Wood, M. (1959). *J. gen. Microbiol.* **21**, 385-388

The Clotting of Rabbit Plasma by Group D Streptococci

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SUMMARY: Some strains of Group D streptococci are capable of coagulating citrated rabbit plasma. This reaction is not due to citrate utilization but to a specific agent elaborated by the organisms; this agent is probably enzymic in nature. The addition of 5 units heparin/ml. to the citrated rabbit plasma inhibits coagulation by streptococci and makes the reaction more specific for staphylococci.

It is now widely recognized that the coagulation of human, rabbit or horse plasma is one of the more reliable diagnostic criteria for the recognition of pathogenic staphylococci. It is also generally inferred that when plasma is clotted as the result of bacterial action, the organism responsible is *Staphylococcus aureus*. But it has been reported that other micro-organisms, particularly Gram-negative coliform organisms, are capable of coagulating plasma (Harper & Conway, 1948; Mushin & Kerr, 1954; Rita, 1954). These workers agreed in concluding that the ability of these bacteria to clot plasma is not due to production of a coagulase enzyme, but to their utilization of the citrate used as the anti-coagulant in the plasma; the release of calcium ion leads to clotting by the normal physiological process. Evans, Buettner & Niven (1952) reported that some Group D streptococci would clot citrated plasma and suggested that this characteristic might, in routine tests used in the food industry, give rise to their diagnosis as late coagulase-positive staphylococci. They also attributed the observed slow coagulation to citrate utilization. Some experiments which invalidate the latter assertion are reported here.

RESULTS

Initial work confirmed the descriptive findings of the American workers, namely that some Group D streptococci would cause plasma coagulation. Citrated rabbit plasma was prepared by mixing equal volumes of a 4% (w/v) sodium citrate solution and whole blood; thus an approximately $\frac{1}{2}$ dilution of the plasma was obtained in which the citrate concentration was between 2 and 3%. The inoculum used was 4 drops of a suspension washed from an overnight culture on a blood agar slope by nutrient broth, and this was added to 0.5 ml. of $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{16}$ dilutions of the plasma in saline. The tubes were observed for production of a solid clot after fixed time intervals ($\frac{1}{2}$, 2, 5 hr.) of incubation in a water bath at 37°, and then after 24 hr. at room temperature. Coagulase-positive, late positive and coagulase-negative staphylococcal strains were used as controls and a plasma control with nutrient broth was incubated with each test. About half of the 29 streptococcal strains tested caused plasma to clot within 24 hr. and occasionally within 5 hr.

The organisms tested included *Streptococcus faecalis*, *S. faecalis* var.

liquefaciens, *S. faecalis* var. *zymogenes* and *S. durans*; clotting activity was characteristic of the strain and not of the species.

Citrate utilization. Koser's citrate medium was an unsuitable test medium for the nutritionally demanding organisms being studied and the use of sodium citrate peptone water (Kauffmann, 1954) did not give repeatable results. A chromatographic method proved most satisfactory for detecting citrate under the conditions of the coagulase test.

Samples of citrated plasma subjected to bacterial action, taken at 0, 24 and 48 hr., were macerated when necessary and 8 μ l. quantities spotted on to Whatman No. 1 paper. The chromatogram was developed with a tertiary amyl alcohol + formic acid + water mixture (90:20:90 vol.) for 6 hr. The papers were dried and sprayed with a 4% (w/v) solution of *p*-dimethylaminobenzaldehyde in acetic anhydride. The citrate then appeared as a pink spot, the size and intensity of which gave some indication of the amount of citrate present.

Of those streptococci tested, only one (strain CN 2438) showed evidence of citrate utilization. Other strains which had coagulated plasma within 24 hr., e.g. strains CN 2540 and CN 2538, utilized amounts of citrate insufficient to be detectable by the chromatographic method. In most cases the ionized citrate concentration after 24 hr. appeared to be little changed, and in all instances a final concentration of at least 1% remained. Clotting could not therefore be due to liberation of calcium ion following destruction of citrate.

Coagulation of decalcified plasma. To confirm that citrate utilization was not the operative factor, plasma stabilized by removing Ca ions with ion exchange resin was used. Rabbit heart blood was pulled through a short column of cation exchange resin (Amberlite 1R/120/H) held in the barrel of the syringe used for withdrawing the blood and then diluted with an equal volume of saline. The blood was centrifuged and plasma stabilized without the addition of anticoagulant was thus obtained. Table 1 shows the results obtained with this plasma and with citrated plasma. The two plasma preparations gave similar results, indicating that citrate metabolism was not responsible for the clotting process.

