no effect on the activation of chymotrypsinogen by trypsin, in the presence of ionic calcium. If this were to hold true over a wide range of conditions, it would offer a possible distinction between activation by trypsin and by thrombokinase. Further tests are now indicated, for experience has emphasized the variability of the phosphatide effect with changing conditions.

Phospholipids augment other enzyme reactions(9-11). The present case may be the first in which phosphatide accelerates the enzymatic activation of a zymogen other than a clotting factor.

Summary. Highly purified thrombokinase, derived from bovine plasma, activated chymotrypsinogen. Chymotrypsin was assayed by the hydrolysis of BTEE, which was not split by thrombokinase under the conditions used. Activation of chymotrypsinogen may have been due to thrombokinase, *per se,* or to an impurity, since a large quantity of thrombokinase was required. However, phosphatide accelerated the process, provided that ionic calcium was included. Without thrombokinase, phosphatide plus ionic calcium failed to activate chymotrypsinogen.

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Anticomplementary Effects and Complement Activity of Human Sera. (29561)

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The relationship between low complement (C′) activity and anticomplementary action of serum has been a source of considerable confusion. Pronounced anticomplementary action of fresh human serum is observed with a small percentage of sera, chiefly from patients with conditions associated with hyperglobulinemia(1). When unheated, these pathological sera are not anticomplementary since they invariably possess C′ activity. Anticomplementary action of such sera becomes manifest, however, only after the serum has been heat inactivated, usually at 56°C or above for 30 minutes. With the standard sheep cell hemolytic system, C′ activity can be found in most fresh unheated mammalian sera, although wide variations exist among different species. In humans, C′ levels below normal have been found among patients with acute glomerulonephritis(2), systemic lupus erythematosus(3), and even from a healthy person(4). In these individuals slight anticomplementary action may be independent of C′ titer, however, as exemplified in this

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report by a serum with pronounced anticomplementary action, but with a normal C′ level. A recent report by Laurell and Nilsson(5) indicating differences between the anticomplementary effects of isolated and heated gamma globulin from myeloma sera and normal gamma globulin provides a nice model for explaining some of the variations noted in this report between complementary activity and anticomplementary effects of different sera.

Materials and methods. Blood was collected by venipuncture, allowed to clot at room temperature, and the serum separated by centrifugation in the cold. Sera were stored for a few days at −60°C in a mechanically refrigerated freezer until tested for C′ activity. Sera from 15 healthy males, age 18 to 25, were pooled to provide a source of normal human C′ (HuC′). In addition, 3 normal sera were individually tested after heating to 52°C or 56°C for 30 minutes. These were designated Normal #1, #2, and #3. Sera from 2 acute glomerulonephritis patients having low C′ levels were designated Patient GN-A and GN-B. Serum from a systemic lupus erythematosus patient (SLE-C) also had a very low C′ titer. A fourth pathological serum, from Patient WB, possessed a high C′ level but the serum became very anticomplementary upon heating to 56°C. This patient has a record of several hospital admissions for gout, stable discoid lupus and organic heart disease but showed no evidence of multiple myeloma nor disseminated lupus erythematosus.

Hemolytic system. Sheep erythrocytes were collected, preserved, washed and sensitized as in(6). However, erythrocytes were standardized to contain 1.5 × 10^8 rbc/0.3 cc (approximately a 2% suspension) rather than the 10^9 rbc/cc as specified. The erythrocyte suspension was sensitized with an equal volume of an optimal dilution of rabbit hemolysin chosen to provide a suitable excess of antibody which will not cause agglutination of the erythrocytes.

Titration of C′. Hemolytic C′ titers were determined by a procedure adapted from a method used for the precise standardization of reagents for a complement fixation test(6). The probit transformation(7) was used to plot the results of each titration for calculation of titer in 50% hemolytic units of C′ per ml of serum ($C'H_{50}$/ml). Titers were expressed as the reciprocal of the amount of test serum (ml) required to lyse 50% of 1.5 × 10^8 optimally sensitized sheep erythrocytes in a reaction volume of 1.5 ml after 30 minutes of incubation at 37°C. Triethanolamine buffered saline (TBS) containing optimal amounts of Ca^{++} and Mg^{++} was used as the reagent diluent(6). Tests were performed in 12 × 75 mm cuvettes and optical density measurements were made at a wavelength of 550 mμ with a Coleman Model 6A spectrophotometer. An initial titration of unknown serum diluted 1:60 in TBS was made as illustrated in Table I.

TABLE I. Titration of Human Complement.

Reagent	Tube 1	2	3	4	5	Hemolysis controls 100%	0%
Unknown C′ (1:60)	.25	.30	.36	.43	.51	—	—
TBS	.65	.60	.54	.47	.39	—	.9
Distilled water	—	—	—	—	—	.9	—
Sensitized RBC	.6	.6	.6	.6	.6	.6	.6
Incubate at 37° for 30 min							
TBS	.5	.5	.5	.5	.5	.5	.5
Centrifuge at 1000 *g* for 5 min. Read optical density at 550 mμ.							

