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Degradation of ^{14}C-Labeled Streptococcal Cell Walls by Egg White Lysozyme and Lysosomal Enzymes

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The resistance of native and trypsin-treated [^{14}C]glucose-labeled cell walls to degradation by lysozyme and human lysosomal enzymes was confirmed. In contrast, chemically *N*-acetylated cell walls undergo significant degradation by these enzymes in the pH range of 4.5 to 5.5 without prior removal of the group-specific carbohydrate. *N*-acetylation after removal of the group A carbohydrate by formamide extraction renders the cell walls considerably more susceptible to these enzymes than by formamaide extraction alone. It appears, therefore, that unless *N*-acetylation can occur in vivo, streptococcal cell walls are minimally degraded, if at all, by human peripheral blood leukocytes or lysozyme. Examination of leukocyte extracts from normal subjects and patients with post-streptococcal syndromes revealed no qualitative differences in ability to dissolve streptococcal cell walls.

The isolated cell walls of group A streptococci have been shown to be relatively resistant to degradation by mammalian enzymes (1, 2, 4, 10) and to persist in tissues for prolonged periods. Such cell wall preparations produce acute and chronic inflammation in skin, myocardium, and skeletal muscle (21, 24). In addition, it has been shown that streptococci may be trapped in tissues previously damaged by streptococcal exotoxins or in delayed hypersensitivity lesions (8). The role of such phenomena in the pathogenesis of acute rheumatic fever is poorly understood. Intrigued by the possible contribution of genetic factors to host susceptibility to rheumatic fever and early observations of a constant incidence of rheumatic fever during epidemic streptococcal pharyngitis (23), we have undertaken a study to determine whether differences in degradative capabilities exist between normal humans and patients with acute rheumatic fever. This report describes a sensitive technique for detecting solubilization of nanogram quantities of ^{14}C-labeled streptococcal cell walls by lysozyme and human lysosomal enzymes.

Other investigators have shown that the lysozyme sensitivity of various *Bacillus* species (11) and group A streptococci (10) is related to the degree of acetylation of free amino groups and is inversely related to *O*-acylation of carbohydrate hydroxyl groups (10, 11, 13). Also, in streptococci, lysozyme resistance may in part be due to steric hindrance, which is related to the presence of the peptidoglycan-bound, group-specific carbohydrate (14).

It has been demonstrated that streptococci are very rapidly killed after phagocytosis (27, 28) and that varying degrees of cytoplasmic disintegration occur, as shown by electron microscopy (3, 9, 10). Cell wall thickness and integrity, however, appear to remain unchanged (9, 10). Because studies involving radioisotope-labeled streptococci have dealt mainly with whole organisms (2), we have chosen to examine the degradation of purified cell wall preparations prior to and after chemical modification, as suggested by Glick et al. (10), addressing ourselves initially to native cell walls that were not formamide treated.

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MATERIALS AND METHODS

Organisms. Group A streptococci, types 1 (T1-AV), 3 (C203S), 6, 12, and 19, were obtained from R. M. Cole of the Laboratory of Streptococcal Diseases, National Institutes of Health, Bethesda, Md. The parent strain of T1-AV, T1/155, was obtained from L. Pine of the Center for Disease Control, Atlanta, Ga. Streptococci of groups B and C were chosen from clinical isolates at Duke Hospital.

Preparation of radiolabeled bacteria. Bacteria were grown to stationary phase (usually 18 h) in Todd-Hewitt broth (Difco) containing 0.5 μCi of [U-^{14}C]glucose per ml (2 to 4 mCi/mmol; Amersham/Searle) and heat killed at 60 C for 60 min. Cells were sedimented by centrifugation and washed with deionized water (DW).

