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Effects of Human and Rabbit Serum on Viability, Permeability, and Envelope Lipids of *Serratia marcescens*

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The major action of serum on gram-negative organisms is thought to be on the microbial envelope. We compared the effects of normal human and rabbit serum on the envelope lipids of two strains of *Serratia marcescens*, one sensitive and one resistant to the bactericidal effects of serum. During killing by either serum, the sensitive strain underwent rapid permeability changes coincident with degradation of microbial phospholipids. The resistant strain exhibited none of these effects. The phospholipid degradation that accompanies killing of the sensitive strain by serum could be caused by phospholipases present in serum or by Serratia's own phospholipid-splitting enzymes. The results indicate that phospholipid breakdown is caused by activation of bacterial phospholipases and not by serum phospholipases. This conclusion is based opon the following findings. (i) Although rabbit serum phospholipase A was at least 10 times more active than human serum phospholipase A, phospholipid degradation in the sensitive *Serratia* strain was comparable during (equally rapid) killing by human or rabbit serum. (ii) Heat treatment (56 C) of both sera eliminated bactericidal activity as well as microbial lipid degradation but abolished phospholipase activity of human serum only. (iii) Virtually complete removal of phospholipase A activity from human serum by adsorption onto autoclaved *Micrococcus lysodeikticus* had no effect on the extent of phospholipid hydrolysis or on bactericidal activity. Activation by serum of endogenous phospholipase activity in *S. marcescens* was accompanied by enhanced incorporation of lipid precursors into bacterial lipids. No evidence was found for increased turnover of protein or ribonucleic acid during killing by serum.

Serratia marcescens strains, like other gram-negative microbial species (20), may vary widely in their susceptibility to the bactericidal effects of serum (M. S. Simberkoff and J. J. Rahal, Jr., Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 12th, Atlantic City, N. J., Abstr. 194, p. 102, 1972).

It is well established that killing of susceptible gram-negative organisms is associated with alterations of the microbial envelope (12, 13, 19, 30). Effects have been observed on each of the three main layers of the envelope, the outer membrane (12,13), the cell wall peptidoglycans (5,12), and the cytoplasmic membrane (19). Different constituents of serum appear responsible for effects on the individual layers (12, 13).

The molecular events that accompany structural alterations that result, for example, in release of lipopolysaccharides (17, 30) and of periplasmic and cytoplasmic enzymes (13) are not well understood. Of the antimicrobial constituents of serum, only lysozyme has a known mode of action on the microbial envelope. However, this enzyme by itself causes limited structural and functional impairment in most gram-negative bacterial populations.

Killing of *Escherichia coli* by granulocyte preparations, as by serum, is accompanied by early permeability changes (2,28). These changes coincide with altered bacterial lipid metabolism.

In this study we examined the effects of normal human serum (NHS) and normal rabbit serum (NRS) on phospholipid metabolism of two *S. marcescens* strains, one sensitive and one resistant to the bactericidal action of serum. The results indicate that killing of *S. marcescens* by serum is associated with a substantial increase in turnover of microbial lipids. Further, evidence is presented suggesting that

the degradation of *Serratia* phospholipids is the result of activation of the organism's endogenous phospholipases.

MATERIALS AND METHODS

S. marcescens strains. Two smooth, nonpigmented strains were isolated from human sources, the sensitive strain (type 01:H12) from a urine culture and the resistant strain (type 012:H12) from a blood culture.

Growth and labeling. The organisms were grown in a triethanolamine-buffered (TEA) medium at pH 7.5, as described before (31). After growth overnight, the bacterial suspension was diluted 1:10 and subcultured for 2.5 h at 37 C. This subculture, after centrifugation at 10,000 × *g* for 10 min, was resuspended in 0.9% saline in the desired concentration. Bacterial phospholipids were labeled by adding 0.125 μCi of [1-^{14}C]palmitic acid per ml (specific activity, 59.9 mCi/mmol; Amersham-Searle Corp. Arlington Heights, Ill.) complexed with 2% bovine serum albumin (BSA) to the subculture. After 2.5 h, the cells were washed and resuspended in fresh TEA medium with 2% BSA and reincubated for 30 min so that remaining unincorporated free fatty acid would be incorporated. The labeled bacteria were centrifuged and resuspended in saline.

