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TITLE OF THESIS... A Study of the Composition and Structure of Cell Walls of Micrococcus sedentarius

UNIVERSITY... of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED.... Ph. D.

YEAR THIS DEGREE GRANTED... 1971

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NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

A STUDY OF THE COMPOSITION AND STRUCTURE OF CELL WALLS OF *MICROCOCCUS SODONENSIS*

by

© Kenneth G. Johnson

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A Study of the Composition and Structure of Cell Walls of Micrococcus sodonensis" submitted by Kenneth G. Johnson in partial fulfilmcnt of the requirements for the degree of Doctor of Philosophy.

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Date 23 November 1970

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External Examiner

ABSTRACT

Cell walls of Micrococcus sodonensis were isolated and purified from cultures grown on two different media. Chemical analyses revealed that the preparations were free of cell membrane contaminants, and were qualitatively identical. Glutamic acid, glycine, alanine, and lysine were present in a 1:1:2:1 ratio. N-acetylmuramic acid and N-acetylglucosamine were present in equimolar amounts, and represented the only two amino sugars present in the wall preparations. Basal-grown cell walls contained almost twice as many hexosamine residues as Trypticase soy broth-grown cell walls. Futher analyses employing enzymic and chemical techniques disclosed that both peptidoglycan and non-peptidoglycan composition and structure varied under different growth conditions.

The subunit peptide structure of M. sodonensis cell walls was determined to be N^{α}-[L-alanyl-γ-(α-glutamyl-glycine)]-L-lysyl-D-alanine. Sequential enzymic digestions and isolation of peptide fragments revealed that peptide cross-bridging was accommodated by "head-to-tail" assembly of peptide subunits. Such assembly was facilitated by N^{ε}-(D-alanyl)-L-lysine and D-alanyl-L-alanine linkages. The distribution and length of such cross-bridges was found to vary significantly in the two preparations.

Lysozyme digestion disclosed that neither preparation was ever completely solubilized, although varying degrees of lysozyme-mediated solubilization were observed. Forty-nine and 75% of the Trypticase soy broth- and basal-grown cell walls respectively were so solubilized. A greater degree of peptide substitution of the glycan and shorter peptide bridges were seen in the lysozyme-insoluble than in the lysozyme-soluble

portions of cell walls. Overall, the organization of the peptidoglycan was more complex in the TCS- than in the basal-grown cell walls. Electron microscopic analysis disclosed that the material imparting structural integrity to the cell wall is composed of at least two peptidoglycan matrices, one of which is insensitive to lysozyme by virtue of its higher levels of esterification and complexity of structure.

The presence of O-acetyl substitution in M. sodonensis cell walls was established. Removal of O-acetyl groups increased the lysozyme susceptibility of the cell walls.

Polysaccharides were isolated from the lysozyme-soluble and -insoluble portions of each preparation. Polysaccharides from the lysozyme-insoluble portions contain rhamnose, glucose, and glucuronic acid in a 1:3:2 ratio and were 83 and 44 residues long in TCS- and basal-grown cell walls respectively. The polysaccharide of the lysozyme-soluble portion of TCS-grown cell walls was an octasaccharide composed of glucose. The polysaccharide of the analogous basal-grown cell wall fraction contained glucose and rhamnose in a 3:1 ratio and was 47 residues long. None of the isolated polysaccharides were covalently associated with the peptidoglycan.

Immunological studies indicated that cell wall antigenicity of the intact cell resides predominantly in the non-peptidoglycan components. Moreover, the M. sodonensis cell wall and the extracellular nuclease elaborated by this organism were found to be antigenically related. Nuclease activity was also demonstrated to occur on the cell wall surface.

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. J. N. Campbell for his empathy, scientific acumen, and unfailing humor that have made these years not only stimulating but rewarding. In addition, the author wishes to express thanks to Drs. D. W. S. Westlake and S. A. Berry for their friendship, guidance, and direction, particularly whilst Dr. Campbell was absent on sabbatical leave.

The author also expresses gratitude to Drs. J. M. Ghuysen, R. Wolf, and R. Tinelli for their gifts of enzymes, Mr. J. Kinnear for his assistance in performing gas-liquid chromatographic analyses, and Drs. T. Yamamoto and S. Shahrabadi, and Mrs. Colleen Dmytriw for their advice and assistance in electron microscopic studies.

The author acknowledges the financial support of the National Research Council of Canada, and the University of Alberta, without which this work would not have been possible.

Finally, the author expresses sincere gratitude to his wife, Wendy, for her encouragement, understanding, and moral support.

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ABBREVIATIONS

Å	-	Ångstrom, (10^{-8} cm)
5'-AMP	-	adenosine-5'-monophosphate
°C	-	degrees Centigrade
cm	-	centimeter
DAP	-	diaminopimelic acid
DEAE	-	diethylaminoethyl
DNP	-	dinitrophenyl
EDTA	-	ethylenediaminetetraacetate
FDNB	-	fluorodinitrobenzene
g	-	gram
x g	-	gravity (centrifugal force)
hr	-	hour
K_{av}	-	distribution coefficient between liquid phase and gel phase
l	-	liter
M	-	molar
m	-	meter
mA	-	milliampere
ml	-	milliliter
mM	-	millimolar
µg	-	microgram (10^{-6} g)
µl	-	microliter (10^{-6} l)
N	-	normal

NAD	-	nicotinamide-adenine dinucleotide
nm	-	nanometer (10^{-9} m)
nmoles	-	nanomoles (10^{-9} moles)
PTC	-	phenylthiocarbamyl
TCA	-	trichloroacetic acid
TCS	-	trypticase soy
TMS	-	trimethylsilyl
Tris	-	tris(hydroxymethyl)aminomethane
V	-	volt
V_e	-	elution volume
V_o	-	void volume
V_t	-	total volume
v/v	-	volume per volume
veronal	-	N,N-diethylbarbituric acid
w/v	-	weight per volume
w/w	-	weight per weight

LIST OF OFFICIAL ENZYME NOMENCLATURE

TRIVIAL NAME	E. C. NUMBER	SYSTEMATIC NAME
Myxobacter AL-1 Protease	Unassigned	
Galactose oxidase	1.1.3.9	D-galactose:O_2 oxidoreductase
Glucosamine-6-phosphate N-acetylase	Unassigned	
Glucosamine-6-phosphate kinase	Unassigned	
Glucose oxidase	1.1.3.4	β-D-glucose:O_2 oxidoreductase
Glycerol dehydrogenase	1.1.1.6	Glycerol:NAD oxidoreductase
Lysozyme	3.2.1.17	General Class: N-acetylmuramide-glycanohydrolases
Streptomyces N-acetylmuramic acid-L-alanine amidase	Unassigned	As above
Streptomyces ML-Endopeptidase	Unassigned	
Peroxidase	1.11.1.7	H_2O_2 oxidoreductase
Trypsin	3.4.4.4	General Class: peptide peptidohydrolases

INTRODUCTION

One of the unique features of bacteria as biological entities is that each bacterium possesses a characteristic rigid, water-insoluble cell wall structure surrounding the cytoplasmic membrane. Isolation of cell walls as homogeneous, morphological entities was not possible until Salton and Horne described the first practical method for their isolation from whole bacteria on a preparative scale (Salton and Horne, 1951). Application of physical, biochemical, and electron microscopic techniques has shown these cellular components to be extremely resistant, complex structures whose essential role is the maintenance of structural integrity of bacterial cells in hypotonic environments.

Various mechanical methods of cell disruption produce an insoluble residue, the properties of which suggest that it represents the material surrounding intact cells and which imparts rigidity. Electron photomicrographs support this premise in that the residue consists of fragments which look exactly like flattened bacteria lacking cytoplasmic components. The cell wall, therefore, must be a reasonably thick layer around a soft interior since removal of the latter causes only flattening, while the shape of the parent bacterium is retained in the isolated wall. Confirming proof of the cell wall as a structural entity was established as early as 1953 when Weibull observed that walls of Micrococcus lysodeikticus and Bacillus megaterium could be rendered soluble by treatment with hen's egg-white lysozyme (Weibull, 1953). Gymnoplasts so produced were highly sensitive to osmotic shock and underwent

immediate lysis upon dilution of the medium with water. Lederberg demonstrated that when the ability of growing bacteria to synthesize new cell walls is impaired by selectively toxic agents such as the penicillins, the cells undergo lysis (Lederberg, 1957). It is, therefore, obvious that the wall provides a physical support which allows the relatively fragile cytoplasmic membrane to withstand the osmotic pressure exerted by intracellular components. Such a role is of dynamic significance when one considers that the internal osmotic pressure of bacterial cells has been estimated at 5 to 6 atmospheres for Gram-negative rods and between 20 to 25 atmospheres for Gram-positive cocci (Mitchell and Moyle, 1956).

The results of any cell wall analysis are exceedingly susceptible to two sources of error potentially intrinsic in the procedures involved in wall preparation. First, incomplete purification may result in the persistence of residual cytoplasmic, membrane, or capsular material in terminal preparations subjected to analysis. Second, destruction or loss of integral cell wall material may occur during processes of cell disruption and subsequent wall purification. Thus, cell wall analytical data must be interpreted in terms of the purification procedures used and this important basic study has been the subject of considerable investigation. Isolation of cell walls begins with the disruption of whole cells which is normally achieved in one of three ways: osmotic lysis, autolysis, or mechanical disintegration. Osmotic lysis of certain halophilic bacteria such as Micrococcus halodenitrificans has been found to produce cell wall disruption (Takahashi and Gibbons, 1957). However, the technique is not always successful, cannot be used in the preparation

of large amounts of wall material, and possesses the added disadvantage that endogenous enzymic degradation of wall material probably occurs during the process. Weidel was able to obtain pure preparations of Escherichia coli cell walls by allowing whole cells to autolyse in the presence of toluene (Weidel, 1951). Generally, however, autolysis is not considered to be the most acceptable method of cell wall isolation for two main reasons: first, such preparations may contain non-cell wall material trapped within the cell wall structure and not released upon autolysis, and second, since autolysis results from the breakage of numerous cell wall bonds, these will remain unidentifiable.

Several mechanical methods of cell disruption have been successful in obtaining pure cell walls. Hughes ruptured frozen preparations of a variety of microorganisms employing the high pressure press which bears his name (Hughes, 1951). McCarty was able to prepare pure streptococcal cell walls by using a ball mill to achieve cell breakage (McCarty, 1952). Sonic oscillation has been used for the preparation of several Bacillus spp. cell walls (Salton, 1953b) but this technique is not always satisfactory. Application of high frequency sound has been found to cause extreme fragmentation or even solubilization of Streptococcus pyogenes cell walls (Slade and Vetter, 1956) and the cell walls of Azotobacter vinelandii (Marr and Cota-Robles, 1957). Shaking thick bacterial suspensions with glass beads to effect cell rupture possesses several advantages and has become the most extensively employed technique for cell wall purification. Since this method was first used to prepare cell walls (Dawson, 1949; Salton and Horne, 1951), several devices have been designed to allow more efficient disruption of larger masses of

cells at low temperatures (Shockman et al., 1957; Merkenschlager et al., 1957). Such procedures minimize undesirable enzymic modification of the cell walls. The grinding method possesses a further advantage in that larger wall fragments are obtained by this than any other procedure with the possible exception of the Hughes press.

After rupture of the cells, a mixture is obtained that contains a few unbroken cells, fragmented cell membranes either free or adhering to the walls, flagella and fimbriae if present, ribosomes, and any other cytoplasmic material, as well as the cell walls themselves. Separation of walls from this mixture is, in essentially all cases, effected by differential centrifugation and extensive washings with water or saline (Salton, 1953b). Often a brief heat treatment to inactivate lytic enzymes is given early in the purification sequence (Mandelstam and Rogers, 1959; Janczura et al., 1961). Further treatments designed to clean the material necessitate a decision on the definition of the cell wall. Untreated, cell walls may have cytoplasmic substances adsorbed to them or present because they possess the same sedimentation properties; when treated further, material more properly regarded as part of the cell wall structure may be removed. Several workers have used such enzymes as trypsin, pepsin, and nucleases to facilitate removal of cytoplasmic materials without destroying the rigidity of or supposedly degrading the cell wall structure (Cummins and Harris, 1956; Mandelstam and Rogers, 1959; Janczura et al., 1961). Whereas these have proved useful in helping to define the nature of polymeric components present in the wall, such enzyme digestions are generally avoided since they can remove material properly regarded as part of the cell wall. Extraction of cell

walls with organic solvents provides a means by which one may obtain enriched preparations of the structural polymers of cell walls. Long term extraction with cold trichloroacetic acid removes structurally dispensable materials (teichoic acids and polysaccharides) from the cell walls of Bacillus subtilis (Armstrong et al., 1958), Lactobacillus plantarum, Streptococcus faecalis, and Lactobacillus casei (Ikawa, 1961). McCarty found a similar enrichment of the structural components of streptococcal cell walls by using an extraction with hot formamide (McCarty, 1960). Although these treatments are widely used, they do have disadvantages. Such extractions can cause random cleavage of some of the covalent linkages within the cell wall (Ghuysen, 1968). Moreover, Perkins has shown that various chemical modifications, such as formylation of free amino groups of cell wall amino acids, can occur during formamide extraction. Finally, structural polymers which have been chemically denuded by such techniques cannot yield any information about the way in which various constitutive polymers are integrated into the bacterial cell wall.

The best available criterion of purity is the study of cell wall preparations by electron microscopy. Pure preparations of Gram-positive cell walls normally appear homogeneous except for the occasional findings of organized patterns such as are observed in B. megaterium cell walls (Lamanna and Mallette, 1965). Since walls do not contain nucleic acids, with the exception of certain streptococcal group A wall preparations (Barkulis and Jones, 1957), purified wall material will not contain detectable amounts of 260 nm absorbancy material. In most cases, the presence of pigments can justifiably be viewed with suspicion. However,

the carotenoid pigments of Anacystis nidulans and Microcoleus vaginatus are located within the cell wall (Salton, 1960), as is the pigment of Micrococcus roseus (Campbell, personal communication). Thus, presence of pigments in cell walls and preparation purity are necessarily not mutually exclusive.

In any discussion of cell wall chemistry, it is necessary to distinguish between Gram-positive and Gram-negative cells. Early work on the gross chemical composition of cell walls revealed that the cell wall of Gram-negative organisms were distinct from those of Gram-positive organisms as a group in that they are more complex in composition, containing significant amounts of lipids, lipopolysaccharides, and proteins in addition to structural components (Salton, 1953b; Cummins and Harris, 1956). Subsequent more detailed studies disclosed that both Gram-positive and Gram-negative organisms possessed high molecular weight amino sugar-peptide polymers which impart structural integrity (Weidel and Primosigh, 1958; Weidel et al., 1960; Mandelstam, 1962). These structural components account for 50 to 90% of the cell wall of Gram-positive organisms but only 5 to 15% of the Gram-negative cell wall. Other non-peptidoglycan or non-structural components such as lipopolysaccharides, polysaccharides, or proteins are located externally (Murray et al., 1965) and are involved with functions apart from maintenance of bacterial shape. This discussion, in the interests of brevity and application to this thesis, will be restricted to the composition and structure of the cell wall of Gram-positive organisms.

In the course of studies to elucidate the composition and nature

of the structural polymers of bacterial cell walls, they have been named and renamed numerous times. Terms like mucopeptide (Perkins and Rogers, 1959), mucopolymer (Primosigh et al., 1961), glycopeptide (Strominger, 1962), glycosaminopeptide (Salton, 1964), murein (Martin, 1966), and peptidoglycan (Ghuysen et al., 1968b) have been used to designate the biopolymer imparting structural integrity to cell walls. The term peptidoglycan will be used henceforth to indicate such material.

Ubiquitous and indispensible peptidoglycan components usually found in equimolar amounts are: two acetamido sugars, 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine), 2-acetamido-2-deoxy-3-(D-1-carboxyethyl)-D-glucose (N-acetylmuramic acid), and a limited number of amino acids, some of which occur as the D-isomer rather than the L-isomer which is normally found in other cellular polypeptides. These are L-alanine, D-alanine, D-glutamic acid, and a diamino acid, such as L-lysine or 2,6-diaminopimelic acid (DAP), the latter occurring most frequently in either the meso- or LL-form. Diamino acids usually play key roles in intramolecular cross-linkage of peptidoglycans by virtue of their capacity to be involved in more than two peptide bonds. Although lysine or DAP are the usual diamino acids found in cell walls, it has been demonstrated that hydroxyl-L-lysine is incorporated into peptidoglycans of S. faecalis, Leuconostoc mesenterioides (Smith and Henderson, 1964), and Staphylococcus aureus (Smith et al., 1965) when this compound is supplied in the growth medium. L-ornithine occurs in Micrococcus radiodurans (Work, 1964), D-ornithine in some plant pathogenic Corynebacteria, and 2,4-diaminobutyric acid in Corynebacterium tritici (Perkins and Cummins, 1964). Other amino acids found on occasion in the

peptidoglycans of bacteria are glycine, present in practically all species of Micrococcus, Staphylococcus, and Sarcina, and D-serine, found in only some species of Micrococcus and Staphylococcus (Perkins, 1963).

General agreement exists that the peptidoglycan network is composed of three components: a glycan polymer of alternating N-acetylglucosamine and N-acetylmuramic acid residues, peptide subunits, and peptide cross-bridges. Assembly of these small components results in the formation of an immense macromolecule that completely envelopes the cell.

Characterization of the glycan backbone was largely a result of intensive studies of the products of lysozyme digestion of M. lysodeikticus cell walls. Appreciable amounts of a disaccharide composed of the aforementioned amino sugars were found in such digests. The disaccharide was initially identified as N-acetylglucosamine-β,1:6-N-acetylmuramic acid (Salton and Ghuysen, 1960). Exhaustive re-investigation of the problem using chemically synthesized N-acetylglucosamine-β,1:6-N-acetylmuramic acid disclosed that the two disaccharides were not identical, and that likely the natural linkage was in fact β,1:4 (Jeanloz et al., 1963). Further studies of the M. lysodeikticus glycan ascertained that the β,1:4 linkages between N-acetylglucosamine and N-acetylmuramic acid occur in repeating units throughout the entire glycan and not just in the disaccharide fraction of lysozyme digests (Park and Griffith, 1964). Similar studies of the cell wall of S. aureus led to the same conclusions (Ghuysen and Strominger, 1963; Tipper et al., 1965).

The chemical nature of Gram-positive cell wall subunit peptides was considerably elucidated by the work of Park who showed that a complex of amino acids and uridine-5'-pyrophosphate-N-acetylmuramic acid,

the so-called "Park's nucleotide," accumulated in penicillin-inhibited staphylococci (Park, 1952). The sequence and configuration of the amino acids in the peptide were established by analysis of the products of partial acid hydrolysis, enzymic techniques, and by biosynthesis (Ito and Strominger, 1964). The structure of the nucleotide deduced by such methods was found to be UDP-N^{α}-(N-acetylmuramyl-L-alanyl-γ-D-glutamyl)-L-lysyl-D-alanyl-D-alanine.

It was early postulated that the UDP-muramyl-pentapeptide served as subunit precursors for cell wall biosynthesis and that, therefore, intact disaccharide-peptide units were inserted into the cell wall during biosynthesis (Park and Strominger, 1957). In vitro synthesis of peptidoglycan using cell-free extracts and the UDP-muramyl-pentapeptide as substrate has unequivocally validated this hypothesis (Chatterjee et al., 1967). Discovery of similar cell wall precursors in a variety of organisms led to the concept that basic peptidoglycan structure must be ubiquitous throughout the bacterial world (Strominger, 1959; Anwar et al., 1963a, 1963b).

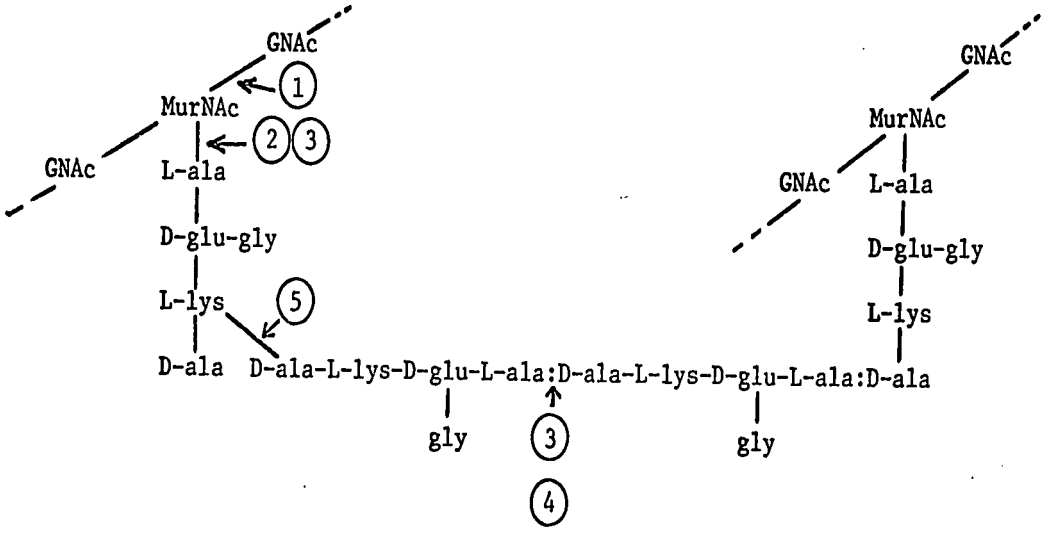
Aside from the study of Park's nucleotide, the most definitive information about subunit peptide structure has been facilitated by the use of highly specific enzymes. Using the Streptomyces spp. N-acetylmuramic acid-L-alanine amidase which hydrolyzes the D-lactyl-L-alanine linkage between N-acetylmuramic acid and L-alanine, Ghuysen clearly established that the peptide subunit is bound to the carboxyl group of N-acetylmuramic acid through the α-amino group of L-alanine (Ghuysen, 1968). Other enzymes, to be discussed later, have been employed to obtain information about unit peptide and cross-bridging structures.

Although basic peptidoglycan structure may be universal, minor variations between subunit peptides have been observed. To date, five types of peptide subunits have been found in a wide variety of bacterial species (summarized by Ghuysen, 1968). Type A, N^{α}-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanine is found in E. coli, B. megaterium, and B. subtilis. Type B, N^{α}-L-alanyl-D-glutamyl-L-lysyl-D-alanine, occurs in S. aureus, M. roseus, S. pyogenes, Arthrobacter crystallopoietes, S. faecalis, Lactobacillus acidophilus, and L. casei. Type C, N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine is found in Sarcina lutea, Micrococcus flavus, Micrococcus citreus, and M. lysodeikticus. Type D, N^{α}-glycyl-D-glutamyl-D-homoseryl-D-alanine is restricted to Corynebacterium poinsettiae, Corynebacterium flaccumfaciens, and Corynebacterium betae. Type E, N^{α}-L-seryl-D-glutamyl-L-ornithinyl-D-alanine, occurs in Butyribacterium rettgeri.

Peptidoglycans of different bacterial cell walls vary not only in the type of subunit peptides, but also in the degree of such peptide substitution. In the peptidoglycan of S. aureus for example, virtually every N-acetylmuramic acid residue is peptide substituted (Ghuysen, Strominger, and Tipper, 1968). On the other hand, at least 40% of the disaccharide units in the peptidoglycan of M. lysodeikticus are not peptide substituted, and can form large segments reaching the size of octasaccharides (Leyh-Bouille et al., 1966). Understandably, such disparities in the degree of peptide substitution markedly affect the tensile strength and response of cell walls to lytic enzymes.

The third component of the peptidoglycan matrix is the peptide cross-bridge. Chemical composition and length of the peptide bridges

vary according to the bacterial species: pentaglycine in S. aureus, tri-L-alanyl-L-threonine in M. roseus R27, diglycine in M. radiodurans, and di- or tri-L-alanine in S. pyogenes group A (Petit et al., 1966; Muñoz et al., 1966). Cross-bridging generally extends from the N^{ε}-amino group of lysine or the amino group on the D-carbon end of meso-DAP of one subunit to the C-terminal D-alanine carboxyl group of another peptide subunit. Direct cross-bridging between peptide subunits is frequently encountered in meso-DAP-containing peptidoglycans. The bridge here, therefore, is D-alanyl-(D)-meso-DAP (Ghuysen, 1968). In M. lysodeikticus and likely many other micrococci, cross-bridging is facilitated by "head-to-tail" assembly of several peptides, each having the same sequence of amino acids as the unit peptide (Ghuysen et al., 1968). Two types of bridges, namely N^{ε}-(D-alanyl)-L-lysine and D-alanyl-L-alanine, accomodate binding between peptide subunits. The structure of such a cross-bridge may have application in Micrococcus sodonensis cell walls and is given below:



As previously stated, highly specific enzymes have been valuable

tools in the elucidation of cell wall structure. The preceeding figure shows linkages which are hydrolyzed by several of these enzymes:

1. Endo-N-acetylmuramidases such as hen's egg-white lysozyme, the F_1 enzyme from Streptomyces spp. (Dierickx and Ghuysen, 1962), and the enzyme from Chalaropsis spp. (Hash, 1963),
2. N-acetylmuramyl-L-alanine amidase from Streptomyces spp. (Ghuysen et al., 1962),
3. AL-1 Protease from Myxobacter spp. (Ensign and Wolf, 1966),
4. SA endopeptidase from Streptomyces spp. (Ghuysen et al., 1967),
5. ML endopeptidase from Streptomyces spp. (Ghuysen et al., 1968a).

A number of other enzymes have been described (Ghuysen, 1968) which are specifically active on some of the unique cross-bridges found in the cell walls of certain microorganisms. All of these can, under the appropriate conditions, be employed to dismantle the peptidoglycan to obtain useful fragments, such as the peptide subunit, for further study.

A unique feature of the peptidoglycan of certain Gram-positive organisms involves O-acetylation of some of the N-acetylmuramic acid residues. The presence of O-acetyl groups in cell walls has been linked with their diminished response to lysozyme (Brumfitt and Wardlaw, 1958). In S. aureus cell walls, approximately half of the N-acetylmuramic acid residues are substituted with O-acetyl groups on carbon 6 and such walls are exceedingly resistant to lysozyme activity (Ghuysen and Strominger, 1963a, 1963b). Little is known of the mechanism by which peptidoglycans are acetylated or what conditions promote this chemical modification.

Peptidoglycan biosynthesis has been extensively studied and the general reaction sequence is well documented (Ghuysen et al., 1968a;

Strominger and Tipper, 1965). Cell wall assembly occurs in three major successive stages. The nucleotide precursors uridine diphospho-N-acetylglucosamine and UDP-N^{α}-(N-acetylmuramyl-L-alanyl-γ-D-glutamyl)-(L-lysyl or meso-diaminopimelyl)-D-alanyl-D-alanine are synthesized by soluble enzyme catalyzed reactions. Once formed, these precursors undergo a complex reaction sequence which produces uncross-linked peptidoglycan strands. Three successive transfer reactions catalyzed by particulate enzymes are involved in this second stage of peptidoglycan synthesis: first, phospho-N-acetylmuramyl-pentapeptide from the UDP-N-acetylmuramyl-pentapeptide is transferred to a C_{55}-isoprenoid phosphate (Higashi et al., 1967) which results in the formation of UMP and a lipid-P-P-N-acetylmuramyl-pentapeptide; second, N-acetylglucosamine is transferred by a transglycosylase from UDP-N-acetylglucosamine to the product of the first reaction with the concomitant release of UDP and the formation of β,1:4-N-acetylglucosamine-N-acetylmuramyl-pentapeptide; finally, the disaccharide-pentapeptide is transferred from the lipid intermediate to an endogenous acceptor, probably an incomplete peptidoglycan. It is at this second level of peptidoglycan assembly that cross-bridging amino acids such as glycine in S. aureus, L-threonine in M. roseus R27, and L-alanine in A. crystallopoietes are inserted. In each case, a charged transfer-RNA molecule bearing the appropriate amino acid serves as donor (Bumsted et al., 1968; Roberts, Petit, and Strominger, 1968; Roberts, Strominger, and Söll, 1968). The third and final stage of peptidoglycan synthesis involves closure of the peptide cross-bridges between peptide subunits. It is thought that this closure is the result of a transpeptidation involving the D-alanyl-D-alanine

sequence of one pentapeptide subunit and a second peptide subunit as acceptor with the simultaneous loss of the terminal D-alanine. This system has yet to be fully elucidated and is the subject of much current investigation.

Cell walls are never pure peptidoglycan; in fact, 10 to 50% of the cell walls of Gram-positive bacteria are composed of non-peptidoglycan material. This may be protein, polyol phosphate polymers (teichoic acids), or polysaccharides. Such non-peptidoglycan components usually occur on the external surface of bacterial cells. Several roles have been assigned to these components, e.g., they may be involved with phage fixation or contribute to the virulence of pathogenic bacteria. By and large, these functions are speculatory with respect to Gram-positive organisms in contrast to the Gram-negative organisms where the roles of at least some of the components have been established.

Non-peptidoglycan protein is found as a cell wall component in several Gram-positive microorganisms. The M proteins of Group A streptococci are determinants in virulence (Lancefield, 1962) and since they can be removed only by mild acid hydrolysis or treatment of the walls with lytic enzymes, are likely covalently bound to the peptidoglycan (Krause, 1963). Bacillus licheniformis 6346 contains a protein constituting 7 to 10% of the wall by weight which is not released by digestion with lysozyme, trypsin, or pepsin, although the former enzyme completely solubilizes the wall (Hughes, 1965). Cell walls of Corynebacterium diphtheriae contain at least one protein antigen (Cummins, 1954), and S. aureus contains an immunologically active cell wall peptide polymer of indeterminant size (Hisatsune et al., 1967).

Polyol phosphate polymers, the so-called "teichoic acids" were first discovered in Micrococcus pyogenes (Mitchell and Moyle, 1951). Since then, cell wall polyribitol or polyglycerol phosphates have been found in a number of bacteria. S. aureus cell walls contain a polymer of 1,5-phosphodiester-linked ribitol units, each of which possesses an N-acetylglucosamine residue on carbon 4, and about half of which are esterified on carbons 2 or 3 by D-alanine (Baddiley et al., 1962). The substituent N-acetylglucosamine residues have been identified as the specific antigenic determinants responsible for cell wall agglutination (Nathenson et al., 1966), and some of these residues are involved with fixation of phages 3C and 71, infectious for S. aureus strain Copenhagen (Coyette and Ghuysen, 1968). Trichloroacetic acid extracts of B. subtilis contain a similar ribitol teichoic acid carrying instead of an N-acetylglucosamine residue on carbon 4, a β-D-glycosyl residue (Armstrong et al., 1961). Bacillus stearothermophilus cell walls contain a 2,3-linked polyglycerophosphate polymer of 18 to 19 residues chain length, with an α-D-glucose on carbon 1 (Wicken, 1966).

The work of Cummins and Harris has shown that purified cell walls of most Gram-positive organisms contain carbohydrate components distinct from peptidoglycan carbohydrates (Cummins and Harris, 1956). Cell walls of M. lysodeikticus contain a polysaccharide composed of equimolar amounts of D-glucose and 2-acetamide-2-deoxymannuronic acid (Perkins, 1963b) and linked to the peptidoglycan by a phosphodiester bond originating from the carbon 6 of an N-acetylmuramic acid residue (Ghuysen, 1968). Streptococcal group A cell walls contain a polysaccharide composed of 2:1 rhamnose and N-acetylglucosamine (McCarty and Morse, 1964). A short-

chain polysaccharide containing arabinose, mannose, and galactose has been found in C. diphtheriae (Ghuysen et al., 1968b). Several other cell wall polysaccharides have been discovered, such as the cell wall polysaccharides of Groups B and C lactobacilli (Sharpe, 1955), and invariably are found to be associated with antigenicity of the parent microorganisms.

Integration of the aforementioned non-peptidoglycan material into intact cell walls, and their syntheses remain, by and large, unsolved problems.

Initially the sole role assigned to the cell wall was, with few exceptions, the pedestrian albeit essential one of an inert "corset" to maintain the cell against the high internal osmotic pressure exerted by intracellular components. Recently, however, the wall has emerged as the site of considerable physiological activity, primarily as the result of the detection of enzymic activity intimately associated with it. Most of the initial work concerns several endogenous autolytic systems which are fixed in bacterial cell walls and must, therefore, be considered cell wall constituents. Autolytic systems have been studied in the cell walls of B. subtilis (Young, 1966; Fan, 1970), S. faecalis (Shockman, Pooley, and Thompson, 1967; Shockman, Thompson, and Canover, 1967), and S. aureus (Cripps and Work, 1967) to name but a few. There is gathering evidence that autolysins, first thought solely as a means of self-destruction, may be powerful agents in the regulation of cell wall biosynthesis. The autolysin of S. faecalis, which has been characterized as an endo-N-acetylmuramidase, is involved in wall extension and cell separation in addition to its role of initiator of new sites

for wall extension (Higgens et al., 1970). Yet another function has been ascribed to autolysins found in the cell walls of B. subtilis. Young noted that strains of B. subtilis highly susceptible to transformation contain a higher autolytic activity than do poorly transformable strains (Young, 1967). It has been suggested that the autolytic system loosens the peptidoglycan net sufficiently to permit passage of DNA to the recipient cell. Other enzymes whose activities are not involved with modification or biosynthesis of walls have been found to be closely associated with the cell wall. The 5'-nucleotidase of E. coli is localized on the cell wall (Nisonson et al., 1969), and recent investigations in this laboratory indicate that the extracellular nuclease of M. sodonensis is cell wall-bound at some point during its elaboration (Berry et al., 1970). Whereas the relationship of such proteins to the wall is unknown, their intimate association with the cell wall supports the hypothesis that the latter possesses a more significant role than that of an inert support.

