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Effect of Sodium Polyanetholesulfonate on Antimicrobial Systems in Blood

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Sodium polyanetholesulfonate (SPS), an anticoagulant which inhibits the antimicrobial systems of blood, is used widely in blood culture media. The addition of SPS to experimental blood cultures inoculated with small numbers of a variety of organisms caused a striking increase in recovery of these organisms. Sodium fluoride also increased the incidence of positive blood cultures with some organisms. SPS completely inhibited serum antibacterial activity and serum-dependent phagocytosis (and killing) by isolated leukocytes at a concentration usually employed in blood culture media. SPS also stimulated both glucose C-1 oxidation in resting leukocytes and formate oxidation in both resting and phagocytosing leukocytes in serum-free systems. These in vitro studies support the concept that SPS is a useful additive to blood culture media and further elaborate on the mechanism of its inhibition of the microbicidal activity of blood.

The recovery of microorganisms from the blood of patients with bacteremia is essential for good medical management. Among the factors which limit the recovery of viable organisms are their destruction by serumcidal factors and by leukocytes after the blood is drawn. Sodium polyanetholesulfonate (SPS, Liquoid), a polyanionic anticoagulant, inhibits the microbicidal activity of blood. Von Haebler and Miles (27), in 1938, showed that relatively small numbers of organisms inoculated into defibrinated blood in the presence of SPS multiply during incubation at 37 C, whereas the organisms were rapidly killed when SPS was not present. Subsequent studies by others have suggested that SPS is a useful additive to blood culture media (3-7, 14, 19-21, 25), and it is currently widely used for this purpose.

In this study, we examined the effect of SPS on the recovery of small numbers of a variety of organisms added to whole blood. Sodium fluoride, an inhibitor of phagocytosis, was also tested in this way, both alone and in conjunction with SPS. We also examined the effect of SPS on serumcidal activity, on the phagocytic and microbicidal activity of isolated leukocytes, and on certain leukocytic metabolic parameters.

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MATERIALS AND METHODS

Recovery of organisms added to whole blood. Human venous blood was collected from healthy adult volunteers and immediately added to 10 sterile test tubes (10 by 150 mm) containing either SPS (Hoffman-La Roche, Inc., Huntley, N.J.), at a final concentration of 0.05% (when the effect of this substance was being tested), or heparin (Connaught Laboratories, Toronto, Can.), at a final concentration of 20 units per ml, as an anticoagulant.

Streptococcus fecalis (ATCC no. 19433), *Escherichia coli* (hospital strain), *Staphylococcus aureus* (502A), *Klebsiella pneumoniae* (hospital strain), *Proteus mirabilis* (hospital strain), *Diplococcus pneumoniae* (hospital strain), *Pseudomonas aeruginosa* (hospital strain), *Enterobacter aerogenes* (hospital strain), *Streptococcus pyogenes* (hospital strain), *Streptococcus mitis* (ATCC no. 6249), *Serratia marcescens* (hospital strain), *Candida tropicalis* (hospital strain), and *Candida albians* (hospital strain) were grown in Trypticase soy agar and broth, and *Neisseria gonorrheae* and *N. meningitidis* were grown in chocolate agar and in Mueller-Hinton broth enriched with 2% Iso Vitalex (BBL), 10% agamma horse serum (Gibco, Grand Island, N.Y.), and 10% yeast dialysate. All organisms were isolated from an overnight broth culture except the neisseriae, which were harvested after 6 hr of growth. Unless otherwise indicated, the organisms were washed twice and diluted with physiological saline until a 0.1-ml sample of the bacterial suspension yielded less than 20 organisms when plated on appropriate media. *S. pyogenes*, *S. mitis*, and *D. pneumoniae* were washed

with Ca^{2+}-free Krebs Ringer phosphate buffer, *p*H 7.4 (KRP); the neisseriae were not washed because of their fragility, but were diluted in saline to a concentration of less than 40 organisms per 0.1 ml.