Protein and ribonucleic acid (RNA) were labeled in the same way, using either 0.1 μCi of [1-^{14}C]leucine per ml (specific activity, 55 mCi/mmol; International Chemical and Nuclear Corp., Waltham, Mass.) or 0.1 μCi of [2-^{14}C]uracil per ml (specific activity, 56 mCi/mmol; New England Nuclear Corp., Boston, Mass.), with no added BSA. To prepare substrate for assay of phospholipase activity, *E. coli* cells were labeled during growth with 0.125 μCi of [1-^{14}C]oleic acid per ml (specific activity, 58.9 mCi/mmol; Amersham-Searle Corp.). After labeling, the organisms were autoclaved to inactivate bacterial phospholipases. This procedure also renders the *E. coli* phospholipids susceptible to degradation by a wide range of phospholipases (9, 14, 15, 23).

Collection of serum. Blood from normal human volunteers and from New Zealand White rabbits was collected in sterile glass tubes. After clotting at 37 C for 45 min and clot retraction in ice for 30 min, the serum was taken and used the same day.

Incubation procedure. From 6 × 10^8 to 12 × 10^8 organisms (either the serum-sensitive or the serum-resistant strain) were incubated in a total volume of 1 ml of physiological sterile saline that also contained 10 mM $CaCl_2$, 40 mM tris(hydroxymethyl)aminomethane-maleate buffer at pH 7.5, and NHS or NRS in the concentration indicated.

Viable counts. At various time intervals, 10-μl samples were taken from the suspension, serially diluted in sterile saline, and plated on nutrient agar. After incubation overnight at 37 C, the number of colony-forming units on the plates was determined.

Phospholipid degradation. To determine degradation of phospholipids during exposure to serum, incubation was carried out as described above except that bacterial populations were used that had been labeled during growth with [1-^{14}C]palmitic acid (see labeling procedure). At the end of the indicated time periods, reactions were stopped by addition of 6 volumes of chloroform-methanol (1:2, vol/vol). Lipids were extracted overnight at room temperature according to the procedure of Bligh and Dyer (4).

Phospholipid synthesis. Incorporation of various lipid precursors([2-^{14}C]glycerol, specific activity, 13 mCi/mmol, Amersham-Searle Corp., 0.4 μCi/ml, in a final concentration of 0.58 mM; [1-^{14}C]palmitic acid, 0.6 μCi/ml; or [1-^{14}C]oleic acid, 0.6 μCi/ml) was studied during incubation with serum in 50% TEA medium (C. Mooney and P. Elsbach, Fed. Proc. **33**:632, 1974). Reactions were interrupted and lipid was extracted as in lipid degradation studies. Lipid extracts of fatty acid-labeled cells were washed once and those of [2-^{14}C]glycerol-labeled cells were washed three times. The washed chloroform phases of the lipid extracts were dried under a nitrogen stream, and the labeled lipid was transferred to commercial Silica Gel F 254 plates (Brinckman Instruments, Westbury, N. Y.) with chloroform-methanol (1:2 vol/vol). Fatty acids and phospholipids were separated in a solvent system consisting of petroleum ether/ether/glacial acetic acid (80:20:1, vol/vol), and individual phospholipid species were separated by using the solvent mixture chloroform/methanol/glacial acetic acid (65:25:6, vol/vol). Lipid species were identified by comparison of the R_f with that of authentic standards after visualization following exposure to iodine. Liquid scintillation counting of thin-layer fractions scraped off the plates directly into counting vials was carried out as previously described (6).

Assay for permeability changes. Effects of serum on the permeability of the envelope of *S. marcescens* was examined by determining: (i) the susceptibility of leucine incorporation into microbial protein to inhibition by actinomycin D, as recently described in detail (2, 28); and (ii) the release of the inducible periplasmic enzyme alkaline phosphatase (16). Since synthesis of readily detectable levels of alkaline phosphatase takes more than 4 h, the *Serratia* populations were incubated overnight, in TEA medium without added inorganic phosphate (13), to achieve adequate induction. Total alkaline phosphatase in induced *S. marcescens* was determined after sonication for three 30-s periods at 70 W by a Sonifier cell disruptor model W185 (Ultrasonics Inc., Plainview, N. Y.). The composition of reaction mixtures of 1 ml was as described above. All experimental samples were filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass.), and 0.2 ml of each filtrate was incubated for 30 min at room temperature with 1.8 ml of *p*-nitrophenyl phosphate, disodium, 200 μg/ml (no. 104, Sigma Chemical Co., St. Louis, Mo.), in 1 M tris(hydroxymethyl)-aminomethane-hydrochloride buffer at pH 8.0. *p*-Nitrophenol product was determined by optical density readings at 410 nm in a Spectronic 20 (Bausch & Lomb, Rochester, N.Y.).

