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The Rockefeller University

THE STREPTOCOCCAL CELL WALL*†

MACLYN McCARTY

The Rockefeller University,
New York, New York

THE detailed analysis of pathogenic microorganisms has become progressively a less fashionable endeavor in the science of microbiology. Today's microbiologist is more likely to be involved in the study of basic biological problems in fields such as genetics, enzymology, or synthetic mechanisms, and to this end he attempts to select the microorganisms that can be most readily manipulated experimentally for his specific purposes. However, if one is concerned with the pathogenetic mechanisms of an infectious disease, there remains no substitute for a direct attempt to understand the biology of the causative organism. Parenthetically, it is worth recalling that this direct approach has throughout the history of microbiology repeatedly yielded extra dividends in the form of discoveries that have enriched biology in general. Nonetheless, the primary aim is to define those attributes of the organism that determine its pathogenicity.

The group A hemolytic streptococcus, an object of study since the early days of the science of bacteriology and long before it was given this designation, appears to be well adapted to a parasitic existence in man. While its menace as the cause of life-threatening acute disease has been largely ameliorated since the introduction of the sulfonamide drugs, followed by a host of antibiotics, the organism remains widely prevalent so that sporadic fatal mishaps occur to remind us of its potential virulence. An even more important reminder of the persistent threat of the streptococcus is the continued occurrence of those serious sequelae of streptococcal sore throat:

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rheumatic fever and glomerulonephritis. It is the unraveled mystery of these diseases that has provided the primary stimulus for the extensive studies of the streptococcus carried out in recent decades.

In keeping with the declining popularity of the investigation of infectious bacteria, reports on studies of the biology of streptococci have been little represented in the Harvey Lecture series of recent years. In fact, the last Harvey Lecture that dealt primarily with this problem happens to have been the first that I ever attended after coming to New York in the summer of 1940. This lecture was delivered by Rebecca Lancefield on May 15, 1941 at a time when I was getting my first intensive exposure to laboratory study of the pneumococcus under Dr. William S. Tillett. This was before I could know that I would spend something over two decades in research on the streptococcus, all in close association with Dr. Lancefield.

Dr. Lancefield's lecture was entitled "Specific Relationship of Cell Composition to Biological Activity of Hemolytic Streptococci," and it represented a masterly review of the current knowledge of the organism, cast in the framework of her fundamental contributions of group and type differentiation of streptococci and of the nature and biological significance of the antigens concerned (Lancefield, 1941). In the intervening years there have been many additions to our knowledge of the streptococcus, including the discovery of previously unrecognized cellular and extracellular components, but it seems inappropriate and cumbersome to attempt a catalog of these developments. Rather, I propose to focus on those findings that have emerged as a result of the recognition of the cell wall as a well defined structural entity and the analysis of its several components. As will become apparent, this analysis involves a strong emphasis on immunochemical approaches. The wall components concerned include the most important cellular antigens that were considered by Dr. Lancefield in her lecture, and to this extent this presentation has continuity with her review. Needless to say, a number of my colleagues, in addition to Dr. Lancefield, have collaborated in the studies to be reported, as I will indicate in the appropriate context.

Because of the rather long period over which these studies extend, it will be necessary to resort in part to a historical presentation, though I will try to avoid a purely chronological recital of the work. The line of investigation to be described had its origin over twenty

years ago in some experiments designed to provide a new and more effective procedure for solubilization of the cellular components of group A streptococci. These organisms are notoriously resistant to ordinary extraction procedures, and the time-honored methods used for solubilizing streptococcal cellular substances were relatively drastic and nonbiologic. Maxted had reported in 1948 that soluble enzymes released from a strain of *Streptomyces albus*, an organism that had been extensively studied by Welsch (1942) for its bacteriolytic properties, would lyse hemolytic streptococci of most serological groups, and he applied the material to the preparation of streptococcal extracts for serological grouping. On the assumption that these enzymes might prove more widely applicable to the study of streptococci, purification and further characterization of the *Streptomyces* products were undertaken (McCarty, 1952a).

In the course of the studies on the streptolytic enzymes, attention was also directed to the nature of the bacterial substrate that was being attacked. The clue came from the study of disrupted preparations of streptococcal cells. One of the few successful methods that had been applied to the disruption of group A streptococci was grinding of dried cells in a ball mill. After extensive extraction of such disrupted cell preparations, followed by treatment with proteases and nucleases to remove denatured material, an insoluble residue remains which resists solubilization in a wide variety of solvents. However, this residue is promptly dissolved by the *Streptomyces* enzyme under the same conditions that the intact cell is lysed. Electron micrographs of the residue revealed that the material retained a recognizable coccoid appearance despite the considerable fragmentation resulting from mechanical grinding. The flat, relatively empty structures clearly seemed to represent isolated cell walls (McCarty, 1952b). The interpretation of these data was also assisted by the elegant studies from Salton's laboratory on the bacterial cell wall which began to appear while this work was in progress (Salton and Horne, 1951). A directly applicable finding that appeared concurrently with our observations was Salton's demonstration that the substrate for the lysis of *Micrococcus lysodeikticus* by lysozyme is the cell wall (Salton, 1952).

Analysis of the streptococcal cell wall material prepared as described revealed that it was primarily carbohydrate in character

a
b
c

and furthermore that the largest component was the group-specific carbohydrate, the antigen on which the serological identification of group A streptococci is based. Thus it was established that this antigen is localized in the cell wall and is the major single antigen of the cell, accounting for as much as 10% of the dry weight of the cell. In addition, it appeared to be a relatively simple polysaccharide. Schmidt (1952) reported that the polysaccharide was composed of two monosaccharides, *N*-acetylglucosamine and rhamnose, and this was independently confirmed in our analysis of the antigen obtained from the isolated cell wall. Taken together, these facts stimulated further study of the group A polysaccharide, and I will return to this subject to describe briefly the immunochemistry of the antigen.

