

The effect of the environment on the growth and
composition of the cell wall of Corynebacterium
fascians

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ABSTRACT

The effect of environmental factors, temperature, aeration, medium, ultraviolet irradiation and presence of penicillin were studied in respect to the growth and morphology of strains of Corynebacterium fascians. The main aim was to study filament formation under these conditions.

Growth and morphology of any one strain varied under each of these conditions and further, there was variation in the response of different strains to each factor studied.

Formation of filaments was good with strain 505 (smooth variant) growing in nutrient agar containing 10 μ g/l penicillin and in Thornton's medium from which either magnesium sulphate, sodium chloride or asparagine had been omitted. The greatest effect was observed in static liquid cultures of Thornton's medium lacking magnesium sulphate.

The cell wall mass of one strain studied in detail, varied under different environmental conditions. In addition similar variations occurred between different strains under the same environmental conditions, although this study was limited to solid media.

The cell wall of strain 505 (smooth variant), was found to contain ribitol teichoic acid, glucosamine, galactosamine and muramic acid as amino sugars and lysine, histidine, asparagine, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine and leucine as amino acids.

The environmental factors found to affect the morphology also influenced the composition of the cell wall. Significant changes observed in the major cell wall components, teichoic acid, amino sugars and amino acids, glutamic acid, methionine, alanine and lysine, were also found to occur with minor wall components, in particular with serine and histidine in this organism.

In general, increases in certain wall components were associated with corresponding decreases in other components. The amount of any single specific component was observed to vary independently according to the environment, and in particular to the presence of magnesium sulphate, sodium chloride and penicillin in the medium.

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I

INTRODUCTION

A. Effect of the environment on growth and composition of bacteria

The chemical composition of microorganisms is so affected by environmental conditions that, as Herbert (1961) emphasised, it would be virtually meaningless to describe this without, at the same time, specifying the environmental conditions that had produced it. For example, the RNA content of Bacillus cereus, can vary between 3 and 30% depending on the environmental conditions of growth.

An appreciation of the importance of the environment on bacterial cell composition did not occur until the 1950's and most of the early work on cell composition (reviewed by Porter, 1946), is of little value since the environmental conditions were not controlled. Both physical and chemical aspects of the environment are important and these may have a qualitative effect with the production of a cell component or components absent in other environments or a quantitative effect with the production of more or less of a component always present. The former is seen with the production of new types of antigens or adaptive enzymes and will not be considered in this thesis.

Quantitative changes have been observed in nucleic acids, proteins, lipids and polysaccharides. RNA and DNA changes occur in bacteria in any batch culture when the environment changes as nutrients are exhausted and metabolic products accumulate. This results in fluctuations in DNA and RNA content such that when the RNA content is high, DNA content is at its lowest (Schaechter, et al., 1958).

Earlier, Henrici (1923), demonstrated significant changes in cell volume during the course of cultivation, and Hershey (1938, 1939) gave a clear distinction between growth rates e.g. a decrease in cell nitrogen which was due to a decline in growth rate and to a rapid division rate.

The rate of growth is not influenced by the physiological state of the cell and the lag in rate of multiplication may be due to changes in the factors limiting the size at which fission occurs during growth. Henrici (1928), observed that the bacterial cell increases in size during the lag phase prior to the commencement of cell division. It has also been demonstrated that increase in size and enrichment in RNA go hand in hand (Malgren and Heden, 1947; Morse and Carter, 1949; Wade, 1952a and Gale and Folkes, 1953).

Mitchell and Moyle (1951), demonstrated the time sequence of changes in percentage RNA, DNA and nucleic acid fragment e.g. nucleotide and nucleoside, and of their absolute rates of synthesis in Staphylococcus aureus throughout a complete normal growth cycle and compared them with the changes that occur when penicillin G is added to the culture in the late logarithmic phase of growth. They reported the inhibition of the synthesis of an unidentified phosphate ester at an early stage in the penicillin-treated cultures and they also observed that there are visual changes in the surface of the penicillin treated cells which accompany the chemical changes.

Wade (1952a, b) in his study of the pattern of growth of bacteria, demonstrated that the fluctuations in phosphate concentration observed during the phases of growth could be largely accounted for by the differences in concentration of RNA, and during the first generation could be associated quantitatively with the increase in average cell weight. He suggested that the bacterial cell requires a certain concentration of RNA, dependent on its size, before it can divide. He also explained the temporary drop in RNA towards the end of the first generation where part of the RNA phosphorus is metabolized into other phosphates or is lost from the cell during the course of the first division.

Growth of the bacteria under a steady state, may be characterized as balanced, i.e. on average each cell constituent doubles in mass between

successive divisions. This may be achieved either by maintaining a maximum growth rate by repeated dilution with fresh medium or by growth at a constant growth rate below the maximum, by limiting an essential metabolite e.g. chemostat culture.

Work on steady state growth was reviewed by Maaløe (1960) and this included the studies of Schaechter et al. (1958) which showed that in Salmonella typhimurium there is a relationship between the RNA and DNA content of the bacteria; cell mass and average number of nuclei per cell and the growth rate in different media at a given temperature. This work was supported by that of Kjeldgaard et al. (1958), who studied the effect of transfer of bacteria from a simple to a complex medium. Mandelstam and McQuillen (1973) reported that in balanced growth the DNA synthesis is not restricted to part of the growth cycle of the bacteria but is continuous. This may, however, be a consequence of rapid growth and the short period of time between divisions. They also demonstrated the relationship between the synthesis of RNA, proteins, and cell envelopes, the mean cell size and number of nuclei per cell to the rate of bacterial growth.

Large differences in the growth rate of a bacterial culture can be achieved by altering the composition of the medium or the temperature of incubation where the rate of synthesis of the cell components must be correspondingly adjusted and coordinated with each other (Pritchard et al., 1969).

In addition, the work of Malette (1969) on evaluation of growth by physical and chemical means, concluded that cell numbers and total mass of cells are not always related in the same way. This relation is affected by all factors influencing cell size at division and must be evaluated whenever cell counts are taken as the measure of total cellular material.

Moore et al. (1966), studied S. faecalis and observed that protein synthesis increased during ageing of the culture and was accompanied by an increase in the percentage of membrane bound ribosomes and by a decrease in ribosomal monomers and subunits. At the time, modifications in membrane composition could be observed, among which were increased concentrations of phospholipids.

The work of Virtanen and Deley (1948), on the nitrogen content of the medium and its effect on the cellular nitrogen, suggested the possibility that cells utilize nitrogenous reserves (Novick and Szilard, 1950). Tepper (1965), using several different media, found, the highest N_2 content in cells grown in complex media. He noted, however, that cells grown in media containing glycerol gave a higher dry weight than those grown in a glucose medium, but that the N_2 content per cell was the same in both cases. The glycerol increased the storage of lipid and carbohydrate.

Lemoigne (1923), showed that Bacillus megaterium formed quantities of β -hydroxy butyric acid in aerobic suspensions in the absence of an external carbon and energy source. In 1925, Lemoigne concluded from the quantitative estimations of this acid, that it accounted for the major part of the acidic substances produced under his experimental conditions. He then demonstrated in 1927, that a polymer of β -hydroxybutyric acid could be extracted from the bacilli by chloroform. Lemoigne et al. (1944), reported the presence of a correlation between the amount of this polymer and the amount of refractile "fatty" cytoplasmic granular material present in the bacilli. This same substance was found later in other Bacillus species (Lemoigne, 1946).

Weibull (1953), in his observations on the nature of the granules isolated after dissolution of the cell wall of B. megaterium by lysozyme, confirmed that the granules in the cells of this organism consist of lipid material, found to be polymerized β -hydroxybutyric acid. Williamson and Wilkinson (1958), using alkaline hypochlorite solution, liberated these

"lipid" granules, which were shown to consist largely of poly- β -hydroxybutyrate.

Macrae and Wilkinson (1958), showed that in carbon limiting conditions, the poly- β -hydroxybutyrate content of the cells remains fairly uniform and low throughout the growth of the culture but when nitrogen is limiting, poly- β -hydroxybutyrate is rapidly synthesized to a high level in the period preceding and following the end of growth due to the exhaustion of nitrogen.

Studies have been made on the influence of the environment on the total content and composition of the free amino acid pools. Tempest et al. (1970), studied this in Gram positive bacteria in chemostat cultures where the pool was found to remain constant unless the growth rate or nature of the limiting substance was changed. The influence of growth rate was completely predictable, an increase in growth rate producing a progressive increase in total concentration of intracellular free amino acids, although not all the detectable amino acids increased proportionately. The overall increase was principally due to changes in the levels of free glutamate and alanine. On the other hand, Tempest and co-workers, reported that the total concentration of free amino acids present in the pool was not markedly altered by the nature of the growth-limiting component. However there were obvious differences in the concentration of the individual amino acids particularly glutamate and alanine.

When either the nitrogen source or the carbon source was limiting, the only amino acids that were present in appreciable amounts were glutamate followed by alanine. Valine, glutamine and lysine were present in lesser amounts.

In magnesium-limiting condition there was an obvious effect on the individual amino acids. This was mainly on alanine, lysine, asparagine, valine and glycine. No direct effect was found on glutamate synthesis although the ratio of glutamate to alanine in the pool has been changed from 10 : 1 to equimolar amounts of each.

The pool glutamate level was increased substantially by the addition of sodium chloride irrespective of the nature of the growth-limiting component of the medium. Thus they concluded that the addition of sodium chloride to the environment directly affected the rate of glutamate synthesis.

B. Walls of Gram positive bacteria

(a) Isolation and purification

Vicenzi (1887), was one of the first to attempt to isolate the cell wall of a bacterium, when he found that the alkali resistant fraction of B. subtilis retained the original shape of the cell.

Techniques now employed rely on the disintegration of the cell followed by purification of the cell wall pieces.

Electron microscopical observations were carried out by Mudd and Lackman (1941), who observed that the cell wall of species of Bacillus and Streptococcus after sonic disintegration remained as a rigid structure in the shape of the original bacterium. There was an absence of electron dense cytoplasm and only electron transparent wall material was present. King and Alexander (1948), and Dawson (1949), repeated this observation on cells that had been broken by shaking with minute glass spheres (ballotini) and obtained a residue of unbroken cells, empty cell walls and small electron dense particles of cytoplasmic origin.

Isolation of the bacterial cell wall as a single morphological entity in a reasonably pure form was first carried out by Salton and Horne (1951).

Once the cell has been broken open, the problem arises of separating the wall from the remainder of the cell. Further, the question as to what represents the cell wall has been a matter for

much discussion, as there is no absolute definition of the term. If little purification is carried out, then contaminating substances from other parts of the cell may appear in the cell-wall preparation. On the other hand if drastic techniques of purification are applied they may remove substances that should properly be regarded as part of the wall. This problem can be simplified by regarding the wall as an organelle that has the same shape as the original cell and which corresponds in appearance to the outer layer of the undisrupted cell as seen in the electron microscope. This includes the cytoplasmic membrane which, using recent critical methods, can usually be distinguished clearly in sections of the whole organism.

Knaysi (1951) and Salton (1956) discussed the early techniques used for the isolation of the rigid cell wall. Two main methods have been used; mechanical disintegration of the cells, or autolysis and digestion of the cells to allow isolation of the more resistant cell walls. It is clear from the results of Salton and Horne (1951), that the alkali-insoluble residues of bacteria, formerly assumed to be the cell wall structures (Vicenzi, 1887), do not correspond to the walls isolated by mechanical disintegration. The supernatant obtained after centrifugation of suspensions of bacteria which have been mechanically disintegrated, is largely composed of cell wall fragments, and a number of workers have reported on chemical analyses of such fractions (Mitchell and Moyle, 1951; Holdsworth, 1952 and Salton, 1952, a,b,1953). Abundant evidence has been accumulated to show that extraction of bacteria, or indeed isolated cell walls, with alkali or acids will lead to a degradation of parts of the wall structure (Salton, 1956). Thus dilute sodium hydroxide removes o-acetyl groups from the walls (Abrams, 1958), and TCA extracts a number of compounds from bacterial walls including teichoic acids (Brown, 1958; Baddiley et al., 1958).

Walls may be purified by differential centrifugation followed by digestion with trypsin, deoxyribonuclease and ribonuclease at pH 7.0. Purification of cell walls by pepsin digestion (pH 1-2, 38°C, 15 hrs.), employed by many early investigators (Salton, 1953; Cummins and Harris, 1956_{a,b}, 1958; McCarty, 1952, and Salton and Marshall, 1959), has more recently been found by Mandelstam and Strominger (1961), to remove about 40% of the teichoic acid. They also found the same removal of teichoic acid to occur in 0.01 N HCl in the absence of pepsin. Brookes et al. (1959), found trypsin reduced the amino acids obtained from the wall of B. megaterium to three only, and at the same time reduced the overall yield of amino acids by approximately 50%. It is obvious therefore that the method of purification has an influence on the composition of the wall sample obtained.

(b) Composition

All analyses of bacteria cell walls have shown that large amounts of alanine and glutamic acid are present in addition to a dibasic amino acid which is either lysine or 2,6-diaminopimelic acid but never both in the same bacterium. A few additional amino acids such as aspartic acid, glycine, serine and threonine may be present, but only one of these is usually present in appreciable amounts (Rogers and Parkins, 1968). In addition two amino sugars are present. This general pattern can be observed in most of the Gram positive bacteria that have so far been examined, and has led to the conclusion that they are organized into some unique complex polymer to which the term mucopeptide, has been given (Rogers and Perkins, 1968). This forms the backbone of the cell and gives it its rigidity.

(i) Amino Acids

The first observation made on the amino acid composition of walls, was from a Gram negative organism which was found to contain amino acids similar to those encountered in proteins (Weidel, 1950, 1951; Salton and Horne, 1951, and Salton, 1952 a, b), and differed from those of walls from the Gram positive bacterium Micrococcus lysodeikticus, which contained only alanine, glycine, lysine and glutamic acid (Salton, 1953). Subsequently extensive investigations in this field have been carried out by Cummins and Harris (1956a, b, 1958, ; Cummins (1956), Cummins et al. (1957) and Cummins (1962). The amino acid, α , β -diaminopimelic acid, was discovered by Work (1949, 1951), and shown to be a common component of bacterial cell walls. In Corynebacterium diphtheriae this amino acid was almost exclusively present in the cell wall (Holdsworth, 1952). The presence of a high proportion of alanine and glutamic acid in the D-configuration, was first established in the lactic acid bacteria by Ikawa and Snell in 1956 and later these were found in other bacteria (Salton, 1957). Toennies et al. (1959), demonstrated the presence of D-aspartic acid in Streptococcus faecalis.

(ii) Amino sugars

Muramic acid, glucosamine and galactosamine are the three amino sugars that have been isolated from bacterial cell walls. Muramic acid, identified as 3-o-carboxyethyl glucosamine (Strange, 1956), has been found to be the most consistent component of cell walls (Perkins, 1963) and it is only found in bacterial cell walls or wall precursors.

Glucosamine is also widely distributed in bacterial cell walls, but is also found in extracellular capsules and slime (Salton, 1960). Galactosamine is less commonly present but occurs for example in some strains of B. subtilis (Young et al., 1964 ; Young et al., 1963; Young, 1966; and Tempest et al., 1968).

(iii) Lipids

A characteristic of the cell walls of Mycobacterium tuberculosis is their high lipid content which amounts to 61.1%. Gram negative bacteria generally contain about 20% (Kotani et al., 1959). On the other hand, in Gram positive bacteria the lipid content seldom exceeds 1 - 2% (Salton, 1952b, 1953).

The biosynthesis of several bacterial wall polymers utilises lipid intermediates that aid in the transport of sugar residues and related components from intracellular nucleotide precursors through the membrane to the polymer chains in the wall. All these lipid intermediates so far isolated and identified have been shown to be C₅₅ polyisoprenol, undecaprenol. The lipid intermediates, which participate in the synthesis of both o-antigen (Wright et al., 1967) and peptidoglycan (Higashi et al., 1967, 1970), have been identified as the phosphomonoester of a C₅₅ polyisoprenoid alcohol.

Polyisoprenol has been demonstrated to participate in the synthesis of mannan (Scher et al., 1968). Related lipid intermediates participate in the synthesis of the wall teichoic acids of S. lactis (Douglas and Baddiley, 1968, and Brooks and Baddiley, 1969), and of several other bacteria (Stow et al., 1971).

The peptidoglycan carrier lipid in S. aureus is effective in the synthesis of o-antigen of Salmonella (Lennarz, 1970), and in Aerobacter, the carrier lipid for capsular polysaccharide synthesis can function in mannan synthesis in Micrococcus lysodeikticus (Watkinson et al., 1971). This suggested that the lipids may also be chemically identical in these organisms.

Such lipid intermediates are used in the biosynthesis of both teichoic acids and peptidoglycan, which is a polymer containing N-acetylmuramic acid, N-acetylglucosamine, alanine, lysine, glutamic acid and glycine (Hughes et al., 1971). Thus the synthesis of several different polymers by the same cell may proceed through closely related lipid intermediates.

Baddiley and co-workers have further suggested the possibility of a single lipid that may be common to both pathways and that this lipid plays some part in the regulation of cell wall synthesis.

The amount of these lipids in Gram positive bacteria is very small, and their direct study by isolation and purification presents difficulties.

(iv) Phosphorus

The phosphorus content of the cell walls represents a further difference between Gram negative and Gram positive bacteria. Phosphorus is a component of a group of polymers known collectively as teichoic acids. They are generally considered to be of intracellular origin, and form a significant part of the cell wall of Gram positive bacteria.

(v) Cell wall heteropolymers

The amino acid, amino sugar, monosaccharide and phosphorus components of the cell wall are built up to form characteristic heteropolymers, those of some bacteria include peptidoglycan (mucopeptide), teichoic acid and teichuronic acid.

(vi). Peptidoglycan (mucopeptide)

This is the most widespread component of bacterial cell walls, and is responsible for the shape and rigidity of the bacterial cell. It is a complex polymer containing acetamido sugars and a few characteristic amino acids.

By examining the cell walls of over one hundred Gram positive bacteria, Salton (1953) and Cummins and Harris (1956a, b, 1958) demonstrated a similarity in composition especially in the presence of D-alanine, L-alanine, D-glutamic acid and a basic amino acid, usually L-lysine or meso-diaminopimelic acid in molar ratio of roughly 1 : 1 : 1. Similarly the two hexosamines, muramic acid and glucosamine (Salton, 1964) were generally present. Thus a common type of 'basal' unit was recognised (Work, 1957). In addition, the presence of amino acids, amino sugars and monosaccharides superimposed on the 'basal' structure are characteristic in each bacterial genus, or even each species. Hughes et al. (1968), found that autolysis of walls of B. subtilis 168, gave a pure glycan fraction containing 60 - 70% of the total glycan in the wall.

The development in recent years of enzymatic techniques for controlled, systematic degradation of the peptidoglycan, in conjunction with improved methods for isolation and microanalysis of the products, has revitalized

fine structure analysis and comparative biochemistry of the polymer. These results, have considerably clarified, extended and corrected earlier ideas of the structure (Salton, 1964), and although only a limited number of organisms have been studied in detail, a clear concept of the common features and viable aspects of the structure now exists (Ghuysen et al., 1968 and Ghuysen, 1968).

The peptidoglycan was found to have relatively invariant major structural features in most organisms studied so far : A glycan portion consisting of a polymer (Polysaccharide chains) made up of alternating units of N-acetyl-D-glucosamine and N-acetylmuramic acid (3-o-lactyl-N-acetyl-D-glucosamine) in β -1,4 linkage (Leyh-Bouille et al., 1966 ; Jeanloz et al., 1963 and Tipper et al., 1965). This structure was also found in the isomeric disaccharide, MurNAC - GlcNAC (Leyh-Bouille et al., 1966; Tipper and Strominger, 1966 and Browder et al., 1965), and a peptide portion formed of subunits based on the tetrapeptide structure, $H_2NL-Ala-\gamma-D-Glu-L-Lys$ (or meso-DAP)-D-Ala.COOH.

The glycan chain is attached to the peptide subunits by amide linkages between the α -amino groups of L-alanine and the lactylcarboxyl groups of muramic acid residues, and the peptide subunits are at least partially cross linked to each other, the C-terminal D-alanine residue of one unit being linked either directly or through an oligopeptide bridge to the free amino groups of lysine (or DAP) in a second unit.

However, elaborations and variations on this basic structure appear to be more common than not (Osborn, 1969).

The basic peptide subunit in peptidoglycan has a tetrapeptide sequence, L-Ala- γ -D-Glu-L-lys(or DAP)-D-Ala and this has been shown to exist in a wide variety of Gram positive

and Gram negative bacteria (Ghuysen, 1968). So far this sequence terminating with a single D-alanine residue has been found in the majority of peptide subunits from peptidoglycan analysis. Recently an additional substituent was discovered in some organisms on the α -carboxyl of the glutamic residue. In S. aureus Copenhagen, the structure of the peptide subunit was found to be N^2 [L-alanyl-D-isoglutaminyl] -L-lysyl-D-alanine (Muñoz et al., 1966a and Tipper et al., 1967). This isoglutamine structure was also found in other Gram positive bacteria (Tipper, 1968; Muñoz et al., 1966b; Tipper et al., 1967; Ghuysen et al., 1967 and Hungeres, 1968), and in C. diphtheriae (Kato et al., 1968). In the latter the free carboxyl group of the DAP residue is also substituted by an amide group. An additional amide group was found in the peptide of S. faecalis ATcc 9790, in this case it was an isoasparaginyll residue which cross-linked peptide monomer units (Ghuysen et al., 1967). In Lactobacillus casei R094, a similar structure was shown, and a different type of substituent on the D-glutamate residue in Micrococcus lysodeikticus whose peptide contains no amide nitrogen, but a single glycine residue in peptide linkage to the α -carboxyl of glutamate. However, it has also been observed in some organisms that a significant amount of tripeptide lacking D-alanine and of pentapeptide containing D-Alan-D-Ala residues at the carboxyl terminus are present (Petit et al., 1966; Veerkamp et al., 1965; Muñoz et al., 1966b; Tipper and Strominger, 1968; Kato et al., 1968 and Van Heijenoort et al., 1968).

The loss of the D-alanine residue at the carboxyl terminus in some organisms is thought to result during biosynthesis of the peptidoglycan, from the action of endogenous

D-alanine carboxypeptidase activities, and this may represent a chain stopping mechanism in peptide cross-link formation (Kazue and Strominger, 1968). The major site of variability in peptidoglycan structure appears to be the nature of the bridging elements in the cross-linked peptide subunits. From the extensive studies of Ghuyssen, Tipper and Strominger and their co-workers, a structure emerged for S. aureus copenhagen in which cross-linking is accomplished by pentaglycine bridges between the terminal carboxyl group of D-alanine in one tetrapeptide unit and the ϵ - NH₂ of lysine in another. Later results of the investigation on S. epidermidis peptidoglycan were very similar to those of S. aureus except for the occurrence of substantial amounts of serine in non-random positions in the pentapeptide cross bridges (Tipper, 1968).

(vii) Polysaccharides

In addition to peptidoglycan, teichoic acids or other polysaccharides are usually present in the cell walls or are of intracellular origin in Gram-positive bacteria, and are responsible for specific properties of the cell (Baddiley, 1964). They are rarely present in the cell wall of Gram-negative bacteria (Lilley, 1962).

The teichoic acids are polymers of glycerol or ribitol phosphate.

During the study of the teichoic acids several workers have found D-alanine and various carbohydrate substituents which include glucose, glucosamine and galactosamine are attached to the polymer, and that these vary between organisms (Armstrong et al., 1960; Davison and Baddiley, 1963; Hay et al., 1965; Ghuyssen et al., 1965 and Chin et al., 1966).

In recent years evidence has accumulated rapidly, that these wall polysaccharides are covalently linked to peptidoglycan. In one instance the mode of attachment has been definitively established. The atypical teichoic acid of Staphylococcus lactis is linked to muramic acid residues by phosphodiester bonds (Button et al., 1966).