RESULTS

Effect of NHS or NRS on viability of two strains of S. marcescens. One of the two

strains was highly and equally sensitive to the cidal effect of NHS and NRS (Table 1). Less than 5% of a population of the sensitive strain was capable of multiplication after 1 h at a serum concentration of 25%. With increasing serum concentrations, both the rate and extent of killing increased. By contrast, the viability and growth of the other strain (resistant) was unaffected by serum concentrations as high as 80%.

Effect of serum on permeability of the S. marcescens envelope. During killing by serum, incorporation of [1-^{14}C]leucine by the sensitive strain proceeded at 50% of control levels for 1 h (Fig. 1). However, in the presence of serum, normally impermeant actinomycin D gained access to intracellular deoxyribonucleic acid and within 5 min caused cessation of incorporation of [1-^{14}C]leucine (2) into acid-precipitable material. Serum also caused a dose- and time-dependent release of the periplasmic enzyme alkaline phosphatase (Fig. 2). Neither NHS nor NRS causes these permeability changes in the resistant strain.

Effects of serum on phospholipids of S. marcescens. Permeability changes during killing of gram-negative organisms (*E. coli*) by serum (13) may be associated with loss of phospholipids and/or lipopolysaccharides (30). It has been suggested that this release is triggered by action of a bacterial enzyme (30). However, we are not aware of evidence indicating that an enzymatic attack on the phospholipids

TABLE 1. *Effect of NHS or NRS on viability of two strains of Serratia marcescens*[a]

Incubation time (min)	Survival (%)					
	Sensitive				Resistant	
	0[b]	10	25	50	0	80
0	100.0	100.0	100.0	100.0	100.0	100.0
5			52.1 (45.9)	9.4		
15			28.4 (4.9)	2.5		
30			12.3 (0.8)	2.2		
60	147.3	42.9	4.8 (0.2)	0.9	141.7	141.8
120	149.7	66.1	4.0 (0.1)	0.5	140.9	331.8

[a] Colony-forming units were determined as described in Materials and Methods. Results are presented as the mean of at least three experiments. Results of experiments with NRS are shown in parentheses.

[b] Percentage of serum.

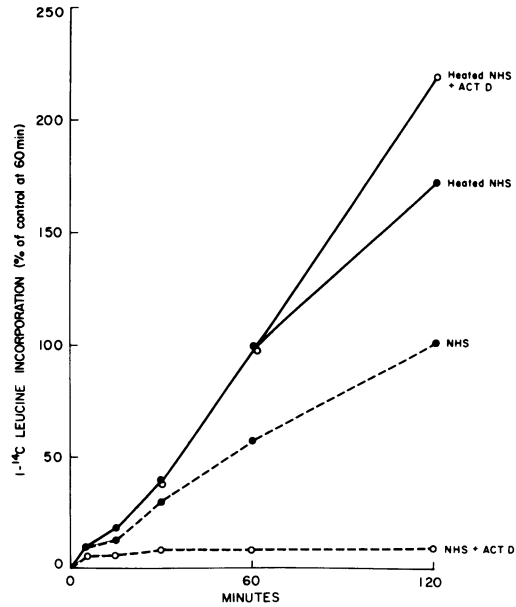

FIG. 1. *Effect of NHS and actinomycin D on [1-^{14}C]leucine incorporation by a serum-sensitive strain of Serratia marcescens. Incorporation of [1-^{14}C]leucine into bacterial protein was determined as described before (2). At the indicated times, 0.1-ml samples of the incubation mixtures were pipetted into 2 ml of ice-cold 10% trichloroacetic acid. Precipitates were collected 15 min later by filtration through 0.6-μm filters (Millipore Corp., Bedford, Mass.) and washed three times with ice-cold 5% trichloroacetic acid. Radioactivity on the filters was determined by liquid scintillation counting (2). All controls were incubated with NHS heated at 56 C for 30 min. Results are expressed as percentage of control at 60 min and are shown as the mean of at least three experiments. NHS concentration = 25%.*

themselves accompanies the envelope changes caused by serum.

To examine the fate of bacterial lipids during killing of the sensitive strain by serum, the organisms were grown with [1-^{14}C]palmitic acid and degradation of labeled bacterial lipids was investigated in the absence and presence of serum. In Table 2 the effects of normal and heated (56 C for 30 min) NHS or NRS on the release of 1-^{14}C-labeled fatty acid from the serum-sensitive and -resistant strains are compared. Whereas both NHS and NRS at a concentration of 25% caused substantial degradation of the phospholipids of the sensitive *Serratia*, degradation of the resistant strain's lipids under the same conditions was minimal. At high serum concentrations (50 to 80%), the phospholipids of the resistant strain underwent a small amount of hydrolysis (less than 10%).