Preparation of cell walls. Bacteria from 1 liter of

media were washed, resuspended in 25 ml of DW, and shaken for 20 min with no. 13 glass beads (EM Co., St. Paul, Minn.) in a Mickle apparatus at 4 C. Whole cell walls (WCW) were isolated by differential centrifugation as described by Salton and Horne (25) and treated with trypsin, chymotrypsin, and ribonuclease, as described by McCarty (18).

Fractionation of cell walls. Protease- and ribonuclease-treated cell walls (CW) were extracted two times with formamide as described by Heymann et al. (12-14). The residual peptidoglycan (PG) was washed three times with DW and lyophilized. Group A polysaccharide was precipitated from the formamide supernatant with 5 volumes of acetone, washed two times with acetone, and air dried. CW preparations were stored at −10 C in the lyophilized state or in suspensions (1 mg/ml) in DW. Before use, aggregates were disrupted by brief sonication (1 to 2 s). No release of radiolabel was noted with either repeated freezing and thawing or brief sonication.

***N*-acetylation.** CW and PG were acetylated in saturated sodium bicarbonate with acetic anhydride and de-*O*-acylated with 0.01 N NaOH as described by Heymann et al. (13).

Preparation of lysosomal enzymes. Twenty-five to thirty milliliters of heparinized venous blood was combined with an equal volume of 250,000-molecular-weight dextran (Pharmacia), 3% in normal saline. Erythrocytes were sedimented by standing at room temperature for 30 min. The leukocyte-rich plasma was separated and centrifuged at 900 × *g* for 15 min, and the leukocytes were washed once with normal saline (10). Erythrocytes were lysed with 0.2% NaCl for 30 s, and the suspensions were brought to isotonicity by the addition of an equal volume of 1.6% NaCl. Leukocytes were sedimented, resuspended in 0.34 M sucrose, and sonicated in ice for 30 s or until all cells were disrupted when observed microscopically. Membranes and granules were sedimented at 17,000 × *g* for 20 min at 4 C, resuspended in 2 ml of 0.1 M sodium phosphate buffer, pH 8.0, and frozen and thawed 10 times. Membranes were removed by centrifugation for 30 min at 80,000 × *g* at 4 C and stored at −70 C. Total protein was determined by the Lowry method (17). Lysozyme activity was estimated by the lysoplate method of Osserman and Lawlor (22), using *Micrococcus lysodeikticus* as the substrate.

Enzymatic degradation. Lysozyme (egg white, twice crystallized; Worthington) and lysosomal enzymes, as prepared above, were utilized for enzymatic degradation of CW preparations. Lysosomal enzymes were adjusted to a final concentration of 25 μg of lysozyme per ml and incubated with 20 μg of CW per ml (25 to 150 to counts/min per μg) or 50 μg of PG per ml (25 to 50 counts/min per μg). Reactions were carried out in 0.1 M sodium acetate buffer, pH 5.0, at 37 C for 4 h. Siliconized tubes were used to prevent adherence of the cell walls to glass. Mixtures were aspirated with a 1-ml tuberculin syringe and passed through a 0.45-μm membrane filter (Millipore Corp.) in a Swinney adaptor directly into scintillation vials. Incubation tubes were washed with 0.5 ml of DW, which was passed through the same filter into the vials. Filters were then washed with an additional 1 ml of DW, removed with forceps, and placed into separate scintillation vials. Percentage of solubilization of CW was expressed as filtrate counts divided by total counts per tube. Controls containing no enzymes were performed simultaneously with each assay, and less than 1% of the radioactivity passed through the filters.

Radioisotope counting procedure. Preparations to be counted were added to scintillation vials containing 10 ml of fluid containing 100 g of naphthalene and 8 g of Omnifluor (New England Nuclear Corp.) per liter of dioxane. One milliliter of DW was added to vials that contained filters, since these filters are insoluble in anhydrous dioxane. Filtrates and filters were counted overnight in a Beckman LS-150 liquid scintillation counter.

