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Plasma Coagulation by Organisms Other Than *Staphylococcus aureus*[1]

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ABSTRACT

BAYLISS, BERENICE G. (Washington State University, Pullman), AND ELIZABETH R. HALL. Plasma coagulation by organisms other than *Staphylococcus aureus*. J. Bacteriol. 89:101-105. 1965.—Approximately 200 organisms were investigated for their ability to clot human and rabbit plasma. Various anticoagulants were used in preparing the plasma: acid-citrate-dextrose, ethylenediaminetetraacetate, balanced oxalate, potassium and sodium oxalates, and heparin. Twelve organisms were found which coagulated citrated plasma in less than 8 hr: four strains of *Streptococcus faecalis*; two strains of *S. faecalis* var. *zymogenes*; three strains of *S. faecalis* var. *liquefaciens*; and one strain each of *S. pyogenes*, *Escherichia coli*, and *Serratia marcescens*. Six strains of coagulase-positive *Staphylococcus* were selected for use as controls. Experiments were performed to determine the mechanism by which these microorganisms coagulated citrated plasma. As this was the only plasma clotted, it was presumed that the citrate was utilized by the microorganisms, thereby releasing the calcium which was then made available so that normal physiological clotting could occur. To test this hypothesis, a chromatographic method was employed to determine the presence or absence of citrate. Coagulation tests, by use of increasing amounts of citrate, showed a linear relationship between the amount of citrate in the plasma and the coagulation time. It was demonstrated that the organisms must be actively metabolizing to clot citrated plasma. Proof for this was obtained by using a cell-free filtrate, to which thimerosal had been added to inhibit growth, instead of whole cultures for the coagulation test. Only the coagulase-positive staphylococci coagulated the citrated plasma under these conditions. From the results obtained, it was concluded that plasma coagulation by these organisms was by citrate utilization.

The coagulation of plasma by the majority of the pathogenic strains of *Staphylococcus aureus* is a well-established phenomenon (Blair, 1939; 1962). Likewise, the coagulation of plasma by organisms other than *S. aureus* has been reported. Burrows (1959) listed strains of *Pseudomonas pyocyanea*, *Serratia marcescens*, *Escherichia coli*, and *Bacillus subtilis* as organisms that coagulated plasma. Harper and Conway (1948) reported that 11 of the 13 gram-negative rods they tested clotted citrated plasma in 18 hr. Neither heparinized nor oxalated plasma was clotted by these organisms, nor could a filtrate of the culture clot the citrated plasma. Hoff and Drake, in a paper given in 1960 before the Laboratory Section of the American Public Health Association, mentioned the coagulation by some strains of *Pseudomonas aeruginosa* of plasma treated with citrate and ethylenediaminetetraacetate (EDTA).

Krech (1952) investigated the coagulation of human and animal plasma by *Bacterium coli* (*E. coli*). Of the 41 strains tested, 11 were able to coagulate citrated horse, cow, guinea pig, and human plasma. Mushin and Kerr (1954) observed no plasma coagulation of citrated plasma by strains of *Proteus* or *Shigella*. In contrast, all the strains of *E. coli*, and the majority of the strains of *Salmonella* and of the paracolons, coagulated citrated plasma in 24 hr. Eisler (1961) discovered some strains of *Pasteurella pestis* that were able to coagulate human citrated and oxalated plasma, but not plasma in which heparin had been used. The coagulation of plasma by strains of streptococci has been reported by Duthie (1953), Evans, Buettner, and Niven (1952), and by Wood (1959). Strains of *Streptococcus faecalis*, *S. faecalis* var. *liquefaciens* and *zymogenes*, and *Streptococcus durans* have all been

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reported as clotting plasma (Campbell and Gunsalus, 1944; Young and Leitner, 1964).

