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Studies on the Teichoic Acids of the Pneumococcus

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STUDIES ON THE TEICHOIC ACIDS OF THE PNEUMOCOCCUS:

I. Segregation of the Wall Teichoic Acid during Cell Growth and Division;

II. Identification of the Pneumococcal Forssman Antigen as a Membrane Lipoteichoic Acid

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Preface

To give full credit where credit is due would more than fill this page ... and would mark me as a sentimentalist. I shall therefore try to be brief, and limit my acknowledgments to those individuals without whose contributions to my graduate-school education this thesis would never have been written:

1 - my thesis advisor, Prof. Alexander Tomasz: I want to thank him for permitting me to work in his laboratory; and even more importantly, I want to thank him for taking seriously his role as educator during my graduate-school training.

2 - my husband, David Briles: Not only did the fellow marry me, but he also taught me how to bleed rabbits!

ABSTRACT

The investigations described in this dissertation are presented in two parts.

Part I deals with the mode of segregation of the pneumococcal wall teichoic acid during cell growth and division. Teichoic acid was labeled with ^{3}H-choline (a teichoic acid-specific constituent of pneumococci) or an analogue, and its distribution among progeny cells over several generations was followed by autoradiography. The results confirm the zonal-growth model for Gram-positive coccal cells.

In Part II, the pneumococcal Forssman antigen is identified as a membrane lipoteichoic acid. The following lines of evidence suggest that the teichoic acid chains of the molecule may be identical to those found in the cell wall: (1) they are both composed of the same chemical constituents, including choline; (2) they are both cleaved by periodate and by nitrous acid into choline-containing fragments of similar size; (3) previous investigators demonstrated serological cross-reactivity between the two (Goebel, W. and Adams, M.H., 1943, J. Exp. Med. 77:435-449). Membrane localization of the F-antigen is suggested by the following observations: (1) the F-antigen is accessible from the surface of whole pneumococci; (2) it is not solubilized by muralytic enzymes; (3) it contains lipophilic regions, and is associated with a particulate cell fraction by hydrophobic interactions. The possibility that the lipoteichoic acid may be a precursor to the wall teichoic acid is ruled out by pulse-chase studies.

TABLE OF CONTENTS

Page

INTRODUCTION

The purpose of this Introduction is to offer a brief overview of the current general knowledge concerning bacterial surfaces, in order to provide an appropriate perspective for the investigations which form the basis of this dissertation. A great deal of information is currently available on this subject; this overview is by no means intended to cover the field with any degree of thoroughness. Many review articles, on various aspects of bacterial surfaces, have recently appeared in the literature, and the reader is referred to several of these reviews in the paragraphs that follow.

Bacterial Envelopes

General remarks: From a simple standpoint, the bacterial envelope consists of two parts: the plasma membrane, which surrounds the cytoplasm of the bacterial cell; and the cell wall, which is located just exterior to the plasma membrane. A mucilaginous "capsule" may or may not be located exterior to the cell wall.

The plasma membrane of bacteria is essentially similar to the membranes of eukaryotic cells. It is composed primarily of lipids and proteins. In this sections, viewed by electron microscopy, the membrane is $\sim$ 75 Å thick, and under appropriate fixation and staining conditions appears as a "double track." A substantial number of enzyme activities which are generally associated with membranes in eukaryotic cells have been shown to be associated with bacterial plasma membranes; these include phosphorylation (e.g., ATPase, hexokinase), transport of molecules and ions, electron transport, lipid metabolism, etc. (Kaback, 1973; Salton, 1964; 1967). Also, the biosynthesis and extracellular transport of wall components (e.g., peptidoglycan, lipopolysaccharide) has been shown to be associated with the plasma membrane (Ghuysen, et al., 1968; Glaser, 1973; Salton, 1967). Bacterial membranes have been the subject of several recent review articles (e.g., Machtiger, et al., 1973; Salton, 1967).

The bacterial plasma membrane differs significantly from that of eukaryotic cells in that, in eukaryotic cells, the membrane serves as the direct communicator between the cell and its environment, whereas in bacteria, this role is largely assumed by the cell wall. The cell wall is also responsible for the maintenance of cell shape (Salton, 1964; Weidel & Pelzer, 1964; Henning & Schwarz, 1973), and for at least some osmotic protection of the bacterial cell; the latter is demonstrated by the fact that spheroplasts and protoplasts (bacteria which have "lost" their normal walls, through the action of either wall-lytic enzymes, such as lysozyme, or inhibitors of wall synthesis, such as penicillin) require much greater osmotic protection from their environment (e.g., high salt or sucrose concentrations) than do the bacteria from which they were derived (Mitchell & Moyle, 1957; Salton, 1964). Bacterial walls seem to be of two general types, correlated with whether the bacterium is Gram-positive or Gram-negative. This distinction is based on the Gram staining procedure: bacteria which retain the purple color of the crystal violet-iodine complex, despite washing with alcohol, are Gram-positive, while those which are discolored by the alcohol extraction are Gram-negative. The correlation between Gram-type and wall structure is based on empirical observation; to date, the actual basis for the Gram-stain differentiation is not known (Salton, 1964; Bartholomew & Mittwer, 1952).

The Gram-positive wall: In electron-microscopic cross-section, the walls of Gram-positive bacteria generally appear as uniformly-staining structures, 150-800 Å thick (Salton, 1964). The plasma membrane appears to make frequent contact with the inner face of the wall. The walls exhibit no obvious differentiation into layers, although sometimes the inner and/or outer faces appear slightly darker-staining than the rest of the wall.

The cell wall is composed of cross-linked polymers; it is the high degree of cross-linking which presumably imparts the characteristic rigidity of shape to the wall. The "ubiquitous" cell wall structural polymer is the peptidoglycan (murein); in a typical Gram-positive

organism (Diplococcus pneumoniae), this comprises ∿ 50% of the dry weight of the isolated wall (Mosser & Tomasz, 1970). The peptidoglycan consists of "glycan" strands of alternating N-acetyl-D-glucosamine and N-acetyl-muramic acid (unique to bacterial walls) in β-1,4 linkage (Ghuysen, 1968; Ghuysen, et al., 1968; Salton, 1964). Attached via amide linkage to the carboxyl groups of at least some of the muramic acid residues are short peptides whose amino acid sequences vary from species to species; examples of typical structures for such peptides are: muramyl-L-alanyl-D-glutamyl-γ-L-lysyl-D-alanine (e.g., Staphylococcus aureus, Streptococcus pyogenes), or muramyl-L-alanyl-D-glutamyl-γ-diaminopimelyl-D-alanine (e.g., Bacillus subtilis). Cross-linking of the peptidoglycan is achieved by the covalent linkage of these peptides to one another, either directly (via amide linkage between the carboxyl terminal of one peptide and a free amino group, e.g., on lysine or diaminopimelate, on the other), or through a "bridging" peptide (Ghuysen, 1968; Ghuysen, et al., 1968; Salton, 1964).

In addition to the peptidoglycan, the Gram-positive wall may also contain additional polymeric wall components, such as carbohydrates (e.g., the Group-specific somatic carbohydrates of streptococci) (Lancefield, 1941; McCarty, 1970; Salton, 1964), proteins (e.g., the type-specific M proteins of streptococci) (Lancefield, 1941; 1962; Salton, 1964), teichoic acids (polymers of ribitol phosphate or glycerol phosphate, discussed in greater detail below), and teichuronic acids (uronic acid-containing polymers) (Glaser, 1973; Salton, 1964; Wu & Park, 1971). Such "auxiliary" wall polymers are probably all covalently bound into the wall.

The Gram-negative wall: In electron-microscopic cross-section, the Gram-negative cell envelope is seen to consist of three distinct layers: the rigid, thin peptidoglycan layer (Salton, 1964; Weidel & Pelzer, 1964) (∿ 20-30 Å) is sandwiched between two membranes, the plasma membrane and the outer, or wall, membrane. As is the case in Gram-positive bacteria, the plasma membrane makes frequent contact with the peptidoglycan layer (Bayer, M.E., 1968; Bayer, M.E., 1968). The outer membrane is a true membrane: under appropriate staining conditions, it

appears as a 75-85 Å bilayer in cross-section, and it is rich in phospholipids and proteins; the structural integrity of this membrane is highly dependent on divalent cations. The outer membrane contains the lipopolysaccharide (LPS) of the Gram-negative envelope. The LPS is a complex heteropolysaccharide, covalently bound to a specific glycolipid known as "lipid A." The polysaccharide portion consists of a "core" region of invariant structure, and an "O-antigen" region which determines the antigenic specificity (serotype) of the bacterial strain. The structure and biosynthesis of LPS has been extensively reviewed elsewhere (Osborn, 1969; Nikaido, 1973).

In E. coli, an interesting "structural protein" has been described, which is covalently bound at one end to peptidoglycan and at the other end to a lipid which is believed to be associated with the outer membrane; this protein thus serves as a "bridge" between these two envelope layers (Braun & Bosch, 1972; Glaser, 1973). It is likely that such "vertical" components exist in the envelopes of other bacteria.

The Walls of extreme halophiles: The halobacteria (Halobacterium halobium, H. salinarium) require high environmental salt concentrations (4M) in order to maintain the structural integrity of their walls; in solutions of low molarity, these bacteria lyse (Stoeckenius & Kunau, 1968; Stoeckenius & Rowen, 1967). The extremely halophilic cocci (Sarcina litoralis, S. morrhuae) do not lyse in dilute solutions, but do lose their Gram-positive character (Brown & Cho, 1970; Henning & Schwarz, 1973). These halophiles contain no peptidoglycan and no muramic acid. The walls of the halobacteria are composed of proteins (Stoeckenius & Kunau, 1968; Stoeckenius & Rowen, 1967); those of the halophilic Sarcinae seem to consist largely of polysaccharides containing relatively little amino sugars (Brown & Cho, 1970).

Teichoic acids: Teichoic acids are found among the Gram-positive bacteria (and some closely-related Actinomycetes) (Archibald & Baddiley, 1966). In their simplest form, they consist of either polyglycerophosphate or polyribitolphosphate. Most teichoic acids contain additional substituents, usually amino acids (e.g., D-alanine in ester linkage) and/or

sugars (in glycosidic linkage) as side chains attached to hydroxyl groups of the glycerol or ribitol residues; less frequently, substituents may be intercalated into the linear backbone of the polymer (Archibald & Baddiley, 1966; Archibald, et al., 1968; Knox & Wicken, 1973).

The teichoic acids occur in two forms: as "wall" teichoic acids and "membrane" teichoic acids. The wall teichoic acids are covalently associated with the peptidoglycan, probably through a phosphate bridge to muramic acid residues in most or all cases (Knox & Wicken, 1973; Archibald, et al., 1968); they may account for up to 50% of the dry weight of the wall (Archibald & Baddiley, 1966; Salton, 1964). It has been reported that walls of Gram-positive bacteria do not always contain teichoic acids (Archibald & Baddiley, 1966; Glaser, 1973; Knox & Wicken, 1973) and it has been reported that sometimes more than one "species" of teichoic acid may occur in the wall of a single bacterium (Glaser, 1973; Knox & Wicken, 1973); some bacteria produce wall teichoic acids only under particular growth conditions (Glaser, 1973; Knox & Wicken, 1973), and it may be that some bacteria produce different kinds of teichoic acids under different conditions. In general, each species of Gram-positive bacteria seems to produce a characteristic wall teichoic acid (Archibald & Baddiley, 1966; Archibald, et al., 1968). The teichoic acids are usually antigenic, and sometimes account for certain species-characteristic antigenic activities (e.g., the somatic C carbohydrate of pneumococci, the group D antigen of streptococci, the "polysaccharide A" of staphylococci) (Archibald & Baddiley, 1966; Knox & Wicken, 1973; Salton, 1964). In some bacteria, the wall teichoic acids serve as receptor sites for bacteriophages (Glaser, 1973).

The membrane teichoic acids, or lipoteichoic acids, are associated with the plasma membranes of the cells; they are presumably anchored in the membrane structure by the lipid moieties (glycolipid) to which they are covalently bound (Coley et al., 1972; Knox & Wicken, 1971; 1973; Toon et al., 1972). Lipoteichoic acids are believed to occur in all Gram-positive bacteria, whether or not the bacteria contain wall teichoic acids (Knox & Wicken, 1973; Archibald & Baddiley, 1966; Archibald, et al., 1968). Until

recently, the only lipoteichoic acids known were all of the polyglycerophosphate variety (Archibald & Baddiley, 1966; Archibald, et al., 1968; Knox & Wicken, 1973); although some ribitol-containing teichoic acid was occasionally found in non-wall fractions, such materials were considered to be contaminants due to partial disintegration of walls during the cell-fractionation procedure (Archibald & Baddiley, 1966). However, it is now known that pneumococci possess a ribitol-containing lipoteichoic acid (Briles & Tomasz, 1973; see Part II of this dissertation), and it is likely that ribitol-containing lipoteichoic acids will be found in other bacteria also.

Not very much is known about the biosynthesis of teichoic acids. Teichoic synthetases have been found in Bacillus subtilis and B. licheniformis (Glaser, 1973; Mauck & Glaser, 1970) which catalyze the formation of polyglycerophosphate from CDP-glycerol precursors; these enzymes are firmly associated with the plasma membranes of these bacteria. Recently, the B. subtilis enzyme has been solubilized with non-ionic detergents (Mauck & Glaser, 1970), and was shown to require a heat-stable "acceptor" molecule which is probably the membrane lipoteichoic acid; the significance of the requirement of this enzyme for a "primer" is not yet clear. Undecaprenol phosphate, a lipid involved in the biosynthesis of peptidoglycan (Ghuysen, et al., 1968; Osborn, 1969), has been implicated in the biosynthesis of wall teichoic acids (Glaser, 1973), but its role is still a matter of controversy.

The teichoic acids have been the subject of several recent review articles, to which the reader is referred for more detailed information (Archibald & Baddiley, 1966; Knox & Wicken, 1973; Archibald, et al., 1968).

Growth of the Bacterial Surface

Growth of the membrane: The problems of membrane growth and assembly are far too complicated to be dealt with in this Introduction; the reader is referred to a recent review article which provides a good summary of the questions involved and the kinds of experiments which have been performed in efforts to answer them (Machtiger & Fox, 1973). The

interpretation of many of these experiments, particularly those which try to answer the question of whether or not membrane growth occurs at a limited number of fixed sites on the cell surface (Green & Schaechter, 1972; Lin, et al., 1971; Machtiger & Fox, 1973), is made especially difficult by the fact that the plasma membrane is fluid; that is, diffusion readily occurs in the plane of the membrane, and thus randomization of membrane elements may occur even if the initial incorporation of these elements into the membrane is not itself a random process.

In the special case of the outer membrane of Gram-negative organisms, the elements of this membrane (e.g., lipopolysaccharide) are synthesized at the plasma membrane, and must be transported through the rigid layer of the wall to the outer membrane (Glaser, 1973; Kulpa & Leive, 1972; Nikaido, 1973); again, the possibility of diffusion in the planes of both membranes makes detection of fixed sites of transport (should they exist) virtually impossible.

Growth of the wall: The biosynthesis of peptidoglycan and of lipopolysaccharide (LPS) are understood in rather elaborate detail (Osborn, 1969; Nikaido, 1973); however, the mode of assembly of the cell wall is not nearly so well understood. The method by which bacterial cells increase their surface area during growth and division has been a subject of investigation and some controversy for some time now.

It is generally believed that coccal-shaped bacterial walls grow in a manner that is significantly different from rod-shaped walls. This belief is based in part on empirical evidence (see below), and in part on the notion (which is probably an oversimplification) that the rod-shape is more complex than the coccus, because it consists of "coccus-type" ends plus cylindrical midportions. There is some evidence for this topological distinction: Hartmann, Höltje, and Schwarz (1972) have observed that there are two distinct targets of penicillin action in E. coli, one of which seems to be involved with cellular elongation (cylinder formation), while the other seems to be involved with cell division (septum or "coccus-shaped end" formation).

The early experiments on cell-wall growth are reviewed in detail by Cole (1965). Studies in which "old" and "new" cell surfaces were visually differentiated by staining with fluorescently-labeled antibodies suggested that, in Gram-positive cocci (e.g., streptococci, pneumococci) (Cole & Hahn, 1962; Wagner, 1965), old walls were conserved as intact hemispheres, while new wall material was inserted between the old hemispheres of the growing cells. Similar immunofluorescence studies on Gram-negative bacilli (e.g., E. coli, Salmonella) suggested that "new" and "old" wall materials were randomly mixed on the cell surface (Cole, 1965; May, 1963). Such a result might have been expected, because the antigenic sites to which the labeled antibodies were directed were situated in the outer membrane and could thus probably diffuse in the plane of that membrane. However, the autoradiography experiments of Van Tubergen and Setlow (1961), in which tritiated diaminopimelic acid was used to specifically label the peptidoglycan of E. coli, seemed to be in agreement with the immunofluorescence findings on this organism.

The conclusion drawn from these results was that Gram-positive cocci increased their surface areas by the addition of new wall materials at "growing zones" located at the bacterial equators, while Gram-negative rods increased their surface areas by the addition of new wall material at points all over the surfaces of the cells.

More recent experiments, in which "old" wall was distinguished from "new" wall by wall-specific radiolabel (Barak & Tomasz, 1970; Briles & Tomasz, 1970; see Part I of this dissertation), resistance to a muralytic enzyme (Tomasz, et al., 1971), or differences in wall thickness (Shockman & Higgins, 1971), have amply confirmed the zonal-growth model for coccal walls. Interestingly, this model seems to apply only to the normal growth of the wall surface area; e.g., under abnormal growth conditions, wall synthesis per se need not necessarily be limited to an equatorial region of the cell (Higgins & Shockman, 1970; Shockman, 1965; Shockman & Higgins, 1971).

Unfortunately, the question of how the wall surface area is increased during cell growth and division has not yet been as unambiguously

answered for rod-shaped bacteria as it has been for cocci. Uniform dispersion of old wall among E. coli progeny cells, first observed by Van Tubergen and Setlow (1961), has recently been confirmed by Lin, et al. (1971). However, by labeling "new" wall with a pulse of tritiated diaminopimelic acid, Ryter, et al. (1973) have shown that, in isolated sacculi (rigid peptidoglycan layer) of E. coli, new material first appears at a "growth zone" at the center of the cell, but that this new wall material becomes rapidly redistributed over the whole surface (within a quarter of a generation-time). Mauck and Glaser (1972) and Glaser and Mauck (1973) have reported that "old" wall material of B. subtilis and B. megaterium (Gram-positive rods) becomes uniformly dispersed among progeny cells, as is the case for Gram-negative rods. However, these bacteria are unusual in that they exhibit a remarkably high rate of wall turnover; 50% of the peptidoglycan and teichoic acid is lost per generation, and this loss seems to occur randomly over the whole cell surface (Mauck & Glaser, 1970; Glaser, 1973).

Muralytic enzymes probably play a significant, though as yet relatively obscure, role in wall growth and morphogenesis; the reader is referred to several recent articles in the literature for more detailed information (Heptinstall et al., 1970; Tomasz, 1968; Tomasz & Westphal, 1971; Tomasz, et al., 1971; Glaser, 1973).

With respect to the question of whether the entire wall is assembled in a synchronous fashion, the early immunofluorescence data on streptococci suggested that this was the case (Cole, 1965); however, more recent studies by Swanson, et al. (1969) and by Shockman and Higgins (1971) and Higgins and Shockman (1970) on M-protein incorporation and peptidoglycan incorporation, suggest that, at least under special circumstances, wall components can be added to pre-existent wall.

The Pneumococcal Cell Surface

Capsules: Bacterial capsules are composed of polymer gels located exterior to the cell wall. These gels are usually composed of highly-hydrated polysaccharides (containing 90% water), and shrink drastically upon desiccation; fully hydrated, they are usually at least 2000 Å thick.

Their presence on the bacterial surface is not readily visualized by direct microscopy, due to their low density and high water content; but they can be made visible by light microscopy, either by negative staining with India ink, or by reaction with capsule-specific antibodies (Neufeld's "swelling" reaction). Capsules are generally not necessary for bacterial viability; encapsulated bacteria ("S," or smooth-colony-forming strains) can be stripped of their capsules, either mechanically or enzymatically, with no loss of viability; and non-encapsulated ("R," or rough-colony-forming) mutant strains can be isolated (Salton, 1964; Burger, 1950; Davis, et al., 1967; White, 1938; Wilkinson, 1958).

The pneumococcal capsule consists solely of the type-specific polysaccharides plus water. The type-specific capsular polysaccharides are of tremendous historical significance, since they figure prominently in the development of modern immunochemistry, genetics, and the pre-antibiotic methods of treatment and prevention of bacterial diseases (Burger, 1950; Davis, et al., 1967; White, 1938; Heidelberger, 1956; Corner, 1964).

Until the discovery, by Avery and Heidelberger (Avery & Heidelberger, 1923; Heidelberger & Avery, 1923), that the pneumococcal capsular antigens were carbohydrates, it had been generally believed that all immunogens were proteinaceous. This fundamental breakthrough in immunochemistry paved the way for the now-classical studies on the relationship between the specific chemical structure of an antigen and the recognition of that antigen by antibodies (Heidelberger, 1956; Davis, et al., 1967).

The ability of pneumococci to undergo genetic "transformation" had been recognized as early as 1928. The experiments of Griffith (1928), Dawson and Sia (1931), and Alloway (1933) demonstrated the existence of a "transforming principle" which was capable of transferring heritable capsule properties from one strain of the bacteria to cells of another strain. The classical work of Avery, MacLeod, and McCarty (1944) established that the "transforming principle" consisted of DNA; their study represents the first implication of DNA as the source of genetic information.

The pneumococcal capsule is responsible for the pathogenicity of this bacterial species; rough strains are nonpathogenic, presumably because the absence of the capsule permits the bacteria to be ingested by

the phagocytic cells of the immune system. Encapsulated bacteria resist phagocytosis unless they are coated with capsule-specific antibodies. Prior to the introduction of antibiotics (e.g., sulfonamides, penicillin) in the late nineteen-forties, pneumococcal pneumonia was treated (in the first half of the twentieth century) by the administration of type-specific antiserum (usually obtained from horses). Such treatment required the precise serological identification of the infecting pneumococcal "type" (Burger, 1950; Corner, 1964; White, 1938; How, et al., 1964).

Studies of the type-specific antigenic properties of pneumococci led to the discovery, by Tillet and co-workers in 1930, of a "somatic" or "C"-antigen, which is common to all pneumococci regardless of encapsulation (Tillet & Francis, 1930; Tillet, et al., 1930). The C-antigen is now known to be the wall teichoic acid of the pneumococcus, and is discussed in greater detail below.

The pneumococcal cell wall: The pneumococcal cell wall is typical of Gram-positive coccal walls in general. It is 170 Å thick (Tomasz, et al., 1964), and contains a peptidoglycan essentially similar to those of Staphylococcus aureus or Streptococcus pyogenes (see above)(Mosser, 1970; Mosser & Tomasz, 1970). The peptidoglycan accounts for ∿ 50% of the dry weight of isolated (trypsin-treated) cell walls (Mosser, 1970; Mosser & Tomasz, 1970); the remaining 50% is accounted for by the teichoic acid (Mosser, 1970; Mosser & Tomasz, 1970). The pneumococcal teichoic acid is perhaps the most complex teichoic acid studied to date, and contains phosphate, ribitol, glucose, galactosamine, choline, and a trideoxydiaminohexose (in molar ratio 2:1:1:1:1:1) (Brundish & Baddiley, 1968). The precise structure of this teichoic acid is not yet known; however, Brundish and Baddiley have demonstrated that the ribitol (as ribitol phosphate) and diaminotrideoxyhexose residues are both in the linear backbone of the chain, and that there is a β-linked phosphogalactosaminylglucose disaccharide unit either within the linear backgone or as a side-chain substituent (Brundish & Baddiley, 1968). The teichoic acid chains are covalently linked to the glycan strands of the peptidoglycan, probably through phosphate bridges at muramic acid residues (Liu & Gottschlich, 1967; Knox & Wicken, 1973). The wall is susceptible to the action of an endogenous

muralytic amidase (autolytic enzyme) (Mosser, 1970; Mosser & Tomasz, 1970). This enzyme hydrolyzes the amide bond between the muramic acid of the glycan and the N-terminal alanine of the peptide (Mosser, 1970; Mosser & Tomasz, 1970), releasing soluble peptides and soluble glycan-teichoic acid complexes. The glycan-teichoic acid complex has been shown to be identical to the "C-carbohydrate" isolated by Tillet, and others (Mosser, 1970; Mosser & Tomasz, 1970; Brundish & Baddiley, 1967; 1968; Tillet & Francis, 1930; Tillet, et al., 1930); the C-antigenic activity has been shown to reside in the teichoic acid (Brundish & Baddiley, 1967; 1968). The wall is also believed to contain protein antigens (Austrian & MacLeod, 1949), analogous to the M proteins of the Group A streptococci; these proteins might account for the presence of small but significant amounts of certain non-peptidoglycan amino acids in acid hydrolysates of isolated pneumococal walls (Mosser, 1970; Mosser & Tomasz, 1970).

The presence of choline residues in the teichoic acid is very interesting, since this is not a usual component of polysaccharides. Pneumococci have an absolute nutritional requirement for choline (Tomasz, 1967); they can neither synthesize nor degrade this molecule. 85% of the choline taken up from the medium becomes incorporated into the wall (Tomasz, 1967; Briles & Tomasz, 1973). In Part II of this dissertation, evidence will be presented indicating that most, if not all, of the remaining choline of the cell is found in the membrane lipoteichoic acid (Briles & Tomasz, 1973). There is no detectable lecithin or sphingomyelin in the pneumococcus (Tomasz, 1967; unpublished results); the only other choline-containing compounds detected in the cell to date are phosphorylcholine and CDP-choline (Bean, 1970), which are presumed to be biosynthetic intermediates in teichoic acid synthesis. This unusual situation with respect to choline offers the opportunity of using radioactively-labeled choline as a tag for teichoic acid; in this dissertation, experiments will be described which take advantage of this opportunity.

