Growth and Division of *Bacillus subtilis*: Biochemical and Electron Microscopic Analysis of Autolysins and Minicells

Margaret M. Manson (BSc)

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I declare that this thesis was composed by me, and that the work described in it is my own.

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ABSTRACT

In an attempt to clarify some of the conflicting data on growth and division in rod-shaped bacteria, two aspects of the growth of a Gram-positive rod (B. subtilis) were chosen for study: autolysins and minicells.

Several properties of the two autolytic enzymes, an amidase and a glycosidase, were examined. Neither enzyme could be assayed using a synthetic substrate, so that the only specific routine assay which was found. was for glycosidase, using M. lysodeikticus walls at pH 6.0. Total lytic activity at various stages of growth was measured, and it was concluded that active enzyme was always present, but with increasing age, cells became more resistant to lysis. Stationary phase cultures excreted enzyme into the medium. Walls which were free of teichoic acid were more resistant to degradation by lytic enzymes than walls containing teichoic acid, which probably resulted from the fact that rebinding of autolytic enzymes was much weaker when walls did not contain teichoic acid. Some structural alteration of the wall may also have been involved. A purification procedure for the amidase was developed, using Sephadex chromatography and gel electrophoresis. Crude enzyme extracted from cell walls by 3M LiCl, and also purified lytic enzyme were used to raise antibodies for ferritin labelling experiments to localise amidase on the cell wall by electron microscopy. Preliminary labelling experiments with ferritin-conjugated antibody to crude enzyme and the lytic fraction indicated labelling all over the wall.

Crude enzyme extracted from minicells (which represent cell poles) was compared to that from normal cells, and found to be very similar, containing both enzyme activities. Thus it was concluded that both lytic enzymes were present at the cell poles.

Sections of cells autolysing under conditions optimal for each enzyme (pH 6.0 and pH 8.6), showed very similar degradation patterns, although at the optimum for glycosidase activity (pH 6.0) the effect was much slower. Either degradation by glycosidase activity was similar to that by amidase activity, or amidase was the major activity at both pH's. A cycle of lytic activity appeared to operate at cross-walls. Under normal conditions, no degradation occurred until the cross-wall was complete, then 'V-shaped' notches were removed from the outside wall, and the cells separated from the outside towards the centre. Once formed, the ends became resistant to further degradation. Electron microscopic evidence suggested a structure for the wall where sheets of peptidoglycan were arranged with glycan chains running perpendicular to the axis. A model was proposed for the growth of the wall by insertion of material all over the inner surface, and loss of old material from the outer surface.

A study of the growth and division of a minicell-producing mutant in exponential phase, indicated that this strain might well fit the model proposed by Teather et al. (J. Bact. 118, 407-413) for an E. coli minicell-producing strain.

CHAPTER I

INTRODUCTION

Inspite of a great deal of work published, very little is yet known about the actual process of bacterial growth and division. Some areas, however, are well documented with the evidence in good agreement, whereas in others exists a mass of confusing and conflicting data. Still other areas have been little investigated.

In the work to be discussed, an attempt has been made to gain some further information about the growth and division of a Gram-positive organism, Bacillus subtilis. This has been done by analysing its autolytic enzymes biochemically, looking at the effects of their action by electron microscopy, and by using minicells both for enzyme localisation and for growth and division studies. As a background to these aspects of biochemistry and morphology, the following topics are discussed; biochemistry of the cell wall, lytic enzymes associated with the wall and possible roles for these enzymes. Several models for three-dimensional peptidoglycan structure and for wall growth of both cocci and rods are considered. An outline of minicell production and characteristics is also given.

Biochemistry of the Cell Wall

The biochemistry of the cell wall is one of the better documented areas. The wall exists outside the plasma membrane and is considered to maintain the rigidity and osmotic stability of the cell. However, it is possible for an organism to survive and even grow and divide without a wall, as with protoplasts, L-forms and some marine organisms, although often these require special conditions for growth. Bacteria with walls may be divided into two classes on the basis of their overall wall structure, namely Gram-positive and Gram-negative organisms. Gram-positive walls appear fairly homogeneous in the electron microscope, and contain predominantly peptidoglycan and teichoic acids,

with other molecules, e.g. polysaccharides, peptidoglycolipids and external protein layers in smaller and varying amounts. Gram-negative walls, on the other hand, consist of an outer membrane of organised lipopolysaccharide-protein complex, and an inner layer of peptidoglycan, which is much thinner than the peptidoglycan of Gram-positive walls. Together with the plasma membrane, these layers are collectively known as the cell envelope.

Gram-positive organisms were used in the work to be discussed, and so the wall structure of this class is considered in more detail. The polysaccharide backbone of the peptidoglycan consists of repeating disaccharide units of 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine, NAG) linked by a β1-4 bond to 2-acetamido-2-deoxy-O-(D-1-carboxyethyl)-D-glucose (N-acetylmuramic acid, NAM). This basic unit is present universally in walls of both Gram-positive and Gram-negative organisms. The disaccharide units, formed from uridine nucleotide precursors of NAG and NAM, are transferred from a membrane lipid carrier to the wall. Polymerisation occurs at the non-reducing end of an NAG residue already present in the wall. At the time of transfer, the disaccharide units already have a pentapeptide attached, which is subsequently used in cross-linking adjacent polysaccharide chains. These peptides are strikingly constant throughout different species, consisting in most cases of L-alanine linked through its N-terminus to the lactyl group on the muramic acid, D-glutamic acid, a diamino acid and two D-alanine residues. During the cross-linking process in the wall, the terminal D-alanine is lost. The diamino acid is usually meso-diaminopimelic acid (DAP) or L-lysine, the former being found in many species of Gram-positive and some Gram-negative rods, and the latter in cocci. The carboxyl groups on residues not involved in peptide bonds may be amidated. In B. subtilis the carboxyl

groups on the D carbon of DAP of some peptide subunits are amidated (Ghuysen, 1968). Varying the amounts of amidated side groups therefore, can significantly alter the net charge of peptidoglycans of otherwise similar composition (Hughes, Pavlik, Rogers & Tanner, 1968).

The other major constituent of Gram-positive walls are the teichoic acids. These are linear polyolphosphate polymers of glycerophosphate or ribitolphosphate carrying sugar or D-alanine substituents, and as such are negatively charged. They are linked to NAM residues through phosphodiester bonds. Because teichoic acids are not found in Gram-negative or even all Gram-positive bacteria, it is difficult to assess the importance of these molecules. But as Baddiley (1972) suggested, the lipopolysaccharide of Gram-negative bacteria is structurally similar and may serve the same function. Some Gram-positive walls contain acidic polysaccharide containing uronic acid residues in place of, or as well as, teichoic acids. Teichoic acids do not appear to contribute much to the rigidity of the cell wall (Archibald, Baddiley & Blumsom, 1968), as isolated walls retain their shape after teichoic acids are removed, and as stated above, some walls lack them entirely. But they are thought to play a role in magnesium binding and uptake by the cell, as phage receptors (Doyle, Birdsell & Young, 1973; Burger, 1966), and as antigenic determinants (Burger, 1966). There is evidence that the teichoic acid biosynthesis is closely linked to that of the peptidoglycan (Mauck & Glaser, 1972). Watkinson, Hussey and Baddiley (1971) suggested that the synthesis of teichoic acids is coupled to that of peptidoglycan by sharing the membrane lipid carrier, undecaprenol phosphate. B. subtilis walls contain a polyglucosyl glycerol phosphate teichoic acid and it appears to be bound to approximately 50% of the glycan polymers (Hughes et al., 1968).

Cross-linking gives rise to the characteristic rigid outer layer

of the cell. Again this process is species specific, varying in the degree of cross-linking and also in the actual amino acids involved. In general, cell walls with L-lysine as the diamino acid are more cross-linked than those with DAP. B. subtilis belongs to the latter group and the bond in this organism is formed directly between the terminal D-alanine of one peptide chain and the DAP of another (Ghuysen, 1968). In more complicated types of cross-linking, an extra bridge of amino acids is involved, such as a pentaglycine bridge in Staphylococcus aureus strain Copenhagen (Ghuysen, 1968).

Especially useful in the studies of cell wall biosynthesis have been antibiotics and enzymes. Each of the three stages of biosynthesis a) synthesis of the uridine nucleotide precursors, b) utilisation of these to form linear peptidoglycan chains, and c) cross-linking of these strands, is susceptible to inhibition by specific antibiotics. For example, the most commonly used antibiotic in cell wall studies, penicillin, is known to inhibit the cross-linking reaction, although the manner in which it does this is not understood. Penicillin has no effect on material which is already cross-linked, and so is only active on growing bacteria. At sub-inhibitory concentrations it has been shown to give rise to an accumulation of three nucleotides, UDP-NAM, UDP-NAM-L-alanine, and UDP-NAM-tripeptide. Bacitracin inhibits the dephosphorylation of the lipid pyrophosphate to lipid monophosphate, thus preventing the lipid carrier from re-entering the cycle of wall synthesis (Siewert & Strominger, 1967). Vancomycin and ristocetin also inhibit a reaction in the second stage, namely release of disaccharide units from the lipid carrier to endogenous acceptors (Anderson, Haskin, Meadow & Strominger, 1966). Bacitracin and vancomycin were the two antibiotics used by Watkinson et al. (1971), to show the interdependence of teichoic acid and peptidoglycan synthesis.

Lytic Enzymes Associated with the Wall

Ghuysen (1968) has reviewed the extensive use of enzymes in the studies of walls. Many enzymes of known specificity from bacteria and other sources have been purified, and because it is known exactly which bond they break in the intact wall, this greatly simplifies analysis of the resulting fragments. And analysis of the reverse process sheds light on how the cell wall is put together. Lysozyme is one of the best characterised of these enzymes- its three-dimensional structure has been worked out to the extent of fitting the substrate into a cleft in the molecule (Blake, Koenig, Mair, North, Philips & Sarma, 1965; Johnson & Philips, 1965; Philips, 1966 and 1967). This enzyme attacks the peptidoglycan backbone of the wall, breaking the bond between NAM and NAG, releasing fragments with NAM residues at the reducing end. Eventually most of the wall is broken down into disaccharide units with the peptides still in place, so analysis of these fragments can give information on the peptide chains and cross-linking. Since the substrate has to fit into a cleft in the enzyme molecule, this also might give some clue as to how the peptidoglycan is arranged in the wall. This will be discussed more fully later. Lysozyme is an example of a group of enzymes known as endo-N-acetylmuramidases. A similar group of enzymes are the endo-N-acetylglucosaminidases, which hydrolyse the glycosidic bonds between NAG and NAM, leaving NAG at the reducing end.

A third group of enzymes are the N-acetylmuramyl-L-alanine amidases. As their name describes, they hydrolyse the linkages between the NAM lactyl group and the L-alanine of the peptide side chain, releasing an N-terminal group on the L-alanine. By attacking the wall specifically with an enzyme of this type, it should be possible to answer such questions as - how long are the glycan chains and what %

of the peptide side chains are involved in cross-linking? Lastly there are the endopeptidases, most of which hydrolyse linkages involving the C-terminal D-alanine of the peptide subunits.

Lytic enzymes of all four specificities have been found in bacteria, associated with the wall, in the membrane or cytoplasm, or excreted into the growth medium. As such they are known as autolytic enzymes. Many have now been partially or wholly purified and characterised (Ghuysen, Leyh-Bouille & Dierickx, 1962; Ghuysen, Tipper & Strominger, 1966; Shockman Thompson & Conover, 1967b; Brown, Fraser & Young, 1970; Huff, Silverman, Adams & Awkard, 1970; Chan & Glaser, 1972; Fan & Beckman, 1972; Ortiz, Gillespie & Berkeley, 1972; and Herbold & Glaser, 1975). A few bacteria have been reported to contain only one autolytic activity; Streptococcus faecalis ATCC 9790 (Shockman & Cheney, 1969), Lactobacillus acidophilus strain 63 AM Gasser (Coyette & Ghuysen, 1970) and Arthrobacter crystallopoietes (Krulwich & Ensign, 1968). In all these the enzyme identified was an N-acetylmuramidase. In many others, more than one activity has been identified, for example, in some Gram-positive rods, including B. subtilis (Fan & Beckman, 1972), an amidase and a glycosidase have been found, and of these the amidase is the major activity. Still others, such as Escherichia coli (Weidel & Pelzer, 1964) and S, aureus (Tipper, 1969; Huff et al., 1970), have been shown to have a number of activities including N-acetylglucosaminidases, amidases and cross-bridge splitting peptidases.

Roles for Autolytic Enzymes

Because these enzymes are potentially damaging to the cells in which they occur, the idea arose that they had some specific function in the normal process of growth and division. Clues as to their possible role have been obtained from studies of mutants deficient in

one or more of their lytic activities, and also from inhibition of peptidoglycan synthesis by addition of penicillin , or deprivation of a necessary wall component. Under these last two conditions, autolytic activity in the absence of normal growth, eventually causes lysis of the cell (Shockman, 1965).

One requirement in many of the growth models for bacterial walls which have been put forward, is that newly synthesised material is added to existing wall, but for growth to occur by insertion of new material into a covalently linked, fairly rigid structure, bonds must be broken. So it has been suggested that this may be a possible role for lytic enzymes. The disaccharide-pentapeptide unit being transferred from the membrane lipid carrier requires a non-reducing terminus of NAG as an acceptor. This will be present at the growing end of a glycan chain and would also arise from the action of an N-acetylmuramidase. However this type of enzyme activity has only been detected in a few species, S. faecalis, L. acidophilus and A. crystallopoietes (as mentioned above), and Bacillus thuringiensis (Kingan & Ensign, 1968). Thus it does not seem likely that this is a major site of incorporation in most species. Another effective means of breaking the existing covalent network would be lytic action on the peptide cross-links. In order for this to result in formation of new cross-links at the site where old ones have been broken, it would seem necessary to break the bond between the D-alanine and the diamino acid, to which it is linked, in the other chain, or between the D-alanine and the cross-bridge linking two peptide chains. An endopeptidase would fulfil either role. Even then the result would be a tetrapeptide linked to a muramic acid in the glycan chain, lacking the second D-alanine which was lost in the original cross-linking, and so, any one peptide chain may only be capable of forming one cross-link during

its lifetime. The peptidoglycan may be broken in the above manner to allow formation of completely new cross-links. Thus two groups of lytic enzymes, the two most common in Bacilli, N-acetylglucosaminidases and amidases, do not seem to produce suitable acceptor or cross-linking sites. Also, in species producing relatively large amounts of amidase, the product of such action in the peptidoglycan, namely unsubstituted NAM residues and N-terminal L-alanine peptides have not been detected (Warth & Strominger, 1971). In S. aureus, the number of N-terminal L-alanine residues is only about 6% of the L-alanine of the wall (Tipper, 1969).

A related role for these enzymes has been postulated in remodelling (Rogers, 1970), which to some extent has the same difficulties with bond specificities discussed above. Rogers suggested that one way in which the autolysin may ensure that new wall is added at an appropriate site could be by breaking bonds to allow rearrangement of existing peptidoglycan. This could provide a suitable site for cross-wall formation for instance. Rearrangement of H-bonds as well as covalent bonds could be important. In A. crystallopoietes a change in morphology from rods to spheres was observed accompanied by a transient increase in N-acetylmuramidase activity (Krulwich & Ensign, 1968). Consistent with this enzyme action, the average chain length of the glycan appeared to decrease during the conversion (Krulwich, Ensign, Tipper & Strominger, 1967). Another aspect of remodelling is the change in shape of cell poles, especially in rods, which occurs during division of the cells (Figure 1).

A widely supported role for the autolytic enzymes is in the actual process of cell separation. The length of this stage of the growth cycle varies between species and even with growth conditions in one organism. If it happens rapidly after septum formation is completed,

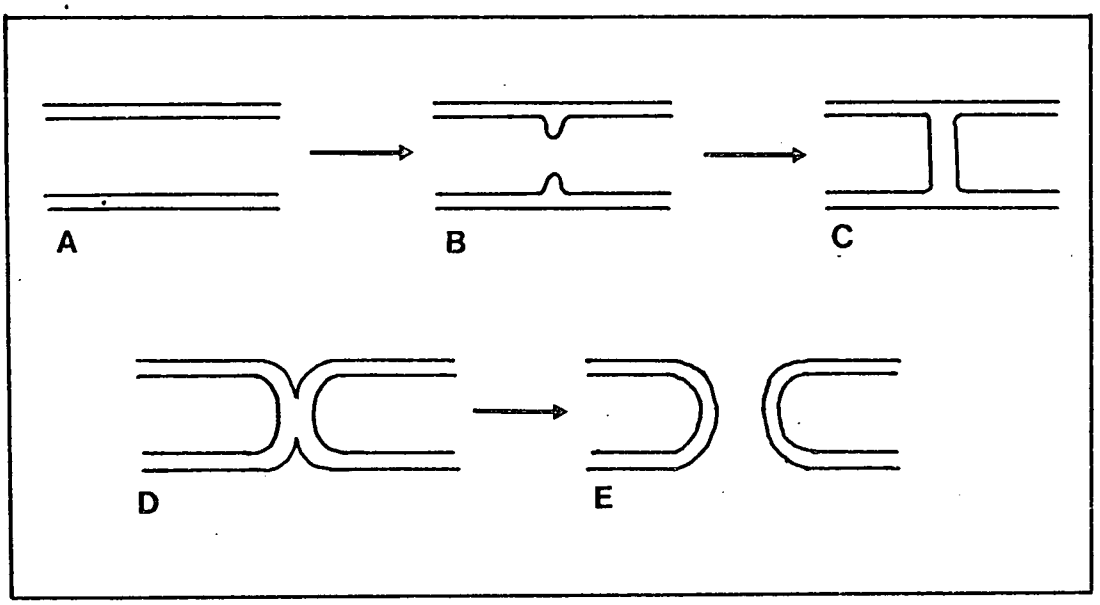


Fig. 1 Stages in cross-wall formation and cell separation in a typical rod. The change in cell shape may require remodelling.

the result is single cells. However, if the separation is slow, such that new septa are initiated before others are completed, the result is filament formation. Paulton (1970) showed for B. subtilis that the average time between production of a septum and cell separation, regardless of growth rate, was 138 minutes. Fan (1970) reported that a strain of B. subtilis grew as single cells at 30°C and as filaments at 48°C. In the light of Paulton's results discussed above, filamentation could be due partly to an increased growth rate at the higher temperature, but Fan has attributed it to a temperature sensitive autolytic activity which is ineffective at 48°C. He showed that after addition of egg white lysozyme or partially purified autolysin (of which amidase was the major activity) from the cells themselves, considerable filament shortening was achieved. However, as he pointed out, the results did not prove that the autolysin acts similarly in vivo, but preliminary experiments with mutants indicated that as the level of autolysins decreased, filament formation became more pronounced. An interesting point arising from Fan's work is that two enzymes with completely different specificities appear able to do the same job.

Forsberg and Rogers (1971 and 1974) isolated mutants of Bacillus licheniformis deficient in autolysin activity. The cells appeared to contain a normal low amount of glycosidase activity, but the amidase activity was greatly reduced. Two of the mutants with least lytic activity grew as very long chains. The walls of these mutants, however, also differed in chemical composition from the wild type. In E. coli subjected to low levels of penicillin, which caused inhibition of septum formation, splitting of the cell has been seen to occur at one particular site. This was due to the action of lytic enzymes and appeared to be associated with a division site or a potential division

site (Schwarz, Asmus & Frank, 1969; Donachie & Begg, 1970). This effect could have resulted from localisation of enzymes at this point for septum formation and/or cell separation. Inhibition of septum formation might have resulted in disorganised lysis giving rise to the split observed.

It has now been shown that for a number of species, wall turnover occurs. Mauck, Chan & Glaser (1971), in studies on B. subtilis W23 during exponential phase, concluded that freshly synthesised wall did not become available for turnover for one half to one generation, the turnover rates for peptidoglycan and teichoic acids were 50% per generation, and the products of turnover isolated and characterised, resulted from cleavage by an N-acetylmuramyl-L-alanine amidase. A peptidoglycan turnover rate of 30% per generation was also observed in Bacillus megaterium strain KM in exponential phase. S. aureus exhibited a turnover rate of approximately 15% per generation for both peptidoglycan and teichoic acids, but both new and old wall material showed similar rates and no significant portion of the walls appeared to be resistant to turnover (Wong, Young & Chatterjee, 1974). It is likely that here also an amidase was involved, as this is the major activity in this strain, and the walls of a mutant deficient in autolytic enzyme did not turn over. Wall turnover has also been shown in L. acidophilus but not in S. faecalis (Boothby, Daneo-Moore, Higgins, Coyette & Shockman, 1973) or E.coli (Van Tubergen & Setlow, 1961). These results suggest that wall turnover is not essential for normal growth, but when it occurs, lytic enzymes, especially the amidase, are involved.

Another more specialised role for autolysins has been suggested by competence studies. Akrigg and Ayad (1970) demonstrated that in competent cells of B. subtilis 168 the competence inducing factor

exhibited a powerful lytic effect on isolated walls of 168, and analysis of lysates suggested that the enzyme might be N-acetylmuramyl-L-alanine amidase. Stewart and Marmur (1970) found that the extractable lytic activity of B. subtilis 168 was markedly increased after uptake of DNA. No such effect was observed in B. subtilis W23 and non-competent cells of 168, neither of which take up DNA. Young and co-workers also presented data suggesting that the autolytic enzyme of B. subtilis might have a role in DNA uptake during transformation, perhaps by hydrolysing a portion of the wall to provide a path of entry for DNA (Young & Spizizen, 1963a and 1963b; Young, Tipper & Strominger, 1964).

From the evidence sited, it seems almost certain that autolytic enzymes play a part in the normal cell cycle, perhaps most clearly in the processes of turnover and cell separation. But it is far from established why some species appear to have multiple activities, while others can survive with only one detectable autolysin. It is possible that in some species one enzyme may have multiple roles, whereas in others, more than one enzyme may effectively do the same job.

Three-Dimensional Arrangement of Peptidoglycan.

There is little biophysical evidence to support or discount any molecular models of cell walls, and studies which might have been particularly useful for the determination of three-dimensional structure, such as X-ray crystallography, have so far had little success. However a number of models have been constructed to explain the available data and quite a lot of work has been done on specific parts of the problem.

The bulk of evidence now suggests that the glycan chains are fairly short, in contrast to the very long strands envisaged by Weidel and Pelzer (1964). Estimates vary and great care must be taken during

preparation of fragments for analysis to ensure that in vitro action of N-acetylglucosaminidases or N-acetylmuramidases does not make the chains smaller. Also the oligosaccharides obtained are often polydisperse and there is no means of knowing the distribution of lengths in different cells of a culture, or in different parts of a cell wall. In S. aureus estimates of 12 to 16 disaccharide units per glycan chain were made (Tipper, Strominger & Ensign, 1967; Ghuysen & Strominger, 1963a). Walls of A. crystallopoietes grown as spheres contained glycan strands which averaged 17 units in length, while from the same organism grown as rods, they averaged 65 units (Krulwich et al., 1967). Kolenbrander and Ensign (1968) investigated the spiral shaped Spirillum serpens and results indicated that the chain length was about 50 units. B. licheniformis 6346 was found to have an average chain length of 11 units and B. subtilis 168 ind⁻ a range of 5 to 21 units with an average chain length of 10 units (Hughes et al., 1968). There is no real evidence to suggest that a relationship exists between cell shape and the average chain length of the wall peptidoglycan (Ghuysen, 1968), although it is possible that the localisation of long and short chains within a wall could be important.

Cross-linking of the peptide subunits is necessary to maintain an intact structure. However, the degree of cross-linking varies greatly. Analysis of E. coli walls revealed 50% unlinked monomers and 50% cross-linked dimers (Weidel & Pelzer, 1964; Van Heijenoort, Elbaz, Dezelee, Petit, Bricas & Ghuysen, 1969). L. acidophilus had 10% monomers, 37% dimers, and 30% trimers (Coyette & Ghuysen, 1970). S. aureus was found to be one of the most cross-linked organisms with about 75% of the possible peptide cross-linkages made (Tipper & Berman, 1969). In S. aureus (Ghuysen, Tipper, Birge & Strominger, 1965), and L. acidophilus the uncross-linked peptides ended in D-alanyl-D-alanine,

while in most other species one or both were absent, presumably removed by carboxypeptidase. Tipper (1970) quoted 55 to 65% of possible cross-links in B. subtilis (Warth, 1968), Bacillus sphaericus (Hungerer & Tipper, 1969), Lactobacillus casei (Hungerer, Fleck & Tipper, 1969) and A. crystallopoietes (Krulwich et al., 1967).

Previc (1970) has produced a theory, which unfortunately has quite a weight of evidence against it, in which he compares coccoid and bacilliform bacteria with respect to distinctive biochemical differences in their peptidoglycan. He considers that a strong correlation exists between morphology and the type of amino acid found in position 3 of the peptide subunits, namely lysine in many cocci and DAP in many rod-shaped cells. He maintains that spherical cells require a radial expansion which could be accomplished by a relatively simple net of polysaccharide chains, linked by linear oligopeptides. The spherical shape could result from pressure within. With rods the poles could also be moulded under the influence of internal pressure, but he considers the cylindrical part to be more complex, requiring a constant diameter and an increase in length. To maintain and propagate the cylindrical shape might require a coplex form of cross-linking, which could be provided by a tetrafunctional amino acid such as DAP. Reports so far are not in favour of this, however, as data suggest that the second carboxyl group of DAP is either free or amidated. Also DAP is replaced in a number of rod-shaped lactic acid bacilli by lysine or ornithine which are linked through their ε- or δ- amino group to aspartic acid. This latter amino acid , however, has an extra carboxyl group which potentially could cross-link in another dimension, but again it appears to be amidated (Ghuysen, 1968). Nor is this type of peptide restricted to rods. Nevertheless, Previc has proposed that trifunctional lysine provides the simpler form of cross-link, while

the more complicated form required for cylinders could be provided by a tetrafunctional amino acid such as DAP, with the possibility of this forming less complicated cross-links at the rounded poles. He has further suggested that if DAP is the key to bacterial shape, a transition from tetrafunctional to trifunctional cross-linking could be responsible for rounding off at the division site, or the difference between cylinder and pole could depend on the presence of a single enzyme capable of cross-linking the ε- carboxyl of DAP.

Attempts have been made to localise teichoic acids within cell walls. Hughes et al. (1968), studying wall fragments of B. licheniformis, which contains teichoic acid and teichuronic acid, concluded that three possibilities existed, 1) teichoic and teichuronic acid molecules might be clustered together in only part of the peptidoglycan, 2) they might occur separately as islands in the wall, or 3) individual cells in a culture might have one or the other.

Archibald, Baddiley and Heckels (1973) looked at walls of Staphylococcus lactis 13, and found nearly 40% of the peptidoglycan covalently attached to teichoic acid, and only one teichoic acid chain attached to any one glycan chain. All the wall teichoic acid was attached to peptidoglycan and the average chain lengths for the polymers were 24 and 9 repeating units respectively. The glycan chains without teichoic acid attached were also 9 units in length. This suggests that there is a considerable degree of order in wall structure and implies restrictions on the location of teichoic acid. If the glycan chains are parallel to the surface then the proportion of chains to which the teichoic acid is attached is more than would cover the outer layer. Thus in this case, teichoic acid would have to be distributed throughout the layers of the wall. Only if glycan and teichoic acid chains lie perpendicular to the surface, presumably in a contracted

conformation, could the teichoic acid occupy a discrete region on the outer or inner edge of the wall.

Concanavalin A binds specifically to the teichoic acid of B. subtilis 168 and ultrastructural studies were carried out using this as a label (Birdsell, Doyle & Morgenstern, 1975). Their studies showed that at least a portion of the teichoic acid was localised on the surface of the cell. Mauck and Glaser (1972) reported that new teichoic acid was added to new peptidoglycan which, in a wall several layers thick, suggests that teichoic acid should also be found throughout the wall. So far the exact location of teichoic acids has not been determined, and is another point for speculation in a model of overall peptidoglycan structure.

Models 1. Higgins and Shockman (1971) have produced a molecular model which they consider applies reasonably well to the variety of peptidoglycan structures determined. Hydrogen bonding and covalent bonding both contribute to structural properties. Their space-filling models show the glycan chains to be flat, rather dense, fairly rigid ribbons. The peptide folds over one face of the amino sugars, resulting in three H-bonds between the peptide side chain and the disaccharide, and additional H-bonds within the peptide chain. The configuration of the peptide is like a letter 'T'. The two arms of the 'T' form an almost straight line that runs at 90^{0} to the long axis of the glycan strands. The distance between the amino group of lysine at one end of the arms and the carboxy terminal of D-alanine at the other end equals the width of a glycan chain. The model therefore allows cross-linking between parallel glycan strands without extra amino acids in a cross-bridge between two tetrapeptides. Bridging amino acids would only increase the distance between parallel glycan strands. The model predicts sheets of peptidoglycan in which glycan strands and peptide chains

are in two separate planes, one above the other, and run at 90° to each other. These sheets stack easily in a parallel or antiparallel direction (see later) with H-bonding between the sheets. They claim that cross-linking between the sheets is also possible.

2. The problem has also been approached by Keleman and Rogers (1971) with bacilli in mind. Their ideas differ quite considerably from those discussed above. Again using molecular models, they found that if glycan chains are H-bonded as in chitin, all the carboxyl groups (to which peptides are linked) of NAM protrude from the same side of the stack of polysaccharide chains, and the 6-hydroxyl groups (to which wall polymers other than peptidoglycan are likely to be joined), are exposed on the opposite side of the stack. Two such stacks are cross-linked by the peptides to give a sheet two layers thick. Keleman and Rogers have pointed out the differences between peptides of peptidoglycan and those of protein, namely - a) some of the amino acids are in the D configuration, b) D-glutamic acid and D-aspartic acid, when it occurs, have their γ- and β- (respectively) carboxyls involved in peptide linkage and not their α- carboxyl groups, c) some of the diamino acids are linked into the peptide by both amino groups, d) the peptides linking two glycan chains are made of two parts joined head to tail. The effect of these differences on peptide configuration makes α-helices and random coil structures unlikely and a pseudo-β- conformation likely, since the latter creates the possibility of multiple interpeptide H-bonding.

3. Tipper (1970) has noted that glycan chains of NAG-NAM repeating units offer only half the intrachain H-bond possibilities between adjacent sugar residues available in chitin, but the substitutions on NAM may offer other possibilities, such as the bond between the lactyl carbonyl group of NAM and the adjacent C_6 hydroxyl group of NAG. As

in the models discussed above, a flat linear glycan chain is stabilised by H-bonds with identically oriented peptides on one side and hydroxyls for attachment of other wall polymers protruding on the other side. In contrast to the other models, he draws special attention to a structure which is common to all known peptidoglycans, the strict DLDLD sequence of the peptide chain, including the D-lactyl substitution of the NAM, in which the glutamyl linkage is always γ, giving a flexibility that might allow the chain to double back on itself. A comparison is drawn between the repeating structure here and that of some antibiotics, whose most striking property is the stability of their folded conformations, which in some cases form a hexadentate co-ordination shell around an unhydrated alkali metal ion. Tipper considers it possible that appropriate folding of the common peptidoglycan peptide might result in a preferred conformation, perhaps stabilised by metal chelation, which could be important in two ways; a) for binding to the active sites of enzymes and b) for structural function since a fixed peptide structure along with that of glycan could fix the network in an expanded yet fairly rigid configuration that might be economical in material and leave gaps for insertion of other cell wall polymers and diffusion of nutrients etc.

4. Braun, Gnirke, Henning and Rehn (1973) have presented a model, which they were careful to designate as being for the shape-maintaining layer of the E. coli cell envelope. They do not believe that peptidoglycan is necessarily shape-determining, since peptidoglycan of one and the same composition can assume virtually any shape (Henning, Rehn, Braun, Hoehn & Schwarz, 1972; Schwarz & Leutgeb, 1971). Braun et al. measured the surface area per molecule of DAP and found it to be the same for a mutant growing as a sphere as for the rod-shaped parent. Their model specifies a monomolecular layer with the polysaccharide

chains all parallel and the cross-linking peptides arranged above or below the polysaccharide, as proposed by Higgins and Shockman. The peptide side chains, according to Braun et al., probably have a stable conformation because of the steric requirement of peptidoglycan biosynthesis, so that the amino group of DAP and the carboxyl of D-alanine of a neighbouring chain can come into close contact to allow transpeptidation. Within the polysaccharide chain no rotation of the repeating units is possible. In a β1-4 linked polysaccharide chain, all peptides extend in the same direction.

The structure of peptidoglycan in all wall models is based on that of chitin (Carlström, 1957), with its linear conformation stabilised by H-bonds. No substitutions have been found in the glycan chains which would alter their conformation. This suggests that the conformation may be essential for the survival of the bacteria (Ghuysen & Shockman, 1973). An α-helix in the peptide seems unlikely. Keleman and Rogers favour the β-pleated sheet for H-bonding, while Tipper suggests flexibility of the peptide chain, and a similarity between some antibiotics and the repeating peptide structure of the peptidoglycan. The model discussed by Higgins and Shockman differs from the others in that it involves extensive H-bonding between peptide chains and glycan chains, thus resulting in a more compact structure. However the models of Higgins and Shockman and Braun et al. are essentially similar in the structure proposed for a sheet of peptidoglycan. They offer the possibility of extension by covalent linkage in two dimensions. Keleman and Rogers however, propose that both the reactive groups of a peptide cross-link to the same glycan chain. Thus only pairs of glycan chains can be linked, and a sheet of peptidoglycan must be formed by stacks of these H-bonded together. This structure does not make use of the full potential of peptide cross-linking, and only allows for extension by

covalent linkage in one dimension. The model proposed by Braun and co-workers caters for a Gram-negative organism with a very thin layer of peptidoglycan, and when expanded to accomodate more than one layer, does not allow for binding between the layers to give a stable three-dimensional peptidoglycan (cf Higgins & Shockman). None of the available data has been able to establish whether the glycan chains run parallel to the surface or perpendicular to the surface, whether if they are parallel, they run along the long axis of the cell or round the diameter, or whether the sugar rings are parallel or perpendicular to the surface.

5. H. Wawra, S. Formanek and H. Formanek presented a three-dimensional model of the murein layer at the Luntern Lectures (1974). This was based on model building and preliminary X-ray diffraction patterns of murein foils (dried sacculi of Spirillum laid one on top of the other with random orientation). The periodicities obtained resembled those from chitin, so again this was taken as the basic structure of the glycan chains. They preferred to think of the peptides as having 2.2_7 helical conformation, claiming that a pleated sheet structure did not fit according to a Ramachandran Plot. They pointed out that this arrangement of glycan and peptide allowed all modifications and substitutions found in peptidoglycan, such as substitution of the C_6 of NAM, formation of muramic acid lactam in spores, incomplete or omitted peptide subunits and formation of the different interpeptide bridges. In their model, they visualised the peptidoglycan as being tightly packed and therefore, H-bonded, but with holes in the structure where permeation and lysozyme digestion could occur. The model allowed for addition of teichoic acid, and they considered it most likely that teichoic acid would be present in the more crystalline parts of the structure, since the more the peptidoglycan is substituted, the more rigid is the structure.

Cell Wall Growth.

With regard to the mechanism of cell growth, the problem which has received most attention over the years is the number and location of growth sites. The problem has been approached by direct and indirect techniques such as immunofluorescent and ferritin antibody labelling, incorporation of labelled wall components followed by autoradiography, and ultrastructural studies. Some of the techniques are more reliable than others, but in all cases great care must be taken in interpreting the results. Cole (1965) has discussed the advantages and limitations of immunofluorescent techniques. The main advantage is the availability of a specific label to the wall of a living organism. The presence of labelled antibodies does not appear to stop or alter growth. When the micro-organisms are removed from the label and undergo subsequent growth a clear differentiation can be obtained between old and new wall. However, the technique itself has several limitations, such as progressive quenching of fluorescence on exposure to ultra-violet light, poor initial fluorescence under some conditions, and an indirect effect of UV during observation preventing further growth without killing the cells. But the main disadvantage is the frequently made assumption that all the constituents of the cell wall are replicated simultaneously and at the same sites. Antibodies are usually produced to specific cell surface antigens rather than to the mucopeptide itself, and these may be unrepresentative of overall cell growth, especially if they are not covalently bonded to the peptidoglycan. A synchronous culture would be most useful for ease of interpretation, but in practice an exponentially growing population is satisfactory. In any case careful observation of an adequate sample is essential for correct interpretation.

A further complication, to be considered in species where it occurs,

is wall turnover. It is difficult to imagine excluding turnover from the growing process of the cell, but loss of label from the outer surface due to turnover may not represent the growing process occuring at the inner edge of the wall. Thus, especially in Gram-positive bacteria which have such a thick cell wall, it is important to look as closely as possible at the area where new material must be incorporated, rather than at the outer surface where old material may be randomly lost.

To date it seems that no one model will fit all the species studied. Current evidence suggests that rods and cocci represent different systems and should be considered separately. Cocci have been considered as representing a more primitive situation, with the cells consisting of two hemispherical poles to be separated by a cross-wall. Rods, on the other hand, may result from a more sophisticated system, evolved to overcome limitations of a coccal form, consisting of a cylindrical portion with a hemispherical pole at either end. The cross-wall normally develops in the centre of the cylinder to give rise to two new hemispherical poles.

Three possibilities exist for the distribution of growth sites in a cell. Wall deposition and thus growth occurs a) at only one site per cell, b) at a finite number of sites distributed throughout the cell, or c) all over the surface of the cell. The evidence at present is still very confused, but indicates that for cocci, one site per cell may be involved in growth, whereas at least in some species of rods, growth may occur all over the surface.