This study was undertaken to isolate, purify, and characterize M. sodonensis cell walls and compare their properties with the cell walls of other members of Micrococcaceae. Differences in the response of walls derived from cells grown under varying conditions to lytic enzymes have been investigated. In addition, attempts have been made to localize the extracellular nuclease produced by this microorganism. While investigation is by no means complete, several fundamental aspects of the problem have been brought to light.

MATERIALS AND METHODS

I. Reagents

All chemicals employed in this investigation were of reagent grade and were obtained from commercial sources as indicated. Enzymes were prepared or obtained from sources indicated in the text.

II. Culture and Growth Conditions

A. Culture: Micrococcus sodonensis ATCC 11880 was the organism used throughout this investigation. Stock cultures were maintained on Trypticase Soy agar (Baltimore Biological Laboratories) at 4°C with monthly transfers or in lyophilized form. All cultures were grown aerobically at 30°C.

B. Growth Media:

1. Trypticase Soy Broth (TCS), pH 7.3. 1.5% (w/v) Bacto-agar was added when solid medium was required.

2. Synthetic medium employed was that described by Campbell and co-workers (Campbell et al., 1961). The amounts indicated are per 100 ml final volume.

$CaCl_2$	1.0	mg
$(NH_4)_6\ Mo_7O_{24}\cdot 4\ H_2O$	0.08	mg
$ZnSO_4$	1.0	mg
$MnSO_4\cdot H_2O$	0.8	mg
H_3BO_3	0.4	mg
$CuSO_4\cdot 5H_2O$	0.1	mg
$CoCl_2\cdot 6H_2O$	0.1	mg
$FeSO_4$	0.4	mg
$MgSO_4\cdot 7H_2O$	20.0	mg
KCl	50.0	mg
Versene	0.05	mg
Sodium-β-glycerol phosphate	10.0	mg
Biotin	0.001	mg

Lactic acid	500.0	mg
Tris	365.0	mg
Glutamic acid	500.0	mg
NH_4Cl	50.0	mg

The pH was adjusted to 8.0 prior to autoclaving. The final pH was 7.3.

III. Cell Wall Purification Procedures

A. Production of Cells: Cells were grown in 14 liter batches on a Microferm Fermentor (New Brunswick Scientific Co.) aerated at 2 liters per minute air and using as inoculum [0.3% (w/v)] an 18 hr culture of M. sodonensis. Cultures were permitted to grow 36 hr at which time they were harvested by centrifugation, washed three times with distilled water, and if not required immediately, stored at -20°C.

B. Cell Breakage: Equal weights of wet cell paste and 0.11 to 0.12 mm diameter glass beads (B. Braun Apparatebau Melsungen) were placed in a Sorvall Omni-Mixer vessel and stirred at maximum speed at 0°C for one hour. After allowing the beads to settle, the resulting homogenate was decanted and centrifuged at 4,100 x g for 10 minutes. The pellet, containing unbroken cells and residual glass beads, was discarded, while the supernatant, designated as "Crude Wall Fraction," was retained.

C. Purification of Crude Wall Fraction: Centrifugation at 35,000 x g for 20 minutes of the crude wall fraction yielded a pellet having a light yellow upper layer. This layer was carefully removed, washed three times with M NaCl, and held at 90°C for 30 minutes to inactivate lytic enzymes. Following heat treatment, the cell wall material was washed 15 to 20

times with 0.5 M NaCl, and then at least 10 times with distilled water. Between washings, the suspensions were subjected to 30 seconds sonic oscillation in a Bronwill Biosonik III set at maximum intensity to remove adhering protein. This material was designated "Pre-phenol Cell Walls."

D. Phenol Extraction: This procedure was a modification of a previously documented technique (Westphal and Jann, 1964). Equal volumes of cell walls in distilled water and 90.5% phenol (Fisher) preheated to 70°C, were combined and violently agitated for 10 minutes. The emulsion was then centrifuged at 27,000 x g for 15 minutes, the resulting interface material carefully removed, resuspended in water, and the extraction repeated twice. Continuous dialysis against tap water of the cell walls was performed until no detectable phenol odour remained. Phenol-extracted walls were then washed repeatedly with M NaCl, 0.5 M NaCl, and finally, distilled water. This material was designated "Pre-trypsin Cell Walls."

E. Trypsin Digestion: Cell walls were digested with 50 µg per ml bovine pancreatic trypsin (E. C. 3.4.4.4) (Pierce Chemicals) in 0.01 M tris-HCl buffer, pH 8.25 for 4 hr at 37°C. Walls so digested were then alternately subjected to sonic oscillation and washings with M NaCl as previously described. Residual salt was removed by dialysis against distilled water, and the wall preparations lyophilized. These preparations were designated "Pure Cell Walls" and stored at room temperature in closed containers until required.

IV. Analytical Methods

A. Protein: A colorimetric technique employing the Folin phenol reagent was used (Lowry et al., 1951). Standard curves were prepared using cyrstalline bovine serum albumin (Sigma).

B. Inorganic phosphate: A standard, colorimetric procedure employing potassium phosphate as standard was used (Ames and Dubin, 1960).

C. Carbohydrates: All carbohydrate analyses were performed upon whole lysozyme digests of cell walls unless otherwise indicated.

1. Total neutral sugar content was determined by the indole and anthrone techniques described by Ashwell (Ashwell, 1957), and the phenol-sulfuric acid technique (Dubois, 1956). Glucose was employed as standard.

2. Total methyl pentose was determined by the cysteine-sulfuric acid technique (Ashwell, 1957) using a rhamnose standard.

3. Uronic acids were assayed by the carbazole technique (Davidson, 1966). Since this assay is affected by contaminating hexoses, electrophoretic separation of uronic acids from other sugars was performed in 0.01 M veronal-HCl buffer, pH 9.0. The compounds were located with alkaline $AgNO_3$ and untreated parallel strips containing known amounts of samples were eluted with water and reacted in the carbazole assay. Glucuronic acid was employed as standard.

4. Sialic acids were determined by the thiobarbituric acid assay (Warren, 1959) following hydrolysis of samples in 0.1 N H_2SO_4 for one hour at 80°C. N-acetylneuraminic acid was employed as standard.

5. Glucose and galactose were determined after hydrolysis of samples

for 2.5 hr in 3 N HCl at 100°C by means of the glucostat and galactostat reagents (Worthington Biochemical Corp.). Each of these prepared reagents contains the specific glucose oxidase (E. C. 1.1.3.4) or galactose oxidase (E. C. 1.1.3.9), peroxidase (E. C. 1.11.1.7), and a chromogen. The reaction was followed by measuring the formation of oxidized chromogen which had an absoprtion maximum in the 400-425 nm region.

6. Total hexosamines were determined by the Morgen-Elson reaction modified for microdeterminations (Ghuysen et al., 1966). The N-acetyl-glucosamine standard and test samples were hydrolyzed in sealed tubes in 3 N HCl for 3 hr at 100°C, and assays performed after chemical re-acetylation (vide infra) of the samples.

7. Glucosamine was specifically determined by the glucosaminostat reagent (Luderitz et al., 1964). The glucosaminostat is a prepared reagent containing, in addition to the three yeast enzymes glucosamine kinase, acetate-activating enzyme, and glucosamine-6-phosphate-N-acetylase, appropriate cofactors to catalyze the specific re-acetylation of glucosamine only. This reagent was the kind gift of Dr. Regina Tinelli, Pasteur Institute, Paris.

Samples and the glucosamine-HCl standard were hydrolyzed in 3 N HCl in sealed tubes at 100°C for 3 hr and evaporated to dryness prior to the addition of 25 µl of the glucosaminostat. After a one hour incubation the usual Morgen-Elson reaction was performed omitting the chemical re-acetylation step and using Morgen-Elson reagent diluted 1:8 with glacial acetic acid.

8. Reducing sugars were determined by the modified Park-Johnson ferricyanide procedure (Ghuysen et al., 1966).

9. Glycerol was determined by means of a technique employing glycerol dehydrogenase (E. C. 1.1.1.6) (Hagen and Hagen, 1962). Samples and a tripalmitin standard were hydrolyzed in 3 N HCl at 100°C for 3 hr, evaporated to dryness in vacuo, and the glycerol content determined relative to a glycerol standard.

10. Ketohexoses were determined by the cysteine-carbazole assay (Dische and Borenfreund, 1957) relative to a fructose standard.

D. Esters: The hydroxylamine technique of Hestrin (Hestrin, 1949) was employed using ethyl acetate as standard.

E. Free Amino Groups: Total free amino groups were determined by the previously documented direct fluorodinitrobenzene assay (Ghuysen et al., 1966).

F. Amino Acids: Total amino acids were determined as their dinitrophenyl derivatives (Ghuysen et al., 1966). Samples were hydrolyzed in sealed tubes in 6 N HCl at 100°C for 18 hr. DNP-amino acids were then formed by reaction of the amino acid hydrolysates with 1-fluoro-2,4-dinitrobenzene (Fisher) under alkaline conditions at 60°C for one hour. The DNP-derivatives were removed from the reaction mixture by extraction with diethyl ether, evaporated to dryness, resuspended in methanol, and spotted on thin layer plates. Chromatographic separation was effected by development in Solvent 1 as described in Section V, B. After drying, spots were carefully removed from the plates, placed in small tubes, and the derivatives eluted with 1.0 ml of ethanol:water:ammonia (370:370:2). Tubes were vigorously agitated, centrifuged for 5 minutes at maximum speed in a Sorvall clinical centrifuge, and the resultant supernatant

fractions estimated colorimetrically at 360 nm in a Gilford 240 Spectrophotometer. Standard amino acid mixtures were subjected to all these manipulations during quantitative analysis.

G. Free N-terminal Amino Acids: Estimation of the total amount of free N-terminal amino acid groups was achieved by means of a previously documented technique (Ghuysen et al., 1966). This method is essentially the same as the estimation of total amino acids with the following exceptions: 1. Cell wall preparations so analyzed were solubilized enzymically.

2. Derivative formation preceeded hydrolysis.

3. Mono-N^{ε}-DNP-lysine remains in the aqueous phase and is removed by extraction with water-saturated n-butanol. This fraction is resolved by thin layer chromatography of the derivative in Solvent 2 as described in Section V. B.

H. Free C-terminal Amino Acids: Estimation of the total amount of free C-terminal amino acid groups was achieved by means of a technique involving hydrazinolysis, benzaldehyde extraction of resultant hydrazides, and quantification of free C-terminal amino acids as their dinitrophenyl derivatives (Ghuysen et al., 1966).

1. Hydrazinolysis: Hydrazine, 99+% pure (Fisher) was stored in sealed tubes in the dark. Samples to be analyzed were thoroughly dried and held at 100°C for 10 minutes prior to the addition of 100 µl of hydrazine. Hydrazinolysis proceeded at 80°C for 20 hr in sealed tubes. When complete, the samples so treated were opened and dried in vacuo over concentrated H_2SO_4. Extreme caution was employed at each stage to

prevent introduction of water.

2. Benzaldehyde Extraction: Commercial benzaldehyde (Fisher) was purified by fractional distillation (Vogel, 1966), and stored in sealed tubes in the dark. To the dried hydrazinolysis mixture was added 250 µl of distilled water and 100 µl of benzaldehyde. Tubes were sealed and agitated continuously for one hour then subjected to centrifugation which produced an upper aqueous and a lower benzaldehyde phase. The upper aqueous phase was carefully transferred to a fresh tube and the extraction repeated twice.

3. Quantification: When benzaldehyde extractions were complete, samples of the aqueous phase were removed, extracted three times with benzene to remove excess benzaldehyde, and the C-terminal amino acids estimated as their dinitrophenyl derivatives as previously described.

I. Peptide Sequencing: The amino acid sequence of monomer peptides was established by a modification of the Edman Degradation (Tipper et al., 1967). In this procedure, phenylisothiocyanate is coupled to the N-terminus of a peptide chain to form a phenylthiocarbamyl peptide derivative. Exposure of the PTC-peptide to trifluoroacetic acid results in cyclization and elimination of the original N-terminal amino acid as a 2-anilino-5-thrazalinone derivative and the concomitant formation of a new N-terminus. The following protocol describes the sequence of manipulations involved.

1. Coupling Procedure: Lyophilized sample $(Peptide)_n$

↓ + N-ethylmorpholine buffer....70 µl
↓ + Phenylisothiocyanate..... 1 µl

Incubation at 37°C for 45 minutes

↓

↓ + distilled water.....100 µl

Two extractions with ether to remove excess phenylisothiocyanate

↓ Lyophilization of aqueous phase

Phenylthiocarbamyl derivative of peptide

2. Cyclization and Formation of a New N-terminus:

PTC-peptide

↓ + trifluoroacetic acid..... 50 µl

45 minutes at 25°C in sealed tubes

↓ + 0.2 N acetic acid.....100 µl

Two extractions with benzene to remove derivatized N-terminus

↓

$(Peptide)_{n-1}$

3. Quantification: Material passed through one cycle of Edman Degradation was analyzed for its content of total, free N-terminal and free C-terminal amino acids by techniques previously described. Samples containing between 40 and 100 nmoles of each amino acid were withdrawn for such estimation and the remainder was taken through another cycle of the degradation and similarly analyzed.

V. Chromatographic Techniques

A. Paper Chromatography: Descending chromatography and Whatman No. 1 paper were used in all cases. All solvents are described in terms of their proportions by volume.

1. Developing Solvents

a. Amino acids were separated by development of chromatograms

in phenol:water:ammonia (80:20:0.5) (Smith, 1960).

b. Neutral sugars were separated by development of chromatograms in n-butanol:ethanol:water (52:32:16) (Putman, 1957).

2. Location Reagents

a. Amino acids were located by the ninhydrin reagent (Cummins and Harris, 1956).

b. Carbohydrates were detected with the alkaline $AgNO_3$ reagent (Smith, 1960). The background was cleared with 5% (w/v) sodium thiosulfate.

c. Radioactivity was located on paper strips by means of a Nuclear Chicago Actigraph III Strip Scanner.

B. Thin Layer Chromatography: Thin layer plates of Silica Gel H (Stahl's) were prepared on glass plates 10 x 20 cm or 20 x 20 cm. All plates were heated at 100°C for one hour prior to use. The following developing solvents were employed.

1. N^{α}-mono-DNP-amino acids and $N^{\alpha,\varepsilon}$-di-DNP-lysine were resolved by development of thin layer plates in chloroform:methanol:acetic acid (85:13:2), a modification of a previously described solvent (Ghuysen et al., 1966).

2. N^{ε}-mono-DNP-lysine was isolated by development of thin layer plates in benzyl alcohol:chloroform:methanol:ammonia:water (30:30:30:2:6) (Ghuysen et al., 1966).

C. Gas-liquid Chromatography: All analyses were performed with a Varion 700 Gas Chromatograph utilizing a thermal conductivity detector and helium as the gas carrier.

1. Lipid Analysis

a. Extraction: Samples were continuously extracted at 75°C with 2:1 chloroform:methanol (v/v) for 72 hr in a Soxhlet apparatus (Bobo and Eagon, 1968). The extract was evaporated to dryness under reduced pressure and retained for saponification.

b. Saponification and liberation of free fatty acids: A modification of the technique of Hammarstrand was employed for saponification (Hammerstrand, 1966). Ten ml of ethanol:ether 3:1 (v/v) plus 0.5 ml of 10 N KOH were added to the dried extract and the mixture refluxed at 100°C for two hours. Water was then added to give a 50% ethanol-water solution of the soap. Free fatty acids were liberated by the addition of 10 ml of 1.5 N HCl, and quantitatively extracted into petroleum ether. This fraction was evaporated to dryness and retained for methylation. Trioctinin was used as a standard to estimate the efficacy of saponification and fatty acid liberation procedures.

c. Methylation: The BF_3-methanol reagent (Applied Sciences Laboratories) (Metcalf and Schmitz, 1961) was employed for the methylation of fatty acids. The methylation technique of Hammarstrand was modified by extending the boiling time from 2 to 30 minutes (Hammarstrand, 1966). Methylated fatty acids were extracted from the reaction mixtures into petroleum ether, concentrated by evaporation, and chromatographed.

d. Chromatography: Methyl fatty acids were chromatographed on a 1/8" x 20' diethyleneglycol succinate column at 180-195°C. Detector temperature was 265°C, while injector temperature was 250°C. Filament current was maintained at 175 mA and gas flow at 33.0 ml per minute.

2. Acetate Analysis: Samples were extracted three times with 0.5 ml portions of diethyl ether, concentrated to a final volume of 0.5 ml, and chromatographed on a 1/4" x 5' 15% FFAP on 60/80 Chromosorb W column. FFAP is the liquid phase of Carbowax 20 M when treated with 2-nitroterephthalic acid (Byars and Jordan, 1964). Column temperature was held at 135°C while detector and injector temperatures were maintained respectively at 228 and 170°C. Filament current was held at 175 mA and gas flow at 60.0 ml per minute.

3. Sugar Analysis

a. Silyation of Samples: Silyation was effected by means of the Tri-sil reagent (Pierce Chemical Co.) (Sweeley et al., 1963). This reagent converts sugars into their trimethylsilyl derivatives. Dried samples containing approximately 0.5 mg of hydrolyzed carbohydrates were shaken vigorously with 0.2 ml of Tri-sil reagent and allowed to stand for a minimum of five minutes. Silyation mixtures were evaporated to dryness by a 70°C air stream, and then dissolved in hexane.

b. Chromatography: The hexane-soluble TMS-sugars were chromatographed on a 1/4" x 5' stainless steel column of QF_1 on 60/80 Chromosorb W. Operating column temperatures varied from 170 to 200°C. Detector and injector temperatures were maintained respectively at 270 and 235°C. Filament current was held at 175 mA and gas flow at 35.0 ml per minute. Standard sugars were subjected to all procedures and identification was based upon relative retention times. Further confirmation was achieved by adding known standards to silyation mixtures and examining tracings of the chromatograms for increased detector response.

D. Ion Exchange Chromatography: Dowex ion exchange resins were obtained from Bio-Rad Laboratories and DEAE-Cellulose was purchased from Eastman Kodak Company.

1. Separation of neutral and charged sugars: Dowex-50W-X4 (200-400 mesh) in H^+ form was employed for the separation of charged from neutral sugars. Dried samples were resuspended in distilled water and the pH adjusted to 1.0 with HCl. Samples were then placed on 1 x 25 cm columns of the resin and eluted with distilled water to remove neutral sugars. Charged sugars were removed from the resin by elution with 0.1 N HCl.

2. Separation of polysaccharides from peptidoglycan components: DEAE-cellulose was prepared according to the method of Peterson and Sober (Peterson and Sober, 1962). Samples were adjusted to pH 10.0 with NaOH, and placed on a 2.5 x 60 cm column which was then washed with 0.01 M sodium carbonate-bicarbonate buffer, pH 10.0. When three column volumes of effluent had been collected, 0.5 M NaCl in the same buffer was employed to remove resin-bound components.

Dowex-1 (200-400 mesh) was prepared in the OH^- form. Samples were adjusted to pH 10.5 with NaOH and placed on 2.5 x 48 cm columns. Neutral components were removed by washing the column with water, pH 10.5. Resin-bound components were eluted with 0.1 M NaCl in water, pH 10.5.

E. Gel Filtration: Sephadex gels were obtained from Pharmacia Corp. and Bio-gels were purchased from Bio-Rad Laboratories. Gel filtration properties of compounds are expressed in terms of the distribution

coefficient, K_{av} .

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume of the compound, V_o is the void volume of the system, and V_t is the total volume of the system.

1. Fractionation of Enzyme Digests: The products of enzyme digestion were fractionated by gel filtration in 0.1 M LiCl on columns of Sephadex G-50 (Medium) and Sephadex G-25 (Medium) connected in series (Ghuysen, 1968a). Void volume was determined by applying 1.0 ml of a 1 mg/ml solution of Dextran Blue 2000 and monitoring effluent fractions for absorbance at 620 nm. Total volume was established by applying 1.0 ml of a 1 mM solution of glycine and assaying effluent fractions for total free amino groups as described previously. The operating pressure was 1.25 m and flow rate 60.0 ml per hour.

2. Desalting: Materials to be desalted were eluted from calibrated Sephadex G-25 or Bio-gel P-20 or P-2 columns with distilled water. Conductivity of effluent fractions was determined by means of a Type CDM 2 Bach-Simpson conductivity meter.

VI. Electrophoretic Techniques

Paper electrophoresis was carried out on 4 x 41 cm LKB filter paper strips in an LKB Electrophoresis Apparatus.

A. Electrophoresis Buffers:

1. Amino sugars were resolved by electrophoresis in pyridine: acetic acid:water (4:2:1000), pH 5.0 (Campbell et al., 1969). The electrophoresis was performed at 380 V and 3.6 mA for 2 hours.

2. Glycan and peptide constituents of sequential Streptomyces ML-endopeptidase, N-acetylmuramic acid-L-alanine amidase, and Myxobacter AL-1 Protease digests were separated by electrophoresis in 0.1 N formic acid, pH 2.0 (Campbell et al., 1969). Electrophoresis was performed at 380 V and 3.6 mA for two hours.

B. Location Reagents:

1. Amino sugars were located with alkaline $AgNO_3$ (Smith, 1960).

2. N-acetylamino sugars were located by fluorescence after alkaline treatment of the paper with the reagent of Sharon (Sharon, 1964).

VII. Immunological Studies

A. Preparation of Antigens: The preparation of pre-trypsin and pure cell walls has been previously described. Purified M. sodonensis extracellular nuclease was prepared as described by Berry (Berry, 1969).

B. Preparation of Rabbit Anti-sera: Anti-pre-trypsin cell wall, anti-pure cell wall, and anti-nuclease sera were prepared in albino rabbits weighing approximately 2 kilograms. Anti-nuclease serum was prepared by tri-weekly intravenous injections of purified nuclease for 6 weeks. Anti-pre-trypsin and anti-pure cell wall sera were prepared by weekly intravenous injections of 1.0 ml of 1 mg per ml suspensions of basal-grown cell walls for 6 weeks. At the end of the injection schedule, the rabbits were bled out, the sera defibrinated, and the plasma stored at -20°C until required.

C. Purification of Rabbit Anti-sera: DEAE-Sephadex A-50 was employed for the separation of the immune globulin fraction from other serum proteins (Dedmon et al., 1965). Ten ml portions of whole rabbit serum were placed on a DEAE-Sephadex A-50 column of dimensions 1 x 25 cm, and eluted with 0.02 M sodium phosphate buffer, pH 7.4 at 4°C. Three column volumes of effluent were collected. This portion constituted the immune globulin fraction of whole serum, and was concentrated to a volume of 5.0 ml.

D. Slide Agglutination Tests: Doubling dilutions of immune globulin or whole antisera were prepared in buffered saline (95 ml of 0.85% NaCl + 5 ml of 0.1 M borate buffer, pH 8.4). Equal 50 µl volumes of antigen and immune globulin were mixed on a glass slide which was then examined microscopically for agglutination. The titre of the immune globulin was the highest dilution causing visible agglutination of the antigen.

E. Tube Precipitation Tests: Into each of a series of Durham tubes was placed 100 µl of soluble antigen. To each tube was added 100 µl of doubling dilutions of the immune globulin in buffered saline. Tubes were sealed with parafilm, incubated one hour at 37°C, refrigerated overnight, and examined for the presence of visible precipitate.

F. Gel Diffusion: The Oudin single gel diffusion method was employed (Oudin, 1946). Glass tubing 70 mm in length was coated with 0.1% agar and sealed at one end. A 0.6% solution of Noble Agar (Difco) was prepared in buffered saline and held at 50°C. Equal volumes of immune globulin and agar were mixed and added to the tube to form a 2.5 cm column. After solidification, the appropriate antigens or buffers were

layered on the columns. Tubes were sealed, incubated at 30°C, and examined periodically up to 72 hr for bands of precipitation.

G. Ferritin-conjugation of Antibody Preparations: Horse anti-rabbit serum (Difco) and the immune globulin fraction of rabbit anti-nuclease serum were conjugated to horse spleen ferritin (PolyScience) employing m-xylene diisocyanate and a previously documented procedure (Singer and Schick, 1961).

VIII. Enzyme Digestions

Lyophilized hen's egg white lysozyme (three times crystallized) (E. C. 3.2.1.17), was obtained from Sigma. Myxobacter AL-1 Protease was the kind gift of Dr. R. Wolf, Dept. of Microbiology, University of Illinois, Urbana, Illinois. Purified Streptomyces N-acetylmuramic acid-L-alanine amidase and partially purified Streptomyces ML-endopeptidase were the generous gifts of Dr. J. M. Ghuysen, Service de Bacteriologie, Université de Liège, Liège, Belgium. Bovine pancreatic trypsin (E. C. 3.4.4.4) was purchased from Pierce Chemical Co.

A. Lysozyme: All digestions were performed in 0.01 M phosphate buffer, pH 7.0, at 37°C with 1:100 lysozyme:cell walls (w/w). Activity was followed by reduction in turbidity of reaction mixtures and release of reducing groups.

B. Myxobacter AL-1 Protease: All digestions were performed in 0.01 M veronal-HCl buffer, pH 9.0, containing 10^{-3} M EDTA, at 37°C with 1:1000 enzyme:cell wall (w/w). Activity was measured by reduction in turbidity

of reaction mixtures and by release of free N- and C-terminal alanine groups.

C. Streptomyces N-acetylmuramic acid-L-alanine amidase: 5 µl of a 1:100 dilution of the preparation is sufficient to completely hydrolyze 30 nmoles of S. aureus disaccharide-peptide in 4 hr at 37°C. All digestions were performed with 100 µl diluted enzyme solution per mg of cell walls in 0.02 M acetate buffer, pH 5.5 at 37°C. Hydrolysis was monitored by assaying for the release of N-terminal alanine.

D. Streptomyces ML-Endopeptidase: The partially purified preparation was fully purified by ion exchange chromatography on carboxymethyl cellulose and gel filtration on Sephadex G-50 (Ghuysen et al., 1969). All digestions were performed in 0.01 M veronal-HCl buffer, pH 9.0 with 1:50 enzyme:cell wall (w/w). Activity was followed by reduction in turbidity of reaction mixtures and release of C-terminal alanine groups.

E. Trypsin: Pre-trypsin cell walls were incubated at 37°C with 1:100 trypsin:cell walls (w/w) in 0.01 M borate buffer, pH 8.25. Activity was followed by assaying for release of protein.

IX. Electron Microscopy

Samples were examined in a Phillips Model 200 Electron Microscope. Where intact samples were observed, negative staining was achieved by the application of 3% phosphotungstic acid. Where thin-sections were studied, the following protocol was employed (Kellenberger et al., 1959).

A. Fixation: Samples were doubly fixed with glutaraldehyde and osmium

tetroxide. Five percent glutaraldehyde was added to samples [1:5 glutaraldehyde:sample (v/v)] and samples allowed to sit at room temperature for 2 to 3 days. Samples were then washed 4 times with Kellenberger buffer, and 1.0 ml of 1% osmium tetroxide (in Kellenberger buffer) added to the pellets of washed materials. Following 20 hr fixation, samples were again washed as described above.

B. Embedding: To washed, fixed samples was added a few drops (about 0.03 ml) of 2% Noble agar maintained at 45°C in a water-bath. Pellets were resuspended in the agar with a Pasteur pipette, and drops of this suspension spread to a thickness of approximately 1 mm on pre-labelled microscope slides. When the agar was solidified, small cubes about 1 mm^3 were cut out with a clean, sharp razor blade. Specimens were dehydrated by 15 minute passages through increasing concentrations of alcohol, propylene oxide, and finally placed in a 1:1 propylene oxide: Epon mixture and embedded. Once embedded, samples were placed at 60°C for 48 hours.

C. Thin-sectioning: Embedded samples were cut on a Porter-Blum Ultra Automatic Microtome (Model MT-2) and affixed to Formvar-treated copper grids.

D. Negative Staining: Preparations were stained with 5% uranyl acetate alone, or with both 5% uranyl acetate and 0.4% lead citrate.

EXPERIMENTAL

I. Purification

Since M. sodonensis is a highly pigmented organism and this pigment is membrane-associated, loss of the yellow pigment during wall purification served as a qualitative criterion of the efficacy of the procedures involved. Phenol-extraction was found to completely remove all pigment from the walls. The purification sequence employed resulted in preparations which were white and which represented approximately 4% (w/w) of original cell input. Various analyses were performed upon the final products to assess the efficiency of the purification sequence.

A. Phosphate Analysis

Samples were removed at various stages of purification, hydrolyzed 24 hours in 6 N HCl at 100°C, and assayed for their content of inorganic phosphate. As the results of Table I indicate, there was a marked decrease in phosphate content during each successive stage of purification. Terminal preparations contained less than 0.01% by weight of phosphate. The purification sequence was, therefore, considered effective in removing cell membrane contaminants.

B. Lipid Analysis

To further substantiate the hypothesis that pure preparations contained little, if any, cell membrane contaminants, cell walls were subjected to gross lipid analysis. Chloroform:methanol extracts of 200 mg of each wall preparation were prepared and analyzed by gas-liquid chromatography. Escherichia coli cell walls were likewise treated to

TABLE I

PHOSPHATE CONTENT OF M. SODONENSIS CELL WALLS

AT VARIOUS STAGES OF PURIFICATION

Cell Wall Material	TCS-grown cell walls	Basal-grown cell walls
Whole cells	92	87
Crude Wall Fraction	34	68
Pre-phenol	18	26
Pre-trypsin	1	1
Pure	0.09	0.09

Data are expressed as µg per mg material analyzed.

serve as a positive control.

M. sodonensis contained no detectable fatty acids. Solvent extracts of E. coli cell walls, however, contained 6 fatty acids, the major one of which was palmitic acid. Fatty acid composition of these latter cell walls agreed closely with previous analysis (O'Leary, 1962), and attested to the efficiency of the techniques employed for the investigation of the presence of lipids.

Acid hydrolysis of glycerides results in the formation of free fatty acids and glycerol. Chloroform:methanol extracts of pure cell walls, when acid hydrolyzed and analyzed by glycerol dehydrogenase contained no detectable glycerol. However, the presence of glycerol in a tripalmitin standard subjected to the same analytical sequence was demonstrated. For the above reasons, pure cell wall preparations were deemed to be free of any cell membrane contaminants.

C. Electron Microscopic Analysis

Electron microscopic examination of negative-contrast stained cell wall preparations showed them to be devoid of gross physical contamination. Such preparations consisted predominantly of structures the same size and shape as intact cells lacking their cytoplasmic contents. As seen in Plate I, preparations were quite homogeneous, although there were in evidence some structures which had been fragmented at some point in the process of isolation and purification. Backgrounds were significantly free of cellular debris.

PLATE I

ELECTRON PHOTOMICROGRAPH OF PURIFIED M. SODONENSIS

CELL WALL PREPARATION

Specimens were negative-contrast stained with 3% phosphotungstic neutralized with KOH.

Magnification = 45,900 x

II. Peptidoglycan Composition and Structure

A. Total Amino Acid Composition

1. Identification of Amino Acids: The identities of peptidoglycan amino acids were established by descending chromatography, co-chromatography with ^{14}C-labelled amino acids, and by isolation of the amino acids as their dinitrophenyl derivatives.

Two-hundred µg of pure cell walls were hydrolyzed in 6 N HCl at 100°C for 18 hours, evaporated to dryness, and resuspended in 1.0 ml of distilled water. Twenty µl of the hydrolysate were spotted on Whatman No. 1 paper and developed for 24 hours in the phenol:ammonia:water system. Standard amino acids were run with each chromatogram. Four spots were detected in both cell wall hydrolysates corresponding to glutamic acid, glycine, alanine, and lysine.

Further confirmation of these amino acids was obtained by co-chromatography with ^{14}C-labelled standards. Twenty µl of the 18 hour hydrolysate were applied to 4 cm Whatman No. 1 filter paper strips along with 1 µl of the ^{14}C-standards, and the strips developed for 24 hours. As shown in Figures 1 and 2, the amino acids previously identified as glutamic acid, glycine, alanine, and lysine co-chromatographed exactly with their respective radioactive standards.