A further unusual situation, which is exploited in some of the experiments to be described, is that under conditions of choline deprivation, the pneumococcus can be coerced into incorporating certain choline analogues (e.g., ethanolamine; N-monomethylethanolamine; N,N-dimethylethano-

lamine) in lieu of choline. The substitution of ethanolamine (or N-monomethylethanolamine) for choline is accompanied by several dramatic pleiotropic effects: the bacteria become unable to completely separate after cell division (thereby forming chains), they lose their characteristic "solubility" in bile acids and detergents, they become resistant to the action of penicillin, and they become unable to become genetically transformed by exogenous DNA (Tomasz, 1968; Tomasz & Westphal, 1971). It is possible that most, if not all, of these effects can be explained by the observation that ethanolamine-containing walls are completely resistant to the action of the pneumococcal autolytic enzyme (Tomasz & Westphal, 1971). The significance of this observation is not entirely clear; it may be that the choline-containing teichoic acid serves as an allosteric "recognition" site for the enzyme. In this regard, it is interesting to note that normal (choline-containing) walls which have been stripped of their teichoic acid by TCA or hot formamide treatment do not serve as substrates for the autolytic enzyme (Tomasz & Westphal, 1971). Furthermore, when ethanolamine-containing walls, which are resistant to the enzyme, are chemically methylated under conditions whereby the ethanolamine residues become fully N-methylated (i.e., converted to choline), they become susceptible to the action of the autolytic enzyme (J.-V. Höltje and A. Tomasz, personal communication).

The pneumococcal Forssman antigen: Many bacteria contain antigenic determinants which cross-react with the Forssman mammalian cell surface antigens (Buchbinder, 1935; Jenkins, 1963; Springer, 19). Pneumococci, regardless of capsular type, possess such an antigen (Bailey & Shorb, 1931; Goebel & Adams, 1943; Goebel, et al., 1943).

Forssman antigens are generally defined as anything that is immunologically cross-reactive with sheep red blood cells (or boiled sheep RBC stromata). Such antigens are distributed widely through nature (Buchbinder, 1935). Recently, the Forssman hapten of sheep red cells and of horse kidney has been identified as a ceramide polyhexoside, with a specific hexose sequence of α-N-acetyl-D-galactosaminyl-1,3-β,D-galactosyl-1,4-β,D-galactosyl-1,4-D-glucosyl-ceramide (Ando & Yamakawa, 1970). It is likely that antibodies may recognize this hapten in more than one way,

since many Forssman antigens in nature cross-react with the hapten but not with each other (Buchbinder, 1935); therefore, it should not be expected that all Forssman antigens are ceramide polyhexosides. Not all mammals carry the Forssman determinant; those that do are called Forssman-positive (e.g., sheep, horses, cats, guinea pigs, foxes, dogs, goats, whales; human type A blood group; etc.) (Buchbinder, 1935), while those that do not are Forssman-negative, and are capable of producing anti-Forssman antibodies when injected with Forssman antigens (e.g., rats, pigs, rabbits, cattle, baboons; human types B and O blood groups; etc.) (Buchbinder, 1935). Anti-Forssman antibodies are occasionally called "heterophile" antibodies; however, the term "heterophile" can also be used to describe any immunologically cross-reacting response between dissimilar organisms, regardless of whether or not the antigens in question are Forssman cross-reactive.

The pneumococcal Forssman antigen (F-antigen) was first accurately described in detail by Goebel, Shedlovsky, Lavin, and Adams in 1943 (Goebel & Adams, 1943; Goebel, et al., 1943). They reported that the F-antigen was serologically cross-reactive with the C-carbohydrate (wall teichoic acid; see above) (Goebel & Adams, 1943). Upon elemental analysis, they found that the F-antigen was similar to the C-carbohydrate; however, the F-antigen contained 6 1/2% (by weight) of fatty acids which the C-carbohydrate did not contain, and the two had different electrophoretic mobilities (Goebel, et al., 1943).

Recently, Fujiwara (1968; 1969) re-investigated and confirmed the cross-reactive relationship between the pneumococcal "F-antigen" and the mammalian Forssman haptens. Anti-pneumococcal-F-antigen-antibodies (of rabbit origin) were shown to bind to the Forssman antigen of guinea pig kidney; this binding was inhibitable with the Forssman hapten (ceramide polyhexoside) of sheep red cells. However, the binding of anti-sheep red cell antibodies to guinea pig kidney was not inhibitable with the isolated pneumococcal F-antigen (Fujiwara, 1968); this is consistent with the hypothesis that the pneumococcal F-antigen determinant shares some structural features with the mammalian hapten, but that the two are not identical. Fujiwara also reported that pneumococcal F-antigen was unable

to elicit a hemolytic antibody response (as tested against sheep red cells) in two Forssman-positive species (guinea pig and mouse), but did elicit a hemolysin response in two Forssman-negative animals (rat and rabbit); this is consistent with Forssman specificity (Fujiwara, 1969).

Fujiwara also corroborated and extended the findings of Goebel, et al. (1943), and showed that the F-antigen contains ribitol, phosphate, glucose, and galactosamine (in molar ratio 1:2:1:1), plus additional nitrogen which he could not account for (Fujiwara, 1967). Simultaneously and independently, Brundish and Baddiley (1968) found the same components in the C-carbohydrate that Fujiwara (1967) had found in the F-antigen, plus choline and diaminotrideoxyhexose (see above). If it is assumed that the diaminotrideoxyhexose and choline are also present in the F-antigen, then that would account for almost all (80%) of the nitrogen that Fujiwara could not account for. The presence of choline residues in the F-antigen has now been established (Briles & Tomasz, 1973; see Part II of this dissertation).

Studies presented in this dissertation: The investigations which form the basis for this dissertation are presented in two parts.

Part I deals with the mode of segregation of the pneumococcal wall teichoic acid during cell growth and division. Teichoic acid was labeled with tritiated choline (or an analogue), and its distribution among progeny was followed by autoradiography over several generations. The results confirm the zonal-growth model for Gram-positive coccal walls (see above) (Barak & Tomasz, 1970; Briles & Tomasz, 1970).

In Part II, the pneumococcal Forssman antigen is identified as a membrane lipoteichoic acid (Briles & Tomasz, 1973); evidence is presented suggesting that the teichoic acid chains of this molecule may be identical to those found in the pneumococcal wall (C-carbohydrate). The possibility that the lipoteichoic acid may be a precursor to the wall teichoic acid is ruled out by pulse-chase studies.

PART I

METHODS

Chemical compounds used in these studies were all commercial reagent-grade products, except as specifically otherwise noted.

Growth of Bacterial Cultures

For the studies described in Part I of this dissertation, Diplococcus pneumoniae, R36A (Avery, et al., 1944), clone R6, was routinely grown in a chemically-defined liquid medium (Cd-enriched). This medium contains, per liter in distilled water: glycine, 47.5 mg; L-alanine, 87.5 mg; L-valine, 180 mg; L-isoleucine, 190 mg; L-proline, 290 mg; L-serine, 147.5 mg; L-threonine, 112.5 mg; L-methionine, 77.5 mg; L-tryptophan, 35 mg; L-aspartic acid, 180 mg; L-glutamic acid, 550 mg; L-cysteine, 37.5 mg; L-histidine, 80 mg; L-tyrosine, 15.25 mg; L-arginine, 100 mg; L-leucine, 250 mg; L-phenylalanine, 137.5 mg; L-lysine, 217.5 mg; sodium chloride, 2 g; sodium acetate (crystals), 2 g; magnesium chloride (crystals), 500 mg; manganese sulfate (crystals), 22.5 µg; calcium chloride (anhydrous), 2.5 mg; glucose (anhydrous), 2 g; sucrose, 250 mg; adenosine, 20 mg; uridine, 20 mg; L-glutamine, 25 mg; sodium pyruvate, 250 mg; L-asparagine, 35 mg; biotin, 0.001 µg; nicotinic acid, 0.96 mg; pyridoxine HCl, 1.12 mg; calcium pantothenate, 3.84 mg; thiamine HCl, 0.73 mg; riboflavin, 450 µg; manganese chloride (crystals), 500 µg; ferrous sulfate (crystals), 800 µg; copper sulfate (crystals), 800 µg; zinc sulfate (crystals), 800 µg; potassium phosphate, pH 8, at a final concentration of either 0.05 M or 0.03 M; plus either 5 mg of choline chloride or 40 mg of ethanolamine hydrochloride (EA medium).

This medium was either used as described above, or modified in the following ways:

(a) no amino alcohol included (neither choline nor ethanolamine).

(b) ^{3}H-choline medium: the C_d-enriched choline-containing medium was supplemented with ^{3}H-methyl choline chloride (New England Nuclear). In a typical experiment, the final choline concentration was 7 µg/ml, with a specific activity of 0.7 mCi/ml.

(c) ^{3}H-EA medium: medium containing ethanolamine hydrochloride in place of choline was supplemented with 2-^{3}H-ethan-1-ol-2-amine (Amersham/Searle).

Experimental Procedures

General Procedure: Cultures were grown overnight from low innocula at 37°C in 10 ml of ^{3}H-labeled medium. At mid-log phase, the cells were harvested by Millipore filtration, washed with one or two volumes of either amino-alcohol-free medium or chasing medium, and finally resuspended in non-radioactive chase medium, all at 37°C. The cultures were maintained at mid-log phase concentrations for the duration of the experiments by appropriate dilutions with chase medium. Growth of the cultures was monitored with a Coleman nephelometer. Doubling-time was slightly more than 1 hour in EA-medium and slightly less than 1 hour in choline-containing medium. Drop-sized samples were removed at appropriate intervals and processed for either light microscopy or electron microscopy, as described below.

Light microscopy: Samples were either (a) spread immediately on clean glass slides, using a blood-smear technique, or (b) fixed with 1/2- or 1-x-volume of 9% formaldehyde in 0.15 M NaCl, and spread at some later time. Smeared preparations were air-dried, either at room temperature or at 60°C, and then exposed to a heat lamp 1/2 m away for 1/2 hour, to promote adhesion to the glass. The slides were then washed extensively in distilled water, and air-dried.

Ilford L-4 gel emulsion (photographic emulsion) was melted at 45°C in an equal volume of water in a Coplin jar (in the dark); slides were coated with the emulsion by dipping. Dried coated slides were stored in darkness under a CO_2-enriched atmosphere over Drierite (W.A. Hammond Drierite Company) at 4°C for 2-9 days (for cells chased in EA-medium) or for 21 days (for cells chased in choline medium). The autoradiographs were developed in Kodak Rapid Fixer for 5 minutes. Slides were viewed through a Zeiss RA microscope fitted with a Planachromat 100/1.25 oil-immersion phase-contrast objective and 12.5 x binocular lenses, and photographed on 35 mm Kodak Panatomic-X film.

Electron microscopy: For electron microscopy, formaldehyde-fixed samples were used (see above). Copper grids, 200 mesh, pre-coated with Formvar and carbon, were floated briefly on drops of the samples, partially drained, and allowed to dry. They were then affixed to glass slides with a very small drop of nail polish at one edge, and coated with Ilford L-4 gel emulsion according to the "loop method" of Caro and Van Tubergen (Caro, 1962; Caro & Van Tubergen, 1962). Exposure time was 29 days over Drierite at 4°C in a CO_2-enriched atmosphere. Development was carried out for 5 minutes in Kodak Microdol X. Specimens were viewed at 75 KV in a type HU-11C-1 Hitachi electron microscope, and photographed on Ilford or Kodak plates.

Grain counting: (for cells chased in choline medium). Radioautographic silver grains over cells were counted under phase-contrast optics; the cells appeared dark, and the grains appeared as bright granules. To visualize all the grains at a cell, it was usually necessary to view the specimens at a continuum of focal planes; even so, when many grains were clustered about a single cell, it was difficult to determine the precise number of grains present. Cells were characterized as having 0, 1, 2, or many grains. The proportion of cells with only 1 or 2 grains was essentially stable over the whole experiment (see Table I), and hence were regarded as background; this was compatible with background grain levels estimated from fields containing no bacteria.

RESULTS

The results described below are based on the following type of experiment: pneumococci were grown for several generations in ^{3}H-labeled amino-alcohol-containing medium (^{3}H-choline or ^{3}H-ethanolamine), in order to uniformly label the teichoic acid; subsequently, the label was "chased" by transferring the cells to nonradioactive medium and allowing them to grow for several further generations. The fate of the labeled teichoic acid was followed during the chase period by autoradiography.

Preliminary experiments indicated that turnover of amino-alcohol label in pneumococci was negligible (less than 10% per generation).

Table I

Slide number*	% Cells with One Grain	% Cells with Two Grains	Total Number of Cells Observed
1	-	-	-
2	-	-	-
3	20	50	197
4	33	6	589
5	33	12	292
6	33	7	297
7	7	1	591
8	25	7	456
9	30	5	1146

* Slide number corresponds to the number of generations elapsed in chase medium.

Case A

In this case, cells were pre-labeled with ^{3}H-choline, and chased in nonlabeled ethanolamine medium (EA medium). It should be recalled (see Introduction) that when pneumococci are grown with ethanolamine as their sole amino alcohol source, they lose the ability to completely separate after division, and hence form chains. Each chain is, in effect, an oriented clone derived from a single choline-grown parent cell (or viable unit). The ancestry of each daughter cell can be traced by observing the spatial position of each cell in the chain. Therefore, in this experiment, the distribution of the teichoic acid label among progeny cells during the chase period can be followed directly.

The results of this experiment are summarized in Figures 1 and 2. As the pneumococci grow and divide in ethanolamine medium, the chains elongate; the radioactive label, identified by autoradiographic silver grains, is seen to segregate to the distal ends of the chains. Occasionally (Fig. 2e), silver grains are also observed in the very middle of the chains.

These results are consistent with the model for cell wall growth indicated in Figure 3. There are two significant features of this model: first, the wall is considered to be rigid, with no mixing or diffusion between the "old" and "new" surface components; secondly, the new teichoic acid is incorporated into the growing wall at a specific "growing zone" at the equator of the cell. As a result, the model predicts that the "old wall" would be conserved as hemispheres, while new wall material becomes inserted between these hemispheres at the equator. After each round of division, a new "equatorial growth zone" becomes established for each daughter cell.

Figure 3A depicts the fate of a single choline-labeled cell chased in ethanolamine medium. As the cell grows and divides, the original choline-labeled wall hemispheres are pushed apart by the accumulating new wall at the equator; since the new wall contains ethanolamine rather than choline, the daughter cells remain attached. As the chain elongates as a result of further growth and division, the choline-labeled hemispheres are

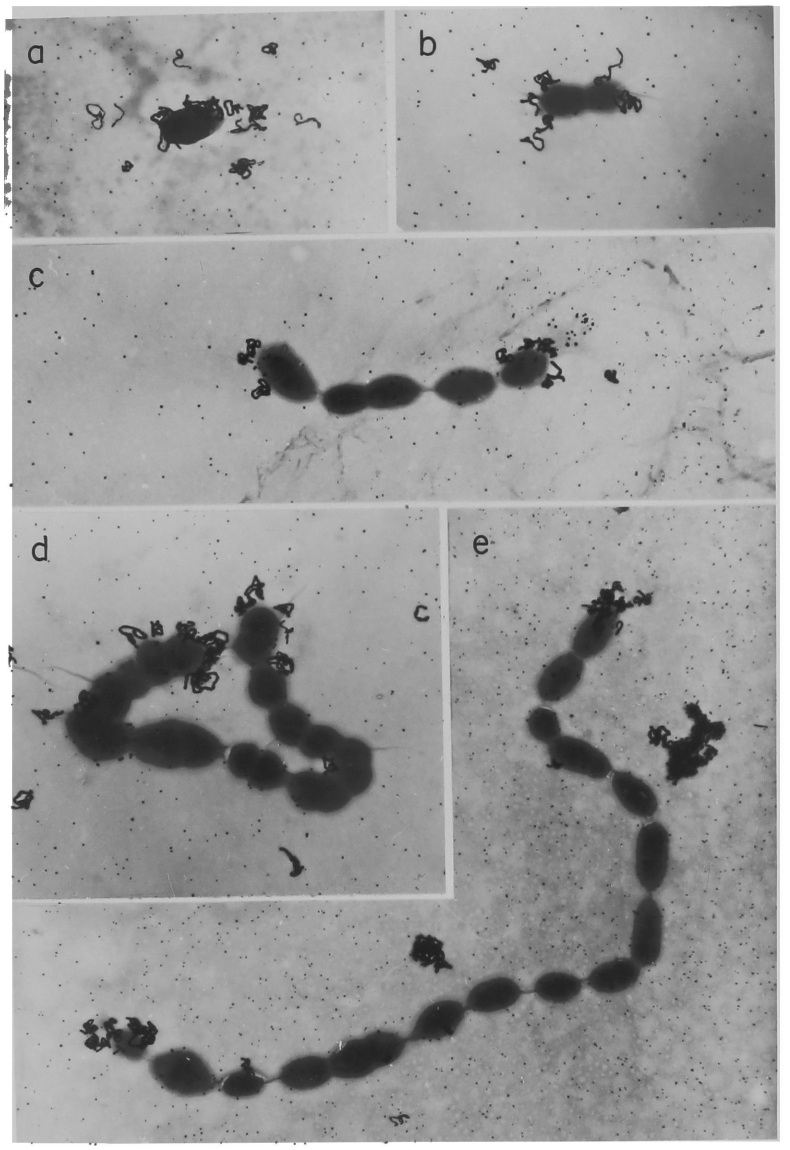


Fig. 1. D. pneumoniae cells pre-labeled with ^{3}H-choline, then shifted to chase medium containing ethanolamine as the sole amino-alcohol source; whole-mount electron microscope autoradiographs; unstained. a - immediately after shift; b - less than one generation (1 hour) after shift; c, d, e - four to five generations (7 hours) after shift. Note localization of silver grains (black threads) at distal ends of chains. (Figure reproduced from Briles, E.B. and Tomasz, A., J. Cell Biol. 47 (1970), p. 789)

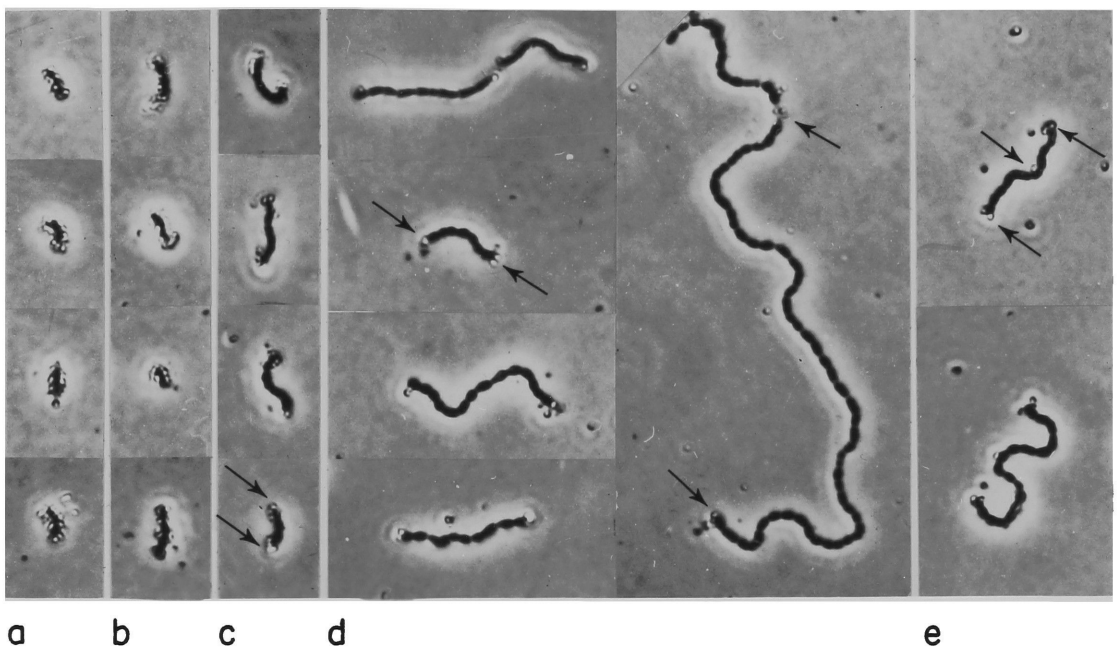


Fig. 2. D. pneumoniae cells, pre-labeled with ^{3}H-choline, then shifted to ethanolamine chase medium. Light-microscope autoradiography; phase-contrast optics. Arrows indicate silver grains. a - less than one generation of growth after shift; b - one to two generations after shift; c - two to three generations after shift; d - several generations after shift; e - several generations after shift; note grains at centers of chains. Compare location of grains at ends and middles to segregation pattern in Figure 3. (Figure reproduced from Briles, E.B. and Tomasz, A. J. Cell Biol. 47 (1970), p.788)

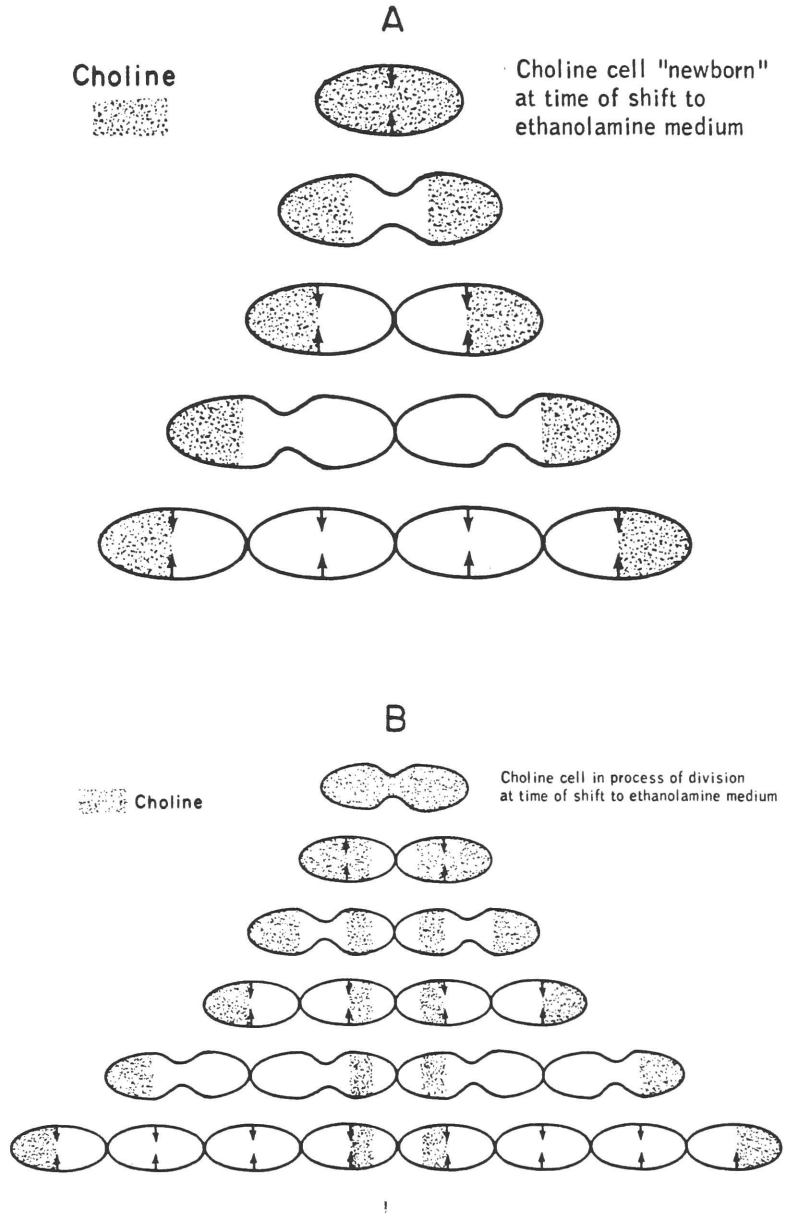


Fig. 3. Diagram of wall segregation after shift from choline to ethanolamine media, assuming zonal growth and no diffusion between old and new surfaces. Choline-containing wall is indicated by shading; ethanolamine-containing wall is unshaded. Arrows indicate equatorial growing zones. Nonseparation of cells begins immediately upon shift to ethanolamine medium. A - single parental choline-labeled cell gives rise to chain in which choline-containing hemispheres segregate to the distal ends. B - parental cell, in process of division at moment of shift, gives rise to chain in which choline-containing wall segregates to the ends and to the middle. (Figures reproduced from Briles, E.B. and Tomasz, A., J. Cell Biol. 47 (1970), p. 787)

seen to segregate to the ends of the chain.

Figure 3B depicts the fate of a choline-labeled cell which is in the process of dividing at the moment of shift to ethanolamine medium. Some choline-labeled wall material is seen to be segregated to the center of the chain in this case, as well as to the distal ends. The amount of choline-labeled wall at the center of the chain would vary: if the original parent cell was just beginning a new division cycle at the moment of shift to the chase medium, there would be very little label at the center of the chain; if the cell was near completion of the division cycle, the amount of label at the center of the chain would be almost as much as at the ends. The pneumococcal cultures used in these experiments were not synchronous; thus, according to the model, some but not all of the chains would be expected to show autoradiographic grains at their centers (and this was, in fact, observed).

The following additional experiments were performed in order to eliminate the possibility that the results observed for Case A, above, were artifacts due to the shift in amino alcohol of the medium or of the wall.

Case B

In this experiment, the cells were uniformly labeled with ^{3}H-choline, and subsequently chased in nonlabeled choline-containing medium. Since the cells separate normally in this case, the progenitorial information derived from the spatial relationships of chained cells (as in Case A) is not available. Thus, the segregation patterns predicted by the model cannot be tested in this case. However, the model predicts that, as the number of generations in the chase increases, the proportion of cells bearing autoradiographic grain clusters should decrease geometrically, and this can be tested (refer to Fig. 3). The observed results, summarized in Figure 4, are in accordance with the model. There was no apparent dilution of label concentration among the labeled cells, over the generations observed.

Case C

In this experiment, the cells were uniformly labeled with ^{3}H-ethanolamine, and subsequently chased with nonlabeled ethanolamine medium.