Salton's procedure for isolation of bacterial cell walls, based on the high velocity shaking of bacterial suspensions with minute glass beads, provided a method that yielded better preparations with less fragmentation and less damage to labile components. An electron micrograph of such a preparation from group A streptococci is illustrated in Fig. 1b. By the use of this preparative method, it was possible to establish that not only the group-specific polysaccharide, but also the major protein antigens, defined by Lancefield and designated by the letters M, T, and R, are part of the cell wall structure. Thus, the type-specific M protein, which plays a major role in the virulence of group A streptococci, is attached to the protective cell envelope.

The proteins of the cell wall can be largely removed by treatment with proteolytic enzymes and the composition of the remaining wall is comparable to that obtained in our earlier studies—that is, composed largely of carbohydrate (Fig. 1c). Somewhat later, Krause and I (1961) found that by application of the method of extraction with hot formamide, which had been introduced some years ago by Fuller (1938) for the preparation of grouping extracts, the group-

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FIG. 1. Chromium-shadowed preparations of isolated streptococcal cell walls. Magnification approximately ×10,000. (a) Walls extracted with hot formamide. This procedure removes all components except the mucopeptide. (b) Untreated cell walls. (c) Walls treated with proteolytic enzyme to remove surface proteins. Figure 1a is reprinted from Krause and McCarty (1961, facing p. 140).

TABLE I

THREE-LAYERED STRUCTURE OF GROUP A STREPTOCOCCAL CELL WALL

Outer layer	—Type-specific M protein T and R proteins
Middle layer	—Group-specific polysaccharide (rhamnose and *N*-acetylglucosamine)
Inner layer	—Peptidoglycan (or mucopeptide) (*N*-acetylglucosamine—*N*-acetylmuramic acid polymer; L-ala, D-glu, L-lys, D-ala side chain with L-ala-L-ala chain interbridge)

specific carbohydrate could be removed from walls of this type, leaving a residue that retains a recognizable wall structure. This residue, illustrated in Fig. 1a, represents the peptidoglycan or mucopeptide of the wall. As the result of the combined efforts of several laboratories, the peptidoglycan is now recognized as the basic structural unit of the wall in all bacteria, with modifications in the details of peptide composition from species to species.

From these considerations and other similar data, we evolved the concept of the group A streptococcal cell wall as a three-layered structure. As indicated in Table I, the outer layer is thought to consist of the protein antigens. There are several pieces of indirect evidence for the surface distribution of the proteins, the most important being Lancefield's demonstration (1943) that M protein can be completely removed by trypsin treatment of living cells without affecting their viability.

The middle layer is the group-specific carbohydrate, forming a major portion of the total structure as previously noted. Soluble preparations of the group-specific polysaccharide can be obtained by a variety of procedures: mild acid (pH 2) hydrolysis, hot formamide extraction, or enzymatic lysis of the wall either by the *Streptomyces* enzymes or by a phage-associated lytic enzyme found by Krause (1957) and by Maxted (1957) to be released during bacteriophage lysis of group C streptococci. The carbohydrate is attached to the third, or peptidoglycan, layer by linkages that have not yet been identified with certainty. I do not mean to imply that the components included in this scheme in Table I represent all the substances that reside in the wall of the living cell. However, they are clearly the major structural components, and I will come back later to recent

morphologic evidence for the reality of the three-layered arrangement.

Not only are the type-specific M proteins a *sine qua non* for virulence of group A streptococci, but they serve as the determinant of the prevalent pattern of human streptococcal disease. Post-infection immunity is largely type-specific in character, directed toward neutralization of the antiphagocytic property of the M antigen; and since there are many distinct serological types of group A streptococci, the stage is set for repeated streptococcal infections in a single individual. These considerations have led investigators in various laboratories to come back repeatedly to the difficult problem of purification and characterization of the M proteins. We still have little information on the structure of the proteins, however, and none on the nature of that common feature of this serologically diverse family of antigens that confers the property of resistance to phagocytosis.

The finding that the group-specific polysaccharide is a major constituent of the cell wall led to the reexamination of certain mutants of group A streptococci that appear to lack this antigen. A number of these mutants have been isolated, the first by Armine Wilson in 1945, all appearing in the course of animal passage and having in common the property of being indistinguishable from the parent strain, except in their failure to yield extracts which react with group A antisera. Analysis of cell walls of these mutants showed that they possessed a carbohydrate layer quantitatively comparable to that of ordinary group A strains and that soluble polysaccharide preparations derived from them were also composed solely of *N*-acetylglucosamine and rhamnose (McCarty and Lancefield, 1955). However, there were significant differences in quantitative monosaccharide composition. As shown in Table II, giving representative figures for formamide preparations, the group A polysaccharide has a rhamnose:glucosamine ratio of approximately 2:1, while the A variant polysaccharide is composed primarily of rhamnose with only a minimal complement of glucosamine.

It proved possible to produce antisera that react specifically with the carbohydrate of these mutant strains, and thus the materials were at hand for an immunochemical analysis of the serological specificity of these related streptococcal polysaccharides. In these

TABLE II

COMPOSITION OF GROUP A AND A VARIANT STREPTOCOCCAL POLYSACCHARIDES (FORMAMIDE PREPARATIONS)

	N-acetylglucosamine	Rhamnose
Group A	35%	60%
A Variant	4%	85%

studies an approach was used that was first introduced by Dubos and Avery (1931) in investigations of the type III pneumococcal polysaccharide: The isolation of a soil organism capable of producing an enzyme that hydrolyzes the polysaccharide. Two strains of aerobic soil bacteria were isolated, one of which elaborates an induced enzyme that acts on the group A polysaccharide with resultant loss of reactivity with rabbit antisera, and the other an induced enzyme which destroys the A variant polysaccharide (McCarty, 1956). These enzymes proved to be invaluable tools in establishing the chemical basis for serological specificity of the antigens. The findings can only be briefly summarized here.

