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Biogenesis of the Wall in Bacterial Morphogenesis

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I. Introduction

For many years the insoluble material remaining after disruption of bacteria was discarded as "debris" by biochemists interested in preparing soluble enzymes from microbes. Material deposited during high- but not low-speed centrifugation, likewise unidentified, was used as the source of

particulate enzymes. New attitudes, towards the "debris" dawned about 20–30 years ago after Dawson (1949) and Cooper *et al.* (1949) had examined it under the electron microscope, and Salton (1953) had analysed it chemically. Likewise the origin and significance of the "particles" became clearer after Weibull's (1953, 1956) study of protoplasts and of the ghosts that could be obtained from them. Despite the sophistication of the subject that has since grown up around studies of bacterial cell envelopes, which comprise cell walls, the "debris" and cytoplasmic and other membranes—the "particles", large areas of ignorance still exist. The further the studies have progressed, the more intricate and numerous have become the structures and biosynthetic process necessary to make them. It has also become apparent that, far from being inert "hulls" or "cases" to the cells, they are dynamic, plastic and growing organs which control major cell functions ranging from cell growth and division through the export and uptake of molecules to the antigenic and adhesive properties of bacteria.

Numbers of reviews have been written describing the structure and biosynthesis of wall components and polymers or summarizing knowledge of bacterial membranes; some of these are quite recent (see Rogers *et al.*, 1978). There would therefore be little point in writing another one. Instead, the present article will be aimed at trying to understand more about the role of the envelope in growth, division and morphogenesis of cells. Growth of bacterial cells necessarily involves increase in the area and volume of wall material. It might be thought legitimate, therefore, to include a review of our knowledge of the growth of cells during the cell cycle and its relation to other major cell events such as DNA replication. Fortunately, these aspects have already been dealt with in a review by my colleague Sargent (1979); for information about these aspects of the problem, I shall lean heavily on his review. Greater attention will be given to ways in which cell division and the shape of the bacterial cell can be disturbed. This knowledge will be related to physiological and molecular knowledge about the envelope, but much will necessarily have to remain speculative.

II. Shape Maintenance

A. THE SHAPE OF BACTERIA

We are familiar enough with the appearance of bacteria at a magnification of about 1000-fold, and we think in terms of cocci, bacilli,

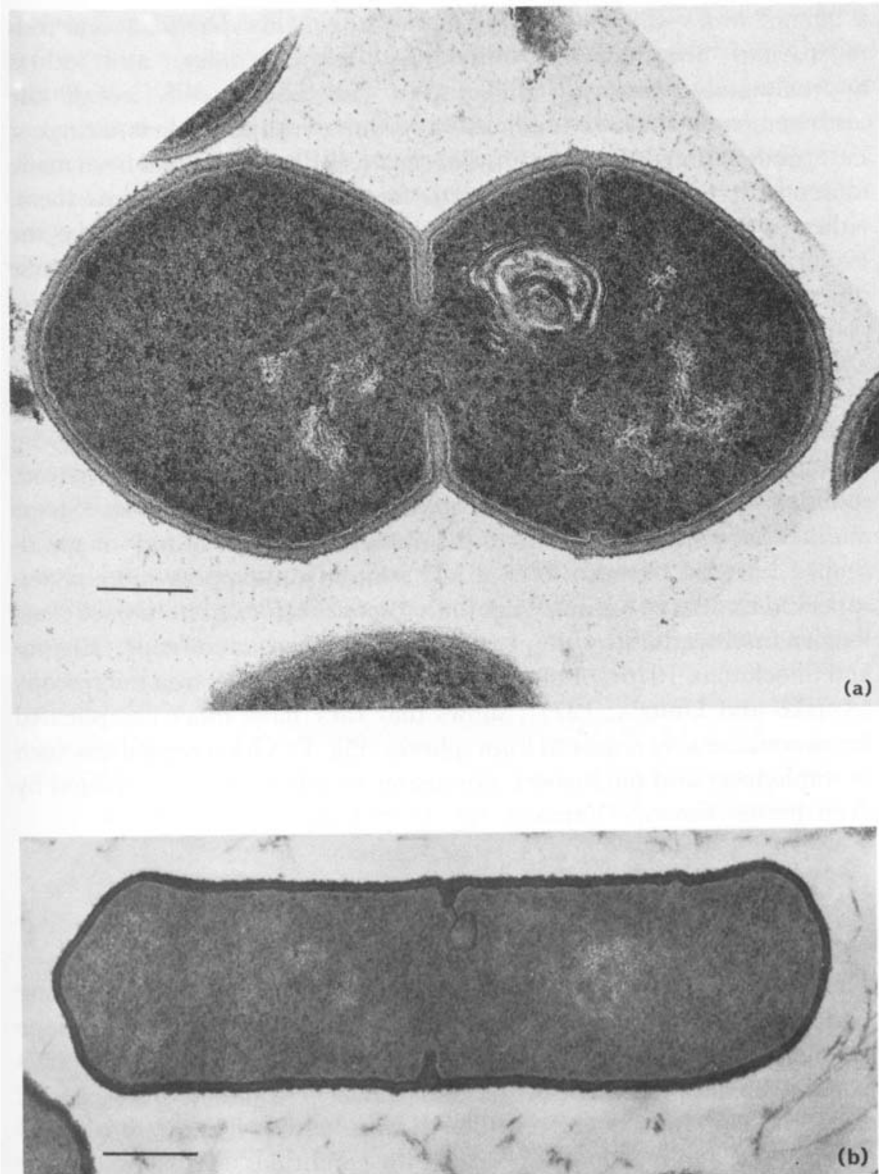

FIG. 1. (a) Section of *Streptococcus faecalis* obtained from a mid-exponential phase culture. The micrograph was kindly provided by Dr M. L. Higgins, Microbiology Department, Temple University, Philadelphia, U.S.A. (b) Section of *Bacillus subtilis* showing asymmetric modelling of cell poles. From Rogers *et al.* (1978). In both micrographs, the bar is equivalent to 0.25 μm.

salmonellae or sprilla knowing that some are roughly spherical, some rods of various lengths with differently shaped poles, and others approximately spiral in shape. We can also readily recall the arrangement of the individual cells as groups, regular packets, strings or independent units. However, until recently, little attempt had been made to designate precise cell-shapes or the forces required to maintain them, either as individuals or in specific arrangements. Still less have the evolutionary significance and the possible biological advantages of these different morphological forms yet been considered. The sphere is, of course, the form of minimum free energy and without constraints, is that adopted by fluids, or fluids contained within plastic envelopes such as membranes. One obvious function of coats of cells external to the cytoplasmic membrane is, therefore, to provide the constraints giving bacteria their characteristic shapes. When the walls are removed, spherical protoplasts or sphaeroplasts are usually formed. Shape maintenance applies not only to the obvious examples of rods or spiral-shaped bacteria but also to cocci. The latter may appear more or less spherical under the ordinary light microscope, but examination of exact longitudinal sections by the transmission electron microscope (Higgins and Shockman, 1976), or of whole cells by scanning electron microscopy (Amako and Umeda, 1977), shows that they have more complicated forms considerably removed from spheres (Fig. 1). Other organisms, such as staphylocci and micrococci, are nearer to spheres but are divided by deep division furrows (Yamada *et al.*, 1975; Koyama *et al.*, 1977.

B. THE ROLE OF THE WALL

The effects of removing or damaging the wall are in themselves strong evidence for functions as supporting structures and in shape maintenance, but a few experiments have been done in which protoplasts from bacilli adopted shapes other than spheres (Abram, 1965; Rogers *et al.*, 1967; van Iterson and Op den Kamp, 1969). These are unusual occurrences, and may happen because the conditions used have stiffened the membrane or even the cytoplasm itself. Protoplasts must be suspended in isomolar solutions to avoid lysis, and so the forces needed to maintain a rod shape, for example, are greatly decreased compared with those needed when the organisms are growing in nutrient media. The possible role of changes in lipid composition found when rod-shaped

protoplasts have been obtained from *Bacillus megaterium* and *B. subtilis*, grown or treated at low pH values, have not be examined further (Op den Kamp *et al.*, 1965, 1967, 1969). Despite these observations, acceptance or assumption of the shape-maintaining function of bacterial walls has been fairly general. The wall components supplying even the supportive strength, particularly in Gram-negative bacteria, have however been the subject of some discussion.

C. PEPTIDOGLYCANS

Isolated wall preparations from Gram-positive bacteria contain 40–90% of peptidoglycan, the remainder being composed of a variety of phosphate-containing teichoic acids, teichuronic acids and other polysaccharides. Preparations from Gram-negative organisms, on the other hand, may have as little as 1% of the polymer, the remainder mostly consisting of proteins and phospholipids. The fine structure of the peptidoglycans from a wide range of bacterial species is by now rather well understood (Schleifer and Kandler, 1972). They all consist of long glycan strands made from residues of *N*-acylglucosamine and *N*-

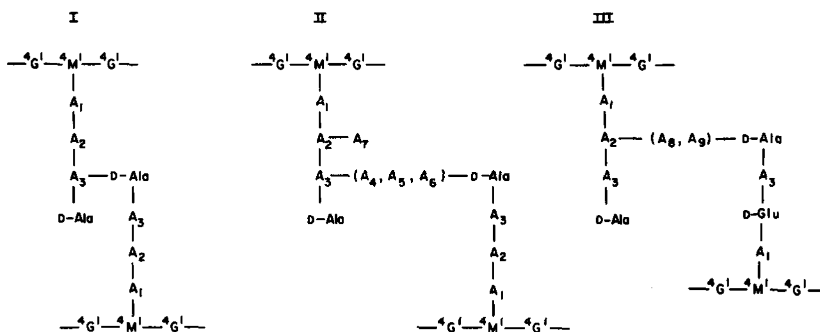

FIG. 2. General structures of peptidoglycans, G indicates *N*-acylglucosamine; M, *M*-acylmuramic acid. In most peptodoglycans, the acyl group is acetyl. A_1—A_3 are amino acids alternating in stereospecificity other than when glycine is involved. The amino acids A_4—A_6 form the so-called bridge between peptides directly attached to the glycan strands. A number of species, including all known Gram-negative organisms and bacilli, have type I peptidoglycan with A_1 indicating L-alanine; A_2, D-isoglutamine; and A_3, 2, 6 mesodiaminopimelic acid. Many cocci have a type II peptidoglycan where A_1 indicates L-alanine; A_2, D-isoglutamine; and A_3, L-lysine. The bridge amino acids (A_4, A_5, A_6) may be all glycyl residues, glycyl and seryl or glycyl and threonyl. In *Micrococcus luteus* there are five instead of three bridge amino acids which repeat the main chain (i.e. -L-ala-D-glu-L-lys-D-Ala-). Fragments have been obtained from this peptidoglycan with up to four repeats of this peptide.

acylmuramic acid linked together by 1—4β bonds. The amino sugars are usually *N*-acetylated although, in some species, other acyl groups are involved. These polysaccharide chains are then joined together by short peptides each containing a very limited number of amino-acid residues with either the L- or D-configuration (Fig. 2). It is generally thought that these polymers form giant, cross-linked, mesh-like structures covering the whole surface of the cell. In Gram-negative organisms, the thin sacculus of peptidoglycan would have only 1–3 such meshwork layers (Braun *et al.*, 1973) whereas a Gram-positive organism, like *B. subtilis*, would have 30–40 (Kelemen and Rogers, 1971). Molecules of the other polymers, such as teichoic acids, are attached to the 6-hydroxl groups of muramyl residues in glycan strands of the peptidoglycan by single terminal bonds. Linker groups interposed between teichoic acid and peptidoglycan are now fully characterized in some species, and may be very common. The distribution of these substituents on glycan chains is as yet unclear. Likewise, the distribution of polymers through the thickness of the wall is uncertain (see Rogers *et al.*, 1978).

In most instances, enzymes that hydrolyse specific bonds in the peptidoglycans lead to lysis or to formation of protplasts or sphaeroplasts under suitable conditions. However certain organisms, such as pseudomonads, or even *Escherichia coli*, can be lysed by chelating agents, such as EDTA, which would not be expected to affect the peptidoglycan. The remaining "ghosts" are rod-shaped and not spherical. This work has been briefly discussed before (Rogers, 1974), and the suggestion made that damaged membranes may allow small activities of autolytic enzymes sufficient access to the thin layer of peptidoglycan present in Gram-negative bacteria for a hole or holes to be punched. This would either allow the contents of the cell to leak out or to a shattering of the "rigid" layer of peptidoglycan. The observations would not seem to provide a strong argument against a dominant role for peptidoglycan in providing strength. Nevertheless, that a variety of hydrolases specific for bonds in peptidoglycan can lead to loss of shape of non-spherical bacteria is not in itself sufficient evidence that peptidoglycan is solely responsible for shape maintenance. It is quite possible to argue that the characteristic shapes of bacterial cells can only be maintained by a contribution from all of the polymers in the walls, rather than by any one of them. The nature of the molecules attached to the peptidoglycan in the walls of Gram-positive species can be changed without apparently altering the function of the wall. For example, wall

teichoic acids can be almost wholly exchanged for teichuronic acids in many organisms by growth under phosphate limitation (Ellwood and Tempest, 1969, 1972; Forsberg *et al.*, 1973). The organisms do not change their shape as a result of this substitution. However, if certain mutant organisms are grown under phosphate limitation, their walls contain very little negatively charged polymer, and are made almost entirely of peptidoglycan; gross shape changes then occur. Instead of growing as rods, for example, bacilli grow as cocci. This change in morphology involves growth and probably is a problem of shape determination. Formal proof is lacking.

One argument commonly advanced for the dominant role of peptidoglycan in shape maintainance is not strictly valid. It is argued that, because molecules such as teichoic acids can be removed from peptidoglycan in isolated preparations without altering morphology of the walls, the latter is the only important shape-maintaining polymer. Walls in these preparations, however, are no longer subject to the osmotic pressure exerted upon them in the living cell. Therefore, even if teichoic acids had a role in maintaining the shape of the cell, little change in isolated wall preparations might be expected following their removal. Unfortunately, the conditions necessary for removal of polymers associated with peptidoglycan are too drastic to apply to living cells without damaging cytoplasmic membranes. Such damage would lead to a collapse of the osmotic forces due to leakage of internal solutes.

Among Gram-negative bacteria, mutants of *E. coli* entirely lacking detectable amounts of the lipoprotein attached to the peptidoglycan (Hirota *et al.*, 1977) provide some evidence for the latter's dominant role. These have abnormalities in the positioning of the outer membrane and in the process of cell division, consistent with a function for the lipoprotein in holding the peptidoglycan and the outer membrane together (Braun and Rehn, 1969). They, however, show no abnormality in their rod shapes despite reports of abnormal morphology in other mutants partially lacking lipoprotein (Wu and Lin, 1976). Another argument in favour of peptidoglycan as the sole shape-maintaining component of the wall is possibly to be found in a marine pseudomonad (Forsberg *et al.*, 1972). All the weight of the very thin insoluble layer of the wall that was isolated from "mureinoplasts" could be accounted for by peptidoglycan constituents, and it had the same shape as the original cell. However, the detection of leucine, isoleucine, glycine, serine and aspartic acid before treatment with proteolytic enzymes during isolation suggests the

presence of a covalently attached polypeptide in the living organism, similar to the lipoprotein of *E. coli.*

There can be no doubt about the importance of peptidoglycan in providing the strength of walls of bacteria necessary to resist the internal osmotic pressure of cells. The arguments for peptidoglycan as the sole shape-maintaining polymer in the wall are nevertheless rather weak.

III. The Physical Properties of Walls

A. WALLS OF THE LIVING ORGANISM

Knowledge of the chemistry of the components of wall preparations is well advanced and now presents no major difficulties of principle. However, when we come to try to decide their arrangement and distribution in the growing organism, major problems arise. Even our knowledge of the volumes in which they are contained in the walls of the living cell is uncertain. As has so frequently been stated, walls of Gram-positive bacteria appear, in sectioned material, to vary from about 15–30 nm in thickness, according to the species of organism and growth conditions. In Gram-negative organisms an outer wavy membrane is seen, separated by a less dense region from a very thin layer identified as peptidoglycan. How far does the appearance of sectioned material reflect the situation in the living cell? Frozen-etched preparations of whole cells provide some basis for comparison since it is likely that a very high proportion of the population of an organism, such as *E. coli*, survives the freezing procedure; in one test 85% survived (Bayer and Remsen, 1970). Freeze-fractured preparations of the Gram-positive *Strep. faecalis* have been compared (Higgins, 1976) with bacteria first fixed in glutaraldehyde followed by osmium tetroxide and then embedded and dehydrated. The walls of these latter organisms, when sectioned, appeared about 30% thinner than in the freeze-fractured material but the other dimensions of the cells did not seem to differ. *Escherichia coli*, on the other hand, shrank 30% in overall dimensions when fixed with osmium tetroxide, prepared and sectioned (Bayer and Remsen, 1970). More subtle changes also occurred. For example, the outer membrane became wavy in the sectioned material but, after freeze-etching, presented an almost smooth surface with a little residual waviness which was said to be influenced in degree by the growth medium (Nanninga, 1970).

These effects are not negligible but may only be indicative of greater

difficulties if the results obtained by application of physico-chemical methods to bacteria are correct. For example, a value for the wall thickness of living *M. luteus* cells of 80 nm was recorded from measuring the difference between the cell volumes impermeable to a dextran of molecular weight 150,000 and to sucrose or phosphate (Carstensen and Marquis, 1968). Such a value is two or three times greater than that seen in sections of the micro-organism. Likewise, a value of 86 nm was deduced for the wall thickness in *Staph. aureus* from light scattering and refractive index measurements (Wyatt, 1970). In this instance, the measured thickness in sectioned material was 18 nm. Again, calculations of wall density from the chemically measured content of wall substances and the approximate volumes of walls measured from electron-microscope pictures, seem to differ very much more from directly determined values than would be expected (Kelemen and Rogers, 1971; Ou and Marquis, 1972). Moreover, densities of isolated walls determined by various more direct methods differ more among themselves than seems easy to explain, except by very considerable expansion and contraction of the preparations according to their treatment.