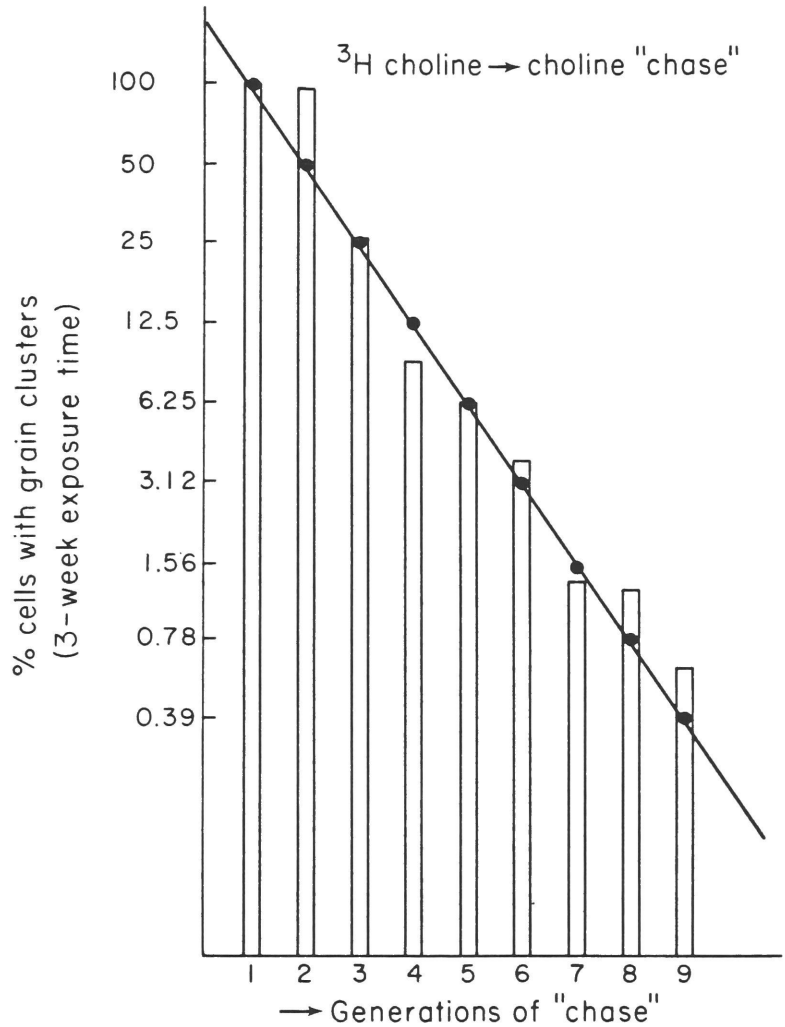


Fig. 4. Pneumococci, pre-labeled with ^{3}H-choline, then shifted to unlabeled choline chase medium. Bar graphs indicate the percentage of cells with autoradiographic grain clusters (labeled cells) observed after each indicated generation of growth in the chase medium. The dots (connected by a straight line; please note that this is a semi-logarithmic plot) represent the percentage of labeled cells that would be expected, assuming that the labeled wall is inherited as intact hemispherical units.

As can be seen in Figure 5, as the cells grow and divide in the chase medium in this case, the silver grains become separated along the chain by lengths of unlabeled cells.

The situation is essentially that of single parental cells (as depicted in Fig. 3), except that the parents are tandemly attached to each other. According to the model, then, growth in the chase medium would be expected to give rise to a tandemly-attached series of chains, each with silver grains at their distal ends (and possibly also at their centers). In Figure 5, the lower frame depicts such a chain after 4-5 generations of growth in the chase medium; the results are entirely consistent with the model. It should be kept in mind that the cells do not grow synchronously.

DISCUSSION

In the experiments presented above, the manner of inheritance of wall teichoic acid was investigated. Pneumococci were uniformly pre-labeled with a teichoic acid-specific radiolabel (tritiated choline or an analogue), and the distribution of this label among progeny cells, over several successive generations of growth in the absence of further label, was followed by radioautography.

Substantially most (85%) of the choline incorporated by pneumococci is found in the wall teichoic acid (Briles & Tomasz, 1973; Tomasz, 1967). For the purposes of the radioautography experiments presented above, essentially all of the choline of the cell can be considered to be associated with the wall teichoic acid, since the silver grain contribution of the relatively small amount of "other" choline would not be expected to be appreciably above "background" under the experimental conditions employed. The wall teichoic acid is covalently linked to the peptidoglycan (Knox & Wicken, 1973; Mosser, 19 ; Mosser & Tomasz, 1970), presumably through a phosphodiester bridge to muramic acid (Liu & Gottschlich, 1967). Mauck and Glaser (1972) have shown that, in Bacillus subtilis, teichoic acid and peptidoglycan which are incorporated into the wall at the same time remain covalently associated with each

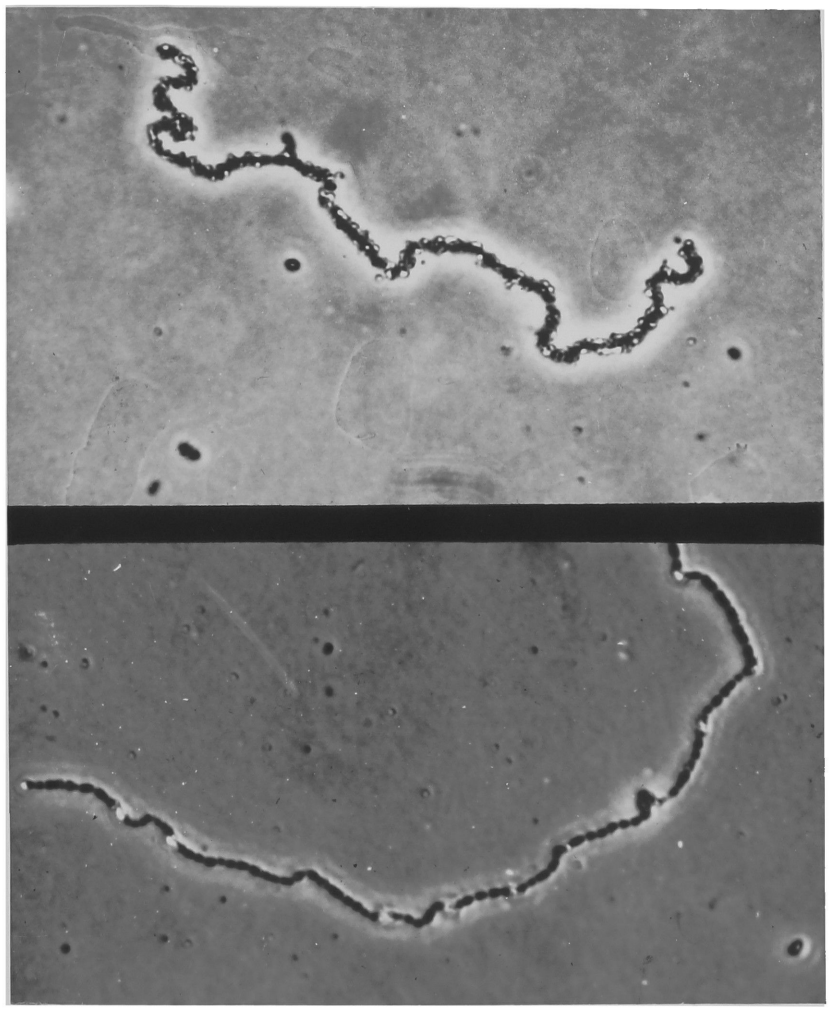


Fig. 5. D. pneumoniae cells, pre-labeled with ^{3}H-ethanolamine, then shifted to unlabeled ethanolamine chase medium. Light-microsope autoradiography; phase-contrast optics. Silver grains appear as white spots over regions containing ^{3}H-radioactivity. Upper frame: after 1 generation of growth in the chase medium; note that every cell in the chain is associated with silver grains. Lower frame: after 4-5 generations of chase; silver grains occur at more-or-less uniform spacings along the chain.

other, and are not translocated to sites that were incorporated at other times, this situation is probably true for Gram-positive bacteria in general. Therefore, segregation of choline label observed in the experiments described in this dissertation most likely coincides with the distribution of peptidoglycan, and thus with the whole wall.

The observed pattern of distribution of the label was found to be consistent with a model of zonal wall growth. The significant features of the model are two-fold: new wall material is incorporated into the growing wall at an equatorial growth zone; and the wall is a rigid (rather than fluid) structure, in which no mixing or diffusion occurs in the plane of the surface. The model also assumes negligible turnover of wall components; this is known to be true for pneumococci, and is probably also true for most other bacteria (a significant exception is discussed below).

One might ask whether the new wall (the nascent "new" hemispheres) is incorporated at the septal region (i.e., at a single zone at the center of the growing cell), or at the edges of each of the two "old" hemispheres (i.e., two "zones," at the sites which are destined to become the equators of the daughter cells). In Figure 3B, the former case is depicted; however, the radioautographic methods employed do not offer a level of resolution sufficient to differentiate between these two possibilities (a pneumococcal cell is $\sim$ 1 micron in length, while the limit of resolution of electron micrographic autoradiography is $\sim$ 0.2 microns at best).

Segregation of wall materials in a pattern suggesting a zonal mode of growth was first demonstrated by Cole and Hahn (1962) for Streptococcus pyogenes, and some time later for pneumococci by Wagner (1965); in their studies, "old" or "new" wall was distinguished by fluorescently-labeled antibodies. However, this same technique, when applied to Gram-negative bacilli (e.g., Salmonella, E. coli) suggested a uniform incorporation of new wall materials over the entire surface (Cole, 1965; May, 1963). Van Tubergen and Setlow (1961) followed the distribution of tritiated diaminopimelic acid (a pdptidoglycan-specific label) in E. coli walls by radioautography, using a statistical evaluation method, and also concluded that the old wall material is distributed uniformly and randomly among progeny cells in that bacterial species.

The use of antibodies to label the wall is not entirely satisfying. Cole (1965) points out the need to assume that the antigenic site to which the antibody is binding is actually segregating along with the entire wall. It is also necessary to assume that the binding of the antibody to the cell surface does not of itself create artifacts, either by affecting the normal growth of the cell, or by causing a fixation or abnormal cohesion of surface components that might not normally occur. In the case of the Gram-negative bacteria, the possibility exists that the antibodies bind to components of the outer membrane of the envelope which are capable of diffusion in the plane of that membrane. The use of radioactively-labeled specific wall components eliminates these difficulties.

The results presented in Part I of this dissertation were first reported in 1970 (Barak & Tomasz, 1970; Briles & Tomasz, 1970). Subsequent studies have been reported in the literature which further support the conclusion that Gram-positive cocci increase their surface area by intercalation of new wall material at equatorial growing zones. In Streptococcus faecalis, continued wall synthesis in the absence of protein synthesis causes an abnormal two- to three-fold thickening of the cell wall (such a condition can be induced by chloramphenicol, or by starvation for a non-wall amino acid such as valine or threonine) (Higgins & Shockman, 1970; Shockman, 1965; Shockman & Higgins, 1971); Higgins and Shockman (1971) have shown that when normal growth ("balanced" growth) is resumed by cells with "thickened" walls, the new wall that is synthesized is of the normal thinness, and that this new wall is inserted at the equator between the thick-walled hemispheres. In pneumococci, Tomasz, et al. (1971) have visualized the equatorial growing zone by electron microscopy. They pulsed ethanolamine-grown cells (whose walls, as may be recalled from the Introduction, are resistant to the action of the pneumococcal wall-lytic enzyme) with choline for 5-15 minutes (generation time $\sim$ 45-60 minutes); they then heat-killed the cells, and treated them with the wall-lytic enzyme. The choline-containing "new" wall was digested by the enzyme, thereby causing gaps at the sites of new wall incorporation; these gaps were observed at the equators of the cells.

The manner in which rod-shaped bacteria increase their surface area during growth is still a matter of controversy and active investigation. In two Gram-positive bacilli, B. subtilis and B. megaterium, Glaser has observed random incorporation of new wall over the whole surface area of the cell (Glaser, 1973; Mauck, et al., 1972); these bacteria also exhibit an unusually high rate of wall turnover (50% of the old wall peptidoglycan and teichoic acid is lost per generation), and this turnover seems also to occur randomly over the whole surface (Glaser, 1973; Mauck & Glaser, 1970). The significance of this turnover is not yet clear. The random incorporation of ^{3}H-diaminopimelate into the wall of E. coli, first reported by van Tubergen and Setlow (1961), has recently been confirmed by Lin et al. (1971). These investigators pre-labeled the cells, and then chased them in a highly-viscous medium in order to induce "chains" in a manner analogous to the situation reported for Case A in this dissertation; at each successive generation of chase, they found that the label was randomly distributed among the daughter cells. However, Schwarz (Henning & Schwarz, 1973; Ryter, et al., 1973) has suggested, on the basis of pulse-label studies, that E. coli actually does possess an equatorial growth zone, but that the newly-incorporated label becomes rapidly redistributed over the entire wall. Certainly, the available evidence indicates that there is a genuine difference in the manner in which rod-shaped and coccus-shaped bacteria increase their surface area during growth and division.

PART II

MATERIALS AND METHODS

Chemical compounds used in these studies were all commercial reagent-grade products unless specifically noted otherwise.

Growth of Bacterial Cultures

The strain of Diplococcus pneumoniae (pneumococcus) used in these studies is R36A, clone R6. This is a rough (unencapsulated) strain, originally isolated in the laboratory of O.T. Avery (1944). Stock cultures were maintained at -20°C or at -70°C in culture medium supplemented just prior to freezing with 10% glycerin.

Usually, the bacteria were grown in a semi-defined medium ("c"-medium) which is used routinely in the laboratory in which this work was performed. Per liter, this medium continas: Difco Bacto Vitamin-free Casamino acids, 5 g; $NaC_2H_3O_2 \cdot 3H_2O$, 2 g; L-tryptophan, 5 mg; L-cysteine, 50 mg; $MgCl_2 \cdot 6H_2O$, 500 mg; $MnSO_4 \cdot 4H_2O$, 22.5 µg; anhydrous $CaCl_2$, 2.5 mg; anhydrous glucose, 2 g; sucrose, 250 mg; adenosine, 20 mg; uridine, 20 mg; L-glutamine, 25 mg; sodium pyruvate, 250 mg; L-asparagine, 35 mg; choline chloride, 5 mg; biotin, 0.001µg; nicotinic acid, 0.96 mg; pyridoxine HCl, 1.12 mg; Ca pantothenate, 3.84 mg; thiamine HCl, 0.73 mg; riboflavin, 450 µg; $MnCl_2 \cdot 4H_2O$, 500 µg; $FeSO_4 \cdot 7H_2O$, 800 µg; $CuSO_4 \cdot 5H_2O$, 800 µg; $ZnSO_4 \cdot 5H_2O$, 800 µg; plus potassium phosphate pH 8 buffer at a final concentration of either 0.05 M or 0.03 M. Cultures were grown at 37°C without aeration; culture growth was monitored with a Coleman nephelometer. The components of "Cd enriched" medium are detailed in Part I of this dissertation.

Immunological Procedures

Materials: Sheep red blood cells were obtained either from freshly-drawn whole sheep's blood preserved in sterile Alsever's solution (Kabat & Mayer, 1967), or commercially from Microbiological Associates.

Guinea-pig complement was obtained from Microbiological Associates in lyophilized form; upon reconstitution, it was absorbed with sheep red blood cells (Kabat & Mayer, 1967) and stored at -20°C. Complement prepared

in this way was good for 1 month. Titration of complement was performed according to the method of Kabat and Mayer (1967).

Anti-pneumococcal F-antigen-sera were produced in New Zealand Red (NZR) or Chinchilla rabbits. Vaccine was prepared in the following manner: pneumococci were grown in 910 ml of medium, harvested at midlog phase by centrifugation at 0°C washed once with 0.86% NaCl at 0°C, resuspended to 20 ml with 0.86% NaCl, heat-killed at 60°-70°C for 20 minutes, and stored in 3 ml aliquots at -20°C. Young rabbits (5-8 lbs) whose preimmune sera showed 20 AbH_{50}/ml or less were immunized by a course of intravenous injections consisting of from 0.5 to 1.0 ml of undiluted vaccine on each of days #1, 2, 4, and 7; blood was drawn for serum on or about day #12. Endogenous complement was inactivated at 56°C for 15 minutes prior to storage of the antisera at -20°C. Hemolytic antibody was titrated by the method of Kabat and Mayer (1967). Antiserum obtained from a single rabbit at a single bleeding, designated NZRA-11-16-71, was used for all routine determinations of F-activity except as noted in the text.

All complement-mediated immunological assays were performed in barbital-buffered saline, pH 7.5, containing 5×10^{-4}M $MgCl_2$ and 1.7×10^{-4}M $CaCl_2$. This diluent was prepared as a five-fold concentrate according to the procedure of Kabat and Mayer (1967), and was stored at 5°C.

TEPC-15 ascites fluid, preserved with 0.25% NaN_3, was a generous gift of Dr. Michael Potter of NIH (Potter & Lieberman, 1970). Phosphocholine chloride·Ca·3 1/2 H_2O, A grade, was purchased from Calbiochem.

Methods: F-antigen was titrated on the basis of its Forssman crossreactivity by means of a hemolysis-inhibition assay performed in the following manner: a dilution of rabbit anti-pneumococcal F Antigen-serum containing 7 AbH_{50}/ml was added to an equal volume of serially-diluted test sample, and incubated at room temperature for 1 hour. 0.5 ml of this was then added to 0.5 ml of a suspension of sheep red cells containing 1×10^9 cells/cc (cell density determined by the laking method of Kabat and Mayer(1967)) at 0°C; this was incubated at 37°C for 15 minutes, then chilled in an ice-water bath. Each sample then received

0.25 ml of a dilution of prepared guinea-pig complement containing 12 $C'H_{50}$/ml, and was incubated at 37°C for 15 minutes. The lytic reaction was terminated by chilling the samples in ice water and immediately adding 2.5 ml of ice cold saline-citrate (4 parts 0.15 M NaCl plus 1 part 0.075 M sodium citrate). The extent of hemolysis was determined by measuring the absorbance of the supernatant at 541 mμ with a Zeiss PMQ II Spectrophotometer. The number of units of F-antigen activity per 0.5 ml of a sample was arbitrarily defined as that factor of dilution which results in 50% inhibition of hemolysis under the assay conditions described.

All other procedures were performed as described by Kabat and Mayer (1967).

Isolation of F-antigen

The F-antigen and C-carbohydrate were isolated by procedures adapted from the methods of Goebel, et al. (1943). The C-carbohydrate is solubilized during autolysis, whereas the F-antigen remains associated with the particulate detritus. The F-antigen can be solubilized from the detritus after a sequence of proteolytic and defatting steps.

Preparation #1: A culture of pneumococci was grown in 1.8 liters of C medium with initial pH 8. Another culture was grown in 450 ml of C, but containing only 35% of the usual amount of choline chloride and supplemented with 0.5 mCi (in 0.07 mg) ^{3}H-methyl choline chloride. The two cultures were harvested separately at midlog phase and resuspended together in 25 ml of 0.05 M sodium acetate. A few drops of toluene were added, and the mixture was incubated at 37°C for 72 hours. At this point, the culture had a characteristic "autolyzed" appearance when viewed in a phase-contrast microscope.

The pH was then adjusted to 4.5 with 0.05 M acetic acid, and the suspension was centrifuged. The supernatant was dialyzed against 0.15 M NaCl and set aside as a source of ^{3}H-choline labeled C-carbohydrate (see below). The pellet was washed in 0.05 M acetate pH 4.5 buffer, resuspended to 12.5 ml, and dialyzed against 0.15 M NaCl. Then 0.5 ml of phosphate buffer (1 M, pH 8), 0.05 ml of 0.1 M $CaCl_2$, 0.5 mg trypsin

(Worthington), and a few drops of toluene were added; this mixture was incubated overnight at 37°C. For the next six days, an additional 0.5 mg trypsin per day was added, and the mixture incubated further. Finally, the mixture was dialyzed against several changes of distilled water. The material remaining in the dialysis sac was concentrated to 22 ml with Aquacide II (Calbiochem). The suspension appeared yellow and opalescent at this stage.

The suspension was then added to 420 ml of acetone, with stirring, and left to stand overnight, at room temperature; the resulting precipitate was collected by centrifugation and further defatted, first with ethanol:ether (3:1, v/v) and then with chloroform:methanol (1:1, v/v). Residual solvent was air-dried over hot water. The dry defatted powder was next extracted with 4.5 ml of distilled water at room temperature for 1 minute; the suspension was centrifuged and the extraction was repeated on the precipitate once again.

To the pooled aqueous extracts were added 20 μg of DNAse I, 1,000 unites of T_1RNase (Worthington), and a few drops of toluene; the solution was incubated at 37°C for 48 hours. Then 4 volumes of ethanol were added and the solution was refrigerated overnight. The resulting precipitate was collected by centrifugation, redissolved in water, and dialyzed overnight against water. After concentration to 4.4 ml with Aquacide II, the aqueous solution was added to 150 ml of acetone. After overnight refrigeration, the acetone suspension was centrifuged, and the collected precipitate was allowed to air-dry. The precipitate was then redissolved in 6 ml of distilled water. This solution, designated "Preparation #1," was stored at -20°C. The properties of this preparation are listed in Table II and are discussed in the text.

Preparation #2: Washed autolytic detritus prepared from a 1.8 liter culture of pneumococci labeled with 4 mCi of ^{3}H-methylcholine, and from a 20 liter culture (unlabeled), were stored separately at -20°C prior to use. These were dialyzed together against saline, and then trypsinized in Tris pH 8 buffer, with fresh trypsin added every other day for 13 days. Dialysis, concentration against Aquacide II, and defatting with organic solvents was essentially the same as for Preparation #1. The dried defatted powder

TABLE II

Properties of Several F-Antigen Preparations

Preparation Number:	1	2	3
Starting Material, Bacterial Culture:	2.2 liters	20 liters	192 liters
F-Activity, Total Units Isolated:	2.64×10^3	1.64×10^5	3×10^6
Dry Weight, Total:	N.D.	22.2 mg ± 1.6	238 mg
Protein, Total:[1]	<60 µg	1.8 mg	8.3 mg
Protein, % by Weight:	N.D.	8.2%	4%
Total Radioactivity, DPM: (^{3}H methylcholine)	1×10^7	9.6×10^7	not labeled
Elemental Analysis:[2]			
Nitrogen			7.05%
Carbon			46.71%
Hydrogen			7.62%
Phosphorus			4.36%
(total phosphate)			(1.42 µmoles/mg)

[1] Protein determinations were performed by the method of Lowry, et al. (1951), using trypsin (Worthington) as reference standard.

[2] Nitrogen, carbon, and hydrogen determinations (corrected for 10.9% ash) were performed by Mr. Theodore Bella of The Rockefeller University. Phosphorus was calculated from total phosphate, which was determined according to the method described by Ames (1966).

was extracted with water for 6 hours at 5°C, and a second time for 10 minutes. Nuclease treatment was omitted for this preparation. The alcohol-precipitated material was taken up in 5.5 ml water, and formed a clear, yellowish, foamy solution. After acetone precipitation, the material was redissolved in a total of 2 ml of distilled water, and stored at -20°C. The properties of this preparation are summarized in Table II.

Preparation #3: Washed autolytic detritus from 192 liters of unlabeled culture were stored at -20°C prior to use, and was then dialyzed in the presence of trypsin against Tris pH 8 buffer (0.025 M, containing 0.01 mg/ml $CaCl_2$). The suspension was trypsinized in a total volume of 300 ml in the presence of 3 ml chloroform at 37°C: 100 mg trypsin was added every 2-4 days over a 12-day period. The suspension was then dialyzed, concentrated against Aquacide, and sequentially defatted as described above. The air-dried powder was then extracted three times with a total volume of 65 ml of distilled water; since much of the defatted material was heavily clumped, it was next extracted with 30 ml containing 3 µg/ml trypsin and 1 mg/ml $CaCl_2$ in 0.005 M Tris HCl, pH 8.2, overnight at 5°C. After centrifugation the clear trypsin extract was allowed to incubate at 37°C for 9 hours. Meanwhile, the water-extracted material was treated with 25 µg/ml each of RNase A and DNase I (Worthington) in 0.0012 M phosphate buffer, pH 7.5, containing 5 mg/ml $MgCl_2 \cdot 6H_2O$, for 4 hours; then trypsin (to 60 µg/ml), K_2HPO_4 (to 0.05 M), and $CaCl_2$ (50 µg/ml) were added and incubation at 37°C was continued for 5 hours.

Both extracts were then precipitated with 4 volumes of ethanol (5°C, overnight). The collected precipitates were dissolved in water, clarified by centrifugation (15,000 RPM, 5°C, 10 minutes, SORVALL Rc2-B), and alcohol-precipitated again. The precipitates were then resuspended together in 20 ml containing 2 mg DNase I, 3 mg RNase A, 6 mg soybean trypsin inhibitor II (Miles laboratories), and 20 mM $MgCl_2$ in 25 mM Tris HCl, pH 8, and incubated at 37°C for 35 minutes. A massive precipitate was formed. The suspension then received 7.5 mg subtilisin (N.B. Co.) and 1.7 mg pronase (Worthington), and was incubated at 37°C for 30

minutes and then at 5°C overnight. The suspension was clarified by centrifugation, and the clear yellow supernatant was dialyzed extensively against water at room temperature in the presence of a few drops of toluene. The clear, straw-colored liquid was treated with 4 volumes of ethanol, and the collected precipitate was dissolved in 6 ml of distilled water. The properties of this preparation are summarized in Table II.

Determination of Radioactivity

Radioactivity was determined by one of three methods: (A) Samples were dried onto Whatman GF/A 2.4 cm filter discs and counted in toluene scintillation fluid containing 0.3% 2,5-dipenyloxazole (PPO), and 0.01% 1,4-bis-2-(5-phenyl-oxazolyl)benzene (POPOP); (B) samples were added to vials containing Beckman Ready-Solv Solution VI; or (C) samples were dried onto Whatman 3MM filter paper discs and combusted in an Oxymat apparatus (Intertechnique) which automatically transfers the ^{3}H-water or 14-C-carbon dioxide combustion products to scintillation fluid. Counting was performed in a liquid scintillation counter (usually Nuclear Chicago Mark II).

Radioactively-Labeled Compounds

2-^{3}H-ethan-1-ol-2-amine hydrochloride was purchased from Amersham/Searle; all other radioactively-labeled compounds were obtained from New England Nuclear Corp.

Phosphate Determinations

Orthophosphate was assayed by the molybdate-ascorbate method; for total phosphate determinations, orthophosphate was generated by ashing with magnesium nitrate. Details of these procedures are given by Ames (1966).

Hydrolysis of ^{3}H-Choline-Labeled F-Antigen

0.3 ml (c. 5×10^{5} dpm ^{3}H) of Preparation #1 was hydrolyzed in 6 N HCl (total volume was 0.66 ml) in a sealed tube at 100°C for 10 hours. The hydrolyzate was evaporated to dryness under vacuum and redissolved in 0.25 ml distilled water.