A 0.1-ml sample of each bacterial suspension was added to 0.9 ml of either blood alone (control) or blood which contained either 0.05% SPS, 0.1 M sodium fluoride, or both SPS and fluoride. The tubes were incubated for 24 hr at 37 C in air or, for the neisseriae, in a CO_2-enriched environment. Samples (0.1 ml) were then removed for determination of the presence of viable organisms in duplicate plates by the pour plate method. Trypticase soy agar was used for all organisms except the neisseriae, which were streaked on prewarmed chocolate agar plates. Trypticase soy broth (4 ml) was then added to the remainder of the blood in the culture tubes (0.8 ml), incubation at 37 C was continued for another 24 hr, and the presence of viable organisms was again determined. Plates were labeled either positive or negative. Positive plates, in general, had essentially confluent growth. Plates containing less than 10 colonies were considered negative. Tubes containing no added bacteria were incubated with each experiment. If any contained viable organisms, the experiment was discarded.

Serum bactericidal activity. Blood from a human volunteer was allowed to clot at room temperature for 1 hr, and the serum was collected by centrifugation for 30 min at 2,000 × *g* and 4 C. A serum-sensitive *E. coli* (ATCC 11775), grown overnight in Trypticase soy broth, was collected by centrifugation, washed twice, and suspended in water to a final concentration of 5.0×10^6 organisms per ml. The organisms were incubated at 37 C with serum and SPS as described in Fig. 1. Samples were removed at intervals, and the viable cell count was determined, after appropriate dilution in water, by using duplicate Trypticase soy agar pour plates.

Isolation of leukocytes. Blood was collected from healthy volunteers in a syringe moistened with heparin (Invenex, San Francisco, Calif., 1,000 USP units per ml), and the leukocytes were isolated by dextran sedimentation as previously described (16). A leukocyte count and chamber differential were performed prior to the last wash, and the cells were suspended in KRP to a final concentration of 5×10^7 polymorphonuclear leukocytes (PMN) per ml.

Iodination by intact leukocytes. Intact leukocytes were incubated with the components described in Table 3 for 60 min, and the conversion of iodide to a trichloroacetic acid-precipitable form was determined as previously described (16), except that the reaction was terminated by the addition of trichloroacetic acid without the prior addition of sodium thiosulfate. The results are expressed as nmoles of iodide converted to a trichloroacetic acid-precipitable form per 10^7 PMN per hr. A blank with all reagents except leukocytes was subtracted from the experimental values. Carrier-free $Na^{131}I$ in 0.1 M sodium hydroxide was obtained from New England Nuclear Corp., Boston, Mass.

Leukocyte microbicidal activity. *S. aureus* and *S. fecalis*, grown and isolated as described above, were incubated with the components described in Fig. 2 in siliconized test tubes (12 by 75 mm) which were tumbled 24 times per min in a Fisher Roto Rack. Samples (0.1 ml) were removed at intervals, and the viable cell count was determined, after appropriate dilution with water, by the pour plate method using Trypticase soy agar. Cover slip smears were prepared at each time interval and stained with either azure II-methylene blue (18) or Wright's stain. The percentage of neutrophils which contained ingested organisms and the average number of organisms per cell were determined.

Glucose C-1 and formate oxidation. The conversion of glucose-*1*-^{14}C and ^{14}C-formate to $^{14}CO_2$ by intact phagocytosing leukocytes was determined as previously described (11). The components of the reaction mixture are indicated in Tables 4 and 5.

RESULTS

Effects of SPS on the recovery of microorganisms from blood. When small numbers of organisms were added to heparinized blood and incubated for 24 hr at 37 C, the blood cultures were frequently negative (Table 1), and an additional 24-hr incubation in the presence of added Trypticase soy broth did not increase the number of positive cultures. The presence of 0.05% SPS in the blood greatly enhanced the survival and recovery of these organisms. In many instances, a heavy growth of organisms, as indicated by viable cell count, was noted at both 24 and 48 hr. In a few instances, a light growth of organisms at 24 hr was followed by a heavy growth at 48 hr. In no instance was there an absence of growth at 24 hr followed by growth at 48 hr. Of the 15 organisms tested, 14 were recovered more frequently from SPS blood with a significance level of $P < 0.01$. Sodium fluoride (0.01 M) significantly increased the yield of positive blood cultures with 9 of the 15 organisms tested. The remaining six were enteric bacilli, with the exception of *N. gonorrheae*. The addition of fluoride to SPS blood did not significantly affect the high yield of positive cultures observed with SPS alone, although small increases were seen with certain organisms. Although 100% of the SPS or fluoride tubes inoculated with *P. mirabilis* were positive, this was not significantly different from the control tubes, which also yielded a high percentage of positive cultures.

