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Lysis of Grouped and Ungrouped Streptococci by Lysozyme[1]

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Thirty strains of streptococci were tested for lysis with lysozyme, and 29 of these could be lysed by the following method: (i) suspension of the cells to a Klett reading of 200 units (no. 42 filter) in 0.01 M tris(hydroxymethyl)aminomethane buffer, *p*H 8.2, after washing twice with the buffer; (ii) addition of lysozyme to a final concentration of 250 μg/ml with incubation for 60 min at 37 C; (iii) addition of sodium lauryl sulfate (SLS) to a final concentration of 0.2% and incubation up to an additional 15 min at 37 C. Significant lysis was obtained only after the addition of SLS. (Strains of groups A, E, and G were treated with trypsin at a concentration of 200 μg/ml for 2 hr at 37 C before exposure to lysozyme.) These parameters for optimal lysis of streptococci by lysozyme were established by testing the group D *Streptococcus faecalis* strain 31 which lyses readily with lysozyme and the group H strain Challis which is less susceptible to the action of the enzyme. Viability of *S. faecalis* decreased 96% after 3 min of exposure to 250 μg of lysozyme per ml, whereas the more resistant strain Challis retained 27% of the initial viability after the same period. After 60 min, there was almost total loss of viability in each case. Variations of three methods of lysing streptococci with lysozyme were compared with respect to the decrease in turbidity and the release of protein and deoxyribonucleic acid (DNA) effected by each variation. The method presented in this paper allowed the greatest release of these cytoplasmic constituents from *S. faecalis* and strain Challis. Transformation experiments using DNA obtained from strain Challis (streptomycin-resistant) by this method showed that the DNA released is biologically active.

Research in such areas as the genetics and metabolism of streptococci often has been hindered by the lack of gentle, efficient methods that will allow lysis of most members of this genus. Lysis of streptococci usually has been accomplished by mechanical means or by the use of enzymes specific for cell wall degradation. McCarty (8) reported the use of enzymes produced by *Streptomyces albus* that hydrolyze cell walls of group A streptococci. Krause (6) and Maxted (10) reported lysis of groups A and C streptococci by phage-induced lysin from lysates of group C streptococci, and Bleiweis and Zimmerman (1) isolated a phage-associated lysin for production of protoplasts of the group D *Streptococcus faecalis*. Mechanical lysis, however, is still employed widely. Colman (3) used a Mickle disintegrator for lysing a variety of viridans-like streptococci to isolate transforming deoxyribonucleic acid (DNA). Perry and Slade (12) used the French press and Sagers press in preparing transforming DNA from strain Challis.

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Recently Metcalf and Deibel (11) reported lysis of *S. faecium* and *S. faecalis* in distilled water by treatment with lysozyme followed by the addition of anions. In an attempt to isolate DNA from a variety of streptococci, we found that 29 of 30 strains tested could be lysed when the cells were suspended in tris(hydroxymethyl)aminomethane (Tris) buffer for treatment with lysozyme and followed by the addition of sodium lauryl sulfate (SLS). The findings reported in this paper suggest that most streptococci can be lysed by lysozyme under these conditions.

MATERIALS AND METHODS

Bacteria. Strains of groups B, E, F, G, L, N, and O were obtained from Rebecca C. Lancefield, Rockefeller University; groups A and C from L. William Clem and Hermann Baer, University of Florida; *S. faecalis* strain 31 from L. N. Zimmerman, Pennsylvania State University; group H strain Challis from Dennis Perry, Northwestern University; the human cariogenic strains AHTj, BHT, CHT, and HHT from James M. Jablon, University of Miami; and the other cariogenic and noncariogenic oral strains from Robert

J. Fitzgerald, Veterans Administration Hospital, Miami.

Enzymes. Egg white lysozyme, crystallized three times and lyophilized, with an activity of 20,000 units/mg was purchased from the Sigma Chemical Co., St. Louis. Trypsin, Type 1 from bovine pancreas, twice crystallized, with an activity of 9,800 BAEE units/mg, was also purchased from Sigma Chemical Co.

Lytic systems. All strains were cultured for 12 hr at 37 C in Todd-Hewitt broth (Difco) adjusted to a final *p*H of 7.2. The bacteria were washed twice with 0.01 M Tris buffer (2-amino-2-hydroxymethylpropane-1,3 diol), *p*H 8.2, and resuspended in the same buffer to a turbidity of 200 Klett units by using a no. 42 filter. A 0.1-ml amount of lysozyme (25,000 μg/ml) was added to 9.8 ml of cell suspension (final concentration, 250 μg/ml), and the contents were incubated at 37 C for 60 min. A 0.1-ml amount of 20% SLS was added (final concentration, 0.2%), and incubation was continued for 15 min before readings were made.