Dinitrophenyl derivatives of the amino acids were formed by reacting 100 µg of acid hydrolyzed cell walls with 1-fluoro-2,4-dinitrobenzene as described in Methods. Thin-layer chromatography of the ether-extracted DNP-derivatives was performed in the chloroform:methanol:acetic acid solvent system with known DNP-amino acid standards. As shown in Table II, the R_f values of the four spots in the hydrolysate

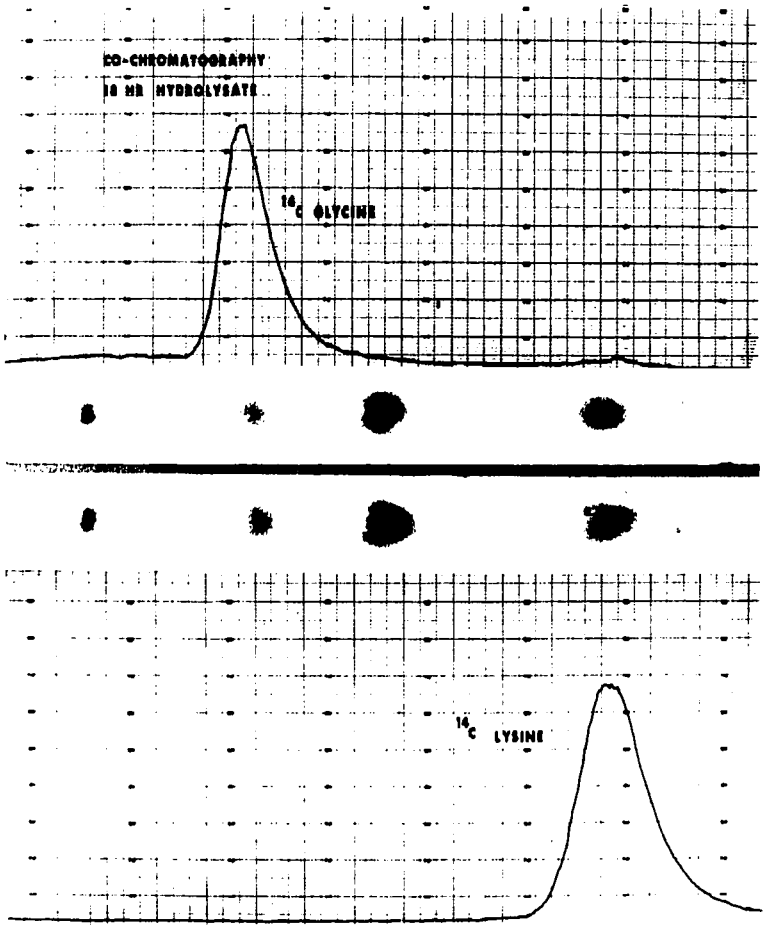
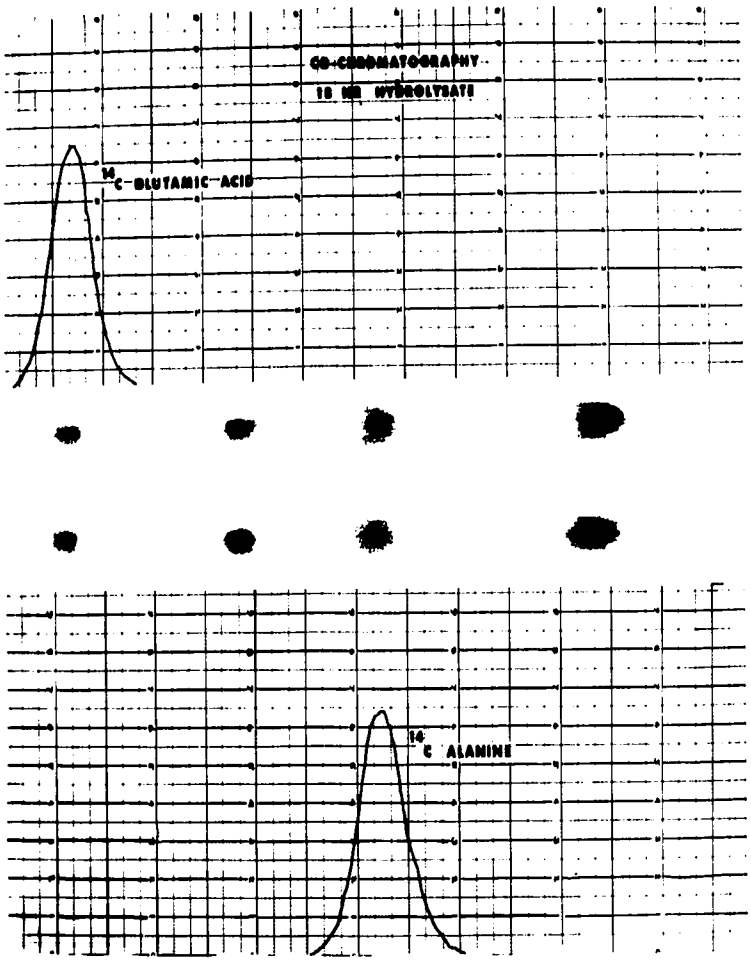

CO-CHROMATOGRAPHY
18 HR HYDROLYSATE
^{14}C GLYCINE
^{14}C LYSINE

FIGURE 1

CO-CHROMATOGRAPHY OF AN 18 HR HYDROLYSATE OF M. SODONENSIS CELL WALLS WITH ^{14}C-GLYCINE AND ^{14}C-LYSINE

Twenty µl of an 18 hr hydrolysate were applied to 4 cm strips of Whatman No. 1 paper along with 1 µl of ^{14}C-standard, and the strip developed 24 hr in the phenol:water:ammonia system. Spots were located with ninhydrin reagent and radioactivity was detected by means of a Nuclear Chicago Actigraph III Strip Scanner.


CO-CHROMATOGRAPHY
^{14}C-GLUTAMIC-ACID
^{14}C ALANINE

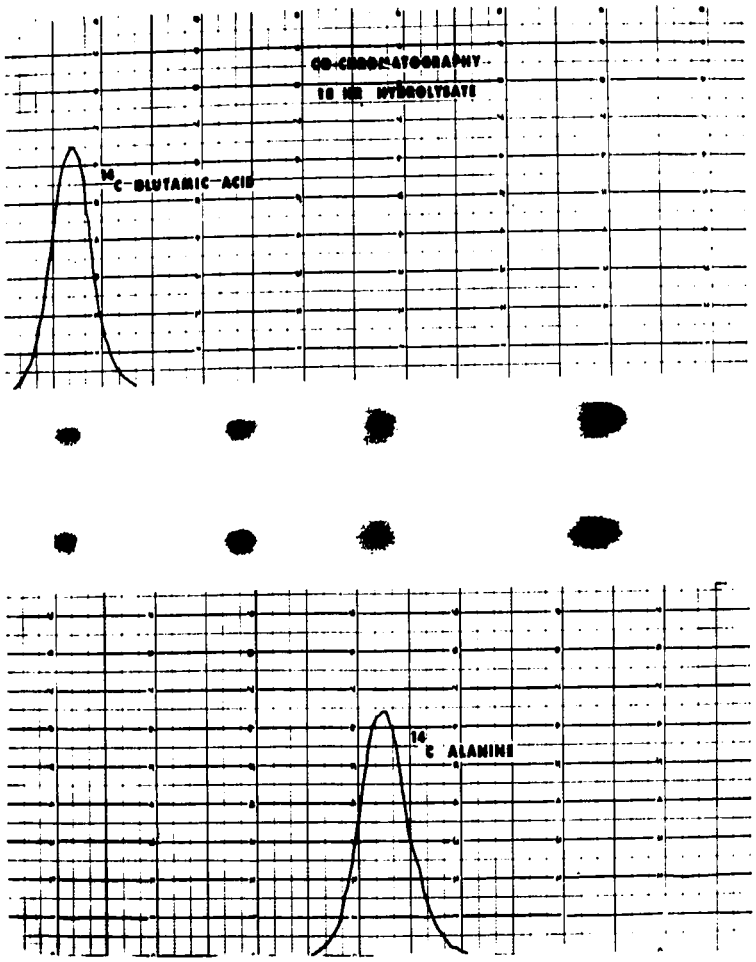

CO-CHROMATOGRAPHY
^{14}C-GLUTAMIC-ACID
^{14}C ALANINE

FIGURE 2

CO-CHROMATOGRAPHY OF AN 18 HR HYDROLYSATE OF M. SODONENSIS CELL WALLS WITH ^{14}C-GLUTAMIC ACID AND ^{14}C-ALANINE

Twenty µl of an 18 hr hydrolysate were applied to 4 cm strips of Whatman No. 1 paper along with 1 µl of ^{14}C-standard, and the strip developed 24 hr in the phenol:water:ammonia system. Spots were located with ninhydrin reagent and radioactivity was detected by means of a Nuclear Chicago Actigraph III Strip Scanner.

TABLE II

R_f VALUES OF KNOWN AND M. SODONENSIS CELL WALL

DNP-AMINO ACID DERIVATIVES

Derivative	R_f	Unknown	R_f
DNP-glutamic acid	0.36	1	0.36
DNP-glycine	0.50	2	0.51
DNP-alanine	0.66	3	0.66
Di-DNP-lysine	0.73	4	0.73

corresponded to the R_f values of the DNP-derivatives of glutamic acid, glycine, alanine, and lysine.

2. Estimation of Amino Acids: Quantification of amino acids was performed as described in Methods. One hundred µg amounts of pure cell walls were acid hydrolyzed along with a standard amino acid mixture containing 50 nmoles each of glutamic acid, glycine, alanine, and lysine. Formation of dinitrophenyl derivatives, and their subsequent separation and quantification were performed as previously described. Standards and samples were assayed in triplicate.

Table III shows that the amino acid composition of both TCS- and basal-grown cell walls was approximately the same. Molar ratios for glutamic acid:glycine:alanine:lysine of 1:1:2:1 are typical for amino acids found in the peptidoglycans of some other microccal cell walls (Ghuysen, 1968). The precision of the technique is evident in that the experimental values closely approximate the theoretical 1:1:2:1 ratio.

3. Estimation of Free N- and C-terminal Amino Acids: Cell walls were digested with lysozyme prior to the analysis for free N- and C-terminal amino acids to loosen the peptidoglycan net such that the maximum number of free amino and free carboxyl groups would be exposed for chemical assay. Five mg of each wall preparation were digested for 24 hours with lysozyme under previously specified conditions. The final volume of each digest was 2.0 ml. Triplicate 50 µl samples of each digest were analyzed for N-terminal amino acids by the dinitrophenylation technique. A standard solution containing 50 nmoles of L-alanine was subjected to the same procedures and served as an internal quantitative control. For the estimation of free N^{ε}-amino groups of lysine, a

TABLE III

AMINO ACID COMPOSITION OF M. SODONENSIS CELL WALLS

Amino Acid	TCS-grown cell walls		Basal-grown cell walls	
	Amount*	Molar Ratio	Amount*	Molar Ratio
Glutamic acid	514	1.00	546	1.03
Glycine	523	1.02	531	1.00
Alanine	1,070	2.08	1,060	2.01
Lysine	514	1.00	530	1.00

*Data are expressed as nmoles per mg cell wall.

solution containing 50 nmoles of N^{ε}-mono-DNP-L-lysine-hydrochloride was subjected to all procedures excluding dinitrophenylation.

The total number of free C-terminal amino acid groups was estimated using the same preparations employed for estimation of N-terminal amino acid groups. Triplicate 100 µl samples of each digest were evaporated to dryness and analyzed for their content of free C-terminal amino acids by the previously described hydrazinolysis technique. Standard solutions containing 100 nmoles each of glycine and L-alanine were treated identically to serve as internal quantitative controls.

The data presented in Table IV indicate that both cell wall preparations contained no detectable amino acids with free α-amino groups. While over 95% of the N^{ε}-amino groups of lysine were free in TCS-grown cell walls, nearly 25% of these groups were substituted in basal-grown cell walls. Alanine and glycine are the only amino acids possessing free C-terminals in intact cell walls. While more than 90% of the carboxyl groups of glycine are unsubstituted in TCS-grown cell walls, only 66% of these groups are free in basal-grown cell walls. The fact that about one-quarter of the N^{ε}-amino groups of lysine and C-terminal groups of glycine are substituted in basal-grown cell walls suggests that several N^{ε}-(glycyl)-L-lysine linkages might occur in this preparation. A very small percentage of the total cell wall alanine of either preparation was found to possess free C-terminals, but TCS-grown cell walls contained more unsubstituted C-terminal alanine groups than did basal-grown cell walls. These data indicate that varying conditions of growth have a definite effect on peptidoglycan peptide bonding.

TABLE IV

FREE N- AND C-TERMINAL AMINO ACIDS† OF

M. SODONENSIS CELL WALLS

	TCS-grown cell walls			Basal-grown cell walls		
	Ala	Gly	Lys	Ala	Gly	Lys
N-terminal amino acids	*	*	490 (95%)	*	*	387 (73%)
C-terminal amino acids	88 (8%)	487 (93%)	*	30 (3%)	352 (66%)	*

† Data are expressed as nmoles per mg cell wall.
* Below levels of detection.

B. Hexosamine Composition

1. Identification of Hexosamines: Identification of cell wall hexosamines was established by separation of charged from neutral sugars and subsequent electrophoretic resolution of the former fraction. Two and one-half mg of cell walls were hydrolyzed in 3 N HCl at 100°C for 3 hr, evaporated to dryness in vacuo, and resuspended in 2.0 ml of water. Ion exchange chromatography was then performed on Dowex-50W-X4 columns as described in Methods. The "Charged Sugar Fraction" eluted from such columns was evaporated to dryness to remove HCl, and resuspended in 0.2 ml of distilled water. Twenty µl of this preparation were spotted on 4 x 41 cm strips of LKB filter paper along with glucosamine and muramic acid standards. Electrophoresis was performed at pH 5.0 as described in Methods. Figure 3 shows a typical electrophoretogram. The Charged Sugar Fraction contained only two sugars, both of which gave positive reactions with ninhydrin and alkaline $AgNO_3$. The two sugars corresponded exactly to muramic acid and glucosamine.

Further confirmation of muramic acid and glucosamine as cell wall components was achieved by gas-liquid chromatography. Component monosaccharides of the Charged Sugar Fraction were silyated and chromatographed on 10% QF_1 on 60/80 Chromosorb W as described in Methods. Figure 4 shows the elution profile of the trimethylsilyl derivatives of the Charged Sugar Fraction. The first peak corresponded exactly to a glucosamine standard which had been silyated under identical conditions while the second corresponded exactly to muramic acid. Addition of TMS-glucosamine to test mixtures resulted in a heightened detector response of Peak 1. Similarly, addition of TMS-muramic acid resulted

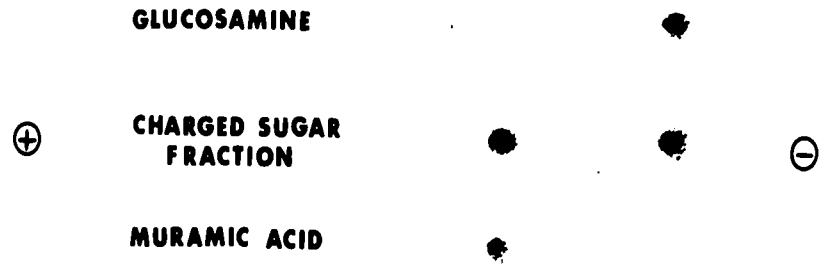

GLUCOSAMINE
⊕
CHARGED SUGAR FRACTION
⊖
MURAMIC ACID

FIGURE 3

ELECTROPHORETIC SEPARATION OF M. SODONENSIS CELL WALL HEXOSAMINES

Twenty µl of the Charged Sugar Fraction obtained from 2.5 mg cell walls were spotted in 4 x 41 cm LKB strips along with sugar standards. Electrophoresis was performed at 400 V and 2.8 mA in the pyridine:acetic acid:water buffer, pH 5.0, for one hour. Spots were located with alkaline $AgNO_3$ reagent.

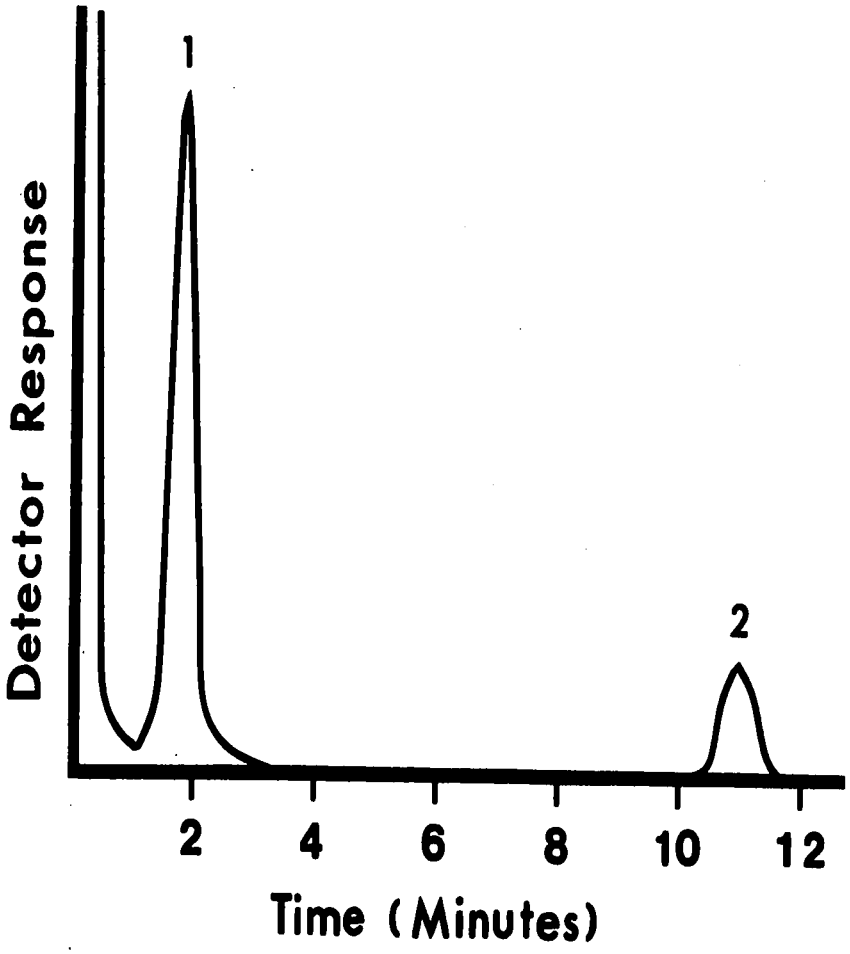

1
2
Detector Response
2
4
6
8
10
12
Time (Minutes)

FIGURE 4

GAS-LIQUID CHROMATOGRAPHY OF M. SODONENSIS CELL WALL HEXOSAMINES

Fifty µl of the TMS-sugars of the Charged Sugar Fraction obtained from 20 mg of cell walls were chromatographed on 10% QF_1 on 60/80 Chromosorb W. Column conditions are described in Materials and Methods.

Peak 1 - Glucosamine Peak 2 - Muramic acid

in a greater amount of Peak 2 material. No other amino sugars sometimes encountered in cell walls of other bacteria such as galactosamine, vanosamine, or mannosamine were detected in the Charged Sugar Fraction. Therefore, muramic acid and glucosamine represent the only two amino sugar components of M. sodonensis cell walls.

2. Estimation of Cell Wall Hexosamines: Quantification of the cell wall hexosamines was achieved by means of the modified Morgan-Elson reaction as described in Methods. Since this technique requires free hexosamines, it is essential to subject samples to acid hydrolysis. To establish the optimal hydrolysis time for maximal release of hexosamines, a series of sealed tubes, each containing 100 µg of cell walls (whole or subjected to 24 hours lysozyme digestion) in 3 N HCl were held at 100°C. At intervals, tubes were removed, their contents evaporated to dryness, and assayed for total hexosamines relative to an unhydrolyzed glucosamine-HCl standard. As shown in Figure 5, the optimum hydrolysis time under these conditions was 3 hours.

Total hexosamine assays were performed upon both intact and lysozyme digested cell walls relative to an N-acetylglucosamine standard. The results are given in Table V. The whole cell walls gave significantly lower total hexosamine values than walls which had been pre-treated with lysozyme. This was attributed to incomplete hydrolysis of the wall in its native state.

Although both TCS- and basal-grown cell walls contained approximately the same amounts of glutamic acid, glycine, alanine, and lysine in a 1:1:2:1 ratio, the latter contained almost twice as many hexosamines per mg cell wall. This implied that either the degree or nature of

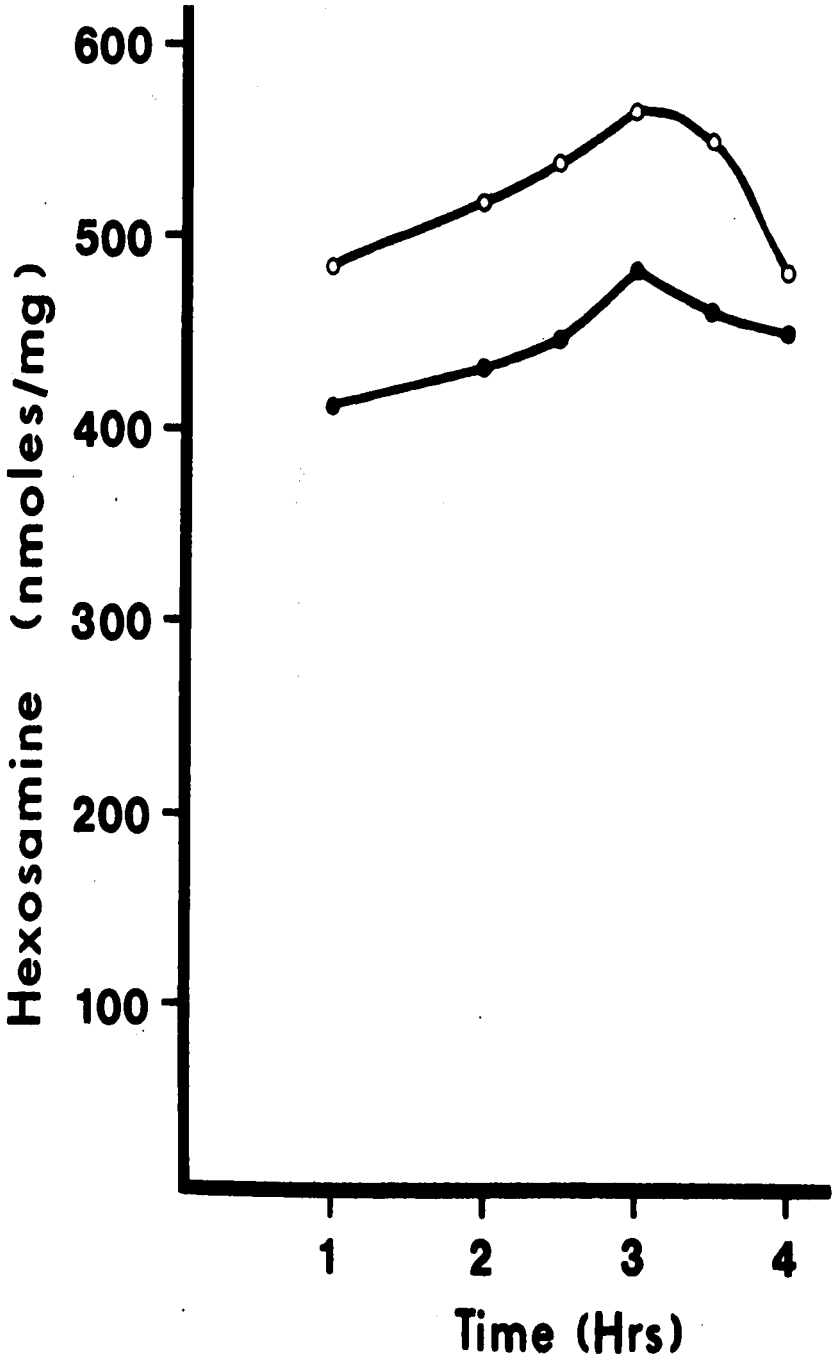

600
500
400
300
200
100
Hexosamine (nmoles/mg)
1
2
3
4
Time (Hrs)

FIGURE 5

KINETICS OF HEXOSAMINE RELEASE BY ACID HYDROLYSIS OF M. SODONENSIS CELL WALLS

One-hundred µg of intact cell walls or whole lysozyme digests of cell walls were hydrolyzed in sealed tubes in 3 N HCl at 100°C. Morgen-Elson assays were performed on samples which were removed at intervals and the total amount of hexosamine determined relative to a glucosamine-HCl standard.

O——O Lysozyme digests of cell walls

●——● Intact cell walls

TABLE V

TOTAL HEXOSAMINE CONTENT* OF M. SODONENSIS CELL WALLS

Wall Preparation	TCS-grown cell walls	Basal-grown cell walls
Intact	489	843
Lysozyme digested	567	1,070

* Data are expressed as nmoles per mg cell wall.

peptide substitution of the glycan was more complex in the TCS-grown cell walls, or that an amino sugar-containing co-polymer might occur in basal-grown cell walls.

3. Estimation of Glucosamine Content: The glucosamine content of cell walls was estimated by means of the previously described glucosaminostat reagent. Cell walls were digested for 24 hours with lysozyme under the standard specified conditions. Samples containing 62.5 µg and 125 µg of digested material were assayed for glucosamine. Appropriate samples (containing between 10 and 50 nmoles glucosamine) of the lysozyme-soluble and -insoluble portions of each cell wall preparation were likewise so assayed. (Lysozyme-soluble and -insoluble portions of cell walls are defined in Section III, A 3). Muramic acid values were obtained by subtraction of the glucosamine content from the total hexosamine content. Results are presented in Table VI.

Both cell wall preparations and their respective lysozyme-soluble and -insoluble portions contained glucosamine and muramic acid in equimolar amounts. It is, therefore, unlikely that an amino sugar-containing co-polymer occurs in basal-grown cell walls. Excess glucosamine and muramic acid residues likely occur in very sparsely peptide substituted glycan strands.

4. N-acetyl Substitution of Cell Wall Hexosamines: N-acetyl substitution of glycan hexosamines was established qualitatively by electrophoretic separation of cell wall glycan and peptide components. Ten mg of pure cell walls were digested sequentially with Streptomyces ML-endopeptidase for 60 hours, Streptomyces N-acetylmuramic acid-L-alanine amidase for 24 hours, and Myxobacter AL-1 Protease for 24 hours.

TABLE VI

GLUCOSAMINE AND MURAMIC ACID CONTENT*

OF M. SODONENSIS CELL WALLS

A. TCS-Grown Cell Walls	Whole Cell Walls	Lysozyme-soluble portion	Lysozyme-insoluble portion
Total Hexosamines	567	273	295
Glucosamine	278	137	144
Muramic acid	289	136	151
Mur:Glu	1.04	0.99	1.05
B. Basal-Grown Cell Walls			
Total Hexosamines	1,070	796	271
Glucosamine	540	399	136
Muramic acid	528	397	135
Mur:Glu	0.98	0.99	0.99

* Data are expressed as nmoles per mg cell wall or portion thereof. Mur, Muramic acid; Glu, Glucosamine

The digest was evaporated to dryness, resuspended in 1.0 ml of 0.1 N formic acid, and 100 µl of the preparation applied to 4 x 41 cm strips of LKB filter paper strips. Electrophoresis was performed in 0.1 N formic acid as described in Methods.

Dried strips were sprayed with Sharon's reagent, specific for N-acetylamino sugars, and examined under ultraviolet light. A broad band of fluorescence, indicative of the presence of N-acetylamino sugars had migrated 1 cm from the origin toward the cathode. This represented glycan material. A parallel ninhydrin-reacted strip exhibited a band of peptide material 7 cm from the origin in the cathode direction.

Additionally, the absence of DNP-muramic acid and DNP-glucosamine in hydrolysates of dinitrophenylated cell walls indicate that constitutive hexosamines of the glycan are N-acetylated.

C. Controlled Digestion of Cell Walls by Myxobacter AL-1 Protease

1. Kinetics of Digestion: Digests were prepared as described in Methods, and aliquots removed therefrom at various times. These were centrifuged 5 minutes in a Beckman Microfuge, and portions of the supernatant assayed for their content of N- and C-terminal alanine. The absorbance of the digest at 550 nm was determined simultaneously to follow loss of turbidity of the digestion mixtures.

Enzyme-mediated release of N- and C-terminal alanine as a function of time is presented in Figure 6. In both cell wall preparations, more N- than C-terminal groups of alanine were released by digestion with Myxobacter AL-1 Protease. This finding implied that there might be few D-alanyl-L-alanine linkages or that such linkages were unavailable for

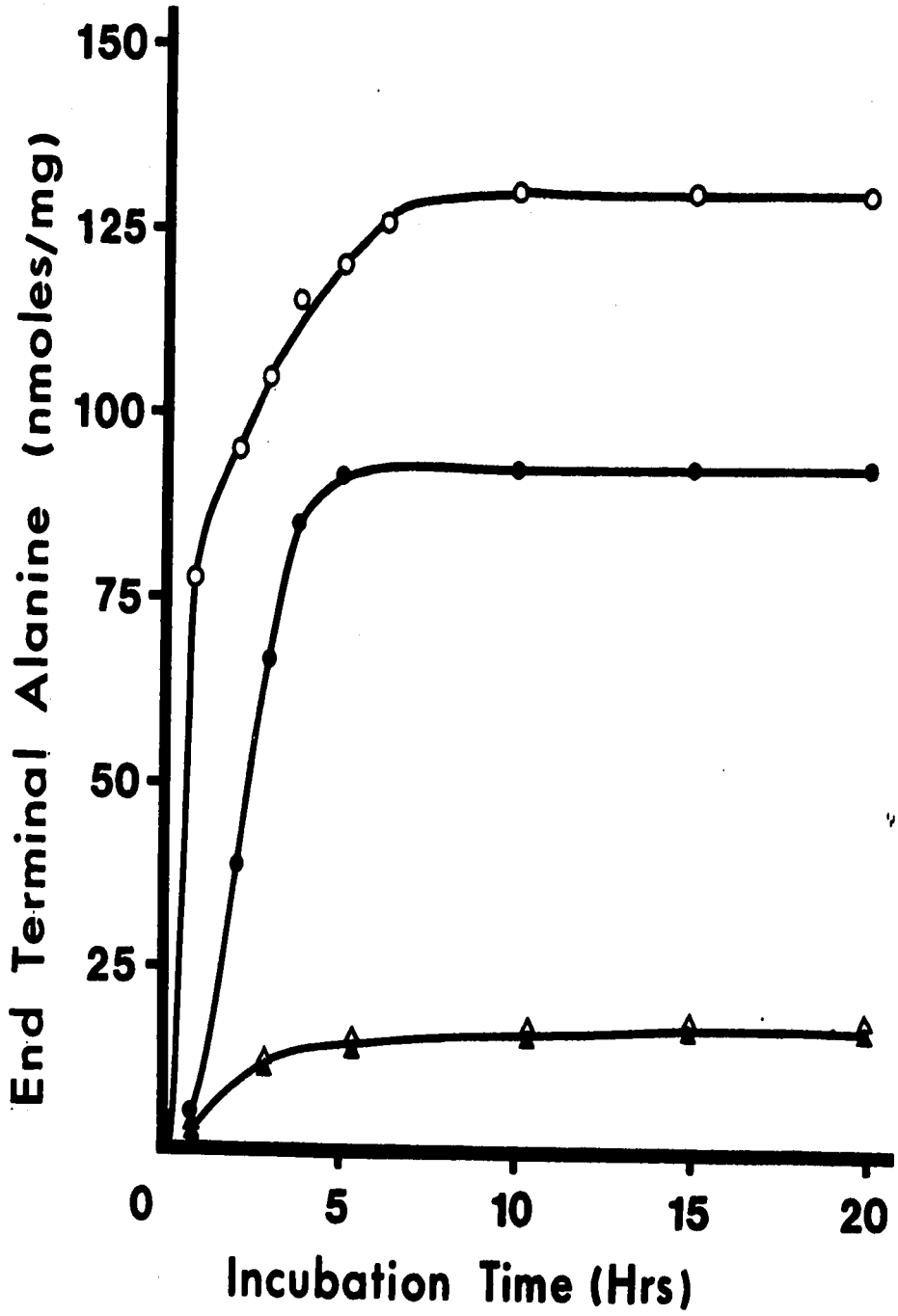

150
125
100
75
50
25
End Terminal Alanine (nmoles/mg)
0
5
10
15
20
Incubation Time (Hrs)

FIGURE 6

KINETICS OF MYXOBACTER AL-1 PROTEASE DIGESTION

OF M. SODONENSIS CELL WALLS

Five mg of each wall preparation were digested with Myxobacter AL-1 Protease under standard specified conditions Triplicate 100 µl samples were withdrawn at intervals and assayed for their content of N-terminal alanine. Triplicate 300 µl samples were simultaneously withdrawn for assay of their C-terminal alanine content.


N-terminal alanine release in TCS-grown cell walls


N-terminal alanine release in basal-grown cell walls


C-terminal alanine release in TCS-grown cell walls


C-terminal alanine release in basal-grown cell walls

enzymic hydrolysis. Moreover, TCS-grown cell walls released more N-terminal alanine groups even though both preparations contain almost equal amounts of this amino acid. This finding strongly suggests that more N-acetylmuramic acid residues are peptide substituted in TCS-grown cell walls.

Clarification of cell wall suspensions digested with Myxobacter AL-1 Protease was compared with the rate of release of N-terminal alanine. As is shown in Figure 7, clarification of basal-grown cell walls was effected by the enzyme before any significant release of N-terminal alanine groups occurred. Thus, the hydrolysis of a relatively few sensitive bonds results in solubilization of the walls with the concomitant release of large fragments. This suggests that long peptide cross-bridges occur in basal-grown cell walls. However, the nature and degree of peptide cross-linking would appear to be less organized and complex than in the TCS-grown cell walls. In the latter case, clarification of wall suspensions and release of N-terminal alanine groups occurred almost simultaneously. Thus, many bonds are cleaved and N-terminal alanine groups exposed prior to complete wall solubilization.

2. Free N- and C-terminal Amino Acid Groups Released by Digestion with Myxobacter AL-1 Protease: Five mg of each wall preparation were digested for 24 hours with the Myxobacter AL-1 Protease under previously specified conditions. The final volume of each digest was 2.0 ml. Triplicate 50 µl aliquots were assayed for their content of free N-terminal amino acid groups as previously described. Triplicate 100 µl aliquots of each digest were evaporated to dryness and analyzed for their content of free C-terminal amino acids by the hydrazinolysis technique.