Analysis of CW components. PG, obtained by formamide treatment, was hydrolyzed in 4 N HCl for 18 h at 100 C in a sealed glass ampoule. The hydrolysate was dried repeatedly with absolute ethanol in a rotary evaporator at 25 C, dissolved in DW, and applied to a column (4.0 by 0.5 cm) of Dowex-50-H^+. This material was eluted sequentially with 40 ml of DW, 40 ml of 1 N HCl, and 40 ml of 4 N HCl, and the fractions were dried as above. The 4 N HCl fraction contained lysine, and the combined DW and 1 N HCl fractions contained the other CW components. Individual components of PG in the 1 N HCl fraction were separated by chromatography on a Dowex-50-H^+ column (60 by 1 cm) eluted with 0.3 N HCl according to Gardell (6). Concentrations of purified components were determined by ninhydrin reagent, using known concentrations of alanine, glutamic acid, lysine, glucosamine, and muramic acid as standards, and the ^{14}C content was determined. Molar ratios and specific radioactivities of each component were then calculated.

Formamide-extracted group A polysaccharide, 1.5 mg, was hydrolyzed for 2 h in 2 N H_2SO_4 at 100 C in a sealed glass ampoule. The hydrolysate was neutralized with Dowex-1-HCO_3-resin, separated from the resin by washing, and dried by evaporation. Rhamnose and glucosamine were separated on Dowex-50-H^+ by elution with DW (rhamnose) and 1 N HCl (glucosamine). Rhamnose was determined according to Dische and Shettles (5).

Thin-layer chromatography. Thin-layer chromatography of PG and group A polysaccharide components was performed on microcrystalline cellulose Avicel plates in ethyl acetate-pyridine-acetic acid-water (5:5:1:3). Spots were developed with ninhydrin spray for amino acids and amino sugars and alkaline silver nitrate for reducing sugars.

Electron microscopy. Acetylated CW (CW-A), 100 μg in 0.5 ml of 0.1 M acetate buffer, pH 5.0, were incubated at 37 C for 4 h with and without 100 μg of egg white lysozyme. These treated and control cell walls were washed two times at 22,000 × *g* and resuspended in 0.1 ml of DW. Specimens were placed on carbon-stabilized, collodion-coated grids, negatively stained with uranyl acetate, and viewed in a Philips EM 300 electron microscope.

RESULTS

Radiolabeling. Streptococci ferment glucose almost entirely to lactic acid, and only 4 to 7% on the ^{14}C-labeled glucose was incorporated into cellular components. Only very low levels of $^{14}CO_2$ were detected, i.e., <0.01%. Some 25 to 32% of the ^{14}C-labeled cell material remained with protease-treated CW (Table 1) and was approximately evenly distributed between PG and formamide-extractable material (e.g., Table 2). Of the latter, 10% remained in soluble form after acetone precipitation of the group A polysaccharide and was not characterized further. The molar ratios and specific radioactivities of PG and group A polysaccharide components are given in Table 2. The formamide-treated cell wall, or PG, retained 8.5% rhamnose by direct analysis, which agrees well with previously reported data (e.g., reference 16). Hydrolysates of the isolated group A polysaccharide revealed only rhamnose and glucosamine by thin-layer chromatography, and the radioactivity in each fraction approached the 2:1 molar ratio reported for these components (15, 16). Specific radioactivities were calculated to be approximately 4×10^4 counts/μmol for both rhamnose and glucosamine. It appears, therefore, that most of the glucose incorporated into these cell wall components is associated with glucosamine, muramic acid, and rhamnose, with less than 10% incorporated into the amino acids comprising the peptide subunits of the PG.