Cocci. An extensive study of growth in S. faecalis, a Gram-positive coccus which divides in only one plane, has been carried out by Higgins and Shockman's group and provides convincing evidence that this organism has a restricted growth zone. From an electron microscopic analysis of serial and random sections, (Higgins & Shockman, 1970),

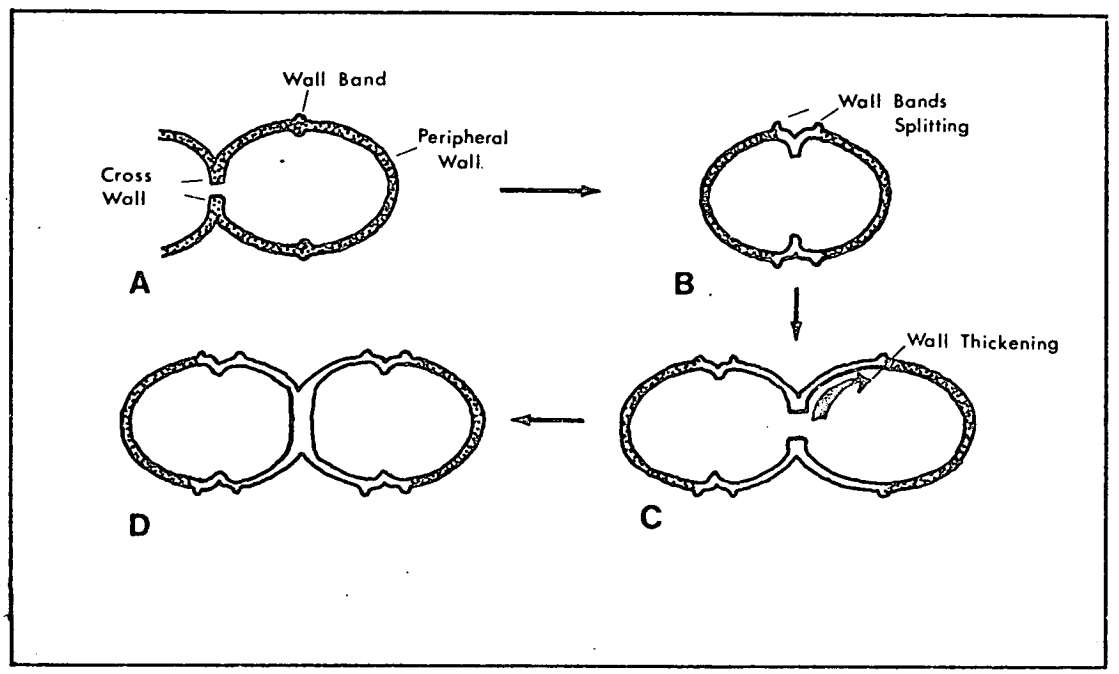


Fig. 2 Model for the growth of S. faecalis (after Higgins & Shockman, 1970)

New wall is represented by unshaded areas. Between stages B and C, centripetal penetration of the cross-wall remains relatively constant, while the wall bands which mark the separation of new wall from old, move apart. When the wall band reaches the equator in C, cross-wall extension resumes to separate the daughter cells. Often initiation of a new cross-wall, before completion of the old one, is observed as in D.

they have constructed a growth model (Figure 2). This proposes that in exponentially growing and dividing cells of S. faecalis, the leading edge of the annularly closing cross-wall is the point of extension for both cross-wall and peripheral wall. Extension of the latter is thought to be produced by separation and splitting of the cross-wall at its junction with peripheral wall. This results in movement of the equatorial bands, fortuitously found on S. faecalis walls, to sub-equatorial positions. Two bands therefore mark the separation of old wall from new. Superimposed on the addition of new material at the growing point is wall thickening (C in Figure 2). A significant proportion of precursors are used to thicken the newly extended tip to about twice that of the peripheral wall, the most rapid thickening occurring at the leading edges of the cross-wall. A continuous gradient of peripheral thickness extends to the wall bands, but as the wall becomes older, that is farther and farther from the cross-wall, the thickening process seems to decay so that there appears to be little or no thickening at the poles.

Immunofluorescent techniques had previously suggested this mode of growth in Streptococci, but lack of resolution and the specificities of the antibodies to accessary wall components, rather than to peptidoglycan, made conclusions less convincing (Cole & Hahn, 1962; Chung, Hawirko & Isaac, 1964b). Swanson, Hsu and Gotschlich (1969), using electron microscopy and ferritin labelled antibody, provided direct evidence that Group A Streptococcal M protein, which had been removed by trypsin, was resynthesised primarily at the equatorial region of growing cells. Briles and Tomasz (1970), by specifically labelling the teichoic acid of Diplococcus pneumoniae with H^3 choline, demonstrated the Zonal growth and conservative segregation pattern of teichoic acid which is probably covalently linked to the peptidoglycan.

A further feature of interest arising from the work of Higgins

and Shockman was the close association of mesosomes with DNA replication and possibly cross-wall initiation. Less apparent was their requirement for DNA segregation or completion of the cross-wall. Because of their frequent association with cross-wall and DNA in electron micrographs, a role for mesosomes in cross-wall growth and DNA replication and/or segregation has often been postulated.

Shockman, Pooley and Thompson (1967) also showed that in S. faecalis the major lytic enzyme, an N-acetylmuramidase, existed in a latent and active form. Action of the active wall-bound form caused preferential lysis of newly formed wall, while action of trypsin- activated latent form (about 85% of which is not wall-bound) led to random hydrolysis all over the wall. The effect appeared to be due to localisation of the active form. Shockman and Martin (1968) following autolysis of S. faecalis, identified initial attack due to active enzyme at the leading edges and tip of the growing cross-wall, followed by dissolution of the entire cross-wall, ultimately releasing hemispherical pieces of wall. Dissolution then continued from the equator toward the poles at a decreasing rate, which suggested that either older wall was more resistant to autolytic attack, or the activity of the enzyme decreased.

Autolysis of S. faecalis recovering from threonine starvation, (Higgins, Pooley & Shockman, 1971) substantiated the results already discussed. During starvation the walls became thicker and resistant to autolysis. There was also a decrease of active and latent enzyme per unit mass of wall. The appearance of recovering cells was consistent with the idea that separation of the cross-wall to generate peripheral wall, normally requires both linear wall elongation and autolytic activity. New, thin, centrally located, peripheral wall was inserted between the old thickened poles. Thickened walls produced during

starvation appeared to be conserved and segregated to the poles rather than thinned out, implying that in terms of cell age, each new-born cell was asymmetric, with one pole at least one generation younger than the other.

Based on the above evidence, and their model for peptidoglycan structure discussed previously, Higgins and Shockman (1971) have proposed two possible schemes for peptidoglycan growth in S. faecalis, which they have designated parallel and antiparallel (Figure 3). Both schemes are based on four assumptions, that 1) peptidoglycan structure is non-random, 2) H-bonding as well as occasional inter-layer cross-bridges are the bonding forces between layers, 3) assembly of the peptidoglycan may result from the transfer of soluble linear disaccharide-peptide oligomers from a membrane site to the wall itself, and that 4) a mechanism for the location of the site of layer separation, within the wall must exist.

The following points arise from their reasoning. During autolysis experiments, ribbons of wall 10 to 20nm wide were released from the leading edge of hemispherical pieces of cell. These could have resulted from the hydrolysis of a few strategically located sensitive 1-4 bonds and may represent the macromolecular arrangement in the intact wall. (A glycan chain of 10 disaccharide units would be about 17nm). The direction of enzyme hydrolysis is consistent with glycan chains running radially from the centre of the cross-wall and perpendicular to the long axis of the cell.

Since glycan chains in the wall are probably fairly short, it is possible that uncross-linked linear oligomers may be transferred from the membrane to points of growth in the wall. Accessory wall polymers may be added to the oligomers before incorporation.

Incorporation itself could occur by one or more of several

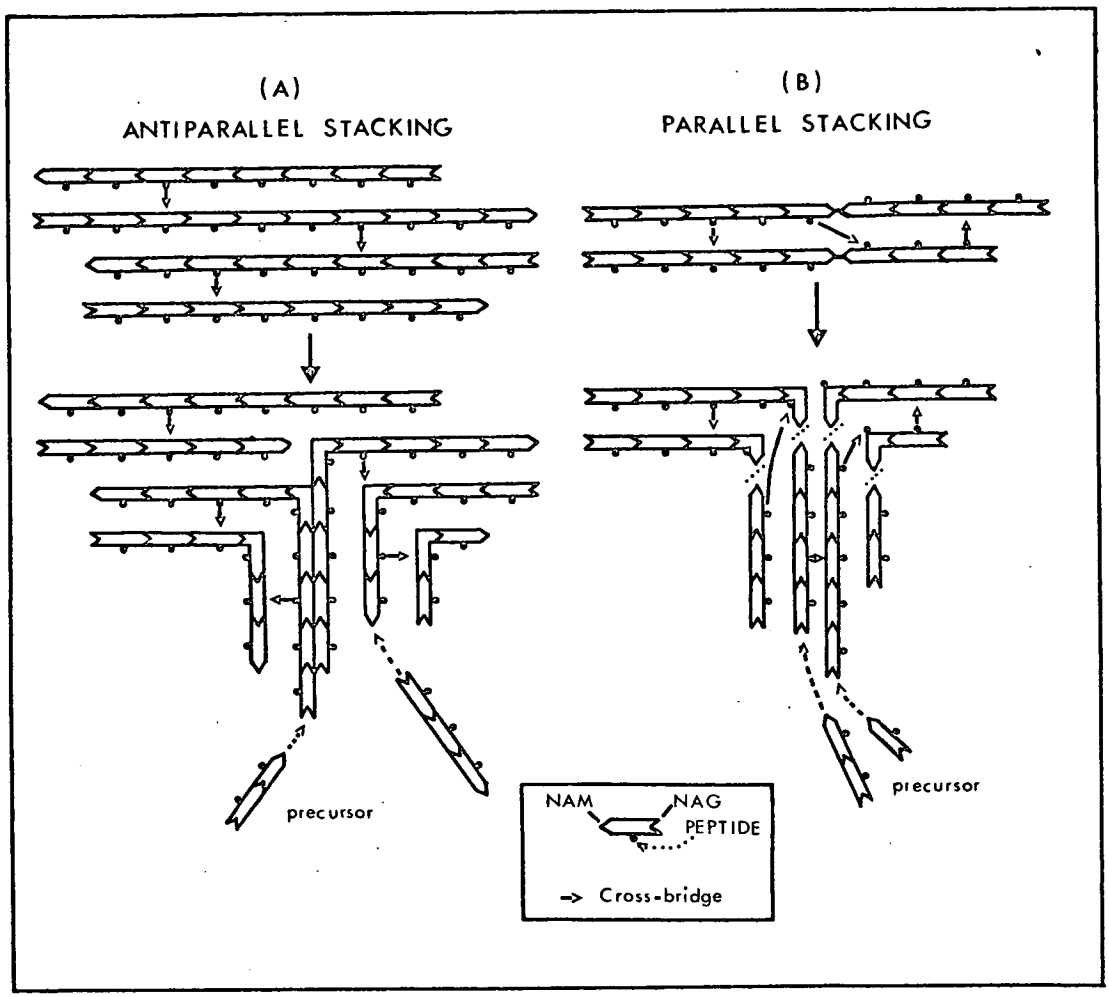


Fig 3. Model for peptidoglycan growth in S. faecalis (after Higgins & Shockman, 1971).

Antiparallel and parallel refer to the direction of the glycan chains in one sheet relative to another. The glycan chains run in the plane of the paper, while the peptide chains are perpendicular to the plane of the paper. The peptides would normally link glycan strands in the same plane, and the resulting sheet would be held to another by H-bonds and an occasional peptide cross-bridge between sheets. According to this model, a cross-wall is initiated at the equator where old and new wall meet. Initiation in either A or B above may require hydrolysis of some existing glycan or peptide linkages. B could result in a discontinuity when the centre of the cross-wall is reached. In A cross-wall could be initiated at any point on the wall. The precursors added would be held in place first by H-bonds, and later by covalent cross-bridges, and perhaps also by the formation of additional glycosidic bonds.

mechanisms. Glycosidic bonds to existing wall glycan could be formed using the energy in the pyrophosphate linkage of the lipid carrier, or by transglycosidation perhaps catalysed by the reversal of an N-acetylmuramidase. More important might be transpeptidation with formation of peptide cross-bridges after the stacking of layers of H-bonded oligomers.

Cell separation might be a physical phenomenon resulting from the presence of a natural shearing plane and internal pressure. However, differences in peptide cross-bridging and glycan chain length could contribute, as could concentrations of highly charged polymers, e.g. teichoic acid, causing a repulsion effect within the cross-wall. The occurrence of mutants, deficient in teichoic acid, which fail to divide or separate properly (Cole, Popkin, Boylan & Mendelson, 1970) is consistent with a role for accessory wall polymers in cell division. Cross-wall separation may also require the action of autolytic enzymes. Activity of the autolysin in S. faecalis is stimulated by the presence of teichoic acid.

Higgins and Shockman do not present any data to show how peptide cross-linking between sheets of glycan is sterically possible. Presumably it requires a completely different conformation from that required for intra-sheet bonding, and Braun et al. have argued that more than one steric conformation is unlikely to suit one enzyme. The antiparallel scheme as it is drawn, involves addition of new material to an acceptor in the wall which is the reducing terminus of NAM, as well as the more conventional non-reducing NAG acceptor. The latter involves addition of an NAM reducing terminus attached to lipid pyrophosphate to a non-reducing end of NAG. Addition of the non-reducing terminus of NAG to a reducing terminus already in the wall means that the lipid pyrophosphate is no longer present on the NAM in the wall and is

attached to the wrong end of the disaccharide (or oligosaccharide) being transferred from the membrane. Thompson (1971) has commented on this problem. He suggested that growth from the NAM reducing end would require an extracellular polymerase which would gradually work its way out from the membrane. Growth would presumably become less and less efficient as precursors would have farther to diffuse from the membrane the farther the chain extended away from it. There would thus be a continuing need to start new chains. Higgins and Shockman do not envisage these chains growing away from the membrane, but they would still require a different enzyme action.

Rods. Among the approaches used to localise surface growth in rods are a) labelling of the wall with radioactivity, immunofluorescence, flagella or thickened wall, and then looking for the appearance of new unlabelled wall, b) direct observation of new cell wall growth with respect to fixed external markers, and c) use of penicillin to modify sites of transpeptidation.

Immunofluorescent studies have been carried out on B. cereus and B. megaterium by Chung, Hawirko and Isaac (1964a). From their results, they concluded that these organisms grew by discrete intercalation, near the poles or sometimes centrally in B. megaterium. However, this work has been strongly critised by Cole (1965) and other workers. For example, antiserum was prepared to whole cells and no information was available on possible labile antigens which may only be transitory surface components. No control was carried out using the reverse labelling technique. Trypsin and ribonuclease were added to the medium and possible effects of these enzymes on wall components were not considered. Resolution in the photographs was poor, and no evidence was presented to show that the label was actually in the wall, or that single cells rather than chains were examined.

Also using immunofluorescence, both May (1963) and Cole (1964) studying Salmonella concluded that no discrete intercalation was visible and the most likely process for wall replication was by diffuse intercalation. Beachey and Cole (1966) reached the same conclusions for four strains of E. coli. The organisms in these three studies were young inocula growing in rich medium, leading to rapid regeneration times.

Meynell and Lawn (1965) used the inheritance of capsule in Bacillus anthracis to study the possible mechanism of wall replication. During growth in broth, this organism became encapsulated towards the end of exponential phase, and when the encapsulated cells were inoculated into fresh medium, they grew without formation of new capsule. The capsule was inherited in blocks by the daughter cells, so that wall growth did not occur by diffuse intercalation if the parental wall and its capsule remained joined.

A more recent study (Hughes & Stokes, 1971) has been carried out on B. licheniformis (his^- lyt^-). Ideally growth in bacteria should be represented by growth of the peptidoglycan matrix, and so these workers used antisera to pure peptidoglycan preparations of B. licheniformis NCTC 6346. Exponential phase cells were labelled, washed, resuspended in fresh medium, and removed at intervals thereafter for analysis. Results suggested that mucopeptide synthesis in B. licheniformis 6346 (his^- lyt^-) occurred at very few sites during growth. They also noted that in these poorly lytic strains there was very little wall turnover.

Mauck et al (1970 and 1971) presented evidence for extensive wall turnover in B. subtilis and B. megaterium, incompatible with a single site of synthesis in these organisms. The growth of the wall in B.megaterium KM was examined by Mauck, Chan, Glaser and Williamson (1972) by labelling the cells uniformly with tritiated DAP and detecting

the distribution of labelled wall material in daughter cells. From their observations, they concluded that growth did not occur at a single central or polar site in B. megaterium. The results did not indicate the number of such sites per cell, but suggested many. Mauck and Glaser (1972) produced further evidence for random distribution of new cell wall material by carrying out a'nearest neighbour' analysis on walls of B. subtilis. During exponential phase this organism synthesised teichoic acid, but under conditions of phosphate limitation teichoic acid synthesis was replaced by teichuronic acid synthesis. Experiments showed that both these polymers were attached only to peptidoglycan synthesised at the same time, and so by suitable labelling techniques, they were able to demonstrate that glycan chains adjacent to newly inserted chains represented a nearly random selection of old and new chains.

A further observation incompatible with a single site of synthesis in many organisms is wall thickening, which occurs uniformly all over the surface in the presence of protein synthesis inhibition (Frehel, Beaufils & Ryter, 1971). Cultures of B. subtilis and B. megaterium grown under such conditions were observed during release of inhibition. The outer portion of the wall fragmented into small pieces, while polar regions remained thick and smooth for at least 2 generations. The results suggested that wall synthesis occurs at a large number of sites uniformly distributed along the cylindrical parts of the wall but not at polar regions. Frehel et al. suggested that a reason for the discrepancy between their results and those of others for B. megaterium might in part be due to the presence of a capsule which could complicate immunofluorescent data. However, this argument does not apply to B. licheniformis, a related organism.

Results obtained by Highton and Hobbs (1972) ,working with B.cereus during recovery after exposure to penicillin, were interpreted to mean

that elongation occurs by addition of material to a large and continuously increasing number of growing points uniformly distributed over the cylindrical surface. Cross-walls grow by addition to their inner edge, and on completion, the two new rounded ends of the daughter cells are formed by splitting at the outer edge and continued addition at the centre. The ends are conserved.

Van Tubergen and Setlow (1961) labelled exponentially growing cultures of a DAP-requiring mutant of E. coli with tritiated DAP and examined the distribution of label in the progeny by autoradiography. The experiments were carefully controlled, and results indicated that the wall did not turn over and label was randomly distributed throughout the daughter cells. This is consistent with a non-localised synthesis of DAP-containing wall peptidoglycan, but resolution was not sufficient to localise grain distribution within the wall.

On the other hand, Chung et al. (1964b), using slightly different immunofluorescent techniques from May (1963) and Beachey and Cole (1966) interpreted their results showing localised growth as indicating that the first step in wall growth is the formation of cross-wall followed by elongation of the cylindrical wall on either side. Additional discrete sites of wall elongation later develop.

Penicillin, because it inhibits the cross-bridging reactions in wall synthesis, has been used in some studies of cell growth in the belief that newly synthesised areas of the wall , which are as yet uncross-linked, and are prevented from becoming so by the presence of penicillin, will be most susceptible to autolytic attack, and therefore represent sites of incorporation of new material. However, caution is needed in equating sites of autolysis with sites of wall growth. Penicillin also appears to affect the site of wall thickening and the lytic effect is due to action of lytic enzymes as well as penicillin

inhibition of peptidoglycan synthesis (Higgins & Shockman, 1971). Thus under some conditions, it is possible that a site of lysis may be due only to action of localised lytic enzymes. Schwarz et al. (1969), in a study of E. coli, found that penicillin-induced bulges occurred in the sacculi at sites where normally new wall was formed in cell division. It was assumed that when synthesis of peptidoglycan was partially inhibited by low concentrations of penicillin, more bonds were broken by localised action of hydrolases than could be closed by cross-bridging of normal peptidoglycan synthesis, leading to a weakening of the wall at this point. From their results using different concentrations of penicillin, they concluded that the growth of the sacculus is by several functionally different systems, one involved in cell elongation, others in cell division, as revealed by their different sensitivity to penicillin. The action of localised hydrolases revealed zonal growth of the sacculus at least during cell division. Their results do not exclude addition of new material by diffuse intercalation, possibly during cell elongation.

Donachie and Begg (1970) measured growth in E. coli by two indirect methods, firstly by measuring the direction of growth of intact living cells relative to fixed external markers, and secondly by measuring the position of an internal marker, in this case the point at which the cell wall split in the presence of penicillin relative to the ends of the cells. Results showed that in cells shorter than a certain critical length, growth was asymmetrical, while in longer cells growth was symmetrical. The smaller cells had one growth site at or near the pole, and grew in one direction; larger cells had more than one growth site, the number increasing with increasing length, and these cells grew in both directions.

Ryter (1973) has tried to reconcile the seemingly contradictory results obtained for E. coli. Van Tubergen and Setlow (1961) and Lin,

Hirota and Jacob (1971), using radioactive DAP, concluded that there was a random distribution of growth sites. Schwarz et al. (1969) and Donachie and Begg (1970), using penicillin, demonstrated growth in one preferential zone. Ryter believes that the complexity of the growth process in rods is the cause of contradictory results. She attempted to verify this by carrying out two types of experiment. The first involved labelling with DAP for several generations, followed by growth in unlabelled medium for one or two generations, and the second, labelling of the peptidoglycan with short pulses. The first type of experiment confirmed results of Van Tubergen and Setlow and Lin et al., while the second type was in agreement with Schwarz et al. and Donachie and Begg. She concluded that from overall results, insertion of new material into the wall takes place essentially in the central part of the cell, but that newly formed peptidoglycan is rapidly mixed with old and is dispersed over the whole sacculus. In a third type of experiment, attempts were made to find out how quickly new peptidoglycan was redistributed. Although not conclusive, results suggested that radioactive DAP molecules, of which 65 to 75% were located in the central portion of the sacculi just after the pulse, were homogeneously distributed within half a generation.

These results raise several points of interest. The overall growth of E. coli, without separating crosswall formation from cell elongation, would show up in labelling studies as diffuse intercalation. Considering that turnover has not been observed in E. coli, the concept of continual rearrangement of peptidoglycan is surprising, although it is possible that because the peptidoglycan is between two membrane layers, complete reutilisation of turnover products may occur. Also it is hard to imagine how a seemingly rigid and thin structure (peptidoglycan is only a few layers thick at most in E. coli) can undergo so much

reorganisation and still retain its integrity. Ryter suggests that a similar complex process as that discussed here for E. coli could account for the conflicting results in Bacillus species, but in this case, the process is further complicated by the thickness of the peptidoglycan layer.

Reeve and Mendelson (1973 and 1974) have approached the problem in a different manner, using mutants of B. subtilis. A temperature sensitive rod$^-$ mutant was isolated which grew as spheres at 45 C and as rods at 30 C. A double mutant carrying the rod$^-$ mutation in combination with a minicell producing mutation was constructed. Rod$^-$ morphology appeared as a swelling in the filamentous rod-shaped cells when they were transferred to 45 C. Data indicated that double mutants assembled new surface when transferred to 45 C primarily in the region close to one cell pole. Prolonged incubation led to additional regions of rod$^-$ morphology at other locations along the cell length. They have compared the initial site of rod$^-$ morphology to the location of the growth zone proposed by Donachie and Begg (1970), for slow growing cells. But Reeve and Mendelson's data differed from Donachie and Begg's in three respects; a) Reeve and Mendelson's growth zone was not always associated with the youngest pole, b) the sub-terminal growth zone was not restricted to short cells similar to the unit cells of E. coli, and c) rod$^-$ morphology sites arose in cells at different locations, suggesting that new growth zones could be initiated in regions not immediately adjacent to older growth zones, and thus need not be derived directly from old zones. Reeve and Mendelson were careful to point out that demonstration of a growth zone does not necessarily imply that the surface is synthesised only in this region. Thus their data are not incompatible with findings which suggest wall and membrane synthesis all over the surface. Their findings are interpreted strictly as a

growth zone; a region in which new material accumulates. Because of the dynamic nature of cell surface components, they emphasised the importance of drawing a distinction between synthesis and growth.

Thus from the conflicting data accumulated, the questions arise - what is actually being measured and what is it important to measure? A distinction has just been made between synthesis and growth. It is possible with some techniques, that only growth i. e. elongation which occurs in restricted zones can be visualised, and this effect can be most clearly seen in organisms which do not exhibit turnover. Obviously material must accumulate and be inserted into growing cross-walls; the question is whether this zone is only responsible for cross-wall formation or whether it is also involved in peripheral elongation as looks to be the case in Streptococci. It is reasonable to assume that wherever turnover is occurring, synthesis is also occurring at the same or a closely related site, unless turnover simply involves reorganisation of existing wall polymers. Evidence suggests that turnover products are released into the medium and not reincorporated (Mauck et al., 1970 and 1971). It therefore seems reasonable that cell growth involves growth at the site of cross-wall formation, peripheral elongation, and turnover when it occurs. Workers should perhaps be more careful to indicate which aspect(s) they are confident their techniques can demonstrate.

The weight of evidence for rod-shaped organisms indicates that the overall growth of these cells is not localised at discrete sites. It may be that in organisms where there is conflicting data, one technique has picked up one aspect of growth, while another may have shown up a different aspect or more than one aspect.

Growth Models for Rods. A suitable model for rods should account for the following, a) elongation of the peripheral wall to enable the cell

to approximately double in length prior to division, b) formation of the cross-wall, c) separation of the completed cross-wall giving rise to two hemispherical poles, d) turnover where it occurs, e) uniformly distributed wall thickening under some conditions, f) the presence of autolytic enzymes and g) the number of layers of peptidoglycan.

One of the main difficulties in postulating a model is the fact that practically nothing is known about control of division, for instance, how the cell knows where to form a septum, and how it ensures that each daughter cell receives a completed copy of the genome. Higgins and Shockman (1971) have suggested three alternative methods of growth with this in mind. 1. The cell could grow out in both directions from its midpoint, and when two half cell lengths have been fed out, the midpoint could be converted to a cross-wall. This is what seems to happen in Streptococci, and the midpoint is a junction between wall from two different generations. 2. There could be multiple surface growth sites, which would mean that the cell must find its next division site without the help of envelope markers, which would seem to require a much more complicated regulation. 3. Growth could occur at the end of the cylindrical part of the cell feeding out an equivalent length of new cylinder towards the nearby pole. As cited above, the weight of evidence favours 2. However Higgins and Shockman have presented a model designed for E. coli (Figure 4), based on the third alternative, their interpretation of the data on rods, information gained from Streptococci and their three-dimensional structure for peptidoglycan (discussed in the last section). They have also taken into consideration the observations of Donachie and Begg, the model which suggests that the cellular chromosome is spatially oriented via an attachment to the cell surface, so that synthesis and partition of sister chromosomes is physically coupled to surface extension and cell

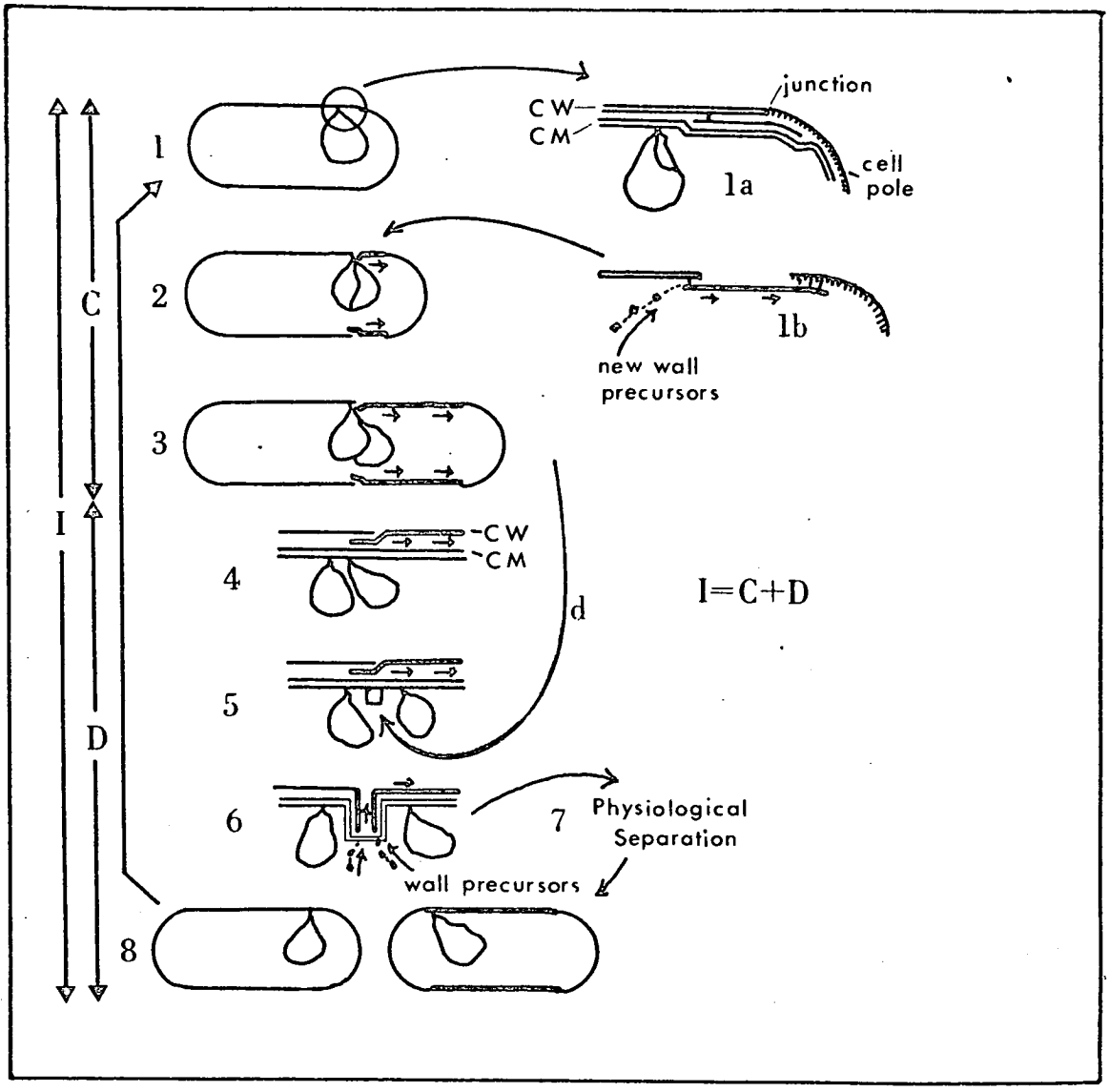


Fig. 4 Model for cell wall growth in E. coli (after Higgins & Shockman, 1971).

This represents a cell growing with a doubling time of 60 minutes. I is the time for accumulation of enough of a hypothetical initiator to allow initiation of a round of chromosome replication. C is the time required for replication of one chromosome. D is the time from completion of replication until division occurs. The cell shown has a single growth point. Simultaneously with the initiation of a round of chromosome replication, a wall elongation site is initiated (1a) at the junction of polar and cylindrical wall. The layer of new wall is begun under the old, and they are linked covalently or by electrostatic interaction or H-bonding. The old wall is cleaved to allow elongation, either by a closely regulated biosynthetic and hydrolytic sequence, or by the shearing of H-bonded layers. When replication is complete, approximately one unit of cell length has been fed out towards the pole, and the division trigger 'd' reacts to convert the elongation site to a cross-wall site. The membrane, carrying wall synthesising enzymes and precursors, envaginates forming an annulus. Precursors are now added at this point to both old and new wall. The cross-wall is completed, strengthened, and the walls separate. At faster growth rates, elongation sites can be initiated at the junction of a nascent cross-wall and cylindrical wall.

division (Jacob, Brenner & Cuzin, 1963) and the model for chromosome replication (Helmstetter, Cooper, Pierucci & Revelas, 1968). Higgins and Shockman's model is the most detailed available for rods.

This model accounts for elongation of cylindrical wall and formation of the cross-wall, but does not discuss any of the problems at the molecular level associated with the change of direction in cross-wall formation, except to say that the process is different from cylindrical wall growth. The model does not allow for diffuse intercalation: the only concession is that in faster growing cells more than one elongation site can be present. The model is designed for E. coli which has a very thin peptidoglycan layer and parts of the model are not easily translated to a peptidoglycan structure some 10 to 20 times thicker, such as is found in Gram-positive organisms. However, the model does account very neatly for the location of the division site, assuming that wall and membrane synthesis are closely related.

Mauck and Glaser (1972) from their data on 'nearest neighbour' analyses and turnover, have sketched an outline of how rods, especially B. subtilis and B. megaterium might grow. They, understandably, are in favour of diffuse intercalation. According to them, the septum, including the area where it connects to pre-existing wall, is completely new. Peripheral wall elongation occurs by random insertion of glycan chains into pre-existing wall. The walls of B. subtilis and B. megaterium turn over, so that new wall is the sum of wall required for growth and that needed to replace material lost by turnover. The structure of the wall must enable random excision of material over the whole surface without causing cell lysis. Mauck and Glaser consider that glycan strands arranged perpendicular to the cell surface rather than parallel to it, allow most easily for random insertion and random excision of material (Figure 5). This arrangement would allow random

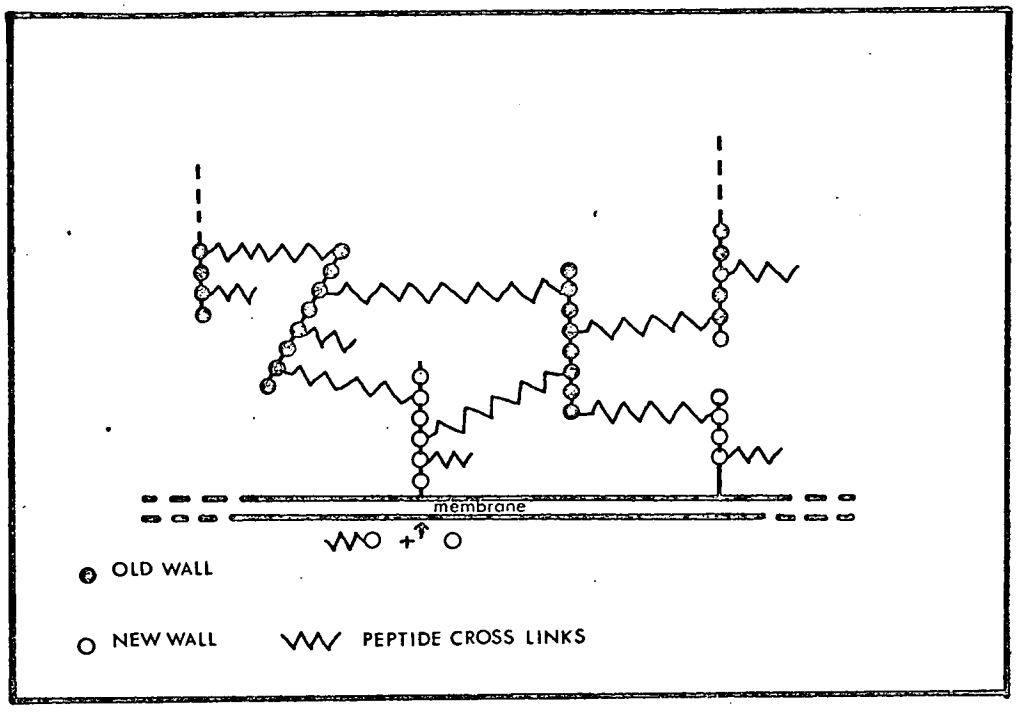


Fig. 5 Model of cell wall synthesis (after Mauck & Glaser, 1972).
It is considered in this model that the peptide cross-links can assume different conformations so that the distance between glycan chains, and the orientation of the strands are variable. For growth, some cross-links must be broken. Cell wall thickening can occur in the absence of lytic activity.

cross-linking of nascent glycan chains to pre-existing chains while the former are still attached to the membrane-lipid carrier. Mauck and Glaser consider that the evidence for a single growth zone in Gram-positive bacilli is not nearly so convincing as it is for cocci. They suggest that wall growth in cocci may correspond to septum formation in bacilli, and that elongation as such does not occur in cocci. Highton and Hobbs (1972) also suggested that the poles of B. cereus were formed by a process similar to that proposed by Higgins and Shockman (1971) for S. faecalis, but that growth of the cylindrical walls involved a different process.

Minicells

As well as the rod-shaped *B. subtilis* 168 used in the work to be discussed, a minicell-producing mutant, *B. subtilis* Cu403 div IV B1 has been studied.

Strains of bacteria producing minicells have been recognised for some time, and have been reported in *E. coli*, *Salmonella*, *B. subtilis* and other less well known species. Most of the information to date has been summarised by Frazer and Curtiss (1974). Minicells are produced continuously in the appropriate mutants and also very rarely in wild type strains. They are small non-growing bodies, produced by aberrant cell divisions at the poles of rod-shaped bacteria, and as such, they lack DNA. (Adler, Fischer, Cohen & Hardigree, 1967; Reeve, Mendelson, Coyne, Hallock & Cole, 1973). They do however, contain RNA and protein (Frazer & Curtiss, 1974), and are capable of transporting amino acids across their membranes, although they do not appear to incorporate these residues into macromolecules (Reeve & Mendelson, 1974). Minicells therefore, provide an opportunity to determine the kinds of functions which genomeless cells can perform. Since they represent a compartmentalisation of cell pole regions, they can be analysed for the pole-specific location of cell components, and also for surface composition of the poles compared to cylindrical wall. Because of their size, they are easily separated from the parental cells. They also offer a unique system for studying the expression of transformed genetic material.

The growth phase of a culture, as well as the growth medium, can influence minicell production. For example, minicell formation is most frequent in late exponential phase and early stationary phase in *B. subtilis* (Reeve, 1974; Mendelson & Reeve, 1973). Various studies suggest that the septation process resulting in the formation of minicells is normal in all respects except for placement of the septum.

for example, Reeve et al. (1973) found that the ultrastucture of such a septum was identical to that of one between two cells undergoing normal division, and indistinguishable from the side wall.

However, most workers seem to agree that there is a difference between cylindrical walls and cross-walls or poles in rods. Evidence for this comes from several observations. The cross-wall synthesising system of E. coli has been shown to have a preferential sensitivity to penicillin (Schwarz et al., 1969). Highton and Hobbs (1971) found that the cross-wall region of B. licheniformis also differed in sensitivity to penicillin from the cylindrical walls, although their results were the reverse of those obtained by Schwarz et al. for E. coli. In B. licheniformis the cross-wall synthesising system appeared to be less sensitive to penicillin. In B. subtilis the polar regions of the cell are much more resistant to the action of autolytic N-acetylmuramyl-L-alanine amidase (Fan, Pelvit & Cunningham, 1972) although Fan, Beckman & Beckman (1974) have subsequently found, by measuring the change in wall buoyant density of the cell ends of B. subtilis grown in D_2O and shifted to H_2O, that walls from the polar regions turned over extensively, similar to the cylindrical wall. Frehel et al.,(1971) found that cell ends remained smooth and thickened, while side walls started to fragment and become thinner, during recovery from protein synthesis inhibition. Such evidence has led to the idea that cell poles once formed are conserved, and indeed it is not obvious why a cell need alter its poles once they are completed.

After consideration of the evidence available, I am in favour of a mode of growth for Gram-positive rods which involves peptidoglycan synthesis all over the cylindrical surface, and it is with this idea in mind that I have interpreted the results to be discussed.

CHAPTER II

PROPERTIES OF THE AUTOLYSINS AND PURIFICATION OF THE AMIDASE

INTRODUCTION

It was decided to approach the problem of cell growth and division by looking at the lytic enzyme system of *B. subtilis*, with the intention of purifying one of the enzymes, and subsequently, localising it within the cell. From the distribution in the cell, it was hoped to gain some information about the stages in the growth cycle in which the enzyme is involved.

B. subtilis has been reported to contain at least two lytic enzymes, an amidase and a glycosidase (Brown & Young, 1970; Fan & Beckman, 1972), with the amidase the most active (Fan & Beckman, 1972; Herbold & Glaser, 1975). In fact, Fan claimed that the amidase activity was ten times greater than that of the glycosidase. Thus amidase was chosen for purification.

At the time this work was initiated, several reports of purification of amidase from other species (Singer, Wise & Park, 1972; Chan & Glaser, 1972) and three reports of partial purification from *B. subtilis* (Brown, Fraser & Young, 1970; Brown, 1972; Fan & Beckman, 1972) had been published. Brown *et al.* used cells from post-exponential phase cultures and allowed them to autolyse. Their attempts to purify the crude enzyme extract by ammonium sulphate precipitation and gel filtration on Sephadex G100 and G200 were unsuccessful. The enzyme appeared to be associated with teichoic acid, the bulk of which could be removed from the autolysate by precipitation with ethanol, followed by gel filtration on agarose columns. The remaining 5% of teichoic acid persisted through a variety of purification techniques.