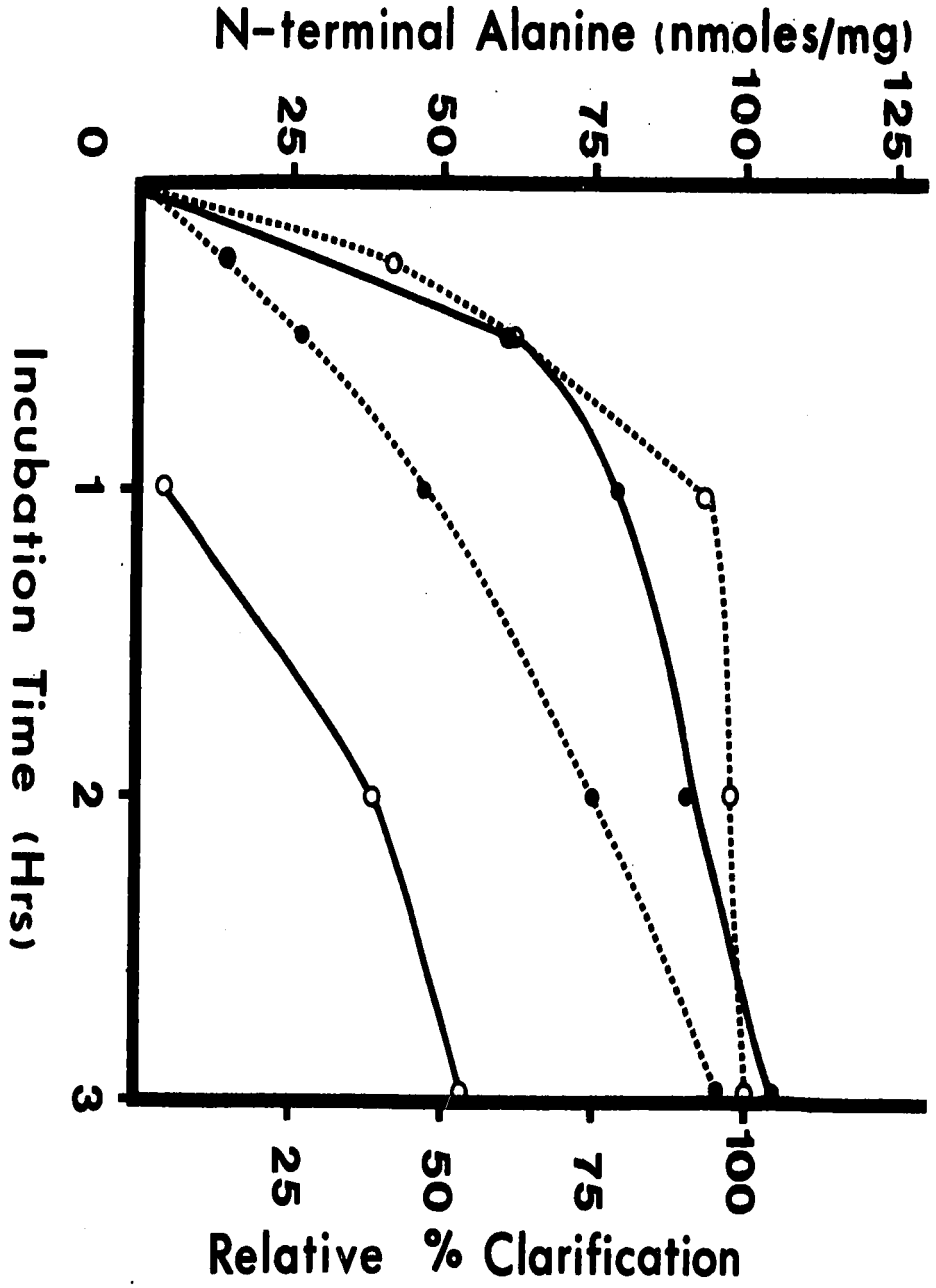

N-terminal Alanine (nmoles/mg)
0
25
50
75
100
125
Incubation Time (Hrs)
1
2
3
25
50
75
100
Relative % Clarification

FIGURE 7

COMPARISON OF N-TERMINAL ALANINE RELEASE AND CLARIFICATION OF MYXOBACTER AL-1 PROTEASE DIGESTS OF M. SODONENSIS CELL WALLS

Two and one-half mg per ml cell walls were digested with Myxobacter AL-1 Protease under standard specified conditions. Triplicate 100 µl aliquots were withdrawn at intervals and assayed for their content of N-terminal alanine. The turbidity of the digestion mixture was simultaneously determined at 550 nm

●——● N-terminal alanine release in TCS-grown cell walls

○——○ N-terminal alanine release in basal-grown cell walls

●·····● Relative % clarification of TCS-grown cell wall digest

○·····○ Relative % clarification of basal-grown cell wall digest

Standards and controls were the same as those employed for the estimation of free N- and C-terminal amino acid groups in lysozyme digests of whole cell walls. Data are presented in Table VII.

Although only a small percentage of the total alanine content of each cell wall preparation was released as N-terminal alanine, Myxobacter AL-1 Protease digestion released more of these groups in the TCS- than in basal-grown cell walls. While essentially all of the carboxyl groups of glycine were free in such digests of TCS-grown cell walls, 25% of these groups still remained substituted in basal-grown cell walls. Higher levels of free N^{ε}-amino groups of lysine and carboxyl groups of glycine appeared in Myxobacter AL-1 Protease digests than those observed in intact cell walls (Table VI). This finding lends support to the postulated existence of an N^{ε}-(glycyl)-L-lysine linkage in the peptidoglycan.

It was possible to calculate the number of N-acetylmuramyl-N^{α}-L-alanine linkages, i.e., the number of N-acetylmuramic acid residues which were peptide substituted in intact cell walls. The simplifying assumption was that the total N-terminal alanine groups released by Myxobacter AL-1 Protease digestion minus the amount of N-terminal alanine groups released by hydrolysis of D-alanyl-L-alanine linkages represented this figure. N-terminal alanine groups released by cleavage of D-alanyl-L-alanine bonds was taken as the difference between free C-terminal alanine content before and after Myxobacter AL-1 Protease digestion. These results are summarized in Table VIII.

One can approximate that , on the average, two in every five muramic acid residues are peptide substituted in TCS-grown cell walls

TABLE VII

ESTIMATION OF FREE N- AND C-TERMINAL AMINO ACIDS† IN M. SODONENSIS CELL WALLS DIGESTED BY MYXOBACTER AL-1 PROTEASE

	TCS-grown cell walls			Basal-grown cell walls		
	Ala	Gly	Lys	Ala	Gly	Lys
N-terminal amino acids	134 (12%)	*	501 (99%)	86 (8%)	*	415 (78%)
C-terminal amino acids	106 (10%)	518 (99%)	*	46 (4%)	397 (75%)	*

† Data are expressed as nmoles per mg cell wall.
* Below levels of detection.

TABLE VIII

N-ACETYLMURAMIC ACID-PEPTIDE SUBSTITUTION

IN M. SODONENSIS CELL WALLS*

	TCS-Grown Cell Walls	Basal-Grown Cell Walls
Total N-terminal alanine released by Myxobacter AL-1 Protease (A)	134	86
N-terminal alanine released by cleavage of D-alanyl-L-alanine linkages (B)	18	16
Total N-acetylmuramyl-N^{α}-L-alanine linkages (A - B)	116	70
Total Muramic Acid	289	528
% Muramic acid substituted	40.1	13.3

* Data are expressed as nmoles per mg cell wall.

whereas only one in ten muramic acid residues is so substituted in basal-grown cell walls.

3. Fractionation of Myxobacter AL-1 Protease Digests: Myxobacter AL-1 Protease digests of cell walls were fractionated by gel filtration in 0.1 M LiCl on the linked Sephadex G-50, G-25 system described in Methods. Fifty mg of each wall preparation were subjected to 36 hours digestion with Myxobacter AL-1 Protease under previously specified conditions. The digest was concentrated to a final volume of 5.0 ml and applied to the column. Five ml fractions were collected and 100 µl samples of these assayed for their content of free amino groups relative to a solution containing 50 nmoles of L-alanine. Figure 8 shows a typical elution profile of such digests fractionated in this manner.

The free amino groups of TCS-grown cell walls treated in this way fractionated into four main peaks having K_{av} values of 0.75, 0.6, 0.32, and 0 (excluded). These first two peaks corresponded to the peptide monomer and dimer fractions observed in a similar type of fractionation of S. lutea cell walls (Campbell et al., 1969). In digests of basal-grown cell walls, there was no such clear fractionation of free amino groups suggesting that altered peptidoglycan structure yields altered types of digestion products.

The material from TCS-grown cell walls having a K_{av} value of 0.75 was provisionally designated "Peptide Monomer" and further analyzed. This material was desalted on a 2.5 x 30 cm column of Bio-gel P-2 eluted with distilled water, and then concentrated to a volume of 3.0 ml. Twenty-five µl aliquots of this solution were analyzed for their content of total amino acids, and free N- and C-terminal amino acid groups.

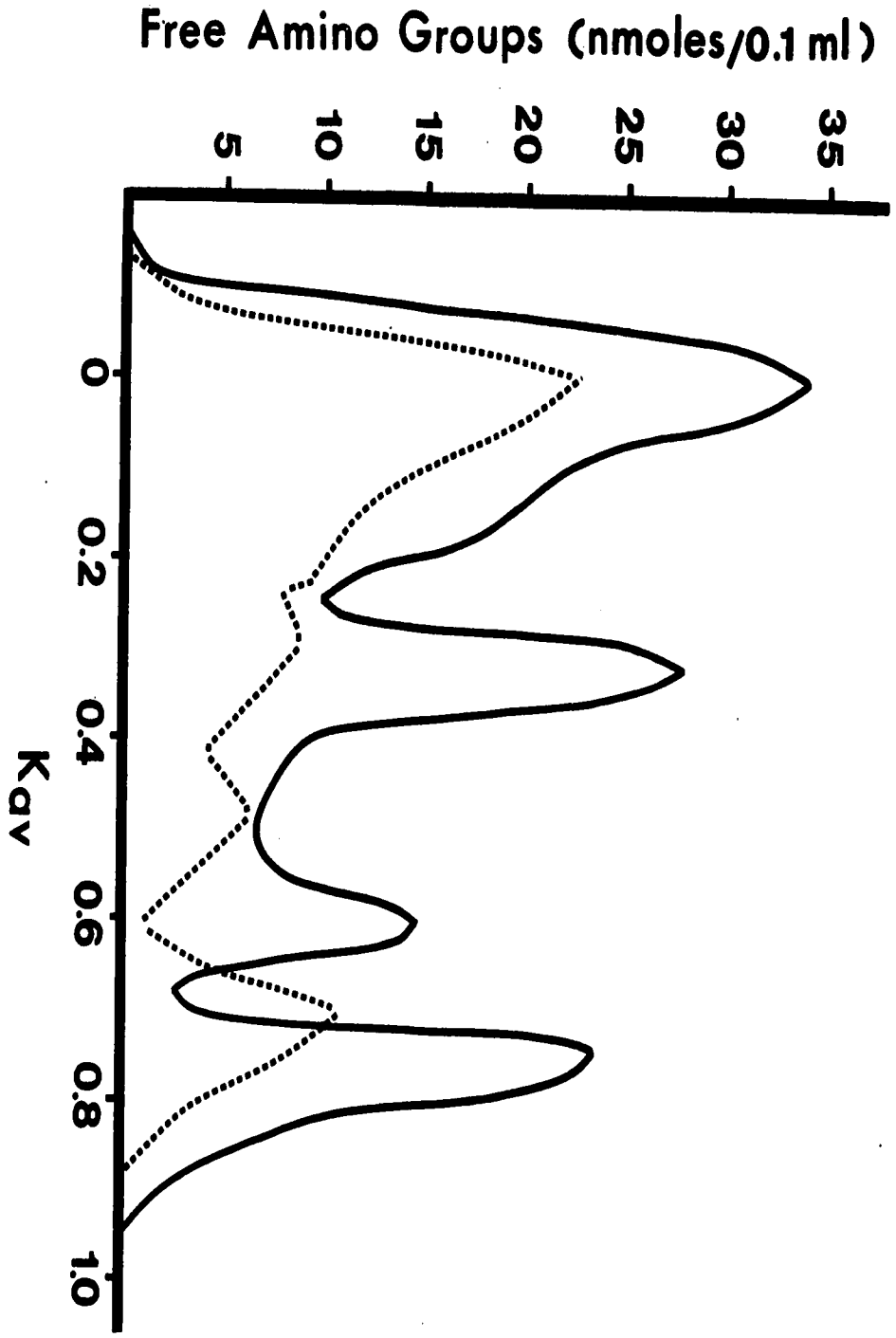

Free Amino Groups (nmoles/0.1 ml)
5
10
15
20
25
30
35
Kav
0
0.2
0.4
0.6
0.8
1.0

FIGURE 8

FRACTIONATION OF MYXOBACTER AL-1 PROTEASE DIGESTS OF M. SODONENSIS CELL WALLS

Fifty mg of each cell wall preparation were digested with Myxobacter AL-1 Protease under standard specified conditions. Digests were concentrated to a volume of 5.0 ml and applied to the linked Sephadex G-50, G-25 system and eluted with 0.1 M LiCl. Five ml fractions were collected and 100 µl of each assayed for its content of free amino groups relative to an L-alanine standard.

——— TCS-grown cell walls

..... Basal-grown cell walls

Data are presented in Table IX.

The peptide monomer contained only glutamic acid, glycine, alanine, and lysine in a 1:1:2:1 ratio. From the N- and C-terminal amino acid analysis of this material, it was evident that approximately half of the alanine was N-terminal and the other half was C-terminal. All the N^{ε}-amino groups of lysine were free.

To establish the amino acid sequence of the peptide monomer, the material was subjected to sequential Edman Degradation, a procedure which removes amino acids from the N-terminal end of a peptide one at a time. A 500 µl aliquot of the peptide monomer solution containing approximately 900 nmoles of peptide, was subjected to one cycle of the Edman Degradation as previously described. The peptide, shortened by one amino acid, was resuspended in 100 µl of distilled water and designated "Edman I" material. Ten µl aliquots of this material were analyzed for their total amino acid content, and their content of free N- and C-terminal amino acids. The remaining 70 µl of Edman I material was passed through yet another cycle of the process, resuspended in 50 µl of distilled water, and designated as "Edman II" material. Ten µl aliquots of this material were subjected to total and N-terminal amino acid analysis. Data are presented in Table IX.

After compensating for amino acid destruction resulting from the technique, a calculated figure of 1,180 nmoles per ml was obtained for the total alanine content of Edman I material. This figure, based upon the average percentage destruction of glutamic acid, glycine, and lysine before and after one cycle of Edman Degradation, compared favorably with the experimental value for total alanine content of 1,160 nmoles per ml.

TABLE IX

ANALYSIS OF THE M. SODONENSIS CELL WALL PEPTIDE MONOMER

SUBJECTED TO SEQUENTIAL EDMAN DEGRADATION

Material Assayed	Total amino acids†	Molar Ratio	N-terminal Groups†	C-terminal Groups†
A. Peptide Monomer				
Glutamic acid	1,840	1.03	*	*
Glycine	1,780	1.00	*	1,790
Alanine	3,640	2.04	1,790	1,850
Lysine	1,780	1.00	1,770	*
B. Edman I				
Glutamic acid	1,160	1.00	1,130	*
Glycine	1,200	1.04	*	1,190
Alanine	1,160	1.01	*	1,160
Lysine	1,160	1.00	1,150	*
C. Edman II				
Glycine	790	-	790	-

† Data are expressed as nmoles per ml of peptide monomer solution

* Below levels of detection.

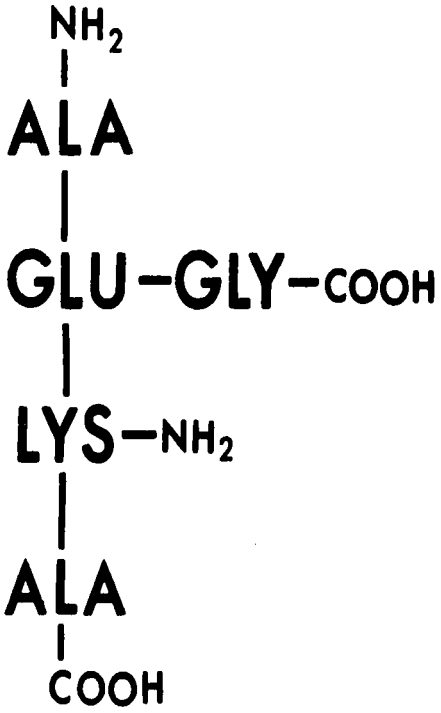

NH2
ALA
GLU-GLY-COOH
LYS-NH2
ALA
COOH

FIGURE 9

STRUCTURE OF THE PEPTIDE MONOMER OF M. SODONENSIS CELL WALLS

AS DEDUCED BY SEQUENTIAL EDMAN DEGRADATION

No N-terminal alanine could be detected in Edman I material. Glutamic acid was the N-terminal of the new peptide which now contained glutamic acid, glycine, alanine, and lysine in an approximate 1:1:1:1 ratio.

Analysis of Edman II material revealed that glycine was the only remaining amino acid with a free α-amino group. Moreover, the glycine existed as a free amino acid since values obtained by both total amino acid assay and total N-terminal amino acid assay were equivalent. The only peptide structure which is consistent with these observations is one in which a peptide linkage occurs between the α-carboxyl group of glutamic acid and the α-amino group of glycine. Since glutamic acid of Edman I material does not possess a free C-terminal, it must also be linked through its γ-carboxyl group to the α-amino group of lysine, which is the only amino acid in the Edman I peptide lacking both a free α-amino group and a free α-carboxyl group. Since the Edman Degradation follows α-peptide bonds, the structure of the monomer presented in Figure 9 is established.

III. Non-peptidoglycan Components of Cell Walls

A. Carbohydrates

1. Identification of Cell Wall Sugars: The identities of remaining cell wall sugars were established by paper and gas-liquid chromatography of the Neutral Sugar Fraction of cell wall hydrolysates. Two and one-half mg of pure cell walls were hydrolyzed in 3 N HCl at 100°C for 3 hr, evaporated to dryness, suspended in 1.0 ml of water, and the primary separation of neutral and charged sugars performed by ion exchange chromatography on Dowex-50W-X4 as previously described. The Neutral Sugar

Fraction so obtained was dried, and resuspended in 1.0 ml of water. Two-hundred µl samples of the Neutral Sugar Fraction containing about 0.5 mg of material were spotted on Whatman No. 1 filter paper along with sugar standards. Figure 10 shows a typical chromatogram which had been developed for 24 hours in the butanol:ethanol:water system. Glucuronic acid, fructose, and rhamnose were clearly separated from other monosaccharides. Galactose and glucose were both present but were never resolved in this or any other solvent systems tried.

To further confirm the identity of these five sugars, portions of the Neutral Sugar Fraction containing approximately 0.5 mg of material were subjected to gas-liquid chromatographic analysis. Conditions for formation of trimethylsilyl sugar derivatives and chromatographic separation have been previously described.

Figure 11 shows the elution profile obtained from the TMS-sugars of the Neutral Sugar Fraction. Known standards of silyated sugars were added in sequence one at a time to the Neutral Sugar Fraction and the resultant elution profiles examined for change in detector response, and alteration, if any, in retention time. In this way, Peaks 1 and 2 were confirmed as being α- and β-rhamnose respectively, Peak 3, fructose, Peaks 4 and 5, α- and β-glucose respectively, and Peak 6, glucuronic acid. Peaks 7 and 8 were trace components which corresponded to none of the standard sugars tested. Galactose could not be detected by the gas-liquid chromatographic procedures employed. Standard TMS-galactose was found to elute fractionally before glucose but its presence was masked by the large amounts of glucose present in the Neutral Sugar Fraction. Estimation employing the highly specific galactostat reagent

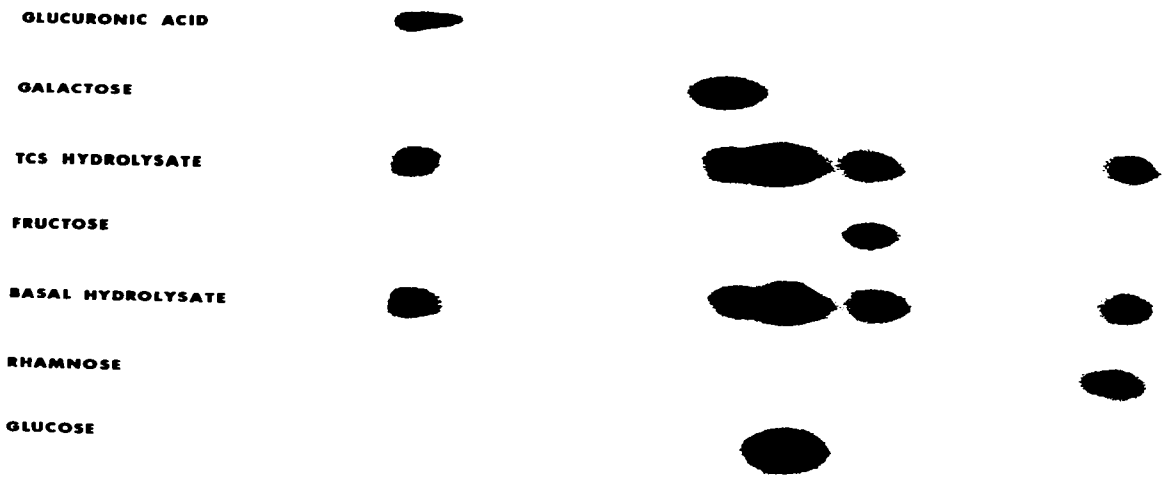

GLUCURONIC ACID
GALACTOSE
TCS HYDROLYSATE
FRUCTOSE
BASAL HYDROLYSATE
RHAMNOSE
GLUCOSE

FIGURE 10

NEUTRAL SUGAR COMPONENTS OF M. SODONENSIS CELL WALLS

Two-hundred µl of the Neutral Sugar Fraction of cell walls containing approximately 0.5 mg material were spotted and the chromatogram developed for 24 hr in the butanol: ethanol:water system. Spots were located with the alkaline $AgNO_3$ reagent.

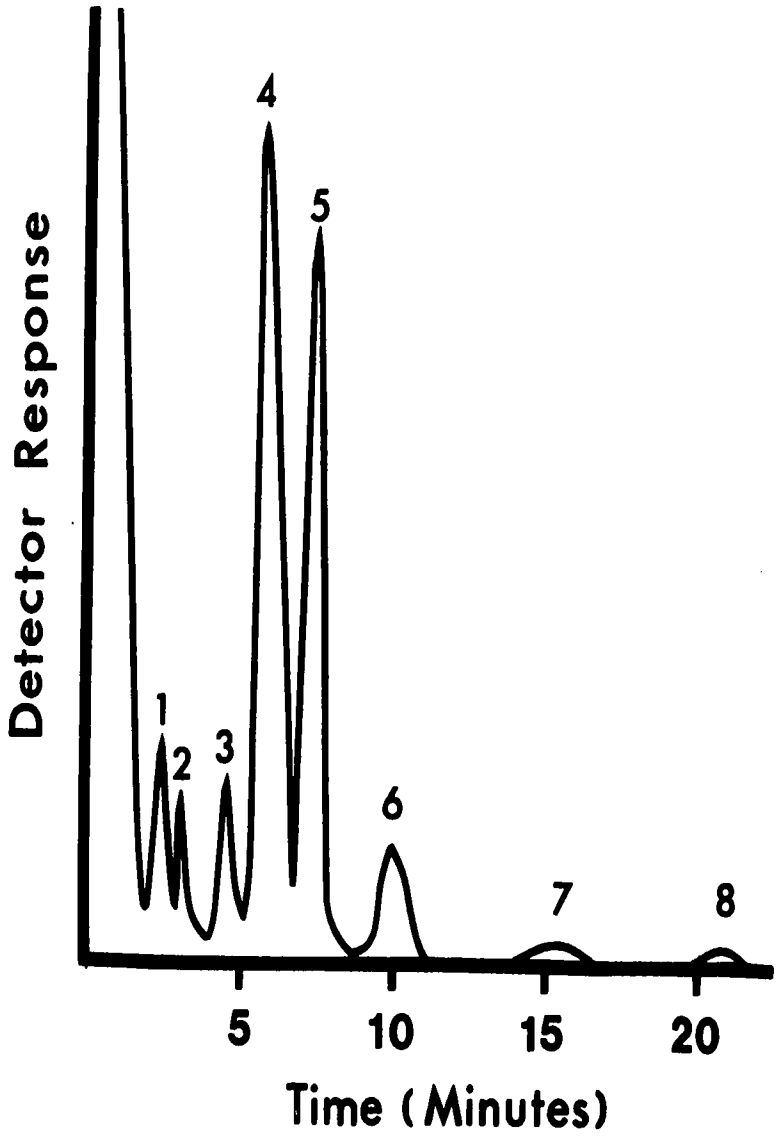

Detector Response
1
2
3
4
5
6
7
8
5
10
15
20
Time (Minutes)

FIGURE 11

GAS-LIQUID CHROMATOGRAPHY OF M. SODONENSIS CELL WALL NEUTRAL SUGARS

Dried samples of the Neutral Sugar Fraction containing approximately 0.5 mg of hydrolyzed material were silyated and chromatographed on QF_1 on 60/80 Chromosorb W as described in Materials and Methods.

Peak 1 - α-rhamnose	Peak 5 - β-glucose
Peak 2 - β-rhamnose	Peak 6 - glucuronic acid
Peak 3 - fructose	Peak 7 - unknown
Peak 4 - α-glucose	Peak 8 - unknown

has, however, confirmed the presence of galactose in the Neutral Sugar Fraction.

2. Estimation of Cell Wall Neutral Sugars: Quantification of cell wall neutral sugars was achieved by chemical or enzymic assays of whole lysozyme digests of cell walls. Table X summarizes these results. As can be seen, both cell wall preparations were qualitatively identical with respect to their neutral sugar content. However, basal-grown cell walls contained larger total amounts of every sugar component than did TCS-grown cell walls. Glucose represented the major sugar component of both cell wall preparations. While the sum of the total hexose and methyl pentose assays was equivalent to the total carbohydrate values for both preparations, only 85% and 93% of the total hexoses could be accounted for in the TCS- and basal-grown cell walls respectively. This may be a result of the techniques involved or the small quantities of unidentified trace sugars seen in gas-liquid chromatographic studies. Sialic acids were not detectable by either chromatographic or chemical techniques.

3. Analysis of Cell Wall Polysaccharides: Isolation of cell wall polysaccharides was effected by gel filtration and ion exchange chromatography. The initial step in obtaining polysaccharide material involved a 24 hour lysozyme digestion of 50 mg of pure cell walls. Lysozyme-soluble material was defined as that portion of the lysozyme digested cell walls which was not sedimented by one hour's centrifugation at 39,000 x g. Such material was fractionated by gel filtration on the previously described linked Sephadex G-50,G-25 system. Effluent fractions were analyzed for their content of free amino groups, hexosamines, glucose,

TABLE X

NON-PEPTIDOGLYCAN CARBOHYDRATES OF *M. SODONENSIS* CELL WALLS*

Component	Assay	TCS-Grown Cell Walls	Basal-Grown Cell Walls
Total Carbohydrate	Phenol-Dubois	128	180
	Anthrone	127	181
	Indole	127	178
Total Methyl Pentose	Cysteine-sulfuric acid	15	19
Total Hexose	Cysteine-sulfuric acid	112	161
Glucose	Glucostat	63	105
Galactose	Galactostat	5	7
Fructose	Cysteine-carbazole	5	7
Glucuronic acid	Carbazole	22	31

* Data are expressed as μg per mg cell wall.

and reducing groups. Carbohydrate-containing fractions were pooled, desalted by passage through 2.5 x 30 cm Bio-gel P-2 columns eluted with distilled water, and retained for further purification.

The lysozyme-soluble polysaccharide from TCS-grown cell walls was found to be free of peptidoglycan components and was designated "Polysaccharide TCS-S." However, the lysozyme-soluble polysaccharide from basal-grown cell walls still contained hexosamines and amino acids. Separation of this material from peptidoglycan components was facilitated by subsequent ion exchange chromatography on DEAE-cellulose. Figure 12 shows the separation of the lysozyme-soluble polysaccharide of basal-grown cell walls on DEAE-cellulose. This polysaccharide was designated "Polysaccharide BS."

Lysozyme-insoluble polysaccharide material was prepared from 24 hour Myxobacter AL-1 Protease digests of the lysozyme-insoluble portions of each cell wall preparation. Such polysaccharides were separated from peptidoglycan components by ion exchange chromatography on Dowex-1 as described in Methods. The polysaccharides of the lysozyme-insoluble portions of basal- and TCS-grown cell walls were found to be free of peptidoglycan components after such treatment and were designated, respectively, "Polysaccharide BI," and "Polysaccharide TCS-I."

Isolation of cell wall neutral sugars as polysaccharides free of peptidoglycan components justifiably allows their classification as non-peptidoglycan components. Moreover, it is obvious that such components are not covalently bound to the peptidoglycan of intact cell walls. Further substantiation of this fact was attained by quantification of carbohydrate material in whole wall digests and in the isolated

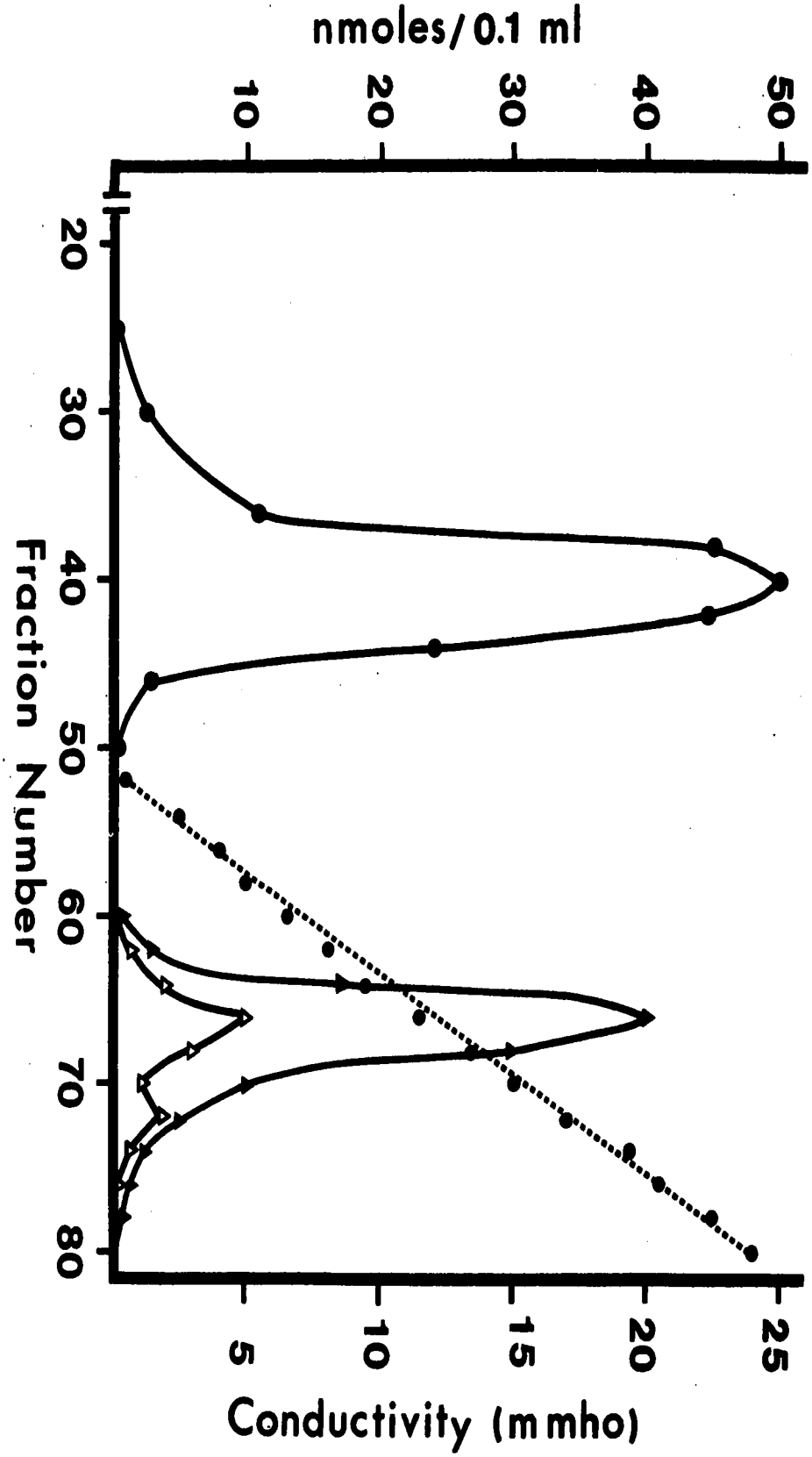
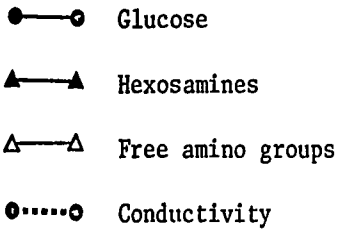

nmoles/0.1 ml
10
20
30
40
50
Fraction Number
20
30
40
50
60
70
80
5
10
15
20
25
Conductivity (mmho)

FIGURE 12

SEPARATION OF POLYSACCHARIDE BS FROM PEPTIDOGLYCAN COMPONENTS

The glucose-containing peak of the lysozyme-soluble portion of 50 mg of basal-grown cell walls was concentrated to a volume of 5.0 ml and applied to a 1 x 50 cm DEAE-cellulose column which was eluted with 0.01 M sodium carbonate-bicarbonate buffer, pH 10.0. Five ml fractions were collected. After three column volumes had been collected, a 0.5 M NaCl gradient was applied. One-hundred µl portions of each fraction were assayed for their content of glucose, hexosamines, and free amino groups. Conductivity was determined with a Type CDM 2 Bach-Simpson Conductivity Meter.



polysaccharide preparations. In every case, more than 95% of the carbohydrate material placed on ion exchange columns was accounted for in the isolated polysaccharides free of peptidoglycan components.