Lysozyme. Each CW and PG preparation was incubated with 25 μg of egg white lysozyme per ml. The pH optimum for lysozyme with both CW-A and acetylated PG (PG-A) was found to be between 4.0 and 5.0 in a 0.1 M sodium acetate buffer (Fig. 1). Lysozyme activity was examined at pH 5.0 with five different group A CW preparations and with one strain each of groups B and C. The results are shown in Table 3. Native group A cell walls (WCW)

TABLE 1. *Distribution of radioactive label within cellular fractions of S. pyogenes strain T1/AV*[a]

Fraction	Total counts	% Whole cells
Whole cells	9.5×10^7	100
3000 rpm pellet (unbroken cells)	0.7×10^7	7
3000 rpm supernatant (broken cells)	8.7×10^7	92
10,000 rpm supernatant (cytosol, membranes)	4.7×10^7	49
Washes	0.8×10^7	8
Enzyme washes	0.7×10^7	7
10,000 rpm pellet (cell walls)	2.8×10^7	29

[a] Percentage of each fraction was calculated on basis of recovery from whole cells. Numbers are averages of two experiments, each using 2 liters of Todd-Hewitt broth, 0.5 μCi of [^{14}C]glucose per ml.

TABLE 2. *Molar ratios and specific radioactivity of CW carbohydrates and amino acids, S. pyogenes strain T1/AV*

Compound	Molar ratio	Counts/μmol
PG		
Glucosamine	1.0	5.3×10^4
Muramic acid	1.15	3.2×10^4
Alanine	3.33	0.24×10^4
Glutamic acid	0.76	0.31×10^4
Lysine	0.76	0.52×10^4
Group A polysaccharide		
Glucosamine	0.5	4.0×10^4
Rhamnose	1.0	3.9×10^4

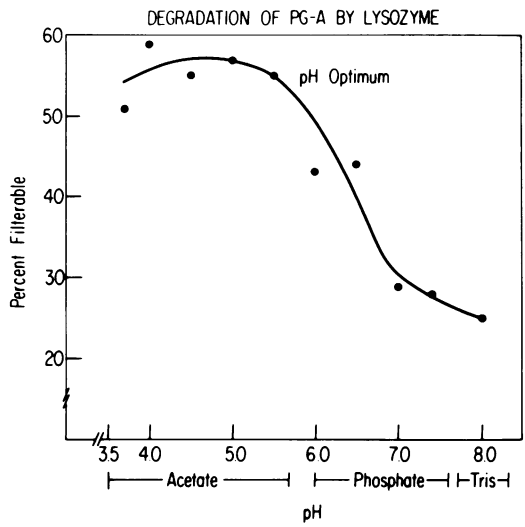

FIG. 1. *pH optimum for degradation of PG-A by egg white lysozyme.*

TABLE 3. *Percentage of degradation of CW fractions by egg white lysozyme, averages of two determinations*

	WCW	McCarty CW	CW-A	PG	PG-A
Group A					
Type 1, A-V		0.9	35	17	56
Type 1/155	0.4	0.6	20	8	37
Type 6	0.5	0.6	24	14	37
Type 12	1.1	0.6	25	14	38
Type 19	0.0	0.2	25	15	33
Group B	5.1	5.6	16	13	57
Group C	3.2	1.9	21	16	55

and protease- and ribonuclease-treated CW were unaffected; however, minimal degradation of WCW and enzyme-treated walls of group B and C streptococci did occur. With each strain, PG, CW-A, and PG-A, respectively, appeared to be more susceptible to lysozyme.

Lysosomal extracts. Lysosomal granule extracts were adjusted to a final volume of 2 ml. Protein determinations ranged from 360 to 1,700 μg/ml and lysozyme activity from 22 to 225 μg/ml. Total leukocyte counts ranged from 1.8×10^8 to 8.6×10^8. Lysozyme activity per 10^8 leukocytes ranged from 16 to 75 μg. The pH optima of lysozyme and lysosomal enzymes from a patient with rheumatic fever versus CW-A and PG-A were determined. Maximal activity was observed in 0.1 M sodium acetate buffer from pH 3.5 to 5.0. Assays, therefore, were routinely performed at pH 5.0. Degradation of CW preparations from strain T1-AV is shown in Table 4, comparing extracts from three normal persons, six patients with acute rheumatic fever, and one patient with acute post-streptococcal glomerulonephritis. Although these data were obtained from a small number of individuals, there seem to be no appreciable differences. PG and PG-A were also examined, and mean values ranged from 3 to 5% with the former and 21 to 28% with the latter.

