either whole kidney emulsion or kidney tissue culture cells. The cytotoxic effect in tissue culture of nephrotoxic sera appears to be species specific, though there is some cross reaction between closely related species of animals. This is significant in view of the species specificity of nephrotoxic serum nephritis (9).

Latta and Kutsakis(10) recently reported that a heat-labile, complement-like factor present in guinea pig anti-chick-embryo-heart serum was necessary for the immediate cytotoxic effect on chick-embryo-heart fibroblast grown in tissue culture. We are currently studying the effect of complement-like factors in this cytotoxic system.

Summary. Rabbit antisera to both rat kidney saline homogenate and the tryptic digest of rat kidney adsorbed on streptococci caused cytotoxic effects upon rat kidney cells in tissue culture as well as renal pathology *in vivo* following intravenous injection of rats. The titers of the cytotoxic effect in tissue culture paralleled the nephrotoxic effect *in vivo* and the anti-kidney hemagglutinin titers *in vitro*. The kidney cells grown in tissue culture tubes were effective in absorbing the potent nephrotoxic factor from serum. The cytotoxic effect of the nephrotoxic serum appears to be markedly species specific.

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1. Lippman, R. W., Cameron, G., and Campbell, D. H., *Proc. Nat. Acad. Sci.*, 1950, v36, 576.
2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.
3. Ehrich, W. E., Forman, C. W., and Seifter, J., *A.M.A. Arch. Path.*, 1952, v54, 463.
4. Cole, L. R., Cromartie, W. J., and Watson, D. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 498.
5. Glynn, L. E., and Holborow, E. J., *Lancet*, 1952, v2, 449.
6. Boyden, S. V., *J. Exp. Med.*, 1951, v93, 107.
7. Rothenberg, M. B., Stavitsky, A. B., and Heymann, W., *Pediatrics*, 1956, v18, 455.
8. Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1948, v88, 521.
9. Baxter, J. H., and Goodman, H. C., *ibid.*, 1956, v104, 467.
10. Latta, H., and Kutsakis, A., *Lab. Invest.*, 1957, v6, 12.

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A Plate Technic for the Conglutinative Complement Fixation Test. (23211)

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The term conglutination (*L. conglutinatio*, act of glueing together) was applied by Bordet and Streng(1) to describe clumping of red blood cells sensitized with antibody in the presence of complement (C′) and of a substance called conglutinin, existing in serum of normal bovines. Realizing that C′ was necessary for conglutination, Streng(2) developed a new serological test applied to the diagnosis of numerous diseases such as syphilis, glanders, dourine, brucellosis, etc. Interest in this test was revived recently largely as a result of the investigations of Hole and Coombs(3-6) and of Rice *et al.*(7,8). Hole and Coombs(3) suggested the expression "Conglutinating Complement Absorption" for Streng's serodiagnostic test to indicate the analogy to and difference from the hemolytic complement fixation (C′F) test. However, use of the word absorption instead of fixation does not seem entirely justifiable, since the same basic phenomenon, *i.e.* disappearance of C′, is involved in both cases. We shall refer,

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therefore, to "Conglutinative Complement Fixation" (CC'F). Recent applications of the conglutination C'F test have been made in serodiagnosis of rickettsial and viral infections(9-12), such as foot-and-mouth disease (Solovieff.9), Q fever (Wolfe and Kornfeld.10), psittacosis-lymphogranuloma, vaccinia and influenza (Stoker, Coombs and Bedson.12); (Hilleman, Haig & Helmold, 11). Experiments of this kind are frequently handicapped by the small amounts of antigen or antiserum available and this led Fulton and Dumbell(13) to devise a drop method for performing the hemolytic C'F test. This technic has been successfully applied to C' fixation studies with influenza(13), Coxsackie(14.15) and poliomyelitis viruses (16).

In the present paper a drop method is also described for the conglutinative C'F test. The conglutinative indicator permits clearer readings than those afforded by the hemolytic test. Furthermore, preparation of glass dropping pipettes, as well as elaborate racks and air-tight boxes required for Fulton and Dumbell's technic are not necessary for the conglutinative C'F microtechnic herein described.

