Further studies will be necessary to establish the relationship.

Summary. Employing a modified McNall procedure for determining serum properdin it was shown that in C57/BL mice growing Ehrlich ascites carcinoma there was a progressive drop of serum properdin after 3 days. Similar decreases of serum properdin values in C3H and A/He mice occurred when they were growing Gardner lymphosarcoma and L No. 2 lymphoma, respectively. On the contrary, when C57/BL mice were given either repeated injections of 2000 r X-irradiated Ehrlich carcinoma, or repeated injections of X-irradiated tumor and 3 to 5 challenges of viable tumor, there was a transient drop with a return to a normal blood level of properdin. However, numerous challenges(11-16) of mice that received repeated injections of X-irradiated tumor resulted in a significant lowering of the serum properdin, but not to levels produced with actively growing tumor. There appeared to be a correlation of serum properdin with cancer growth. During cancer growth there was a fall in serum properdin, while regression was accompanied by a rise back to control levels of serum properdin.

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Detection of Antibody to Urease by Hemagglutination.* (27102)

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Since the demonstration by Kirk and Sumner(1) that rabbit homologous antisera will precipitate jackbean urease, the antigenicity of enzymes has become well established(2). The serologic assay apparently depends upon the antigenicity of the protein moiety. It is independent of the prosthetic group of the enzyme(3) and therefore may be considered to be indirect. The *in vitro* antigen-antibody reaction is demonstrable as specific precipitation of the enzyme by its homologous antibody in the equivalence zone or in the region of slight antigen excess. In the case of urease-antiurease, it has been quantitated by Marucci and Mayer(4) to give precipitin units as antibody nitrogen. The precipitin test is not suitable, however, for quantitative assay of small amounts of antibody. Assay of antibody by the agglutination of inert particles coated with soluble antigen has proved to be a more sensitive method than the precipitin test. Of such particles the red blood cell has been the most widely used(5). Both protein and polysaccharide antigens are readily and firmly adsorbed by tannic acid treated fresh cells which are agglutinated, often in

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high dilution, in the presence of homologous antibody. Formalin treated and preserved cells have been used by a number of workers (6). In our hands the method of Ingraham (7) has proven the most satisfactory and has given stable suspensions of erythrocytes which are not subject to spontaneous agglutination. The assay of antibody to urease by this method, and its correlation with the quantitative precipitin test, is described here.

Materials and methods. Urease extracted from finely ground jackbean meal†, using the method of Kirk and Sumner, was recrystallized at least 3 times using Dounce's procedure(8). Twice distilled water from an all-glass apparatus was used in preparation of the enzyme and all other reagents. The crystalline enzyme was stored at minus 10°C in 0.85% NaCl. Urease activity in Sumner units‡ was determined using as substrate a urea phosphate buffer(9). Kjeldahl determinations of protein N were found to range from 1.20 to 1.25 μg of N per unit of urease activity. Antibody was determined as antiurease units,§ using the method of Sumner and Kirk(1) with attention to the principles put forth by Marucci and Mayer(4). Known amounts of urease were added to antisera for estimation of the zone of equivalence. Residual enzyme activity was determined upon the supernatant following centrifugation under refrigeration at 2200 × gravity. Replicate checks with a slight excess of antigen were made on the tubes approximating the equivalence zone.

Human type O erythrocytes were used for mammalian species. Antibody studies on chickens utilized pooled chicken erythrocytes.

Antiurease sera for development of the technic were obtained from rabbits immunized to jackbean urease. Immunity to the enzyme was developed by subcutaneous injections twice weekly for 2 months. Dosages ranged from 0.5 unit/kg body weight to a maximum in the final week of 8 units/kg of body weight. Antisera from rats, mice and chicks were produced by subcutaneous injections of the crystalline enzyme on 3 successive days each week for a period of 4 weeks as elsewhere described(10).

Neutral formalin in 0.85% NaCl was prepared according to Ingraham(7).

Citrate buffer pH 3.9-4.1 was made by combining solutions of 0.5 M sodium citrate with 0.5 M citric acid in a ratio of 2.0 : 1.7.

