control, while those treated at pH 6.4 and in saline showed no such instability and exhibited agglutination only in the higher concentrations of the positive serum (1:5,000 to 1:80,000).

Therefore, in subsequent experiments the cells were exposed to tuberculin in saline buffered at pH 6.4 instead of in unbuffered saline, the pH of which is liable to variation.

Necessity of Washing after Treatment with P.P.D.—As the lysis referred to above only occurred during the process of washing after exposure to P.P.D., experiments were carried out to determine whether this washing was necessary.

Cells treated with tannic acid were exposed to decreasing concentrations of P.P.D. (in twofold steps), ranging from a considerable excess to a concentration much too small to sensitize the cells to agglutination by antibody in the method generally used, which involved washing the cells. After 30 minutes at room temperature, the red cells were added, without washing, to the usual serum dilutions, and in no case did complete agglutination occur, although partial agglutination was observed in two tubes which contained cells treated with amounts of P.P.D. in the region of the minimum amount necessary to sensitize the cells under the usual conditions of the test.

The results of this experiment clearly showed that washing after addition of P.P.D. was essential.

Protective Effect of Normal Serum on Erythrocytes Treated with P.P.D.—It was found that N.R.S. in various dilutions exhibited a protective effect on the red cells during washing after exposure to P.P.D. A dilution of 1:250 N.R.S. was found satisfactory in preventing the hemolysis referred to above if care were taken not to agitate too vigorously while resuspending the cells after centrifugation.

It should be mentioned that, although this procedure was adopted throughout subsequent experiments with P.P.D. and with other antigens, it was probably not necessary in the case of the latter, since the cells treated with these substances were less fragile than those treated with P.P.D. However, it aided in the resuspension of cells after centrifugation in all cases.

The possibility exists that the cells treated with tannic acid and then with the antigen solution may not become completely saturated with the specific protein and, consequently, might adsorb proteins of the rabbit serum in which they are washed; it is also possible that the rabbit serum proteins might replace some of the antigen molecules on the cell surface. Components of rabbit serum adsorbed in this way would be available at the surface of the cells to react with antibodies against them in the event of these being present in the test serum, and thus a possible source of error would be introduced. As a precaution against such errors, it might be advisable in some cases to wash only in a dilution of normal serum from the same species as that which is being tested. However, N.R.S. was used throughout the experiments reported in this paper.

Order of Adding the Reagents.—Experiments were designed to determine the effects of changing the order in which the reagents were added and also of not washing between the treatment with tannic acid and the exposure to P.P.D. It was found that no sensitization to antibody agglutination occurred when the P.P.D. was put in contact with the tannic acid before the addition of the red cells when the same amounts of these substances were used as in the standard technique.

When the P.P.D. (in solution in buffered saline at pH 6.4) was added to red cells that had already been exposed to 1:20,000 tannic acid for 10 minutes, but not washed until after contact with the P.P.D., sensitization occurred, but agglutination did not take place in quite such high dilutions of antiserum.

Concentration of N.R.S. Optimum for Titration of Antibodies.—Because

erythrocytes treated with tannic acid and P.P.D. agglutinate in saline, N.R.S., diluted 1:100 in saline, has been used as a diluent in titrating test sera. As stated above, under such conditions agglutination occurs only in the presence of antibodies.

Experiments involving different concentrations of N.R.S. as a diluent in titrating the serum of a tuberculous patient showed that a solution of 1:100 N.R.S., as used in the original experiments, was as satisfactory as any. If a 3 per cent solution was used, there occurred considerable interference with the formation of a typical agglutination pattern. This was noted especially in the higher concentrations of the test serum. When N.R.S. 1:100 was used as a diluent, there was some slight inhibition of the formation of the typical agglutination pattern in the tube containing the highest concentration of human serum; however, in all other tubes agglutination was complete up to a dilution of 1:800 of the test sera, after which all tubes were negative. With 1:300 and 1:900 N.R.S. as diluent, some agglutination was observed in all tubes, including the normal serum controls.