It was found that autolysins could be extracted from whole cells and walls by strong salt solutions (Fan, 1970a; Pooley, Porres-Juan & Shockman, 1972). It was also shown that sodium dodecyl sulphate (SDS) treated walls bound autolysin (Shockman, Thompson & Conover, 1967; Fan, 1970b). Brown (1972) used these two facts in his purification

technique. He extracted enzymes from whole cells in 5M NaCl, rebound them to SDS-treated walls, and then re-extracted them from the latter three times with 5M NaCl. This resulted in an 11 fold purification with a 55% yield.

Fan and Beckman (1972) also used salt extraction in their centrifugation technique to separate the amidase and the glycosidase of B. subtilis. They centrifuged mid-exponential phase walls through a linear gradient of 0 to 2M LiCl and 10% to 25% sucrose, which resulted in banding of the two autolysins at separate positions in the gradient, because they were removed by different concentrations of LiCl (0.5M for glycosidase and 1.5M for amidase). Fan and Beckman also separated the enzymes by chromatography of 3M LiCl-extracted enzyme mixture through a Sephadex G75 column. In both cases , they recovered one third of the autolytic activity. The enzymes were characterised with respect to pH optima and molecular weights; the glycosidase had a pH optimum between 5 and 8, was relatively heat sensitive and had a molecular weight of 60,000 (from a calibrated G75 column), while the amidase had a pH optimum around 8, was relatively heat stable, and had a molecular weight of 35,000. Because very little protein could be detected in the region of lytic activity, they concluded that their enzymes were largely uncontaminated by other proteins.

During the latter stages of the work described in this thesis, a report was published by Herbold and Glaser (1975) of their purification of B. subtilis N-acetylmuramyl-L-alanine amidase. They used a culture at the end of exponential phase and the enzyme was extracted from the harvested cells with 5M LiCl. After extensive dialysis and treatment with DNAase and RNAase, the enzyme was eluted from a hydroxyapatite column. Subsequently elution from a Bio-gel A-1.5m column separated the enzyme from a modifier protein which was found to stimulate the activity

of the amidase. The modifier subunit was highly insoluble at low ionic strength, as was the impure enzyme-modifier complex. Therefore, high ionic strength was required at all stages of the purification, until enzyme and modifier protein were separated. Thus the high salt concentration used to extract the enzyme served both to elute the enzyme from the wall and to disocciate the enzyme-modifier coplex. The activity of the purified enzyme was shown to be that of an amidase, and both the enzyme and the modifier ran as single bands on SDS gels. From these, the calculated subunit molecular weight was 51,000 for amidase and 80,000 for the modifier. Sucrose density gradients showed the enzyme to be a monomer with a molecular weight of 47,000. The enzyme bound modifier in a 1:1 ratio when the ionic strength was decreased, giving a faster sedimenting complex. Herbold and Glaser calculated that about 1 in 1,000 glycan chains were capable of binding enzyme at any given time. They found that the enzyme activity was lower in tris buffers than in others and that it required a divalent cation. By using radioactively labelled walls, they showed that once the enzyme was bound to the walls, these had to be completely hydrolysed before the enzyme was released and was bound to other walls. They suggested that the molecular weight of the amidase is small enough to allow it to pass easily through the wall. But binding of the modifier brings it into a size range where it might no longer escape into the growth medium.

MATERIALS AND METHODS

1. Growth of B. subtilis 168. B. subtilis 168 obtained from Dr M. Masters, was stored as spores at 4°C. Cultures were grown in S broth (Evans peptone 10g, Lab lemco powder, Oxoid, 2.4g, NaCl 2.0g in 1 l H_2O) at 37°C with shaking. Growth was followed by optical density (O.D.) measurements at 675nm, with a 1cm path length, on a Zeiss (PM QII) spectrophotometer. An inoculum from a growing culture showed a lag of less than one hour, compared to three hours for a culture inoculated from spores.

2. Preparation of Cell Walls. Cultures were harvested in mid-exponential phase in a Sorvall RC2-B centrifuge at room temperature. All further operations were carried out at 4°C. The cells were washed in H_2O prior to sonication. Since walls were required for enzyme assays and electron microscopy, as well as for preparation of the enzyme, a compromise was reached between maximum breakage of whole cells and the size of wall fragments. The optimum conditions were found to be as follows; cells from a 2 l culture were resuspended in 5ml of H_2O and sonicated (MSE probe sonicator, 220V output) for 3 x 15s. This gave between 60 and 70% cell breakage as checked by phase microscopy. Whole cells were removed by centrifugation (2 x 5min at 500g) and then walls were spun down(15min at 12,000g), and washed 3 times with H_2O. If necessary the low speed spin was repeated to reduce the number of whole cells to less than 1%.

3. Buffers. (As per Fan & Beckman, 1972). The following buffers were used, T buffer (tris,pH8.0), TK buffer (tris/KCl, pH8.6), TM buffer (tris/maleic anhydride, pH6.0) and TMB buffer (tris/maleic anhydride/boric acid, at the required pH).

4. Preparation of Autolysin-free Walls (SDS-Walls). Before walls could be used as a substrate for enzyme assay, it was necessary to inactivate

their endogenous autolysins. It was found that walls which were boiled for 10 to 15 min were reduced to 70% of their original O.D. in 3h. Walls treated with 3M LiCl in TK buffer (the method used in removing enzyme for purification) lost 40% of their O.D. in 1h and 90% in 3h. However, walls treated with 1% SDS in T buffer at 100 C for 10min, lost only 5% of their O.D. in 4h. SDS inactivation of both walls and cells was tried for various times. It was found that whole cells did not form a good suspension in SDS, and the most effective method of inactivation was suspension of washed walls in 1% SDS overnight at 37 C, followed by thorough washing in H_2O. After this treatment, walls underwent virtually no autolysis when incubated in TK buffer at 45 C. A solution of B. subtilis walls of concentration 1mg/ml had an O.D. at 540nm of approximately 4 (Fan & Beckman, 1973).

5. Assay for Autolytic Activity. A sample of fresh, untreated walls was suspended in TK or TM buffer at 45 C or 37 C respectively, and autolysis was followed by measuring the decrease in O.D. For comparative purposes, lytic activity was expressed in terms of half-life or half-life^{-1}. Half-life was the time required for the O.D. of the wall suspension to be reduced by 50% at the initial reaction velocity.

6. Crude Autolysin Preparation (Crude Enzyme). The method using high salt concentrations discussed in the introduction was used. For small preparations, washed walls were resuspended in 3M LiCl in TK buffer in an ice bath (1ml solution/ 1 original culture). After 15min the walls were spun out (27,000g for 10min) and the extraction repeated twice more. Extracts were combined and spun at 27,000g for 20min to bring down any remaining pieces of wall, and were stored at -20 C for several months without loss of activity. For larger preparations, batches of 100 or 200 l were grown on a fermenter and harvested in a continuous flow centrifuge. The resulting cell paste was washed in H_2O before

sonication. And walls were isolated as described in section 2 above, except that one or more extra minutes of sonication were usually required, and repetition of the differential centrifugation was essential. Extraction of the enzyme was as above.

7. Protein Estimation of Enzyme Preparations. Routinely the method of Warburg and Christian (1942) was used, but for more accurate determinations or for very low levels the Lowry method was used (Lowry, Rosebrough, Farr & Randall, 1951).

8. Standard Rebinding Assay for Autolysins extracted with 3M LiCl. SDS-walls were resuspended in the appropriate buffer to give an O.D. of approximately 0.2 (0.05mg/ml). One ml of wall suspension and 0.1ml of extracted enzyme solution were mixed in a centrifuge tube. The mixture was diluted with at least 10ml H_2O to allow rebinding (on ice for 5min). The suspension was then spun at 27,000g for 5min, the walls were washed and resuspended in 1ml of buffer. O.D. decrease was followed as described in section 5. One unit of enzyme activity caused an O.D. decrease of 0.001 per min.

9. Removal of Teichoic Acid from Walls. The method of Hughes et al., 1968, where SDS-walls were suspended in 0.1M NaOH, was used.

10. Reducing-group Assay for Glycosidase. A modification of the original Park and Johnson method was used (Thompson & Shockman, 1968).

11. p-Nitrophenyl Assay for Glycosidase. The method is similar to that used by Ortiz, Gillespie and Berkeley (1972), using the substrate p-nitrophenyl-2-acetamido-2-deoxy- -D-glucopyranoside (Koch-light). Enzyme solutions (0.2ml) were incubated with 0.2ml substrate(2mM) in 0.2ml sodium phosphate buffer (0.1M, pH5.9) at 37 C. After 30min, the reaction was stopped by the addition of 0.8ml borate buffer (0.2M, pH9.8). The absorption of p-nitrophenyl released was measured at 400nm. One unit of enzyme activity was defined as that which released 1uM p-nitro-

phenyl per min at pH 5.9 and 37 C. Controls were set up with addition of enzyme after the borate buffer.

12. Glycosidase Assay using Micrococcus lysodeikticus. M. lysodeikticus (obtained from the Microbiology Dept., Edinburgh University) was grown to exponential phase in nutrient broth(Oxoid No. 2, 25g/l) on a shaker at 37 C. Cells were then harvested and whole cells or walls (prepared as for B. subtilis, but with longer sonication times) were treated with SDS as described in section 4, and washed. Rebinding assays were done in TM or TK buffer at 37 C. Controls were run containing no enzyme. M. lysodeikticus walls also had an O.D. of approximately 4 at a concentration of 1mg/ml (Fan & Beckman, 1973).

13. Amidase Assay using an Amide Substrate.

Reagents-	a) 0.1M tris buffer pH 7.2	0.9ml
	b) 2M hydroxylamine pH 7.2 (freshly adjusted)	0.5ml
	c) enzyme	0.1ml
	d) H_2O	0.0 to 0.5ml
	e) 0.5M amide substrate	0.0 to 0.5ml

Tubes containing a) to d) above were set up in a water bath at 37 C, and the reaction was started by addition of the amide substrate. The reaction was stopped after 15min by addition of colour reagent to each tube ($FeCl_3$ 6% (w/v)in HCl 2% (w/v)). A series of tubes with the enzyme omitted was treated in the same way to estimate chemical reaction. After shaking to remove bubbles, the O.D.'s of the reaction mixtures were measured at 500nm. Concentrations of hydroxylamine and substrate were varied and different enzyme preparations were used, e.g. crude enzyme, whole cells, and walls with enzyme still attached.

14. Perret Assay for Amidase.

Reagents- Substrate: 0.007M penicillin G (sodium salt, Glaxo) in 0.1M phosphate buffer pH 7. Kept on ice.

Enzyme: in 0.5% buffered gelatin

Iodine: 0.0166N in 1.75M sodium acetate buffer pH 4

Thiosulphate: 0.0166N in H_2O

Starch: 1% (for end point of titration)

Penicillin was first warmed to 30 C, and then 5ml of substrate, 0.5ml of gelatin (0.5%) and 1ml of enzyme solution were added to a 50ml conical flask, and gently shaken in a water bath at 30 C for 100min. Ten ml of iodine were added and the mixture was allowed to stand for a further 10min so that the iodine could react with any penicilloic acid formed. Any unreacted iodine was then titrated with thiosulphate. A control flask was set up, to which the enzyme was added after the iodine. The enzyme activity was calculated from;

$$2 \times \Delta \times \frac{60}{\text{Time(min)}} \times \frac{1}{\text{Vol(enzyme)}} = \text{units/ml}$$

where Δ is the difference between the volume of thiosulphate used to titrate the control flask and the flask containing enzyme.

15. Concentration of Large Volumes. Unless otherwise indicated, enzyme solutions were first dialysed overnight at 4 C against tris or TK buffer to remove high concentrations of LiCl.

a) Dialysis against glycerol. The enzyme solution was dialysed overnight at 4 C against 50% glycerol (w/v) in TK buffer.

b) Dialysis against polyethylene glycol (PEG). The enzyme solution was dialysed overnight at 4 C against a 20% solution (w/v) of PEG, M. W. range 15,000 to 20,000, in TK buffer.

c) Sephadex G200. The enzyme solution in a dialysis sack was laid in a petri dish and covered with Sephadex G200 (Pharmacia). This method was more rapid than the previous two, requiring only a few hours at 4 C.

d) Ammonium sulphate precipitation. A saturated solution of ammonium sulphate was added dropwise, with stirring in the cold, to give the

required saturation in the enzyme solution. After standing in the cold for 15min to allow a precipitate to form, the solution was spun at 27,000g for 15min. Any precipitate was redissolved in an appropriate amount of TK buffer. Protein concentration was estimated by the method of Warburg and Christian (1942), and lytic activity of redissolved precipitate and supernatant was tested by the rebinding assay.

e) Ethanol precipitation. Absolute alcohol was added to the enzyme solution as described in d). The solution was left in the cold for 30min to allow a precipitate to form. It was then spun at 27,000g for 15min. Any precipitate was redissolved in the appropriate amount of TK buffer.

f) Amicon filtration. In this case it was unnecessary to dialyse out LiCl before the enzyme solution was concentrated. Filtration was done in an Amicon cell, 10 or 50ml size depending on the volume of solution, using a PM10 diaflo membrane, which excluded any molecules of molecular weight greater than 10,000. Before use, the membranes were soaked for at least 1h with frequent changes of H_2O, to remove the protective coating of glycerin. If required for re-use, the membranes were washed in 2M NaCl or dilute NaOH, thoroughly rinsed with H_2O and stored in 10% ethanol in water (v/v) at 4 C. The Amicon cell was operated at 4 C under pressure (N_2) not exceeding 70psi.

16. LiCl Sucrose Gradients. Walls were prepared using H_2O instead of buffer throughout. Three l of original culture provided enough sample for 1 gradient. Gradients were made in 34ml tubes using a gradient maker (Buchler Instruments) containing the following solutions; a) 10% sucrose and b) 25% sucrose/2M LiCl in TK buffer, giving a gradient of 0 to 2M LiCl. Runs were done at 15,000rpm, 10,000rpm or 8,000rpm for 70min, in a Beckman L2-50 centrifuge using an SW 25.1 rotor. Fractions (2ml) were collected downwards by piercing the tube. Amidase is

released at around 1,5M LiCl i.e. near the bottom of the tube, whereas glycosidase is released at around 0.5M LiCl, much nearer the top of the tube (Fan & Beckman, 1972).

17. Sephadex Chromatography. A column (90 x 2.5cm) contained Sephadex G75 or G100 equilibrated with 3M LiCl/TK buffer. The samples were loaded in 3M LiCl/TK buffer with a trace amount of 0.5% blue dextran when a marker was required. Fractions were collected from the top of the column using an LKB peristaltic pump and fraction collector. The flow rate was approximately 0.5ml/min, and 2ml fractions were collected. Fractions were assayed for protein and lytic activity.

18. Acrylamide Gel Electrophoresis. The following solutions were required;

Running buffer (Tris-glycine reservoir buffer). Tris HCl (12g) and 57.6g glycine were dissolved in 1 l of H_2O, adjusted to pH8.6 with NH_4OH, and diluted x 4 for use, giving 0.025M tris and 0.192M glycine, with mercaptoethanol added to a final concentration of 0.1%.

10% SDS. Ten g of SDS (BDH, specially pure) was dissolved in 100ml H_2O.

Sample buffer. Glycerol (20ml), 2-mercaptoethanol (5ml), 10% SDS (30ml) and upper tris (12.5ml) were made up to 100ml with H_2O.

Lower tris. Tris HCl (18.17g) and 4ml of 10% SDS were made up to 100ml with H_2O and adjusted to pH 8.8 with conc. HCl.

Upper tris. Tris HCl (6.06g) and 4ml of 10% SDS were made up to 100ml with H_2O and adjusted to pH 6.8.

Ammonium persulphate. 100mg of ammonium persulphate in 1ml H_2O was freshly prepared.

Temed. N,N,N',N'-Tetramethylethylenediamine (Koch-light).

Acrylamide. Thirty g acrylamide (recrystallised) and 0.8g of N,N-methylene-bis-acrylamide were made up to 100ml with H_2O.

SDS Slab Gel (15 x 10cm)

Lower gel

H_2O	16.6ml
Lower tris	10.0ml
Acrylamide	13.3ml
Ammonium persulphate	0.12ml
Temed	15ul

Upper (Stacking) Gel

H_2O	3.2ml
Upper tris	1.25ml
Acrylamide	0.5ml
Ammonium persulphate	0.12ml
Temed	15ul

Gel solutions were degassed before use. When polymerised, the gel was set up in the tank and the running buffer was poured in. Samples were heated for 4min at 100 C in sample buffer and were then loaded on to the stacking gel with a syringe (up to 50ul could be accomodated in each well). A current of about 15mA was passed until the samples had left the stacking gel and entered the lower gel. The current was then increased to about 20mA. After the run was completed, the gel was removed and fixed in 20% sulphosalicylic acid for several hours, followed by staining overnight at room temperature or 37 C.

Stain

50% TCA (tri-chloro acetic acid)	100ml
H_2O	400ml
5% Coomassie blue.	2ml

The gel was then destained in 10% TCA.

Non-SDS Gel. These gels were made from the same recipes as SDS gels, omitting the SDS, with the amount of ammonium persulphate and temed

adjusted to give similar gelling times. These gels were run at 4 C and so slight adjustment of the current was also necessary.

RESULTS AND DISCUSSION

Properties of the Autolysins

Treatment of walls of B. subtilis with 3M LiCl was known to release autolysins, and the only specificities so far detected were those of an amidase and a glycosidase. Very little information was available about either of these enzymes other than that published by Fan and Beckman, (1972). Also there did not appear to exist a specific routine assay to measure one enzyme in the presence of the other. Because of this, it was decided to attempt to find such an assay and to look at some of the properties of the two enzymes.

Assays for Amidase and Glycosidase. Assays for lytic activity such as autolysis of whole cells, and rebinding assays, do not distinguish between different specificities, and assays which do, suffer from several disadvantages. For example, detection of N-terminal L-alanine groups released by amidase (Ghuysen, Tipper & Strominger, 1966) is too time-consuming to be used to assay a large number of fractions from a Sephadex column. The reducing group release assay is complicated by the background readings from other reducing groups already present in the wall, and so very small amounts of glycosidase activity may not be detected. Several approaches were used in an attempt to find a specific assay.

1. Amidase assay using an amide substrate. This was based on an assay for transferase activity (Lipmann & Tuttle, 1945; Brammar, 1965), in which estimation of the active enzyme depended on the formation of acylhydroxamate from hydroxylamine and an amide substrate in a standard time. The acyl hydroxamate formed a red complex with trivalent iron and could therefore be measured spectrophotometrically. Brammar (1965) used this assay for an intracellular amidase (an aliphatic amide amidohydrolase) of Pseudomonas aeruginosa. However, with the wall amidase

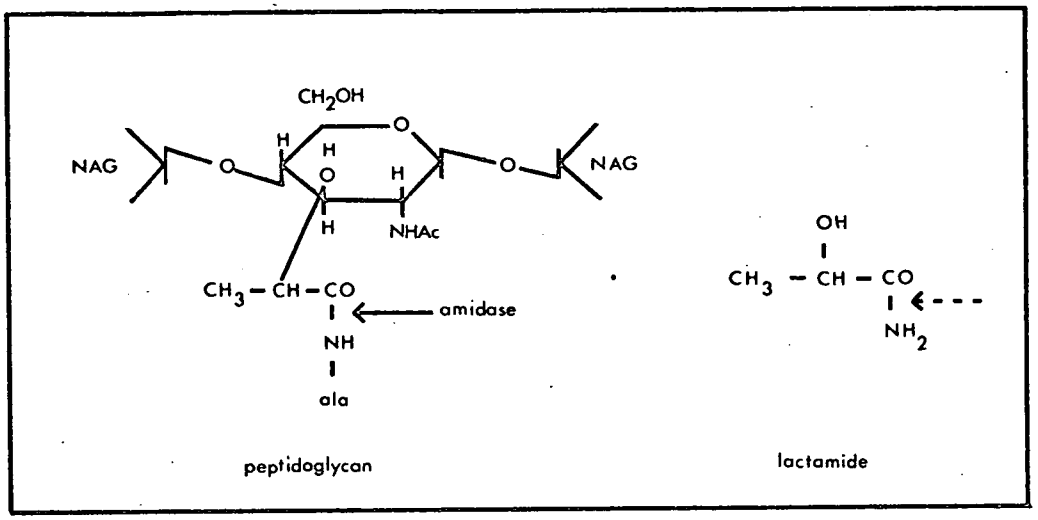


Fig. 6 Similarity between the structure of lactamide and the bond in the cell wall attacked by amidase.

of B. subtilis, no enzymic reaction took place, and any product formed was due only to chemical reaction. A variety of amide substrates were tested, acetamide, propionamide, butyramide, hexamide and lactamide. Lactamide in particular offered a similar bond to that broken by the amidase in the cell wall (Figure 6), but it was not similar enough to act as a substrate. Varying concentrations and reaction conditions only affected the amount of chemical product formed. To check the results, the assay was carried out using a suspension of P. aeruginosa C142 and B. subtilis 168. Results obtained were as follows;

Table 1.

Substrate	Enzyme source	Reaction
acetamide	C142	+++
acetamide	168	-
lactamide	C142	-
lactamide	168	-

Lactamide was a good inducer of the enzyme in P. aeruginosa, but a poor substrate.

2. Perret assay for amidase. Penicillinase splits a bond in the penicillin molecule between a nitrogen and a carbon as does the amidase in the cell wall. Also the structure of penicillin has been likened to that of D-alanyl-Dalanine, and although the bond in the wall involves an L-alanine, it was hoped that there might be enough stereochemical resemblance for the amidase to mimic penicillinase when penicillin was used as a substrate. The standard assay for penicillinase is the Perret assay in which penicilloic acid, formed as a result of enzyme action, is reacted with iodine and any remaining iodine is titrated with thiosulphate. The assay was carried out as described and the results were negative in all, but one assay, where the result was too low to be significant. This result was not surprising, since if amidase activity gave a positive result with the Perret assay, this would complicate penicillinase assays in species which contained both enzymes, and no such complication has been reported. A further point on which no data could be found was whether the penicillin inhibited or activated the lytic enzymes. Thus the following experiment was done; to 1ml suspensions of SDS-walls (O.D. 0.2), were added varying volumes of a crude enzyme solution (5mg/ml) and a penicillin solution (50mg/ml). The assay was carried out at 45 C in TK buffer. Results are shown in Table 2.

Table 2

Volume of enzyme in ul	Volume of pen. in ul	Half-life^{-1} in min^{-1}
0	0	∞
40	0	0.029
40	100	0.035
40	50	0.037
40	40	0.038
40	20	0.040
40	10	0.042
40	2	0.042

Similar results were found using lysozyme (0.1mg/ml) in place of crude enzyme.

Thus there was no evidence for inhibition of activity at the concentrations used, and all the rates measured in the presence of penicillin were faster than in its absence. However, when the experiment was repeated using whole autolysing cells, it was found that at concentrations of penicillin of 5mg/ml and above, there was increasing inhibition of autolysis.

These results showed at least that the reason for negative results with the Perret assay for amidase was not the inhibition of the enzyme by penicillin.

Because of lack of success with amidase, it was decided to try to find a specific assay for glycosidase, since this would also facilitate separation of the two activities.

3. p-Nitrophenyl assay for Glycosidase. p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside is a substrate for an N-acetyl-β-D-glucosaminidase. The enzyme reaction releases p-nitrophenol, which can be measured spectrophotometrically. Ortiz et al. (1972) used such a system to assay the activity of an exo-β-N-acetylglucosaminidase from B. subtilis B. However, when the assay was applied to the endo-N-acetyl-glucosaminidase of B. subtilis 168, no activity could be detected. This result was also obtained by Fan and Beckman (1973).

4. M. lysodeikticus walls as a substrate for glycosidase. Fan and Beckman (1973) reported that M. lysodeikticus cells or walls could be used to assay specifically the glycosidase of B. subtilis 168 in the presence of amidase at pH 6 . They suggested that the inability of the amidase to act on these walls could result from the differences in the peptide subunits of B. subtilis (Hughes, 1970; Warth & Strominger, 1971) and M. lysodeikticus (Ghuysen, Bricas, Lache & Leyh-Bouille, 1968).

The assay was therefore carried out using freshly prepared crude enzyme from B. subtilis 168. The rebinding technique was used on

M. lysodeikticus walls at pH 8.6 and 6.0, incubated at 37 C, and also on B. subtilis walls under the same conditions. Results are shown below;

Table 3

Walls	pH	Half-life in min.
M. lysodeikticus	6.0	13
M. lysodeikticus	8.6	21
B. subtilis	6.0	70
B. subtilis	8.6	35

The optimum pH for activity on M. lysodeikticus walls was 6.0 (that of glycosidase), while for B. subtilis it was 8.6 (that of amidase). The results indicated that either there was still considerable glycosidase activity at the higher pH, or amidase was acting on the Micrococcus walls at this pH. To distinguish between these, a sample of the crude enzyme was heated to 52 C to inactivate the glycosidase.(Fan & Beckman, 1972), and the assays were repeated as above with enzyme samples removed after 5, 15 and 30min of heating.

Table 4

Walls	pH	Inactivation time at 52 C (min)	Half-life (min)
M. lysodeikticus	6.0	0	13
	6.0	5	15
	6.0	15	∞
	6.0	30	∞
M. lysodeikticus	8.6	0	26
	8.6	5	72
	8.6	15	73
	8.6	30	∞
B. subtilis	6.0	0	62
	6.0	5	63
	6.0	15	73
	6.0	30	85
B. subtilis	8.6	0	35
	8.6	5	34
	8.6	15	38
	8.6	30	58

These results indicated that the activity attacking M. lysodeikticus walls at pH 6.0 was destroyed after heating for 15min at 52 C. At pH 8.6 however, loss of activity was slower, suggesting that amidase may also be involved. When B. subtilis walls were used, a small decrease in activity was observed at both pH values, presumably due to the destruction of glycosidase.

The experiment was repeated using two separate extractions from B. subtilis walls, one in 1M LiCl which should release mainly glycosidase (Fan & Beckman, 1972 and 1973), and a second in 3M LiCl which should release mainly amidase.

Table 5 Walls	pH	Enzyme	Half-life (min)
M. lysodeikticus	6.0	1M	31
	6.0	1M 20'@ 52 C	∞
	6.0	3M	∞
B. subtilis	8.6	1M	86
	8.6	3M	47

The above results suggest that there was no amidase acting on the Micrococcus walls at pH 6.0, thus providing a useful routine assay for the glycosidase in the presence of amidase.

It therefore appeared that neither enzyme would act on a synthetic substrate, and that any assay system required bacterial cell walls as a substrate. In the case of glycosidase, it was possible to use M. lysodeikticus walls which were apparently resistant to attack by the amidase at pH 6.0. It was decided that the most efficient means of detecting amidase activity, throughout a purification procedure for instance, was to use the rebinding assay on B. subtilis walls at pH 8.6 and to resort to a specific amidase assay only in the last stages of purification. A suitable substrate for the latter would be the fraction of peptidoglycan resulting from lysozyme digestion of the wall, designated C6 (Primosigh, Pelzer Maass & Weidel, 1961).

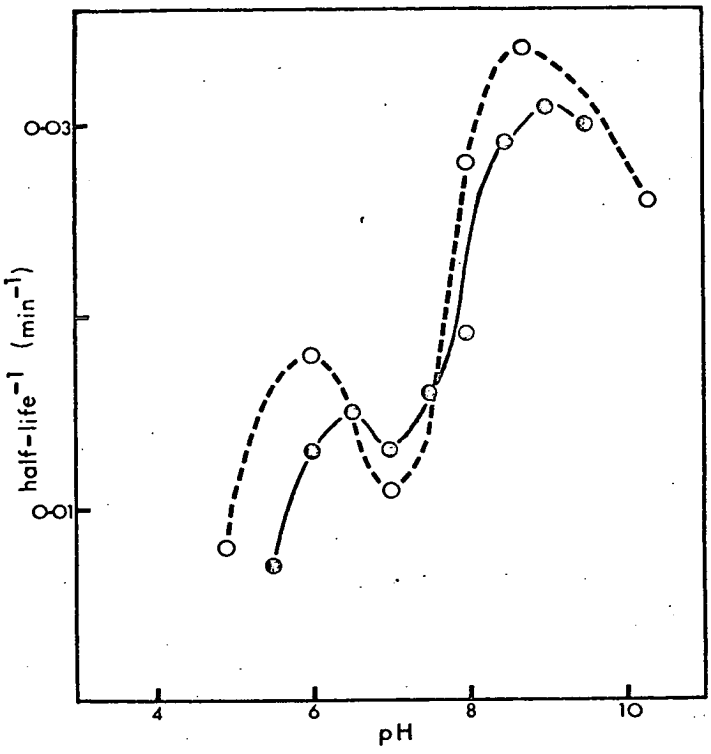


Fig. 7 pH Optima of the Autolysins. The assays were done in TMB buffer at 45 C using whole cells (●) and SDS-walls with rebound enzyme (O).

pH Optima of the Autolysins. Freshly prepared walls and SDS-walls with enzyme rebound were incubated at 45 C in TMB buffer at various pH values (checked immediately before use, since the buffer did not store well), and the decrease in O.D. was followed. The results are plotted in Figure 7, and they indicated two pH optima almost the same for whole cells as for walls with rebound enzyme. These were in agreement with the results of Fan and Beckman (1972) who identified the enzyme with a pH optimum of around 6.0 as glycosidase and that with an optimum between 8.0 and 9.0 as amidase.

Lytic Activity during Growth of a Culture. Cultures of strain 168 were grown at 37 C for 3h (exponential phase), 4.5h (late exponential-early stationary phase) and 15.5h (late stationary phase). In each culture the following samples were analysed for autolytic activity; a) autolysing whole cells, b) SDS-walls + crude enzyme from walls of the culture,

c) walls washed after removal of crude enzyme, d) SDS-walls + supernatant from sonicated cell suspension (in contact with the walls throughout incubation) and e) SDS-walls + supernatant as in d) but rebound to the walls which were then washed before incubation. The results are shown in Figure 8. It was concluded that 1) as the age of the culture increased, autolysis slowed down, 2) the enzyme removed by LiCl and rebound to SDS-walls was active at all three times, 3) the activity of the enzyme remaining on the wall after LiCl extraction, decreased with increasing cell age, and 4) there was activity in the supernatant from the broken cell suspension and this activity was able to rebind to SDS-walls. It therefore appeared that there was loss of autolytic activity with increasing cell age. This may have been due to loss of enzyme or to modification of the wall structure in older cells making them more resistant to autolysins, so that only when enzyme was removed and rebound to exponential phase SDS-walls, would it exhibit normal activity. The latter alternative was supported by the difficulty in sonicating older cells.

A further experiment was carried out to test for activity in the culture supernatant. Three h, 4h and 17h cultures were examined. Supernatant was added to enough SDS-walls to give an O.D. of 1.0 to 1.2 and the O.D. followed with time. It was concluded from the results in Figure 9 that there was very little, if any, detectable activity in the supernatant of exponential phase cultures, but considerable activity in that of stationary phase cultures. It is not known whether this activity is the same as that bound to the walls during the earlier stage of growth.

Lytic Activity on Teichoic Acid-Free Walls. Hughes et al. (1968) claimed that 0.1M NaOH at 35 C, in the absence of oxygen, removed rapidly and completely the teichoic acids from B. subtilis W23 and 168.

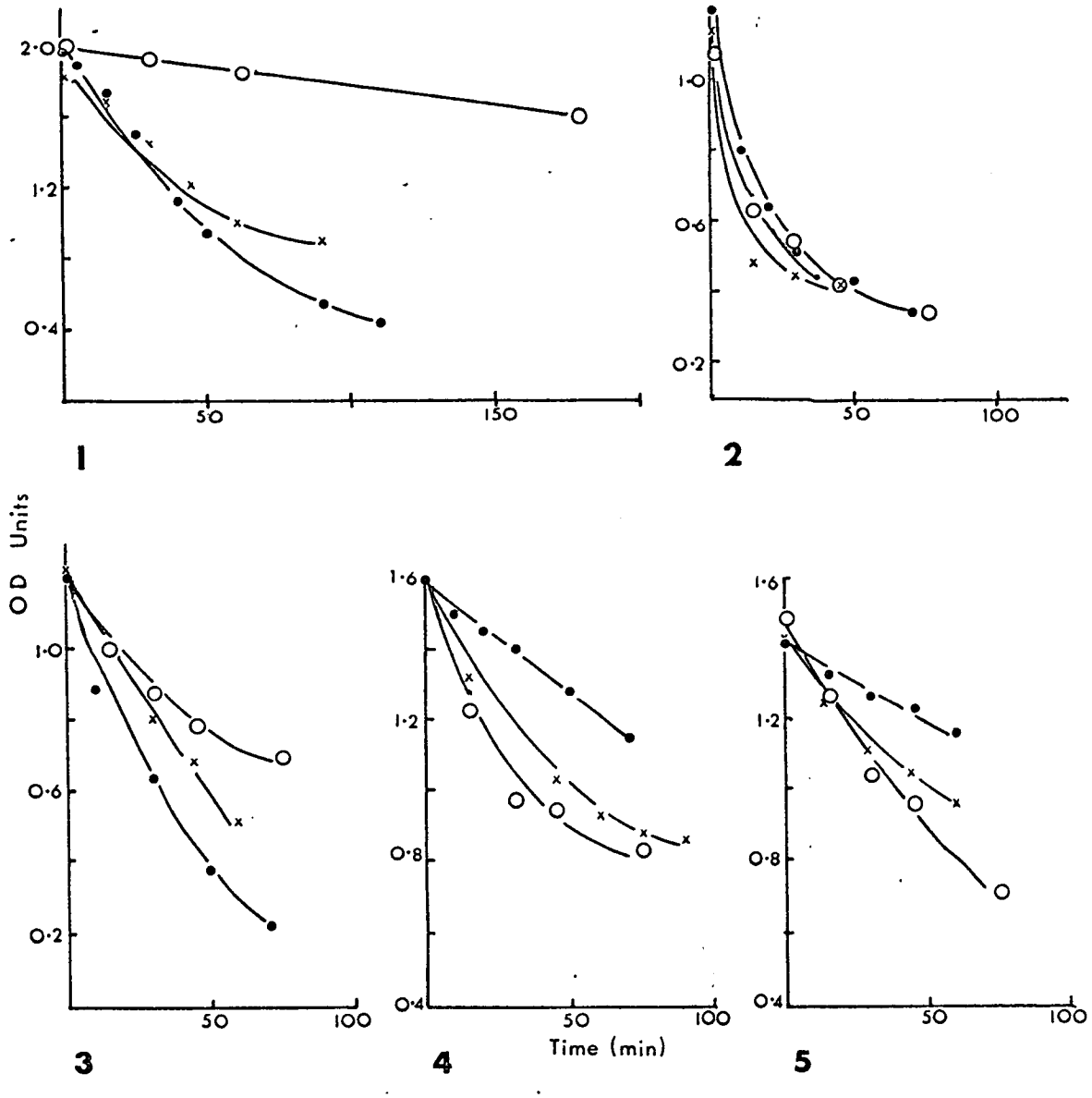


Fig. 8 Lytic Activity during Growth of a Culture
1) autolysing whole cells, 2) SDS-walls + crude enzyme from the walls of the culture, 3) walls washed after removal of crude enzyme, 4) SDS-walls + supernatant from sonicated cell suspension, 5) SDS-walls + supernatant as in 4) but rebound to the walls. The assays were done in TK buffer at 45 C. The cultures are represented by •—3h, x——4.5h and O—15·5h.

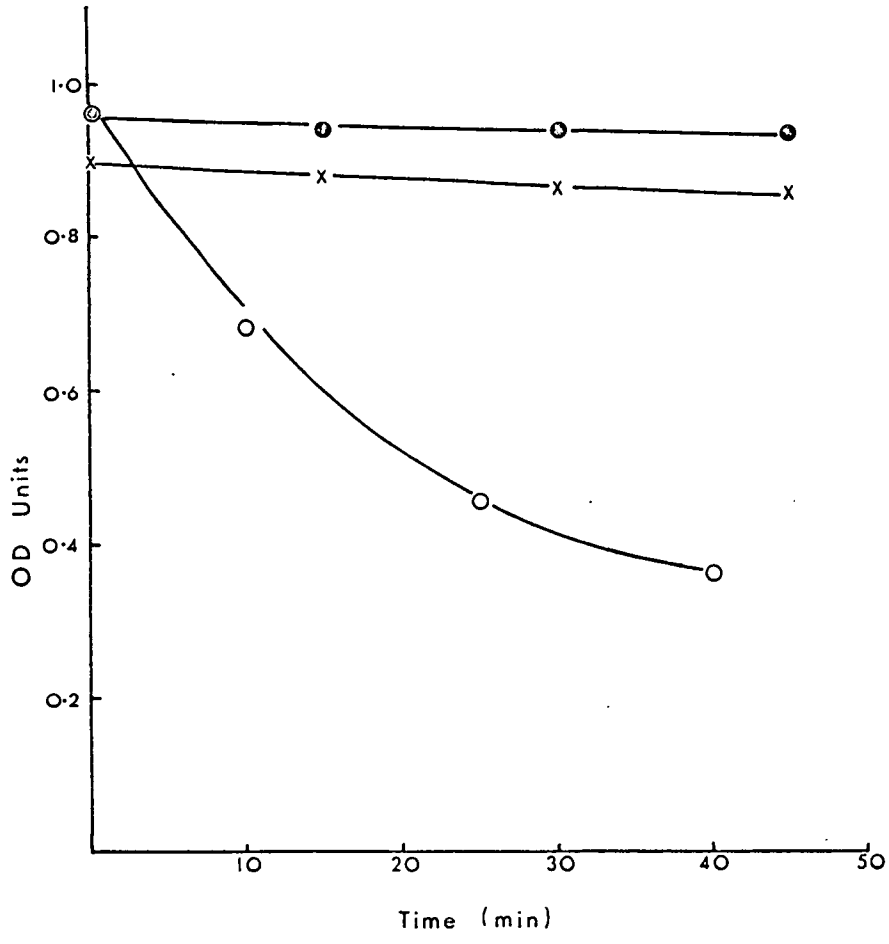


Fig. 9 Activity in the Culture Supernatant
The assays were done at 45 C in TK buffer, without rebinding enzyme to the walls. The cultures are represented by ●—3h, x—4h and ○—17h.

Negatively stained walls (for method see Chapter III) which had been treated with NaOH had a much flimsier appearance than normal walls (Plates 1 and 2).

SDS-walls and SDS,NaOH-walls were treated with B. subtilis autolysins and lysozyme to find out what effect removal of teichoic acid had on degradation of the walls by these enzymes.

Since autolytic enzymes can be removed from the wall by high concentrations of salt, this suggests that they are ionically bound to the wall, and since teichoic acids are negatively charged, they could be important in this binding. The results showed that when teichoic acid was removed, the walls were more resistant to digestion by autolysins and lysozyme. However, with increasing concentrations of lysozyme, the effect decreased until at high enough concentrations it disappeared and both SDS- and SDS,NaOH-walls were degraded equally rapidly (Figure 10).

