STAPHYLOCOCCAL CLUMPING WITH SOLUBLE FIBRIN MONOMER COMPLEXES*

By B. LIPIŃSKI, Ph.D., J. HAWIGER, M.D., and J. JELJASZEWICZ, Ph.D.

(From the Department of Bacteriology, State Institute of Hygiene, Warsaw, Poland)

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Most coagulase-positive staphylococci exhibit an ability to clump in plasma or fibrinogen solution. This phenomenon, discovered by Much (1), is attributed to a factor located on the cell surface and called clumping factor (2). This designation seems to be preferable to the others used before ("plasma agglutination," "bound coagulase"), for the latter terms suggest an immunological or blood-clotting phenomena involvement and this is not the case.

Most of significant work on the nature of clumping factor was done by Duthie (2, 3). He demonstrated clearly the separateness of staphylocoagulase (SC) and clumping factor (CF) by pointing out the direct action of clumping factor on fibrinogen and its antigenic distinction from staphylocoagulase. According to Duthie (2), clumping factor is a peptide complex which is heat-resistant, stable within a wide pH range, and sensitive to some proteolytic enzymes. Staphylococcal clumping occurs in two stages, the first being an adsorption of fibrinogen phase within pH 2–11, whereas the second concerns clumping within pH 5.1–10.0 only. Duthie (2) demonstrated that as small amounts of normal or denatured fibrinogen as 1 mg/liter are sufficient to produce clumping of staphylococci. He also obtained antisera against clumping factor, as later confirmed by Kato and Omori (4) and Rotter and Kelly (5).

Clumping factor detection was applied as a so-called "slide coagulase test" for replacement of the tube coagulase test, and this technique is well described (6–9). Another method for clumping factor detection, described by Finkelstein and Sulkin (10) and Alami and Kelly (11), is based on the appearance of different colonies of staphylococci producing staphylocoagulase (diffuse) and clumping factor (compact) in soft agar containing albumin and plasma supplemented with fibrinogen.

Identification of techniques for SC and CF detection should be considered unsuitable, however, because both factors clearly concern different phenomena. Moreover, the existence of SC+ and CF− staphylococcal strains and vice versa has been demonstrated by some authors, as for instance in a large survey of strains isolated from different sources (12).

However, because the understanding of clumping factor reaction is poor, it has been

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questioned whether clumping factor and staphylocoagulase are separate entities (13–15). The situation became even more obscure when Brown and Faruque (16) reported that clumping of staphylococci may occur in sera and fibrinogen-free plasmas.

We have attempted to elucidate the nature of the staphylococcal clumping factor reaction and of its substrate. This report presents results postulating that clumping of staphylococci occurs with soluble fibrin monomer complexes.

Materials and Methods

Reagents.—Heparin, 2-mercaptoethanol, mucin, and protamine sulfate were obtained from Light Co., Colnbrook, England; merthiolate from Eli Lilly and Co., Indianapolis, Ind. All other reagents used were of local origin and of analytical grade.

Enzymes and Inhibitors.—Plasmin was a commercial preparation (Lysofibrin, Novo Laboratories, Copenhagen, Denmark). Thrombin (bovine) and streptokinase (Distreptase) were products of Warsaw Serum and Vaccines Laboratory. Soybean trypsin inhibitor (STI) was supplied by Sigma Chemical Co., St. Louis, Mo.

Plasma and Sera.—Human plasma was obtained by collecting venous blood into 0.1 M sodium citrate (1:10). Sera were obtained by separation from clots after incubation of fresh blood with 1:10 volume of saline, STI (1 mg/ml), and streptokinase (1000 units/ml) at 37°C for 90 min.

Fibrinogen.—Purified fibrinogen was prepared from citrated bovine plasma according to Kekwick et al. (17).

Fibrin Lysate.—To 6 ml of 1.5% fibrinogen in pH 7.4 tris buffer, 1 ml (10 mg) of plasmin was added. This was immediately followed by addition of 0.4 ml (500 units/ml) of thrombin solution. The mixture clotted within 5 sec. Clot lysis occurred after 10 min of incubation at 37°C. Plasmin action was stopped by soybean trypsin inhibitor (final concentration 0.2 mg/ml) at 14 min of digestion.

When necessary, the fibrin lysate was heated at desired temperatures in a water bath, centrifuged, the precipitate discarded, and the supernatant used for determinations.

