Again the points fell close to a straight line whose slope, determined by the usual least squares method, is equal to the Michaelis constant(9). Since the molecular weight of the true substrate, *i.e.*, the constituent of the bacterial cell containing the linkage upon which lysozyme acts, is unknown, the constant cannot be given in the usual units.

Simultaneously, a second series of determinations was run which differed from the first only in that the reaction mixture contained in addition heparin in a final concentration of 10 mg per ml. The initial velocity was again plotted against the velocity constant (Fig. 2), and it is obvious to the eye that the slope has changed. The change was found statistically to be highly significant. The intercept on the Y axis is V_{max}, and the difference in the two values for V_{max} is not significant. Inhibition by heparin of the action of lysozyme on *M. lysodeikticus* is therefore competitive(10).‡

Summary. The inhibition of lysozyme by heparin is shown by the usual criteria of the Michaelis-Menton formulation to be competitive in type.

‡ Additional experiments have shown an inhibiting effect of large amounts of heparin on the lysozyme activity of lysates of human leukocytes also. No inhibition is evident at heparin concentrations in the range used in prior experiments for *in vitro* anticoagulation(8).

1. Fleming, A., and Allison, V. D., *Brit. J. Exp. Path.*, 1922, v3, 252.
2. Fleming, A., *Proc. Roy. Soc. Med.*, 1932, v26, 71.
3. Thompson, R., *Arch. Path.*, 1940, v30, 1096.
4. Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, v163, 723.
5. McClean, D., *J. Path. Bact.*, 1942, v54, 284.
6. Astrup, T., and Alkjaersig, N., *Nature*, 1950, v166, 568.
7. Bergamini, L., and Ferrari, W., *Boll. Inst. Sieroterap. Milan*, 1948, v27, 89.
8. Kerby, G. P., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 129.
9. Eadie, G. S., *J. Biol. Chem.*, 1942, v146, 85.
10. ———, *Science*, 1952, v116, 688.

Received April 13, 1953. P.S.E.B.M., 1953, v83.

Measurement of Complement Fixation in Small Volumes.* (20282)

EDWARD A. ECKERT,† ROBERT H. REEVE, AND J. W. BEARD.
From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

The applicability of the complement fixation reaction to the assay and immunological characterization of biological materials is frequently limited or entirely impractical because of the scarcity of the antigen or antiserum available. This is particularly the case in the virus field in which many useful studies have not been possible with the amounts of these materials needed for complement fixation as usually practiced. A specific problem encountered recently in this laboratory has stimulated an investigation of the adaptability of the standard reaction to quantitative work with small volumes. As will be described in this report, the results of the study indicate the promise of considerable success with the reaction in total volumes as small as 12 mm^3.

Materials and methods. The development of the procedure described here has depended principally on modifications of the standard method(1) for complement fixation necessary to overcome the technical difficulties introduced by the minute volumes employed. These have been circumvented in the main by the construction of apparatus eliminating the principal adverse factors. An initial prerequisite to use of the small volumes was

* This work was supported by research grants to Duke University from the National Institutes of Health, U. S. Public Health Service; and from the American Cancer Society on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

† Scholar in Cancer Research of the American Cancer Society.

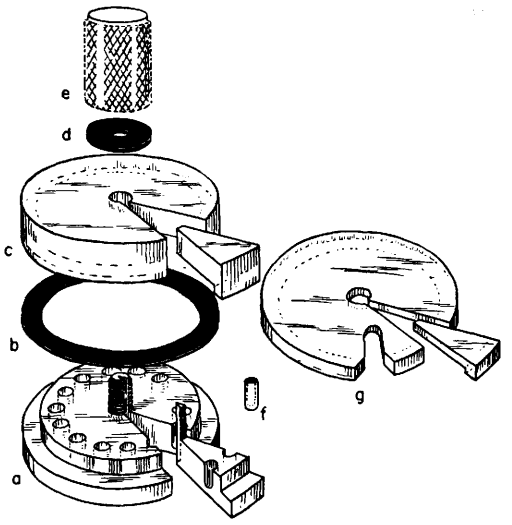

FIG. 1. The water-tight tube rack and the reaction tube used in micro complement fixation.