These data show, for the first time, significant solubilization of CW components of whole, protease-treated, group A CW, but only after *N*-acetylation. Because the procedure of *N*-acetylation also involved de-*O*-acylation with 0.01 N NaOH, the effect of alkali treatment alone was examined with a non-acetylated preparation of T1-AV CW. These CW (treated for 2 h at 27 C with 0.01 N NaOH and washed two times with DW) were reacted with lysozyme, resulting in release of 4% of the radioactivity as compared to 35% seen with the *N*-acetylated CW preparation.

Kinetics of degradation. A timed incubation of CW-A with lysozyme and lysosomal enzymes is shown in Fig. 2. Reactions were virtually complete after 2 h. The early phase was examined in detail with lysozyme and was linear during the first 60 min. No further degradation occurred with increased concentrations of lysozyme or lysosomal enzymes or by addition of more enzyme at 2 to 4 h. Lysosomal enzymes were less active against all preparations when compared to lysozyme (Fig. 2; Tables 3 and 4).

Analysis of solubilized degradation products. T1 CW-A, 5 mg, was reacted with 6.25 mg of lysozyme in 250 ml of DW (same proportions as above experiments) for 4 h at 37 C (DW could be substituted for acetate buffer without altering results). The reaction mixture was passed through a 0.45-μm filter (Nalgene) and dried by evaporation. This filtrate was subjected to thin-layer chromatography both before and after acid hydrolysis (4 N HCl, 18 h, 100 C). The prehydrolysis filtrate material gave seven strongly positive ninhydrin spots, which did not correlate with glucosamine, muramic acid, alanine, glutamic acid, or lysine and probably represented larger sugar-peptide units (7). After 4 N HCl hydrolysis this material gave a chromatography pattern that correlated perfectly with the amino acids and amino sugars of similarly hydrolyzed whole protease-treated CW-A.

A similar 0.45-μm filtrate of a lysate from 12

TABLE 4. *Percentage of degradation of CW and CW-A by human lysosomal enzymes*

Extract (no. of subjects)	CW	CW-A
Acute rheumatic fever (6)	1.3 ± 1.95 (7)[a]	11.5 ± 1.76 (10)
Acute glomerulonephritis (1)	1.2 ± 1.15 (6)	12.9 ± 2.01 (5)
Normal controls (3)	0.5 ± 0.72 (4)	11.2 ± 1.20 (2)

[a] Average of group. Numbers in parentheses represent total number of determinations. All patients were studied at least one time and some on several occasions. Means and standard deviations are given in each group.

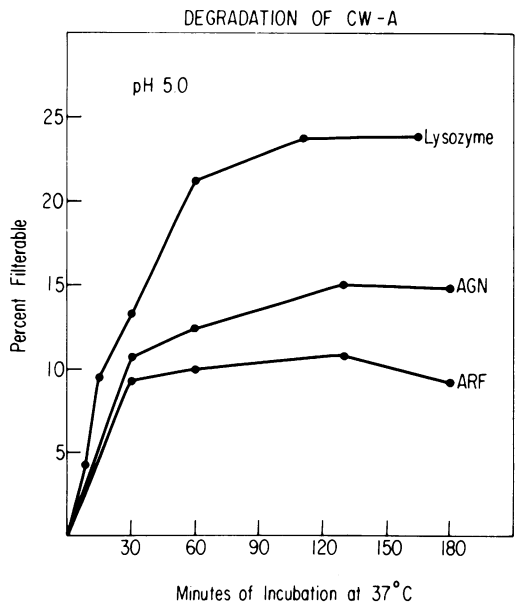

FIG. 2. *Kinetics of degradation of CW-A by lysozyme and lysosomal enzymes from a patient with acute post-streptococcal glomerulonephritis (AGN) and a patient with acute rheumatic fever (ARF). Maximal degradation occurred between 0 and 60 min. The reaction with lysozyme was linear during this period. This figure also demonstrates the increased activity of lysozyme versus lysosomal enzymes against CW-A.*