Effect of the Presence of Complement in the Final Test.—A hemolytic modification of the direct sensitization hemagglutination method with tuberculin has been described (12, 13). Accordingly, the effect of complement on cells sensitized with antigen by the tannic acid technique and exposed to homologous antiserum was investigated.

It was found that the presence of guinea pig complement in anti-H37Rv serum dilution and in an N.R.S. control, to which tannic acid-P.P.D.-treated cells had been added, resulted in hemolysis in all tubes, including the normal serum controls. This outcome might have been anticipated in view of Reiner's observation (7) that tannic acid acted as "amboceptor" in rendering erythrocytes susceptible to lysis by complement, although the conditions of his experiments were not strictly the same as those described above.

Efficacy of Other Tuberculin Preparations.—The Lederle preparation of old tuberculin was found less effective than P.P.D. (Weybridge) in sensitizing tannic acid-treated red cells to agglutination by anti-H37Rv serum. The agglutination was weaker and was confined to higher concentrations of the antiserum. Tuberculin P.P.D.-S. was even less effective than O.T., and only a trace of agglutination by anti-H37Rv serum was observed in the case of red cells which had been treated with tannic acid and subsequently exposed to this P.P.D. preparation.

Comparative Titrations of Human Sera for Antibodies against Antigens of the Tubercle Bacillus by the Tannic Acid Method and the Hemagglutination Method of Middlebrook and Dubos

A number of human sera from individuals with various disease conditions, including tuberculosis, were titrated for antibodies against antigens of the tubercle bacillus by the tannic acid-P.P.D. technique. The results were compared

with those obtained by the hemagglutination method of Middlebrook and Dubos (3), which was slightly modified as described under Materials and Methods. The technique of the tannic acid method used in these titrations was based on the results of the foregoing experiments and was as follows:—

Preparation of Erythrocytes with Tannic Acid.—One volume of 2.5 per cent sheep erythrocytes in buffered saline (pH 7.2) was added to 1 volume of 1:20,000 tannic acid and the mixture incubated for 10 minutes at 37°C. The mixture was then washed once in buffered saline (pH 7.2), centrifuged at 1,500 R.P.M. for 3 minutes, and resuspended in 1 volume of saline.

Adsorption of Antigens by Erythrocytes.—One volume of this tannic acid-treated erythrocyte suspension in saline was added to 4 volumes of buffered saline (pH 6.4), containing the dissolved antigen (e.g., 0.15 mg. P.P.D. per cc. of erythrocyte suspension), and left for 15 minutes at room temperature. It was then washed in 1:250 N.R.S., centrifuged at 1,000 R.P.M. for 3 minutes, and finally resuspended in 1 volume of 1:250 N.R.S. Care was taken at this stage not to agitate the cells too vigorously during the washing.

Exposure of the Cells to the Test Serum.—The desired dilutions of test sera were made in 1:100 N.R.S. in 0.5 cc. quantities, and one drop (0.05 cc.) of the cell suspension was added to each tube; the tubes were shaken immediately. A satisfactory reading could be made 2 hours later, but somewhat more clear cut results were obtained by placing the tubes at this time at 0–4°C., allowing them to stand overnight, and reading the results the following morning.

The results of the titration of thirty-five human sera by the two techniques are shown in Table II. Patients 1 to 25 were suffering from pulmonary tuberculosis. The sera of patients 5 to 11 were especially selected because they were known to have high titers by the direct sensitization hemagglutination technique. The sera of patients 22 to 25 were included because they had been shown to be negative, or very weakly positive, by that method. Patients 1 to 7 were described as “far advanced” cases, and Nos. 21, 23, and 25, as “with minimal disease.” In the remainder the disease was “moderately advanced.”

Patients 26 to 31 were non-tuberculous individuals, No. 27 (chronic lymphatic leukemia) and No. 29 (syphilis) being included because their sera had given positive, or weakly positive, agglutination by the direct sensitization method.

As far as could be ascertained, tuberculin skin tests had not been carried out on any of the patients, at least within the previous 6 months.

Untreated red cells and cells treated with tannic acid only were added to 1:5 dilutions of each serum as controls. Agglutination was not observed in any of these tubes.

