TABLE I. Highest Killing Dilution of Antibiotic for Embryonic Chick Heart Tissue and *Micrococcus pyogenes* var. *albus* and Corresponding Toxicity Index.

Antibiotic	Tissue (A)	Bacteria (B)	Toxicity index (A/B)
Iodine	1:2000	1:20,000	.10
Penicillin G sodium	1:12	1:5000	.0024
Streptomycin sulfate	1:20 growth	1:1000	<.02
Polymyxin B sulfate	1:50 growth	1:80	<.63
Terramycin	1:600	1:900	.67
Bacitracin	1:10 growth	1:10 growth	—
Chloramphenicol	1:100 growth	1:100 growth	—

parison for a number of well-known antibiotics. Of the antibiotics tested (Table I) penicillin G sodium exhibited the greatest degree of germicidal efficiency, combining high potency with low tissue toxicity. A toxicity index of 0.0024 indicated that penicillin was more than 400 times more toxic to the test bacteria than to the tissue cells, and approximately 42 times more efficient than iodine. Terramycin with an index of 0.67 was inferior to iodine as a germicide. Streptomycin sulfate was nontoxic to tissue in the highest concentration used and gave an index less than 0.02. A more concentrated solution could not be prepared without sacrificing accuracy. Likewise, Polymyxin B sulfate was nontoxic to tissue in the highest concentration used but killed the test organism in a dilution of 1:80 giving an index of less than 0.63. Chloromycetin and Bacitracin had no effect on tissue or bacteria in the concentrations used which would indicate that their actions were bacteriostatic rather than bactericidal.

Summary. A new method was proposed recently for the evaluation of germicides intended for clinical application. Iodine, with a toxicity index of 0.10 exhibited the greatest degree of germicidal efficiency. The same procedure was followed for the evaluation of a group of antibiotics. Penicillin G sodium gave an index of 0.0024 which indicated that it was more than 400 times more toxic to the test organism, *Micrococcus pyogenes* var. *albus*, than to the tissue cells, and approximately 42 times more efficient than iodine. Results on other antibiotics in order of decreasing efficiency are: Streptomycin sulfate, 0.02—; Polymyxin B sulfate, 0.63—, Terramycin, 0.67. Bacitracin and Chloramphenicol were bacteriostatic and could not be evaluated. Likewise, Aureomycin HCl, Achromycin HCl, and Erythromycin could not be evaluated; the former two precipitated from solution shortly after being prepared, and the latter failed to dissolve in saline.

1. Salle, A. J., *Applied Microbiol.*, 1955, v3, 63.

Received July 16, 1956. P.S.E.B.M., 1956, v93.

Quantitative Determination of C-Reactive Protein by Complement Fixation.*†‡ (22666)

MAURICE M. RAPPORT AND LISELOTTE GRAF

Division of Laboratories and Research, New York State Department of Health, Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York City.

Interest has been growing in the measurement of serum levels of C-reactive protein as an indicator of inflammatory processes, in particular, those associated with rheumatic fever, myocardial infarction, and cancer. The original determination of this substance(1) was based on the precipitate it forms with pneumococcal C-polysaccharide. Lack of sensitivity led to an immunochemical method,

* Presented before Am. Assn. Immunol., Atlantic City, N. J., April 17, 1956.

† We wish to express our appreciation to Dr. Alfred Halpern and Schieffelin and Co. for their cooperation, to Dr. Maclyn McCarty for a specimen of pneumococcal C-polysaccharide, and to Mr. Nicholas Alonzo, Mr. Bernard Schiffer, and Mrs. Evelyn Abeshouse for their excellent technical assistance.

‡ Mailing address: Sloan-Kettering Institute, 410 East 68th St., N. Y. 21, N. Y.

the reaction between C-reactive protein antigen and specific rabbit antibody. The degree of reaction is visually estimated from the quantity of specific precipitate(2,3). More recently, estimation by complement fixation (4) and gel diffusion(5) have been reported. In this communication we describe our modifications of the complement fixation method of Muschel and Weatherwax and the theoretical and practical reasons for them. Our purpose was 2-fold: to establish an objective index of the serum level of C-reactive protein, and to control the variables inherent in the quantitative determination of antigens by complement fixation.

The method described was designed to measure C-reactive protein in the range determined in the microprecipitation method, using approximately the same quantity of antiserum, and furnishing the result within a short time.

Materials and methods. Human test sera. Test sera were inactivated at 56°C for 10 minutes(4). Specimens so treated showed no residual complement activity at a dilution of 1:10. No loss was observed in the standard CRP serum after this treatment.