Materials and methods. Six samples of rabbit anti-bovine serum albumin, 21, 22, 24, 25, M and O, containing respectively 0.707, 0.510, 1.080, 0.169, 0.151 and 0.350 mg antibody N ml were used. The sera were diluted so as to contain 40 μg AbN ml and preserved frozen at –20°C. Hyperimmunized guinea pig sera were utilized in experiments with foot-and-mouth disease virus, types A and C. The rabbit anti-C-reactive protein (anti-CRP) was a commercial preparation from Schieffelin & Co., New York City. Crystallized bovine serum albumin obtained from Pentex Inc., Kankakee, Ill., was used without further purification. The foot-and-mouth disease antigens were prepared from guinea pig membranes.‡ Beef serum obtained from recent bleeding, and inactivated for 30 minutes at 56°C was used as source of *conglutinin*. The *conglutinative complement* used was fresh, unheated horse serum. Both *complement* and conglutinin were stored frozen in 1 ml amounts at –20°C. The *diluent* used for all reagents was isotonic veronal bicarbonate buffer, pH 7.3-7.4, containing 0.144 *M* NaCl, 0.00015 *M* $CaCl_2$ and 0.0005 *M* $MgCl_2$(17). The *indicator system* was prepared by mixing 2 volumes of sheep red cell suspension containing approximately 1.5 x 10^8 cells ml with 1 volume of appropriate dilution of rabbit amboceptor and 1 volume of selected dilution of conglutinin. The red cell suspension was first standardized so that an optical density of 0.56 was obtained when 1 ml of the suspension was lysed with 14 ml of distilled water. The O.D. = 0.56 refers to the reading in a Coleman Junior spectrophotometer, model 6, at a wave length of 550 mμ and with matching square cuvettes, 13 x 13 x 100 mm. From this standardized suspension a 1:6.6 dilution was prepared to yield approximately 1.5 x 10^8 erythrocytes/ml. The amboceptor dilution corresponded to the optimal sensitizing dose as usually determined in hemolytic tests with a 10^9/ml red cell suspension. The test-dose of conglutinin was ascertained in bloc titration experiments as indicated in the experimental part. Most tests were performed simultaneously by the usual tube method and by the drop technic. In the latter instance, the container was a plastic polysterene molded plate of the type commonly utilized for cellular cultures and virus neutralization studies.§ During the test, the molded chambers of the plastic panel were partially immersed in ice water, a precaution which was essential to avoid C' deterioration. After distribution of the reagents and careful mixing of the contents, the panel was covered with a glass plate whose edges were sealed with a strip of Scotch tape. The molded panel was then placed in a metal box whose bottom was covered with a layer of moist absorbent cotton. The unit volume of antiserum, antigen, C', conglutinin and sensitized cells was 0.2 ml for tests in tubes and 0.05 ml for the plate test, giving final

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§ May be purchased from Dow Chemical, Midland, Mich., or from Fabri-Kal, Kalamazoo, Mich.

TABLE I. Selection of Test-Dose of Conglutinin.

Complement	Tube method: Conglutinin 1:20	1:40	1:80	0	Plate method: Conglutinin 1:20	1:40	1:80	0
1: 20	L3	L3	4	0	4	4	4	0
1: 40	4	4	4	0	4	4	4	0
1: 80	4	4	4	0	4	3	2	0
1:160	4	4	2	0	2	±	0	0
1:320	±	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0

L = lysis; 0 = negative; ± to 4 = increasing degrees of conglutination.