Identification of constituent monosaccharides and their relative proportions in Polysaccharides BS, BI, and TCS-I was achieved by gas-liquid chromatography. Samples of each polysaccharide containing approximately 0.5 mg of carbohydrate were hydrolyzed in 3 N HCl for 3 hr at 100°C, evaporated to dryness, and analyzed by gas-liquid chromatography as previously described. Relative proportions of the composite sugars were calculated from the areas under effluent peaks. Figure 14 shows the elution profile of the constitutive monosaccharides of the three polysaccharides so analyzed.

Peaks 1 and 2 corresponded, respectively to α- and β-rhamnose, Peaks 3 and 4 corresponded, respectively to α- and β-glucose, and Peak 5 corresponded to glucuronic acid. Polysaccharide BS contained only rhamnose and glucose, while Polysaccharides BI and TCS-I, both from the lysozyme-insoluble portions of cell walls, contained rhamnose, glucose, and glucuronic acid in a 1:3:2 ratio. These data are summarized in Table XI.

Mean chain length of each polysaccharide was established on the assumption that the polysaccharides were linear and not branched-chain polymers. Calculations were based upon the total carbohydrate content per ml of solution divided by the total number of reducing groups per ml of solution. These data are presented in Table XI.

Gel filtration of the individual polysaccharides on columns of Bio-gel P-30 (2 x 45 cm) established that they were single macromolecules

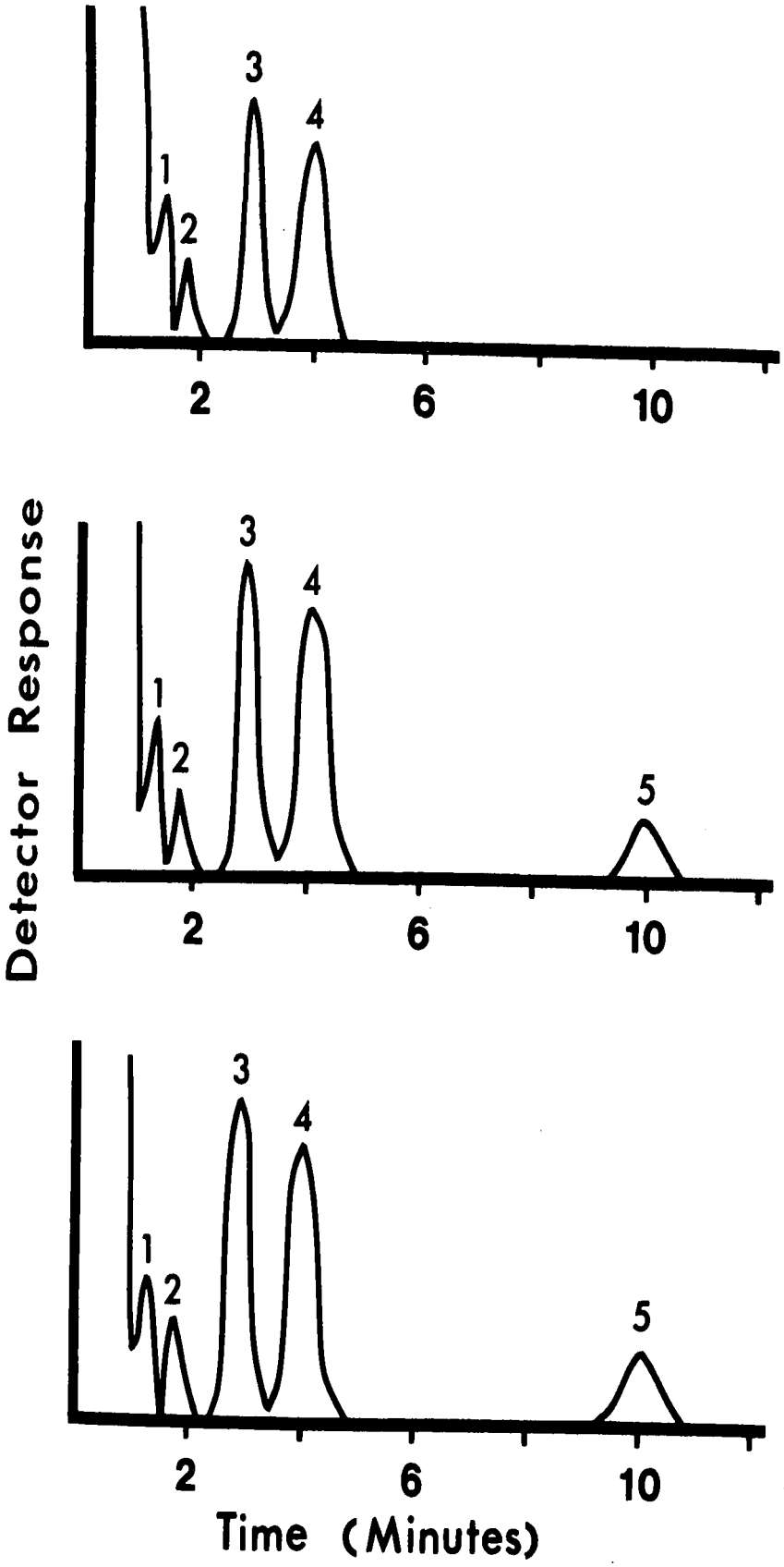

Detector Response
1
2
3
4
5
2
6
10
Time (Minutes)

FIGURE 13

MONOSACCHARIDE COMPOSITION OF M. SODONENSIS CELL WALL POLYSACCHARIDES

Samples containing approximately 0.5 mg of each polysaccharide were hydrolyzed in 3 N HCl at 100°C for 3 hr. Hydrolysates were evaporated to dryness and silyated and chromatographed on 10% QF_1 on 60/80 Chromosorb W as described in Materials and Methods.

Top profile: Monosaccharides of Polysaccharide BS

Middle profile: Monosaccharides of Polysaccharide BI

Bottom profile: Monosaccharides of Polysaccharide TCS-I

Peak 1 - α-rhamnose

Peak 2 - β-rhamnose

Peak 3 - α-glucose

Peak 4 - β-glucose

Peak 5 - glucuronic acid

TABLE XI

PROPERTIES OF M. SODONENSIS CELL WALL POLYSACCHARIDES

Polysaccharide	Components	Ratio	Amount*	Reducing Power*	Chain Length
BS	Rhamnose, glucose	1:3	4,020	86	47
TCS-S	Glucose	-	706	89	8
BI	Rhamnose, glucose glucuronic acid	1:3:2	1,110	25	44
TCS-I	Rhamnose, glucose glucuronic acid	1:3:2	830	10	83

* Data are expressed as nmoles per ml of polysaccharide solution.

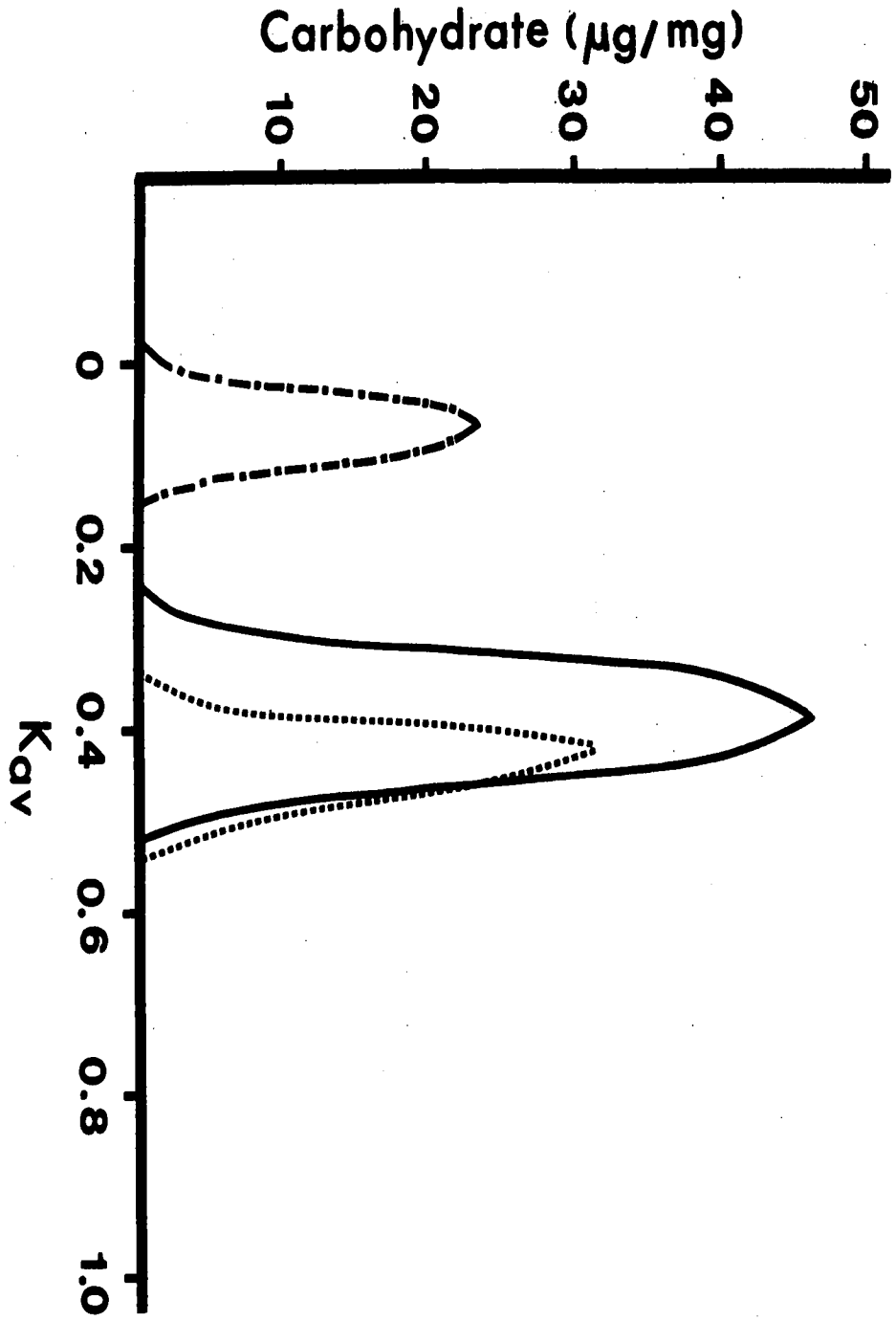
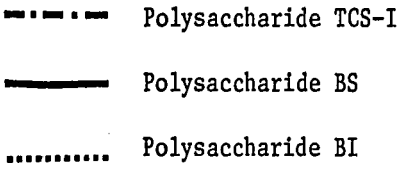

Carbohydrate (µg/mg)
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1.0
Kav

FIGURE 14

GEL FILTRATION OF M. SODONENSIS CELL WALL POLYSACCHARIDES BS, BI, AND TCS-I ON BIO-GEL P-30

Isolated polysaccharide preparations in a final volume of 1.0 ml were applied in sequence to a Bio-gel P-30 column having a V_o of 85 ml and a V_t of 225 ml. Effluent fractions were assayed for their carbohydrate content by the phenol-Dubois technique relative to a glucose standard.



rather than collections of small heterogeneous fragments. Figure 14 shows the elution profiles of the polysaccharides on Bio-gel P-30 columns.

B. Protein: It has been noted that cell walls contain demonstrable monoesterase and diesterase activities during all but the last stage of purification and that serological cross-reaction between purified M. sodonensis extracellular nuclease and rabbit anti-cell wall serum occurs (Berry et al., 1970). Bearing these points in mind, attempts were made to estimate the amount of protein present in cell walls.

1. Trypsin Digestion of Cell Walls: Trypsin-mediated release of protein (peptides) from pre-trypsin cell walls was demonstrable. Pre-trypsin cell walls were digested with bovine pancreatic trypsin under previously specified conditions. Samples were withdrawn from digests at designated intervals, centrifuged to sediment insoluble material, and assayed for Lowry-positive materials and for hexosamines by the modified Morgen-Elson reaction. Parallel reaction mixtures containing trypsin but no cell walls, and cell walls but no trypsin were analyzed simultaneously. Minimum values for the release of protein were obtained by subtraction of the sum of the values of the negative controls from test values. Release of Lowry-positive material from pre-trypsin cell walls as a function of time is presented in Figure 15.

At no time were hexosamines detected in the supernatants of trypsin digests. Thus, the release of protein was real and did not represent peptidoglycan net disintegration. Basal-grown cell walls released significantly more protein than did TCS-grown cell walls.

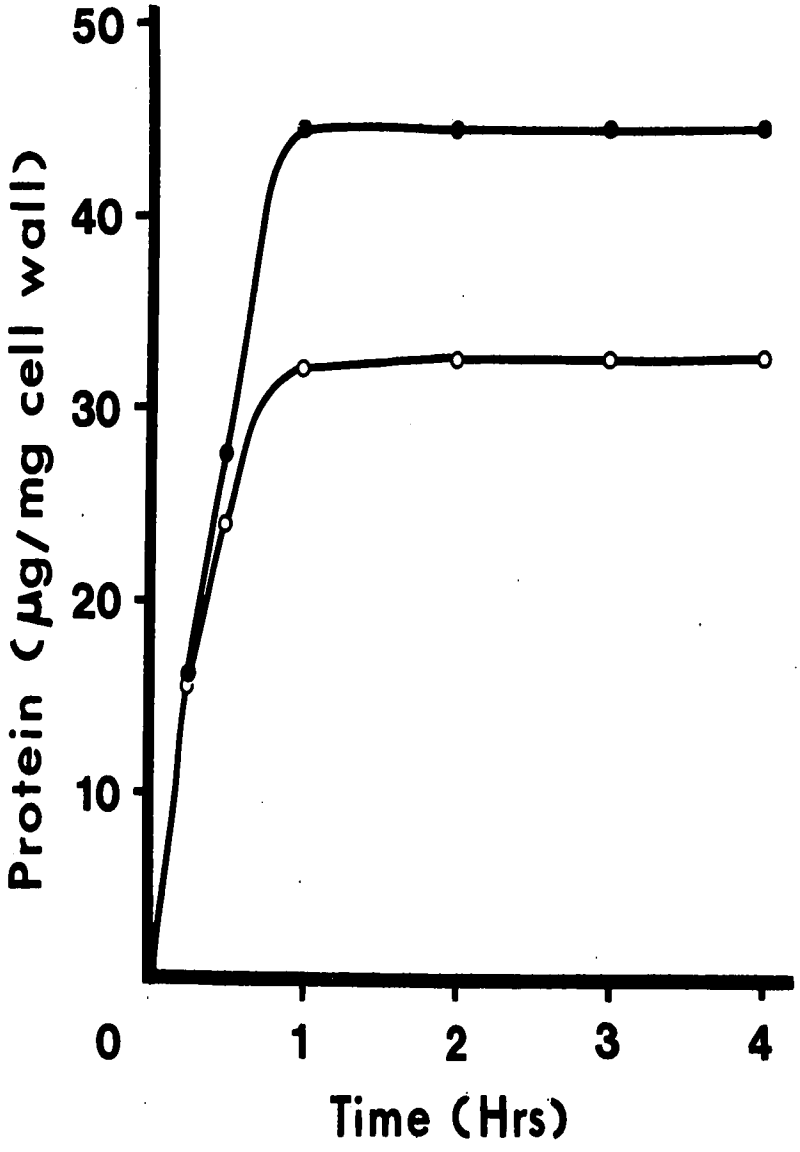

50
40
30
20
10
0
1
2
3
4
Protein (µg/mg cell wall)
Time (Hrs)

FIGURE 15

RELEASE OF M. SODONENSIS CELL WALL PROTEIN BY TRYPSIN DIGESTION

Five mg pre-trypsin cell walls were digested at 37°C with bovine pancreatic trypsin under standard specified conditions. The final volume of each digest was 2.0 ml. At intervals, 250 µl samples were removed, centrifuged to sediment insoluble material, and portions of the supernatant fractions assayed for their content of protein as described in the text.

●——● Protein released from basal-grown cell walls

○——○ Protein released from TCS-grown cell walls

2. Alkali Digestion of Cell Walls: Alkali-mediated release of cell wall protein was employed to estimate the amount of protein bound to cell walls since the enzyme experiment yielded only a minimum protein value and was subject to error introduced by trypsin autocatalysis. Five mg of each pre-trypsin cell wall preparation were incubated in 0.5 N KOH at 37°C. Samples containing 200 µl of digest were removed at intervals, and centrifuged for 5 minutes in a Beckman Microfuge to sediment insoluble material. Assays for protein, hexosamines, and free amino groups were then performed on 100 µl, 25 µl, and 40 µl portions respectively, of the supernatant fraction. Since alkali is known to catalyze lactyl-peptide elimination from N-acetylmuramic acid of glycan strands (Tipper, 1968) thereby giving rise to materials reactive in the Lowry protein assay, a parallel reaction mixture of post-trypsin cell walls was prepared in 0.5 N KOH and simultaneously analyzed. The amount of protein in cell walls was calculated by subtraction of the amount of Lowry-positive material released in post-trypsin cell walls by alkali digestion from the amount of such material in alkali digests of pre-trypsin cell walls.

Up to 5 hours, no soluble Lowry-positive materials were detected in the post-trypsin cell wall control. Samples withdrawn from both test and control digests after this time contained hexosamines and free amino groups, indicating that peptidoglycan components were being solubilized. However, the calculated protein content was the same at 5 hours as it had been at 3 hours. Therefore, protein release from cell walls was essentially complete before the initiation of peptidoglycan solubilization. The final values of 38 µg per mg and 53 µg per mg protein in

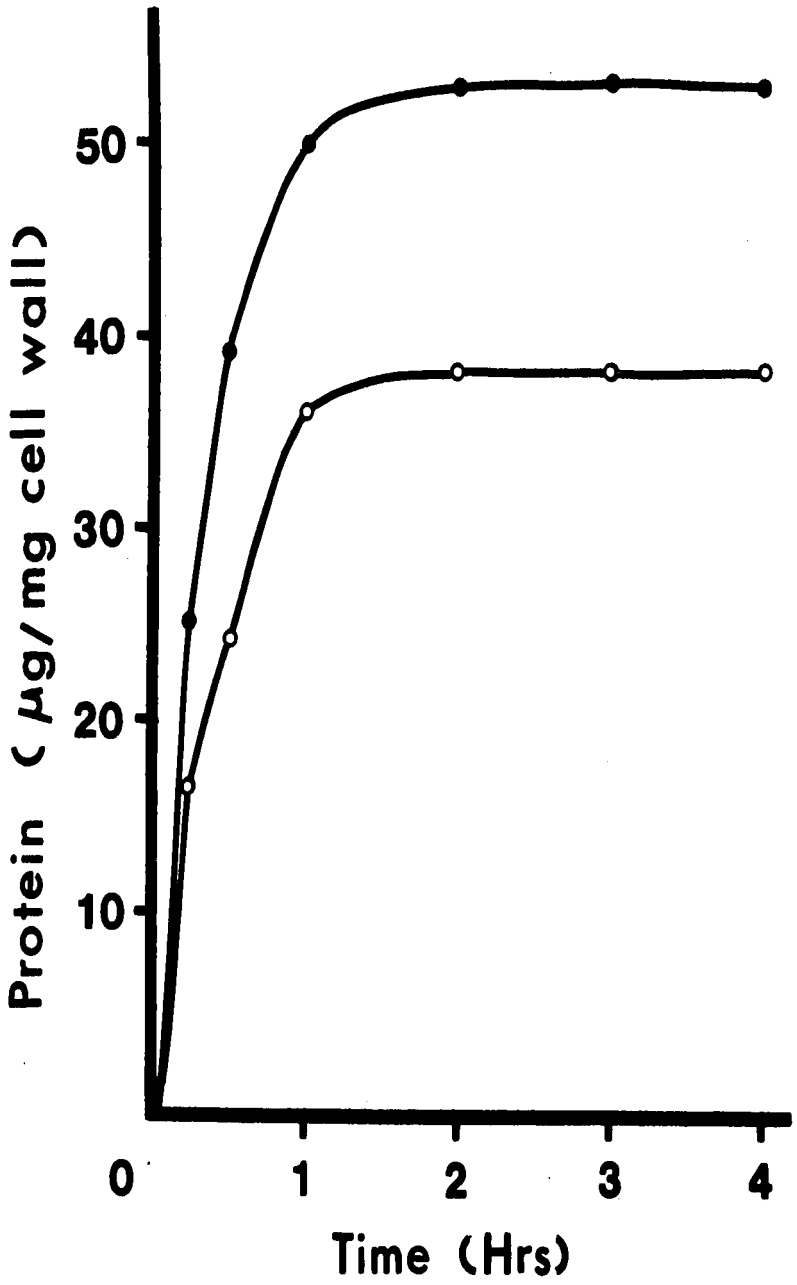

Protein (µg/mg cell wall)
50
40
30
20
10
0
1
2
3
4
Time (Hrs)

FIGURE 16

RELEASE OF M. SODONENSIS CELL WALL PROTEIN BY ALKALI DIGESTION

Five mg of pre-trypsin cell walls were incubated in 0.5 N NaOH at 37°C, and 250 µl aliquots of the 2.0 ml hydrolysis mixture withdrawn at intervals. These were centrifuged to sediment insoluble material, and the supernatant fractions assayed for their content of protein as described in the text.

O—O Protein release from TCS-grown cell walls

O—O Protein release from basal-grown cell walls

TCS- and basal-grown cell walls respectively are slightly higher than values obtained from trypsin digestion data.

C. A Flavin-like Substance Found in the Cell Walls

During preparation of the lysozyme-soluble and -insoluble portions of basal- and TCS-grown cell walls, a slight yellow coloured material was observed at the bottom of lysozyme-insoluble pellets. Lysozyme-insoluble material was carefully removed by scraping to leave the yellow material. The material was extracted into 95% ethanol, evaporated to dryness, and resuspended in 2.0 ml of 95% ethanol. The absorbance spectra of these preparations were determined in a DBG-Spectrophotometer. A standard solution containing 50 µg of riboflavin was likewise scanned from 900 nm to 200 nm.

The absorption spectra of material extracted from both cell wall preparations were identical. Tracings of absorbance versus wavelength revealed that the unknown yellow, ethanol-soluble material had absorption maxima at 340 nm, 260 nm, and 215 nm. Riboflavin had absorption maxima at 340 nm (the typical absorption region), 270 nm, and 215 nm. Such absorption maxima are typical of flavin compounds. Since this flavin-like substance is present in minute amounts, it would have been impractical to prepare in sufficient quantity for a complete analysis.

IV. Studies on the Controlled Enzymic Degradation of Cell Walls

A. Lysozyme Activity on Cell Walls

1. Kinetics of Lysozyme Digestion: Five mg of each cell wall preparation were digested with lysozyme under the standard specified conditions.

The final volume of each digestion mixture was 2.0 ml. At designated intervals, 50 µl aliquots were removed, and centrifuged for 5 minutes in a Beckman Microfuge to sediment insoluble material. Ten or 20 µl portions of the resultant supernatants were assayed for reducing groups as described in Methods. Pellets were resuspended in 400 µl of distilled water and turbidity measured as absorbance at 550 nm. One-hundred percent clarification was based on the terminal turbidity of the digest which exhibited the greatest degree of clarification. All other values were expressed relative to this figure.

Clarification of digests and lysozyme-mediated release of reducing groups as a function of time is presented in Figure 17. Response of the two cell wall preparations to lysozyme was markedly different. Basal-grown cell walls exhibited rapid clarification accompanied by a concomitant release of reducing groups. Lysozyme-digestion of TCS-grown cell walls, on the other hand, produced only about 10% clarification and released less than half as many reducing groups as were observed in basal-grown cell walls. These findings suggest that TCS-grown cell walls contain fewer lysozyme-sensitive linkages or that such bonds are protected from enzymic hydrolysis in some manner.

Neither cell wall preparation was ever completely solubilized by lysozyme digestion. The fact that digestion was complete, and that it stopped because of unavailability of substrate and not because of product inhibition (Johnson and Phillips, 1965), was established by the fact that addition of fresh lysozyme to digestion mixtures or resuspension of lysozyme-insoluble material in fresh enzyme did not increase the amount of reducing groups liberated per mg cell wall.

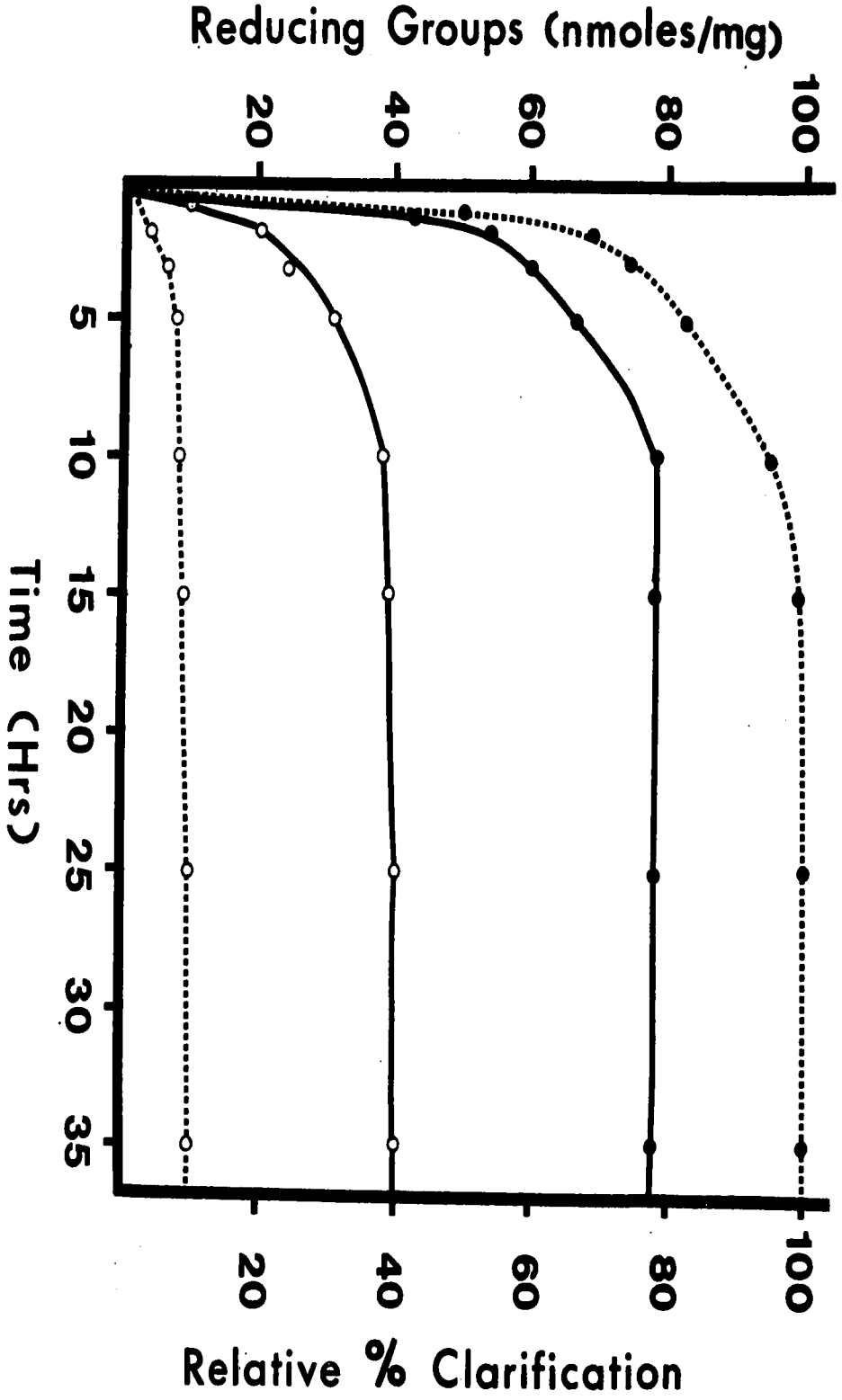

Reducing Groups (nmoles/mg)
20
40
60
80
100
Time (Hrs)
5
10
15
20
25
30
35
20
40
60
80
100
Relative % Clarification

FIGURE 17

KINETICS OF LYSOZYME DIGESTION OF M. SODONENSIS CELL WALLS

Five mg of each cell wall preparation were digested at 37°C with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At intervals, 50 µl aliquots were withdrawn, centrifuged to sediment insoluble material, and 10 or 20 µl portions of the supernatant fractions assayed for their content of reducing groups relative to a glucosamine standard. The pellet was resuspended in 400 µl of distilled water and the turbidity at 550 nm determined.

O——O Reducing groups liberated in TCS-grown cell walls

O——O Reducing groups liberated in basal-grown cell walls

O·····O Relative % clarification of TCS-grown cell wall digests

O·····O Relative % clarification of basal-grown cell wall digests

2. Electron Microscopic Examination of Lysozyme Digests: Conditions for the staining, fixation, and thin-sectioning of specimens have been previously described. Plate II shows an electron photomicrograph of a preparation of basal-grown cell walls which was lysozyme digested until there was no detectable increase in reducing power. The material represents, therefore, the lysozyme-insoluble portions of cell walls. The matrix of the wall was perceptibly more diffuse than that seen in the whole, undigested cell wall. However, structural integrity of the walls was maintained even though 75% of the wall had been solubilized. Plate III shows an electron photomicrograph of the same preparation in thin-section. The wall was found to have a thickness of 920 Å, only slightly less than the thickness of the intact cell wall which has been calculated from similar thin-sections to be 980 Å.

3. Distribution of Lysozyme-soluble and -Insoluble Components: Five mg of each wall preparation were digested for 24 hours with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. The following triplicate samples were removed for chemical assay: 200 µl for total ester assay, 50 µl for total hexosamines assay, 50 µl for total glucose assay, 50 µl for total amino acid assay, and 25 or 50 µl for total glucosamine assay. Parallel digests were centrifuged at 39,000 x g for one hour to yield the lysozyme-soluble and -insoluble portions. The lysozyme-soluble material was carefully decanted into a fresh container. The same quantities of this material were subjected to the assays given above. The lysozyme-insoluble material was resuspended in a final volume of 1.0 ml with distilled water. Again, the same quantities of this material were assayed for total esters, amino acids,

PLATE II

ELECTRON PHOTOMICROGRAPH OF THE LYSOZYME-INSOLUBLE PORTION OF BASAL-GROWN M. SODONENSIS CELL WALLS

Basal-grown cell walls were digested with lysozyme under standard specified conditions until addition of fresh lysozyme produced no increase in the release of reducing groups. The preparation was negative-contrast stained with 3% phosphotungstic acid.

Magnification = 36,600

PLATE III

ELECTRON PHOTOMICROGRAPH OF THE LYSOZYME-INSOLUBLE PORTION OF BASAL-GROWN M. SODONENSIS CELL WALLS IN THIN-SECTION

The lysozyme-insoluble portion of basal-grown cell walls was obtained as previously described. Procedures for fixation, embedding, thin-sectioning, and staining are given in Materials and Methods.

Magnification = 64,800 x

hexosamines, glucose, and glucosamine as previously described.

Data presented in Tables XII and XIII indicate that gross cell wall composition varies under altered growth conditions. Intact cell walls of the TCS-grown preparation contained more esters, fewer hexosamines, and less glucose than preparations of basal-grown cell walls. Attention is directed to the excellent agreement between the total amount of each component and the sum of components in the respective lysozyme-soluble and -insoluble portion of each wall preparation. On the basis of hexosamine content, TCS-grown cell walls were 49% solubilized by lysozyme and basal-grown cell walls were 75% solubilized by lysozyme digestion.

In the TCS-grown cell walls, the most insensitive to lysozyme, the ester:muramic acid ratio of the insoluble portion was double that found in the analogous portion of basal-grown cell walls. These findings suggest that esterification in the form of O-acetylation is a key factor in the diminished response of these cell walls to lysozyme. O-acetylation of cell walls is the subject of subsequent discussion.

These data indicate that the distribution of peptidoglycan components into their lysozyme-soluble and -insoluble cell wall portions also varies under differing growth conditions. Although a 1:1 ratio of muramic acid to glucosamine and a 1:1:2:1 ratio of glutamic acid, glycine, alanine, and lysine were preserved in intact cell walls and their respective lysozyme-soluble and -insoluble portions, in TCS-grown cell walls, the lysozyme-insoluble portion contains 87% of the total cell wall amino acids, slightly more than twice the amino acid content of the analogous portion of basal-grown cell walls.