Figure 10a shows that with enzyme rebound to the walls, SDS,NaOH-walls were digested much more slowly than SDS-walls. In Figure 10b, where rebinding was not necessary, since the enzyme was left in contact with the walls, the same effect was observed. This might mean that for the enzyme to work, it first has to rebind to the walls, rather than just be in close contact with the walls, and the teichoic acid-free walls have largely lost this ability; or it may mean that the structure of the walls lacking teichoic acid is such that they are more resistant to digestion. The lysozyme results (Figure 10c, d and e) fit more easily into the second explanation, since this enzyme does not rebind ionically to the walls.

Similar results were later published by Herbold and Glaser (1975) who found that teichoic acid-free B. subtilis walls were hydrolysed at a slower rate than walls containing teichoic acid. Also enzyme pre-bound to these walls could be instantly displaced by teichoic acid-containing walls. They concluded that binding to teichoic acid-free

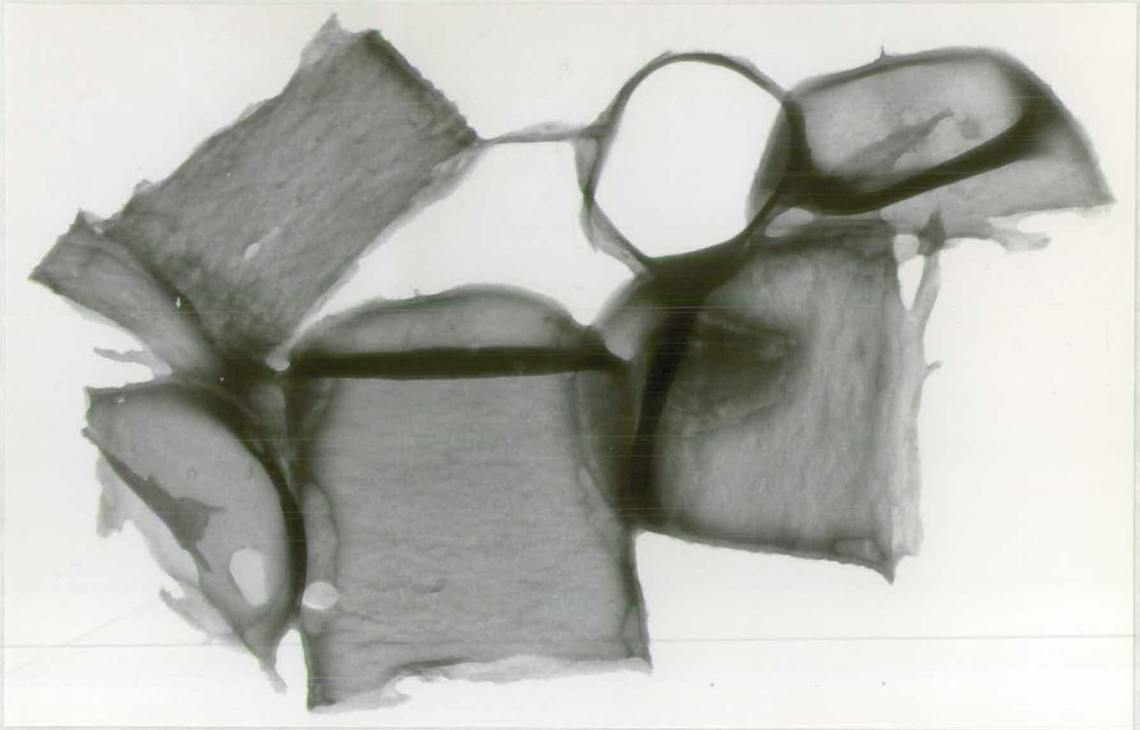


Plate 1 Negatively stained SDS-walls of B. subtilis.
Magnification x 40,000.

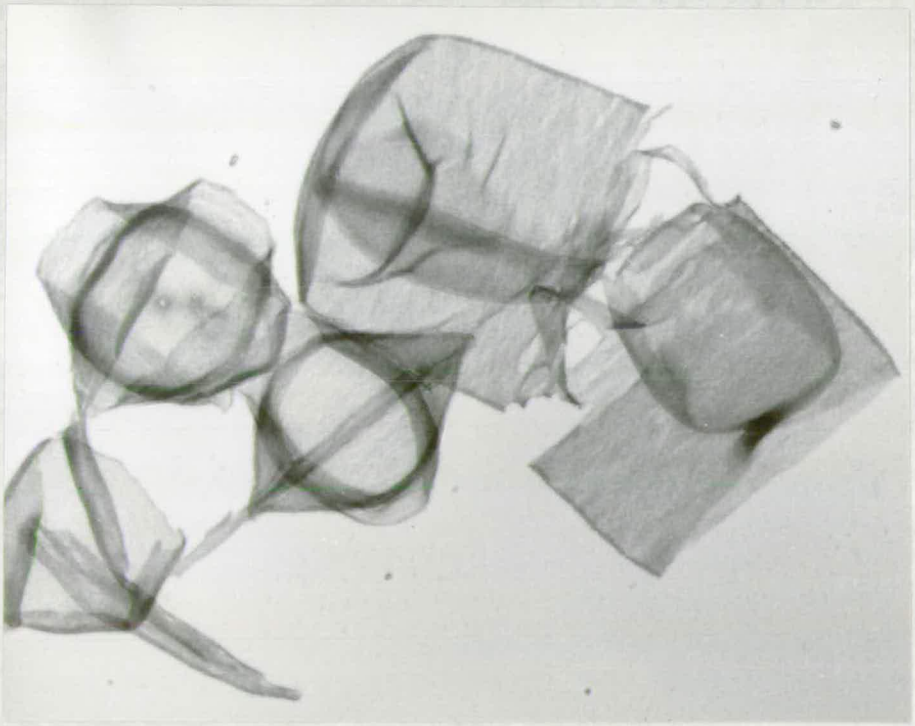


Plate 2 Negatively stained SDS,NaOH-walls of B. subtilis.
Magnification x 40,000.

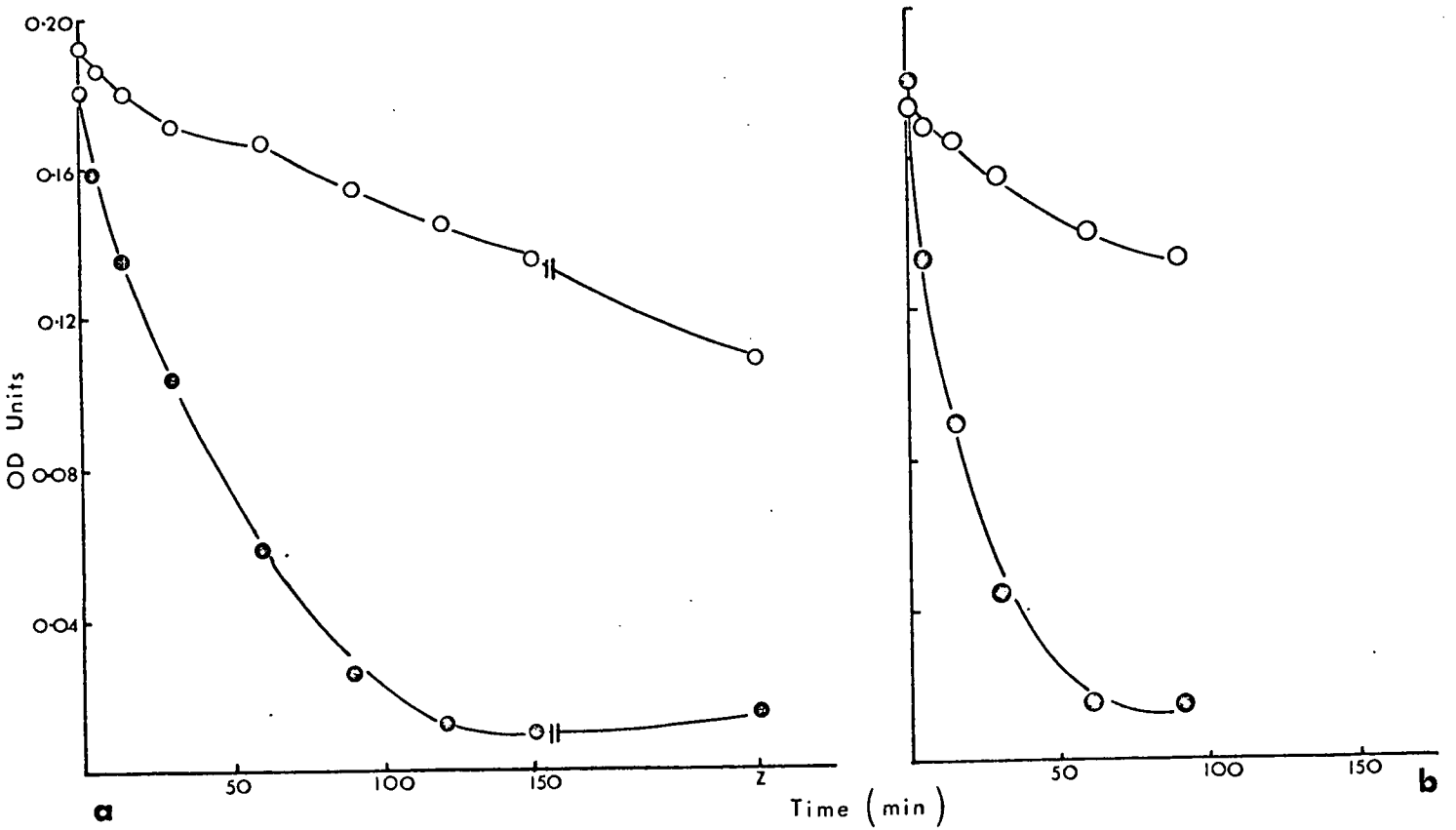


Fig. 10 Lytic activity on Teichoic Acid-free walls. a) shows activity when crude enzyme was rebound to SDS- (●) and SDS,NaOH-walls (○) and b) shows that a similar result is obtained when the enzyme was left in contact with the walls throughout the experiment, so that rebinding was not required. The assays were done at 45 C in TK buffer. z in graph a) represents an O.D. reading after the samples had been left at room temperature overnight.

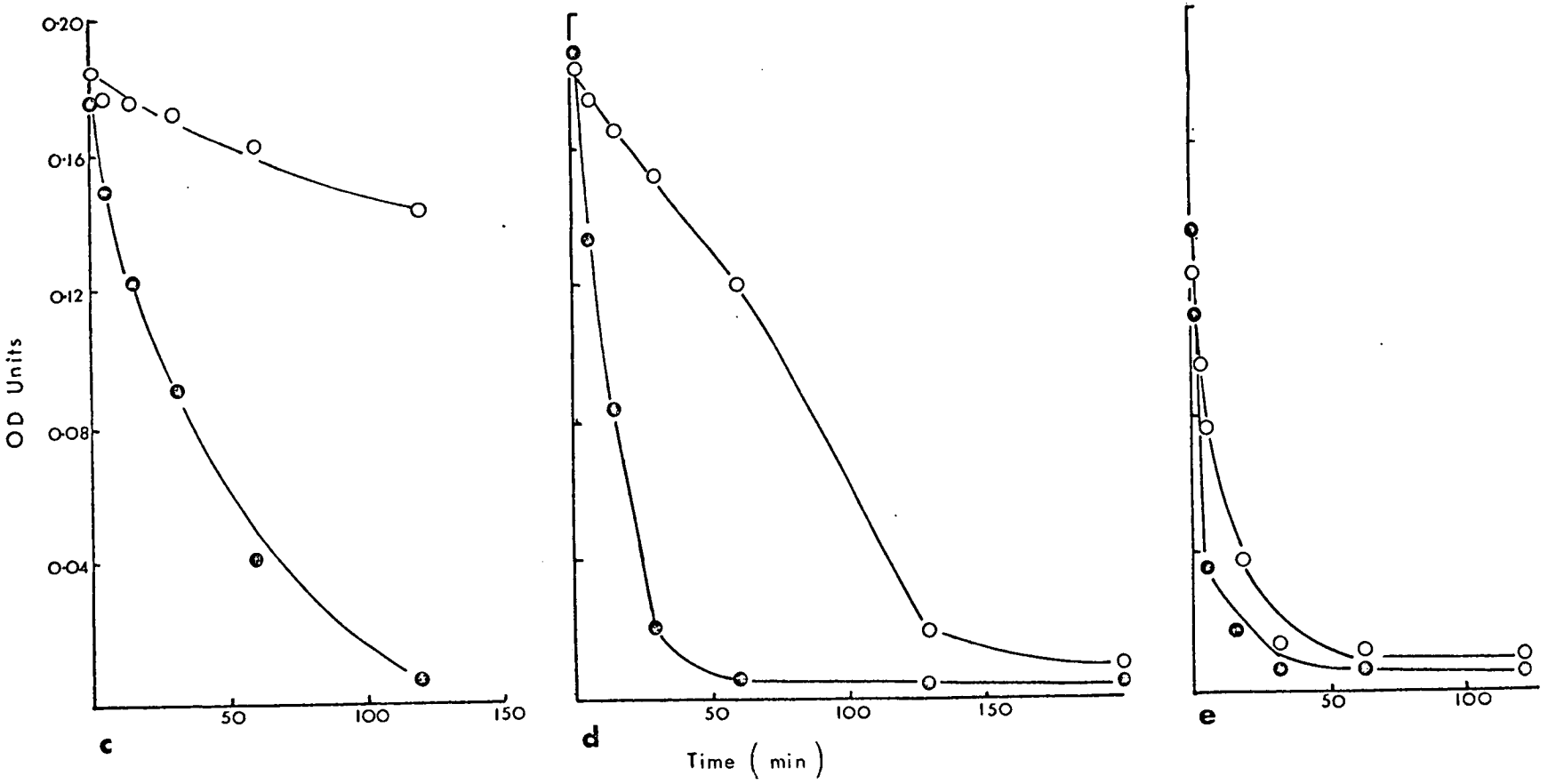


Fig. 10 continued c), d) and e) show the lysis of SDS- (●) and SDS,NaOH-walls (○) with increasing concentration of lysozyme. Lysozyme in c) is 360 units/mg wall, in d) 720 units/mg wall and in e) 3,600 units per mg wall.

walls was much weaker than to walls with teichoic acid. It was also found that B. subtilis teichoic acid-free mutants had less autolysin than wild-type cells.(Boylan, Mendelson, Brooks & Young, 1972; Fiedler & Glaser, 1973; Hughes, 1970).

It therefore seemed that tight binding of the autolysins was dependent on the presence of teichoic acid. This might represent an electrostatic effect on enzyme binding, but could also reflect the fact that in the absence of teichoic acid the wall had a more compact structure. Herbold and Glaser (1975) favoured the first alternative because a low molecular weight teichoic acid-glycan complex was a good inhibitor of enzyme activity. They also pointed out that these observations meant that amount of lytic enzymes in a cell could not be estimated from isolated walls. Walls lacking teichoic acid would absorb activity poorly, and the enzyme would be removed during wall preparation. Mutants lacking teichoic acid may still produce the same amount of enzyme but release it into the growth medium.

Purification of the Amidase.

It was decided to use salt extraction rather than autolysis of whole cells in the first stage of enzyme purification to avoid contamination with intracellular proteins and other material which absorb at 260 and 280nm and could complicate purification. It was also desirable to avoid enzyme that might be bound to teichoic acid as reported by Brown et al. (1970), since the enzyme was required for antibody production. For similar reasons walls were used in preference to whole cells, since the latter might lyse during the extraction procedure. At the same time it was necessary to extract the maximum amount of activity since mg quantities were required to raise antibodies.

Percentage of Autolytic Activity removed from the Walls. To ensure that the maximum amount of enzyme was removed from the walls by salt, three extractions were carried out, and the activity of each extract and of the walls was measured and compared with the activity of walls before extraction. Results showed that after the first extraction, 35% of the enzyme had been removed and 65% remained bound to the wall. However, after the second extraction, the total activity removed was 57%. A third extraction did not result in a significant release of further activity. The result of the first extraction was in agreement with Fan (1970) who reported that he was able to remove approximately one third of the total lytic activity. He did not attempt a second extraction but the above results indicate that this was worthwhile, and for large quantities of walls a third extraction was advisable. Plate 3 shows samples from three such extractions run on an SDS gel.

Rebinding as a Means of Purification. Fan and Beckman (1972) reported that glycosidase could be removed from B. subtilis walls with 0.5M LiCl while amidase required 1.5M LiCl. It was therefore probable that other

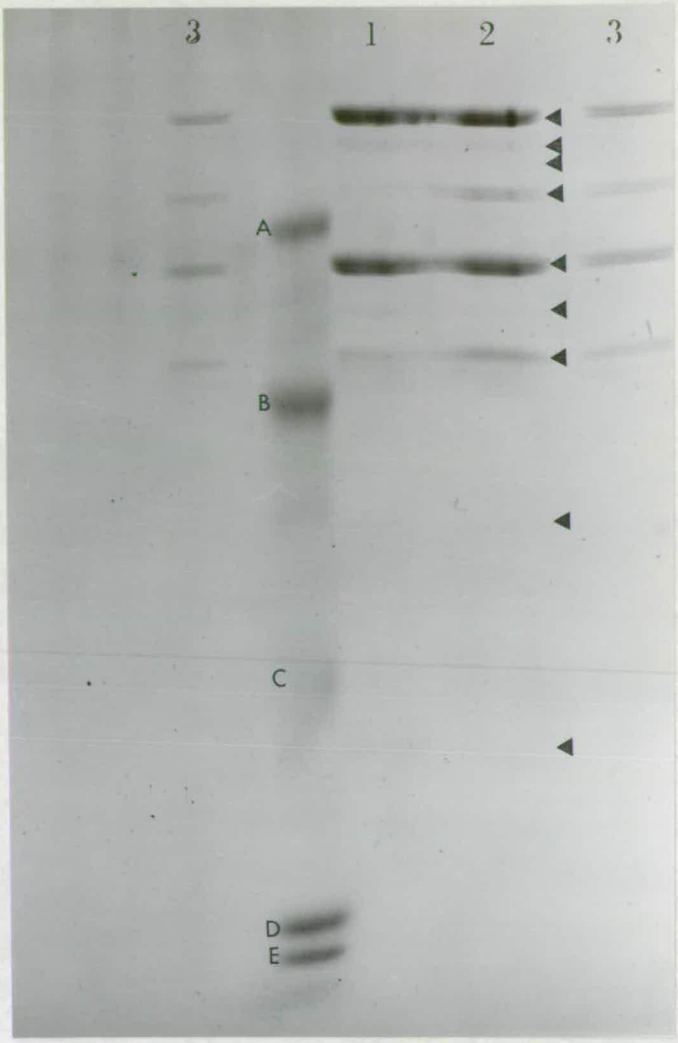


Plate 3 Proteins Extracted from the Wall with LiCl.
The first, second and third extractions of protein from B. subtilis walls are shown by 1, 2 and 3 respectively. It is clear that most of the material was removed during the first two extractions. The samples were run on an SDS gel (10% acrylamide) and protein bands which were visible in the gel after staining are marked with arrows. Scans of the gel showed that samples 1 and 2 contained the same proteins while for sample 3, only 4 protein bands could be detected. Standards labelled A to E were as follows:

Protein	Molecular Weight
A. Bovine serum albumin (Sigma)	68,000
B. Ovalbumin (Sigma)	43,000
C. Pepsin (Sigma)	35,000
D. Myoglobin (Sigma)	17,000
E. Cytochrome c (horse heart, Sigma)	12,000

proteins could be selectively extracted from the wall with salt. Thus rebinding and re-extraction of walls with different concentrations of LiCl might offer a means of purification.

For rebinding, SDS-walls of B. subtilis 168 were washed twice with H_2O and twice with 3M LiCl/TK buffer. The amount of walls was equivalent to that from which the enzyme was extracted. A concentrated crude enzyme extract was added to the SDS-walls and diluted x 100 to allow rebinding to occur for 20min on ice. The walls were then spun out and the supernatant retained. Re-extraction of the SDS-walls was then carried out as follows; with a) 1M LiCl/TK b) 3M LiCl/TK and c) 3M LiCl/TK. Extractions b) and c) were combined and then the following samples were concentrated in an Amicon cell to about 1mg of protein per ml, dialysed overnight against 0.1M tris pH 8.0 and run on an SDS gel (Plate 4);

1. supernatant after initial rebinding (i.e. all proteins which did not rebind to the walls)
2. 1M LiCl/TK extract
3. 3M LiCl/TK extract.

The gel was scanned with a Vitatron TLD 100 densitometer and a schematic representation of the major peaks is show in Figure 11, with the position of the standards indicated. Samples 1 and 2 contained relatively more of the higher molecular weight materials than 3, which suggested that these did not rebind so efficiently to the walls. Peak X in particular showed that much of this protein did not rebind and most of that which did was released by 1M LiCl. All samples showed lytic activity on B. subtilis SDS-walls at pH 8.6 and peak Y which was present in all, corresponded to the M.W. of amidase subsequently determined by Herbold & Glaser (1975). There was no peak corresponding to the value quoted by Fan and Beckman (1972).

It was concluded from this experiment that rebinding of crude

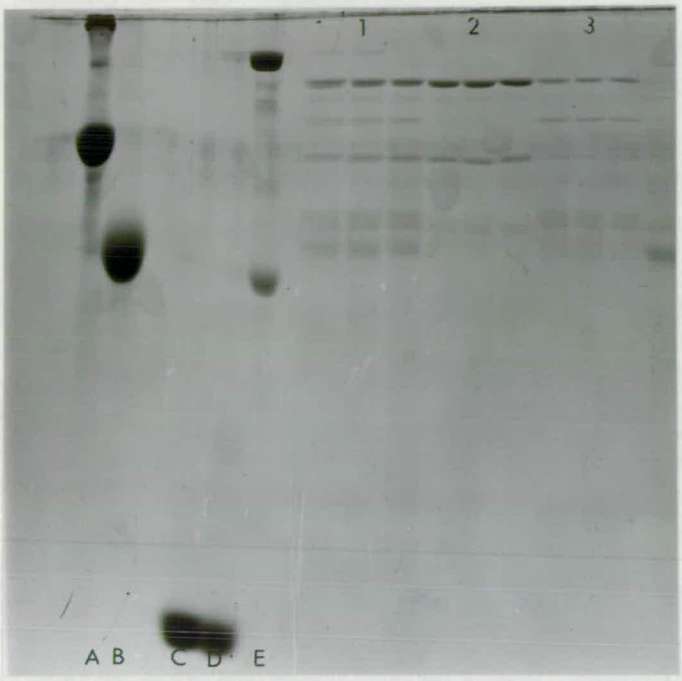


Plate 4 Rebinding and Re-extraction of Crude Enzyme.

The samples were run on the gel as follows; 1) the supernatant after initial rebinding (i.e. all proteins which did not rebind to the walls) 2) 1M LiCl/TK extract and 3) 3M LiCl/TK extract. The standards labelled A to E were

Protein	Molecular Weight
A. Bovine serum albumin (Sigma)	136,000 68,000
B. Ovalbumin (Sigma)	86,000 43,000
C. Egg white lysozyme (Sigma)	14,000
D. Cytochrome c (horse heart, Sigma)	12,000
E. RNA polymerase (Dr G. Peters)	165,000 155,000
	90,000 40,000

The scans of this gel are represented in Figure 11.

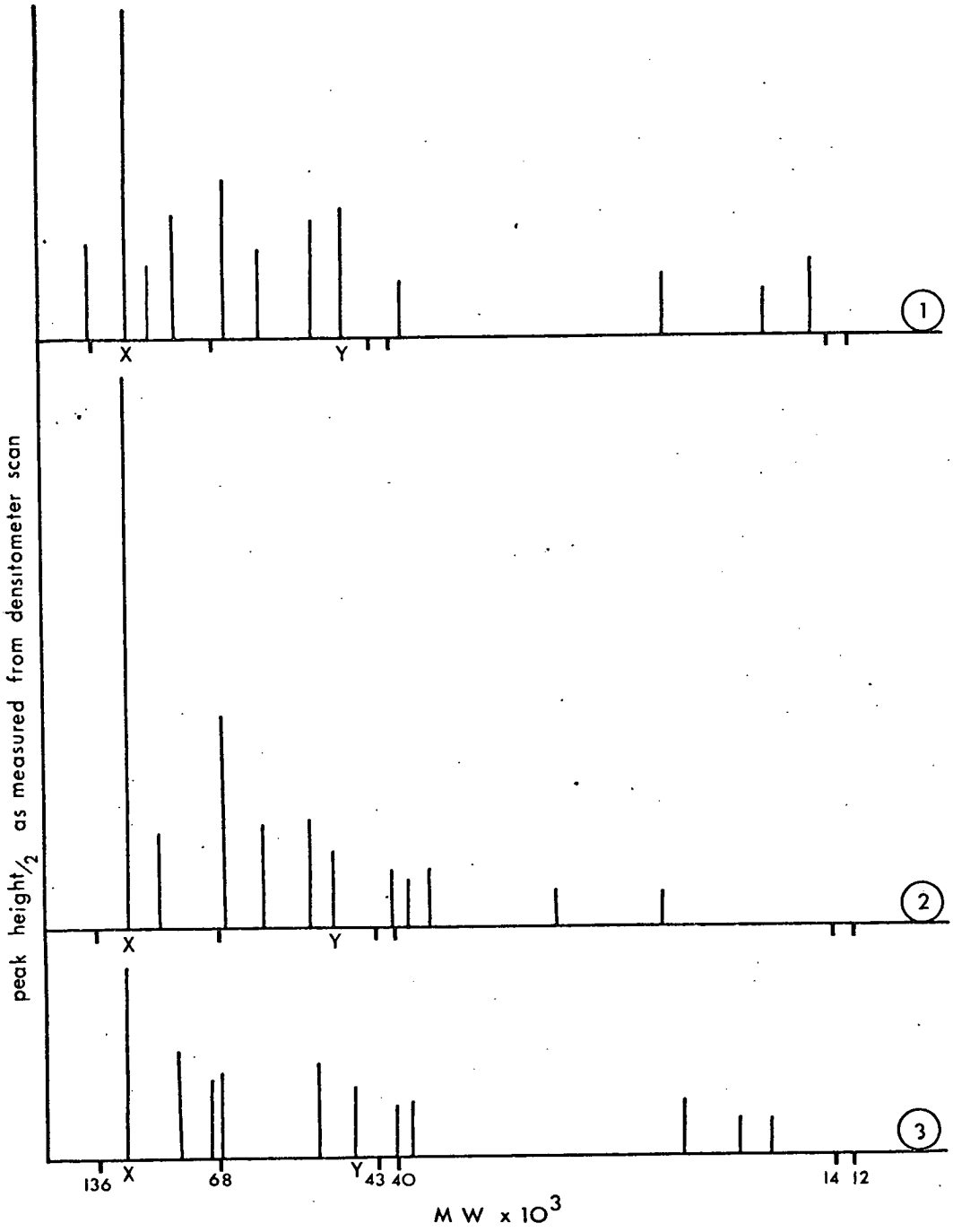


Figure 11 This is a schematic representation of the scans of the gel in Plate 4, shown in order to compare the proteins in each sample. 1, 2 and 3 are as for Plate 4, and the position of the standards is indicated by their molecular weights. Peak X, a high M.W. protein, is an example of a protein which does not appear to rebind well to the walls. Peak Y is at the expected position for amidase.

enzyme to SDS-walls and subsequent stepwise elution was an effective means of reducing the concentration of some of the high M.W. proteins in the crude enzyme (Plate 3). However, rebinding of lytic activity was not complete and even when more SDS-walls were used, activity was still lost. Thus in a purification procedure designed to obtain the maximum amount of pure enzyme, the disadvantages of this method were considered to outweigh the advantages.

Fan and Beckman (1973) reported that M. lysodeikticus walls bound amidase less well than glycosidase, so rebinding to these walls was tried in an attempt to remove glycosidase from the crude enzyme. When the rebound enzyme was extracted from M. lysodeikticus walls and run on a Sephadex column, it was found that the concentration of glycosidase had increased greatly, but the extract also contained considerable amidase activity. Thus again this was not a successful method for separating the two enzymes, but rather for increasing the concentration of glycosidase relative to the amidase.

Concentration of Large Volumes. Several different techniques were examined in the hope that this process might also serve as a purification step.

Concentration against 50% glycerol was satisfactory for small volumes of enzyme solution, but since this method only reduced a sample to one half or one third of its original volume, it was not suitable for large volumes. When PEG was used there was up to 70% loss of activity, a disadvantage which also applied to the Sephadex method, which in any case became costly for large volumes. More than 67% (V/V) ethanol in the enzyme solution caused a precipitate to form. The resulting supernatant contained no lytic activity, but the activity in the precipitate accounted for less than 50% of that originally present.

Results obtained by ammonium sulphate precipitation are shown qualitatively in the following table:

% Saturation	Decrease in total protein in supn't.	Activity in supn't	Activity in ppt
0	-	+++++	-
25	-	+++++	-
40	+	+++++	-
50	+	+++++	-
57	+	++++	+
70	++	++++	+
79	++	+++	++
85	+++	++	++

Table 6.

From these it could be seen that even at 85% saturation, there was still considerable lytic activity in the supernatant, and at this concentration of ammonium sulphate, it was not possible to account for all the activity present initially. Samples of the active precipitates were heated to 52 C for 20min to remove glycosidase activity, but in no instance was activity completely destroyed or completely unaffected, indicating the precipitates probably all contained a mixture of the two enzymes. There was not a significant precipitation of non-active protein before lytic activity started to be lost from the supernatant. Thus this method, which might have been a useful purification step, was not effective in separating the lytic activity from other proteins, or in separating the two enzymes.

By far the most useful of all the methods was ultrafiltration in an Amicon cell. No dialysis to remove LiCl was required, a step which led to some loss of activity. Large volumes of enzyme solution could be easily dealt with. Measurements of lytic activity showed no detectable enzyme passing through the membrane and as much as 80 to 85% of the activity was recovered in the concentrated solution. Activity lost was

probably due to adsorbtion on to the membrane filter, or to the enzyme becoming slightly less stable in very concentrated solutions. This method however, was not of much value as a purification step since the membrane only excluded molecules of molecular weight less than 10,000.

LiCl Sucrose Gradients. In all cases the separation was unsatisfactory, activity being found to some degree in most of the fractions. In a graph of protein concentration of the fractions, large peaks were always present over the first few and last few fractions collected. A large protein peak from the last fractions collected was unexpected because this represented the portion of the gradient with little or no LiCl, thus not much protein could have been removed from the walls in this region. However, it may have been that small pieces of wall with enzyme still attached were dragged through with the last of the fractions. Assays of pH optima, lytic activity, and reducing group activity failed to show any clean separation of the enzymes (as claimed by Fan and Beckman, 1972), and also this method was considered unsuitable for large scale preparations, since one gradient could only accomodate material from 3 l of original culture.

Sephadex Chromatography. Sephadex G75 was used first (as per Fan & Beckman, 1972) in an attempt to purify the amidase in a crude enzyme extract. One of the better separations is illustrated in Figure 12, where the peaks are fairly distinct. The reducing group assay indicated that glycosidase activity was concentrated in fractions 10 to 21 (shown by the arrows in Figure 12), although traces of reducing group activity were picked up in later fractions. Otherwise separations were much less distinct, and in all cases, the first active fractions came off the column very close to the large non-active protein peak which came off just after the void volume. Samples of this large peak indicated that it contained protein of molecular weight 85,000 and upwards. It was

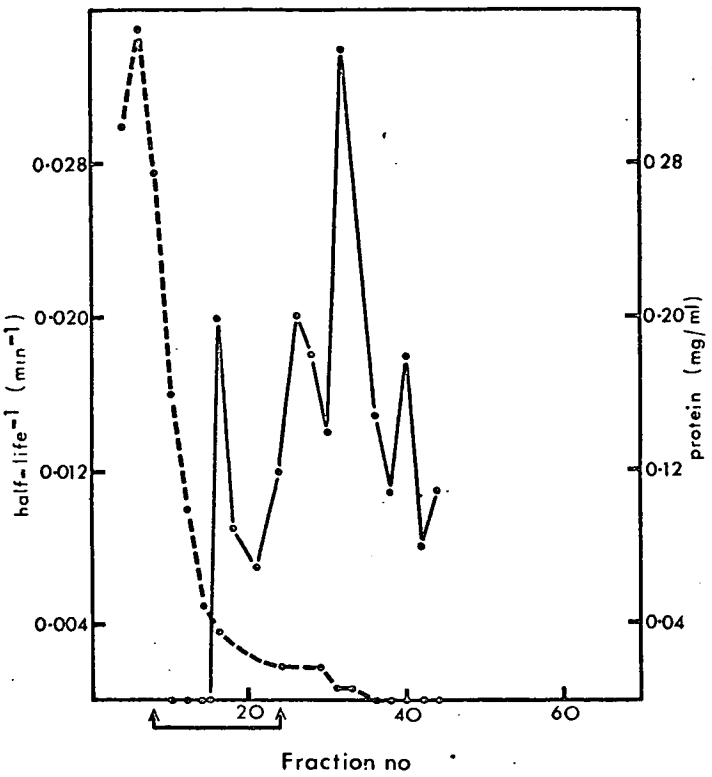


Fig. 12 Separation of the Crude Enzyme on Sephadex G75
Lytic activity of the fractions is represented by •——• and protein concentration by •----• . The arrows indicate the fractions in which most of the glycosidase activity was found as measured by reducing group release.

thus decided to use a system which would retain the activity on the column longer.

Therefore Sephadex G100 was substituted for G75. A typical separation on this column is shown in Figure 13a. The lytically active fractions in this case were more completely separated from the first large protein peak. Because of the lack of a routine specific assay for the amidase, and because it was important to obtain as much pure enzyme as possible, it was decided at this stage to combine all the fractions from the column which contained lytic activity. Lytic fractions from several such separations were combined, concentrated in an Amicon cell, and re-run on a G100 column. The result of this

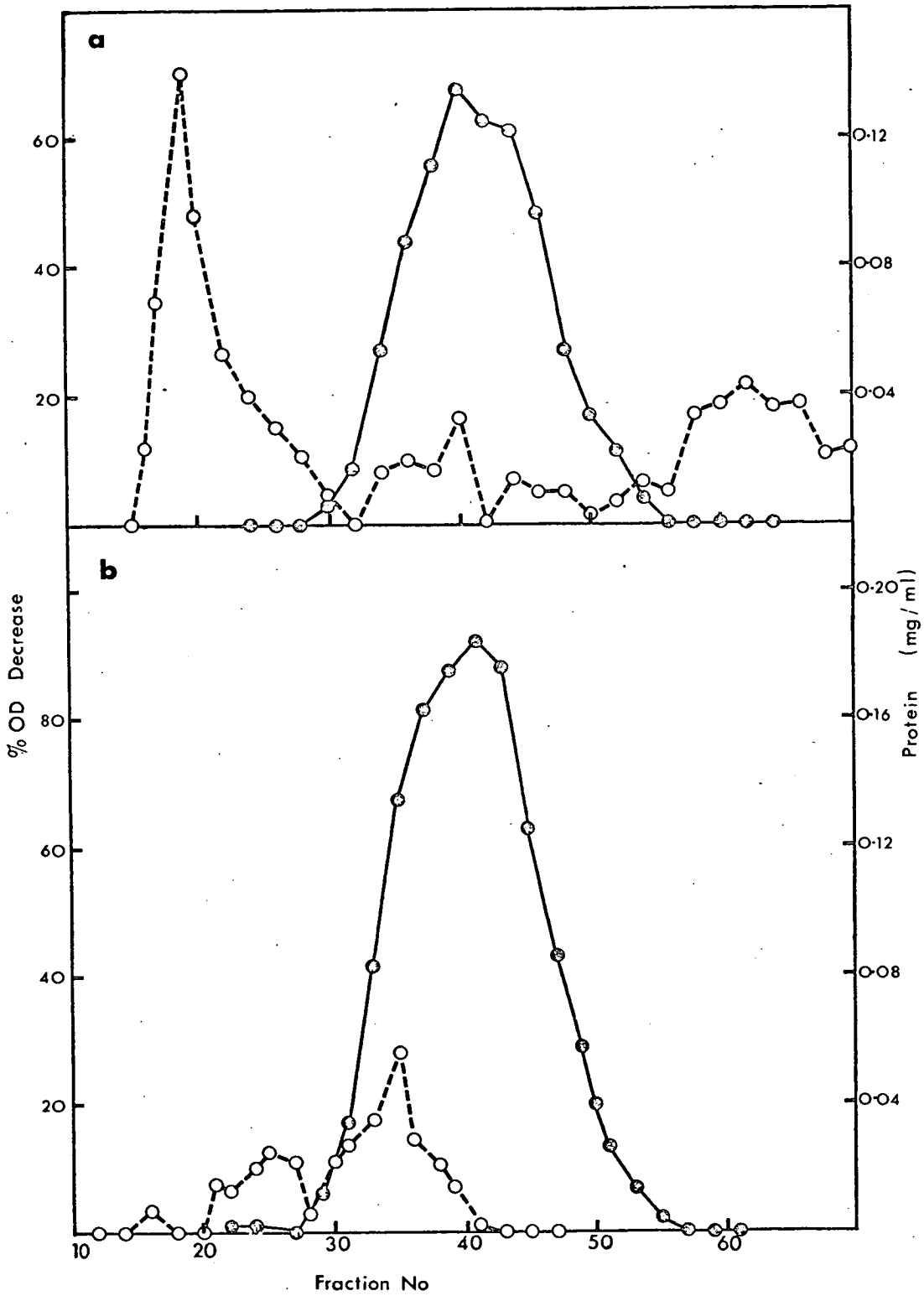


Fig. 13 Separation of the Crude Enzyme on Sephadex G100
Lytic activity of the fractions is represented by ●——● and protein concentration by O - - - - O . a) shows the separation after one run through the column and b) the separation obtained when pooled lytic activity from the first separation is re-run on the column.

separation is shown in Figure 13b. The peak of activity was now free of most of the protein measured in 13a, and by pooling fractions 30 to 55 in the second separation, it was further purified. The major activity in this fraction was that of an amidase as measured by activity at pH 8.6, lack of reducing group activity and lysis of Micrococcus walls at pH 6.0, and relative stability to heat. This fraction was then dialysed to remove LiCl, and was used to raise antibodies, and was also run on acrylamide gels. When the gels were analysed, a strong band was equivalent to a M. W. of 45,000, close to the value obtained for amidase by Herbold and Glaser(1975). There was some evidence of other protein in the fraction and the antibody production suggested that the enzyme was not completely pure (see Chapter III). There was no band corresponding to the value of 35,000 quoted by Fan and Beckman (1972) for amidase. Their estimated M.W. of 60,000 for glycosidase would mean that this enzyme would be well separated on the gel from the band at 45,000 (Plate 5 and Figure 14).

This was as far as the purification could be taken in the time available, but it was hoped that by running the lytic fraction on non-SDS slab gels at 4 C the amidase activity could be recovered pure.