Chromatography

Samples of the above hydrolyzate (5-20 microliters) were streaked onto Chromatographic sheets and developed as follows: (a) descending paper chromatography: was performed on Whatman Grade #1 paper, in ethanol: conc. NH_4OH:H_2O (90:5:5, v/v/v) for 10 hours (Tomasz, 1967); (b) ascending thin-layer chromatography: was performed on Eastman Chromagram Sheet #6060 (silica gel), in either 0.25 M Tris pH 8 in 50% ethanol for 8 hours (Kennedy, 1956), or in methanol: conc. HCl (95:5, v/v) (Reismann & Wieske, 1967), for 4 hours.

Developed chromatograms were cut into vertical strips and sliced horizontally at 1/2-cm (silica gel) or 1 cm (paper) intervals. Slices were assayed for radioactivity by counting directly in toluene-PPO-POPOP. For localization of L-serine (run as a reference standard), the appropriate vertical strip was sprayed with ninhydrin reagent (E M Reagents, Brinkmann Instruments, Inc.) and heated to 100°C for 10 minutes.

Sucrose Density Gradient Centrifugations

In a typical experiment, 0.1 ml of sample was layered onto a 5 ml gradient of 5-20% sucrose in 0.15 M NaCl; gradients were either unbuffered, or buffered with 0.18 M Tris HCl, pH 8, or with 0.25 M sodium acetate, pH 4.5, or according to the Tris-EDTA procedure of Haseltine and Fox (1971). The behavior of purified F-antigen and of C-carbohydrate on sucrose gradients were unaffected by the different buffers. Gradients were centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm for 13-18 hours at 13°C. Usually, ten-drop fractions were collected through a hole pierced in the bottom of the gradient tube (Buchler Instruments); occasionally, fractions were collected from the top with an Isco Model 182 Gradient Fractionator.

In experiments in which the gradients also contained 0.4% SDS throughout, 20-drop fractions were collected.

Column Chromatography

For characterization of the F-antigen, Sephadex G-100 columns, 1 x 25 cm, were eluted with either 0.15 M NaCl or with 0.15 M NaCl containing

0.4% sodium dodecyl sulfate. Blue Destran 2000 was used to determine void volumes (exclusion volumes); glycine (detected with ninhydrin) or orthophosphate (detected with concentrated $CaCl_2$) was used to determine total retention volumes. The Bio-Gel A-5m column, 100-200 mesh, 1 x 25 cm column was eluted with 0.15 M NaCl containing 10 mM K_2HPO_4.

Characterization of the degradation products obtained by periodate or nitrous acid treatment or by boiling at pH 2 was performed on 1 x 25 cm columns of Sephadex gels eluted with 0.15 M NaCl containing 20 mM potassium phosphate, pH 8.

Removal of Sodium Dodecyl Sulfate

In order to assay sodium dodecyl sulfate-containing fractions for F-activity, it was necessary to first remove the detergent. The detergent-containing samples were chilled in an ice-water bath for 10 minutes, and the resulting precipitate was removed by centrifugation at 0°C. If necessary, this was repeated until sheep red cells would no longer be lysed when mixed with an equal volume of the supernatant. This cold-precipitation of the detergent does not cause any co-precipitation of the F-antigen (as determined by radioactivity).

Periodate Oxidation

560 micrograms of ^{3}H-choline-labeled F-antigen (Preparation #2) in 50 microliters of distilled water were added to 1.0 ml of 0.025 M paraperiodic acid in 0.5 M sodium acetate, pH 4.5, at 0°C and incubated in the dark at 5°C for 67 hours. Excess periodate was consumed by adding 0.2 ml of 10% (w/v) glycerin and incubating 1 1/2 hours further at 5°C in the dark.

Nitrous Acid Deamination

56 micrograms of ^{3}H-choline-labeled antigen in 2 ml of 0.4 M sodium acetate, pH 3.5, was mixed with 2 ml of 2% (w/v) $NaNO_2$ and allowed to stand at room temperature for 12 hours.

Isolation of C-Carbohydrate

The autolytic supernatant from F-antigen Preparation #1 (see above) was clarified by a brief centrifugation, and added to 4 volumes of ethanol. After overnight refrigeration, the precipitate was collected by centrifugation and redissolved in 5 ml of water. To this solution was added 0.4 ml of 1 M phosphate buffer, pH 8, plus 3 µg DNase I and 1000 units T_1RNase. The solution was incubated at 37°C for 4 hours; next, 50 µl of 0.1 M $CaCl_2$ and 0.5 mg trypsin were added and the solution incubated first at 37°C for 48 hours and then at 4°C for 12 hours in the presence of a few drops of chloroform. The solution was then dialyzed against water. Total final volume was 10.5 ml. 70% of the ^{3}H-radioactivity of the autolytic supernatant was recovered in the final product.

Degradation at pH 2

Small samples of ^{3}H-choline labeled F-antigen or C-carbohydrate, in either 0.05 M glycine -HCl, pH 2, or 0.10 M glycine, pH 7, were heated at 100°C for 30 minutes in a boiling water bath. Samples were allowed to cool, and then were either assayed directly for F-activity by the hemolysis-inhibition assay, or characterized on sucrose gradients or on a Sephadex G-100 column.

Analysis of Autolytic Products

450 ml of pneumococci in C medium were harvested, resuspended in 0.05 M sodium acetate plus a few drops of toluene, and allowed to autolyze at 37°C for 72 hours. The turbid suspension (which could not be rendered less turbid with lysozyme) was adjusted to pH 4.5 with 0.05 M acetic acid; the soluble and detritus fractions were then separated by centrifugation, and dialyzed separately against several changes of distilled water. The dialyzed samples were then lyophilized; total yields were 10.5 mg lyophilized soluble products and 31.2 mg lyophilized detritus products. Weighted samples of each fraction were hydrolyzed in 6 N HCl and analyzed on a Durrum D-500 amino acid analyzer by Dr. Paul Fletcher of this University.

Microscopic Examination of Autolytic Detritus

For phase-contrast microscopy, a drop of autolysate or washed autolytic detritus suspension was wet-mounted and observed directly in a Zeiss R.A. microscope fitted with a Planachromat 100 x/1.25 N.A. oil-immersion phase contrast objective. Photographs were taken on Kodak Panatomic-X film.

For electron microscopy, the autolytic detritus was fixed in gluteraldehyde (2.5% gluteraldehyde in 0.15 M NaCl containing 0.025 M phosphate, pH 7.3) for 17 1/2 hours at 0°C. The detritus was then washed extensively in acetate-Veronal buffer (Juniper, et al., 1970) (diluted 1:1 with distilled water) by repeated centrifugation and resuspension, collected on a Millipore filter, covered with OsO_4 fixative (prepared by mixing equal volumes of fresh 2% OsO_4 and acetate-Veronal buffer), and allowed to stand at 0°C for approximately 4 hours. The filter was washed with 7 changes of distilled water:acetate-Veronal buffer (1:1). Dehydration proceeded in a graded ethanol series. The material on the filter was imbedded in Epon 812, sectioned on a SORVALL Porter-Blum ultramicrotome with a diamond knife, stained with uranyl acetate and examined at 75 kv in a Hitachi Type HU-11C-1 electron microscope.

Chalaropsis Lysozyme

This enzyme was prepared by Dr. Owen Garrigan of Seton Hall University, according to the published procedures of Hash and Rothlaug (1967).

Preparation of F-Antigen from Cells Labeled with ^{14}C-Palmitic Acid

Pneumococci were grown to logarithmic phase in 1.8 liters of C medium; 7.5 ml of 10% albumin (Armour Fraction V Bovine Serum Albumin) containing 6.3×10^8 dpm of 1-^{14}C-palmitic acid (New England Nuclear; 20.5 mg/mCi; flakes were dispersed in the albumin by sonication) was added to the culture, and growth was allowed to continue at 37°C for 45 minutes (one generation), whereupon the cells were chilled and harvested. 1.4×10^8 dpm of ^{14}C label remained in the culture medium supernatant. After autolysis, the detritus was washed with distilled water, and then defatted, first with 80% ethanol

and then with four washes of acetone, in order to minimize nonspecific carryover of ^{14}C-labeled lipids. The defatted detritus was then extensively trypsinized in Tris buffer. After removal of insoluble material by centrifugation, the soluble fraction was precipitated with 80% ethanol; at this point, 60% of the solubilized radioactivity dissolved in the alcoholic supernatant, and was discarded. The precipitate was treated with nucleases and additional proteases, essentially as for Preparation #3, above, and finally was dialyzed, precipitated with 80% ethanol, washed with acetone, and redissolved in water. Final yield was ≃ 50,000 units of F-activity and 36,000 dpm of ^{14}C radioactivity (0.0073% yield with respect to cell-associated ^{14}C label).

Wheat Germ Lipase

The wheat germ lipase (esterase) activity of a commercial preparation of wheat germ acid phosphatase (Worthington) was used without further purification of the enzyme(s). The esterase reaction was run in 0.10 M potassium phosphate buffer at pH 7.5, according to the methods described by Singer (Singer, 1948; Singer & Hofstee, 1948).

^{3}H-Choline Pulse Experiments

In a typical experiment, pneumococci were grown to mid-logarithmic phase in 25 ml of "Cd-enriched" medium. Cells were harvested by centrifugation at room temperature and resuspended in 30 ml of "pulse" medium (Cd enriched medium modified to contain 0.14 µg/ml choline chloride including 1.16 µC/µg ^{3}H-methylcholine). After a brief (1/2-1 min) incubation at 37°C, the culture was centrifuged again (each centrifugation lasted ≃ 3-5 min), and the cells were resuspended in 35 ml of "chase" medium (Cd enriched medium modified to contain 30 µg/ml choline chloride, non-labeled). Aliquots, 1 ml each, were removed at various times after resuspension in the chase medium, and immediately heat-inactivated at 80-90°C for 15 minutes to prevent further biosynthetic activity. The aliquots were then fractionated by one of the following methods:

(a) Chalaropsis lysozyme digestion – the aliquots were washed once with 0.05 M acetate buffer, pH 5.0, containing 0.02% NaN_3, and resuspended

in 0.2 ml of the same buffer. To each were added 0.1 ml of a 20 μg/ml solution of Chalaropsis lysozyme. Digestion proceeded for 22 hours at 30°C, whereupon the samples were centrifuged. The supernatant was taken to be the C-carbohydrate containing fraction, and the pellet was taken to be the F-antigen-containing fraction (see Table IX, p.81). The two fractions were each scored for radioactivity.

(b) Autolysis method - the heat-killed aliquots were washed once with 0.05 M sodium acetate, and then resuspended with 1 ml of a uniform suspension of live (unlabeled) pneumococci in 0.05 M sodium acetate (pneumococci from 200 ml of culture, washed and resuspended with 24 ml, 1 hour prior to use). Each sample then received 1-2 drops of toluene and was incubated at 37°C for ≈ 80 hours (control experiments showed that heat-killed cells, uniformly labeled with ^{3}H-choline, underwent apparently normal wall autolysis when incubated together with an excess of normally-autolyzing cells). The samples were then centrifuged, and the supernatant and pellet fractions were scored separately for radioactivity, as in (a) above.

Competent and Incompetent Cells

Competent and incompetent twin cultures of pneumococci were prepared according to published procedures used routinely in the laboratory of Prof. A. Tomasz (1973). The level of competence of the cultures was tested by transformation with exogenous pneumococcal DNA bearing a streptomycin-resistance marker. The cells were heat-killed at 65-90°C for 10 minutes to inactivate the endogenous hemolysins, and stored at -20°C before use. The F-antigen assays were performed on whole (thawed) cells.

Determination of F-Antigen Content per Cell as a Function of Growth

Pneumococci were grown up in 400 ml of C medium, from a 10 ml inoculum at mid-logarithmic phase. Growth of the culture was monitored with a Coleman Nephelometer (Nephelo-Spectrophotometer). At various times during growth of the culture, known sample volumes were removed; the

samples were harvested at 0°C by centrifugation, resuspended in 5 ml of saline, heated to 90°C for 10 minutes, and stored at -20°C.

The thawed saline suspensions were standardized to suspensions whose cell densities corresponded to 800 Nephelos units (2×10^8 cells/cc) by appropriate dilution with saline (for example, a sample, in 5 ml of saline, containing cells originally collected from 50 ml of culture harvested at 126 Nephelos units was diluted with 0.4 volume of saline; a sample, in 5 ml, containing cells collected from 10 ml of culture harvested at 824 Nephelos units was diluted with 1.06 volumes of saline, etc.). Each standardized suspension was then assayed for F-antigen by the hemolysis-inhibition assay (two-fold serial dilutions of the suspensions were employed).

RESULTS

Presence of Choline in the F-Antigen

The following experiments show that choline is a component of the pneumococcal F-antigen.

Copurification of F-antigen and ^{3}H-choline-containing material: Autolysis of pneumococci yields a particulate detritus fraction which can be separated from the soluble fraction by centrifugation. The detritus fraction contains essentially all of the F-antigen of the bacteria; the C-carbohydrate is found in the soluble fraction (Fig. 6).

In order to determine whether or not the F-antigen contains choline residues, F-antigen was prepared from pneumococci uniformly labeled with ^{3}H-choline; for comparative purposes, C-carbohydrate was also prepared from the same culture. In the experiment described below, portions were sampled for radioactivity and F-activity (hemolysis-inhibition activity) after each major step of the isolation procedure. The procedure, described in detail in Methods, is presented schematically in Figure 6 for ease of discussion.

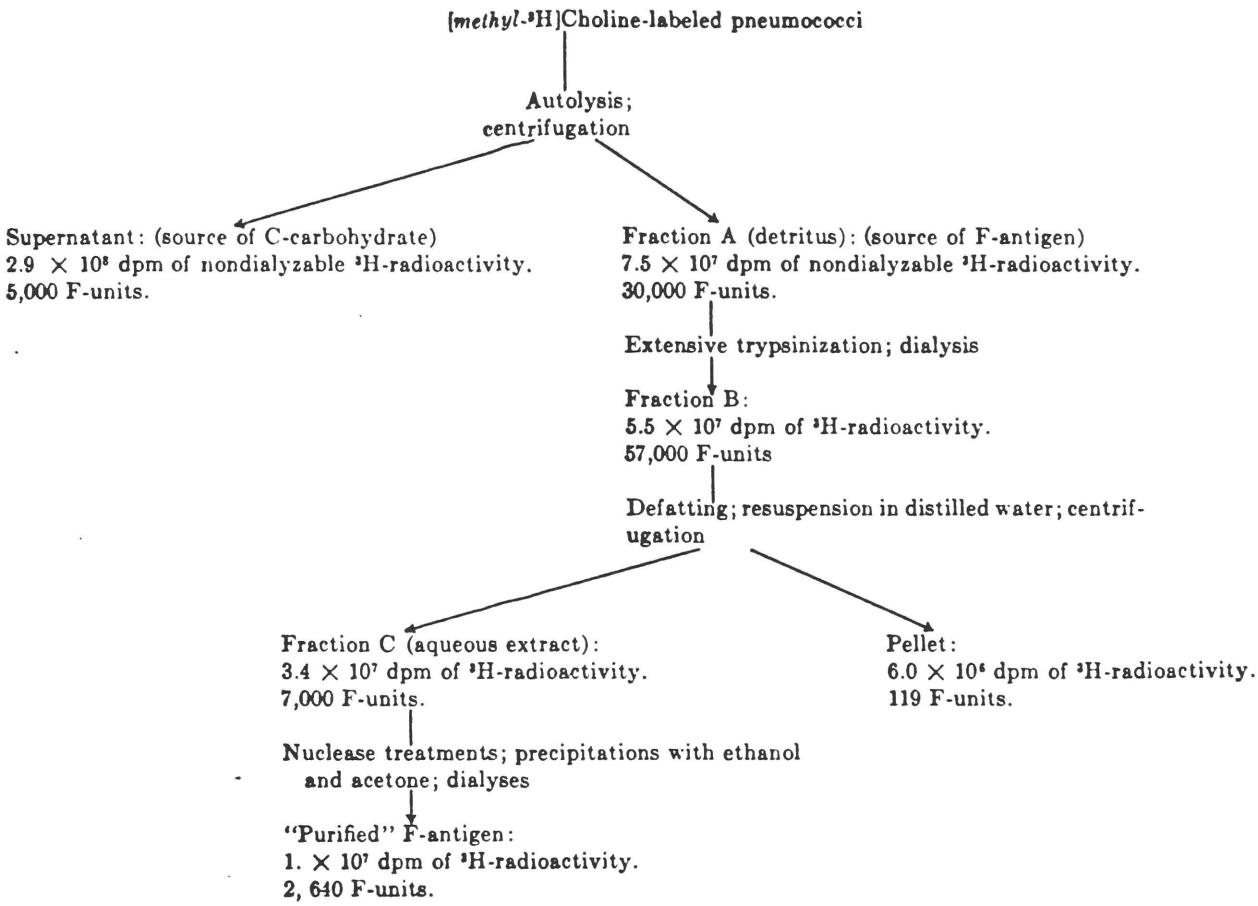


Fig. 6. Co-fractionation of radioactive choline and antigenic activity during isolation of F-antigen (Preparation #1; see Table I). See text for details. (Figure reproduced from Briles, E.B. and Tomasz, A. J. Biol. Chem. 248 (1973), p. 6395)

After centrifugation of the autolyzate of bacteria from 2.2 liters of culture, two fractions (detritus and supernatant) were obtained; each were dialyzed. The detritus was found to contain 21% of the total non-dialyzable ^{3}H-label and 86% of the F-activity; 79% of the ^{3}H-label and 14% of the total F-activity was associated with the supernatant. Amino acid analysis (kindly performed by Dr. Paul Fletcher) showed that 100% of the nondialyzable muramic acid and 95% of the nondialyzable glucosamine was associated with the supernatant, indicating that all of the cell wall peptidoglycan was solubilized during autolysis (this particular point will be discussed in greater detail later on). 70% of the ^{3}H-label associated with the supernatant was recovered as C-carbohydrate; the non-recovered ^{3}H-label from the supernatant fraction probably represents mechanical losses of the material during the preparation procedure.

Extensive trypsinization and dialysis of the detritus (yielding Fraction B) resulted in the loss of only 27% of the detritus-associated radioactivity. This probably represents largely mechanical losses.

The apparent increase in amount of F-activity in going from Fraction A to Fraction B deserves comment: the hemolysis inhibition assay, used for determination of the F-antigen, is dependent on the ability of the hemolytic antiserum to recognize the F-antigen and bind to it in preference to sheep red cells. This ability, in turn, is greatly affected by the condition or microenvironment of the F-antigen being assayed (e.g., particle-associated vs. soluble). Thus, no attempt should be made to infer quantitative yield of F-antigen on the basis of F-activity titer; the assay was used merely to follow the course of the isolation procedure.

When Fraction B, which was microparticulate in nature, was defatted with organic solvents and then resuspended in distilled water, 62% of the radioactivity was recovered in water-soluble form and 11% remained sedimentable by centrifugation; the unrecovered 27% of the radioactivity once again most likely represents mechanical losses.

In this experiment, approximately two-thirds of the water-soluble F-antigen (Fraction C) was lost during the final steps of the procedure; this loss is probably due to incomplete precipitations and mechanical losses during dialyses and handling. The final product, or "purified" F-antigen,

was dissolved in 6 ml of distilled water. The preparation contained no detectable protein (less than 10 μg/ml) according to the assay of Lowry, et al. (1951). There was insufficient material in this particular preparation for a dry-weight determination (Preparation #1). However, similar preparations were made from larger amounts of starting material and were analyzed more fully; the results of these experiments are summarized in Table II.

In order to identify the ^{3}H-labeled constituent(s) of the "purified" F-antigen, a sample was hydrolyzed and subjected to paper and thin-layer chromatography (see Methods). The ^{3}H-labeled hydrolysis product travelled as a homogeneous compound with the R_f value of choline in each case (Table III).

Identity of ^{3}H-choline-labeled material with F-antigen as determined by sucrose density gradient centrifugation and by column chromatography: The purified ^{3}H-choline-labeled F-antigen was characterized on sucrose gradients and by gel column chromatography. Fractions were assayed for F-activity and ^{3}H-radioactivity. For comparative purposes, ^{3}H-choline-labeled or ^{14}C-choline-labeled C-carbohydrate was characterized in the same gradients and columns; in the case of the C-carbohydrate, the fractions were assayed for radioactivity only.

When the purified ^{3}H-choline-labeled F-antigen was run on sucrose gradients, the F-activity and the ^{3}H-radioactivity always travelled together. Under the conditions employed (see Methods), the F-antigen was found in the lower third of the gradient. By comparison, the C-carbohydrate always remained at the top of the gradient (see Fig. 7).

In one experiment, purified ^{3}H-choline-labeled F-antigen and ^{14}C-choline-labeled C-carbohydrate were mixed together and run on the same gradient; fractions were assayed for radioactivity only. The bands of radioactivity associated with the ^{3}H- and ^{14}C-radioactivity were found to be well separated (Fig. 8).

When the F-antigen was run on Sephadex G-100 columns, both the F-activity and the ^{3}H-radioactivity eluted with the void volume (Fig. 9). By comparison, the C-carbohydrate elutes from G-100 columns in a double peak, one part of which coincides with the exclusion volume (Fig. 10).

TABLE III

Chromatography of ^{3}H-Labeled Component of F-Antigen

Purified ^{3}H-labeled F-antigen, prepared from pneumococci grown in the presence of [methyl-^{3}H]choline chloride (New England Nuclear) was hydrolyzed in 6 N HCl at 100° for 10 hours. The hydrolysate was subjected to chromatography in the following systems: A, descending paper chromatography, Whatman grade No. 1, in concentrated NH_4OH-ethanol-water (5:90:5, v/v), for 10 hours (Tomasz, 1967); B, thin-layer chromatography on silica gel, Eastman Chromagram sheet No. 6060, in methanol-concentrated HCl (95:5, v/v) for 4 hours (Reismann & Wieske, 1967); C, Eastman Chromagram sheet No. 6060, in 0.25 M Tris, pH 8, in 50% ethanol, 8 hours (Kennedy, 1956). [1,2-^{14}C]Choline chloride (New England Nuclear), [2-^{3}H]ethan-1-ol-2-amine (Amersham/Searle), and L-serine were employed as standards. Developed chromatograms were cut into vertical strips and sliced at 0.5 cm (silica gel) or 1 cm (paper) intervals. Slices were placed in scintillation fluid and radioactivity determined in a Nuclear Chicago Unilux scintillation spectrophotometer. For localization of L-serine, the appropriate vertical strip was sprayed with ninhydrin (Brinkmann Instruments) and heated to 100° for 10 min.

Sample	R_F values		
	Solvent A	Solvent B	Solvent C
Serine	0.125	0.85	0.69
Ethanolamine	0.550	0.81	0.19
Choline	0.400	0.44	0.06
Hydrolysate of F-antigen	0.400	0.44	0.06

(Reproduced from Briles, E.B. & Tomasz, A., J. Biol. Chem. 248 (1973) p. 6396).

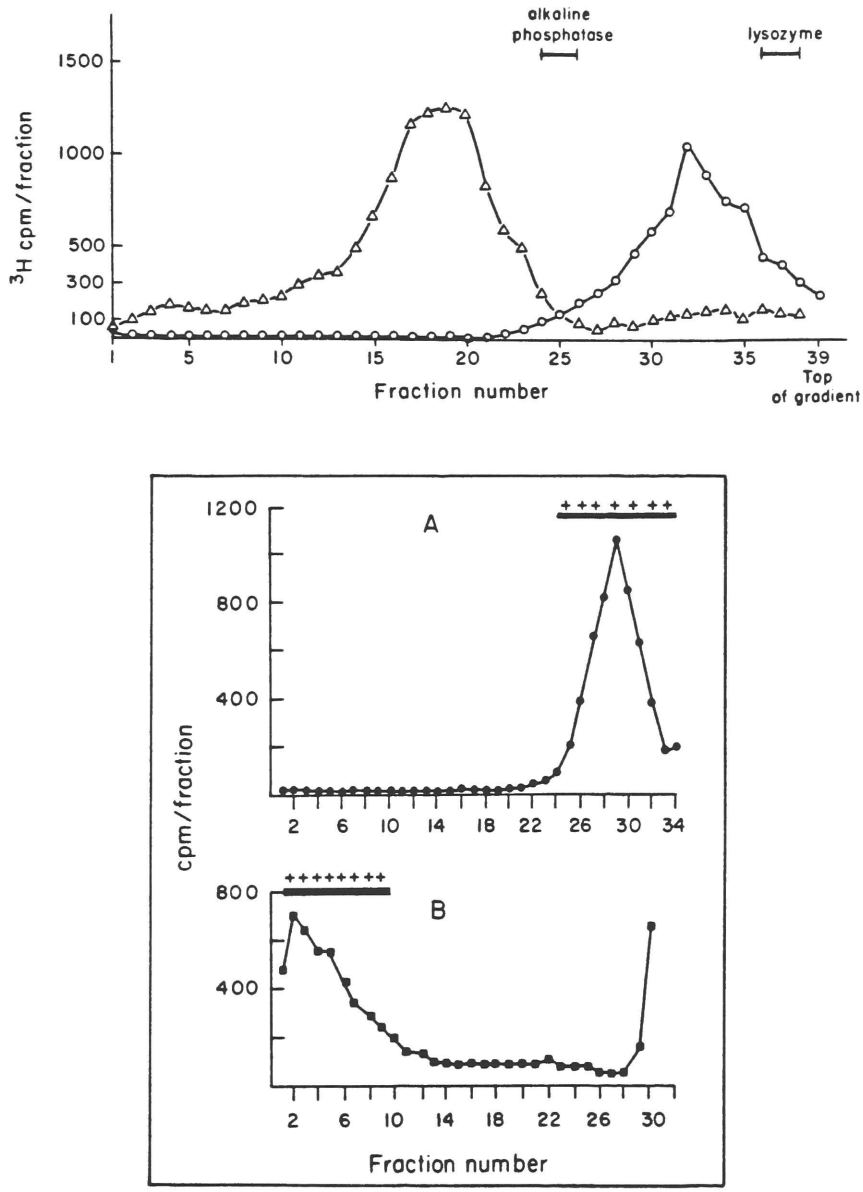


Fig. 7. Sucrose-gradient banding patterns. Upper figure: 5-20% sucrose gradients prepared according to Haseltine and Fox (1971), in NaCl-EDTA-Tris pH 8; centrifuged 14 hours at 35,000 RPM in a Beckman SW 50.1 rotor at 15°C. Alkaline phosphatase (MW ≈ 80,000) and lysozyme (MW ≈ 14,000) banding positions are indicated. Δ-Δ-Δ, ^{3}H-choline-labeled F-antigen; 0-0-0, ^{3}H-choline-labeled C-carbohydrate. Blue Dextran 2000 travels to bottom of tube under these conditions. Lower figure: 5-20% sucrose gradients in 0.15 M NaCl, in presence (A) or absence (B) of 0.4% sodium dodecyl sulfate. Top of each gradient is at right. Horizontal bars with plus signs indicate region of each gradient containing F-antigen activity; curves denote ^{3}H-choline-labeled radioactivity of the F-antigen preparation.