The marked increase in the yield of positive cultures produced by SPS prompted a study of the effect of this agent on the serum and cellular microbicidal systems of blood and on the metabolic activity of leukocytes.

Effect of SPS on serumcidal activity. Figure 1 demonstrates the striking inhibitory

TABLE 1. *Recovery of organisms added to whole blood*[a]

Organism	Average no./tube	Control		SPS (0.05%)		Fluoride (0.01 M)		Fluoride (0.01 M) + SPS (0.05%)	
		Total cultures	No. positive	Total cultures	No. positive	Total cultures	No. positive	Total cultures	No. positive
Streptococcus fecalis	4	18	4	18	18**	36	36**	18	18**
Escherichia coli	7	12	0	12	12**	12	1	12	12**
Staphylococcus aureus	2	18	10	24	23**	12	12**	24	24*
Klebsiella pneumoniae	6	12	1	12	12**	12	0	12	12**
Proteus mirabilis	8	18	16	18	18	12	12	18	18
Diplococcus pneumoniae	12	12	0	12	12**	12	11**	18	18**
Pseudomonas aeruginosa	4	12	0	12	12**	12	12**	12	12**
Enterobacter aerogenes	8	12	1	18	18**	12	3	18	18**
Streptococcus pyogenes	3	12	0	24	24**	12	12**	24	24**
Streptococcus mitis	9	12	1	18	18**	12	6*	12	12**
Serratia marcescens	6	12	0	12	12**	12	1	12	12**
Candida tropicalis	3	12	1	12	12**	12	12**	12	12**
Candida albicans	4	12	7	12	12**	12	12**	12	12**
Neisseria gonorrheae	28	12	2	12	9**	12	3	12	11**
Neisseria meningitidis	11	12	0	12	11**	12	5*	12	12**

[a] Significance level by chi-square test, additive versus control: $*P<0.05$; $**P<0.01$; remainder not significant.

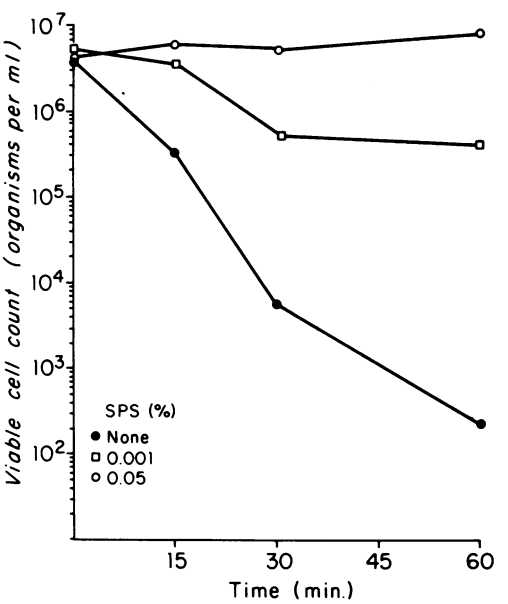

FIG. 1. *Effect of SPS on serum bactericidal activity. The reaction mixture contained 1.0 ml of serum, 5 × 10⁶ Escherichia coli, and SPS at the concentrations indicated. Total volume, 1.02 ml.*

effect of SPS, at a final concentration of 0.05%, on the killing of a serum-sensitive strain of *E. coli* by normal human serum. A decrease in SPS concentration to 0.001% decreased but did not abolish the protective effect.

Effect of SPS on the microbicidal activity of isolated leukocytes. Figure 2 demonstrates the rapid fall in viable cell count when *S. aureus* or *S. fecalis* were incubated with isolated human leukocytes and serum. SPS, at a final concentration of 0.05%, completely protected both organisms from the microbicidal activity of the leukocytes. SPS was slightly less protective at a concentration of 0.005%, whereas no effect was observed when the SPS concentration was decreased to 0.0005%. This effect of SPS could be due to an inhibition of phagocytosis or to an inhibition of intracellular killing. Smears prepared from the 15-min samples revealed a marked reduction in the percent of leukocytes which contained ingested organisms and in the average number of organisms per cell, as a result of the addition of 0.05% SPS (Table 2, Fig. 3). This inhibition of phagocytosis by SPS could account for the decrease in leukocytic microbicidal activity.

