BACTERIOLYSIS OF *ENTEROBACTERIACEAE*

I. Lysis by Four Lytic Systems Utilizing Lysozyme[1, 2]

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In recent years, an expanding body of information on the lysis of gram-negative bacteria has developed. Much of the work in the area of lytic phenomena has resulted from interest in the structural differences between gram-negative and gram-positive bacteria and in the determination of lytic mechanisms. Lysozyme has been used extensively in these studies because of its ability to degrade structural components of both gram-negative and gram-positive cell walls. While this enzyme is capable of depolymerizing its substrate in many gram-positive bacteria without previous cell treatment, the mucopolysaccharide substrate is protected in gram-negative cell walls by an outer layer or a complex of lipoprotein which must be dissociated or removed before lysozyme may act. Cell treatments which permit the access of lysozyme to its substrate include Nakamura treatment (Grula and Hartsell, 1957*a*), heat pretreatment (Becker and Hartsell, 1954), freezing and thawing (Kohn, 1960), extraction with lipid solvents or alkali (Becker and Hartsell, 1955), and treatment with Versene (Repaske, 1956, 1958), certain detergents (Colobert, 1957), or polybasic antibiotics (Warren, Gray, and Yurchenco, 1957).

Many of the lytic studies have included *Escherichia coli* or some other member of the *Enterobacteriaceae* as a typical gram-negative test organism. However, there has been little effort to compare the lytic response of several strains of *E. coli* and other enteric bacilli to the various systems containing lysozyme under standardized conditions. Such a comparison is desirable since it would aid in the development of new cytochemical tools for determining cell structure, provide information on the mechanism(s) by which pre- and co-lytic treatments potentiate lysozyme action, and minimize erroneous conclusions regarding lytic responses of taxonomic groups based on single-strain studies.

In the present study, the lytic response of 41 members of the *Enterobacteriaceae* was investigated using Nakamura's technique (Nakamura, 1923); lysozyme and Versene; lysozyme and polybasic antibiotics; and lysozyme, trypsin, and butanol.

MATERIALS AND METHODS

Bacterial strains. Species of all genera in the family *Enterobacteriaceae* except *Alginobacter* were included in this study. Strain designations and their sources are indicated in Table 6.

Culture maintenance and preparation of cell suspensions. All cultures were maintained by monthly transfer on glucose-yeast extract-veal infusion agar containing glucose, 0.1%; yeast extract (Difco), 0.3%; peptone (Difco), 0.5%; trypticase (BBL), 0.5%; NaCl, 0.5%; agar, 2%; pH 7.0. Cells for lytic tests were grown on the same medium for 16 to 18 hr at 37 C (25 C for *Erwinia* and *Serratia* species). Cells were harvested, washed twice with distilled water, resuspended, and then diluted with water to give an optical density at 610 mμ of 0.50 (Coleman Universal spectrophotometer) when diluted 1 to 10. In experiments using cell walls of bacteria, washed whole cells were ruptured by shaking with glass beads (Grula and Hartsell, 1954) followed by isolation of walls by differential centrifugation. Cytoplasmic debris was removed from the walls by washing with molar sodium chloride. Sodium chloride was removed by rinsing the walls with distilled water until the supernatant fluid no longer gave a positive test for chloride ion with silver nitrate.

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Determination of lysis. The term "lysis" will be used to indicate a decrease in the optical density (OD) of a cell suspension after exposure to the test systems. Per cent lysis = $\frac{OD\ (initial) - OD\ (final)}{OD\ (initial)} \times 100$. In those cases where lysis was observed microscopically, untreated cells were air-dried to the undersurface of a cover glass supported above a microscope slide by two thin parallel bands of paraffin. Lytic reagents were run under the cover glass and their effects on the attached cells observed with an American Optical Microstar microscope equipped with a phase condenser and a 97× bright medium contrast objective.

Nakamura's technique for lysis. Tubes containing 10 μg/ml lysozyme (crystalline from egg-white, Armour Research Division, Chicago) in water adjusted to pH 3.5 and sufficient cells to give an optical density of 0.50 were incubated for 1 hr at 45 C. Sufficient 0.1 N NaOH was then added to adjust the system to pH 10 to 10.5. Optical density was read immediately and per cent lysis calculated.

Lysozyme and Versene. Tubes containing 20 μg/ml lysozyme and 133 μg/ml Versene (disodium salt of ethylenediaminetetraacetic acid, Bersworth Chemical Company, Framingham, Massachusetts) in 0.03 M tris [tris(hydroxymethyl)aminomethane, Sigma Chemical Company, St. Louis] buffer at pH 8 and sufficient cells to give an optical density of 0.50 were incubated at 45 C. Per cent lysis was determined after incubation for one hour.