One hundred per cent hemolysis controls were made in triplicate to obtain a mean. It was necessary to decant the supernatant from the 0% hemolysis control and this supernatant was used as a blank. Optical density measurements of other supernatants were made without decanting and % hemolysis was calculated for each tube from the equation:

$$\%\ \text{hemolysis} = \frac{\text{Observed O.D.}}{\text{O.D. of 100\% controls}} \times 100$$

Percent hemolysis was plotted *vs* ml diluted C′ on logarithmic and probability graph paper (Keuffel & Esser Co. #358-22) as shown in Fig. 1. If 3 or more points fell within the range of 20-80% hemolysis the titer was computed directly from this plot by use of the relationship:

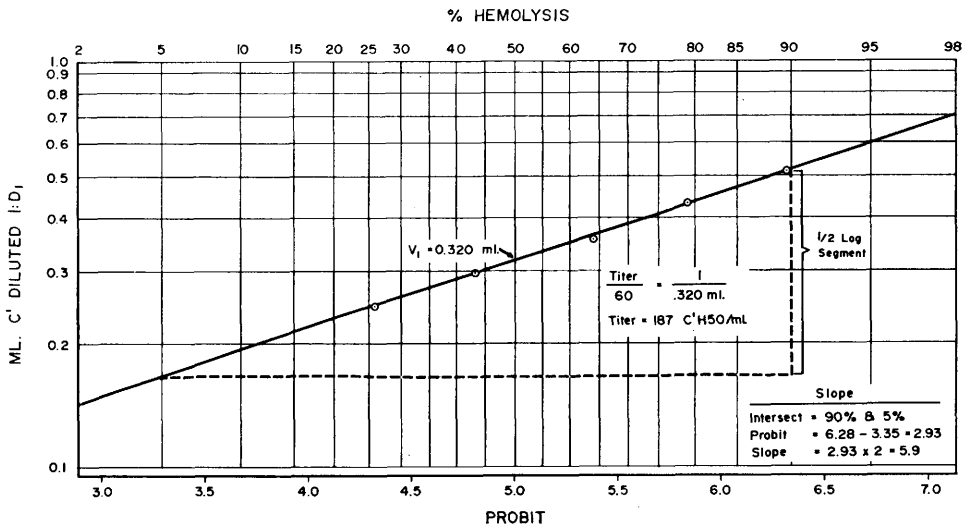

FIG. 1. Plot of percentage hemolysis against amount of C′.

$$\frac{\text{Titer}}{D_1} = \frac{1\text{ ml}}{V_1}$$

$$\text{Thus, Titer} = \frac{D_1}{V_1}$$

Where: Titer = number of 50% hemolytic units (C′H50) contained in 1 ml of undiluted test serum.

D_1 = dilution factor of serum, *i.e.* 60.

V_1 = ml diluted C′ giving 50% hemolysis (taken from plot).

An example of the plot of such a titration is given in Fig. 1.

If less than 3 points fell within the 20-80% hemolysis range the titration was repeated using an appropriate dilution of test serum. This was calculated from the results of the preliminary titration by use of the formula:

$$\frac{D_2}{D_1} = \frac{.360\text{ ml}}{V_1}$$

where: D_2 = dilution of C′ to use for second titration so that 0.36 ml yield approximately 50% hemolysis.

D_1 = dilution used in preliminary titration.

V_1 = ml diluted C′ which gives 50% hemolysis (taken from plot).

Most normal human sera have a titer in the range 150-250 C′H50/ml. A portion of a standard serum pool stored at −60°C was tested with each day's titration as a control. Reproducibility was found to be excellent, with only occasional variations greater than ± 10 C′H50 observed with the control serum. Such variations have sometimes been observed when the source of erythrocytes was changed.

Slope of the response was obtained graphically by noting probits corresponding to % hemolysis on the probit scale of the graph paper or by referring to the table of Bliss(8). The constructed line was extrapolated so that probit values for any measured ½ log segment were obtained. Using Fig. 1 as an example, the line crossed at 90% and 5% hemolysis. Subtracting the corresponding probit values (6.28-3.35) and multiplying by 2 gave a slope of 5.9. Normal human sera were found to generally have a slope between 5.5 and 6.5 whereas pathological sera sometimes varied noticeably from this range.

When titrations were performed on mixtures, *i.e.*, serum from patient WB plus HuC′, equal quantities of each serum were combined and diluted to an appropriate volume so that approximately 50% hemolysis was

TABLE II. Anticomplementary Activity of Serum from Patient WB (Gout, Discoid Lupus and Pleural Effusion) Appearing as a Result of Heating.