Phagocytosis of *S. aureus* and *S. fecalis* by human leukocytes requires serum for opsonization under the conditions used in Fig. 2. The phagocytosis of latex particles, however, does not require serum. Table 2 indicates that SPS, at a final concentration of 0.05%, had no effect on the percent of leukocytes which contained ingested latex particles although the number of particles per cell was moderately decreased. The ingestion of latex particles by leukocytes in the presence of SPS is visualized in Fig. 3. These studies suggest that the inhibition of the phagocytosis of the microorganisms by SPS is

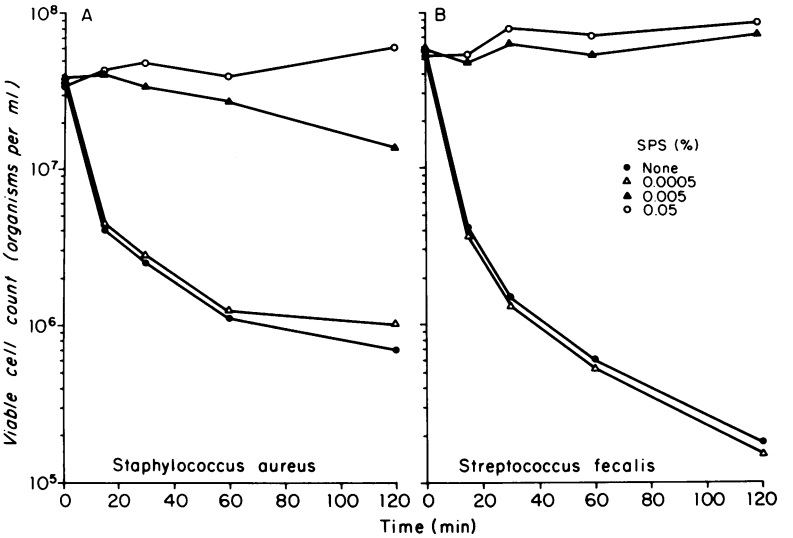

FIG. 2. *Effect of SPS on leukocytic bactericidal activity. The reaction mixture contained 0.03 ml of KRP, 1 × 10⁷ PMN, 10% AB serum, 2 μmoles of glucose, either 4 × 10⁷ Staphylococcus aureus (A) or 5 × 10⁷ Streptococcus fecalis (B), SPS at the final concentrations indicated, and water to a final volume of 0.5 ml.*

TABLE 2. *Effect of SPS on phagocytosis by isolated leukocytes*[a]

SPS (%)	*Staphylococcus aureus*		*Streptococcus fecalis*		Latex particles	
	Organisms per cell	Percent of cells with organisms	Organisms per cell	Percent of cells with organisms	Particles per cell	Percent of cells with particles
None	2.8	81	3.3	76	10.4	83
0.0005	3.2	83	3.3	86		
0.005	0.1	6	0.1	4		
0.05	0.1	3	0.3	11	4.4	85

[a] Average numbers of organisms or latex particles per cell and the percent of the cells which contained organisms or particles were determined by using smears prepared from the 15-min samples in Fig. 2 for the bacteria and from 30-min samples of the following incubation mixture for the latex particles: 0.3 ml of KRP, 10^7 PMN, 1,000 nmoles of glucose, 0.03 ml of a 1% suspension of latex particles (1.1 μm diameter, Dow Chemical Co., Midland, Mich.), SPS at the concentrations indicated, and water to a final volume of 0.5 ml.

related to the requirement for serum.

Further evidence for an inhibition of serum-mediated phagocytosis by SPS was obtained from a study of the effect of SPS on iodination by intact phagocytosing leukocytes. Inorganic iodide is converted to a trichloroacetic acid-precipitable form by intact leukocytes which have ingested an appropriate particle such as zymosan (16). Serum is required for opsonization. Table 3 demonstrates the marked inhibitory effect of 0.005% SPS on this reaction. Smears prepared at the end of the incubation period indicated a complete inhibition of phagocytosis by SPS. This inhibition was abolished when the leukocytes were preincubated with zymosan and serum for 15 min prior to the addition of SPS and labeled iodide. Phagocytosis occurred during the preincubation period; thus, the inhibitory effect of SPS on iodination in the absence of a preincubation period is due to the inhibition of phagocytosis rather than to the inhibition of the intracellular iodination reaction.