Lysozyme and polybasic antibiotics. The lytic procedure of Warren et al., (1957) was modified to eliminate pretreatment of cells with polybasic antibiotics. Instead, the lytic effect of a combination of lysozyme with the polybasic antibiotics was determined. Polymyxin B sulfate (7,105 u/ml, Pitman-Moore Company, Indianapolis) and circulin sulfate (5,300 u/mg, Upjohn Company, Kalamazoo) were used as typical polybasic antibiotics. Since the polybasic antibiotics have strong cationic surfactant properties, the effects of the polybasic peptide, salmine (protamine sulfate, Eli Lilly and Company, Indianapolis) and of a quaternary disinfectant, Hyamine 1622 (Rohm and Haas Company, Philadelphia) were compared with those of the antibiotics. The influence of pH, buffer type and concentration, and concentration of the four cationic surfactants was tested to determine optimal conditions for lysis of bacterial cells. Comparisons of the lytic response of *Enterobacteriaceae* were carried out using three systems (all in 0.0067 M phosphate buffer, pH 7): 10 μg/ml circulin plus 5 μg/ml lysozyme, 20 μg/ml Hyamine 1622 plus 5 μg/ml lysozyme, and 10 μg/ml polymyxin plus 5 μg/ml lysozyme. The extent of cell lysis by these systems was determined after 5 min incubation at 45 C.

Lysozyme, trypsin, and butanol. Early during these studies, it was found that the addition of 5% v/v *n*-butanol to cell suspensions in the presence of lysozyme would permit lysis of the cells by lysozyme. Addition of trypsin (crystalline, lyophilized, Worthington Biochemical Corporation, Freehold, New Jersey) caused increased cell lysis. This system was studied with regard to the effects of other solvents in place of butanol, other isomers of butanol, pH, and concentrations of *n*-butanol and the enzymes. The lytic system found to be optimal for lysis then used in determining the lytic response of *Enterobacteriaceae* contained 20 μg/ml lysozyme, 10 μg/ml trypsin, and 5% v/v *n*-butanol in 0.0267 M phosphate buffer, pH 7. Per cent lysis was determined after incubation of the suspensions at 45 C for 5 min and 1 hr.

RESULTS

Nakamura lysis. The majority of the 30 cultures tested for lysis by the Nakamura technique (Table 1) were extensively cleared by this treatment. Only six cultures lysed less than 65% and only one culture less than 35%. In all cases when lysis was greater than 66%, the degree of lysis of acid pretreated cells in the absence of lysozyme was much less than if lysozyme had been present. Since lysozyme was essential for maximal lysis to occur, the extent of final lysis must reflect the amount or manner of distribution of the substrate in the cell wall. The spectrum of quantitative lysis ranging from less than 35% to greater than 90% is in agreement with the findings of Peterson and Hartsell (1955) and Grula and Hartsell (1957*a*). In their study of the lysis of gram-negative bacteria by Nakamura's technique, Grula and Hartsell (1957*a*) calculated the per cent lysis that was due to lysozyme by substracting per cent lysis due to alkali from per cent total lysis. When such calculations were made on the two *E. coli* cultures used in both

TABLE 1

Lysis of Enterobacteriaceae by Nakamura's technique

Genus	Number of Cultures	Number of Strains Showing Indicated Per Cent Lysis			
		11–35	36–65	66–89	⩾90
Aerobacter	4			1	3
Erwinia	2			1	1
Escherichia	9	1	2	3	3
Paracolobactrum	4		1	1	2
Proteus	3		1	2	
Providence	2			2	
Salmonella	2			1	1
Serratia	2			1	1
Shigella	2		1		1

Lytic system. Cells incubated for 30 min at 45 C, pH 3.5, in the presence of 10 μg/ml lysozyme. NaOH (0.1 N) was then added to give pH 10.0. Per cent lysis was determined from optical density change.

TABLE 2

Lysis of Escherichia and Aerobacter by lysozyme and Versene

Organisms	Optical Density, 45 C			Per Cent Lysis, 1 Hr
	Initial	15 Min	1 Hr	
A. aerogenes (884)	0.50	0.05	0.03	94
A. aerogenes (Haj)	0.50	0.15	0.12	76
A. aerogenes (PU-2)	0.49	0.18	0.14	71
E. coli (H-52)	0.50	0.05	0.04	92
E. coli (4157)	0.46	0.05	0.03	93
E. coli (Haj)	0.46	0.05	0.04	91
E. coli (19)	0.49	0.08	0.06	88
E. coli (U-2)	0.51	0.07	0.04	92

the present studies and by Grula and Hartsell, the per cent lysis values due to lysozyme agreed within two percentage units. Hence, per cent lysis due to lysozyme appears to be a stable cultural characteristic that was not lost during the 4 years of culture maintenance that elapsed between these two studies.