B. EXPANSION AND CONTRACTION OF THE WALL

The physical properties of the peptidoglycan meshwork are likely to be greatly modified by attachment to the glycan chain of highly negatively-charged teichoic or teichuronic acid molecules. The effect of the removal of teichoic acid from wall preparations on their physical properties has been studied. For example, the dextran-impermeable volume of walls of *Staph. aureus* is regulated not only by the presence or absence of teichoic acid but markedly by the amount of D-alanine that is ester linked to teichoic acid (Ou and Marquis, 1970). Removal of esterified amino acid, leaving the *N*-acetylglucosaminyl substituted polyribitol phosphate still in place, doubled the wall volume impermeable to large dextrans; it changed from 5.1 to 10.1 ml per g. Subsequent removal of the substituted polyribitol phosphate lowered this value to 6.2 ml per g. Changes in surface location of teichoic acids, or even more of the D-alanine substituent on it, within the wall of growing organisms could possibly have profound effects upon the morphology of the wall and hence upon cell shape.

Extensibility of the envelope has been demonstrated by other approaches. Examples from two groups of workers (Knowles, 1971;

Matts and Knowles, 1971; Marquis, 1968; Ou and Marquis, 1970) show that the dimensions of living bacteria can be changed by altering the balance of forces exerted on the envelope. This can be done by increasing the concentration of solutes on the outside, using compounds such as sucrose. Volumes of the cells can be deduced from changes in the extinction value of the suspension (Koch, 1961). This method must be applied with circumspection, and possibly a better one is to use measurements of the volume of the suspension that is impenetrable to high molecular-weight substances such as dextrans. Applied both to isolated wall preparations and to whole cells of *Micrococcus luteus*, *Staphylococcus aureus* (Ou and Marquis, 1970) and *B. megaterium* (Marquis, 1968), this method showed a small decrease of 10–33% in cell volume for the whole bacteria when transferred to concentrated sucrose solutions. This change was deduced to be associated with a degree of plasmolysis. Definite plasmolysis could not be seen under the microscope, but could be estimated from the decrease in the sucrose-impermeable volumes of cells. Leakage of cytoplasmic contents was not detected, so that gross damage to the cytoplasmic membrane had presumably not occurred. The volumes of cell-wall preparations, at least of *B. megaterium* (Marquis, 1968), were not affected by concentrated solutions of sucrose. Greater alterations in the volumes of bacteria could be obtained by varying the ionic strength of the medium. Decreases of the order of 25–30% were found with *B. megaterium* and *M. luteus* in progressing from an external ionic strength of 0.001 to 1.0. The changes obtained with staphylococci were much smaller, of the order of 9% (Ou and Marquis, 1970). Similar changes in the volumes of isolated cell-wall preparations from the former two organisms were obtained with altered ionic strength. The patterns of change with wall preparations and increases in ionic strength differed considerably from those obtained with whole cells. They started at lower ionic strengths and decreased more rapidly. Similar, rather large, effects on the volumes of isolated wall preparations from these two organisms could also be obtained by varying the pH value of the suspending media at constant ionic strength. Significant changes could be obtained by varying the pH value but not the ionic strength of suspending media for wall preparations from *Staph. aureus*. These latter results were complicated, and are possibly explained by removal of esterified D-alanine from teichoic acid at the higher pH values. Such losses incur their own increase in wall volume, as has been noted, and cause a hysteresis in the volume/pH value curves. It seems reasonable to conclude from these

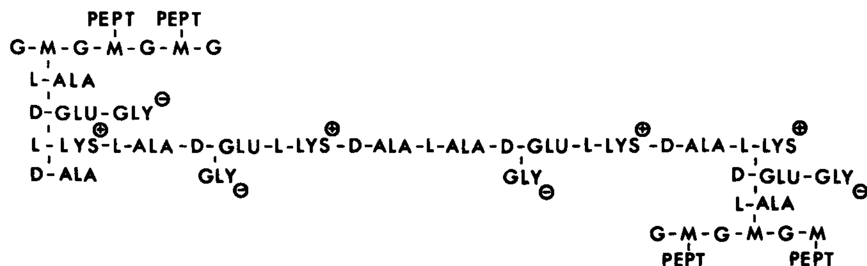

FIG. 3. One part of the peptidoglycan of *Micrococcus luteus* showing potential fixed charges in the structures. PEPT indicates peptidoglycan.

results that small volume changes in the whole cells of *M. luteus*, *B. megaterium* or *Staph. aureus* can occur due to gross alterations in the osmotic forces acting on the envelope. Larger changes in the two former organisms can be obtained by varying the ionic strength or pH value of the medium. The effects on the walls are consistent with alterations in ionization of groups fixed within the wall polymers.

As can be seen from Fig. 3, a number of charged groups exist within the peptidoglycan structure, apart from those due to polymers attached to it. When charged groups of like sign are relatively close together, they will repel each other, tending to expand the wall. Such expansion presumably occurs at the expense of the conformation of the peptide chains in the peptidoglycan. The $1 \rightarrow 4\beta$ linkages of the sugar residues in the glycan chains are such as to make impossible a tightly coiled configuration which would allow expansion. The differences between the behaviour of walls and cells at different ionic strengths probably reside in the absence of osmotic pressure on wall preparations which would oppose wall contraction in whole cells. Thus, the volume changes in cells are the result of a balance between osmotic effects and electrostatic forces in the walls.

The mechanisms underlying volume changes of Gram-negative bacteria have been the subject of some disagreement between the protagonists of purely osmotic forces and purely electrostatic ones. In general, when Gram-negative bacteria are moved to fluids containing higher concentrations of low molecular-weight solutes, the optical density of the suspensions increases rapidly (e.g. Mager *et al.*, 1956). This should indicate a decrease in the volume occupied by the micro-organisms, if due precautions have been observed (Koch, 1961). Careful study has been made of the relationship of such increases in optical

density of suspensions and cell volumes of a marine pseudomonad and of *Pseudomonas aeruginosa* suspended in various concentrations of sodium chloride (Matula and MacLeod, 1969a, b); cell volumes were measured by using ^{14}C-inulin. The intracellular volumes of the organisms decreased up to a concentration of 150–200 mM NaCl in the fluid; they then had about 76% of the volumes they had when suspended in the absence of sodium chloride. Thereafter, there was a small increase in volume. The decrease was rapid and completed in less than 30 s. Other work (Matts and Knowles, 1971) with *E. coli*, using stop-flow methods, shows that the volume changes are likely to occur several orders of magnitude faster than indicated by a time of 30 s. A first-order reaction was found for the increase in turbidity with a rate constant of 0.825×10^{-3} m sec/mg dry weight of bacteria, using a range of monovalent and divalent salts. This result is surprisingly similar to a very much earlier and approximate determination for the rate of shrinkage (Mager *et al.*, 1956).

A discrepency in interpretation, not at the moment resolvable, arises from these two pieces of work. When the rates of increase in optical density with several different salts in the suspending fluid were plotted against the osmolality of the solutions, a single straight line was obtained (Matts and Knowles, 1971). This was not true when the optical densities were plotted against molarity or ionic strength. The authors therefore concluded that the contraction was wholly due to the osmotic effects of the salts. In the work with pseudomonads, however, the organism was found to be permeable to Na^+ and Cl^- ions and their internal concentration one hour after incubation (this was the time required for the test) reached more that 80% of the external value. Moreover, providing Mg^{2+} was absent, isolated envelopes from the marine organism also showed increases in optical density comparable with those of the organisms. It was argued that the observed effects must be entirely due to electrostatic effects on the envelopes of the type already discussed. However, sucrose could shrink cells of the marine organism. Presumably, this must be due to an osmotic effect. Some degree of plasmolysis of the terrestrial pseudomonad was also found following addition of sodium chloride. One is then inclined to ask whether, because "osmotic shrinkage" can occur so quickly, it may not be that osmotic effects were also involved in the initial shrinking of both micro-organisms, with a subsequent slower gain of intracellular ions. The decreased volumes initially caused by osmotic effects might then be maintained by cation neutralization or shielding of mutually repulsive fixed-charge groups in

the envelope. The two explanations propose very different properties for the envelope. If the cell shrinks as soon as the internal osmotic force is released, this implies that the wall, or probably the peptidoglycan network, is normally held under tension and collapses as soon as the force is released. If, on the other hand, shrinkage is due only to the reduction of electrostatic repulsion within the network, the wall of the living organisms must be held in extended form by its own properties of contiguity of charged groups.

A correct explanation would seem to be important, since the decrease in volume of bacteria with increasing external salt concentration up to about 100–200 mM appears to be a very general phenomenon for bacteria (Mager *et al.*, 1956). Unfortunately, little has been attempted in the way of measuring changes in the individual cell dimensions as distinct from average cell volumes. Combination of rapid methods for measurement, such as those adopted by Matts and Knowles (1971), together with measurements of cell dimensions might be used to tell us more about the physical properties of wall materials and perhaps of the orientation of polymers.

A last aspect of the physical chemistry of walls involves the question of how far wall properties are maintained by secondary valency forces, such as hydrogen bonding. The purely speculative models that have been suggested (Kelemen and Rogers, 1971; Braun *et al.*, 1973; Oldmixon *et al.*, 1974) have all depended, to varying extents, on hydrogen bonding to maintain the conformation of the peptidoglycan. Measurements by infrared spectroscopy (Formanek *et al.*, 1974, 1976) have confirmed that there is abundant hydrogen bonding in cell-wall preparations, although not that necessary to maintain the β conformation of the peptides as originally suggested (Kelemen and Rogers, 1971). It would then seem reasonable that reagents weakening such bonds, like strong urea solutions, should cause swelling of walls. Indeed treatment of wall preparations from *M. luteus* and *Staph. aureus* with 8 M urea led to an increase in their dextran-impermeable volumes from 10 ml/g up to 15 ml/g (Ou and Marquis, 1972). Such large changes were, however, highly pH dependent, and occurred at pH values above 7.0. Below neutrality but above pH 4–5, a change of the order of only 10–12% occurred. When the pH value dropped below 4–5, the volume again increased. Likewise, raising the temperature of wall suspensions from 22°C to 62°C led to increses in volume of the order of 35%, providing the pH value was between 6 and 9.2. These changes are consistent with the results from infrared

spectroscopy, and suggest that hydrogen bonding plays a major part in keeping the wall structure reasonably compact. However, the pH value dependence also suggests that electrostatic interactions within and between wall polymers play a major regulatory role in expansion of the wall.

As a general conclusion, it would appear that envelopes of micro-organisms can expand and contract as a result of shear forces applied by altering osmotic forces or by varying the strength of hydrogen bonding. The extent of these changes, however, is dominated and regulated by electrostatic forces which are influenced by the ionic environment of the cells. As will be seen later (p. 44) the importance of electrostatic changes for the behaviour of the wall may be important in shape determination apart from shape maintenance.

IV. Shape Determination

A. PHILOSOPHY

Both of the terms "maintenance" and "determination" have been borrowed from the common stock of English words for specialized use in describing morphogenesis of living forms. "Determination" carries with it particularly confusing and baleful overtones (Henning, 1973). If we consider two rubber balloons, one spherical and the other sausage-shaped, most would agree that the shapes are maintained by interaction of the skins with the air-pressure inside. When we come to consider determination of the shapes, we quickly find that the trap-door opens to First Causes. At one level of explanation, the topology of properties in the skins is sufficient but, if we press the question, we have to retreat to the machine that made the skins and, if we persist, we arrive at the designer of the machine that made the skins. Finally, one is left with the designer's mind or with the Grand Designer's Mind. The terms "define" or "determine" therefore have most of the philosophic problems of the term "Cause" and should be used with due appreciation of the dangers. The thought of investigating a First Determinant may daunt the boldest experimentalist.

In thinking about growth of bacteria with relatively simple shapes, four problems of somewhat different types can be seen: (1) replication of shape in an exponential steady-state culture; (2) changes that take place when the growth rate of a culture is changed; (3) larger changes of shape

found in some species, like *Arthrobacter* spp. and in conditional morphological mutants; (4) regulation of shape occurring when wall-less protoplasts or L-forms revert to bacteria.

It would seem that each of these stages needs rather different thinking, and may involve different aspects of structure and biosynthesis at the molecular level. In this article, most attention will be paid to problems (3) and (4), since problems (1) and (2) have been recently considered in depth by Sargent (1979).

B. BIOSYNTHESIS OF WALL POLYMERS

As is now well known, bacterial wall polymers are made from soluble nucleotide intermediates by membrane enzymes. Our knowledge of the later stages in the process have been derived from the use of various types of broken or damaged-cell preparation. In peptidoglycan synthesis, the uridine nucleotide intermediates are transferred to the C_{55}- isoprenoid alcohol phosphate intermediate to give a disaccharide-peptide pyrophosphate. Membrane preparations from species such as *Staph. aureus, M. luteus* or *B. licheniformis* then build up glycan strands bearing peptides which are not cross-linked together by the action of the transpeptidase responsible for doing so in whole cells. If small amounts of membrane are left attached to walls, as in so-called membrane-wall preparations, then the transpeptidase is active and cross-linked peptidoglycan results. Toluene-treated cells of some micro-organisms will also carry out the complete reaction (Reynolds, 1977). As far as can be seen, the material biosynthesized by these systems appears to be closely similar to that in walls from whole organisms. Only very small amounts, however, can be formed and caution is necessary in interpreting from the *in vitro* to the *in vivo* situation. Most of the preparations can also biosynthesize free teichoic acid and, where examined, teichuronic acids, from the appropriate soluble nucletide precursors.

Two relatively recent additions to knowledge of wall biosynthesis are relevant to considerations of how the bacterial surface grows. These concern the way in which the glycan strands are built and the method by which teichoic acid is linked to peptidoglycan. Examination of the direction of growth of glycan strands synthesized by a strain of *B. licheniformis* (Ward and Perkins, 1973) showed them to be built up from the reducing ends in a way similar to the lipopolysaccharides on the surface of *Salmonella typhimurium* (Osborne, 1969). In the latter system, the

polysaccharide chain grows whilst still attached to the lipid intermediate. Likewise, it appeared that the muramyl reducing ends of the glycan chains were blocked during biosynthesis of uncross-linked peptidoglycan (Ward and Perkins, 1973). They could be liberated by brief treatment of the preparation with 0.1 M hydrochloric acid at 60°C. Evidence was produced to show that this treatment was not due to non-specific rupture of glycoside bonds. More recent work has, however, shown that treatment of wall preparations with acid under the same conditions liberates equal numbers of muramyl and glucosaminyl reducing groups (S. M. Fox, J. B. Ward and C. Taylor, unpublished work). Thus, although it is reasonable that the glycan chains should remain attached to the lipid intermediate, further proof is necessary. Growing chains in the bacilli are then incorporated into the existing wall in membrane-wall preparations, or in toluenized cells (Reynolds, 1977) by transpeptidation, the reaction inhibited by β-lactam antibiotics. In some organisms, such as *M. luteus* but not bacilli, a small amount of penicillin-resistant transglycosylation also occurs (Mirelman *et al.*, 1974a, b; Weston *et al.*, 1977). The remainder of the wall synthesis occurs by transpeptidation and, in the presence of high concentrations of penicillin, uncrosslinked peptidoglycan is secreted into the medium as with *B. licheniformis* (Tynecka and Ward, 1975).

Linkage of teichoic acids to peptidoglycan has been generally thought to be directly to the 6-hydroxy group of the muramyl residues of glycan strains. The one situation examined thoroughly (Button *et al.*, 1966) appeared to confirm this supposition. It is now clear, however, that in many organisms linkage takes place through short linker groupings which, in the few organisms so far examined, contain two of three glycerol residues and a molecule of *N*-acetylglucosamine (Bracha and Glaser, 1976; Heckels *et al.*, 1975; Coley *et al.*, 1976; Hancock and Baddiley, 1976; Hancock *et al.*, 1976; Ward, 1977; Wyke and Ward, 1977). This is an actively developing area, and it may well be that other types of linker groups will be found. Among important aspects of these observations are the necessary involvements of UDP-*N*-acetylglucosamine and CDP-glycerol, irrespective of the presence or absence of the latter as the main glycol residue in the teichoic acid. Both intermediates can be seen as control points in synthesis of walls by Gram-positive bacteria, the former for both peptidoglycan synthesis and the attachment of teichoic acid, and the latter for attachment or synthesis of teichoic acid, according to the nature of the teichoic acid. Whether or not other substances, such as the

teichuronic acids or polysaccharides, are attached *via* linker groups has not so far been examined.

Once the intermediates for wall synthesis have been transferred to their lipid intermediates, chain extension, modification and presumably transpeptidation into the wall take place by action of membrane-bound enzymes. Moreover, although as yet unproven, it is reasonable to suppose that the chains may actually stay linked to the lipid intermediate dissolved in the membranes, whilst they are fed into the expanding wall. It is therefore perverse to argue whether the wall or the membrane defines the shape of the growing cell. The topology of the insertion of newly synthesized enzymes into the membrane may well influence, in a major way, the direction of wall growth. On the other hand, if glycan chains remain attached to the lipid intermediate, the expanding wall may move the intermediate, together possibly with its associated enzymes, through the "lipid-sea" of the membrane. One can see a closely integrated system which could account, for example, for the very late morphogenesis of reverting protoplasts (see p. 26).

C. RELATIONSHIPS BETWEEN WALL SYNTHESIS AND SURFACE GROWTH

Exponentially growing cultures of bacteria synthesize their walls in a regulated manner, so that the proportions of wall polymers to each other and of wall components to cell mass remain constant. If the dilution rate in chemostat cultures of *B. subtilis* var *niger* is varied, the proportion of wall decreases with increasing growth rate (Ellwood and Tempest, 1972). This result would be expected if wall thickness remains constant, since the size of organisms increases, a deduction which is confirmed by the very much smaller changes shown by *M. luteus* and *Staph. aureus* that also alter much less in size. One potential factor for regulation of the proportion of wall components in Gram-positive bacteria might, one would suppose, be wall turnover. However, surprisingly, the amounts of wall components in exponentially growing cultures of autolytic enzyme-deficient mutant strains are not necessarily different from those in wild-type cultures growing at similar rates. Such mutants do not carry out wall turnover. If protein synthesis is stopped, either by including suitable antibotics such as chloramphenicol or by omitting suitable amino acids required for protein but not wall formation, wall synthesis continues unabated (Mandelstam and Rogers, 1958; Shockman, 1959; Shockman

et al., 1958; Rogers and Mandelstam, 1962). The relationship between peptidoglycan synthesis and that of other macromolecules such as DNA and RNA has not been subject to sufficient investigation to allow dogmatic statements.

