be produced in female mice with chloroform and 1,1,2-trichloroethane, even at lethal doses. Male and female mice were also tested for their susceptibility to the hepatotoxic properties of carbon tetrachloride. Liver dysfunction, as determined by elevated serum glutamic-pyruvic transaminase activity, could be demonstrated in both male and female mice at 0.009 ml/kg. No sex difference was observed in the 24-hour LD_{50} values for the three agents tested. This indicates that with these agents nephrotoxicity is not an important contributing factor in the deaths occurring during the first 24 hours.

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Hemagglutination with Aldehyde-Fixed Erythrocytes for Assay of Antigens and Antibodies.* (31953)

D. H. BING,† J. G. M. WEYAND, ‡ AND A. B. STAVITSKY
(Introduced by I. H. Lepow)
Department of Microbiology, Western Reserve University School of Medicne, Cleveland, Ohio

Passive hemagglutination methods have proved very useful for the assay of small amounts of antigen and antibody(1). Fresh or formalinized erythrocytes have been conjugated to proteins by the tannic acid or bis-diazotized benzidine and other procedures. Formalin-fixed, antigen-conjugated cells have been especially used because they can be frozen for long periods of time without loss of sensitivity to agglutination by specific antibody(2). Often, however, such fixed and sensitized erythrocytes tended to clump upon freezing and thawing, making them unsuitable for the assay of antibody. It, therefore, seemed desirable to develop other procedures for preservation and sensitization of sheep erythrocytes. Glutaraldehyde fixation of erythrocytes was investigated and found to be superior to formalin fixation of red blood cells. Conditions for the conjugation of the glutaraldehyde-preserved red blood cells to proteins by the tannic acid and bis-diazotized methods were compared and the stability of these preparations ascertained. The application of these preparations to the detection of antibody to human serum albumin and to bovine beta lactoglobulin is described.

Materials and methods. Antigens and antisera. Bovine beta lactoglobulin (BLG) and human serum albumin (HSA) were purchased from Pentex Laboratories, Kankakee, Ill.

Antisera were prepared in 6-lb male or female white New Zealand rabbits. Rabbits

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† Predoctoral trainee supported by USPHS Training Grant 2T1-GM-171. Present address: Dept. of Bacteriology and Immunology, Univ. of California, Berkeley.

‡ Senior medical student. These studies form part of a thesis submitted to Western Reserve University School of Medicine in partial fulfillment of requirement for the degree of Doctor of Medicine, January 1966.

were injected 3 times a week for 3 weeks with 20 mg of BLG or HSA into each hind foot pad. At the end of the fourth week they were bled from the lateral ear vein every day for 3 days. Sera from the 3 bleedings of an individual rabbit were pooled.

Hemagglutination assay. a) *Preparation of antisera and diluent.* All sera were collected by centrifugation at 5000 RPM for 20 minutes and stored at —20°C until used. The antisera were heated at 56°C for 30 minutes to inactivate the complement. All dilutions were made in buffer containing .25% normal rabbit serum (NRS—Cappel Laboratories, West Chester, Pa.). The normal rabbit serum was heated for 30 minutes at 56°C and absorbed with an equal volume of .15 M NaCl-washed fresh, formalinized or glutaraldehyde preserved sheep erythrocytes, depending on which preparation of conjugated cells was being employed. A 10-fold dilution of the antisera was absorbed for 10 minutes at room temperature with the suitable non-conjugated erythrocyte preparation just prior to dilution for the hemagglutination or hemagglutination inhibition assay.

b) *Hemagglutination assay.* Hemagglutination assays were performed as described previously(1). The end point of the assay was defined as the last dilution of the antiserum which gave a definite 2-plus pattern. Titers were expressed as the reciprocal of the dilution of antiserum at the end point.

c) *Hemagglutination inhibition assay.* Hemagglutination inhibition was carried out by 2 procedures. First, the antigen was added to each tube in a duplicate series of the antiserum dilutions prior to adding the antigen conjugated erythrocytes. Second, the inhibitor was serially diluted in the diluent containing .25% NRS, the final volume of each dilution being 0.5 ml. A constant amount of antibody was then added to each tube. This was 0.1 ml of a dilution of the antiserum which still caused specific agglutination of the conjugated cells. For exapmle, the hemagglutination titer of an antiserum was 5120. For the inhibition assay it was diluted 1:500 in buffered saline containing .25% NRS and 0.1 ml of this dilution added to each dilution of inhibitor. The volume of the inhibitor was 0.5 ml. Therefore, the final dilution of antiserum was 1:3000, a concentration which still caused agglutination of the antigen conjugated cells. Five-hundredths ml of a 2% suspension of the protein conjugated cells were added to each dilution of inhibitor, the tubes mixed, and the cells allowed to settle. In both assays, the end point of inhibition was the last dilution of inhibitor which produced a definite plus-minus pattern(1).