Analysis of the action of the enzyme active against the group A polysaccharide revealed that the loss of precipitating reactivity with group A antisera was associated with limited degradation of the antigen. About two-thirds of the total hexosamine was released as free monosaccharide *N*-acetylglucosamine, leaving the remainder of the molecule as an intact, nondialyzable unit. This type of result is illustrated in Fig. 2, showing that the reactivity of the polysaccharide with specific antiserum decreased progressively as *N*-acetylglucosamine was released. A second revealing finding is illustrated by the curve which demonstrates that at the same time that the treated polysaccharide lost group A reactivity it became precipitable with A variant antisera. Thus, in removing *N*-acetylglucosamine from the group A polysaccharide, the enzyme converts it to a product that simulates the serological behavior of the polysaccharide of mutant strains.

By constrast with the limited action of the A enzyme, the enzyme active against the variant polysaccharide caused extensive degradation of the molecule with release of dialyzable oligosaccharides and left little nondialyzable residue. In addition, the residue remaining

after removal of *N*-acetylglucosamine from group A polysaccharide with the A enzyme became susceptible to the second enzyme, so that the newly developed reactivity with A variant antisera was destroyed. These findings are illustrated in Fig. 3. In brief, group A polysaccharide treated with A enzyme is like the variant polysaccharide in enzymatic susceptibility as well as in serological reactivity.

The dominant role of *N*-acetylglucosamine in the serological specificity of the group A polysaccharide was confirmed by other procedures. For example, the monosaccharide *N*-acetylglucosamine is a potent and specific inhibitor of the precipitin reaction between the polysaccharide and rabbit antisera. In addition, it was possible to apply Landsteiner's azo-antigen technique to provide further evidence. *p*-Aminophenyl-β-*N*-acetylglucosaminide was synthesized

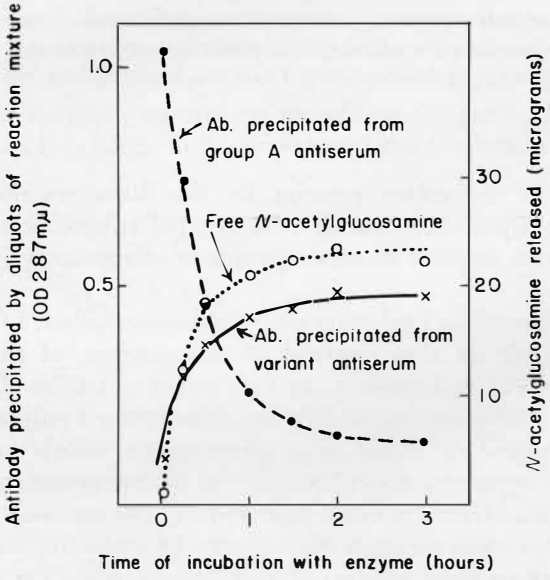

FIG. 2. Action of the soil bacillus enzyme (A enzyme) or group A carbohydrate. Samples were tested at intervals for the release of *N*-acetylglucosamine and for serological reactivity. *Ab.*, antibody.

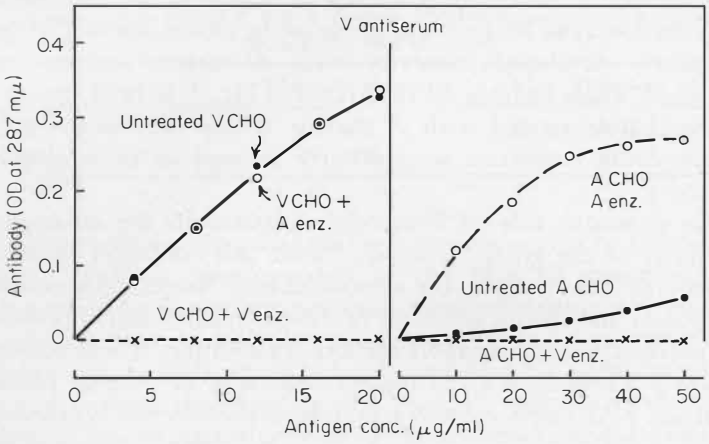

FIG. 3. The action of A and V enzymes on group A carbohydrate (A CHO) and group A Variant carbohydrate (V CHO). The reaction of the treated carbohydrates with V antiserum is depicted in quantitative precipitin curves. The reactivity of A CHO with V antiserum is markedly enhanced by treatment with A enzyme and then completely destroyed by treatment with V enzyme. Reprinted from McCarty (1956, p. 633).

and coupled to various proteins by the diazo reaction. These synthetic antigens cross-reacted with group A streptococcal antisera but not with antisera to other groups of streptococci (McCarty, 1958).

Without dwelling further on the experimental detail, I will move on to the picture that emerged of the structure of these polysaccharides. Figure 4 presents the most recent of a series of attempts to provide a schematic representation of the group A polysaccharide. As in the case of the others, this representation, which was devised by Krause, cannot be taken literally but incorporates features that can be deduced from the experimental data. The molecule is looked upon as a branched structure with *N*-acetylglucosamine forming the nonreducing terminal residues of each branch. Since the A enzyme has been shown to be a β-*N*-acetylglucosaminidase, it is apparent that the linkages of these terminal units to the subterminal rhamnose have the β-configuration. At least part of rhamnoses are combined

with one another by 1→3 linkages as demonstrated both by immunological cross-reactivity and by the direct chemical studies of Estrada-Parra *et al.* (1963) and of Heymann *et al.* (1963). Little is known concerning the backbone of the molecule. There is no evidence for a uniform repeating unit, and the apparent heterogeneity of the molecule which is suggested in the diagram could be a result of the necessity of obtaining soluble preparations by breaking them out of the giant cell wall molecule by chemical or enzymatic means.

