In the digested smears the cytoplasm of the 12 basophiles studied was almost transparent and without color. It is therefore concluded that the material in the basophilic leucocytes which stained red in leucofuchsin after periodic acid is glycogen. Furthermore, since glycogen can be demonstrated in the human basophilic leucocytes, they do "differ from mast cells which contain periodic acid-Schiff positive material which is insoluble in saliva." (Wislocki, Rheingold and Dempsey[1]).

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A Quantitative Method for Measuring Staphylococcal Anticoagulase.* (17382)

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The study of infections produced by *Staphylococcus aureus* in man or in experimental animals has been hampered by the lack of a suitable serological test. Since staphylococcal coagulase will stimulate the production of an inhibitor substance, termed anticoagulase,[1,2] it has been possible to devise a serological test that could be used in the study of infections caused by this organism.

The mechanism whereby staphylococcal coagulase clots plasma has been partially clarified by the recent work of Smith and Hale,[3] Kaplan and Spink,[4] and Tager.[5] Most strains of *S. aureus* produce an extracellular substance, coagulase, which, added to plasma, results in the formation of a clot. Coagulase does not clot fibrinogen directly, for it has been found that a second factor, termed activator[3] or reacting factor,[5] is also required. This substance is found in the blood of man and of certain animal species. Although it is not entirely clear at the moment whether coagulase, activator, or a product of these two substances is responsible for the clotting of fibrinogen, antibodies against coagulase develop following immunization with cell-free coagulase. By employing measured amounts of coagulase, fibrinogen and activator, it has been possible to develop a quantitative method for the measurement of coagulase inhibitor, anticoagulase.

Materials and methods. Buffered Saline. A solution of 0.01 M phosphate in 0.85% sodium chloride at a pH of 7.4 was prepared for dilution of all sera and for preparation of fibrinogen, activator and heparin solutions.

Activator. The source of activator was 300 ml of human serum from one donor. This standard serum was stored in glass containers containing 50-75 ml at 4°C.

Two strengths of activator were employed. For standardization of coagulase, a final dilution of 1:3500 of the serum in a solution of fibrinogen was employed. In the coagulase inhibition test a dilution of standard serum of 1:1000 was used.

Heparin. A solution of heparin containing 100 mg per 10 ml of buffered saline solution was prepared and stored at 4°C until used. A 1:100 dilution of this was added to the fibrinogen-activator mixture.

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1 Rammelkamp, C. H., *Am. J. Med.*, 1948, **4**, 782.

2 Tager, M., and Hales, H. B., *J. Immunol.*, 1948, **60**, 475.

3 Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, **25**, 101.

4 Kaplan, M. H., and Spink, W. W., *Blood*, 1948, **3**, 573.

5 Tager, M., *Yale J. Biol. and Med.*, 1948, **20**, 269.

Coagulase. Cell-free coagulase was prepared as follows: From 5-10 ml of an overnight culture of a coagulase-positive strain of *S. aureus* in tryptose broth were placed in a flask containing 500 ml of tryptose broth to which 10 ml of bovine albumin† had been added. This broth culture was incubated for 72 hours at 37°C, following which the cells were sedimented in a refrigerated centrifuge for 2 hours at 2,000 RPM. The supernate was removed and stored at 4°C overnight. The supernate was then passed through a Seitz filter, the first 50 ml being discarded. The filtrate was tested for sterility and stored in the frozen state in ampules. At the time of use it was thawed rapidly and the necessary dilutions made with sterile tryptose broth.

Fibrinogen. For standardization of coagulase a solution of 100 mg of Fraction I (bovine fibrinogen‡) in 63 ml of buffered saline solution was employed. After incubation at 37°C for 30 minutes with occasional agitation, 1 ml of the 1:100 dilution of heparin was added.

To 50 ml of this solution was added 0.1 ml of the standard activator. Using volumetric pipettes, this was further diluted with the fibrinogen solution so that the final concentration of activator was 1:3500.

For the coagulase-inhibition test, 100 mg of fibrinogen was dissolved in 31 ml of buffer and following incubation at 37°C for 30 minutes, 1 ml of 1:100 heparin was added. Activator was added to this solution so that there was a final concentration of 1:1000.