Fixation of the erythrocytes. Sheep erythrocytes were preserved by formalin as described previously(2). They were also fixed with glutaraldehyde as follows:

Fresh sheep erythrocytes suspended in Alsever's solution were centrifuged for 20 minutes at room temperature at 3000 RPM and washed 3 times with .15 M NaCl. After the last wash the packed cells were chilled to 4°C in an ice bath. Twenty-five percent glutaraldehyde (Technical grade, J. T. Baker Co., Phillipsburg, N. J.) was diluted to 1% (v/v) with a solution containing one volume of .15 M $NaPO_4$, pH 8.2, nine volumes of .15 M NaCl and five volumes of distilled water. The glutaraldehyde-salt solution was chilled to 4°C and used to dilute the packed red blood cells to 1 to 2% (v/v). The mixture of cells and glutaraldehyde was incubated for 30 minutes at 4°C with occasional mixing. The fixed cells were collected by centrifugation at 3000 RPM at room temperature and washed 5 times with .15 M NaCl and 5 times with distilled water. The volume of the wash was about one-tenth the volume of the glutaraldehyde-red blood cell mixture. The cells were suspended to a final concentration of 30% in distilled water, merthiolate was added to final concentration of 1:10,000 (w/v) and the cells stored at 4°C.

The formalinized cells were dark brown in color and morphologically indistinguishable from unfixed cells. They sedimented rapidly and required vigorous shaking for dispersion. Upon prolonged storage at —20°C, they tended to clump, and required vigorous shaking and/or straining through three layers of gauze in order to obtain a uniform suspension. The glutaraldehyde-fixed cells were bright red immediately after fixation, and gradually became brown with succeeding

washes. The final color, however, was lighter than a corresponding suspension of formalinized erythrocytes. They did not lyse when suspended in distilled water, upon freezing and thawing 20 times, or when lyophilized and reconstituted. Clumping of the cells occurred after a few weeks storage at —20°C, but they could be resuspended by vigorous agitation in a Vortex mixer. Specific sensitization of the cells with a protein antigen was still possible even after 6 months storage of the cells at 4°C in 1:10,000 merthiolate.

Sensitization of erythrocytes. The fresh formalinized and glutaraldehyde preserved erythrocytes were sensitized by the tannic acid method as previously described(1,2). The pH of sensitization was 6.4 for BGG and 5.6 for HSA. One-tenth milligram of HSA was employed for sensitizing one ml of a standardized suspension of erythrocytes. Sensitization of glutaraldehyde-treated cells with bis-diazotized benzidine (BDB) was performed in the following manner: An aliquot of cells was removed from the stock suspension of 30% cells and collected by centrifugation at 3000 RPM for 10 minutes. They were washed 2 times with .11 M $NaPO_4$ buffer, pH 7.3, and suspended to a final concentration of 25% in the same buffer. The volume of the washes was 10 times the volume of the cells removed from the stock solutions of cells. The BDB was prepared according to a previous description(3) and stored at —20°C in 0.5 ml aliquots. Assuming complete reaction there was 4.08 mg of BDB/ml of solution. For use, each aliquot was thawed quickly at 37°C and diluted 1:15 with the .11 M sodium phosphate buffer, pH 7.3. Cells, antigen and .11 M $NaPO_4$ buffer, pH 7.3, were mixed and then the BDB was added. The final concentration of erythrocytes in the reaction mixture was 1% (v/v). The mixture of cells BDB and antigen was incubated for 15 minutes at 37°C and the cells collected by centrifugation at 3000 RPM for 5 minutes. The cells were washed twice with .15 M NaCl, and then once with .15 M NaCl-$NaPO_4$, pH 6.4 containing .25% NRS. The volume of the washes was one-half the volume of the reaction mixture. Finally, the cells were suspended in .15 M NaCl-$NaPO_4$ buffer pH 6.4 containing .25% normal rabbit serum to a final concentration of 2% (v/v). Freezing and thawing of the BDB conjugated cells caused clumping. The cells were, therefore, stored at 4°C in 1:10,000 merthiolate. At this temperature they maintained sensitivity for at least 4 weeks. That is, they were specifically agglutinated by the same dilution of a standard antiserum.