The effect of salt on lysis after lysozyme treatment as described by Metcalf and Deibel (11) was tested. The bacteria were suspended in distilled water or in Tris buffer and, after 60 min of exposure to lysozyme, sodium chloride was added to a final concentration of 0.4 M.

A modification of the method used by Schaechter et al. (14) for gentle lysis of *Bacillus megaterium* was tested with two strains. The cells were suspended in 0.01 M Tris buffer, (*p*H 8.2) containing 20% sucrose, exposed for 30 sec or for 5 min to 250 μg of lysozyme per ml, and immediately chilled to 0 C by addition to crushed, frozen, sucrose buffer. After thawing, the preparations were centrifuged and resuspended in sucrose-buffer, and 0.2% SLS (final concentration) was added to effect lysis.

Assays. The amount of lysis occurring in suspensions of cells was estimated by measuring the decrease in turbidity in a Klett colorimeter with a no. 42 filter and the release of protein and DNA into the supernatants. Protein was measured by the method of Lowry et al. (7). After treatment with lysozyme and SLS, the suspensions were centrifuged at 12,100 × *g* for 10 min, and 1 ml of the supernatant was mixed with 3 ml of distilled water. One milliliter of this dilution was used in the protein assay (7). Total soluble protein for each streptococcal strain was measured by boiling the pellets obtained from 9.8 ml of control suspensions in 5 ml of 1 N NaOH for 5 min and mixing 1 ml of this with 2 ml of distilled water and 1 ml of 1 N NaOH. One milliliter of this dilution was used in the protein assay (7) that was modified in this case by preparing the solution of 2% Na_2CO_3 in distilled water.

DNA was measured by the method of Ceriotti (2) as modified by Keck (5). The soluble DNA released by lysis of the cell suspensions was determined by precipitating 3 ml of the supernatant (obtained after centrifugation at 12,100 × *g* for 10 min) with 3 ml of cold 20% trichloroacetic acid overnight. After washing three times with cold 10% trichloroacetic acid, the precipitate was heated in 2 ml of 0.5 N perchloric acid for 40 min at 70 C, and 1.5 ml was used in the assay (2, 5). Total soluble DNA for each strain was measured by precipitating the control pellets in cold 10% trichloroacetic acid overnight, washing as described, and heating them in 4 ml of 0.5 N perchloric acid for 40 min at 70 C; 1.5 ml of this extract was used for the assay (2, 5).

The amount of lysozyme remaining in the supernatants was measured by the method of Shugar (15). Calculations for the amount of protein released from the cells by lysis were corrected for the lysozyme remaining in the supernatants.

Plate counts for viability studies were made on Todd-Hewitt agar (Difco) to which 0.5% glucose was added. Each determination of colony-forming units was an average of the counts of colonies on three plates.

Pretreatment with trypsin. Group A streptococci and others that did not lyse initially were pretreated with 200 μg of trypsin per ml in saline-phosphate buffer (0.067 M), *p*H 7.5, for 2 hr at 37 C, washed three times with 0.01 M Tris buffer, *p*H 8.2, and tested for lysis with lysozyme as described.

RESULTS AND DISCUSSION

Optimal conditions for lysis. Initial attempts to lyse streptococci suspended in phosphate buffer with lysozyme were unsuccessful. This is in accordance with the finding of Metcalf and Deibel (11) that the presence of salts during exposure of *S. faecalis* to lysozyme inhibited lysis. We found, however, that many strains of streptococci lysed when they were suspended in Tris buffer containing lysozyme and then exposed to the detergent SLS. Significant lysis was never obtained until addition of SLS. To determine optimal conditions for lysis (buffer molarity, *p*H, concentrations of lysozyme and SLS, and times of exposure), *S. faecalis* strain 31 and the

TABLE 1. *Effects of pH and molarity of buffer on lysis by lysozyme*[a]

Tris buffer, *p*H 8.2	Decrease in turbidity[b]		0.01 M Tris buffer	Decrease in turbidity[b]	
	S. faecalis	Challis		*S. faecalis*	Challis
M	%	%	*pH*	%	%
0.002	88	72	7.8	91	58
0.01	86	74	8.0	90	74
0.02	81	76	8.2	91	74
0.05	82	64	8.4	90	66
0.1	80	54	8.6	87	52
0.2	77	30	9.0	89	54

[a] Bacterial suspensions were exposed to 250 μg of lysozyme per ml for 1 hr at 37 C and lysed by the addition of sodium lauryl sulfate at a final concentration of 0.2%.

