Complement Fixation: A Modified Procedure Designed to Eliminate Anticomplementary and Procomplementary Effects. (30537)

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(Introduced by J. L. Fahey)
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In standard, 2-stage complement fixation (C'F) tests(1, p209), antigen and antibody interact in the presence of complement (C') and therefore anticomplementary or procomplementary effects of antigen or antiserum may prevent valid interpretation. In addition, after absorption with antigen, antisera frequently become so anticomplementary that residual antibody content cannot be measured by C'F. These effects limit the amounts of antigen and antiserum which can be used in C'F tests(1, p211). The modified, 3-stage C'F test presented here does not suffer from these difficulties, for antigen and antiserum are mixed in the absence of added C', the aggregates are collected by centrifugation, washed, and then resuspended in a standard dose of C'. Examples of the usefulness of this method will be presented.

Materials and methods. Preparation of the following reagents has been described in(1, p149): Veronal buffered saline diluent (alternative procedure), sheep erythrocytes (E), hemolytic antibody (A), sheep erythrocytes sensitized with hemolytic antibody (EA), and guinea pig complement (C').

The modified C'F test is performed in 3 stages. *First stage:* Before use, antisera are clarified(1, p67) by centrifugation for about 20 minutes at a minimum of 800 *g* (RCF at tip). Equal volumes of diluted antigen and clarified antiserum are mixed in 13 × 100 mm test tubes. Generally, 0.4 ml of each is convenient. However, with relatively weak antisera these amounts may be increased in order to obtain measurable complement fixation. Controls consist of antigen mixed with diluent and of antiserum mixed with diluent. As in the standard test, titrations can be carried out with varying concentrations of antigen and a fixed concentration of antiserum or varying concentrations of antiserum and a fixed concentration of antigen. The concentration selected for each reagent is based on the results of a checkerboard (2-dimensional) titration in which the concentrations of both antigen and antiserum are varied.

Reaction mixture (antigen plus antiserum) and control tubes are capped and are shaken overnight at 4°C in a motor driven reciprocating shaker. The displacement and frequency of shaking are adjusted to maintain particulate matter in suspension.

The following day all tubes are centrifuged in the cold at a minimum of 800 *g* (RCF at tip) for 20 minutes, and supernatant fluids poured off. If particulate matter is present which might be poured off with the supernatant fluid, the fluid should be removed by aspiration.

Diluent (usually 3 ml) is added to all tubes as well as to each of 3 additional test tubes which are to be used for C' titration (see *Stage 2*). After mixing their contents, all tubes are centrifuged and the supernatant fluids are removed.

Second stage: The 3 tubes to be used for C' titration are set aside, and three 50% units* of C' (3 C'H50) are added to all other tubes. In calculating the concentration and volume of C' to be added at this stage, it is necessary to account for the volume of liquid remaining in the tubes after the supernatant fluids have been removed. This residual volume of fluid averages 0.1 ml, and the desired test volume is 0.65 ml. Thus, to each tube 0.55 ml of C' is added containing a concentration sufficient to yield 3 C'H50 when mixed with 0.1 ml of diluent. One of the C' titration tubes receives 0.55 ml of C' (3 C'H50), another receives 0.37 ml of

* Three C'H50 are recommended because this is a convenient amount of C' which causes more than 99% hemolysis. Use of larger amounts of C' decreases sensitivity.

TABLE I. Results of Checkerboard Complement Fixation Test with Homogenate of Guinea Pig Hepatoma and Rabbit Antiserum to Guinea Pig Hepatoma Using the 2-Stage Technic.

Antigen dilution	Antiserum dilution						Control with:	
	1/10	1/30	1/90	1/270	1/810	1/2430	5 C'H50	3 C'H50
1/10	4	1½	½	0	0	1	4	4
1/30	4	2	½	0	0	½	4	4
1/90	4	2½	1	½	½	1	4	4
1/270	4	3	3	3	3½	4	4	4
1/810	4	3	3	3½	4	4	4	4
Control with:								
5 C'H50	4	3	3	4	4	4		
3 C'H50	4	2	1	3	4	4		

Antiserum titer estimated to be about 1/5000. Antiserum not heat inactivated, and both procomplementary and anticomplementary effects are evident. 0 = no lysis; 4 = complete lysis. Experimental procedure was the same as that in(1, p. 224).

diluted C′ and 0.18 ml of diluent (2 C′H50), and the last receives 0.18 ml of diluted C′ and 0.37 ml of diluent (1 C′H50). All tubes are capped and shaken overnight at 4°C.