Serum. Blood was collected and allowed to clot at room temperature. After separation, the serum was stored either at −20°C or at 4°C. The day before testing, 0.25 ml of sera were placed in small tubes and inactivated in a water bath at 60°C for 20 minutes. The sera were then stored at 4°C for 60 minutes and again inactivated at 60°C for 20 minutes. To each serum was added 6 ml of buffered saline solution and the solution was stored at 4°C overnight.

Standardization Procedure. A preliminary standardization of the coagulase preparation is performed by making serial two-fold dilutions in 1 ml volumes, using tryptose broth as a diluent. To each tube is added 1 ml of the fibrinogen solution containing 1:3500 activator. The rack containing the tubes is shaken, and placed in the 37°C water bath for 3 hours. The preliminary titer is then read as the highest dilution of coagulase which results in the formation of a visible clot.

The final titration employs the proper dilution of coagulase in tryptose broth as indicated by the preliminary titration. The coagulase is added to a duplicate series of 6 tubes in decrements of 0.1 ml. Tryptose broth is added to bring the volume up to 1 ml.

One ml of the fibrinogen standard is added, and incubation carried out as above. A unit of coagulase is then defined as the smallest amount which results in the slightest visible shred of fibrin after 3 hours' incubation. Reading of the test is facilitated by the use of a hand lens.

Coagulase-inhibition Test. Previous studies[6,7] have indicated that the union of coagulase and inhibitor is practically complete after 90 minutes' incubation at 37°C. The procedure employed for the titration of anticoagulase includes a preliminary and a fine titration as follows: to a series of 10 x 125 mm tubes containing 1 ml of various dilutions of serum previously inactivated as described above, is added 0.5 ml of coagulase containing 1 unit. After shaking, the mixtures are incubated at 37°C for 90 minutes and 0.5 ml of fibrinogen solution containing 1:1000 activator and heparin is added with an automatic pipette. The tubes are placed again in a water bath at 37°C for 3 hours. The titer of anticoagulase is read as the greatest dilution (initial) of serum which completely inhibits the formation of any visible shred.

In the preliminary titration the initial dilutions of serum employed are 1:25, 1:125, and 1:625. The final titer is then determined using 4 or 5 tubes of the proper dilutions as indicated in Table I.

Accuracy. To establish the accuracy of the

† Armour and Co.

‡ The authors wish to thank Armour and Co., Chicago, Ill., for supplying this product.

[6] Lominski, I., and Roberts, A. B. S., *J. Path. and Bact.*, 1946, **58**, 187.

[7] Rammelkamp, C. H., unpublished observations.

TABLE I.
Method for Preparing Preliminary and Fine Titrations of Sera for Anticoagulase Titrations.

Preliminary dilution of serum	Serum, ml	Buffered saline, ml	Initial dilution
1/25	1.0	0	25
0.25 ml of serum	0.7	0.3	36
+	0.45	0.55	56
6.0 ml of buffered saline	0.3	0.7	83
1/125	1.0	0	125
1.0 ml of 1/25 serum	0.7	0.3	179
+	0.45	0.55	278
4.0 ml of buffered saline	0.3	0.7	417
1/625	1.0	0	625
0.5 ml of 1/25 serum	0.7	0.3	900
+	0.45	0.55	1400
12.0 ml of buffered saline	0.3	0.7	2100

test 4 control sera are included in all titrations of anticoagulase. The results of repeated tests on 2 sets of control sera are shown in Table II. Titers of sera run on dffierent days varied only 2 dilution increments. It would appear that acute and convalescent sera exhibiting a difference in titer of 2 or more dilution increments, as determined on a single titration, represent a true difference in inhibitory substance. Occasionally it may be desirable to determine the amount of antibody in dilutions less than 1:25; in such cases the reproducibility of the titers is somewhat less accurate.

Discussion. The serological test described in the present report has been used for the past 2 years and numerous sera have been examined. The technic is simple; 100 or more sera may be titrated in one day. The test has the advantage that the various reacting substances are carefully controlled. A fixed amount of coagulase is allowed to react with dilutions of the serum to be tested, following which known amounts of fibrinogen and activator are added. The end-points are reproducible and easily read.