[b] Initial turbidity was 200 Klett units which represented 3.3×10^8 colony-forming units (CFU) of *S. faecalis* and 5.2×10^8 CFU of strain Challis.

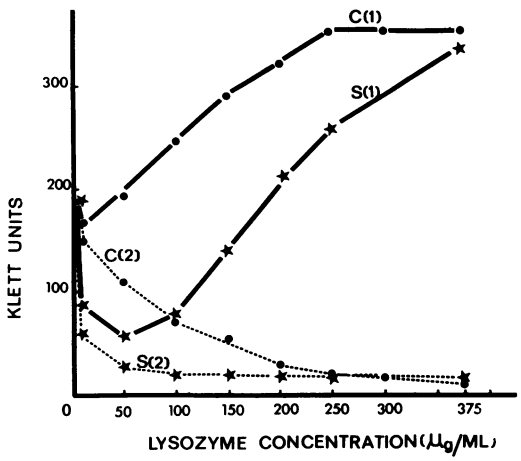

FIG. 1. *Effect of lysozyme concentration on lysis of S. faecalis (S) and strain Challis (C) suspended in 0.01 M Tris buffer, pH 8.2. C(1) and S(1) represent the changes in turbidity of cell suspensions after incubation for 1 hr at 37 C with each concentration of lysozyme. C(2) and S(2) represent the changes in turbidity in the same tubes after the addition of 0.2% SLS (final concentration) and further incubation for 15 min to effect final lysis.*

transformable group H strain Challis were employed as test organisms.

Table 1 shows the effects of changes in molarity and *p*H of Tris buffer on lysis as determined by measuring decrease in the turbidity of cell suspensions. Strain Challis was more sensitive than *S. faecalis* to changes in molarity and *p*H. From these results, 0.01 M Tris buffer, *p*H 8.2, was selected for use in testing strains of streptococci for lysis by lysozyme.

Figure 1 illustrates the changes in turbidity that occur after 60 min of incubation at increasing concentrations of lysozyme: (i) before addition of SLS and (ii) in the same tubes after the addition of SLS. The turbidity of strain Challis [C(1)] decreased slightly at a concentration of 10 μg of lysozyme per ml but increased considerably up to 250 μg of lysozyme per ml, after which the turbidity remained constant. The turbidity of suspensions of *S. faecalis* [S(1)], however, decreased at concentrations up to 150 μg of lysozyme per ml after which there was a progressive increase in turbidity. Similar results with high concentrations of lysozyme were explained by Friedberg and Avigad (4) as being due to the formation of aggregates caused by the electrostatic interaction of the enzyme with acidic components of the cellular surface. The cells do not lyse until dissolution of the aggregates is effected through the addition of anions from sources such as salts or detergents. In suspensions of *S. faecalis* that have been treated with lysozyme, an immediate clearing of the suspensions occurs after the addition of SLS. In suspensions of strain Challis exposed to lysozyme, however, maximal decrease in turbidity occurs after 10 to 15 min of incubation with SLS.

As shown in Fig. 1 [C(2)], turbidity decreased progressively in suspensions of strain Challis as the lysozyme concentration increased. In each case the change in turbidity was measured 15 min after addition of SLS. In contrast, there was a marked decrease in the turbidity of suspensions of *S. faecalis* at lower concentrations of lysozyme with little additional decrease occurring above a concentration of 50 μg/ml [Fig. 1, S(2)]. A lysozyme concentration of 250 μg/ml was selected for further studies.

Figure 2 illustrates the lysis of *S. faecalis* and strain Challis by 250 μg of lysozyme per ml measured at 10-min intervals over a period of 60 min. With strain Challis the turbidity of the suspensions decreased progressively (after addition of SLS to each tube) over the 60-min interval [Fig. 2, C(2)]. Suspensions of *S. faecalis* lost turbidity rapidly after only 1 min of exposure to lysozyme followed by the addition of SLS [Fig. 2, S(2)].