Measurements in terms of the mass of peptidoglycan formed per unit mass of cells can now be supplemented by looking at the average length to which the glycan chains have been biosynthesized in wall preparations (Ward, 1973). Such averages are controlled by rates of chain extension and rates of initiation of new chains. Likewise, inspection of the average length at which they exist in the wall can give an indication of any alterations in the autolytic breakage of chains. The effect of inhibiting protein and DNA synthesis on these parameters has now been examined (S. M. Fox, M. G. Sargent and J. B. Ward, unpublished work). In neither case was the average chain length significantly different from the value in walls of the wild-type organism. Also, there was no change in the average chain lengths in walls prepared from bacteria in cultures having a four-fold difference in growth rate and, as a result, a three-fold difference in cell length. On its face value, these results should mean that rates of initiation of new glycan chains and of growth of existing ones are not affected by inhibiting either protein or DNA synthesis, or by the growth rate of the organism.

After synthesis has proceeded in the absence of protein formation, walls are evenly thickened over the whole surface (Shockman, 1963, 1965; Higgins and Shockman, 1970; Miller *et al.*, 1967, Hughes *et al.*, 1970; Giesbrecht and Ruska, 1968). This process can continue for long periods until the walls are many times as thick as in exponentially growing cultures. Thickening has always been assessed by looking at sectioned material. It would be helpful to have measurements of cell-wall volume by exclusion methods such as have been discussed earlier (see p. 10). Increase in the amount of chemically measured wall substance parallels the increase in wall thickness (Higgins, 1976, Hughes *et al.*, 1970) in *Strep. faecalis* and *B. subtilis*. Nevertheless, problems arise as to just how these large thickenings of walls are accommodated and, indeed, how they can occur. If new glycan chains are initiated and old ones continue extending at the same rate as in an exponentially growing culture, there must presumably be many new non-functional sites in the growing cell which can be pressed into use when protein synthesis is shut off. In *Strep. faecalis*, where the biosynthetic glycan chain lengths have not yet been studied for technical reasons, the new sites may exist in the earlier formed pole of the

cell (see p. 20). This does not thicken or grow whilst the new pole is being formed (Higgins and Shockman, 1976) but does so when protein synthesis is shut off (Higgins and Shockman, 1970). In *B. subtilis*, however, there may be no hard and fast division into growing and non-growing parts of the main cylindrical region of the cell thereby retaining a reserve of unused sites. A further problem arises as to how the new wall substance is accommodated, since all available evidence suggests that the new wall is extended out from the membrane under the old existing wall. If the old wall is pushed outwards, it must expand in area. How does it do this and has this a relation to similar processes occurring during cell growth?

Peptidoglycan synthesis by Gram-negative bacteria such as *E. coli* also proceeds in the absence of protein synthesis (Rogers and Mandelstam, 1962; Braun *et al.*, 1974). Evidence, however, as to whether the appropriate structural layer in the envelope becomes thicker is difficult to obtain because its normal thickness is near the level of resolution of the electron microscope. Whether synthesis of the lipoprotein attached to the peptidoglycan in *E. coli* is dependent upon overall protein synthesis is also not wholly clear. In the very early work (Rogers and Mandelstam, 1962) incorporation from ^{14}C-glucose into glycine (and serine), which were still attached to the peptidoglycan presumably as part of the lipoprotein, was not inhibited by high concentrations of chloramphenicol (100 μg/ml). However, interpretation of this result is questionable because there is now known to be a large pool of free lipoprotein in the outer membrane which, although not having a precisely precursor-product relationship with the bound material, can be drawn upon and become linked to peptidoglycan. Careful and detailed investigation (see Inouye *et al.*, 1974, and Braun *et al.*, 1974) has shown, nevertheless, considerable abnormalities in the biosynthesis of lipoprotein compared with that of protein in general, including resistance to some antibiotics. Among other factors is the long life of the messenger RNA for this polypeptide.

D. TOPOLOGY OF WALL SYNTHESIS

One of the important aspects of surface growth of micro-organisms that helps to decide the shape and size of the individuals might be expected to be the site, or sites, at which new material is added. Attempts to identify such sites by distinguishing new from old surface met with great success when immunofluorescence methods were applied to streptococci (Cole

and Hahn, 1962). Unfortunately, the genus *Streptococcus* has remained the only successfully explored one despite the application of this and many other methods to a number of species of rod-shaped bacteria, both Gram-positive and negative. A wide range of approaches to surface growth of *Strep. faecalis* has given a consistent picture for this organism; the results of these observations have been summarized elsewhere (Rogers *et al.*, 1978). Briefly, the peripheral walls of two new streptococci are fed out from the septum of the dividing cell, the septum splitting into two layers (Fig. 4).

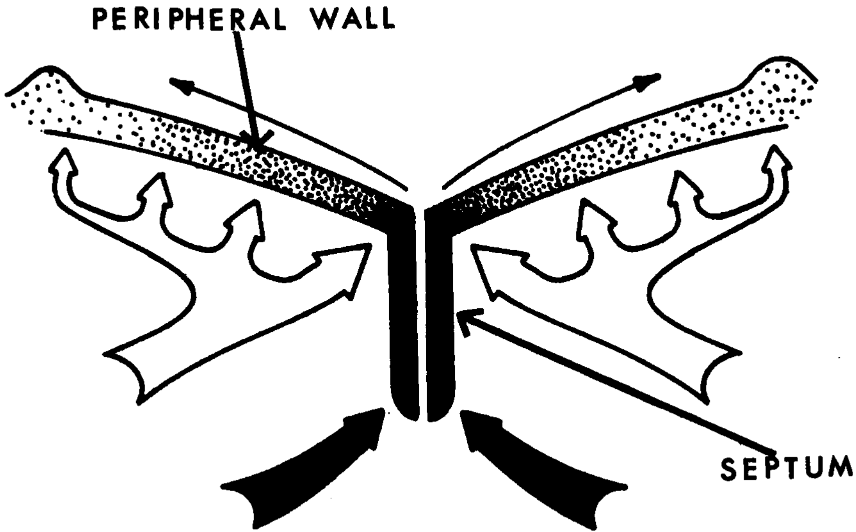

FIG. 4. A model of the expansion of the peripheral wall of *Streptococcus faecalis*. Taken from Higgins and Shockman (1976).

The area of the septal wall itself, meanwhile, remains approximately constant until the new poles of cells are almost completed, and the septum then closes and cells separate. The precise morphological analysis (Higgins and Shockman, 1976) of this growth process has shown deficiencies in the detection of newly inserted material by other methods. For example, the new peripheral wall shows an important gradient of thickening in progressing from the base of the septum to the hemispheroidal surface marker that divides the old from the new wall. Presumably, new material is being continuously inserted over the whole new surface, which could not be deduced from earlier immuno-

fluorescence work or from autoradiography. The newly added material may not be available to antibodies at the surface of the wall and may be too dispersed to be easily demonstrated by autoradiography.

Growth of rod-shaped bacteria might be expected to show vectorial components in addition to those involved with coccal-shaped cells. Whilst formation of the ends or poles of the rod could be by a "streptococcal-like" mechanism, some additional features must be necessary to regulate growth in length and the diameter of the cylindrical parts. Examination of the formation of poles of *B. subtilis* rods (Burdett and Higgins, 1978) has indeed shown that they are fed out from the base of the new septum. However, in contrast to streptococcal growth, the septum closes at a relatively early stage in the process. There is again a gradient of thickness in the developing pole, and a good deal of remodelling during and after cell separation. Because the septum closes considerably before pole formation is complete, cell separation and closure appear as much more distinct processes than in streptococci. The poles of *B. subtilis*, however, account for only about 15% of the peripheral wall instead of all of it as they do in streptococci. Attempts to identify discrete growth zones in the cylindrical walls of Gram-positive rod-shaped organisms by methods so successful for streptococci have failed (see Shockman *et al.*, 1974; Doyle *et al.*, 1977 for a summary of these attempts) in all but three experiments (Chung *et al.*, 1964; Hughes and Stokes, 1971; Pooley *et al.*, 1979). In one of these, the immunofluorescence method, as used by Cole and Hahn (1962), was applied to an autolytic deficient-phosphoglucomutase-negative strain of *B. licheniformis* (Hughes and Stokes, 1971); conserved zones of new wall were demonstrated in the centre of the cell or 25% along the length of the cells from the poles. Several aspects of this experiment differentiated it from other apparently similar ones with bacilli. Perhaps the most relevant point was that, being a strain deficient in autolytic enzymes, its wall turnover was much decreased (Rogers *et al.*, 1974a). In view of the failure of streptococci also to turnover their wall components (Boothby *et al.*, 1973), it is tempting to find in this an explanation of the positive identification of growth zones in a bacillus, in contrast to the results from much other work which has favoured the introduction of new material by diffuse intercalation over the whole cylindrical region of the rods. Setting aside for a moment this tempting correlation, other work with bacilli, both on the distribution of bacteriophage receptor sites (Archibald and Coapes, 1976; Archibald, 1976) and on the distribution of radioactivity after application of very

short pulses of radioactive 2, 6 diaminopimelic acid (De Chastellier *et al.*, 1975a, b), has drawn attention to a difference in the behaviour of the polar regions of the cells from the cylindrical region.

Evidence was obtained some years ago (Young, 1967) that some bacteriophages adsorb specifically to the glycosyl substituents of wall teichoic acids. Making use of this observation, together with the possibility of reversibly exchanging wall teichoic acid for teichuronic acid by growing *B. subtilis* in a chemostat with a phosphate-limiting medium (Ellwood and Tempest, 1969, 1972), the disappearance and re-appearance of phage adsorption sites on the walls of *B. subtilis* was examined (Archibald and Coapes, 1976, Archibald, 1976). Whereas the sites appeared and disappeared in a random fashion along 90% or so of the cylinder of the rods, the wall of the poles of the cells appeared to be conserved for a number of generations. This was particularly apparent on changing from potassium to phosphate limitation of growth, when the phage receptor sites disappeared from the cylindrical region of the cells but the poles could still actively fix the particles.

In the autoradiographic work (De Chastellier *et al.*, 1975a, b), pulses of equivalent to 0.05% of a generation were supplied to *B. megaterium* and the distribution of radioactivity examined in both walls and whole cells.

As much as thirty per cent or more of the grains emmenating from wall preparations was associated with the developing cross-walls, i.e. regions of pole formation, whilst it was thought that no discrete zones were present along the cylinders of the rods. In whole cells, 50% of the silver grains in the autoradiographs were associated with the cytoplasm in the form of material that the authors considered to be wall precursors. The distribution of this material was also not random, being concentrated around the developing cross walls and generally near the walls of the organism. The apparent differences in behaviour of the polar regions of the cells, or where the poles are forming, reinforces many observations showing subtle differences are likely to distinguish the wall in these areas from the rest of the surface. For example, they are relatively resistant to autolysis and persist intact when the rest of the wall has already been badly damaged (e.g. Fan *et al.*, 1972; Fan and Beckman, 1973).

Another possible source of confusion in trying to identify growth zones in the walls of Gram-positive bacteria is to be found in the observations of Pooley (1976a, b) and Archibald and Coapes (1976). In both studies, it was shown that new material, laid down on the inner side of the wall

contiguous with the cytoplasmic membrane, passes only slowly through the wall and reaches the outer surface after a time equivalent to or greater than one generation of growth. Pooley (1976a) considered that, during this passage through the wall, the newly-synthesized material must spread to form a thin layer on the surface of the cells whence it is ultimately lost as turnover products. Such spreading, if it occurs, is clearly liable to diffuse any discrete zones of new glycan chains initiated in the membrane surface. Moreover, unless the orientation of the glycan chains of the peptidoglycan in the thick wall is remarkably uniform, pulses of radioactivity other than exceeedingly short ones, might be randomized. Nevertheless, if the glycan chains grow from their reducing ends, discrete zones of synthesis ought to have been preserved using pulses as short as those of De Chastellier *et al.* (1975a, b). Whether or not localized synthesis is indeed evident in the work of De Chastellier *et al.* (1975a, b) depends on the statistical analysis applied to the distribution of grains along the cells, and on acceptance or otherwise of their very high proportion of the synthesis apparently involved in septal growth. In very recent work, Pooley *et al.* (1979) non-Poissonian distribution of radioactivity was demonstrated along the cylinders of *B. subtilis* cells as well as conservation of the poles. A strain with decreased turnover of its wall components was used and labelled with *N*-acetyl-D-[1-^{3}H]glucosamine which was chased through the wall with unlabelled material. Non-random distribution was only seen after several generations of chase. The grains in the conserved poles shown in autoradiographs amounted to 13% of the total, in good agreement with the value of 15% obtained by the image-rotation technique already described.

The involvement of turnover as a confusing factor has been somewhat minimized by use of a phosphoglucomutase-negative mutant of *B. subtilis* (Doyle *et al.*, 1977). Glycosylation of teichoic acid will not occur in such mutants because the necessary intermediate, glucose 1-phosphate, cannot usually be formed. The lesion can, however, be partially circumvented by supplying the organisms with galactose as well as an assimilable form of carbon such as glycerol (Fukasawa *et al.*, 1962; Young, 1967; Birdsell and Young, 1970, Forsberg *et al.*, 1973; Forsberg and Rogers, 1974). In the absence of galactose, these strains are also defective in autolytic activity, and they turnover their wall components very inefficiently (Rogers *et al.*, 1974a). Doyle *et al.* (1977) followed glycosylation of teichoic acid in the walls of such strains with

concanavalin A. No evidence of discrete zones could be found along the cylinder of the cell, even in the experiments in which galactose was removed from the growth medium, and therefore wall turnover was presumably decreased. However, in the published pictures, resolution did not seen to be very high.

More work is clearly needed with strains totally defective in wall-component turnover. The balance of evidence favoured the idea that length extension of Gram-positive rods occurs by some sort of diffuse intercalation of material over the cylindrical part of the cells until the very recent experiments by Pooley and his colleagues (1979). The process of pole formation takes place by a different mechanism, more akin to formation of the streptococcal cell wall.

A different type of problem of surface topology is raised by the isolation of mutant strains of *B. subtilis* that grow as helices (Mendelson, 1976; Tilby, 1977). The question is whether the mutations impose a helical form on the cells or only disply a normal helical component in the arrangement of wall polymers present in wild-type strains. Mendelson (1976) suggests that the helices in his mutant, which had a quite different pitch from those in Tilby's (1977) strain, arise by rotation of cells disturbed in division and which had the poles of the original cells still fixed to the spore coats from which they germinated. In support of this suggestion, it was found possible to generate helices with the same pitch as those of Mendelson (1976) by constructing double *rod* A *lyt* or *rod* B *lyt* strains of *B. subtilis* (see pp. 28–33 and 43–55) and changing cultural conditions so that rods were generated from cocci (Rogers and Thurman, 1978a). Since the cocci were formed by the *rod* mutations in an autolytic enzyme-deficient strain, they remained fixed together as did the rods that were generated. Thus, two of Mendelson's (1976) criteria were fulfilled in that the poles of the first rods generated remained fixed together and the new rods could not separate. Neither *rod* nor *lyt* mutants, when the lesions are not expressed in the same organism, grow as helices, and it seems possible, therefore, that helical growth was the expression of a character normally present in the parent wild-type. If so, it would seem that a helical arrangement of the wall polymers is possible, a situation so common for cellulose in plant cell walls. To hypothesize further, may it not be that the essential part of length extension, growth in girth and septation in rod-shaped organisms are reflections of the pitch of such helices? If, for example, the pitch were shallow in the cylindrical part of

the rods to give length extension but steepened at the point where septation or swelling (see pp. 43–55) is to occur, the distinction in direction of cell growth might be provided.

Gram-negative bacteria, such as *E. coli*, present a quite different set of problems. The wall is multilayered, with an outer membrane of limited permeability and a very thin layer of peptidoglycan next to the cytoplasmic membrane. Certain data (Spratt, 1975, 1977a, b; Spratt and Pardee, 1975) may suggest that separate cytoplasmic-membrane proteins, that are supposedly in some unknown way concerned with peptidoglycan synthesis, are responsible for septum formation and length extension of these organisms. Turnover of peptidoglycan is not generally thought to be a problem.

Experiments have been done in which pulses of radioactive 2, 6-diaminopimelic acid were supplied to *E. coli* and the distribution of radioactivity studied in isolated sacculi of peptidoglycan (Ryter *et al.*, 1973; Schwarz *et al.*, 1975). Intense concentrations of silver grains in autoradiographs were found in the central regions of cells, irrespective of the age of the cells or the growth rates of the cultures. In faster growing cultures, more diffuse bands were also found between the central region and the poles. If these bands, at which the radioactive precursor had been taken up, represent sites of length extension, then similar discrete zones should appear in filamentous strains. When a temperature-sensitive mutant was grown under restrictive conditions as filaments, and treated with a pulse of radioactive 2, 6-diamonopimelic acid, such zones were found in only some of the isolated peptidoglycan sacculi but not in others. Nevertheless, experiments designed to study the mode of growth of the outer membrane of *E. coli* also produced results suggesting growth from the septal region of the cell (Ryter, 1974; Ryter *et al.*, 1975). In these experiments, the distribution of new receptors for λ bacteriophage was studied after expression of the *lam*B gene by supplying the micro-organism with maltose and cAMP. Even in the longest cells, the zone of new receptors appeared in the septal region, and in only a few very short, presumably immediately, freshly divided cells was there evidence for asymmetric polar labelling. This result, of course, stands in frank contradiction to the interpretation put by Begg and Donachie (1973, 1977) on their experiments on surface growth using fixation of T6 bacteriophage. In these experiments done under different conditions from those with λ phage, the authors considered that they had evidence for growth of cells from the poles.

E. REVERSION OF PROTOPLASTS AND L-FORMS

The change from a spherical protoplast or a relatively unorganized wall-less L-form to a regularly dividing rod-shaped bacterium with a wall perhaps most clearly illustrates the necessarily integrated function of membrane and wall in recreating the bacterial shape. It also makes it probable that our analytical approach to morphogenisis of bacteria, certainly at the molecular level, is as yet quite inadequate. Landman and Halle (1963) first clearly showed that bacterial protoplasts, in their experiments produced from *B. subtilis* strain 168, could develop in one of either two ways according to the nature of the solid medium on to which they were plated. On soft agar medium they grew as wall-less L-forms but changed back to bacteria if placed on media containing high concentrations of either gelatin or agar. The ability of bacteria to follow two different paths has, by now, been shown to be true for a number of species. The L-forms can also revert to bacteria rather rapidly, particularly if they are subcultured on to hard agar or gelatin-containing media lacking antibiotics that inhibit wall synthesis (Landman and Halle, 1963; Fodor and Rogers, 1966; Wyrick and Rogers, 1973; Chatterjee *et al.*, 1967). For this reason such strains have come to be called "unstable" L-forms. However, a semblance of permanency can be given to them, either by repeated transferrance on soft agar or, much better, by continuing to include in the growth medium antibiotics, such as the penicillins or cycloserine, that stop the formation of walls. When continually subcultured in the presence of such antibiotics, a point arrives, however, at which reversion to bacteria will no longer occur even when the antibiotic is removed, and such forms have been called stable L-forms. This appears likely to be due to selection of mutants from the population with lesions affecting enzymes in the wall biosynthetic pathway (Wyrick *et al.*, 1973; Ward, 1975).