The presence of substantial amounts of muramic acid 6-phosphate in the walls of a variety of organisms, suggests that this type of linkage is of frequent occurrence (Liu and Gotschlich, 1967). The most accepted linkage is a phosphoramidate bond between the terminal phosphate groups of a wall teichoic acid unit and the amino group of the muramic acid of the peptidoglycan. This occurs in several Gram-positive bacteria (Young et al., 1964; Archibald and Baddiley, 1965; Hay et al., 1965; Button et al., 1966; Knox and Hall, 1965; Liu and Gotschlich, 1967; Heymann et al., 1967; Archibald et al., 1968; Ghuyssen et al., 1968 and Ghuyssen, 1968).

An example of a structure which can be regarded as polymers of D-ribitol-5 phosphate (L-ribitol-1-phosphate), were found to be composed of chains in which ribitol phosphate units are joined to each other through phosphodiester linkages. Carbohydrate residues are usually attached to the D-4 (L-2) position, and alanine is in labile ester linkage with a hydroxyl group at position 2 or 3 on ribitol. The size of these polymers is thought to vary from 7 to 15 or so units in length (Baddiley, 1968).

The structural variation in the early studies was thought to be largely confined to the number and nature of the carbohydrate residues. The substituent in many cases is D-glucose, but N-acetyl-D-glucosaminyl substituents are often found. Such polymers have been found in the wall of a

large range of Gram-positive bacteria.

The composition of such teichoic acids is usually fairly constant but variation has been noted in organisms grown in different media. The suggestion that nucleotides are involved in the biosynthesis of teichoic acids was confirmed and extended by demonstrating the transfer of polyolphosphates from nucleotides to teichoic acid in a cell free system. It has been shown that the nucleotide cytidine diphosphate ribitol (CDP-ribitol) is formed by a reversible action between cytidine triphosphate and the ribitol phosphate (Shaw, 1962). Baddiley et al. (1968), demonstrated that in Staphylococcus lactis wall teichoic acid could be synthesized exclusively from the two nucleotides CDP-glycerol and UDP-N-acetylglucosamine.

The enzyme responsible for the transfer of ribitol phosphate residues from the nucleotide to the polymer is located in the protoplast membrane. The mechanism whereby D-alanine is incorporated into teichoic acid is not known, but analogy with other biosynthetic routes suggests that a transfer RNA derivative might be involved.

Sugar residues are attached to the polymer by indirect transfer from the appropriate nucleotide diphosphate sugar after the transfer of polyolphosphate (Glaser, 1964 and Ishimoto and Strominger, 1966).

The sugars are transferred from nucleotides to a phospholipid in the particulate enzyme, thereby producing a lipid intermediate. Although the structure of this lipid intermediate is not known for certain, it contains an N-acetylglucosamine 1-phosphate residue. As a lipid intermediate participates in the biosynthesis, in this respect the synthesis of the teichoic acid shows some similarity to the synthesis of peptidoglycan, lipopolysaccharide

and probably other wall polysaccharides (Baddiley et al., 1968).

Teichoic acids are found associated with the cell wall, the cell membrane and the intervening space in Gram-positive organisms. They are synthesized by stepwise polymerization of the corresponding sugar alcohol - CDP derivatives (Mahler and Cordes, 1971).

Archibald et al. (1973) were able to demonstrate that only one teichoic acid unit was associated with any one glycan chain in the peptidoglycan of the walls of Staphylococcus lactis. The fact that in complexes of teichoic acid - glycan the two components are present in equimolar amounts, suggests a degree of controlled order in their biosynthesis. Watkinson et al. (1971), showed that the synthesis of teichoic acid is coupled with that of peptidoglycan through mutual sharing of the lipid carrier-undecaprenol phosphate. This sharing might ensure attachment of only one teichoic acid molecule to a glycan chain. The actual point of attachment of the teichoic acid molecule on the glycan chain has not been found but Archibald and his colleagues considered that this attachment was unlikely to be random and favoured attachment to the non-reducing terminal end of the glycan.

The actual arrangement of the polymers within the cell wall is not known. Immunochemical studies of various bacilli (Burger, 1966) and an electron microscopical study of B. megaterium (Nermut, 1967), concluded that the teichoic acid is present on the external but not internal surface of the walls. The observed thickness of the wall and of the derived peptidoglycan in most Gram-positive bacteria implies the presence of several layers of

peptidoglycan which may be arranged either parallel or perpendicular to the cell surface. If the peptidoglycan strands are arranged perpendicular to the surface, the teichoic acid could readily form an outer layer. If the peptidoglycan strands are parallel, then the teichoic acid could only exclusively form an outer layer if each glycan strand carried more than one teichoic acid unit. Archibald and his colleagues consider that for S. lactis 13 both systems are consistent with their findings. The latter system would however give a fairly uniform distribution of teichoic acid throughout the wall. Archibald and Baddiley (1966), have suggested that the function of the membrane teichoic acids might be protective. These highly charged polymers might control the approach of ions towards or through the wall membrane or act as a barrier against harmful substances. It is possible that these membrane teichoic acids are essential for survival and so are of reasonably constant general structure. The wall teichoic acids provide additional security and consequently their detailed structure is less important. These suggestions are supported by the observations that membrane teichoic acids have been found in all Gram positive bacteria examined; bacteria containing large amounts of wall teichoic acid are particularly able to withstand high concentrations of salts, in the surrounding medium (Archibald et al., 1961). Teichoic acids may also afford protection to the wall against lytic enzymes. In certain strains of Streptococcus zymogenes a ribitol teichoic acid containing glucose and D-alanine has been shown to act as a specific inhibitor of the lytic agent produced by these strains (Davie and Brock, 1966). The integrity of the D-alanine ester residues was necessary for the

activity of the inhibitor. A further function of wall teichoic acids is associated with their charge. The surface charge of bacteria can probably be attributed in many cases to teichoic acid and the maintenance of repulsive charges may enable cell populations to disperse in the medium and thereby utilize nutrients efficiently (Heptinstall et al., 1970).

C. The effect of environment on cell wall composition

Inhibition of bacterial cell wall synthesis often causes a corresponding intracellular accumulation of Uridine nucleotides that contain N-acetyl muramic acid and some or all of the amino acids found in the peptidoglycan of the wall. This accumulation can be brought about in several ways, as for example, by the addition of certain antibiotics to the growth medium (Park, 1952). The UDP derivatives that Park isolated from S. aureus treated with penicillin were later identified as UDP-N-acetylmuramic acid, UDP-N-acetylmuramyl-L-alanine and UDP-NAMur-L-Ala-D-Glu-L-lys-D-Ala-D-Ala (UDP-NAMur-pentapeptide) (Strominger, 1959 and Strominger and Threnn, 1959).

The striking similarity of the composition of accumulated UDP-NAMur-pentapeptide to that of the peptidoglycan, led to the suggestion that the former might be an intracellular precursor of cell-wall peptidoglycan (Park and Strominger, 1957 and Salton, 1964). Using cell-free enzyme systems this hypothesis was substantiated by the work of Chatterjee and Park (1964) and Meadow et al. (1964).

This work led to the hypothesis that the mechanism of action of penicillin is related to inhibition of a step in biosynthesis of the cell wall (Park and Strominger, 1957). Baddiley et al. (1956), observed that teichoic acid synthesis is inhibited when growth is limited by the presence of antibiotics. Park (1958) and Mandelstam and Rogers (1959), produced evidence that penicillin and other antibiotics which inhibit the

synthesis of cell-wall peptidoglycan would bring about the accumulation of peptidoglycan precursors. Sunkkonen (1961), has demonstrated the accumulation of cytidine diphosphate ribitol in cells inhibited by penicillin.

Penicillin has been shown by Wylie and Johnson (1962), to inhibit incorporation of carbon from C^{14} -labelled sugar in the medium into the walls of E. coli. Synthesis of wall glucosamine, muramic acid and diaminopimelic acid (DAP) was reduced, and as this constitutes a major part of the mucopeptide backbone of the wall, it was concluded that the abnormal structure formed would lack the mechanical strength of the normal wall.

Studies on B. subtilis by Roberts and Johnson (1962), indicated a similar pattern, the main components affected being wall muramic acid and DAP with some reduction in teichoic acid content. Reynolds (1966), suggested that penicillin might inhibit an enzyme that transferred the muramyl-peptide portion of the uridine muramylpentapeptide to an acceptor molecule. Later a study of peptidoglycan synthesis in S. aureus, showed that penicillin at high concentrations had little or no effect on incorporation of either murNAC-pentapeptide or glycine into the lipid intermediates. At 20°C glycine was readily incorporated into the lipid intermediate and the rate was higher still at 37°C. However, at 37°C peptidoglycan synthesis ceased indicating that this utilization step, unlike the formation of the lipid intermediate, is very temperature sensitive (Matsushashi et al., 1967).

Strong evidence has been obtained from studies both in vivo and in vitro that penicillin acts at the level of the final transpeptidation reaction in peptidoglycan synthesis. Wise and Park (1965), found that walls isolated from penicillin-treated S. aureus contained increased amounts of D-alanine and NH_3 -terminal glycine. Tipper and Strominger (1965, 1968), were able to show directly the accumulation of uncross-

linked monomer units containing open pentaglycine bridges and D-alanine residues, providing evidence that incorporation of monomer units into the final cross-linked product is blocked by penicillin.

Araki et al. (1966b) and Izaki et al. (1966, 1968), using E. coli were able to demonstrate inhibition of transpeptidation in vitro. Total incorporation of UDP-MurNAc pentapeptide into polymer was unaffected by penicillin but release of free D-alanine was completely and irreversibly inhibited, the product consisting entirely of uncross-linked monomer units with intact D-alanyl-D-alanine carboxyl termini. Tipper and Strominger (1965), have proposed that penicillin acts as an analogue of the normal substrate to acylate the transpeptidase thereby irreversibly inactivating the enzyme. In addition to the transpeptidase, a penicillin-sensitive D-alanine carboxypeptidase was found which specifically removes the D-alanine residue from the nucleotide sugar (Araki et al., 1966a and Izaki et al., 1968). This inhibition was reversible and competitive with the substrate and the concentration required for inhibition was much lower than the minimal growth inhibitory level. The physiological significance of the carboxypeptidase is not clear, as the product is utilized only poorly for peptidoglycan synthesis in a particulate enzyme system (Izaki et al. 1968).

It may be concluded that the primary action of penicillin on bacteria is to interfere with a step in the formation of peptide chains (Baddiley, 1968)

Describing unpublished work of Archibald, Baddiley (1968), has stated that the number of glycosyl substituents on the wall ribitol teichoic acid depends partly upon the concentration of glucose in the growth medium.

Later it was found that the amount of teichoic acid in walls, in contrast to their general composition, can be markedly influenced by the composition of the medium, and probably by other factors concerned with growth conditions (Ellwood and Tempest, 1967). Bacilli grown in phosphate deficient medium fail to synthesize wall teichoic acid producing instead large amounts of uronic acid residues (Tempest et al., 1968 and Ellwood and Tempest, 1967), although membrane teichoic acid is still produced.

Webb (1949 a, b), was the first to notice that a critical level of magnesium ions is necessary for cellular separation to occur in several species of Gram positive rods. Later it was demonstrated that the UDP-NAMur-pentapeptides of several bacteria including C. xerosis are synthesized by the sequential enzymic addition of L-alanine, D-glutamic acid, L-lysine (or DAP) and D-alanyl-D-alanine to UDP-N-acetylmuramic acid (Ito and Strominger, 1962 a, b ; Comb, 1962 and Ito et al., 1966). All of these reactions are catalysed by soluble enzymes that are activated by magnesium ions or manganese ions.

Baddiley et al. (1968), have found that the enzyme system for the biosynthesis of teichoic acid shows a fairly sharp pH optimum at 8.0 and requires a high concentration of magnesium ions. Ellwood and Tempest (1969), did pioneering work when they described modifications in the anionic polysaccharide content of the walls of *Bacillus* which are dependent on magnesium ions and phosphate.

Garrett (1969 a, b), observed that in B. subtilis strain W₂₃ magnesium ions or manganese ions starvation causes accumulation of bound amino sugars that are soluble in trichloroacetic acid. This accumulation increased in the presence of chloramphenicol or puromycin, but these components were unable to stimulate the accumulation when magnesium ions are present. On the other hand peptidoglycan synthesis was inhibited between 35 and 50% under conditions of magnesium ions starvation.

The amount of N-acetylhexosamine that accumulates is balanced exactly by the associated fall in peptidoglycan synthesis. "Chase" experiments show that the accumulated N-acetylhexosamine compound is utilized in peptidoglycan synthesis. He concluded that the protoplasmic membrane loses some of its structural and functional integrity when the cells are starved of magnesium ions, resulting in membrane damage and the enzymes essential for mucopeptide synthesis could be denied the

conditions required for their maximum activity.

It has been found that Gram positive bacteria contain teichoic acids covalently attached to glycolipids or phosphatidylglycolipids in the membrane (Hay et al., 1963; Wichen and Knox, 1970; Toon et al., 1972 and Coley et al., 1972), and wall teichoic acids or structurally related sugar 1-phosphate polymers attached covalently to the peptidoglycan in the walls (Archibald and Baddiley, 1966 and Baddiley, 1972). These observations together with the results of Ellwood and Tempest (1968), on the effect of the chemical nature of the environment on cell wall composition, in particular cell wall teichoic acid to phosphate and magnesium availability in the medium, suggest that Gram positive bacteria require membrane teichoic acid for their normal cellular activity and that they also require an anionic polymer in their walls preferably a teichoic acid, although in some bacteria other anionic polymers may suffice (Heptinstall et al., 1970).

The presence of membrane teichoic acid between the cell wall and surface of the cytoplasmic membrane could provide an integrated cation exchange system between the exterior of the cell through the wall to the membrane itself. These certain concentrations of magnesium ions are required for membrane stability (Weibull, 1967 and Reavely and Rogers, 1969), and a variety of enzymic reactions, including in species of *Bacillus*, phospholipid synthesis (Patterson and Lennarz, 1971), teichoic acid synthesis (Burger and Glaser, 1964) and peptidoglycan synthesis (Anderson et al., 1972). It was found by Hughes et al. (1971), that a membrane bound enzyme system that required magnesium ions for optimum activity was fully activated by the magnesium ions bound to the endogenous teichoic acid and was insensitive to externally added magnesium ions. A multi-enzyme system is believed to be responsible for complete wall synthesis and although it is not known which individual enzymes require bivalent cations, it is thought likely that all reactions directly involving sugar nucleotides are cation-dependent. In cells of *B. licheniformis*,

Hughes et al. (1973), conclude that the ordered array of anionic wall and membrane teichoic acids provides a constant reservoir of bound bivalent cations with which the membrane preferentially interacts. Further, the anionic polymers in the wall scavenge cations from the medium and maintain a constant environment for the membrane teichoic acid. Thus a function of wall and membrane teichoic acids is to maintain the correct ionic environment for cation-dependent membrane enzymes.

Hughes et al. (1971), showed that teichoic acids bind magnesium ions and make them available to the membrane enzymes involved in teichoic acid synthesis. The biosynthesis of several bacterial wall polymers uses lipid intermediates that aid in the transport of components through the membrane to the polymer chains in the wall. These intermediates are used in the biosynthesis of both teichoic acids and peptidoglycan and are likely to be polyisoprenols (Watkinson et al., 1971).

Baddiley and co-workers have postulated that the same lipid intermediate is used for teichoic acid and peptidoglycan synthesis. Competition would occur for the limited lipid in this case. Addition of peptidoglycan precursors inhibits teichoic acid synthesis. The antibiotics bacitracin and vancomycin inhibit the formation of peptidoglycan and prevent the return of the lipid intermediate to the pool : the antibiotics have no direct effect on teichoic acid synthesis. When peptidoglycan precursors and the antibiotics are added together, the lipid intermediate is trapped and accumulates and a sharp inhibition of teichoic acid synthesis results.

Heptinstall et al. (1970), showed that the cell walls of S. aureus grown in a medium supplemented with a high concentration of sodium chloride, contained less alanine ester residues and bound a substantially greater amount of magnesium ions than those grown in a medium not supplemented with sodium chloride.

Ellwood (1971), showed that cells of B. subtilis var. niger when grown in a phosphate limiting medium containing sodium chloride, contain

both teichoic acid and teichuronic acid in their walls. This was in contrast to earlier results (Ellwood, 1970), where these polymers were found to be mutually exclusive. The amount of teichoic acid increased as the sodium chloride concentration in the medium was increased. Meers and Tempest (1969), showed that isolated cell walls of B. subtilis var. niger, bound increasing amounts of magnesium as the sodium chloride concentration was increased. These results indicate that there is a correlation between magnesium binding capacity and the teichoic acid content of the cell walls (Ellwood, 1971), and it was concluded that the phosphodiester residues of the teichoic acid were affecting the binding of the magnesium to the cell wall (Heptinstall et al., 1970).

D. Effect of the environment on morphology

From studies made on morphology and growth in different culture media, it has been found that the transfer of E. coli and other bacteria from a complex medium to a minimal medium causes an immediate arrest of RNA and protein synthesis (Kjeldgaard et al., 1958 and Rogerson and Freundlich, 1970). The variation in length of cells in a culture, is due to the fact that the mean length of an organism at the moment of division is twice that of the daughter cells at the moment they are formed. Thus there is a steady increase in length between divisions (Collins and Richmond, 1962).

Studies on the effect of thymine starvation and U.V. irradiation on bacteria, and their failure to divide with a consequent tendency to form long filaments, were carried out by Howard et al. (1964), Van de Putte et al. (1963), Adler and Hardigree (1965), Donachie et al. (1968), Donachie (1969), Fisher et al. (1969) and Leighton and Donachie (1970). The general conclusion was that the effect on cell division is probably not a direct one but an indirect effect through temporary inhibition of DNA synthesis, as they found that cell division

recommences as soon as the DNA to mass ratio in the cell is restored to its normal value.

The peptidoglycan part of the wall has been thought to be chiefly responsible for the mechanical strength and morphology of the cells (Rogers et al., 1968). These authors found an increase in the degree of cross-linking in peptidoglycan when certain mutants of B. subtilis were converted from the spherical to the rod form by supplementing the medium with specific amino acids.

Krulwich et al. (1967 a, b), studied the polysaccharides and peptides of the cell wall peptidoglycan in A. crystallopoietes, using lag phase sphere and rod cells. The polysaccharide backbones of the peptidoglycan of the cell wall of the spherical cells were heterogeneous in size and averaged less than 40 hexosamines per chain. In the rod-shaped cells these were more homogeneous in size and averaged 114 to 135 hexosamines per chain indicating a more rigid cell wall than that found in spherical cells. When cell walls from both types of cells were treated with an N-acetylmuramidase the mixture on analysis showed that the pattern of distribution of cross-bridges in glycopeptides of spheres and rods is quite similar. Both types show large amounts of peptide-linked monomer and dimer as well as a considerable amount of highly polymerised glycopeptide. In the rod cells all peptide bridges in the glycopeptide fractions are formed by one L-alanine residue but in the higher oligomers of the sphere glycopeptide glycine was also present in their cross bridges.

Roberts et al. (1968), were able to obtain a particulate enzyme system that catalyses the synthesis of the glycopeptide of the rod-form from the uridine nucleotide substrates and the simultaneous incorporation of L-alanine into the interpeptide bridge. The incorporation of L-alanine requires L-alanine transfer RNA.

Krulwich and Ensign (1968), in an attempt to explain how the specific changes associated with sphere to rod morphogenesis are brought

about, found that the activity of an N-acetylmuramidase within the cell correlates positively with the observed changes in the polysaccharide backbones.

Morphogenesis was induced by the addition of peptone to spherical cells growing in a glucose-minimal salts medium. Very shortly after this addition, autolytic activity decreased and remained low when the cells were growing as rods. When the rod-shaped cells were later fragmented to spherical cells, the cell wall fractions again showed high autolysin activity. The regulation of this activity is as yet unknown.

In *Pneumococcus* the dietary requirement for choline arises through the presence of choline phosphate residues in the ribitol teichoic acid in the cell wall. Ethanolamine can be substituted for choline in the medium and under these conditions ethanolamine residues are incorporated into the wall polymer. The effect of this structural change is not to impair reproduction but to inhibit cell separation and long chains of many hundreds of unseparated cell are formed (Tomasz, 1968).

Although the mechanism has not been established, it is believed that at the time of cell division, changes occur in the behaviour of lytic enzymes required for separation.

In *S. zymogenes* also, D-alanine ester residues in the wall teichoic acid provide resistance to its own extracellular lytic agent (Davie and Brock, 1966).

In both cases small changes to the structure of wall teichoic acids can have large effects on the behaviour of the lytic enzymes. It is possible that the effect results from an alteration in the cation balance, causing changes in the activity of the enzymes, but this has not been confirmed.

E. Anatomical studies

In thin sections of Gram-positive bacteria observed in electronmicroscopy, the protoplasm is seen to be surrounded by a triple-

layered membrane (unit membrane) in close contact with a thick amorphous cell wall. In the vast majority of bacteria, the wall is rather amorphous in appearance, however Archibald et al. (1961), detected the presence of an electron dense layer along the inner as well as the outer surface of isolated walls from Lactobacillus plantarum.

At the surface of Gram positive organisms more complex arrangements have been encountered. Ghosh and Murray (1967), indicate that in Listeria monocytogenes three layers of cell wall are separated by a space from the two layers of the unit-membrane and cross bridges between the cell wall and the outer membrane layer were observed. Nermut and Murray (1967), described an orderly array of 70 Å globules in the wall of B. polymyxa and a similar ordered substructure was found on the cell surface of B. cereus (Eller and Lundgren, 1967) and Clostridium sporogenes (Betz and Zeikus, 1968).

Electron microscopic observations demonstrated that intra-cytoplasmic membranes are found in many Gram positive organisms and are located at the sites in the cell where new cell wall and spores are formed and also near the nucleoplasm and in the cell periphery. These organelles or mesosomes appear in different forms and undergo structural changes during development of the organism (Remsen, 1968).

Reyn et al. (1966), observed large vesiculotubular membranous structures in C. diphtheriae. Hard (1969), reported that with increasing incubation time of C. ovis cultures, there was an increase in cellular polyphosphate granule content and a decrease in mesosomes. In C. fascians many membranous regions were observed in the peripheral cytoplasm (Glauert and Hopwood, 1960). Most of these membranes appear to be invaginations of the plasma membrane.

Braun and Shwarz (1969), in their work on the interaction between peptidoglycan and lipoprotein in E. coli noticed under the electron microscope that cell walls treated with trypsin were seen to be split into two layers. This was coupled with a rapid decrease in the optical

density of the treated material. These findings suggested that the lipoprotein too plays a significant part in stabilizing of cell wall.

Fiil and Branton (1969), used a freeze-etching technique to study the structure of membranes of E. coli cells grown in conditions of magnesium starvation. In normal lag phase cells there can be detected by this method, a net-like arrangement of particles. During magnesium starvation a paracrystalline pattern appeared on the plasma membrane and large areas devoid of particles were seen. Although these changes were reproducibly associated with magnesium starvation no decrease in the magnesium content of the cell envelope was detected.

These structural changes appeared to involve specific and permanent alterations in membrane development and the authors suggested that the magnesium deprivation effect on the membrane was an indirect effect via other perturbances in cell metabolism.

Highton and Hobbs (1971), used the electron microscope to study structural changes in B. licheniformis brought about by penicillin. In one strain of B. licheniformis studied, it was found that after penicillin treatment, the appearance of the wall suggested accumulation of wall material at the plasma membrane and that the existing wall stretched to accommodate the increase in cell mass. This is consistent with inhibition of transpeptidase preventing cross-linking of polymers to form the normal wall structure (Izaki et al., 1966).

An accumulation of material around developing cross-walls has been observed in B. megaterium (Fitz-James and Hancock, 1965) and this was interpreted by Strominger (1967), as unlinked mucopeptide.

Also thin walls produced by penicillin treatment and considered to have resulted from stretching have been seen in S. aureus (Murray et al., 1959), although it was suggested by Perkins (1963), that they may have resulted from the removal of material by autolytic enzymes, mucopeptidases. It also seems possible that such enzymes could have simply 'loosened' the structure allowing stretching.

Penicillin has been shown to inhibit the transpeptidation reaction which forms links between peptide side chains on different sugar chains (Tipper and Strominger, 1965, 1968 and Wise and Park, 1965). In S. aureus this linkage is via a penta-glycine cross bridge and in E. coli the linkage is directly between alanine on one chain and DAP on the other.