Enzymatic removal of the terminal *N*-acetylglucosamine residue leaves short rhamnose side chains, which now become the determinants of a new serological specificity. The available evidence indicates that in the A variant polysaccharide these terminal residues are replaced by additional rhamnose, so that the side chains are longer than those of the group A polysaccharide after removal of glucosamine but with essentially the same conformation and specificity. In both cases the specificity is determined by rhamnose oligosaccharides, as demonstrated by the ability of a rhamnose disaccharide, isolated from the enzymatic split products, to inhibit the precipitin reaction (McCarty, 1956). The nature of the mutation resulting in the changed polysaccharide would appear to involve a loss of the synthetic mechanism for adding the terminal *N*-acetylglucosaminide residues to the branches of the polysaccharide.

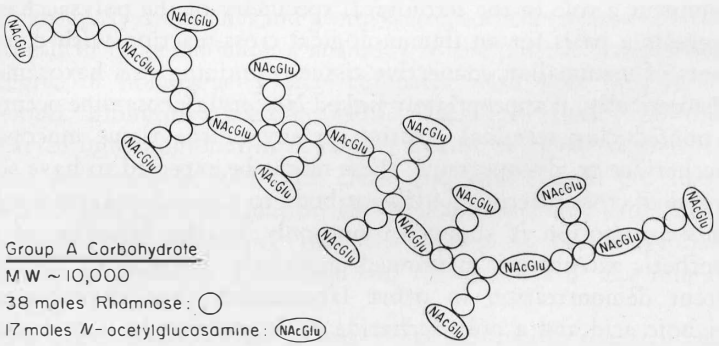

FIG. 4. Diagrammatic representation of group A carbohydrate molecule. From Krause (1970, p. 60).

Group C streptococci, which are found most commonly in animal infections but also occur in man, have been found to be closely related biologically to group A streptococci. It was therefore of interest to find that the group-specific cell wall polysaccharide of group C, although serologically distinct from group A polysaccharide, differs from it primarily in the fact that the immunodominant terminal hexosamine residues are *N*-acetylgalactosamine rather than *N*-acetylglucosamine (Krause and McCarty, 1962). The similarity between group A and group C polysaccharides is fu ther emphasized by the isolation of mutants of group C streptococci which lack the *N*-acetylgalactosamine determinants in the cell wall (Araujo and Krause, 1963). The cell wall polysaccharide of these mutant organisms is nearly identical in serological reactivity, chemical composition and enzyme susceptibility with the A variant polysaccharide. It would appear that the major distinguishing feature of these two groups of streptococci is in the configuration around the fourth carbon atom of the terminal hexosamine units of the cell wall carbohydrate.

The immunochemistry of the cell wall carbohydrates of certain other groups of streptococci has also been examined, notably in Krause's laboratory, but the details are not immediately pertinent to this discussion. It seems preferable to touch upon some of the implications of the findings concerning the group A polysaccharide. That terminal, β-linked *N*-acetylglucosaminide residues have so dominant a role in the serological specificity of the polysaccharide suggests a basis for an immunological cross-reaction with components of mammalian connective tissue containing this hexosamine. Theoretically, if appropriately linked *N*-acetylglucosamine occurs in a nonreducing terminal position in any of the tissue mucopolysaccharides or glycoproteins, these might be expected to have some degree of cross-reactivity with antibody to group A polysaccharide. This assumption is supported not only by the behavior of the synthetic azo-antigen mentioned previously, but also by the more recent demonstration in other laboratories that staphylococcal teichoic acid and a polysaccharide of *Streptococcus bovis* both cross-react with group A streptococcal antisera on the basis of terminal β-linked *N*-acetylglucosaminide residues. We were not successful in isolating a connective tissue component of this type, but some work

carried out in France during the past few years indicates that such a component may exist. Goldstein and his collaborators (1967) have reported that a glycoprotein extracted from mammalian heart valves by the use of trichloroacetic acid and urea gives striking cross-reactions with group A polysaccharide. However, it has not yet been established unequivocally that an *N*-acetylglucosamine determinant is involved in this case, and the possible pathological significance of the cross-reaction is unknown.

Regardless of the reality of these considerations, it is clear that human subjects commonly produce antibodies to the group-specific carbohydrate, presumably as a part of their response to repeated streptococcal infection. One recent intriguing development that has emerged from the renewed interest in this part of the antibody response to streptococci is the finding of Dudding and Ayoub (1968) that elevated levels of antibody to the group A polysaccharide appear to persist for long periods of time in patients with rheumatic heart disease in contrast to a normal rate of decline in rheumatic patients without carditis and those with glomerulonephritis. This is an observation that warrants further investigation in order to assess its significance in the pathogenesis of rheumatic fever.

One final comment on the cell wall polysaccharides relates to a dividend of broader biological importance that has come from the studies on the immunochemistry of these substances. Krause and his co-workers have found that a small proportion of rabbits immunized with group A, A-variant, and group C streptococci respond with the synthesis of large amounts of antibody to the polysaccharides with a degree of homogeneity that compares with that seen in the myeloma globulins (Krause, 1970). Detailed studies of these restricted immunoglobulins of known specificity have confirmed the initial observations, and it is clear that this system provides a powerful tool for examination for several important problems in immunobiology, including the relationship of immunoglobulin structure to antibody specificity and the genetics of the immune response.