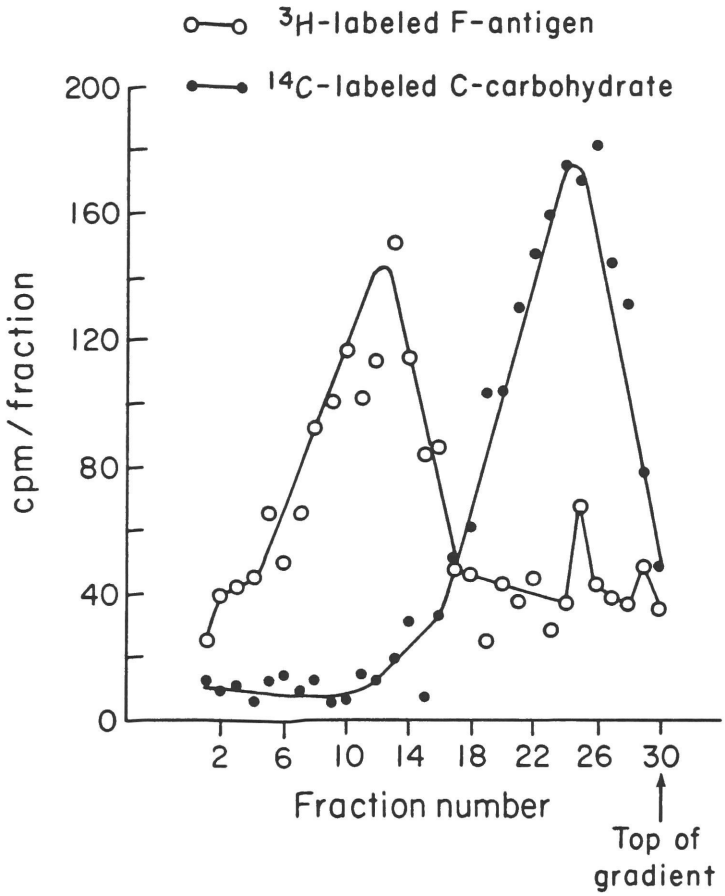


Fig. 8. Banding pattern of ^{3}H-choline-labeled F-antigen and ^{14}C-choline-labeled C-carbohydrate run simultaneously on the same sucrose gradient. The gradient consisted of 5-20% sucrose in 0.15 M NaCl containing 0.10 M potassium phosphate, pH 8; centrifugation was for 15 hours at 35,000 RPM in a SW 50.1 rotor at 10°C.

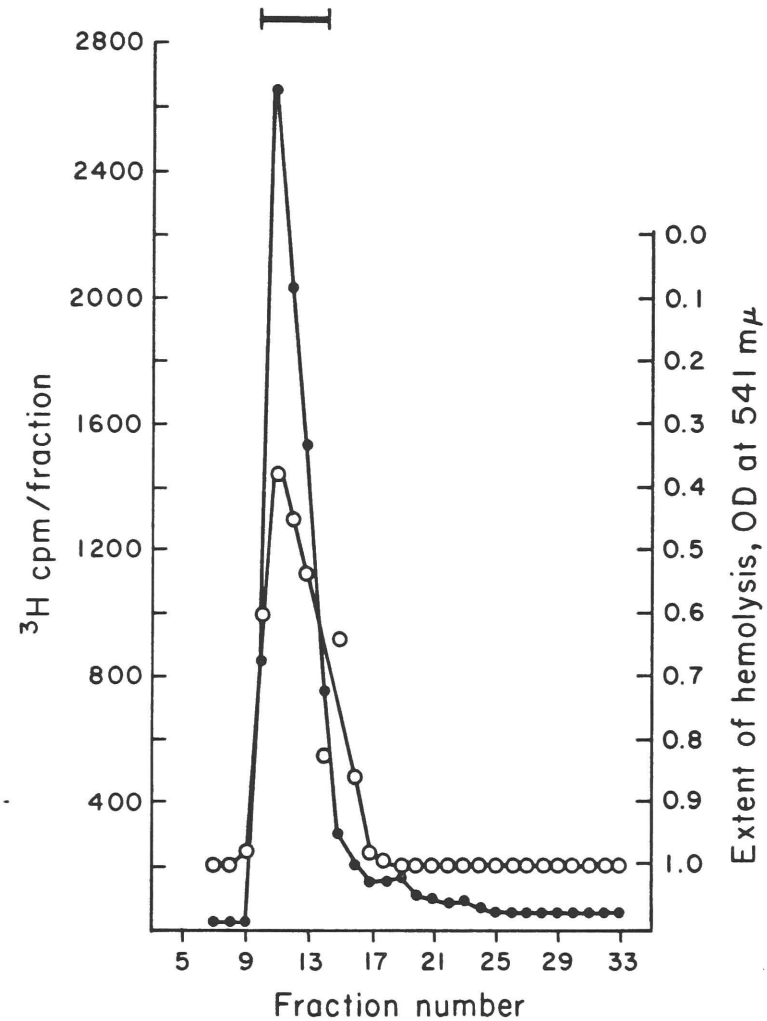


Fig. 9. Elution profile of isolated ^{3}H-choline-labeled F-antigen on Sephadex G-100 (1 x 25 cm column). ●-●-●, radioactivity; 0-0-0, extent of hemolysis (release of hemoglobin to the assay supernatant, as determined colorimetrically). The eluant was 0.15 M NaCl; the horizontal bar denotes the elution position of Blue Dextran 2000 (void volume). Fraction volumes were approximately 0.5 ml.

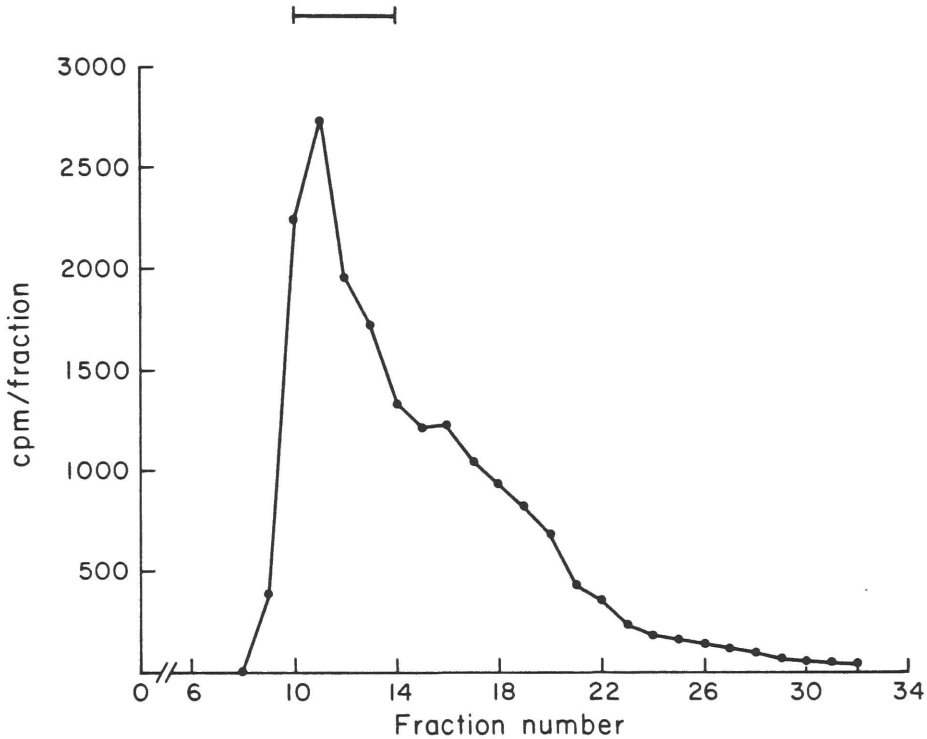


Fig. 10. Elution profile of ^{3}H-choline-labeled C-carbohydrate on Sephadex G-100 (1 x 25 cm column). Horizontal bar indicates elution position of Blue Dextran 2000 (void volume). Eluant was 0.15 M NaCl. Fraction volumes were approximately 0.5 ml.

On Bio-Gel A-5m (Agarose) columns, most of the F-antigen co-elutes with Blue Dextran 2000 while some of it elutes in a trailing shoulder; again, the radioactivity and F-activity co-elute. On this column, the C-carbohydrate elutes in a broad double peak well separated from the peak position of the F-substance (Fig. 11).

Recognition of phosphocholine residues in the F-antigen by a myeloma protein: The TEPC-15 myeloma protein is one of several well-characterized myeloma proteins known to specifically bind phosphocholine (Potter & Lieberman, 1970). It is a serum immunoglobulin of the IgA class, and is produced by a plasma-cell tumor originally induced in a BALB/c mouse by intra-peritoneal injection of Pristane. Since the original induction of the TEPC-15 plasma cell tumor in 1969, the tumor cell line has been converted to ascites form and cultured in vivo by intraperitoneal passage of the cells in BALB/c hosts. The myeloma protein can be precipitated from the ascites fluid or serum of such tumor-bearing mice with ammonium sulfate at 50% saturation (Potter & Lieberman, 1970).

The purpose of the experiments that follow was to utilize the specificity of the TEPC-15 myeloma protein to provide further evidence for the presence of choline residues on the F-antigen.

If phosphocholine were present on the F-antigen, it would be expected that the TEPC-15 myeloma protein would bind to it. Such an event might be expected to render the F-antigen molecule unavailable for binding by the heterophile (hemolytic) antibody, and thus undetectable by the hemolysis-inhibition assay. It would furthermore be expected that the binding of the TEPC-15 myeloma protein would be inhibitable with phosphocholine. Also, all of the choline-containing F-antigen should be precipitable with the myeloma protein, either by immunoprecipitation at equivalence, or by ammonium sulfate precipitation of the complex under conditions of antibody excess. This precipitation should also be inhibitable by the appropriate hapten (phosphocholine).

Whole ascites fluid (preserved with 0.25% NaN_3) containing the TEPC-15 myeloma protein was generously provided by Dr. Michael Potter of NIH. Neither the ascites fluid (hereafter referred to simply as

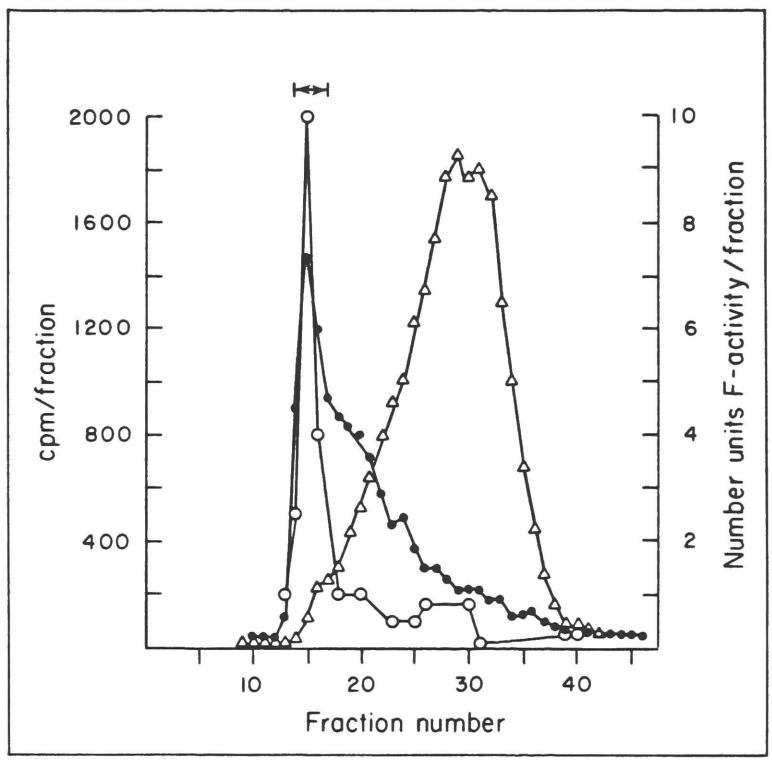


Fig. 11. Elution patterns of ^{3}H-choline-labeled macromolecules on Bio-Gel A-5m (1 x 32 cm column). Δ-Δ-Δ, ^{3}H-radioactivity associated with C-carbohydrate; ●-●-●, ^{3}H-radioactivity associated with F-antigen; 0-0-0, units of F-antigen activity as determined by hemolysis inhibition assay. Eluant was 0.15 M NaCl containing 0.010 M K_2HPO_4. The horizontal arrow indicates elution position of Blue Dextran 2000. Fraction volumes were approximately 0.5 ml. (Figure reproduced from Briles, E.B. and Tomasz, A., J. Biol. Chem. 248 (1973), p. 6396)

"TEPC-15"), nor phosphocholine, in the concentrations employed, had any effect on the hemolysis assay. Preliminary experiments indicated that TEPC-15 prevents inhibition of hemolysis by whole heat-killed pneumococci, and that this TEPC-15 effect is inhibitable with phosphocholine.

The experiments summarized in Table IV demonstrate the ability of TEPC-15 to inhibit detection of the "purified" F-antigen by the hemolysis inhibition assay. In part A, four units of "purified" F-antigen (Preparation 1) in 0.5 ml were incubated with an equal volume of a dilution of TEPC-15 ascites fluid for 1 hour at room temperature (no precipitate was formed). This mixture was then tested for residual F-activity by the hemolysis inhibition assay. In this experiment, maximal F-activity (in the absence of TEPC-15) gave 82% inhibition of hemolysis. A decreased inhibition of hemolysis is indicated as a proportional decrease in residual F-activity. As can be seen from Table III, TEPC-15 at 100-fold dilution had a marked effect on apparent F-activity; at the lower concentrations of TEPC-15 tested, there was no observed effect.

In part B, aliquots of 100-fold diluted TEPC-15 containing various amounts of phosphocholine were prepared and pre-incubated at room temperature for 1 hour; samples were then tested for their ability to bind the F-antigen, exactly as in part A. The results obtained indicate that the phosphocholine was able to prevent the TEPC-15-mediated depression of apparent F-activity.

The experiment summarized in Table V demonstrates the ability of TEPC-15 to precipitate the purified F-antigen in a hapten-inhibitable manner. In this experiment, 0.5 ml portions of undiluted TEPC-15 were pre-incubated with either 0.5 ml saline or 0.5 ml of a 20 mg/ml solution of phosphocholine Cl·Ca·3 1/2 H_2O at room temperature for 30 minutes; 5 µl of a solution of ^3H-choline-labeled F-antigen (Preparation #2) containing 1.2×10^5 cpm ^3H and 400 units of F-activity were added to each sample (as well as to controls containing either 1.0 ml saline or 0.5 ml saline plus 0.5 ml of 20 mg/ml phosphocholine), and the mixtures were incubated overnight at 5°C. Each sample then received an equal volume of either saline or saturated (room temperature) ammonium sulfate and

TABLE IV

Blocking of F-antigen activity by a choline-specific myeloma protein

Preliminary experiments showed that neither phosphocholine nor TEPC-15 ascites fluid, in the concentrations used, had any effect on the hemolysis inhibition assay.

Part A—Four units of purified F-antigen in 0.5 ml were incubated with an equal volume of a dilution of TEPC-15 ascites fluid for 1 hour at room temperature. This mixture was then tested for residual F-antigen activity by the hemolysis inhibition assay. In this experiment, maximal F-activity (in the absence of TEPC-15) gave 82% inhibition of hemolysis. A decreased inhibition of hemolysis in the assay, due to binding of the F-antigen by the myeloma protein, is indicated as a proportional decrease in residual F-antigen activity.

Part B—Aliquots of 100-fold diluted TEPC-15 ascites fluid containing varying amounts of hapten (phosphocholine chloride · calcium 3½ H_2O) were preincubated for 1 hour at room temperature, and then tested for their ability to bind F-antigen, exactly as in Part A.

	Residual F-antigen activity
	%
A. Dilution of TEPC-15 used	
No TEPC-15 (maximal F-antigen activity).....	100
10,000-fold diluted.....	100
1,000-fold diluted.....	100
100-fold diluted.....	31
10-fold diluted.....	29
B. Concentration of phosphocholine present during preincubation	
No phosphocholine.....	29
0.01 mg per ml.....	80
0.10 mg per ml.....	83
1.0 mg per ml.....	94
10.0 mg per ml.....	97
No TEPC-15 (maximal F-antigen activity).....	100

(Reproduced from Briles, E.B. and Tomasz, A., J. Biol. Chem. 248 (1973) p. 6397).

TABLE V

SAMPLE	% RADIOACTIVITY in precipitate	% RADIOACTIVITY in supernatant	F-ACTIVITY units per 0.5 ml supernatant
^{3}H-choline-labeled F-antigen	--	100%	65
" " " +phosphocholine	--	100%	50
" " " +ammonium sulfate	--	100%	25
" " " +TEPC-15	77%	23%	<5
" " " +TEPC-15 + ammonium sulfate	99%	1%	<<5
" " " +TEPC-15 pre-incubated with phosphocholine	2%	98%	17
" " " +TEPC-15 pre-incubated with phosphocholine + ammonium sulfate	38%	62%	10

incubated at 5°C for an additional 20 hours. The samples were then centrifuged. Each precipitate was washed once with 1.0 ml 50% saturated ammonium sulfate, resuspended in saline, and assayed for radioactivity; each supernatant was assayed for both radioactivity and for F-activity (it was necessary to dialyze the ammonium-sulfate-containing supernatants against saline before performing the hemolysis-inhibition assays).

At the concentrations used in this experiment, substantial immunoprecipitation occurred when the F-antigen and TEPC-15 were mixed. When TEPC-15 was pre-incubated with phosphocholine, a cloudiness formed in the test tube which was not affected by the presence or absence of the F-antigen; TEPC-15 alone did not cloud.

As can be seen from Table V, neither phosphocholine nor ammonium sulfate alone have any appreciable effect on the F-antigen. TEPC-15 alone caused 77% of the ^{3}H-radioactivity to be precipitated, and virtually eliminated any detectable F-activity from the supernatant; the action of TEPC-15 on the ^{3}H-choline-labeled F-antigen is partially inhibited (at the concentrations used) by pre-incubation of the myeloma protein with phosphocholine.

The discrepancies between the results obtained with or without ammonium sulfate precipitation warrant comment. There was no attempt, in this experiment, to form an immunoprecipitate at equivalence; the concentrations employed were chosen arbitrarily, and it was expected that there would be an antibody (myeloma) excess. The fact that 77% of the radioactivity was immunoprecipitable in the absence of hapten suggests that this antibody excess was, in fact, not very large. The difference between the amount of radioactivity precipitated directly by the TEPC-15 and the amount of radioactivity precipitated by ammonium-sulfate treatment of the antigen-antibody complex probably represents that portion of the ^{3}H-choline-labeled F-antigen bound by the myeloma protein but not incorporated into the immunoprecipitate lattice. When the F-antigen is subjected to TEPC-15 pre-incubated with hapten, a significant amount of F-activity remains in the supernatant. No direct immunoprecipitate is formed, due to the large excess of monovalent hapten molecules which are bound by the meyloma protein in lieu of the F-antigen and thus prevent

lattice formation; however, ammonium sulfate precipitation of the myeloma protein co-precipitates a substantial amount of radioactivity, indicating that the hapten inhibition at the concentration employed is only partial.

In a control experiment, the fraction of the TEPC-15 ascites fluid not precipitable at 50% saturated ammonium sulfate was found to be completely devoid of any ability to block F-activity as detected by the hemolysis-inhibition assay.

Effect of Detergents on the Isolated F-Antigen

Since the apparent molecular size of the F-antigen was much larger than that of the C-carbohydrate, we performed certain experiments in the presence of 0.4% sodium dodecyl sulfate (SDS), in order to see if such detergent treatment would transform the F-antigen into units of smaller apparent size.

As can be seen from Figure 12, in the presence of SDS, the isolated F-antigen is retained on a Sephadex G-100 column. The effect of the SDS is to change the elution pattern of the F-antigen on G-100 from exclusion (in the absence of SDS) to retention. The elution pattern of C-carbohydrate from such a column is not affected by SDS.

A similar phenomenon is observed when the F-antigen is characterized on sucrose gradients. The single band containing both the F-activity and the ^{3}H-radioactivity moves considerably more slowly in the presence of the detergent than in its absence (Figs. 13, 14), and in fact assumes the same banding position as that characteristic of the C-carbohydrate. The C-carbohydrate bands at the same position along the gradient in either the presence or absence of SDS.

The same dissociation of the F-antigen was observed on sucrose gradients containing 1.0% sodium desoxycholate.

To test whether the observed dissociation effect was reversible, the detergent was removed (by precipitation at 0°C; see Methods) from those fractions of an SDS-sucrose gradient which contained the ^{3}H-choline-labeled F-antigen (see Fig. 14). This "detergent-treated" material was then characterized on a sucrose gradient in the absence of SDS; fractions

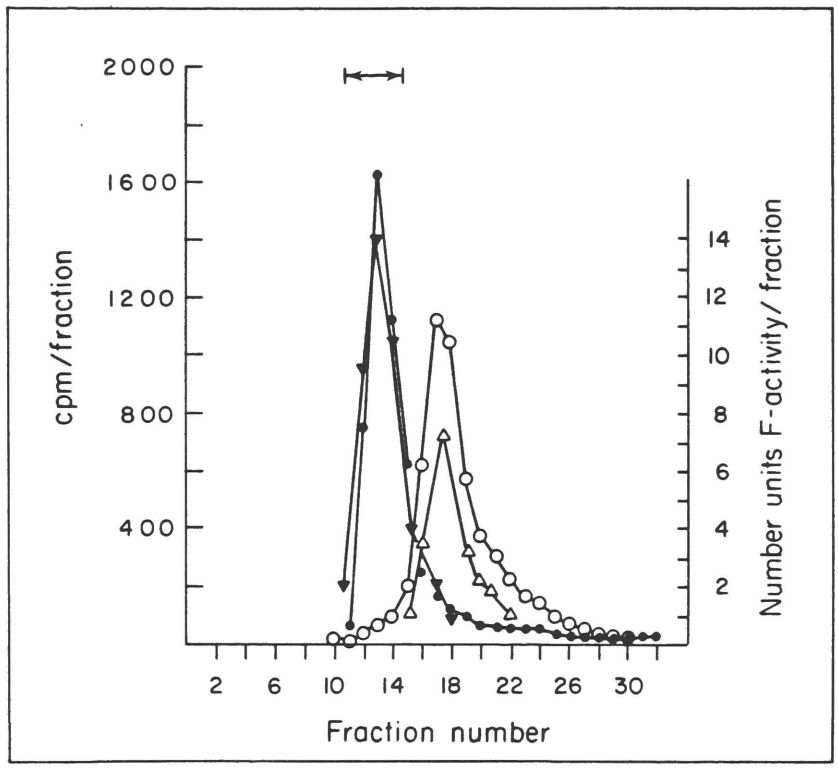


Fig. 12. Elution patterns of ^{3}H-choline-labeled F-antigen on Sephadex G-100 (1 x 25 cm column) in the absence or presence of sodium dodecyl sulfate (SDS). ●-●-●, ^{3}H-radioactivity, eluted with 0.15 M NaCl; 0-0-0, ^{3}H-radioactivity, eluted with 0.15 M NaCl containing 0.4% SDS; ▲-▲-▲, F-antigen activity eluted with 0.15 M NaCl; Δ-Δ-Δ, F-antigen activity eluted with 0.15 M NaCl containing 0.4% SDS. Horizontal arrow indicates elution position of Blue Dextran 2000 (void volume). Fraction volumes were approximately 0.5 ml. (Reproduced from Briles, E.B. and Tomasz, A., J. Biol. Chem. 248 (1973), p. 6396)

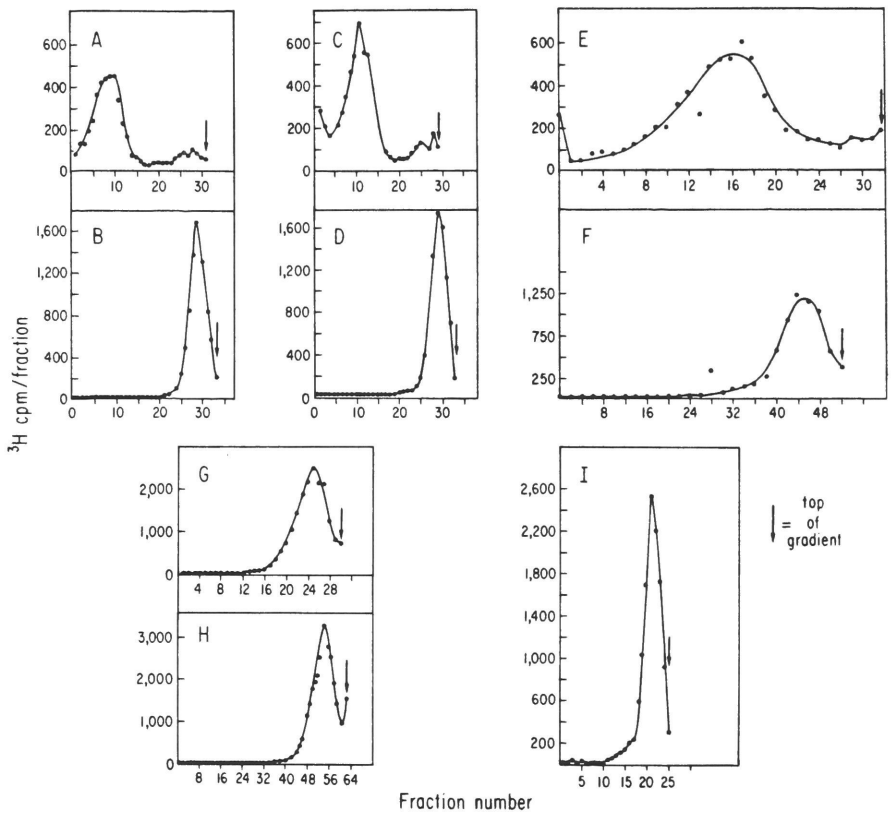


Fig. 13. Banding patterns on 5-20% sucrose gradients in the absence or presence of sodium dodecyl sulfate (SDS). All gradients contain 0.15 M NaCl; for each pair of gradients (A-H), the lower gradient (B, D, F, H) contains 0.4% SDS while the upper one (A, C, E, G) does not. A-F: ^{3}H-choline-labeled F-antigen. A,B: gradient buffered with 0.25 M acetate, pH 4.5. C,D: buffered with 0.18 M Tris Cl pH 8. E,F: unbuffered. G,H: ^{3}H-choline-labeled C-carbohydrate on unbuffered gradients. I: ^{3}H-choline-labeled F-antigen run on an unbuffered gradient containing 1.0% sodium deoxycholate throughout. Arrows indicate top of gradient.