Lysis by lysozyme and Versene. Three strains of *Aerobacter aerogenes* and five strains of *E. coli* were found to be extensively lysed by a combination of lysozyme and Versene in 0.03 M tris buffer at pH 8 (Table 2). While two of the Aerobacter strains were not quite as sensitive as the third strain, Aerobacter cultures certainly

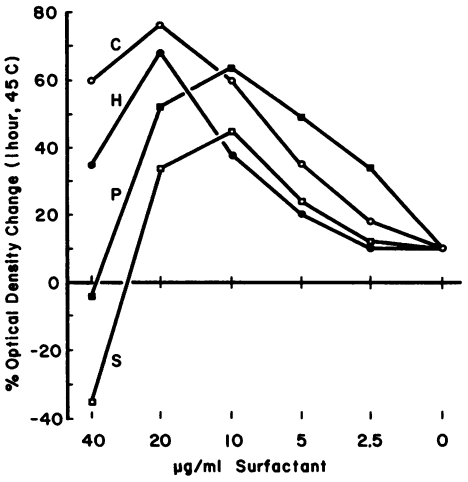

Fig. 1. Lysis of *Escherichia coli* strain U-2 by polybasic antibiotics and other surfactants. ○——○ = Circulin (*C*); ●——● = Hyamine (*H*); ■——■ = polymyxin (*P*); □——□ = salmine (*S*).

cannot be said to be resistant to these lytic conditions. Moreover, suspensions of isolated cell walls of *A. aerogenes* strain PU-2 were lysed approximately 60% in 1 hr by the same system. Thus, the suggestion of Repaske (1958) that the lytic response of *Aerobacter* and *Escherichia* species to lysozyme and Versene appears to be differential has not been supported. It is possible that the one Aerobacter culture used in his studies may have been resistant, but resistance to lysozyme-Versene action is not characteristic of the genus.

Lysis by lysozyme and polybasic antibiotics. When tested individually in 0.0067 M PO_4 buffer at pH 7, polymyxin B, circulin, salmine, and Hyamine 1622 were all found capable of lysing *E. coli* strain U-2 at a specific concentration that was critical for each agent (Fig. 1). Decreased lysis at low concentrations is undoubtedly due to an insufficiency of the surfactant, whereas increased turbidity at high concentration may be due to agglutination of cells caused by neutralization of surface charge or by denaturation of protein. The extent of lysis was found to be lower at pH 6 than at pH 7. At pH 8, total lysis increased but in the control lysis was also much higher. Higher concentrations of buffer also tended to increase the amount of control lysis. The use of tris-acid-maleate buffer in place of phosphate buffer at the same pH values resulted in somewhat decreased lysis.

TABLE 3

Enterobacteriaceae lysable by polybasic antibiotics

Surfactant	Sensitive Cultures (Greater than 50% Lysis)	Per Cent Lysis (1 Hr, 45 C, pH 7)
Circulin (20 μg/ml)	*Aerobacter aerogenes* (Haj)	53
	Aerobacter cloacae (Haj)	72
	Erwinia amylovora (7398)	62
	Escherichia aurescens (12814)	56
	Escherichia coli (Haj+)	58
	E. coli (U-2)	76
	Paracolobactrum sp. (Haj Vi+)	71
	Paracolobactrum sp. (Haj Vi−)	75
Polymyxin (10 μg/ml)	*E. aurescens* (12814)	52
	E. coli (U-2)	63
	Paracolobactrum sp. (Haj Vi+)	69
	Paracolobactrum sp. (Haj Vi−)	68
Salmine (10 μg/ml)	*E. coli* (Haj I−)	52
Hyamine (20 μg/ml)	*E. coli* (U-2)	69

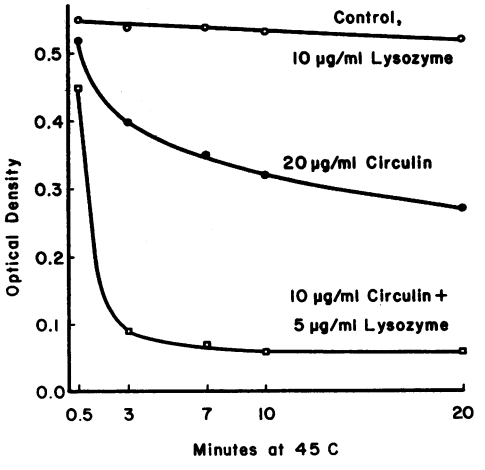

Fig. 2. Lysis of *Escherichia coli* strain U-2 by circulin and lysozyme.