Rabbit anti-CRP serum. Native antiserum preserved with sodium azide (1:1000) was obtained from Schieffelin and Co. Antibodies to normal serum components were absorbed by adding lyophilized normal human serum, 0.05 g/ml. After incubation at 40°C for 2 hours and 4°C for 2 days, a small quantity of precipitate formed. The treatment to this point was based on the procedure Schieffelin and Co. employs to make its reagent specific. Removal of the precipitate by the usual centrifugation results in an antiserum which is anticomplementary because of the presence of soluble immune complexes. This is the source of the anticomplementary property reported by Muschel and Weatherwax(4). The absorbed antiserum was centrifuged in the #40 rotor of a Model L Spinco ultracentrifuge for 2 hours at 35,000 rpm (110,000 x g). The floating lipid layer (4 mm) was rejected and the next 42 mm of fluid was withdrawn in several portions which were subsequently combined. The lowermost 12 mm of clear supernatant was then removed and kept separately. Examination of anticomplementary property showed that this increased with sample depth, but the recombined specimen described was relatively free of this interference (hemolysis greater than 55% with two 50% hemolytic units of complement at antiserum dilution 1:10). The antiserum, preserved with merthiolate (1:10,000) and stored at —20°C, is used without inactivation, since its complement is removed during the absorption. Antiserum from the lowest fifth of the tube was too anticomplementary for complement fixation tests but was useful for microprecipitation and gel diffusion analyses.

Standard CRP serum. A specimen of serum with a high CRP level was stored in small quantities at —20°C and left at 4°C after being thawed. This standard was assigned a titer of 160 after comparison with an earlier standard which had been arbitrarily set at 100. The quantity of CRP in the standard, determined by precipitation with C-polysaccharide according to the method of Wood and McCarty(3) was 280 μg/ml.

Complement fixation experiments. Reagents and procedure have been described in detail(6).

Evaluation of reactions. The results are expressed as the quantity of CRP serum (antigen) required to obtain 50% hemolysis with a constant quantity of antiserum and a constant quantity of complement. When partial hemolysis greater or less than 50% was actually measured, the quantity for this endpoint was obtained by graphic interpolation from a plot of log dilution of antigen versus log (% hemolysis ÷ 100 — % hemolysis). The routine complement fixation test was set up in 8 tubes with dilutions of CRP test serum 1:10, 1:40, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200. The quantity of complement used was four 50% hemolytic units (about 0.005 ml undiluted guinea pig serum). Antiserum was used at a dilution of 1:40 (0.00125 ml). The standard CRP serum was run with each test at dilutions of 1:800, 1:1200, 1:1600, and 1:2400. Anticomplementary property of test sera at 1:10 dilution was examined with 2 units of complement.

Incidence of AC property (hemolysis of less than 55%) at this dilution is very low (less than 1%).

Microprecipitation reaction. This test was performed in capillary tubes(3) with the following modification to insure complete mixing of reagents. 0.01 ml of antiserum was placed in the well of a clear glass spot plate. 0.01 ml of test serum was added. The entire mixture was run into the capillary tube by tipping the plate. The contents of the capillary were then almost completely returned to the spot plate well by gentle (mouth) pressure. This procedure was repeated and the capillary tube was sealed after the third filling. Tubes were read by comparison against the standard CRP serum (equal to 160) at concentrations of 80, 40, 20, 10, 5 and 2½ after incubation (2 hours at 37°C, overnight at 4°C). With the antiserum used, the supernatant from the "80" tube produced no additional precipitate on adding either antiserum or CRP serum. The quantity of precipitate in the "2½" tube was less than 1 mm but more than a trace.

Gel diffusion. This test was carried out by the modification of the Ouchterlony technic described by Korngold and Lipari(7) which uses 1.5% concentration of agar. Plates were kept at room temperature for one week and then, as described by Libretti, *et al.*(5), at 4°C for 2 weeks.