Bovine serum albumin (Fraction V, Armour Pharmaceuticals‖ was diluted to required concentrations using 0.85% NaCl. The reactions were carried out in agglutination trays measuring 5-11/16 × 7-1/16 inches and containing 80 cavities of three-fourths inch in diameter¶

Preparation of erythrocytes. Citrated erythrocytes are washed with 30 volumes of 0.85% NaCl at room temperature 4 to 6 times. Removal of the wash solution is accomplished by centrifugation at 3500 times gravity. After washing, the erythrocytes are resuspended in an equal volume of neutral formalin in 0.85% NaCl according to the method of Ingraham(7). Storage is at 2-5°C and the erythrocytes are resuspended daily by gentle manual shaking for at least 10 days. Erythrocytes may be stored in this formalin medium for at least a year at 2-5°C without loss of usefulness.

Prior to sensitization with urease, the formalin is removed from the erythrocytes by 4 to 6 washings with 20 to 30 times their volume of 0.85% NaCl. Although greater precautions for removal of formalin were followed by McKenna(6), 4 to 6 washings have been found satisfactory in our work and corroborate the experience reported by Csizmas (11). The washed formalinized erythrocytes are resuspended in 10 ml of 0.4% NaCl and the red cell concentration is adjusted to 10% by volume. Two-tenths ml of citrate buffer (pH 3.9-4.1) are added to each ml of cell suspension. Crystalline urease (50-60 units) in a volume not to exceed 2 ml or 0.4 to 0.5

† Sigma Chemical Co., St. Louis, Mo.

‡ One unit of urease activity is that amount of urease required to liberate one mg of ammonia N in 5 min. in a phosphate buffer at pH 7.0 at 20°C.

§ One unit of antiurease activity is that quantity of antibody which will inhibit one unit of urease activity.

‖ Armour Pharmaceuticals, Kankakee, Ill.

¶ Prestware Ltd., Southdown Work, Kingston Road, Raynes Park, London SW 20, England.

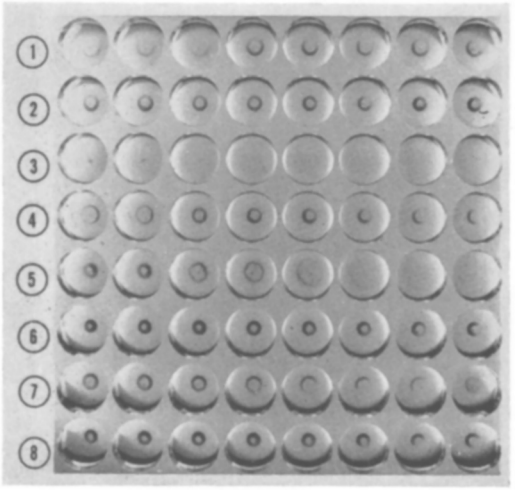

FIG. 1. Hemagglutination reactions with pooled sera from mice immunized to jackbean urease in dilutions of 1:10 through 1:5000 proceeding from left to right. Rows are numbered from top to bottom. Formalin treated human type "O" erythrocytes were used.

Row 1. Normal serum with negative titer.
Row 2. Normal serum as used in Row 1, using identical dilutions with unsensitized erythrocytes.
Row 3. Immune serum showing agglutination through a dilution of 1:5000.
Row 4. Serum used in Row 3 with unsensitized erythrocytes.
Row 5. Highly positive antiserum demonstrating prozone phenomenon and agglutination throughout all dilutions.
Row 6. Serum used in Row 5 with unsensitized erythrocytes.
Row 7. Urease sensitized erythrocytes with phosphate buffer and 1% beef serum albumen.
Row 8. Unsensitized erythrocytes with phosphate buffer and 1% beef serum albumen.

mg of protein is added to 10 ml of the buffered cell suspension. The resultant mixture is placed in a small polyethylene bottle (30 ml) and rotated in a horizontal position at 60 rpm for 5 days at room temperature. Following 4 washings with 0.85% NaCl the cell concentration is adjusted 2.5% with 0.85% NaCl containing 0.005 M dibasic sodium phosphate and 1% bovine serum albumen (Fraction V Armour Pharmaceuticals). This reagent will retain satisfactory activity for about 5 days at 2-5°C.

