A SEROLOGICAL STUDY OF THE CELL WALLS OF LACTIC ACID BACTERIA

by

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ABSTRACT

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Cell walls of three species of Lactobacillus and three of Streptococcus, cultured in a synthetic medium, were isolated by mechanical disintegration and purified by washing. Suspensions of cell walls, and also of intact cells, were injected into rabbits--three replicates with cell walls and three with intact cells of each species. Antigens from these species were demonstrated by agglutination, by hemagglutination, and by precipitation tests. A greater number of antigens was detected by cell-wall antisera than by intact-cell antisera. Certain antigens combined with antibodies even though they did not induce them.

A large proportion of the antigens was shared by the different species in the two genera. S. faecalis proved to be an exception.

A new approach for detecting protein-type antigens by the hemagglutination technique was attempted. Cell-wall suspensions were used to sensitize tannin-treated erythrocytes.

The evidence presented shows the possibility of using antisera from species-specific cell-wall antigens for the identification of the six species. The close relationship of these species is discussed.

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INTRODUCTION

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Intensive studies on the physical and chemical properties of bacterial cell walls were initiated by Salton and Horne (1951 a, b). Electronmicrographs showed cell walls of Gram-positive bacteria to be thicker than the walls of the Gram-negative bacteria, the former 15 to 20 mμ, and the latter 10 to 15 mμ in thickness (Dawson, 1949; Salton and Horne, 1951 a, b; Birch-Anderson, Maale and Sjostrand, 1953). Two layers were recognized in the cell walls of Spirillum sp, the inner layer composed of spherical macromolecules in a hexagonal pattern (Houwink, 1953).

Chemical studies on cell walls of bacteria have been carried out. Walls of Gram-positive bacteria contained a low concentration of lipids, and a limited range of amino acids but no proline, histidine, arginine, aromatic, or S-containing amino acids. Salton (1952 a, 1953 a) reported lysine, glutamic acid, alanine, glucose, rhamnose and galactose to be the chief components of a strain of Streptococcus faecalis. Cummins and Harris (1956), working with two strains of this species, reported similar findings with two exceptions. Mannose was present, but galactose was not. Species of lactobacilli contained essentially the same components, with only minor differences. L. plantarum lacked alanine and galactose but contained diaminopimelic

acid. L. casei contained aspartic acid and mannose (Cummins and Harris, 1956). L. lactis contained aspartic acid, but neither rhamnose nor galactose (Ikawa and Snell, 1960).

Cummins and Harris (1956) noted a correlation between cell wall composition and taxonomic patterns. The amino acid components appeared to differentiate genera, whereas the sugars and amino sugars to differentiate species within the genera. They expressed the opinion that the chemical constituents and the serological characteristics of the bacterial cell wall are closely related.

Little information concerning the serological properties of the cell wall has been published. The study reported herein involves a comparison of the antigenic components of cell walls and intact cells in certain species of Lactobacillus and of Streptococcus, the two genera being chosen because of their close relationship.

HISTORICAL

HISTORICAL

In the isolation of bacterial cell walls, Vincenzi (1887) treated whole cells of Bacillus subtilis with 0.5 per cent NaOH, and obtained residues similar in outline to those of the original cells. However, alkaline extraction may have altered the chemical properties of the cell walls. Salton and Horne (1951 b), using electron microscopy, demonstrated that cell wall residues prepared by the alkaline method differed from those prepared by other methods. Degradation of walls was thought to have taken place (Salton, 1952 a).

Mechanical disintegration of microorganisms was reported by Bucher (1897). MacFadyen, Harris-Morris and Rowland (1900) used silver sand to rupture yeast cells. MacFadyen and Rowland (1901) employed fine sand to grind cells of typhoid bacilli. Barnard and Hewlett (1901) ruptured yeast cells by grinding in a self-constructed disintegrating device of five steel balls confined in a rotary phosphor-bronze vessel. Thick cell walls were observed when a smear of the cell debris was examined microscopically.

Curran and Evans (1942) disintegrated bacterial spores by violent agitation with small inert particles. King and Alexander (1948) reported that mechanical

destruction was obtained by subjecting bacterial cells to violent shaking with minute round glass beads. Dawson (1949) ruptured intact cells of Staphylococcus aureus with glass beads in the Mickle tissue disintegrator. Cell walls were separated from intact cells and other cytoplasmic components by centrifugation at 8000 r.p.m.. Electron microscopic examination made it clear that intracellular cytoplasmic material had been separated from the cell walls. However, Cooper, Rowley and Dawson (1949) estimated that approximately seven per cent of the cells subjected to the disintegrator remained intact. King and Alexander (1948) observed that a small but definite proportion of organisms was not disintegrated by mechanical treatment, and that the resistant cells did not seem to differ from normal cells in any of the other characteristics investigated.