Paracoagulation Reaction with Protamine Sulfate (PS).—0.9 ml of the tested sample was mixed with 0.1 ml of 1% PS. The precipitate was washed with tris buffer and dissolved in 40% urea with 0.2 N NaOH. Protein concentration was measured by determination of optical density of the alkaline urea solutions at 280 mμ. Standard curve was prepared with solutions of purified fibrinogen.

Bacterial Strains.—Strains of *Staphylococcus aureus* were obtained from the following sources: Smith compact and Smith diffuse from Dr. D. E. Rogers, Vanderbilt University School of Medicine, Nashville, Tenn.; 524 from Dr. H. J. Rogers, National Institute for Medical Research, London, England; Newman D-2-C from Dr. E. S. Duthie. Strain Zak of *Staphylococcus epidermidis* was isolated from a case of human conjunctivitis. The bacteria were grown in 40 ml aliquots of Hartley broth at 37°C for 18 hr, centrifuged, and suspended in 1 ml amounts of tris buffer.

Coating of Bacteria with Protamine Sulfate.—Staph. epidermidis Zak, prepared under conditions described above, was suspended in 1 ml of 1% solution of protamine sulfate. The suspension was incubated for 20 min at 37°C and centrifuged. The bacterial sediment was washed several times with distilled water, until no PS was present in washings, as controlled by paracoagulation reaction with fibrin lysate. The coated bacteria were finally suspended in 1 ml of tris buffer.

Standard Bacterial Suspension.—Strain Newman D-2-C was grown in Hartley broth at 37 °C for 18 hr. The bacteria were centrifuged, suspended in water, and heated for 90 min at 60° C. After centrifugation, the bacterial sediment was washed three times with water and freeze-dried. Control tests revealed no viable cells.

Standard bacterial suspension was freshly prepared by suspending 10 mg of lyophilized bacterial cells in 1 ml of pH 7.4 tris buffer. The amount of bacterial cells in 1 mg was 2.1×10^8, as calculated from viable counts performed before heating.

Clumping Reaction (CR) Test.—Standard bacterial suspension (0.04 ml) was mixed on a slide with 0.04 ml of a tested solution. The slide was then gently rocked for 2 min. Clumping occurring within that time was considered a positive result. The control test employed standard bacterial suspension and tris buffer.

TABLE I

Reaction of Staph. aureus Newman D-2-C Strain Clumping Factor with Various Substrates

Substrate	Unheated	Heated 56°C, 10 min	Heated 60°C, 10 min
Human citrated plasma	+	+	−
Human serum	+	+	−
Human serum, heated at 60° + fibrin lysate	+	+	−
0.3% bovine fibrinogen	+	−	−
Bovine fibrin lysate	+	+	−

TABLE II

Clumping Reaction of Different Staphylococcal Strains with Fibrin Lysate

Strain	Fibrin lysate		
	Unheated	Heated 56°C, 10 min	Heated 60°C, 10 min
Smith diffuse (SC+, CF−)	−	−	−
Smith compact (SC+, CF+)	+	+	−
Newman D-2-C (SC−, CF+)	+	+	−
524 (SC+, CF+)	+	+	−
Staph. epidermidis Zak (SC−, CF−)	−	−	−
As above, coated with protamine sulfate	+	+	−

SC, staphylocoagulase; CF, clumping factor.

The clumping titer is a reciprocal of the highest dilution of the tested substrate, giving positive clumping reaction with standard bacterial suspension.

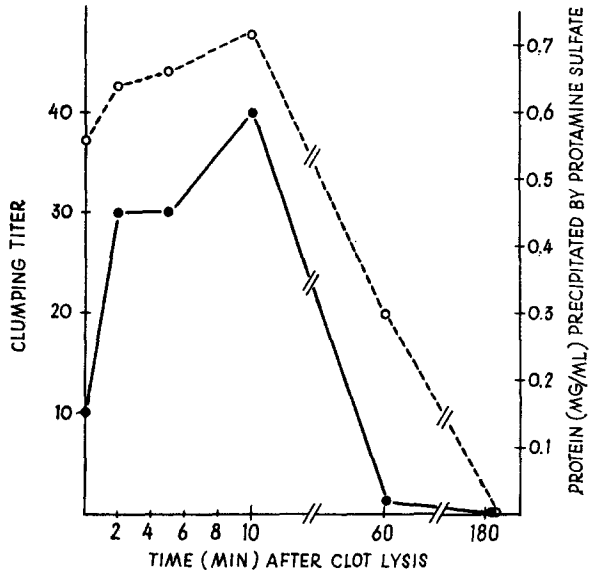
RESULTS

Demonstration of a Substrate for Clumping Reaction (CR).—It is evident (Table I) that clumping reaction of Newman D-2-C standard suspension occurs not only in purified bovine fibrinogen solution, but also in fibrin lysate and human serum. Moreover, this reaction is positive in supernatants of fibrin lysate, human plasma, and human serum heated for 10 min at 56°C. On the contrary, 0.3 % purified fibrinogen heated under the same conditions is completely devoid of ability to produce clumping reaction.