obviously the prevention of significant evaporation. This was accomplished with the apparatus shown in Fig. 1. Here there are illustrated the parts making up a rack or holder which carries the small reaction tubes during the preparation and incubation of the mixtures. The base, A, 2-1/16 in. in diameter, was turned from a lucite block 1/2 in. thick. At the periphery a shoulder was turned in the upper surface 1/4 in. deep and 3/16 in. wide. Holes, equally spaced and 13 in number, in the present instance, were drilled as indicated 0.177 in. in diameter and 0.280 in. deep. Numbers were engraved above alternate holes. At the center, a hole was drilled part way through the disc and threaded to take a 20-thread brass stud 1/4 in. in diameter and 11/16 in. long, which was cemented in with water proof glue. Item B is a rubber gasket 1/16 in. thick cut to fit over the shoulder of A. The lid, C, was turned also from a 1/2 in. plate of lucite and machined in the under surface to fit snugly over the shoulder of A. The inner surface of C and the upper surface of A were machined, and the depression in C was of a depth, slightly less than 3/16 in., such that when pressure was exerted on the gasket B, closed joints were made both at the contacting surfaces of A and C as well as at the surfaces of the gasket. A water-tight joint was formed by gasket D between the upper surface of C and the knurled knob E when the knob was drawn down on the stud. Another lid, G, was made for use during preparation of the mixtures. It was of the same diameter as A and, in the inferior surface, a depression was machined to fit the shoulder of A. The depth of this depression was only 3/32 in. deep. A notch was cut into the edge as indicated at G of a width and depth to allow access to a single reaction tube while all the others were covered. A hole was drilled in the center to allow free passage of the stud. At F is shown one of the reaction tubes which is a piece of pyrex glass tube 5/32 in. O.D. and about 3/32 in. I.D., closed in the flame at one end and cut of a length to bring the top of the tube exactly flush with the surface of A when the tube is put into one of the holes.

Measurement of the small volumes was effected easily, though somewhat tediously, by means of homemade pipets hand-drawn from standard pyrex tubing. The tubing was 3 mm O.D. (wall thickness 0.6 mm) for 1 and 2 lambda (λ) volumes, both in the same pipet; 4 mm O.D. (0.8 mm wall thickness) for 5 and 10 λ volumes, each in a separate pipet; 5 mm O.D. (0.8 mm wall thickness) for 20 λ volumes; and 8 mm O.D. (1.0 mm wall thickness) for 45 and 50 λ volumes, a separate pipet for each volume. The tips were left as fine as possible compatible with reasonable treatment on use, a point of some importance. A piece of plastic tube sealed with heat at one end made a good nipple for manipulating the fluid in the pipet. Commercial pipets could not be used because of the large tips. Calibration was accomplished with weighed volumes of mercury. Manipulation of the pipets was greatly facilitated through use of a jeweler's loupe. *Stirring of the mixtures* during incubation after the addition of red cells was of paramount importance; because of the very short distances in the small volumes, the cells settled out within a few minutes. After many trials with various methods it was possible to evade this difficulty by rotating the tubes in their containers in the device shown in Fig. 2, the construction of which is evident from the photograph. The size was determined by that of a small standard constant temperature

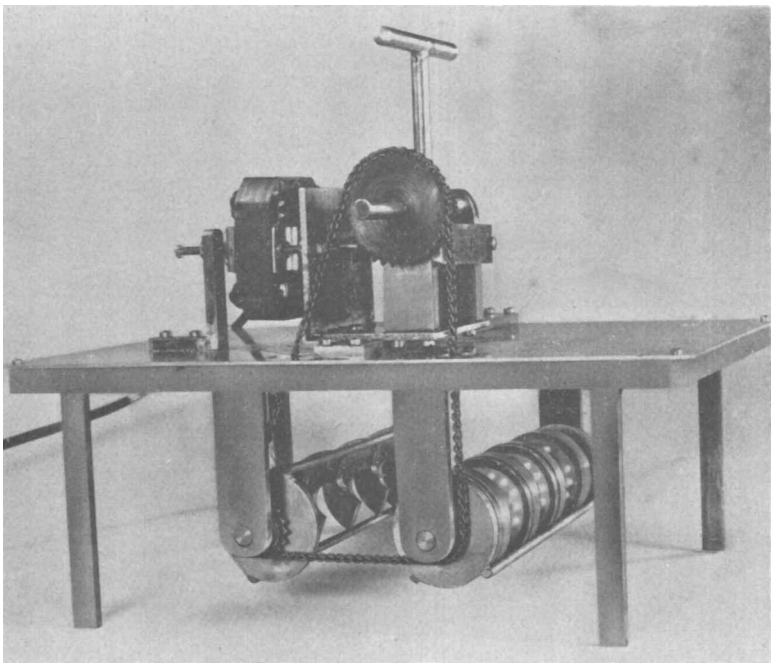

FIG. 2. Device for rotating the tube rack in the water bath.

water bath into which the device snugly fitted, resting on the four legs and effectively covering the bath. The speed of rotation of the tube containers, completely submerged in the water at 37°C, was about 100/min. In order to effect stirring, a single lead pellet from a 22 caliber long rifle shot cartridge was added to each tube. No adverse effects were observed with the use of lead; glass beads gave about the same results but tended to be caught in the meniscus of the liquid. It was found necessary to treat the glass tubes with silicone; otherwise, the cells gathered in the long angle made by the meniscus of the fluid and the tube wall.