Purification by Gel Electrophoresis. Preliminary experiments were done to ascertain that activity could be recovered from the gels. At the end of the run a thin strip, containing standards and a sample of the enzyme fraction was cut off the slab, and stained with coomassie blue. Meanwhile the rest of the gel was frozen with dry ice on a perspex sheet and stored at -20 C until use. The gel was the thawed out and the section containing the activity was cut out and ground up with TK buffer. Activity in the buffer was detected using the rebinding assay. A fairly sensitive method for detecting activity in the gel was later developed, using agar plates. Nutrient broth (2.5g) and agar (1.25g) were added

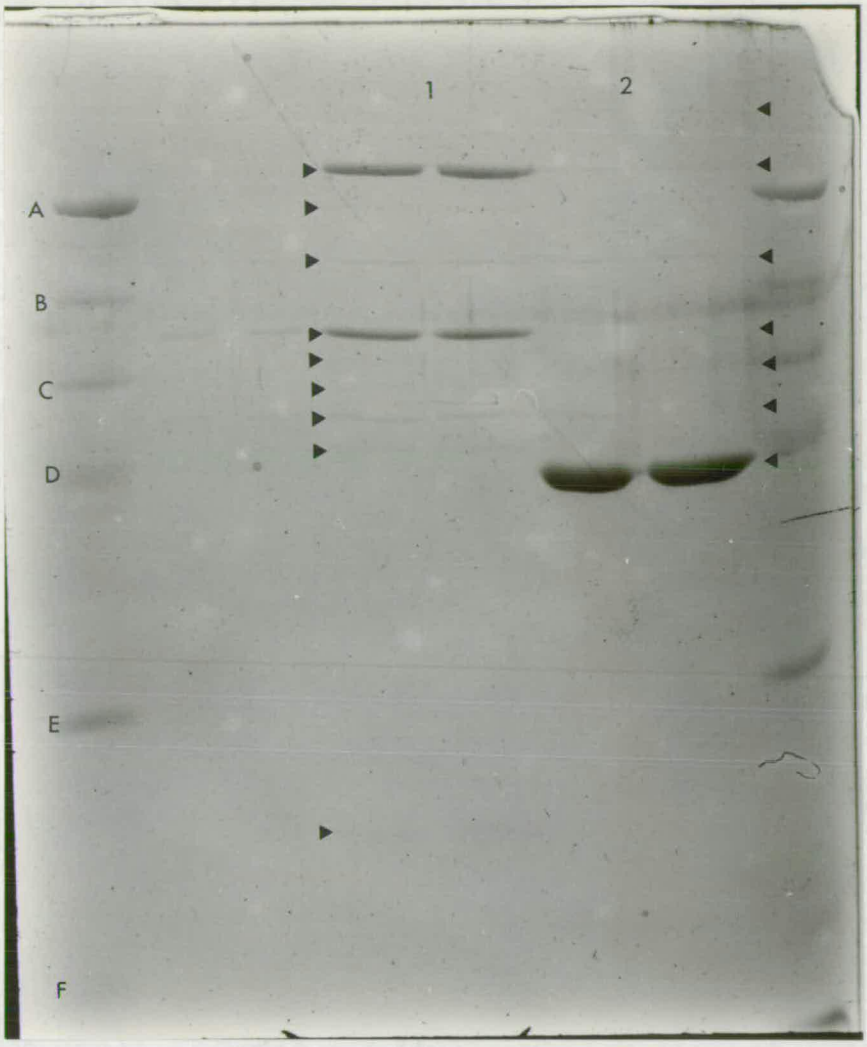


Plate 5 Purified lytic fraction run on an SDS Gel

1 is a sample of crude enzyme run with 2, the lytic fraction after two runs on a Sephadex G100 column. The major band in 2 corresponds to a M.W. of 45,000. The standards A to F are as follows;

Protein	Molecular Weight
A. Phosphorylase a (Sigma)	92,000
B. Bovine serum albumin (Sigma)	68,000
C. Hexokinase (Sigma)	52,000
D. Ovalbumin (Sigma)	43,000
E. Penicillinase (Wellcome)	28,000
F. Lysozyme (Sigma)	14,000

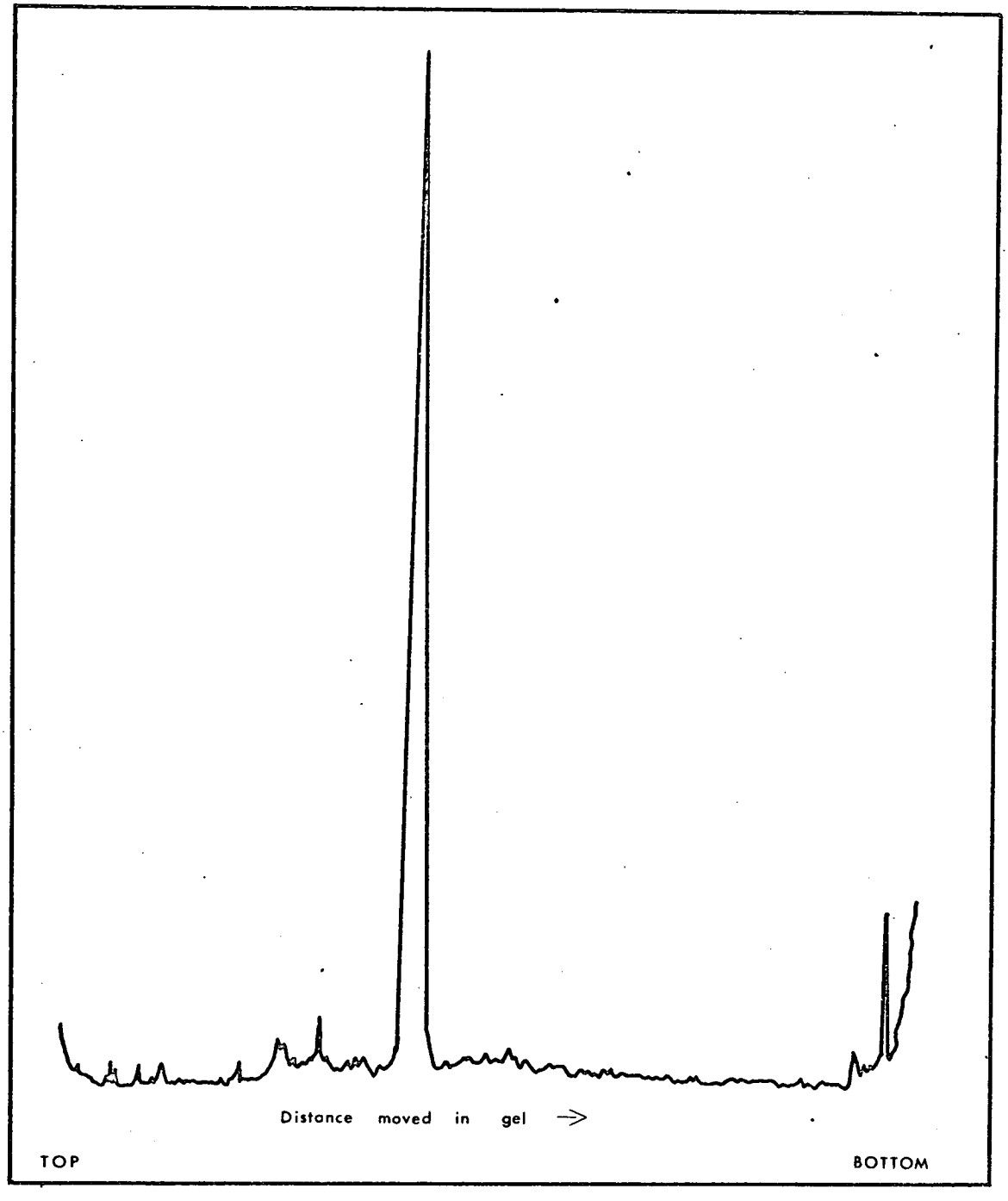


Fig. 14 This shows a scan of the lytic fraction run on the gel in Plate 5. The large peak corresponds to the band with a M.W. of 45,000. Several other very small protein peaks are visible, mainly at higher M.W.'s. Thus this scan gives an idea of the amount of impurities still comtaminating the protein believed to be the amidase.

to 100ml of hot TK buffer. When the agar had dissolved, the solution was cooled slightly and 5mg of SDS-walls were added. Thin layers of agar were poured into petri dishes, which could then be stored at 4 C for several weeks. To assay for enzyme activity, a plate was placed just above the water level in a covered bath at 45 C. To test the sensitvity, a drop of enzyme solution was placed on the surface of the agar, and after suitable incubation, activity was detected by a zone of clearing. Activity in quantities as small as 5ul of crude enzyme solution (0.02mg protein) could be detected, so that it should be possible to detect the active band in a strip of gel laid on top of the agar (in this case 50ul volumes of lytic fraction containing 0.05mg of protein, most of which is amidase, would be layered on to the gel). If each slab gel will take 0.5mg protein, then by running several gels, it ought to be possible to obtain mg quantities of pure enzyme.

Expected Enzyme Yield. From a 200 l culture, approximately 200g (wet weight) of cells were obtained. After sonication, these yielded 32g (wet weight) of walls. LiCl (3M) released about 80mg protein from the walls. The lytic fraction from the first Sephadex run contained 8 to 10mg of protein, and after the second column had been run, this was reduced to 2 to 3mg. At this stage the enzyme was contaminated with several impurities, but all in very low amounts as shown in Plate 5 and Figure 14. Thus, providing most of the enzyme could be re-extracted from the gel, 200 l of culture should yield between 0.5 and 1,0mg of enzyme. Three or four such cultures ought to provide enough material for antibody production. The results of a typical purification are shown in Table 7:

Fraction	Volume ml	Units/ml	Total Units	Specific Act, units/mg	Yield
Cell wall extr.	340	17.8	6.0×10^3	49	100%
Conc' extract	130	26.1	3.4×10^3	50.1	56.6%
Lytic fraction II (concentrated)	5	135	6.8×10^2	260.5	11%

This represented a 5 fold purification between the step where the crude enzyme was concentrated and the lytic fraction obtained after the second Sephadex run.

When Herbold and Glaser (1975) published their results earlier this year, it was possible to look at the above results in a slightly different light. They found evidence of a modifier protein which enhanced the activity of the amidase, and which had a M.W. of 80,000 as compared to the M.W. for amidase of 47,000 to 51,000. As can be seen from Figure 14, most, if not all, of any such protein must have been removed from the amidase and so the specific activity for the last stage in the purification may be as much as 2 to 2.5 fold too low. Herbold and Glaser, starting with the same volume of culture (but harvested at an O.D. of 2.0 instead of 0.3 to 0.5), and extracting enzyme from whole cells, instead of walls, finished up with a yield of 12% and a specific activity of 250 units/mg. However they had almost 10x the total number of enzyme units, compared to the above results.

Summary

It was found that neither amidase nor glycosidase could be assayed using a synthetic substrate, so that for routine assays, rebinding had to be used. Lytic activity at various stages of growth was examined, and it was concluded that active enzyme was always present, but with increasing age, the cells became more resistant to lysis. Stationary phase cultures excreted enzyme into the medium. Walls which were free of teichoic acid were more resistant to degradation by lytic enzymes than walls containing teichoic acid, which probably resulted from the fact that rebinding of autolytic enzymes was much weaker when walls did not contain teichoic acid. Some structural alteration of the wall may also have been involved.

A purification procedure for the amidase is described, using Sephadex chromatography and gel electrophoresis. Although the yield was not as good as that reported by Herbold and Glaser (1975), the specific activity obtained was similar, and during the first steps of purification, the volumes handled were considerably smaller.

CHAPTER III

LOCALISATION STUDIES

It was hoped that by localising one of the lytic enzymes in the cell it would be possible to discover more precisely which stages of the cell cycle required the enzymes, and to learn more about the growth and division processes. Several approaches were used, purification of the amidase to produce ferritin conjugated antibodies, analysis of minicells, and autolysis of cells under conditions optimal for each enzyme.

By labelling cells with ferritin-conjugated antibodies to amidase, it should be possible to determine the distribution of the enzyme, for instance, whether it is more concentrated along the cylindrical walls, or at the ends, whether it is evenly distributed all over the cell, or whether it occurs in discrete areas. It might even be possible with a good labelling technique, to count the individual ferritin molecules, and thus get an idea of the quantity of enzyme present. Because of the postulated role of amidase in cell separation discussed in the introduction, it would be expected that a considerable concentration of amidase would be found at ends and associated with developing cross-walls.

Minicells, because they are formed at the poles of parental cells, represent polar material which can be easily isolated from cylindrical wall material because of the different sedimentation properties of the minicells and their parents. Thus isolation of proteins, in particular the lytic enzymes, from minicells, and comparison of these with the proteins of the parental cells (representing mostly cylindrical wall because the parental cells are unusually long) is another way of examining which enzymes are present in different parts of the cell.

The third approach was more indirect, and looked not at the enzymes, but at the result of their action on the cells. By comparing autolysis patterns under conditions optimal for glycosidase and for amidase, it

should be possible to gain information about both the position and the function of the enzymes.

MATERIALS AND METHODS

1. Preparation of Antigen. Crude enzyme and the lytic fraction obtained after 2 runs on Sephadex G100 were freeze dried and stored at -20 C. Both of these preparations were used to raise antibodies, by injection into rabbits. Antigen was dissolved in sterile distilled H_2O at a concentration of 2mg/ml, and 0.5ml was added to 1.5ml of complete Freunds adjuvant, and mixed thoroughly until emulsified. The mixture was then stored at 4 C and during the first month, the rabbit was given 2 x 0.25ml injections into the thigh muscle. Over the next 4 months, the rabbit received intravenous injections (0.2 to 0.5ml) of alum precipitated antigen at fortnightly intervals. The precipitate was prepared by dissolving 5mg of antigen in 3.5ml of sterile 0.01M phosphate buffer, and adding 0.2ml of 1% potassium alum. The solution was mixed, and the precipitate allowed to form at 4 C.

2. Antibody Titre. About 4 months after the first injection, a small sample of blood was tested for antibodies. Plates were made in petri dishes from 0.8% agarose gel in veronal buffer pH 8.3 (Kwapinski, 1972), and wells of 5.5mm diameter were made in the plate with a distance of 0.8 to 0.9cm between the well containing antibody and those containing antigen. Antigen of the following concentrations was made up in veronal buffer; 4mg/ml, 2mg/ml, 0.5mg/ml and 0.05mg/ml. A fresh crude enzyme extract, which had not been freeze dried, was also used. The plates were kept at 4 C for several days to allow precipitation lines to form.

3. Fractionation of Serum Globulins. (i) A saturated solution of ammonium sulphate in water was prepared at room temperature well in advance of use. (ii) To a measured amount of undiluted serum in an ice bath was added an equal amount of the above solution, dropwise with stirring. (iii) The solution containing precipitated globulin was allowed to stand overnight at 4 C, and was then centrifuged for 30min

at 3,000g. The supernatant was discarded. (iv) The precipitated globulin was dissolved by slowly adding measured amounts of water. Usually the volume of water required to dissolve the precipitate was less than the original volume of serum. (v) To the dissolved globulin was added an amount of saturated ammonium sulphate equal to the volume of water used to dissolve the precipitate as described in step ii). The precipitated globulin was centrifuged immediately, and precipitation was carried out a third time. (vi) The dissolved globulin was dialysed at 4 C against 0.85% NaCl, using frequent changes of saline. Dialysis was continued until sulphate was no longer detectable in the liquid outside the sac following overnight dialysis. This was determined by adding a few ml of saturated barium chloride to an equal equal amount of dialysate. If the mixture did not become cloudy or opalescent, the globulin was considered to be free of sulphate. The globulin fraction was stored at -20 C.

4. Conjugation of Ferritin to Antibody. Ferritin (horse spleen, 6x recrystallised) was obtained from Calbiochem and stored at 4 C. Conjugation was carried out according to the method of Sri Ram, Tawde, Pierce and Midgley (1963). The ferritin-antibody conjugate was purified on an agarose column according to the method of Nicolson, Marchesi and Singer (1971) and was stored at 4 C.

An alternative to this procedure was to use unlabelled globulin, and label it after binding to cell walls. with ferritin-conjugated goat anti-rabbit IgG (obtained from Cappel Laboratories, USA.).

5. Labelling Cells or Walls with Ferritin Conjugate. Whole cells or walls were washed with H_2O and resuspended in 0.1M phosphate buffer. pH 7.6. To them was added either ferritin-conjugated antibody or unlabelled globulin, and the solution was left at room temperature for 30min or overnight at 4C with gentle stirring. If unlabelled globulin

was used, a further period of incubation was carried out after addition of ferritin-conjugated goat anti-rabbit globulin. After labelling was complete, cells or walls were spun at 1,000g for 20min, which was not sufficient to bring down unbound ferritin, and were washed twice with buffer. In order to ensure maximum labelling, saturation experiments were done, varying the amounts of ferritin conjugate and serum added. Electron microscopy revealed the concentration required for maximum binding.

6. Negative Staining. Copper grids were coated with a carbon film made in a Balzers BA3 vacuum evaporator. A drop of specimen solution was placed on the grid and was washed off with several drops of 2% uranyl acetate (U.A.). The grid was then dried. Often cell walls were not truely negatively stained using this technique, and it was found that if a film of cytochrome c was first spread over the carbon film, this improved the staining. Occasionally less concentrated solutions of U.A. were used.

7. Embedding and Sectioning. Samples were pre-fixed and fixed with OsO_4, and post-fixed with U.A. (Ryter & Kellenberger, 1958). CN was added to OsO_4 (Highton, 1969). The samples were dehydrated in acetone (Glauert, 1965), embedded in araldite (Glauert, Rogers & Glauert, 1956; Glauert & Glauert, 1958), and post stained (Reynolds, 1963), for 30s. Sections were cut on a Reichert Om U2 ultra microtome, using a diamond knife. Electron microscopy was done on a Siemens Elmiskop 101 operating at 80 kV.

8. Growth of the Minicell Mutant, B. subtilis Cu 403 div IV B1 thy^-, met^-, a minicell producing strain derived from strain 168, was obtained from Dr J. Reeve. Cultures were grown at 30 C in L Broth (Difco tryptone 10g, yeast extract 5g, NaCl (1%) 10g in 1 l H_2O. pH 7.2) with added thymine (20ug/ml), or in Spizizen minimal salts (Spizizen, 1968) with

added thymine and methionine (20ug/ml) and glucose as a carbon source.

9. Purification of Minicells. The sucrose gradient method was similar to that used by Teather (1974) to purify E. coli minicells. The culture was grown to late log phase in minimal medium to maximise the number of minicells free in solution, and was then harvested by spinning at 10,000g for 15min. Large cultures were grown in a fermenter and harvested using a continuous flow Sharples centrifuge with a flow rate adjusted such that at least 80% of minicells were retained in the centrifuge. All subsequent operations were carried out at 4 C. The pellet was resuspended in cold growth medium, 40ml/l original culture, and was spun at 1,500g for 8min to bring down most of the parental cells. The supernatant was set aside, and the pellet was resuspended and spun again. The supernatants were then combined, spun at 1,500g for 8min to bring down any remaining parental cells, and then spun at 6,000g for 10min to pellet the minicells. This pellet was resuspended in 5% sucrose in 10mM tris (pH 8.0) using 1ml/l original culture, and was layered on to sucrose gradients made from 6ml each of 10%, 20%, 30%, 40% and 60% sucrose in 10mM tris (pH 8.0). The gradients (one/3 to 4 l original culture) were spun at 3000g for 10min (SS 34 rotor, Sorvall RC2B centrifuge). The clearly distinguishable minicell band was removed with a Pasteur pipette, checked by phase contrast microscopy for homogeneity, and if necessary, run again on a gradient. The minicells were then washed thoroughly with H_2O.

The sonication of minicell cultures, extraction of enzymes from minicells and their parents, determination of lytic activity and pH optima of the enzymes was carried out as described for strain 168, using 168 SDS-walls as a substrate in the assays.

RESULTS AND DISCUSSION

Production of Antibodies

Pure amidase was not available to prepare antibodies. However, antibodies were raised to the crude enzyme fraction and to the lytic fraction pooled from the second Sephadex G100 separation discussed in the last chapter.

After 4 months of injections with antigen, the titre of antibodies in the blood was tested by a double diffusion technique. For the rabbit injected with crude enzyme the result is shown in Plate 6. Precipitation bands formed between the antibody well in the centre and the antigen wells 1 and 2 containing 4mg/ml and 2mg/ml of protein respectively. A very faint precipitation line occurred also at position 6, the well containing fresh crude enzyme with a protein concentration of about 0.9mg/ml. A series of dilutions was used because antigen-antibody precipitation occurs most efficiently when the concentrations of each are approximately equal. At positions 1 and 2 one very strong precipitation band formed with a faint band inside, suggesting the formation of more than one antibody.

When the test was carried out on the blood from the rabbit injected with the lytic fraction (Plate 7), it was found that only one precipitation band formed opposite the wells with lytic fraction (1mg/ml), whereas 4 bands could be distinguished opposite the wells containing crude enzyme (1mg/ml). This suggested that only one protein in the lytic fraction was present in sufficient concentration to react with its antibody, but the impurities in the lytic fraction acted as antigens forming enough antibody to precipitate with their antigens present at greater concentrations in the crude enzyme.

Antibodies were purified from the serum of both rabbits, and were subsequently used in preliminary labelling experiments.

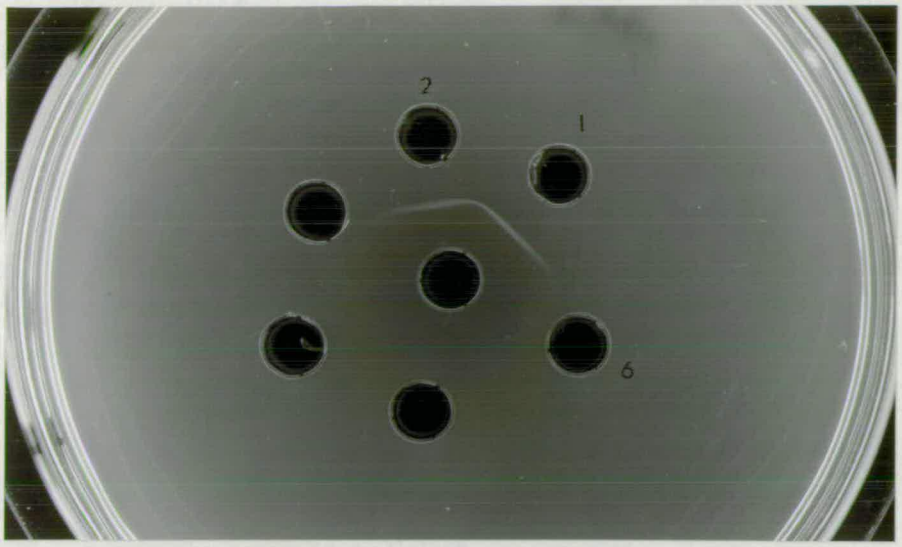


Plate 6. Double Diffusion Technique with Antibody to Crude Enzyme.
Wells 1 and 2 , containing 4mg/ml and 2mg/ml of antigen respectively, showed a strong precipitation line, accompanied by a very faint line on the side nearest the centre well. Well 6, containing fresh crude enzyme (0.9mg/ml), showed a very faint precipitation line.

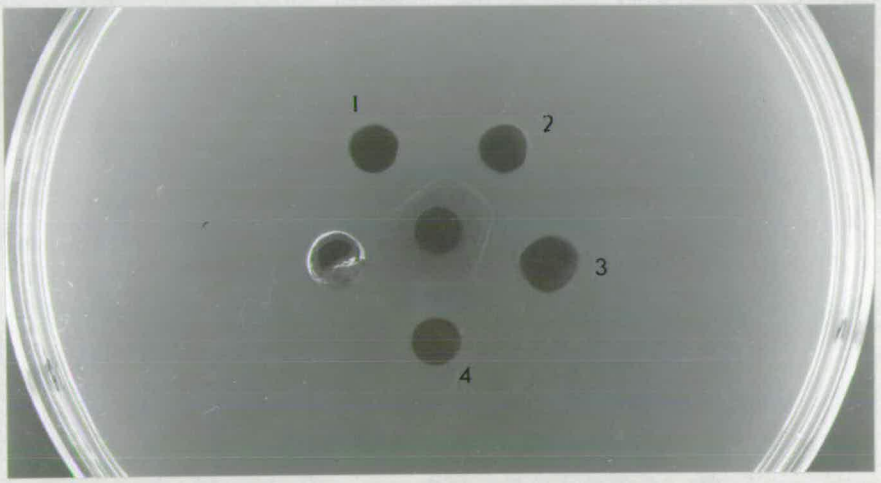


Plate 7. Double Diffusion Technique with Antibody to the Lytic Fraction.
Wells 1 and 3, containing crude enzyme (1mg/ml), showed 4 precipitation lines, while for wells 2 and 4, containing lytic fraction (1mg/ml), only one line was visible.

Labelling of Cells and Walls

Walls of B. subtilis were examined for non-specific binding of ferritin by using ferritin-conjugated antibody to B. cereus penicillinase and unconjugated ferritin. Results in both cases were negative.

When walls were negatively stained on carbon-coated grids, it was extremely difficult to make out the individual ferritin molecules (Plate 8).

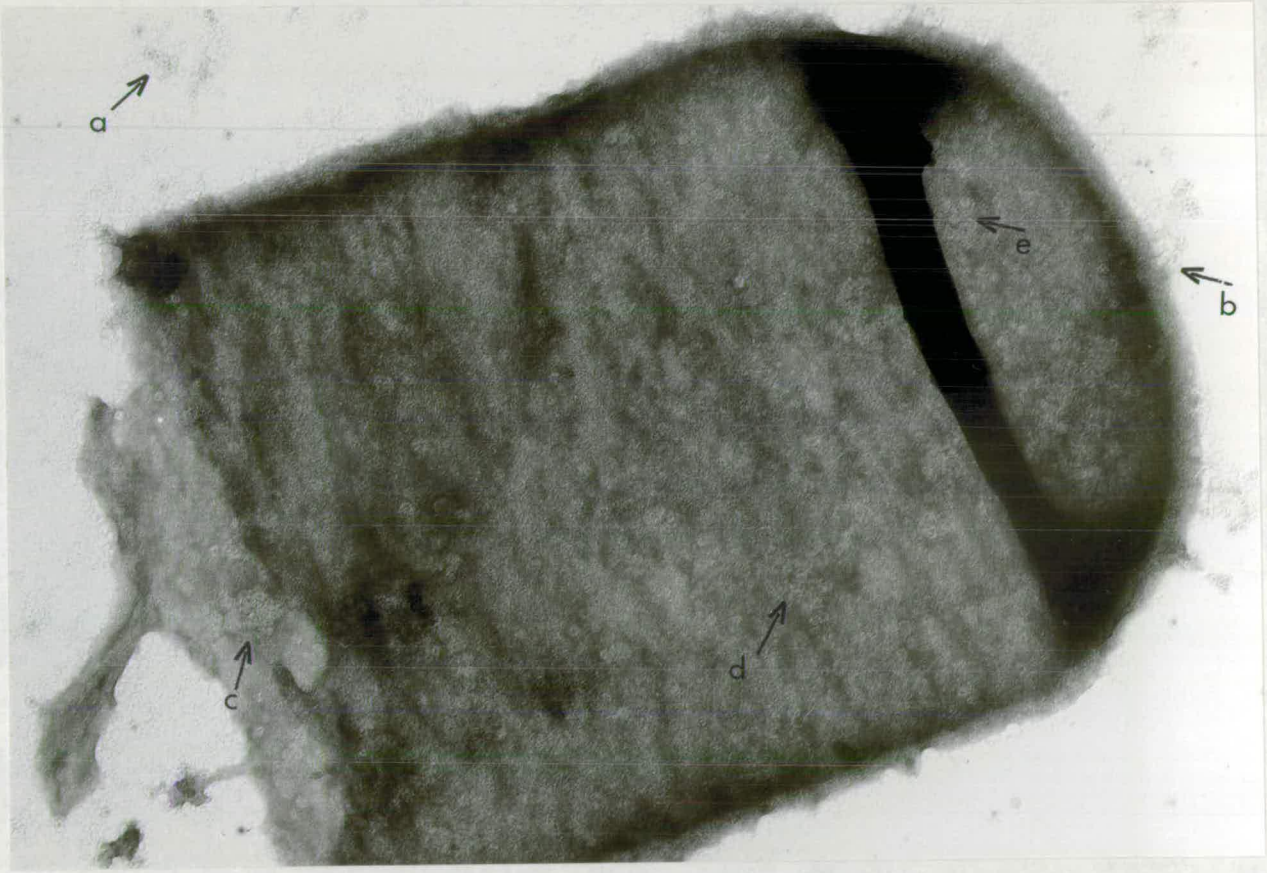


Plate 8 Negatively stained wall labelled with ferritin-conjugated antibody to crude enzyme. a) clearly defined ferritin molecules in the background, b) ferritin bound to material loosely attached to the wall, c) a clump of ferritin on a single thickness of wall, d) a clump on a double thickness of wall and e) a clump on the cell pole. Magnification x 100,000.

Visibility was enhanced by using carbon-coated grids with a film of cytochrome c (Plate 9).

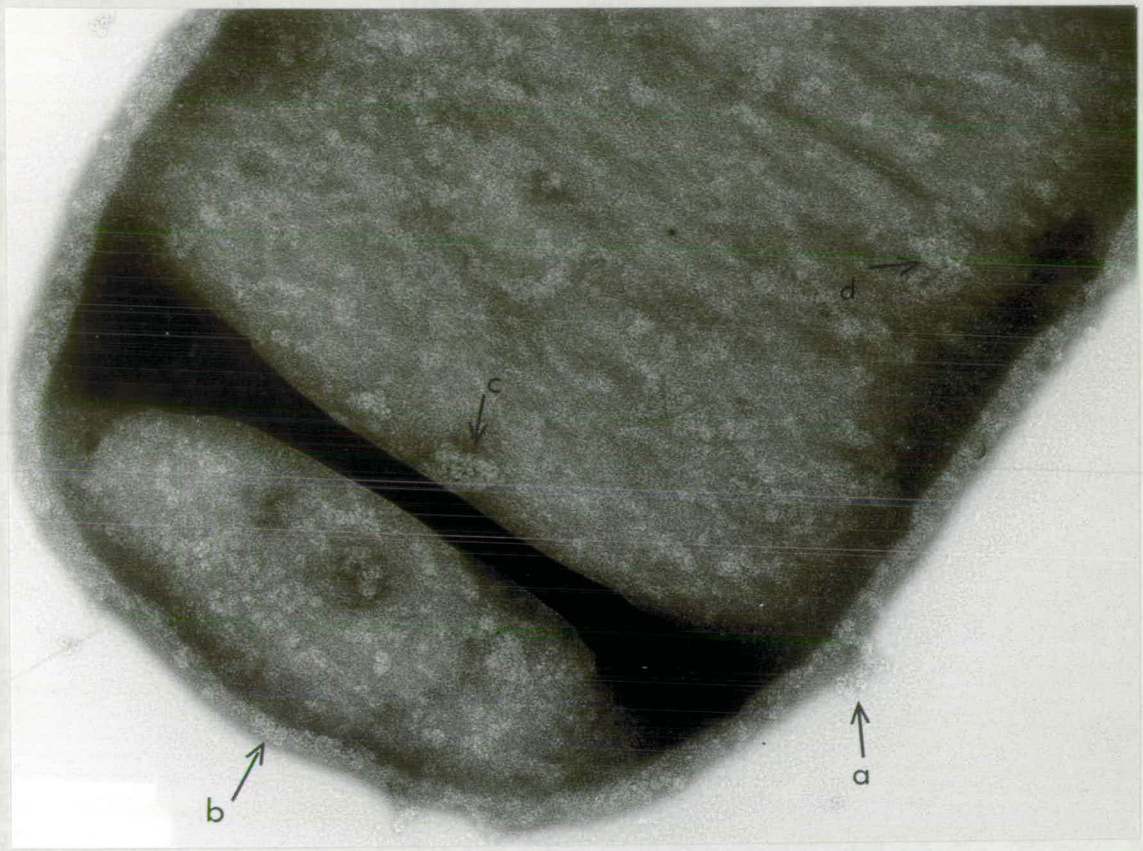


Plate 9 Negatively stained wall on a carbon-coated grid covered with a film of cytochrome c. a) well defined ferritin molecules bound to the edge of the cell, b) a clump of ferritin within the less darkly staining edge of the wall, c) and d) clumps on double thickness of wall. Magnification x 100,000.

Whole cells were labelled, fixed, and embedded, and sections were examined for label. The ferritin molecules showed up more clearly when the sections were not post stained with lead citrate (Plate 10).

Labelling with antibody to crude enzyme and to lytic fraction occurred along the cylindrical wall and at the ends, and although neither preparation contained antibody only to amidase, results described elsewhere in this chapter, suggest that a similar pattern of labelling

would be found when antibody to pure amidase was used. From the results obtained, it was impossible to say whether the amidase was present in discrete areas over the surface rather than randomly distributed.

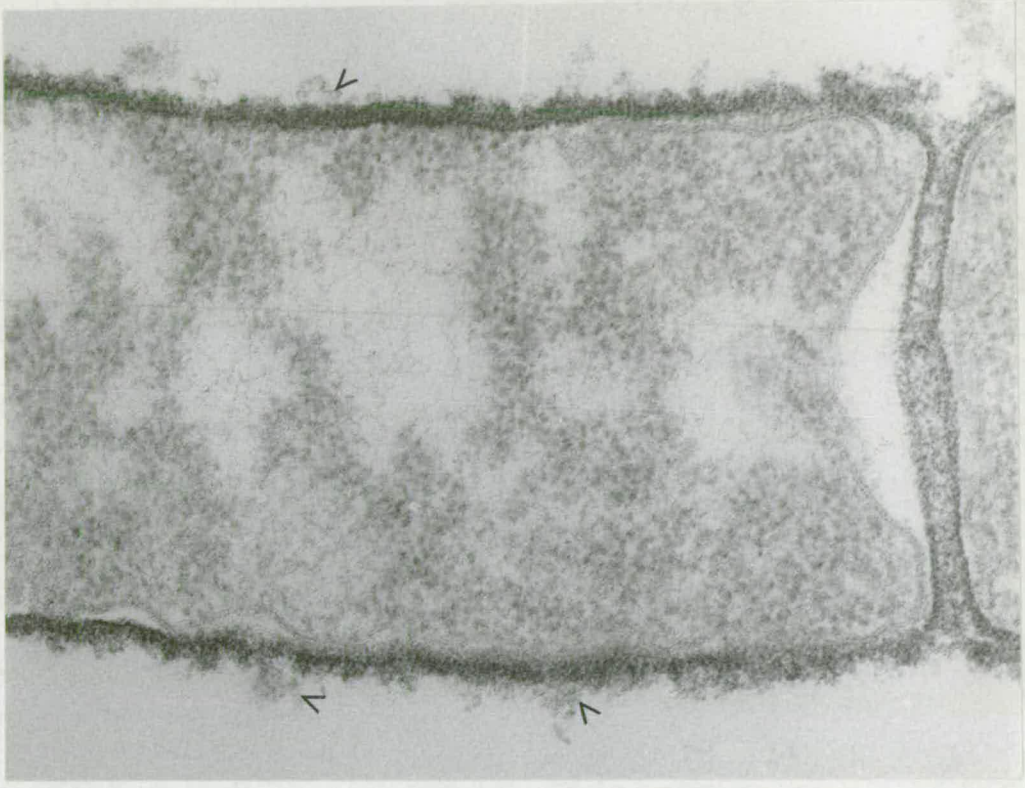


Plate 10 Section of a cell wall, labelled with antibody to lytic fraction. Clumps of ferritin label attached to the wall are indicated by arrows. Magnification x 100,000.

Localisation of Enzymes in Minicells

A culture of strain Cu 403 was harvested in mid exponential phase, and the enzymes extracted from the whole cells. The extract was assayed for its rebinding to strain 168 SDS-walls and subsequent lytic activity. Both were efficient making strain 168 SDS-walls a suitable substrate for assaying strain Cu 403 enzymes. Similar results were obtained with enzyme extracted from cells in late exponential phase.

A 10 l culture of strain Cu 403, grown in minimal salts, was harvested in late exponential phase, and the minicells separated from the parental cells. It was difficult to compare the amount of enzyme removed from minicells with that from parental cells, since not all the minicells were recovered during harvesting, more minicells were lost on the sucrose gradients, and the separation of minicells from parental cells was not complete, i.e. some minicells were present in the parent cell preparation often still attached to parental cells, and a small number of parental cells were present in the minicell preparation. However a typical extract produced 31 units of activity from the minis and 693 units from the parental cells, but the minicell extract contained more units per mg of protein than the parental extract. A possible reason for this is discussed later.

A pH optimum experiment was carried out on both extracts at 45 C. The results are shown in Figure 15. The minicell extract appeared to have three peaks so the experiment was repeated with a different extract and the same result was obtained. This raised the question as to whether a third lytic enzyme was present, whose pH optimum was masked in extracts from parental cells where the pH 8.5 to 9.0 peak was so high. Apart from this, pH optima at 6.0 to 6.5 and 8.5 to 9.0 were obtained, similar to those for strain 168 shown in Figure 7.