F. The growth and cell wall composition of Corynebacteria with reference to C. fascians

Corynebacterium fascians is a characteristically slow growing rod shaped organism, frequently showing granular or uneven staining, with pleomorphic forms common in older cultures. Morphology and staining characteristics vary with the strain and are influenced by the composition of the medium (Lacey, 1936_{a, b}, 1955), and the age of the culture (Mohanty, 1947).

Environmental conditions and nutrient supply, influence growth and division as has been reported by Mohanty (1951) and Ahmed (1959). The composition of the cell wall of Corynebacteria, as determined by Cummins and Harris (1956_a), is shown in table 1 (Cummins, 1962). Both C. betae and C. poinsettiae, were found to have rhamnose as a cell-wall sugar, and alanine, glutamic acid, lysine and glycine as principle amino acids, a combination of sugars and amino acids which has not been noted before in other organisms.

The three strains of C. fascians are the only plant pathogens whose walls have arabinose and galactose as major components, a pattern found in C. diphtheriae. Both amino acids, alanine and glutamic acid, and amino sugars, muramic acid and glucosamine, are present in the cell walls of Corynebacterium. Galactosamine is present only in the cell walls of C. pyogenes and C. hofmannii L.H. Perkins (1965), demonstrated that the cell wall peptidoglycan of C. poinsettiae (N.C.P.P. 177) contains approximately equimolecular proportions of glutamic acid, glycine, alanine, homoserine and ornithine.

Table 1. Composition of the cell walls of Corynebacteria (C.S. Cummins J.gen. Microbiol. (1962) 28 35 - 50)

Name of strain	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Unidentified methyl pentose	Glucosamine	Galactosamine	Iuramic acid	Aspartic acid	Alanine	Glutamic acid	Lysine	Glycine	Serine	Isomers	α -E-D.A.P.	
																	DL	LL
<i>C. diphtheriae</i> mitis 2764	+++	---	++	---	+	---	+	---	+		++	++				(++)		
<i>C. diphtheriae</i> gravis XY	+++	---	++	---	+	---	+	---	+		++	++				(++)		
<i>C. ulcerans</i> NCTC 7910	+++	---	+++	---	++	---	+	---	+		++	++				(++)		
<i>C. ovis</i> NCTC 3450	+++	---	++	+	++	---	+	---	+		++	++				(++)		
<i>C. xerosis</i> NCTC 9755	+++	---	++	+	++	---	+	---	+		++	++				(++)		
<i>C. renale</i> NCTC 7448	+++	---	++	+	tr.	---	+	---	+		++	++				(++)		
<i>C. equi</i> NCTC 1621	+++	---	+++	---	tr.	---	+	---	+		++	++				(++)		
<i>C. hofmannii</i> L.H.	+++	---	++	+	tr.	---	+	+	+		++	++				(++)		
<i>C. fascians</i> NCPP 156	+++	---	++	---	++	---	+	---	+		++	++				(++)		
<i>C. fascians</i> NCPP 188	+++	---	++	---	---	---	+	---	+		++	++				(++)		
<i>C. fascians</i> NCPP 467	+++	---	++	---	---	---	+	---	+		++	++				(++)		
<i>C. tritici</i> NCPP 471 *	---	---	---	+	+	---	+	---	+		++	++		++		(++)		
<i>C. betae</i> NCPP 373	---	+	---	---	---	+	+	---	+		++	++	++	++		(++)		
<i>C. poinsettiae</i> NCPP 177	---	++	+++	---	+	---	+	---	+		++	++	++	++		(++)		
<i>C. insidiosum</i> NCPP 308	---	---	---	+	---	---	+	---	+		++	++	++			(++)		
<i>C. flaccumfasciens</i> var. auranticum NCPP 558	---	---	---	+	+	---	+	---	+		++	++	++	++		(++)		
<i>C. simplex</i> ATCC 8373	---	---	++	+	+	---	+	tr.	+		++	++		++		(++)		++
<i>C. pyogenes</i> Wye 1	---	++	---	++	tr.	---	+	+	+		++	++	++			(++)		
<i>C. pyogenes</i> Wye 2	---	++	---	+	+	---	+	+	+		++	++	++			(++)		
<i>C. pyogenes</i> Wye 3	---	++	---	+	+	---	+	+	+		++	++	++			(++)		
<i>C. pyogenes</i> 5224	---	++	---	+	+	---	+	+	+		++	++	++			(++)		

* ninhydrin positive spot tentatively identified as β -alanine.

tr. = trace.

Results for DAP in brackets indicate that the acid is present and is assumed to be the isomer indicated.

SCOPE OF THE THESIS

Lacey (1955), observed that in certain media, C. fascians grows as long or even filamentous cells and it was decided to study the growth of this organism in different defined media, in an attempt to relate morphological form with specific nutrient factors. Following a general study, one or two specific nutrient factors were selected for further quantitative studies. This forms the first part of the work reported in this thesis.

In the second part, an attempt has been made to determine the influence of specific nutrient factors on the composition of the cell wall of C. fascians and to relate this to changes in cell morphology.

II

MATERIALS AND METHODS

A. Origin and Maintenance of cultures

The bacterial cultures of C. fascians were obtained from the laboratory collection. In the majority of experiments, a single strain, designated strain 505, was used, although in some early observations, other strains were included.

Initially stock cultures were grown on nutrient agar and incubated at 25°C for 48 hours. A smooth type colony was isolated and this cultured for further experimental work. Cultures were stored at 4°C in a refrigerator and subcultured every 4 weeks.

B. Media

A list of media used with their constituents and method of preparation is given below.

(a) Thornton's medium (T) (Thornton, 1922)

Constituents	: (g/litre distilled water)
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.1
NaCl	0.1
$FeCl_3 \cdot 6H_2O$	0.002
KNO_3	0.5
L- Asparagine	0.5
Mannitol	1.0

The potassium phosphate, potassium nitrate and asparagine were dissolved in approximately 700 ml of distilled water, and the $MgSO_4$, $CaCl_2$, NaCl and $FeCl_3$ added from stock solutions in the

order given. The medium was then heated in a water bath at 90°C for 10 minutes, allowed to cool to 70°C and filtered through a Whatmann No. 41 filter paper. The volume was made up to one litre with distilled water, the mannitol added and the pH adjusted to 7.4, before dispensing 200 ml into each 2 litre conical flask. After plugging, the medium was sterilized at 15 lbs. pressure for 15 minutes.

Where a solid medium was required 2% "Oxoid" No. 3 agar was added and dissolved by steaming before dispensing the medium.

(b) Synthetic medium (S) (Mohanty, 1947)

Constituents : (g/litre of distilled water)

K_2HPO_4	0.5
KH_2PO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
$FeCl_3 \cdot 6H_2O$	0.001
NH_4Cl	2.0
Sucrose	1.0
Thiamine	0.001

The potassium phosphate, thiamine and ammonium chloride were dissolved in 900 ml of distilled water, and the $MgSO_4$, NaCl and $FeCl_3$ added from stock solutions in the order given.

The volume was made up to 995 ml, and the pH adjusted to 7.0, before dispensing 199 ml into each 2 litre conical flask. After plugging, the medium was sterilized at 15 lbs. pressure for 15 minutes. The sucrose was added aseptically after sterilization as 1 ml of a stock solution previously sterilized at 10 lbs. pressure for 20 minutes.

Where a solid medium was required, 2% "Oxoid" agar No. 3 was added and dissolved by steaming before dispensing the medium.

The above media have been modified in various experiments as follows :-

Medium T_1 = as for Thornton's medium, but with asparagine omitted.

Medium T_2 = as for Thornton's medium, but with $MgSO_4 \cdot 7H_2O$ omitted.

Medium T_3 = as for Thornton's medium, but with NaCl omitted.

Medium C = as for Thornton's medium, but with asparagine, $MgSO_4 \cdot 7H_2O$ and NaCl replaced by thiamine, nicotinic acid, biotin and p-aminobenzoic acid each at a concentration of 1 mg/ml.

The addition of these components were made aseptically after sterilization using filter sterilized solutions.

Medium D = as for Thornton's medium, but with the omission of asparagine, $MgSO_4 \cdot 7H_2O$ and NaCl and the addition of thiamine, nicotinic acid and p-aminobenzoic acid only.

Medium S_2 = as for Synthetic medium (S), but with $MgSO_4 \cdot 7H_2O$ omitted.

Medium S_3 = as for Synthetic medium (S), but with NaCl omitted.

Medium S_4 = as for Synthetic medium (S), but with NH_4Cl omitted.

Amino Acid media = as for Synthetic medium (S), but with amino acids added one at a time at a concentration of 0.001% of the L-isomer.

Whenever 0.1% of "Difco" yeast extract was added to any one of the above media, the letter Y was added to denote this, e.g.

TY_1 is medium T_1 plus 0.1% "Difco" yeast extract.

(c) Rhizobium medium (R)

Constituents : (g/litre distilled water)

K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.2
$MnSO_4$	trace
$FeCl_3 \cdot 6H_2O$	trace
KNO_3	0.5
$CaCO_3$	3.0
Sucrose	10.0
Yeastral	1.0
"Oxoid" Agar No. 3.	10.0

This was prepared by dissolving the constituents in 990 ml distilled water in the order given. The medium was sterilized at 15 lbs. pressure for 15 minutes and cooled to 50°C. The sucrose was then added, aseptically, as 10 ml of a stock solution previously sterilized at 10 lbs. pressure for 20 minutes. The medium was then poured into petri dishes.

(d) Nutrient broth (N.B.)

This was prepared from "Oxoid" dehydrated nutrient broth No. 2 according to the manufacturer's instructions.

(e) Penicillin nutrient agar (P)

Constituents :

"Oxoid" Nutrient broth No. 2.	25.0 g
"Oxoid" Agar No. 3.	20.0 g
Benzyl Penicillin (Sodium) B.P.	10.0 µg
Distilled water	1 litre

Using a sterile disposable 1 ml syringe, 1 ml of sterile distilled water was added to a vial containing 1 g of penicillin and the vial was shaken to dissolve the contents. From a serial dilution of this the appropriate quantity of solution was added aseptically to the sterile nutrient agar medium at 40°C, to give a final penicillin concentration of 10.0 µg/litre. 200 ml aliquots of this were then measured into sterile penicillin flasks and allowed to solidify.

(f) Penicillin nutrient broth

This was prepared from "Oxoid" nutrient broth No. 2 by the addition of benzyl penicillin after sterilization of the nutrient broth by autoclaving at 15 lbs for 15 minutes, it was allowed to cool to room temperature and stock penicillin solution (1 g/ml) was added aseptically to give the desired concentrations.

(g) Penicillin Synthetic Medium

This was prepared from synthetic medium (medium S) by the addition of the desired concentrations of penicillin.

C. Buffers

(a) Citrate buffer pH 2.2

Constituents :

Sodium citrate 2H ₂ O	19.6 g
Conc. HCl	16.5 ml.
Thiodiglycol (TG)	5.0 ml.
n-Caprylic acid	0.1 ml.
Distilled water to	1 litre

(b) 0.1 M Phosphate buffer pH 7.6

Two stock solutions were prepared. A 0.2 M solution of di-sodium hydrogen orthophosphate was prepared by dissolving 71.64 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1 litre of distilled water. And a 0.2 M solution of sodium dihydrogen orthophosphate was prepared by dissolving 31.21 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 1 litre of distilled water.

To prepare the buffer, 87 ml of the stock solution of Na_2HPO_4 was added to 13 ml of the stock solution of NaH_2PO_4 and the volume made up to 400 ml with distilled water.

(c) 0.1 M Phosphate buffer pH 7.0

39 ml of the stock solution of NaH_2PO_4 prepared as given above was added to 61 ml of the stock solution of Na_2HPO_4 and the volume made up to 400 ml with distilled water.

D. Combined staining of Nucleus and Cell Wall

A dilute suspension of the bacteria was smeared onto a slide and allowed to dry. The cells were then fixed in osmic acid vapour for 20 seconds, and stained as follows :-

Dehydrate with methanol for 2 minutes, followed by immersion in 60% HCl for 8 minutes. Wash with distilled water, then stain with Giemsa (dil c. $\frac{1}{100}$) for 2 - 3 minutes. Wash with distilled water, blot dry and examine. The field examined under the microscope was marked on the underside of the slide with indian ink. The number of nuclei per rod was recorded and the staining procedure then continued.

Decolourise completely with acid-alcohol for 6 minutes, wash with distilled water, then cover the slide with 5 - 10% tannic acid for 6 minutes. Stain with 0.2% aqueous crystal violet for 1 minute, wash and blot dry. The same cells in the marked area were examined for the number of unit cells (a single cell, a number of which may join together to form a rod or filament) per rod, number of septa per rod, rod length

and other morphological features.

E. Preparation of Cell Walls

Culture and harvesting of bacteria

The organism was grown on nutrient agar for 48 hours at 25°C and a standard inoculum containing 8×10^7 /ml taken from this culture, was used to inoculate each 2 litre flask containing 200 ml of the appropriate medium. Normally 20 such flasks were set up for each medium to provide sufficient material for analysis. The flasks were incubated for 48 hours at 25°C and at the end of this period the cells were harvested by centrifugation at $4000 \times g$ for 15 minutes. After washing twice with distilled water, the bacteria were resuspended in a known volume of distilled water and 0.1 ml of the suspension taken and diluted $1/10$ with distilled water. 1 ml of this dilution was taken for a whole cell dry weight estimation (as described later). A further 1 ml was taken and diluted $1/10$ and used for a direct count of number of cells.

(a) Disruption

The volume of the remainder of the original suspension was measured. This was then transferred to 2 glass 'Mickel' cups, mixed with an equal volume of cold ballotini and the cups were carefully balanced, placed in the Mickel Tissue Disintegrator. The cups were cooled before shaking and at intervals during the procedure.

Breaking was continued until 99% of the cells were broken, as determined by microscopic examination under phase contrast. The time needed for this to occur depended on the concentration of the bacterial suspension and the rigidity of the cell wall which appears to vary with the medium in which the bacteria are grown.

(b) Purification

After breakage, the suspension of broken cells was decanted off and remaining cell material removed from the ballotini by washing it with several aliquots of distilled water, these washings being added to the broken cell suspension.

Whole cells and any ballotini were removed by centrifugation at 2000 x g for 5 minutes, the supernatant was decanted off into a clean centrifuge tube and the cell wall material deposited by centrifugation at 20,000 x g for 1 hour.

The sediment was washed with M. NaCl, distilled water; and 0.1 M phosphate buffer pH 7.0 in that order, the cell walls being sedimented after each washing by centrifugation as given above. Then the crude wall material was digested with trypsin and ribonuclease (0.5 µg/ml per 10 µg cell wall) in 0.1 M phosphate buffer pH 7.6 for 16 hours at 37°C after which the suspension was centrifuged at 2000 x g for 5 minutes to remove debris. The supernatant was decanted off and centrifuged at 20,000 x g for 1 hour to sediment the cell walls. After washing once with distilled water, the cell walls were treated with pepsin (1 µg/ml per 10 µg cell wall in 0.02 N HCl) for 18 hours at 37°C. Centrifugation at 2000 x g for 5 minutes, was then carried out to remove debris and the purified cell walls sedimented as previously described. A final washing in distilled water completed the purification.

The cell walls were transferred to a clean dry weighed pyrex test tube, frozen by dipping the tube in an acetone-solid CO₂ mixture and dried over phosphorous pentoxide in vacuo.

It was noticed that following harvesting the whole procedure of breaking and purification of the cell wall fraction had to be carried out continuously without interruption until treatment with the first proteolytic enzyme at which point the cells were left overnight. The procedure was continued with the second enzyme

treatment and from then continued without interruption to the end of the procedure when the purified cell walls were frozen and dried in vacuo over phosphorous pentoxide.

If this procedure was interrupted immediately after harvesting, the cells stored frozen overnight, and then thawed, a change in cell form was observed. Further, if the procedure was interrupted at any stage following breaking and the material stored frozen, when it was thawed, a granular precipitate was obtained which was almost impossible to restore to a homogenous suspension. This was useless for the remaining stages of the purification procedure, as most of the cell wall material was lost with the debris during centrifugation.

The problem of cell wall isolation and its contamination by cytoplasmic proteins, nucleic acids and membrane material was overcome by the use of enzymes in the cell wall purification procedure. This practice has been widely adopted (Salton, 1964).

(c) Estimation of purity

During the purification procedure, the purity of the wall material was evaluated spectrophotometrically. The presence of contaminating nucleic acid was determined by measuring the absorption of wall material over the range 220 m μ to 300 m μ . The presence of a peak at 260 m μ indicates contamination by nucleic acid (Salton, 1964). The detection of contaminating membrane material was similarly determined by measuring the absorption of a suspension of wall material over the range 360 m μ to 440 m μ . If cytochrome was present, a peak at 410 m μ would be obtained. This method was similar to that used by Keeler et al. (1966), and assumes that cytochrome is associated with the cell membrane.

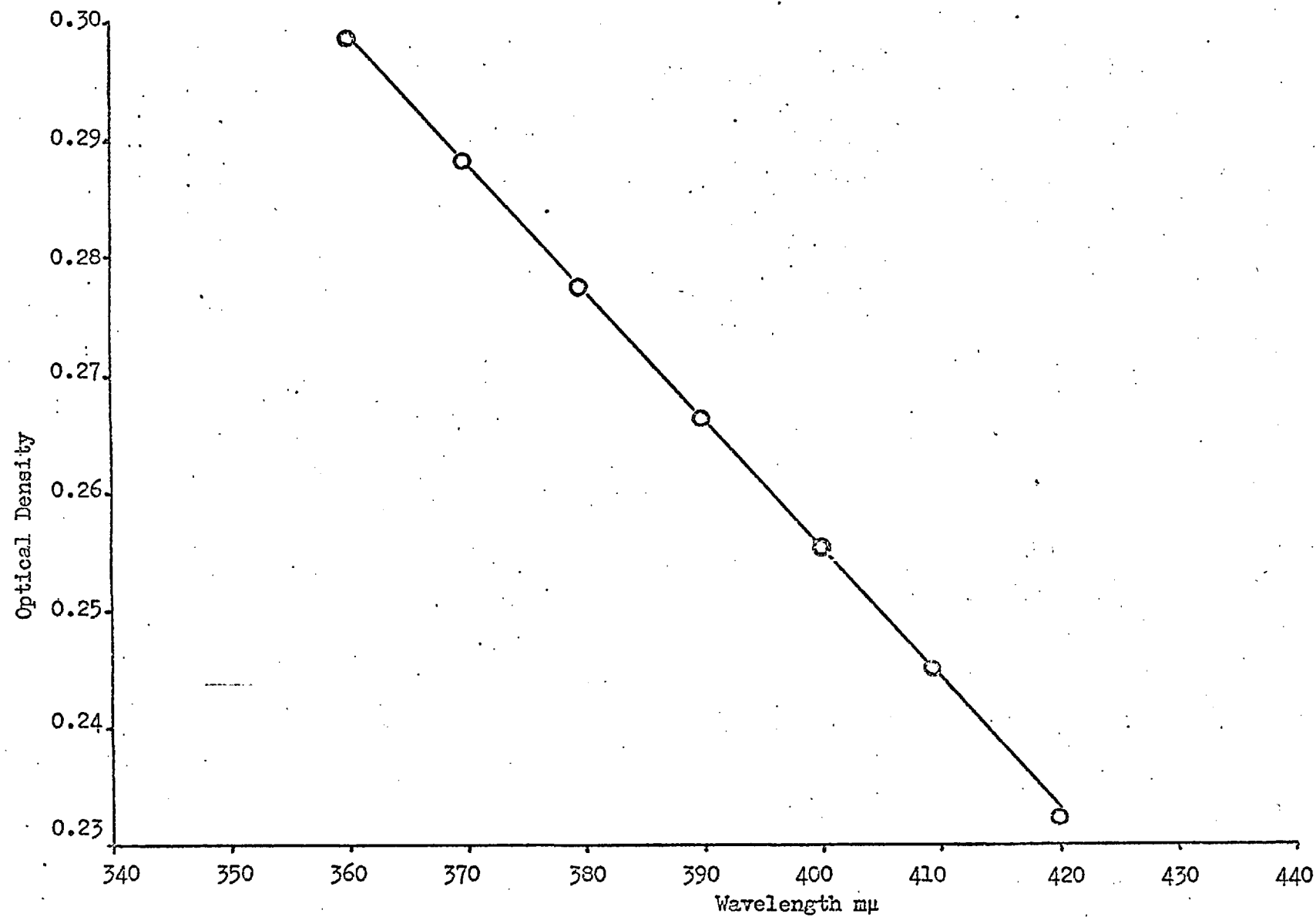


Fig. 1. Purity of cell wall sample.

(d) Estimation of whole cell dry weight

This was determined on 1 ml samples taken from a suspension whose concentration was determined by a direct count. The samples were dried at 100°C for 48 hours and then allowed to cool in a dessicator over calcium chloride and silica gel to constant weight.

(e) Estimation of cell wall dry weight

A known volume of cell suspension, whose whole cell dry weight had been determined, was broken. The cell wall fraction obtained was purified and dried as previously described. After removal from the vacuum, the wall material was placed in a dessicator containing anhydrous calcium chloride and silica gel, and weighed at intervals until a constant weight was obtained. The cell wall dry weight was expressed as a percentage of the dry weight of the whole cell, i.e. $\frac{\text{Cell wall dry weight}}{\text{Whole cell dry weight}} \times 100$

F. Identification of cell wall components

(a) Amino acids

A known weight (about 5 mg) of purified dried wall material was hydrolysed in 4 ml 6N HCl for 16 hours at 105°C in vacuo, and then dried in a vacuum over phosphorus pentoxide and sodium hydroxide pellets. The material was then extracted with acetone containing 5% 6N HCl and applied to Whatmans chromatography paper (12 in x 12 in) as a spot 2 in. from one corner.

Separation was achieved by two dimensional chromatography. In the first phase, n-butanol/acetic acid/water (120:30:50) was used and run for 16 hours. The paper was then dried for 2 hours at room temperature and subjected to chromatography using phenol/ammonia (200:1) at right angles to the first phase. This was run for 12 hours. The paper was sprayed with acetone containing 0.05%

ninhydrin and then heated for 5 minutes at 100°C.

The position of the spots containing the amino acids were identified using the system of R_f values (Smith, 1960). The identity of the amino acids was checked against a marker which was run parallel with the samples.

(b) Amino sugar

5 mg of cell wall material was hydrolysed in 2.5 ml 4N HCl for 4 hours at 100°C (Forrester and Wicken, 1966). Then a chromatographic procedure was followed similar to that described for amino acids. Using the method described by Smith (1960), the amino sugars were located by dipping the chromatogram through a solution containing :

1 volume of a solution prepared by dissolving
5 g of potassium hydroxide in 20 ml of distilled water,
then adding 80 ml of absolute ethanol

and

10 volumes of a solution prepared by adding
1 ml of acetylacetone to 99 ml of absolute ethanol.

Following this the chromatogram was heated for 5 minutes at 110°C and finally immersed in a second solution which contained :

1 volume of 10% p-dimethylamino-benzaldehyde in concentrated
HCl.

and

9 volumes of absolute ethanol.

Two purple spots were obtained, one of which corresponded to muramic acid (R_f value 32) and the other to glucosamine and/or galactosamine (R_f value 20).

For more accurate identification of this second amino sugar, the spot was cut out and eluted with water. Then the amino sugar was chromatogrammed in ethylacetate/pyridine/water (120:50:40) as described by Smith (1960). This solvent system produces clearly different R_f values for galactosamine and glucosamine i.e. 38 and 52 respectively.

A standard for glucosamine and galactosamine was run parallel with the unknown sample.

(c) Teichoic acid

To analyse the composition of teichoic acid polymer, it was necessary to separate it from the glycosaminopeptide.

Following the method described by Janczura et al. (1961), 5 mg of cell wall material was extracted with 3 ml. of 10% trichloroacetic acid for 3 days at 0°C.

Separation was achieved by centrifugation at 4000 x g for 30 minutes when the teichoic acid was located in the supernatant fraction. This fraction contained 95% of the total cell wall phosphorus with small amounts of alanine, glutamic acid and lysine.

Teichoic acid was precipitated from the supernatant with 3 volumes of absolute ethanol at 0°C for 24 hours. The precipitate was then washed 8 times with ether.