Technic of antiurease assay. The hemagglutination reactions are carried out in agglutination trays. Serial dilutions of sera are made using 0.85% NaCl containing 0.005 M dibasic sodium phosphate. Each cup receives 0.5 ml of diluted sera and one drop of 10% bovine serum albumen (BSA) to give an approximate concentration of 1% BSA. These reactants are thoroughly mixed by placing the tray on a flat surface and moving it in a circular motion. A drop of sensitized erythrocytes is added and the contents of the reaction cups are again mixed. The trays are incubated at 2-5°C for 12-18 hours. Positive reactions cause the erythrocytes to form a "veil" whereas negative reactions are indicated by a "button" of erythrocytes which is formed in the base of the reaction cup. (Fig. 1).

Results. Table I presents results of assays upon sera from rabbits, rats or mice immunized with jackbean urease. Assays were made upon the same sera using erythrocytes sensitized on 3 different occasions. Precipitin titers determined in triplicate upon the same sera by the method of Kirk and Sumner (1) are also shown. Fig. 2 shows the hemagglutination titers plotted as a function of precipitin titers in 3 mammalian species.

Sensitization of the formalinized erythrocytes was found to be optimal at pH 3.9 to 4.1 in studies covering a pH range from 3.0 to 8.7. At a pH value below 3.9 false positive results were obtained, whereas false negative results were common at pH values greater than 4.1. The optimal concentration of antigen used to sensitize the formalin

TABLE I. Hemagglutination Titers Obtained in Duplicate on Antisera of 3 Species Immunized to Jackbean Urease Utilizing 3 Different Batches of Human Group "O" Erythrocytes Sensitized with the Antigen. Precipitin titers were determined in triplicate upon the same sera.

Hemagglutination titer	Precipitin titer, antiurease units/ml	No. of assays*		
		Rabbits	Rats	Mice
1: 10	0	8	3	4
20	.7 ± 1.1†	2		
50	5.1 ± 1.2	2		1
100	10.6 ± 2.0	1		1
200	no sample			
500	14.2 ± 3.6	2		1
1000	17.9 ± 1.5	3		
2000	22.0 ± 2.0	3		1
5000	25.9 ± 2.8	3	1	
10000	29.1 ± 2.1	1		1
20000	37.7 ± 1.8	2		

* Sera from rats and mice were pooled samples.
† Stand. dev.

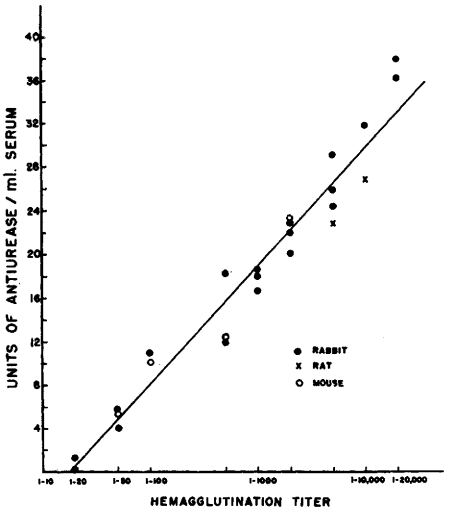

FIG. 2. Each point represents hemagglutination titer plotted as a function of precipitin titer of an antiserum. Duplicate hemagglutination titers determined with 3 different lots of urease sensitized erythrocytes. Precipitin titers (antiurease units/ml serum) were determined in triplicate. Species represented are rabbits, rats and mice.

treated cells was found to be 0.05 mg of urease (6-8 units) per ml of cell suspension. Lower concentrations gave inconsistent titers on positive sera and higher concentrations gave positive results on sera known to be negative for antiurease. Human type O erythrocytes cross-reacted nonspecifically with sera from immunized and non-immunized chickens. Chicken erythrocytes, however, gave reliable results in this avian species.

Studies upon the most desirable pH for dilution of the sera were conducted using phosphate buffered 0.85% NaCl ranging from pH 7.1 through 8.7. Throughout this range satisfactory results were obtained although dilution of all of the sera was carried out routinely with phosphate buffered 0.85% NaCl at pH 7.25-7.45.