Clumping reaction is negative when all these systems are heated for 10 min

at 60°C. CR in serum treated as above occurs when fibrin lysate is added. Clumping reaction with various substrates is shown in Fig. 1.

The specificity of clumping reaction with fibrin lysate is indicated by the data presented in Table II. In this system, fibrin lysate and different staphylococcal strains were used. Reaction is positive when staphylococci possess clumping factor regardless of staphylocoagulase production. Also in this case,



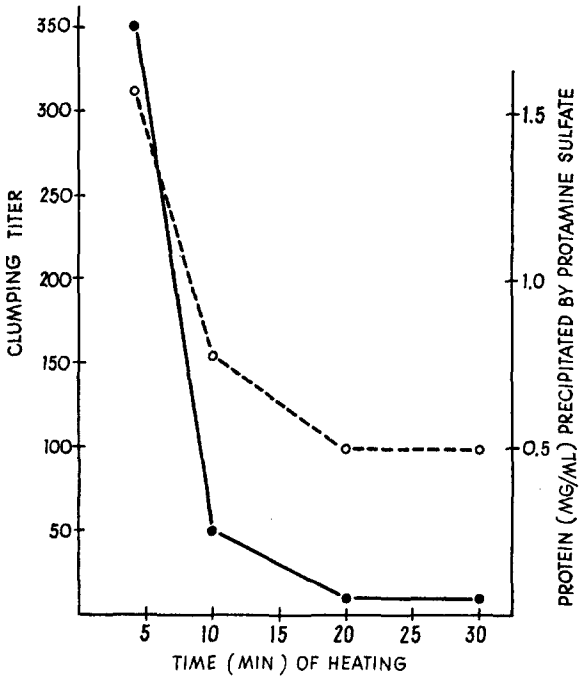
TEXT-FIG. 1. Clumping reaction (solid line) in fibrin lysate at various stages of plasmin digestion in comparison with protamine sulfate paracoagulation (dashed line).

heating of fibrin lysates at 56°C had no effect on clumping reaction. However, CR was negative after heating of lysates for 10 min at 60°C.

When a strain is SC− and CF−, positive clumping reaction can be induced by coating such bacteria with protamine sulfate.

Kinetics of Substrate Formation during Proteolysis of Fibrin Clot.—The results (Text-fig. 1) indicate that the substrate for CR is formed during proteolysis of fibrin clot by plasmin. The titer of this substrate depends on time of proteolysis, reaches maximum 10 min after fibrin clot lysis and becomes negative after 180 min. It is noteworthy that CR-substrate formation, as measured by clumping titer, is parallel to the amount of protein "paracoagulated" by protamine sulfate. At the stage of fibrin lysate digestion at which CR is negative, no protein is precipitated by protamine sulfate. Regardless of the duration of proteolysis, heating at 60°C destroys the ability of substrate to produce a positive clumping reaction.

Effect of Heating and of Various Substances on the Clumping Reaction.—It was found that temperature has a significant effect on ability of fibrin lysate to clump staphylococci. Thermostability of substrate at 56°C is time dependent. As shown in Text-fig. 2, the titer of clumping was 1:350 after heating for 4 min at 56°C; it decreased to 1:50 after heating to 10 min and reached 1:10 after 20 min. At the same time, protamine sulfate-precipitable protein content also fell from 1.56 mg/ml to 0.50 mg/ml.



TEXT-FIG. 2. Effect of heating of fibrin lysate at 56°C for various lengths of time on clumping (solid line) and paracoagulation (dashed line) reactions. Digestion was stopped 5 min after clot lysis.

Heating at 60°C completely destroyed the ability of fibrin lysate to produce staphylococcal clumping reaction and "paracoagulation" with protamine sulfate.

None of the tested substances, such as heparin, sodium citrate, 2-mercaptoethanol, 3 M urea, merthiolate, or mucin, inhibited CR in undiluted fibrin lysate (Table III). However, "paracoagulation" reaction with protamine sulfate was prevented by heparin and 2 M urea.