Using the method described by Baddiley et al. (1958), about 2 mg of teichoic acid was hydrolysed in 2N HCl for 3 hours at 100°C in vacuo.

The hydrolysate was then subjected to chromatography similar to that for amino sugars, except that the solvents used in this case were propanol/ammonia/water (6:3:1) as used by Armstrong et al. (1960), to determine if ribitol or glycerol was a component. R_f values for glycerol, ribitol and glucose

in this system are 75%, 65% and 61% respectively.

A duplicate sample was run using the second solvent, butanol/ethanol/water/ammonia (40:10:48:1), where the corresponding R_f values are 1.47, 1.00 and 0.65. By using this second solvent greater separation, particularly of ribitol and glucose, was obtained.

Duplicate samples were set up to allow two different detection methods to be used for each solvent system.

Glucose was detected on the chromatogram by dipping the paper into a mixture of 1 volume of 85% phosphoric acid and 10 volumes of (1% aniline plus 1% diphenylamine in acetone).

The chromatogram was then heated at 100°C for 5 minutes. A blue colour is produced by glucose.

For the detection of ribitol using the method described by Buchanan et al. (1950), the chromatogram was first lightly sprayed with 2% sodium metaperiodate and oxidized for 7 minutes at 60°C under nitrogen. It was then placed in an atmosphere of sulphur dioxide. Finally the chromatogram was sprayed with Schiff's reagent (1 g rosoline in 50 ml of distilled water) and heated at 60°C for 10 minutes.

G. Quantitative analysis of cell wall compounds

(a) Amino acids

A known weight of dry cell wall material was weighed into a pyrex test tube, 0.5 ml of concentrated HCl (Analar) was added followed by 0.5 ml of distilled water. With an oxygen-gas flame, the neck of the tube was worked at a point about 1.5 cm. from the end in such a way as to draw it down to a capillary. During the entire operation, care was taken that the contents did not come into contact with the hot regions of the glass.

The tube was sealed off under vacuum with a pin-point oxygen flame and the sealed evacuated tube was placed vertically in a drying oven regulated at 110°C for 24 hours. After cooling the tube to room temperature it was opened to allow estimation of amino acids.

The hydrolysate was centrifuged to remove a visible precipitate and the supernatant passed through a membrane filter (supported in a Hemming's filter holder with 5 ml bijou bottles on either side) and placed in a centrifuge at $8000 \times g$ for 30 minutes to induce passage through the filter.

Water and HCl were removed by placing in a dessicator in vacuo over NaOH pellets and phosphorus pentoxide. Then the dried pellet so obtained was dissolved in 2.5 ml of citrate buffer pH 2.2 and this was used for analysis.

Samples were analysed in a Beckman Unichrom amino acid analyser using 1.0 ml of hydrolysate per column.

(b) Amino sugars

Chromatography was found to be a more convenient method in this work for the estimation of glucosamine and muramic acid. When the analyser was used for the estimation of these two substances, difficulties were encountered due to the superimposition of the peaks of muramic acid and one of the amino acids, and the practical difficulties of estimating the peak height of glucosamine from the resulting broad based peak obtained on the trace. The cell wall material was hydrolysed as for amino acids except 4N HCl was used instead of 6N HCl and the heating was for 4 hours only.

The hydrolysed cell wall material after it had been dried and dissolved in acetone containing 5% 6N HCl, was applied to the chromatography paper as a 3 in. band, 2 in. from one edge

of a 12 in. x 12 in. sheet of Whatman 3MM chromatography paper.

Using a solvent system composed of n-butanol/acetic acid/water (3:1:1) as described by Ingram and Salton (1957), the chromatogram was run for approximately 16 hours, the paper then being dried for two hours at room temperature. The position of the amino sugars were detected using the method described by Smith (1960), and their levels estimated according to the method of Rondle and Morgan (1955).

The identity of muramic acid was confirmed by observing that the colour formed varied with time of standing. When measured at 505 m μ , the intensity of colour approximately doubled during a 24 hour period. This specific observation for muramic acid has been demonstrated by Crumpton (1959). Using Rondle and Morgan's method (1955), the paper containing the amino sugar was chopped into pieces and placed in test tubes. One ml of acetyl acetone solution was added, followed by 1 ml of the freshly prepared reagent (1 ml of concentrated acetylacetone plus 50 ml of 0.5 N sodium carbonate) and 2 ml of distilled water. The tubes were sealed and then heated in a water bath for 20 minutes at 100°C. After cooling quickly to room temperature 5 ml of absolute ethanol were added followed by 1 ml of Ehrlich's reagent (prepared by dissolving 0.8 g of p-dimethyl amino benzaldehyde in 30 ml of absolute ethanol and adding 30 ml concentrated HCl). This reagent was stored at -15°C when not in use.

Finally, 4 ml of absolute ethanol were added to produce a total fluid volume of 13 ml. The contents were then mixed and heated at 65°C for 10 minutes. After cooling to room temperature, the colour intensity was measured in a Uvispek at 530 m μ with a 2 cm light path and the intensity compared with that obtained using known amounts of glucosamine (Figure 2). At 530 m μ muramic

acid produces 27% of that colour intensity given by an equal weight of glucosamine (Crumpton, 1959), and hence the muramic acid quantity could be estimated.

(c) Teichoic acid

Following the method used by Chen et al. (1956), teichoic acid was estimated as cell wall phosphorus. The cell wall material was placed in a pyrex test tube 6 in x $\frac{5}{8}$ in, graduated to 25 ml. Four drops of concentrated sulphuric acid were added and the tube heated until complete ashing had occurred. This was decolourized by a further heating with 2 drops of 72% perchloric acid. The volume was then made up to 25 ml with distilled water, of which 4 ml were mixed with an equal volume of the test reagent for the estimation of phosphorus. The test reagent consists of :

- 1 volume of 6 N sulphuric acid,
- 2 volumes of distilled water,
- 1 volume of 2.5% (w/v) solution of ammonium molybdate
in distilled water

and

- 1 volume of 10% (w/v) solution of ascorbic acid in
distilled water.

The tubes were sealed with parafilm, incubated at 37°C for 90 minutes and then allowed to cool for a few minutes to attain room temperature. The intensity of the blue colour that developed was measured in a Uvispek at 820 mμ with a 1 cm light path. The intensity was compared with that obtained with known quantities of phosphorus run parallel with the unknown sample.

A blank consisting of 4ml of distilled water plus 4 ml of the test reagent was included in each determination (Figure 2).

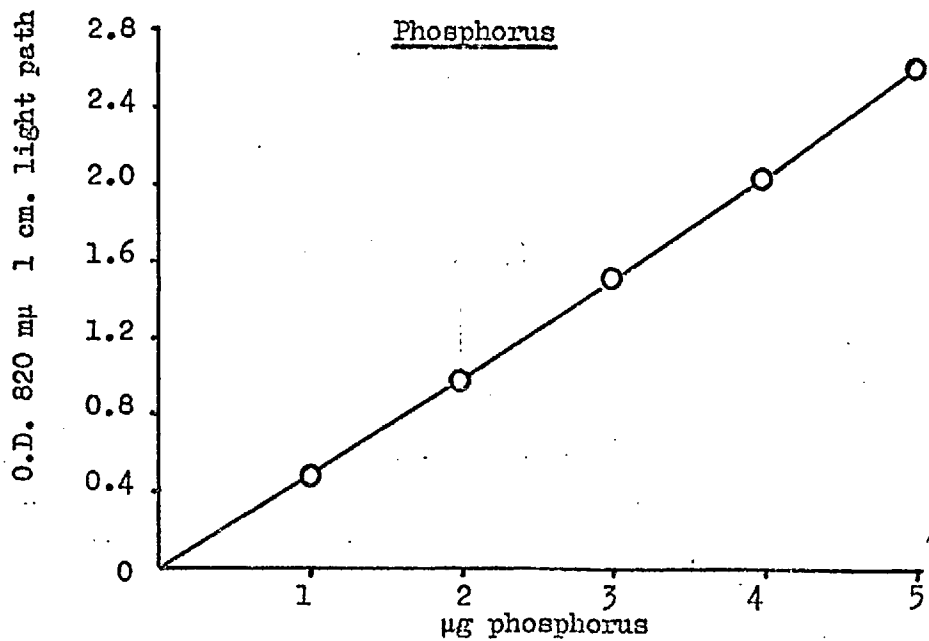
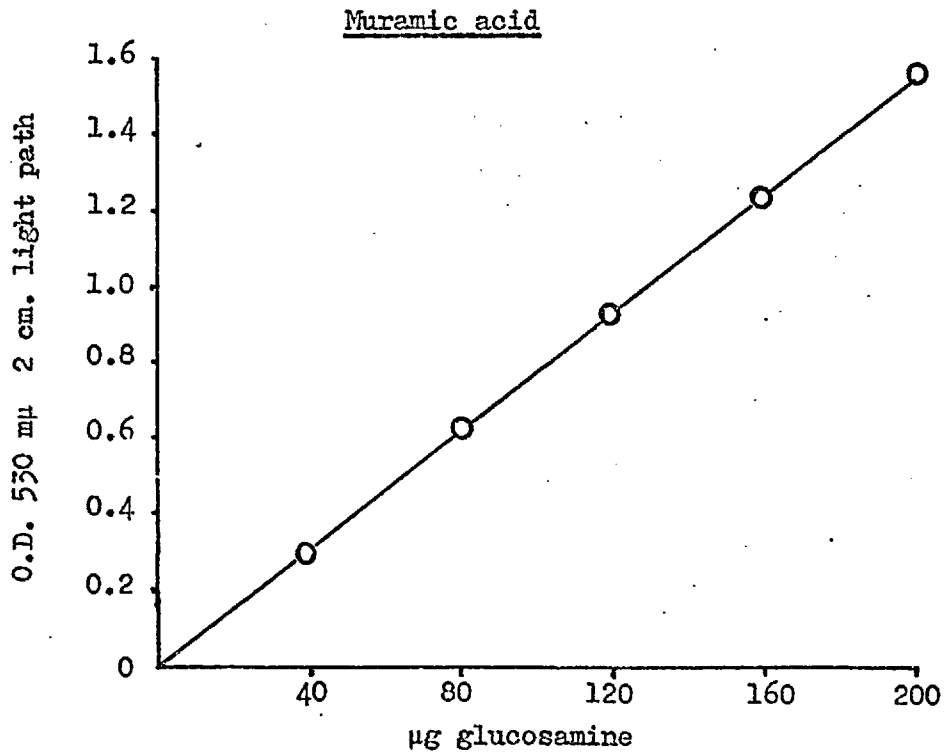
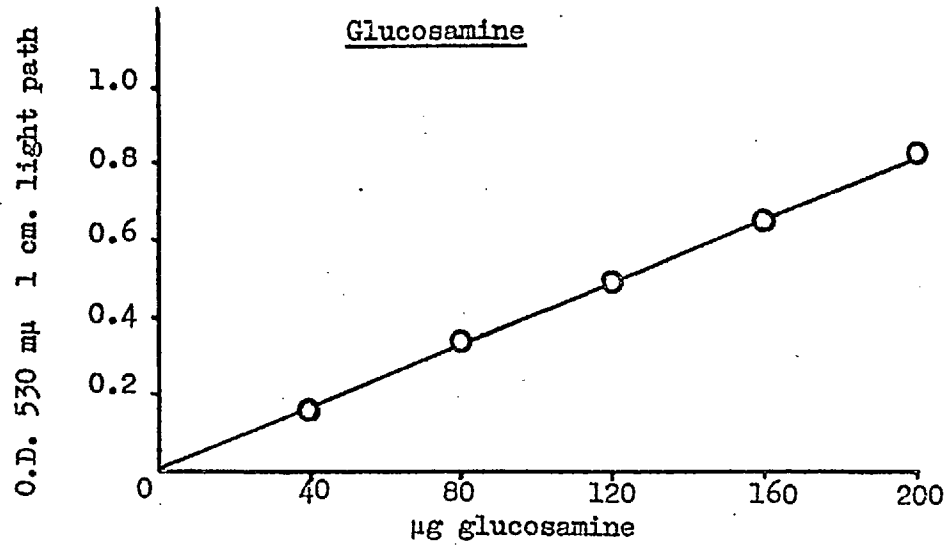


Fig. 2. Calibration curves for glucosamine, muramic acid and phosphorus.

III.

EXPERIMENTS AND RESULTS

A. Morphology and growth

General observations

The morphology of bacteria has most frequently been studied using cultures grown in nutrient broth or nutrient agar and this practice was followed with C. fascians by Tilford (1936), Lacey (1936, a, b) and Mohanty (1951), who established that the organism is a slow growing one which shows a typical 'coryneform' morphology, with a certain degree of irregularity of form, uneven staining by Gram's method, and a marked tendency for the cell to be arranged in X, Y and V and palisade arrangements. Variations between strains exists, and although the cells are generally typical rods, a tendency to form filaments was observed by Lacey (1955) and confirmed by Ahmed (1959), although the latter reported on old cultures.

In this first part, a study has been made of the effect of the environment on the morphology of the cells. The environmental factors studied were temperature, ultra-violet irradiation, penicillin, nutrients and aeration, and particular attention has been given to the appearance of filamentous cells.

The inoculum used which is referred to as a standard inoculum throughout this work contained 8×10^7 bacteria per ml. This inoculum was taken from two day cultures on nutrient agar.

In the early work a range of C. fascians strains was used, but in later experiments this was reduced on the basis of the results that had been obtained.

(a) The effect of temperature

Cultures of strains 197, 194, 214, 110, 12, 137 and the rough and smooth variants of strain 505, were set up on nutrient agar medium (N.A.).

The plates were inoculated with a standard inoculum (as defined in Materials and Methods), spread evenly over the surface, ten replicates of four plates being used for each strain or variant. One plate of each strain was incubated at 25°C as a control, and one plate of the remaining three, at each of 28°C, 30°C and 37°C. After 2 days, the cultures were examined and morphology observed in stained preparations. The results are shown in table 2a.

It was found that at 28°C, the growth of all the strains was very good. With strains 197 and 194, the cells were mostly normal but a few were bulged and a very small number of filaments were present. The remaining strains were similar to strain 197 and 194 except that no filaments were observed. Staining showed that all cells were septate. At 30°C all strains showed fair growth, except strain 137, in which growth was poor. The cells were mostly normal but with some long cells, and a small number of short spheroid cells. Filaments were not observed in the rough form of strain 505, although a very few were observed in the smooth form. More were observed in strain 197 and some of these filaments were bulged. No filaments were observed in the other strains. Staining showed septa present and part of the filament wall stained darker than the rest of the wall.

At 37°C, growth was fair in strains 194 and 214, poor in strains 12 and 505 both smooth and rough variants and very poor in strains 197, 137 and 110. In all cases it consisted of mostly small cells, sometimes bulged with, in the case of the smooth and rough variants of strain 505, a few filaments. Occasional branched filaments were found in the smooth form. Staining showed the presence of septa in the cells and a dark cell wall staining in a few of the filaments.

This experiment was continued with those strains where a few filaments occurred. Plates of each strain, i.e. 505, 197 and 194 at each of the previous temperatures, were subcultured onto fresh nutrient

Table 2. The effect of temperature of incubation on growth and morphology of strains of *C. fascians*

a.

Incubation Temperature The effect of different temperatures of incubation, on the growth and morphology of strains of *C. fascians*

Incubation Temperature	505						Strain																	
	rough			smooth			197			194			214			110			12			137		
	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B
28°C	4	0	0	4	0	0	4	1	0	4	1	0	4	0	0	4	0	0	4	0	0	4	0	0
30°C	3	0	0	3	r	r	3	1	0	3	0	0	3	0	0	3	0	0	3	0	0	2	0	0
37°C	2	1	0	2	1	r	1	0	0	3	0	0	3	0	0	1	0	0	2	0	0	1	0	0

- b. The effect of subculturing at 25°C, after incubation at different temperatures, on the growth and morphology of strains of *C. fascians*

Incubation Temperature	Strain											
	505						197			194		
	rough	smooth		rough	smooth		rough	smooth		rough	smooth	
28°C - 25°C	3	0	0	3	0	0	3	0	0	4	0	0
30°C - 25°C	3	0	0	3	0	0	3	0	0	4	0	0
37°C - 25°C	3	0	1	3	0	2	1	0	3	4	0	0
*		4	2	2								
+		1	r	1			1	1	1			

* 3 successive subcultures at 37°C then subcultured at 25°C.

+ Cultured at 37°C then 2 successive subcultures at 25°C.

G = growth.

4 = 80 - 100% , 3 = 50 - 80%

2 = 30 - 50% , 1 = 10 - 30%

- c. The effect of successive subcultures at the same temperature, on the growth and morphology of strains of *C. fascians*

Incubation Temperature	Strain								
	505						197		
	rough	smooth		rough	smooth		rough	smooth	
28°C - 28°C	1	0	0	1	0	0	1	r	0
30°C - 30°C	1	r	r	1	0	0	1	0	0
37°C - 37°C	1	2	3	1	0	0	0	0	0

F = filamentation } 4 = 80 - 100%
 B = branching } 3 = 60 - 80%
 2 = 30 - 40%
 1 = 10 - 20%
 0 = none
 r = rare

agar plates and incubated at 25°C for 2 days, to determine whether or not there would be an improvement in either filament formation or growth. The results are shown in table 2b. These show that in subcultures from 37°C, growth was improved in strains 505 and 194, although no change was observed in strain 197. No filaments were observed in all the three strains, but branching was increased in both strains 505 and 197. Growth was decreased in subcultures from 28°C with strains 505 and 197, but no change was observed with strain 194. No filaments were observed in all the three strains.

In subcultures from 30°C, no change in growth was noticed with both strains 505 and 197, although an improvement was noticed in strain 194. No filaments were observed in either of the strains.

In strain 505 (smooth variant), three subcultures at 37°C followed by a subculture at 25°C resulted in increased filamentation and branching.

Strain 197 and 505 (smooth variant) grown at 37°C and subcultured twice at 25°C, strain 505 (smooth variant) grew very poorly and showed a decrease in filamentation and slight increase in branching. In strain 197, no change in growth was observed, although an increase in both filamentation and branching was observed.

The two strains 505 and 197, which were the only strains found to produce a few filaments at the higher temperatures, were subcultured onto fresh nutrient agar plates and the incubation continued at the same temperatures as used previously for two days, to see whether or not any increase in filamentation would occur. The results are shown in table 2c. These show that in subcultures made for both strains 505 and 197, from all the temperatures used, growth was decreased significantly with no change in filamentation or branching at 28°C, but an increase was observed at 30°C and 37°C in strain 505 (rough variant), although no filaments or branch cells were observed in either strain 505 (smooth variant) or 197.

(b) The effect of irradiation with Ultra-violet Light

This was studied by exposing a known number of bacteria on nutrient agar plates, to ultra-violet light at a distance of 25 cm from a Hanovia ultra-violet lamp, 90% of the output of which is at 237 nm wavelength. Thirty replicates of a series of inoculations at different levels and times of exposure from 10 - 60 minutes were used. After exposure, the petri dish lids were replaced and the dishes incubated at 25°C for two days. The plates were then examined for resistant colonies growing on the surface of the agar plates. Each colony was examined and the ones showing filamentous cells were subcultured onto fresh nutrient agar plates several times at two day intervals and incubated at 25°C. Before each subculture the growth was examined for the existence of filamentous cells.

The colonies which produced filaments after several subcultures occurred following 60 minutes treatment of the smooth variant of strain 505, 30 minutes treatment of strain 197, 50 minutes treatment of strain 194 and 60 minutes treatment of strain 214. These were selected for the measurement of length, number of septa and number of nuclei per cell and these results are shown in table 5 together with those obtained in studies on the effect of solidified media, for comparative purpose.

The maximum filamentation was obtained from the smooth variant of strain 505 and this was selected for studies on cell wall dry weight (table 13).

(c) The effect of penicillin

An experiment was set up to determine the effect of different concentrations of penicillin on morphology and growth in nutrient broth medium (N.B.). The medium was prepared as described in the previous section with a series of concentrations of penicillin ranging from 10 - 100 µg/litre, ten replicates of 2 litre flasks containing 200 ml each were used for each of the concentrations. After inoculation the cultures were incubated for two days at 25°C and then examined. The results in table 3 show that with

Table 3. The effect of penicillin concentration ($\mu\text{g/l}$) in nutrient broth medium on growth and morphology of strains of C. fascians

Strain	Penicillin concentration µg/l																													
	10			20			30			40			50			60			70			80			90			100		
	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B			
197	4	3	3	3	3	3	2	2	1	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	0	1
505 smooth	4	2	1	4	2	1	4	1	1	4	0	0	3	2	0	3	0	0	3	0	0	3	0	0	3	0	1	3	0	0
505 rough	4	r	r	4	1	0	2	0	1	2	0	0	1	0	1	1	0	1	1	0	1	1	r	1	1	r	1	1	r	1

G = growth 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation)
B = branching } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, 0 = none, r = rare.

strain 197, and penicillin at a concentration of 10 $\mu\text{g/l}$, good growth occurred, the majority of cells being long, branched and filamentous. A few cells of normal size and a few irregular cells or bulged and tree-like branched cells were also present. When the concentration of penicillin was doubled, growth remained fairly good but the majority of cells appeared as bulged and branched filaments. At a penicillin concentration of 30 $\mu\text{g/l}$, growth was poor and the cells showed the same range of form as in the previous concentration, but all were smaller in size. Increasing the penicillin concentration up to 100 $\mu\text{g/l}$, only decreased growth and increased the formation of bulged cells.

With strain 505 (smooth variant), the pattern was similar except that filamentation was less at penicillin concentrations of 20 and 30 $\mu\text{g/l}$ and growth was not generally influenced by concentrations up to 100 $\mu\text{g/l}$. At 50 $\mu\text{g/l}$, an abnormally high proportion of long cells which did not reach filamentous length were observed. The rough variant of strain 505 behaved similar to strain 197 except that filaments were very rarely formed. Staining showed, with penicillin, the induced filaments were septate and had thin deformed walls.

This experiment was repeated using synthetic medium (S) but, as growth was poor at all penicillin concentrations, it was considered unsuitable and the study discontinued.

(d) The effect of different solid media

Four strains (197, 194, 214 and the rough and smooth variants of strain 505), were grown on plates of the following media : penicillin nutrient agar (P), synthetic agar (S), synthetic mannitol agar (Sm) and Rhizobium agar (R). The penicillin concentration selected for the penicillin nutrient agar (P) was that which showed induction of a few filaments in the previous experiment (10 $\mu\text{g/l}$). The plates were

inoculated with a standard inoculum taken from two day cultures on nutrient agar. All plates were then examined after two days of incubation at 25°C and compared with control cultures on nutrient agar plates. The information from fifteen replicates presented in table 4 shows that all strains grew well on all media and filamentation occurred mainly with strains 197 and 505 (smooth variant) on penicillin nutrient agar (P), synthetic agar (S) and Rhizobium agar media (R), being best on penicillin nutrient agar (P). Strain 197 produced more filaments on penicillin nutrient agar (P) and large numbers of branched cells on penicillin nutrient agar (P), synthetic agar (S) but fewer on the other media. Strain 505 (smooth variant), produced few branched cells, but the rough variant, showed little response as did both strains 194 and 214.

As synthetic mannitol agar (Sm) was unfavourable for filament induction, this was not included in the rest of the work.

From this experiment it was concluded that no significant effect of medium occurred with strains 194 and 214 and these strains therefore were not included in further experiments.

In view of the results on penicillin nutrient agar medium (P), fresh penicillin nutrient agar plates were used. Here the plates were inoculated with an inoculum taken from a two day old culture on plates of nutrient agar containing 10 µg/l penicillin, to see if filamentation was improved by successive subculturing. After two days incubation at 25°C the cultures were examined and it was found that filamentation was increased only with the smooth variant of strain 505, no change occurring with the other strains. Thus this inoculation procedure was adopted whenever penicillin nutrient agar medium (P) was used.

Smears were made from the growth of strains 505 (smooth variant), 197 and 194 on penicillin nutrient agar (P), synthetic agar (S) and Rhizobium agar media (R) and strain 214 on penicillin nutrient agar medium (P). The smears were stained by the combined nucleus/cell wall staining method as described in the Materials and Methods. The length of

Table 4.