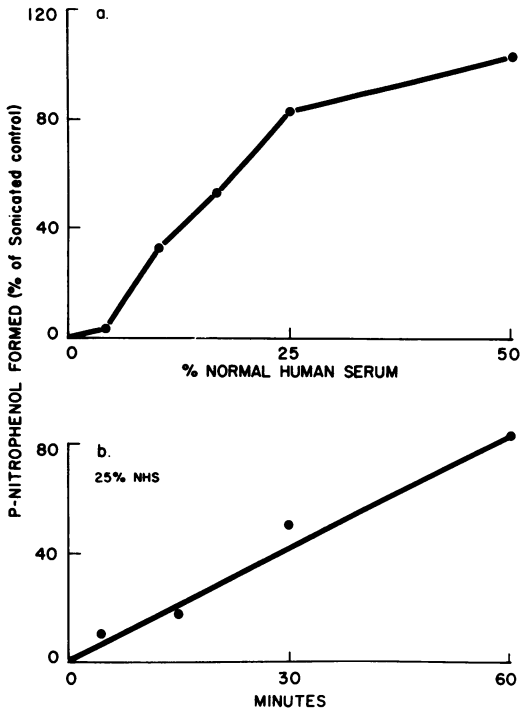

FIG. 2. *Effect of NHS on release of alkaline phosphatase by serum-sensitive Serratia marcescens. Alkaline phosphatase was assayed as described in Materials and Methods. Values are expresses as percentage of sonicated controls. Time of (a) = 60 min.*

TABLE 2. *Effect of normal or heated NHS or NRS on fatty acid release from sensitive or resistant Serratia marcescens*[a]

Organism	Fatty acid released (%)				
	Alone	25% NHS	25% Heated NHS	25% NRS	25% Heated NRS
Sensitive	1.0 ± 0.2 (9)	26.1 ± 2.3 (9)	3.1 (3)	20.7 ± 5.4 (6)	2.7 (3)
Resistant	0.9 ± 0.2 (5)	3.1 ± 0.7 (4)	4.3 (3)	3.7 ± 0.2 (4)	3.7 (3)

[a] The two strains of *S. marcescens* were labeled during growth with [1-^{14}C]palmitic acid as described in Materials and Methods. Incubation was carried out for 2 h at 37 C and terminated by addition of chloroform-methanol (1:2, vol/vol). For determination of fatty acid release, see Materials and Methods. 1-^{14}C-labeled fatty acid release is expressed as percentage of total phospholipid radioactivity. Numbers in parentheses indicate number of observations.

Heat-treated sera had no appreciable effect on phospholipids (nor on viability or permeability in either strain). Figure 3a shows that increasing amounts of serum caused increasing degradation (in parallel with a serum concentration-dependent increase in the rate of killing [Table 1]), and Fig. 3b shows that the degradation of phospholipids of the sensitive strain occurred linearly with time (Table 1). In line with observations by others (30), a small amount of intact diacylphosphatides (less than 10% of total labeled phospholipids) was released into the medium. Under the conditions of these experiments, there was no appreciable formation of lysocompounds or of labeled diglycerides.

Phospholipid-splitting activities of serum and of S. marcescens and substrate properties of S. marcescens lipids. The presence of phospholipase A_2 in serum from various mammalian sources has been reported by several laboratories (9, 11, 22, 24). The phospholipase A_2 of rabbit serum is approximately 10 times more active per unit volume than the phospholipase of human serum (unpublished observations), using [1-^{14}C]oleic acid-labeled autoclaved *E. coli* as substrate (9, 14, 15, 23).