Results. The complement fixation test for CRP involves 3 main variables—namely, the quantities of antiserum and complement which are required for the most effective measurement of a given quantity of CRP. The interrelationships are best studied by means of a block titration in which the quantity of complement is held constant and antiserum and antigen are varied. The data can be integrated into a line curve expressing the relationship between antigen and antibody over the full range of antibody excess, equivalence, and antigen excess by plotting the quantities of these reactants which always produce the same hemolytic endpoint(8). The relationship for the rabbit antibody-CRP system is shown in Fig. 1a. The curve is a non-rectangular hyperbola with asymptotes in the zones of antibody excess and antigen excess and a minimum close to the zone of equivalence. This curve is representative of systems in which the zone of antigen excess is characterized by marked inhibition of immune complex formation(8). It can be seen that for the quantity of complement with which the curve is determined, a minimum quantity of CRP can be measured, no matter how large an excess of antibody is used. In Fig. 1b there is shown on expanded axes the zone of antibody excess determined with each of 3 different quantities of complement: 3, 4, and 5 units. These curves show how sensitivity of the determination (minimum quantity of measurable CRP) depends on quantity of complement used in the test, and further, how much antiserum must be used to insure antibody excess, once the sensitivity level has been selected. The choice of a particular set of quantities is a balance between supply of antiserum on one hand, and on the other, both quantity of CRP which must be determined and degree of interference from anticomplementary properties of the reactants.

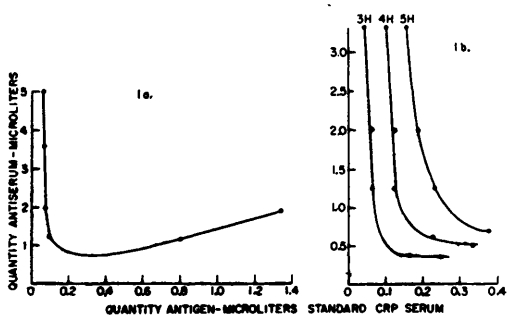

FIG. 1a. Antigen-antiserum reactivity curve for human CRP-rabbit antibody system.

FIG. 1b. Curves in zones of antibody excess and equivalence determined with 3, 4, and 5 hemolytic units of complement.

A correlation of CRP titers determined for 192 specimens by both complement fixation and precipitation methods is shown in Fig. 2. It is evident that sensitivity of the method based on complement fixation has been set to conform exactly to sensitivity of that based on microprecipitation. Twenty-four specimens gave zero titers with both methods, 8 showed a CF titer of 1 without producing any precipitate, while 4 showed a precipitate corresponding to 1, 2 a precipitate correspond-