Coagulation of heparinized plasma. Group D streptococci able to clot citrated plasma, did not clot plasma containing 25 units heparin/ml. At this heparin concentration clotting by late coagulase-positive staphylococci was also delayed. It seemed that heparin might inhibit enzymes responsible for the coagulation phenomenon. Lower concentrations of heparin (5 units/ml.) in citrated plasma effectively eliminated the streptococcal reaction without interfering with the staphylococcal reaction. This observation, of practical diagnostic importance, has not been further investigated.

Extracellular activity. The supposition that streptococci could produce a coagulating enzyme would be confirmed should cell-free preparations be active. Supernatant fluids from centrifuged broth cultures were found to have no detectable activity. However, failure might have been due to poor growth, and other cultural methods were tried. Streptococci were grown inside a cellophan membrane with an external supply of nutrients. Under these conditions better growth was obtained and any extracellular enzymes

formed would be retained within the membrane. The centrifuged supernatant fluids from these cultures clotted plasma, with positive reactions in less than 5 hr. in many cases and sometimes in less than 2 hr. The results are summarized in Table 2. Addition of 0.1 % (w/v) sterile bovine albumin solution to cultures within the cellophan bag appeared to increase coagulase activity. Cultures sterilized by filtration were also effective as clotting agents.

Table 1. *The clotting of citrated and decalcified rabbit plasma by Group D streptococci*

Time (hr.)	CN	Decalcified plasma									Citrated plasma								
		$\frac{1}{2}$			5			24			$\frac{1}{2}$			5			24		
Reciprocal of plasma dilution ...		2	6	12	2	6	12	2	6	12	2	6	12	2	6	12	2	6	12
Staphylococci																			
Coagulase +ve	49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulase late +ve	69	-	-	-	±	-	-	+	+	+	-	-	-	±	-	-	+	+	+
Coagulase -ve	1366	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	±	-	-
Group D streptococci																			
	2536	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	±	±
	2385	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2538	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+
	2540	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	+
	2530	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2626	-	-	-	-	-	-	+	+	+	-	-	-	-	-	±	-	+	+
	2438	-	-	-	-	±	+	-	+	-	-	-	-	-	-	-	-	+	+
Control		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, ± = degree of clotting; - = no clot; CN = strain number.

Table 2. *The clotting of citrated rabbit plasma by supernatant fluid from Group D streptococcal cultures*

Time (hr.)	CN	Whole culture									Supernatant fluid								
		$\frac{1}{2}$			5			24			$\frac{1}{2}$			5			24		
Reciprocal of plasma dilution ...		2	6	12	2	6	12	2	6	12	2	6	12	2	6	12	2	6	12
Staphylococci																			
Coagulase +ve	49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulase late +ve	69	-	-	-	+	-	-	+	+	+	-	-	-	+	-	-	+	+	+
Coagulase -ve	1366	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group D streptococci																			
	2540	-	-	-	-	+	-	+	+	-	-	-	-	+	±	-	+	+	+
	2438	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
	2442	-	-	-	-	+	-	+	+	+	-	-	-	-	+	-	-	+	+
	2538	-	-	-	+	+	+	+	+	-	-	-	-	-	+	-	+	+	±
	2530	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2541	-	-	-	-	+	-	+	+	±	-	-	-	-	+	+	+	+	+
Control		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, ± = degree of clotting; - = no clot; CN = strain number.

The coagulase activity of the streptococcal filtrate was heat labile. A temperature of 60° for 5 min. had little effect, but heating at this temperature for 15 min. caused clotting to be much delayed; 70° for 15 min. virtually destroyed activity. The preparations were active between pH 5 and pH 9 with little indication of an optimum pH for activity. Nutrient media of pH 8 or slightly above appeared to yield preparations of high activity.

The preparations would not clot solutions of bovine fibrinogen; other plasma factors are involved and the mode of action has not yet been determined.

DISCUSSION

These results show clearly that clotting of plasma by streptococcal filtrates cannot be due to citrate utilization with subsequent clotting by Ca ion as in the normal physiological process. Clotting is due to a specific agent elaborated by the streptococci and this agent is likely to be enzymic in nature. The capacity to produce these enzymes is obviously not limited to the staphylococcus but is more widely distributed. Fredericq (1946 *a, b*) reported the production of such reactive agents by *Serratia marcescens* and *Actinomyces* species. Krech (1952) described a cell-free filtrate of *Escherichia coli* which would clot plasma and Billaudelle (1955) listed some strains of *Haemophilus (Bordetella) pertussis* which had coagulase activity.

It seems unlikely that the streptococcal enzyme functions in precisely the same way as the staphylococcal enzyme, for its action on horse and human plasma does not parallel that on rabbit plasma. This might be because inhibitors which are apparently produced (often there appears to be an optimal concentration) are activated differently in plasmas of the different species. Fractionation of the filtrates would help to resolve this problem.

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(Received 18 March 1959)