The third layer of the streptococcal cell wall, the structural framework composed of the peptidoglycan or mucopeptide, has also been subjected to immunochemical analysis. We did little more than to determine that this material appeared to induce an antibody

response, but studies from Krause's laboratory have pursued the matter in detail. These studies have demonstrated a considerable degree of cross-reactivity between the mucopeptides of several gram-positive cocci, a fact that conforms to the known chemical similarity. The specificity of antibodies to a streptococcal mucopeptide appears to be most commonly referable to the peptide moiety, although there is also evidence for the occurrence of antibody directed toward the *N*-acetylmuramic–*N*-acetylglucosamine polymer (Karakawa and Krause, 1966; Karakawa *et al.*, 1967). Because of the common structural features and broad serological cross-reactivity, bacterial mucopeptide can be looked upon as a kind of heterophile antigen. In addition, it has toxic properties that may not be wholly dependent upon its antigenicity. The nodular, remittent skin reaction produced by streptococcal cell wall fragments in the studies of Schwab *et al.* (1959) is dependent on this component of the cell wall, and the investigations of isolated streptococcal mucopeptide by Rotta and Bednar (1969) has revealed that this material has properties analogous to those of the gram-negative endotoxins, including pyrogenicity, induction of the localized Shwartzman phenomenon, etc. The role that immune reactions play in these effects needs further exploration.

It is evident that each of the three layers of the cell wall is composed of antigenic substances, although a consideration of the full range of antigenic diversity would require a fuller discussion, especially of the surface proteins. In addition, as noted earlier, a description of these major structural components does not suffice to give a complete or dynamic picture of the cell wall. For example, no mention has been made of lipid in the streptococcal wall, and this subject has received little attention, although it seems certain that the type of isoprenoid lipid intermediate in cell wall synthesis described by Higashi *et al.* (1967) in gram-positive organisms and by Wright *et al.* (1967) in gram-negative must be present. Furthermore, there is evidence that at least one of the substances ordinarily included as one of the extracellular products, streptolysin S, is attached in some fashion to the cell surface (Ginsburg and Harris, 1963). Even the carbohydrate-mucopeptide structure is oversimplified in this presentation, and the occurrence of a glucose-containing polysaccharide in the wall has been reported by Munoz *et al.* (1967),

with the suggestion that it serves as the anchor point for the surface proteins. Since the amount of glucose in walls free of cytoplasmic membrane is no more than a few tenths of 1%, this component is quantitatively minor in relation to the group-specific polysaccharide.

One additional substance that is localized in the cell wall deserves mention, since it represents a rather major component with a function that has not yet been clarified. This component differs from those already discussed in that it has no covalent attachment to the wall structure, and when isolated cell walls are prepared, regardless of the method used, it appears in the soluble supernatant fraction. The general name now applied to this type of substance is glycerol teichoic acid, on the basis of the work from Baddiley's laboratory (1962), although we first encountered the group A streptococcal representative of this class of polymers on the basis of its antigenic properties (McCarty, 1959).

Certain streptococcal rabbit antisera were found to give strong precipitin reactions with an unidentified component of streptococcal extracts prepared by a variety of techniques. Using these antisera, appropriately absorbed with known antigens such as the group A polysaccharide, to monitor fractionation procedures, the new antigen was isolated and found to consist of a polymer made up almost exclusively of glycerol phosphate. The various lines of evidence suggesting that this rather simple polymer was responsible for the serological activity observed were strongly supported by experiments using synthetic polyglycerophosphate preparations that were supplied by Michelson. These preparations, with average chain lengths of from six to twelve glycerophosphate units, proved to be extremely potent and specific inhibitors of the reaction between the streptococcal antigen and precipitating antisera.

It is worth noting, also, that similar substances with some degree of glycerophosphate specificity occur in a variety of other gram-positive bacterial cells. Crude extracts of these organisms precipitate with the streptococcal antisera, and the reaction is inhibited by the synthetic polyglycerophosphate. Thus, this is another component with the properties of a heterophile antigen.

The work of Baddiley's group on glycerol teichoic acids of other bacterial species showed that they commonly carry as substituents in the basic glycerophosphate chain glycosidically linked sugars and

ester-linked amino acids. A reexamination of the group A streptococcal antigen prepared by milder procedures showed that it followed this pattern and in the native state has a significant number of ester-linked D-alanine residues. Actually, this reexamination was also stimulated by immunologic considerations and arose from the finding of Wilson and Wiley (1963) in immunoelectrophoretic studies of an antigen distinct from polyglycerophosphate but related to it. It led to the finding that the D-alanine could serve as a determinant of serological specificity, and that with certain antisera the presence of the D-alanine will mask the expression of glycerophosphate specificity in a manner analogous to the effect of the terminal *N*-acetylglucosamine residues on the expression of rhamnose specificity of the group A polysaccharide (McCarty, 1964).

In view of the fact that the glycerol teichoic acid of the group A streptococcus is not found in the isolated wall, one might ask why it is assumed to be present in the wall in the living cell. There are several independent pieces of information that lead to this assumption, one of the most important being the finding that a large part of the total teichoic acid can be removed from some strains of streptococci by the simple expedient of extracting viable organisms with acetate buffer. This is accompanied by no appreciable loss of other cellular components. Ultrastructural evidence relating to this matter is discussed below.