The morphological events involved in changing from spherical osmotically-labile protoplasts of *B. subtilis* and *B. licheniformis* to normally growing rod-shaped bacilli show a well marked succession (Landman *et al.*, 1968; Elliot *et al.*, 1975a). In the very early stages, a fibrillar coat of material is built up that reacts with antibodies raised against peptidoglycan; it is also lysozyme-sensitive. This material already contains all of the normal components of the wall. Despite the often curious and contorted shapes of the bodies at this stage, they can be reconverted to spheres by suspending them in solutions containing high

concentrations of solutes, such as sucrose. They are still osmotically fragile and burst if the sucrose solution is diluted. At the next stage of development, a relatively thick wall wall can be seen in sections, but long radially arranged fibres penetrate out beyond it. Such fibres can be seen both in sections of cells treated with ferritin-labelled peptidoglycan antibody, or in freeze-etched preparations. These fibres also have the chemical composition of normal walls. The spheroidal cells then become increasingly oval, grow many aberrant septa, and slowly transform themselves into normal dividing rods. The most chastening aspect of the work is that examination of wall composition, such as the proportions of peptidoglycan, teichoic acid and teichuronic acid, the degree of cross-linking of the peptides in the peptidoglycan or the chain lengths of the glycan in the latter (Elliot *et al.*, 1975b), shows it to be normal by the time the cells have reached the thick-walled spheroidal form, despite the totally abnormal morphology. The walls in all compositional respects are closely similar to those in rapidly multiplying, morphologically normal, vegetative cells. The impression gained is that strong "normal" walls form around whatever shapes the cells have adopted at the time, and the presence of the strong wall *per se* has no effect on morphology. It is only sometime after this that the cells gradually stretch out to rods and septate normally. Thus, neither the cytoplasmic membrane nor the wall itself "determines" the shape but some subtle interplay between functioning cytoplasm, membrane and wall does so.

Attempts have been made to dissect the relative importance of protein, DNA and wall biosynthesis during the process (Landman and Forman, 1969) and, as far as is allowed by the difficulties of the rather complicated technique and nomenclature, the situation may be described as follows. Synthesis of DNA and protein, but not wall formation, can be halted during early stages of the reversion process, without affecting the ability of protoplasts eventually to change back to bacilli when they are plated on media containing high concentrations of solute. Inhibition of protein synthesis, however, prevents the development on media not containing high concentrations of salt. This suggests that protein synthesis is necessary to allow sufficient wall to form in the early stages to prevent lysis of the cells. Presumably, the necessity for protoplasts to undertake protein synthesis is related either to keeping the membrane enzymes properly ordered on the surface, or to making more biosynthetic enzymes available to replace those lost during preparation of the protoplasts. It is difficult to draw precise conclusions because Landman and Forman (1969) applied

their inhibitory situations to protoplasts suspended in 25% gelatin incubated in tubes. Morphological events were then not monitored (Landman *et al.*, 1968) until after these suspensions were spread on to the surface of agar-containing medium. It is not possible to say what was happening to wall formation when the inhibitors were present.

Examination (Elliot *et al.*, 1975b) of the agar medium on which the reverting protoplasts of *B. licheniformis* had been incubated showed that autolytic degradation products of peptidoglycan had been secreted during the process. These products were eliminated in the early stages of reversion, and greatly reduced in quantity in the later stages, by including a cell-wall preparation in the reversion medium. When reversion of a strain of *B. licheniformis* deficient in autolytic enzymes was studied, no soluble products could be detected in the medium. Thus, during reversion of protoplasts to bacteria, autolytic enzymes are active and a form of turnover of the peptidoglycan is proceeding, but neither is necessary for the change from the primitive spherical protoplast to the organized dividing bacillus.

Further study of this system, that has been recently reviewed elsewhere in greater detail (Ward, 1978), is clearly worthwhile. It provides the rare opportunity to study evolution of organized cellular forms from a relatively unorganized state. Also possible are comparative studies of the division processes and nuclear segregation in wall-less apparently chaotically organised L-forms.

V. Autolytic Enzyme Function and Cell-Cell Interaction

It was widely believed until a few years ago that autolytic enzymes played a vital part in expanding the walls of bacteria during growth and division but, whilst such a role is still possible, it would seem increasingly unlikely. In Gram-positive species, these enzymes seem rather to be involved with three other major aspects of bacterial behaviour, cell separation, turnover of the walls and formation of organised flagella. Although perhaps less exciting, these functions should by no means be dismissed. Both cell separation and motility are very directly concerned with colonization of natural habitats and autolysins have, for these reasons alone, considerable evolutionary survival value. Turnover of wall polymers is equally important since it accelerates alterations in the nature of the surface, such as in the change from teichoic to teichuronic acids that occurs as a result of phosphate-limitation of growth, as has already been

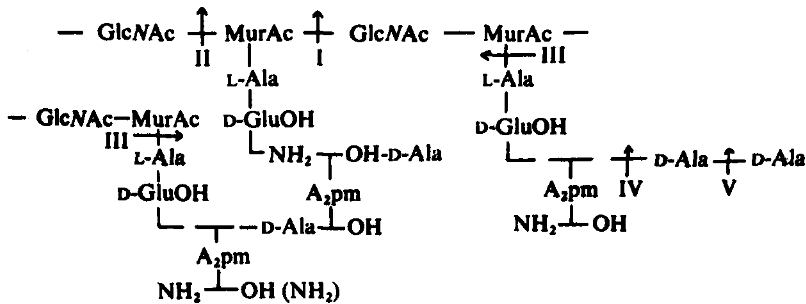

FIG. 5. Structures of the peptidoglycan found in the majority of bacilli and all known Gram-negative species (cf Fig. 2 p. 5) showing the bond specificity of the autolysins. Bacilli have autolysins hydrolysing bonds II and III, *Streptococcus faecalis* and *Lactobacillus acidophilus* have a single autolysin hydrolysing bond I, but note that the peptide in the former organism is different, being of type II with asparagine as the bridge amino acid.

mentioned (p. 24). Such polymers are again related to the interaction of microbes with their environment, most obviously when they are dominant cell antigens as, for example, are the teichoic acids in a number of species. The role of autolysins in Gram-negative species is less clear and is so far a relatively unexplored field. However, *env* A mutants of *E. coli* which grow as chains have grossly decreased autolytic activity (Wolf-Watz and Normark, 1976).

Autolysins are enzymes hydrolysing specific bonds in peptidoglycans (Fig. 5) and, of course, their action can lead to cell disaster in the form of lysis. Indeed, they probably determine the bactericidal rather than bacteriostatic action of those antibiotics that inhibit wall synthesis, such as the penicillins (Rogers, 1967; Fosberg and Rogers, 1971; Tomasz *et al.*, 1970). Their involvement in separating bacterial cells after septation has been completed was first indirectly raised when it was found (Lominski *et al.*, 1958) that either culture supernatants or lysozyme would break up chains in cultures of *Strep. faecalis*. The argument was reinforced by the observed (Tomasz, 1966) correlation between chain formation and loss of autolytic activity when choline in the walls of pneumococci was replaced by ethanolamine. An exactly similar correlation was found in phosphoglucomutase-deficient mutants of *B. licheniformis* (Forsberg and Rogers, 1971, 1974) in which glycosylation of wall polymers did not occur, and autolytic activity was greatly decreased. Such organisms formed long chains of unseparated cells.

The situation in teichoic acid-deficient staphylococci is more complicated. They also have difficulties in cell separation (Chatterjee *et*

al., 1969) but they are not apparently deficient in autolytic enzyme activity. Indeed, treated with 1.0 M sodium chloride, such mutants autolyse even faster by action of an *N*-acetylmuramyl-L-alanine amidase and a glycosidase than does the parent strain (Gilpin *et al.*, 1972). Under these conditions, however, clumps of unseparated cells break up. The unusual ultrastructure of the mutant which had thickened ragged walls and abnormal septa was also corrected by growth in media containing 1.0 M sodium chloride. The impression obtained from this work is that the distribution of autolysins in the mutant between the "cytoplasm" and wall is abnormal (Chatterjee *et al.*, 1969). When cells were disrupted, the autolytic activity in the soluble fraction was 6–10 times less than that in the soluble fraction from the parent. Isolated native walls of the mutant strain correspondingly had about 10 times more activity.

Despite this somewhat confusing picture, deliberate isolation of mutants of *Staph. aureus* deficient in autolytic activity gave rise to organisms that grew as large clumps of unseparated cells, and neither the cytoplasm nor wall preparations showed significant lytic activity (Chatterjee *et al.*, 1976). Thus, again in this mutant strain, there is a close direct relationship between the function of autolytic enzymes and separation of staphylococcal cells. The work of Koyama *et al.* (1977) is particularly interesting in showing another possible role of autolytic enzymes. When staphylococci are grown in the presence of low concentrations of detergents, such as SDS or Triton X-100, the organisms form large regular packets of cells, in strong contrast with the irregular clumps of wild-type cells. Supernatant fluids from wild-type cultures disaggregated these clumps and caused lysis; the factor present was inactivated by the detergents. A mutant which was isolated by selecting cells which fell to the bottom of a tube containing a culture which had been treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine also grew as regular packets and did not produce the disaggregating lytic factor.

Tzagoloff and Novick (1977) suggested from their phase-contrast study of division of staphylococci that the division planes were regular and alternated at right angles to each other. This should lead to formation of regular packets of cells. It would appear that, when the cell separation enzyme is either not present or present with greatly decreased activity, this indeed happens. In its presence, the organisms are able to remain only partially attached together and, presumably, the junction becomes distorted in such a way that the regularity of the groups is destroyed. According to Koyama *et al.* (1977), there may also be some

slight irregularity in the division planes. It may be noted that the teichoic acid-deficient mutant of Chatterjee *et al.* (1969) grew as very large irregular clumps. There is no record of the form of the clumps in these worker's deliberately selected autolytic-deficient mutants (Chatterjee *et al.*, 1976). In all observations recorded, the growth rate of autolytic-deficient strains is similar to, or identical with, that of the wild-type despite the loss of up to 95% of autolytic activity. This gives little support to any essential role of autolysins in growth of cells. Moreover, since the two known autolytic enzymes in staphylococci are an *N*-acetylmuramyl-L-alanine amidase and an endo-β-*N*-acetylglucosaminidase, it would be difficult to propose a role for them in biogenesis of walls.

The same situation, in this respect, applies to mutants of *B. subtilis* (Fein and Rogers, 1976) which are also grossly deficient in both of the known autolytic enzymes having the same bond specificities as those in staphylococci. Data would strongly support the idea that these strains have only a single genetic lesion, and they grow at exactly the same rate as the isogenic wild-type in five different media. Very long chains of unseparated bacilli are formed in media allowing fast growth rates (see Fig. 6) and shorter ones in those giving slow growth rates. This would be predicted from Paulton's (1970) calculations assuming a greatly prolonged separation time for the individual bacilli. Walls of the mutant bacteria were as readily lysed as those from the wild-type by autolytic enzyme preparations made from the latter, and their chemical composition was in no detectable way abnormal. Bacteria of the mutant strain were, however, non-motile and formed no flagella. When motile revertants were selected they had various levels of autolytic activity usually similar to those present in the wild-type but, in some instances, they were more active. Since the activities of two autolytic enzymes are affected, and the revertants showed a range of activities, it seems likely that the original mutations were of a regulatory nature. Unlike the partially autolytic enzyme-deficient non-motile chain-forming mutants of *B. subtilis* isolated by Ayusawa *et al.* (1975) and Yoneda and Maruo (1975), they were not excessive producers of exo-enzymes such as amylase and protease. Mutants isolated by the Japanese workers were of course non-flagellated but, interestingly, a pool of the protein flagellin was found in their cytoplasm. The strains isolated by Fein and Rogers (1976) were not examined in this respect.

The phosphoglucomutase-deficient mutants of *B. licheniformis* are also autolysin-deficient and are non-motile but, when supplied with galactose

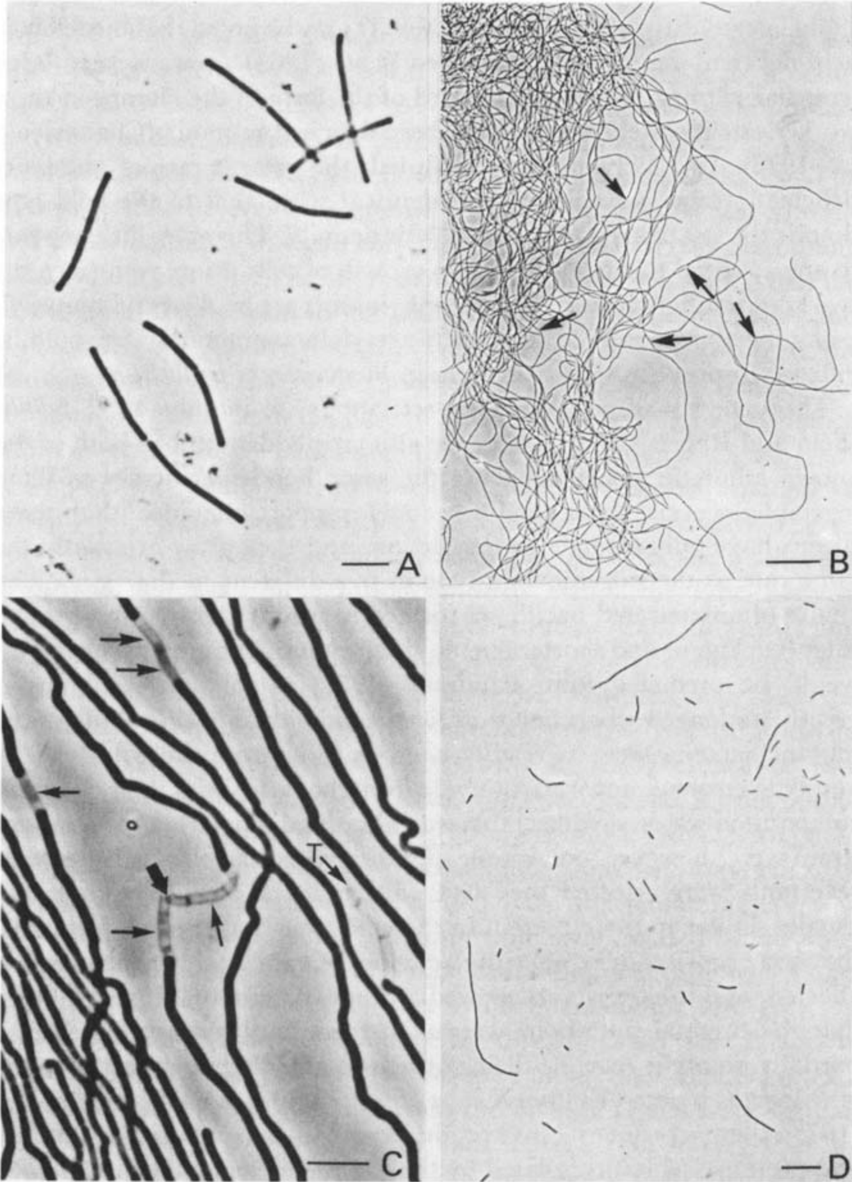

FIG. 6. The appearance (A) of the wild-type growing in the broth medium; (B) of a temperature-sensitive mutant of *Bacillus subtilis* grossly deficient in autolytic enzymes (i.e. a *lyt* mutant) growing at 30°C in the same medium; (C) the mutant at a higher magnification. The arrows indicate empty cells and an apparant break in a cell. (D) Mutant growing at 17°C. Scale lines represent 5 μm (A and C), 100 μm (B) and 20 μm (D) (from Fein and Rogers, 1976).

as well as an assimilable carbon source in the growth medium (see p. 23), glycosylation of the wall, autolytic activity, flagellation and motility are all partially restored (J. E. Fein, unpublished work). It is therefore tempting to suggest that one function of autolytic enzymes is to aid penetration of the wall, so that the flagellin can polymerize to make organized flagella but, since some form of regulation is involved in all of the mutants, dogmatic conclusions are not justified. This work would seem to confirm that the bulk of the autolytic activity in micro-organisms is related to cell separation, wall turnover and organization of cell appendages, but not to the growth rate of cultures or to morphology of individual cells in them, despite claims to the contrary (Fan and Beckman, 1971; Fan *et al.*, 1972; see Fein and Rogers, 1976 for a discussion of this work). Nevertheless, the results to date do not completely exclude some roles in these latter respects for very low activities of autolysins of known or unknown specificity. In discussing the topology of growth of steptococcal cells, it was pointed out (p. 20) that the new cell wall is derived by an apparent splitting and pealing apart of the septal wall. It is reasonable to suppose that some wall-hydrolysing enzyme should be involved in this process. Observations on the ultrastructural effects of autolysin in *Strep. faecalis* (Higgins *et al.*, 1970) show it to be the most active near, but not quite at, the right place. The observed position is at the leading edge of the septum rather than at its base where pealing apart of the wall occurs. Mutants of this organism with partially decreased autolytic enzyme activity also showed normal morphology, but the cells did not separate so well as did those of the wild-type (Pooley *et al.*, 1972). *Rod* mutants of *Bacillus subtilis* (see p. 52) growing as cocci do not have their morphology disturbed, apart from failure of the cells to separate, by a reduction of 95% in their autolytic activities. Further investigation is clearly necessary both to obtain structural mutants more completely deficient in autolytic activity, and to search for other relevant enzymes splitting peptidoglycan that possibly have not so far been recognized.