Effect of SPS on the metabolic activity of isolated leukocytes. Glucose C-1 oxidation by PMN is a measure of hexose monophosphate pathway activity. It is normally low in resting leukocytes and is increased many fold during the respiratory burst associated with phagocytosis (24). Table 4 demonstrates the stimulatory effect of SPS on glucose C-1 oxidation by resting human leukocytes. Maximal stimulation occurred at an SPS concentration of 0.005%. Glucose C-1 oxidation by phagocytosing leukocytes was unaffected by SPS under the conditions employed in Table 4.

H_2O_2 formed in intact PMN can be utilized, in part, for the conversion of ^{14}C-formate to $^{14}CO_2$, and this reaction has been employed as

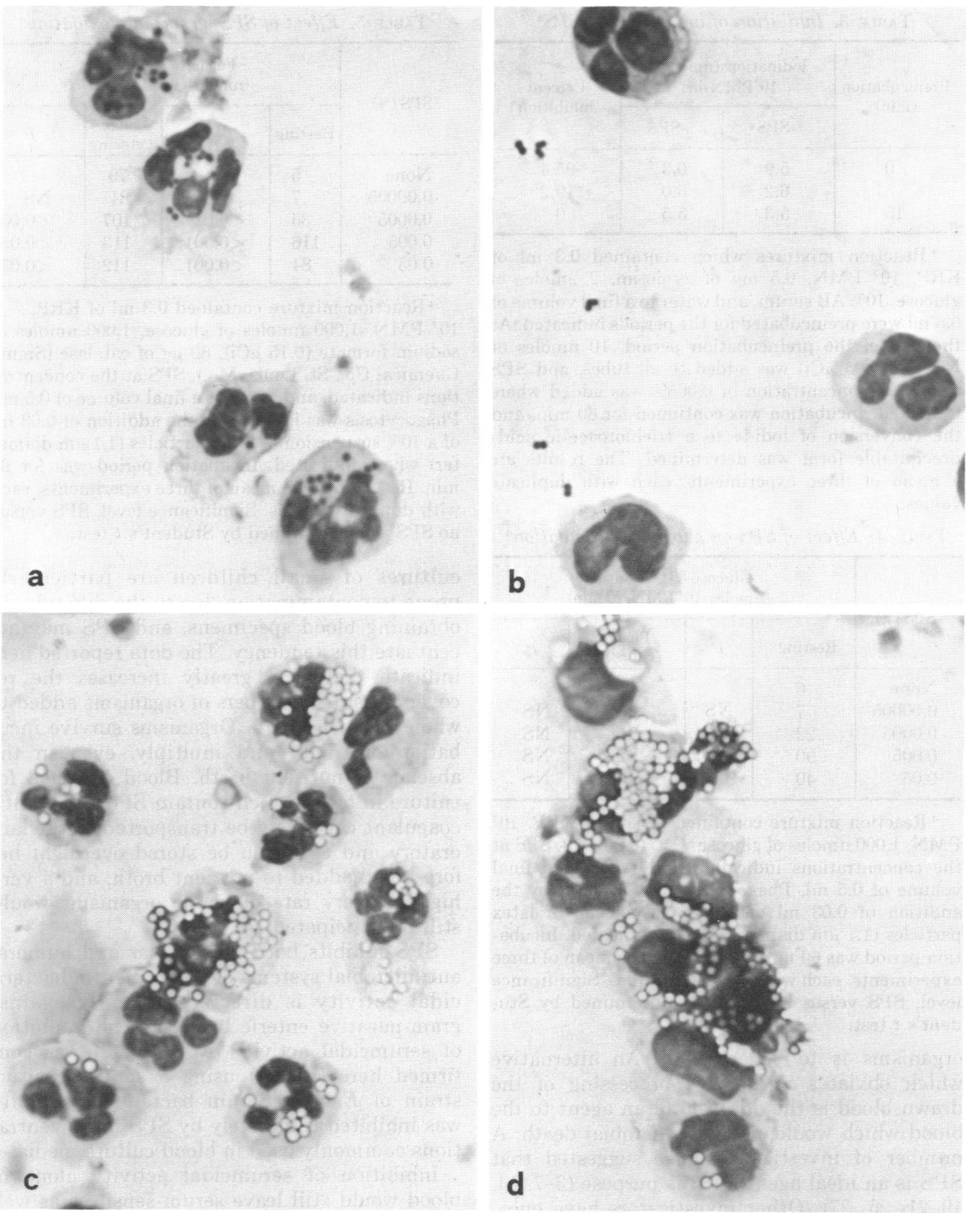

FIG. 3. *Effect of SPS on phagocytosis of Staphylococcus aureus and latex particles: a, PMN + serum + S. aureus; b, PMN + serum + S. aureus + SPS (0.05%); c, PMN + latex particles; d, PMN + latex + SPS (0.05%). Incubation conditions are as described in Table 2.*