Time will not permit a discussion of certain other approaches that were used in study of the cell wall. These include an analysis by Krause (1958) of the action of the phage-associated lysin mentioned previously and, in collaboration with Freimer, the application of this enzyme to the preparation of cell wall free forms, or protoplasts, of group A streptococci (Freimer *et al.*, 1959). In addition, the interaction between bacteriophage and streptococci has proved to be a valuable tool for a study of the cell surface in the hands of Krause (1957) and of Fischetti and Zabriskie (1968), and the evidence indicates that the group-specific polysaccharide acts as at least part of the phage-receptor mechanism.

The accumulated information concerning the cell wall stimulated a second look at certain other streptococcal problems. As an example, I would like to review briefly our findings concerning the opaque colony mutants of group A streptococci. These mutants, which are

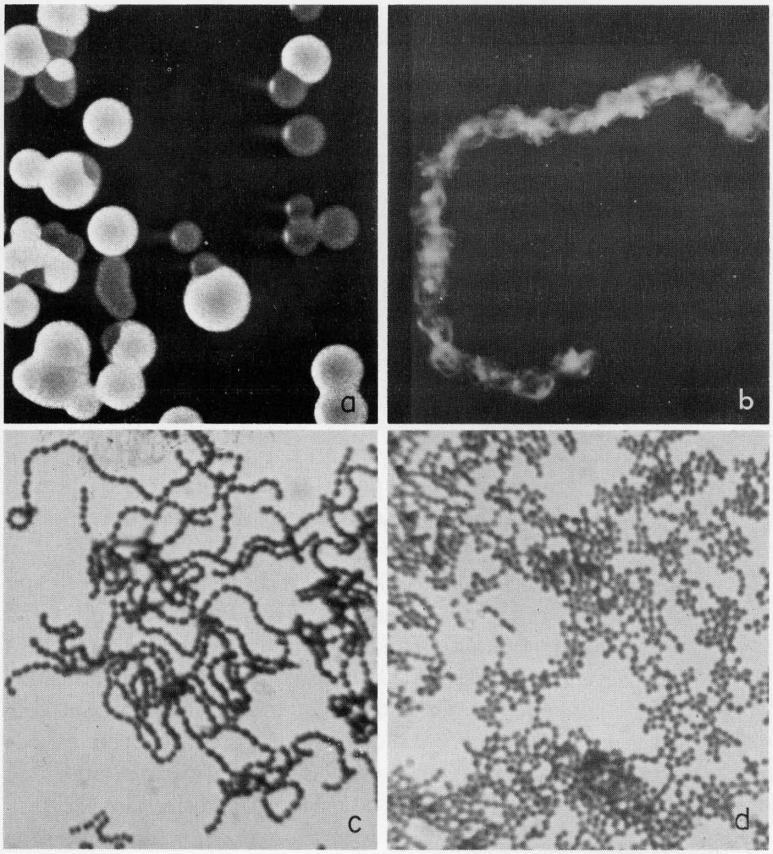

FIG. 5. Opaque-blue colony variation. (a) Appearance of opaque and blue colonies on transparent agar viewed with obliquely transmitted light. Opaque colonies appear white and granular. (b) Formamide extracted chain of streptococci from opaque colony, illustrating chaining of mucopeptide. (c) Gram-stained smear of suspension of organisms from opaque colony. (d) Gram-stained smear of suspension of organisms from blue colony. Reprinted from McCarty (1966, facing p. 190).

illustrated in Fig. 5a can be isolated from strains of group A streptococci of various serological types, and indeed quite similar forms are encountered in other bacteria. The colonies are most readily distinguished with obliquely transmitted light on clear agar, on which they appear white and coarsely granular in contrast to the

more common bluish and translucent colonies. The opaque colony forms had been studied in a number of laboratories, including our own, without a clear indication of their nature.

Since colony configuration of bacteria is markedly influenced by surface constituents, it seemed worthwile to reexamine the opaque colony mutants from the point of view of cell wall composition. However, an extensive chemical and serological comparison of the cell walls of paired opaque and blue colony-forming strains revealed neither quantitative nor qualitative differences. In the course of handling the strains for these studies, it was observed that suspensions of cells of the opaque mutants were consistently composed of tangles of exceedingly long chains of streptococci (Fig. 5c) in contrast to the short chains or single cocci found in suspensions of blue mutants (Fig. 5d). Microscopic visualization of growing microcolonies indicated that this property of exaggerated chaining persisting on the agar surface is responsible for an ordered pattern of growth that results in the granular appearance of the mature colonies (McCarty, 1966).

The long chains are highly resistant to mechanical disruption, and even after extraction of the organisms with hot formamide, the residual material (composed primarily of mucopeptide) occurs in chains (Fig. 5b). This suggests that an overdeveloped intercellular bridge at the mucopeptide level determines the chaining behavior of opaque mutants. Dr. John Swanson has prepared electron micrographs from thin sections of organisms of typical opaque and blue colonies which confirm the proposed basis for the difference between the two forms. The cells of the blue colonies are usually seen as single or diplococcal forms, while those from opaque colonies are arranged in chains in which the individual cells have distorted, flattened appearances. The flattened cocci have a larger diameter than the cells of blue colonies, and the exaggerated intercellular septa are nearly as long as the maximal width of the cells (Fig. 6a) (Swanson and McCarty, 1969). The isolated cell walls of these organisms retain the same bizarre configuration (Fig. 6b). The electron micrographs revealed further anomalies of the opaque strains relating to contact between the nucleoid and the cytoplasmic membranes beneath the intercellular septa, but the significance of these findings is not clear and they cannot be presented in detail here.