VI. Morphological Mutants and Shape Changes in Bacteria

A. HOPES AND DIFFICULTIES

Depressing pictures are sometimes drawn about the value of studying mutants disturbed in division or morphology. The difficulties of

TABLE 1. Broad classification of mutations and conditions leading to disturbed morphology

Morphological phenotype	Disturbance	Examples
Filamentous	Failure to form septa	Div$^-$ mutants of *Bacillus subtilis* and *Escherichia coli* (e.g. van Alstyne and Simons, 1971; Reeve *et al.*, 1970; Ricard and Hirota, 1973; Mendelson and Cole, 1972)
		Inhibition or interference with DNA synthesis (see Sargent, 1979 for a summary)
		Interference with peptidoglycan synthesis (Gardner, 1940; Hughes *et al.*, 1949; Schwarz *et al.*, 1961)
Mini-cell formation	Misplacing of septa	*div*A51, *div*A27, *div*A35, C403, *div*IV-A1 and B1 mutants of *Bacillus subtilis* (van Alstyne and Simons, 1971; Reeve *et al.*, 1973). Miniature cells of *Escherichia coli*; (Adler *et al.*, 1967)
Spherical	Increase in width	Rod$^-$ mutants of *Bacillus subtilis* and *Escherichia coli* (Rogers *et al.*, 1968; Boylan and Mendelson, 1969; Adler *et al.*, 1968)
		Effect of FI1060 on *Escherichia coli* (Lund and Tybring, 1972; Melchior *et al.*, 1973)
Failure to separate	Autolytic enzyme	*Lyt* strains of *Bacillus subtilis* (Fein and Rogers, 1976), *pgm*$^-$ strains of *Bacillus licheniformis* (Forsberg and Rogers, 1971) and *Bacillus subtilis*, (C. W. Forsberg and H. J. Rogers, unpublished work). Lyt$^-$ strains of *Staph. aureus* (Chatterjee *et al.*, Koyama *et al.*, 1977)
		Replacement of choline by ethanolmine in medium for growth of pneumococci (Tomasz, 1966)

interpretation are summarized by Henning (1973) as "The as yet unsolved problem inherent to all such problems is that of causality". A search for "causality" sets one on a logical and metaphysical pathway that leads inevitably into general consideration of "causes'. Science has usually progressed by formulation of hypotheses based on reproducible correlations, and attempts, successful or otherwise, to dispose of them. The problems inherent in studying the molecular basis of morphological events are immensely complicated compared with those involved in studying, say, a metabolic pathway. Nevertheless, it is surely a retreat into mysticism to regard them as of a different kind. Rather, must even more care than usual be taken in analysing the genotypes and phenotypes of any mutants obtained (cf. Slater and Schaechter, 1974) and more patience be exerted when gaps between demonstrated physiological and morphological events are revealed. Problems of the magnitude of cell division, sporogenesis or ordered surface growth are unlikely to be soluble in terms of any one concept or experimental approach. If in doubt, one only has to remember that there are already 55 identified gene functions for assembly of a seemingly simple structure like T4 bacteriophage (Wood and Revel, 1976) and that 30–40 operons have been computed to be involved in spore formation in *B. subtilis* (Hranueli *et al.*, 1974). Each operon almost certainly controls a number of genes.

B. TYPES OF MORPHOLOGICAL DISTURBANCE

Inspection of mutants with abnormal morphology allows the broad classification suggested in Table 1. A similar but more elaborate classification suggested elsewhere (Mendelson, 1977) differs in one major respect in its designation of the spherical shaped mutants as ones of "division plane" orientation. In Table 1, the width control aspect of these mutants has been emphasized on the basis of recent work with them (see p. 53).

C. FILAMENT FORMATION

A large variety of treatments of bacteria, particularly Gram-negative species, result in organisms failing to form septa and growing as filaments. The opinion has even been expressed (Slater and Schaechter, 1974) that any substance applied at some concentration will cause filamentation. Nevertheless, analysis of the literature suggests that effects on two

processes are the commonest cause of the phenomenon. These are upon aspects of DNA replication and upon peptidoglycan synthesis. It has been known almost since the discovery of penicillin that this antibiotic, applied in sublethal doses, will lead to filamentation of a wide variety of Gram-negative species (Gardner, 1940; Hughes *et al.*, 1949). The effect of penicillin on septation in relation to peptidoglycan synthesis was explored in greater detail by Schwarz *et al.* (1961). Other agents affecting peptidoglycan synthesis are also effective, for example, cycloserine, vancomycin and D-serine (Grula and Grula, 1964). The role of internal osmotic pressure on the phenomenon is of interest. Agents such as 0.66 M propylene glycol or 0.5 M methyl-D-glucoside, reverse the filament-forming effect of inhibitors of peptidoglycan synthesis in *Erwinia* sp. (Grula and Grula, 1964). On the contrary, some mutants of *E. coli*, dependent for growth on the presence of 0.5 M sucrose, grew as filaments in its presence but as normal lengthened cells in the presence of D-alanine, an essential component of peptidoglycan (Mangiarotti *et al.*, 1966). The effect of partially inhibiting cell-wall synthesis is not so universally effective in producing filaments from Gram-positive species. None is formed from *B. subtilis* with penicillins, cycloserine or vancomycin (H. J. Rogers, unpublished work). When low doses of benzylpenicillin acted on a penicillinase-negative mutant of *B. licheniformis*, there appeared to be a loss of control of wall formation with uneven thickening and gross distortion of septa, but no filament formation (Highton and Hobbs, 1971). Filaments are however formed from some streptococci with penicillin (Lorian, 1976; Lorian and Atkinson, 1976) but not from others (Higgins and Shockman, 1971). *Clostridium welchii* also forms filaments (Gardner, 1940; Crofts and Evans, 1950), although part of the "snake formation" described is due to failure of cell separation (R. Williamson, J. B. Ward and H. J. Rogers, unpublished work).

Analysis of the effects of penicillin-binding proteins (Spratt and Pardee, 1975; Spratt, 1975) may be able to rationalize this somewhat chaotic situation. By using radioactively-labelled benzylpenicillin and competition experiments with other penicillins and cephalosporins for the six penicillin-binding proteins present in the cytoplasmic membranes of *E. coli*, three were selected as being connected with maintaining the normal morphology of cells. One, designated protein 3, was thought to control septation and was inactivated by combination with β-lactams. This conclusion has been strengthened by isolation of two temperature-sensitive filamentous mutants of *E. coli* which both had a temperature-

TABLE 2. Properties of the penicillin-binding proteins in the cytoplasmic membrane of *Escherichia coli*. From Spratt (1975)

β-Lactam	Relative affinity constant for the penicillin-binding protein	Morphological effect of β-lactam	
		low concentrations	intermediate concentrations
Cephalosporidine	$1 > 2$ or 3	Lysis	Lysis
6-Aminopenicillanic acid	$2 > 1 > 3$	Ovoid cells	Lysis
Mecillinam (FL1060)	$2 \gg 1$ or 3	Ovoid cells	Ovoid cells
Benzylpenicillin	$3 > 1 > 2$	Filaments	Lysis
Ampicillin	$3 > 2 > 1$	Filaments	Filaments with bulges; lysis at higher concentrations

sensitive penicillin-binding protein 3 (Spratt, 1977a). None of the *fts* filamentous mutants of Ricard and Hirota (1973) was so affected.

The other two penicillin-binding proteins in the membrane of *E. coli* concerned with morphology are protein 1, related to cell length extension and since been shown to be complex both in nature and action (Tamaki *et al.*, 1977), and protein 2 concerned with shape determination (see Table 2). If proteins with similar functions but different affinities for penicillins exist in Gram-positive cells, then this together with the different physicochemical properties of their walls and their different internal osmotic pressure could account for the different behaviour of reagents affecting wall synthesis in these species of bacteria.

D. MINI-CELLS

Small anucleate cells are formed by strains of a number of species including *E. coli* (Adler *et al.*, 1967) and *B. subtilis* (Reeve *et al.*, 1973) bearing the appropriate genetic lesions. Two rather widely separated genes in *B. subtilis* have been found to cause mini-cell formation (Reeve *et al.*, 1973). Since these small cells, which are produced from about 30% of the divisions of *B. subtilis* (Coyne and Mendelson, 1974), have no DNA, they are unable to grow or to synthesize most macromolecules unless episomal DNA which can replicate is introduced into them. However, those from both *B. subtilis* (Mertens and Reeve, 1977) and *E. coli* (Reeve, 1977) are able to synthesize peptidoglycan thus providing very clear evidence for the independence of this process from other cellular macromolecular syntheses.

Because mini-cells are so very short, a high proportion of their surface should be derived from the old pole of the parent cell and from the new misplaced septum. Unless the latter is abnormal in it chemistry—which it may be—the surface of the mini-cell is thus essentially made of polar wall. In this respect, it is of interest that mini-cells only autolyse very slowly under a number of different conditions despite rapid autolysis of the parent cells (Reeve *et al.*, 1973; Mendelson *et al.*, 1974). This may be due either to the known resistance to autolysis of the polar walls of bacilli (see p. 22) or the absence of autolysins; they were only shown to be sensitive to the action of lysozyme. Very preliminary examination of the composition of the walls of isolated mini-cells (J. N. Reeve and J. E. Fein, unpublished work) showed no differences from those of vegetative cells.

E. SPHERICAL CELLS FROM ROD SHAPES

1. Arthrobacter *spp.*

Arthrobacter species are naturally occurring conditional morphological "mutants" that can grow either as rods or ovoid cocci. The common method of achieving the change in shape is either to grow bacteria in glucose-containing salts media, when they grow in the exponential phase as spheres, or to grow them as rods during the exponential phase of cultures in media containing organic nitrogen, such as peptone, or a salts medium with succinate as carbon source and then let them grow into the resting phase when the cells become coccoid. The use of better steady-state systems would clearly be desirable and chemostat cultures seem to offer such possibilities. In such glucose-limited cultures, the transition from rod to coccal morphology occurs over a limited range of growth rates that has a fixed relationship to the maximum (Luscombe and Gray, 1971, 1974). This is true for *A. crystallopoites* when the temperature of the culture is varied, and for other species of *Arthrobacter* with very different maximum growth rates. Whether the change is related to growth rate *per se* or to the steady-state concentration of metabolites in the culture is not clear.

The ultrastructural events involved in cell division and the morphological change have been investigated for *A. crystallopoites*, both by sectioning the organisms (Krulwich and Pate, 1971) and by using the carbon-replica technique (Kolenbrander and Holman, 1977). Both approaches suggest that, as the cocci multiply or become rods, a rotation of the cells occurs, giving rise to the "snapping" division seen under the light microscope, and the appearance of V forms in cultures of rods. Carbon replicas of the surfaces of the cocci showed bands, similar to those seen by a variety of techniques on *Strep. faecalis* and *B. subtilis* cells. In sections, scars were seen which resembled those on the surface of *B. subtilis* but which were different from the wall-band markers of *Strep. faecalis*. In *Strep. faecalis*, the compact wall-band markers move apart in a symmetrical fashion until they reach hemispheroid positions on the two new cocci. In *A. crystallopoites*, the bands moved asymmetrically across the surface forming an increasing angle with the old septum (see Fig. 7). This is due, it is suggested, to asymmetric insertion of new wall material after septum closure but before cell separation, in a manner illustrated in Fig. 7.

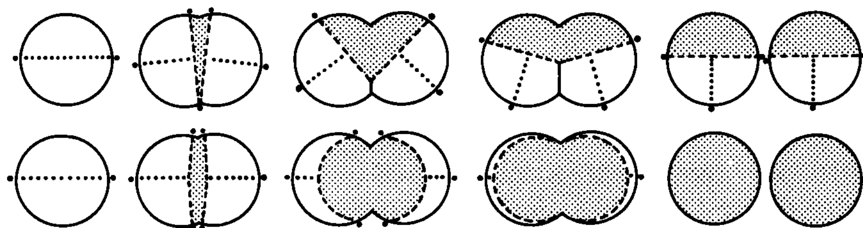

FIG. 7. Diagrammatic representation of the proposal for cell-surface growth and division of *Arthrobacter crystallopoites*. The horizontal line on the initial coccus represents the wall-band marker remaining from the previous division (modified from Kolenbrander and Holman, 1977).

A study of sectioned material clearly showed the presence of a double layer of wall, particularly in the region of the forming septum. Only the inner layer appears to invaginate and, when this has happend, the outer layer ruptures presumably because peripheral wall is fed out from the septal region at a faster rate into the inner layer than into the outer. The ruptured outer layer leaves the characteristic scars already mentioned, presumably also giving the bands seen in carbon replicas. Because inner wall synthesis does not always occur symmetrically, rupture of the outer wall occurs on one side only with the rotation of the cell, to give the snapping division already mentioned. The biosynthetic processes that give rise to these two layers of wall with apparently different growth rates is not understood. During the change from cocci to rods, similar asymmetric synthesis rotates the rod relative to the coccus. It is suggested (Kolenbrander and Holman, 1977) that the rod itself grows from the pole distal to the coccus.

A marked difference between the lengths of the glycan chains in the peptidoglycan from rod and spherical forms has been claimed (Krulwich *et al.*, 1967a). Those in the cocci were short whilst those in the rods were long. This appeared to be reasonably explained by the greater activity in the spherical form (Krulwich and Ensign, 1968) of an autolytic enzyme with lysozyme-like specificity. However, the situation probably merits further examination in the light of more recent observations (Ward, 1973) suggesting that the short chains found in other organisms are sometimes artefactual. Very early inactivation of autolytic enzymes in other bacteria allows the subsequent demonstration of long chains in peptidoglycans which had otherwise been thought to consist of short ones.

No correlation has been found between either glycan-chain length or autolytic enzyme activity and morphology in rod- and coccal-shaped mutants of *B. subtilis* after adequate precautions have been taken to inactivate autolytic enzymes (C. Taylor, J. B. Ward and H. J. Rogers, unpublished observations). A difference has also been claimed between the amino-acid composition of interpeptide bridges in the peptidoglycan from spheres and rods of *Arthrobacter* sp. (Krulwich *et al.*, 1967b). The rod form contained only L-alanine residues, but a proportion of the bridges in the peptidoglycan from the spherical form contained glycine residues. Recent studies, however, have not confirmed this difference (Schleifer *et al.*, 1976).

An interesting difference in the degree of compactness of wall material from the spherical and rod forms of *A. crystallopoietes* has been claimed (Marquis, 1973). The dextran-impermeable volume of walls from the rods was 11.1 ml/g whereas that from the coccal form was only 8.3 ml/g. This difference correlated with a similar difference between the walls from other cocci, such as streptococci and staphylococci, as compared with those from rods such as bacilli. The effect of growth medium and the growth phase of cultures upon wall compactness, however, has not been investigated.

2. *Proteus* "L-*forms*"

Before dealing with up-to-date work concerned with mutants of rod-shaped bacteria that can be grown as spheres, attention should be drawn to an early example which sometimes escapes notice and contains important lessons. Whilst isolating L-forms by a classical method involving growing *Proteus vulgaris* on penicillin-containing osmotically protective media, coccal-shaped variants that readily reverted to rods were isolated. Stable ones that would not revert were also isolated, and they were all called L-forms. However, all the unstable ones but, remarkably, none of the stable ones were found to contain a characteristic component of peptidoglycan, namely 2, 6-diaminopimelic acid (Kandler and Zehender, 1959). A relatively stable strain was eventually isolated (Martin, 1964) from the unstable variants. A spherical peptidoglycan layer could then be isolated which was cross-linked, despite the ready growth of the strain in the presence of high concentrations of penicillin (Martin, 1966, 1967). It is clear that these variants do not fit within the present-day definition of L-forms as wall-less cells, but they fit excellently

with the definition of Rod⁻ mutants and should be so regarded. Their affinity to some of the mutants isolated from *E. coli* strains, particularly those resistant to the unique penicillin mecillinam (FL1060), makes them worthy of further attention within this context.

3. *Round mutants from* Escherchia coli

Mutants of *E. coli* growing with approximately spherical morphology have been repeatedly described (Adler *et al.*, 1968; Normark, 1969; Henning *et al.*, 1972; Olden and Wilson, 1973; Matsuzawa *et al.*, 1973; Lazdunski and Shapiro, 1972; Matsuhashi *et al.*, 1974). Those which have been examined have peptidoglycans that do not differ from the wild-type; many if not all are more sensitive to lysis by neutral detergents than the wild-type, possibly indicating some abnormality in the outer membrane. Apart from these fairly general properties, the physiological phenotypes of the various mutants would seem to show considerable diversity. One was temperature sensitive, growing as rods at 30°C but spheres at 42°C (Henning *et al.*, 1972). Magnesium ions were necessary, and growth was improved by the presence of 12% sucrose. If neither was present, the rods changed to spheres, but they did not multiply and DNA synthesis stopped. This result agrees with the finding (Goodell and Schwarz, 1975) that the morphological change took place in the presence of nalidixic acid. In changing to spheres, the diameter of the cells approximately doubled with little or no increase in length of the original rod. Protein synthesis was necessary for the change. The spherical morphology of two of the mutants (Lazdunski and Shapiro, 1972; Olden and Wilson, 1973) could be corrected by rather high concentrations of D-alanine despite the normal kinetic parameters for uptake of the amino acid for L-alanine racemase and D-Ala-D-Ala synthetase. One of these mutants (Lazdunski and Shapiro, 1972) resembled the *Arthrobacter* sp. in having the morphology and the carbon source for energy in some way connected.

Isolation of a number of sphere-shaped mutants among those resistant to the antibiotic FL 1060 (Matsuhashi *et al.*, 1974) raises interesting problems. This unique penicillin itself produces ovoid cocci when acting on *E. coli* (Lund and Tybring, 1972; Melchior *et al.*, 1973) but does not inhibit either the transpeptidase or the D-alanine carboxypeptidase of the organisms (Park and Burman, 1973), which are the usual targets for the penicillin. Spratt (1975) considers that, in combining with a rather low-

affinity constant with penicillin-binding protein 2 (see Table 2), it inactivates the latter's ability to determine shape. If so, then it is reasonable to think that spherical mutants isolated as resistant to the antibiotic have lost functional shape-determining protein 2 from the cytoplasmic membrane, and indeed they appear to have done (Spratt, 1977b). Examination of the temperature-sensitive Rod^- mutants studied by Henning *et al.* (1972) showed them also to have an altered binding protein 2. Further temperature-sensitive Rod^- mutants were isolated and had modified proteins 2 such that they bound [^{14}C] benzylpenicillin at 30°C but not at 42°C (Spratt, 1977b).