Growth and morphology of strains of C. fascians grown on different solid media

Solid medium	Strain														
	505						197			194			214		
	smooth			rough			G	F	B	G	F	B	G	F	B
P	4	2	1	4	r	r	4	3	3	4	0	0	4	0	0
S	4	2	1	4	0	0	4	2	3	4	r	1	4	0	0
S(m)	4	1	0	4	1	1	4	1	2	4	0	0	4	0	0
R	4	2	1	4	0	0	4	2	2	4	r	0	4	0	0
N.A.	4	0	0	4	0	0	4	0	0	4	0	0	4	0	0

P = Penicillin nutrient agar

S = Synthetic agar

S(m) = Synthetic medium but mannitol replacing sucrose

R = Rhizobium agar

N.A. = Nutrient agar

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%

F = filamentation)

B = branching } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%

the cells, number of nuclei/cell and number of septa per filament were then counted under the microscope. Twenty filaments were counted for each strain/medium. These data together with those obtained from the ultra-violet irradiation experiments are shown in table 5.

The percentage of the cell as cell wall was determined for these cells of strain 505 (smooth variant) and for strain 197 on nutrient agar medium (N.A.) and the results are shown in table 13 for comparative purpose.

From the results shown in table 5, it was concluded that the length of the unit cell was the greatest with strain 505 (smooth variant) in the media tested. The control cells (grown on nutrient agar (N.A.)), showed differences between these strains, the greatest length of unit cell was shown by strain 505 (smooth variant), the greatest length of control cell (rod) was shown by strain 197.

(e) The effect of individual amino acids on growth and morphology

This was studied (a) using synthetic medium (S), with both NH_4Cl and mannitol replaced by a single amino acid, and (b) using synthetic medium (S) with the addition of one of the amino acids, glycine, L-alanine and L-asparagine.

In both cases, the concentration of the amino acid used was 0.001% of the L-isomer. The media were prepared in 5 two litre flasks each containing 200 ml. After inoculation with a standard inoculum of strain 505 (smooth variant), the flasks were incubated at 25°C for two days. They were then examined and the results are recorded in table 6. It will be seen that no filaments, long cells, or branched cells were observed in any culture.

In the flasks containing alanine, serine, or asparagine as sole carbon and nitrogen source good growth occurred, but in the remainder only moderate or poor growth, was recorded. Poor growth was recorded in all the flasks of complete synthetic medium with the addition of either alanine, glycine or asparagine. In view of these results it was decided to discontinue any further work with these media.

Table 5. The measurements of cell length, number of septa nuclei of induced filaments of *C. fascians* under different environmental factors

Strain	Medium	Mean No. of septa per rod	Mean length of rod (μ)	Mean No. nuclei per rod	Mean No. cells per rod	Mean No. nuclei per cell	Mean length of unit cell (μ)
505 smooth	P	8.7	21.24	8.8	11.5	1.72	2.43
	R	9.67	23.09	9.5	10.67	0.89	2.16
	N.A.	0.45	2.84	2.25	1.45	1.55	1.96
	S	1.25	6.75	3.25	2.25	1.44	3.0
U.V. mutants of 60' exposure	N.A.	5.3	19.17	9.01	6.2	1.45	3.09
197	P	10.5	16.2	13.5	11.5	1.17	1.41
	R	10.0	12.6	10.33	11.0	0.94	1.14
	N.A.	4.3	6.66	5.3	5.3	1.0	1.26
	S	10.0	22.8	9.67	11.0	0.88	2.07
U.V. mutants of 30' exposure	N.A.	9.67	12.6	10.67	10.67	1.0	1.18
194	P	16.0	19.8	17.0	17.0	1.0	1.16
	R	18.0	22.14	14.6	19.0	0.77	1.16
	N.A.	3.53	4.8	4.53	4.53	1.0	1.06
	S	11.33	15.0	10.0	12.33	0.81	1.22
U.V. mutants of 50' exposure	N.A.	24.5	25.2	25.5	25.5	1.0	0.99
214	P	10.0	13.8	12.0	11.0	1.09	1.25
	R	-	-	-	-	-	-
	N.A.	-	-	-	-	-	-
	S	-	-	-	-	-	-
U.V. mutants of 60' exposure	N.A.	8.0	16.8	10.67	9.0	1.18	1.87

P = penicillin nutrient agar
N.A. = nutrient agar

R = Rhizobium agar S = synthetic agar U.V. = ultra violet - = data not available
Unit cell = a single cell, a number of which may join together to form a rod or filament

Table 6. The effect of individual amino acids on growth and morphology of *C. fascians* strain 505 (smooth variant)

amino acid added (0.001 %)	Synthetic Medium (S)					
	With NH ₄ Cl and mannitol ⁴ omitted			Complete		
	G	F	B	G	F	B
D-L-homo-cysteine	1	0	0	-	-	-
L-Glutamic acid	2	0	0	-	-	-
L-monolysine	2	0	0	-	-	-
L-cystine	2	0	0	-	-	-
D-L-alanine	4	0	0	2	0	0
D-L-serine	4	0	0	-	-	-
D-L-valine	3	0	0	-	-	-
L-tyrosine	3	0	0	-	-	-
L-glycine	3	0	0	2	0	0
D-L-phenylalanine	3	0	0	-	-	-
L-asparagine	4	0	0	2	0	0

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%,

B = branching) 0 = none, r = rare.

- = data not available.

(f) The effect of individual media constituents on growth and morphology

This was studied using Thornton's medium with the modifications described in the Materials and Methods section, to examine either the effect of the presence or absence of a specific single substance or group of substances in the culture medium or the effect of concentration of a component of the medium on the growth and morphology of C. fascians. In some instances this was limited to the replacement of a single constituent by another or even a single cation or anion by another.

The media were prepared as explained in the previous section and dispensed into five 2 litre conical flasks each containing 200 ml. Fourty replicates were used for this experiment. After inoculation with a standard inoculum containing a known number of bacteria, they were incubated at 25°C for 2 days before being examined. The results shown in table 7 indicate that good growth occurred in all media, although this was less than maximal in medium C and medium D with the rough variant of strain 505.

Filamentation was best with strain 505 (smooth variant) in medium TY₂ but also good in TY₁ and TY₃. With strain 197, filamentation occurred in CY and D media, but with strain 505 (rough variant) it was good only in medium TY₂. Although no filaments were observed in TY medium, long cells were common with strains 505 (smooth variant) and 197.

Branched cells occurred frequently in TY and D media with strain 197, in TY, TY₂, TY₃ and CY media with strain 505 (smooth variant) and in TY₂ medium with strain 505 (rough variant), although they were found to a lesser extent also in media T, CY and C.

This experiment was repeated using the same media and number of replicates except that 2% 'Oxoid' agar No. 3 was added to solidify the media which were then poured into petri-dishes. After the plates had solidified they were inoculated with a standard inoculum and incubated at 25°C for 2 days. At the end of this period all the plates were

Table 7. The effect of individual Thornton's medium constituents on growth and morphology of two strains of *C. fascians* in static culture

Strain number	Thornton's medium and its variants																	
	T			TY			TY ₁			TY ₂			TY ₃			CY		
	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B
197	4	0	0	4	0	3	4	1	1	4	1	1	4	1	2	4	2	2
505 smooth	4	0	1	4	0	3	4	2	1	4	4	4	4	3	3	4	1	3
505 rough	4	0	2	4	0	0	4	0	1	4	3	3	4	0	1	4	0	2

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, o = none, r = rare.
B = branching }

T = Thornton's medium.

TY = Thornton's medium, but with yeast extract added.

TY₁ = Thornton's medium, but with yeast extract added and asparagine omitted.

TY₂ = Thornton's medium, but with yeast extract added and MgSO₄ omitted.

TY₃ = Thornton's medium, but with yeast extract added and NaCl omitted.

C³ = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

D = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid and p-aminobenzoic acid only.

CY = Thornton's medium, with added yeast extract, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

examined. The results in table 8 show that growth is good in all media, but lower in media C and D, except for strain 505 (rough variant) in medium C.

Filamentation is best with strain 197 in medium TY_3 in most other cases, filamentation was poor or not observed at all. Branched forms occurred frequently in media TY_2 , TY_3 , CY and C with strain 197, in media TY_2 and CY with strain 505 (smooth variant) and with strain 505 (rough variant) in media TY_1 , TY_2 , TY_3 and CY.

From the results obtained it was concluded that the omission of any one of three components, i.e. $MgSO_4$, NaCl and asparagine from the complete medium induced filaments to be formed. To confirm the effect of omission of $MgSO_4$ or NaCl, and to study the omission of NH_4Cl , the previous experiment was repeated using synthetic medium (S) to study each salt separately. The results shown in table 9, where $MgSO_4$ and NaCl were omitted, concur with those previously obtained with the Thornton's medium except here filamentation was slightly less. The omission of NH_4Cl resulted in very poor growth and filamentation could not be observed.

Further studies were therefore made to determine whether the anion or cation is responsible for the induction. This was done by replacing one anion or one cation at a time with an equivalent salt, e.g. NaCl was replaced by KCl or Na_2SO_4 . Where the anion or cation used for this substitution was already present in another salt this medium served to provide information on the effect of the increased concentration of this ion. The effect of sucrose was also studied. Strain 505 (smooth variant), was used as inoculum and forty replicates of the cultures were examined after two days of incubation at $25^{\circ}C$.

From the results shown in table 10, it appears that where KNO_3 was replaced by $NaNO_3$, an increase in the sodium ion concentration occurred and no long cells were observed, especially with the TY medium where they previously occurred. On the other hand in the TY_3 medium where NaCl was originally omitted, this replacement resulted in sodium ions being

Table 8. Growth and morphology of strains 197 and 505 (smooth and rough variants), of *C. fascians*, on Thornton's Agar (T) and its variations

Strain	T			TY			TY ₁			TY ₂			TY ₃			CY			C			D		
	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B
197	4	0	r	4	0	2	4	2	2	4	2	3	4	3	4	4	r	3	3	0	3	3	0	1
505 smooth	4	0	r	4	0	1	4	1	2	4	2	3	4	1	2	4	r	3	3	0	2	3	0	1
505 rough	4	0	0	4	0	1	4	0	3	4	0	3	4	0	3	4	0	3	4	0	2	3	0	2

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation }
 B = branching } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, r = rare.

T = Thornton's medium.

TY = Thornton's medium, but with yeast extract added.

TY₁ = Thornton's medium, but with yeast extract added and asparagine omitted.

TY₂ = Thornton's medium, but with yeast extract added and MgSO₄ omitted.

TY₃ = Thornton's medium, but with yeast extract added and NaCl⁴ omitted.

C³ = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

D = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid and p-aminobenzoic acid only.

CY = Thornton's medium, with added yeast extract, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

Table 9. The effect of omission of individual constituents of synthetic medium (S) on growth and morphology of two strains of *C. fascians* in static culture

Strain number	Synthetic medium														
	S			SY			SY ₂			SY ₃			SY ₄		
	G	F	B	G	F	B	G	F	B	G	F	B	G	F	B
197	4	0	0	4	0	3	4	1	1	4	1	2	2	0	0
505 smooth	4	0	r	4	0	3	4	3	4	4	2	3	2	0	0
505 rough	4	0	2	4	0	0	4	2	3	4	r	1	1	0	0

SY₂ = Synthetic medium (S) with MgSO₄ omitted and yeast extract added.

SY₃ = Synthetic medium (S) with NaCl omitted and yeast extract added.

SY₄ = Synthetic medium (S) with NH₄Cl omitted and yeast extract added.

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, r = rare, 0 = none.
B = branching }

Table 10. The effect of replacement of one component in Thornton's medium on growth and morphology of
C. fascians strain 505 (smooth variant) in static culture

Replacement number	1			2			3			4		
	T	TY	TY ₃	T	TY	TY ₃	T	TY	TY ₃	T	TY ₂	TY ₃
Strain number	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B
505 smooth	4 0 0	4 0 r	4 0 2	4 0 r	4 1 3	4 3 3	4 0 r	4 0 3	4 2 3	4 0 r	4 2 3	4 3 3

1 = replacement of KNO₃ with NaNO₃

2 = replacement of NaCl with KCl

3 = replacement of mannitol with sucrose

4 = replacement of NaCl with Na₂SO₄

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, r = rare, 0 = none.
 B = branching }

T = Thornton's medium.

TY = Thornton's medium with yeast extract added.

TY₂ = Thornton's medium with yeast extract added and MgSO₄ omitted.

TY₃ = Thornton's medium with yeast extract added and NaCl⁴ omitted.

present so that no filaments occurred.

Where NaCl was replaced by KCl, there was no change in the type of growth in TY₃ medium where NaCl was already absent, but in the complete medium longer cells were observed and a few filaments occurred in the TY medium.

Where sucrose replaced mannitol at the same concentration, little difference was observed but the cells were generally shorter. Where NaCl was replaced by Na₂SO₄, there was an increase in both the sodium and sulphate ions in the complete medium and the cells were shorter. This did not affect the TY₃ medium where NaCl was already omitted, but in the TY₂ medium where MgSO₄ was omitted, it reduced filamentation.

No change in amount of growth was observed with any of the four replacements. Thus it appears that the omission of the cations Mg²⁺ and Na⁺ as well as asparagine from the media induce filament formation. Yeast extract appears to encourage both growth and filament formation and the omission of the nitrogen source (NH₄Cl), as expected, reduced growth significantly. Sucrose is apparently unfavourable to filament formation. To find out whether the process of filament formation can be reversed, the missing element was added to a 12 hr culture, 24 hr culture and 48 hr culture. This was studied in both synthetic medium (S) and Thornton's medium and in both static and aerated cultures incubated at 25°C. All cultures were examined before and after the addition was made, i.e. 12 hrs, 24 hrs, 48 hrs and finally after 4 days. No reduction in filamentation was observed, once elongated cells had been found after 12 hrs.

It may be concluded therefore, that the formation of filaments and long cells once established in a culture cannot be reversed by the addition of the missing ion.

(i) Effect of carbon source

The effect of altering the carbon source was further studied,

by using mannitol and glucose to replace sucrose in the synthetic medium (S), and sucrose and glucose to replace mannitol in Thornton's medium (T).

The media were prepared as explained in the previous section, five 2 litre conical flasks, each containing 200 ml. being set up in each case. Strain 505 (smooth variant) was used and ten replicates of the cultures were examined after 2 days incubation at 25°C.

In synthetic media S and S₂, when 0.1% mannitol replaced 0.1% sucrose, the cells were slightly longer than normal. When 1% mannitol was added, even longer cells were produced. On the other hand when glucose was used to replace sucrose in the synthetic medium (S) at a concentration of 1%, cells of normal size and shape were produced. At 2%, however, although no change in size occurred, the cells were thicker and a few were bulged.

In Thornton's media, T, T₁, T₂ and T₃, when 1% sucrose replaced 0.1% mannitol, the cells were normal in the complete medium T, but shorter in its variants T₁, T₂ and T₃. When glucose was used, in this case to replace 0.1% mannitol, no effect was observed at either a concentration of 1% or 2%. When 1% sucrose was examined with 1% yeast extract as a medium, good growth occurred and the cells were generally longer than normal. Thus it appears that the yeast extract, not the sugar, was the component which encouraged the formation of elongated cells in the media tested.

(g) The effect of aeration

C. fascians is known to be strongly aerobic and it was considered of interest, therefore, to study the effect of aeration on its growth and morphology. It was observed that when the organism is grown in one of the media which induces the formation of filamentous cells, those growing in

the depths of an undisturbed liquid medium, which are at a reduced oxygen tension, are long and filamentous, whereas surface cultures on the same medium solidified with agar, exhibit less filamentous and elongated cells. This was further investigated in liquid media.

Two litre conical flasks each containing 200 ml of Thornton's medium (T) or one of its variations were set up. They were divided into three series each containing two flasks of each medium. Each series was inoculated with one of the strains of C. fascians, 197, 505 (smooth variant) and 505 (rough variant). One flask of each medium and strain was incubated on a reciprocating shaker at 25°C, for two days and the second was incubated statically at the same temperature for the same time. At the end of this period, all cultures were examined and the results obtained from fifty replicates are given in table 11. These show that growth was generally best in the aerated cultures, with the exception of strains 197 and 505 (rough variant) in C and D media. Cells were shorter in the aerated cultures of all the strains tested in both T and TY media and filamentation was less frequent in all strains in the aerated TY₂, CY and D media. On the other hand aerated cultures of all strains produced more filaments in TY₁ and TY₃ media.

(h) Dry weight

(i) As a measure of growth in any medium, the dry weight of 1 ml of culture was determined.

These determinations were carried out as described in the previous section on 1 ml samples taken from two day old cultures of strain 505 (smooth variant) in T, TY, TY₁, TY₂ and TY₃, i.e. in the complete Thornton's medium and its variations and from media with individual components replaced by equivalent ones containing an alternative ion or anion.

The cells here were sedimented by centrifugation at 4000xg for 15 minutes, washed twice with distilled water, resuspended in

Table 11. Growth and morphology of strains of *C. fascians* grown in Thornton's medium and its variations in static

		<u>and aerated cultures</u>															
		T		TY		TY ₁		TY ₂		TY ₃		CY		C		D	
		static	aerated	static	aerated	static	aerated	static	aerated	static	aerated	static	aerated	static	aerated	static	aerated
Strain number		G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B	G F B
197		4 0 0	4 0 0	4 0 3	4 0 3	4 1 1	4 4 1	4 1 1	4 0 1	4 1 2	4 3 1	4 2 2	4 0 1	4 0 0	3 0 0	4 2 3	3 0 0
505 smooth		4 0 r	4 0 0	4 0 3	4 0 0	4 2 1	4 4 1	4 4 4	4 3 3	4 3 3	4 3 1	4 1 3	4 0 1	4 0 1	4 0 0	4 0 1	4 0 0
505 rough		4 0 2	4 0 1	4 0 0	4 0 3	4 0 1	4 4 3	4 3 3	4 2 4	4 0 1	4 3 3	4 0 2	4 0 2	3 0 2	3 0 2	3 0 1	3 0 2

G = growth. 4 = 80 - 100%, 3 = 50 - 80%, 2 = 30 - 50%, 1 = 10 - 30%.

F = filamentation } 4 = 80 - 100%, 3 = 60 - 80%, 2 = 30 - 40%, 1 = 10 - 20%, r = rare, 0 = none.
B = branching

T = Thornton's medium.

TY = Thornton's medium, but with yeast extract added.

TY₁ = Thornton's medium, but with yeast extract added and asparagine omitted.

TY₂ = Thornton's medium, but with yeast extract added and MgSO₄ omitted.

TY₃ = Thornton's medium, but with yeast extract added and NaCl omitted.

CY₃ = Thornton's medium, with added yeast extract, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

C = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid, biotin and p-aminobenzoic acid.

D = Thornton's medium, but with the omission of asparagine, MgSO₄ and NaCl, and the addition of thiamine, nicotinic acid and p-aminobenzoic acid only.

one ml of distilled water, dried at 100°C for 2 days, then cooled and weighed. These determinations were made to investigate the effect of these media on growth.

The results given in table 12 expressed in micrograms weight per ml, show that both yeast extract and aeration gave a higher yield of weight at 25°C than those in both yeast extract absence and static cultures. On the other hand, higher temperature (37°C), gave a very sharp decrease in weight in all media tested. The omission of one component from the medium gave the lowest weight when MgSO_4 was omitted, than when NaCl and asparagine, in that order. In the cultures where the replacement of components were made, the replacement of KNO_3 with Na_2CO_3 (1), gave no significant difference in the complete media T and TY, but in TY_3 where NaCl was originally omitted, gave higher weight than that in the complete medium TY. When NaCl was replaced with KCl (2), a decrease in weight occurred in the complete media T and TY, although no change occurred in TY_3 where NaCl was originally omitted. On the other hand, when sucrose replaced mannitol in the media (3), there was an increase in weight in TY and TY_3 media, but not a significant one in T medium.

When Na_2SO_4 replaced NaCl in the media (4), a slight increase in weight occurred in both TY and TY_2 media, although no change occurred in TY_3 medium, where NaCl was originally omitted.

(ii) Cell wall dry weight

Determination was carried out to ascertain whether the proportion of the cell as cell wall varied with changes in media. Some indication had been obtained from stained preparations that the walls were thicker under certain growth conditions.

Accepted methods of purification of bacterial cell walls range from washing the broken cell walls in various ways to, in addition, treating with the enzymes, trypsin and ribonuclease,

Table 12. Dry weight of one ml culture of *C. fascians*, strain 505 (smooth variant), grown in different media under different conditions of temperature and aeration, expressed as mg/ml dry weight

Replacement number	1					2					3					4				
Conditions of culture	T	TY	TY ₁	TY ₂	TY ₃	T	TY	TY ₃	T	TY	TY ₃	T	TY	TY ₃	T	TY ₂	TY ₃			
Static at 25°C	4.28	7.65	7.40	3.95	6.94	4.73	7.50	8.90	1.90	6.20	6.35	4.62	10.08	8.55	5.10	4.45	6.47			
aerated at 25°C	7.90	10.30	9.88	8.05	11.60	7.80	10.50	10.45	5.40	8.50	10.50	9.15	10.67	10.80	7.90	6.65	9.45			
static at 37°C	2.20	1.65	0.90	0.39	1.00	-	-	0.80	-	-	0.98	-	-	1.50	-	0.90	1.16			
1 = replacement of KNO ₃ with NaNO ₃ . 2 = replacement of NaCl with KCl. 3 = replacement of mannitol with sucrose. 4 = replacement of NaCl with Na ₂ SO ₄ . - = not tested.																				
T = Thornton's medium. TY = Thornton's medium, but with yeast extract added. TY ₁ = Thornton's medium, but with yeast extract added and asparagine omitted. TY ₂ = Thornton's medium, but with yeast extract added and MgSO ₄ omitted. TY ₃ = Thornton's medium, but with yeast extract added and NaCl ⁴ omitted.																				

and sometimes also pepsin. It was decided, therefore, to carry out this experiment using wall samples taken from three stages in purification to determine the effect each stage has on the amount of wall material obtained. These samples were taken (a) after washing in M NaCl, distilled water, 0.1 M phosphate buffer pH 7.0 and finally in distilled water. (b) After washing as above and treatment with trypsin and ribonuclease, and finally (c) after further treatment with pepsin.

These samples were from cultures of C. fascians, strain 505 (smooth variant) in different media, both liquid, static and aerated, and solid. Determinations were also made on samples taken from cultures of strain 197 grown on nutrient agar and solid media S and R to investigate if any differences between species occur. All the samples taken at different stages of purification, were frozen and dried in vacuo over phosphorus pentoxide (see previous section), before they were used for the different studies carried out during this work.

The results of the determinations expressed as % of whole cell dry weight, in table 13 showed that differences in yield of cell wall dry weight by different purification procedures occurred. With enzyme treatment, consistently low yield of cell wall dry weight have been obtained. It appears that after enzyme treatment, cell wall material dispersed easily during the washing procedure. It was almost translucent in appearance and appeared to be more difficult to recover upon centrifugation. On the other hand, the cell was bound with other cell materials prior to enzyme treatment. Thus, it did not disperse easily during the washing procedure but readily sedimented on centrifuging.

Also the cell wall dry weight appears to differ from one strain to another. In this case, strain 197 gave a higher percentage than strain 505. In addition, the purified cell wall dry weight

Table 13. Cell wall dry weight of strains 197 and 505 (smooth variant), of *C. fascians*, grown under different environmental conditions, expressed as % of cell as cell wall, at different stages of wall purification

Strain 505(smooth variant)						
Liquid media	Untreated		Treated with ribonuclease + trypsin		Treated with ribonuclease + trypsin + pepsin	
	static	aerated	static	aerated	static	aerated
N.B.	20.9	23.2	18.4	20.1	11.5	12.6
TY	25.8	28.3	16.3	18.9	9.1	11.3
TY ₁	17.6	22.5	13.9	15.6	3.7	5.6
TY ₂	26.0	29.4	15.8	16.9	7.3	9.1
TY ₃	22.8	25.8	14.3	17.3	6.5	7.9

N.B. = nutrient broth.
 TY = Thornton's medium with yeast extract added.
 TY₁ = Thornton's medium with yeast extract added and asparagine omitted.
 TY₂ = Thornton's medium with yeast extract added and MgSO₄ omitted.
 TY₃ = Thornton's medium with yeast extract added and NaCl omitted.