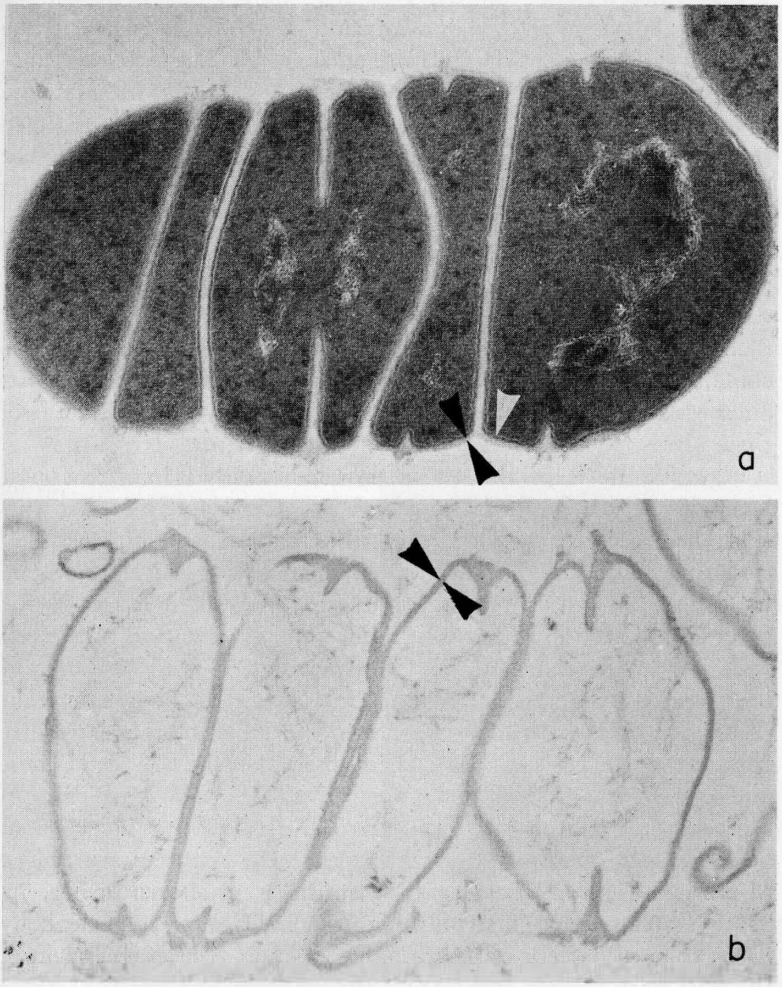

FIG. 6. Electron micrographs of opaque colony streptococci. (a) Section of chain of streptococci from opaque colony showing flattened cocci and exaggerated, long intercellular septa. For comparison with ordinary streptococcal cells, see Figs. 7a and b. Black arrows indicate the homogeneous layer of the cell wall. (b) Section of isolated cell walls of opaque organisms, showing retention of shape after disruption. Arrows indicate thickness of homogeneous isolated wall. Note absence of dense line. Electron micrographs by Dr. John Swanson.

These observations on the opaque colony mutants provide a suitable introduction to a presentation of additional data on the multilayered nature of the cell wall emanating from further collaborative studies with Dr. Swanson. These studies were designed to develop ultrastructural information which could be correlated with the type of immunochemical information concerning the wall that has already been described. This fruitful collaboration had its origin during the time that Dr. Emil Gotschlich of our group and Dr. Swanson were in the service at the Walter Reed Army Institute of Research. This led to a continuing association with members of our group after Dr. Swanson joined the Department of Microbiology at the College of Physicians and Surgeons. I am happy to say that the collaboration has continued since Dr. Swanson moved to the Mt. Sinai Medical School in the summer of 1969. Only a small sample of these ultrastructural studies can be presented here.

Figure 7a illustrates that the M protein not only is in fact an outer layer of the cell wall, as suggested by other data, but that this substance occurs in the form of hairlike fimbriae extending radially from the cell surface. These cells are prepared by primary glutaraldehyde fixation, which is not suitable for clear delineation of the cytoplasmic membrane but is excellent for visualization of the cell wall after appropriate staining. Shown in Fig. 7a is an M+ mutant of a type 6, group A streptococcus, in contrast to the M− mutant illustrated in Fig. 7b, and the presence of the fimbriae is readily apparent on the M+ cell, as is their absence on the M− cell. These findings were consistent for a number of M− and M+ pairs of different serological types (Swanson *et al.*, 1969). Other independent evidence was obtained to establish that the fimbriae are indeed M-protein. Ferritin-labeled anti-M antibody, as shown in Fig. 7c, neatly emphasizes the distribution of the fimbriae; and the antibody is not so fixed by cells of heterologous M-type. A brief treatment of the cells with trypsin eliminantes the fimbriae, and ferritin-labeled antibody is no longer fixed by the cells. When the trypsin-treated cells are washed and transferred to fresh medium, fimbriae begin to reappear after a few minutes of incubation, and the cells have regained their original appearance within an hour.

It can also be observed in Figs. 6a, 7a, and 7c that the cell wall underlying the M-protein layer is composed of two lamina: a homo-