Estimation of the *degree of hemolysis* was made by counting the number of red blood cells at the end of the reaction. These counts were made in duplicate in bright-line Neubauer counting chambers. It was convenient to use the 10 λ pipets for filling the chambers. Errors due to movement of the cover slip by touching it with the pipets were avoided by the use of clips made of thin-sheet phosphor bronze which held the slips firmly to the chamber. A thin sheet of aluminum was fastened to each end of the chamber to hold the latter above the stage, preventing displacement of the clips. In the standardization of the cell-counting procedure, a comparison was made of end points obtained by cell counts and estimation of hemolysis by hemoglobin determination in the standard way. The latter was done with reaction mixtures of 3.3 ml volumes, and hemoglobin was estimated in the Coleman spectrophotometer after sedimentation of the red cells. The percent hemolysis has been plotted on the probit scale in the graphs presented in this report. The sheep red blood cells, kindly provided by Mr. H. W. Craig, were obtained by the collection, washing and storage procedures described by Kabat and Mayer(1). A stock suspension, approximately 5% cells in Alsever's solution(1) stored in the refrigerator, could be used for several months. Cells for about 3-days work were washed with veronal-$MgCl_2$ buffer(2) (without $CaCl_2$) through 4 centrifugations immediately before each set of experiments, and cell concentration was determined by count. The cells were sensitized by the addition to the suspension of sufficient antisheep cell rabbit serum to yield 4 100% units of hemolysin. Complement was pur-

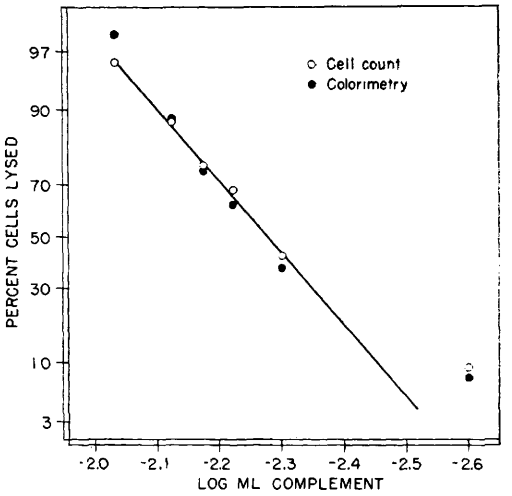

FIG. 3. Comparison of estimates of hemolysis in complement titration made by cell counts and colorimetry.

chased in the lyophilized state (Carworth Farms, New City, N. Y.), dried from carefully measurered 1.0 ml volume per ampoule from large volumes of guinea pig serum and reconstituted to the initial volume with the diluent supplied by the manufacturer. A fresh solution was made for each experiment, and its activity was of such uniformity that preliminary titration was not necessary. The test *antigen and antiserum*, obtained through the generosity of Dr. H. Piersma, Lederle Laboratories, were pneumococcus polysaccharide type VIII and the corresponding rabbit antiserum. A solution of the dry polysaccharide was prepared in a concentration of 0.759 mg per ml in veronal-$MgCl_2$ buffer and kept in the refrigerator without preservative. The fluid antiserum was parcelled in 10 λ volumes in melting point tubes (Kimble Glass, Toledo, Ohio), sealed at each end and stored in the frozen state at −20°C. At the time of its use, the tube and contents were heated at 56°C for 30 minutes, and a volume of 5 λ was diluted to appropriate concentrations with the veronal buffer.

Estimation of hemolysis by cell count and by colorimetry. As a preliminary to use of the small volumes, a series of studies was made to determine the correlation between the results of cell count and those of colorimetric measurement of the degree of hemolysis. The findings in one experiment are shown in Fig. 3. It is seen that the 50% end points of hemolysis estimated by the two methods were essentially identical, a finding encountered in a total of four experiments carried out in this way.