A sample of minicell extract was run beside a sample of crude

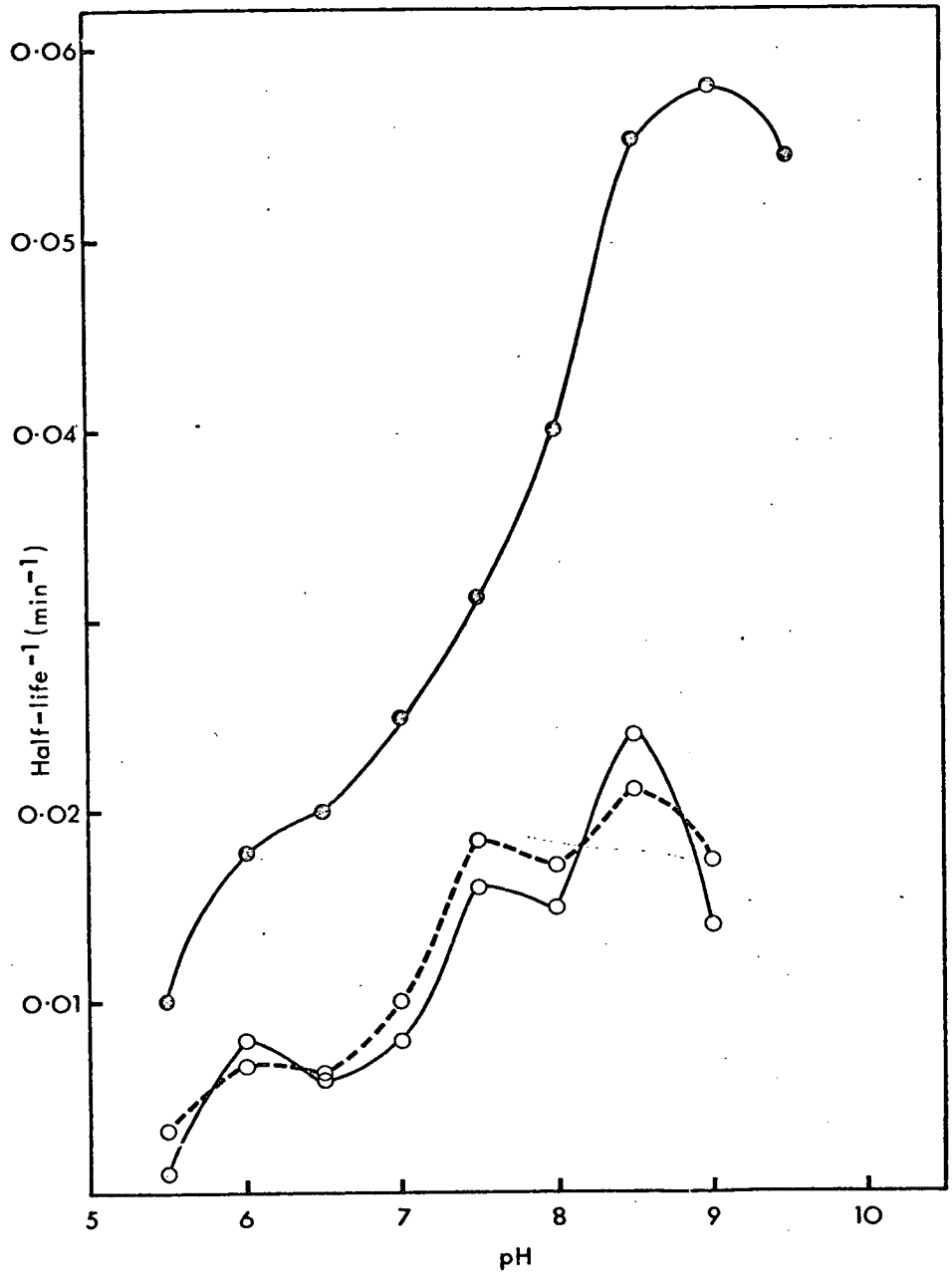


Fig. 15 pH Optima of Enzyme extracted from strain Cu 403 parental cells (●) and minicells (○).

enzyme from strain 168 on an SDS gel (Plate 11). Unfortunately, the minicell extract was not so concentrated as the crude enzyme. However, bands were found in the minicell extract corresponding to all but the faintest in the crude enzyme extract, and no new bands were found in the minicell extract. In the crude enzyme, the highest M.W. band (X) (see Figure 11) was the most concentrated, followed by the band (Z) between standards B and C. However, in the minicell extract, band X

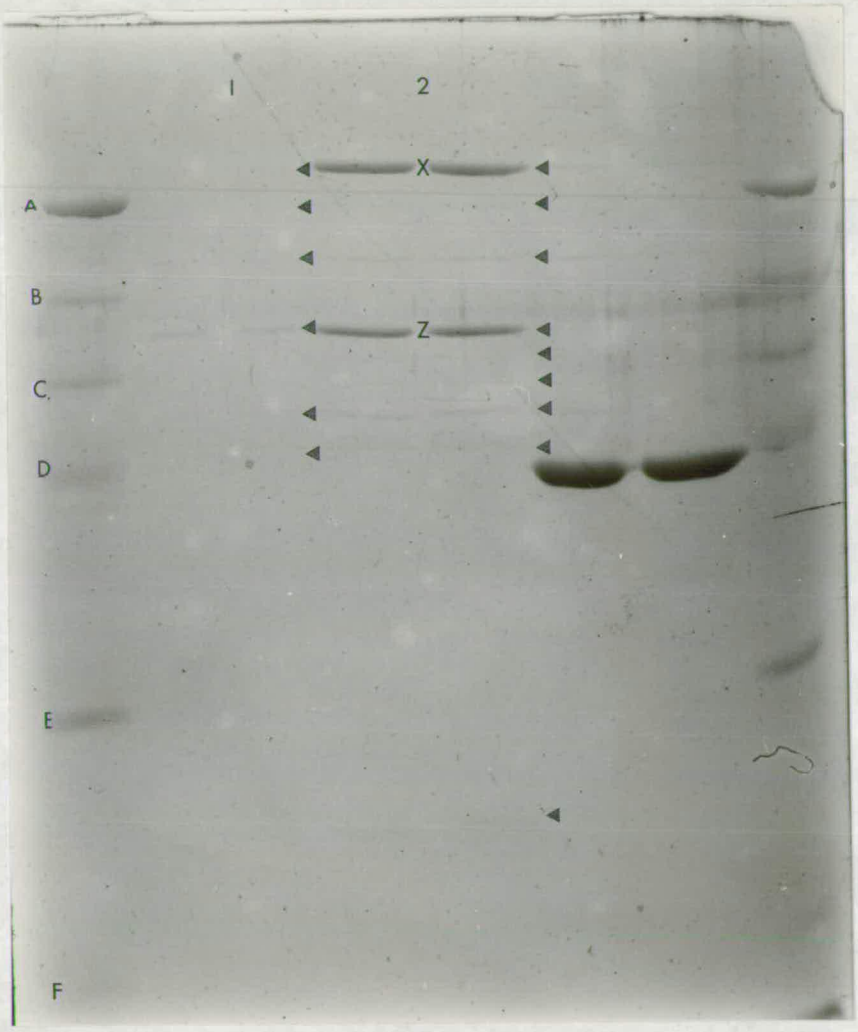


Plate 11 A crude enzyme extract from strain Cu 403 minicells (1) was run alongside an extract from strain 168 cells (2) on an SDS-gel. The bands which were visible on the gel are indicated with arrows. X is the most concentrated protein band and Z the next most concentrated. Standards A to F are as shown in Plate 5.

is barely visible while Z is quite distinct. This may account in part for the fact that the minicell extract contained more units/mg of protein than did the parental extract.

It was concluded from these experiments on minicells, that the proteins of the minicell wall were very similar to those of the parent and to those of strain 168, although the proportions differed. Minicells contained considerable lytic activity with the same pH optima as the parental cells and strain 168. However an extra peak appeared in the pH curve for minicells, which remained unexplained.

Autolysis of Whole Cells

As discussed already, Fan and Beckman (1972) showed that glycosidase and amidase have different pH optima, 6.0 and 8.6 respectively, and that glycosidase is less heat stable. An experiment to follow autolysis under these different conditions was carried out , using strain 168 in exponential phase. Half of the culture was heated to 52 C for 15min to inactivate the glycosidase. The following samples were then incubated at 37 C;

1. untreated cells in TK buffer (pH 8.6)
2. untreated cells in TM buffer (pH 6.0)
3. heated cells in TK buffer (pH 8.6)
4. heated cells in TM buffer (pH 6.0)

Samples 1 and 3 showed a rapid decrease in O.D.,while samples 2 and 4 showed a very slow decrease. At the beginning of incubation, all four samples had the same appearance, consisting of single cells, pairs and chains of up to 10 or more. There was no sign of lysis. However, after 15min, samples 1 and 3 contained many light cells indicating that autolysis was occurring, but there were still many chains present. At this time samples 2 and 4 showed no change.

After 30min, almost all the cells in 1 and 3 had lysed, and only short chains remained, along with an increased number of pairs and single cells. Samples 2 and 4 showed the first signs of lysis, but only in a few cells, and long chains were still present.

After 45min, only pairs,single cells and fragments remained in samples 1 and 3, and 3 appeared to be more degraded than 1. At this stage in samples 2 and 4, some of the longer chains were breaking up, and more lysis had occurred.

At 60min, samples 1 and 3 consisted mostly of fragments with very few intact cells, while samples 2 and 4 still contained chains of four cells (in sample 4 some chains of eight), and little more lysis than at 45min .

By 105min, nothing was visible in samples 1 and 3 by phase microscopy. Samples 2 and 4, on the other hand, still contained many pairs, and chains of four cells, although by this stage there was quite extensive lysis.

The samples were left overnight at room temperature, and were then examined by electron microscopy using negative staining. Samples 1 and 3 contained mainly very small fragments of wall, some cell ends and an occaisional whole cell. Samples 2 and 4, on the other hand, contained as well as many small fragments, many more whole cells, single and in pairs, many ends and large pieces of wall, and walls retaining their original shape, but with a very 'moth-eaten' appearance.

These results suggested that under conditions optimal for glycosidase activity, a much slower, but similar process to that under conditions optimal for amidase is taking place. If glycosidase was localised at the polar regions and involved in cell separation, then at pH 6.0 it would be expected that chains of cells would be rapidly broken down, with little lysis. This did not occur, and the maximum rate of cell

separation was achieved under conditions optimal for amidase activity, in agreement with evidence for amidase involvement in cell separation discussed in the introduction. Thus the results did not agree with the idea that, since Cocci have been found containing only one demonstrable lytic activity, an N-acetyl muramidase (Shockman & Cheney, 1969), whereas Bacillus species have both an amidase and a glycosidase activity (Fan and Beckman, 1972), then the amidase might be associated with the cylindrical wall and the glycosidase with the polar regions.

The only difference between samples 2 and 4 (glycosidase inactivated) was that the degradation pattern in 4 was slightly slower. This seemed to indicate that, even here, most of the degradation was due to amidase acting slowly at the lower pH. There was no apparent slowing up of the degradation rate at pH 8.6 when glycosidase was inactivated, indicating that under these conditions, the latter contributed very little to the autolytic process. Thus amidase appeared to be responsible for cell separation, degradation of the cylindrical wall and ultimately degradation of the polar region.

These results were confirmed and extended by examining sections of autolysing cells in the electron microscope. An exponential phase culture of strain 168 was allowed to autolyse at 37 C in TK buffer, pH 8.6. The following samples were removed, and immediately pre-fixed, spun down and fixed; cells growing in culture medium (control 1), and cells resuspended in TK buffer for 0min (control 2), 15, 30, 45, 60, and 75min. A similar experiment was carried out in TM buffer, pH 6.0, and samples were taken at 0, 20, 40, 60, 80, 100, 120 and 165min.

A typical control 1 cell is shown in Plate 12. The walls were about 50nm thick, and the sides often thicker than the ends and not so smooth. All round the wall, a trilayered structure was visible, consisting of an inner dark layer, an intermediate light layer, and an

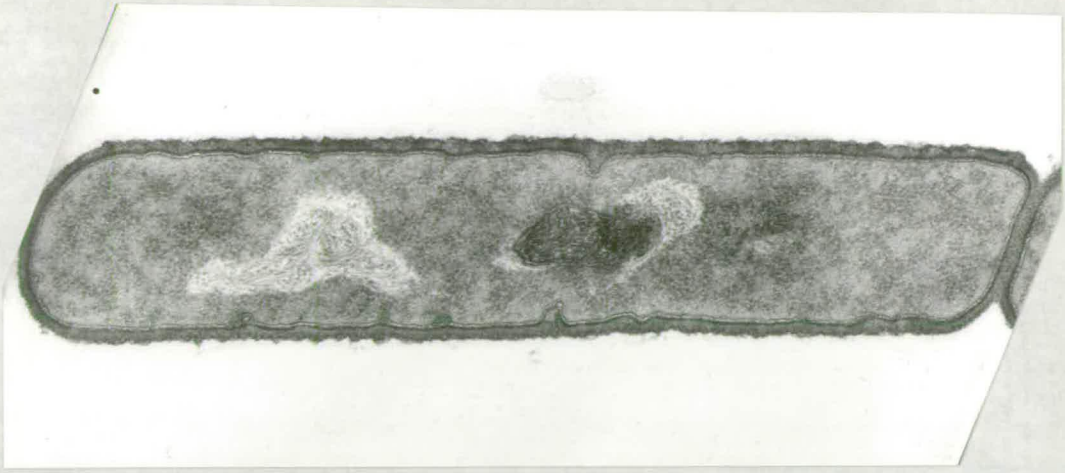


Plate 12 Section of a control 1 cell, used as a standard for comparison throughout the autolysis experiment. The side walls are 50nm wide, and thicker and smoother than the ends. There is no sign of degradation at the developing cross-wall, but the completed cross-wall is undergoing separation. The tri-layered structure is visible all round the wall. Magnification x 40,000.

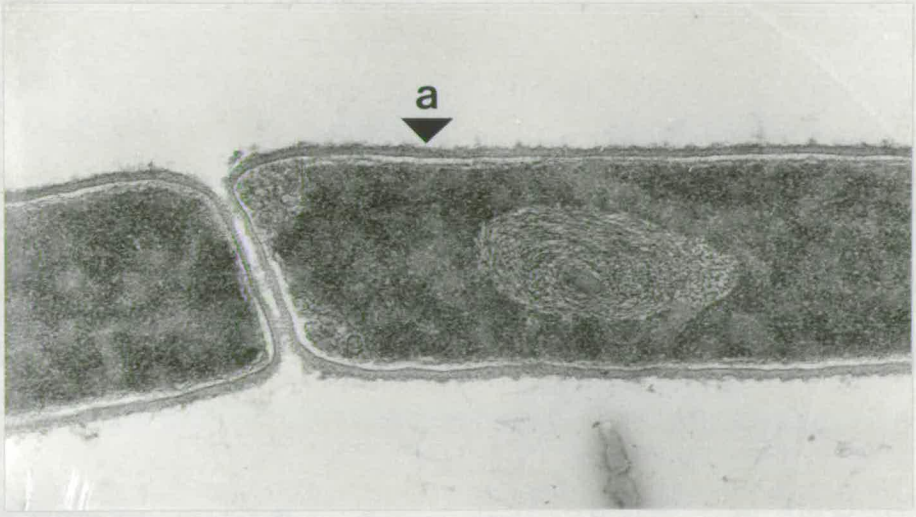


Plate 13 Section of control 2 (pH 8.6). a) shows shrinkage of the cytoplasm away from the wall. The walls are thinner than in Plate 12, and degradation is occurring within the completed cross-wall. Magnification x 50,000.

irregular dark layer on the outside. Where cross-walls were forming, there was no sign of indentation of the side wall. When the cross-wall was complete, it appeared to separate from the outside towards the centre, forming rounded ends. As can be seen from Plate 12, the finished cross-wall was less than twice the width of the side walls, implying that ends once formed, required to be thickened if they were to reach the width of the side walls. Some ends were found which were as thick, if not thicker than the side walls.

Control 2 (pH 8.6) was already considerably altered from control 1. Some shrinkage of the cytoplasm away from the wall had occurred, and the walls themselves were generally thinner. Degradation was now visible within completed cross-walls, instead of separation occurring in a controlled manner from the outside (Plate 13). There was however, no evidence of degradation of incomplete cross-walls. The appearance of cells in control 2 is assumed to have resulted from the continued action of lytic enzymes in the absence of wall synthesis.

Plate 14 shows stages in cross-wall formation which might be considered in terms of a lytic cycle. a and e represent the oldest cross-walls formed, followed by c, then d, and then b, (the cross-wall d divided cell c-e, and cross-wall b, the newest, was just beginning to divide cell a-c). In b and d there is no sign of autolysis, which might indicate that the autolytic enzymes were not yet present, or were inactive. However, at position c the normal separation process had started, and under the experimental conditions, considerable autolysis had occurred inside the cross-wall, indicating that at this stage, the enzymes were present and active right across. Position e represents a later stage of this process, and position a the last. If such a cycle existed, it could be due to variation either in the amount of enzyme present, or the activity of the enzyme. Other evidence discussed in this chapter and the last, for example, the presence of autolytic

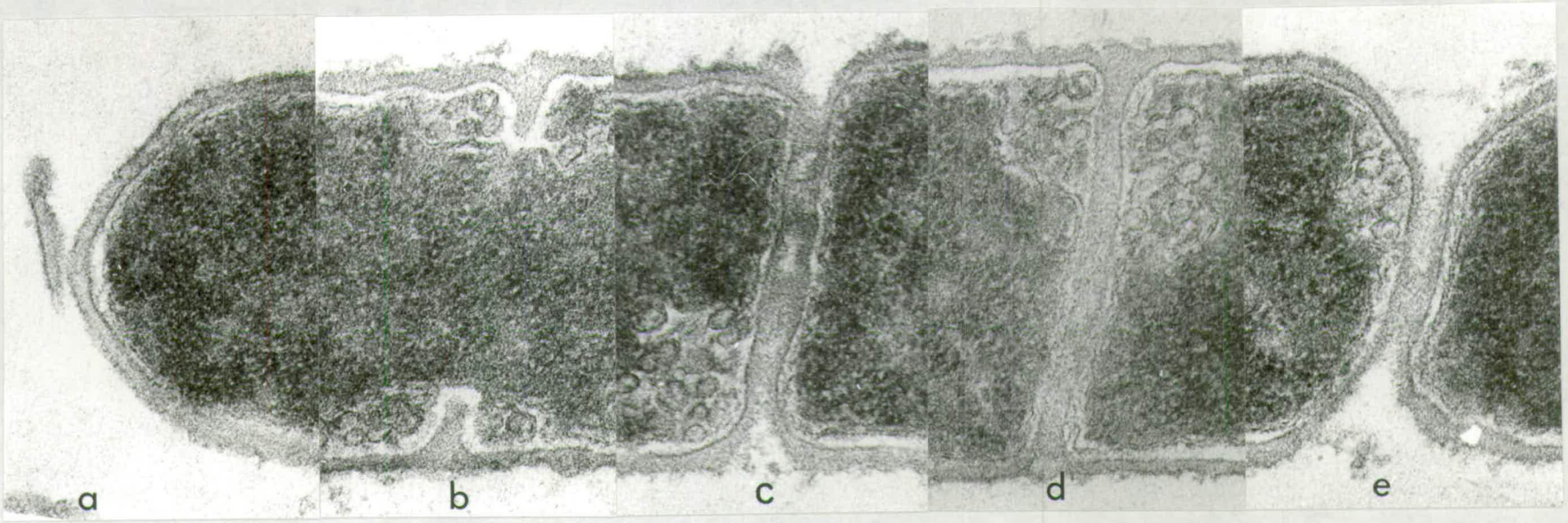


Figure 14 Sections of cells from control 2 (pH 8.6), showing stages in cross-wall formation. a to e were consecutive cross-walls in a chain of cells. a and e represent the oldest cross-walls, followed by c, then d, and then b. Magnification x 100,000.

enzymes in minicells, which are resistant to autolysis, does not support the first alternative. Therefore it is more likely that the enzyme is always present, but its activity varies (perhaps according to the wall structure). Since amidase is the major lytic activity under the experimental condition used, it follows that no activity will be evident until after cross-linking has taken place. Under normal growth conditions, it seems reasonable that cross-linking should occur from the outside of the cross-wall towards the centre, probably some distance behind the leading edge of the cross-wall where new peptidoglycan units are being inserted. This could explain why b and d showed no sign of attack, although to differentiate between d and c would still require some control of amidase activity, which could involve the modifier described by Herbold and Glaser (1975). Otherwise one would expect to see some signs of degradation from the outside, unless no crosslinking occurs until the cross-wall is completed. This could also explain the difference between the results of these experiments and those of Higgins, Pooley and Shockman (1970), who found that the primary site of autolytic activity in S. faecalis was the leading edge of the nascent cross-wall. Here the major lytic activity was an N-acetylmuramide glycanhydrolase, which could presumably attack peptidoglycan chains as soon as they were formed. Infact, chains which were not cross-linked might be more susceptible to attack. However, the explanation does not fit the results obtained by Higgins, Coyette and Shockman (1973) for L. acidophilus. The major lytic activity in this case was also an N-acetylmuramide glycanhydrolase. but the pattern of autolysis was more similar to that obtained in B. subtilis.

Since minicells are resistant to the autolytic enzymes on their walls, and ends are much more resistant to autolysis than cylindrical walls in strain 168, this suggests that once the end is formed, it

becomes resistant to autolysis, presumably due to structural modification (see Chapter IV for further discussion).

Plate 15 (15min incubation at pH 8.6) shows another feature of the separation process, namely 'V-shaped' pieces of wall removed from the outer edge of the cross-wall. The cylindrical walls are ragged in comparison to the smooth thinner poles. In places the inner dense layer of the wall has disappeared, and occasionally a break can be seen extending right through the wall.

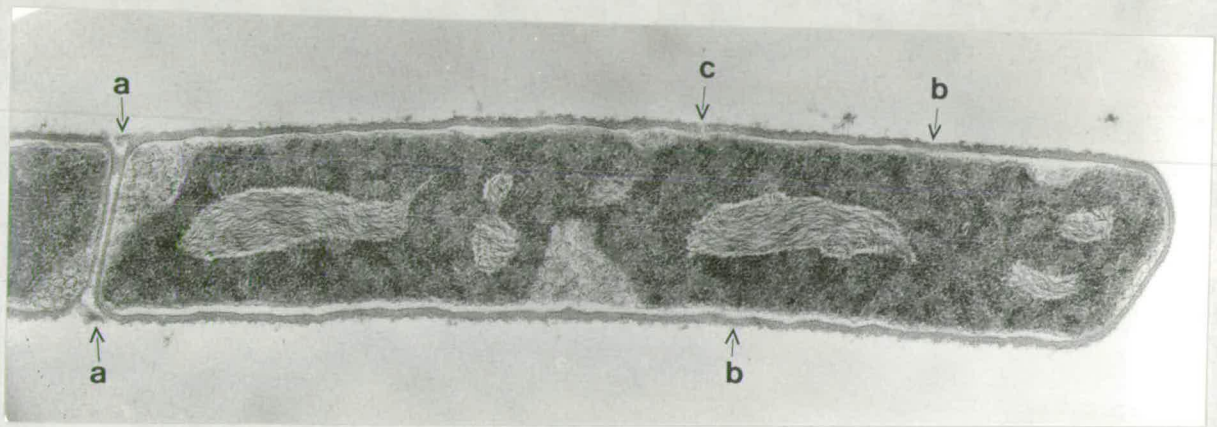


Plate 15 Section of a cell after 15min incubation at pH 8.6. V-shaped notches of wall were removed from the outer edge of the cross-wall (a), in places the inner dense layer had disappeared (b), and a break can be seen extending right through the wall (c). Magnification x 40,000.

After 30min incubation, it was very obvious that autolytic activity varied from cell to cell. Some cells were completely lysed at this stage, while others showed little difference from control 2. Lysis was not closely correlated with wall thickness, for in many cells, the point of lysis was not at the thinnest part, and some thin-walled cells were not lysed. However, the inner dense layer was always absent at points of lysis. Plate 16 shows the cytoplasm about to burst through the wall.

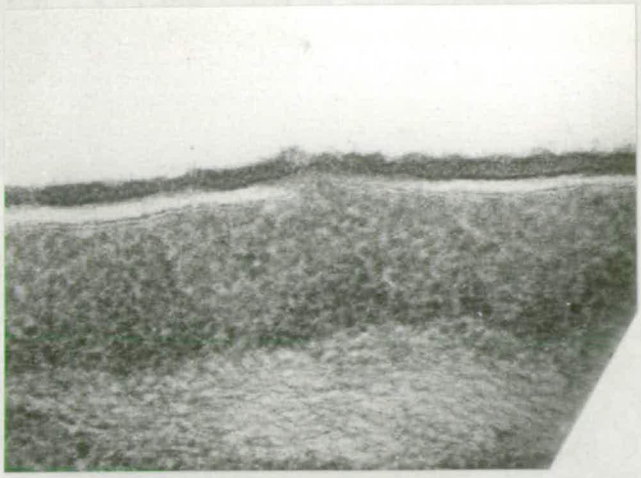


Plate 16 Section of a cell after 30min incubation at pH 8.6. It shows the cytoplasm about to burst through the wall. Magnification x 100,000.

Plate 17 (45min incubation at pH 8.6) illustrates clearly the greater resistance to lysis of the poles. This cell was at an advanced stage of autolysis, and the unfinished cross-walls had been eaten away. Many points of lysis occurred, indicating that the amidase was still active after the cell contents were lost. In contrast, Plate 18, taken from the same sample, showed signs of degradation right across the cross-wall, but little evidence of any enzyme action on the side walls.

After 60 and 75min of incubation, most of the cells were lysed. Plate 19 illustrates just how fragile some of the walls had become.

Thus it appeared that in the normal process of division and separation, the cross-walls were completed before separation began, and that when this occurred, it began at the outer edge from two directions, cutting out a 'V-shaped' piece of wall, and then continued on down the centre of the cross-wall (Figure 16). Sometimes a small bump was visible on one of the ends where the 'V-shaped' piece had not been completely removed. In three-dimensional terms, this meant that a ring of material of roughly wedged shape cross-section must have been removed from the outside of the cross-wall, although this may have occurred in many

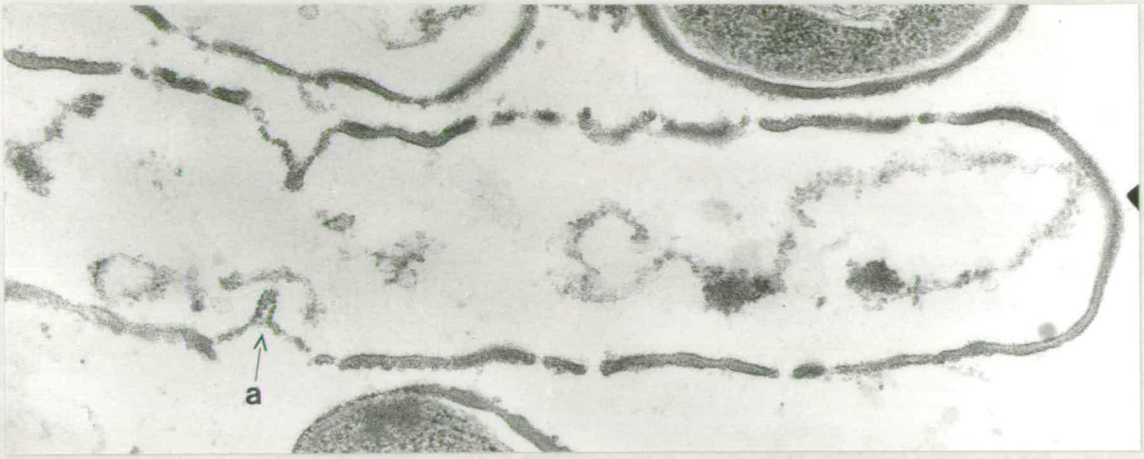


Plate 17 Section of a cell after 45min incubation at pH 8.6. The ends are more resistant to autolysis than sides, and there has been degradation of an unfinished cross-wall (a). Magnification x 50,000.

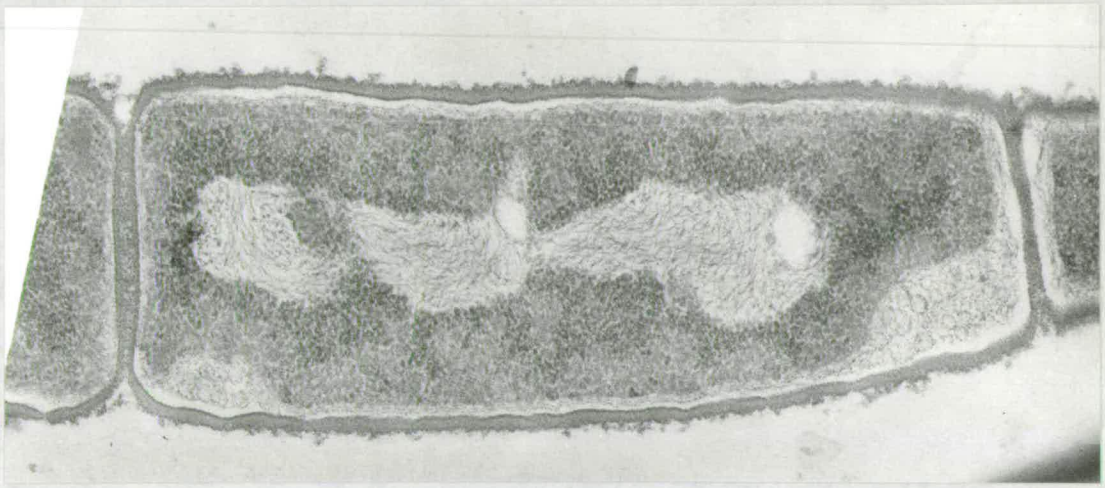


Plate 18 Section of a cell also after 45min at pH 8.6, showing very little degradation of the sides. Magnification x 50,000

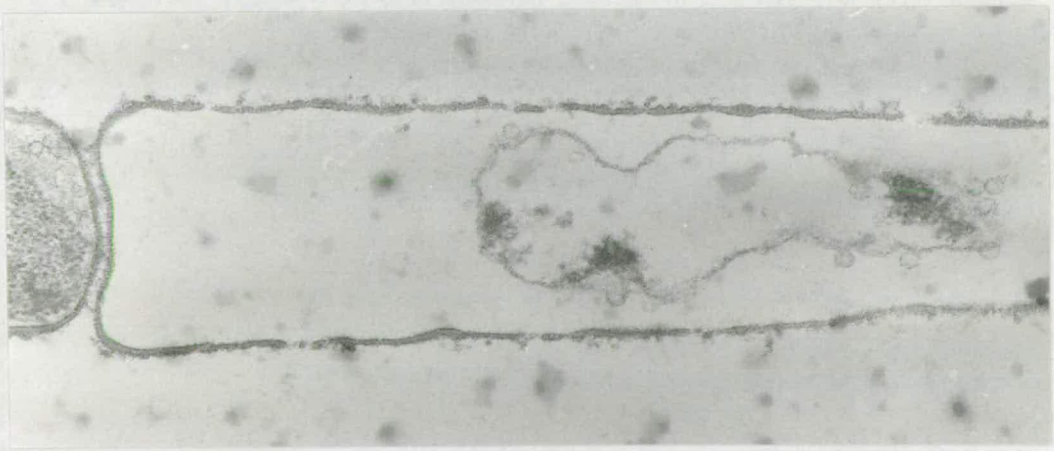


Plate 19 Section of a cell after 75min at pH 8.6. The walls have become very fragile. Magnification x 50,000.

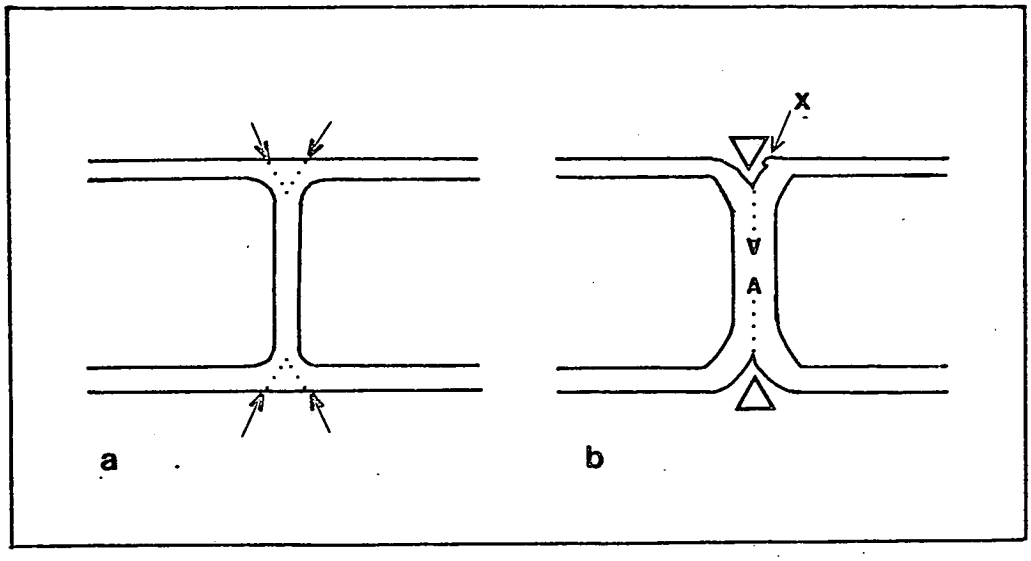


Fig. 16 The first stages of cell separation
The first signs of degradation appear to occur as indicated by the dotted line in a). 'V-shaped' notches are removed as shown in b) and then separation continues towards the centre of the cross-wall. X indicates the position of the bump which appears to result from the incomplete removal of the 'V-shaped' notch.

small fragments. There must be some very precise means of controlling where the cross-wall splits, since even under the abnormal conditions of autolysis, the splitting occurred in the same place, and the ends were not degraded before the sides. Ends produced from cross-walls were thinner and smoother than sides, and were probably thickened subsequent to their formation, as ends of various thickness were normally seen.

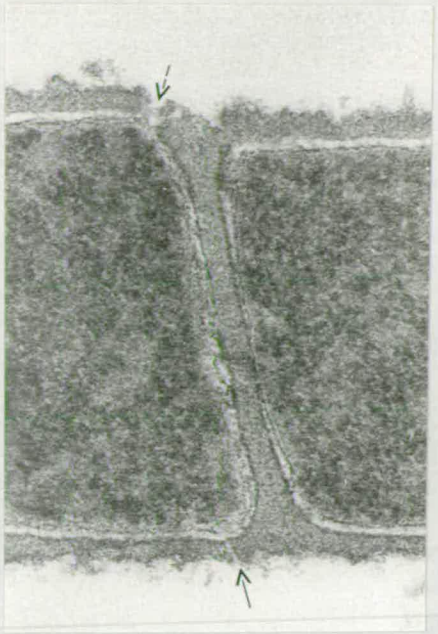
Under conditions of autolysis at pH 8.6, where amidase was the major activity, separation of the cells was no longer under such strict control, and enzyme right across the cross-wall was able to act, so that often the cells were separated in the middle but still joined nearer the outside. And a point was reached in autolysis where even unfinished cross-walls were degraded from the outside. Amidase was also acting on the sides, making them progressively thinner, but at some places attacking the wall in such a way as to form a split right through, resulting

in lysis. Before lysis occurred., the inner dense layer was lost.

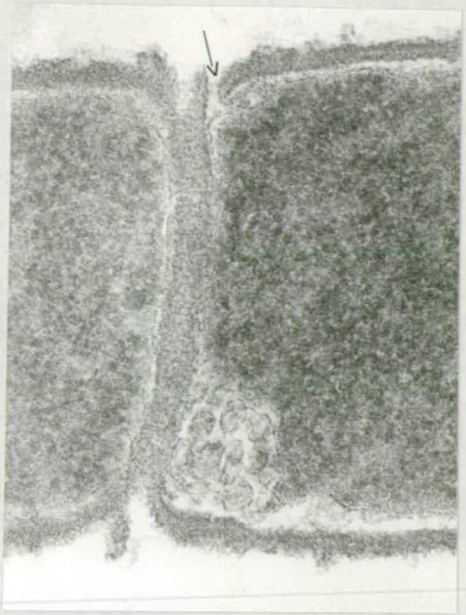
When the experiment was repeated under conditions optimal for glycosidase activity (pH 6.0), no such immediate degradation of cross-walls and thinning of sides was visible. The most noticeable effect in control 2 and the first two incubation samples, was breakage of the wall near poles and cross-walls, which appeared to have been caused by a structural weakness. In some cells a thin split was visible, in others, parts of the wall had been completely dislodged; and in one instance the fracture lines could be seen in transverse section (Plate 20). Not until after 40min was much degradation of the cross-walls evident. Again the 'V-shaped' notches removed from the outside of the cross-wall were visible (Plate 21). After 80 or 100min, degradation of developing cross-walls occurred, as had happened at pH 8.6 after only 45min. As before, the sides were more ragged and often thicker than the ends, which remained smooth and more resistant to attack. There was no obvious degradation of nascent cross-walls at their inner edge, as might be expected if the glycosidase were acting like the N-acetyl muramidase in S. faecalis.

Thus under conditions optimal for glycosidase activity, the main difference from the amidase results was the appearance early in the incubation of an apparent structural weakness next to poles and cross-walls. This occurred to a very limited extent in control 1, but was more evident once the cells were resuspended in TM buffer. Otherwise, the pattern of autolysis was similar to that where amidase was acting, but at a greatly reduced rate. In fact results in the previous section showed lysis at pH 6.0 even after heating to inactivate glycosidase, suggesting amidase activity at this pH. The appearance of the 'V-shaped' notches was no less marked than previously.

If the activity at pH 6.0 is from glycosidase, these results could



a



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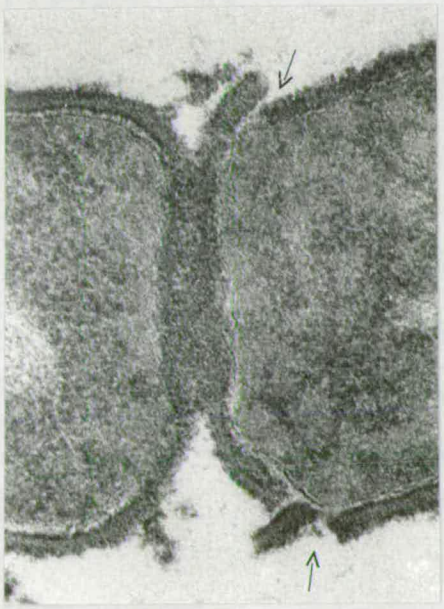
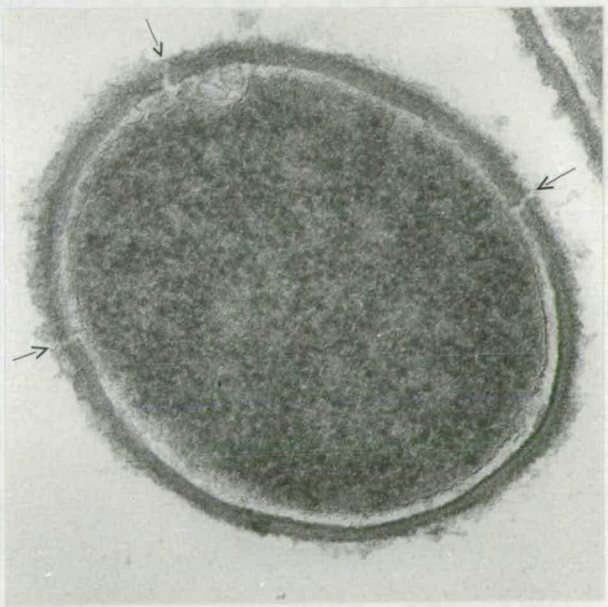


c



d

Plate 20 Sections of cells from control 2 at pH 6.0. Fractures in the wall are indicated by arrows. d) shows them as seen in transverse section. Magnification x 100,000.

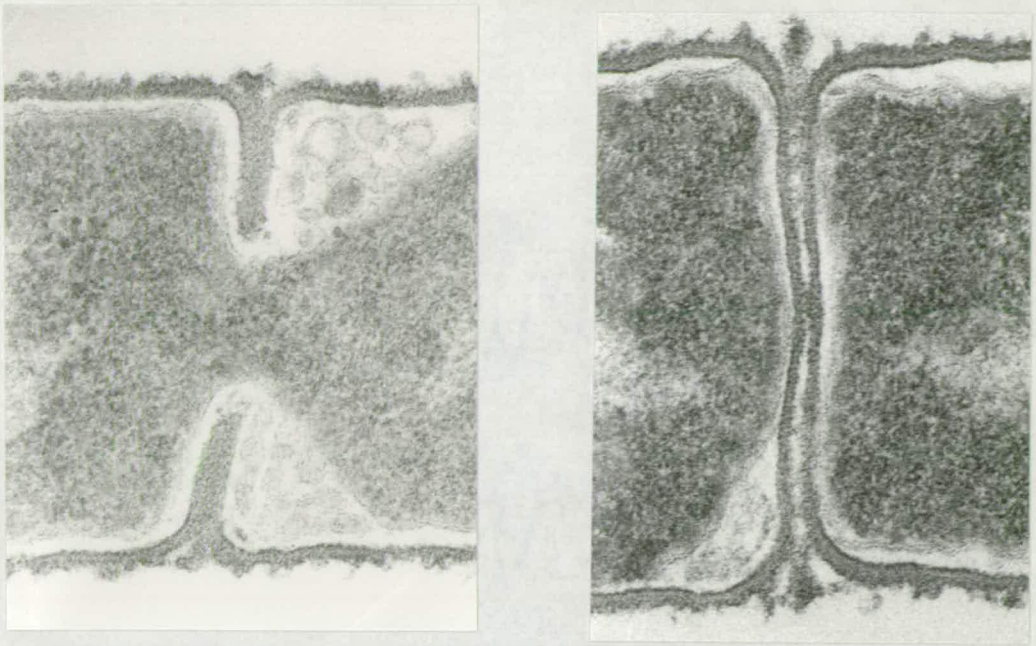


Plate 21 Sections of cells after 80min incubation at pH 6.0. V-shaped notches are visible at the outside edges of cross-walls. Magnification x 100,000.

indicate that by breaking different bonds, glycosidase can achieve the same results as amidase, as far as can be detected at the resolution of the electron microscope, but requires a much longer period of time, perhaps because it is present in very small amounts. This means that glycosidase is situated at the poles and along the cylindrical walls, and is in agreement with the results of Fan (1970b), who found that both the 'amidase' and lysozyme could act as dechaining enzymes, and with the presence of glycosidase in minicells.