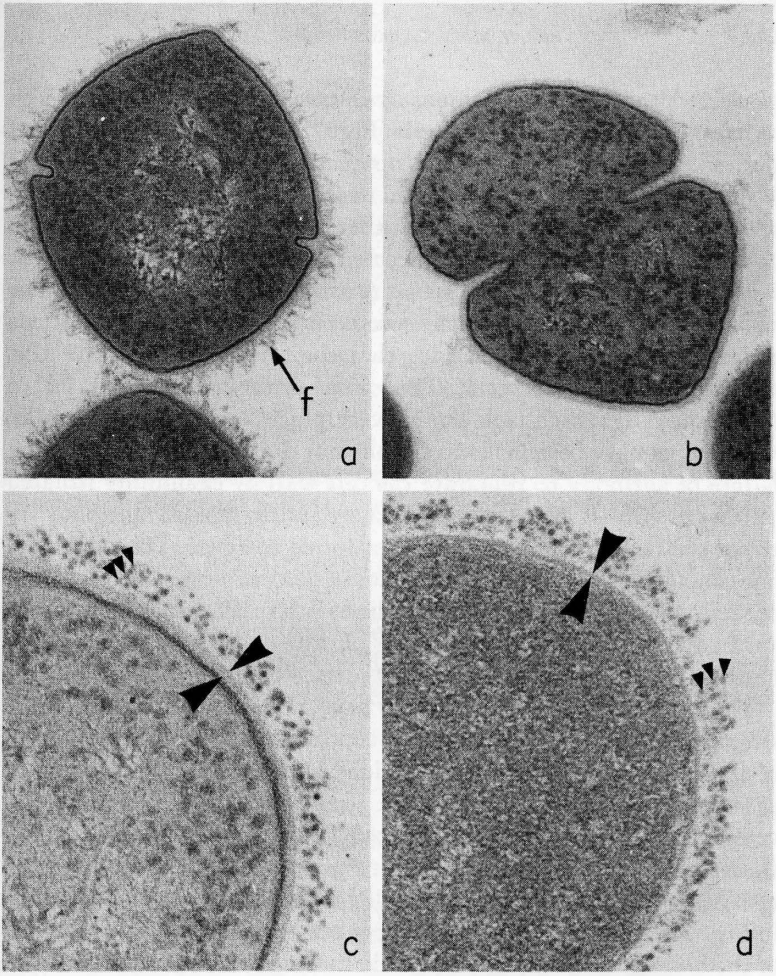

FIG. 7. Electron micrographs of thin sections of streptococci. (a) M-positive strain, showing hairlike fimbriae of M-protein (*f*). (b) M-negative strain lacking surface fimbriae. (c) Higher magnification of M-positive streptococcus tagged with ferritin-labeled antibody to M-protein. Large arrows indicate thickness of homogeneous layer of cell wall. Small arrows indicate alignment of ferritin label along M-protein fimbriae. (d) Section similar to (c) after treatment of organisms with nitrous acid. Reduction in homogeneous outer zone of wall is correlated with removal of large part of group-specific carbohydrate, although M-protein remains intact (ferritin-labeled M-antibody). Large arrows indicate substantial reduction in thickness of wall associated with removal of carbohydrate. Note also that the dense line of the inner portion of the wall has been removed by nitrous acid treatment. Electron micrographs by Dr. John Swanson.

geneous outer layer and an intensely electron opaque inner layer. The same appearance is seen in M- cells (Fig. 7b). This is characteristic of the appearance of the wall in cells prepared by this method, and one of our aims is to relate what is seen to the known components of the wall. Ferritin-labeled antibody to other components does not provide clear evidence on this point since it does not penetrate the wall. Ferritin-labeled antibody to group A carbohydrate coats the surface but does not penetrate the homogeneous layer, and its distribution is at least consistent with the idea that the carbohydrate is the next layer under the M-protein. The same antibody does not fix to A-variant cells which lack the *N*-acetylglucosamine determinant.

Attempts have been made to circumvent the difficulty presented by failure of antibody to penetrate the cell wall by examining isolated cell walls which have been interacted with labeled antibody to either the carbohydrate or the mucopeptide antigens. The results are not conclusive because of ambiguities in determining the orientation of sectioned, isolated walls, but in line with other data, the group A antibody is found predominantly on the outer surface and the mucopeptide antibody on the inner surface of the walls.

The finding of Gotschlich that brief nitrous acid treatment of streptococci under relatively mild conditions removes a large part of the cell wall carbohydrate provided another tool for this study, as well as giving a possible clue concerning the linkage of the carbohydrate to the wall structure. The effect of nitrous acid is shown in Fig. 7d, which depicts nitrous acid-treated cells that have also been tagged with ferritin-labeled antibody to the M-protein as in Fig. 7c. The nitrous acid treatment removes a substantial portion of the homogeneous layer without disturbing the M-protein, and the indications are that the protein penetrates the carbohydrate layer and is fixed to a deeper portion of the wall.

You will also note that the electron-dense layer of the wall disappears completely from nitrous acid treated cells. The other wall component encountered in large amounts in the supernatants after nitrous acid treatment is the glycerol teichoic acid. Since the dense line also disappears when isolated cell walls are prepared (as in Fig. 6b), the current view is that this opaque layer may represent the glycerol teichoic acid, a reasonable possibility in view of the very high phosphorus content of this antigen.

It was noted earlier that a large portion of the glycerol teichoic acid can be removed from streptococci by simple buffer extraction. The obvious experiment is to test the effect of this type of extraction on the appearance of the opaque line. Dr. Swanson and I do not yet have conclusive evidence on this point, although in one test the buffer extraction resulted in a clear decrease in the opaque layer without other changes. Unfortunately, the proportion of teichoic acid removed by this procedure is too variable to provide a crucial test.

The current picture of the streptococcal wall can be summarized as follows:

1. The type-specific M-protein occurs in the form of hairlike fimbriae on the outer surface, but penetrating more deeply into the wall.
2. The group-specific polysaccharide composes the outer homogeneous layer of the cell wall.
3. The mucopeptide forms an inner structural framework.
4. The glycerol teichoic acid, which lacks covalent attachment to the wall structure, may be reflected as an electron-opaque layer over the inner portion of the wall (i.e., in the region of the mucopeptide).

It is anticipated that further clarification of these relationships will come from a continuation of this type of combined ultrastructural and immunochemical analysis. Hopefully, the end result will be better understanding of structure-function relationships in the complex cell wall of this important pathogen.

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