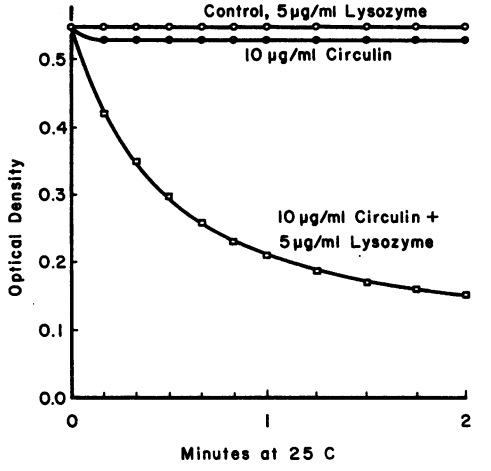

Fig. 3. Lysis of *Aerobacter aerogenes* strain PU-2 by circulin and lysozyme.

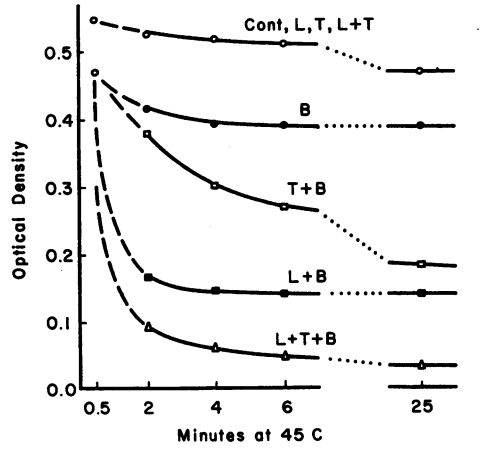

Fig. 4. Lysis of *Escherichia coli* strain 19 by lysozyme, trypsin, and butanol. *Cont*=control; *L*=20 μg/ml lysozyme; T=10 μg/ml trypsin; *B*=5% v/v *n*-butanol.

Of the 41 cultures tested at concentrations of the various surfactants found optimal for lysis of *E. coli* strain U-2 (Table 3), only 9 were lysed more than 50%. Of the compounds tested, circulin was found to be the most lytic.

Combinations of the polybasic surfactants with lysozyme, illustrated with *E. coli* in Fig. 2, were found to cause rapid and extensive lysis of most enteric bacilli. The synergistic lysis of cells caused by combinations of salmine and lysozyme or Hyamine and lysozyme, although evident, was not as great as that of circulin and lysozyme. Experiments with polymyxin and