volumes of 1.0 and 0.25 ml respectively. After the fixation period, the indicator system (sensitized cells plus conglutinin) was added and the conglutination reaction was allowed to proceed in the incubator at 37°C for 30 minutes, with occasional shaking to insure resuspension of red cells. Antigen and antisera controls, as well as a confirmatory C′ titration were always included in the tests and subjected to the same incubation period as the fixation mixtures. When the conglutinative C′F test was performed in tubes, results were read after centrifugation at 1000 rpm for 1 minute. Tubes were held between thumb and the index and middle fingers of the left hand and repeatedly tapped at the bottom with the index and middle fingers of the right hand to disperse gently the sedimented or conglutinated cells. Unconglutinated erythrocytes formed a homogeneous suspension, while conglutinated cells remained either as a solid disc (4 reaction) or as clumps of varying size (± to 3 reactions). Readings on the plates were also recorded as ± to 4, the last indicating complete conglutination. A gentle circular movement of the plate favors clumping of erythrocytes into the center of the molded chamber and so affords a more clear cut reading. In doubtful cases, readings were repeated 3-5 minutes after complete resuspension of the cells.

Results. a) *Selection of test-dose of conglutinin.* It is well known that amboceptor and C′, as well as C′ and conglutinin are mutually supplementary in conglutination(8). If we desire to titrate C′ or conglutinin, it is then necessary to use maximally sensitized cells in order to remain with only 2 variables (conglutinin and C′), so that one may be titrated with a convenient, arbitrarily chosen dose of the other. Table I summarizes a bloc titration showing this effect. With the exception of the fixation tests carried out with C-reactive protein, all the tests were performed simultaneously with tube and plate methods to check reliability of the latter.

The results obtained with the plate technic were only slightly weaker than those of the tube method in the conglutinin range of 1:20 to 1:80 (Table I). By setting the test-dose of conglutinin at dilution of 1:20, the single batch of horse C′ used here always titrated 1:160 by either the tube or plate technics. In all fixation experiments, conglutinin and horse C′ were used at dilution of 1:20.

b) *Determination of optimal amount of antigen.* Table II gives the results of a bloc titration designed to determine optimal amount of BSA required to measure the C′-fixing potency of a rabbit anti-BSA serum

TABLE II. Determination of Optimal Amount of Antigen.

BSA, 10 μg N/ml	Tube method: Rabbit serum anti-BSA 22, 40 μg AbN/ml 1:5	1:10	1:20	1:40	1:80	0	Plate method: 1:5	1:10	1:20	1:40	1:80	0
1: 5	0	3	4	4	4	4	0	2	3	4	4	4
1: 10	0	0	3	4	4	4	0	0	1	3	4	4
1: 20	0	0	2	4	4	4	0	0	0	3	4	4
1: 40	0	0	0	4	4	4	0	0	0	0	4	4
1: 80	0	0	0	3	4	4	0	0	0	1	4	4
1:160	0	0	3	4	4	4	0	0	0	2	4	4
1:320	2	3	4	4	4	4	0	2	2	2	4	4
0	4	4	4	4	4	4	4	4	4	4	4	4
	Complement titration											
	1:10	1:20	1:40	1:80	1:160		1:10	1:20	1:40	1:80	1:160	
	L1	4	4	4	4		L1	4	4	3	3	

TABLE III. Effect of Fixation Time and Temperature on C′-Fixing Potency of Rabbit Antisera.

Time and temp. of fixation	Tube method Rabbit anti-BSA No. 25	M	0	Plate method Rabbit anti-BSA No. 25	M	0
I. ½ hr, room temp.	10*	10	10	10	10	16
	C′ titer: 160†			C′ titer: 40-80		
II. 1 hr, 37°C	63	25	40	40	25	63
	C′ titer: 80			C′ titer: 20		
III. 20 hr, 0°C	40	40	63	63	40	63
	C′ titer: 80			C′ titer: 40		

* Reciprocal of antiserum dilution required for complete inhibition of conglutination in presence of optimal amount of antigen.

† Reciprocal of C′ dilution giving complete conglutination (4) without serum or antigen, but after same incubation as for fixation mixtures.