It will be seen that, among the sera from tuberculous individuals, all that were positive by the direct sensitization technique also agglutinated cells treated with tannic acid and P.P.D. In most cases the titer of the serum was considerably higher by the latter method. However, in Nos. 7, 12, 23, the titers were the same by both methods. Serum from patient 24 had a titer of 1:405 by the tannic acid method and was negative at 1:5 by the other technique. Serum from No. 25 showed no agglutination by either technique.

The sera from the non-tuberculous individuals were negative by both methods, with the exception of Nos. 27 and 29, which had been especially selected as being “false positives” in the direct sensitization test. These showed some agglutination in 1:5 dilution in both cases.

TABLE II

Titration of Sera from Tuberculous and Non-Tuberculous Individuals by the Tannic Acid Method as Compared with the Direct Sensitization Technique of Middlebrook and Dubos

Patient No.	Tannic acid technique										Direct sensitization technique					
	Serum dilutions										Serum dilutions					
	5	15	45	135	405	1,215	3,645	10,925	33,875	101,625	5	15	45	135	405	1,215
1	4	4	3	2	2	1	0	0	0	0	4	3	1	0	0	0
2	4	4	4	4	2	0	0	0	0	0	3	2	0	0	0	0
3	4	4	4	4	4	3	0	0	0	0	4	1	1	0	0	0
4	4	4	4	4	4	4	3	2	0	0	4	3	1	0	0	0
5	4	4	4	4	4	4	3	3	0	0	4	4	4	3	0	0
6	4	4	4	4	4	3	2	0	0	0	4	4	4	2	0	0
7	4	3	3	2	0	0	0	0	0	0	4	4	3	2	0	0
8	4	4	4	4	4	4	4	3	2	0	4	4	4	4	3	0
9	4	4	4	4	2	1	0	0	0	0	4	4	4	3	1	0
10	4	4	4	4	3	1	0	0	0	0	4	4	3	3	1	0
11	4	4	4	4	3	3	2	0	0	0	4	4	4	4	3	0
12	4	4	4	3	0	0	0	0	0	0	4	4	4	2	0	0
13	4	4	4	1	1	0	0	0	0	0	4	3	2	1	0	0
14	4	4	4	4	4	1	0	0	0	0	4	2	0	0	0	0
15	4	4	4	4	3	2	1	0	0	0	3	3	1	0	0	0
16	4	4	2	1	1	1	0	0	0	0	4	3	1	0	0	0
17	4	4	4	3	1	1	0	0	0	0	4	2	0	0	0	0
18	3	3	3	2	1	0	0	0	0	0	2	1	0	0	0	0
19	4	4	3	1	0	0	0	0	0	0	4	4	3	1	0	0
20	4	4	3	1	0	0	0	0	0	0	4	4	4	1	0	0
21	4	4	4	1	1	1	0	0	0	0	3	2	0	0	0	0
22	2	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0
23	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
24	3	4	4	4	3	1	0	0	0	0	2	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	2	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The lack of correlation between the agglutination titers obtained by these two methods suggests that antibodies of more than one kind are involved, perhaps directed against different antigens or differing in some of their other properties, although directed against the same antigens.

The Application of the Tannic Acid Method to Some Other Antigen-Antibody Systems

Experiments were carried out to determine whether tannic acid-treated erythrocytes would be able to take up antigenic proteins in general and consequently undergo agglutination in the presence of the corresponding antisera. For this purpose ovalbumin, horse serum globulin and albumin, chicken serum globulin, and a streptococcus protein preparation (strep. type 28) were used.