N.A. = nutrient agar.
 S = synthetic agar.
 R = Rhizobium agar.
 P = penicillin agar.
 N.A.*= U.V. mutants grown on nutrient agar.
 - = not determined.

Strain 505 (smooth variant)			Strain 197		
aerated	Treated with ribonuclease + trypsin	Treated with ribonuclease + trypsin + pepsin	Untreated	Treated with ribonuclease + trypsin	Treated with ribonuclease + trypsin + pepsin
8	19.0	12.0	30.0	22.0	15.5
0	15.2	7.4	38.8	17.8	10.5
2	16.3	4.5	36.6	18.7	11.7
3	15.9	5.9	31.7	13.2	7.8
8	14.4	15.8	-	-	-

was generally higher in both nutrient broth and nutrient agar media than those in all the other media, with the exception of that from the ultra-violet treatment, which was the highest.

Aeration also seems to affect the percentage of the purified cell wall dry weight, being generally higher in the aerated cultures in all media tested. Differences were also noticed between different media. In the solid media, using nutrient agar as a control, the lowest occurred in Rhizobium medium. In penicillin it was half the amount of that present in nutrient agar and in synthetic two thirds of that present in nutrient agar.

In the liquid media, the highest being in the complete medium and lowest in TY₁ medium, where asparagine was omitted. A decrease also occurred in TY₂ and TY₃ media, where MgSO₄ and NaCl were omitted.

From an examination of the results of these experiments and those previously described, it was concluded that of the strains and media used, strain 505 (smooth variant) in static culture in Thornton's medium, or one of its variants, TY₁, TY₂, TY₃ and penicillin agar, gave maximum filamentation and these were the media selected for further study. Also TY₂ medium was used for further investigation on the aeration factor.

B. Cell wall composition

(a) Qualitative analysis

Before studies could be made on the effect of different environmental conditions on cell wall composition, it was necessary to identify the components present in the cell wall of strain 505 (smooth variant) of C. fascians growing in nutrient broth. A culture was grown for 2 days at 25°C and harvested by centrifugation. The cells were broken and the walls purified as

described in 'Materials and Methods'. After hydrolysis the hydrolysate was subjected to two dimensional paper chromatography. The amino acid wall components were located by the use of an appropriate spray reaction, the amino sugar components by dipping the chromatogram into the reagents and drying in an oven. The components were identified by their R_f values, these being checked against known markers which were run parallel to the samples (Smith, 1960).

As both glucosamine and galactosamine have the same R_f value, the developed spot of the amino sugar was cut out from the original chromatogram, eluted with water and then subjected to further chromatography as described in 'Materials and Methods'.

The results in table 14 show the composition of the purified cell wall and wall fragment that was soluble with 10% trichloroacetic acid, where teichoic acid was separated from the peptidoglycan by the method given in the previous section.

From the results in table 14 it will be seen that the purified cell wall contains glutamic acid, alanine and methionine as the major amino acid constituent. Serine, leucine, threonine and glycine were also present in significant amounts together with valine, isoleucine, asparagine and histidine in small amounts only. A small amount of lysine, as a dibasic amino acid was also detected. Muramic acid was shown to be a major constituent together with the amino sugars, glucosamine and galactosamine in significant amounts.

Furthermore, the wall teichoic acid of this organism was found to contain ribitol. The amino acids present in the purified cell wall were confirmed in the quantitative analysis carried out using an amino acid analyser, and some of these amino acids (e.g. histidine and arginine) hardly detectable by the paper chromatographic technique, were identified.

The amino sugars, glucosamine and galactosamine, gave separate peaks by the amino acid analyser technique, although muramic

Table 14. Cell wall composition of strain 505 (smooth variant) of
C. fascians, grown in static cultures in nutrient broth
at 25°C for two days

Components	Level of purified cell wall components
<u>Amino acids</u>	
Lysine	+
Histidine	tr.
Arginine	-
Proline	-
Asparagine	+
Threonine	++
Serine	++
Glutamic acid	++++
Glycine	++
Alanine	++++
Valine	+
Methionine	++++
Isoleucine	+
Leucine	++
<u>Amino sugars</u>	
Glucosamine	+
Galactosamine	+
Muramic acid	+++
<u>Teichoic acid</u>	
Phosphorus	(+)

Each + indicates 1 µg of component per mg dry weight wall, i.e.
tr. = present but not measurable, + = 1-10, ++ = 10-20, +++ = 20-30,
++++ = 30-40, +++++ = over 40.
- = 0. (+) = 0.1-1.

acid did not separate.

(b) Quantitative analysis

A previous result showed that different purification procedures affect the amount of the cell measurable as cell wall but it was considered essential therefore, to establish a standard purification procedure, such that the composition of different wall samples could be compared. This was studied using the cell walls of strain 505 (smooth variant), the organism being grown in 20, 2 litre flasks each containing 200 ml of nutrient broth and incubated for two days at 25°C, before being harvested. The cells were broken and the wall fraction recovered as described in the Materials and Methods section.

This material was divided into three samples : the first was washed successively in M saline, twice in 0.1 M phosphate buffer pH 7.0 and finally twice in distilled water. The second sample was washed as described above, but subjected to digestion with trypsin and ribonuclease (0.5 µg/ml per 10 µg cell wall), before being hydrolysed. The third sample was given an additional treatment with pepsin (1 µg/ml per 10 µg cell wall), before being hydrolysed. After drying, the wall samples were hydrolysed and the amino acids present estimated in the Unichrom amino acid analyser.

The results of the analyses shown in table 15, demonstrate that walls which had only been washed, contained the whole range of amino acids present in a typical protein hydrolysate. When the walls were treated with ribonuclease and trypsin, in general, the level of the amino acids dropped significantly, this being especially so with proline and arganine. This treatment brings out glutamic acid, alanine and methionine as the major amino acids of the cell wall, followed closely by serine, leucine and threonine. Purification by trypsin, ribonuclease and pepsin, gave a pattern of amino acids

Table 15. The amino acid composition of the cell wall of strain 505 (smooth variant) of *C. fascians* at three different stages of the wall purification procedure

µg amino acid per mg dry weight wall			
<u>Amino acids</u>	Sample 1 Untreated	Sample 2 Ribonuclease and trypsin treated	Sample 3 Ribonuclease, trypsin and pepsin treated
Lysine	22.0453	6.6424	2.9188
Histidine	0.8885	tr.	tr.
Arginine	48.5561	tr.	-
Proline	20.8868	2.6394	-
Asparagine	38.3368	9.8297	4.8145
Threonine	38.5362	21.5134	12.6677
Serine	38.9778	31.3770	18.1630
Glutamic acid	67.5488	64.6164	41.5241
Glycine	28.3247	15.0315	10.3922
Alanine	49.1746	54.2578	36.1612
Valine	30.9880	10.9057	6.7783
Methionine	15.0133	55.5226	37.3499
Isoleucine	20.0341	9.9733	5.8378
Leucine	44.6047	25.6520	16.6524

- = 0

tr. = present but not measurable

similar to that obtained by treatment with trypsin and ribonuclease. However, the quantities of all these amino acids were lower than that found after only trypsin and ribonuclease treatment, indicating that there has been some loss during the additional purification stage. It was considered, however, in this work, that it was more important to have a standard procedure for maximum purity of the cell wall rather than to be concerned with the yield. Thus the third stage of the purification procedure, which removed all traces of proline (as an indicator of cytoplasmic contamination), was included in the purification procedure adopted in all future work.

C. The effect of the environment on cell wall composition

In the previous part of this section, an appreciable effect of different environmental conditions on the growth and morphology of C. fascians, strain 505 (smooth variant), was observed. This was seen in the cell mass and proportion of the cell measurable as cell wall. It was, therefore, decided to continue this study by investigating the effect of different media and media constituents on the chemical composition of the cell wall.

(a) Quantitative analysis of the cell wall from bacteria grown in different media

For each of the media, i.e. nutrient broth (N.B.), Thornton's medium, with and without yeast extract (TY and T respectively) and synthetic medium (S), twenty, 2 litre flasks were set up each containing 200 ml of the appropriate medium. After sterilization they were inoculated with a standard inoculum and incubated at 25°C for 2 days. At the end of this period the cells were harvested and the cell wall purified and prepared for quantitative analysis as previously described in 'Materials and Methods'. The results are shown in table 16, the quantity of each component being expressed in µg per mg dry weight wall. It was found that the level of all

Table 16. Analysis of the cell wall of *C. fascians*, strain 505
(smooth variant), grown in different media⁺

Cell wall components	µg of component per mg dry weight wall in different media ⁺			
	N.B.	TY	T	S
<u>Amino acids</u>				
Lysine	2.9188	1.1551	0.6551	0.2095
Histidine	trace	5.1009	3.8647	0.7495
Asparagine	4.8145	8.8470	9.4553	0.9555
Threonine	12.6677	13.4751	13.8962	3.1442
Serine	18.1630	36.3679	31.4362	7.9075
Glutamic acid	41.5241	22.0210	30.3372	11.9809
Glycine	10.3922	8.8624	9.0905	1.4076
Alanine	36.1612	52.2343	49.0846	15.6986
Valine	6.7783	4.9520	7.7596	1.1673
Methionine	37.3499	44.7983	45.1400	15.6142
Isoleucine	5.8378	3.8765	3.8159	0.8141
Leucine	16.6524	7.2878	20.9239	5.0103
<u>Amino sugars</u>				
Glucosamine	7.7970	7.7960	-	7.2730
Muramic acid	20.4730	28.2490	-	24.2420
<u>Teichoic acid</u>				
Phosphorus	0.6700	0.4920	0.2380	0.6340

⁺ N.B. = nutrient broth.

T = Thornton's medium.

TY = Thornton's medium with the addition of yeast extract.

S = synthetic medium (S).

- = not determined.

amino acids was lower in the synthetic medium (S) than in the other media. When the levels of each of the components in the TY and T media were compared with those in nutrient broth (N.B.), a decrease was found in lysine, glutamic acid, isoleucine and teichoic acid (phosphorus), but an appreciable increase occurred in histidine, asparagine, serine and alanine and to a lesser extent in methionine and muramic acid. The glucosamine level remained constant in all media except in medium T.

(b) The effect of individual constituents of the medium

(i) The addition of yeast extract to Thornton's medium

Table 17 compares the results obtained for medium T (yeast extract omitted) with those obtained for TY (yeast extract added) and shows the percentage increase or decrease for each cell wall component.

It will be seen that a pronounced decrease was found in the levels of teichoic acid (phosphorus), lysine and histidine followed by serine and alanine in that order, and an appreciable increase in the levels of leucine, valine, glutamic acid and to a lesser extent methionine, isoleucine, glycine and threonine.

(ii) The omission of asparagine from Thornton's medium containing yeast extract (TY)

For both the media TY and TY₁ (asparagine omitted), a batch of twenty, 2 litre flasks was set up, each containing 200 ml of the appropriate medium. Inoculation and analysis were carried out as previously described and the results are given in table 18. Here TY₁ medium has been compared with TY medium. An appreciable increase was found in the levels of leucine, isoleucine and valine followed by teichoic

Table 17. Comparative analysis of cell wall of *C. fascians*, strain 505 (smooth variant), grown in the presence and omission of yeast extract from Thornton's medium

Cell wall components	µg component per mg dry weight wall		
	TY	T	% increase or decrease against medium TY
<u>Amino acids</u>			
Lysine	1.1551	0.6551	- 43.0
Histidine	5.1009	3.8647	- 24.0
Asparagine	8.8470	9.4553	+ 6.9
Threonine	13.4751	13.8962	+ 3.1
Serine	36.3679	31.4362	- 13.6
Glutamic acid	22.0210	30.3372	+ 37.8
Glycine	8.8624	9.0905	+ 2.6
Alanine	52.2343	49.0846	- 6.0
Valine	4.9520	7.7596	+ 56.7
Methionine	44.7983	45.1400	+ 0.8
Isoleucine	3.8765	3.8159	- 1.6
Leucine	7.2878	20.9239	+187.1
<u>Amino sugars</u>			
Glucosamine	7.7960	(-)	(-)
Muramic acid	28.2490	(-)	(-)
<u>Teichoic acid</u>			
Phosphorus	0.4920	0.2380	- 52.0

T = Thornton's medium.

TY = Thornton's medium with the addition of yeast extract.

(-) = not determined.

Table 18. Comparative analysis of cell wall of *C. fascians*, strain 505
(smooth variant), grown in the presence and omission of
asparagine from Thornton's medium with yeast extract

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium TY
	TY	TY ₁	
<u>Amino acids</u>			
Lysine	1.1551	0.9990	- 13.5
Histidine	5.1009	trace	->100.0
Asparagine	8.8470	7.3349	- 17.0
Threonine	13.4751	12.6060	- 6.9
Serine	36.3679	16.0805	- 56.0
Glutamic acid	22.0210	19.0587	- 13.5
Glycine	8.8624	8.4082	- 5.0
Alanine	52.2343	28.1419	- 46.0
Valine	4.9520	7.1242	+ 44.0
Methionine	44.7983	25.0930	- 44.0
Isoleucine	3.8765	6.3941	+ 65.0
Leucine	7.2878	13.2296	+ 82.0
<u>Amino sugars</u>			
Glucosamine	7.7960	9.8390	+ 26.0
Muramic acid	28.2490	23.9000	- 15.0
<u>Teichoic acid</u>			
Phosphorus	0.4920	0.6770	+ 27.0

TY = Thornton's medium with the addition of yeast extract.

TY₁ = Thornton's medium with the addition of yeast extract and the
omission of asparagine.

acid (phosphorus) and glucosamine in that order, and a pronounced decrease in the levels of histidine, serine, alanine and methionine and to a lesser extent the remainder of the components estimated.

(iii) The omission of magnesium sulphate from Thornton's medium containing yeast extract (TY)

This was studied by comparing the levels of components in the cell walls of bacteria grown in TY₂ medium (magnesium sulphate omitted) against those grown in TY medium. The procedure adopted was similar to that described for the previous experiment. The results are shown in table 19. The maximum increase was observed in the levels of teichoic acid (phosphorus), glucosamine, muramic acid, lysine and histidine. An appreciable decrease was found in the levels of serine, alanine, asparagine and methionine. No significant effect on the levels of glycine, glutamic acid and valine was observed.

(iv) The omission of sodium chloride from Thornton's medium containing yeast extract (TY)

This was studied in media TY and TY₃ (sodium chloride omitted), the experimental details being similar to those for the previous experiment. The results, comparing the two media, are shown in table 20. In TY₃ a pronounced decrease was found in the levels of lysine, serine, muramic acid, glucosamine, alanine, glycine and to a lesser extent threonine, (phosphorus), methionine, glutamic acid, teichoic acid / asparagine and valine. An appreciable increase was found in the level of histidine followed by leucine and isoleucine.

The omission of magnesium sulphate in TY medium,

Table 19. Comparative analysis of cell wall of *C. fascians*, strain 505 (smooth variant), grown in the presence and omission of magnesium sulphate from Thornton's medium with yeast extract

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium TY
	TY	TY ₂	
<u>Amino acids</u>			
Lysine	1.1551	2.1151	+ 83.0
Histidine	5.1009	7.4650	+ 46.0
Asparagine	8.8470	5.2006	- 41.0
Threonine	13.4751	9.8141	- 27.0
Serine	36.3679	1.7640	- 95.0
Glutamic acid	22.0210	22.4143	+ 1.8
Glycine	8.8624	8.9503	+ 0.99
Alanine	52.2343	26.2899	- 49.7
Valine	4.9520	5.0359	+ 1.7
Methionine	44.7983	26.5193	- 40.8
Isoleucine	3.8765	5.0403	+ 30.0
Leucine	7.2878	11.0825	+ 52.0
<u>Amino sugars</u>			
Glucosamine	7.7960	28.3330	+263.0
Muramic acid	28.2490	57.6130	+104.0
<u>Teichoic acid</u>			
Phosphorus	0.4920	5.3000	+977.0

TY = Thornton's medium with the addition of yeast extract.

TY₂ = Thornton's medium with the addition of yeast extract and the omission of magnesium sulphate.

Table 20. Comparative analysis of cell wall of *C. fascians*, strain 505
(smooth variant), grown in the presence and omission of sodium
chloride from Thornton's medium

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium TY
	TY	TY ₃	
<u>Amino acids</u>			
Lysine	1.1551	trace	- 991.0
Histidine	5.1009	11.2121	+ 120.0
Asparagine	8.8470	6.2321	- 29.6
Threonine	13.4751	8.1911	- 39.2
Serine	36.3679	9.9212	- 73.0
Glutamic acid	22.0210	13.6197	- 38.0
Glycine	8.8624	5.1737	- 41.6
Alanine	52.2343	26.5298	- 49.2
Valine	4.9520	4.3300	- 12.6
Methionine	44.7983	27.5804	- 38.4
Isoleucine	3.8765	4.2453	+ 9.5
Leucine	7.2878	8.8716	+ 21.7
<u>Amino sugars</u>			
Glucosamine	7.7960	3.5100	- 55.0
Muramic acid	28.2490	10.5360	- 63.0
<u>Teichoic acid</u>			
Phosphorus	0.4920	0.3250	- 34.0

TY = Thornton's medium with the addition of yeast extract.

TY₃ = Thornton's medium with the addition of yeast extract and the omission of sodium chloride.

produced large differences in the level of certain amino acids, amino sugars and teichoic acid (phosphorus) in the cell wall, when compared with those of cells grown in complete medium (TY). Differences here were generally greater than in other media when comparisons were made. Further, morphology has been previously shown to be profoundly influenced by the omission of magnesium ions from Thornton's medium. It was, therefore, decided to further investigate this effect.

(v) The omission of magnesium sulphate from different media

This was studied in those media where the levels of certain cell wall components had been shown to vary from the levels measured for nutrient broth (N.B.). In the first experiment, medium T and medium T₂ (magnesium sulphate omitted) were used. Twenty, 2 litre flasks were set up for each medium, each flask containing 200 ml of medium. Inoculation and preparation of cell wall samples for the quantitative analysis, were prepared as previously described. The results shown in table 21, give a comparison between the two media. It was again found that a pronounced increase in the levels of lysine, histidine and teichoic acid/ ^(phosphorus) and an appreciable decrease in serine, alanine, methionine, threonine and asparagine occurred. Although a pronounced decrease in the levels of glutamic acid, glycine, valine, isoleucine and leucine occurred in this medium, this decrease was not found in TY₂ medium. It was thought that this difference might have been due to the presence of yeast extract, a second factor affecting the levels of components.

In view of these results it was decided that to repeat this experiment using a third medium to see if the results

Table 21. Comparative analysis of cell walls of C. fascians, strain 505
(smooth variant), grown in the presence and omission of magnesium
sulphate from Thornton's medium

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium T
	T	T ₂	
<u>Amino acids</u>			
Lysine	0.6551	3.1004	+373.3
Histidine	3.8647	21.9337	+467.5
Asparagine	9.4553	7.0068	- 26.0
Threonine	13.8962	8.0851	- 41.8
Serine	31.4362	2.3182	- 92.6
Glutamic acid	30.3372	13.0334	- 57.0
Glycine	9.0905	2.0620	- 77.3
Alanine	49.0846	28.6138	- 41.7
Valine	7.7596	4.3295	- 44.2
Methionine	45.1400	26.0324	- 42.3
Isoleucine	3.8159	1.5351	- 59.8
Leucine	20.9239	12.5714	- 39.9
<u>Amino sugars</u>			
Glucosamine	(-)	(-)	(-)
Muramic acid	(-)	(-)	(-)
<u>Teichoic acid</u>			
Phosphorus	0.238	1.790	+652.0

T = Thornton's medium.

T₂ = Thornton's medium with the omission of magnesium sulphate.

(-)= not determined.

showed a similar pattern. In this, the experimental procedure was again similar to the previous one except that the medium used, synthetic medium (S) was that which showed the lowest levels of all wall amino acids in a previous experiment. The results for S_2 medium compared against those for S medium are shown in table 22. It was found that changes in the level of the components were generally similar to those observed between T and T_2 except for the level of leucine, which changed in a similar way to that observed between TY and TY_2 .

(vi) The effect of concentration of magnesium sulphate and sodium chloride in Thornton's medium containing yeast extract (TY)

In the previous work, the effect of complete omission of added magnesium sulphate from Thornton's medium was studied. A similar study was also carried out with sodium chloride. It was considered useful to investigate the effect of different concentrations of these salts from zero to that present in Thornton's medium on the composition of the cell wall.

The contamination of chemicals used in the media was not taken into account in describing the concentration of the salts. In Thornton's medium for example, the concentrations were compared with those in the complete medium and expressed as constituents added or omitted. Zero concentration, therefore, means that no addition of the salt was made to the medium, i.e. the salt was omitted from the medium.

1. Magnesium sulphate concentration

For each of the concentrations of magnesium sulphate (0 - 0.2 g/l), a batch of twenty, 2 litre flasks was set

Table 22. Comparative analysis of cell wall of *C. fascians*, strain 505
(smooth variant), grown in the presence and omission of magnesium
sulphate from the synthetic medium (S)

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium S
	S	S ₂	
<u>Amino acids</u>			
Lysine	0.2095	0.4313	+105.9
Histidine	0.7495	4.5467	+506.6
Asparagine	0.9555	0.2810	- 70.6
Threonine	3.1442	1.5549	- 50.1
Serine	7.9075	1.0412	- 86.8
Glutamic acid	11.9809	5.4339	- 54.7
Glycine	1.4076	0.6900	- 51.0
Alanine	15.6986	6.9633	- 55.6
Valine	1.1673	0.6554	- 43.9
Methionine	16.6142	4.5752	- 72.5
Isoleucine	0.8141	0.2052	- 74.8
Leucine	5.0103	8.0380	+ 60.0
<u>Amino sugars</u>			
Glucosamine	7.2730	16.1110	+121.5
Muramic acid	24.2420	67.5020	+178.5
<u>Teichoic acid</u>			
Phosphorus	0.6340	1.8750	+195.7

S = synthetic medium (S).

S₂ = synthetic medium (S) with the omission of magnesium sulphate.

up, each flask containing 200 ml of the appropriate medium as previously described. After inoculation with a standard inoculum, the media were incubated at 25°C for two days. The cell wall samples were prepared for quantitative analysis as previously described.

The results are shown in table 23. A similar change in the component levels was found at each of the three concentrations tested as that at zero concentration. It was observed that the majority of the components were affected progressively by the addition of the increasing amount of magnesium sulphate from 0 - 0.2 g/l. However, with glutamic acid, although at zero concentration no change occurred, at 0.1 and 0.15 g/l magnesium sulphate, a significant decrease in the level occurred. In addition, it was observed that the level of glycine also decreased at a magnesium sulphate concentration of 0.1 g/l.

2. Sodium chloride concentration

A similar experiment to that described previously was set up using different concentrations of sodium chloride from zero to 0.1 g/l. The results are shown in table 24. The changes in the levels of the components at each successive increase in sodium chloride concentration, were similar to the levels found at 0 g/l. With glutamic acid, however, the change in level at 0.025 g/l was greater even than that found at 0 g/l.

