tivity and that "the substances responsible for the specificity of the transplantation antigens are probably similar to the blood group mucoids". There is an indication that the blood group A and B antigens are involved in the sloughing of homogenous skin grafts and clouding of corneal transplants(8). The presence of these antigens in human cancellous bone and their absence from human cortical bone has been observed by Weinberg, Makin, Nelken and Gurevitch(9). Curtiss, Chase and Herndon(10) showed that a blood group A antigen-antibody interaction had no demonstrable effect on the acceptance of an iliac bone graft. Thus the successful transplantation of bone, in contrast to soft tissues, occurs even in the presence of known antigen-antibody interaction.

Cortical bone is relatively acellular and reports of apparently successful transplants made with autogenous and homogenous bone (11,12) would suggest the low order of antigenicity of bone tissue. Therefore, it is not surprising that most of the antigenicity of cortical bone observed in the present study comes from its blood content rather than from its cellular composition. If the antigens of bovine serum and bovine red cells could be removed from the bovine cortical bone, it is possible that an immunologically unreactive bone could be made available clinically for heterogenous bone transplantation.

Summary. Rabbits were immunized with finely ground, pulverized bovine cortical bone. It was demonstrated that the antibodies in the sera of these rabbits reacted with the bovine serum and the bovine red cell antigens present in the bone; however, after absorbing from these rabbit sera the bovine serum and bovine red cell antibodies, hemagglutination titers of low order still persisted. Grabar immunoelectrophoresis confirmed the results obtained by the hemagglutination assay and indicated the presence of 2 distinct antigens in bovine cortical bone unrelated to its serum and red cell content.

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Hypersensitivity to Chemical Allergens I. Use of Allergen-Erythrocyte Conjugates in Detection of Antibodies.* (27269)

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Despite the many advantages afforded by the use of simple chemicals in studies of hypersensitivity, critical investigations have been limited by the absence of accurate and sensitive methods for ascertainment of small amounts of antibody. Most reports(1,2,3) concerned with proof of antibody production to chemical allergens (*e.g.*, picryl chloride, citraconic anhydride, etc.,) have been based on results obtained by *passive cutaneous anaphylaxis* (PCA). While this method is presently considered more sensitive for detection

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of antibody to chemical allergens, various *in vitro* procedures, including complement-fixation and precipitin tests, have been successfully applied(4-8). Sensitive and reproducible *in vitro* methods of determining antibodies to allergenic chemicals have not been reported, although hemagglutination has been used in demonstration of antibody to both azodyes and azoproteins(9,10). This report describes a reliable *in vitro* procedure for detection of specific hemagglutinating antibodies to chemical allergens (*i.e.,* simple chemicals capable of inducing both antibody production and dermal sensitivity). The method utilizes the principle of direct conjugation of simple chemicals to formalinized erythrocytes.

Methods. Formalinization of erythrocytes. Blood from sheep was collected in an equal volume of modified Alsever's solution (11) and cells were washed 5 to 6 times in 10 volumes of chilled (0-4°C) 0.85% NaCl. A 10% saline suspension of washed erythrocytes was added to an equal volume of buffered (pH 6.8) 10% formalin containing 0.85% NaCl. This mixture was subjected to continuous gentle agitation with a magnetic stirrer for 48 to 72 hours with a temperature of 0-4°C being maintained throughout the procedure. At the end of the period of formalinization, cells were washed 8 times in 10 volumes of saline. In contrast to procedures described previously(12-16), erythrocytes prepared by this method did not agglutinate non-specifically and morphological characteristics were similar to those observed in normal, but unformalinized cells. Although our cell suspensions were somewhat lighter in color than those prepared by Czismas(16), they did not appear to be more suitable for the test. It should be noted that cells prepared in this manner were used several months prior to the report of Czismas. Moreover, these cells appear to be more susceptible to chemical conjugation than those prepared by other methods.