Duthie (1953) attributed the coagulation of plasma by the streptococci to the production of a coagulase similar to the bound coagulase produced by the staphylococci. Smith and Conant (1960) assumed that the coagulation of plasma by the enteric bacilli and by *B. subtilis* was due to coagulase production. The strains of streptococci used by Wood (1959) did not appear to coagulate plasma by the utilization of citrate. Chromatographic studies for citrate utilization showed too little citrate disappearance to be detected by this method. These organisms also coagulated plasma which had been decalcified by drawing the blood through a short ion-exchange column (Amberlite) in the syringe, and then mixing the blood immediately with equal volumes of saline. The plasma was clotted even by cell-free filtrates of the culture medium in from 2 to 5 hr. Heparinized plasma was not coagulated, which showed that the enzymes responsible for the coagulation by the streptococci do not function in the same way as does the coagulase produced by the staphylococci. Young and Leitner (1964) made no attempt to determine the mechanism of plasma coagulation by the organisms they isolated from such clinical sources as blood, tissues, wounds, and urine, but described them as coagulase-positive enterococci. With these exceptions, the coagulation of citrated plasma, by microorganisms other than *S. aureus*, has been attributed to the utilization of citrate with the release of the calcium which is then available for the conversion of prothrombin into thrombin, and the completion of the normal physiological clotting of the plasma (Mushin and Kerr, 1954; Evans et al., 1950).

As a preliminary to the present investigation, several hundred cultures were tested for their ability to coagulate plasma. Those which clotted citrated plasma within a reasonable length of time (2 to 8 hr) were then selected for further study regarding the mechanism involved. A comparison was also made between the coagulation of plasma by these organisms and that produced by the coagulase formed by strains of *S. aureus*.

MATERIALS AND METHODS

Cultures. Cultures to be tested for their ability to coagulate plasma were grown in Infusion Broth (BBL), and on Infusion Agar slants. A semisolid medium, Infusion Broth plus 0.3% agar, was substituted for the Infusion Broth for some of the fastidious pathogenic bacteria in an effort to obtain a more luxuriant growth. With the exception of the yeasts, which were incubated at room temperature, all media after inoculation were incubated at 35 to 37 C for 18 to 21 hr prior to the coagulation test. All cultures were maintained on Stock Culture Agar (Difco), and kept in screw-cap tubes in a refrigerator. These were transferred to freshly prepared tubes of medium at intervals of from 2 to 3 months. A known coagulase-positive strain of *S. aureus* was always inoculated into the broth and onto the slant. The same strain was used as a control throughout the experimental work.

Plasma. Acid-citrate-dextrose (ACD) plasma was obtained from outdated blood-bank blood. Citrated rabbit plasma was prepared by placing 35 to 40 ml of blood obtained by cardiac puncture into a sterile flask containing the dried residue from 1 ml of a 20% solution of sodium citrate. EDTA rabbit plasma contained 0.01 ml of a 10% solution of EDTA (disodium) per milliliter of whole blood. Plasma treated with balanced oxalate, potassium oxalate, or sodium oxalate was prepared in the conventional manner. Heparinized plasma was obtained from blood mixed with a commercial preparation of heparin (Anticlot) according to the directions prescribed by the manufacturer (Clinton Laboratories, Los Angeles, Calif.). The plasma from at least two different animals was pooled, dispensed in samples (2 to 5 ml) in small screw-capped bottles, and stored in a conventional deep freeze at approximately −20 C. Rabbit plasma was diluted with three parts of 0.9% saline, and human plasma was diluted with one part of 0.9% saline before being used for coagulation tests.

Coagulase testing. The method used was as follows. Into a sterile, aluminum-capped test tube was placed 0.5 ml of an 18- to 21-hr broth culture of the organism. To this was added 0.5 ml of diluted plasma. Organisms taken from the agar slant were added directly to 0.5 ml of diluted plasma. The contents of the tubes were mixed and placed in a water bath (37 C). Readings were made in 30 min, and at hourly intervals thereafter, unless the exact time of clotting was desired. If no clotting was observed at the end of 8 hr, the rack of tubes was refrigerated for an additional 16 hr before the final readings were made. Controls for each test included 0.5 ml of the uninoculated broth plus the diluted plasma, 0.5 ml of the diluted plasma alone, and a known coagulase-positive strain of *S. aureus* treated in the same manner as were the organisms being tested.