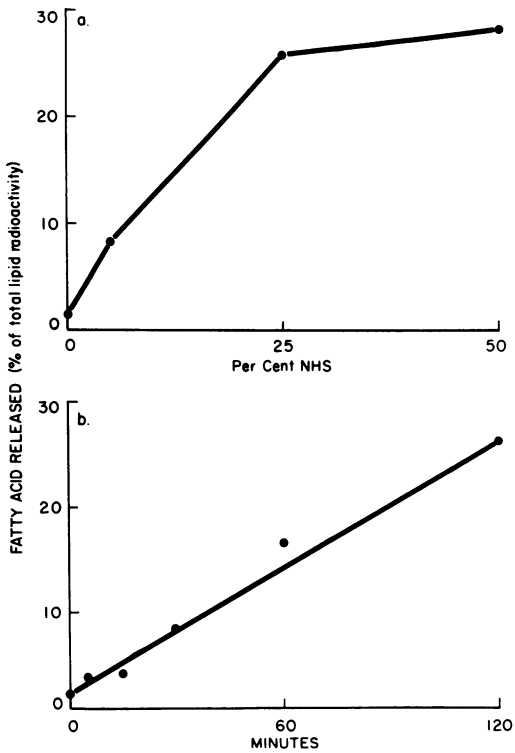

FIG. 3. *Effect of NHS concentration (a) and time (b) on fatty acid release by serum-sensitive Serratia marcescens. Experiments were carried out as described in Materials and Methods. Release of ^{14}C-labeled free fatty acid from [1-^{14}C]palmitic acid-labeled S. marcescens is expressed as percentage of total lipid radioactivity. Results shown represent the mean of at least three experiments. Time of (a) = 60 min; (b) NHS concentration = 25%.*

Further, whereas NHS phospholipase was inactivated by heating at 56 C for 30 min, NRS phospholipase was unaffected by such treatment. Nevertheless, heating of both sera resulted in elimination of all effects on the serum-sensitive *Serratia* strain (Table 2). Snake venom phospholipase A_2 alone or added to heat-inactivated serum had no effect on the viability or on the labeled phospholipids of the two strains.

As is the case for other gram-negative rods (27), *S. marcescens* cells have phospholipid-splitting enzymes. Under normal growth conditions, the [1-^{14}C]fatty acid-labeled phospholipids of both the serum-sensitive and -resistant strains are quite stable. When the organisms were heated at 56 C for 30 min, however, both strains degraded their own phospholipids to about the same extent (Table 3).

The distributions of palmitate among the major lipids of the two *S. marcescens* strains after labeling during several divisions were quite similar (Table 4). Since labeling under such conditions is known to reflect rather closely the chemical composition of the bacterial phospholipids (31), no gross differences in phospholipid composition between the two strains were apparent.

Further, autoclaving of *S. marcescens* (inactivating the bacterial phospholipases) after labeling with 1-^{14}C-labeled fatty acid rendered the phospholipids of the two strains equally susceptible to degradation by several exogenous phospholipases A, including snake venom and granulocyte phospholipase A_2 (Table 5). These observations reveal no major differences in the phospholipid-degradative apparatus of the two strains or in the substrate properties of their phospholipids.

Effect of human bactericidal, phospholipase-free serum on the sensitive strain. To determine whether the degradation of phospholipids that accompanies killing of the sensitive *Serratia* strain is caused by the phospholipase activity in serum and/or the *Serratia*'s own phospholipases, an attempt was made to remove the phospholipase activity from NHS without loss of bactericidal activity. This was achieved by incubating NHS with autoclaved *Micrococcus lysodeikticus* for 5 min (in the presence of 10 mM ethylene glycol tetraacetic acid to prevent activation of the complement system) (Table 6). This procedure resulted in removal of more than 98% of the phospholipase activity (uncorrected for background) from the serum after sedimentation of the bacteria by centrifugation for 10 min. The phospholipase activity apparently was removed from the serum by adsorption onto the autoclaved *M. lysodeikticus*, because the resuspended pellet contained all the activity of the untreated serum when tested against autoclaved [1-^{14}C]oleic acid-labeled *E. coli* as substrate. The bactericidal activity of phospholipase-depleted

TABLE 3. *Effect of temperature on activation of endogenous phospholipase in two strains of Serratia marcescens*[a]

Organism	Fatty acid released (%)	
	37 C	56 C
Sensitive	0.4	38.2
Resistant	0.7	49.5

[a] Sensitive and resistant *S. marcescens* were labeled with [1-^{14}C]palmitic acid as described in Materials and Methods. The labeled organisms were then incubated for 30 min at either 37 or 56 and then extracted for lipids.

TABLE 4. *Distribution of radioactivity among major phospholipid species of two strains of Serratia marcescens after labeling with [1-^{14}C]palmitic acid*[a]

Organism	Total radioactivity (%) in:			
	LPE	PE	PG	CL
Sensitive	3.0	76.2	10.8	5.9
Resistant	2.5	73.0	14.1	6.0

[a] *S. marcescens* strains were labeled with [1-^{14}C]palmitic acid as described in Materials and Methods. LPE, Lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidyl glycerol; CL, cardiolipin.