Conjugation. All of the chemical allergens used (*e.g.,* picryl chloride, 2:4-dinitrochlorobenzene, 2:4-dinitrofluorobenzene, and citraconic anhydride)†‡ were either recrystallized or redistilled 2 to 3 times prior to use in procedures for either conjugation or immunization. Allergens (12.5 mg) were dissolved in either 0.2 ml of acetone or 1 ml of absolute alcohol. This solution was added to 150 ml of chilled (0-4°C) buffered (pH 8.3) 0.85% saline containing 5 γ of heparin/ml. After adjusting pH to 9.3 with 20% sodium carbonate, 0.5 ml of formalinized erythrocytes was added. The reaction-mixture was subjected to constant agitation with a magnetic stirrer for 20 to 30 minutes and the temperature was maintained at 0-4°C, after which the pH was adjusted to 7.3 with 1 N HCl. Conjugated erythrocytes were washed 8 times with 10 volumes of 0.85% saline containing 5 γ of heparin/ml. Cell suspensions prepared in this manner were usable for several weeks without appreciable loss in effectiveness or any increase in incidence of non-specific agglutination. *Immune sera.* Albino guinea pigs of either sex, weighing approximately 450 g were immunized by the "combination method" of Chase(2) utilizing dinitrophenyl guinea pig erythrocyte stromata, or by successive daily topical application (7 times) of 5 drops of a 2% alcoholic solution of DNCB. In both instances, animals were bled out 7 days after final administration of the allergenic chemical. Certain sera were subjected to quantitative precipitin tests and subsequent analysis for antibody nitrogen by the Kjeldahl procedure(17),§ and the method of Eisen, *et al.*(18). For quantitative studies, dinitrophenyl-bovine gamma globulin (DNP-BGG) was employed as the antigen.

Hemagglutination. Immune sera were inactivated at 56°C for 15-30 minutes and absorbed with an equal volume of packed formalinized erythrocytes for 15-30 minutes. Despite the resistance of these cells to mechanical and chemical injury, varying degrees of hemolysis were observed after absorption with inactivated sera. Similar findings have been previously reported(13).

† Obtained from Eastman Kodak Co., Rochester, N. Y.

‡ PCl = picryl chloride; DNCB = 2:4 dinitrochlorobenzene; DNFB = 2:4 dinitrofluorobenzene; CA = citraconic anhydride.

§ Some of these sera were analyzed by Dr. Abram B. Stavitsky, Western Reserve Univ.

Small test tubes measuring 10 × 76 mm were used. Serial dilutions of the immune sera were then made in a diluent consisting of 0.85% saline containing 5 γ of heparin/ml in 0.5% normal rabbit serum, with 0.5 ml representing the final volume in all tubes. Following completion of the dilution of serum, 0.1 ml of a 0.5% suspension of the cells in the diluent was added to each tube. The tubes were thoroughly shaken and incubated at room temperature for 3 to 5 hours, followed by overnight storage at 0-4°C. Tubes were removed from the refrigerator and after 2 to 3 hours at room temperature, readings were made. Control tests consisted of allergen-erythrocyte conjugates *vs.* normal guinea pig sera and/or diluent. For inhibition studies, 64 γ of DNP-BGG N contained in 0.1 ml of saline was added to the diluted anti-dinitrophenyl sera and incubated at 37°C for 30 to 60 minutes prior to addition of dinitrophenyl erythrocytes. It should be mentioned that agglutination of erythrocytes did not occur in this instance. Other attempts to demonstrate the specificity of the test employed the use of erythrocytes conjugated to simple chemicals with related and non-related structures.

Passive cutaneous anaphylaxis. Albino guinea pigs, weighing approximately 250 g, of either sex, were used as recipients for intradermal sensitization. Animals were challenged, following a latent period of either 4 or 17 hours, by intravenous injection of 2 mg of DNP-BGG and 0.5 cc of Evans Blue. Readings were made 15-30 minutes later(19).