Table 23. Comparative analysis of cell wall of *C. fascians*, strain 505 (smooth variant), grown in Thornton's medium with yeast extract and at different concentrations of magnesium sulphate added

Cell wall components	Concentration of magnesium sulphate g/l added									
	0		0.050		0.100		0.150		0.200 (TY medium)	
<u>Amino acids</u>	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY
Lysine	2.1151	+ 83	0.8863	- 23	0.6560	- 43	0.6068	- 47.5	1.1551	
Histidine	7.4650	+ 46	6.4862	+ 27.2	5.9644	+ 16.9	5.2470	- 2.9	5.1009	
Asparagine	5.2006	- 41	6.8598	- 22.5	7.9623	- 10	7.7917	- 11.9	8.8470	
Threonine	9.8141	- 27	8.8106	- 34.6	8.1559	- 39.5	12.5458	- 9.7	13.4751	
Serine	1.7640	- 95	19.4395	- 46.5	27.1412	- 25.4	28.8960	- 20.6	36.3679	
Glutamic acid	22.4143	+ 1.8	24.9351	+ 13.2	14.6741	- 33.4	13.3207	- 39.5	22.0210	
Glycine	8.9503	+ 0.99	5.5340	- 37.6	3.4983	- 60	7.6041	- 14.2	8.8624	
Alanine	26.2899	- 49.7	47.3894	- 9.3	43.8801	- 16	52.2310	0	52.2343	
Valine	5.0359	+ 1.7	2.9385	- 40.7	3.9374	- 20.5	5.4887	+ 10.8	4.9520	
Methionine	26.5193	- 40.8	39.4518	- 11.9	54.7215	+ 22.2	57.4727	+ 28.3	44.7983	
Isoleucine	5.0403	+ 30	2.0128	- 48.1	2.3871	- 38.4	4.2107	+ 8.6	3.8765	
Leucine	11.0825	+ 52	12.0878	+ 65.9	8.9899	+ 23.4	11.6856	+ 60	7.2878	
<u>Amino sugars</u>										
Glucosamine	28.3330	+263	10.0100	+ 28	8.0000	+ 2.6	7.9100	+ 1.5	7.7960	
Muramic acid	57.6130	+104	46.7000	+ 65	30.8330	+ 9.2	28.3270	+ 0.3	28.2490	
<u>Teichoic acid</u>										
Phosphorus	5.3000	+977	4.4230	+800	0.7080	+ 44	0.4440	- 9.8	0.4920	

Table 24. Comparative analysis of cell wall of *C. fascians*, strain 505 (smooth variant), grown in Thornton's medium with yeast extract at different concentrations of sodium chloride added

Cell wall components		Concentration of sodium chloride g/l added							
		0	0.025	0.050	0.075	0.100(TY medium)			
<u>Amino acids</u>	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall	% increase or decrease against medium TY	μg component /mg dry wt. wall
Lysine	trace	-991	0.7855	- 32	0.8201	- 29	1.7953	+ 55	1.1551
Histidine	11.2121	+120	10.5401	+107	5.2279	+ 2.5	5.3507	+ 4.9	5.1009
Asparagine	6.2321	- 29.6	10.4648	+ 18.3	7.9673	- 10	2.7862	- 68.5	8.8470
Threonine	8.1911	- 39.2	10.3181	- 23.4	9.5362	- 29	11.7907	- 12.5	13.4751
Serine	9.9212	- 73	20.1620	- 44.6	22.5214	- 38	26.2750	- 27.75	36.3679
Glutamic acid	13.6197	- 38	5.8545	- 73.4	12.1446	- 44.9	21.4936	- 2.4	22.0210
Glycine	5.1737	- 41.6	4.2897	- 50	4.3309	- 51	8.7973	- 0.7	8.8624
Alanine	26.5298	- 49.2	25.8208	- 50	30.8420	- 41	41.6861	- 20.2	52.2343
Valine	4.3300	- 12.6	5.0025	+ 1	4.5303	- 8.5	3.8599	- 22	4.9520
Methionine	27.5804	- 38.4	38.5497	- 14	33.8226	- 24.5	31.7468	- 29	44.7983
Isoleucine	4.2453	+ 9.5	4.4856	+ 15.7	3.3546	- 13.5	1.7444	- 55	3.8765
Leucine	8.8716	+ 21.7	8.9015	+ 22	11.7726	+ 61.5	16.7391	+129.7	7.2878
<u>Amino sugars</u>									
Glucosamine	3.5100	- 55	5.3100	- 2.4	7.0100	- 10	7.6100	- 31.9	7.7960
Muramic acid	10.5360	- 63	13.0210	- 54	24.4590	- 13	28.6060	+ 1.3	28.2490
<u>Teichoic acid</u>									
Phosphorus	0.3250	- 34	0.3400	- 30.9	0.3910	- 20.5	0.4450	- 9.6	0.4920

(c) The effect of aeration

This was studied using TY₂ medium. This medium was selected because of the large differences in the level of components previously found.

Two batches each consisting of twenty, 2 litre flasks were set up, each flask containing 200 ml of TY₂ medium. The first batch was incubated statically at 25°C and the second incubated at 25°C on a reciprocal shaker for two days. Harvesting of the cells and the preparation of cell walls for the quantitative analysis was carried out as previously described. The results are shown in table 25. Aeration produced a large increase in the level of serine, glycine, histidine, lysine, asparagine, leucine and valine, and a decrease in the level of methionine, teichoic acid/ (phosphorus), glucosamine, glutamic acid and isoleucine. The level of muramic acid remained constant and no significant change in the level of threonine occurred.

When the results from the aerated TY₂ medium are compared with those from the static TY medium (previously reported in table 19), it can be seen that a larger increase in the levels of lysine and histidine accompanied by a great decrease in methionine, than that in the static TY₂ medium were recorded. The level of glutamic acid also decreased in the aerated TY₂ medium.

(d) The effect of penicillin

This was studied using twenty penicillin flasks, each containing 200 ml of nutrient agar containing penicillin at a concentration of 10 µg/litre, a concentration at which maximum effect on morphology had previously been recorded. The cultures and samples for the quantitative analysis were prepared as described in Materials and Methods. As there was no significant difference observed in the morphology and percentage of cell as cell wall, between cells grown in nutrient broth (N.B.) and

Table 25. Comparative analysis of cell wall of *C. fascians*, strain 505
(smooth variant), grown in static and aerated TY₂ medium

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium TY ₂ static
	Static	Aerated	
<u>Amino acids</u>			
Lysine	2.1151	6.7115	+217.3
Histidine	7.4650	39.8115	+433.3
Asparagine	5.2006	10.0210	+ 92.7
Threonine	9.8141	9.4326	- 3.9
Serine	1.7640	19.5286	+1007.1
Glutamic acid	22.4143	11.9809	- 46.6
Glycine	8.9503	18.7675	+109.7
Alanine	26.2899	34.9611	+ 33
Valine	5.0359	8.2769	+ 64.4
Methionine	26.5193	2.8572	- 89.2
Isoleucine	5.0403	2.9070	- 42.3
Leucine	11.0825	18.9492	+ 71
<u>Amino sugars</u>			
Glucosamine	28.3330	13.3330	- 52.9
Muramic acid	57.6130	57.6130	0
<u>Teichoic acid</u>			
Phosphorus	5.3000	1.3194	- 75.1

TY₂ = Thornton's medium with the omission of magnesium sulphate.

nutrient agar (N.A.) media, the results for the cell wall components estimated in the penicillin nutrient agar medium were compared against those in nutrient broth medium and are shown in table 26.

In the presence of penicillin, a pronounced increase was observed in lysine, asparagine and valine followed to a lesser extent by leucine, isoleucine, glycine and threonine. A large decrease was observed in the level of teichoic acid (phosphorus) and an appreciable decrease in both the amino sugars glucosamine and muramic acid and in glutamic acid, methionine and alanine. No significant change occurred in the levels of histidine and serine.

D. Electronmicroscopical examination

A short study was made to see if differences in chemical make-up were reflected as observable differences in the fine structure of the wall.

Three samples were taken for this study of C. fascians, strain 505 (smooth variant), grown in : a - nutrient broth (N.B.), b - Thornton's medium with yeast extract added (TY) and c - Thornton's medium with the addition of yeast extract and the omission of magnesium sulphate (TY₂).

The cells were fixed in Osmiumtetroxide according to Kellenberger et al. (1958), embedded in 'Araldite', sectioned and stained with lead citrate and uranyl acetate in the usual manner. The sections were examined using the Cambridge No. 1 electronmicroscope. This work was carried out in the Electronmicroscope Unit of the Botany Department at Imperial College of Science and Technology.

The resulting electronmicrographs, figures 3, a, b, c, following features : In a (N.B.), the cell wall is straight with three layers clearly observable. The septum is complete and well formed. In b (TY), the three layers of the wall are still distinguishable, but here the walls are not straight but slightly convoluted (wrinkled).

Table 26. Comparative analysis of cell wall of *C. fascians*, strain 505
(smooth variant), grown in nutrient broth (N.B.) and on
penicillin nutrient agar (P)

Cell wall components	µg component per mg dry weight wall		% increase or decrease against medium N.B.
	N.B.	P	
<u>Amino acids</u>			
Lysine	2.9188	12.6641	+334.0
Histidine	trace	trace	0
Asparagine	4.8145	24.5020	+409.0
Threonine	12.6677	18.5405	+ 46.4
Serine	18.1630	17.6802	- 2.6
Glutamic acid	41.5241	24.6743	- 40.6
Glycine	10.3922	16.1073	+ 50.0
Alanine	36.1612	27.6848	- 22.0
Valine	6.7783	13.4266	+ 98.1
Methionine	37.3499	24.5321	- 34.0
Isoleucine	5.8378	9.1840	+ 57.3
Leucine	16.6524	27.7997	+ 67.0
<u>Amino sugars</u>			
Glucosamine	7.7970	3.2000	- 59.0
Muramic acid	20.4730	10.5100	- 48.7
<u>Teichoic acid</u>			
Phosphorus	0.6700	0.0230	-966.0

N.B. = nutrient broth.

P = nutrient agar containing 10 µg/l penicillin.

In c (TY₂), the walls are completely distorted and convoluted. The septum is much wider consisting mainly of a wide electron-transparent central area. The inner layer of the wall is abnormal, the middle layer is much thicker in this sample and it seems as if it is pulled apart from the other two layers by this electron-transparent area. The differences in the septal regions of cells grown in the media a, b and c, are further illustrated in Figure 4.

Figure 3. A typical cell of C. fascians, strain 505

(smooth variant), grown in :

a - nutrient broth (N.B.). 30 000 x

b - Thornton's medium with yeast extract added (TY). 40 000 x

c - Thornton's medium with the addition of yeast extract and
the omission of magnesium sulphate (TY₂). 36 000 x

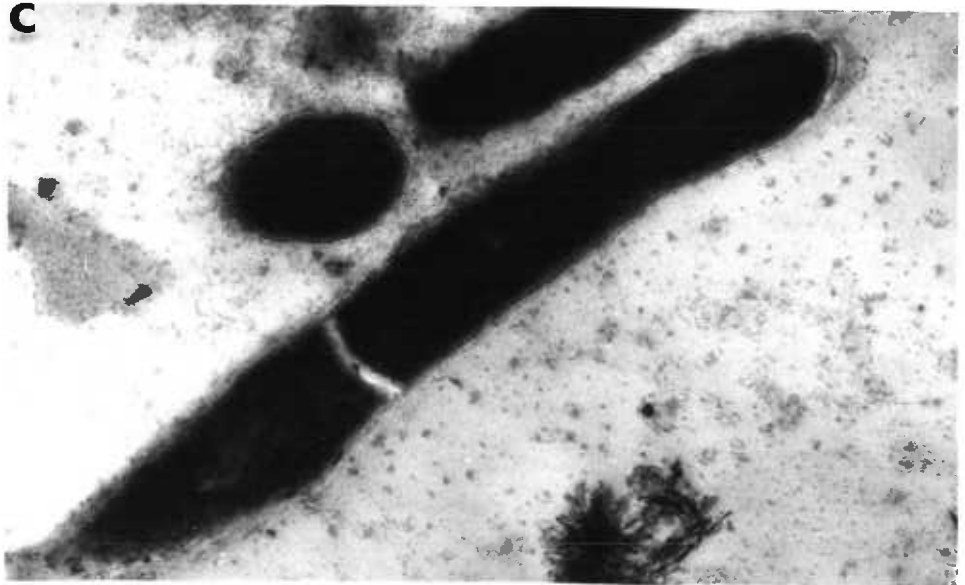
a



b



c



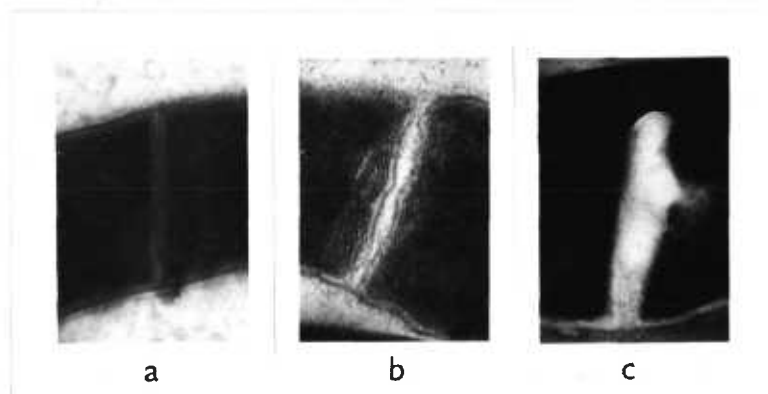


Figure 4. The septal regions of cells of C. fascians, strain 505
(smooth variant), grown in,

a - nutrient broth (N.B.). 33 800 x

b - Thornton's medium with yeast extract added (TY). 60 000 x

c - Thornton's medium with the addition of yeast extract and the omission
of magnesium sulphate (TY₂). 80 000 x

DISCUSSION

The observation by Lacey (1955) and Ahmed (1959), on the induction of filamentous forms in C. fascians under certain environmental conditions, has been demonstrated on both strains 197 and 505 and in part on the remainder of the strains used.

Variation in the effect of the different environmental factors on the growth and morphology of different strains and even between the smooth and the rough variant of the same strain was demonstrated in this work. This difference in the response of the smooth and the rough variant individually to the same treatment might be one of the reasons for the pleomorphic character of this organism when both variants are present in the culture under the same conditions.

Mandelstam and McQuillen (1973), reported that, bacterial growth response to physical and chemical factors can be explained in very general terms since these factors are numerous and most bacterial species exhibit different responses to different factors.

In this work, filament formation was achieved by omitting magnesium ions, sodium ions, or asparagine and was slightly encouraged by yeast extract. This process is also affected by factors other than those mentioned above, notably the presence of penicillin and the degree of aeration. Differences in the response of the organism to its environment were also represented in the amount of growth and in the whole cell dry weights, where it seems that heavier growth occurs in a complex medium, aerated medium and in the complete medium. These differences were also shown in the proportion of cell occurring as cell wall where more cell wall was found in the cells grown in complete medium and aerated medium although this differed between the specific media employed. Similar differences found earlier suggested that growth can be increased or decreased according to the addition or omission of one or more of the medium constituents (Mandelstam and McQuillen, 1973). Thus, in this work, the differences found in the response of the organism to its environment

might be explained on the basis of the conclusion drawn by Mandelstam and McQuillen (1973). They concluded that in complex undefined media such as nutrient broth or yeast extract, where the best growth occurred, most of the small molecular weight building blocks, peptides and other more complex substances, which would otherwise have to be synthesized, are already present. On the other hand, although in chemically defined media such as Thornton's medium (T) or Synthetic medium (S), containing only mineral salts and a single organic component as the carbon source, all the essential nutrients for the growth of the bacteria are present, the amount of growth would be expected to be reduced when any one of the major nutrients was omitted from the medium. However, growth may not be significantly affected by the omission of any one of the other medium constituents, but morphological changes might occur. In such situations reported earlier by other workers, where cell dimensions are not related in a simple way to the growth rate, morphological changes occurred. For example, cell division might be prevented without substantially inhibiting the synthesis of cellular elements, by low levels of many physical and chemical agents such as ultra violet (Walker and Pardee, 1968 and Adler and Hardigree, 1965) and penicillin (Tipper and Strominger, 1965 and Highton and Hobbs, 1972), which at higher levels are bacteriostatic. This leads to the formation of very long filaments which grow by synthesizing the cellular material at nearly normal rates (Mandelstam and McQuillen, 1973). In other cases the cell wall is weakened, leading to cells that are irregular in shape (bulged) (Rogers et al., 1970 and Cole et al., 1970). It is assumed that these situations are similar to that observed in C. fascians also the formation of filaments occurred under many of the environmental conditions studied. According to Mandelstam and McQuillen (1973), bacterial growth rate has an optimum temperature and pH, which varies with the species, being lower on each side of the optimum. Therefore, the range of temperature over which a bacterium can grow must also be the range over which its enzyme machinery can function, so that the optimum temperature for growth results from a balance between the increased

rate of thermal inactivation of the enzymes and the increased rate of the synthetic reactions. It is probable that the changes found in growth and morphology of C. fascians, when subjected to variations in temperature of incubation were to some extent the result of modifications in the enzyme functioning of the cells. Although the intracellular pH of the bacteria is different from its optimum pH value for growth, a drop in the pH of the medium creates an acidic environment which eventually leads to a cessation of the reactions occurring in the cell (Mandelstam and McQuillen, 1973). In this work, although no significant change in the pH of the media was noticed except in the synthetic medium (S), and no significant change in the growth occurred, a large decrease in the amount of cell wall amino acids was observed. The change in the pH in this medium is believed to be due to the presence of ammonium chloride (Thornton, 1922). The average number of nuclear bodies per bacterium is directly proportional to the growth rate which is in turn likely to be related in some manner to the process of cell division. At slower growth, bacteria have a higher amount of cell membrane and cell wall than when growing fast. Consequently, at a fast growth rate synthesis of the cell envelopes might be limited. The cells then adjust to the relatively small amount of envelope material by increasing in volume and hence decreasing the ratio of surface to volume (Mandelstam and McQuillen, 1973). In this work, a similar case appears to happen during filament formation, where the number of septa and nuclei increased indicating that an increase in volume as well as cell division is occurring, although no separation of the cells takes place. This was assumed from the observed increase in the number of unit cells per rod. Although the size of the unit cell increased, the cell wall portion is decreased, suggesting that there is some sort of limitation on the synthesis of the cell envelope. The cells have adjusted to enclose the increased cytoplasmic volume by increasing the surface area through stretching of the wall and thus filaments are formed. This will be further discussed later.

Many workers have studied the cell wall composition of different bacteria, after purifying and digesting the cell wall with ribonuclease, trypsin and pepsin, in order to remove "contaminating" proteins and RNA (Salton, 1953 and Cummins and Harris, 1956 a.b., 1958). The aim of the purification procedure is to remove cytoplasmic contamination and in this work, it was demonstrated that different purification treatments did not affect the composition of the cell wall, although there was a general decrease in quantity of some of the components when pepsin was used in addition to ribonuclease and trypsin. When ribonuclease and trypsin only were used, a similar decrease occurred when compared with washing alone. In this organism, removal of cytoplasmic contamination only occurred when pepsin was used, as shown by the fact that no protein was detected and proline and arginine were shown to be absent following treatment.

Brookes et al. (1959), have reported the presence of only three principle amino acids in cell walls treated with trypsin and ribonuclease and although all the common amino acids were detected, the yield of purified cell wall was only half that of the crude preparation. It was found in this work that none of the amino acids present in the untreated control were lost on treatment with trypsin, except arginine which was reduced to a trace amount.

Although Mandelstam and Strominger (1961), have reported a 40% loss in teichoic acid when pepsin is used under acid conditions, the differences in levels of teichoic acid obtained from cells grown under different environmental conditions were still very large and thus it was considered that any loss in teichoic acid sustained was acceptable in that the final result was not obscured.

Pepsin has been used in the purification procedure by most of the workers who have studied the cell wall composition of Corynebacterium (Holdsworth, 1952; Cummins and Harris, 1956 b; Cummins, 1962 and de Herrera and Moreno, 1969). All the analyses that have been made of the cell wall of Gram positive bacteria so far examined have shown that

large amounts of alanine and glutamic acid are present plus a dibasic amino acid which is either lysine or di-aminopimelic acid but never both in the same wall. Often other additional amino acids like aspartic acid, glycine, serine and threonine are found, but only one of these may be present in noticeable amounts (Rogers and Perkins, 1968).

Holdsworth (1952), Cummins and Harris (1956a) and Cummins (1962), have shown in their study of the composition of the cell wall in different species of Corynebacterium, only three amino acids, alanine, glutamic acid and glycine and in some cases only alanine and glutamic are present plus a dibasic amino acid. No information was provided as to the presence of any other amino acids in small amounts. However, they expressed the importance of the presence of one or another amino acid in each species, for example, glutamic acid in C. glutamicum (Somerson and Philips, 1961; Matsuo et al., 1966; Birnbaum and Demain, 1969 and Chatterjee and Perkins, 1966). Although in the analysis of the cell wall of C. michiganensis made by de Herrera and Moreno (1969), aspartic acid, glutamic acid, glycine, threonine, valine, leucine and isoleucine were regarded as major constituents and alanine, methionine and lysine as minor constituents, they specified particularly the presence of valine in large quantities. Their analysis of the whole cell revealed the presence of the above amino acids with diphenylalanine, tryptophan and arginine in addition.

In both analyses de Herrera and his colleague, found the sugars arabinose, glucose, galactose, mannose, ribose, rhamnose and the amino sugar glucosamine. In this work, the cell wall of C. fascians strain 505 (smooth variant), was found to contain glutamic acid, alanine and methionine in large quantities followed by serine, isoleucine, threonine and glycine, and finally valine, leucine, asparagine, histidine, plus lysine as a dibasic amino acid in minor quantities.

Arginine, although present in the unpurified cell wall, was not present after the cell wall had been purified. This suggests that arginine is of cytoplasmic origin in this organism. According to de Herrera and Moreno (1969), the cell wall composition of bacteria is useful as a feature for classification. Thus they considered that the amino acids present in the cell wall seem to be characteristic of the genus, and that the sugar and amino sugars characterize the species, with the exception of ribose which has a special significance in the genus Corynebacterium. A comparison on this basis shows that, except for serine and histidine found in the purified cell wall of this organism, the other amino acids present were also found by de Herrera and Moreno in the cell wall of C. michiganensis. Arginine is present in the two organisms in the whole cell analysis. Also, the amino sugar glucosamine, is found in both the whole cells and the cell walls of the two organisms.

In addition, the amino sugars muramic acid and galactosamine were found in the cell wall of the organism studied in this work. The presence of galactosamine in this organism is of special significance as it has been pointed out by Cummins (1962), that muramic acid and glucosamine are present in the majority of Corynebacterium species studied, galactosamine being present in the cell walls of C. pyogenes and C. hofmannii l.H.

The cell wall teichoic acid found in the organism studied in this work, was identified as a ribitol polymer.

In this work on C. fascians, the large amount of methionine present and the additional amino acids, serine and histidine found, distinguishes this organism from the other species of Corynebacterium studied earlier. The fact that one amino acid characterises a species has been pointed out previously by Rogers and Perkins (1968), and de Herrera and Moreno (1969).

In addition, the ratio of the molar amounts of methionine, serine and histidine present in this organism in relation to the other amino acids

might well be of significant importance in the formation of the peptide structure and might affect their organization with the amino sugar to form some sort of structurally unique peptidoglycan polymer for this organism. The latter is in agreement with the suggestion of Rogers and Perkins (1968) and Perkins (1965), who demonstrated that the cell wall peptidoglycan of C. poinsettiae contains approximately equimolar proportions of glutamic acid, glycine, alanine homoserine and ornithine.

Breed et al. (1957), reporting on Corynebacterium, remarked that the genus was "generally highly exacting in their nutritional demand". Keddie et al. (1966), considered that a correlation existed between the cell wall composition and the growth factor requirements. In this work, the level of each of the cell wall components varied in different media as well as in media with different constituents varied individually. It appears from the results in this work that the environmental conditions determine the morphology and size of the organism as well as its cell wall composition. The latter is in agreement with the findings of Ellwood and Tempest (1967), who found that in B. subtilis, the amount of teichoic acid in walls, in contrast to their general composition, can be markedly influenced by the composition of the medium and probably by other factors concerned with growth conditions. This has also been reported by Mandelstam and McQuillen (1973). In this work, the proportion of the components of the cell wall varied in all of the media used. In general, the most significant changes were found in teichoic acid, amino sugars and the amino acids lysine, histidine, serine, glutamic acid, alanine and methionine in that order, less significant changes being found in the other amino acids. However, in any one medium, a change in other environmental conditions resulted in a more specific change in the composition of the wall which did not follow the general pattern above.

In general, the differences found in the levels of these cell wall components under the different environmental conditions studied and especially the relation between the changes occurring in methionine,

histidine and serine with those occurring in the rest of the wall components might be explained as the result of some diversion in the metabolic pathways of the biosyntheses. It is accepted that the metabolic pathways of biosynthesis of valine, leucine and isoleucine from pyruvate are closely related by proceeding through the common intermediate, α -oxoisovaleric acid. The transformation of pyruvate to the α -oxoisovaleric acid requires Mg^{2+} and a reduced pyridine nucleotide. Threonine is connected to the above metabolic pathways where it is converted to the key intermediate in the biosynthetic route to isoleucine. Also, the biosynthesis of threonine, methionine and lysine from aspartic acid is controlled by the common intermediate aspartic- β -semialdehyde. The conversion of the intermediate to the appropriate amino acid is catalysed by aspartokinases. In addition, the biosynthesis of lysine in bacteria, is derived from pyruvate and aspartate.