(No. 22). Here again, the difference in results obtained with plate and tube technics is negligible. The optimal amount of antigen was either a 1:40 or 1:80 dilution of the stock solution. Deterioration of C′ was slightly greater in the plates than in tubes and this probably accounts for the higher titer given for anti-BSA in the plate (1:40), as compared with titer of 1:20 observed in the tube series.

c) *Effect of fixation time and temperature on CC′F with rabbit antiserum.* It is customary to use a fixation period of ½ hour at room temperature for the CC′F test. In fact, Blomfield, Coombs and Hole(18) have shown that the C′-fixing potencies of anti-mallein sera from different species (horse, cattle, pig, goat, sheep, dog, cat, guinea pig, rabbit, ferret, hamster, rat and human), when assayed by CC′F with horse C′, were only slightly enhanced if incubated overnight at 4°C instead of for ½ hour at room temperature. However, our experiments with rabbit anti-BSA and BSA have shown the former to be preferable (Table III).

As seen in Table III, CC′F titers were considerably higher after 20 hours at 0°C or 1 hour at 37°C than after ½ hour at room temperature. C′ deterioration was about the same when tests were incubated in tubes for 1 hour at 37°C or for 20 hours at 0°C (C′ titer: 80). However, if C′ titrations were carried out in the plate, more deterioration occurred, particularly after 1 hour at 37°C (C′ titer: 20), probably due to deficient protection against evaporation under conditions of the plate test.

d) *Relative titers in hemolytic and conglutinative C′F tests.* Table IV summarizes data which permit a comparison of the fixing potencies of 6 rabbit anti-BSA sera, as measured by the CC′F test (tube and plate technics) with horse C′ and by a refined method of hemolytic C′F using 2 50% units of guinea pig C′. Details concerning the last method will be presented elsewhere. The fixation period for all tests was 20 hours at 0°C.

e) *Use of CC′F in a virus system.* The CC′F plate and tube technics were also used in parallel to demonstrate specific antibodies in sera of guinea pigs hyperimmunized with A and C types of foot-and-mouth disease virus. Fairly good agreement was observed in results obtained with 2 different immune sera and their corresponding antigens. With type A antiserum and its homologous antigen diluted 1/5 and 1/10, antiserum end points (complete fixation) of 1/160 and 1/320 were obtained by the tube and the plate method respectively. Tested under the same conditions, type C antiserum showed the same titer (1/160) by both methods.

f) *Estimation of C-reactive protein in human sera.* The CC′F microtechnic was also used for estimating C-reactive protein in human sera. The results of this study will be reported separately.

Discussion. The results reported here show that the CC′F test may be easily performed on plastic molded plates in a final

TABLE IV. Comparison of Titers* Given by Conglutinative and Hemolytic C′-Fixation.

Rabbit anti-BSA sera diluted to 40 μg AbN/ml	Conglutinative C′F Tube	Plate	Hemolytic C′F
21	40	63	77
22	63	63	110
24	63	100	137
25	40	63	93
M	40	40	93
0	63	63	200

* CC′F titers are expressed as reciprocals of serum dilutions giving complete inhibition of conglutination; HCF titers, as reciprocals of dilutions, required 50% lysis.

