New Anticoagulant for Combating Antibacterial Activity of Human Blood (36648)

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Antibacterial agents have been recognized in human blood and serum for decades. However, the exact nature of the components involved in the bacteriocidal system has not yet been clearly elucidated. Evans, Cekoric and Searcy (1) call attention to three blood constituents that are probably instrumental in the destruction of bacteria. These include beta-lysin, normal antibody–complement systems and lysozyme.

Recently sodium polyanethol sulfonate was shown to abolish the antibacterial activity of blood (2, 3) and serum (4). Information gained from the study of agents that influence the bacteriocidal properties of blood may lead to their identification and characterization. A newly synthesized sulfated polysaccharide was found to be a weak chelating agent for calcium and anticoagulant (5). Because of its structure and its polyanionic character, it was suspected of influencing the bacteriocidal factors in human blood. The present report describes survival patterns of organisms in blood or serum treated with the sodium amylosulfate.

Materials and Methods. The stock laboratory cultures included *Escherichia coli* 136-E; *Salmonella oranienburg* WL277; *Bacillus subtilis* ATCC6633; *Streptococcus fecalis* ATCC10541; *Streptococcus pyogenes* ATCC10402; *Diplococcus pneumoniae* M-2; *Micrococcuc lysodeikticus* M-1, as well as *Staphylococcus aureus* and *Pseudomonas aeruginosa* which were clinical isolates from Lutheran General Hospital, Park Ridge, IL.

Citrated blood was obtained from Michael Reese-Research Foundation, Chicago, IL. Fresh human blood was drawn from laboratory personnel. The serum was removed aseptically from the clotted blood and refrigerated in screw cap tubes.

The media used for growth and dilution was trypticase soy broth (TSB) or trypticase soy agar (TSA) (Baltimore Biological Laboratories, Baltimore, MD). Sodium polyanethol sulfonate (Grobax) was obtained from Roche Diagnostics, Nutley, NJ[1]

Sodium amylosulfate (SAS) was prepared in 5% (w/v) aqueous solution and autoclaved. SAS or Grobax was added to blood at a concentration of 0.05% (w/v) and then an equal volume of a serial dilution of bacteria was added to each tube. Blood without additives served as a control. The number of organisms in each dilution was determined by plate count. All tubes were incubated 3 hr at 37° and then 1 ml was transferred to 50 ml of TSB with and without additives and incubated overnight at 37°. Growth was determined by visual inspection of turbidity and verified by culture on TSA.

Serum was used at a final concentration of 25% (v/v). In these studies 10^4 to 10^5 organisms/ml were routinely added to serum with and without additives and incubated 90 min at 37°. Serum heated at 56° for 30 min was also used. Viable organisms were determined by plate count on TSA.

Results. All of the organisms used in this study showed luxuriant growth in 0.025 to 10% (w/v) SAS in TSB within 24 hr. The concentration of SAS required to neutralize the antibacterial effect of 25% fresh serum toward *E. coli* at 10^5 organisms/ml within 90 min was 0.025%.

With *E. coli* as the test culture (Table I), 1.4×10^5 cells/ml were required for growth without SAS in whole blood, whereas only 1.4 organisms/ml were necessary when the ad-

[1] TEKIT® S-A-S™ available from Searle Diagnostic Inc., G. D. Searle & Co., Chicago, Illinois 60680.

TABLE I. A Representative Determination of the Number of Organisms to Initiate Growth After Incubation in Whole Blood.

Organisms added[a]	1.4×10^7	1.4×10^6	1.4×10^5	1.4×10^4	1.4×10^3	1.4×10^2	14	1.4	0
SAS-treated blood	+[b]	+	+	+	+	+	+	+	−
Untreated blood	+	+	+	−	−	−	−	−	−

[a] Organism used was *E. coli*. Plate count on trypticase soy agar. Results in organisms per milliliter.
[b] + = growth in trypticase soy broth after overnight incubation at 37°; − = no growth.

ditive was present. *S. oranienburg* was also sensitive to the lethal effects of blood. When added to SAS-treated blood, only 1 organism/ml was needed to demonstrate growth and 3.4×10^4 organisms/ml were required for growth without SAS treatment.

The viability of *S. fecalis, S. pyogenes, S. aureus, P. aeruginosa* and *B. subtilis* was not affected by treatment with blood and grew with 1 or 2 organisms/ml without additive. *M. lysodeikticus* was apparently inhibited by citrate in the whole blood with or without SAS, but would grow in freshly drawn blood with SAS.

Two typical experiments are shown in Table II using fresh serum; SAS and *E. coli* as the test organisms. Fresh serum rapidly killed all the organisms within 30 min. However, this lethal effect could be eliminated by adding 0.05% SAS or heating the serum at 56° for 30 min.

The series of organisms were added to serum and the results were similar to that of the blood study (Table III). The growth of *E. coli* and *S. oranienburg* was strongly inhibited by fresh serum. This effect was overcome by the addition of SAS or heating serum at 56° for 30 min. By contrast, *M. lysodeikticus* was susceptible to heated as well as fresh serum and SAS inhibited the action of the serum.