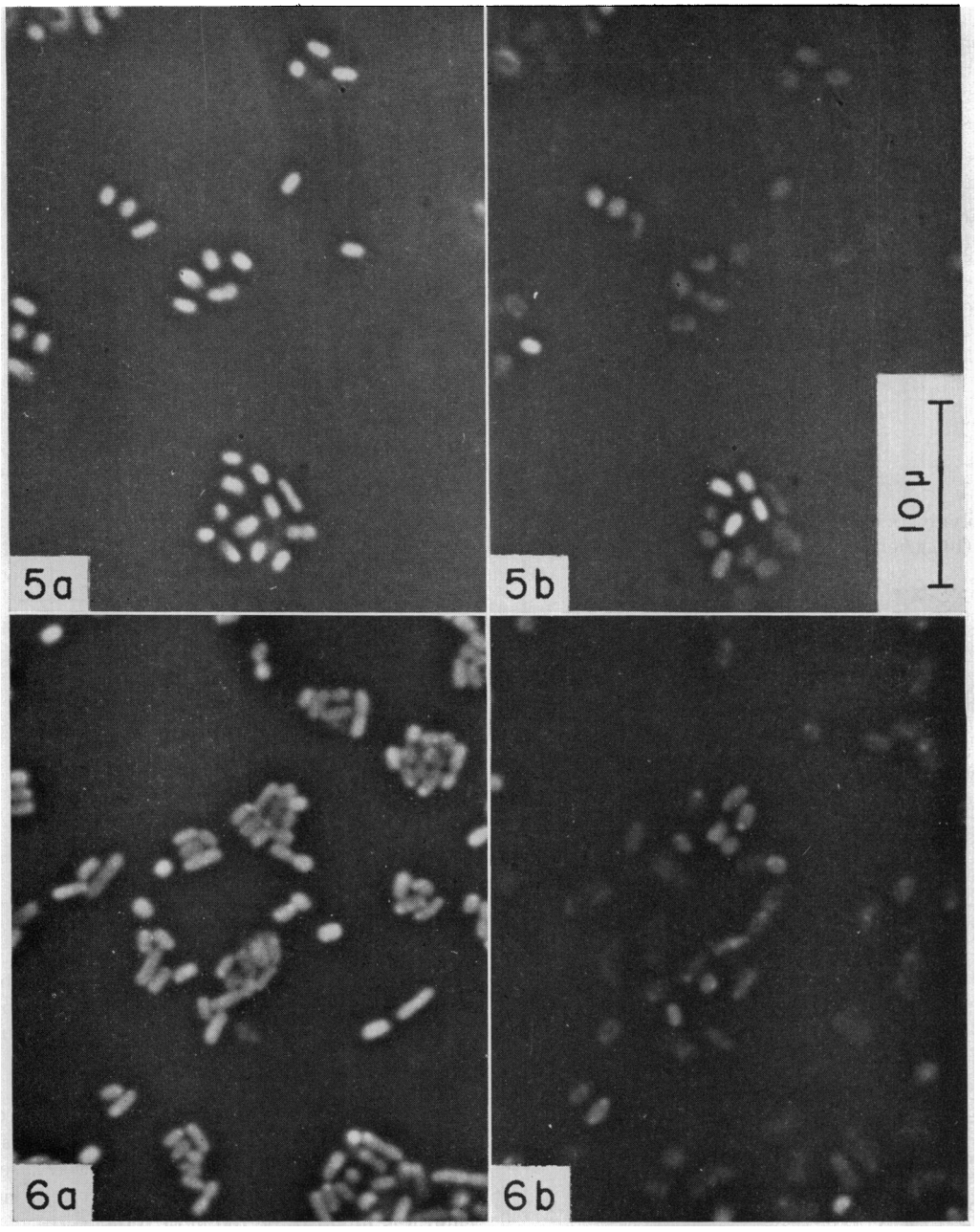

Fig. 5. Lysis of *Aerobacter aerogenes* strain PU-2 by circulin and lysozyme. *a*) Cells in water; *b*) cells after treatment with circulin and lysozyme for 10 min at 25 C.

Fig. 6. Lysis of *Escherichia coli* strain U-2 by lysozyme, trypsin, and butanol. *a*) Cells in water; *b*) cells after treatment with lysozyme, trypsin, and butanol for 5 min at 25 C.

TABLE 4

Lysis of Enterobacteriaceae by polybasic antibiotics and lysozyme

Genus	Number of Cultures	Number of Strains Showing Indicated Extent of Lysis* (5 min, 45 C, pH 7)														
		Circulin 10 μg/ml + lysozyme 5 μg/ml					Hyamine 20 μg/ml + lysozyme 5 μg/ml					Polymyxin 10 μg/ml + lysozyme 5 μg/ml				
		0	+1	+2	+3	+4	0	+1	+2	+3	+4	0	+1	+2	+3	+4
Aerobacter	4				3	1	3	1							1	3
Erwinia	2		1	1				1	1					1	1	
Escherichia	18		3	3	12		7	6	4	1			1	5	10	1
Klebsiella	2		2				2						1	1		
Paracolobactrum	4	1	1		2		2	2				1	1		2	
Proteus	3		1	2			1	1	1				1	2		
Providence	2		2				1	1					1	1		
Salmonella	2			1	1			1	1						2	
Serratia	2		1	1			1		1			1		1		
Shigella	2	1			1		1	1						1	1	

* Extent of lysis: 0 = 10% or less; +1 = 11–35%; +2 = 36–65%; +3 = 66–89%; +4 = 90% or greater.

lysozyme gave identical results (synergism) with those of circulin and lysozyme. Dual combinations of circulin, salmine, and Hyamine, in the absence of lysozyme, indicated that only circulin plus salmine exhibited lysis much greater than the additive effects of their individual action.

The lytic effect of circulin and lysozyme is not limited to bacteria that are sensitive to circulin alone. *A. aerogenes* strain PU-2 is lysed only 19% by 20 μg/ml circulin in 1 hr at 45 C, but it is almost as sensitive to the action of circulin plus lysozyme (Fig. 3) as is *E. coli* strain U-2. Lysis of *A. aerogenes* strain PU-2 by 10 μg/ml circulin and 5 μg/ml lysozyme was observed with the phase microscope (Fig. 5). Rapid loss of refractility is apparent for the majority of cells following addition of the circulin-lysozyme system. The end point of lysis is not complete cell dissolution and some cells do not appear to be affected by the lytic system. Suspensions of isolated cell walls of *A. aerogenes* strain PU-2 and *E. coli* strain U-2 were not appreciably lysed by circulin and lysozyme. The cell residues may be degraded by trypsin at pH 8 to the extent that they no longer retain a rodlike morphology. Hence, it would appear that the ghosts remaining after lysis of whole cells are composed of at least the protein portion of the cell wall. Since lysozyme substrate is presumably degraded, it would appear that cell wall protein also contributed to the rigidity and form of the gram-negative cell wall.