CHAPTER IV

STRUCTURE AND GROWTH OF *B. SUBTILIS*

RESULTS AND DISCUSSION

In this chapter, I have attempted to summarise the information on *B. subtilis*, and using this, to put forward some ideas about the structure and growth of this organism.

Structure

B. subtilis is a Gram-positive rod, requiring many layers of peptidoglycan to make up its thick wall. Much evidence suggests that there is a difference, probably structural, between the cylindrical side wall and the ends (Frehel *et al.*, 1971; Highton & Hobbs, 1971 and 1972; Fan, Pelvit & Cunningham, 1972). The only difference which can be seen in sections of cells is that the ends are often thinner than the sides, and are always smoother on their outer edge. But they do have the same trilayered structure as the side walls. In negatively stained preparations, the sides appear more granular and uneven than the ends. During autolysis, or on the addition of autolytic enzymes to SDS-walls, the ends are always more resistant to attack.(Fan *et al.*, 1972). During autolysis the O.D. fell steadily to about 10% of its original value, and then much more slowly (Figure 17). Negatively stained preparations made at various times during the autolysis (Plate 22a, b and c), showed that the persistence of ends was responsible for this residual absorption. Very little other wall material remained. Eventually the ends developed holes and were gradually degraded.

Considerable wall turnover occurs in *B. subtilis*. Mauck and Glaser (1970) reported about 50% per generation, although newly synthesised material did not become available for turnover for at least one half of a generation. It has usually been assumed that cell ends are conserved, while turnover occurs in the cylindrical portion. If turnover is a feature of growth, then there is no reason for it to

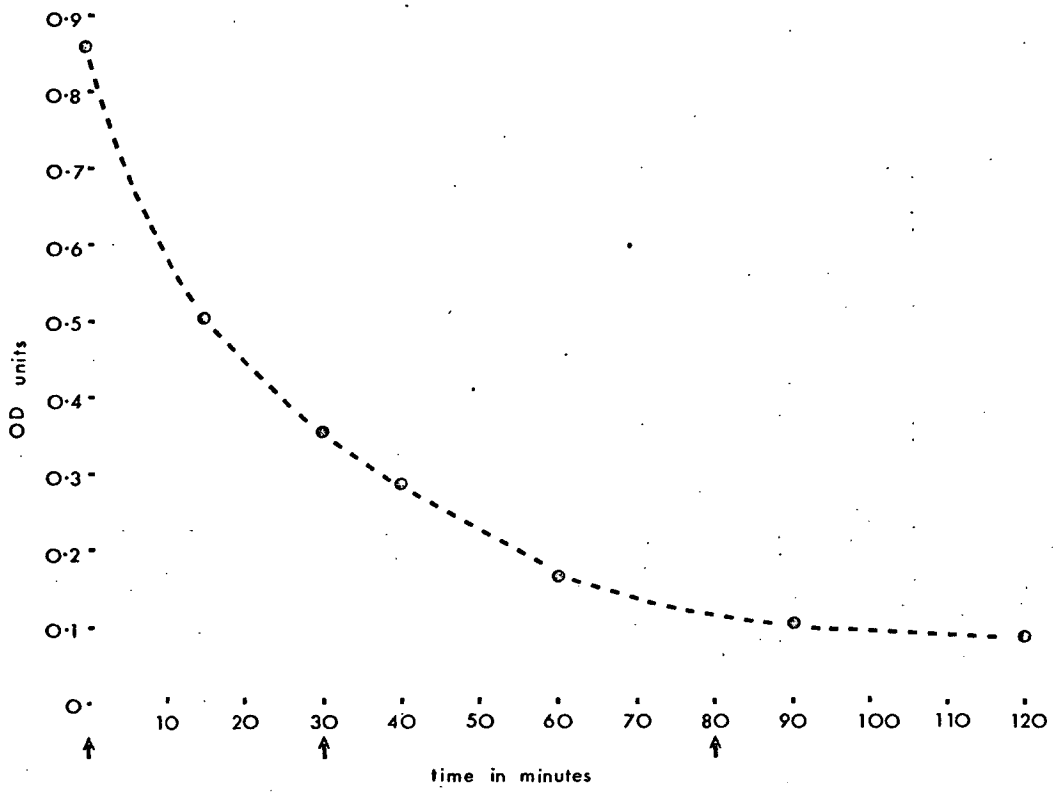


Figure 17 O.D. curve for autolysing B. subtilis cell walls. Samples were taken at 0, 30 and 80min (indicated by arrows), negatively stained and photographed in the electron microscope (Plate 22). The autolysis was done at 45 C at pH 8.6. The remaining material after 60min is predominantly from cell ends.

occur in ends since once formed, they need not grow. However Fan, Beckman and Beckman (1974) have reported turnover to occur in B. subtilis ends.

Results presented in this thesis, indicate that amidase and glycosidase are present in both the cylindrical wall and the poles, although exact distribution has not been determined. However, amidase is the major activity, and appears responsible for the separation of cells after completion of the cross-wall, and also for dissolution of

Plate 22a Autolysing B. subtilis walls (45 C, pH 8.6) at zero time. (see Figure 17). Magnification x 40,000.

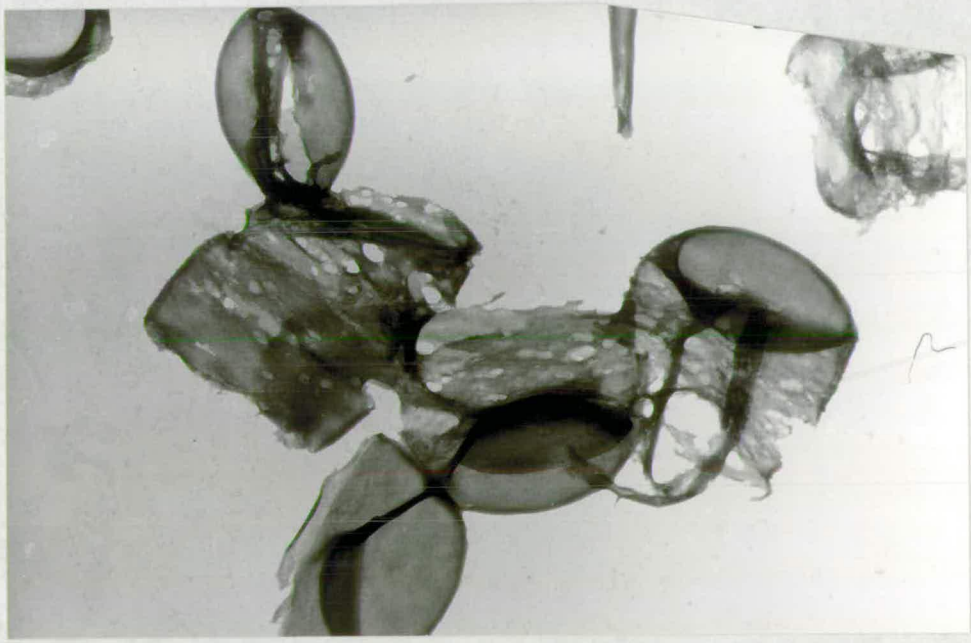


Plate 22b Autolysing B. subtilis walls (45 C, pH 8.6) at 30min. Magnification x 30,000.

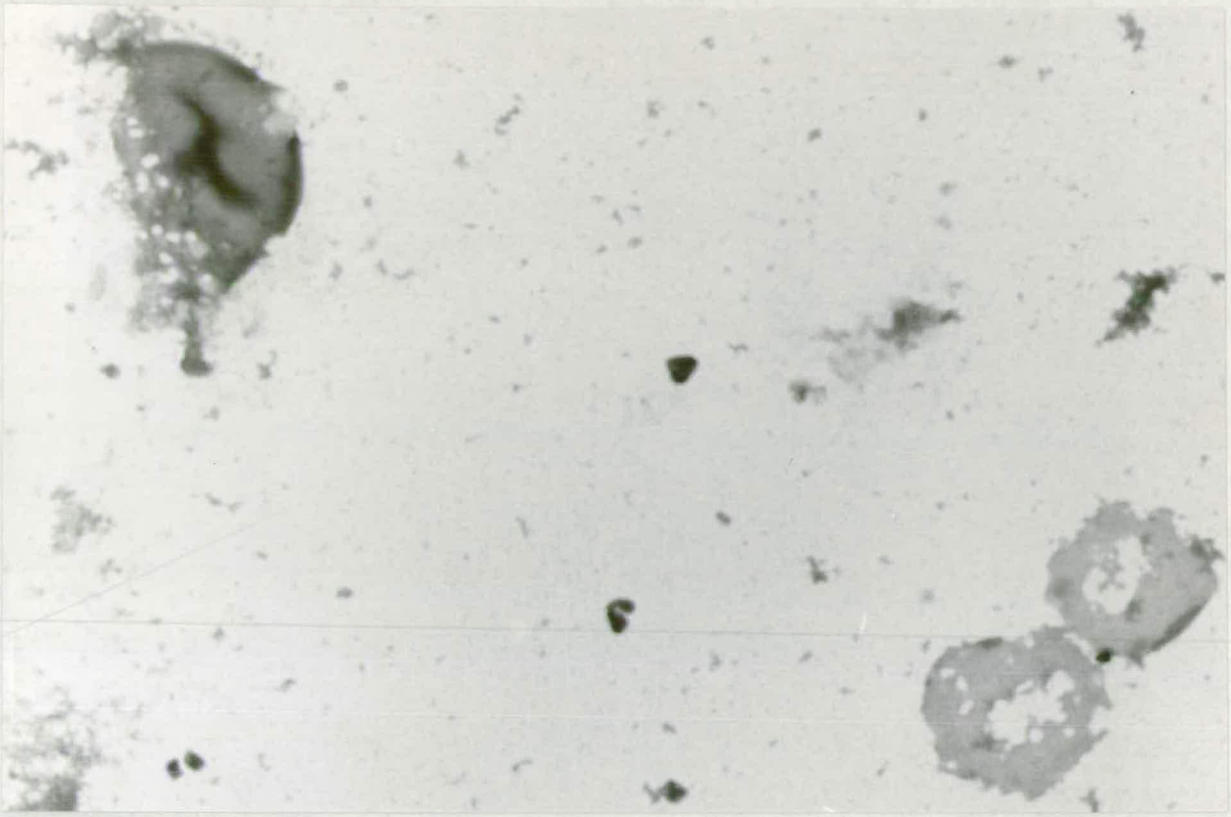


Plate 22c Autolysing B. subtilis walls (45 C at pH 8.6) at 80min. Magnification x 30,000.

cylindrical walls during autolysis. This could well indicate that amidase is the enzyme involved in turnover during normal growth. The reason for the existence of glycosidase has not become clear during these studies. No specific site of activity could be attributed to it in the presence of amidase, and under conditions optimal for its activity a similar, although much slower pattern of events to that with amidase occurred, except perhaps for some localised weakening of the wall close to the ends and cross-walls. Glycosidase activity does not provide a suitable acceptor site for addition of new material, so it is unlikely to be involved directly in growth. However, it could still be important for breaking glycan chains in the process of end formation. If glycosidase can achieve the same results as amidase by breaking different bonds, e.g. cell separation and degradation of outer layers,

it might be useful to the cell under adverse conditions when the amidase cannot work so efficiently, such as in an acid pH environment.

It is not known whether the glycan strands are arranged parallel to the surface of the wall or perpendicular to it. If they were parallel to the surface, they could be perpendicular or parallel to the cylinder axis. However, the fact that the wall is such a good substrate for lysozyme may give a clue as to the arrangement of the peptidoglycan. It has been shown by Philips et al. that a hexasaccharide substrate of NAG fits into the cleft in the lysozyme molecule with the plane of the sugar rings such that one edge of the rings is buried in the cleft, while the other edge is exposed. The bulky lactyl groups of NAM would be attached to the exposed surface. This suggests that peptidoglycan might be arranged in the wall with the glycan chains lying parallel to the surface of the cell, with the plane of the sugar rings perpendicular to the surface, and with the peptide subunits extending towards the inside.

With regard to whether the glycan chains are arranged parallel or perpendicular to the cell axis, it is difficult to equate structural changes at the resolution of the electron microscope with what is actually happening at the molecular level. However, several structural features became apparent from electron micrographs taken during the course of my work.

1. Cells, which were sonicated, always split perpendicular to their long axis, and most of the initial splits occurred near an end or a developing cross-wall, or in the centre of the cell. Central splits became more common in older, shorter cells i.e. as they approached stationary phase. Also, when cells or walls were fragmenting due to amidase activity, ribbons of wall were very often lost from around the diameter of the cell (Plate 23). This may result from the reported

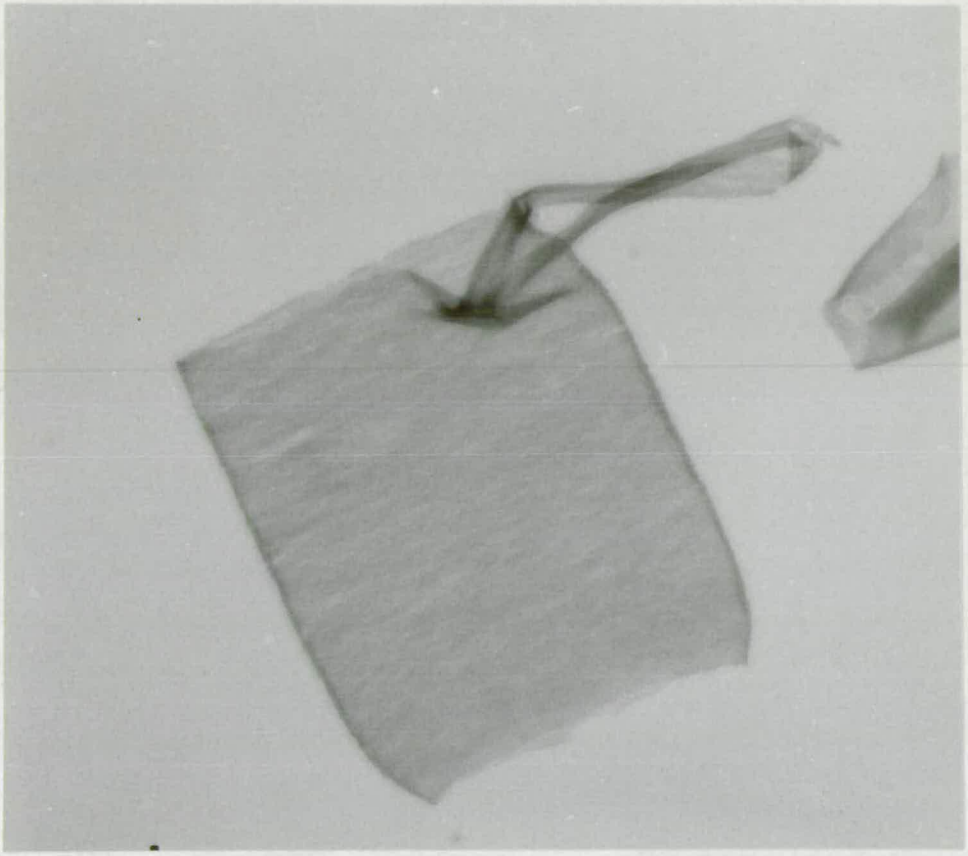


Plate 23 Negatively stained wall of B. subtilis showing characteristic degradation pattern. Magnification x 50,000.

behaviour of amidase, namely that once it binds to the wall, it breaks all the bonds in the vicinity, before moving on to attack another piece of wall (Herbold & Glaser, 1975). This pattern of degradation suggested that the glycan chains were perpendicular to the cylinder axis.

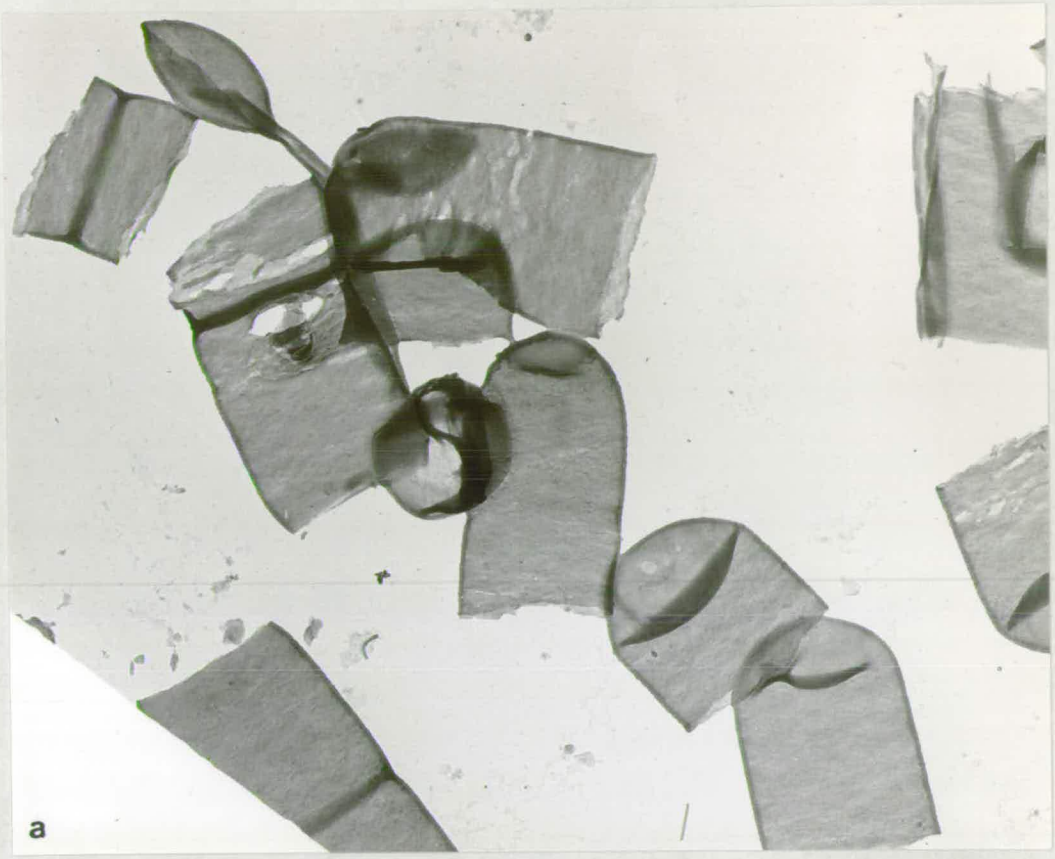
On the other hand, wall being degraded by lysozyme, and also (although not so clearly) by glycosidase, had a much more 'moth-eaten' appearance (Plate 24a and b), indicating a more random type of degradation.

2. Cell ends also often showed a characteristic pattern, In many negatively stained preparations, they appeared to have a circular pattern (Plate 25), and degradation usually took place in a circular manner (Plate 26), with ribbons of material being lost from around the circumference. Also areas of apparent weakness were often seen in the centre of the end, such as the hole in Plate 27. Fan and Beckman, (1973) found that walls from partial septa of B. subtilis were more sensitive to digestion by amidase than completed ends. This they interpreted as indicating that septal walls were modified to an amidase resistant form after completion. The hole in Plate 27 could thus be the result of amidase action.

3. Walls in negatively stained preparations often showed some kind of surface pattern. The 'honeycombs' shown in Plate 28 a and b occurred in many samples. They were thought at first to be an artifact due to drying down of stain, but on closer inspection, the structure of the surface inside the 'honeycombs' was seen to be the same as outside, indicating that perhaps the lines of the pattern were due to genuine irregularities on the surface of the wall.

Another more generalised surface pattern is shown in Plate 29, where again the ends appear much smoother than the sides.

4. While examining sections of cells, one transverse section was found which appeared to have a definite layered structure in its wall (Plate 30).



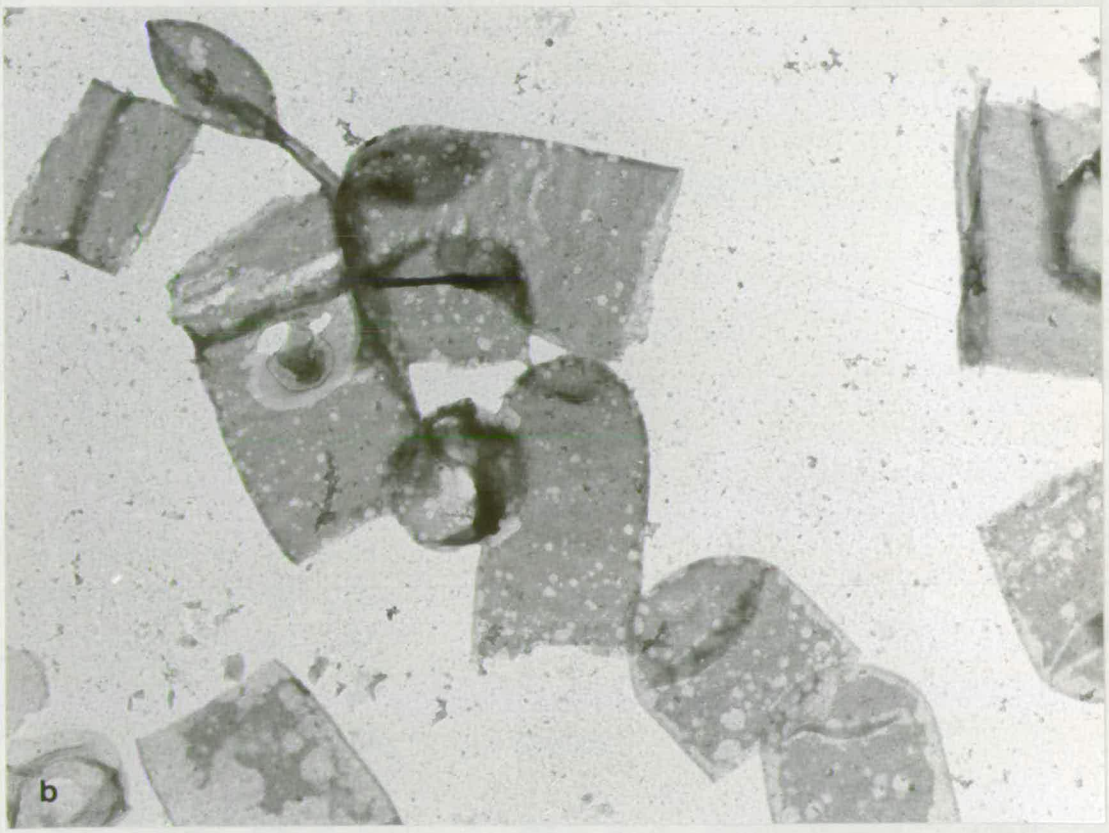


Plate 24 Negatively stained walls of B. subtilis. a) shows the walls before addition of lysozyme, and b) shows the same walls after the grid had been floated on lysozyme (1mg/ml, pH 6.0, 37 C) for 5min. Magnification x 15,000.

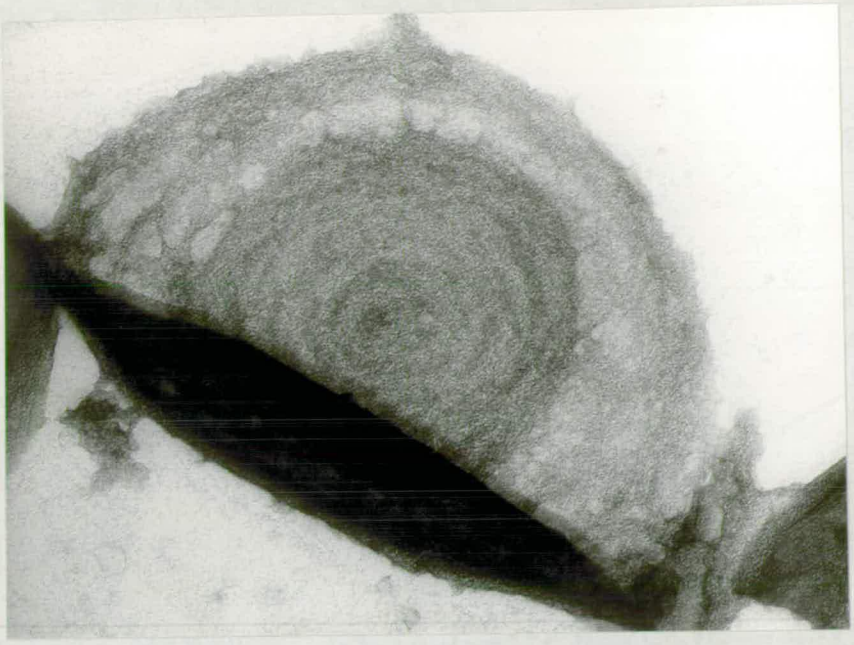


Plate 25 Negatively stained wall of B. subtilis treated with NaOH, after removal of crude enzyme with 3M LiCl. Magnification x 120,000.

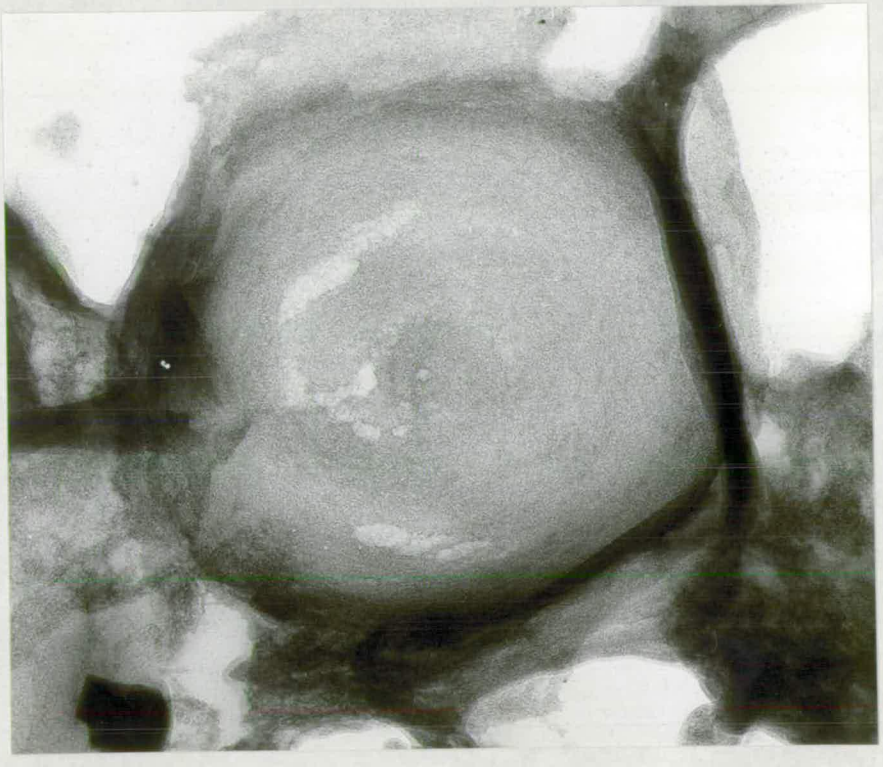


Plate 26 as above. Magnification x 120,000.

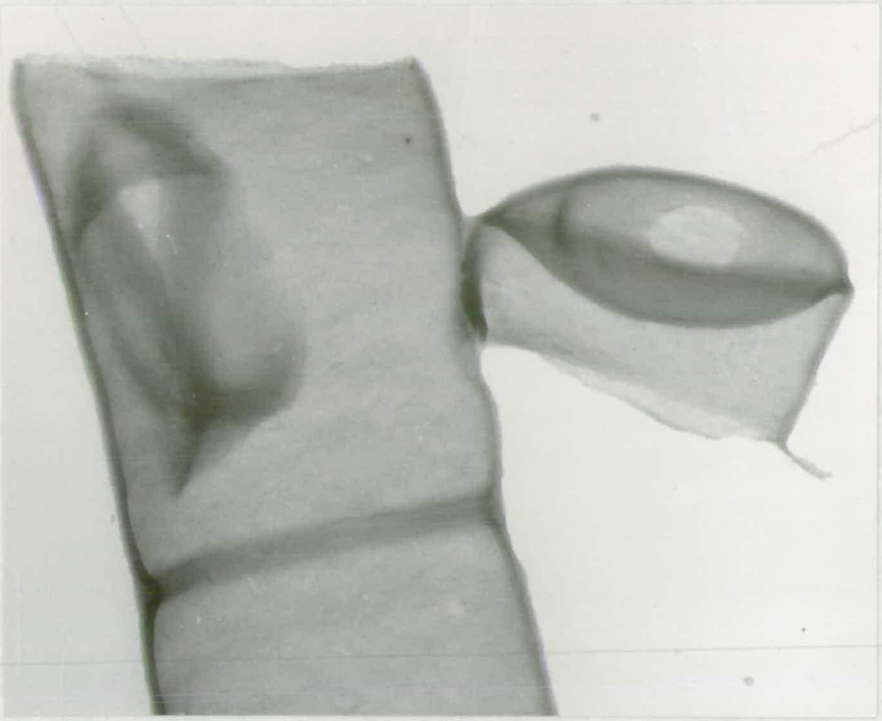


Plate 27 Negatively stained SDS-walls of B. subtilis. Magnification x 50,000.

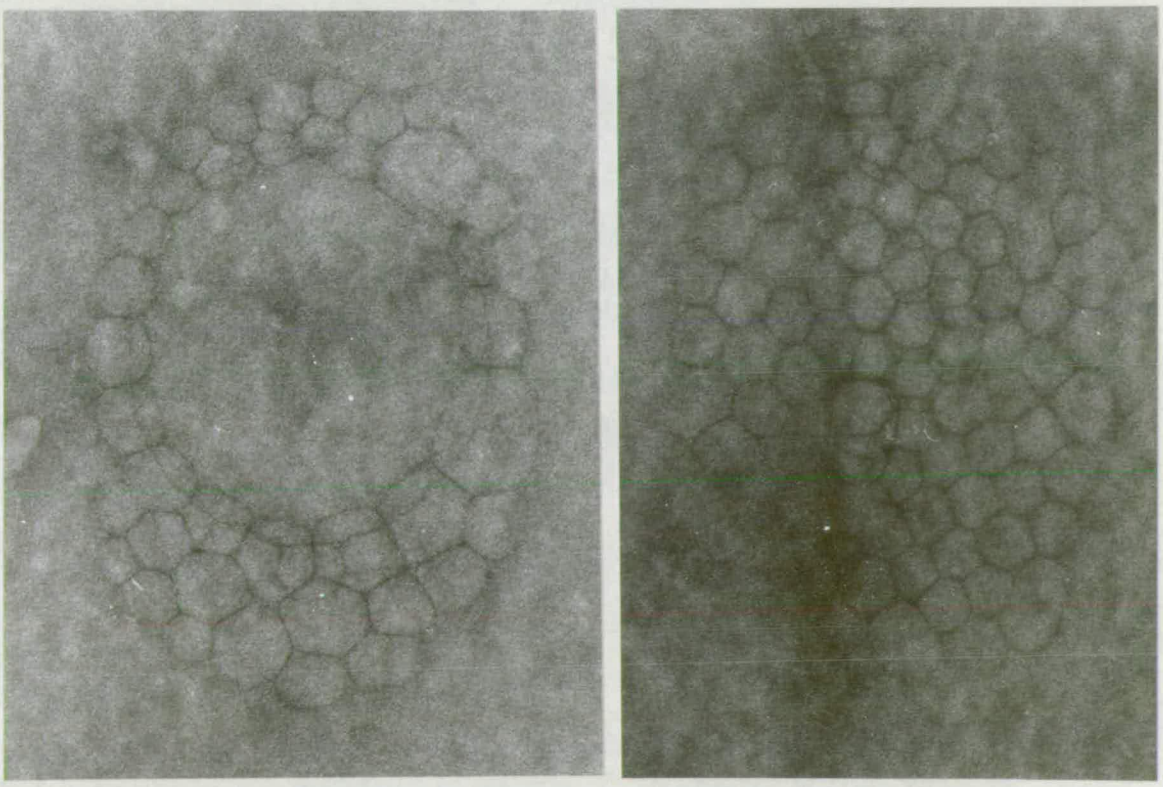


Plate 28 Negatively stained walls of B, subtilis showing a typical 'honeycomb' pattern. Magnification x 150,000.

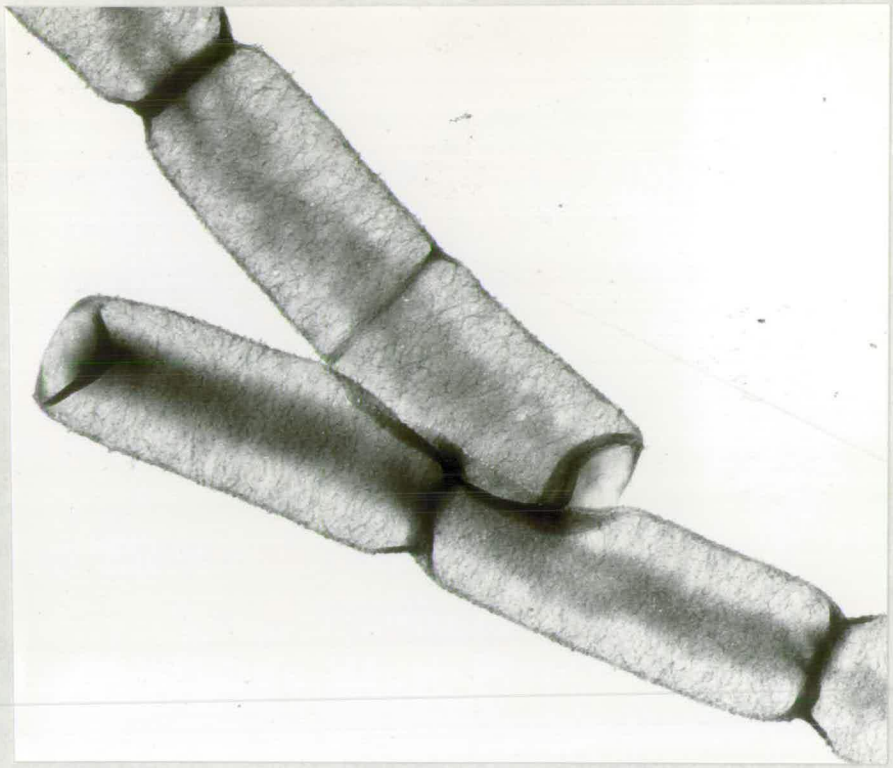


Plate 29 Negatively stained cells of *B. subtilis* after treatment with SDS and incubation at pH 8.6 for 15min at 45 C.
Magnification x 20,000.

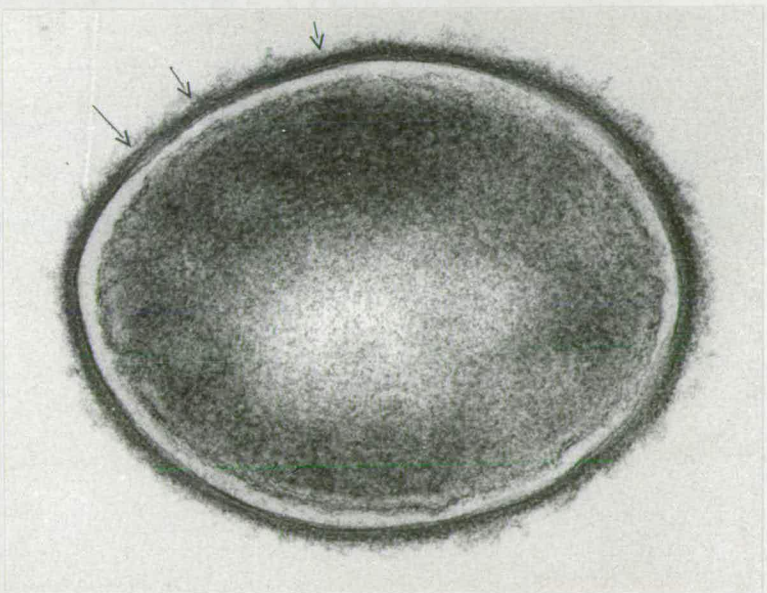


Plate 30 Transverse section of a *B. subtilis* cell from control 2 pH 8.6, showing a layered structure(arrows) in the wall.
Magnification x 120,000.

Subsequently, many other sections were examined, but none were found which demonstrated the effect so convincingly.

The above observations have been taken into account when considering possible models for structure and growth.

Peptidoglycan is probably a linear polymer (analogous to chitin, Carlström, 1957), with the repeating disaccharide-peptide units related simply by a translation along the chain, and if all the peptide subunits have the same shape, there is only one possible form of bond between them, cross-linking the glycan chains. Thus the only covalently bonded, extended structure would be a sheet with the glycan chains parallel to one another in the plane of the sheet, and the peptides all on the same side. Curvature in one direction would produce a covalently bonded, open-ended cylinder. But the thickness of a Gram-positive wall would require some 10 to 20 such cylinders. If the sheets were all in the same orientation, covalent bonding between them would only be possible if some of the peptide subunits were arranged in a different orientation from that required for intra-sheet bonding. This would be necessary even if some glycan chains were reversed to bring their peptides on to the other side. However, there is the further possibility of H-bonding between the sheets. The glycan chains have been reported to be only 5 to 21 units long with an average length of 10 (Hughes et al. 1968) and 55% to 65% of the possible cross-links are formed (Tipper, 1970).

If the degradation patterns observed are representative of what is occurring at the molecular level, then the results of amidase action can be explained by considering the glycan chains either parallel or perpendicular to the cylindrical axis. If the chains were perpendicular to the axis, then as the amidase split the bonds between the chains, the latter could move apart causing a split to be seen around the diameter of the cell. The same action on chains running parallel to the axis would

be expected to result in splitting of the wall parallel to the axis, which was never observed. However, if the amidase only broke the bonds at the ends of glycan chains which attached them to neighbouring chains, then it is possible that chains could have moved apart, giving the appearance of a split perpendicular to the axis. Higgins and Shockman (1971) have discussed observed degradation patterns in S. faecalis in terms of orientation of glycan chains.