Organisms. From the organisms screened for the coagulation of the various plasmas, 12 were chosen for further study regarding the mechanism involved. Four of these were different strains of *S. faecalis*, three were strains of *S. faecalis* var. *liquefaciens*, two were strains of *S. faecalis* var. *zymogenes*, and one strain each was of *Streptococcus pyogenes*, *Serratia marcescens*, and *E. coli*. In addition to the strain of *S. aureus* used as a control with all coagulation tests, five other coagulase-positive strains were tested to be sure the strain used was typical for the species.

Chromatographic test for citrate. The fluids to be tested were spotted on Whatman no. 1 filter paper in 0.010-ml amounts. A hand hair dryer was used to dry the material as it was applied so that the

area covered did not exceed 5 mm in diameter. These were spotted 3 cm from the lower edge of the paper, and at intervals of 2 cm. Ascending chromatography was employed by use of a developing solution composed of *tert*-amyl alcohol–formic acid–water (90:20:90, v/v). This was a diphasic system of which the alcoholic phase was used. The paper and atmosphere in the developing jar were hydrated for 2 hr before the developer was poured into the bottom of the jar. A developing period of 6 to 8 hr gave good separation of the citrate from the plasma. Discrete citrate spots were obtained from the citrated plasma if the plasma proteins were precipitated by the addition of a very small amount of 50% trichloroacetic acid (two small drops of the trichloroacetic acid to 1 ml of the plasma-culture mixture), and the precipitated protein was removed by centrifugation. Citrate concentrations as low as 0.06% could be demonstrated by this method. The pink color which was specific for citrate (demonstrated with plasma containing citrate, but not with plasma containing any other anticoagulant) developed after the dried chromatogram was sprayed with a 4% (w/v) solution of *p*-dimethylaminobenzaldehyde in acetic anhydride, again dried, and then heated at 140 C in an electric oven for 2 min.

RESULTS

Effect of various anticoagulants upon the coagulation of plasma by microorganisms. Of the 18 organisms selected for study regarding the mechanism involved in plasma coagulation, only the 6 strains of *S. aureus* coagulated plasma which had been obtained by use of anticoagulants other than citrate. To be sure that the anticoagulant was the only variable factor, rabbit and human bloods were collected, and samples were added to each anticoagulant. Here, too, only the sample containing citrate was coagulated by these organisms. Also tested was EDTA plasma plus added sodium citrate. This was done to check the effect that the sodium citrate might have upon the coagulation. The final concentration of sodium citrate was 0.125%, the same concentration that is used to prevent clotting during the collection of citrated plasma. This amount did not stimulate or inhibit the plasma coagulation by any of the organisms tested.

Nutrients necessary for the coagulation of citrated plasma. A loopful of organisms from a 24-hr agar slant was suspended in 0.5 ml of each of the following solutions: 0.9% saline, Infusion Broth, and 2% peptone in water. To this suspension was added 0.5 ml of diluted citrated rabbit plasma. A routine coagulation test was done as a control. Only the *Staphylococcus* clotted the plasma in the tube containing saline. The strain of *E. coli* used clotted the plasma in 3 hr when suspended in the Infusion Broth, and in 6 hr in the 2% peptone.

TABLE 1. *Coagulation times, in hours, of citrated rabbit plasma containing various concentrations of sodium citrate*