Titration of complement. A lengthy series of experiments was made in the simple titration of complement in order to develop the various technical procedures and to test the precision of the method finally adopted. The results of 5 such studies are given in the points of Fig. 4. These experiments were made over a period of 2 weeks; for each, a different ampoule of complement was diluted first to 1.0 ml, and further dilutions were made with the veronal buffer so that in the range expected to include the 50% hemolysis point, there were about 10 dilutions of complement available for study. These were spaced evenly with 6 dilutions covering the range of a 2-fold dilution. The reaction mixture consisted of 4 λ of buffer solution plus 5 λ of the appropriate dilution of complement. Mixing was effected by pumping buffer and complement solution several times with the pipet delivering the complement. These operations were carried out with all solutions chilled in ice water and with the tube rack standing on a cake of ice. The rack was sealed and immersed in a water bath at 37°C for 20 minutes. The rack was dried with a cloth, opened, and each tube

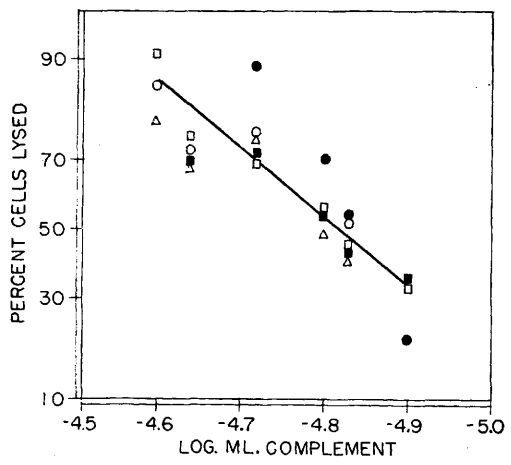

FIG. 4. The results of titration of separate batches of complement from the same pool over a period of 2 wk. The different symbols designate the findings in 5 different experiments.

received 2 λ of cell suspension containing a total of 20,000 red cells in the 2 λ volume (total volume of the reaction mixture was now 11 λ). Into each tube was placed the lead bead, and all were stirred with an inch-long piece of nichrome wire fixed in the handle of a bacteriological inoculating needle. The wire was washed and dried between tubes. After the rack was again sealed, it was placed in the rotator, submerged in water at 37°C and rotated for 30 minutes. Finally, the container was opened and, by inspection of the tubes, the range of hemolysis appropriate for cell counts was selected. To make the dilutions for counting, 20 λ volumes of buffer solution were measured into clean tubes and to each were added 5 λ of the respective, well-stirred reaction mixtures. The 25 λ volumes were thoroughly stirred by pumping with the pipet delivering the complement-red cell suspension into the diluent.

It is seen that the points of the different experiments shown in Fig. 4 are arranged in an apparently linear fashion and that those of 4 of the 5 experiments are a relatively close fit to the line drawn through the grouping by sight. The points of one experiment seem to lie along a line of a somewhat different slope from that through the others. Despite this difference in the slope, the position of the hypothetical linear relation at the level of the 50% point was very close to the analogous points in the other experiments. Analysis showed that the mean 50% unit of complement was 2.76×10^{-5} ml of the reconstituted material with a standard error of $\pm 0.23 \times 10^{-5}$ ml. It should be emphasized that these variations include not only possible differences in the properties of various specimens of reconstituted complement employed but also those to be expected in the repeated technical manipulations. It is notable also that this is a series of 5 successive experiments.

Fixation of complement by pneumococcus polysaccharide. A group of experiments were now made to learn the behavior of the system under those practical working conditions suitable to the study of complement fixation by a specific antigen and its antiserum. The procedure employed was modeled after that used by Mayer *et al.*(3). For this the pneumococcus polysaccharide, type VIII, and rabbit anti-polysaccharide serum were employed. The relations of complement, antigen and antiserum and the results of 4 consecutive experiments are given in Table I. *Immediately before each experiment,* the stock polysaccharide solution described before was diluted further 1-400 with veronal buffer to provide the standard working solution for the day. With this preparation, different additional dilutions were made to yield, in 10 λ volumes, the amounts of antigen designated in the first column of Table I. Through this point the volumes were of the usual magnitude. The antiserum, after inactivation at 56°C, was diluted by adding 5 λ antiserum to 1.0 ml buffer solution. Reconstituted complement was diluted 1 part with 2 parts of buffer solution. The mixtures were now prepared by placing in the micro tubes in the chilled rack 10 λ of antiserum, 10 λ of complement and finally 10 λ of the various dilutions of antigen. Mixing was done with the pipet transferring the antigen. These mixtures, in the sealed racks, together with appropriate controls were incubated at 4°C for 16 hours. The racks were now opened, kept chilled and a series of twofold dilutions was made of each mixture, employing 10 λ volumes of mixture and buffer solution. The rack was again sealed and warmed in the water bath for 20 minutes at 37°C. To each tube there were now added 2 λ of red cell suspension (a total of 20,000 cells). A lead shot was put into each tube, the mixtures were

TABLE I. Fixation of Complement by Pneumococcus Type VIII Polysaccharide in Presence of Specific Rabbit Antiserum.