Clumping Reaction in Human Serum.—In order to determine the conditions of appearance of a CR substrate in serum, whole fresh blood was collected in

saline, streptokinase, and soybean trypsin inhibitor. CR titers in sera obtained under above conditions are presented in Table IV. The highest titer was found in serum derived from blood collected in streptokinase. Soybean trypsin inhibitor had little effect on formation of CR substrate in normal serum. The CR-substrate formed after streptokinase activation of the fibrinolytic system is diminished after heating at 56°C and is completely eliminated at 60°C.

TABLE III

Effect of Various Substances on Clumping Reaction and Paracoagulation with Protamine Sulfate

Substance	Clumping reaction	Paracoagulation*
Heparin		
10 mg/ml	+	−
1 mg/ml	+	−
Sodium citrate		
1 M	+	+
10^{-1} M	+	+
Mercaptoethanol 10^{-1} M	+	+
Urea		
1 M	+	+
2 M	+	−
3 M	+	−
Merthiolate 1:10,000	+	+
Mucin 5%	+	+

* Protamine sulfate.

TABLE IV

Clumping Reaction with Human Serum Obtained under Different Conditions

Substance added to blood before clotting started	Clumping titer		
	Unheated	Heated 56°C, 10 min	Heated 60°C, 10 min
Saline	1:10	1:10	Negative
Streptokinase	1:150	1:100	Negative
Soybean trypsin inhibitor	1:10	Undiluted	Negative

DISCUSSION

Results of the present work show that ability to produce a positive clumping reaction is a property not only of purified fibrinogen preparations, but also of soluble forms of fibrin monomers. Fibrin monomers represent fibrinogen molecules devoid of fibrinopeptides. Thus, splitting only one out of four fibrinopeptides (two A and two B) leads to transformation of fibrinogen into fibrin monomers. At appropriate concentration, fibrin monomers polymerize resulting in a fibrin clot. It is known (18) that fibrinogen degradation products form soluble complexes with fibrin monomers. Moreover, a similar type of

fibrin monomer complexes are formed at an early stage of fibrin clot lysis by plasmin.[1] Shainoff and Page (19) have found that fibrinogen forms a complex with fibrin monomers deprived of fibrinopeptides A only. By this technique, fibrin monomers are kept in solution either by complexing with fibrinogen or with fibrin degradation products. Complexes of fibrin monomers with fibrinogen are thrombin-clottable, whereas those formed with fibrin degradation products are thrombin-unclottable. A common feature of these fibrin monomer complexes is their ability to exhibit "paracoagulation" with protamine sulfate.[2]

The model system for testing the clumping reaction with substrate other than purified fibrinogen was the fibrin lysate. Such lysate is solubilized, unclottable by thrombin, and precipitable by protamine sulfate. In contrast to results obtained with 0.3% purified fibrinogen, heating the lysate for 10 min at 56°C preserved clumping and paracoagulation reactions. At this temperature, fibrinogen is completely and irreversibly denatured (21). In a supernatant of 0.3% purified fibrinogen, no clumping and paracoagulation occurs after heating at 56°C. The thermostability of fibrin lysate at 56°C depends on duration of heating. This fact can be explained as the protective effect of fibrin degradation products on fibrin monomer molecules. The thermostability of fibrin lysate is probably related to the optimal ratio of fibrin monomers and fibrin degradation products. Such complexes with optimal ratio are resistant to heating at 56°C even for 30 min. Other complexes precipitate at this temperature. All types of fibrin monomer complexes precipitate after heating at 60°C for 10 min, thus eliminating clumping and paracoagulation reactions.

The titer of clumping reaction in fibrin lysate during proteolysis varies with time of digestion and follows the amount of protein precipitable by protamine sulfate. Prolonged digestion of fibrin monomer complexes leads to formation of fibrin degradation products.[1] Such products neither give clumping nor paracoagulation reaction. It seems, therefore, that soluble fibrin monomer complexes are the substrate for both staphylococcal clumping and protamine sulfate paracoagulation. Clumping reaction cannot therefore be used for titration of fibrinogen in body fluids as suggested by Duthie (2) and applied by Kapral (22).

Clumping reaction in fibrin lysate is specifically dependent on the presence of clumping factor in staphylococci. Clumping reaction is positive only with the staphylococcal strains that possess this factor. Staphylocoagulase has no relation to this reaction. However, *Staph. epidermidis* strain Zak (SC−, CF−) produces positive clumping reaction when the bacterial cells are coated with protamine sulfate. It can be assumed, therefore, that staphylococcal clumping factor possesses paracoagulating properties.