TABLE 5. *Hydrolysis of phospholipids of autoclaved [1-^{14}C]palmitic acid-labeled sensitive or resistant Serratia marcescens by exogenous phospholipases*[a]

Autoclaved organism	Fatty acid released (%)			
	Alone	25% NRS	Snake venom A_2	Granulocyte phospholipase A_2
Sensitive	0.4	22.7	32.4	13.6
Resistant ...	1.0	19.2	42.4	12.7

[a] Labeled organisms were exposed to 120 C at 2.7 kg/cm^2 for 15 min. The autoclaved bacteria were then washed in 2% bovine serum albumin in saline to remove radioactive free fatty acid (less than 10% of original total phospholipid radioactivity). Reaction mixtures of 1 ml contained 6×10^8 autoclaved organisms, 10 mM $CaCl_2$, and 0.02 units of Russell viper venom phospholipase A_2 (Sigma Chemical Co.). or phospholipase A_2 in a partially purified granulocyte preparation (1.2×10^7 cell equivalents) (2), or NRS.

NHS towards the sensitive strain was fully preserved and, moreover, the extent of phospholipid degradation of [1-^{14}C]palmitic acid-labeled *Serratia* was identical to that observed with unabsorbed NHS (Table 6). The serum phospholipase-rich resuspended *M. lysodeikticus* pellet caused neither loss of viability of the sensitive strain nor degradation of its lipids. These findings strongly suggest that Serratia's own phospholipases account for the observed breakdown of the bacterial phospholipids during killing by serum.

Effect of serum on protein and RNA of S. marcescens. In contrast to the substantial degradation of phospholipids during killing of *Serratia* by serum, labeled bacterial protein and RNA did not undergo appreciable degradation (Table 7), indicating that lipid breakdown is not simply part of a generalized autolytic process triggered by serum.

The observation that leucine incorporation by the sensitive organism during killing by serum continued at only moderately reduced rates, remaining linear for at least 2 h, further suggests that both structural and functional disorganization are relatively limited under these conditions. This might also mean that phospholipid degradation is part of a more complex change in bacterial phospholipid metabolism rather than solely an expression of a catabolic process that accompanies loss of viability. To examine this possibility, we studied phospholipid synthesis by *Serratia* in the absence and presence of serum.

Effect of serum on incorporation of [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, or [2-^{14}C]glycerol. Whereas NHS (or NRS; not shown) had no effect on incorporation of either palmitic or oleic acid into phospholipids of the resistant strains, the initial rate of incorporation of both fatty acids by the sensitive strain during killing by serum was stimulated (Fig. 4). The distribution of the labeled precursors among the major phospholipid species of both *Serratia* strains exposed to normal serum was the same as that of control populations incubated with heated serum.

Incorporation of [2-^{14}C]glycerol into lipids of the sensitive strain was also enhanced by serum (Fig. 5). This stimulation was statistically significant at all three time periods studied but clearly was most pronounced at 2 h. The increased incorporation of exogenous 1-^{14}C-labeled fatty acid at 30 min, in the face of a concomitant release of endogenous fatty acid (into the medium, see above), probably means that the stimulation of synthesis of phospholipid from fatty acid by the sensitive strain was underestimated. Whether or not the increased incorporation of [2-^{14}C]glycerol actually represents enhanced de novo synthesis is uncertain. Endogenous labeled glycerol pools may well have decreased, either owing to loss through a leaky envelope or as a consequence of decreased

TABLE 6. *Effect of removal of phospholipase A$_2$ from NHS on sensitive Serratia marcescens*[a]

Organism	Fatty acid released (%)			
	Alone	NHS	Supernatant fraction	Resuspended pellet
Sensitive *Serratia*	1.0 (100.0)[b]	16.9 (7.2)	16.9 (8.1)	1.2 (92.8)
Autoclaved *E. coli*	1.6	19.0	1.7	22.6

[a] One milliliter of NHS was incubated at 37 C for 5 min with 10^{10} autoclaved *Micrococcus lysodeikticus* in 1 ml of saline with 10 mM ethylene glycol tetraacetic acid. The suspension was subjected to centrifugation at 4 C at 10,000 × *g* for 10 min. The supernatant fraction was taken off and the sedimented bacteria were resuspended in 2 ml of saline. Amounts of supernatant and resuspended sediment to provide a final concentration of 25% were incubated with [1-^{14}C]palmitic acid-labeled sensitive *Serratia* or with 6 × 10^8 autoclaved [1-^{14}C]oleic acid-labeled *E. coli* as described in Materials and Methods. To all incubation mixtures 20 mM $CaCl_2$ was added. Results shown are mean of two virtually identical experiments.

[b] Numbers in parentheses indicate percentage of survival.