One of the difficulties when considering glycan chains parallel to the cylindrical axis, is to visualise what happens during formation of a cross-wall. One possibility is that at the start of division, the glycan chains are laid down perpendicular to the surface of the cell and grow inwards towards the centre (as in Figure 3). When visualised in transverse section, the chains would then no longer be growing parallel to each other and would eventually reach a point where they could extend no further. Thus to prevent a hole remaining in the centre of the cross-wall, a less regular array would have to be postulated. Another problem with this model is direction of chain extension discussed by Higgins and Shockman (1971). Also it does not easily explain the observed pattern of lytic activity during cell separation. 'V-shaped' notches of material are often seen in the process of being removed in sections of completed cross-walls (Plate 21). This would suggest that glycosidase activity was required to break the glycan chains in the outer layers, since they are unlikely to be aligned suitably to produce this shape from amidase action alone. Although the model provides a definite site for separation, it does not require amidase if the peptide cross-bridges are within a glycan sheet and two sheets are only H-bonded together. However, if there were cross-linking between sheets, then amidase would be required.

A second possibility for the growth of the cross-wall is essentially localised thickening, with the chains laid down parallel to the wall,

as shown in Figure 18. This model is unattractive for a number of reasons. Firstly it provides no obvious site for cell separation, unless glycan chains are oriented as in b). a) requires predominantly glycosidase for separation, both a) and b) would probably require glycosidase to split the outer layers of the wall, and unless the glycan chains of the cross-wall are staggered to allow cross-linking, it is unlikely that amidase will feature prominently in the separation process. This model also gives rise to cell ends whose glycan chains are arranged perpendicular to the surface. Although this could explain some of the observed differences between ends and sides, it is not easy to visualise how the ends and sides would be continuous with each other.

Another possibility is that the formation of cross-walls is completely different from that of side walls, such that, as with the last alternative, structural weakness between end and side might be expected. Although indications of this were observed in on e experiment under conditions optimal for glycosidase activity (see Chapter III), there was no other evidence for it. For instance when whole cells were sonicated, cell poles were often isolated with a small section of cylindrical wall attached, and there was no evidence of discontinuity in sections viewed in the electron microscope.

If the glycan chains are visualised as being arranged perpendicular to the cylindrical axis, then a model can be proposed in which the formation of cross-walls is an extension of side wall formation. If the glycan chains are short, then many will be required to form the circumference of the cylinder, so that each one only requires to be bent very slightly. As already discussed, this arrangement explains the results of amidase activity as seen in electron micrographs. It also fits with the layered structure seen in Plate 30 (which of course

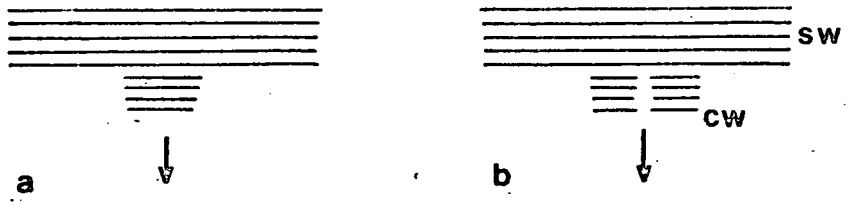


Fig. 18 One possible method of laying down glycan chains to form the cross-wall. When arranged as in b) they provide an obvious site for cell separation.

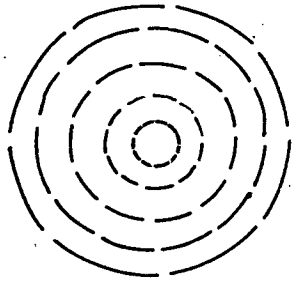


Figure 19 A transverse view of the cross-wall when glycan chains running perpendicular to the axis of the cell are laid down as annular rings one inside the other. It would probably be necessary for the glycan chains to become progressively shorter as they got nearer the middle in order to avoid excessive bending of the chains. If bonding allowed, it might be possible to reach a hole in the centre which was a square with sides the length of a disaccharide unit.

would also allow glycan chains to be parallel to the axis). In this model, cross-walls would be formed by localised wall thickening. At the appropriate time and place, presumably determined genetically, glycan chains would be laid down inside existing wall, and in transverse section would appear as shown in Figure 19. This method of laying down cross-walls is consistent with there being no detectable difference or discontinuity between cross-walls and cylindrical walls in sections, and with the appearance of negatively stained ends(Plates 25 and 26). The cross-wall could be made of several rows of glycan chains linked into sheets in each annular ring, and amidase would then be required for separation.

As the cells begin to separate, the annular rings could be gradually pushed out by internal pressure into the characteristic dome-shape of the pole (rather like a paper light shade which will fold into a flat circle and expand to a sphere). The glycan chains in the end would then be running parallel to the surface, but with the plane of the sugar rings parallel to the surface, instead of perpendicular as in the side walls, resulting in sheets of glycan perpendicular to the surface. Alternatively, the orientation of the glycan chains could be slightly altered during expansion, so that after appropriate cross-linking, sheets of glycan could be formed over the cell end continuous with the sides. Subsequent to their formation, new material could be laid down on the inside of ends, making them thicker.

This type of arrangement for cell poles is consistent with observed degradation patterns (Plates 26 and 27), and avoids the difficulty of direction of growth of the chains forming the cross-wall. It provides a role for both the lytic enzymes found at the pole, and amidase would be a suitable enzyme to produce 'V-shaped' notches. The greater resistance of cell poles to autolytic attack, and their smoother appearance,

could be due to extra cross-linking required to keep all the short glycan chains together. The fact that lysozyme can separate cells (Fan, 1970b) would have to be explained by breakage of glycan chains, such that the peptide cross-links were no longer sufficient to hold together neighbouring chains in a sheet. But cell separation using lysozyme would be expected to be a much more random process.

In negatively stained preparations of walls, structures were observed which appeared to be developing cross-walls with projections. These remain unexplained, but suggest that the cross-wall may not be built up evenly all round the cell, but may be fed out from specific points. (Plate 31).

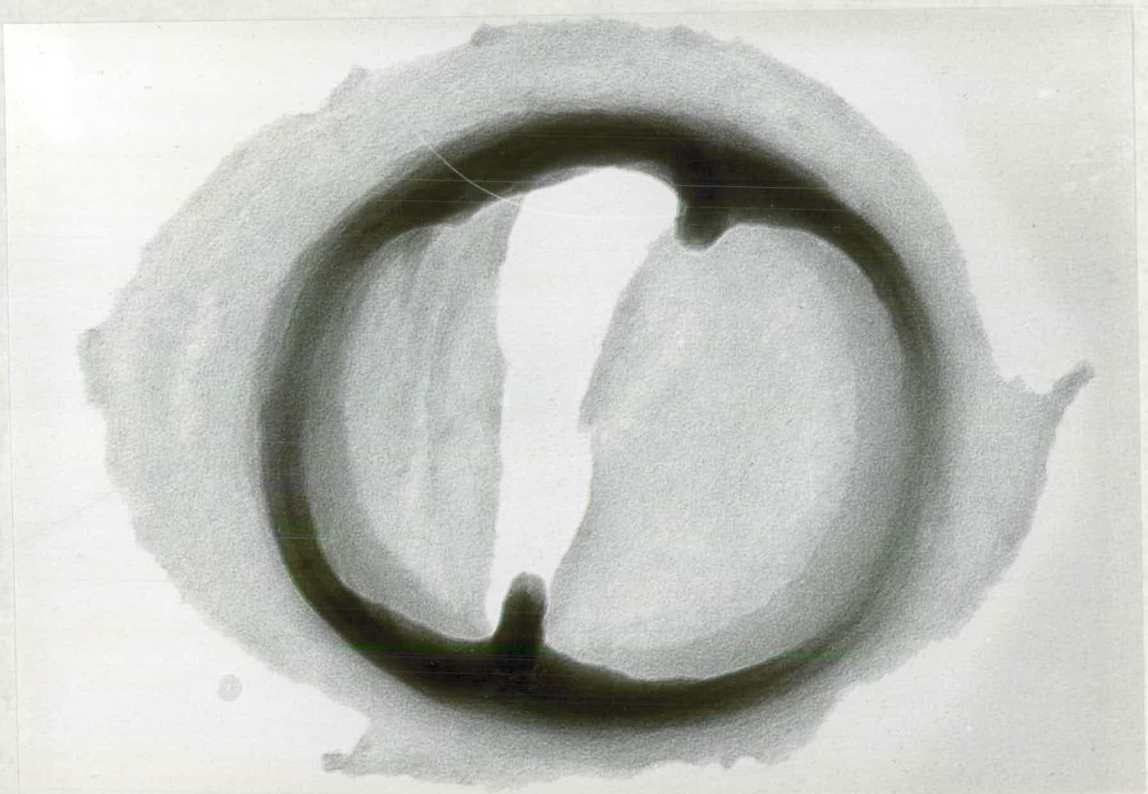


Plate 31 Negatively stained wall of B. subtilis which appears to show a developing cross-wall. Magnification x 140,000.

Growth

Very little is known about the control mechanisms of cell division, for instance, what determines when and where the cross-wall is formed, or what the diameter of the cell should be. But the most important part of the cell is the genetic material, and the essential process for survival is the replication of this material, so that information for cell division must arise during the replication process, in order that one complete genome is passed on to each daughter cell. Minicells are an example of what can happen, when there is a flaw in this system. However, even without understanding the control mechanism, it is possible to suggest a model of cell growth.

A model for *B. subtilis* has to be able to account for the following observations. The cell doubles in length, while the diameter remains constant, and a cross-wall is formed. Under normal conditions, cell separation does not begin until the cross-wall is completed. Wall material turns over at a rate of 50% per generation (Mauck & Glaser, 1970) but newly incorporated material does not become available for turnover for half a generation. However, wall turnover is not essential for growth, since some species show no signs of it, and strains of *B. subtilis* have been found with very low rates of turnover.[1]

Analysis of sections of penicillinase negative mutants of *B. licheniformis* and *B. cereus* (Highton & Hobbs, 1971 and 1972), after exposure to penicillin, and during recovery after addition of penicillinase, led to the conclusion that cylindrical walls grew by addition of material all over their surface. The same conclusions were reached from the appearance of *B. subtilis* and *B. megaterium* recovering from protein synthesis inhibition (Frehel *et al.*, 1971) and from turnover and incorporation of peptidoglycan in *B. subtilis* (Mauck & Glaser, 1972). Also it has been found that any lytic enzymes likely to be involved

[1] Fiedler & Glaser 1973

in growth are present all over the cell.

A model has therefore been proposed which allows for addition of new material along the whole length of the cell. Formation of disaccharide-peptide units takes place at the plasma membrane, and it is from there that new material is inserted into the existing wall. The model proposes that new material is added to the innermost layers of the wall. This avoids the conceptual problem of transporting subunits of wall and all the necessary enzymes through 10 or more layers of peptidoglycan to expand the whole thickness of the wall by insertion of new material. Instead, new material goes predominantly into the formation of new layers, which use as a template the completed layers outside them. If the innermost layers are made in a contracted form, then as new layers are formed inside them, they can be pushed outward and expanded slightly. Eventually they will reach the limit of their expansion, and with the help of amidase, could be randomly degraded and gradually lost. This would account for the untidy appearance of the cylindrical wall in many sections. To fit Mauck and Glaser's data, the time for a layer to move through the wall would have to be at least half a generation. The percentage of turnover would depend on the frequency of insertion of new layers, and on the wall thickness. Incorporation of material into existing layers would also influence the rate. This model would predict that walls which showed little or no turnover might be slightly thinner, since the outermost layer would not have to stretch so far that it would be degraded. In this case for the cell to double in length, new material may have have to be inserted into more layers than where turnover occurs. A feature of this model is that it does not require lytic enzyme to provide sites for insertion of new material, but rather that lytic enzymes accomodate the new material more indirectly, by getting rid of old material.

The reason for the differences observed at cell poles may be that they do not turn over, and new material is only added for thickening. The model is applicable irrespective of the orientation of glycan chains.

In summary, the essential features of the model are;

1. Growth occurs all over the cylindrical surface and new material is added to the inside of the wall.
2. The innermost layers of the wall are shape determining and will maintain the rigidity of the cell.
3. Turnover is explained, and the percentage turnover per generation depends on the thickness of the wall, and on the frequency of insertion of new layers.
4. Lytic enzymes, probably amidase, are involved in degradation of the outer layers where turnover occurs, or where new material is inserted into existing layers.
5. The length of the cell increases between insertion of each new layer.

(Part of this work is being published in abstract form for the Society of General Microbiology Symposium, 1975.)

CHAPTER V

ANALYSIS OF GROWTH AND DIVISION IN A MINICELL PRODUCING MUTANT OF *BACILLUS SUBTILIS*

INTRODUCTION

During the normal process of division in B. subtilis , the cell doubles in length and divides in the centre. Several lines of evidence suggest that the divisions in mutant strains, giving rise to minicells, are normal in all respects, except for their location along the axis of the cell (Reeve & Mendelson, 1973; Khachatourians, Clark, Adler & Hardigree, 1973). However, because minicells are unable to grow and divide (Reeve, Mendelson, Coyne, Hallock & Cole, 1973), division resulting in a minicell does not increase the number of viable cells in a population.

Teather, Collins and Donachie (1974) analysed E. coli K12 P678 and its minicell producing derivative P678-54, and from their results proposed a model to account for the difference in cell size distribution which they found between the normally dividing strain and the mutant. The model, which applies to an exponential population, proposes that i) potential division sites (PDS) arose during cell growth in the same way in both wild type and mutant strains, but that such sites were active for only one division in the wild type, while they remained active indefinitely in the minicell producing strain, ii) the probability of division occurring at any one PDS was equal, and iii) enough 'division factor' was produced at each unit cell doubling for one division, and this 'division factor' was utilised entirely in the formation of a single cross-wall. The site where 'division factor' was expressed was chosen at random out of all the available PDS. A polar division giving rise to a minicell, therefore prevented a normal central division, causing an increase in the average cell length in the minicell producing strain. Thus the length of a new-born cell in the mutant could be a multiple of the new-born cell size (c) of the wild type strain. If the length of a particular new-born cell were nc (where n is an integer),

then after one generation, the cell would have grown to 2nc and would have produced sufficient'division factor' to undergo n divisions. The number of division sites available would have been the normal number of division sites (2n-1), plus the two polar sites, giving 2n+1 PDS. From the model they predicted the length distribution of an exponentially growing population. The distribution of lengths depended on a) the distribution of lengths at birth, and b) the pattern of length increase during the cell cycle (Collins & Richmond, 1962). The parental strain used by Teather et. al. grew with a mean cell size of 1.46c, very close to the theoretical value of 1.44c (Powell, 1956) for exponential length increase. The theoretical distribution of lengths for the minicell producing population, was very close to that measured experimentally, with a predicted average cell length of 2.52c and an experimental result of 2.46c. Calculations based on the model also predicted that minicells would be produced at the rate of 0,75 per nucleated cell per generation. From the predicted pattern of daughter cell lengths, it became apparent that there was a very high probability that a large cell would give rise to smaller progeny, e.g. the probability of a new-born cell of length 8c giving rise to a minicell was 0.94. Minicells were observed to be produced with an approximately equal frequency at both old and new poles. Electron microscopic analysis gave a minicell production of 0.67 ± 0.08 per cell per generation, while observations on nutrient agar brought the value closer to the theoretical one, giving 0.72 ± 0.11.

An alternative to the first proposal of this model was put forward by Adler and Hardigree (1972), who suggested that the polar division sites in the minicell strain were 'immature' sites which, in the normal course of events, would be moved to the centre of the cell by wall growth before they became available for septum formation. Thus minicell formation would result from the premature inactivation of these sites. Teather (1974) however, pointed out that there would already be

a mature PDS or at least part of one present at the pole from the division which gave rise to the pole.

Coyne and Mendelson,(1974) in a study of a minicell producing mutant of B. subtilis, maintained that, although the location of the first abnormal division site giving rise to a minicell appeared to be random, thereafter, location became non-random, with new minicells being formed preferentially adjacent to those already existing. This clustering of abnormal events suggested to them that division site location might be related to pole age. They also calculated that the probability of production of minicells was 0.31 per nucleated cell. However, their studies were carried out on cells newly germinated from spores and followed through only a few generations. Thus it is unlikely that the clones were in a balanced state of growth, since just after germination, shorter cells would predominate, and the number of minicells per nucleated cell would be lower than in a steady state of growth. Two other difficulties arise with B. subtilis which are not present with E. coli, both of which make cell measurements very difficult. One is the long period of time required to complete division and separation, even when the cells are in balanced growth, and the other is the difficulty of identifying nascent cross-walls. Mendelson and Coyne (1975) also reported that certain minicell strains of B. subtilis were suppressed in division ability, From their observations, they distinguished three phenotypic aspects of minicell producing strains, i) division site location, ii) frequency of minicell producing divisions and iii) division ability, presumably all resulting from a single mutation. On the other hand, Teather et.al. (1974) attribute i) to the mutation and consider ii) and iii) to result from the mutation.

Coyne and Mendelson (1974) noted that any model attempting to define the mechanism for selection of division site location in

B. subtilis minicell producing mutants should not overlook the fact that this species, unlike E. coli, Salmonella and Haemophilus, which also produce minicells, contains genetic information regulating events similar to the initial stages of division in close proximity to a pole, namely during sporulation.(Balassa, 1971) Although normally turned off during vegetative growth, the genetic potential for partitioning the cell in the polar region always exists in B. subtilis cells. However, unlike minicell formation, the partitioning in spore formation consists initially of laying down only a membrane, and enclosure of the nuclear material. But it is still possible that minicell formation results from the expression of some of the spore information during vegetative growth.

It was decided to study the growth of the minicell producing mutant B. subtilis Cu 403 div IV BI, to see if it was similar to E. coli, and would fit the model proposed by Teather et. al. (1974).

MATERIALS AND METHODS

1. Organisms. *B. subtilis* 168 and a minicell producing mutant derived from it, Cu 403 div IV B1 thy^-, met^- were grown to exponential phase in L broth at 30 C (see previously).

2. Wall Stains. Crystal violet. A thin film of culture was made on a grease-free slide, and was fixed with 1% formalin for 1min. The slide was then flooded with 10% tannic acid for 20min, washed carefully with H_2O, and stained with 0.2% crystal violet for 2 to 3min. The slide was washed and dried, and immediately before viewing, a drop of H_2O was placed under the coverslip.

Methylene blue. A film was made as above, and fixed in a flame. The slide was then flooded with 0.03% methylene blue for 30 to 45s. washed and dried. Immediately before viewing, 1 drop of 67% alcohol was placed under the coverslip. This had the effect of decolourising the cell ends and septal regions.

3. Preparation of Unstained Cells for Microscopy. Cells were spread on thin layers of 1.2% agar, containing 0.05% sodium azide, on a microscope slide, and the cover slip was pressed down firmly.

4. Microscopy and Photography. Cells were photographed with a Zeiss Ultraphot microscope using phase contrast optics. Measurements were made on enlarged projections of the negatives. For electron microscopy, the cells were spun at 10,000g for 5min, washed with H_2O and resuspended in H_2O, all at 4 C. Negative staining (using 1% uranyl acetate) and sectioning were carried out as already described.

RESULTS AND DISCUSSION

Visualisation of Cross-walls

As mentioned in the introduction, the major problem in analysing cultures of Bacilli, was visualising nascent cross-walls. Under the growth conditions used, the cells grew in long filaments, and using phase contrast microscopy, only completed and fairly well advanced cross-walls could be detected with certainty (Plate 32). Thus various wall stains were used in an attempt to clarify positions of cross-walls, especially the most recently initiated. The results of these are shown in Plates33, 34 and 35. From negatives such as these, it was much easier to measure cell lengths and to locate minicells within chains.

Distribution of Cell Length

A total of 573 cells of exponential phase strain 168, and 544 cells (excluding minis) of strain Cu 403 were measured, and grouped in size classes of multiples (n) of the new-born cell length (c) of strain 168, The distributions are shown in Figure 20 a and b.

For strain 168, 76% of the population fell between c and 2c, and 95% between c and 3c. This was not so close to the ideal culture as *E. coli* P678 used by Teather *et.al.*, suggesting a more variable new-born cell size or cycle length for *B. subtilis*. The length distribution for strain Cu 403 ranged from c to 6c for 95% of the cells, with some cells measuring up to 9c. This was similar to *E. coli* K12 P678-54. These results gave an average cell length of 1.61c for strain 168 and 2.39c for strain Cu 403, so that the average cell length for the minicell strain was 1.8 times that of the wild type. The minimum cell length for both strains was about the same.

In *E. coli* the average length of strain P678-54 was 1.68 times that of the parent strain, compared with a theoretical value of 1.76 predicted from the model (Teather *et. al.*, 1974). It ought to be possible to

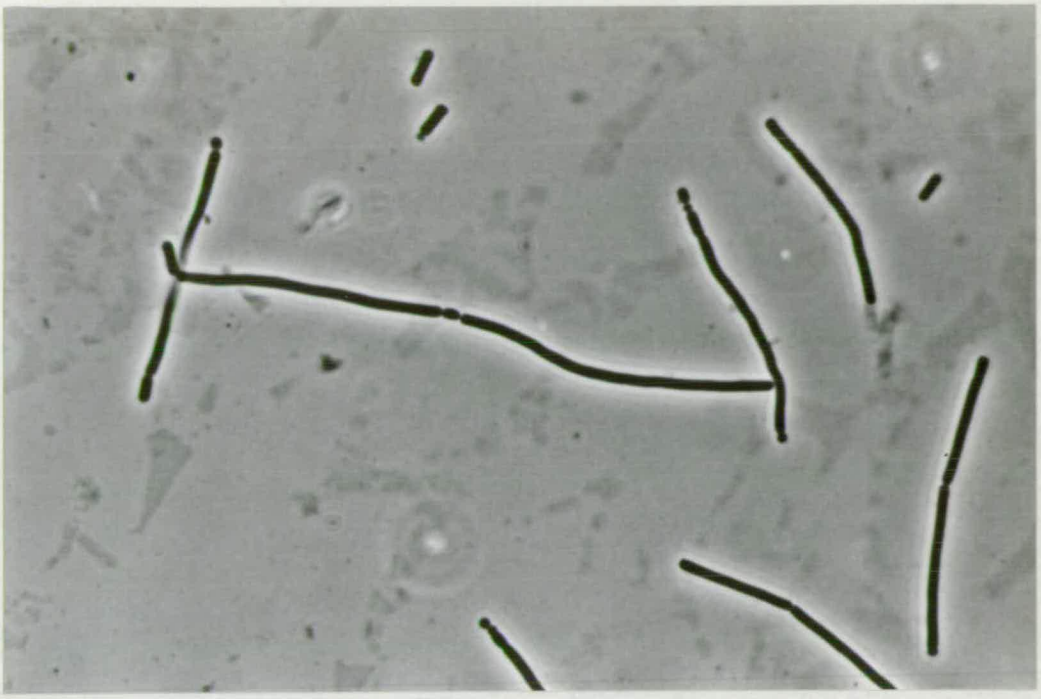


Plate 32 Unstained cells of strain Cu 403 on an agar film.
Magnification x 1,500.

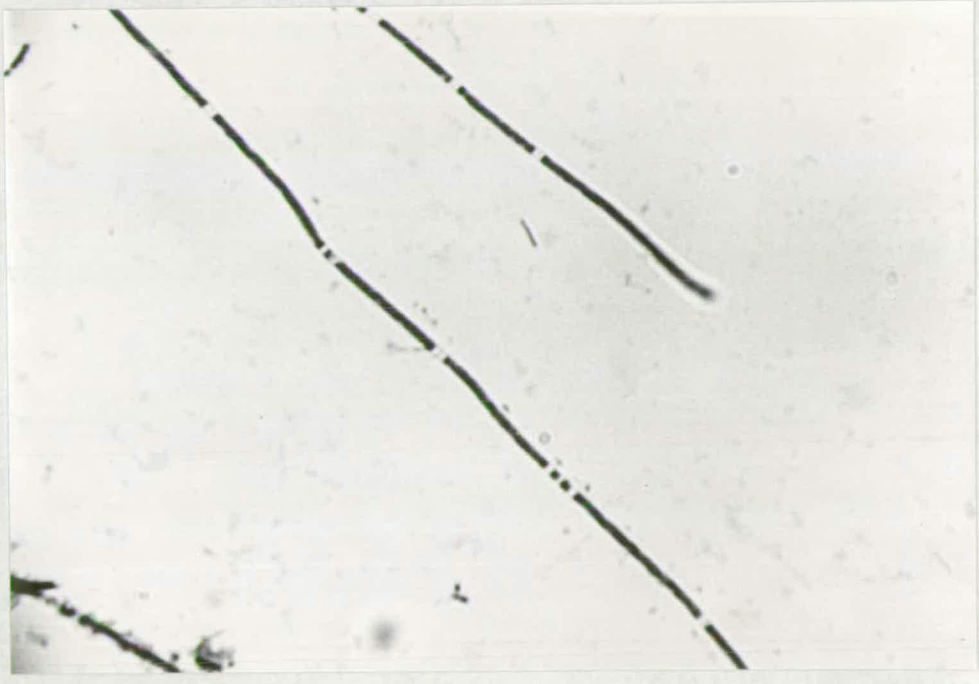


Plate 33 Cells of strain Cu 403 stained with methylene blue.
Magnification x 1,500.

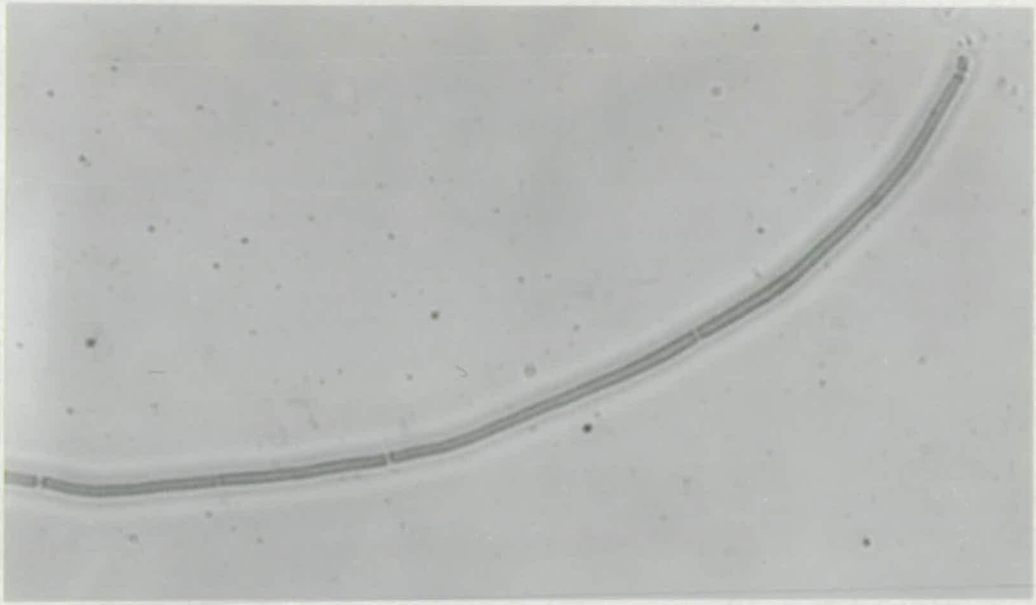


Plate 34 Cells of strain Cu 403 stained with crystal violet.
Magnification x 1,500.

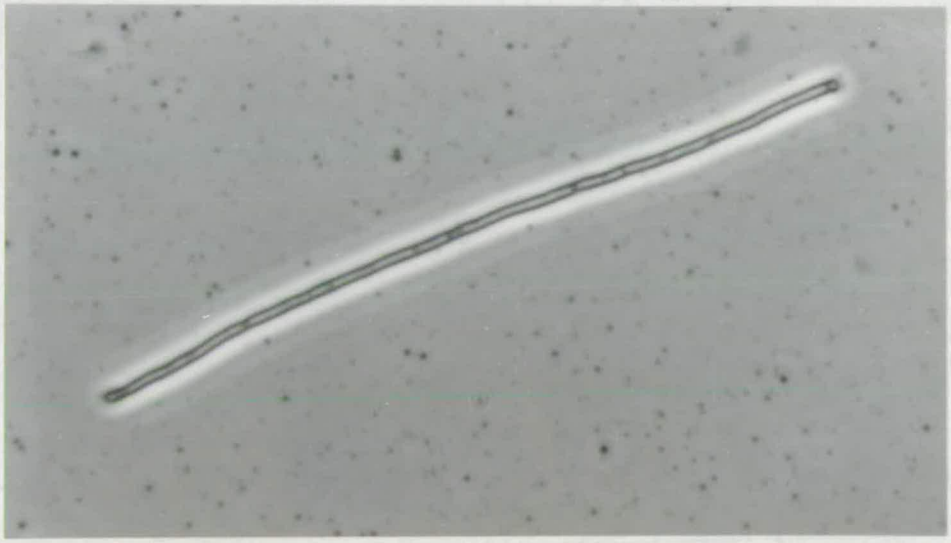


Plate 35 as above. The different effects were obtained by chance, depending on where the cells were lying on the slide.
Magnification x 1,500.

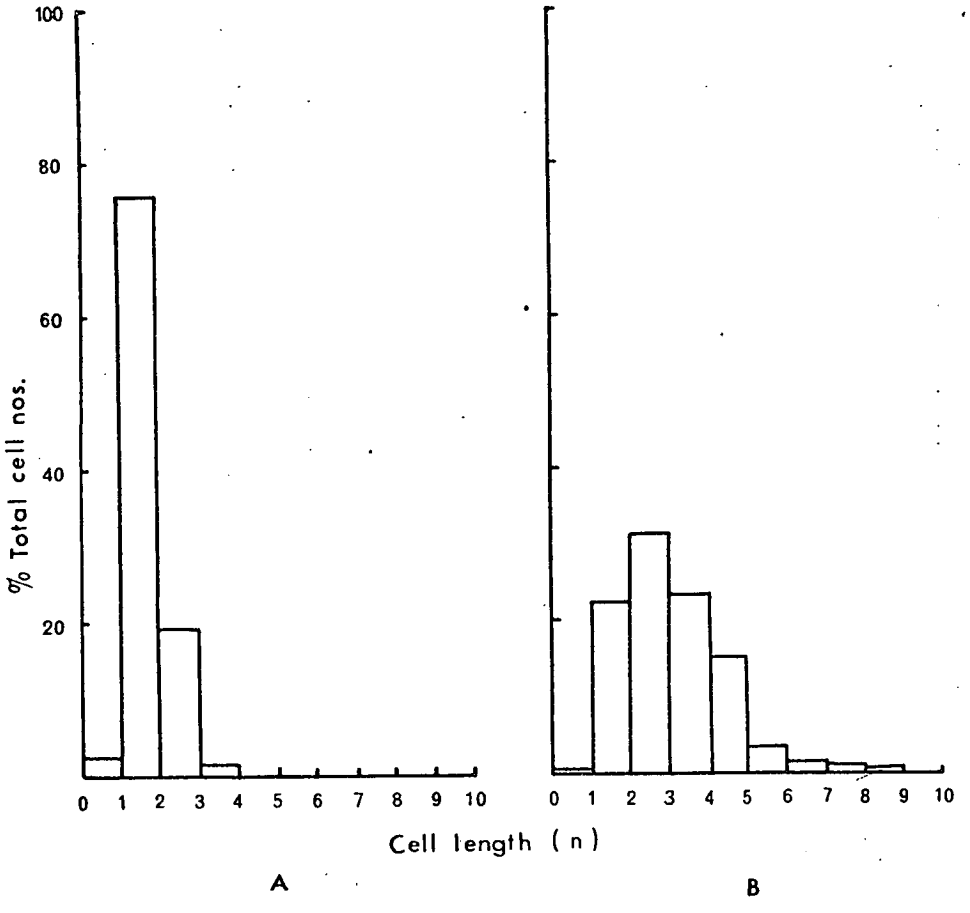


Fig. 20 Cell length distributions for strain 168 (A) and strain Cu 403 (B). Cells were grouped in size classes of multiples of the minimum cell length which was the same for both strains.

compute a theoretical distribution for strain Cu 403 analogous to that produced for E. coli (Teather et al. 1974), but unfortunately this was not available for inclusion. However, the results for strains Cu 403 and K12 P678-54 were in close agreement, indicating that a similar pattern of events may be occurring in both, although the B. subtilis populations were less ideal than those of E. coli, that is there were fewer cells between c and 2c.

Frequency of Minicell Production

The model for E. coli also predicted 0.75 minicells per nucleated cell. Given that the average cell length of the minicell strain was 1.8 times that of the parent strain, the number of minicells per nucleated cell was calculated for strain Cu 403. Consider a new-born cell of length c doubling to length 2c. This cell is then capable of undergoing one division. Using the same reasoning, a cell of length 1.8c doubling to length 3.6c would then be capable of undergoing 1.8 divisions. For every division giving rise to a daughter nucleated cell, 0.8 divisions are available to give rise to minicells. Therefore there should be 0.8 minicells per nucleated cell per generation. Counts of minicell ratios were carried out by several different techniques. Counting populations on agar using phase microscopy, gave a value of 0.33 ± 0.08, very close to the value obtained by Coyne and Mendelson (1974). However, when the cells were stained before counting, the number of minis per nucleated cell increased to 0.47 ± 0.06. Finally the analysis was carried out using negatively stained preparations and electron microscopy. This gave the greatest number of minicells per nucleated cell, namely 0.62 ± 0.09. Plate 36 illustrates nascent cross-walls which may not have been visible under a light microscope, even after staining.

There are several possible reasons why the experimental value was somewhat lower than the calculated one. Firstly, the latter assumed

Plate 36 Negatively stained cells of strain Cu 403 showing nascent cross-walls in minicell formation. Magnification x 50,000.

that all minicells survived for counting, and secondly, that all possible divisions occurred. In view of the report by Mendelson and Coyne (1975) that minicell producing strains of B. subtilis, including Cu 403 div IV B1, were suppressed in division ability, it is possible that a few of the divisions , which could result in minicells, are absent in this strain (i.e. in preference to those giving rise to daughter nucleated cells), although it is unlikely that the number of such non-occurring divisions was nearly as high as these workers reported. Thirdly, all minicells may not have been counted, even in the electron microscope. However, the good agreement obtained between the experimental value and the theoretical value for E. coli, and the calculated value for strain Cu 403 suggested that the number of missing minicells , for whatever reason, was very low.

All measurements of cell lengths of Cu 403 were done from negatives of stained cells taken with a light microscope, so that all nascent cross-walls would not have been detected. It is likely that on average, more may have been missed in the minicell strain than in the parent strain, since in the latter it was much easier to decide about a doubtful cross-wall, because they were occurring at approximately regular intervals. This would then result in the value for the average length of the minicell strain being slightly too high, which in turn means that the ratio of minis to nucleated cells is too high. However, again the close agreement suggests that this source of error may not have been significant.

Randomness of Minicell Production

In view of the apparent disparity between the results of Teather et. al. and of Mendelson and Coyne about the randomness of minicell production, this phenomenon was also studied. Because, under the growth conditions used, strain Cu 403 grew as long filaments with the

minicells remaining attached, at least during most of the exponential phase, this offered an excellent system for studying minicell production. Analysis was done with the electron microscope to ensure maximum visualisation of nascent cross-walls. Using the predicted number (0.8) of minicells per nucleated cell, the probability of nearest neighbours was calculated. This study assumed that all cells were end to end in one long chain, and in a cluster of minis it did not matter from which of two neighbouring parental cells they had arisen.

Table 8

Boundary	Probability of occurring
cell-cell	30.9%
cell-mini	24.68%
mini-cell	24.68%
mini-mini	19.75%

From these values, the probability of increasing numbers of minis in clusters was as follows;

Table 9

No of minis	Probability
2	0.1975 or $19/10^2$
3	0.0392 or $3/10^2$
4	0.0077 or $7.7/10^3$
5	0.0015 or $1.5/10^3$
6	0.0003 or $3/10^4$

An experimental count of minis compared with the theoretical values based on the probabilities above is shown in Table 10;

Table 10

No of minis	Experimental count	Theoretical count
1	220	220
2	68	44
3	8	8.8
4	7	1.76
5	2	0.35
6	0	0.07

If the suggestion of Coyne and Mendelson (1974) that minicells were formed preferentially adjacent to those already existing were true, then

it would be expected that, compared to values predicted for random distribution, the numbers for 1, 2 and 3 minis would be lower than expected, while for 4 or more minis in a cluster, they would be greater. This was not true for the results shown above. In fact, the number of pairs of minis was higher than expected, and the number of 3 minis was the same as predicted. Although the numbers for 4 and 5 minis were slightly higher, they were not high enough to suggest any form of non-random distribution. The results were not analysed in terms of the age of poles where clusters were occurring, except to say that clusters of 3, 4 and 5 minis were found within long filaments as well as attached to the end cell of a filament.

The results discussed in this chapter indicate that there is a great deal of similarity between the control of division in E. coli K12 P678-54 and B. subtilis Cu 403, suggesting that the latter may fit a similar, if not the same, model as proposed for E. coli (Teather et. al.,1974) The results are not in agreement with those of Mendelson and Coyne, who were studying the same strain of B. subtilis. However, two differences in experimental procedure of these workers could well account for the disagreement. Firstly they studied clones immediately after germination from spores, instead of a well established exponential culture, and secondly, their measurements were done on unstained cells, which may imply that a large number of nascent cross-walls were undetected.

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ABSTRACT OF THESIS

Name of Candidate Margaret Mackay Manson

Address 58, Arden Street, Edinburgh, EH9 1BN.

Degree PhD *Date* September, 1976

Title of Thesis Growth and Division of Bacillus subtilis: Biochemical and Electron Microscopic Analysis of Autolysins and Minicells.

In an attempt to clarify some of the conflicting data on growth and division in rod-shaped bacteria, two aspects of the growth of a Gram-positive rod (B. subtilis) were chosen for study: autolysins and minicells.

Several properties of the two autolytic enzymes, an amidase and a glycosidase, were examined. Neither enzyme could be assayed using a synthetic substrate, so that the only specific routine assay which was found was for glycosidase, using M. lysodeikticus walls at pH 6.0. Total lytic activity at various stages of growth was measured, and it was concluded that active enzyme was always present, but with increasing age, cells became more resistant to lysis. Stationary phase cultures excreted enzyme into the medium. Walls which were free of teichoic acid were more resistant to degradation by lytic enzymes than walls containing teichoic acid, which probably resulted from the fact that rebinding of autolytic enzymes was much weaker when walls did not contain teichoic acid. Some structural alteration of the wall may also have been involved. A purification procedure for the amidase was developed, using Sephadex chromatography and gel electrophoresis. Crude enzyme extracted from cell walls by 3M LiCl, and also purified lytic enzyme were used to raise antibodies for ferritin labelling experiments to localise amidase on the cell wall by electron microscopy. Preliminary labelling experiments with ferritin conjugated antibody to crude enzyme and the lytic fraction indicated labelling all over the wall.

Crude enzyme extracted from minicells (which represent cell poles) was compared to that from normal cells, and found to be very similar, containing both enzyme activities. Thus it was concluded that both lytic enzymes were present at the cell poles.

Sections of cells autolysing under conditions optimal for each enzyme (pH 6.0 and pH 8.6), showed very similar degradation patterns, although the optimum for glycosidase activity (pH 6.0) gave a much slower effect. Either degradation by glycosidase activity was similar to that by amidase activity, or amidase was the major activity at both pH's. A cycle of lytic activity appeared to operate at cross-walls. Under normal conditions no degradation occurred until the cross-wall was complete, then 'V-shaped' notches were removed from the outside wall, and the cells separated from the outside towards the centre. Once formed, the ends

Use other side if necessary.