From these data a standard system for testing lysis of streptococci was chosen consisting of: (i) suspension of cells at a Klett reading of 200 units (no. 42 filter) in 0.01 M Tris buffer, *p*H 8.2; (ii) incubation at 37 C for 60 min with lysozyme at a concentration of 250 μg/ml; and (iii) addition of

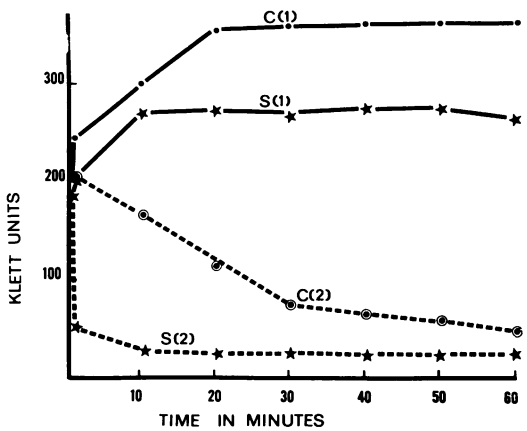

FIG. 2. *Kinetics of lysis by 250 μg of lysozyme per ml of suspensions of S. faecalis (S) and strain Challis (C) in 0.01 M Tris buffer, pH 8.2, at 37 C. C(1) and S(1) represent changes in turbidity after incubation of cells in lysozyme alone for each time period. C(2) and S(2) represent lysis of the same suspensions after the addition of 0.2% SLS (final concentration) and further incubation for 15 min at 37 C.*

TABLE 2. *Lysis of grouped streptococci by lysozyme*[a]

Group and strain	Soluble protein			Decrease in turbidity[c]
	Total	Amt released	Per cent released	
	μg/ml	μg/ml		%
A-486	372 (392)[b]	0 (0)	0 (0)	0 (0)
A-SS	540 (568)	70 (200)	13 (35)	2 (20)
A-3W53	508 (592)	122 (220)	24 (37)	24 (35)
B-090R	372	372	100	88
C-74	388	164	42	56
D-(*S. faecalis* 31)	300	258	86	88
E-K129	396 (392)	0 (156)	0 (40)	0 (55)
F-A575	240	230	96	76
G-D166B	412 (396)	0 (178)	0 (45)	0 (16)
H-Challis	328	286	87	84
L-D167A	420	185	44	45
N-C559	380	300	79	100
O-B357	320	314	98	84

[a] Cell suspensions were incubated for 60 min at 37 C with 250 μg of lysozyme per ml followed by the addition of 0.2% sodium lauryl sulfate (final concentration) to effect lysis.

[b] Values in parentheses were determined after pretreatment of cells with 200 μg of trypsin per ml for 2 hr at 37 C.

[c] Initial turbidity was 200 Klett units (no. 42 filter) ± 10%.

SLS at a final concentration of 0.2% and further incubation up to 15 min at 37 C.

Comparative lysis of streptococci. Lysis of 30 strains of streptococci was measured by determining decrease in turbidity and release of protein from the cells. Table 2 shows the lysis of various serologically grouped streptococci. Strains of group A that bear the M protein layer and the strains of groups E and G that did not lyse initially were pretreated with trypsin. All of the strains except group A-486 lysed to some degree with lysozyme after this treatment, as shown by the values in parentheses. Although the same approximate turbidity (200 Klett units) was used in each case, the values for total soluble protein obtained from each suspension (Table 2) indicate that significant differences exist in cell size, number, aggregation, and composition. Nevertheless, the per cent of protein released by lysis paralleled the per cent of change in turbidity in most of the suspensions.

The lysis of ungrouped cariogenic and noncariogenic oral streptococci from human and animal sources is shown in Table 3. Of these strains, only strain HHT (*S. salivarius*) showed significant resistance to lysis.

It is apparent from these data that the conditions required for optimal lysis may vary from one strain to another. Nevertheless, the method presented here has produced lysis in all but one of 30 strains that were tested and should provide a basis for obtaining lysis by lysozyme in most streptococci.

Viability after treatment with lysozyme. To determine the relative viabilities of strain Challis and *S. faecalis* after exposure to lysozyme and SLS, plate counts were made of colony-forming units (Table 4). Viability of *S. faecalis* decreased markedly after addition of lysozyme, whereas suspensions of strain Challis lost viability less rapidly. The viability of *S. faecalis* remained constant at 4% from 3 to 60 min of exposure to lysozyme, whereas the viability of strain Challis dropped from 27% at 3 min to 3% after 60 min. After the addition of SLS, few viable cells remained.