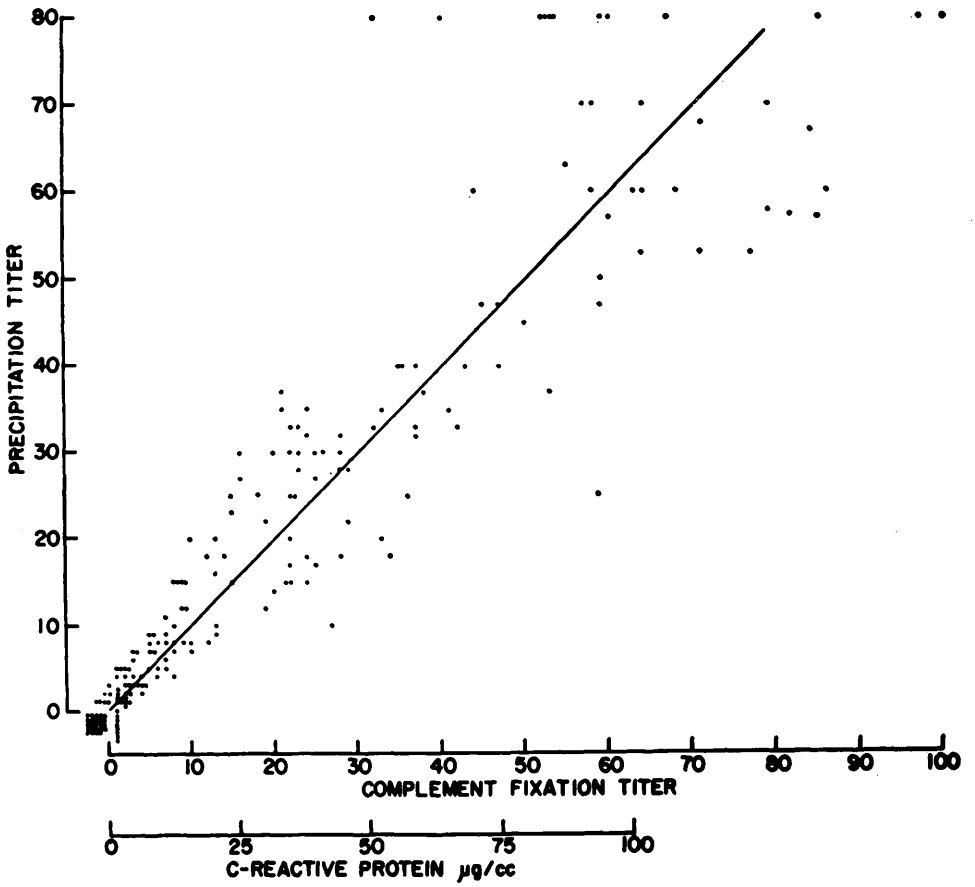

FIG. 2. Correlation of CRP levels determined by microprecipitation and complement fixation showing identical range of both methods. Specimens with very high complement fixation titers are not shown since maximum precipitation titer obtainable with undiluted test specimens is "80." Precipitation titers are the avg of observations by 3 individuals. The line represents the location of points for exact correspondence.

ing to 2, and 1 a precipitate corresponding to 3 without having any CRP determined by complement fixation. If we accept the greater reliability of the complement fixation method (since it is applied at a level considerably above the limit of its sensitivity), these results suggest that very small quantities of precipitate may not be specific for CRP. It has been frequently noted that traces of precipitate tend to redissolve on standing several minutes at room temperature. The relatively poor correlation between CRP titers obtained by the two methods is principally due to the error inherent in the subjective (visual) method of estimating the quantity of precipitate. Distinguishing a titer of "40" from one of "20" or "80" is frequently impossible, and the degree of correlation shown in Fig. 2 could be achieved only by averaging the estimates of 3 observers. The complement fixation method, on the other hand, offers an objective numerical index of the CRP titer. If 2-fold serial dilutions are tested and the exact dilution titer for 50% hemolysis is estimated by the graphical method described, an error of as much as 10% hemolysis produces an error of about 10% in the final result. Closer spacing of dilutions, as described for the standard, can appreciably decrease this source of variation.

The hemolytic unit of complement (quantity of guinea pig serum) which is defined by the indicator reaction serves as a control of the reagents participating in this part of the

test. Fluctuations in the unit result principally from variations in batches of sensitized red cells and guinea pig serum. However, this index of variability in reagents is not applicable to the primary reaction. For this purpose, we have found that the use of a standard is essential(6). A convenient stable standard is a high-titered CRP serum specimen. The data in Table I representing dilution titers of the standard serum determined over a 5 month period and complement unit on the day of each determination show that the standard is stable. They also indicate that with a higher complement unit, a lower dilution titer is observed. This explanation is not sufficient to account for the variation or to eliminate the necessity for the standard, as is seen by inspecting the values on 1/11, 1/18, 1/27 and 1/31. On these days the complement unit was constant but the standard changed 35 to 45%.

The quantity of CRP in the standard was 280 μg/ml. Average titer was about 1500. Since 0.1 ml of antigen dilution is used, the endpoint in the complement fixation test requires about 0.02 μg of C-reactive protein. The sensitivity of the test described here (with limiting dilution of 1:10) therefore corresponds to 2 μg CRP/ml undiluted serum, a result in excellent agreement with the report of Wood and McCarty‡(3). An example of the calculation of CRP titer or concentration based on the standard is as follows. Assuming the test specimen shows a dilution titer of 350 when the standard serum dilution titer is 1500, then

$$\text{Test specimen CRP titer is } \frac{350}{1500} \times 160 = 37.$$

$$\text{Test specimen CRP conc. is } \frac{350}{1500} \times 280\ \mu\text{g/ml} = 65\ \mu\text{g/ml}.$$

In gel diffusion studies by the method of Ouchterlony we have not observed the multiplicity of CRP components reported by Libretti, *et al.*(5), either with 20 high titered specimens from different individuals or a pool of 20 such specimens. On prolonged standing (more than 10 days), a pattern similar to the one they describe was seen, but this is an artifact of the diffusion process, particularly in the region of antibody excess(9).

‡ The values for concentration of CRP in Table 4 of this reference should be divided by 2 according to the authors.

TABLE I. Titers of Standard CRP Serum and Complement over 5 Month Period Showing Stability of Standard and Relative Independence of Fixability and Hemolytic Activities of Guinea Pig Complement.