Bacterial cells ruptured in the Mickle tissue disintegrator (Dawson, 1949) were subjected to differential centrifugation. Salton and Horne (1951 b) separated unbroken cells of Streptococcus faecalis and Salmonella pullorum at 3000 r.p.m.; and separated cell walls by centrifuging the supernatant at 10,000 r.p.m.. Ribi, Milner and Perrine (1958) centrifuged suspensions of ruptured cells of Salmonella enteritidis at 2500 x G. The sediment consisted of a translucent upper layer of cell walls, and an opaque under layer of unbroken cells. The upper layer was removed, and freed from intact cells by repeated washings and

centrifugations. Munoz, Ribi and Larson (1959) centrifuged a suspension of disintegrated cells of Bordetella pertussis at 10,000 r.p.m.. These workers recognized an upper layer of cell walls, a middle layer of a mixture of intact cells and cell walls, and a lower layer of intact cells. The cell wall layer was removed, resuspended and centrifuged repeatedly, discarding in each case the heavier intact cell layer and collecting only the upper layer, until a pure preparation of cell walls was obtained.

The application of sonic energy in the disintegration of bacterial cells was investigated by Mudd, Polevitzky, Anderson and Chamber (1941). These authors subjected intact cells of Bacillus megaterium, B. subtilis and B. anthracis to a sonic oscillator, and examined the disrupted cells by electron microscopy. They demonstrated that cell walls have definite, solid morphological structure.

Of the 16 organisms exposed to ultrasonic disintegration by Stump, Green and Smith (1946), 12 were ruptured easily, and the remaining four, particularly the Gram-positive cocci, were refractory. Citing evidence that some cells in every batch resisted the disruptive force of the sonic oscillator, they postulated that complete disintegration even of the non-refractory cells was impossible. These authors also stressed that the viscosity of the cell suspension was a factor of importance. They recognized that ultrasonic vibration was largely converted

to heat when the thickness of the cell suspension exceeded a certain limit, and that, as a result, the disruptive power was lost.

Salton (1953 a), in his work on the isolation of cell wall by a Raytheon sonic oscillator, showed that Escherichia coli and Bacillus megaterium were ruptured in 10 minutes, whereas micrococci presented no evidence of disruption after treatment for an hour.

Marr and Cota-Robles (1957), in a study of the sonic disintegration of Azotobacter spp., demonstrated that 40 per cent of the surface of the intact cells was fragmented to submicroscopic particles. These workers, as did Slade and Vetter (1956), further demonstrated that when continuous oscillation was maintained the cell walls lost structural features and finally were reduced to a soluble state.

Using a different approach, Salton and Horne (1951 b) ruptured bacteria by heating cell suspensions at 75 degree C and 100 degree C, and then separated cell walls from coagulated protoplasts by shaking with glass beads. This method was considered to have little value due to the possible destruction of surface features of cell walls by heat and to the low yield.

The crude cell wall material, obtained after disintegration by differential centrifugation, has been shown to contain contaminating cytoplasmic substances such as nucleic acids, denatured proteins, and cellular particles

(Salton and Horne, 1951 b; Barkulis and Jones, 1957). Salton (1956) reported that the adherent cytoplasmic material was removed more easily from the walls of Gram-positive than from those of Gram-negative bacteria. Salton and Horne (1951 b), working with cell walls of Streptococcus faecalis and Salmonella pullorum claimed that cell walls, prepared by disintegrating in distilled water and washing a few times in 0.1 M phosphate buffer at pH 7.0, were free of cytoplasmic contaminants. Barkulis and Jones (1957) purified cell walls of type 14 hemolytic streptococci by the same method. Vennes and Gerhardt (1959) reported that washing with saline facilitated the removal of adherent small particles. McCarty (1952 a, b), Salton (1956), and Cummins (1956) obtained cell walls free of cytoplasmic material by digesting crude walls with ribonuclease, pepsin or trypsin. Salton (1956) objected to the unrestricted use of proteolytic enzymes for this purpose. He expressed the opinion that enzymes like trypsin might contaminate wall preparations with insoluble plasteins, and that crude enzymes probably contained small amounts of wall-degrading enzymes. Cummins (1954) showed that prolonged treatment of cell wall material with enzymes like pepsin eventually destroyed certain specific antigens.

The immunological properties of bacterial cell walls have been investigated by McCarty (1952 a, b) and Salton (1952 c, 1953 a), each of whom produced evidence that the group-specific antigen for group A streptococci was located

in the cell walls. Jones and Shattock (1960) reported that for group D streptococci the opposite was true. The group-specific antigen was detected in the cell content after the cell walls had been removed. McCarty (1952 a, b) dissolved the isolated cell walls of several strains of group A streptococci with enzymes extracted from Streptomyces albus. The extract on analysis was found to consist of approximately two-thirds carbohydrate (mainly N-acetyl-glucosamine and rhamnose), and one-third protein. He pointed out that the carbohydrate portion contained the group-specific C carbohydrate.