Thirty-nine additional members of the *Enterobacteriaceae* were tested for their lytic response to the combinations of circulin, polymyxin or Hyamine with lysozyme (Table 4). These data indicate that mixtures of the polybasic surfactants with lysozyme are generally capable of greater lysis of the majority of cultures than are the individual agents. The two antibiotics are far more effective in lysing cells than is Hyamine 1622. One notable separation of genera by lytic response resulted from these studies. The four Aerobacter cultures were readily lysed by circulin and lysozyme whereas Klebsiella cultures were not. If testing of large numbers of Aerobacter and Klebsiella strains supports sensitivity of Aerobacter and resistance of Klebsiella as true generic characteristics, an important differential procedure might be developed. At present, the differentiation of these two genera is unsatisfactory since they are often confused by the limitations of pathogenicity and capsulation tests. Both of these tests are suggested by Skerman (1959) as taxonomically invalid since virulence and capsulation are features that are readily lost.

Lysis by lysozyme, trypsin, and butanol. The initial studies on the lysozyme-trypsin-butanol system were made using *E. coli* strain 19 as the test organism. The enteric bacilli are refractory to lysozyme, trypsin, or lysozyme and trypsin (Fig. 4) and only partially sensitive to the action of 5% v/v *n*-butanol alone at pH 7 (45 C).

TABLE 5

Lysis of Enterobacteriaceae by lysozyme, trypsin, and butanol

Culture	Per Cent Lysis	
	5 Min	1 Hr
Escherichia coli (19)	91	94
E. coli (61)		85
E. coli (SD)		88
E. coli (K12)		87
E. coli (B/M12)		89
E. coli (H-52)		89
E. coli (113-3)		86
E. coli (U-2)		90
E. coli (U-4)		53
E. coli (4157)		85+
E. coli (B/r/o)		88
E. coli (Haj)		85+
E. coli (Haj I+)	91	96
Escherichia aurescens (12814)	86	95
Escherichia freundii (Haj)	90	94
Escherichia intermedia (6750)	87	94
Aerobacter aerogenes (PU-2)		90
A. aerogenes (884)	91	96
A. aerogenes (Haj)	81	94
Aerobacter cloacae (Haj)	92	96
Erwinia carotovora (PU-93)	74	90
Klebsiella pneumoniae (9997)	59	89
Paracolobactrum aerogenoides (11604)		85+
Proteus vulgaris (X-19)	90	94
Providence sp. (Haj U+)	93	93
Salmonella choleraesuis (10708)	85	91
Serratia marcescens (PU-238)	75	77
Shigella dysenteriae (11835)	94	96

However, the presence of butanol permits extensive lysis by 10 μg/ml trypsin and even greater lysis by 20 μg/ml lysozyme. When all three reagents are present in combination, greater than 90% lysis of the cells occurs at 45 C in 5 min at pH 7. The extent of lysozyme and trypsin potentiation by *n*-butanol decreases as its concentration is decreased from 5% v/v. Isomers of butanol with shorter effective chain lengths (*i*-butanol, *s*-butanol, *t*-butanol) are less effective than *n*-butanol at a 5% v/v concentration. The only other solvent found comparable to *n*-butanol in potentiating enzymatic lysis was ethyl acetate. Shorter chain length homologues of the aliphatic alcohols were less effective than butanol or were ineffective. Solubility limitations prevented use of C_5 and higher alcohols. The extensive lysis of cell suspensions by the lysozyme-trypsin-butanol system observed spectrophotometrically was supported by microscopic observations during the course of lysis (Fig. 6).

Testing of other *Enterobacteriaceae* by the lysozyme-trypsin-butanol system (Table 5) indicated that the majority of the enteric bacilli are extremely sensitive to the lytic action of the butanol system. Only *E. coli* strain U-4 was not extensively lysed in 1 hr (pH 7, 45 C), and of those cultures for which lysis was recorded at 5 min, most were approaching the final degree of lysis attained in 1 hr.

Summary of lytic responses of the Enterobacteriaceae. All lytic systems capable of lysing members of the *Enterobacteriaceae* to an extent of 70% or greater are indicated in Table 6. Of the 32 cultures tested for lysis by both Nakamura conditions and by the circulin-lysozyme system, 13 were sensitive and 5 were insensitive to both systems. Hence, a correlation in lytic response to these two systems was found for 18 (13 sensitive, 5 insensitive) out of 32 cultures and may reflect similarity, availability, or amount of lysozyme substrate in these organisms. All cultures lysable by lysozyme and Versene were also lysed by lysozyme-trypsin-butanol, circulin-lysozyme, or polymyxin-lysozyme. Lysozyme-trypsin-butanol and lysozyme-Versene appeared to be the most generally effective lytic systems for those *Enterobacteriaceae* tested. The other lytic procedures produced a range of lysis dependent upon the test species utilized. Replicate tests, when performed, indicated the individuality and constancy of cultural response.