Results. Erythrocytes prepared by this method did not agglutinate spontaneously, were stable for a period up to 11 months and were quite susceptible to conjugation with various chemical allergens. Although properly prepared cells were found to be stable in a variety of protein-containing diluents, it was considered essential to add heparin(16) to 0.85% saline in order to obtain consistently negative results in control tubes.

Hemagglutination patterns were apparent at 4 to 6 hours, but were more definite at 24 to 36 hours. Although positive patterns were detected at 24 hours, shaking the tubes did not reveal clumps, but readily dispersible cellular aggregations. Definite clumps were not apparent until 36 to 48 hours had elapsed. Once developed, patterns were unchanged for several days. In lower dilutions, positive patterns were manifest by gelatinous coating in the center of the tube and "wrinkling" or folding of the edges. Completely gelatinous layers were apparent in higher dilutions and finally, granular sedimentation with rings was observed. Negative patterns were manifest by either solid buttons or "doughnuts" possessing regular margins.

TABLE I. Demonstration of Hemagglutinating Antibodies to a Chemical Allergen.

Sensitizing chemical allergen	Animal No.	Reciprocal of antibody titer: Dinitrophenyl-RBC's	Picryl-RBC's	Citraconyl-RBC's
DNCB	1-60	320	40	0
	1-57	320	40	0
	2-23	160	20	0
	2-25	160	0	0

Arbitrary use of 64 γ of DNP-BGG in 0.1 ml of saline resulted in complete inhibition in all instances of anticipated hemagglutination.

TABLE II. Minimal Amount of Antibody Detected by Hemagglutination and Passive Cutaneous Anaphylaxis.

Serum No.	μg antibody N/ml serum	Reciprocal of hemagglutination titer	μg of antibody N detected by: HA	PCA
3-01	197	5,000	.039	.013
3-00	112	16,000	.007	.011
Pool-III	94	6,000	.015	.018
Pool-I	91	3,000	.031	.018

Although cross agglutination was observed in instances where sera were tested against erythrocytes conjugated to chemically related allergens (*e.g.*, DNCB and PCl), titers were significantly lower than those obtained when conjugates of the specific allergen were used. It should be further noted that agglutination did not occur when use was made of erythrocytes conjugated to CA. A summary of typical results is presented in Table I.

Table II depicts results obtained from repeated tests on sera of known antibody nitrogen concentration, with PCA results being obtained after a latent period of both 4 and 17 hours. This table clearly shows that he-

magglutinating antibody N was detected at levels ranging from 0.015 γ to 0.007 γ, whereas PCA detected antibody N at levels ranging from 0.011 to 0.018 γ.

Discussion. In routine experiments, antibody to chemical allergens is usually determined by the method of PCA, and this method has been considered to be adequately sensitive. In addition to expense, it should be noted that there is some variation in susceptibility of individual animals to PCA. The method described above is relatively inexpensive, sensitive, and readily reproducible. Demonstration of the capacity of erythrocytes to be directly conjugated to chemical allergens suggests that this method may be employed for tracing antibodies to certain allergenic drugs used in treatment of human disease. The ability to use formalinized cells affords the opportunity of applying varied chemical procedures to effect conjugation without disturbing the physical integrity of the cells. Further, a constant and standard source of erythrocytes is available when these procedures are employed. Despite the apparent sensitivity of this *in vitro* method, it is obvious that extensive comparative studies with PCA should be carried out. Animals are currently being immunized to produce a sufficient quantity of hyperimmune sera for this investigation.

Summary. An *in vitro* method for demonstration of antibodies to chemical allergens, using formalinized erythrocytes is described. Results are reproducible and the test can be specifically inhibited. Except for chemicals of related structure, cross reactions were not observed.

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The Seminal Vesicle as the Source of the Spermatozoa-Coating Antigen of Seminal Plasma.* (27270)

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The seminal plasma of man and other mammals contains a strongly antigenic and highly organ and species specific compound (1,2; for additional references see 3). In man and rabbit, this antigen coats the spermatozoa during their passage through the adnexal structures of the male genital tract. This coat dominates the antigenic behavior of seminal spermatozoa in contradistinction

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