4. *The* rod *mutants of* Bacillus subtilis

Conditional mutants of *B. subtilis* that grew as deformed cocci in a minimal salts medium in the absence of 0.8 M sodium chloride but as rods in its presence were first isolated in 1968 (Rogers *et al.*, 1968). These were called Rod^- since they appeared to be changed in having some salt-requiring characteristic necessary for formation of normal rods. Further work (Boylan and Mendelson, 1969; Boylan *et al.*, 1972; Rogers *et al.*, 1970; Rogers and McConnell, 1970; Karamata *et al.*, 1972) made it clear that several classes of mutants could be isolated with very similar morphological phenotypes but different physiological requirements necessary to effect the shape change. Some of the original mutants, although growing as rods in the presence of high concentrations of salt, were better maintained in this shape by addition of glutamate, glutamine or amino acids metabolically closely related to them; others were only sensitive to salt. The Rod^- mutant described by Boylan and Mendelson (1969), on the other hand, was medium-independent but temperature-sensitive and grew as cocci at 45°C and rods at 30°C. As originally described, this mutant failed to form colonies at 45°C. Subsequent work (Reeve *et al.*, 1972) showed that this was due to the presence of a separate mutation affecting DNA synthesis. The genetic lesions in the various types of Rod^- mutant strains mapped in different positions on the chromosome (Boylan *et al.*, 1972; Karamata *et al.*, 1972) and their walls showed different chemical compositions correlated with growth as rods or cocci (Rogers *et al.*, 1971; Boylan *et al.*, 1972). The temperature-sensitive mutation, called *tag*-1 (Boylan *et al.*, 1972) or *Rod* A (Karamata *et al.*, 1972), mapped near *his* A and strains bearing it had grossly decreased

amounts of teichoic acids in their walls when grown as cocci. The glutamate- and salt-requiring ones mapped between *phe* A and *leu*8, and had normal amounts of wall teichoic acid both as rods and cocci, but contained consistently more galactosamine in the walls of the rods (Rogers *et al.*, 1971). Presumably this indicated a higher wall concentration of the teichoic acid containing *N*-acetylgalactosamine and glucose-phosphate residues that was subsequently isolated (Duckworth *et al.*, 1972) from the parent. Two other groups of mutants, one called *rod* C, were also found (Karamata *et al.*, 1972; Rogers *et al.*, 1970) among those originally isolated. *Rod* C mapped in a similar but not identical position to *rod* A. It was only maintained as a rod by high concentrations of salt (glutamate was ineffective) and it had a lower content of wall teichoic acid when growing as a coccus. The other group of mutant strains always grew as cocci and the expression of the phenotype was not conditional. This group was called rod-5 originally (Rogers *et al.*, 1970) but subsequent work (M. McConnell and H. J. Rogers, unpublished work) showed that it was almost certainly a multiple mutant and was not examined further. The properties of various *B. subtilis* mutants showing rod-to-coccus transformations are summarized in Table 3.

The obvious points of departure for investigation of the relationships between morphology and physiology were the gross decrease in teichoic acids in the walls of *rod* A mutants when growing as cocci, and the need for glutamate, glutamine or salt to maintain the group B mutants as rods. However, before describing the detailed work, the eventual hopes of any such investigations need some consideration. A simple correlation between a morphological and a biochemical change in a cell, microbe or otherwise, might tell very little. The results of two other different approaches have to be integrated. Firstly, the crude morphological change must be analysed to give precise topological parameters; in the present example, the change from a rod shape to a spherical shape, we need to know the topology of the change in surface formation.

Fortunately, we already have the well documented growth of the *Strep. faecalis* surface already described (p. 20). In the rod forms, we can do no more than say that the length extension takes place somewhere in the cylinder whereas the poles are fashioned from the septal region. A change from one growth form to the other should nevertheless be relatively easy to demonstrate. Secondly, we need to find the relationship between the topological change in surface formation and the biochemistry; this is likely to be the most difficult part. Investigations of the *rod* mutants have

TABLE 3. Properties of mutants of *Bacillus subtilis* that change from rods to cocci

Genotype	Map position	Conditions for: Rods	Conditions for: Cocci	Wall	Autolysins
rod A (also known as tag-1)	*gta*A-*rod*A-*fla*C	30°C	45°C	cocci low in teichoic acids	low activity in cocci
rod B	*leu*A-*rod*B-*phe*A	high concentration of Mg^{2+}; halide anions or nitrate; low temperature; high concentrations of glutamate	low concentration of Mg^{2+}; high temperature; no glutamate; low ionic strength	normal in rods and cocci	normal
rod C	near *his*A	0.8 M sodium chloride	low ionic strength	cocci low in teichoic acids	not studied
(gtaC)		normal media	growth limitation by inorganic phosphate	cocci low in teichoic acids	low activity in cocci
phenotype is phosphoglucomutase$^-$ (pgm$^-$)					
glu 8332	not mapped	high concentration of glutamate	limiting glutamate	normal	not studied
Aconitase-negative mutant isolated as glutamate-requiring; (D. Karamata, unpublished observations)					

provided much evidence for the first two approaches, but so far only hints at the third.

Returning to the detailed investigation of the biochemistry, first of the *rod* A mutants, the gross decrease in wall-teichoic acid seems to be a firm handle by which to grasp the problem, whatever the reason for its occurrence. In support of the generalized contention that a decrease in negatively-charged wall polymers leads to a change from rod shapes to spheres are the observations with phosphoglucomutase-deficient mutants (pgm$^-$) and others disturbed in the formation of glucose 1-phosphate or UDP-glucose (Forsberg *et al.*, 1973; Forsberg and Rogers, 1974). Such mutants grow as deformed spheres when subjected to phosphate-limited growth. Their walls then have a grossly decreased content of negatively charged polymers like the *rod* A mutants grown at 45°C. The ultrastructural similarity between phosphate-limited pgm$^-$ and *rod* A or B mutants, grown as spheres, is striking. The occasional central incompleted septa formed at right angles to the main one in pgm$^-$ and *rod* A mutants may give some support to the idea that these cocci arise from two rods, first forming a hairpin or like structure with subsequent swelling and division of each cell (Mendelson, 1977). The similarity to this proposition and that for growth of *Arthrobacter* sp. is to be noted (see p. 39). If this occurs, the topology of the change in these mutants is likely to be different from that in the *rod* B mutants which has been explored in detail (Burdett, 1979a, b).

There is a technical complication of interpreting electron-microscope pictures of both the *rod* A and *pgm*$^-$ mutants, however, that may lead to difficulties. When growth of *rod* A mutants is changed from low to high temperatures, not only does the proportion of wall teichoic acids decrease but so also does the autolytic enzyme activity (Brown *et al.*, 1976). Although this latter loss is very unlikely to be connected with the morphological change itself (Rogers and Taylor, 1978), it leads to formation of long chains of unseparated cells which, possibly because of cell rotation (see p. 24), tend to form themselves into very complex masses of cells. It is difficult to cut transverse sections of individuals in such masses, and to be quite sure that aberrant-appearing septa are not parts of other contiguous cells cut in a glancing fashion. The *pgm*$^-$ mutants also have very low autolytic activities and grow in normal media as chains of unseparated rods but, under phosphate limitation in chemostat cultures, the groups of round forms do not seem to be so large as those of the *rod* A mutant, possibly because the chains are

TABLE 4. The phenotype of *Bacillus subtilis rod* A mutants

Mutant strain		Growth temperature 30°C	45°C
rod A (272 200ts)	1 Morphology	rods	cocci in masses
	2 Wall glycerol teichoic acid	normal	greatly decreased
	3 Peptidoglycan/cell mass	normal	increased about 3–4 times
	4 Glycerol teichoic acid/ cell mass	normal	decreased 2–3 times
	5 Galactosamine teichoic acid/cell mass	small	eliminated
	6 Autolysins	normal	very low
rod A *lyt* (230)		characteristics 2–5 as for *rod* A	
	1 Morphology	chains of rods	chains and masses of cocci
	2 Autolysins	very low	undetectable

mechanically broken by agitation of the cultures. In changing from phosphate- to magnesium-limiting growth, comma-like shapes have been seen in sections with one side only of thickened wall. This appearance is suggestive of asymmetric wall synthesis during the change from cocci to rods, and is again reminiscent of the suggestions for explanation of the snapping division of *Arthrobacter* spp. (see p. 39).

Reasons for the decrease in the proportion of wall teichoic acids in *rod* A mutants are themselves complicated. The enzymes concerned with glycerol teichoic acid synthesis are not lost when the growth temperature is raised (Boylan *et al.*, 1972). Examination of the amounts of wall substances formed when the temperature is changed from 30°C to 45°C shows (Table 4) that there is a rapid and large increase in the amount of peptidoglycan per unit mass of cells (Rogers *et al.*, 1974b; Rogers, 1977). This increase is unlikely to be due to diminished wall turnover due to loss of autolytic activity, as has been suggested (Young *et al.*, 1976), since double *lyt rod* A mutants show a similar increase despite the virtual absence of autolytic activity at either the low or high temperature of incubation. Moreover, the increase in peptidoglycan content is inhibited by stopping protein synthesis (Rogers and Taylor, 1978) which should not be so if it is explained by loss of autolytic activity. Wall-membrane preparations made from the mutant grown at 45°C are several times more active in making peptidoglycan than when they are made from organisms grown at 30°C (M. V. Hayes, J. B. Ward and H. J. Rogers, unpublished observations). It is therfore likely that a derepression of peptidoglycan synthesis has taken place, and a large part of the decreased proportion of wall teichoic acid is due to dilution with peptidoglycan. Formation of glycerol teichoic acid per unit mass of cells is, however, also somewhat decreased at higher growth temperatures (Rogers and Taylor, 1978) despite earlier preliminary results (Rogers *et al.*, 1974a). During growth at 45°C, the galactosamine-containing teichoic acid is also entirely lost from walls of *rod* A mutants (Boylan *et al.*, 1972). When membrane preparations are made from the mutant or wild-type grown at 30°C, they actively synthesize this teichoic acid from UDP-*N*-acetylgalactosamine and UDP-glucose. After growth of the mutant strain at 45°C, no synthesis can be obtained with such preparations, although the wild-type grown at this temperature yields fully active membranes (Hayes *et al.*, 1977). These various pleiotropic phenotypic changes are set out in Table 4. Which one is related to the morphological change?

TABLE 5. Effect of temperature, anion and magnesium ions upon the growth rate and morphology of *Bacillus subtilis rod* B-1 (strain 104)

Growth temperature (°C)	Anion species[a] (15 mM)	K_s[b] Mg^{2+} conc^n	Morphology[c] description	Morphology[c] length/radius
25	none	not determined	rod	3.92
30		1.5 mM	coccus	2.10
35		3 mM	coccus	2.10
20	chloride	not determined	long rod	5.24
30		10 μM	rod	3.64
35		1.2 μM	coccus	2.21
30	bromide	2 μM	long rod	4.60
35		200 μM	rod	4.14
45		not determined	coccus	2.10
30	nitrate	2 μM	rod	not determined
35		200 μM	rod	not determined
45		not determined	coccus	not determined

[a]Concentration of anion added to basal medium made to contain only SO_4^{2-} and organic anions (Rogers *et al.*, 1976).
[b]The concentration of Mg^{2+} required to allow growth at half of the maximum rate.
[c]Morphology of the cells determined in media containing 10 mM Mg^{2+} and 15 mM anions.

We may possibly eliminate loss of the *N*-acetylgalactosamine-glucose phosphate wall polymer because pgm$^-$ mutants cannot make it and have no galactosamine in their walls (H. J. Rogers and C. Taylor, unpublished observations) but, nevertheless, they grow as normal rods unless subjected to phosphate limitation. The proportions of total teichoic acid in the walls of *rod* A type mutants and their revertants appear to bear a relationship to cell morphology; the lower it is, the more coccal are the cells (Shifflet *et al.*, 1977). So far, no clear evidence has been obtained for a dissociation between coccal morphology, increased cellular peptidoglycan, and a decreased proportion of teichoic acid in the walls. However, in the presence of chloramphenicol, some evidence for a partial shape change has been seen without the concomitant effects on wall polymers (Taylor and Rogers, 1979). Apart from this isolated observation, the loss of negatively charged wall polymers seems to remain closely correlated with the change to a spherical shape. Teichuronic acids can substitute in this function for teichoic acids, since normal wild-type organisms remain rod in shape despite almost complete substitution of teichoic acids by teichuronic acid during phosphate-limited growth.

The *rod* B mutants (Fig. 8) quickly show that decrease in negative charge in the walls is not the only way to achieve the shift in topology of surface growth necessary to give spheres instead of rods. These mutants do not lose their glycerol teichoic acid when grown as spheres, although under many circumstances their walls show a decrease in the content of galactosamine (Rogers *et al.*, 1971; H. J. Rogers and P. F. Thurman, unpublished observations). This latter change can be eliminated, however, since circumstances can be found under which the morphological change occurs without it happening. Detailed study has shown (Rogers *et al.*, 1976; Rogers and Thurman, 1978b) that the morphology of *rod* B mutants is under the control of two medium constituents, namely Mg^{2+} and certain specific anions, together with growth temperature. These factors are interrelated in such a way that the temperature required for the change from rods to spheres is dependent upon Mg^{2+} concentration and, more critically, on the presence, nature and concentration of the anion. The active anions are Cl^-, Br^-, I^-, NO^{3-} and glutamate or glutamine. In the presence of 10 mM Mg^{2+}, the anions are active at concentrations from 5–15 mM. The rate of growth as well as the morphology of the mutant are likewise affected by the same three factors (Table 6). For example, in the absence of anions, growth is

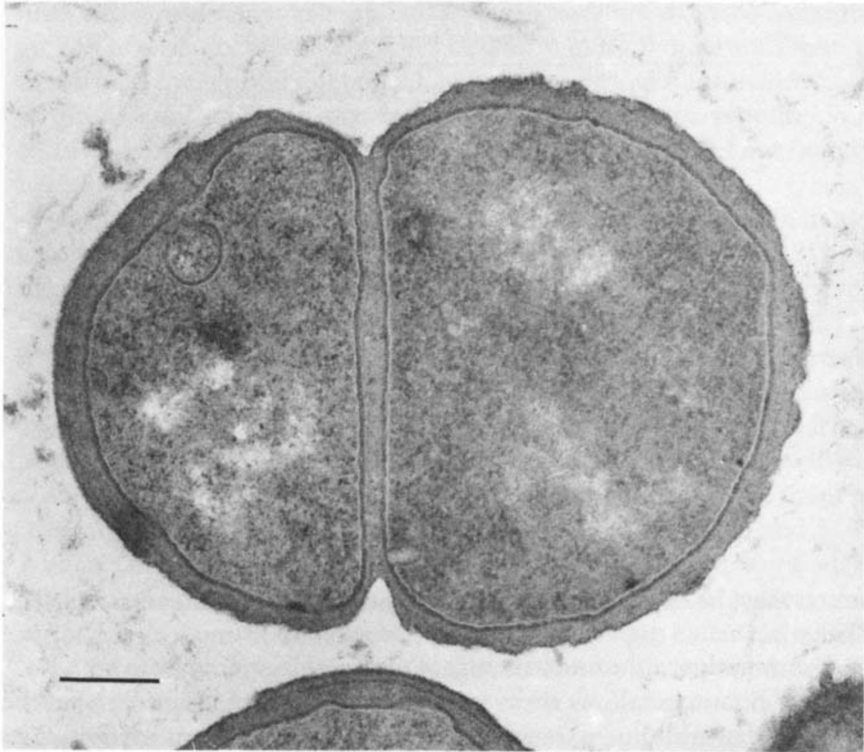

FIG. 8. Transverse section of a *rod* B1 mutant of *Bacillus subtilis* growing at 42°C in the basal medium of Rogers *et al.* (1976) supplemented with 10 mM Mg^{2+}, but with no additional anions. Bar equals 0.2 μM.

slow and the Ks value for Mg^{2+} (the concentration required for half maximum growth rate) is hardly affected by temperature. Addition of 15 mM Br^- decreases the Ks value for Mg^{2+} at 30°C from 1.5 mM to 2μM and, if growth temperature is raised to 35°C, it falls still further to about 0.2 μM a value close to that for the wild type. These dramatic changes are not reflected in the kinetics for transport of $^{28}Mg^{2+}$ and, of course, do not occur at all with the wild type. At half of the maximal growth rate, the mutant strain always grows as cocci irrespective of the absolute growth rate and of which combination of controlling parameters is used to obtain it. The fact that the characteristics for transport of Mg^{2+} into the cell are normal, combined with nearly unchanged composition of the wall and distribution of Mg^{2+} (P.F. Thurman and I. D. J. Burdett, unpublished observations), all tempt one to suggest that the effects of the cation and

TABLE 6. Wall components of *Bacillus subtilis rod* A (strain 172 ts200) in terms of bacterial dry weight

Component	Content (nmoles/mg dry wt) Growth temperature	
	30°C	45°C
Muramic acid	48.1	198.6
Glucosamine	65.0	251.9
2,6-Diaminopimelic acid	67.8	280.1
Glutamic acid	60.7	285.5
Alanine (total)	109.1	451.5
Ester alanine (chemical)	17.9	12.05
Ester alanine (^{14}C incorporation)	13.9	10.0
Galactosamine	21.0	"0.1
Phosphate (total)	204.0	62.7[a]
Glycerol	180.0	56.1[a]
Glucose	159.7	18.3

[a] These values have been found to be variable and in other experiments have been as high as about 100 nmoles/mg.

anion must be expressed at the level of the cytoplasmic membrane in the bacteria.

Examination of the fine structure of the peptidoglycans from both *rod* A and *rod* B mutants shows them to be indistinguishable from those in the walls of the wild-type irrespective of whether the mutants are grown as rods or cocci. Increase in the temperature of growth of *rod* B strains also leads to some increase in the amount of peptidoglycan per unit of mass of cells. This is entirely a temperature effect, however, and conditions can be found under which the morphology changes with very little affect on peptidoglycan synthesis. Again, autolysins are not likely to play a role in the morphology. Double *lyt rod* B mutants which have only 5% or less of the autolytic activity shown by *rod* B strains show the same changes, under the same sets of conditions, as the fully autolytic *rod* B strain, with the difference that long strings of unseparated rods and cocci are formed.

The topology of surface growth of the rod and the coccal forms of *rod* B mutant strains has been examined (Burdett, 1979a, b) by the section-rotation method introduced by Higgins (1976). The rods show the same type of surface growth as the wild type, with the cylindrical part of the surface extending by unknown methods but the poles forming from the septal region. Pole formation accounts for only about 15% of the total wall during growth of the cells. In the cocci, on the other hand, all of the area of the new peripheral wall is derived

from the septal region. One notable difference, however, between the growth of *Strep. faecalis* and of the coccal form of *rod* B mutants is that, in the former, the septum does not close until a very late stage in formation of the new cell pole. In the coccal-shaped mutant, the septum closes relatively early but the new cell pole still continues to be fed out from its base.