Organism	Sodium citrate concn*					
	0	0.125	0.25	0.37	0.50	0.6
Streptococcus faecalis var. *zymogenes*	2.5	4.0	5.0	6.5	7.0	9.5
S. faecalis var. *zymogenes*	2.5	3.5	4.5	5.0	7.5	9.5
S. faecalis	2.0	2.25	3.5	4.5	5.5	7.0
S. faecalis	2.5	3.5	4.5	5.5	6.0	8.0
Escherichia coli	4.0	24.0	24.0	—	—	—
Streptococcus pyogenes	5.0	9.5	—	—	—	—
S. faecalis	1.5	2.5	3.25	4.5	5.0	6.0
Serratia marcescens	3.25	5.5	—	—	—	—
S. faecalis	1.5	2.25	3.0	3.75	4.5	5.25
S. faecalis var. *liquefaciens*	2.0	2.75	3.5	4.25	4.75	6.0
S. faecalis var. *liquefaciens*	3.5	4.75	5.5	6.5	—	—
S. faecalis var. *liquefaciens*	3.75	5.0	6.0	6.75	7.75	10.0
Staphylococcus aureus	0.5	0.5	0.5	0.5	0.5	0.75
S. aureus	0.5	0.5	0.5	0.5	0.5	0.5
S. aureus	0.5	0.5	0.5	0.5	0.5	0.5
S. aureus	1.0	0.5	0.5	0.5	1.0	1.0
S. aureus	0.5	0.5	0.5	0.5	0.5	0.5
S. aureus	0.5	0.5	0.5	0.5	0.5	0.5

* The percentage of concentrations in the final plasma-culture mixture in addition to that used as an anticoagulant in the plasma.

Effect of varying concentrations of sodium citrate upon the coagulation of citrated rabbit plasma. Varying the concentration of citrate in the plasma showed that the time required for a solid clot to form with all the organisms tested, except the coagulase-positive strains of *S. aureus*, was dependent upon the concentration of sodium citrate in the plasma-culture mixture. The relationship between the time required by the organisms to coagulate the plasma and the sodium citrate concentration can be seen in Table 1. From these data, it was evident that the clotting times of these organisms varied directly with the concentration of sodium citrate in the plasma, except for the coagulase-producing *S. aureus* (Table 1).

Coagulation of citrated plasma by cell-free suspensions of the test organisms. The test organisms were grown in thick-walled culture tubes which could be centrifuged. Three coagulation tests were set up for each organism. Tubes 1 and 2 contained 0.5 ml of the 21-hr culture. These differed because the diluted citrated plasma added

to tube 2 was treated with thimerosal to contain a final concentration of 1:5,000 of the thimerosal. This was used to prevent the further growth of the organisms. After removing the material for tubes 1 and 2, the culture tubes were centrifuged, and 0.5 ml of the clear supernatant fluid from each tube was then placed into tube 3. The plasma added to tube 3 also contained thimerosal, but in a final concentration of 1:10,000. All of the six strains of *Staphylococcus* clotted the plasma in all three tubes in essentially the same time. None of the other organisms clotted the thimerosal-treated plasma in 24 hr.

Citrate utilization by microorganisms as demonstrated by paper chromatography. Paper chromatography was used as the final proof that citrate utilization was the mechanism by which the ten strains of *Streptococcus*, one strain of *E. coli*, and *Serratia marcescens* coagulated plasma. The chromatograms were spotted with the supernatant fluid obtained from the plasma coagulation tubes after clotting occurred. None of the organisms tested showed a citrate spot upon these chromatograms. The controls, made from the clots of the six strains of coagulase-positive *S. aureus* and the uninoculated medium-plasma mixture, all gave distinct citrate spots. Chromatograms done at 30-min intervals during the coagulation incubation period showed decreasing amounts of citrate as evidenced by lighter citrate spots until none could be demonstrated. Clotting took place soon after this.

DISCUSSION

During the course of this investigation, about 200 organisms were tested for their ability to clot plasma. The various anticoagulants used were sodium citrate, EDTA, acid-citrate-dextrose, potassium oxalate, balanced oxalate, sodium oxalate, and heparin. The six strains of *S. aureus* clotted all of these plasma preparations within a 2-hr period. Other organisms, if they clotted the plasma at all, clotted only that in which citrate had been used to remove the calcium. With these citrate-utilizing organisms, the animal from which the blood is obtained should not influence the coagulation test. However, none of the organisms tested clotted bovine plasma as readily as either the human or rabbit plasma. Rabbit plasma that was obtained from the pooled bloods of several animals, dispensed in small samples and kept frozen, maintained its coagulability for over a year with no lengthening of the clotting time by the strain of *S. aureus* which served as a control in all experiments. Plasma that was thawed and refrozen several times seemed to be less effective in the coagulase test than that which was not thawed until ready for use.