mg of strain C203S (concentrated to 1 ml) gave a strongly positive precipitin reaction with group A streptococcal antiserum (Burroughs-Wellcome).

Electron microscopy. Electron microscopy of T1 CW-A treated with lysozyme showed significant dissolution when compared to untreated but similarly incubated controls (Fig. 3). Characteristic CW ultrastructure was, for the most part, destroyed in whole cell mounts of lysozyme-treated CW-A, although occasional clumps of unaltered CW were observed.

Size of degradation products. The size of solubilized degradation products was estimated by membrane filtration (Millipore Corp.) and dialysis. CW-A reacted with lysozyme resulted in 21% filterable material at 0.45 μm and 16% at 0.10 μm, whereas lysosomal enzymes from a rheumatic patient gave 17 and 16%, respectively, indicating that greater than 75% of the solubilized material was less than 0.10 μm in size. In addition, 3% of lysozyme-treated CW-A and 36% of PG-A was recovered from dialysates of reaction mixtures after dialysis in acetylated dialysis tubing (effective cut-off limit, approximately 15,000 daltons).

DISCUSSION

Glick et al. (10), have previously demonstrated by electron microscopy and optical den-

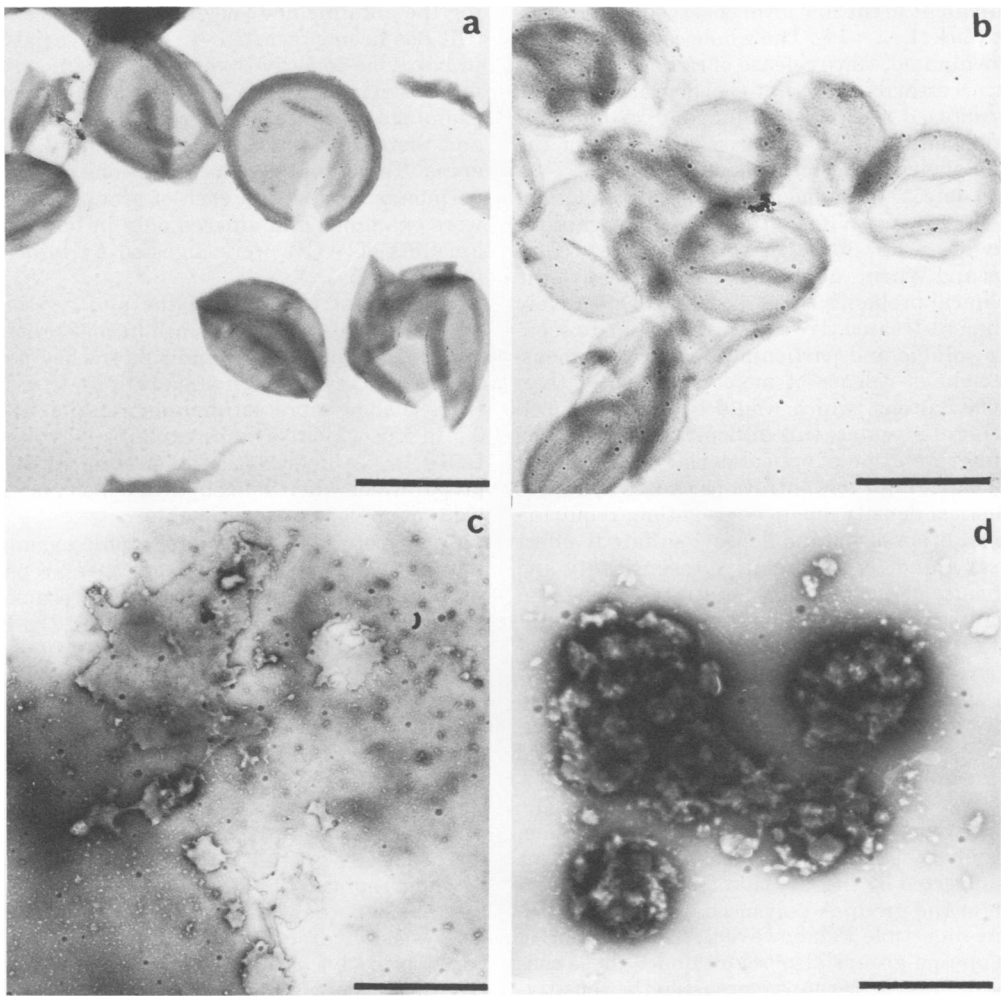

FIG. 3. *Electron micrographs of CW-A from T1/155, control CW (a and b), and lysozyme-treated CW (c and d). Preparations were negatively stained with uranyl acetate. (a) and (b) demonstrate the typical morphology of Mickle-disintegrated group A streptococci. (c) and (d) are representative of grids from lysozyme-treated CW-A, showing particulate matter which has lost recognizable morphology. Occassional areas showed intact CW. Bar, 1 μm.*

sity changes that neither *N*-acetylation nor de-*O*-acylation results in discernible degradation of native streptococcal CW. Complete wall degradation, on the other hand, was accomplished by removal of group A polysaccharide by formamide or hydrochloric acid, followed by *N*-acetylation and de-*O*-acylation (10). Ayoub and Wannamaker (2) have shown release of dialyzable radioactivity from ^{32}P- and ^{14}C-labeled whole streptococci and, to a lesser extent, from isolated native (i.e., non-formamide extracted) CW similar to those prepared in this study. These authors did not report the percentage of total counts released from CW, but it appears probable from the minimal information presented that the level of counts released was equivalent to the low level observed in the present work, i.e., <1%. The whole cell data cannot be evaluated, since release of radioactivity does not necessarily represent CW degradation, and is confused with membrane and cytoplasmic components, which comprise over 60% of the radiolabeled material (this study).