The specificity of action of staphylococcal clumping factor and protamine

[1] Lipiński, B. Data to be published.

[2] Paracoagulation was described by Derechin and Szuchet (20). It consists of the formation of a clotlike precipitate induced by protamine sulfate in thrombin-unclottable solutions of partially digested fibrinogen.

sulfate is different. Clumping reaction is insensitive to heparin and 3 M urea, and thus differs from the protamine sulfate–induced paracoagulation. Merthiolate has no effect on CR in fibrin lysate, which is consistent with the observations of Duthie (3) concerning CR in merthiolate-treated plasma. Lack of sensitivity of clumping and paracoagulation reactions to 2-mercaptoethanol indicates that both phenomena proceed without involvement of labile S—S bonds. Clumping reaction and paracoagulation by protamine sulfate are not inhibited by 0.01 M sodium citrate. It has been recently found that platelet factor 4 (PF_4) is a very potent paracoagulating factor. Paracoagulation reaction induced by PF_4 is not affected by heparin, but is strongly inhibited by EDTA and sodium citrate (23).

Occurrence of clumping in purified fibrinogen preparations cannot be fully explained. There are two possibilities. One is connected with the presence of fibrin monomer complexes with fibrinogen, as described by Shainoff and Page (19); the other is that clumping reaction is related to the intact molecule of fibrinogen. In the latter case, a reactive site for clumping reaction would be located at a position other than the polymerization center. Thus, clumping reaction would require the presence of either an intact molecule of fibrinogen or of fibrin monomer. This problem can be solved with the application of a fibrinogen preparation free of fibrin monomers. Attempts to obtain such a preparation have been so far unsuccessful.

Occurrence of clumping reaction in plasma may originate also from the two mechanisms mentioned above. However, since heating of plasma at 56°C eliminates the effect of fibrinogen, the thermostable activity of heated plasma enabling positive clumping reaction should be considered as indicating the presence of soluble fibrin monomer complexes. The source of these complexes can be intravascular, most probably resulting from clotting followed by fibrinolysis. This possibility under physiological conditions is still controversial (24, 25).

The explanation of clumping reaction in serum may be as follows: Firstly, soluble fibrin monomer complexes of plasma, unclottable with thrombin, may remain in serum as a result of spontaneous blood clotting. Secondly, such complexes may be released during fibrin clot lysis. This is especially possible in the state of activated fibrinolysis. Activation of fibrinolysis in vitro by streptokinase increases the CR titer in serum. The present data explain the findings of other authors (16) concerning the clumping of staphylococci in serum.

Clumping reaction, according to Kapral (22), plays a role in phagocytosis during experimental staphylococcal peritonitis. Clumps of unencapsulated staphylococci are not digested by leukocytes, but are surrounded by them thus preventing alpha-toxin release. In view of findings reported in this paper, it cannot be concluded what the substrate for clumping reaction is in the mouse peritoneum. It is quite possible that the exudate contains soluble fibrin mono-

mer complexes rather than fibrinogen. As shown in this paper, mucin—a substance which enhances staphylococcal virulence in mouse peritoneum—does not inhibit the clumping reaction. It seems that in inflammatory reaction with formation of exudates, staphylococci may be clumped even in the absence of fibrinogen, provided soluble, thrombin-unclottable complexes of fibrin monomers are present. Clumping phenomenon may therefore indicate a role for blood clotting and fibrinolytic mechanisms in staphylococcal infection.

SUMMARY

Clumping reaction, using standard suspension of *Staph. aureus* Newman D-2-C strain and various substrates, was quantitatively tested. It has been shown that clumping occurs in fibrin lysate containing soluble fibrin monomer complexes unclottable by thrombin. The reaction was positive with staphylococcal strains possessing clumping factor regardless of staphylocoagulase production. Clumping reaction is similar to paracoagulation reaction induced by protamine sulfate. The substrate for both reactions is stable at 56°C but is destroyed at 60°C. The kinetics of substrate formation for both reactions during fibrin clot lysis is also similar. Clumping reaction with a strain of *Staph. epidermidis* possessing no clumping factor was positive when these bacteria were coated with protamine sulfate. The effect of heparin, sodium citrate, urea, 2-mercaptoethanol, merthiolate, and mucin on both reactions was tested. The present findings explain the clumping reaction in serum and emphasize the role of blood clotting and fibrinolytic systems in this phenomenon.