Results. Preparation and activity of variously fixed and sensitized erythrocytes. a) *Direct and tannic acid-conjugated and sensitized fixed cells.* One lot of the glutaraldehyde-fixed sheep erythrocytes was sensitized with HSA by the tannic acid method, using 0.1 mg of HSA per ml of a 2.5% solution of fixed cells. Another batch of glutaraldehyde-fixed cells was sensitized directly by addition of 0.0005 to 0.5 mg of HSA per ml of a 2.5% solution of fixed cells. It was found that the optimal concentration of antigen was 0.125 mg of HSA per ml of 2.5% fixed cells. A batch of formalinized cells was sensitized in a similar manner using HSA at a concentration of 0.25 mg per ml of 2.5% fixed cells yielding cells of a comparable or occasionally higher titer. Attempts to directly snesitize formalinized cells with an antigen concentration of 0.125 mg per ml 2.5% fixed cells yielded cells with a markedly reduced titer (Table I).

For assay the cells were stored in 1:10,000 merthiolate at 4°C. The anti-HSA serum was diluted in pH 7.2 buffered saline containing 0.25% NRS and the anti-BLG serum in pH 6.4 buffered saline containing 0.25% NRS.

Table I indicates the results of the assays with the variously sensitized cells and the results of the tests of the stability of the preparations after repeated freezing and thawing or lyophilization and storage for one month. Direct sensitization of the formalinized cells was achieved but necessitated a higher antigen concentration than the glutaraldehyde-fixed cells. Direct sensitization of both cell types resulted in sensitive cells for the detection of anti-HSA antibody. The formalinized cells, however, had somewhat less distinct settling patterns particularly in the hemagglutination inhibition row. Within the

TABLE I. Tests on the Stability of Various Antigen-Conjugated Cell Preparations.

Antigen	Cell preparation	Initial titer*	1 mo	3 mo	4 mo
HSA	Formalinized, tanned	32,000	12,800	6,400	12,800
HSA	Glutaraldehyde-fixed, tanned	3,200	12,800	3,200	6,400
HSA	Formalinized,‡ untanned	102,400	—†	—	—
HSA	Formalinized,§ untanned	100	100	—	—
HSA	Glutaraldehyde,§ untanned	12,800	12,800	25,600	25,600
BLG	Glutaraldehyde BDB	32,000	32,000	32,000	32,000

* The titer is expressed as the reciprocal of the serum concentration at which the end point occurred.
† Not tested.
‡ .25 mg of HSA per ml of 2.5% cells was used in sensitization.
§ .125 mg of HSA per ml of 2.5% cells was used in sensitization.

limits of the error of the hemagglutination method, the glutaraldehyde-fixed cells usually appeared to be as sensitive as other cell preparations for detection of these antibodies. Moreover, the sensitivity was maintained during storage for at least 4 months at —20°C.

Bovine β-lactoglobulin could not be conjugated to formalinized erythrocytes by the tannic acid method unless 1 mg of BLG-A was added per ml of final volume of 2% cells. In addition, the cells were not specifically agglutinated by an anti-BLG-A antiserum after one to two weeks storage at 4°C or —20°C. The experience with fresh cells was similar; more than 2 mg of BLG-A was required for sensitization of 1 ml of a standardized solution of tannic acid-treated fresh erythrocytes, and the cells never proved sensitive for detection of antibody.

b) *Conjugation of HSA and BLG-A to glutaraldehyde-fixed erythrocytes with BDB.* Table II shows the results of varying HSA concentration and BDB concentration. With a high BDB/HSA ratio in the mixture, the cells were not specifically agglutinated by the anti-HSA antiserum; that is, pre-incubation of the antiserum with .2 mg of HSA did not inhibit the agglutination reaction. When the ratio of HSA to BDB was lowered, the resulting protein conjugated cells were agglutinated by very high dilutions of antisera, but were not agglutinated in the absence of antiserum, or when the antiserum was inhibited by the pre-incubation with 75 μg of HSA. When BDB was omitted, the cells were specifically agglutinated by lower dilutions of the antiserum.