Third stage: The following day 0.1 ml of optimally sensitized EA are added to each tube and the mixtures are incubated for one hour at 37°C with occasional mixing. Degree of lysis is estimated by visual inspection as described in reference(1, p212).

Results. The experiments illustrated here were carried out in a total volume (third stage) of 1.5 ml with 5 C′H50 so that the standard and modified tests could be compared directly. The procedure outlined in *Materials and methods* was based on the results of these comparative studies, which showed that in the standard test the antiserum titer was about 8 times greater than in the modified procedure. Antiserum titers comparable to those obtained in the standard method using 5 C′H50 can be obtained in the 3-stage technic by following the procedure outlined in *Materials and methods, i.e.,* by using 3 C′H50 instead of 5 C′H50 in a third stage test volume of 0.75 ml instead of 1.5 ml. Further increases in titer can be achieved by increasing the amounts of reactants used in the first stage. Increases in antiserum titer effected in this manner are not associated with proportional increases in anticomplementary activity.

The results of checkerboard C′F tests with a homogenate of guinea pig hepatoma and rabbit antiserum to hepatoma† are shown using the standard test (Table I) and the 3-stage procedure (5 C′H50 and a third stage volume of 1.5 ml) (Table II).

To test whether the complement fixation seen in the 3-stage procedure is due to specific antigen-antibody interaction, checkerboard 3-stage C′F tests (5 C′H50 and a third stage volume of 1.5 ml) were carried out using the following reactants:

1) Normal rabbit serum and homogenate of guinea pig hepatoma with dilutions of both beginning at 1/10. There was no detectable complement fixation.

2) Rabbit antiserum to guinea pig hepatoma and homogenate of normal rabbit liver with dilutions of both beginning at 1/10. Neither the rabbit liver homogenate alone nor the antiserum alone contained sedimentable anticomplementary material. The resulting antiserum titer was 1/130. This reactivity could have been due either to antigen-antibody interaction or to uptake of anticomplementary material by particulate matter. To distinguish between the two possibilities a checkerboard 3-stage C′F test was carried out using homogenate of normal rabbit liver and a rabbit antiserum to human serum which was highly anticomplementary (1/130 by the standard technic). Dilutions of both reactants were begun at 1/5, and there was no fixation of complement and no anticomplementary action. Thus the fixation of complement in the modified procedure appears to be specific, that is, due to the presence of antigen-antibody aggregates.

† Hepatomas were induced in guinea pigs by oral administration of diethylnitrosamine(2). Anitsera were obtained from rabbits hyperimmunized with homogenates of guinea pig hepatoma.

TABLE II. Results of Checkerboard Complement Fixation Test with Homogenate of Guinea Pig Hepatoma and Rabbit Antihepatoma Serum Using the Modified 3-Stage Procedure.

Antigen dilution	Antiserum dilution						Control with:	
	1/10	1/30	1/90	1/270	1/810	1/2430	5 C'H50	3 C'H50
1/10	0	0	0	0	3½	4	4	4
1/30	0	0	0	½	3	4	4	4
1/90	2	3	3½	4	4	4	4	4
1/270	4	4	4	4	4	4	4	4
Control with:								
5 C'H50	4	4	4	4	4	4		
3 C'H50	4	4	4	4	4	4		

Antiserum titer estimated to be about 1/600. Antiserum not heat inactivated, and there is no evidence of either procomplementary or anticomplementary effects. 0 = no lysis; 4 = complete lysis. Total first stage volume = 0.8 ml; second stage volume = 1.3 ml; third stage volume = 1.5 ml. Test with 5 C'H50.

Discussion. The experiments presented here demonstrate that anticomplementary and procomplementary effects can be avoided by performing C'F tests in 3 stages. This technic also allows greater flexibility than the standard test, for it is possible to increase the amounts of antigen and antibody used in the first stage in order to increase antigen or antiserum titer without proportional increase of procomplementary or anticomplementary action. Furthermore, the modified technic obviates the need for pretreatment of reagents by heat inactivation or by other methods designed to reduce anticomplementary or procomplementary effects. Data have been presented which indicate that C' fixation measured by the 3-stage procedure is due to antigen-antibody aggregates and not due to non-specific binding of anticomplementary material. It is not known whether antiserum titers obtained with the standard method are entirely due to antigen-antibody interaction or whether, in part, they are due to the anticomplementary action of antisera. The antiserum titer obtained with the 3-stage technic, on the other hand, appears to be a valid measure of antigen-antibody interaction, for there is no evidence of anticomplementary action.