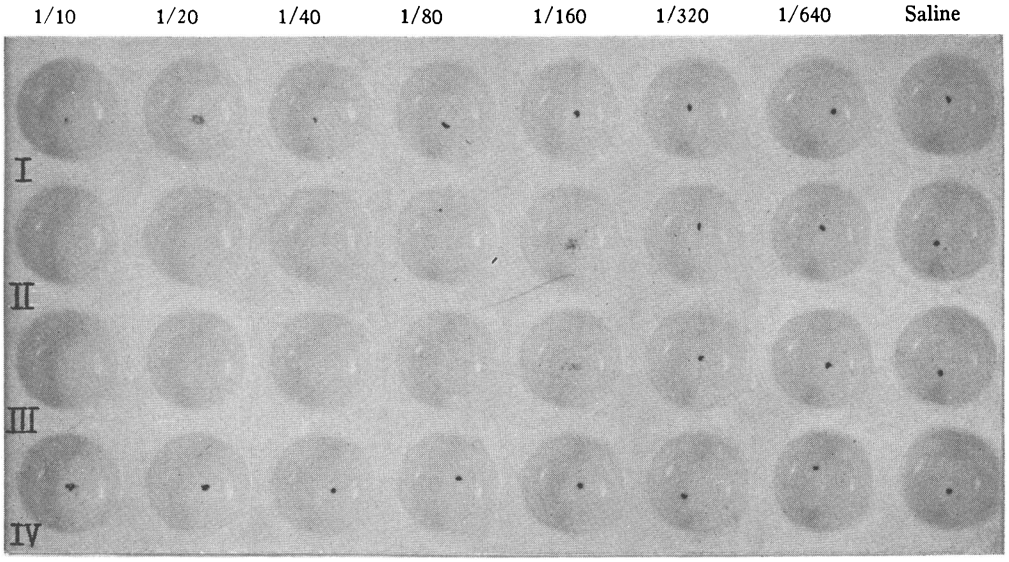

FIG. 1. I—Negative serum. II and III—Complete fixation up to 1/80, partial fixation at 1/160. IV—Saline control

volume of 0.25 ml without any impairment of sensitiveness or specificity. This microtechnic is especially valuable because of the small volumes of reagents used and because of the ease of execution and reading (Fig. 1).

The amounts of antibody N detected by the CC'F microtechnic were in the range of 0.4-1.0 μg/ml for rabbit anti-BSA serum and as little as 0.1 ml of serum suffices for the test, including the serum control. The quantitative information provided by the microtest may be considered as sufficiently accurate for virus typing studies and for estimation of pathological proteins of clinical interest, particularly in cases where only small amounts of material are available.

Summary. 1) A plate technic is described for study of complement fixation with a conglutinative indicator system. The reliability of the new technic was checked by parallel use of the tube technic in experiments with 6 different rabbit anti-bovine serum albumin sera. 2) The conglutinative complement fixation microtechnic has also proved satisfactory for detection of antibodies against foot-and-mouth disease viruses in guinea pig hyperimmune sera, as well as for estimation of C-reactive protein in human sera.

1. Bordet, J., and Streng, O., *Zbl. f. Bakt.,* I Orig., 1909, v49, 260.

2. Streng, O., Konglutinine, in *Kolle, Kraus u. Uhlenhuth, Hdbuch d. path. Mikroorg.,* 3rd edition, 1929, v2, 1117.

3. Hole, N. H., and Coombs, R. R. A., *J. Hyg.,* 1947, v45, 480.

4. ———, *ibid.,* 1947, v45, 490.

5. ———, *ibid.,* 1947, v45, 497.

6. Coombs, R. R. A., and Hole, N. H., *ibid.,* 1948, v46, 296.

7. Rice, C. E., and Avery, R. J., *Am. J. Vet. Res.,* 1950, v11, 98.

8. Rice, C. E., *Canadian J. Comp. Med.,* 1950, 65.

9. Solovieff, P., *Rev. Zootech.,* 1943, v30, 19.

10. Wolfe, D. M., and Kornfeld, L., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 251.

11. Hilleman, M. R., Haig, D. A., and Helmold, R. J., *J. Immunol.,* 1951, v66, 115.

12. Stoker, M. G. P., Coombs, R. R. A., and Bedson, S. P., *Brit. J. Exp. Path.,* 1950, v31, 217.

13. Fulton, F., and Dumbell, K., *J. Gen. Microbiol.,* 1949, v3, 97.

14. Kraft, L. M., and Melnick, J. L., *J. Exp. Med.,* 1950, v92, 483.

15. Lépine, P., Sautter, V., Delphy, J., and Artzet, F., *Ann. Inst. Pasteur,* 1953, v84, 684.

16. Svedmyr, A., Enders, J. F., and Holloway, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 296.

17. Osler, A. G., Strauss, J. H., and Mayer, M. M., *Am. J. Syph. Gon. Ven. Dis.,* 1952, v36, 140.

18. Blomfield, A. M., Coombs, R. R. A., and Hole, N. H., *J. Hyg.,* 1949, v47, 132.

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