Salton (1953 a) and Barkulis and Jones (1957) observed the presence of type-specific M-protein in cell walls of group A streptococci prepared by mechanical disintegration. This protein was isolated from the cell walls and partially purified. In the presence of trypsin, it was destroyed.

Cummins (1954) made a serological study on cell walls of a mitis strain of Corynebacterium diphtheriae, using suspensions of intact cells and cell walls prepared by mechanical disintegration. He demonstrated the presence of two antigens by agglutination and agglutinin absorption techniques. One was a superficial heat-labile specific antigen and the other, a deeper heat-stable group antigen. The former, a protein, was considered to be responsible for the agglutination of intact cells, and the latter for that of cell walls. The author suggested that the deeper group

antigen, which was detected in all cultures of Corynebacterium diphtheriae studied and was also present in one strain of C. ovis, was probably polysaccharide in nature, since it was attacked rapidly by periodate. Cummins considered that these cell wall antigens might be valuable in classification.

Yoshida, Nagayuki, Fukuya, Kakutani, Tanaka, Tegawa and Tadayo (1954) studied cell walls of Bacillus subtilis stained with 0.1 per cent phosphotungstic acid. Electron micrographs revealed the rough appearance of the outer surface of the cell wall, compared to the smooth inner surface. However, the rough structure of the outer surface disappeared when cell walls were treated with pepsin and trypsin. They suggested that these electron microscopical observations supported the assumption that the protein component was situated in the superficial outer layer and the polysaccharide components in the inner layer.

Tomcsik and Guex-Holzer (1954) examined the immunological properties of surface layers of bacterial cells. These workers provided evidence that protoplast and the intact cell, or the cell wall, of Bacillus megaterium were antigenically distinct. Vennes and Gerhardt (1956, 1959), working with B. megaterium also, reported no cross-reaction between intact cells (or cell walls) and antiserum to protoplast (or protoplast membrane). With the agglutinin absorption technique, these authors demonstrated that intact

cells absorbed only a portion of the cell-wall antibodies.

Sharpe (1955) investigated the group-specific antigens of lactobacilli. This author tested crude HCl extracts with group-specific antisera by the precipitin test, and classified 312 of the 442 strains studied into six groups and one subgroup.

The presence of a surface protective antigen in the cell wall was reported by Yoshida and his associates (1955). They demonstrated that cell walls of Bordetella pertussis were as effective as intact cells for protection against experimental infections. Munoz, Ribi, and Larson (1959) confirmed this finding, and in addition found that the protective antigen and the histamine sensitizing factor were predominantly located in the cell wall, whereas the heat-labile toxin was in the protoplasm.

Shafa (1958) detected a surface antigen in the cell walls of Salmonella gallinarum and Vibrio metchnikovi. This antigen was identical with the O-suspensions of these organisms. Ribi, Milner and Perrine (1958) reported on the antigens in cell walls of Salmonella enteritidis, noting that the toxicity, the ability to produce antibody, and the reactivity with O-antibody were associated principally with the cell wall. Other authors who have provided evidence that cell walls contain antigens which participate in immunologic reactions: Salton (1952 c) and Barkulis and Jones (1957), with Streptococcus pyogenes;

Shepard, Ribi and Larson (1955), with Histoplasma capsulatum; Carey and Baron (1958), with Salmonella typhosa; and Ribi, Larson, List and Wicht (1958), with Mycobacterium sp.

The direct adsorption of bacterial extracts on erythrocytes and subsequent hemagglutination by antisera to these extracts was reported by Landsteiner (1945), Keogh, North and Warburton (1948), Middlebrook and Dubos (1948), Norden (1949), and Boyden (1950, 1951). Keogh, North and Warburton (1947, 1948) suggested that polysaccharides from bacterial extracts caused the direct sensitizing of erythrocytes; and that hemagglutination by immune sera was due to a serological reaction between specific antibody and adsorbed polysaccharides. Middlebrook and Dubos (1948), with Mycobacterium tuberculosis, and Alexander, Wright and Baldwin (1950), with Pasteurella tularensis, reported that polysaccharides from these organisms also sensitized erythrocytes; and that the subsequent hemagglutination reaction was highly specific.

Purified protein, likewise, has been adsorbed to erythrocytes. Boyden (1950) noted that treatment of sheep erythrocytes with a suitable concentration of tannic acid rendered them capable of adsorbing protein molecules such as Tuberculin P.P.D., ovalbumin, chicken serum albumin, horse serum albumin, horse serum globulin, and protein preparation from Streptococcus sp. The sensitized erythrocytes