TABLE XII

DISTRIBUTION OF TCS-GROWN M. SODONENSIS CELL WALL COMPONENTS IN LYSOZYME DIGESTS*

Component	Whole Cell Walls	Lysozyme-soluble portion	Lysozyme insoluble-portion
Total Hexosamines	567	273	295
Muramic acid	289	137	151
Glucosamine	278	136	144
Glutamic acid	514	63	458
Glycine	523	64	451
Alanine	1,070	123	913
Lysine	514	63	452
Glucose	350	134	214
Esters	470	116	354
Esters:Muramic acid	1.62	0.85	2.34
Alanine:Muramic acid	3.70	0.90	6.05

* Data are expressed as nmoles per mg cell wall.

TABLE XIII

DISTRIBUTION OF BASAL-GROWN M.SODONENSIS CELL WALL COMPONENTS IN LYSOZYME DIGESTS*

Component	Whole Cell Walls	Lysozyme-soluble-portion	Lysozyme-insoluble-portion
Total Hexosamines	1,070	796	271
Muramic acid	528	397	136
Glucosamine	540	400	137
Glutamic acid	546	300	240
Glycine	531	305	230
Alanine	1,060	590	450
Lysine	530	300	330
Glucose	580	460	125
Esters	338	175	160
Esters:Muramic acid	0.64	0.46	1.19
Alanine:Muramic acid	2.01	1.49	3.29

* Data are expressed as nmoles per mg cell wall.

4. Fractionation of Lysozyme Digests: The lysozyme-soluble portion of 100 mg of cell walls was fractionated by gel filtration on the Sephadex G-50,G-25 system described in Methods. Five ml fractions were collected and 100 µl samples of these assayed for free amino groups, hexosamines, and glucose. Figure 18 shows a typical elution profile of the lysozyme-soluble portion of cell walls.

There was little correspondance between the soluble products of lysozyme digestion of the two cell wall preparations. Only one peak of the lysozyme-soluble portion of TCS-grown cell walls contained peptidoglycan components. This material, which was excluded from the system, contained 13% of the total cell wall amino acids, and 49% of the total cell wall hexosamines. Glucose, which represented only 38% of the total cell wall glucose, consisted of low molecular weight fragments having a K_{av} value of 0.9. These glucose fragments were free of peptidoglycan components. In contrast, lysozyme-soluble peptidoglycan components of basal-grown cell walls fractionated into three main peaks. These soluble products of lysozyme digestion contained 75% and 56% respectively, of the total cell wall hexosamines and amino acids. In this case, the glucose, which was excluded from the system along with peptidoglycan components, accounted for 79% of the total cell wall glucose. Previous studies of this high molecular weight glucose-containing material have shown that it is not covalently associated with the peptidoglycan.

B. The Degree and Nature of Glycan Peptide Substitution

1. Streptomyces N-acetylmuramic acid-L-alanine Amidase Digestions: To obtain some idea of the degree and nature of peptide substitution of

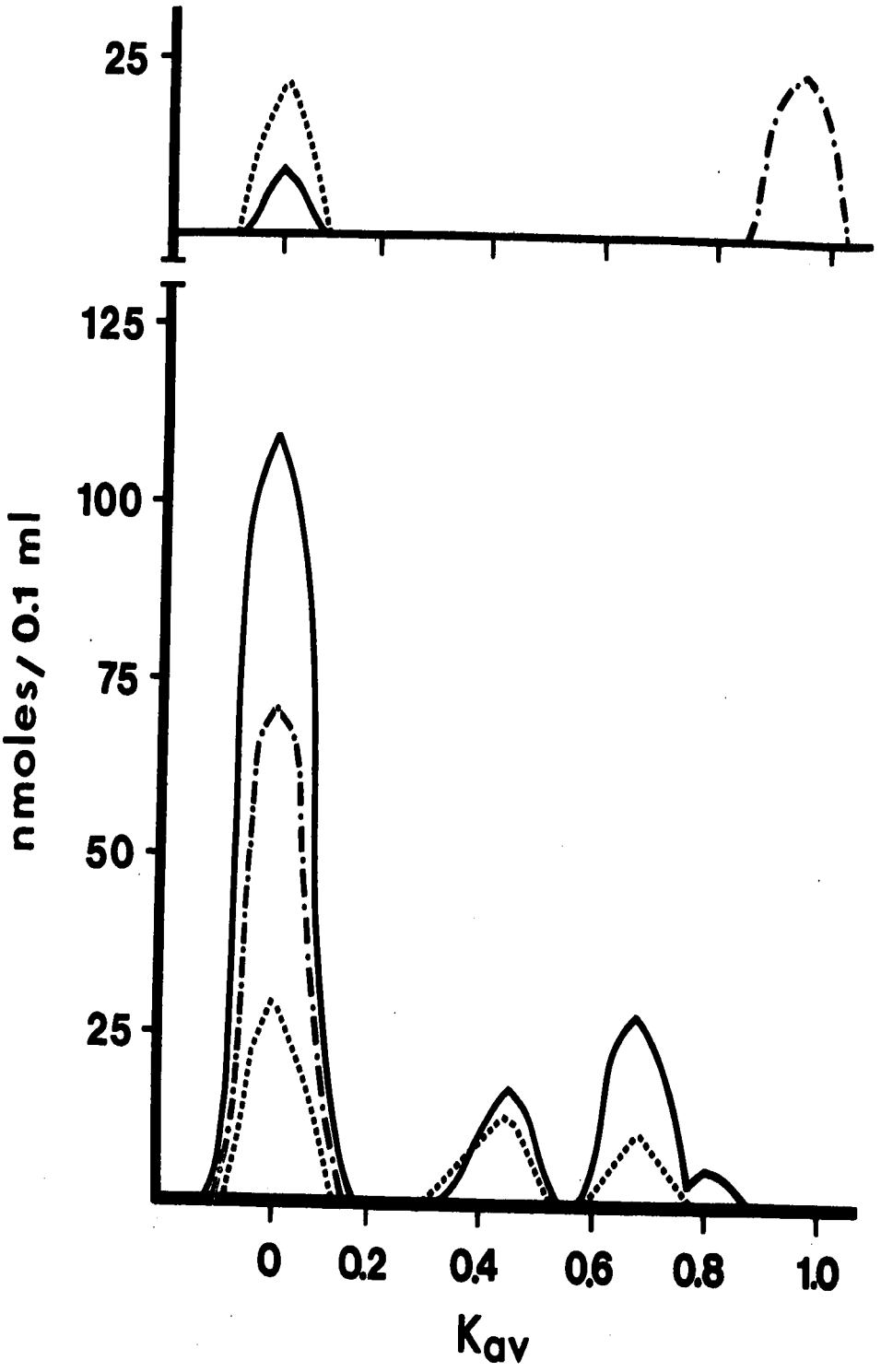

25
125
100
75
50
25
nmoles/0.1 ml
0
0.2
0.4
0.6
0.8
1.0
Kav

FIGURE 18

FRACTIONATION OF THE LYSOZYME-SOLUBLE PORTION OF M. SODONENSIS CELL WALLS

The lysozyme-soluble portion obtained from 100 mg of cell walls was concentrated to a volume of 5.0 ml and applied to the linked Sephadex G-50, G-25 system and eluted with 0.1 M LiCl. Five ml fractions were collected. The content of glucose, hexosamines, and free amino groups in 0.1 ml portions of each fraction was determined as described in Materials and Methods.

Top profile: lysozyme-soluble portion of TCS-grown cell walls

Bottom profile: lysozyme-soluble portion of basal-grown cell walls

Glucose

Free amino groups

Hexosamines

the glycan, the lysozyme-soluble and -insoluble portions from 5 mg of each cell wall preparation were digested with the Streptomyces N-acetylmuramic acid-L-alanine amidase under previously specified conditions. The final volume of each reaction mixture was 2.0 ml. After 24 hours digestion at 37°C, triplicate 100 µl aliquots of each digest were withdrawn and assayed for their content of free N-terminal alanine groups. Results are presented in Table XIV.

This amidasic activity releases only alanine which is covalently bound to N-acetylmuramic acid. The lysozyme-insoluble portions of both cell wall preparations contained significantly more muramic acid-linked alanine than did the lysozyme-soluble portions. Glycans of these portions are, therefore, more highly peptide-substituted than their corresponding lysozyme-soluble portions. On the average, approximately three muramic acid residues in five are peptide substituted in the TCS-grown lysozyme-insoluble portion while about two in five are so substituted in the analogous fraction of basal-grown cell walls.

A greater difference in degree of peptide substitution was observed in the lysozyme-soluble portions of the two preparations: in the soluble portion of TCS-grown cell walls about one in five muramic acid residues are peptide substituted while only one in twenty are so substituted in the lysozyme-soluble portion of basal-grown cell walls. Significantly, both portions of the TCS-grown cell wall, which is the least sensitive to lysozyme, are more highly peptide-substituted than the corresponding basal-grown cell wall fractions. The correlation between degree of peptide substitution of the glycan and lysozyme-sensitivity will be discussed later.

Amidase digestion of these walls was deemed complete in that the total release of N-terminal alanine groups, approximately 117 nmoles per mg in TCS-grown cell walls, and 70 nmoles per mg in basal-grown cell walls, was identical to the values obtained by Myxobacter AL-1 Protease digestion of whole cell walls.

Calculations of the amount of muramic acid-linked L-alanine were based on the assumption that L-alanine constitutes one-half of the total cell wall alanine content (see Discussion). The data presented in Table XIV indicate that relatively low amounts of the total L-alanine were released by amidase digestion. Residual L-alanine must, therefore, be involved in peptide cross-bridging. In the lysozyme-insoluble portion of TCS-grown cell walls, about 80% of the L-alanine was not released by amidase digestion. Such a distribution of L-alanine is consistent with peptide bridges composed, on the average, of four peptide subunits. In the corresponding portion of basal-grown cell walls, approximately 75% of the L-alanine residues are not released by amidase digestion suggesting that the cross-bridges are somewhat smaller, likely only three peptide subunits in length, on the average. About 60% of the L-alanine residues of the lysozyme-soluble portion of TCS-grown cell walls are likely involved in peptide cross-bridging. Such a pattern would result in peptide cross-bridges two to three subunits in length. That approximately 95% of the total L-alanine content of the lysozyme-soluble portion of basal-grown cell walls was not involved in N-acetylmuramyl-L-alanine linkages suggests that very long cross-bridges, 18 to 19 subunits in length might occur. Since this portion constitutes 75% of the whole cell wall, the peptidoglycan matrix of basal-grown cell walls is a much looser network

TABLE XIV

PEPTIDE SUBSTITUTION IN THE GLYCANS OF LYSOZYME-INSOLUBLE AND -SOLUBLE PORTIONS OF M. SODONENSIS CELL WALLS*

	Lysozyme-soluble portion		Lysozyme-insoluble portion	
	TCS-Grown	Basal-Grown	TCS-Grown	Basal-Grown
Amidase-released N-terminal alanine groups (A)	27	18	88	52
Total Muramic acid (B)	137	397	151	136
% Muramic acid peptide-substituted (A/B x 100)	20.0%	4.6%	58.4%	38.0%
Total L-alanine (C)	62	295	457	224
% Muramic acid-linked L-alanine (A/C x 100)	43.6%	6.1%	19.3%	23.1%

* Data are expressed as nmoles per mg original cell wall.

than that found in TCS-grown cell walls. Differences in the degree of peptide substitution and nature of cross-bridging are consistent with the observed response of the two wall preparations to lysozyme digestion.

2. Myxobacter AL-1 Protease Activity on Pre-solubilized Cell Walls:

To determine if the L-alanine not present in N-acetylmuramic acid-L-alanine linkages occurred in D-alanyl-L-alanine bonds, the foregoing amidase digests were subjected to digestion with Myxobacter AL-1 Protease. The amidase digests were adjusted to pH 9.0 with NaOH, and 10 µg of Myxobacter AL-1 Protease and sufficient 0.01 M veronal-HCl buffer, pH 9.0, added to yield a final volume of 3.0 ml. Reaction mixtures were incubated at 37°C for 24 hours after which time triplicate 100 µl aliquots were withdrawn and assayed for their content of N-terminal alanine residues. Two-hundred µl aliquots of the reaction mixtures were assayed for their C-terminal alanine content before and after Myxobacter Protease digestion. Data are presented in Table XV.

Although previous studies of Myxobacter AL-1 Protease digests of whole cell walls indicated that few D-alanyl-L-alanine bonds were cleaved, this was not the case with preparations which had been pre-treated with lysozyme and the amidase. In every case, the amount of free N-terminal alanine groups found in cell wall fractions sequentially digested with lysozyme, the amidase, and Myxobacter AL-1 Protease, accounted for more than 94% of the total L-alanine content. Since such preparations had been pre-digested with the amidase, release of N-terminal alanine by Myxobacter AL-1 Protease represents cleavage of D-alanyl-L-alanine bonds. This is confirmed by the observation that the ratios of C- and N-terminal alanine released by digestion were 1:1 in every case. Moreover, these

TABLE XV

ENZYMIC RELEASE OF N- AND C-TERMINAL ALANINE IN PRE-DIGESTED *M. SODONENSIS* CELL WALLS*

	Lysozyme-soluble portion		Lysozyme-insoluble portion	
	TCS-Grown	Basal-Grown	TCS-Grown	Basal-Grown
Total N-terminal alanine released by Digestion 1 (A)	58	290	450	220
% of total L-alanine	94%	98%	98%	98%
Total N-terminal alanine released by Digestion 2 (B)	27	18	88	52
N-terminal alanine released by hydrolysis of D-alanyl-L-alanine linkages (A - B)	31	272	362	168
C-terminal alanine released by Digestion 1 (C)†	34	285	381	183
Ratio of C- to N-terminal alanine in D-alanyl-L-alanine linkages [C/(A - B)]	1.13	1.05	1.05	1.09

Digestion 1- Sequential digestion of the cell wall fractions with *Streptomyces* N-acetylmuramic acid-L-alanine amidase followed by digestion with *Myxobacter* AL-1 Protease.
Digestion 2- Digestion with *Streptomyces* N-acetylmuramic acid-L-alanine amidase only.
* Data are expressed as nmoles per mg original cell wall.
† Release of C-terminal alanine groups above control level of free C-terminal alanine.

findings strongly support the previous assumption that one half of the total cell wall alanine is present as the D-isomer and the other half occurs as the L-isomer.

3. Streptomyces ML-endopeptidase Activity on Cell Walls: Kinetics of ML-endopeptidase digestion of the two wall preparations were determined. Five mg of each wall preparation were digested with the ML-endopeptidase under standard specified conditions. The final volume of digestion mixtures was 2.0 ml. Reduction in turbidity of the digests with time was measured at 550 nm. One-hundred percent clarification was defined as the terminal turbidity of the reaction mixture which exhibited the greatest degree of clarification. Terminal optical densities of the digests were 0.468 and 0.353 for TCS- and basal-grown cell wall digests respectively. Relative % clarification as a function of time is presented in Figure 19.

Streptomyces ML-endopeptidase digests of basal-grown cell walls were 100% clarified in 45 to 50 hours. TCS-grown cell walls achieved only 82% clarification relative to the basal-grown cell walls, and digestion was complete after 60 hours. A suspension of M. lysodeikticus prepared under identical conditions was 100% clarified by 22 hours.

Estimation of N^{ε}-(D-alanyl)-L-lysine linkages in purified basal- and TCS-grown cell wall preparations was performed by assaying free C-terminal alanine groups released by ML-endopeptidase. A digestion mixture containing 2.5 mg per ml cell walls was prepared and digested at 37°C for 72 hours under previously specified conditions. Duplicate 500 µl aliquots were withdrawn and assayed for their content of C-terminal alanine groups. Separate digests containing 2.5 mg per ml cell walls

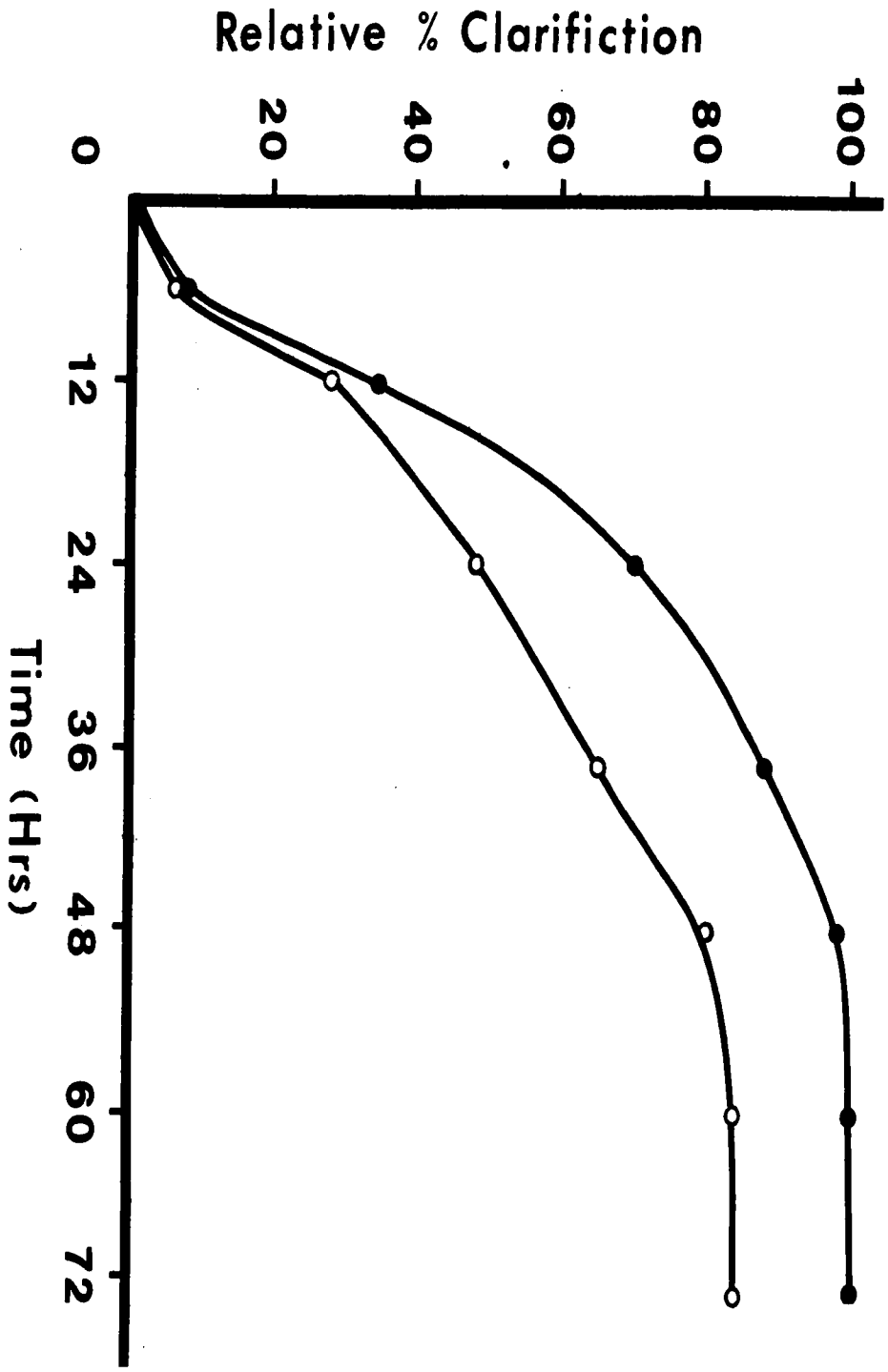

Relative % Clarifiction
0
20
40
60
80
100
Time (Hrs)
12
24
36
48
60
72

FIGURE 19

KINETICS OF *STREPTOMYCES* ML-ENDOPEPTIDASE DIGESTION OF *M. SODONENSIS* CELL WALLS

Five mg of each wall preparation were digested with the *Streptomyces* ML-endopeptidase under standard specified conditions. The final volume of each digest was 2.0 ml. Reduction in turbidity of the digests with time was measured at 550 nm.

O——O Relative % clarification of TCS-grown cell wall digests

O——O Relative % clarification of basal-grown cell wall digests

were prepared and digested for 24 hours with lysozyme under standard conditions. Five-hundred µl portions of such digests were analyzed for their content of free C-terminal alanine groups. Data are presented in Table XVI.

N^{ε}-(D-alanyl)-L-lysine linkages occur in both cell walls albeit they are relatively few in number. TCS-grown cell walls contain 41 such linkages per mg while basal-grown cell walls contain only 34 per mg cell wall. The presence of N^{ε}-(D-alanyl)-L-lysine bonds validates the hypothesis that the "head-to-tail" assembly of peptide subunits to form cross-bridges found in M. lysodeikticus cell walls is also present in M. sodonensis cell walls.

4. Sequential Enzymic Degradation of Cell Walls: Peptide cross-bridge fragments were obtained by sequential digestion of 100 mg of each cell wall preparation with ML-endopeptidase for 60 hours, lysozyme for 36 hours, and finally N-acetylmuramic acid-L-alanine amidase for 24 hours. The previously specified conditions of digestion were employed at each stage. Digests were concentrated to a volume of 5.0 ml and fractionated by gel filtration on the linked Sephadex G-50, G-25 system. Effluent fractions were assayed for their content of free amino groups. Elution profiles of TCS- and basal-grown cell walls so digested are presented in Figures 20 and 21 respectively.

Amino groups of TCS-grown cell walls fractionated into four main peaks having K_{av} values of 0.045 (Peak 1), 0.34 (Peak 2), 0.605 (Peak 3), and 0.75 (Peak 4). The elution profile of basal-grown cell walls subjected to this sequential digestion was similar. Free amino groups fractionated into three peaks having K_{av} values of 0 (excluded)(Peak 1),

TABLE XVI

ESTIMATION OF N^{ε}-(D-ALANYL)-L-LYSINE LINKAGES*

IN M. SODONENSIS CELL WALLS

	TCS-Grown Cell Walls	Basal-Grown Cell Walls
Total C-terminal alanine in ML-endopeptidase digests (A)	129	64
C-terminal alanine in intact cell walls (B)	88	30
Total N^{ε}-(D-alanyl)-L-lysine linkages (A - B)	41	34

* Data are expressed as nmoles per mg cell wall.

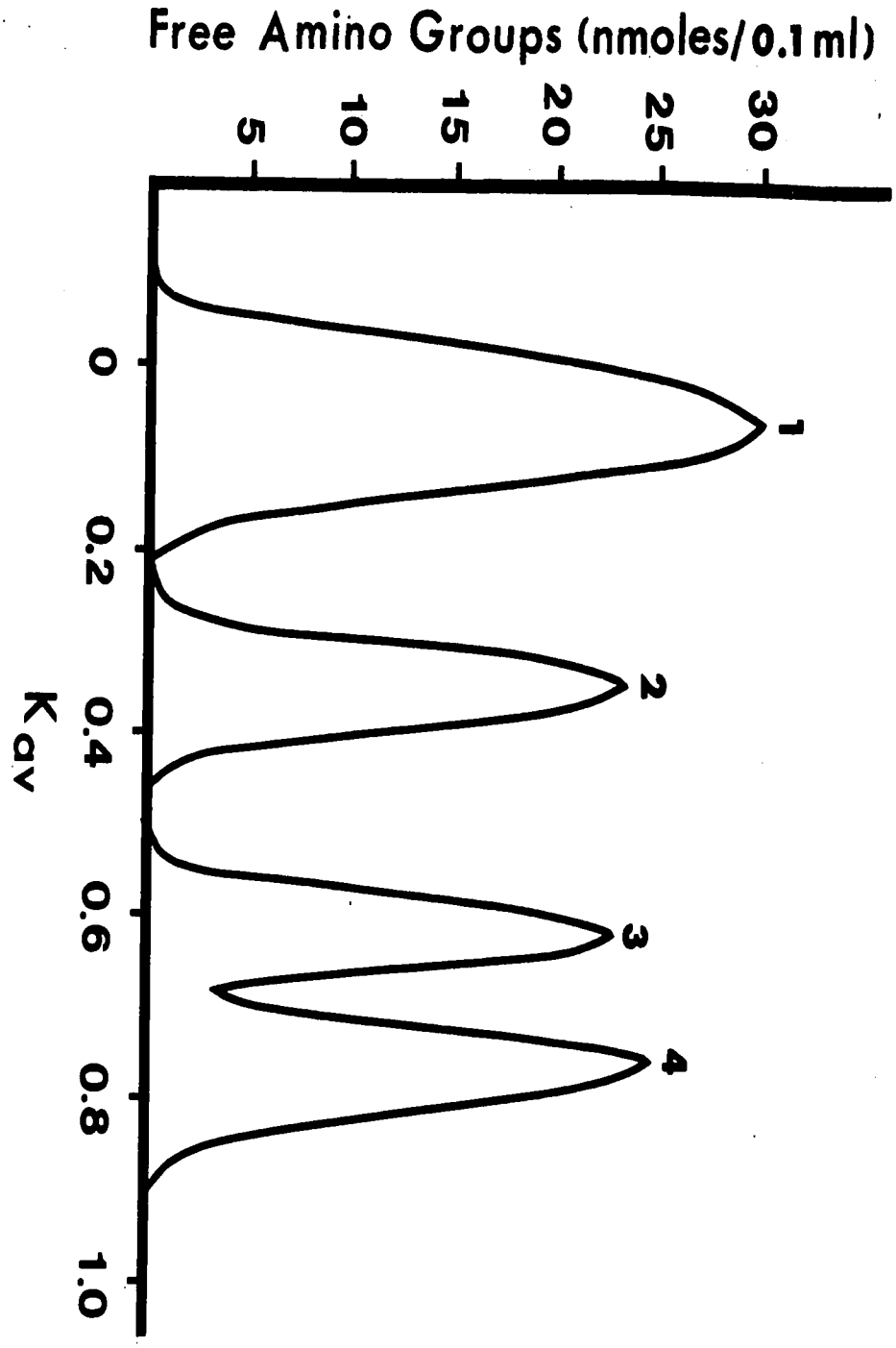

Free Amino Groups (nmoles/0.1 ml)
5
10
15
20
25
30
0
0.2
0.4
0.6
0.8
1.0
Kav
1
2
3
4

FIGURE 20

FRACTIONATION OF PEPTIDE FRAGMENTS OF SEQUENTIALLY DIGESTED TCS-GROWN M. SODONENSIS CELL WALLS

One-hundred mg of TCS-grown cell walls were sequentially digested with Streptomyces ML-endopeptidase for 60 hr, lysozyme for 36 hr, and Streptomyces N-acetylmuramic acid-L-alanine amidase for 24 hr. Standard specified conditions for digestion were employed at each successive stage. The digest was concentrated to a volume of 5.0 ml and applied to the Sephadex G-50, G-25 system eluted with 0.1 M LiCl. Five ml fractions were collected and 0.1 ml portions of each assayed for free amino groups relative to an L-alanine standard.

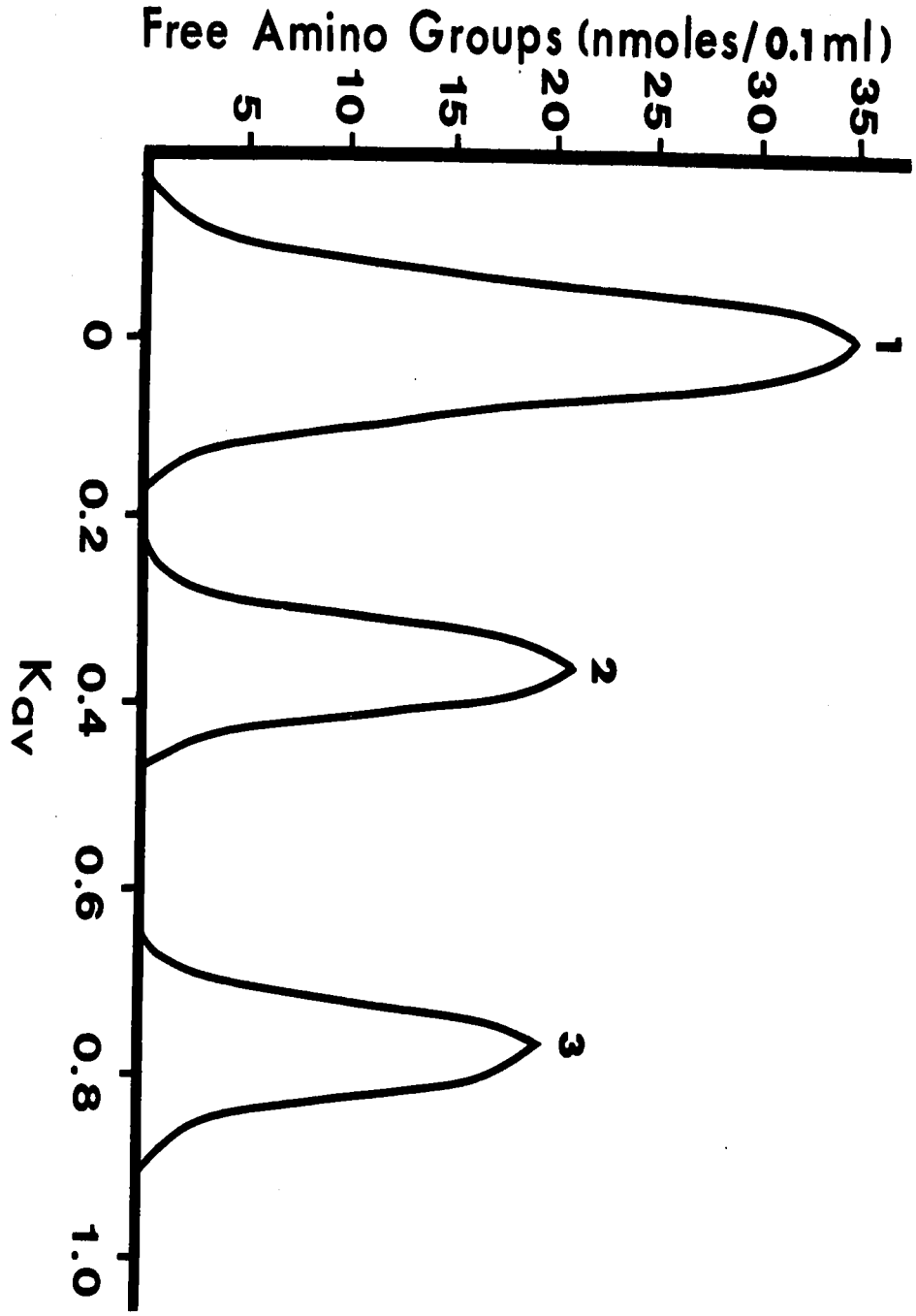

Free Amino Groups (nmoles/0.1ml)
5
10
15
20
25
30
35
Kav
0
0.2
0.4
0.6
0.8
1.0
1
2
3

FIGURE 21

FRACTIONATION OF PEPTIDE FRAGMENTS OF SEQUENTIALLY DIGESTED BASAL-GROWN M. SODONENSIS CELL WALLS

One-hundred mg of basal-grown cell walls were sequentially digested with Streptomyces ML-endopeptidase for 60 hr, lysozyme for 36 hr, and Streptomyces N-acetylmuramic acid-L-alanine amidase for 24 hr. Standard specified conditions for digestion were employed at each successive stage. The digest was concentrated to a volume of 5.0 ml and applied to the Sephadex G-50, G-25 system eluted with 0.1 M LiCl. Five ml fractions were collected and 0.1 ml portions of each assayed for free amino groups relative to an L-alanine standard.

0.34 (Peak 2), and 0.75 (Peak 3). Material having a K_{av} value of 0.75 in this chromatographic system has been previously characterized as the peptide subunit of M. sodonensis cell walls.

The fractions containing each of the peaks were pooled, concentrated, and desalted on Bio-gel P-2 columns (2.5 x 45 cm) eluted with distilled water. Desalted materials were evaporated to dryness and resuspended in 10.0 ml of 0.01 M veronal-HCl buffer, pH 9.0, containing 10^{-3} M EDTA. Triplicate 20 µl aliquots were removed from each sample and assayed for total alanine content. In addition, triplicate 200 µl aliquots were removed from each preparation and analyzed for free N-terminal alanine groups. These data are presented in Tables XVII and XVIII, for TCS- and basal-grown cell wall preparations respectively. Mean chain length of each peptide preparation was determined by dividing the L-alanine content of each fraction (i.e., one-half of the total alanine content) by the total number of free N-terminal alanine groups.

Table XVII indicates that approximately 20% of the TCS-grown cell wall peptides are released as monomeric units. This material represents peptide subunits which were covalently bound to N-acetylmuramic acid in the intact cell wall. These observations are consistent with conclusions drawn from previous studies with cell wall amidase digests. Summation of the muramic acid-linked N-terminal alanine in the lysozyme-soluble and -insoluble portions reveal that 23.3% of the total alanine of the cell wall is involved in covalent linkage with the glycan.

Table XVIII indicates that the length and distribution of peptide bridges differs significantly from those encountered in TCS-grown cell walls. Again, production of 12% of the total cell wall peptide subunits

TABLE XVII

ANALYSIS OF PEPTIDE FRAGMENTS OF SEQUENTIALLY DIGESTED TCS-GROWN *M. SODONENSIS* CELL WALLS*

Material	Total L-alanine	% of total	N-terminal alanine	Fragment length
Peak 1	533	39.5	68	8
Peak 2	335	24.8	83	4
Peak 3	201	14.9	97	2
Peak 4	270	20.0	265	1

* Data are expressed as nmoles per ml of each peptide preparation.