TABLE 7. *Effect of NHS on loss of acid-precipitable radioactivity from [1-^{14}C]leucine- or [2-^{14}C]uracil-labeled sensitive Serratia marcescens*[a]

Conditions	[1-^{14}C]leucine-labeled protein (%)			[2-^{14}C]uracil-labeled RNA (%)		
	0 min	60 min	120 min	0 min	60 min	120 min
Alone	100.0	101.8	105.6	100.0	93.3	96.0
25% NHS	100.0	99.4	96.9	100.0	102.0	104.1
25% NRS	100.0	103.0	95.2	100.0	107.4	101.8

[a] Sensitive *S. marcescens* were labeled during growth with [1-^{14}C]leucine or [2-^{14}C]uracil as recently described (10). Incubation mixtures of 1 ml contained 6 × 10^8 labeled organisms in 0.9% saline, 25% NHS or NRS, 10 mM $CaCl_2$, and 40 mM tris(hydroxymethyl)aminomethane-maleate buffer, pH 7.5. Samples of 0.1 ml were taken at the indicated time intervals and pipetted into 2 ml of ice-cold 10% trichloroacetic acid, and precipitates were collected on 0.6-μm filters (Millipore Corp., Bedford, Mass.) and washed three times with ice-cold 5% trichloroacetic acid. Radioactivity on the filters was counted in a liquid scintillation spectrometer as previously described (10). Radioactivity is expressed as percentage of zero-time values.

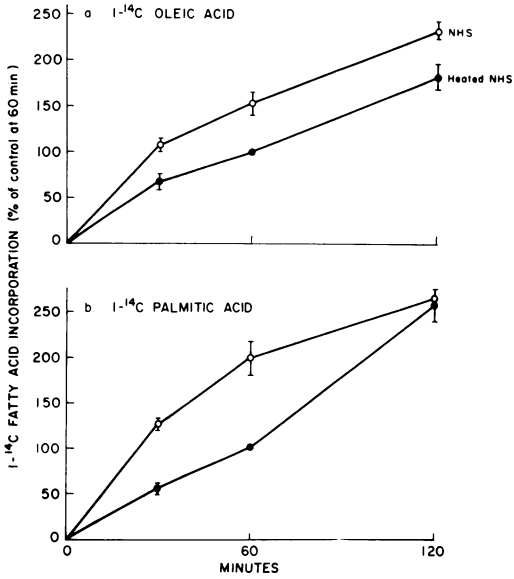

FIG. 4. *Effect of NHS on incorporation of [1-^{14}C]oleic acid (a) or [1-^{14}C]palmitic acid (b) by serum-sensitive Serratia marcescens. The experiments were carried out as described in Materials and Methods. Results are expressed as percentage of 60-min control (heated NHS) and are shown as mean ± standard error of the mean of at least three experiments. NHS concentration = 25%.*

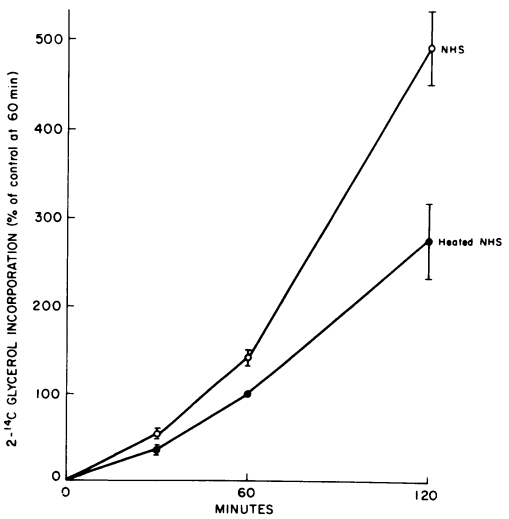

FIG. 5. *Effect of NHS on incorporation of [2-^{14}C]glycerol by serum-sensitive Serratia marcescens. See legend of Fig. 4.*

production, causing an increase in the specific activity of the incorporated [2-^{14}C]glycerol.

Effect of polymixin B on viability of S. marcescens and phospholipid degradation. The bactericidal effect of polymixin B on gram-negative organisms is thought to be related to interaction of the antibiotic with the envelope (probably lipid) of susceptible organisms (1, 26). Therefore, the possibility was explored that killing of *Serratia* by polymixin B, as by serum, is associated with activation of bacterial phospholipases. Although *Serratia* is relatively resistant to polymixin B, at high concentrations (50 μg/ml) more than 80% of both the serum-sensitive and -resistant strains were killed in 60 min (Table 8). However, no degradation of phospholipid occurs.