Living cultures of *S. aureus* have usually been employed in attempts to demonstrate an antibody to coagulase. Such a procedure precludes a quantitative measurement of the inhibitor since variable amounts of coagulase are produced during incubation of the plasma-coagulase mixtures. Cell-free coagulase may be obtained by heating or filtering cultures of *S. aureus*[8] or by the addition of bacteriostatic agents.[9] Variable results have been obtained as regards the resistance of coagulase to heat and filtration.[8] Acid cultures yield no coagulase upon heating, and alkaline cultures yield little or no coagulase on filtration. Lominski and Roberts[6] employed broth containing 10% plasma and found that coagulase would then pass through a filter. Originally we employed plasma in the culture medium, but later it was found that filtrates from such cultures clotted purified fibrinogen directly, indicating that a significant and variable amount of activator from the plasma remained in the culture filtrate. Since the measurement of coagulase is dependent upon the interaction of activator and coagulase, plasma cannot be used in the culture medium. For this reason bovine albumin was employed, and it was determined that coagulase became filterable in the presence of albumin.

Activator was obtained from one source: human serum from one donor known to be free of a coagulase inhibitor. Because this serum was capable of clotting plasma or fibrinogen solutions directly, heparin was added to the system. Experience has shown that most animal sera and an occasional human serum will clot fibrinogen directly. In the titration of antibody, sera to be tested are inactivated in order to remove the activator or reacting substance. It is important that inactivated

8 Lominski, I., and Milne, J. A., *J. Path. and Bact.*, 1947, **59, 516.**

9 Tager, M., and Hales, H. B., *Yale J. Biol. and Med.*, 1947, **20**, 41.

TABLE II.
Repeated Titrations of Anticoagulase of Control Sera.

Serum	Anticoagulase titer										
1	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
2	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
3	278	417	278	278	278	179	179	417	417	278	417
4	278	278	278	278	278	179	278	417	417	278	278
A	179	125	179	125	179	—	125	—	—	—	—
B	900	900	900	900	900	1400	900	900	900	900	900
C	278	278	417	278	417	417	278	278	278	—	—
D	417	278	417	278	417	417	417	—	—	—	—

Anticoagulase titers of sera 1, 2, 3, and 4 were determined on different days between 7/15/48 and 8/2/48.

Sera A, B, C, and D were used as controls between 8/26/48 and 1/29/49.

sera be employed in the antibody titration within 24 hours since within 48 hours small amounts of activator may again be detected.

The system used for measuring inhibitor to coagulase employs 0.5 ml of fibrinogen containing 1:1000 activator, whereas in the titration of coagulase, 1.0 ml of fibrinogen containing a dilution of 1:3500 activator is used. This procedure of increasing the activator concentration in the antibody titrations was employed so that technical variations in the amounts of coagulase or activator added, or residual amounts of activator remaining in the serum being tested, would not alter the end-point appreciably. The present test, as compared to that devised by Lominski and Roberts,[6] controls the concentration of activator in the fibrinogen and therefore more reproducible results should be obtained.

The stability of the various reagents used in this test is not definitely known. The fibrinogen solutions should be employed within a few hours of preparation. When stored at –20° or 4°C in sealed ampules, coagulase retains most of its activity over a period of a year or more, but repeated standardizations are best performed at monthly intervals. Tryptose broth is added as a diluent to the coagulase because it appears to stabilize its action so that results are reproducible. Activator, when stored in sealed ampules at –20°C or –4°C or at 4°C in rubber stoppered flasks for over a year, appeared to have lost little activity. When stored at room temperature, however, a loss of activity was observed.

Summary. A satisfactory serological test for the titration of anticoagulase in human and animal sera has been devised by controlling the various factors which enter into the coagulase-anticoagulase reaction.

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Effect of Dibuline* on Nocturnal Gastric Secretion in Man. (17383)

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It has been shown[1-5] that dibuline (dibutyl urethane of dimethyl ethyl-B-hydroxyethyl ammonium sulfate, Merck) has a qualitative effect similar to that of atropine.

* Supplied by Dr. M. I. Grossman, University of Illinois, Chicago, Ill.

1 Swan, K. C., and White, N. G., *J. Pharm. and Exp. Therap.*, 1944, **80**, 285.

2 Featherstone, R. M., and White, N. G., *J. Pharm. and Exp. Therap.*, 1945, **84**, 105.

3 Peterson, C. G., and Peterson, D. R., *J. Pharm. and Exp. Therap.*, 1945, **84**, 236.