Comparison of methods for lysis by lysozyme. The lysis of two strains of streptococci was compared by decrease in turbidity and the release of protein and DNA (i) by the method described in this paper; (ii) by the use of salt as described by Metcalf and Deibel (11); and (iii) by a modification of the method described by Schaechter et al. (14) for isolating polyribosomes from *B. megaterium*.

Table 5 shows a comparison of the results. Effective lysis of *S. faecalis* with concomitant protein release was obtained by all methods, though less release of DNA occurred when lysis was effected by salt. Strain Challis did not appear to lyse as well when 0.4 M NaCl was used as it did when 0.2% SLS was added to the suspensions after exposure to lysozyme. More DNA was released from each organism when lysis was effected by 0.2% SLS than by 0.4 M NaCl (Table 5). This perhaps is due to more effective release

TABLE 3. *Lysis of cariogenic and noncariogenic oral streptococci by lysozyme*[a]

Strain	Source and description	Soluble protein			Decrease in turbidity[c]
		Total	Amt released	Per cent released	
		μg/ml	μg/ml		%
AHTj	Human, active[b]	304	198	65	50
BHT	Human, active	376	374	100	90
CHT	Human, inactive	360	280	78	68
HHT	Human, slightly active (*S. salivarius*)	340	58	17	17
6715-Parent	Human, active, parent strain	296	120	40	58
6715-Smooth	Human, slightly active, smooth variant	304	264	87	71
HS-6	Hamster, active, related to AHT serologically	304	252	83	97
E-49	Hamster, moderately active, substrain of HS-6	256	256	100	74
6927	Hamster, active, substrain of E-49	288	288	100	72
FA-1	Rat, active, related to BHT serologically	252	252	100	95
GF-71	Rat, moderately active, similar to FA-1	308	308	100	82
OMZ-61	Rat, moderately active, similar to HS-6	316	144	46	80
2M2	Hamster, inactive	308	308	100	80
2M2R	Hamster, inactive, resistant to streptomycin	272	236	87	86
4M4	Hamster, inactive	280	269	96	68
JR-8Lg	Rat, inactive (*S. faecalis* var. *zymogenes*)	240	200	83	91
JR8-Sm	Rat, inactive (*S. lactis* type)	292	292	100	88

[a] Cell suspensions were incubated for 60 min at 37 C with 250 μg of lysozyme per ml followed by the addition of 0.2% sodium lauryl sulfate (final concentration) to effect lysis.
[b] Cariogenicity is rated as active, moderately active, slightly active, and inactive.
[c] Initial turbidity was 200 Klett units (no. 42 filter) ± 10%.

TABLE 4. *Viability after exposure to lysozyme and sodium lauryl sulfate (SLS)*

Bacterium	Time	Treatment[a]	Klett reading	Colony-forming units	Approx viability
	min				%
S. faecalis strain 31	0	Control	200	3.3×10^8	100
	3	Lysozyme, 250 μg/ml	232	1.2×10^7	4
	60	Lysozyme, 250 μg/ml	252	1.2×10^7	4
	60	Control	197	3.1×10^8	94
	75	Lysozyme, 250 μg/ml, for 60 min + 0.2% SLS[b] for 15 min	10	1.2×10^3	<1
Group H strain Challis	0	Control	200	5.2×10^8	100
	3	Lysozyme, 250 μg/ml	242	1.4×10^8	27
	60	Lysozyme, 250 μg/ml	358	1.4×10^7	3
	60	Control	190	4.7×10^8	90
	75	Lysozyme, 250 μg/ml, for 60 min + 0.2% SLS[b] for 15 min	49	5	<1

[a] Cells were suspended in 0.01 M Tris buffer, *p*H 8.2, and incubated at 37 C.
[b] Final concentration, 0.2%.

of DNA from membrane and cell fragments by the detergent.

By using the method of Schaechter et al. (14) with strain Challis, almost no lysis was produced after an exposure of 30 sec to lysozyme, though a small amount occurred after 5 min of exposure. Longer periods of incubation with lysozyme are required for optimal lysis of Challis as shown in Fig. 2. This method, therefore, would seem more useful with strains of streptococci that are more susceptible to the action of lysozyme, such as *S. faecalis* (Table 5). The advantage of using the method of Schaechter et al. (14) is that it is fast and effects lysis in the cold in the presence of an osmotic stabilizer. The lysis of strains of streptococci was inhibited, however, by the salts present in the TMK buffer (0.01 M Tris-0.01 M magnesium acetate-0.1 M KCl, *p*H 7.0) used in the original technique (14) for lysis of *B. megaterium*. Therefore, 0.01 M Tris buffer, *p*H 8.2, supplemented with 20% sucrose, was substituted.