PN VIII in 30 λ reaction mixture ($\mu g \times 10^{-3}$)	50% units of complement fixed — Experiments			
	I	II	III	IV
3.80	47.8	54.9	>30	>48
1.73	31.2	38.0	>30	48.4
1.00	25.2	31.2	23.4	39.8
.70	11.1	25.2	20.4	23.7
.38	5.1	4.2	6.0	10.5
50% units complement in 30 λ reaction mixture	48.6	61.2	36.0	55.9
PN VIII antigen fixing 20 50% units complement —$\mu g \times 10^{-3}$	1.05	.77	.85	.62

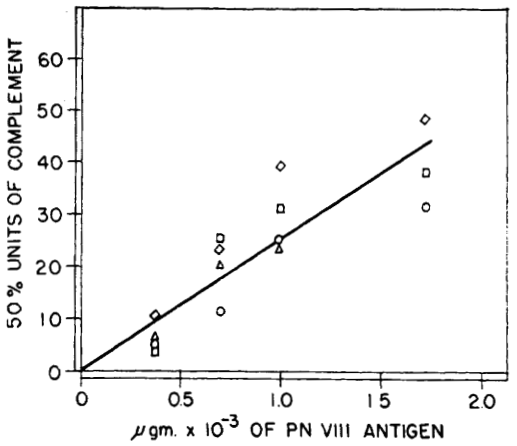

FIG. 5. Repeated estimates, in 4 experiments, of the complement fixing capacity of type VIII pneumococcus polysaccharide.

stirred with the wire, sealed again in the racks, and rotated for 30 minutes submerged in the water bath at 37°C. Appropriate dilutions of these mixtures were then used for counting the residual cells.

In Table I there are seen the various quantities of antigen employed with the constant volume of complement solution used in each experiment. The actual number of 50% point units of complement in this volume in each experiment was determined from the results obtained with the control mixture of inactivated immune serum and complement. The values in the body of the table indicate the number of 50% point units of complement fixed by each of the different quantities of antigen in the presence of a constant volume of antiserum. These results are shown graphically in the points of Fig. 5 which indicate, for practical purposes, a linear relationship between the number of 50% units of complement fixed and the quantity of antigen. From this relation there are calculated the amounts of antigen required to fix 20 50% units of complement. The 20-unit point was chosen for comparison since it lay about midway of the range of values. In the 4 experiments the amount of antigen required varied from 0.62 to 1.05 x 10^{-3} μg. The mean was thus 0.82 x 10^{-9} g with a standard error of the mean of 0.09 x 10^{-9}.

Discussion. The results of these experiments have shown that the complement fixation reaction can be employed on a scale sufficiently small to broaden greatly the range of reacting materials. The procedures described offer, in contrast with other micro methods(4), the advantage of quantitative data. Neither accuracy nor precision in a single experiment is as great as that obtainable with the use of larger amounts of antigen and antiserum; however, the exceedingly minute amounts of these materials needed for an experiment make possible the number of repetitions necessary for the required accuracy.

It was found highly advisable to employ the principle of complement fixation in mixtures of high concentrations of complement as used by Mayer *et al.*(3). In the small volumes, the loss of complement in the higher dilutions during the prolonged fixation period was both great and variable, and could not be eliminated by any means tried in this work. In other respects the technics, except for the greater tedium and care involved, differed little from the methods employing large volumes. The added difficulties are greatly overshadowed by ready accessibility to studies previously entirely impractical.

Summary. The equipment and the procedures have been devised for quantitative complement fixation in volumes of 12 mm^3. The technic, reduced to a routine basis, is illustrated in the results obtained in the titration of complement and in the estimation of complement fixed by pneumococcus polysaccharide type VIII and its specific antiserum induced in the rabbit. With the procedures described the reaction can be carried out with antigen and antiserum in amounts of the order of 100-fold less than those required for the volumes usually employed, greatly extending the range for the study of material available only in small quantities.

1. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* Springfield, Ill., Charles C. Thomas, 1948.

2. Mayer, M. M., Croft, C. C., and Gray, M. M., *J. Exp. Med.,* 1948, v88, 428.

3. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Immunol.,* 1948, v59, 195.

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

Received April 14, 1953. P.S.E.B.M., 1953, v83.