All of the hemagglutination experiments employed the following controls through a complete range of dilutions: (1) unsensitized formalin treated erythrocytes plus known positive serum, (2) unsensitized formalin treated cells plus phosphate buffered 0.85% NaCl diluting fluid, (3) sensitized formalin treated erythrocytes plus phosphate buffered 0.85% NaCl, (4) sensitized formalinized cells plus known positive serum. Using this system of controls, unsensitized and sensitized formalinized cells plus phosphate buffered saline always gave negative reactions. Unsensitized formalinized cells plus known positive or negative sera usually showed agglutination titers of 1:10 and occasionally titers of 1:20. Therefore, titers of 1:20 or lower with sensitized formalinized erythrocytes were considered negative for antiurease.

Discussion. Our studies show that treatment of erythrocytes with suitable concentrations of formalin render them capable of adsorbing crystalline urease at pH 4.0 in citrate buffered saline. Red cells with urease adsorbed by this technic are agglutinated by homologous antisera in high dilution.

A semi-log plot of the hemagglutination titers *vs* precipitin titers gave a straight line relationship over a range of 1:50 to 1:20,000 using sera from 3 species of animals immunized with the same antigen but at different dosing schedules (Fig. 2). Deviations can be accounted for by errors in the 2 tests, but no systematic deviation or scattering is evident. The positive antisera used in these studies, if passively administered, protected normal animals against lethal doses of urease. These findings offer further evidence that the antisera agglutinating the sensitized red cells contained antiurease.

The method has been applied to urine frozen at minus 70°C immediately upon collection from 2-3-month-old rats immunized with jackbean urease by various schedules of injection. The technic has given highly reproducible results on 70 such 24-hour collections of urine. Though urinary excretion of antiurease has been intermittent within the same animal, titers ranging from 1:50 to 1:1000 have been found in over 50% of the samples.

In studies with chicks immunized with jackbean urease using sensitized chicken erythrocytes, antiurease has been determined in sera and fecal extracts. In over 120 fecal samples, titers have been obtained with high precision and the technic is capable of detecting antibodies in quantities of chicken feces equivalent to one mg of fecal dry matter.

Summary. A new method for determination of the antibody to the enzyme urease is

described which can be correlated with the precipitin method for antiurease.

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Accelerated Rejection of Male Skin Isografts by Female C57BL Mice Infected with *Bacillus Calmette-Guérin* (B.C.G.).* (27103)

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Several methods to alter the homograft reaction in a non-specific‡ fashion have been described. Depression of immunologic reactivity as measured by increased survival of grafted tissue has been accomplished with x-rays, cortisone and certain drugs, the degree of success largely depending on experimental conditions(1). On the other hand, little attention has been paid to the possibility of non-specifically accelerating the homograft mechanism. Increased resistance to transplantable homologous and isologous tumors in mice has been demonstrated following zymosan and B.C.G. infection(2-5). The effect was attributed to a host mediated immune response, rather than a direct action on the tumors. This assumption was made in view of the fact that both agents increase immunological reactivity as measured by other means: prior administration of B.C.G. or zymosan enhances the phagocytic activity of the reticuloendothelial system (RES), increases the host's capacity to form antibody, and heightens resistance to infectious challenge(6-12).

To substantiate the assumption that increased host resistance to tumor growth following B.C.G. and zymosan is mediated by a heightened capacity to reject antigenic grafted tissue, it was felt important to demonstrate comparable effects using transplantation of normal tissue.

The present report deals with the effect of B.C.G. on rejection time of male skin isografts by C57BL female recipients.

Materials and methods. Mice. Adult male and female C57BL/6 mice were obtained from Bio-Research Consultants, Inc., Cambridge, Mass. and from a colony of C57BL mice originally obtained from Dr. Daniel S. Martin, Univ. of Miami, and maintained in this laboratory on a strictly inbred basis for the last 2 years. Female mice were grafted exclusively with skin from male mice of the same colony, although reciprocal grafts proved the mice from both sources to be isologous.

B.C.G. The Phipps strain of *Mycobacterium tuberculosis* var. *Bacillus Calmette-Guérin* (B.C.G.) was grown in Dubos fluid medium. Pooled 10- to 14-day cultures were harvested by centrifugation, weighed and di-

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‡ "Non-specific" refers to methods that alter rejection time of transplanted antigenic tissue without prior introduction of specific cellular or non-cellular antigens, sensitized cells or specific antibody.