In this work, when Mg^{2+} was reduced by the omission of magnesium sulphate from the media, it might be reasonable to expect a limitation in α -oxoisovaleric acid produced from pyruvate to occur. This in turn, could reduce the availability of this intermediate for the biosynthesis of valine, leucine and isoleucine, therefore, a reduction in these amino acids would occur. It was found in this work, however, that Mg^{2+} omission, gave no such decreases in these amino acids. Instead, methionine and threonine was decreased. A possible explanation for this could be that through threonine, there might have been an alternative route for their synthesis. Several routes for the biosynthetic pathway of other amino acids were known to occur in other organisms. For example, alanine, could be synthesized either from pyruvate or aspartic acid. In both alternatives, alanine synthesis, would be connected to the amino acids synthesis proceeding from either source. In this situation, where a common intermediate or source is shared in the synthesis of more than one amino acid, the amount of each amino acid will be dependent on the availability of the source and the activity of the enzymes catalysing the

reactions. The latter could be influenced by the environment. Thus it should follow that the more alternatives in the synthetic route available, the less the probability of it being blocked. A similar situation might be occurring in this work, when an increase in lysine was found on the omission of magnesium sulphate and a decrease in alanine on the omission of asparagine, sodium chloride and magnesium sulphate and the presence of penicillin in the medium. This was a reason for the suggested significance of the presence of serine in this organism, where large changes in this amino acid were found to occur under different environmental conditions. These changes were also accompanied by changes in glutamic acid, methionine, glycine, threonine, alanine and histidine.

Glutamic acid is also known to be synthesized from glutamine. The latter acts as a donor of nitrogen atoms for the synthesis of eight end products including, glycine, alanine, histidine and glucosamine.

Because of the interrelation of the biosynthetic pathways of the wall amino acids, it should follow that a blockage or a decrease of one amino acid will allow more precursors to become available for synthesis of other amino acids.

On the other hand, the ability of amino acids to be synthesized by more than one route, might be responsible for the reduced possibility of complete blockage of its synthesis. In practice, however, these interrelations are more complex and a complete understanding of these changes would need a complete knowledge of the biosynthetic pathways of every amino acid involved. One of the most obvious conclusions to be drawn from a survey of the literature on microbial pools - particularly amino acid pools - is that they are extremely variable and markedly dependent on the nutritional complexity of the growth medium (Holden, 1962; Brown and Rose, 1969 a,b; Herbert, 1961; Neidhardt, 1963 and Tempest, 1970). This diversion in the metabolic pathways suggested, may be explained on the basis of the activity of the enzymes involved in the biosynthesis e.g. the enzyme catalyzing the reaction glutamine to glutamate needs ATP and has

an absolute dependence on Mg^{2+} , as do all the kinase reactions. Regular growth and cell division in bacteria require the operation of accurate control processes in the regulation of the amount and composition of wall material. From the literature on the structure and the relationship between the syntheses of teichoic acid and peptidoglycan, where a decrease in rate of synthesis of one polymer is brought about by simultaneous synthesis of the other, it may be concluded that there is a common component which is rate-limiting for both.

It is concluded that this common component is undecaprenol phosphate (lipid phosphate), whose amount controls the rate of synthesis of wall polymers (Anderson et al., 1972). This was supported by the observation that bacteria possess two enzymes which are able to control this common lipid phosphate in the membrane; a kinase for the phosphorylation of undecaprenol, and a phosphatase for its hydrolysis. Thus the amount of phosphate could be controlled by regulating the amounts or activities of either or both of these enzymes (Higashi et al., 1970). The authors further explained that regulating the amount of lipid phosphate, which controls the wall synthesis, would not itself effectively determine the composition of the wall, as changes in wall composition may depend upon the composition of the growth medium and other external factors. The fact that these changes can be brought about rapidly, indicates the existence of sensitive control mechanisms for such regulation. The possibility that a constant cationic environment is required for the enzymes involved in the synthesis of nucleotide precursors has been suggested (Glaser et al., 1966 and Archibald and Coapes, 1971). An alteration of this cationic environment might effect the synthesis of the wall polymers by its influence on the synthesis of the nucleotide precursors. The nucleotide precursors provide sugar residues and related components for transport through the membrane by lipid intermediates used for both peptidoglycan and teichoic acid synthesis (Hughes et al., 1971).

When no complete inhibition of any wall component occurred, by the

environment in this work, this suggests that there is some form of restriction on the return of lipid phosphate to the common pool. This has been interpreted as meaning that the lipid phosphate remains associated with the enzyme system for the synthesis of a polymer until the whole chain has been completed, and only then is it returned to the common pool.

Also, it is suggested that the multi-enzyme systems responsible for the synthesis of the several wall polymers are in fairly close proximity to each other for the purpose of sharing the lipid phosphate (Baddiley, 1972 and Watkinson et al., 1971).

In this work, the more specific changes in the wall composition found under each of the environmental conditions studied, could be interpreted individually. For example, when Mg^{2+} was omitted from the media, a high level of teichoic acid was produced. This may have been caused by a restriction in the synthesis of peptidoglycan giving an increased availability of the common lipid intermediate for teichoic acid synthesis. This enhanced teichoic acid synthesis would allow extra phosphate sites for maximum binding of the cations.

This suggestion has already been proposed by Baddiley (1972), who interpreted the increased content of wall teichoic acid of bacteria grown in a chemostat under Mg^{2+} limitation as a means for these bacteria to scavenge the limited supply of bivalent cations.

In the cell envelope of Gram positive bacteria, teichoic acids are present which are strongly acidic polymers containing phosphate (Archibald and Baddiley, 1966). These are distinctly located covalently linked to the peptidoglycan of the cell wall (Baddiley, 1972) and associated with a glycolipid on the outer surface of the cytoplasmic membrane (Coley et al., 1972 and Baddiley, 1972).

It is found that the wall teichoic acid is responsible for the ability of the cell wall of Gram positive bacteria to bind divalent cations (Heptinstall et al., 1970), the membrane teichoic acid mediating an interaction between Mg^{2+} , bound to the wall, and the cytoplasmic membrane,

thus maintaining the correct optimum ionic environment for the activity of cation-dependent biosynthetic membrane bound enzymes (Hughes et al., 1971, 1973).

Earlier, Ellwood and Tempest (1969), noticed the accumulation of bound amino sugars that are soluble in trichloroacetic acid under Mg^{2+} and Mn^{2+} starvation, on the other hand, peptidoglycan synthesis was inhibited by 35 - 50% under conditions of Mg^{2+} starvation. The amount of N-acetylhexosamine that accumulates is balanced exactly by the associated fall in peptidoglycan synthesis. Garrett (1969 a, b), demonstrated that the accumulated N-acetylhexosamine compound is utilized in peptidoglycan synthesis. Their explanation for this is that the protoplasmic membrane loses some of its structural integrity when Mg^{2+} is lacking, and that the activity of the membrane bound peptidoglycan synthesis is precluded.

Also, there was a general decrease in all the wall components, and especially a large decrease in the major amino acids and amino sugars, when sodium ion was omitted from the media. This decrease might also be due to a restriction in the synthesis of both cell wall polymers, peptidoglycan and teichoic acid. This in turn might also indicate a limitation occurring in the availability of their common lipid intermediate due to the inactivation of the membrane bound enzymes involved in their synthesis because of the lack of a correct ionic environment. Thus, there is an interrelation between the sodium and magnesium ions of the medium and the teichoic acid formed. Heptinstall et al. (1970), demonstrated that the sodium ion concentration in the medium which is inversely proportional to the alanine ester residues, is directly proportional to the teichoic acid and to the Mg^{2+} bound in the cell walls. This modification of the structure of the teichoic acid was presumably to give a more effective binding of the bivalent cations in competition with the sodium ions. Teichoic acid was found to increase as sodium chloride concentration increased (Ellwood, 1971), also, the organism was found to bind increasing amounts of Mg^{2+} as sodium chloride concentration increased in the medium (Meers and Tempest, 1970).

The correlation between Mg^{2+} binding capacity and the teichoic acid content of the cell walls was demonstrated by Ellwood (1971). They concluded that it was the phosphodiester residues affecting the binding of the Mg^{2+} to the cell wall.

The binding process of ions to teichoic acid, can be explained according to the suggestion made in the recent important work of Baddiley and Hancock (1973), on identifying the interactions involved in binding Mg^{2+} to teichoic acid in the bacterial envelope. They showed that although electro static interaction between the cations and negatively charged phosphate groups is a prominent feature, interaction with other components of the envelope can not be ruled out.

Two forms of binding of Mg^{2+} ions to teichoic acid molecules could be demonstrated, i.e. strongly bound and weakly bound. The strongly bound situation was interpreted as a bond between a Mg^{2+} and two phosphate residues on the teichoic acid molecule. The weakly bound situation occurred when the teichoic acid molecule contained alanine. In this case, it was suggested that the $-NH_3^+$ of the amino acid could neutralize the charge on an adjacent phosphate group thus allowing only one of the pair of phosphate groups to interact with the Mg^{2+} ion. Thus in this case, the Mg^{2+} ions might compete less successfully with other cations for the anionic sites on the polymer and thus monovalent ions as well as Mg^{2+} could be expected to be present in preparations from alanylated teichoic acid.

It was thought possible that due to the flexibility of the teichoic acid molecule and the freedom of movement of the alanyl group a dynamic state could exist in which the amino group alternates between several phosphates to give a change in the position on the polymer chain at which Mg^{2+} could bind strongly to two phosphates and thus a net movement of strongly bound Mg^{2+} could occur.

This behaviour would be consistent with the known function of teichoic acid in walls and membranes of providing and maintaining an appropriate divalent cationic environment.

As teichoic acids are found to be indispensable (Ellwood and Tempest, 1968), then it would be reasonable to expect that the inhibitory action of the antibiotic penicillin might result from interference with the synthesis of teichoic acid. This interference was found to occur in this work, where a large decrease especially in teichoic acid, alanine and amino sugars were found. This has been explained by other workers as follows :

In the terminal stage of the biosynthetic pathway of peptidoglycan, transpeptidation occurs, which is a peptide cross-linking reaction, where the carboxyl-terminal of D-alanyl - D-alanine group of one subunit and the free amino group of lysine in an adjacent D-alanine residue are linked. A transpeptidase enzyme is involved. Penicillin, inhibits transpeptidation by blocking the incorporation of monomer units into the final cross-linked product. This has been explained by the fact that the β -lactam ring of penicillin acts as an analogue to the normal substrate (the D-alanyl-D-alanine end of the pentapeptide), thereby irreversibly inactivating the enzyme (Tipper and Strominger, 1965 and Highton and Hobbs, 1971).

The differences in morphology and the levels of components, found in this work, demonstrate how even small changes in the environment can lead to quite striking alterations in both the levels of cell wall components and the gross morphology of the organism. Variation in the cell size was found when cells were grown under a variety of media permitting different rates of balanced growth. This suggests that with regard to structure and composition, bacteria can undergo phenotypic changes of great magnitude (Mandelstam and McQuillen, 1973).

Interchange between the levels of cell wall components, found in this work in the induced filaments, might be the explanation for a distortion in the structure of the cell wall polymers, especially in the binding of the teichoic acid and peptidoglycan, giving rise to a stretched, weakened (distorted) wall in the filamentous cells.

The possible influence of wall teichoic acid on lytic enzymes in the wall which are considered to be associated with the process of cell division is also described by Davie and Brock (1966) and Tomasz (1968).

All the filamentous cells observed in this work, were highly septate. This in turn was found to be accompanied by specific alterations in teichoic acid and alanine. A decrease in alanine was constantly found to occur in the cell walls of filaments induced under different environmental conditions.

Teichoic acid was also found to decrease when sodium ions were omitted and in the presence of penicillin. In contrast, teichoic acid increased when magnesium ions were omitted. The connection between the availability of these two wall components and filament formation was also found under aerated conditions. Aeration was found to reduce both the increase in teichoic acid and the decrease in alanine caused by the omission of magnesium ions from the medium. A decrease in the formation of filaments also occurred. This relationship, could be explained as follows : The lack of alanine, which is found to be essential for the activity of the lytic enzymes (mucopetidases), might be a possible cause of the failure of the cells to split, thus forming filaments. This was also suggested by Davie and Brock (1966).

Also, there was a linear relationship between the maximum rate of lysis and the concentration of Mg^{2+} (Brown and Melling, 1969). This was interpreted to be a consequence of the alteration in the cation balance which may cause changes in the activity of the lytic enzymes.

The teichoic acid residues have also been found to be essential for both the binding of the cations and the movement of these cations (especially the Mg^{2+}) to the membrane bound enzymes (Archibald and Baddiley, 1966). The activity of these enzymes are cation (Mg^{2+}) dependent (Hughes et al., 1971, 1973). The irreversible effect on filament formation brought about by the omission from the medium, of asparagine, sodium ions or magnesium ions, might be explained by the findings of Hughes et al. (1971), that a membrane bound enzyme system that required Mg^{2+} for optimum activity was fully activated by the Mg^{2+} bound to the endogenous teichoic acid and was insensitive to externally added Mg^{2+} .

It was found earlier that the growth of rod-shaped cells involves two processes, one is elongation of the cylinder and the other is the formation of a cross wall in the form of a circular disc which then splits in two increasing in area to form the rounded ends of the daughter cells. Cell wall growth is thought to involve the action of mucopeptidases which break both the sugar chains and the peptide cross-links to permit the insertion of additional molecules (Weidel and Pelzer, 1964). In the filaments found in this work, an interference in this insertion process might have occurred through a disturbance in the activity of these lytic enzymes, as suggested earlier.

Inactivation of these enzymes has also been suggested by Tipper and Strominger (1965) and Highton and Hobbs (1971), to explain the uninhibited growth where a low concentration of penicillin is used. Penicillin was found to inhibit the separation of cells after cross wall formation, and as a result the cells grew in long chains. They also found that at this concentration of penicillin, inhibition of teichoic acid and a change in the amino acid and amino sugar composition occurred. This in turn causes the covalently linked teichoic acid and the peptidoglycan to produce an abnormal structure, in which cross-linking between peptidoglycan chains is prevented.

They also suggested that the wall synthesized in the period after the addition of low concentrations of penicillin, stretched to accommodate the increase in cell mass, while the wall material accumulated at the plasma membrane.

An accumulation of material around developing cross walls has also been observed earlier by Fitz-James and Hancock (1965), after the addition of penicillin. This accumulation was interpreted by Strominger (1967), as unlinked mucopeptide. A similar explanation might be put forward to account for the electron-transparent thickened area seen, especially around the septa, in electron micrographs, of bacteria grown in the absence of added magnesium ions. This might be interpreted as the

accumulation of the unbound wall substituents. The wrinkled walls observed in the same treatment might be explained as the weakened walls. This accumulation of the unbound wall material was suggested to be responsible for changing the density profile of the walls examined under the electron microscope (Highton and Hobbs, 1971).

The stretching process has been considered earlier by Murray et al. (1959), to be responsible for the thin walls produced by bacteria growing in the presence of penicillin. In the filaments observed in this work, although an increase in the length of the unit cell occurred, the proportion of the cell as cell wall decreased. A similar stretching process could be considered here to explain the increase in length by the smaller amount of cell wall present. It was suggested by Perkins (1963), that these thin walls may have resulted from the removal of material by autolytic enzymes, i.e. mucopeptidases.

However, it seems possible that such enzymes could have simply "loosened" the structure allowing stretching.

The effect of penicillin has also been used to study wall synthesis in B. licheniformis and B. cereus (Highton and Hobbs, 1972). From these studies it has been suggested that longitudinal extension is by addition of material to a large and continuously increasing number of growing points uniformly distributed over the cylindrical surface. Addition is only in the longitudinal direction so the cell diameter remains constant. Cross walls grow by addition to their inner edge. After splitting the rounded ends of the daughter cells are formed by continued addition at the centre.

The effect of ionic strength and high temperature was also studied by Rogers et al. (1970) and Cole et al. (1970). In these studies, the loss of wall structure and its reappearance on returning to low temperature was very similar to that described above for exposure to penicillin. Bulges, which developed in the region of septa, were thought to result from weakened wall produced by the continued action of mucopeptidases

after transpeptidase inhibition. A similar situation was noticed in the thin walled filaments found in the presence of penicillin and also in the bulged, distorted septal region of filaments observed in this work under the electron microscope upon the omission of added magnesium ions.

Further, evidence for separate mechanisms of division and elongation, or possibly temporal control of a single mechanism, has been demonstrated by the study of Higgins and Shockman (1970), where they found septum formation appeared to begin and then to stop for a while during cell elongation, and finally to be completed. A similar situation might be occurring, which might explain the filaments observed in this work, where a failure in cell separation appeared to occur, but cell division and cell elongation continued. Higgins and Shockman (1970), also found that wall synthesis occurred only in the septal region. This is a significantly different mechanism from that suggested by the above data, but it may in part explain why one cell is coccoid and another cylindrical, since this must be determined by the nature of the constituent molecules and by the assembly mechanism.

It is probable that a similar situation occurs in the formation of filaments observed in the work reported in this thesis, where variation in the rod length and in the cell unit length within the same culture occurred. Also in the electron micrographs, an electron-transparent area was observed in the septal region of filaments produced by the omission of added magnesium from the medium. This might be interpreted as unbound wall precursors, as cell wall synthesis is occurring in the septal region.

More extensive study is necessary to determine the structure and biosynthetic routes of the major wall polymers and the mechanism of division and elongation in this organism. Unless this is established, no conclusion may be drawn as to the degree of distortion of the wall structure by the disorganisation of the substituted components which will

explain variations in wall composition with respect to the proportion of peptidoglycan and teichoic acid and its relation to changes in growth conditions.

SUMMARY

1. The present knowledge of the influence of the environment on bacterial cell composition and in particular cell wall composition, has been reviewed. Attention has been specifically directed to the cell wall of Gram positive bacteria and in particular that of the genus Corynebacterium.
2. The growth of a number of strains of C. fascians was observed on different media and under different environmental conditions (aeration, temperature, etc.).
3. The formation of filamentous cells was recorded as an indicator that changes in the bacteria, and particularly the cell wall were occurring.
4. A staining method has been modified for combined cell wall and nuclei and used for the measurement of cell length, number of septa per rod and number of nuclei per cell.
5. Considerable variation was observed between strains, including the rough and smooth variants of strain 505, in their response to different environmental conditions.
6. Temperatures above optimum were found to reduce growth, without filament formation.
7. Penicillin induced the formation of filaments at a concentration of 10 µg/litre in nutrient agar.
8. Staining revealed that although the length of the rod and unit

cell differed between strains and variants of the same strain grown under the same conditions, as well as under different conditions, the length of unit cell was higher in penicillin nutrient agar medium with strain 505 (smooth variant).

9. In studies on the effect of specific constituents of the medium on growth and morphology, Thornton's medium with yeast extract was used. Although filaments were observed when either one of three constituents namely asparagine, magnesium sulphate and sodium chloride were omitted, variation in response occurred with different strains and variants of the same strain, being the highest with the omission of magnesium sulphate in the static cultures of strain 505 (smooth variant). No significant effect on the amount of growth was observed when any one of these constituents was omitted.
10. The omission of the cations, Mg^{2+} and Na^{+} was found to be responsible for the induction of filaments.
11. Yeast extract was found to encourage both growth and cell elongation.
12. Aeration was found to encourage growth, but discourage filament formation.
13. Cell disruption was brought about by the use of a Mickle Tissue Disintegrator, after recovery of cell wall pieces, treatment with trypsin, ribonuclease and pepsin was included in the cell wall purification procedure adopted, although this did lead to consistently low yields of cell wall material.
14. The preparation of cell as cell wall was lower in the cells grown

in media other than nutrient broth or nutrient agar. This preparation varied between strains grown under the same conditions as well as under different environmental conditions.

15. Contamination of the wall with protoplasm was detected by the presence of proline.
16. The cell wall of C. fascians, strain 505 (smooth variant), was shown to contain ribitol teichoic acid, glucosamine, galactosamine, and muramic acid as amino sugars and the amino acids histidine, asparagine, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine and leucine plus lysine as a dibasic amino acid.
17. An analysis was made of the wall of cells of strain 505 (smooth variant), grown in nutrient broth, Thornton's medium, with and without yeast extract (TY and T respectively) and synthetic medium (S). The level of each of the cell wall components varied with the medium used, in synthetic medium, the level of estimated components being low. Although levels in both TY and T media were lower than in nutrient broth, the glucosamine level remained constant.
18. An analysis was also made of the wall of cells grown under the environmental conditions found to produce good filamentation i.e. nutrient agar plus 10 µg/litre penicillin and Thornton's medium plus yeast extract with the omission of asparagine, sodium chloride or magnesium sulphate.
19. In general, major differences were observed in the levels of lysine, serine, histidine, glutamic acid, alanine, methionine, glucosamine, muramic acid and teichoic acid.

20. The omission of asparagine results in an increase in leucine, isoleucine, valine, teichoic acid and glucosamine and a decrease in histidine, serine, alanine and methionine.

The omission of magnesium sulphate resulted in a large increase in teichoic acid, glucosamine, muramic acid, lysine and histidine accompanied by a large decrease in serine, alanine, asparagine and methionine.

And the omission of sodium chloride resulted in a pronounced increase in histidine, leucine and isoleucine accompanied by a decrease in lysine, serine, muramic acid, glucosamine, alanine, glycine, threonine, glutamic acid, teichoic acid, asparagine and valine.

21. Omission of magnesium sulphate in both T_2 and S_2 media resulted in similar cell wall modifications to those observed on magnesium sulphate omission in Thornton's medium with yeast extract except for the levels of glutamic acid, glycine, valine, isoleucine and leucine.
22. The omission of yeast extract from Thornton's medium resulted in a substantial decrease in the levels of teichoic acid, lysine, histidine and an increase in the levels of leucine, valine and glutamic acid.
23. Aeration of the TY_2 medium resulted in a large increase in the levels of serine, glycine, histidine, lysine, asparagine, leucine and valine and a decrease in the levels of methionine, teichoic acid, glucosamine, glutamic acid and isoleucine.

24. The changes in the levels of cell wall components at each successive increase of the salt concentration were similar to those observed at zero concentration. However, the amount of a specific amino acid was observed to vary independently according to a particular salt concentration.
25. Penicillin also affected the level of the cell wall components, an increase being observed in lysine, asparagine, valine, leucine, isoleucine, glycine and threonine accompanied by a decrease in teichoic acid, glucosamine, muramic acid, glutamic acid, methionine and alanine.
26. The relationship between serine and histidine and the major cell wall components in this organism is of special interest in the biosynthetic pathways leading to the formation of peptidoglycan and teichoic acid.
27. The results of this work were considered in relation to the work of other investigators on a) the role of magnesium and sodium ions in the synthesis of cell wall polymers, b) the mode of action of penicillin in the transpeptidation step of peptidoglycan synthesis, c) the mode of cell elongation and separation, d) the biosynthetic pathways of cell components and their relation to the environmental conditions.

These investigations are considered in an attempt to explain changes in the composition of the cell wall occurring under different environmental conditions and to link these to changes in morphology.

Some suggestions were made concerning the direction future work might proceed.

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CORRECTIONS

- (a) The figures quoted in tables 15 - 26 inclusive should be amended to one decimal place only to give a true understanding of accuracy.
- (b) On page 53, line 19, delete "and heated at 60° for 10 min".
- (c) Delete in tables 14 - 26 inclusive all reference to asparagine and results relating to it.
- (d) The values quoted for teichoic acid should be multiplied by a factor of 10.
- (e) The identification of methionine as carried out was incomplete.
- (f) The analysis of amino sugars should be viewed with suspicion.
- (g) The reference to Ellwood and Tempest on page 122 line four should be deleted and the work described attributed to Garrett (1969a).
- (h) Although the data presented does not clearly identify the presence of ribitol teichoic acid, this was established during examination of the candidate.