Tannic acid-treated cells were exposed to different amounts of these substances, washed, and added to dilutions of the antisera. The details of technique were the same as those described for P.P.D. in the last section. All the preparations were found to be capable of rendering the cells agglutinable by the corresponding antisera, although the minimum amount of the proteins required to cause maximum sensitivity to antibody (*i.e.*, the "optimum sensitizing amount" of the preparations) varied considerably. For instance, the optimum sensitizing amounts of ovalbumin, streptococcus protein, horse serum globulin, horse serum albumin, chicken serum globulin, and P.P.D. (Weybridge) were in the region of 5 mg., 0.5 mg., 0.5 mg., 1 mg., 1 mg., 0.025 mg., and 0.15 mg. per cc. of red cell suspension, respectively. Amounts greater than this resulted in no increase in the agglutinating titer of the test sera. On the other hand, red cells treated with smaller amounts of the antigen were less sensitive to antibody and resulted in lower titers of the antiserum, until finally no agglutination would occur with the smallest amount, even in 1:10 serum dilution.

There follows an experiment in which each of the six antisera mentioned was tested against all of the antigens by the tannic acid method.

Six sets of different rabbit antisera directed against the following antigens were diluted in fourfold steps in N.R.S. 1:100: anti-ovalbumin, anti-horse serum albumin, anti-horse serum globulin, anti-chicken serum globulin, anti-streptococcus protein 28, and anti-H37Rv. The dilutions commenced at 1:10, 1:100, 1:1,000, 1:10,000, or 1:100,000, according to the anticipated titer of the serum. According to the usual technique, 3 cc. samples of 2.5 per cent tannic acid-treated red cell suspension were exposed to the different antigen preparations and, after contact for 10 minutes, were centrifuged and washed once in N.R.S. 1:250.

One drop (0.05 cc.) of each suspension of treated cells was added to dilutions of all the antisera so that each of the six sets of serum dilutions contained cells sensitized with a different antigen preparation. In Table III the amounts of antigens used per cc. of cells is shown, as well as the agglutinating titers of the sera against cells treated with the various antigens.

It will be seen that in each case the sensitized cells were agglutinated by fairly high dilutions of antiserum directed against the adsorbed antigen. A certain degree of cross-reaction occurred as indicated, and in the case of the horse serum proteins this was probably due to incomplete separation of albumin and globulin during preparation. This, however, does not explain the agglutination of red cells sensitized with horse albumin by dilutions of 1:40 of the heterologous antisera and of the N.R.S. An unexpected cross-reaction was seen in the agglutination of cells sensitized with chicken serum globulin by anti-horse globulin serum up to a dilution of 1:640, which is, however, a serum concentration 4,000 times greater than that of the titer of the homologous anti-

serum. No cross-reactions were observed in the case of cells treated with the two bacterial antigens.

Inhibition of Antibody Hemagglutination by Antigens

As was expected, the addition of a given antigen to the homologous anti-serum resulted in the inhibition of the effect of the latter in agglutinating erythrocytes sensitized with this antigen, and the inhibitory effect was specific. For instance, it was found that if 0.1 cc. of a dilution of N.R.S. and antistreptococcus serum was added to threefold dilutions of the streptococcus antigen (so that each tube had a final concentration of 1:2,000 antiserum and 1:100 N.R.S.), and one drop of tannic acid-treated cells sensitized with the same

TABLE III
Titers of Immune Rabbit Sera against Homologous and Heterologous Antigens by the Tannic Acid Hemagglutination Method

Sera	Ovalbumin (10 mg.)*	Horse serum albumin (1 mg.)	Horse serum globulin (2 mg.)	Chicken serum globulin (0.05 mg.)	Streptococcus protein (1 mg.)	Tuberculin P.F.D. (0.30 mg.)
Anti-ovalbumin	40,000	40	0	0	0	0
Anti-horse serum albumin	0	160,000	16,000	0	0	0
Anti-horse serum globulin	0	40,000	1,600,000	640	0	0
Anti-chicken serum globulin	2,560	40	0	2,560,000	0	0
Anti-streptococcus protein	0	40	0	0	16,000	0
Anti-H37Rv	0	40	0	0	0	64,000
Normal rabbit serum	0	40	0	0	0	0

0 means no agglutination at a dilution of 1:10 and higher. The other figures are the reciprocal of the highest dilution at which agglutination occurred.

* The figures in parentheses refer to the amount of antigen used per cc. of 2.5 percent suspension of tannic acid-treated red cells.

antigen was added, agglutination by antiserum was inhibited by dilutions up to 1:10 million of the antigen. Under the same conditions, the other antigens did not inhibit in this system at a concentration of 1:1,000.