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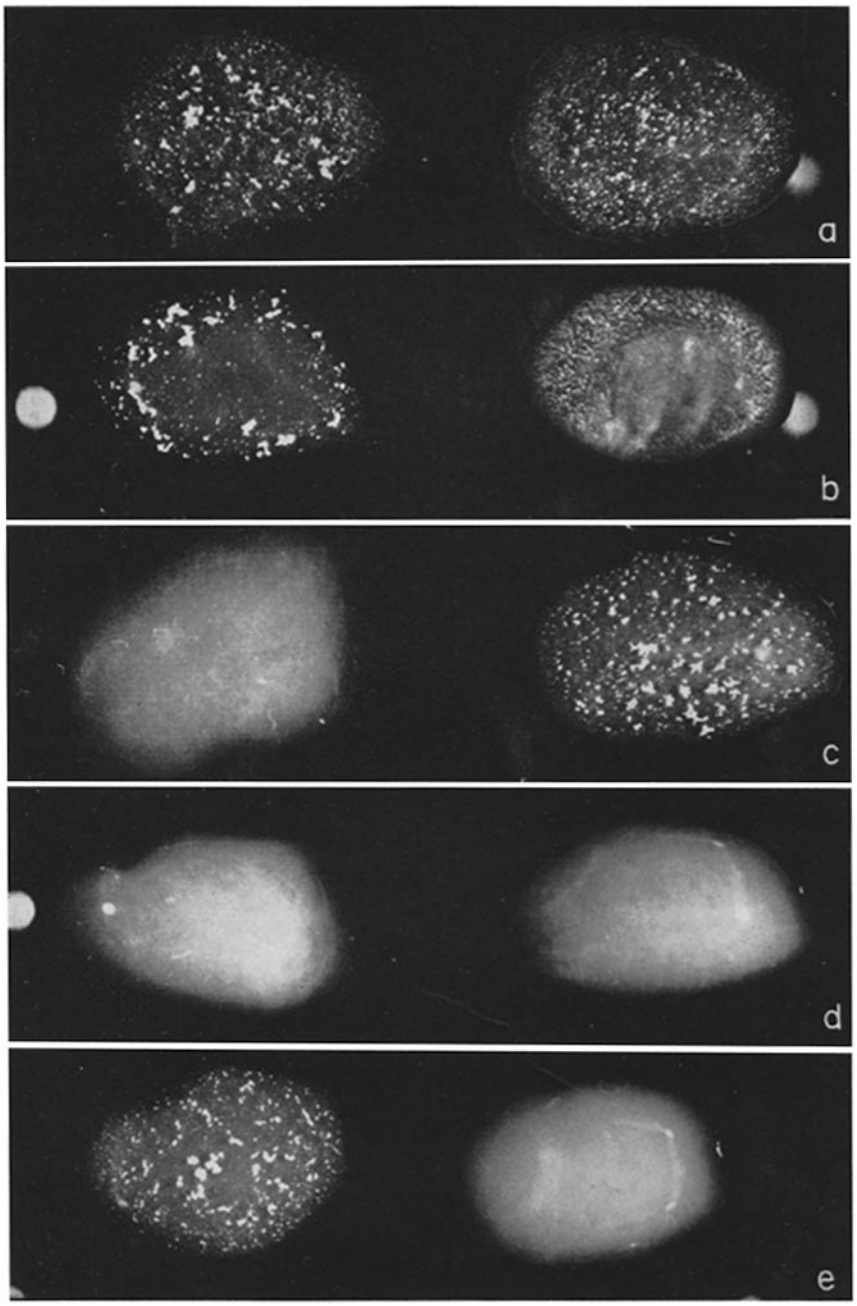
EXPLANATION OF PLATE 71

FIG. 1. Clumping reaction with various substrates performed by the slide test. (*a*) Left, unheated fibrinogen; right, unheated fibrin lysate. (*b*) Left, unheated serum; right, serum heated at 56°C for 10 min. (*c*) Left, fibrinogen heated at 56°C for 10 min; right, fibrin lysate heated at 56°C for 10 min. (*d*) Left, serum heated at 60°C for 10 min; right, fibrin lysate heated at 60°C for 10 min. (*e*) Left, unheated fibrinogen; right, pH 7.4 tris buffer. × 1.5.



(Lipiński et al.: Mechanism of staphylococcal clumping)