DISCUSSION

Prior to these investigations, no information was available on the lytic reactions of Paracolobactrum, Serratia, members of the Providence group, or species of *Escherichia* other than *E. coli*. Hence, this study has broadened the group of gram-negative organisms known to be lysable by systems containing lysozyme and has correlated the effects of several systems on a common group of bacterial species.

Since all of the lytic systems tested included lysozyme as one of their components, and since none of the systems caused extensive lysis in the absence of lysozyme, a portion of cell lysis must be attributable to degradation of lysozyme substrate within the cell wall. The remaining com-

TABLE 6

Lysability of Enterobacteriaceae by systems containing lysozyme

Cultures Lysed by One or More Systems	Source*	System(s)† Causing Lysis‡
Aerobacter aerogenes (PU-2)	PCC	1, 3, 4, 5, 6; (2)
A. aerogenes (884)	ATCC	1, 3, 4, 5, 6; (2)
A. aerogenes (Haj)	ISBH	1, 3, 4, 5, 6; (2)
Aerobacter cloacae (Haj)	ISBH	1, 2, 3, 4, 5
Erwinia amylovora (7398)	ATCC	4, 5; (2, 3)
Erwinia carotovora (PU-93)	PCC	1, 5; (2, 3, 4)
Escherichia aurescens (12814)	ATCC	1, 3, 4, 5; (2)
Escherichia coli(19)	PCC	1, 3, 4, 6; (2)
E. coli (61)	PCC	1; (2, 3, 4)
E. coli (SD)	PCC	1, 3, 4; (2)
E. coli (K-12)	PCC	1; (2, 3, 4)
E. coli (B/M12)	PCC	1; (2, 3, 4)
E. coli (H-52)	PCC	1, 3, 4, 6; (2)
E. coli (113-3)	PCC	1, 3, 4; (2)
E. coli (U-2)	Isolate	1, 2, 3, 6; (5)
E. coli (4157)	ATCC	1, 3, 4, 5, 6; (2)
E. coli (B/r/o)	PCC	1, 3, 4; (2)
E. coli (Haj)	ISBH	1, 3, 4, 6; (2, 5)
E. coli (Haj I+)	ISBH	1; (2, 3, 4, 5)
E. coli (Haj I−)	ISBH	3, 4, 5; (2)
Escherichia freundii (12012)	ATCC	5; (2, 3, 4)
E. freundii (Haj)	ISBH	1, 3, 4, 5; (2)
Escherichia intermedia (6750)	ATCC	1, 3, 4, 5; (2)
Klebsiella pneumoniae (9997)	ATCC	1, 5; (2, 3, 4)
Paracolobactrum sp. (Haj Vi+)	ISBH	2, 3, 4, 5
Paracolobactrum sp. (Haj Vi−)	ISBH	2, 3, 4, 5
Paracolobactrum aerogenoides (11604)	ATCC	1, 5; (2, 3, 4)
Proteus rettgeri (Haj)	ISBH	5; (2, 3, 4)
Proteus vulgaris (X-19)	PCC	1, 5; (2, 3, 4)
Providence sp. (Haj U+)	ISBH	1, 5; (2, 3, 4)
Providence sp. (Haj U−)	ISBH	5; (2, 3, 4)
Salmonella choleraesuis (10708)	ATCC	1, 3, 4, 5; (2)
Salmonella pullorum (PU-216)	PCC	4, 5; (2, 3)
Serratia marcescens (PU-238)	PCC	1, 5; (2, 3, 4)
S. marcescens (4002)	PCC	5; (2, 3, 4)
Shigella dysenteriae (11835)	ATCC	1, 3, 4, 5; (2)
Nonlysable cultures:		
E. coli (U-4)	Isolate	(1, 2, 3, 4)
Klebsiella pneumoniae (PU-123)	PCC	(2, 3, 4, 5)
Paracolobactrum coliforme (PU-158)	PCC	(2, 3, 4, 5)
Proteus morganii (8019)	ATCC	(2, 3, 4, 5)
Shigella alkalescens (PU-247)	PCC	(2, 3, 4, 5)

* PCC: Purdue Culture Collection; ATCC: American Type Culture Collection; ISBH: Indiana State Board of Health.