TABLE XVIII

ANALYSIS OF PEPTIDE FRAGMENTS OF SEQUENTIALLY DIGESTED BASAL-GROWN *M. SODONENSIS* CELL WALLS*

Material	Total L-alanine	% of total	N-terminal alanine	Fragment length
Peak 1	954	72	53	18
Peak 2	212	16	53	4
Peak 3	159	12	156	1

* Data are expressed as nmoles per ml of each peptide preparation.

as monomeric units is consistent with previous results obtained by the study of muramic acid-linked N-terminal alanine in amidase digests of the lysozyme-soluble and -insoluble portions of basal-grown cell walls. Most of the material released by this sequence of digestions was excluded from the linked Sephadex G-50,G-25 system and had a mean chain length of 18 subunit peptides. While 16% of the material was released as tetramer, (Peak 2) indicating that some cross-bridges were three peptide subunits in length, no peptide dimers were present. Foregoing assertions that the peptidoglycan matrix of basal-grown cell walls is less organized in the degree of peptide substitution of the glycan and nature of peptide cross-bridging are further supported by these data.

All peptide fractions so analyzed were then digested with the Myxobacter AL-1 Protease at 37°C for 24 hours under previously specified conditions. Such digests were then concentrated to a volume of 5.0 ml and applied to the linked Sephadex G-50,G-25 system. Effluent fractions were examined for their content of free amino groups. Peaks 2 and 3 of the TCS-grown cell walls (tetramer and dimer, respectively), and Peak 2 of the basal-grown cell walls (tetramer), when digested with Myxobacter AL-1 Protease and so fractionated, produced single peaks having K_{av} values of 0.75. These materials had, therefore, been converted to the peptide monomer.

Peak 1 of the TCS-grown cell walls, when digested with the Myxobacter AL-1 Protease formed some monomer peptides but much material remained having a K_{av} value of 0.045. Likewise, Peak 1 of the basal-grown cell walls released peptide monomer upon digestion with the protease, but much of the material was still undegraded. Elution profiles of Peak 1

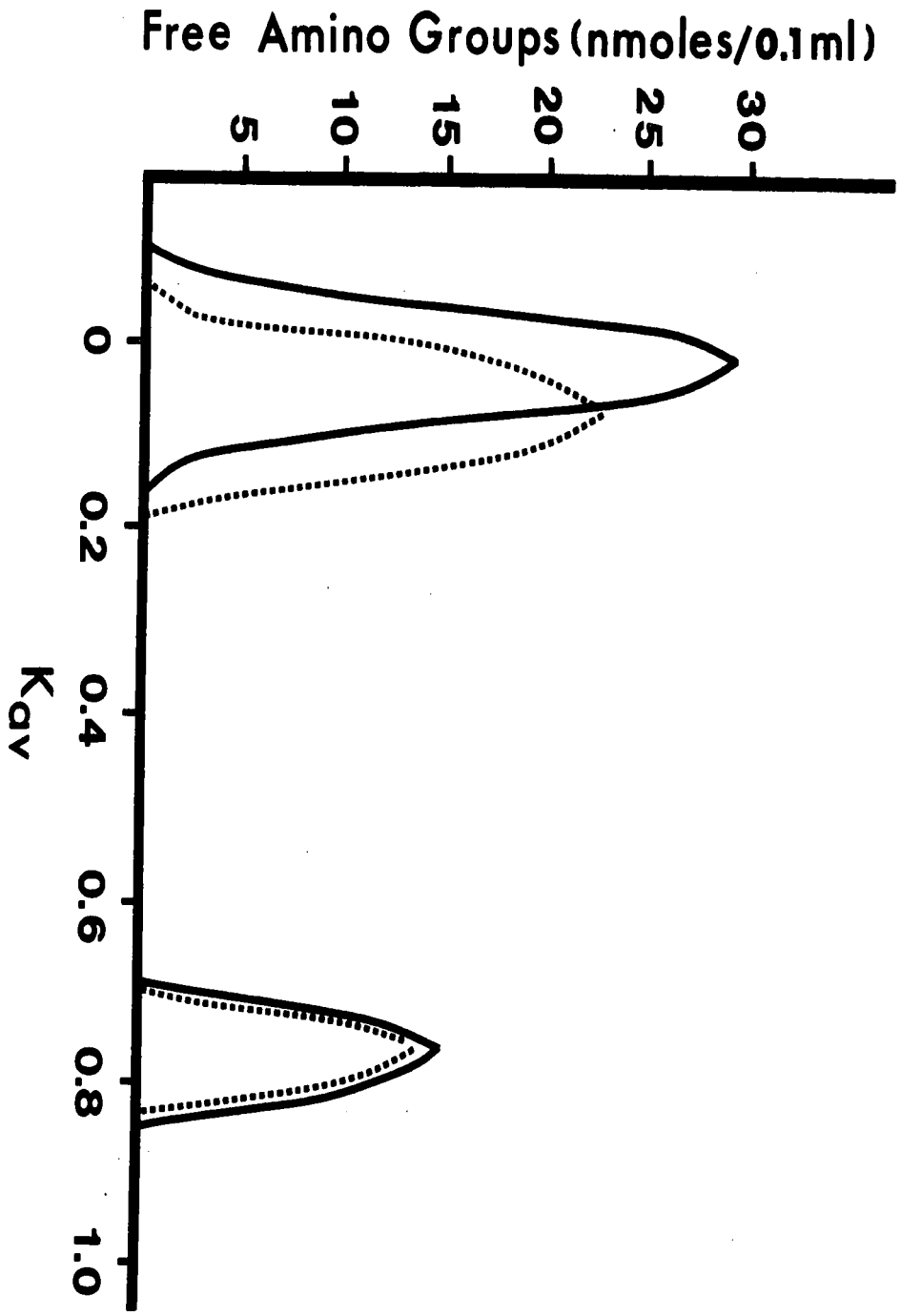

Free Amino Groups (nmoles/0.1ml)
5
10
15
20
25
30
0
0.2
0.4
0.6
0.8
1.0
Kav

FIGURE 22

FRACTIONATION OF HIGH MOLECULAR WEIGHT PEPTIDE FRAGMENTS OF M. SODONENSIS CELL WALLS DIGESTED WITH MYXOBACTER AL-1 PROTEASE

High molecular weight peptide fragments obtained by sequential Streptomyces ML-endopeptidase, lysozyme, and Streptomyces N-acetylmuramic acid-L-alanine amidase digestion of TCS-grown cell walls (Peak 1, K_{av} = 0.045) and basal-grown cell walls (Peak 1, K_{av} = 0), were digested with Myxobacter AL-1 Protease under standard specified conditions. Digests were applied to the linked Sephadex G-50, G-25 system eluted with 0.1 M LiCl. Five ml fractions were collected and 0.1 ml portions of each assayed for free amino groups relative to an L-alanine standard.

..... TCS-grown cell wall peptide fragments

——— Basal-grown cell wall peptide fragments

from the two cell wall preparations are presented in Figure 22. These findings suggest that undiscovered bonds insensitive to any enzymic hydrolysis attempted are present in sufficient quantity to maintain complex peptide structures.

C. O-acetylation of Cell Walls and Lysozyme-sensitivity

1. Establishment of O-acetylation of Cell Walls: Cell wall O-acetylation was established by hydrolyzing cell wall preparations in alkali (Brumfitt, 1959), and analyzing the soluble portions of such mixtures for acetate. Fifty mg of each cell wall preparation were incubated in 0.01 N NaOH at room temperature for 48 hours. Insoluble material was sedimented by centrifugation at 30,000 x g for 20 minutes and the supernatant fraction removed. This fraction was shown to contain no amino acids, neutral sugars, or hexosamines. Following passage of this material through Dowex-50W-X4 (H^+) to remove sodium ions, effluent portions were extracted three times with diethyl ether and analyzed for acetate content by gas-liquid chromatography as described in Methods.

Figure 23 shows the elution profile of the soluble material of alkali-digested cell walls. Two components were detected, one corresponding to acetic acid, and the other to acetic anhydride. This resolution of acetate into two species on gas-chromatographic analysis was likely a result of dehydration of the molecule and spontaneous bimolecular esterification to form its anhydride during ether extraction. Johnson (personal communication, 1970) has observed that α-hydroxyglutaric acid forms its lactide and lactone derivatives during ether extraction. These findings, coupled with the fact that the presence of

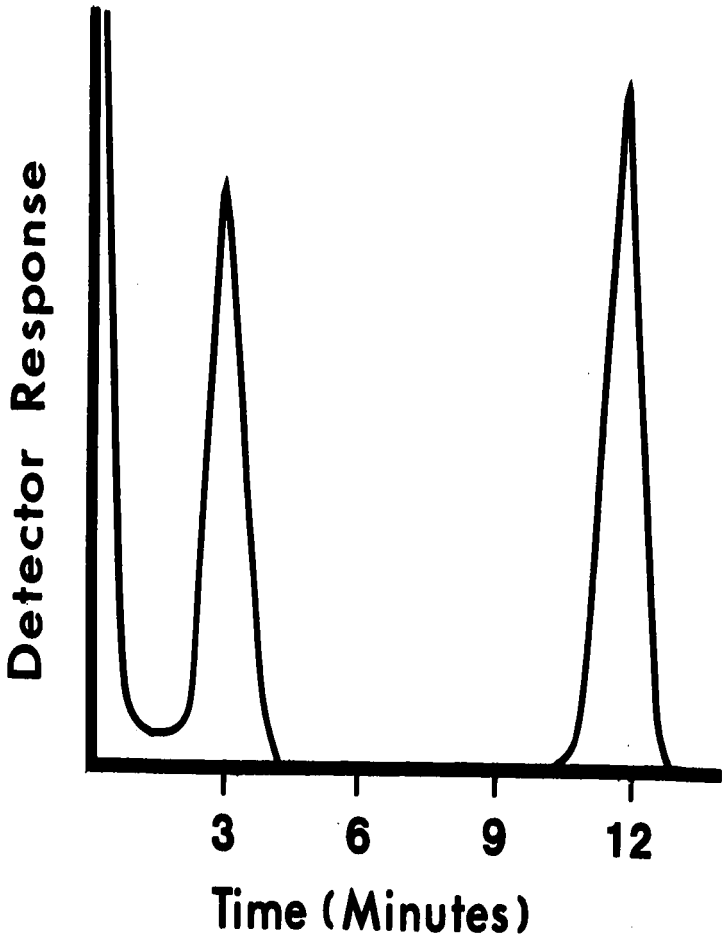

Detector Response
3
6
9
12
Time (Minutes)

FIGURE 23

GAS-LIQUID CHROMATOGRAPHY ANALYSIS OF DEACETYLATION PRODUCTS OF M. SODONENSIS CELL WALLS

The soluble material of 50 mg of cell walls which had been incubated in 0.01 M NaOH for 48 hr at room temperature was passed through Dowex-50 (H^+) to remove sodium ions, concentrated to a final volume of 0.5 ml, and extracted three times with diethyl ether. Ether extracts were chromatographed on 15% FFAP on 60/80 Chromosorb W as described in Materials and Methods.

cell wall esters is demonstrable by the Hestrin technique confirm the presence of O-acetyl substituents in M. sodonensis cell walls.

2. Kinetics of Deacetylation: The kinetics of cell wall deacetylation and deacetylation of the lysozyme-insoluble portions of TCS- and basal-grown cell walls were measured. A suspension of cell walls or the lysozyme-insoluble portions thereof, containing 2.5 mg per ml was prepared in 0.01 N NaOH and incubated at room temperature. At intervals, 200 µl aliquots were withdrawn for ester assay. Percentage of total esters released as a function of time is presented in Figures 24 and 25.

In both cases, deacetylation of cell walls occurred, although to a much lesser extent in whole walls than in their lysozyme-insoluble portions. A surprising and inexplicable observation was that while the lysozyme-insoluble portions of walls were completely deacetylated, whole cell walls were not. Basal-grown cell walls were deacetylated to about half the extent of TCS-grown cell walls.

To ascertain if disappearance of Hestrin-positive material might be caused by "de-N-acetylation" of constituent amino sugars, solutions of N-acetylmuramic acid and N-acetylglucosamine were subjected to 48 hr incubation in 0.01 N NaOH at room temperature and estimated by the Morgan-Elson technique without chemical re-acetylation. No loss of material was observed. Since both of these N-acetylated hexosamines failed to react in the Hestrin assay, it was concluded that alkali-digestion hydrolyzed only the weak alkali-labile O-acetyl linkages and that disappearance of esters was a result of cell wall deacetylation.

3. Effect of Cell Wall O-acetylation on Lysozyme-sensitivity: The effect of cell wall O-acetylation on lysozyme-sensitivity was investigated

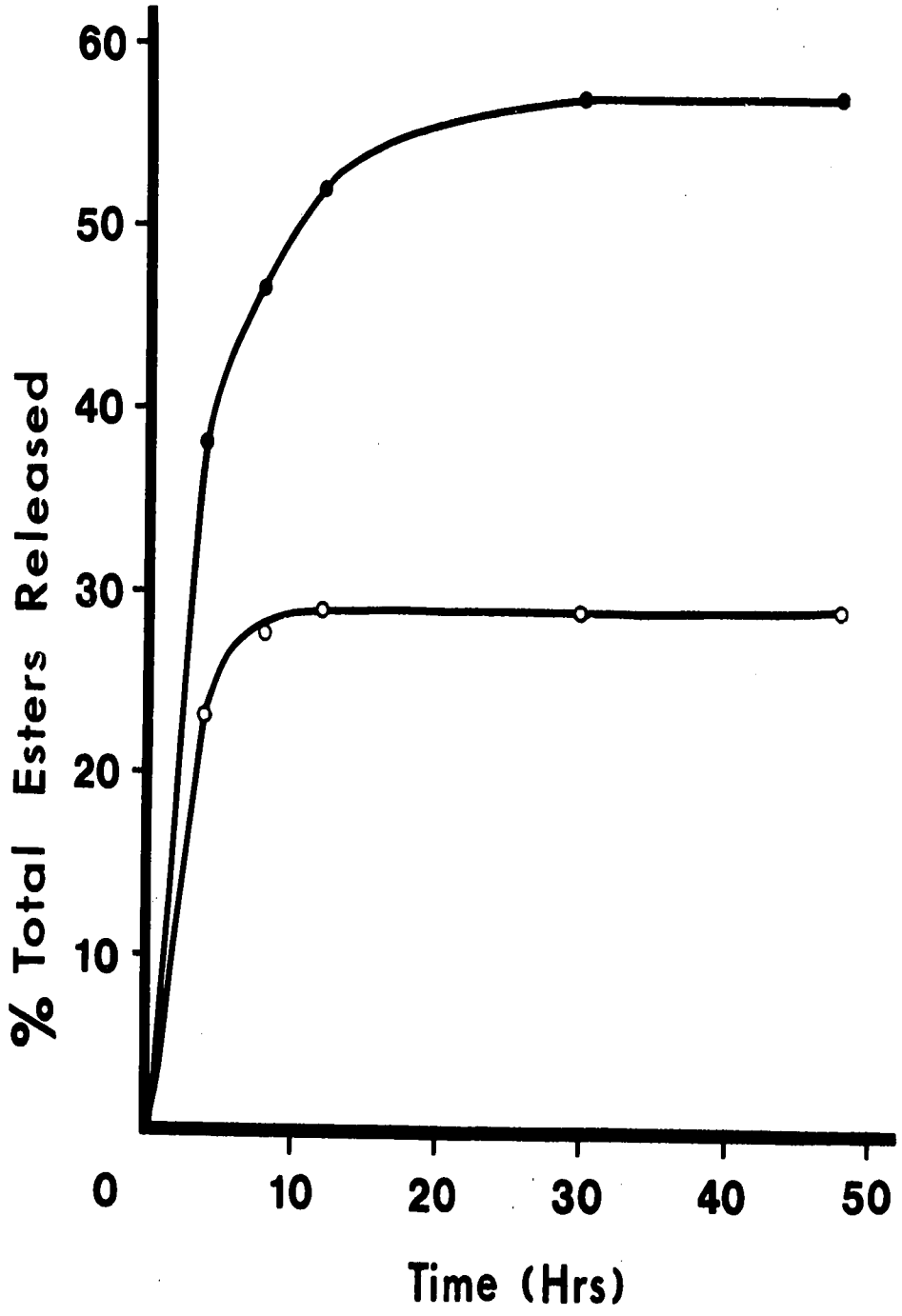

60
50
40
30
20
10
0
% Total Esters Released
10
20
30
40
50
Time (Hrs)

FIGURE 24

KINETICS OF DEACETYLATION OF M. SODONENSIS CELL WALLS

A 2.5 mg per ml suspension of cell walls in 0.01 M NaOH was incubated at room temperature. At intervals, 0.2 ml aliquots were removed and assayed for their content of esters relative to an ethylacetate standard.

●——○ TCS-grown cell walls

○——○ Basal-grown cell walls

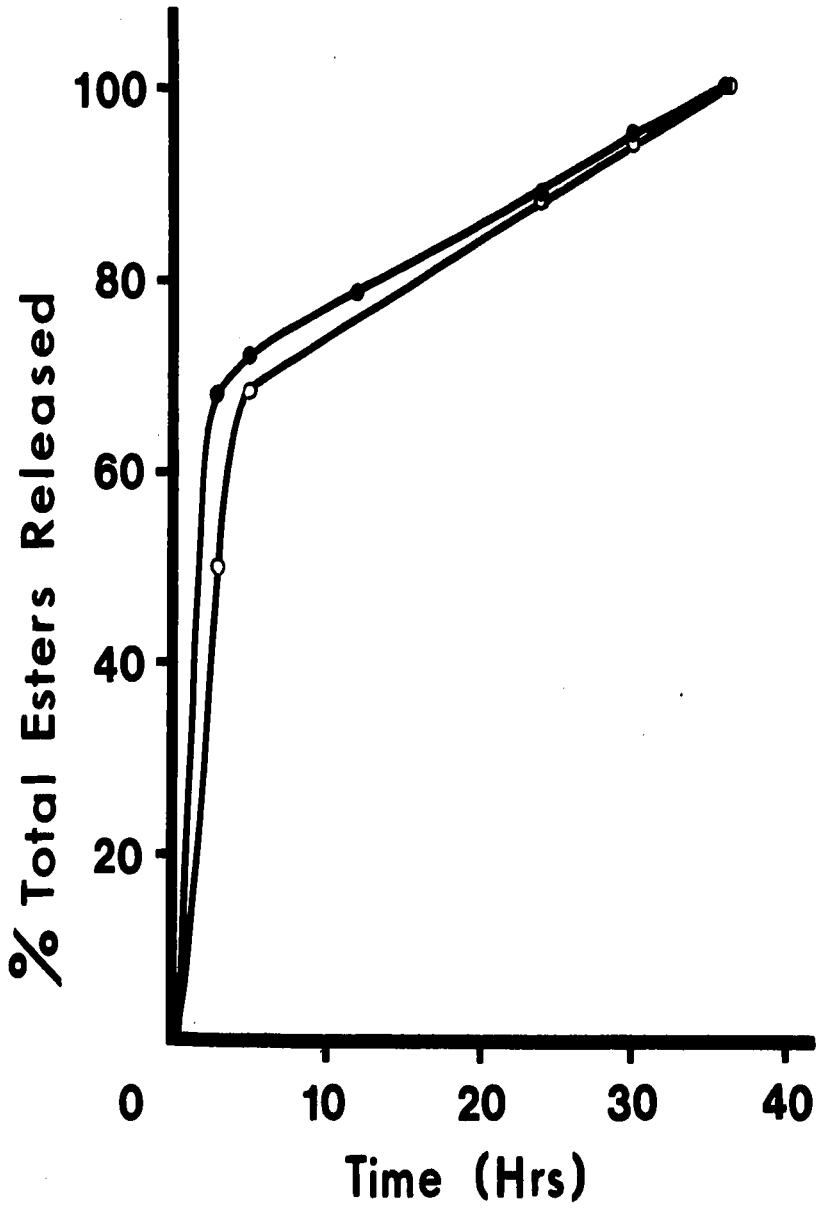

100
80
60
40
20
% Total Esters Released
0
10
20
30
40
Time (Hrs)

FIGURE 25

KINETICS OF DEACETYLATION OF THE LYSOZYME-INSOLUBLE PORTION OF *M. SODONENSIS* CELL WALLS

A 2.5 mg per ml suspension of the lysozyme-insoluble portion of cell walls in 0.01 M NaOH was incubated at room temperature. At intervals, 0.2 ml aliquots were withdrawn and analyzed for their content of esters relative to an ethylacetate standard.

Ester release from the lysozyme-insoluble portion of basal-grown cell walls

Ester release from the lysozyme-insoluble portion of TCS-grown cell walls

in whole deacetylated cell walls and preparations of the deacetylated lysozyme-insoluble portion of cell walls. Kinetics of lysozyme digestion of these deacetylated preparations were established in the same manner as previously described for native cell walls.

Comparison of the lysozyme-mediated release of reducing groups in native and deacetylated cell walls is presented in Figure 26. Deacetylated cell wall preparations were considerably more sensitive to lysozyme than native cell walls. Such walls released 61 and 67 nmoles per mg more reducing groups than native basal- and TCS-grown cell walls respectively. Thus, deacetylation of the walls made approximately an additional 65 glycosidic linkages per mg cell wall sensitive to the action of lysozyme.

Figure 27 shows that deacetylation of the lysozyme-insoluble portion of cell walls conferred upon it a degree of lysozyme susceptibility. The deacetylated lysozyme-insoluble portion of basal-grown cell walls, which is known to be less complex with respect to its glycan substitution and peptide cross-bridging pattern, was more susceptible to the enzyme than the corresponding TCS-grown cell wall fraction. Nonetheless, these data support the hypothesis that O-acetylation plays a key but not the only role in lysozyme-sensitivity of M. sodonensis cell walls.

4. Distribution of Lysozyme-soluble and -Insoluble Components In Deacetylated Cell Walls: Thirty-six hour lysozyme digests of deacetylated preparations were centrifuged to yield their respective lysozyme-soluble and -insoluble portions. Appropriate samples of such materials were assayed for their content of esters, glucose, and hexosamines as has been previously described. Data are presented in Table XIX.

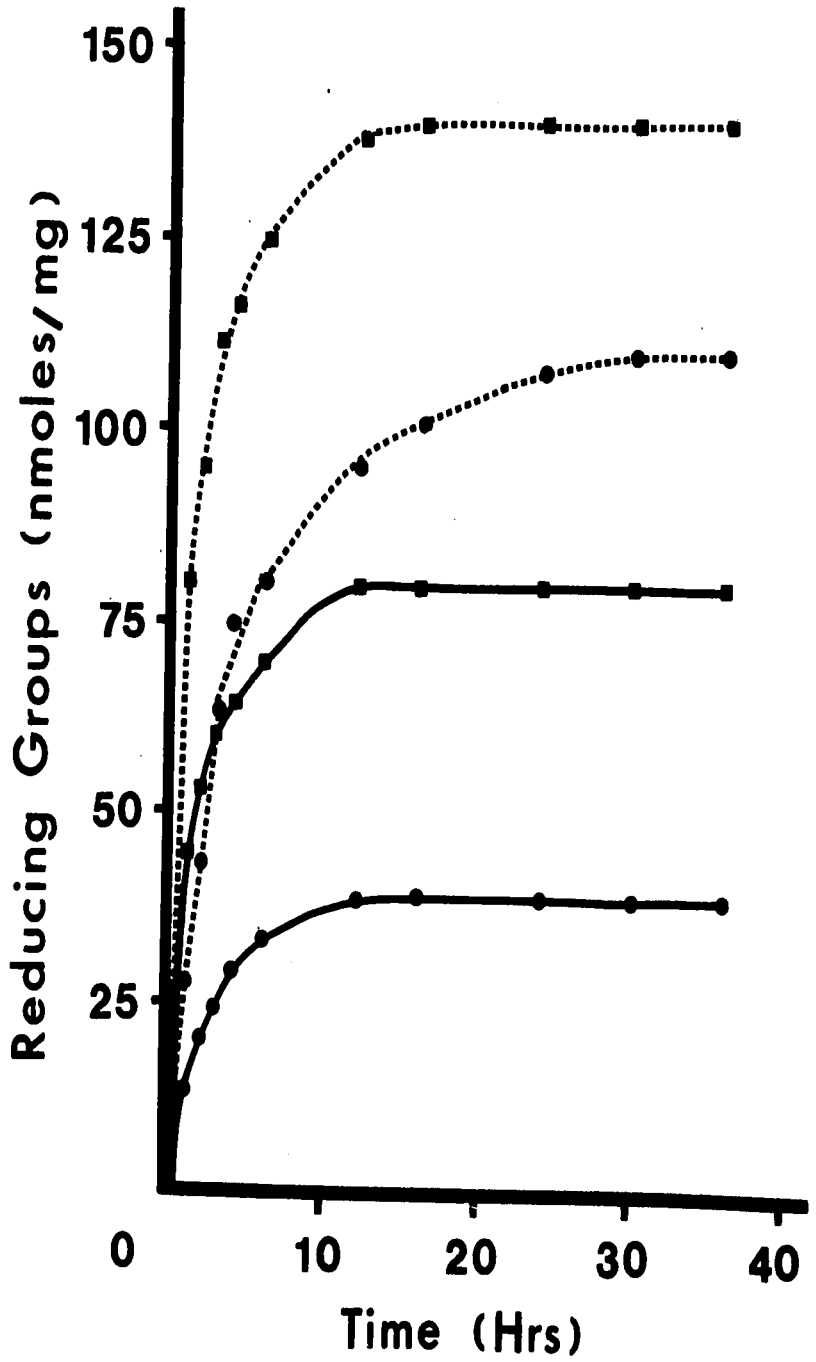

150
125
100
75
50
25
0
10
20
30
40
Reducing Groups (nmoles/mg)
Time (Hrs)

FIGURE 26

COMPARISON OF THE RESPONSE OF NATIVE AND DEACETYLATED

M. SODONENSIS CELL WALLS TO LYSOZYME DIGESTION

Five mg of each wall preparation were digested with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At various times, 50 µl aliquots were removed, centrifuged to sediment insoluble material, and 20 µl of the supernatant fractions assayed for their content of reducing groups.

●——○ Reducing groups liberated in native TCS-grown cell walls

●·····○ Reducing groups liberated in deacetylated TCS-grown cell walls

■——□ Reducing groups liberated in native basal-grown cell walls

■·····□ Reducing groups liberated in deacetylated basal-grown cell walls

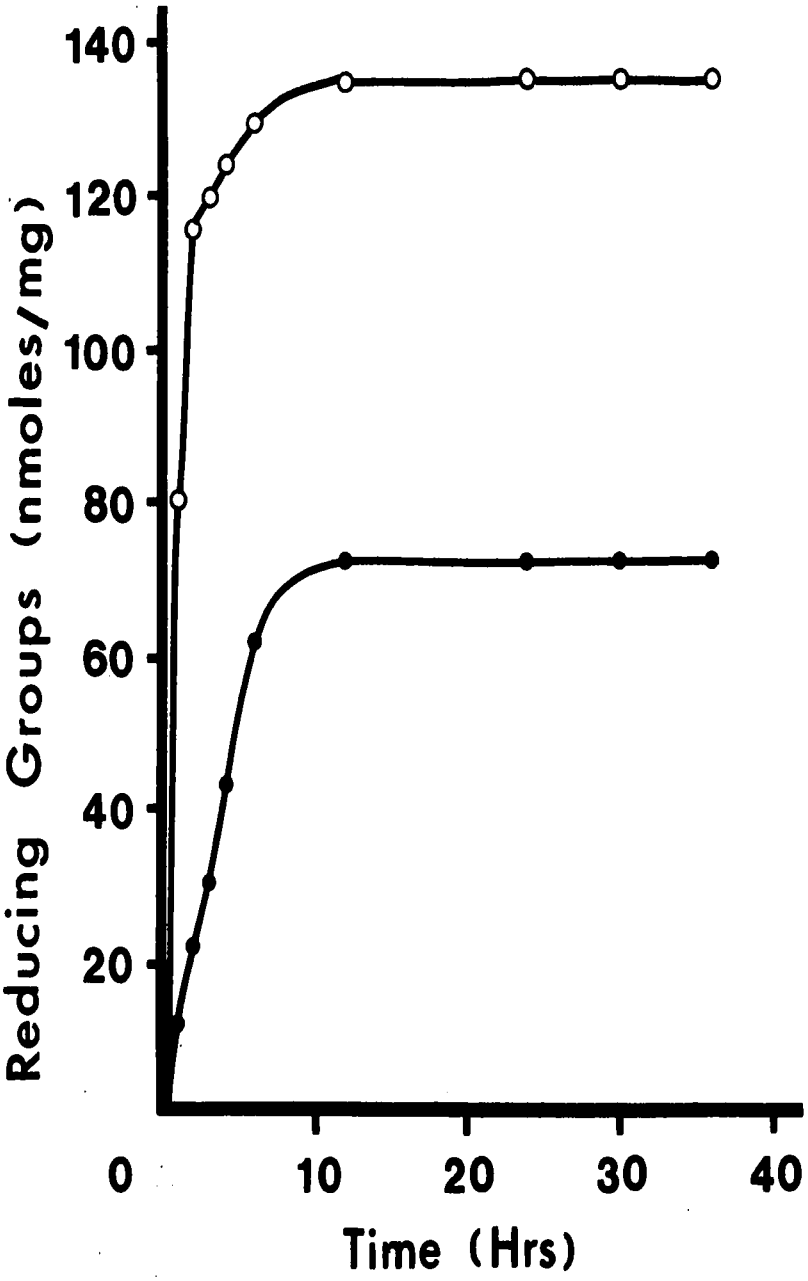

140
120
100
80
60
40
20
Reducing Groups (nmoles/mg)
0
10
20
30
40
Time (Hrs)

FIGURE 27

RESPONSE OF THE DEACETYLATED LYSOZYME-INSOLUBLE PORTIONS OF M. SODONENSIS CELL WALLS TO LYSOZYME DIGESTION

Five mg of each deacetylated lysozyme-insoluble portion of cell walls were digested with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At intervals, 50 µl aliquots were withdrawn, centrifuged to sediment insoluble material, and 20 µl of the supernatant fractions assayed for their content of reducing groups.

●——○ Reducing groups liberated in the lysozyme-insoluble portion of TCS-grown cell walls

○——○ Reducing groups liberated in the lysozyme-insoluble portion of basal-grown cell walls

On the basis of hexosamine content, deacetylated cell walls were 76% and 92% solubilized in the TCS- and basal-grown preparations respectively. Thus, deacetylation of cell walls results in an increase of 28% in lysozyme-mediated solubilization in TCS-grown preparations and of 17% in basal-grown preparations. Residual esters were found primarily in the lysozyme-insoluble portions of deacetylated cell walls. The lysozyme-soluble portion of deacetylated TCS-grown cell walls were exactly twice as highly esterified as the same fraction of basal-grown cell walls on the basis of the esters:hexosamines ratio. However, the situation was reversed in the lysozyme-insoluble portions of the deacetylated cell walls. In the lysozyme-insoluble portion of TCS-grown cell walls the ester:hexosamines ratio was 0.85 while in the analogous portion of basal-grown cell walls it was 1.72. Since both of these ratios are greater than 0.5, one can conclude that acetylation may occur on both N-acetylmuramic acid and N-acetylglucosamine.

Distribution of hexosamines in the lysozyme-soluble and -insoluble portions of the deacetylated lysozyme-insoluble portions of cell walls was also established. On the basis of hexosamine content, lysozyme solubilized 73% and 97.5% of the deacetylated TCS- and basal-grown preparations respectively. Significantly, complete deacetylation did not render the lysozyme-insoluble portions of cell walls wholly sensitive to this enzyme.

D. Involvement of Peptide Substitution with Lysozyme-sensitivity: The involvement of peptide substitution of the glycan with lysozyme-sensitivity was investigated in deacetylated cell walls and the deacetylated lysozyme-insoluble portion of cell walls. Two types of digestions

TABLE XIX

DISTRIBUTION OF COMPONENTS IN DEACETYLATED LYSOZYME-DIGESTED *M. SODONENSIS* CELL WALL MATERIAL*

Deacetylated Material	Whole Cell Walls	Lysozyme-soluble portion	Lysozyme-insoluble-portion
A. TCS-grown cell walls			
Hexosamines	560	426	138
Esters	210	96	120
Glucose	350	190	157
Esters:Hexosamines	0.38	0.23	0.85
B. Basal-grown cell walls			
Hexosamines	1,070	984	86
Esters	260	110	148
Glucose	584	445	145
Esters:Hexosamines	0.24	0.11	1.72
C. Lysozyme-insoluble portion of TCS- walls			
Hexosamines	391	285	105
D. Lysozyme-insoluble portion of basal walls	446	436	10

* Data are expressed as nmoles per mg cell wall material.

were performed. In one case, the deacetylated cell wall materials were digested first with the Myxobacter AL-1 Protease, and then with lysozyme. In the other case, the deacetylated cell wall materials were digested with lysozyme alone. Standard specified conditions were employed for these digestions. The number of glycosidic bonds cleaved by lysozyme was estimated as nmoles reducing groups liberated per mg of cell wall. The results are presented in Table XX.

In every case, pre-treatment of the wall material by Myxobacter AL-1 Protease, which specifically hydrolyzes off peptide substituents but leaves the glycan backbone intact, permitted enhanced lysozyme activity. This suggests that peptide substitution, in some manner, prevents complete lysozyme digestion of M. sodonensis cell walls. Owing to the dependence of lysozyme activity upon stereochemical factors, configuration of peptide cross-bridges of the peptidoglycan likely plays a role in the observed effect.