During the change from rods to cocci, the volume of the cells increases unless bromide anions are present (Rogers and Thurman, 1978b). This is the reverse of the situation in *Arthrobacter* sp. where the volume of the rod is greater than that of the coccus (Luscombe and Gray, 1971, 1974). The spheres of the *rod* B mutants always have a lower ratio of surface area to volume than the rods. It would seem that an essential aspect of the change from rod to spherical morphology is an increase in width of the cell at a point approximately equidistant from the poles. A width increase will still occur when DNA synthesis is stopped by removing the supply of thymine to a rod B1 *thy* $PBSX^-$ strain (H. J. Rogers, P. F. Thurman and R. S. Buxton, unpublished observations), or by adding nalidixic acid to cultures of either *rod* B or *rod* A (Reeve *et al.*, 1972); protein and peptidoglycan synthesis, however, must occur. Length extension of *rod* B cells, either in the presence or absence of DNA synthesis, appears to be greatly diminished when conditions are changed so that swelling occurs. This is an interesting relationship with the observation with the double *rod* A div IV–A1 or –B1 mutants of *B. subtilis* growing at 45°C (Mendelson and Reeve, 1973) where it appeared that length extension of the cell occured only in the region that was swelling. However, no comparison was made of the rate of cell-length extension of the double mutant with that of the wild type growing at the same temperature.

In light of the evidence with *rod* B, it would seem a reasonable, though no doubt an over-simplified, hypothesis to suppose that an essential characteristic of the *rod* B mutation is to code for a cell-length extension protein that can be very easily heat inactivated. Whether this is localized or diffuse in the cylinder of the cell is irrelevant. Presumably such a protein must either be protected by a particular combination of Mg^{2+} and anions that are capable of modifying the temperature at which the morphological change occurs, or the protein remaining at this temperature can work more effectively in the presence of the ions. Decrease in rate of length extension with a normal rate of cell division would be expected to be capable of producing oval or even round cells, essentially consisting of cell poles. However, these cells would have the

same diameters as the rods but much smaller volumes. In the rod mutants, the volumes of the cocci are greater in most circumstances, although in the presence of bromide they have about the same volume; but they are never smaller. Thus wall and membrane growth that would have extended the length of the rods makes the cell girth greater by about two-fold under some conditions (I. D. J. Burdett, unpublished observations). Circumstances can be chosen such that the rate of peptidoglycan synthesis per unit mass of cells remains approximately constant during the morphological change (H. J. Rogers and P. F. Thurman, unpublished observations). The conditions were a constant temperature of incubation of 35°C with a replacement of chloride with bromide ions in the growth medium. There is then only a small increase in the numbers of cells per unit mass, and a correspondingly small change in the rate of synthesis of peptidoglycan per cell. But, since the surface-volume ratio of the cells increases significantly in changing to rods (Rogers and Thurman, 1978a), the average wall thickness in the rods formed should be smaller.

The constant rate of peptidoglycan synthesis during the morphological change suggests that either the supply of precursors for wall synthesis is rate limiting and that it is used by biosynthetic enzyme sites in the region of the septum rather than the cylindrical region of the cell, or that there is activation or formation of enzymes in the septal region to about the extent of those inactivated in the cylinder of the cell. Protein synthesis is necessary for the morphological change, and this may favour the second explanation. The rod-shaped organisms undoubtedly swell in their central region before the septum initials have appeared. Therefore, unless movement of the cell poles apart is constrained by internal structures, some feature, presumably the direction of assembly of wall polymers, must account for the radial expansion of the mid-region of cells. One speculation is that wall polymers of the orientation and cross-linkage, such as normally form septa, may instead be incorporated into the peripheral wall to enlarge the diameter of the cell.

To return to interrelationships between the *rod* A and *rod* B mutants, the common theme between them, apart from the rod-coccus morphology, would seem possibly to be the supply of Mg^{2+} to the membrane. A principal function for teichoic acids, as it has been suggested, is to regulate the supply of cations, and in particular Mg^{2+}, to the cell (Heckels *et al.*, 1977; Heptinstall *et al.*, 1970). The grossly decreased amount of envelope Mg^{2+} due to the smaller number of

fixed negative charges in the wall of *rod* A mutants may prevent normal functioning of the membrane enzymes at the length-extension site. However, as has been pointed out (Ou *et al.*, 1973), divalent cations neutralizing fixed negative charges in the wall are not free to leave the wall, since electroneutrality must be preserved. Teichoic acid in the walls of *Staph. aureus* did not appear to protect the organisms against Mg^{2+} deprival. Other work (Hoover and Gray, 1977) claims, however, that staphylococcal mutants lacking teichoic acid could not grow in a Mg^{2+}-limiting medium. Also to be taken into account, with *rod* A mutants, is the known alteration of the volume of walls that occurs when teichoic acid is missing from them (see p. 9). The contraction may alter the conformation of the peptidoglycan which, in turn, might be perpetuated by addition of newly synthesized material. Such knowledge as we have about the later stages of this process (see p. 15–17) predicts that the conformation of pre-existing peptidoglycan would be expected to have a major effect on that of newly laid-down material. It may be significant that cocci, in general, appear to have more compact walls than rods (Marquis, 1968).

VII. Conclusions

Among the simplest and most easily defined changes in the shape of micro-organisms is that from rods to spherical-shaped bacteria or from spherical protoplasts to rod-shaped bacilli. Spore formation and cyst formation should probably be excluded because they involve specialization as well as the morphological change. Several mutations and physiological conditions can bring about this simple change in a number of different species of bacteria. Evidence at least from *B. subtilis rod* B would suggest that it involves a true modification of growth zones so that the original rod-natured growth of the bacillus is transformed nearly completely to a truly streptococcal-like process. When we look at the correlated physiologies and molecular events in such situations, we find a seemingly bewildering variety of changes. Most chastening of all, however, is the failure to recognize any differences in wall composition during the very significant later morphological stages of reversion of protoplasts to bacilli. Whilst disappointing in not showing immediately and clearly the pathway forward, this picture is, nevertheless, by hindsight not surprising. Most of the results have been culled from experiments designed to measure average phenomena. Wall

composition, biosynthetic mechanisms and the physical properties of walls all refer to large populations of whole bacteria or broken preparations made from them. The phenomena we wish to study can now clearly be seen to affect only parts of the surfaces of individual bacteria. We, therefore, need to design experiments and techiques which will investigate the topological distribution of the bulk phenomena that have been discoverd to be correlated with morphological changes.

A complication of work with morphological mutants is the plethora of molecular and physiological changes that arise from what are apparently single mutations. One way to help in overcoming this difficulty is to find two or more quite different sorts of mutations that have only some of the biochemical changes in common, but which result in the same morphology. An example is that of the *rod* A mutants of *B. subtilis* grown at 45°C and those deficient in the enzyme phosphoglucomutase which, when grown under phosphate limitation, arrive at a closely similiar morphology. The reasons for the absence of negatively-charged wall polymers in the latter but not in the former mutants are easily explicable in terms of a lesion with known biochemical sequelae, yet the morphology of both types is closely similar if not identical; *rod* A mutants have wild-type levels of phosphoglucomutase. It then seems less likely that the lesion leading to the morphological change in *rod* A mutants is unconnected with the alteration in wall composition. More such examples would be useful. The recognition of a protein in the cytoplasmic membrane of *E. coli* that can possibly control the interrelationship between longitudinal and radial growth in the organisms raises hopes of uncovering a general mechanism of shape maintenance. When inactivated by mutation or by a combination with a β-lactam antibiotic, coccoid-like cells result. Result so far obtained with *rod* B mutants would be explicable by the presence of an analogous protein which is temperature-sensitive and which has some special ionic requirements.

Finally, the relations between wall composition and properties in bacteria need further thought. For example, the large changes in wall volume that can be brought by altering their fixed charges could have profound effects if they occur locally in the expanding wall of a growing cell with its high internal osmotic pressure. Moreover, magnification of conformational alterations might be expected to be caused by charge alterations in newly-synthesized material on its way to be incorporated into the old wall. It might be possible that such alterations would even modify the directions of addition of new to old peptidoglycan by

transpeptidation. For example, it could be changed from an addition that is essentially longitudinal to one that is radial. The localized presence of very small activities of an autolysin of appropriate specificity might cut the glycan chains in the peptidoglycan, as we know they are cut *in vivo*, to enable them to lie radially in the wall. The intense osmotic pressure exerted during the process might manage to expand locally the girth of a rod-shaped organism. It will be remembered that alterations in fixed charges can be brought about either by structural modification of the wall, such as alterations in the amount of esterified D-alanine added to the teichoic acids, or by alterations of ionic strength or hydrogen-ion concentration in the environment of the walls. Such changes could be highly localized as well as general and be the basis of the very localized alterations in the bacterial surface that we now know eventually bring about profound general changes in shape.

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Note added in proof: Two important papers have recently appeared about the growth and function of the outer membrane of Gram-negative bacteria. (a) Insertion of an outer membrane protein is random over the surface (Smit and Nikaido, 1978) and occurs at points of envelope adhesion (Bayer, 1968). (b) Mutants of *E. coli* deficient in lipoprotein and one or more of the outer-membrane proteins grow as spheres and have abnormal divalent cation requirements (Sonntag *et al.*, 1978).

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Spermine, Spermidine and Putrescine in Fungal Development

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I. Introduction

Despite the substantial increase in the number of papers concerning polyamines published in the last ten years, this important group of molecules is unrecognized by many people as belonging in the mainstream of biochemistry. Another misunderstanding of a different nature lies in the term "polyamine" which to some people may imply a group of macromolecules. The amines which are generally included under this name are in fact aliphatic diamines, triamines and tetra-amines together with their derivatives. Oligoamine is therefore a more precise terminology (cf. oligosaccharide, oligonucleotide, oligopeptide) and will be used throughout this review.

Unlike most other low molecular-weight organic compounds, such as sugars, amino acids, carboxylic acids and nucleotides which are widely distributed throughout prokaryotes and eukaryotes, oligoamines appear to serve neither as precursors of macromolecular cell constituents nor to supply energy necessary for biosynthetic or other endergonic processes. They form the end of an anabolic sequence. A number of different naturally-occurring oligoamines exist (see Section IIA, p. 66) but only three are known to be widely distributed, namely putrescine (1,4-diaminobutane), spermidine (4 azaoctane-1,8-diamine) and spermine (4,9 diazadodecane-1,12-diamine). No example of a cell devoid of all three of these amines has yet been reported. A fairly comprehensive range of organisms has now been examined and oligoamines have been shown to be present in viruses (Lanzer and Holowczak, 1975; Fukuma and Cohen, 1975), bacteria (Tabor and Tabor, 1972), fungi (Viotti *et al.*, 1971; Nickerson *et al.*, 1977a, b; Hart *et al.*, 1978), algae (Rolle *et al.*, 1971), protozoa (Pösö *et al.*, 1976; Bacchi *et al.*, 1977), higher plants (Smith, 1971, 1975), arthropods (Wyatt *et al.*, 1973), molluscs (Gould and Cottrell, 1974), echinoderms (Manen and Russell, 1973), fish (Seiler and Lamberty, 1973; Sturman *et al.*, 1976; Manen *et al.*, 1976), amphibia (Russell *et al.*, 1969), birds (Raina, 1963; Caldarera and Moruzzi, 1970; Caldarera *et al.*, 1965; Seiler and Lamberty, 1975) and mammals (Bachrach, 1973). The concentrations of oligoamines present in different organisms are quite variable, and range between 10^{-6} and 10^{-2}M.

The reason for the increased interest in oligoamines is that a vast amount of evidence points to a clear correlation between oligoamine synthesis and the onset of growth (Tabor and Tabor, 1976a; Jänne *et al.*,

1976). However, apart from this correlation, the basic question of oligoamine function is still not resolved. Prior to 1970, it was generally thought that isolation of putrescine-requiring auxotrophs of *Esherichia coli* would offer the best experimental approach to understanding the function of oligoamines. The isolation of such mutants in 1970 (Morris and Jorstad, 1970; Hirschfield *et al.*, 1970) has been valuable, but the use of these mutants has so far been unsuccessful in resolving oligoamine function.

Since there is a correlation between oligoamine synthesis and cell growth and differentiation, and since the latter process cannot be easily studied in bacteria, fungi have a number of advantages in studying differentiation. Fungi exhibit a wide variety of developmental patterns many of which can be varied under laboratory conditions. Also, extensive genetic analysis can often be carried out on fungi (see King, 1974). They are therefore a particularly suitable phyllum in which to study the role of oligoamines in development, and this in turn may help to solve the more general question of oligoamine function. There are distinct differences between oligoamine synthesis and nucleic acid synthesis in bacteria as compared with higher eukaryotes, and it is therefore of interest to ascertain how fungi bridge the gap between prokaryotes and higher eukaryotes. The evolution of fungi is a controversial issue (Klein and Cronquist, 1967) and attempts have been made to use biochemical criteria (Hall, 1969; Bartnicki-Garcia, 1970) in classification. Knowledge of oligoamine synthesis and distribution in fungi may help in this respect.

In the first section of this review, we consider briefly the distribution and biosynthesis of oligoamines with particular emphasis on the contrast between prokaryotes and eukaryotes. For more detailed reviews on oligoamines, see Cohen (1971), Williams-Ashman *et al.* (1972), Bachrach (1973), Tabor and Tabor (1972, 1976a), Morris and Fillingame (1974) and Raina and Jänne (1975). The second section considers oligoamine distribution and synthesis in fungi, and is followed by a section on related amino-acid biosynthesis. Fungal development, with emphasis on correlations between nucleic acid, protein and oligoamine synthesis, is the theme of Section V. We discuss possible roles of oligoamines in the final section.

II. Oligoamines in Prokaryotes and Eukaryotes

A. TYPES OF NATURALLY-OCCURRING OLIGOAMINES

The oligoamines which are most widely distributed in organisms, and which occur in the highest concentrations, are putrescine, spermidine and spermine. These usually occur as the free bases although they may also be conjugated as the acetyl or glutathionyl derivatives in, for example, *E. coli* (Tabor and Dobbs, 1970; Tabor and Tabor, 1975) and in certain tissues (Nakajima *et al.*, 1969; Seiler *et al.*, 1973; Blankenship and Walle, 1977). Other more complex derivatives have been found in alkaloids (see Smith, 1971), antibiotics (Hettinger and Craig, 1968) and modified nucleotides (Kelln and Warren, 1973).

Other oligoamines which appear to have a much narrower distribution, or to occur only under certain physiological conditions, have also been identified, e.g. 1,3-diaminopropane (Smith, 1970; North and Turner, 1977), 1,5-diaminopentane (Dion and Cohen, 1972), 4-azaheptane-1,7-diamine (De Rosa *et al.*, 1976; Stillway and Walle, 1977), 4-azanonane-1,9-diamine (Kuttan *et al.*, 1971; Smith, 1977a; Kneifel *et al.*, 1977), 4,8-diazoundecane-1,11-diamine (Oshima, 1975; Stillway and Walle, 1977), 2-hydroxyputrescine (Kullnig *et al.*, 1970) and *N*-(4-aminobutyl)-3-aminopropionic acid (Nakajima and Matsuoka, 1971). It seems likely that some of these oligoamines may serve quite different functions from putrescine, spermidine and spermine, although 2-hydroxyputrescine and, under certain conditions, 1,5-diaminopentane, may substitute for putrescine in the cell (Dion and Cohen, 1972; Kullnig *et al.*, 1970).

B. CHEMICAL PROPERTIES OF OLIGOAMINES

Oligoamines are simple organic compounds and their chemistry is fairly well understood. We mention here only properties which may have relevance to understanding their biological function. Putrescine, spermidine and spermine are all very soluble in water and, at physiological pH values i.e. around neutrality, have an average of 2, 2.5 and 3 positive charges, respectively. The pK values of the amino groups have been determined by different workers and are shown in Table 1. A number of studies have been made on complex formation between oligoamines and transition metals (Bertsch *et al.*, 1958; Schwarzenbach and Szilard, 1962; Barbucci *et al.*, 1970; Paoletti *et al.*, 1973; Ahuja and

TABLE 1. pK Values of putrescine, spermidine and spermine

Oligoamine	Values for				Conditions	Reference
	pK_1	pK_2	pK_3	pK_4		
Putrescine	9.3	10.4			22°C	Rometsch *et al.* (1951);
	9.35	10.8			20°C	Bertsch *et al.* (1958)
	9.6	10.8			25°C; 0.5M $NaNO_3$	Barbucci *et al.* (1970)
Spermidine	8.41	9.81	10.89		25°C; I = 0.1M	Palmer and Powell (1974)
Spermine	8.19	9.05	10.21	10.99	25°C; 0.15M NaCl	Hirschman *et al.* (1967);
	7.96	8.85	10.02	10.8	25°C; I = 0.1M	Palmer (1974)

I indicates ionic strength.

Singh, 1975; Cox *et al.*, 1976). The most stable chelation complexes are usually five-membered rings (Cotton and Wilkinson, 1962); thus, although 1,2-diaminoethane will form stable chelation complexes, the stability decreases with increasing number of methylene groups. Thus, it seems doubtful whether the weak chelating ability of putrescine, spermidine and spermine are important properties in respect of their biological function. Their ability to bind strongly to polyanions such as nucleic acids is almost certainly of much greater importance. This is discussed further in Section D (p. 71).

C. PHYLOGENETIC DISTRIBUTION OF OLIGOAMINES

With few exceptions, bacteria investigated so far contain spermidine and putrescine but not spermine (Tabor and Tabor, 1972). *Bacillus stearothermophilus* contains spermine in addition (Stevens and Morrison, 1968), but it has not been proven that this organism synthesizes spermine as opposed to incorporating it from the medium on which it grows. Thus, spermine synthesis appears to be either rare in or absent from bacteria. Oligoamine concentrations are generally higher in Gram-negative bacteria than in Gram-positive bacteria. *Escherichia coli* is the only prokaryote to have been investigated systematically; when grown on minimal medium, the intracellular concentration of putrescine may be about 0.019 M and of spermidine 0.006 M (Tabor and Tabor, 1972). The putrescine concentration depends on the osmolarity of the growth medium; increasing the osmolarity causes a decrease in the content of intracellular putrescine, although the spermidine concentration is scarcely affected (Munro *et al.*, 1972).