DISCUSSION

The foregoing experimental results have demonstrated that the treatment of sheep erythrocytes with tannic acid brings about a change in the properties of these cells, rendering them capable of adsorbing certain protein molecules when they are suspended in solutions of these substances in saline. Red cells that have thus adsorbed proteins are agglutinated by the homologous rabbit antiprotein sera in high dilutions.

The fact that tannic acid agglutinates erythrocytes has been known for many years, and Reiner and Fischer (7) have discussed the possible mechanism of the

effect. They suggested that tannic acid alters the surface properties of the red blood cells, changing them from a hydrophilic to a hydrophobic state. Freund (14, 15) assumed that tannic acid, like homologous antibody, brings about a change in the surface potential of the cells, which, in the presence of certain electrolytes, results in their aggregation. Whatever the exact mechanism of the agglutination of the red cells by tannic acid, it seems not unlikely that the same factors are involved in the ability of the acid to promote the adsorption of proteins by red cells.

There are certain features of the adsorption of antigens by tannic acid-treated cells that make this process distinct from the direct sensitization of normal erythrocytes by extracts of bacteria, as described by a number of workers (1-6). For instance, in our experience with mallein 3 hours' contact between red cells and a certain amount of mallein was found necessary at room temperature to cause maximum sensitization of the cells to agglutination by antimallein sera, while at 37°C. the same effect was brought about in 10 minutes (5). In contrast, cells treated with tannic acid immediately adsorb the protein antigens to which they are exposed and do so as well at 0-4°C. as at higher temperatures. Another important difference in the behavior of normal red cells and red cells treated with tannic acid is the fact that certain purified protein preparations, for example, horse serum albumin, which are readily adsorbed onto tannic acid-treated red cells, do not sensitize normal cells even when in contact with them for several hours at 37°C.

The work of Keogh, North, and Warburton (2) has suggested that, at least in some cases, polysaccharides are the active principles involved in causing direct sensitization of erythrocytes to agglutination by antibacterial sera. However, even if it can be convincingly shown that the polysaccharide is essential for this sensitization, it does not necessarily follow that this substance alone is responsible for the subsequent reaction with antisera that results in hemagglutination. Products active in causing direct sensitization of erythrocytes like mallein or old tuberculin contain a multitude of molecular species, and it does not seem impossible that certain of these, perhaps polysaccharides, alter the erythrocytes in a manner more or less similar to that of tannic acid, with result that they adsorb other sorts of molecules from their immediate environment. These secondarily adsorbed molecules, unable by themselves to bring about the primary alteration of the cells, would, nevertheless, be available at their surfaces to react with antibody and might play an important part in the subsequent serological reaction. It is obvious, however, that such a situation cannot be strictly analogous to that occurring with tannic acid since this substance, when mixed first with tuberculin, does not appear to promote the adsorption of antigens onto erythrocytes which are added later. In this connection, there is under investigation in these laboratories, a substance which, when mixed with certain protein antigens prior to addition to red cells, causes the latter to become sensi-

tized to agglutination by the homologous antiprotein sera. This substance is present in an impure preparation of fructose and has not been identified.

It is thought that the phenomena described in this paper may find application in the development of serological procedures involving antigens which can be adsorbed onto red cells treated with tannic acid. It should be kept in mind, however, that the technique may be of value only in cases in which relatively pure preparations of the antigens are available, for in the case of impure preparations it is probable that non-specific molecules would interfere with the serological reaction.

SUMMARY

Treatment of sheep erythrocytes with suitable concentrations of tannic acid render them capable of adsorbing certain protein molecules from solution in saline. Red cells which have adsorbed proteins in this way are agglutinated after washing by the homologous antiprotein sera, even by high dilutions.

Through hemagglutination sera can be titrated for antibodies against antigens adsorbed on the cells exposed to tannic acid. Furthermore, small amounts of the antigens can be detected through their power to inhibit hemagglutination of the treated cells.

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