The modified technic is now used routinely in our laboratory to measure residual antibody content of antisera after absorption with antigen. It is frequently impossible to measure small amounts of residual antibody by the standard C'F test because absorption not only lowers the titer but usually increases the anticomplementary level of the antiserum. Thus, the difference between titer and anticomplementary level becomes too small to permit valid interpretation. Despite the fact that the modified procedure may give lower titers than the standard technic, these are more meaningful because anticomplementary activity is not a problem. Thus, the three-stage procedure is especially useful for titrating absorbed antisera, where valid measurement of low levels of antibody activity is required.

In experiments not reported here, it has been found that the 3-stage procedure may also be used to measure the interaction of antibody and soluble antigen (bovine serum albumin). In these experiments it was noted that C' fixation occurred even in the absence of macroscopically visible precipitates. The rabbit antiserum used, a commercially obtained hyperimmune antiserum to bovine serum albumin, showed a trace of anticomplementary activity in the 3-stage test. Thus far, this is the only antiserum which has given measurable anticomplementary activity in the modified procedure.

The major disadvantage of the 3-stage procedure is that an additional day is required to carry out the test. Thus, in experiments in which anticomplementary action is not a problem, it is recommended that standard procedures be used.

Summary. A modified complement fixation (C'F) technic designed to eliminate anticomplementary and procomplementary effects of antigens and antisera is presented. In the modified procedure antigen and antiserum are allowed to interact in the absence of added

complement, the immune aggregates are washed and then resuspended in a standard dose of complement. The relative merits of the standard and modified procedures are discussed.

1. Mayer, M. M., in Kabat and Mayer's Experimental Immunochemistry, Charles C Thomas, Springfield, Ill., 2nd ed., 1961.

2. Argus, M. F., Hoch-Ligeti, C., J. Nat. Cancer Inst., 1963, v30, 533.

Received July 12, 1965. P.S.E.B.M., 1965, v120.

N-Amidino-3-Amino-6-Chloropyrazinecarboxamide: A New Diuretic Which Antagonizes the Renal Actions of Aldosterone. (30538)

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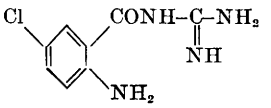
Evidence has been accumulated within the past decade which indicates that aldosterone plays an important contributory role in the salt and water retention associated with nephrosis, liver cirrhosis, toxemia of pregnancy and congestive heart failure(1,2,3,4). In view of the participation of aldosterone in these edematous states, efforts were undertaken which resulted in the discovery of both steroidal(5,6) and non-steroidal(7) antagonists of aldosterone. We wish to report the aldosterone-blocking and other actions of a new series of non-steroidal diuretics. The structure of the compound to be reported upon is shown below.



N-amidino-3-amino-6-chloropyrazinecarboxamide (ACP)

Methods. Male Holtzman rats (130 ± 3 g) were bilaterally adrenalectomized and maintained on sugar cubes and distilled water for 18 hours. The animals were randomized into groups and all except the control group were injected subcutaneously with 12 μg of desoxycorticosterone acetate (DCA) in 0.2 cc of Mazola oil. The test and reference compounds were then administered either subcutaneously or orally in 0.2 cc of dimethyl formamide (DMF). All animals were then given 5.0 cc of 0.85% sodium chloride solution by stomach gavage. The animals' bladders were voided of urine by slight suprapubital pressure and the animals placed in individual metabolism cages equipped with paraffin-coated funnels. The urine was collected in graduated centrifuge tubes with flared tops. At the end of the 7-hour collection period, the voiding of urine was again induced and the volume of the urine recorded. The floors of the cages and the funnels were repeatedly washed with distilled water and the sample and washings made up to a volume of 50 cc. Individual samples were analyzed for sodium and potassium content using a Baird Atomic Clinical Flame Photometer, Model KY. The sodium and potassium concentrations thus obtained were utilized in computing the log (Na × 10)/K ratio.* As both DCA and aldosterone are known to promote Na retention and enhance K loss, the test and reference compounds were evaluated for their ability to reverse the effects of DCA. Either or both of the following compounds were used as reference materials in these studies: 1) spironolactone (Aldactone: 3-[3-oxo-7-α-acetylthio-17β-hydroxy-4-androsten-17α yl] propionic acid α-lactone)† and 2) triamterene (2,4,7-triamino-6-phenyl-pteridine).‡

The ability of ACP to reverse the electrolyte effects of other steroids was also studied. A series of adrenaledtomized rats was given d-aldosterone monoacetate (0.5 μg/0.2 cc of a 20% ethanol solution) subcutaneously and

* Multiplication of Na values by 10 eliminated negative log (Na/K) values.

† G. D. Searle & Co., Aldactone.

‡ Smith, Kline and French Laboratories, Triamterene.