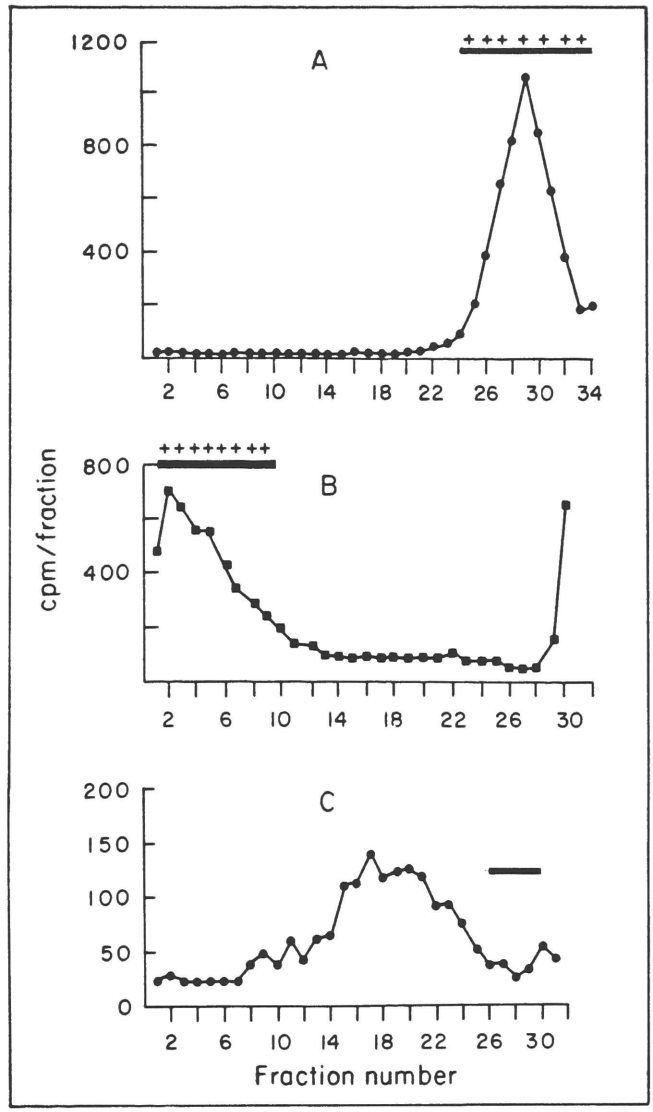


Fig. 14. Sucrose density gradient centrifugation of purified F-antigen in the absence or presence of sodium dodecyl sulfate, and partial reaggregation of sodium dodecyl sulfate-treated F-antigen. 5 to 20% sucrose gradients in 0.15 M NaCl were prepared either with (A) or without (B) 0.4% SDS. Fraction 1 is from the bottom of the tube. Fractions were each assayed for ^{3}H-radioactivity; radioactive and nonradioactive fractions were separately pooled and assayed for F-antigen activity by the hemolysis inhibition assay; results are indicated by horizontal bars with plus signs over the regions of each gradient where the F-activity was found. In C, 0.2 ml of the pooled fractions containing the F-antigen from the SDS-containing gradient (A, Fractions 24 to 34) was layered onto a sucrose gradient in 0.15 M NaCl without SDS. Detergent was first removed from the pooled samples by precipitation at 0°. Centrifugations were performed in a model L3-50 ultracentrifuge at 35,000 rpm in a Beckman SW 50.1 rotor at 13° for 14 hours. Horiziontal bar in C indicates position of C-carbohydrate in similar gradients in either the presence or absence of SDS. (Reproduced from Briles, E.B. and Tomasz, A., J. Biol. Chem. 248 (1973), p. 6397)

were assayed for radioactivity only. The results of this experiment indicate that the detergent-treated material is able to reaggregate, at least partially, after removal of the detergent.

Degradation of the F-Antigen by Periodate and by Nitrous Acid

The effects of periodate oxidation and of nitrous acid deamination on the C-carbohydrate have been suudied by Brundish and Baddiley (1968) and by Mosser (1970) and Mosser and Tomasz (1970). Their results demonstrated that these reagents are able to cause fragmentation of the C-carbohydrate.

Periodate oxidizes vicinal unsubstituted hydroxyl or amino groups to aldehyde groups, with concomitant cleavage of the involved carbon-carbon bond. The only constituent of the C-carbohydrate whose oxidation by periodate would be expected to result in fragmentation of the polymer chain is ribitol.

Nitrous acid effects destructive deamination by reacting with unsubstituted amino groups to form an unstable diazo intermediate whose decomposition results in formation of a carbonium ion. In the case of a 2-amino aldohexose, nitrous acid deamination proceeds by a nucleophilic attack on the carbonium ion at the two position by the ring oxygen, resulting in the formation of a 2,5-anhydroaldohexose. A glycosidic linkage at the C-1 position of the affected amino sugar would be hydrolyzed in the process (Jeanloz & Balazs, 1965). Since the data of Brundish and Baddiley (1968) demonstrate that the galactosamine residues of the C-carbohydrate are stable to the action of nitrous acid (and also to periodate), it is most likely that the only constituent of the polymer affected by nitrous acid is the diaminotrideoxyhexose.

^{3}H-Choline-labeled F-antigen was treated with periodate (see Methods) and the resulting ^{3}H-labeled products were characterized on Sephadex G-100, G-50, and G-25 columns. For comparison, ^{3}H-choline-labeled C-carbohydrate was treated in the same way and characterized on the same columns (Figs. 15, 16). The results demonstrate that the choline-labeled fragments obtained by periodate degradation of F-antigen

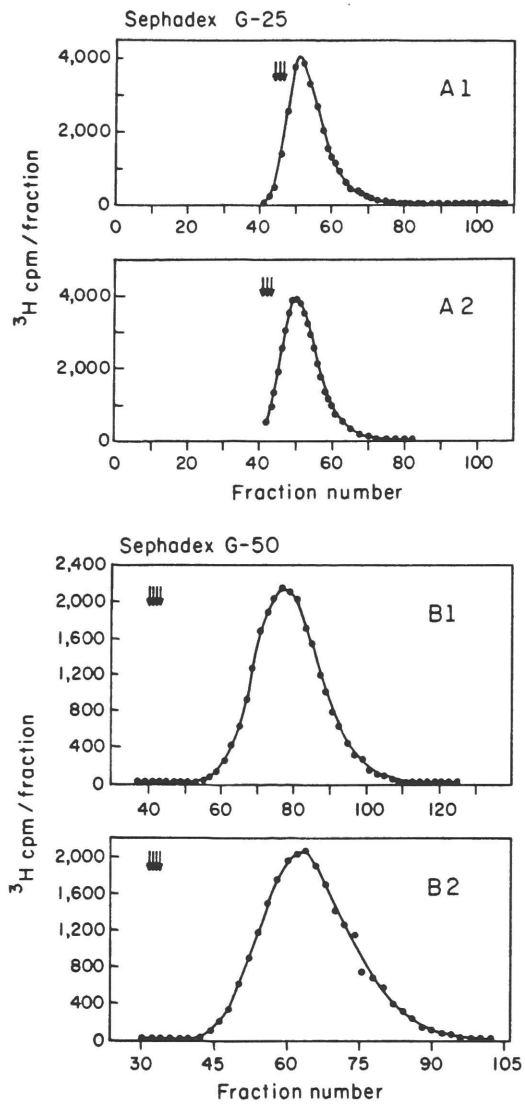


Fig. 15. Periodate-treated ^{3}H-choline-labeled F-antigen and C-carbohydrate: elution patterns on Sephadex columns. A, Sephadex G-25; B, Sephadex G-50. For each pair of curves, the upper (A1, B1) represents periodate-treated F-antigen and the lower (A2, B2) represents periodate-treated C-carbohydrate. The arrows indicate the elution position of Blue Dextran 2000 in each case. (In B1, 6-drop fractions were collected; in B2, 8-drop fractions were collected.)

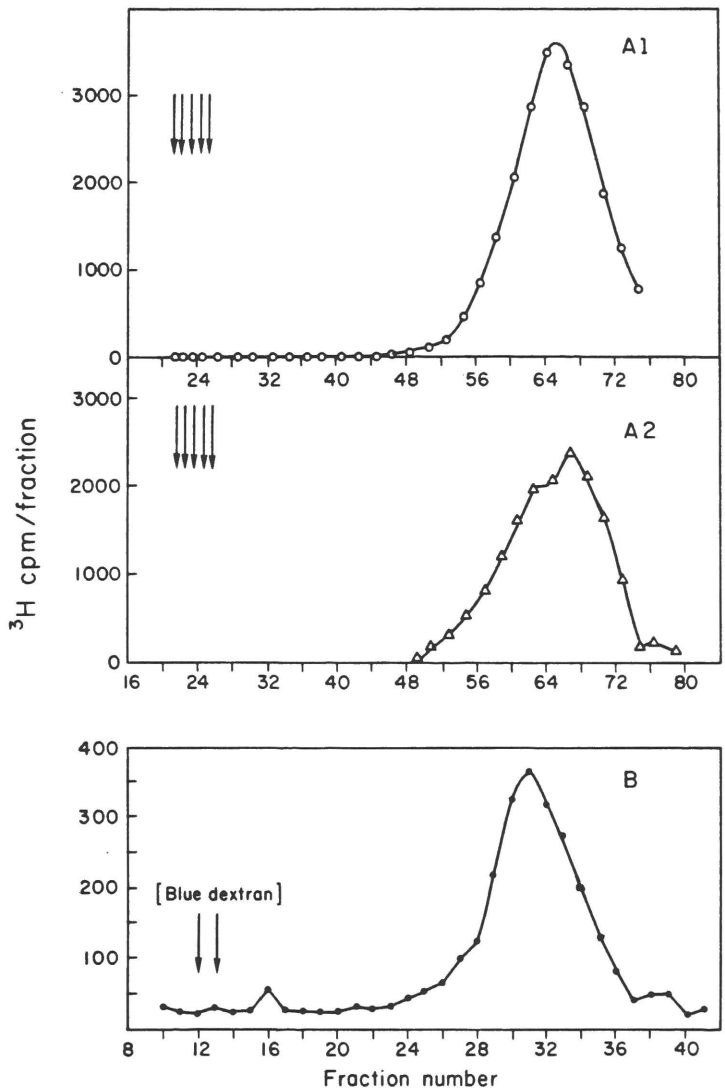


Fig. 16. Characterization of degraded teichoic acids on Sephadex G-100. A1, ^{3}H-choline-labeled F-antigen, degraded by periodate; A2, ^{3}H-choline-labeled C-carbohydrate, degraded by periodate; B, ^{3}H-choline-labeled F-antigen, degraded by nitrous acid. Arrows indicate elution positions of Blue Dextran 2000 in each case. In B, the collected fractions contained twice the volume of those collected in A.

and of C-carbohydrate have identical Sephadex elution properties, and are probably homogeneously-sized fragments with a molecular weight on the order of c. 1000 daltons.

The F-antigen was also subjected to nitrous acid degradation, and the ^{3}H-choline-labeled products were characterized on Sephadex G-100 (Fig. 16). The resulting elution pattern was essentially the same as that obtained for periodate-degraded F-antigen. Such a result was also obtained by Mosser (1970) and Mosser and Tomasz (1970) for the C-carbohydrate.

The work of Brundish and Baddiley (1968) and of Mosser (1970) and Mosser and Tomasz (1970) suggest that the C-carbohydrate is a polymer whose repeating subunits contain one residue each of ribitol, diaminotridesoxyhexose, glucose, N-acetyl galactosamine, and choline, and two residues of phosphate; and furthermore, that at least the ribitol and the diaminotrideoxyhexose residues occur in the linear backbone of the polymer (see Introduction). The data presented above suggests that the F-antigen shares this structure with the C-carbohydrate.

Effect of pH 2 at Elevated Temperature

Goebel, et al. (1943) reported a series of experiments in which the stability of the F-antigen was tested at various pH values at 100°C. Integrity of the antigen was determined by two different criteria: by the ability of the material to inhibit hemolysis (F-activity), and by its ability to be precipitated by Pneumococcus R Antiserum. Their results are presented in Table VI. As a rule, F-activity and immuno-precipitability were both either destroyed or not destroyed in each trial; however, they found that heating at pH 1.97 resulted in a significant (though not complete) loss of F-activity but little or no decrease in precipitability with the antiserum.

The following experiments represent an attempt to take advantage of this "limited lability" of the F-antigen at pH 2 to further characterize the molecule.

TABLE VI

Inhibition of Hemolysins in Pneumococcal Heterophile Antiserum by F Polysaccharide after Heating at Various Values of pH

0 = complete hemolysis inhibition; ++++ = complete lysis, or no hemolysis inhibition.

F carbohydrate heated 30 min at pH	Concentration of F carbohydrate tested					
	1:20,000	1:40,000	1:80,000	1:160,000	1:320,000	1:640,000
1.13	++++	++++	++++	++++	++++	++++
1.97	+	+	+	+±	+++	++++
2.80	0	0	0	0	0	++
7.00	0	0	0	0	0	++
9.54	+	+±	++±	+++±	++++	++++
11.89	++++	++++	++++	++++	++++	++++

Precipitation of F Polysaccharide in Pneumococcus R Antiserum after Heating at Various Values of pH

+++ = heavy disk-like precipitate; 0 = no precipitation.

F carbohydrate heated 30 min at pH	Final concentration of F carbohydrate tested			
	1:500,000	1:250,000	1:500,000	1:1,000,000
1.13	+	0	0	0
1.97	+++	+++	++	+±
2.80	+++	+++	++±	++±
7.00	+++	+++	++±	++
9.54	+±	++	++±	++
11.89	±	0	0	0

Reproduced from Goebel, W.F., Shedlovsky, T., Lavin, G.I., and Adams, M.H. J. Biol. Chem. 148, 13 (1943) by permission of the author.

The material heated at pH 2 exhibited only 15% as much F-activity as the pH 7-heated control, thus suggesting that much of the antigenic material is destroyed at pH 2. On sucrose gradients (Fig. 17), the pH 2-treated material gave rise to two bands of radioactivity: 77% of the radioactivity banded at the usual position of the F-antigen, while 23% appeared near the top of the gradient. In contrast, 92% of the pH 7-treated control banded at the position of the F-antigen (this is characteristic of Preparation #2). When eluted from a Sephadex G-100 column, 57% of the radioactivity of the pH 2-treated material was excluded from the gel, while 43% of the radioactivity appeared in a broad heterogeneous peak well separated from the excluded material. In the case of the pH 7-treated material, 92% of the radioactivity was excluded from the gel (see Fig. 18). These results suggest a physical breakdown of the F-antigen molecular structure as a result of heating at pH 2.

By way of comparison, samples of ^{3}H-choline-labeled C-carbohydrate treated at pH 2 or at pH 7 gave products whose elution characteristics in both cases were virtually identical (Fig. 18). It is of interest to note that those F-antigen "fragments" after pH 2 treatment which are retained on G-100 are, in general, smaller than the G-100 retained fraction of the C-carbohydrate material. When the C-carbohydrate is partially degraded by the action of cold 10% tricholoroacetic acid for 71 hours, the elution profile of the resulting ^{3}H-choline-labeled products on G-100 is remarkably similar to that of the pH 2-degraded F-antigen (Fig. 18).

The results of these experiments indicate that there exists a covalent bond in the F-antigen, but not in the C-carbohydrate, which is labile at pH 2 at 100°C. The serological and chemical evidence of Goebel and Adams (1943) and Goebel, et al. (1943), of Fujiwara (1967), and of Brundish and Baddiley (1967; 1968) together suggest that the "teichoic acid" chains of the F-antigen and C-carbohydrate are probably structurally identical (see Introduction), and the results of the nitrous acid and periodate degradation experiments presented earlier in this dissertation support this suggestion. Therefore, it is highly unlikely that the teichoic acid chain itself contains the mild-acid-labile bond. It is tempting to speculate that this pH 2-labile bond is situated between the

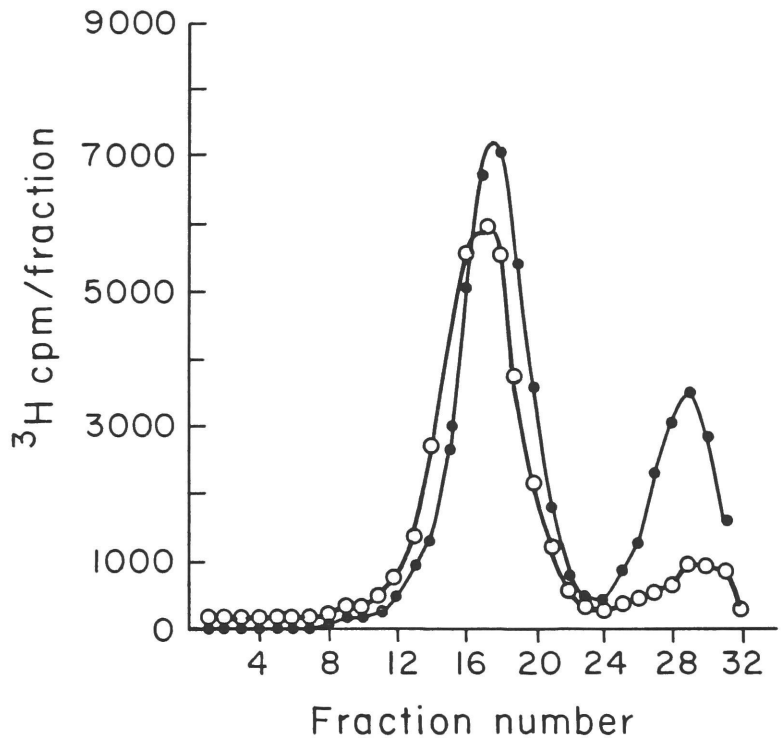


Fig. 17. Effect of heating at pH 2 on ^{3}H-choline-labeled F-antigen: characterization on sucrose gradients. ●-●-●, heated at 100°C at pH 2 for 30 minutes; 0-0-0, control, heated at pH 7. Gradients contained 5-20% sucrose in 0.15 M NaCl buffered at pH 8 with 0.10 M potassium phosphate; they were centrifuged for 15 hours at 35,000 RPM at 2°C in a Beckman SW 50.1 rotor.

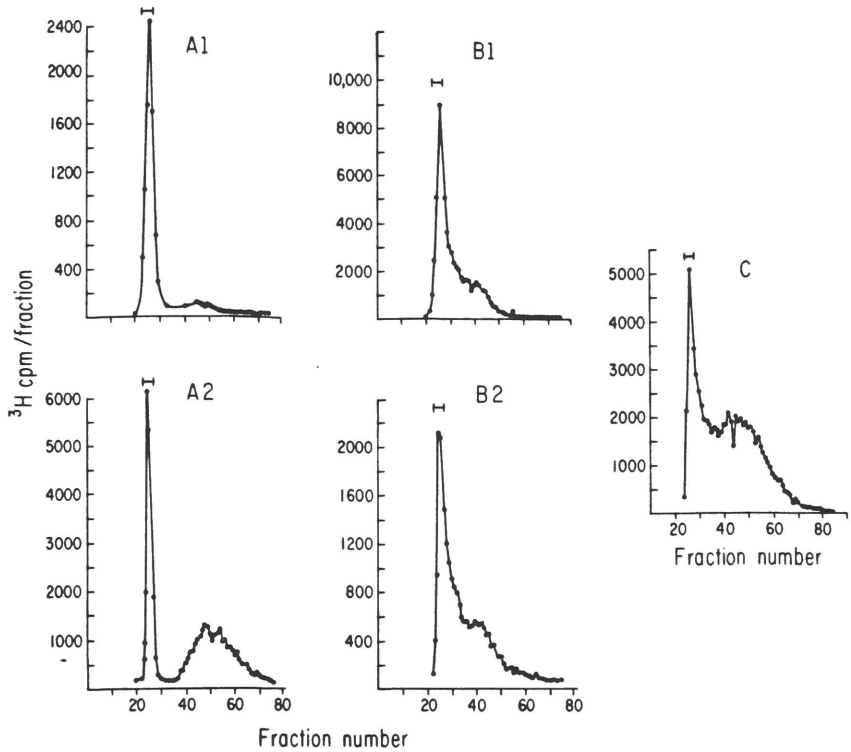


Fig. 18. Effect of heating at 100°C at pH 2 on ^{3}H-choline-labeled F-antigen and C-carbohydrate: elution profiles on Sephadex G-100. A1: F-antigen, heated at pH 7 (control). A2: F-antigen, heated at pH 2. B1; B2: C-carbohydrate, heated at pH 7 and pH 2, respectively. C: C-carbohydrate, partially degraded by cold 10% TCA (5°C, 71 hours). Horizontal bars indicate elution positions of Blue Dextran 2000 (void volume) in each case.

teichoic acid chain and the lipophilic moiety of the F-antigen. If so, then the teichoic acid chains of the F-antigen are of heterogeneous length.

An attempt was made to determine whether the pH 2-labile bond could be a phosphodiester (or possibly a pyrophosphodiester). PH-2-degraded F-antigen was incubated with phosphatase (chicken intestinal alkaline phosphatase, Worthington); orthophosphate was then assayed by the molybdate-ascorbate test (Ames, 1966). Since the F-antigen itself produces a blue color with the molybdate-ascorbate reagent (which is not extractable with isoamyl alcohol), it was necessary to first separate the orthophosphate from the teichoic acid; this was done by passage through Sephadex G-10. No evidence was found for any release of orthophosphate.

Localization of the F-Antigen

The following experiments suggest that the F-antigen may be located in the cytoplasmic membrane of the pneumococcus.

Analysis of products of autolysis: The results of analysis of the autolytic supernatant and detritus fractions on an amino acid analyzer are tabulated in Table VII. All of the muramic acid of the dialyzed autolyzate is found in the supernatant, indicating that the cell wall is completely solubilized by autolysis. Furthermore, the composition of the supernatant is strikingly similar to that observed by Mosser (1970) and Mosser and Tomasz (1970) for the choline-containing products of autolytic enzyme action on purified cell walls (see Table VIII), suggesting that wall materials may be the only cell components released during autolysis. Since the F-antigen does not become solubilized during autolysis, it is reasonable to conclude that the F-antigen is not an integral part of the wall. Possibly, it is part of both the wall and some non-wall structure and becomes dissociated from the wall but not from this non-wall structure during autolysis.

Microscopic observation of autolytic detritus: Electron microscopic observation of the detritus fraction suggests that it consists of membrane-enclosed vesicles (see Figs. 19 and 20).

TABLE VII

Composition* of Autolytic Products

	Supernatant µmoles	Detritus µmoles
ASP	0.20	16.05
THR	0.09	8.69
SER	0.23	7.34
MURAMIC ACID	1.01	0.00
GLU	0.80	18.13
PRO	0.0	trace
GLY	0.25	14.31
ALA	1.19	14.66
CYS	0.0	0.0
VAL	trace	12.27
MET	0.022	7.14
ILEU	0.06	9.38
LEU	0.09	13.38
TYR	0.0	4.56
PHE	0.0	5.79
GLUCOSAMINE	1.01	0.16
GALACTOSAMINE	4.2	1.90
HIS	0.03	2.62
LYS	0.62	11.07
NH_4	8.61	22.46
TRP	trace	0.0
ARG	trace	6.77

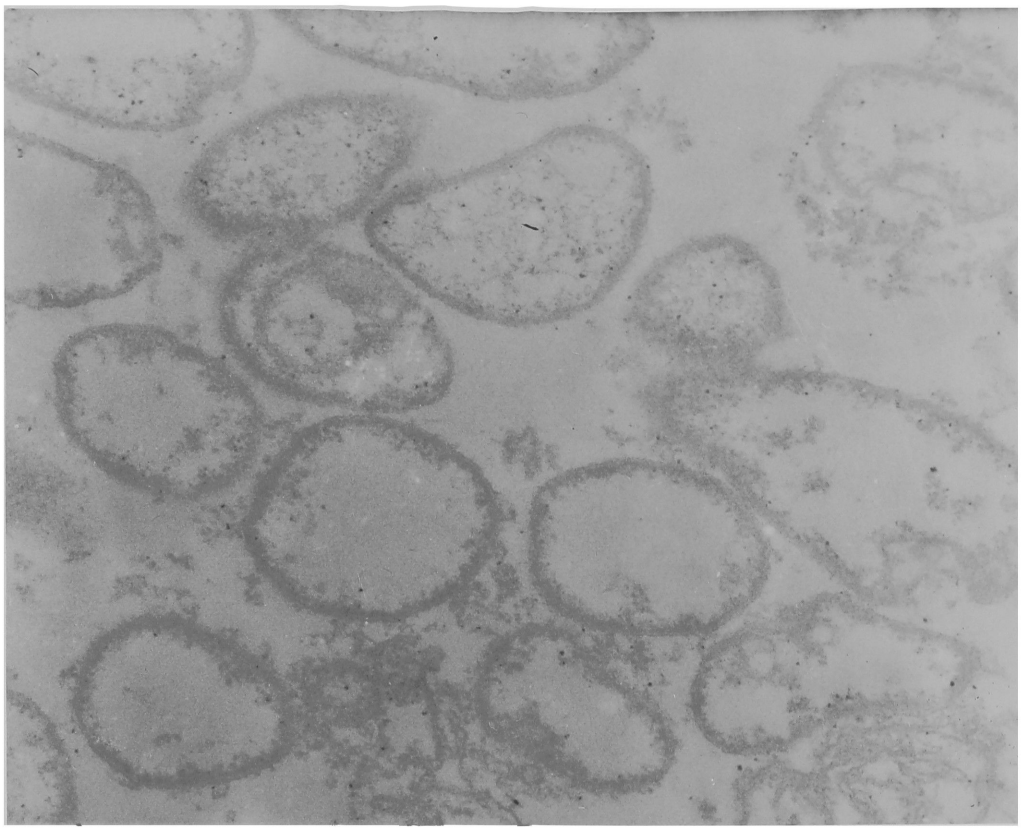
*Values listed are in µmoles for the total supernatant and detritus fractions of a single autolyzate. Analyses were performed on a Durrum D-500 analyzer by Dr. Paul Fletcher of The Rockefeller University.