V. Immunological Studies

Production and purification of the immune globulin fractions of whole rabbit anti-pre-trypsin cell wall, anti-pure cell wall, and anti-nuclease sera have been described in Methods.

A. Tube Precipitation Tests: Tube precipitation tests were performed using two antigens: 1. the lysozyme-soluble portion of pre-trypsin cell walls, and 2. the lysozyme-soluble portion of pure cell walls. Both of these preparations were obtained from basal-grown cell walls. Into each of a series of Durham tubes were placed 100 µl of soluble antigen containing approximately 100 µg of cell wall material. To each tube

TABLE XX

RESPONSE OF DEACETYLATED M. SODONENSIS CELL WALL MATERIAL TO SEQUENTIAL MYXOBACTER AL-1 PROTEASE AND LYSOZYME DIGESTION*

	Deacetylated Material	Reducing Groups Liberated by Digestion 1	Digestion 2	Δ
A.	TCS-grown cell walls	110	142	32
B.	Basal-grown cell walls	134	170	36
C.	Lysozyme-insoluble portion of TCS-grown cell walls	68	140	72
D.	Lysozyme-insoluble portion of basal-grown cell walls	130	152	22

Digestion 1- Digestion of the cell wall material with lysozyme alone.
Digestion 2- Digestion of the cell wall material with Myxobacter AL-1 Protease followed by lysozyme digestion.
* Data are expressed as nmoles per mg cell wall material.

was added 100 µl of doubling dilutions of the immune globulin fraction of anti-pre-trypsin cell wall serum in buffered saline. Tubes were sealed, incubated one hour at 37°C, refrigerated overnight, and examined for precipitation. The titre of the immune globulin was the highest dilution causing visible precipitation. Use of either antigen resulted in a titre of 512 for the immune globulin fraction of anti-pre-trypsin cell wall serum. Therefore, cell wall protein is not involved in cell wall antigenicity since the titres remained constant independent of trypsin-treatment of the antigen.

The experiment was repeated using instead the immune globulin fraction of anti-pure cell wall serum and the same two antigens. In this instance, the observed titre was 512 for both antigens indicating that, indeed, cell wall protein does not contribute to the reaction.

B. Slide Agglutination Tests: The following antigens were employed:

1. whole basal-grown pure cell walls, and
2. the lysozyme-insoluble portion of basal-grown cell walls.

The following antibodies were reacted with the above antigens:

1. the immune globulin fraction of anti-pre-trypsin cell wall serum,
2. the immune globulin fraction of anti-pure cell wall globulin.

Equal 50 µl volumes of antigens containing approximately 100 µg of cell wall material and the immune globulin fractions were mixed on a glass slide and examined microscopically for agglutination. The titre of the immune globulin was the highest dilution causing visible agglutination of the antigen. Results are presented in Table XXI.

These results indicate, as demonstrated previously, that cell wall protein does not participate in the reaction since the observed

TABLE XXI

REACTIONS BETWEEN IMMUNE GLOBULIN FRACTIONS OF ANTI-CELL WALL SERA AND CELL WALL MATERIAL OF M. SODONENSIS

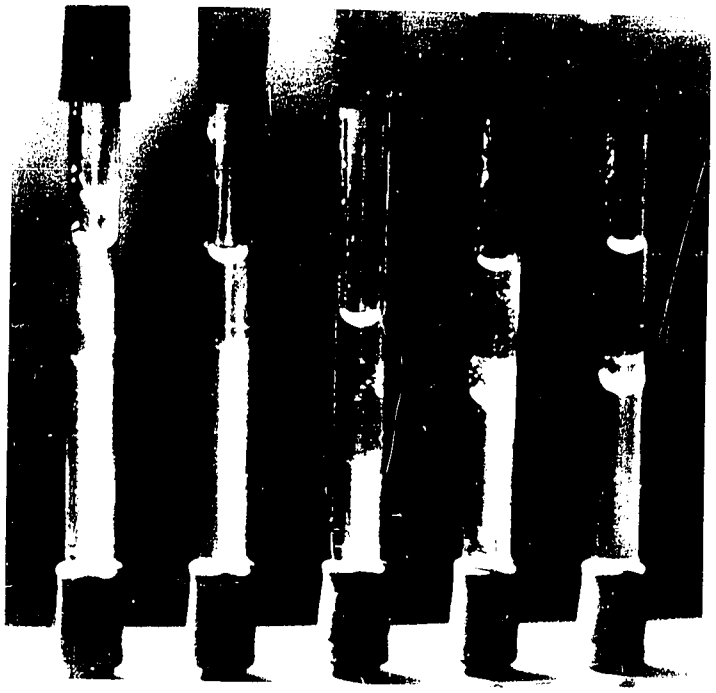
Antigen	Titre of the immune globulin fraction of	
	Anti-pre-trypsin cell wall serum	Anti-pure cell wall serum
Pure cell walls	256	256
Lysozyme-insoluble portion of cell walls	8	8

titres are the same for both trypsin-treated and -untreated cell wall antigens. There was no significant reaction between either of the antibody preparations tested and the lysozyme-insoluble portion of basal-grown cell walls. Therefore, lysozyme removed the components which are the primary determinants of cell wall antigenicity.

C. Gel Diffusion: The Oudin method of single gel diffusion was employed to demonstrate cross-reactivity between cell walls and the immune globulin fraction of rabbit anti-nuclease serum. Pre-trypsin and pure cell walls digested 24 hours with Myxobacter AL-1 Protease under standard specified conditions were used as antigens. Equal 300 µl volumes of the immune globulin preparation and Noble agar were mixed at 50°C and added to tubes as described in Methods. Three-hundred µl of each soluble antigen containing approximately 300 µg of cell wall material were layered over the solidified agar. Tubes were sealed, incubated at 37°C, and examined at intervals for precipitation. As Figure 28 shows, a single band of precipitation appeared in tubes containing both pre-trypsin and pure cell walls. These results indicate that cell walls of M. sodonensis and its extracellular nuclease are immunologically related.

VI. Localization of Nuclease Activity on the Cell Wall

Investigations conducted in this laboratory have shown a small portion of the extracellular nuclease activity elaborated by M. sodonensis to be covalently bound in or on the cell wall (Berry et al., 1970). Attempts were made to visualize the location of M. sodonensis nuclease with techniques employing ferritin-labelled antibodies. The preparation of such ferritin-conjugated materials has been previously described.



1 2 3 4 5

FIGURE 28

OUDIN SINGLE GEL DIFFUSION TEST FOR CROSS REACTIVITY OF NUCLEASE AND PURIFIED CELL WALL OF M. SODONENSIS

The immune globulin fractions of rabbit anti-nuclease serum or rabbit control serum were incorporated into the agar and the solidified columns overlaid with antigens or buffer. Preparation of the antigens is given in the text.

Tube 1 - the immune globulin fraction of rabbit anti-nuclease serum + buffered saline

Tube 2 - the immune globulin fraction of rabbit control serum + pre-trypsin cell walls

Tube 3 - the immune globulin fraction of rabbit control serum + buffered saline

Tube 4 - the immune globulin fraction of rabbit anti-nuclease serum + pure cell walls

Tube 5 - the immune globulin fraction of rabbit anti-nuclease serum + pre-trypsin cell walls

⟶ Diffusion Gel-Soluble antigen interface

In one series of experiments, the ferritin-conjugated immune globulin fraction of rabbit anti-nuclease serum was reacted against thin sections of whole cells or cell wall preparations. In another series of experiments, absorption of the immune globulin fraction of rabbit anti-nuclease serum to thin-sections of whole cell walls or cell wall preparations was followed by treatment with ferritin-conjugated horse anti-rabbit serum. A combination of non-specific binding of the labelled antibody to the cell wall plus the already established low levels of nuclease present in walls rendered the results inconclusive.

A histochemical technique utilizing the deposition of lead that was successful in locating the 5'-nucleotidase of E. coli was modified for use in the M. sodonensis system (Nisonson et al., 1969). Fifty ml of an 18 hour basal-grown culture of M. sodonensis was washed three times in 0.01 M Tris-HCl buffer, pH 8.8, and resuspended in a volume of 3.0 ml of the same buffer. One ml of this washed cell suspension was added to one ml of a solution containing 33 µmoles Tris, 13 µmoles $MgCl_2$, 20 µmoles 5'-AMP, 1.7 µmoles $CaCl_2$, 1.7 µmoles $MnCl_2$, 10 µmoles mercaptoethanol, and 3 mmoles of lead acetate. Two negative controls, one complete except for 5'-AMP, and the other complete except for lead acetate, were also prepared. These reaction mixtures were incubated at 37°C for two hours and the reactions terminated by the addition of 0.4 ml of 5% glutaraldehyde. Specimens were then prepared for electron microscopic analysis as described in Methods.

As shown in Plate IV, hydrolysis of 5'-AMP, as evidenced by the precipitation of lead phosphate, is limited to the exterior of the cell wall. That lead precipitation was specific for 5'-nucleotidase activity

PLATE IV

LOCALIZATION OF THE NUCLEASE ON M. SODONENSIS CELL WALLS

Nuclease activity was detected by the precipitation of inorganic phosphate produced by enzymic hydrolysis of 5'-AMP as lead phosphate. Details of the reaction mixtures are given in the text. Procedures for fixation, embedding, and thin-sectioning are given in Materials and Methods.

Magnification = 64,800 x

was evidenced by the lack of cell surface lead deposits in cells incubated in the absence of the nucleoside-5'-monophosphate substrate. Moreover, since no deposition of lead was found inside the cell, penetration of nucleotides into intact cells did not occur. Thus, the designation of the nuclease as extracellular is not entirely correct since a portion of its activity is associated with the cell wall.

DISCUSSION

The cell wall purification employed in these studies resulted in preparations which were homogeneous upon examination by electron microscopy and chemical analysis. Although terminal preparations contained no demonstrable glycerol or fatty acids, a minute amount of phosphate, almost below the levels of detection, was observed. Lipid analysis of S. lutea, a Gram-positive coccus closely related to M. sodonensis, disclosed that phospholipids constituted 10% of the total lipids (Huston and Albro, 1964) and 1% of the total cell weight (Akashi and Saito, 1960). If the phospholipid composition of M. sodonensis is similar, the observed phosphate content of 0.09 µg per mg cell wall is negligible. Moreover, this value is sufficiently low to exclude the possibility that teichoic acid or phosphodiester-linked polysaccharides are significant cell wall constituents.

M. sodonensis peptidoglycan composition was typical of members of the Micrococcaceae. Glutamic acid, glycine, alanine, and lysine are found in approximate 1:1:2:1 ratios in peptidoglycans of M. lysodeikticus, M. flavus, and M. citreus (Campbell et al., 1969). Based upon glycine content, TCS-grown cell walls contain respectively 523 peptide subunits per mg and basal-grown cell walls contain 531 peptide subunits per mg. These data place M. sodonensis intermediate between M. lysodeikticus and M. flavus cell walls which contain respectively 480 and 610 peptide subunits per mg cell wall. Further, the peptide structure deduced by sequential Edman Degradation of the isolated peptide monomer was found to be N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)-L-lysyl-D-alanine. This is

the Type C subunit found also in S. lutea, M. flavus, M. lysodeikticus, and M. citreus cell walls. In micrococci possessing the Type C peptide subunit, L-alanine is found in linkage with either N-acetylmuramic acid residues or in the D-alanyl-L-alanine bonds which facilitate peptide cross-bridging. Moreover, L-alanine constitutes one half of the total cell wall alanine content. This assumption was made in the estimation of the amount of muramic acid-linked L-alanine (Table XIV) in M. sodonensis cell walls.

Values obtained for hexosamine content were found to vary not only between cell wall preparations, but also under the conditions by which they were obtained. Incomplete acid-mediated release of hexosamine and concomitant destruction of free amino sugars would explain the lower values obtained for intact cell walls. One would expect that acid hydrolysis would be more complete in cell walls partially solubilized by lysozyme. For these reasons, the hexosamine content determined for cell walls treated with lysozyme was considered more representative than values obtained from intact cell walls. Based upon glucosamine content, TCS- and basal-grown cell walls contained, respectively, 278 and 540 disaccharide units per mg cell wall, i.e., under differing conditions of growth, approximately twice as many disaccharide units exist for about the same number of peptide subunits.

On cursory examination, it would appear that the peptidoglycan of the basal-grown cell walls, containing disaccharide and peptide subunits in an approximate 1:1 ratio, is more highly ordered than the TCS-grown cell wall peptidoglycan. However, studies with the Myxobacter AL-1 Protease revealed that in TCS-grown preparations, 40% of the

available N-acetylmuramic acid residues are peptide substituted while only 13% of these residues are so substituted in basal-grown cell walls. Thus, it is likely that residual peptide subunits not in covalent linkage with N-acetylmuramic acid are involved in the "head-to-tail" assembly of peptides found in cross-bridges of M. lysodeikticus cell walls (Ghuysen et al., 1968a). The fact that virtually no N-terminal alanine groups and over 90% of the C-terminal alanine groups in intact cell walls are unavailable for dinitrophenylation or hydrazinolysis supports this hypothesis.

Sequential digestions of the lysozyme-soluble and -insoluble portions of both cell wall preparations with the Streptomyces N-acetylmuramic acid-L-alanine amidase and the Myxobacter AL-1 Protease substantiated this claim. Amidase digestion of these portions disclosed that not all of the L-alanine is covalently associated with N-acetylmuramic acid. Myxobacter AL-1 Protease digestion of amidase-treated cell wall fractions, however, resulted in the release of equimolar amounts of the residual N- and C-terminal alanine. This served not only as a qualitative indication of the existence of the suspected D-alanyl-L-alanine peptide bond, but also of the aforementioned peptide cross-bridging structure. Furthermore, the sum of N- and C-terminal alanine groups released by such digestion, and the amount of free C-terminal alanine groups accounts for 94.4% and 93.6% of the total alanine content of TCS- and basal-grown cell walls respectively.

Analysis of the amount of N-acetylmuramic acid-linked N-terminal alanine in whole cell walls is not an accurate reflection of the situation regarding peptide substitution of the glycan since it implies

that the peptidoglycan is uniform throughout. Whereas there was excellent agreement between the total amounts of muramic acid-linked N-terminal alanine released by Myxobacter AL-1 Protease and the Streptomyces amidase, the lysozyme-insoluble portions of both cell walls were more highly peptide substituted than the corresponding lysozyme-soluble portions. Moreover, the degree of peptide substitution was greater in both the lysozyme-soluble and -insoluble portions of TCS-grown cell walls than in the analogous fractions derived from basal-grown cell walls. Three conclusions may be drawn from these findings: first, that some areas of the peptidoglycan of intact cell walls are more highly organized than others; second, that the peptidoglycan of basal-grown cell walls is much less highly ordered than that of TCS-grown cell walls; and third, that subtle changes in growth conditions have a profound effect on peptidoglycan structure. As will be discussed later, these factors are implicated in the increased susceptibility of basal-grown cell walls to lysozyme activity.

Comparison of the amount of N-terminal alanine covalently bound to N-acetylmuramic acid and the total L-alanine content gave an indication of the length of cross-bridges in different fractions of cell wall preparations. In the lysozyme-soluble and -insoluble portions of TCS-grown cell walls, cross-bridges were calculated to be 2 to 4 peptide subunits in length. While the lysozyme-insoluble portion of basal-grown cell walls has a relatively normal cross-bridge of three peptide subunits length, the lysozyme-soluble portion is highly atypical in that cross-bridges are a calculated length of 18 to 19 subunits. It must be

emphasized that these values permit the construction of models which represent an average and probably not the actual situation.

Sequential digestion of whole cell walls with Streptomyces ML-endopeptidase, N-acetylmuramic acid-L-alanine amidase, egg white lysozyme, and Myxobacter AL-1 Protease clarified the nature of peptide cross-bridging to a large extent. Not only was the presence of the two linkages required for "head-to-tail" assembly of peptides, N^{ε}-(D-alanyl)-L-lysine and D-alanyl-L-alanine, unequivocally established, but it was also possible to obtain an idea of the size and distribution of such cross-bridges (Tables XVII and XVIII). In TCS-grown cell walls, 20% of the peptide subunits are covalently bound to the glycan backbone while the remaining 80% are found in peptide cross-bridges. Of the total peptide subunits, 15% are derived from cross-bridges one peptide subunit in length, 25% from cross-bridges three subunits in length, and 40% from cross-bridges seven subunits in length. From the studies of the distribution of the peptidoglycan amino acids in the lysozyme-soluble and insoluble portions of cell walls, it is probable that most of the short cross-bridges occur in the latter portion of cell walls. There is, however, no direct experimentation to support this hypothesis.

In basal-grown cell walls, only 12% of the total peptide subunits are covalently associated with the glycan. Although 16% of the total peptide subunits are derived from peptide cross-bridges three subunits in length, no smaller cross-bridges which account for 15% of the total peptide subunits in TCS-grown cell walls, are encountered in basal-grown cell walls. The remaining peptide subunits are apparently in cross-bridges of 17 monomeric units length. This final result likely

represents an average situation since chemical analysis were performed upon high molecular weight material which could have been a heterogeneous mixture of large peptide fragments. No attempt was made to study possible heterogeneity of peptide fragments excluded from gel filtration on the linked Sephadex G-50, G-25 system or to further fractionate them. Myxobacter AL-1 Protease digestion of such materials showed that they were not simply long peptide polymers of peptide subunits assembled in "head-to-tail" fashion since all of the material was not released as monomeric peptide subunits. Therefore, unidentified bonds that are not hydrolyzed by any of the enzymes used in this investigation must be present in sufficient quantity to maintain complex peptide structures. These findings do, however, illustrate that the mode of peptide cross-bridging is greatly altered under varying conditions of growth.

The structure of the subunit peptide of M. sodonensis cell walls and the "head-to-tail" assembly of monomeric peptide subunits to facilitate cross-bridging established in this investigation have been recently confirmed in another laboratory (Schleifer and Kandler, 1970). Peptide sequences were established by partial acid hydrolysis of purified cell walls followed by chromatographic separation and identification of the resultant peptides. Use of this technique destroys the sample thus precluding any further test to confirm the distribution and size of peptide cross-bridging structures.

Differences in the peptidoglycan of TCS- and basal-grown cell walls were not restricted to variation in the degree of peptide substitution of the glycan or length of cross-bridging. Studies of Myxobacter AL-1 Protease digests of whole cell walls revealed that nearly all of the

N^{ε}-amino groups of lysine are free in TCS-grown cell walls (95%) while approximately 25% of these groups are involved in unknown linkage(s) in basal-grown cell walls. Whereas the sum of lysine possessing free N^{ε}-amino groups and lysine in N^{ε}-(D-alanyl)-L-lysine linkages accounted for all of the lysine in the TCS-grown cell walls, it accounted for only 80% of the total lysine of basal-grown cell walls. Since approximately 25% of the glycine carboxyl groups are substituted in basal-grown cell walls, it is possible that an N^{ε}-(α-glycyl)-L-lysine linkage might occur. Myxobacter AL-1 Protease digestion of these cell walls released 45 nmoles per mg C-terminal glycine and 28 nmoles per mg of the N^{ε}-amino groups of lysine. A similar but lower release of such groups was also observed in TCS-grown cell walls. Such a lysine-glycine bond is, therefore, possible but another unknown linkage involving residual glycine carboxyl groups must also exist. It is possible that the α-carboxyl groups of glycine are not peptide bonded to other amino acids but are amidated and, therefore, not detectable by the hydrazinolysis technique. Amidation of α-carboxyl groups of DAP occurs in some strains of Corynebacteria, and the α-carboxyl group of glutamic acid is known to be amidated in the cell walls of S. aureus strain Copenhagen, M. roseus R27, S. pyogenes, and C. diphtheriae (Ghuysen et al., 1968b).

These results implied that the Myxobacter AL-1 Protease activity is not restricted to cleavage of the N-acetylmuramyl-N^{α}-L-alanine and the D-alanyl-L-alanine linkages found in intact peptidoglycans. Hydrolysis of linkages other than those described has been observed in S. aureus cell walls in which N- and C-terminal glycine is released in addition to N- and C-terminal alanine (Cole, 1965; Jarvis and Strominger,

1967). Thus, hydrolysis of an N^{ε}-(α-glycyl)-L-lysine linkage by this enzyme is a possibility which cannot be excluded. Furthermore, although this enzyme possesses a broad bacteriolytic spectrum, and completely solubilizes cell walls of M. sodonensis,its activity on the D-alanyl-L-alanine linkages appears to be greatly enhanced by pre-solubilization of walls with other enzymes. Although there have been no reports of the effect of peptide configuration on the activities of this enzyme, it may well be that it is so affected in this system.

When M. sodonensis was grown on different media, significant changes occurred in the cell wall peptidoglycan. Such changes did not simply involve substitution of one peptide amino acid for another as seen in S. faecalis and S. aureus cell walls (Smith and Henderson, 1964; Smith and Gilboe, 1965). Rather, variations were found to be in the degree of peptide substitution of the glycan and length of peptide cross-bridges. These variations were undoubtedly reflections of the response of certain cell wall biosynthetic enzymes to differing environments. Large regions of unsubstituted glycan could result from removal of the peptide from disaccharide-peptide units in the intact peptidoglycan. Although the reaction sequence for the biosynthesis of disaccharide-peptide units and their insertion into cell walls is fairly well documented, little is known of the control mechanisms responsible for its regulation.

A biosynthetic mechanism involving successive alternating trans-peptidation and amidase reactions has been postulated for the formation of the "head-to-tail" assembly of peptide units in the peptidoglycans of M. lysodeikticus and related Micrococcaceae (Ghuysen, 1968; Schleifer

and Kandler, 1967). The sequence of events proposed is as follows: the penultimate D-alanine residue of a peptide (B) on one glycan strand is transferred to the lysine residue of another peptide (A) on an adjacent glycan strand with the concomitant loss of the terminal D-alanine residue. Following this transpeptidation, the N-acetylmuramic acid-L-alanine linkage of peptide B is cleaved. A subsequent transpeptidation results in the penultimate D-alanine residue of yet another peptide (C) on a glycan strand adjacent to the one which carried peptide B being bound to the N-terminal L-alanine residue of peptide B and simultaneous expulsion of one D-alanine residue. These reactions are repeated in sequence until a cross-bridge having several subunit peptides is formed. Not only does this postulated sequence account for head-to-tail assembly of subunit peptides, it also decreases the degree of peptide substitution of the glycan. The extent to which such a system functions must be under the control of a delicate set of checks and balances that can be altered under different environmental (i.e. growth) conditions. In addition to the amidase, two different transpeptidases whose specifities would depend upon the structure of the amino acceptor (i.e., the N^{ε}-amino groups of L-lysine or the α-amino groups of L-alanine) are required for this to be a functional scheme. Proof of the existence of this system and characterization of the enzymes involved is the subject of much current investigation. Elucidation of their nature would do much to explain how peptidoglycan structure is regulated, at least in the Micrococcaceae.

Non-peptidoglycan components of M. sodonensis cell walls were similarly found to vary under differing growth conditions. Four

different polysaccharides were isolated from the two wall preparations. None of the polysaccharides isolated were covalently bound to peptidoglycan components but neither could they be released without prior enzymic digestion of cell walls. They must, therefore, be interwoven into the peptidoglycan matrix in such a fashion as to be designated true cell wall components. Polysaccharides isolated from basal-grown cell walls were fairly homogeneous with respect to chain length, although the polysaccharide of the lysozyme-insoluble portion contained glucuronic acid in addition to rhamnose and glucose. The polysaccharide isolated from the lysozyme-soluble portion of TCS-grown cell walls was an octasaccharide composed entirely of glucose. In contrast, the polysaccharide from the analogous lysozyme-insoluble portion contained glucose, rhamnose, and glucuronic acid and was approximately 83 monosaccharide units in length. Isolation of these moieties was performed, not to fully characterize them, but rather to demonstrate that their composition changed under altered growth conditions and that they were not covalently associated with peptidoglycan components. The physiological role of cell wall polysaccharides is subject to speculation; they are frequently implicated as antigens but the possibility exists that they might be metabolic sinks.

Results of immunological studies indicate that the non-peptidoglycan carbohydrate components of M. sodonensis cell walls are involved with cell wall antigenicity. Cell wall protein made no detectable contribution to any immunological reactions performed. However, lysozyme digestion of cell walls was found to have a profound effect on such reactions. There was no significant reaction between either of the

immune globulin fractions of rabbit anti-cell wall sera and the lysozyme-insoluble portion of basal-grown cell walls. On the basis of alanine: muramic acid ratios (Table XIII), the lysozyme-insoluble portion of basal-grown cell walls is known to be enriched in peptidoglycan components. Furthermore, the organization of peptidoglycan components is much more complex than in the lysozyme-soluble portion. However, the lysozyme-insoluble portion of basal-grown cell walls contains only one-fifth of the total cell wall glucose. Previous studies established that all of the glucose of basal-grown cell walls occurred in high molecular weight non-peptidoglycan heteropolymers. These facts, and the data presented in Table XXI suggest that cell wall antigenicity resides not in the peptidoglycan, but in the non-peptidoglycan carbohydrate components. The high titres observed in tube precipitation tests using the non-peptidoglycan carbohydrate-enriched lysozyme-soluble portion of cell walls support this premise.

Lysozyme-susceptibility of the two cell wall preparations was markedly different. Whereas 49% of the TCS-grown cell wall was solubilized with the concomitant release of only large molecular weight peptidoglycan fragments, basal-grown cell walls, when treated with lysozyme, were 75% solubilized. Furthermore, these digests contained a heterogeneous mixture of wall fragments. Several factors are known to affect the extent to which cell walls are solubilized by lysozyme: the complexity of the peptidoglycan net, esterification of bacterial cell walls, and various stereochemical effects.

The tightness of the peptidoglycan matrix depends upon the frequency with which glycan strands are substituted by peptide subunits,

and upon the frequency with which these peptide subunits are interlinked. Lysozyme-treatment of a cell wall possessing an extensively cross-linked peptidoglycan net need not necessarily produce solubilization of the wall; although many glycosidic linkages may have been hydrolyzed, the high degree of cross-linking serves to keep the matrix intact. Such is the situation in the cell walls of S. aureus (Ghuysen, Strominger, and Tipper, 1968). In organisms possessing a "loose" peptidoglycan, such as M. lysodeikticus (Leyh-Bouille et al., 1966), lysozyme digestion produces rapid and complete clarification of cell wall suspensions.

O-acetylation of bacterial cell walls has been implicated as a mechanism of lysozyme resistance (Brumfitt and Wardlaw, 1958). Walls of M. lysodeikticus cells grown on solid media containing microgram amounts of lysozyme were found to be O-acetylated. Removal of O-acetyl groups restored lysozyme-sensitivity indicating that this organism's ability to acetylate certain cell wall hydroxyl groups was associated with its diminished response to this N-acetylhexosaminidase activity. Similar removal of the O-acetyl groups of S. aureus cell walls, which involve about half of the N-acetylmuramic acid residues, did not confer lysozyme susceptibility (Ghuysen and Strominger, 1963a, 1963b). This may appear paradoxical unless one considers that S. aureus possesses a highly cross-linked or "tight" peptidoglycan matrix in which every glycosidic linkage would have to be cleaved to produce solubilization. Responses elicited to lysozyme are, therefore, varied and depend upon several factors.

The varied response of M. sodonensis cell walls to lysozyme involves peptidoglycan complexity and the presence of cell wall O-acetyl groups. Lysozyme-insoluble portions of both TCS- and basal-grown cell

walls are more highly peptide substituted and have shorter cross-bridges than their respective lysozyme-soluble portions. Moreover, as seen from the ester:muramic acid ratios, these portions are enriched in ester content. Soluble portions were markedly less complex with respect to their peptidoglycan structure, particularly the lysozyme-soluble portion of basal-grown cell walls and contained fewer O-acetyl groups. Overall, the TCS-grown cell walls had a more complex peptidoglycan structure and higher levels of esterification; it was, therefore, not surprising to find a greater portion of this cell wall was not solubilized by lysozyme.

Electron microscopic examination of various lysozyme digested cell wall preparations revealed that bacterial shape was retained even though 75% of the wall had been solubilized. This finding excluded the possibility that the lysozyme-sensitive and -resistant peptidoglycan polymers are covalently integrated to form the structural material of the cell wall. Were this so, lysozyme-mediated solubilization of the susceptible material would have abolished structural integrity. Moreover, the fact that the cell wall thickness is unaltered by lysozyme digestion precludes the theory that the lysozyme-sensitive and -resistant portions exist as separate lamellar structures in the intact cell wall. While wall thickness was unaltered by lysozyme digestion, the cell wall matrix was perceptibly more diffuse than that observed in electron photomicrographs of whole cell wall preparations. These observations suggest that the native cell wall is composed of at least two interwoven peptidoglycan nets, one of which is resistant to lysozyme because of extensive peptide cross-bridging and increased levels of O-acetylation.

Deacetylation of cell walls established unequivocally that

O-acetylation was involved with the lysozyme susceptibility of the cell wall preparations. The alkali labile O-acetyl bond is hydrolyzed by incubation of the cell walls in 0.01 N NaOH. Treatment of cell walls with 0.05 N NaOH at 37°C results in the release of a lactylpeptide (Tipper, 1968). However, since the soluble portion of deacetylation mixtures investigated in this study contained no amino acids, it was assumed that base-catalyzed lactyl-elimination from N-acetylmuramic acid had not occurred.

Deacetylated cell walls and the lysozyme-insoluble portions thereof were examined for their response to lysozyme. Such studies showed that in whole cell walls, deacetylation permitted lysozyme hydrolysis of an additional 65 glycosidic linkages per mg cell wall. Deacetylation of the lysozyme-insoluble portion of cell walls made them sensitive to lysozyme, although complete solubilization of such mixtures did not occur. Again, the greater levels of O-acetylation found in TCS-grown cell walls probably reflects the response of cell wall biosynthetic enzymes to different environments.

The mechanism of lysozyme activity has been widely investigated and its dependence upon stereochemical factors noted (Raferty and Dahlquist, 1969). Binding studies with various compounds have shown that substrate molecules must undergo certain structural modifications before enzymic hydrolysis is initiated. Presence of substituent groups, such as the O-acetyl group, disallows these modifications and therefore, prevents hydrolytic activity. In addition, the inability of the lysozyme molecule to approach the sensitive β1:4 linkage may account for reduced activity. It is probably the outer layers of lipid or lipoprotein of

Gram-negative cell walls that confers upon them lysozyme resistance. Although the polysaccharides of M. sodonensis cell walls are not covalently bound to the peptidoglycan, it is possible that they confer some degree of lysozyme-resistance by physically blocking lysozyme-sensitive linkages. It is difficult to establish this experimentally because these moieties cannot be removed without partially or completely solubilizing the cell walls. Another type of interaction which likely involves physical blocking of lysozyme-sensitive linkages has been encountered in these cell walls. Pre-treatment of deacetylated cell walls with the Myxobacter AL-1 Protease allowed more glycosidic bonds to be hydrolyzed by lysozyme. Since O-acetyl groups had been removed, steric hindrance to lysozyme should have been minimal. Therefore, peptide substitution or the configuration of peptide bridges also plays a decisive role in lysozyme susceptibility of the cell walls of this organism.

Several enzymes are known to be intimately associated with cell walls (Young, 1966; Tipper, 1968a; Nisonson et al., 1969). With the exception of the 5'-nucleotidase of E. coli which is cell wall-bound (Nisonson et al., 1969), all the wall-associated enzymes described to date are autolytic in nature. Their function is supposedly to loosen the peptidoglycan matrix sufficiently to facilitate insertion of new cell wall material. While the presence of nuclease in M. sodonensis cell walls is not a unique phenomenon, the existence of a cell wall-associated enzyme which is not involved in wall biosynthesis or degradation suggests that the wall may have unsuspected metabolic functions. The designation of the extracellular nuclease of M. sodonensis as a cell wall component is justified on several points. Previous data established that

nuclease activity persists in cell walls during all but the last stage of purification (Berry et al., 1970). A histochemical technique employing deposition of lead indicated that this activity was associated only with the cell walls and not the periplasmic space or cell membrane. Third, trypsin- or alkali-mediated release of protein from pre-trypsin cell walls was demonstrable. Undoubtedly, all or a portion of protein so removed represented nuclease or nuclease precursor. This investigation offers further evidence that the role of cell walls is not solely limited to the maintenance of structural integrity. Presence of the flavin-like substance, which could function as an electron acceptor or donor implies that the cell wall is the seat of considerable metabolic activity.

Immunological studies showed that there is a definite relationship between purified cell walls and the extracellular nuclease. Previously, Berry documented a serological cross-reaction between purified nuclease and whole rabbit anti-cell wall serum (Berry, 1969). This reaction had no appreciable effect on enzyme activity and led to the conclusion that serological cross-reaction must be with the carbohydrate moiety of the glycoprotein. A cross-reaction between the immune globulin fraction of rabbit anti-nuclease serum and pre-trypsin and pure cell walls was observed in this investigation. Such behavior substantiates the hypothesis that antigenicity resides in the carbohydrate moiety since reactions occurred in both pre- and post-trypsin cell walls. Presumably, all protein which could have participated in such a reaction was removed by trypsin digestion.

The study of the composition and structure of M. sodonensis cell walls has been highly stimulating and interesting. Although much has

been learned from this investigation, the work is by no means complete. Elucidation of the involvement of wall in nuclease formation and release, identification of still unknown peptidoglycan bonds, and the means by which altered cell wall structures are produced under changes in growth conditions are only some of the more challenging aspects of the problem and merit further investigation.

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