The results with SAS were comparable to those obtained with sodium polyanethol sulfonate in all experiments. Both substances caused a cloudy precipitate to form when added to fresh serum. The precipitate has been partially characterized and will be the subject of a future communication.

Discussion. Examination of sodium amylosulfate, a polyanionic anticoagulant prepared from potato amylopectin, for its ability to inhibit antibacterial substances in blood and serum indicated that it behaved like sodium polyanethol sulfonate. Three of the organisms used in this study showed both blood and serum sensitivity. Several other organisms were examined for their susceptibility to the lethal action of blood and serum, but were shown to be resistant. It is noteworthy that other strains of *P. aeruginosa* and *S. aureus* were shown to be sensitive in

TABLE II. The Activity of Two Sera Against *E. coli*.

Exposure to serum (min)	Initial inoculum, 4.6×10^5 organism/ml			Initial inoculum, 3.2×10^5 organism/ml			
	No serum	50% Serum	50% Serum 0.05% SAS[a]	No serum	25% Serum	25% Serum[b]	25% Serum 0.05% SAS[a]
10	4.6×10^5[c]	0	3.9×10^5	3.2×10^5	11.8×10^4	3.5×10^5	3.7×10^5
30	4.8×10^5	0	5.3×10^5	6.1×10^5	0	5.3×10^5	6.0×10^5
90	13.0×10^5	0	11.6×10^5	12.0×10^5	0	12.7×10^5	11.7×10^5

[a] SAS refers to sodium amylosulfate.
[b] Serum was heat inactivated at 56° for 30 min.
[c] Values represent organisms per milliliter.

blood cultures (2, 3). Whether this sensivity is a strain susceptibility or the difference between artificial as opposed to actual clinical blood culture is unknown.

E. coli and *S. oranienburg* responded similarly to blood and serum. Both organisms were not killed by serum heated at 56° for 30 min. This implies that a heat-labile agent, possibly certain fractions of complement, was involved in the destruction of these organisms.

M. lysodeikticus has a low tolerance to lysozyme. With this organism, the heated as well as unheated sera destroyed the cells. In all cases, SAS or Grobax, prevented the death of the bacteria from serum or blood. The data suggest that at least two substances are involved in the bacteriocidal effect of serum. Moreover, the activity of both lethal agents disappeared from serum treated with SAS.

A precipitate always formed when SAS was added to serum. This insoluble material is presently being characterized. It is known that SAS selectively removes beta-lipoproteins from serum (6). Therefore, the precipitate probably contains lipid-bearing proteins.

Lysozyme is a cationic, low molecular weight, heat-stable protein. Hence, it is likely that a polyanionic substance such as SAS could render this substance insoluble. Heparin, a sulfated polysaccharide, is also an anticoagulant that inactivates antibacterial cationic rabbit reticulocyte proteins, which possess lysozyme-like activity (7).

The heated serum was inactive against *E. coli and S. oranienburg*. Goldman *et al.* (8) showed that the entire complement system was required to kill *E. coli*. Some fractions of complement were found to be heat sensitive. Carrageenin, another sulfated polysaccharide, acts as a powerful inhibitor of guinea pig

TABLE III. Effect of Serum on Organisms With and Without Sodium Amylosulfate.

Organism[a]	No serum	25% Serum	25% Serum[b]	25% Serum 0.05% SAS[c]
E. coli	1.11×10^5	0	1.0×10^5	1.0×10^5
S. oranienburg	1.3×10^5	0	1.4×10^5	1.6×10^5
S. fecalis	8.3×10^4	6.9×10^4	7.9×10^4	8.2×10^4
S. pyogenes	3.8×10^5	3.7×10^5	3.8×10^5	3.8×10^5
D. pneumoniae	2.1×10^4	2.6×10^5	2.2×10^5	2.2×10^5
M. lysodeikticus	7.7×10^4	0	0	8.2×10^4
P. aeruginosa	2.0×10^5	2.2×10^5	2.0×10^5	2.1×10^5
S. aureus	8.0×10^4	7.1×10^4	7.6×10^4	6.5×10^4

[a] Organisms were exposed to serum for 90 min at 37°.
[b] Serum was inactivated at 56° for 30 min.
[c] SAS refers to sodium amylosulfate.

complement (9). It is therefore possible that one or more fractions of complement were inactivated by SAS. Our findings suggest that SAS may be a valuable additive for neutralizing the bacteriocidal agents in blood or serum. Furthermore, SAS may be useful for isolation of certain cationic blood proteins.

Summary. Sodium amylosulfate (SAS), a new anticoagulant, was found to interfere with the antibacterial activity of blood and serum. This agent permitted recovery of 1 organism/ml from artificially inoculated blood, whereas over 10^4 organism/ml were required without SAS. At least two fractions were involved in the bacteriocidal properties of serum. One was heat labile and the other heat stable. Both were eliminated by treatment with SAS.

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