a measure of H_2O_2 formation by the intact cell (10). SPS stimulated formate oxidation by both resting and phagocytosing leukocytes (Table 5). This effect is maximal at an SPS concentration of 0.005%.

DISCUSSION

Inocula of a variety of organisms do not survive incubation in whole blood. This emphasizes the need for rapid transfer of the blood to nutrient broth, if efficient recovery of viable

TABLE 3. *Inhibition of iodination by SPS*[a]

Preincubation (min)	Iodination (nmoles/10^7PMN/hr)		Percent inhibition
	-SPS	+SPS	
0	5.9	0.3	95.4
5	6.2	5.0	19.2
15	5.3	5.5	0

[a] Reaction mixtures which contained 0.3 ml of KRP, 10^7 PMN, 0.5 mg of zymosan, 2 μmoles of glucose, 10% AB serum, and water to a final volume of 0.5 ml were preincubated for the periods indicated. At the end of the preincubation period, 10 nmoles of Na ^{125}I (0.05 μCi) was added to all tubes, and SPS at a final concentration of 0.005% was added where indicated. Incubation was continued for 60 min, and the conversion of iodide to a trichloroacetic acid-precipitable form was determined. The results are a mean of three experiments, each with duplicate values.

TABLE 4. *Effect of SPS on glucose C-1 oxidation*[a]

SPS (%)	Glucose C-1 oxidation (nmoles/10^7 PMN/20 min)			
	Resting	*P*	Phagocytosing	*P*
None	6		151	
0.00005	7	NS	164	NS
0.0005	22	<0.01	168	NS
0.005	90	<0.01	166	NS
0.05	40	<0.02	145	NS

[a] Reaction mixture contained 0.3 ml of KRP, 10^7 PMN, 1,000 nmoles of glucose-*1*-^{14}*C* (0.05 μCi), SPS at the concentrations indicated, and water to a final volume of 0.5 ml. Phagocytosis was induced by the addition of 0.03 ml of a 10% suspension of latex particles (1.1 μm diameter) where indicated. Incubation period was 60 min. Results are the mean of three experiments, each with duplicate values. Significance level, SPS versus no SPS was determined by Student's *t* test.

organisms is to be achieved. An alternative which obviates immediate processing of the drawn blood is the addition of an agent to the blood which would prevent microbial death. A number of investigators have suggested that SPS is an ideal agent for this purpose (3–7, 14, 19–21, 25, 27). Other investigators have questioned its usefulness in certain instances. Hoare (9) reported that SPS decreases the growth of anaerobic streptococci, and suggested that this agent may not be useful for the culture of these organisms. Minkus and Moffet questioned the usefulness of SPS in the detection of bacteremia in small children, since organisms judged to be contaminants were recovered with high frequency (13). Blood

TABLE 5. *Effect of SPS on formate oxidation*[a]

SPS (%)	Formate oxidation (nmoles/10^7 PMN/hr)			
	Resting	*P*	Phagocytosing	*P*
None	5		79	
0.00005	7	NS	81	NS
0.0005	36	<0.01	107	<0.05
0.005	116	<0.001	113	<0.05
0.05	84	<0.001	112	<0.05

[a] Reaction mixture contained 0.3 ml of KRP, 2 × 10^7 PMN, 1,000 nmoles of glucose, 1,000 nmoles of sodium formate (0.15 μCi), 80 μg of catalase (Sigma Chemical Co., St. Louis, Mo.), SPS at the concentrations indicated, and water to a final volume of 0.5 ml. Phagocytosis was induced by the addition of 0.03 ml of a 10% suspension of latex particles (1.1 μm diameter) where indicated. Incubation period was for 60 min. Results are the mean of three experiments, each with duplicate values. Significance level, SPS versus no SPS was determined by Student's *t* test.

cultures of small children are particularly prone to contamination due to the difficulty in obtaining blood specimens, and SPS may accentuate this tendency. The data reported here indicate that SPS greatly increases the recovery of small numbers of organisms added to whole blood in vitro. Organisms survive incubation overnight and multiply, even in the absence of nutrient broth. Blood obtained for culture in tubes which contain SPS as an anticoagulant can safely be transported to the laboratory and can even be stored overnight before being added to nutrient broth, and a very high recovery rate of viable organisms would still be anticipated.