Serum	Complement titer	Slope (probit plot)
Normal human complement source (HuC′)	210	6.4
Unheated serum from patient WB	260	6.2
Idem + HuC′	464	6.3
Serum WB heated 1 min, 56°C + HuC′	260	5.3
" " " 5 " " "	135	5.3
" " " 10 " " "	<10	—
" " " 20 " " "	<10	—
" " " 30 " " "	<10	—
Serum WB heated 30 min, 37°C + HuC′	465	5.3
" " " " " 41°C "	435	5.4
" " " " " 45°C "	217	4.7
" " " " " 49°C "	200	4.4
" " " " " 52°C "	200	4.4
" " " " " 56°C "	<10	—
" " " " " 60°C "	<10	—
Serum WB heated 30 min, 56°C in presence of 9% bovine serum albumin + HuC′	257	5.3
Serum WB (diluted 1:50) after heating 30 min, 56°C + HuC′	70	3.6

obtained with 0.36 ml of the diluted mixture. Titrations were performed immediately after mixing.

Serum electrophoresis patterns were determined using a Spinco Model R paper strip apparatus and a Spinco Model RB densitometer. The protective effect of bovine serum albumin on C′ was determined by heating serum to 56°C for 30 minutes with an equal quantity of albumin (Armour Pharmaceutical Co. #2293) suspended to a concentration of 9% in 0.85% NaCl solution.

Results. Serum obtained from a patient with gout and discoid lupus (Serum WB) was found to become highly anticomplementary after heating to 56°C for 30 minutes. The effect of heating the patient's serum for various time periods and temperatures are given in Table II. This serum was nevertheless highly active when unheated (titer 260 compared to normal HuC′ with titer 210). Moreover, serum WB, when unheated, had no detectable deleterious effect on the normal HuC′, nor did the normal HuC′ affect the activity of serum WB since the combined activity (titer 464) was very nearly equal to the sum of the titers of each individual serum. Anticomplementary activity was evident after 5 minutes of heating at 56°C resulting in a drop in the titer of the normal HuC′ from 210 to 135, and after 10 minutes of heating it was capable of inactivating all detectable hemolytic activity in the added HuC′. Addition of serum WB heated to temperatures between 37 and 52°C to an equal quantity of unheated normal HuC′ did not result in titers significantly below that of unheated HuC′ alone. On the other hand, 60°C, like 56°C, caused a marked loss of HuC′ activity.

Paper strip electrophoresis of unheated serum from patient WB revealed an abnormally large proportion of gamma globulin. Results of integration of the protein strained pattern are given in Table III. No significant differences were seen between patterns obtained with heated (56°C, 30 minutes) and unheated serum.

It seems likely that the anticomplementary activity of this serum, detectable at a dilution of 1:50 (Table II), may be related not merely to its high content of gamma globulin, but to a peculiar molecular species of gamma globulin whose heat aggregation leads to a particularly potent C′ inactivation. Table II also shows that serum WB did not become anticomplementary when heated to 56°C in the presence of an equal quantity of 9% bovine serum albumin. Thus, the albumin appeared to inhibit the anticomplementary activity of gamma globulin(1).

Fresh, normal human serum does not become anticomplementary when heated to 56°C for 30 minutes (Table IV). Heated normal human sera #1, #2, and #3 did not depress the titer of HuC′ to which they were added. All of these sera possessed C′ levels within the normal range, and when added to

TABLE III. Electrophoretic Distribution of Serum Proteins from Serum WB.

Component	Patient WB	Normal human serum pool
γ-Globulin	26.8%	13.3%
β-Globulin	8.0	10.1
α_2-Globulin	13.0	8.6
α_1-Globulin	3.0	2.9
Albumin	49.2	65.1

TABLE IV. Heat Treatment of Normal Sera Producing No Anticomplementary Effect.

Serum	Complement titer	Slope (probit plot)
Human complement source (HuC′)	212	6.2
Normal human serum #1 unheated	218	6.2
" " " #2 "	231	6.1
" " " #3 "	182	6.3
Normal #1 heated 30 min 56°C + HuC′	233	5.9
" #2 " " " " "	261	5.8
" #3 " " " " "	233	6.0
Normal #1 unheated + HuC′	421	7.0
" #2 " "	469	6.5
" #3 " "	415	6.1

HuC′ without prior heating yielded a titer similar to the sum of the titers of the two.

Sera from 3 patients having very low C′ titers became slightly anticomplementary upon heating to either 52°C of 56°C for 30 minutes (Table V). A low slope (5.1) was noted also with serum from patient GN-A. When unheated, these sera were not anticomplementary and enhanced the C′ activity of added HuC′ very considerably. For example, serum of GN-B elevated the HuC′ titer from 177 to 240. Unlike sera from patient WB, sera from patients GN-A, GN-B, and SLE-C showed no significant variations from normal values in their electrophoretic pattern.