Date	Dilution titer of standard CRP serum	Complement unit, ml g.p. serum
9/12	1000	.00142
11/ 1	1800	.00122
11/ 9	1750	.00110
11/16	1450	.00110
11/30	1300	.00110
12/ 7	1750	.00121
12/14	1700	.00110
12/21	1150	.00121
12/28	1450	.00105
1/ 5	1400	.00100
1/11	1900	.00105
1/18	1350	.00105
1/27	1750	.00107
1/31	1300	.00105
2/ 8	1250	.00122

Discussion. In the procedure herein described, we have paid particular attention to 3 aspects of quantitative immunochemical analysis by complement fixation. These are the removal of residual anticomplementary activity from absorbed antisera, the necessity for a standard to eliminate variations as large as 2-fold in "fixability" of complement, and the selection of the most suitable quantity of antiserum for a given purpose. The range of C-reactive protein levels usually found in human serum is 0 to 300 μg per ml(3). The level can rise well above 300 μg after surgery (10). The large range taken in conjunction with the observed inhibition by excess antigen determines minimum number of tubes which must be set up for the complement fixation test. A 10 to 20-fold excess of antigen over the quantity measured at the endpoint is sufficient to produce hemolysis indicating a weak or negative test; the inhibition produced by a lesser degree of antigen excess may still appreciably affect determination of titer.‖ Suitable modifications of the complement fixation

‖ Since titer is usually determined from hemolysis greater or less than 50% by interpolation, a partial hemolysis in the zone of inhibition will incorrectly reduce the titer.

method will permit measurement of considerably lower concentrations of C-reactive protein than 2 μg per ml. However, as sensitivity is increased, two limitations become progressively more important: incidence of anticomplementary activity in test specimens, and C-reactive protein interaction with normal serum constituents which affects its reaction with antibody.

Summary. A quantitative immunochemical method for determination of C-reactive protein concentration in serum is described. The sensitivity of this method, based on complement fixation, has been selected to measure the same concentration range of CRP as determined by microprecipitation. Two important features generally applicable to complement fixation methods are presented; namely, removal of anticomplementary activity from absorbed antisera and use of a reference standard to compensate for variations in fixability of complement.

1. Tillet, W. S., and Francis, T., *J. Exp. Med.*, 1930, v52, 561.

2. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.

3. Wood, H. F., and McCarty, M., *J. Clin. Invest.*, 1951, v30, 616.

4. Muschel, L. H., and Weatherwax, R. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 191.

5. Libretti, A., Kaplan, M. A., and Goldin, M., *ibid.*, 1955, v90, 481.

6. Rapport, M. M., and Graf, L., *Cancer*, 1955, v8, 538.

7. Korngold, L., and Lipari, R., *Cancer Res.*, 1955, v15, 159.

8. Almeida, J. O., *J. Immunol.*, 1956, v76, 259.

9. Korngold, L., personal communication.

10. Rapport, M. M., Schwartz, A., and Graf, L., to be published.

Received July 16, 1956. P.S.E.B.M., 1956, v93.

Pheochromocytomas of Adrenals in Male Rats Chronically Injected with Pituitary Growth Hormone.* (22667)

HENRY D. MOON, ALEXEI A. KONEFF, CHOH HAO LI, AND MIRIAM E. SIMPSON.
Department of Pathology, Anatomy, Hormone Research Laboratory and Institute of Experimental Biology, University of California, Berkeley and San Francisco.

Increased numbers of tumors of the types occurring spontaneously in the rat were observed during the course of chronic injection of normal female rats with pituitary growth hormone. Pulmonary and lymphatic tissues, reproductive organs, pituitary and adrenal glands were involved(1-6). Outstanding was the occurrence of adrenal medullary pheochromocytomas(2). Since none of this work had hitherto been done in male rats, the questions arose as to whether males would be equally responsive in body growth and similarly susceptible to tumor formation, especially as regards reproductive organs and adrenal glands.

Materials and methods. Male rats of the Long-Evans strain, of 2 different age groups, 6 and 14 months, were divided into experimental and control groups of 8 rats each. The purified growth hormone(7) was injected intraperitoneally in saline 6 days weekly. The initial daily dose of 0.5 mg was increased progressively during the first 160 days, reaching a maximum of 3.0 mg in the younger, and 3.5 mg in the older group. These doses were then maintained for the remainder of the 15-month injection period. Complete autopsies were performed on all rats. The weights of all organs were determined, and the entire organ or representative blocks were fixed and prepared for histological study.

Results. Body weight and length. The rats in the 6-month-old group weighed approximately 400 g at the beginning of the experiment and gained steadily, reaching a maximum of almost 700 g at 18 months (Fig.

* Aided by grants from the United States Public Health Services A-366 and C-1098; and The University of California Cancer Research Coordinating Committee.