SPS inhibits both the cellular and humoral antimicrobial systems of blood. Serum bactericidal activity is directed generally against gram-negative enteric bacteria. The inhibition of serumcidal activity by SPS (12) was confirmed here (Fig. 1) using a serum-sensitive strain of *E. coli*. Serum bactericidal activity was inhibited completely by SPS in concentrations commonly used in blood culture media.

Inhibition of serumcidal activity alone in blood would still leave serum-sensitive as well as serum-resistant organisms susceptible to phagocytosis and destruction by leukocytes. However, SPS, in concentrations commonly used in blood culture media, also completely inhibited the killing of *S. aureus* and *S. fecalis* by isolated leukocytes (Fig. 2). This effect of SPS is due to an inhibition of phagocytosis (reference 1, Table 2, Fig. 3) rather than to an inhibition of the intracellular microbicidal

mechanisms. Smears prepared during the incubation of the bacteria with leukocytes in the presence of SPS reveal a striking decrease in intracellular organisms. Serum is required as a source of opsonins for the phagocytosis of *S. aureus* and *S. fecalis* by leukocytes. The inhibition of phagocytosis by SPS is dependent on the serum requirement, since the uptake of latex particles, which occurs in the absence of serum, is not inhibited by SPS.

Iodination by intact leukocytes can be employed as an indirect measure of phagocytosis under conditions in which intracellular components of the iodinating system are unaffected. Iodination by phagocytosing leukocytes is inhibited by SPS, and localization of the defect to phagocytosis is indicated by its abolition when phagocytosis occurs prior to the addition of SPS and radioiodide as a result of the preincubation of cells with particles and serum (Table 3).

Sodium fluoride also inhibits phagocytosis by intact leukocytes (2, 24), and this agent was added to blood, alone and in the presence of SPS, in an attempt to further improve the recovery of viable organisms. Fluoride added alone increased the incidence of positive cultures with 9 of the 15 organisms tested. Of the remainder, five were enteric bacilli, which are susceptible to fluoride-insensitive serumcidal systems. In no instance was fluoride alone preferable to SPS alone. The addition of fluoride to SPS tubes did not significantly increase the high yield of positive cultures observed with SPS alone, although small increases were seen with certain organisms.

Sodium fluoride decreases phagocytosis by its inhibitory effect on glycolysis and energy production by leukocytes (2, 24). SPS, on the other hand, decreases the opsonic capacity of serum, presumably due to its anticomplementary and serum protein-precipitating activity (17, 26, 28). These effects of SPS could also account for its inhibitory effect on serum bactericidal activity.

SPS also exerts a direct effect on the metabolic activity of the leukocytes. It significantly increases the glucose C-1 oxidation of resting leukocytes, but not of phagocytosing leukocytes, and significantly increases formate oxidation by both resting and phagocytosing leukocytes. In the measurement of both glucose C-1 and formate oxidation by intact leukocytes, phagocytosis is induced by the addition of large numbers of latex particles. Serum is not required, and, as a result, phagocytosis is not inhibited by SPS. The mechanism of the metabolic stimulation by SPS is unknown. An increase in resting glucose C-1 oxidation occurs on the exposure of leukocytes to a number of substances, e.g., surface active agents (8, 22, 30), phospholipase C (15), and anticellular antibody (23), which react with the plasma membrane and apparently trigger a response similar to that of phagocytosis. SPS may react similarly. Whatever the mechanism, these metabolic alterations have no apparent relationship to the inhibition of phagocytic function by SPS. Since SPS does not inhibit metabolic activity or iodination by leukocytes if phagocytosis occurs, this agent would not be expected to prevent the killing of microorganisms which are already intracellular when the blood is drawn.

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