TABLE VIII

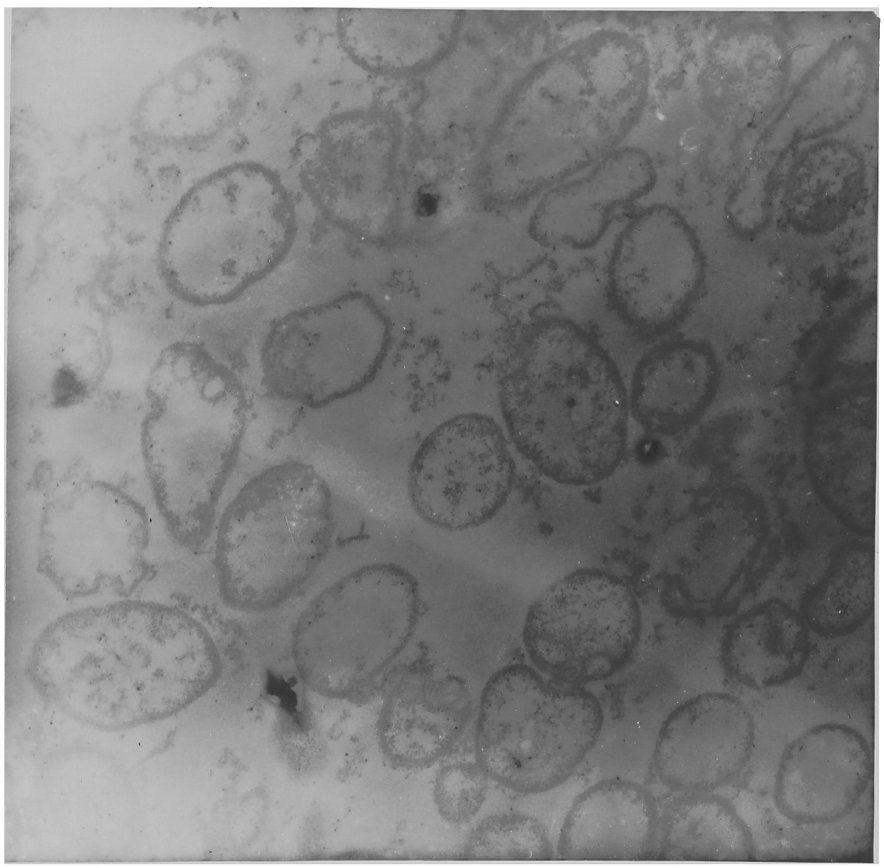
Comparisons between Compositions of Autolytic Supernatant, Cell Walls,* Choline-labeled Autolytic Enzyme Products,* and C-Polysaccharide†

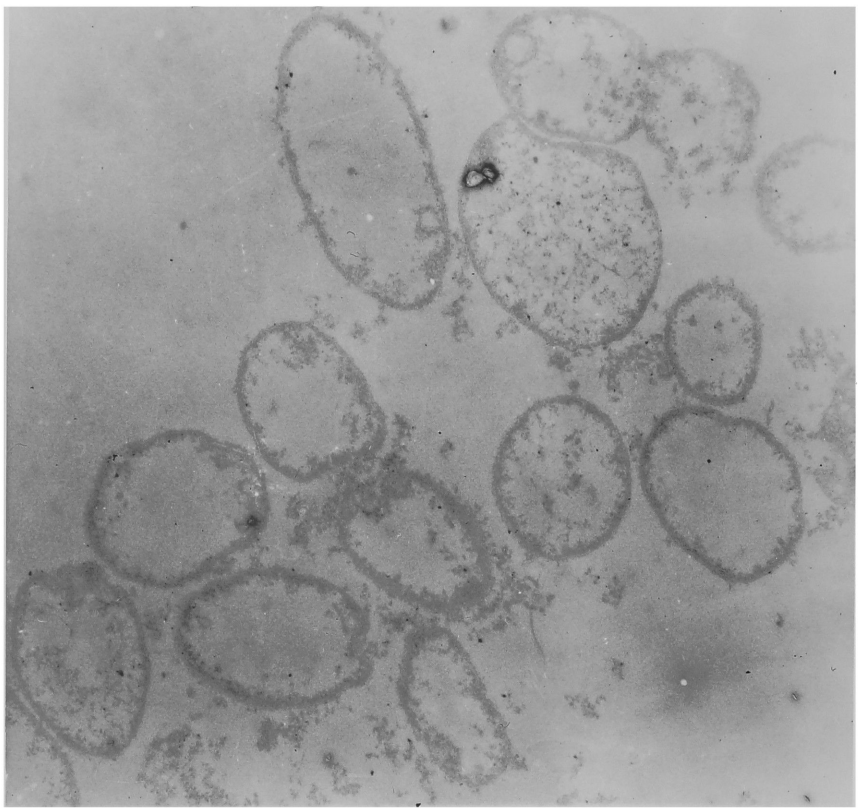
	Molar Ratios with Respect to Muramic Acid			
	Autolytic Supernatant	Choline-Labeled Enzyme Products*	C-poly-saccaride†	Cell Walls*
Galactosamine	4.2	2.5	2.5	2.5
Alanine	1.2	0.98	0.4	3.8
Glucosamine	1.0	0.90	1.2	1.0
Muramic Acid	1.0	1.0	1.0	1.0
Muramic acid Phosphate	(not deter-mined)	0.15		0.2
Glutamate	0.8	1.08	0.22	2.08
Lysine	0.6	0.62	0.18	1.5
Glycine	0.25	0.48	0.03	1.0
Serine	0.23	0.32	0.05	0.95
Aspartate	0.20	0.82	-	0.92
Threonine	0.09	0.32	-	0.52
Leucine	0.09	0.4	-	1.05
Isoleucine	0.06	0.28	-	0.72
Histidine	0.03	none	-	0.01
Methionine	0.02	none	-	0.02
Valine	trace	0.50	-	0.92
Proline	-	none	-	0.38
Tyrosine	none	none	-	0.32
Phenylalanine	none	none	-	0.48
Argine	trace	none	-	0.32

*Adapted from data of Mosser and Tomasz (1970); †adaptation of Mosser and Tomasz's calculations based on data of Liu and Gottschlich (1970).



Figs. 19A, 19B, 19C. Electron micrographs of pneumococcal autolytic detritus. Cells were autolyzed in 0.05 M sodium acetate, in the presence of a small amount of toluene, at 37°C for 72 hours. The washed autolyzate was fixed with 2.5% gluteraldehyde and post-fixed in 1% osmium tetroxide. Magnification: 40,000 diameters.





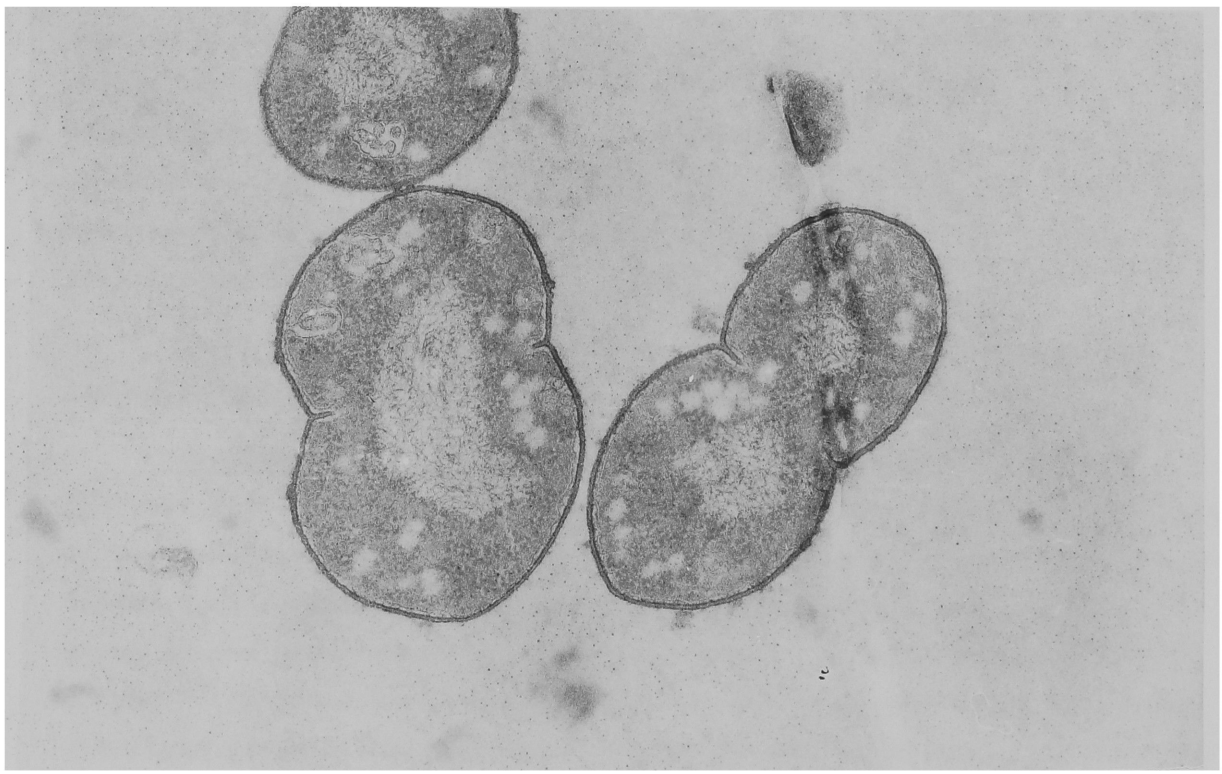


Fig. 20. Electron micrograph of osmium-tetroxide fixed whole pneumococci; compare with Figure 19.
Magnification: 60,000 diameters.

Action of a lysozyme from Chalaropsis: A muralytic enzyme from the fungus Chalaropsis has been described and characterized by Hash and Rothlaug (1967). It solubilizes the peptidoglycan from bacterial cell walls by hydrolyzing the glycosidic bonds at the C-1 position of N-acetyl muramic acid. Preliminary experiments demonstrated that this enzyme (unlike egg-white lysozyme) was able to digest complete pneumococcal walls.

Heat-inactivated pneumococci, uniformly labeled with ^{3}H-choline, were treated with the Chalaropsis lysozyme. The enzyme-treated cells usually had the same appearance under phase-contrast optics as autolyzed cells. As can be seen from Table IX, 75% of the pneumococcal choline is solubilized by the fungal lysozyme, while virtually all of the F-activity is retained by the cellular detritus (it should be recalled at this point that autolysis of pneumococci results in solubilization of 79% of the cellular choline).

These results suggest that the F-antigen is not a covalently-linked component of the cell wall.

Hydrophobicity of the F-antigen: Goebel, et al. (1943) and Fujiwara (1967) have reported that fatty acids comprise 6 1/2% by weight of the F-antigen. The presence of fatty acids in the F-antigen was confirmed qualitatively by studies of F-antigen isolated from pneumococci grown in the presence of 1-^{14}C-palmitic acid. A small amount (0.0073%) of the ^{14}C-radioactivity associated with the cells was recovered in the F-antigen preparation, and could not be removed by washing with acetone. The behavior of this ^{14}C-palmitate-labeled F-antigen was examined on Sephadex G-100 columns in the absence or presence of detergent. The ^{14}C-label eluted with the void volume on G-100 when phosphate-buffered saline was used as eluant, but was retained on G-100 exactly like ^{3}H-choline-labeled F-antigen when eluted with 0.4% SDS in saline (see Fig. 21). Upon treatment with wheat germ lipase (esterase), the ^{14}C-label became chloroform-extractable. These results suggest that the fatty acid constituents are covalently bound to the F-antigen, probably through ester linkages. Fujiwara (1967) reported that the F-antigen shows a small amount of IR absorption at 1740 cm^{-1}, suggesting the presence of esters.

TABLE IX

Treatment of Heat-Killed Pneumococci with Chaloropsis Lysozyme

	Supernatant		Detritus	
	Trial I (3 1/2 hrs treatment)	Trial II (12 hrs treatment)	Trial I (3 1/2 hrs treatment)	Trial II (12 hrs treatment)
^{3}H-radioactivity (choline)	72%	75%	28%	25%
F-activity*	1*	1*	4.7	9.5 (whole detritus) 46.8 (sonicated detritus)

* F-activity in supernatant normalized to 1 for each trial.

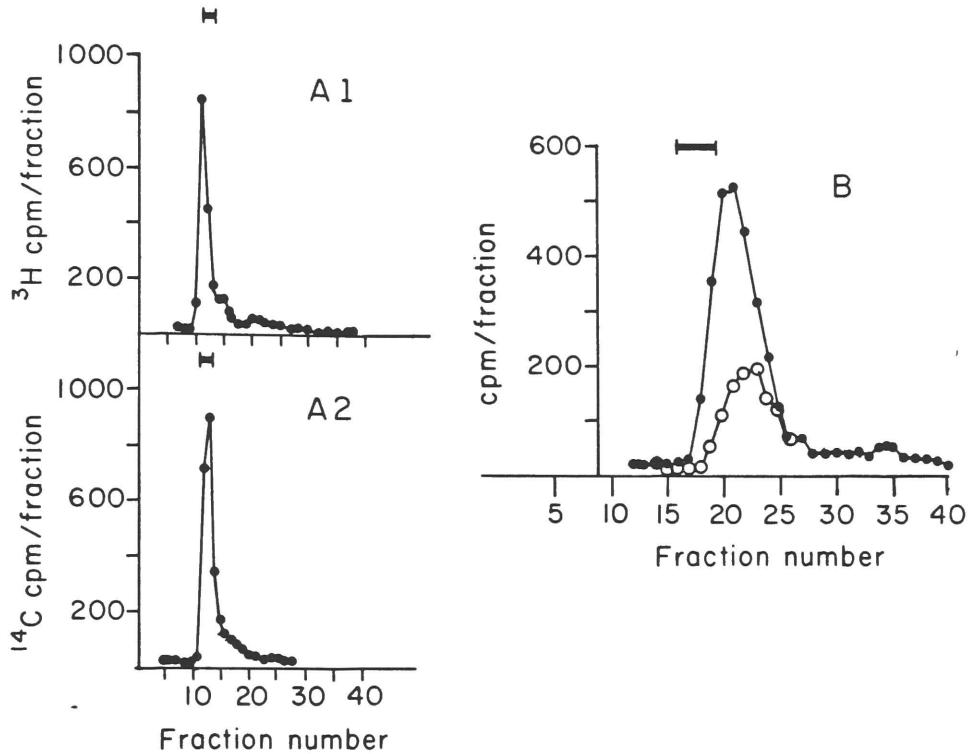


Fig. 21. Behavior of ^{14}C-palmitate-labeled F-antigen on Sephadex G-100, in either the absence or presence of sodium dodecyl sulfate (SDS). A: absence of detergent; column was eluted with 0.15 M NaCl containing 0.020 M potassium phosphate, pH 8. A1: ^{3}H-choline-labeled F-antigen. A2: ^{14}C-palmitate-labeled F-antigen. B: presence of detergent; column was eluted with 0.4% SDS in 0.15 M NaCl. ●-●-●, ^{3}H-choline-labeled F-antigen; O-O-O, ^{14}C-palmitate-labeled F-antigen. Horizontal bars indicate elution positions of Blue Dextran 2000 (void volume) in each case.

The presence of hydrophobic domains in the F-antigen is strongly suggested by the tendency of the purified material to form detergent-dissociable complexes in aqueous solution, as described earlier. The observation, first noted by Goebel, et al. (1943), that in order to solubilize the F-antigen from the detritus it is first necessary to remove free lipids, suggests that the F-antigen is anchored in the detritus through lipophilic interactions.

Ability of anti-F antibodies to bind to whole pneumococci: The ability of whole pneumococci to be bound by the anti-F (hemolytic) antiserum was tested by the hemolysis inhibition assay. Since live bacteria produce hemolysins which interfere with the assay, binding could only be ascertained for killed cells. Bacteria killed either by heat (65°-100°C, 20 minutes) or formaldehyde (2% HCHO in saline, 5°C, 24 hours, followed by extensive washing with saline) exhibited binding activity equivalent to ≃ 5 units of F-activity per ml of culture at mid-logarithmic phase (i.e., per 10^8 bacteria).

These results indicate that at least some of the F-antigen is accessible from the exterior of intact pneumococci, and suggest a cell-surface location for the antigen. Since it has already been shown (see above) to be unlikely that the F-antigen is a part of the wall, and since the hydrophobic character of the F-antigen (see also above) is consistent with a membrane localization, it is suggested that the F-antigen is a part of the cytoplasmic membrane of the bacterium.

Biosynthetic Relationship Between the F-Antigen and the C-Carbohydrate

Incorporation of a pulse of ^{3}H-choline into F-antigen and C-carbohydrate was followed, in order to determine if a precursor-product relationship exists between these two macromolecules. Figure 22 summarizes the results of two such experiments. In each case, the levels of radioactivity in both the C-carbohydrate-containing fractions and the F-antigen-containing fractions are essentially constant over the chase periods, indicating that the F-antigen is not a precursor to the C-carbohydrate.

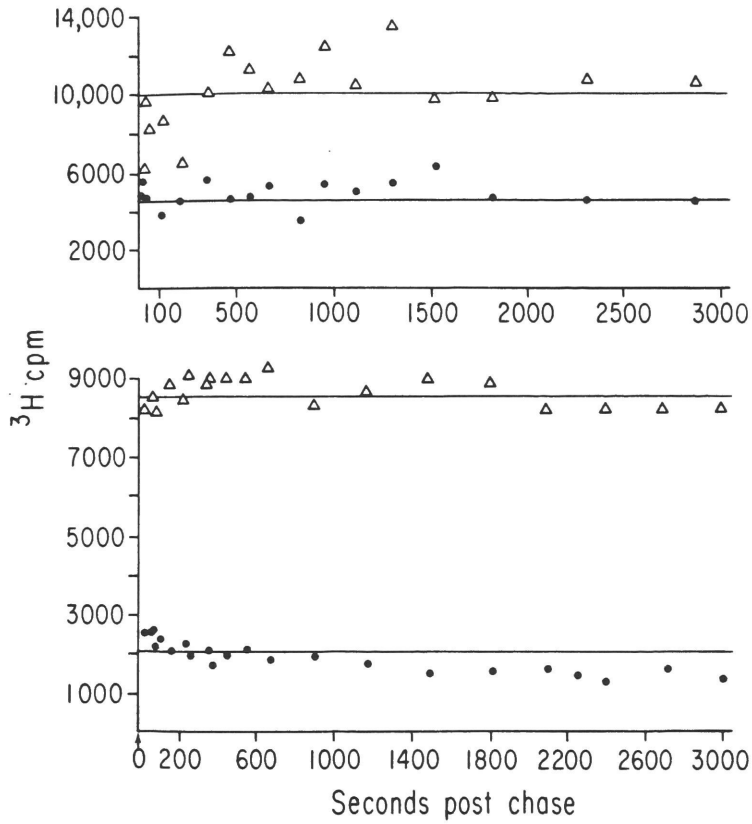


Fig. 22. Choline pulse-and-chase experiments: partitioning of pulsed label between wall and membrane in the bacterium. Pneumococci were briefly pulsed with ^{3}H-choline, then chased with excess unlabeled choline. Samples were heat-killed at various times during the chase; wall and membrane fractions were separated and the radioactivity in each fraction determined. Upper plot: aliquots were treated with Chalaropsis lysozyme to solubilize the wall (see Table VIII). Lower plot: wall was solubilized by the action of excess unlabeled normally-autolyzing pneumococci. Δ-Δ-Δ, radioactivity in solubilized fraction (C-carbohydrate); ●-●-●, radioactivity in pellet fraction (F-antigen). (See Methods).

Immunological Distinctions With Respect to the F-Antigen Between Pneumococci Grown in the Presence of Choline and Pneumococci Grown in the Presence of Choline Analogues

Pneumococci grown with either ethanolamine, N-monomethylethanolamine, or choline as sole amino-alcohol source seem to exhibit differences with regard to both their ability to stimulate heterophile (hemolytic) antibody production in rabbits and in their ability to bind such antibodies. Gholine-grown bacteria seem to crossreact with sheep red blood cells better than do the analogue-grown cells.

Elicitation of heterophile antibodies by choline-grown cells and by analogue-grown cells: Heterophile antisera were produced in rabbits by intravenous injection of vaccines prepared from pneumococci grown with either choline, ethanolamine, or N-monomethylethanolamine as their sole source of amino alcohol. The hemolytic titers obtained in each case are listed in Table X. Although the number of rabbits involved in this comparison is small, a pattern emerges suggesting that the quantitative yield of heterophile (sheep-hemolytic) antibody elicited by pneumococci is dependent on the amino-alcohol composition of the bacteria: choline-grown cells seem to elicit hemolytic titers which exceed those titers induced by analogue-grown cells by roughly an order of magnitude.

Cross-reactivity between choline-grown cells and analogue-grown cells: Preliminary experiments suggested that the ability of analogue-grown cells to inhibit the hemolysis of sheep red blood cells by NZR-A serum (the hemolytic antiserum used routinely for assaying F-activity, and which was produced in response to choline-grown bacteria) was either poor or non-existent. This seemed to be true for both whole heat-killed cells and for sonicated preparations.

An example of such a result is presented in Figure 23. This figure shows F-antigen titration curves for sonicated preparations of choline-grown cells, ethanolamine-grown cells, and mixtures of the two; the hemolytic antibody used in this case was NZR-A (see above). The sigmoid curves are approximately linear in the region around 50% inhibition of hemolysis, and the slopes of these linear portions of the curves reflect the dissociation tendency between the antigen and the

TABLE X

Titers of Heterophile Antisera

Vaccine Type	Rabbit	Titre, AbH_{50}/ml
Monomethylethanolamine-grown cells	NZR-D	400
Ethanolamine-grown cells	NZR-C	520
	Chinchilla B	130
Choline-grown cells	NZR-A	5,000
	Chinchilla A	4,000
	NZR-MA	6,000
	NZR-MB	3,500
	NZR-MC	10,000
	1 DUTCH	≃100
	2 NZR-J1	700
	2 NZR-J2	4,000

[1]This was the only Dutch rabbit used in these studies; all others were either New Zealand Red (NZR) or Chinchilla, as indicated.

[2]These rabbits received no Day 4 injections; titres were determined for day 17 bleedings.

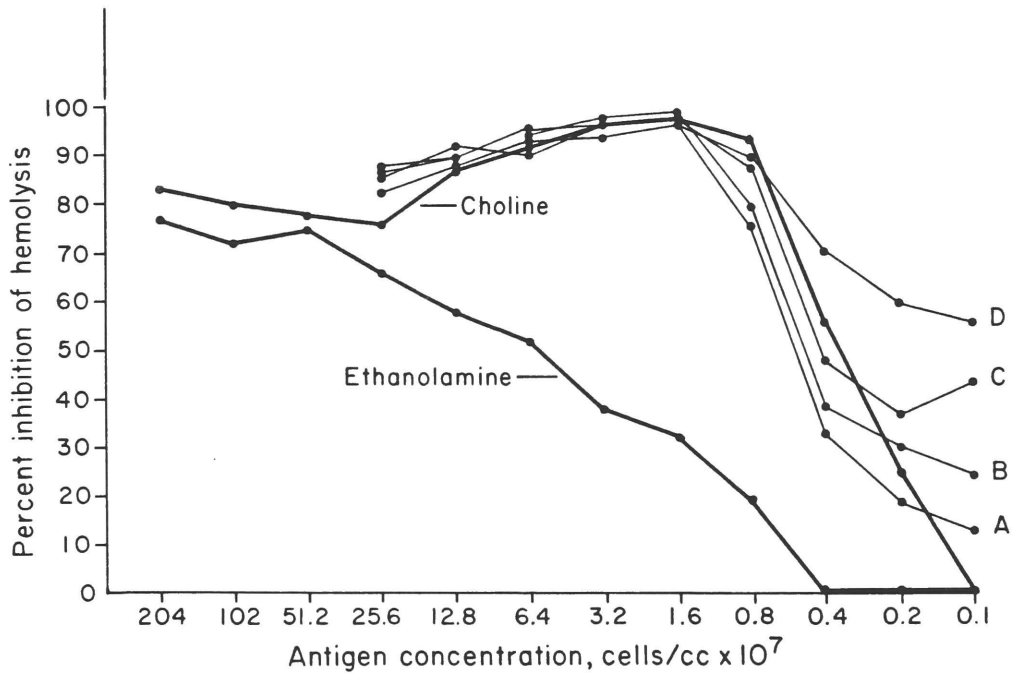


Fig. 23. Titration curves for F-antigen activity of sonicated pneumococcal materials, as assayed using NZR-A antiserum (directed against choline-grown cells). Indicated curves are for choline-grown cells ("choline"), ethanolamine-grown cells ("ethanolamine"), and mixtures; the mixtures contained sonicated choline-grown cells in the concentrations indicated along the figure axis, plus a constant amount of sonicated ethanolamine-grown cells. In mixture A, this was 0.4×10^7 cells/cc of ethanolamine-grown pneumococci; in B, 0.8×10^7 cells/cc; in C, 1.6×10^7 cells/cc; in D, 3.2×10^7 cells/cc.

antibody: the shallower the slope, the greater the tendency toward dissociation; the steeper the slope, the stronger the binding between the antigen and the antibody. As can be seen from the figure, the slope in the case of the choline-containing cells is much steeper than in the case of the ethanolamine-containing cells (35 percentage points change in inhibition per two-fold factor of dilution versus 13 percentage points, respectively), indicating that the choline cells bind much more strongly to the NZR-A hemolytic antibody than do the ethanolamine cells. The choline cells also exhibit a twenty-fold higher titer (based on the point of 50% inhibition of hemolysis) than the ethanolamine cells, but this difference may be an exaggerated one due to the difference in antibody binding. The titration curves obtained for the mixtures (curves A, B, C, and D) indicate that the ethanolamine-containing material does not interfere with the normal behavior of the choline-containing material in this assay.