To detect subtle degrees of CW degradation, we chose to use a (0.45 μm) filtration technique previously developed in this laboratory (Gormus and Wheat, unpublished data) to solve the technical problems of: (i) inability to effectively sediment-treated CW by centrifugation to separate soluble and particulate matter; (ii) measurement of release of molecules greater than 15,000 daltons, which would be missed by collecting dialysates; (iii) difficulties encountered in interpretation of optical density changes (lysozyme in high concentrations causes increases in optical density due to aggregation, requiring the addition of sodium dodecyl sulfate to effect "lysis"); and (iv) the relative insensitivity and difficulty of performing a multitude of electron microscopic measurements. In addition, the filtration technique allows analysis of the filterable products. It should be noted, however, that the siliconizing of glassware was necessary to prevent loss of CW substrate and products (i.e., "counts"), which tended to stick to nontreated glassware.

It has been proposed that the group-specific carbohydrate of streptococci inhibits lysozyme, possibly by steric mechanisms. This argument is supported by the fact that formamide extraction of the group A polysaccharide yields lysozyme-digestible PG; however, *N*-formylation of free amino groups also occurs under these conditions (12–14), perhaps increasing the sensitivity of PG to lysozyme. The latter explanation would fit the appealing alternative possiblity that native streptococcal cell walls contain lysozyme-inhibitory free amino groups, as has been shown in other gram-positive bacteria (11, 26), where lysozyme resistance was correlated with the occurrence of free amino groups in the PG-glucosamine residues. Our data support this hypothesis in that simple *N*-acetylation, without removal of group A polysaccharide, resulted in some 25% solubilization of the WCW by lysozyme. Of interest, also, is the observation that formamide extraction followed by de-*O*-acylation prior to digestion with lysozyme resulted in an 8 to 17% release of radioactivity, whereas subsequent *N*-acetylation of the same PG led to a release of 33 to 56% by lysozyme. These observations are probably explained by the occurrence of partial formylation of both free amino and hydroxyl groups during formamide treatment and removal of the -*O*-formyl esters during the alkaline de-*O*-acylation procedure.