Extraction of transforming DNA. The condi-

TABLE 5. *Comparison of methods of lysis of streptococci by lysozyme*

Method[a]	Decrease in turbidity[b]		Total soluble protein released		Total soluble DNA released	
	Challis	*S. faecalis*	Challis	*S. faecalis*	Challis	*S. faecalis*
	%	%	%	%	%	%
Described in text:						
Tris-lysozyme (60 min)-SLS	88	98	60	100	57	62
Metcalf and Deibel (11):						
Water-lysozyme (60 min)-0.4 M NaCl	42	90	41	100	19	31
Tris-lysozyme (60 min)-0.4 M NaCl	71	87	58	100	35	32
Schaechter et al. (14):						
Tris + sucrose[c]-lysozyme (30 sec)-SLS	12	82	0	100	2	60
Tris + sucrose[c]-lysozyme (5 min)-SLS	27	88	18	100	20	64

[a] Cells were suspended in 0.01 M Tris buffer (*p*H 8.2) or in distilled water as indicated and incubated with 250 μg of lysozyme per ml followed by the addition of 0.2% sodium lauryl sulfate (SLS) or 0.4 M NaCl (final concentrations) for 15 min at 37 C.

[b] Initial turbidity was 200 Klett units which represented 3.3×10^8 colony-forming units (CFU) of *S. faecalis* and 5.2×10^8 CFU of strain Challis.

[c] Sucrose (20%) in 0.01 M Tris buffer, *p*H 8.2.

TABLE 6. *Effect of the addition of citrate on the release of protein and deoxyribonucleic acid (DNA) from strain Challis.*

Conditions[a]	Soluble protein			Soluble DNA			Decrease in turbidity[b]
	Total	Amt released	Per cent released	Total	Amt released	Per cent released	
	μg/ml	μg/ml		μg/ml	μg/ml		%
Tris-lysozyme-SLS	328	197	60	39	22	57	88
Tris + 0.015 M citrate-lysozyme-SLS	360	192	53	42	21	50	57
Tris + 0.1 M citrate-lysozyme-SLS	312	4	1	29	1	4	3
Tris-lysozyme-centrifuged-suspended in SSC-SLS[c]	328	179	55	39	18	46	86

[a] Cells were suspended in 0.01 M Tris buffer, *p*H 8.2, alone or with sodium citrate added, and incubated 60 min with 250 μg of lysozyme per ml followed by the addition of sodium lauryl sulfate (SLS) to a final concentration of 0.2%.

[b] Initial turbidity was 200 Klett units (no. 42 filter) which represented 5.2×10^8 colony-forming units.

[c] Cells were suspended in 0.01 M Tris buffer, *p*H 8.2, incubated for 60 min with 250 μg of lysozyme per ml, centrifuged, and suspended in 0.1 M NaCl + 0.015 M sodium citrate (8) with SLS added to a final concentration of 0.2% to effect lysis.

tions for obtaining optimal amounts of biologically active DNA by this technique were determined. Table 6 shows the effect of the addition of sodium citrate to the cell suspensions as an inhibitor of nuclease activity. The addition of 0.1 M sodium citrate completely inhibited lysis by lysozyme, although the addition of 0.015 M sodium citrate was only slightly inhibitory. If the cells suspended in Tris buffer were centrifuged after 60 min of exposure to lysozyme and suspended in 0.1 M NaCl-0.015 M sodium citrate (SSC) solution, lysis and DNA release could be obtained by adding SLS (final concentration, 0.2%) to the cells in this solution. Marmur (9) recommended that cells be lysed in SSC for isolation of DNA to provide the proper ionic strength for maintaining the native structure of the DNA. DNA from cells lysed in this manner was isolated by the method of Marmur (9) and used to transform cells of strain Challis to resistance to streptomycin employing the method of Perry and Slade (13). Maximal yields of DNA from strain Challis (streptomycin-resistant) were obtained by lysing early stationary cells. The ability to obtain transformation demonstrates that the DNA obtained by lysis of these cells is biologically active.

These results illustrate the variation in susceptibility to lysozyme that occurs among the strains of streptococci studied. Electron micrographs show that extensive degradation of the cell walls of certain strains that are more susceptible to the action of lysozyme occurs after exposure to lysozyme alone (S. E. Coleman, I. van de Rijn, and A. S. Bleiweis, *in preparation*). It would appear that most streptococci can be gently and effectively lysed by this method for isolation of cellular components.

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