Table XI summarizes an experiment in which choline-grown and analogue-grown pneumococci were tested for F-antigen activity using hemolytic antisera produced in response to choline-grown, ethanolamine-grown, and N-monomethylethanolamine-grown cells (NZR-A, NZR-C, and NZR-D, respectively; see Table X). Both the F-antigen titers (based on 50% inhibition of hemolysis) and slopes of the titration curves are tabulated. In the table, slopes are given as changes in percentage of hemolysis inhibition per two-fold dilution of antigen, divided by 100. The higher the numerical value of the slope, the steeper the curve and thus the stronger the binding between the antigen and the antibody. Since the slopes were determined for the part of the curve around 50% inhibition of hemolysis, no slopes could be determined for those curves which did not intersect the 50% inhibition level at the concentrations tested. Also, both whole cells and sonicated preparations were tested for antigenicity. In general, sonication increases the observed F-antigen titer of a preparation, while decreasing the slope of the titration curve. This is reasonable, since sonication would be expected to increase the number of antigen-bearing particles (and possibly expose antigens which may be sequestered in whole cells), while decreasing the number of

TABLE XI

Pneumococci Tested for Hemolysis Inhibition:				ANTISERUM DIRECTED AGAINST: Choline cells	Ethanolamine cells	N-monomethyl-ethanolamine cells
Whole Cells	Choline-grown	Titer* / (slope)		4.7 (0.60)	1.25 (0.64)	5.0 (0.44)
	EA-grown	titer* / (slope)		0.29 (0.19)	0.32 (0.50)	0.80 (0.50)
	MEA-grown	titer* / (slope)		<<0.2 (-)	0.51 (0.60)	0.24 (0.55)
Sonicated Cells	Choline-grown	titer* / (slope)		17.0 (0.34)	9.0 (0.18)	>>6.4 (-)
	EA-grown	titer* / (slope)		<0.2 (-)	2.4 (0.20)	>6.4 (-)
	MEA-grown	titer* / (slope)		<0.2 (-)	4.5 (0.22)	>6.4 (-)

7

* titer = number of F-activity units observed per 5 x 10^7 bacteria or their equivalent.

antigenic sites per particle (hence decreasing the avidity of binding of the antibody). Sonication does not boost the titers of the analogue-grown cells disproportionately with respect to the choline-grown cells, and thus the effect of growth in choline-analogues on the F-antigen activity is not simply due to a decreased accessibility of antigen through the wall.

From the slope values tabulated in Table XI, it can be seen that the hemolytic antiserum directed against choline-grown cells binds relatively poorly to ethanolamine-grown cells, and even more poorly (if at all) to N-monomethylethanolamine-grown cells. However, the hemolytic antisera directed against the analogue-grown cells seem to bind with equal avidity to analogue-grown and choline-grown cells.

These findings suggest that the Forssman-crossreactive determinant that is recognized by the rabbit immune system in analogue-grown cells is also present in choline-grown cells, but that, in addition, there is a Forssman-crossreactive "determinant" recognized in choline-grown cells which is either not present, or masked, or present in a significantly different form, in the analogue-grown cells.

The differences among the F-antigen titers listed in Table X warrant comment. In the case where titers were determined using antiserum against choline-grown cells, the very low titers detected for the analogue-grown cells may be a reflection of the poor binding of these antigens by the antiserum. Where antisera against analogue-grown cells were used in the assay, the differences among titers might represent differences in either the amount of, or accessibility to, the F-antigen determinants of the samples tested. It should be kept in mind that the assays were performed on two-fold serially diluted test samples, and thus the titers obtained are subject to a two-fold possible error.

F-Antigen Content of Competent and Incompetent Cells

A comparison was made between pneumococci which are physiologically capable of becoming genetically transformed by exogeneous DNA ("competent" cells) and those which are not ("incompetent" cells). Whether or not a culture is transformable is determined by a complex variety of variables, including the composition and pH of the growth medium, and the history

and cell density of the culture. Transformability is immediately preceded by, and apparently mediated by, the appearance in the culture medium of a low-molecular-weight protein (Tomasz, 1973; Tomasz & Mosser, 1966), known as "competence factor" or "activator." In C medium at pH 8, supplemented with bovine serum albumin and yeast extract, cells will become competent at a cell density of ≃ 220 Nephelos units (5.5×10^7 cells/cc), provided that they are grown up from a low starting inoculum. Competence is a transient property of the culture, and fades in less than a generation (Tomasz, 1973). In supplemented C medium at somewhat lower initial pH (6.6), cells do not become competent. Incompetent cells will become competent if they are trasnferred to pH 8 medium and given a cell-free extract (of competent cells) which contains the competence factor. Unlike spontaneous induction of competence in growing cultures, induction of competence by the addition of exogeneous competence factor is not dependent on cell density. The effect of competence factors on pneumococci and other transformable bacteria has been extensively studied (Tomasz, 1973). The addition of the factor alone does not render the pneumococcus transformable; instead, it seems to trigger a specific sequence of events which requires protein synthesis and which ultimately enables a cell to take up DNA from the medium and integrate this genetic material within its own chromosome. Fuller discussion of transformation in bacteria is beyond the scope of this dissertation; several recent reviews have appeared on this subject (Tomasz, 1973), to which interested readers are referred.

As can be seen from Table XII, no significant difference was observed, with respect to F-antigen content per cell, between competent and incompetent cultures.

F-Antigen Content of Pneumococci as a Function of Growth

In order to determine whether or not the F-antigen content of pneumococci varies significantly as a function of growth, a single growing culture (in C medium) was sampled periodically during the logarithmic and early stationary phases of growth, and the number of F-antigen units per 10^8 cells was determined for each sample (see Methods). Growth was logarithmic until the culture reached 800 Nephelos units (2×10^8 cells/cc), whereupon the cells entered stationary phase. The results of this experiment, summarized in Table XIII, show no significant differences in F-antigen content per cell at the growth stages tested.

TABLE XII

State of Culture, Transformability	F-Antigen Content Units per 10^8 Cells	
	Trial I	Trial II
Competent	3.2	3.8
Incompetent	3.2	3.0

TABLE XIII

Sampling Time, min after first sample	Culture Concentration		F-Antigen, units per 10^8 cells
	Nephelos Units	Cells/cc	
0	126	3.15×10^7	7
40	224	5.6×10^7	8 1/2
80	324	8.1×10^7	12
100	440	1.1×10^8	8 1/2
120	576	1.4×10^8	8 1/2
140	696	1.7×10^8	7 1/2
160	824	2.1×10^8	7
180	800	2.0×10^8	6
200	720	1.8×10^8	6

SUMMARY AND DISCUSSION

Similarities Between the F-Antigen and the C-Carbohydrate

From the work of Goebel and Adams (1943) and Goebel, et al. (1943), it was already known that the F-antigen and the C-carbohydrate shared a similar elemental composition, and were serologically-crossreactive (suggesting that they shared some structural features). By comparison of the work of Fujiwara (1967) and Brundish and Baddiley (1967; 1968), it was known that both contained ribitol, galactosamine, glucose, and phosphate, in molar ratio 1:1:1:2. Brundish and Baddiley found choline and diaminotrideoxyhexose in the C-carbohydrate, while Fujiwara (1967) found additional nitrogenous material in the F-antigen which he was unable to identify.

Choline is not a usual component of carbohydrates, and its presence in pneumococci is intriguing in several respects. The bacteria have an absolute nutritional requirement for choline (or an analogue), and can neither degrade nor synthesize this molecule. This makes possible the use of radioactively-labeled choline as a tag for choline-containing compounds. 85% of the choline taken up from the medium becomes incorporated into the cell wall (Briles & Tomasz, 1973; Tomasz, 1967). There is no lecithin or sphingomyelin in the bacterium (Tomasz, 1967; unpublished results); the only choline-containing compounds other than teichoic acid detected to date in the pneumococcus are phosphoryl choline and CDP-choline, which are presumed to be intermediates in teichoic acid biosynthesis (Bean, 1970). The choline residues of the wall appear to play a role in the control of the action of the cell wall autolytic amidase, since walls stripped of teichoic acid (by TCA treatment) or containing a non-methylated choline analogue (ethanolamine) instead of choline, do not serve as substrates for the enzyme (Tomasz & Westphal, 1971; Tomasz, 1968).

The results presented in this dissertation establish that choline is a component of the F-antigen. When F-antigen is isolated from pneumococci grown in the presence of radioactively-labeled choline, the isolated product contains choline. The isolation procedure used in these studies

is a modification of the purification procedure of Goebel, et al. (1943); this modified procedure yields a product which is essentially identical to the F-antigen preparation obtained by Goebel, et al. (see Table XIV). This choline-labeled F-antigen preparation was subjected to characterization by various methods, in order to verify that the labeled material was in fact the F-antigen, and not some choline-containing contaminant which may have been co-isolated in the purification procedure. It was found that the F-antigen (as detected by the hemolysis inhibition assay) could not be separated from the choline label by column chromatography, sucrose gradient centrifugation, or treatment with sodium dodecyl sulfate. Furthermore, the F-antigen (and the choline label) is specifically bound in a hapten-inhibitable way by a myeloma protein (TEPC-15) known to be specific for phosphocholine residues (Potter & Lieberman, 1970). Also, the antigenic properties with respect to Forssman crossreactivity are altered if the choline of the cell is replaced by a choline analogue such as ethanolamine or monomethylethanolamine.

From Figure 6, we see that the amount of choline-labeled material which was recovered as purified F-antigen (Preparation #1 of Table II) is only 13% of the amount in the autolytic detritus. However, much of the loss of radioactivity is probably due to rather high mechanical losses during handling at the various stages of the procedure. It should be kept in mind that Preparation #1 was run on a rather small scale. The yield of F-antigen in the other preparations listed in Table II was higher by approximately an order of magnitude; by extrapolation, it can thus be estimated that probably all of the choline-labeled material of the autolytic detritus is F-antigen.

Thus, the F-antigen and the wall teichoic acid are seen to resemble each other not only in that they both contain ribitol, galactosamine, glucose, and phosphate, but also in that they both contain choline (probably as phsophocholine, since the phosphocholine-specific TEPC-15 myeloma protein binds the C-carbohydrate (Potter & Lieberman, 1970) as well as the F-antigen). Probably all of the choline of the pneumococcus is accounted for by these two macromolecules.

TABLE XIV

Elemental Analyses of Isolated F-Antigen

	Preparation #3[1]	Original Preparation of Goebel, et al.[2]
Carbon	46.71%	45.12%
Hydrogen	7.62%	7.11%
Nitrogen	7.05%	5.61%
Phosphorus	4.36%	4.75%
Ash	10.9% (includes phosphorus)	0.61% (does not include phosphorus)
Yield	1.24 mg/liter culture	1.2 mg/liter culture

[1] See Table II.

[2] Taken from Goebel, Shedlovsky, Lavin & Adams (1943), J. Biol. Chem. 148, 6.

No attempt was made, in these studies, to assay directly for the diaminotridesoxyhexose. This is a highly-labile sugar, for which no reliable assay method exists (Brundish & Baddiley, 1968). It is likely that this sugar exists in the F-antigen, since it (together with choline) would account for almost all of the nitrogen-containing material which Fujiwara (1967) could not account for. Also, Goebel, et al. (1943) found that both the F-antigen and the C-carbohydrate contain 13% (by weight) acetyl groups; since this is twice as much acetyl as would be necessary for acetylation of all the galactosamine residues, it is presumed that the diaminotrideoxyhexose is monoacetylated; this is consistent with the findings of Distler, et al. (1966).

A further significant similarity was found between the F-antigen and the C-carbohydrate: the two macromolecules exhibit similar susceptibility to degradation by periodate and by nitrous acid. It was already known, from the work of Mosser and Tomasz (1970), that if choline-labeled C-carbohydrate is treated with either of these reagents, the choline-labeled polymers are fragmented into products having a molecular weight on the order of 1,000 daltons. Presumably, these fragments represent the single repeat subunits of the teichoic acid chain: the sum of the molecular weights of one residue each of choline, ribitol, glucose, N-acetyl-galactosamine, and mono-N-acetyl-diaminotrideoxyhexose, plus two residues of phosphate (the known components of the C-carbohydrate), is approximately 1,000 daltons. The experiments described in this dissertation demonstrated that these reagents also degrade the F-antigen to pieces of molecular weight on the order of 1,000 daltons.

Periodate acts on carbohydrates by oxidizing vicinal unsubstituted hydroxyl or amino groups to aldehydes, with concomitant cleavage of the connecting carbon-carbon bond. Of the periodate-susceptible components known in the pneumococcal C-carbohydrate, all but one are ring-structured pyranohexoses. Cleavage of a carbon-carbon bond by periodate in such ringed structures would simply open the ring, but would not cause any discontinuity in the polymer chain. Ribitol, however, is a linear residue, and cleavage of a carbon-carbon bond in this case would cause a break in the polymer chain. Thus, the ability of periodate to fragment the polymer

implies that ribitol is a regularly repeating unit in the backbone of the polymer chain, and that it contains a pair of vicinal unsubstituted hydroxyl groups.

Nitrous acid is a degradative deaminating agent. It reacts with unsubstituted amino groups to form diazo groups. Alkyl diazo groups are unstable, and split off to form molecular nitrogen, leaving a carbonium ion which is then subject to nucleophilic attack. In the case of 2-amino, 2-deoxy pyranohexoses, reaction with nitrous acid is followed by a nucleophilic attack on the carbonium ion at the C-2 position by the ring oxygen, resulting in the formation of a 2,5-anhydro sugar. If the C-1 position of the amino sugar is involved in a glycosidic linkage, then this linkage is cleaved in the formation of the anhydro sugar.

The only C-carbohydrate component believed to carry an unsubstituted amino group is the diaminotrideoxyhexose, which Distler, et al. (1966) identified as a 2-acetamido, 2-amino, 2,4,6-trideoxyhexose. The galactosamine residues are almost certainly N-acetylated, since Brundish and Baddiley (1968) were able to detect galactosamine in hydrolyzates of nitrous-acid-treated C-carbohydrates. Also, Goebel, et al. (1943) reported sufficient acetyl groups (13% by weight) to account for acetylation of the galactosamine residues and one amino group each of the diaminotrideoxyhexose residues. Therefore, the likely target of nitrous acid degradation of the C-carbohydrate is the diaminotrideoxyhexose, and thus the ability of nitrous acid to fragment the chain implies that this unusual sugar is also a regular repeating unit in the backbone of the polymer chain.

The fact that the F-antigen and the C-carbohydrate behave similarly upon treatment with periodate or with nitrous acid suggests that the two polymers share certain structural features: namely, that they both contain periodate--sensitive ribitol residues in the backbone of the polymer chain, that they both contain nitrous-acid-sensitive amino sugars in the backbone of the chain (probably diaminotrideoxyhexose in each case), and that they are both composed of repeating subunits of the same molecular weight.

The striking similarities, described above, between the F-antigen and the C-carbohydrate (common composition, common susceptibility to selective degradative reagents, serological crossreactivity) strongly suggest that these two polymers consist of identical teichoic acid chains.

Differences Between the F-Antigen and the C-Carbohydrate

There are several significant differences between these two macromolecules. The most obvious of these is that the F-antigen is immunologically crossreactive with the Forssman antigen of sheep red blood cells, while the C-carbohydrate is not (Goebel & Adams, 1943). Goebel, et al. (1943) found that, when pneumococci undergo autolysis, the C-carbohydrate becomes solubilized whereas the F-antigen does not. They also found that the F-antigen contains fatty acid residues which are not present in the C-carbohydrate, and that the two possess somewhat different electrophoretic mobilities.

In this dissertation, several additional differences have been described between these two pneumococcal teichoic acid forms.

The F-antigen, in aqueous solution, exists in large-molecular-weight aggregates which are probably held together by lipophilic (hydrophobic) interactions; these aggregates are dissociable with detergents such as sodium dodecyl sulfate or deoxycholate. The C-carbohydrate does not form such aggregates, and is unaffected by detergents.

Also, the F-antigen and the C-carbohydrate were found to differ in their susceptibility to heating at pH 2. The F-antigen is labile at pH 2 at 100°C: after a half-hour under these conditions, approximately 80% of the F-antigen activity (as detected by the hemolysis-inhibition assay) is destroyed. When choline-labeled F-antigen is heated at pH 2, approximately half of the label becomes associated with heterogeneously-sized fragments, the largest of which are approximately 40,000 daltons (as estimated by Sephadex filtration). The C-carbohydrate is completely stable at pH 2; both the C-carbohydrate and the F-antigen are stable at neutral pH.

These observations suggest that a bond is present in the F-antigen, but not in the C-carbohydrate, which is labile at pH 2. The evidence presented above suggesting that the C-carbohydrate and the F-antigen contain identical teichoic acid chains makes it unlikely that the labile bond is in the chain. Rather, it is likely that the pH 2-labile bond is associated with the lipid moiety of the F-antigen; the lipid moiety is the only chemical feature of the F-antigen known which is not present in the C-carbohydrate. Except for the fact that it contains fatty acids, essentially nothing is known about the lipid moiety, or about how it is linked to the teichoic acid chain. Most covalent bonds in biological macromolecules are reasonably stable at pH 2; certain bonds, however, including the so-called "high-energy bonds" such as pyrophosphodiesters, would be expected to be hydrolyzed under these conditions. Toon, Brown, and Baddiley (1972) have suggested that, in Streptococcus faecalis the lipid moiety (known to be a glycolipid) of the lipoteichoic acid may be linked to the teichoic acid chain through a phosphodiester bridge; they based their suggestion on the observation that the linkage can be broken by treatment with hydrofluoric or tricholoroacetic acid (Toon, Brown & Baddiley, 1972). These authors also noted, interestingly, that this presumed phosphodiester bridge was much more labile than the phosphodiester linkages within the teichoic acid chain. A pyrophosphodiester linkage would be consistent with the HF and TCA sensitivity observed by Toon, et al. (1972). A limited effort was made to determine whether or not any phosphomonoester groups are liberated when the F-antigen is heated at pH 2, but this was unsuccessful.

A further significant difference between the F-antigen and the C-carbohydrate concerns the subcellular localization of these two macromolecules. The C-carbohydrate is known to be a part of the pneumococcal cell wall (Mosser & Tomasz, 1970); in fact, the soluble C-carbohydrate is actually an enzymatic breakdown product of the cell wall, and consists of the teichoic acid polymers of the wall plus the peptidoglycan strands to which they are covalently attached (Mosser, 1970; Mosser & Tomasz, 1970). In this dissertation, evidence was presented suggesting that the F-antigen is associated with the plasma membrane of the cell.

The F-antigen is not a part of the wall, since neither the pneumococcal autolytic enzyme (muramyl-L-alanyl amidase) nor the Chalaropsis lysozyme (muramidase) could solubilize the F-antigen from whole cells. However, anti-F (hemolytic) antiserum binds to whole cells (either heat-killed or formalin-fixed), which suggests that at least some of the F-antigen is at or near the surface of the cell.

The hydrophobicity of the F-antigen is consistent with a membrane localization in vivo. The F-antigen has a tendency to form detergent-dispersible micelles in aqueous solution, suggesting the presence of hydrophobic domains. Goebel, et al. (1943), Fujiwara (1967), and Brundish and Baddiley (1968) reported finding fatty acids associated with the F-antigen; the covalent nature of the linkage of the fatty acids to the F-antigen, first suggested by Goebel, et al. (1943), was corroborated by the finding that isolated ^{14}C-palmitic-acid-labeled F-antigen exhibits the same elution characteristics as ^{3}H-choline-labeled F-antigen on Sephadex G-100, both in the absence and presence of sodium dodecyl sulfate. Furthermore, it is impossible to solubilize the F-antigen from the autolytic detritus without first removing free lipids (by washing with organic solvents, e.g.), which fact strongly suggests that the F-antigen is bound to the detritus through lipophilic interactions. Finally, the autolytic detritus (source of F-antigen in the isolation procedure employed in these studies) was examined by electron microscopy and was found to consist of membranous vesicles.

Identification of the F-Antigen as a Lipoteichoic Acid

The existence of two classes of teichoic acids in Gram-positive bacteria has been recognized for some time (Archibald & Baddiley, 1966; Knox & Wicken, 1973; Salton, 1964); the two classes are distinguished on the basis of their association with either the wall (by covalent linkage to peptidoglycan) or the membrane (by covalent linkage to a lipid) of the cell. The pneumococcal C-carbohydrate was recognized as a wall teichoic acid by Brundish and Baddiley (1967; 1968), on the basis of its ribitol-phosphate content. In view of the various significant similarities and equally-significant differences (summarized above) between the C-carbohydrate and the F-antigen, it is evident that the F-antigen is a lipoteichoic acid.

Wall teichoic acids are known to vary widely in structure, ranging from simple polyglycerophosphate or polyribitolphosphate to highly-substituted polymers containing not only polyolphosphate but also other constituents either intercalated in the repeating backbone or as side chains (or both, as in the case of the pneumococcal teichoic acid) (Archibald & Baddiley, 1966; Knox & Wicken, 1973). Until recently, however, the only membrane lipoteichoic acids reported on in the literature have consisted of relatively simple polyglycerophosphate polymers, varying only with respect to side-chain substitution at the 2-position of the glycerol residues (Archibald & Baddiley, 1966; Knox & Wicken, 1973); the recognition of the pneumococcal F-antigen as a lipoteichoic acid (Briles & Tomasz, 1973) is the first, and so far the only, exception to this (known to this author). It may be that lipoteichoic acids in nature actually reflect exactly the same structural variability as do the wall teichoic acids, but that a wider variety of lipoteichoic acids simply has not yet been discovered, possibly due to technical reasons such as the choice of organisms studied to date or limitations of the isolation procedures currently in use.

Structural Features of the Lipoteichoic Acid

The specific chemical structures of the pneumococcal teichoic acids have yet to be elucidated. Brundish and Baddiley (1968) have shown that the teichoic acid chains of the wall consist of repeating subunits containing at least ribitol phosphate and a diaminotrideoxyhexose in the linear backbone, at least choline phosphate as a side-chain constituent, and a β-linked (based on optical rotation) phosphogalactosaminyl-glucose disaccharide which may be either a side-chain or backbone component. The data presented in this dissertation suggest that the teichoic acid chains of the membrane lipoteichoic acid are probably identical to those of the wall polymer. In addition, the lipoteichoic acid is known to contain fatty acids, presumably as components of a lipid moiety of otherwise unknown composition and structure. By analogy with lipoteichoic acids of other bacteria (Knox & Wicken, 1973; Toon, et al., 1972), this lipid moiety is probably a glycolipid, whose saccharide composition need not necessarily be related to that of the teichoic acid chain.

It is most likely that the Forssman-crossreactive determinant resides in the lipid moiety, since the wall teichoic acid lacks this antigenic activity, and since the Forssman determinant (hapten) of the sheep red cell membrane is itself a lipid. The structure of the sheep Forssman hapten has recently been shown to be N-acetylgalactosaminyl-α-1,3-galactosyl-β-1,4-galactosyl-β-1,4-glucosyl-ceramide (Ando & Yamakawa, 1970); there is no obvious resemblance between the structure of this hapten and that of the pneumococcal teichoic acid chain.

The observation that the amino alcohol constitution of the F-antigen affects its Forssman crossreactivity warrants comment. Pneumococci grown with choline or choline analogues (ethanolamine, N-monomethylethanolamine) as sole amino alcohol source exhibit significant differences with respect to both their ability to stimulate heterophile antibody production in rabbits and in their ability to bind such antibodies. The ability to elicit a heterophile response per se is independent of the amino-alcohol constituent of the cell, suggesting that the amino-alcohol residues are not critical for the Forssman determinant; this conclusion is supported by the fact that the mammalian Forssman hapten contains no amino alcohol residues. However, the affinity of the elicited heterophile antibody seems to be dependent on the amino alcohol of the antigen. The choline-grown cells elicit antibodies which bind only poorly to analogue-grown cells; the analogue-grown cells, however, elicit antibodies which bind well to choline-grown cells. Also, the choline-grown cells elicit more heterophile antibodies in rabbits than do analogue-grown cells. It seems as if the Forssman-crossreactive determinant of the analogue-grown cells is present in the choline-grown cells, but that there is some further "determinant" in the choline-grown cells which is unavailable in the analogue-grown cells.

The effect of amino alcohol composition of the F-antigen on its Forssman crossreactivity (F-activity) is especially intriguing in view of the several other effects that choline-analogue substitution has on the pneumococcus (e.g., inhibition of autolysis, transformation, and daughter-cell separation). Most, if not all, of these effects could conceivably be accounted for by the fact that the cell walls of analogue-grown bacteria

are resistant to the action of the endogenous wall-lytic enzyme (Tomasz, 1968; Tomasz & Westphal, 1971). It is not yet clear whether this resistance is due to a difference in 3-dimensional conformation of the wall polymers (choline-containing vs. analogue-containing), or whether the amino-alcohol residues are part of a specific site which the wall-lytic enzyme must in some way "recognize" in order to be enzymatically active.

Function of the Lipoteichoic Acid in the Bacterium

Although it is believed that all Gram-positive bacteria contain membrane lipoteichoic acids, the functions of these polymers are entirely unknown. Baddiley and co-workers (Heptinstall, et al., 1970) have proposed that lipoteichoic acids serve to bind divalent cations, thereby maintaining the proper ionic environment for membrane functions. Mauck and Glaser (1972) have isolated a lipoteichoic acid from B. subtilis which serves as an acceptor for glycerol phosphate residues (from CDP-glycerol) in the presence of a particle-associated teichoic synthetase; they propose that the teichoic acid chain is assembled in vivo at the membrane, whereupon the completed chain is transferred from its lipid carrier to the wall position. As was noted above, the bond linking the lipid moiety to the teichoic acid chain may be unusually labile, which is consistent with the hypothesis that the membrane polymer is a biosynthetic precursor to the wall polymer. However, the pulse-chase studies (using choline label) presented in this dissertation show that, at least in pneumococci, the lipoteichoic acid (F-antigen) is not a precursor to the wall teichoic acid. The amount of pulse label incorporated into the F-antigen and the C-carbohydrate (wall teichoic acid) remains essentially constant for each over a time period of between 17 seconds and 50 minutes (generation time: 45 minutes), and the percentage of total incorporated label for each is the same as that observed for uniformly-labeled cells. The two polymers thus appear to be biosynthesized simultaneously and independently, at least with respect to the incorporation of choline. The possibility that there may exist a teichoic acid precursor at which subunits are assembled and then transferred to the wall and/or membrane positions is not ruled out.

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