DISCUSSION

Numerous observations indicate that bactericidal concentrations of normal and immune serum cause alterations in the envelopes of gram-negative organisms (12, 13, 19, 20, 30). Similarly, other microbicidal systems and agents include in their effects on the microorganism structural and functional changes in the microbial envelope (1, 2, 5, 7, 8, 10, 17, 26, 28).

The studies presented here also demonstrate that killing by serum of a susceptible strain of *S. marcescens* is associated with early changes in permeability of the microbial envelope. These serum effects are accompanied by substantial degradation of microbial lipids. Although both NHS and NRS possess active phospholipases A_2, the degradation of the lipids of the serum-sensitive strain appears attributable to activation of Serratia's own phospholipid-degradative apparatus. This conclusion is based mainly on the observation that effective removal of NHS phospholipase A_2 activity onto autoclaved *M. lysodeikticus* (a procedure that does not interfere with bactericidal activity of the serum) did not diminish the phospholipid degradation that parallels loss of viability. Similar experiments with *E. coli*(W) (not shown) yielded identical results, indicating that activation of endogenous phospholipase activity oc-

TABLE 8. *Effect of polymixin B on a serum-sensitive and -resistant strain of Serratia marcescens*[a]

Organism	Survival (%)		Fatty acid released (%)	
	Alone	Polymixin B	Alone	Polymixin B
Sensitive	100.0	18.7	1.0	0.5
Resistant	100.0	7.7	0.9	0.8

[a] The experiment was carried out as described in the legend of Table 2. Final polymixin B sulfate (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N.C.) concentration was 50 μg/ml.

curs in other gram-negative organisms susceptible to the bactericidal effects of serum. Further, NRS which was as bactericidal as NHS towards the sensitive strain but which contained a far more potent phospholipase A_2 (unpublished observations) caused no more bacterial phospholipid degradation than NHS.

This activation by serum of autolysis of Serratia's lipids is not part of a general autolytic process, if judged by the lack of evidence of degradation of previously labeled protein and RNA and by the fact that biosynthetic activity by the killed organisms is remarkably well preserved. This is particularly evident with respect to incorporation of exogenous fatty acid into bacterial phospholipid, suggesting that the loss of envelope lipids through hydrolysis (and to some extent as whole phospholipid molecules [30]) is counteracted by formation of new phospholipid.

It is not clear whether this increase in phospholipid synthesis should be seen as an attempt at repair of the envelope or as a nonspecific response of a still-intact biochemical apparatus (17).

It is tempting to relate the rather dramatic changes in the metabolism of lipids, i.e., essential envelope constituents, to the bactericidal and permeability effects. On the other hand, neither killing nor effects on the permeability barrier need to be linked to phospholipid breakdown because bactericidal concentrations of the polypeptide antibiotic polymixin B, which also cause increased permeability (1; unpublished observations), produced no detectable effect on lipid degradation (nor on synthesis [not shown]). However, polymixin B strongly interacts with envelope lipids of several susceptible gram-negative bacterial species (1). This might mean that the physical chemical effects of this interaction and the biochemical effects of serum on microbial lipids produce the same functional changes in the envelope.

The bactericidal effects of serum on gram-negative organisms have been attributed to interaction between the envelope and the antibody-complement system. Muschel believes that this system acts directly on the cytoplasmic membrane (19, 20). Feingold et al., however, have argued that the primary site of action is at the outer membrane and probably involves the lipopolysaccharides and phospholipids (12, 13). The present study does not resolve this issue. On the other hand, the rapid entry of actinomycin D, the release of the periplasmic enzyme alkaline phosphatase, and the prompt activation of phospholipase activity do indicate an early effect on the outer membrane. This layer of the envelope of several other gram-negative organisms contains a major part of the microbial phospholipid-splitting apparatus (3, 25, 27) and also is the site of the permeability barrier to actinomycin D (17). On the other hand, the enzymes of the lipid biosynthetic sequence are located in the cytoplasmic membrane (3, 29), and the early stimulation of incorporation of radioactive lipid precursors, although possibly a secondary phenomenon, may also imply an immediate (coincident) effect on this portion of the envelope.

In any event, whether or not the outer membrane is the primary target of temperature-sensitive serum components (presumably complement) that initiate the train of events leading to death of susceptible organisms, our results lend support to the view that the outer membrane of *S. marcescens* is the major determinant of resistance or susceptibility to serum. Because no obvious differences in the phospholipid-splitting activities and in the substrate properties of the lipids of the two strains were detected, a comparison of other characteristics of their outer membranes seems in order. Such a comparison will be aided by the availability of procedures that permit isolation of the outer membrane (21).

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