TABLE II. Results with HSA-Sensitized BDB-Cells and Varying Concentration of HSA and Amount of BDB.

Conc. HSA, mg/ml	Amt BDB, ml	Titer*	Diluent control	Inhibition control
0.05	.5	163,840	pos†	pos†
10	1.0	1,638,400	"	"
0.5	1.0	1,638,400	"	"
20	.5	1,638,400	neg	neg
10	.5	1,638,400	"	"
5	.5	1,638,400	"	"
10	.00	12,800	"	"

* The titer is expressed as the reciprocal of the serum dilution at which the end point occurred.
† Pos—positive agglutination; neg—negative agglutination. Inhibition was tested by adding 75 μg of HSA to a duplicate series of antiserum dilutions.

The β-lactoglobin—anti-β-lactoglobulin system was similarly studied. Very sensitive and stable preparations of protein conjugated erythrocytes could be obtained using 0.018 mg of protein N and 0.32 mg of BDB per ml of final concentration of 2% cells. The cells were not sensitized in the absence of BDB. These preparations were extremely specific; as little as 10^{-5} μg of BLG would inhibit the hemagglutination reaction when a constant amount of antibody was added to a series of dilutions of the antigen (*cf., Materials and methods*).

Discussion. The use of standardized preparations of antigen-conjugated erythrocytes has greatly facilitated the use of the hemagglutination method in the studies on antibody production. A previous paper has described the preparation and sensitization of formalin preserved sheep red blood cells by the tannic acid method(2). Subsequent studies(4) have shown that the formalinized cells tend to clump after storage for 6 months to one year at —20°C, making them unsuitable for anti-

body assays. In addition, uniform preparations of formalin preserved cells were difficult to reproduce. As a result, other methods were investigated which would still retain the advantage of employing preserved cells in hemagglutination assays.

The glutaraldehyde method for preservation of red blood cells appeared to fulfill the desired requirements. A shorter time of treatment with the fixing reagent resulted in stable, uniform preparations of erythrocytes which were insensitive to freezing and thawing, and changes in pH and osmolarity.

Two antigens, human serum albumin and beta lactoglobulin, were covalently linked to these fixed cells by use of the bifunctional reagent, bis-diazotized benzidine. This method should, therefore, be useful for many types of antigens which contain diazotizable histidine and tyrosine residues.

During the course of these studies, it was noted that human serum albumin was absorbed to the glutaraldehyde-treated cells in the absence of any agent such as tannic acid or bis-diazotized benzidine. The absorption of proteins to glutaraldehyde-treated sheep erythrocytes is not a universal phenomenon, as bovine beta lactoglobulin was not absorbed by the same preparation of cells.

Employing BDB-BLG-glutaraldehyde-fixed cells and anti-BLG sera, the inhibition of hemagglutination was found to be a very sensitive assay for small amounts of BLG; 10^{-5} μg of BLG could be detected. More precision in this assay can be achieved by measuring the settling time of agglutinated *versus* non-agglutinated cells(5).

Summary. Methods have been described for the preservation of sheep erythrocytes by brief treatment with glutaraldehyde. These cells could be conjugated to proteins in the presence or absence of coupling reagents and were specifically agglutinated by specific antiprotein sera. These antigen-conjugated cells were stable for at least 6 months. Utilizing these cells, the inhibition of hemagglutination was found to be a sensitive technique for detection of small amounts of antigen.

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Effect of Steroids on Disposition of Oxyphenbutazone in Man.*† (31954)

MURRAY WEINER, A. A. SIDDIQUI, R. T. SHAHANI, AND PETER G. DAYTON
New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York City

The administration of more than one drug at a time is common medical practice, and numerous examples of synergistic and antagonistic effects are known. Many of these effects are assumed to be interactions at the receptor sites. However, in recent years several instances have been reported in man and animals in which the apparent synergistic or antagonistic effects could be correlated with an alteration in the metabolic fate of one drug induced by the other(1,2). Some of these

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