TABLE V. Anticomplementary Activity of Pathological Human Sera Having Low C′ Titers.

Serum	Complement titer	Slope (probit plot)
Human complement source (HuC′)	177	6.4
Patient GN-A (glomerulonephritis)	33	5.1
" GN-B "	<10	—
" SLE-C (systemic lupus erythematosus)	<10	—
" GN-A heated 30 min, 56°C + HuC′	163	5.0
" GN-B " " " " "	149	4.7
" SLE-C " " " " "	150	5.5
" GN-A heated 30 min, 52°C + HuC′	175	5.2
" GN-B " " " " "	167	4.8
" SLE-C " " " " "	152	5.2
" GN-A unheated + HuC′	234	3.4
" GN-B " "	240	4.5
" SLE-C " "	200	5.4

Discussion. A method for the spectrophotometric determination of C′ is presented. This technique has been used for 5 years in our laboratory and has proven to be quite satisfactory for determination of C′ levels of hospital patients. Use of the probit transformation to plot each titration yields 2 parameters: the C′H50 and the slope. This method was used to study an unusual serum from a patient with gout and discoid lupus erythematosus whose serum showed pronounced anticomplementary activity after heating to 56°C for 30 minutes. When inactivated at the usual temperature (56°C) this serum was highly anticomplementary and was thus unsuitable for testing in complement fixation procedures. When inactivated at 52°C for 30 minutes, however, this serum (patient WB) was not inordinately anticomplementary and did not react in a standard complement fixation test with calf thymus nucleoprotein antigen(6). We have found also with several other sera that an inactivation temperature of 52°C may be useful in avoiding the appearance of anticomplementary substances. However, an inactivation temperature of 52°C was not useful in eliminating anticomplementary action resulting from microbial contamination of serum nor with a serum from a patient with Waldenstrom's macroglobulinemia.

With 3 pathological sera possessing very low complement levels, slight anticomplementary activity was noted upon heating these sera to 52°C or 56°C for 30 minutes (Table V). This common finding is perhaps the basis for the opinion that sera with low C′ activity are lacking in C′ because of their inherent anticomplementary nature(3). Serum from patient WB demonstrated dramatically that a serum may become markedly anticomplementary and yet possess an abundant C′ titer. This result indicates that there need be no relationship between C′ activity and anticomplementary action resulting from heat aggregation. It indicates that certain molecular species of gamma globulin when unheated are not particularly anticomplementary and that the anticomplementary activity of such globulins results from heat aggregation. Earlier observations of Norgaard(9)

indicated that the anticomplementary property arose only after heating in some cases, but that it may be present in some "active" sera. Our results are in agreement with the former concept, but in general have indicated an additive complementary effect with "active" sera rather than any antagonism or synergism. Finally, the finding of Ishizaka *et al*(10) that heat-aggregated gamma globulin fixes complement in the same manner as antigen-antibody complexes suggests that anticomplementary sera act similarly.

On the other hand, serum lacking in detectable C′ activity need not be anticomplementary when heated (Table V). Thus, one can dissociate low C′ levels from anticomplementary activity. The reasons for the decline in C′ levels in systemic lupus erythematosus and glomerulonephritis is not known, but may include *in vivo* fixation, decreased production, increased breakdown or loss, or presence of an inhibitor(11). The lack of anticomplementary activity of sera with low levels of C′ may be presumed to exclude the presence of an inhibitor in these sera. Such an inhibitor, however, might be bound to one or more of the C′ components and unavailable for reactivity with exogenous C′.

Conclusion. The probit transformation for the plotting of immune hemolysis produced by measured amounts of fresh human serum was found to be suitable for titration of complement. Serum from a patient with a high level of C′ activity was found to become highly anticomplementary when heated to 56°C for 30 minutes, but inactivation by heating for 30 minutes at 52°C or below produced little anticomplementary activity in this serum. Addition of an equal quantity of 9% bovine serum albumin prior to heating also prevented the expression of the anticomplementary activity. Ability of serum to become highly anticomplementary as a result of heating is not necessarily associated with diminished activity of its endogenous complement. Conversely, sera with low levels of C′ may not become anticomplementary as a result of heating.

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Comparison of Measurements of Renal Function by an External Monitoring Technique and Renal Clearances. (29562)

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Recently there has been increasing use of I^{131}-labelled organic acids for evaluating renal function by external counting techniques (1-7). Clinical evidence of renal disease appears to correlate qualitatively with tracings obtained by monitoring over the renal areas following injection of I^{131}-iodohippurate. However, no studies have attempted to correlate quantitative data obtained by external monitoring with glomerular filtration rate or renal plasma flow measured by conventional clearance techniques.