From this preliminary work, it appeared that the coagulation of plasma by the organisms included in this study, with the exception of the staphylococci, was caused by the utilization of citrate. This led to the release of the calcium which then became available for normal plasma coagulation. An attempt was made to determine the presence or absence of citrate in the fluid remaining after the removal of the clot. Chemical methods for citrate determination were felt to be not sufficiently sensitive and to present technical difficulties (Pucher, Sherman, and Vickery, 1936; Marier and Boulet, 1958) which made them impractical for routine testing of very small amounts of material containing low citrate concentrations. Reference to the use of chromatography for the determination of citrate in a solution was made by Wood (1959), Scheirer and Hack (1956), Stark, Goodban, and Owens (1951), Block, Durrum, and Sweig (1958), and Eisler (1961). This method proved to be suitable for the detection of the small amounts of citrate remaining in the fluid after the coagulation of plasma by microorganisms. Only 0.010 ml of material was needed, and the sensitivity was in the order of 0.06% sodium citrate. This amount gave a definite citrate spot on the completed chromatogram. Even a spot from material containing only 0.03% sodium citrate could be seen, but its appearance was variable.

Several lines of investigation were followed in an effort to substantiate the assumption that the coagulation of the citrated plasma by the ten strains of *Streptococcus*, the strain of *E. coli*, and the *Serratia marcescens* was accomplished by the utilization of citrate with the resulting release of calcium. No citrate could be demonstrated on the chromatograms spotted with the fluid from the clots formed in the citrated plasma, which contained 0.125% sodium citrate above that used as an anticoagulant. The six strains of *Staphylococcus* showed citrate levels comparable to the plasma-medium control. Other experiments were carried out to show that these other organisms studied must be growing and metabolizing for coagulation of the citrated plasma to occur. In one of these tests, a loopful of *E. coli* from a slant clotted plasma only in the presence of a nutrient, but not when suspended in saline. The *S. aureus* under the same conditions clotted in all of the suspending media tested.

Another reason for concluding that citrate utilization is the mechanism by which the organisms other than the staphylococci clotted plasma was the increased time required for clot formation when additional sodium citrate was added to the plasma. As more citrate was added, the coagulation time increased up to a certain point. In sodium citrate concentration which was

1.25% greater than that used as an anticoagulant, coagulation did not occur with any of the *Streptococcus* strains, the *E. coli*, or the *Serratia marcescens*.

S. aureus coagulated the plasma containing 2.5% sodium citrate as rapidly as that containing no additional sodium citrate. The linear relationship between the coagulation time and the amount of sodium citrate is clearly illustrated in Table 1. When coagulation times for each organism were plotted against citrate concentration on graphs, the slopes varied in steepness with the organisms used. The steepest slope was with the *E. coli*. The least slopes were with the strains of *Streptococcus faecalis*. These varied in the time required to clot the plasma containing 0.60% sodium citrate from 5.25 to 8 hr.

None of the cell-free suspensions of the organisms, with the exception of the six strains of *S. aureus*, clotted the plasma. Also, cultures rendered bacteriostatic by the addition of thimerosal did not clot plasma. The addition of thimerosal to the plasma-culture mixture, in a concentration of 1:5,000 when the whole culture was used and 1:10,000 when the centrifuged supernatant fluid was used, did not interfere with the coagulation of the plasma by the six strains of *S. aureus*. None of the ten strains of streptococci nor the one strain of *E. coli* or *Serratia marcescens* clotted plasma rendered sterile by the thimerosal. This is not in agreement with the reports of Lindsell and Gorrill (1951) who found that some strains of *S. aureus* required a longer time for plasma coagulation if thimerosal was present in the plasma, even in a concentration of 1:100,000. Davies (1951) used thimersalate to stop the production of procoagulase, but stated that it did not affect the action of coagulase.

Thus, the coagulation of citrated plasma by microorganisms may be the result of citrate utilization, and not due to the production of a coagulase. To avoid error, a plasma prepared from blood rendered incoagulable by EDTA, oxalates, or heparin should be used in conjunction with the citrated plasma for coagulase testing of microorganisms.

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