† *Lytic systems:* 1. Lysozyme-trypsin-butanol, 2. Circulin alone, 3. Circulin plus lysozyme, 4. Polymyxin plus lysozyme, 5. Nakamura's technique, 6. Lysozyme plus Versene (pH 8; tris).

‡ Numbers in parentheses indicate test made but no appreciable lysis.

pounds in the systems, with the possible exception of trypsin, must merely render the substrate available for lysozyme degradation, or aid in the final solubilization phase of lysis.

The effect of low pH in potentiating lysozyme activity has been interpreted by Grula and Hartsell (1957*b*) as due to release of the "substrate from a 'bound' state within the cell wall" by acid denaturation of substrate-protecting components of the cell wall. The effect of alkali presumably results from "hydration and dispersion of the cell proteins" following removal of lysozyme substrate.

The effect of Versene as a lysozyme-potentiator has been discussed by Repaske (1958), who considers that metals bound at sites on the cell surface interfere with formation of lysozyme-substrate complexes. The effect of Versene is presumed to be one of rupture of co-ordinate bonds between the cell and the metal, thus exposing lysozyme substrate. However, unless the lipoprotein layer is held in its protective configuration over the muco-complex by metals, it seems unlikely that chelation of surface-bound metals would cause sufficient disorganization of the lipoprotein to allow lysozyme to reach its substrate. A more reasonable explanation of Versene action has been suggested by Colobert (1958), who considers that Versene may act as an effective lipid dissociant through a simple detergent-like action. Further evidence to support this latter concept of Versene action will be included in a subsequent publication.

At concentrations in the range of 10 to 20 μg/ml, the action of circulin and polymyxin (and Hyamine 1622) would also appear to be attributable to an unmasking of lysozyme substrate by lipoprotein dissociation. These antibiotics are known to have properties as strong cationic surfactants. Their bactericidal effects at low concentrations are believed due to disorganization of the plasma membrane resulting in loss of cytoplasmic components vital to growth of the cell (Few and Schulman, 1953; Newton, 1956). The polymyxins appear to have an affinity for gram-negative lipid components due to a higher amount of lipid phosphate in gram-negative cell membranes than in gram-positive species (Few, 1957). Warren et al. (1957) observed that pretreatment of gram-negative bacteria with polymyxin sensitized the cells to the action of lysozyme. Since polymyxin is capable of bonding strongly to the cell surface, it is probable that polymyxin was carried over into the lysozyme system on the surface of pretreated cells. Hence, they observed lysis by a polymyxin-lysozyme system quite similar to the dual systems studied in these investigations.

We have reported (Noller and Hartsell, 1960) that the role of butanol in the lysozyme-trypsin-butanol system is one of lipoprotein dissociation. Butanol is often used in the extraction of lipoprotein-bound enzymes (Morton, 1955) and its unique effect has been attributed to marked lipophilic and concomitant hydrophilic properties of the normal isomer. Orientation of the butanol molecule between lipid and water provides a detergent-like action while preventing re-formation of the lipoprotein complex. Since alcohol homologues of shorter chain length and butanol isomers with shorter effective chain lengths were less active than *n*-butanol in potentiating lysozyme-trypsin action (just as they are less effective in extraction of enzymes from lipoprotein complexes), it seems very likely that *n*-butanol causes sufficient unfolding of the protective lipoprotein layer or complex to permit lysozyme to reach its substrate. Ethyl acetate also has both lipophilic and hydrophilic properties. The fact that this solvent also potentiates lysozyme and trypsin activity speaks for a similarity of action to that of *n*-butanol.

SUMMARY

The lytic responses of 41 members of the family *Enterobacteriaceae* were tested using four lytic systems, all of which contained lysozyme. Extensive lysis of all strains tested was observed with lysozyme-Versene (pH 8; tris) and with lysozyme-trypsin-butanol. Lysis by lysozyme plus polybasic antibiotics and by Nakamura's technique indicated a spectrum of quantitative lysis ranging from negligible to greater than 90%. A positive correlation between lysis by lysozyme plus polybasic antibiotics and by Nakamura's technique was apparent in 18 of the 32 cultures tested.

The resistance of Aerobacter strains to lysozyme and Versene reported by Repaske has not been supported as a typical characteristic of this genus.

Lytic sensitivity of *Aerobacter aerogenes* and resistance of *Klebsiella pneumoniae* to lysozyme plus circulin may serve as a basis for rapid differentiation of these two genera.

The group of gram-negative bacteria known to

be lysable by lysozyme-containing systems has been extended to include the genera *Paracolobactrum* and *Serratia*, members of the Providence group, and species of *Escherichia* other than *Escherichia coli*.

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