It has been suggested (4, 10) that variations in lysozyme sensitivity might occur among different strains of group A streptococci. We have examined four strains of group A streptococci, and there appear to be no outstanding differences (Table 3) under the conditions of our experiments. One strain each of groups B and C were examined and differed only in that small amounts of WCW were digested by lysozyme (3.2 to 5.6%).

The role of pH in lysozyme and lysosomal digestion is also of importance in that previous studies used pH 6.1 (10) and 8.2 (4) when using native or acetylated cell preparations. Our data (Fig. 1) show more optimal degradation at pH 3.5 to 5.0, which perhaps explains why others failed to see degradation of acetylated WCW preparations when using optical density or electron microscope measurements.

Colorimetric and chromatographic examination of acid hydrolysates of our CW preparations revealed only rhamnose, glucosamine, and PG components. We did not detect the PG-linked glucose polymer described by Munoz et al. (19); however, this was expected since our conditions of PG hydrolysis would have destroyed both glucose and rhamnose. Contaminating protein components were not detected since only the PG amino acids were observed. It can be argued that, since these amino acids contained less than 15% of the total radioactivity, release of greater than 15% of the radiolabel must represent hydrolysis of glycosidic linkages rather than simple peptidase activity. Analysis of hydrolyzed membrane filtrates prepared from lysozyme digests of *N*-acetylated CW revealed glucosamine, muramic acid, alanine, lysine, and glutamic acid by thin-layer chromatography, indicating that solubilization was effected by muramidase activity. In addition, these filtrates gave positive precipitin reactions with group A streptococcal antiserum,

indicating that group A polysaccharide was released in soluble form, as observed with *Streptomyces albus* enzyme (18) and phage-associated lysin (15).

The differences observed between human lysosomal extracts and egg white lysozyme in terms of maximal lysis are of interest but cannot be explained. When standardized by the lysopla technique against *Micrococcus lysodeikticus*, 25 μg of lysosomal "lysozyme" activity per ml was not found to be equivalent to 25 μg of egg white lysozyme per ml against group A streptococci, as measured by our radiolabel filtration assay. Increasing the concentration of lysosomal "lysozyme" to 50 to 75 μg/ml did not result in increased lysis of acetylated streptococcal CW. On the other hand, the zones of lysis produced by the lysoplate method (22) were qualitatively different in that egg white lysozyme produced sharp borders, whereas the lysosomal enzymes produced diffuse but measurable borders. It is possible that inhibiting substances present in lysosomal extracts may be removed or neutralized in the agar plate but not in the test tube assay.

Electron microscopy of lysozyme-digested *N*-acetylated CW preparations revealed considerable destruction of normal morphology. Although thin sections were not utilized, it is likely that these changes represent phenomena similar to those reported by Glick et al. (10). Considerable difficulty was encountered in locating recognizable CW remnants in lysozyme-treated, *N*-acetylated preparations compared to untreated CW.

In regard to the significance of these data and post-streptococcal syndromes, we have confirmed previous observations of the total resistance of native and protease-treated CW to lysozyme. We have also shown that patients with post-streptococcal syndromes do not possess inadequate or augmented degradative capabilities against native or acetylated CW preparations. This would suggest that the susceptibility to these syndromes is perhaps due to factors other than those pertaining to CW degradation.

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