In eukaryotes, the predominant oligoamines are generally spermidine and spermine with putrescine being present in much lower concentrations, although the last compound is the precursor of both spermidine and spermine in these organisms. This is discussed further in connection with the properties of *S*-adenosylmethionine decarboxylase (E.C. 4.1.1.50) in Section E (p. 75).

D. INTRACELLULAR DISTRIBUTION OF OLIGOAMINES

Understanding the function of oligoamines would be greatly assisted by information on their intracellular distribution. The problems of studying intracellular distribution have been discussed in many previous reviews

(Tabor and Tabor, 1964; Stevens, 1970; Raina and Jänne, 1975). They stem from the redistribution of oligoamines which occurs during isolation of subcellular organelles due to their high solubility and charge. Autoradiographic techniques are also difficult to apply because oligoamines are very soluble and because there is no precursor which is specific for oligoamines. Attempts to circumvent the problem of redistribution have been made, but none has been very successful so far. The approaches which have been used are: (i) comparison of normal and enucleated cells, (ii) use of cross-linking agents to fix oligoamines, (iii) use of organic solvents to isolate subcellular organelles, and (iv) measurement of binding constants for oligoamines binding to cell components. These approaches will be discussed briefly in turn.

A comparison of normal and mini cells of *E. coli* has been made (Michaels and Tchen, 1968). The mini cells contain about 3% of the amount of DNA present in normal cells. Evidence from this work suggests that there is no preferential binding of putrescine and spermidine to DNA. Recently, McCormick (1977) prepared enucleated mouse L cells by centrifuging monolayers in the presence of cytochalasin B. He found spermine and spermidine to be present in both the nucleus and the cytoplasm, but the concentration of both amines in the nucleus was about three times that present in the cytoplasm. It has been shown that spermidine does not accumulate during development of an anucleolate mutant of *Xenopus laevis* (Russell, 1971).

Attempts have been made to crosslink oligoamines to nucleic acids, proteins (Margolles *et al.*, 1973) and to ribosomes (Stevens and Pascoe, 1972). The crosslinking reagents used (formaldehyde and dinitrodifluorobenzene) both react more readily with proteins than with nucleic acids. But, in view of the ready exchange of oligoamines on and off ribosomes which is known to occur (Tabor and Kellog, 1967), it will be necessary to compare the exchange rate with the rate of covalent fixation of oligoamines. If conditions could be found in which the exchange rate was slow compared with the rate of covalent fixation, then this approach might become more useful.

The use of organic solvents for isolating nuclei can be advantageous for determining the intracellular distribution of low molecular-weight substances which readily leak out of the nucleus if aqueous media are used (Busch, 1967). When this method was applied to rat-liver and calf-thymus nuclei, it was found that there is no preferential concentration of spermine and spermidine in the nuclei as compared with the cytoplasm

TABLE 2. Binding of olagoamines to nucleic acids and ribosomes

Ligand	Nucleic acid	Ionic conditions	K_1 (M^{-1})	n_1	K_2 (M^{-1})	n_2	Reference
Spermine	DNA	0.15M NaCl; pH 6.5	1.31×10^4	0.058			Hirschman *et al.* (1967)
Spermidine	DNA	0.1M NaCl; pH 7.5	2.48×10^3	0.20			Rubin (1977)
Spermidine	DNA	0.2M NaCl; pH 7.5	4.35×10^2	0.19			Rubin (1977)
Spermidine	tRNA	0.05M KCl–0.05M cacodylate; pH 7.0	6.6×10^4	0.031	7.6×10^3	0.19	Sakai *et al.* (1975)
Putrescine	ribosomes	0.1M KCl–0.01M $MgCl_2$ –0.01M tris; pH 7.2	8.0×10^2	0.17			Turnock and Birch (1973)
Spermidine	ribosomes	0.1M KCl–0.01M $MgCl_2$ –0.01M tris; pH 7.2	6.95×10^3		4.2×10^2		Turnock and Birch (1973)
Spermine	ribosomes	0.1M KCl; pH 7.0	5.9×10^4	0.127			Stevens (1969)

K indicates the association constant; n the number of moles oligoamine/mole nucleic acid phosphate.

(Stevens, 1966). The method suffers the disadvantage that it is difficult to prepare clean nuclei since cytoplasmic material is readily absorbed onto the nuclear surface during isolation (Busch, 1967), and also the method can only be used to determine intranuclear and extranuclear distribution.

A number of studies have been made of the binding of oligoamines to nucleic acids, and to nucleic acid-containing organelles. From these studies it is possible to state how many oligoamine molecules can bind to a nucleic acid and how strongly they bind. If oligoamines are free to diffuse and equilibrate within a cell, as seems plausible, then information on the binding constants can indicate to which sites they are likely to bind. The binding constants have been determined by different authors under different conditions. In Table 2 we have given a selection of these binding constants, selected so that comparisons can be made under as similar

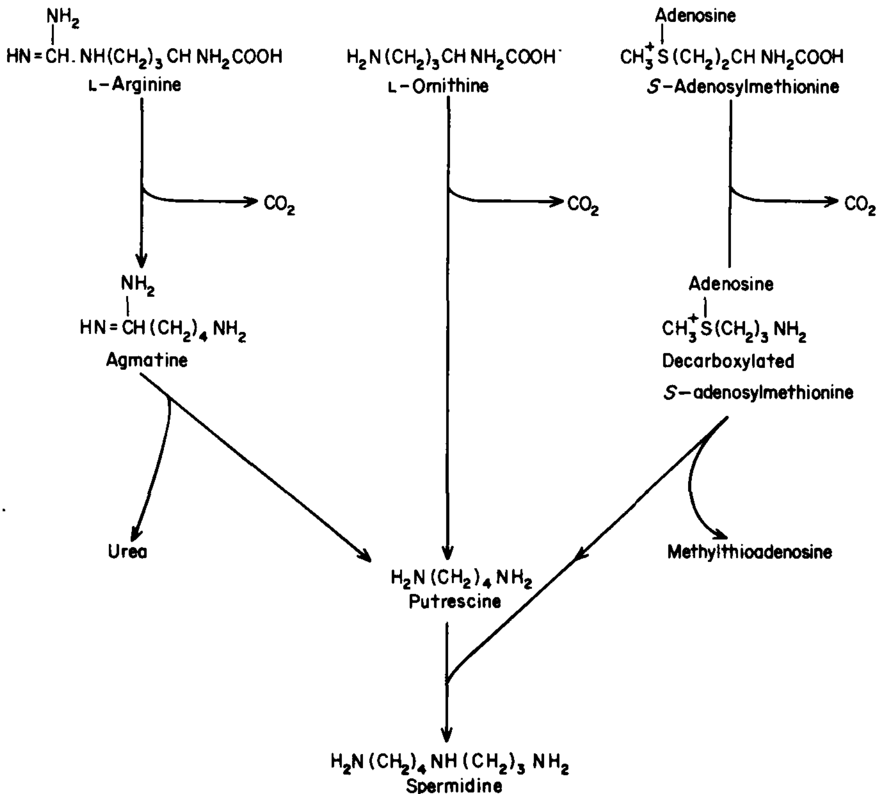


FIG. 1. Oligoamine biosynthetic pathway in *Escherichia coli.*

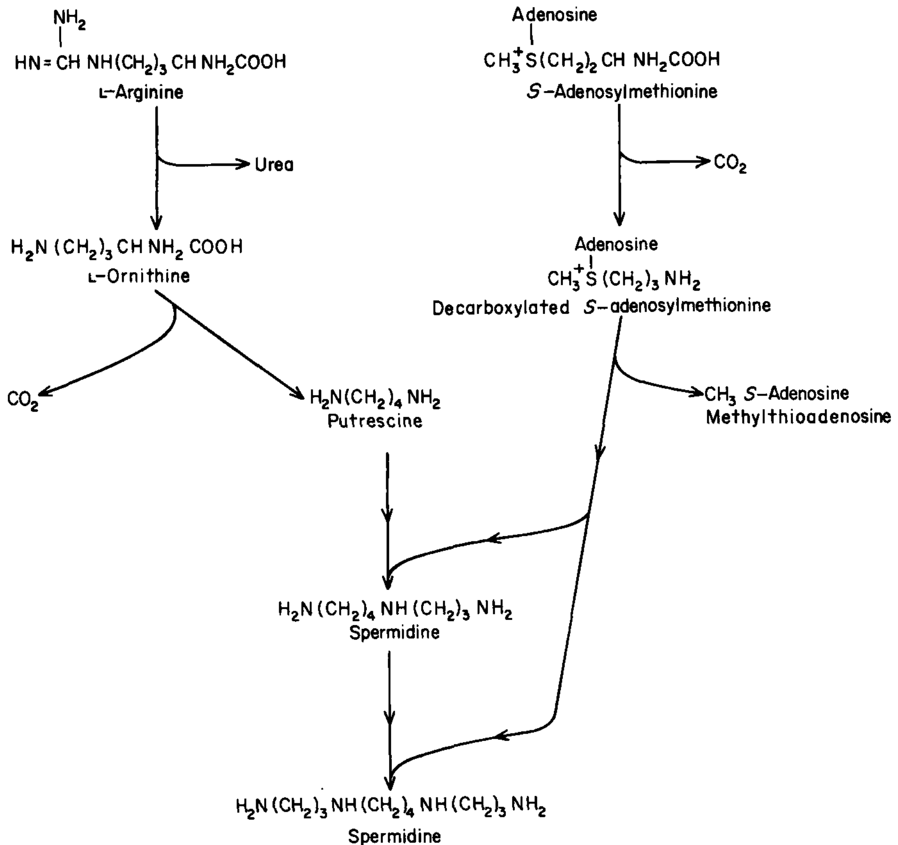


FIG. 2. Oligoamine biosynthetic pathway in rat liver.

conditions as possible. The strength of binding of oligoamines to DNA and RNA varies inversely with the ionic strength of the medium in which measurements are made (Stevens, 1969; Rubin, 1977) and, therefore, comparisons should be made at similar ionic strengths. Binding is clearly strongest with spermine and weakest with putrescine. In the case of spermidine binding to both ribosomes and to RNA, evidence from Scatchard plots indicates the existence of two types of site, namely strong binding sites and weaker binding sites. The weaker sites greatly outnumber the stronger sites, but the latter have binding constants about ten-fold greater. Spermine seems to bind preferentially to double-stranded RNA. This has been shown by comparing the binding of spermine to single-stranded and double-stranded homopolynucleotides (Ikemura, 1969) and also by demonstrating that there is competition

between spermine and ethidium bromide for double-stranded ribosomal RNA (Stevens and Pascoe, 1972). Oligoamines do also bind to single-stranded polynucleotides (Igarashi *et al.*, 1977). Competition between ethidium bromide and spermidine for sites on tRNA has also been demonstrated more recently by Sakai *et al.* (1975). Spermine is also thought to stabilize the conformation of tRNA (Prinz *et al.*, 1976).

Although nucleic acids and nucleic acid-containing structures are the sites usually considered to be the most likely ones to which most oligoamines bind, they will also bind to phospholipids and may bind to proteins. No studies of binding constants have been made. In the case of proteins, where it is possible that a very small number of specific binding sites may exist in the cell, detection may not easily be made until ^{3}H-^{14}C-oligoamines with higher specific activities become available.

E. BIOSYNTHESIS OF OLIGOAMINES

As with many other biochemical investigations, the organism and tissues which have been most used to study the biosynthetic pathway of oligoamines have been *E. coli* and rat liver and, additionally in this case, rat ventral prostate gland. It is from these three sources that most information has been obtained regarding the enzymology of oligoamine synthesis. As can be seen from Figs. 1 and 2, biosynthetic pathways in *E. coli* and in rat liver are similar but there are certain important differences and, in Section III (p. 86), we examine the situation in fungi to see to what extent they resemble prokaryotes, as typified by *E. coli,* and higher eukaryotes, as typified by the rat tissues, in the biosynthetic pathway of their oligoamines.

The biosynthetic pathway was first worked out in *E. coli*. This was achieved mainly by Tabor and Tabor (1964) and helped by the discovery of the second route from arginine by Morris and Pardee (1966). Putrescine is produced both by decarboxylation of ornithine, and by decarboxylation of arginine followed by ureolysis of agmatine. Two ornithine decarboxylases and two arginine decarboxylases have been isolated and purified from *E. coli* (Morris and Fillingame, 1974). These have been described as the biosynthetic and the biodegradative decarboxylases, respectively. Biodegradative ornithine and arginine decarboxylases are inducible enzymes which are produced in response to growth in acidic medium, whereas the biosynthetic enzymes are constitutive. All four enzymes require pyridoxal phosphate as a cofactor.

S-Adenosylmethionine decarboxylase from *E. coli* has been purified to

homogeneity (Wickner *et al.*, 1970); it possesses covalently bound pyruvate at the catalytic site and is activated by Mg^{2} . Propylamine transferase has also been purified to homogeneity (Bowman *et al.*, 1973). The remaining enzyme involved in oligoamine synthesis in *E. coli,* agmatine ureohydrolase, has not yet been purified. Arginase, which links arginine and ornithine biosynthetically, is absent from *E. coli* (Morris *et al.*, 1970). Similarly, *Klebsiella aerogenes* and *B. stearothermophilus* possess arginine decarboxylase activity (Stevens *et al.*, 1978), but arginase activity is undetectable in these organisms. In contrast, in rat liver arginase is present in high concentration. Mutants of *E. coli* deficient in arginine decarboxylase (Morris and Jorstad, 1970), agmatine ureohydrolase (Morris and Jorstad, 1970; Hirschfield *et al.*, 1970) and ornithine decarboxylase (Cunningham-Rundles and Maas, 1975) have been isolated. They all map in a cluster between 56 and 57 min on the chromosome (Maas, 1974).

The biosynthetic pathway in animal tissues was elucidated largely by two groups of workers, namely Raina and Jänne in Finland and Williams-Ashman and Pegg in U.S.A. (see Williams-Ashman *et al.*, 1969). Only one route to putrescine has been detected in animal tissues. In the case of mammalian liver, the high arginase activity associated with the urea cycle connects arginine and ornithine. Ornithine decarboxylase activity was initially more difficult to detect in animal tissues than in *E. coli.* The specific activity of ornithine decarboxylase at its peak in regenerating liver is at least 100-fold lower than that in exponentially growing *E. coli.* Ornithine decarboxylase has been purified from rat liver (Friedman *et al.*, 1972b; Ono *et al.*, 1972; Obenrader and Prouty, 1977a, b) and rat ventral prostate gland (Jänne and Williams-Ashman, 1971). It requires pyridoxal phosphate as cofactor.

S-Adenosylmethionine decarboxylase from rat liver and prostate gland differs from that of *E. coli* in that the rat enzymes are very strongly activated by putrescine (Hannonen *et al.*, 1972; Pegg and Williams-Ashman, 1969) and also they are very sensitive to a specific inhibitor, methylglyoxal bis (amidinohydrazone), in contrast to the enzyme from *E. coli* (Williams-Ashman and Schenone, 1972).* Two propylamine

*This Compound $CH_3-C[=N-NH-C(=NH)-NH_2]-CH[=N\ NH-C(=NH)-NH_2]$, is frequently referred to in the literature as methylglyoxal bis(guanylhydrazone). This is an unfortunate name because it implies a structure different from the one possessed. The word "guanyl" suggests a relationship to guanine. At the suggestion of Dr. H. B. F. Dixon, Department of Biochemistry, University of Cambridge, we use the name methylglyoxal bis(amidinohydrazone) and abbreviate it to MGBA.

transferases have been separated from rat liver (Hannonen *et al.*, 1972) and from rat brain (Raina and Hannonen, 1971), *S*-methyladenosyl-homocysteamine: putrescine aminopropyl transferase (EC.2.5.1.16), and *S*-methyladenosyl-homocysteamine: spermidine aminopropyl transferase, usually called spermidine and spermine synthases, respectively. Both enzymes have high affinities for decarboxylated adenosylmethionine. Earlier reports suggested that *S*-adenosylmethionine decarboxylase could not be separated from aminopropyl transferase activity (Feldman *et al.*, 1972), but, with the purification of rat liver *S*-adenosylmethionine decarboxylase lacking aminopropyl transferase activity (Pegg, 1974), the general consensus of opinion now is that separate enzymes are involved, although in the intact cell they may possibly form a multi-enzyme complex. Not only are there differences in the pathways for synthesis of oligoamines in *E. coli* and in rat tissues, but there are also differences in their control. The main regulatory step in both is the production of putrescine. The intracellular concentrations of putrescine in rat tissues are normally very low, since the strong activation of *S*-adenosylmethionine decarboxylase by putrescine ensures its conversion to spermidine and spermine. Biosynthetic ornithine decarboxylase activity from *E. coli* is modulated by guanine nucleotides. Höltä *et al.* (1972, 1974) have shown that ornithine decarboxylase from *E. coli* is activated by GTP which, at a concentration of 1 mM, can lower the K_m value for ornithine from 2 mM to 0.2 mM. Guanosine tetraphosphate, on the other hand, has an inhibitory effect. Höltä *et al.* (1972, 1974) suggest that the stringent response of *E. coli* CP78 to amino-acid starvation, which alters the rates of RNA and oligoamine synthesis, is mediated by changes in the guanine nucleotide ratios. Using strain B207, Sakai and Cohen (1976) have confirmed the sensitivity of ornithine decarboxylase from *E. coli* to guanine nucleotides, but they doubt whether in this strain the changes in guanine nucleotide ratios alone can account for the changes in putrescine metabolism.

In eukaryotes, there is no evidence so far that ornithine decarboxylase activity is controlled by guanine nucleotides, although there is evidence for the presence of guanosine tetraphosphate in some lower eukaryotes (De Carlo and Somberg, 1974; Pao *et al.*, 1977). Also there is no evidence that mammalian ornithine decarboxylase is modulated by other effectors. Instead, other forms of control seem to operate. The most renowned property of mammalian ornithine decarboxylase is its short half-life and it appears at the top of all the most recent league tables of

TABLE 3. Properties of oligoamine biosynthetic enzymes

Enyzyme	Source	Molecular weight	Cofactors or prosthethic group	Effectors	Kinetic constants	Half-life	Reference
Ornithine decarboxylase (biosynthetic)	*Escherichia coli*	147,000	Pyridoxal phosphate	GTP, dGTP-positive; ppGpp-negative	$K_m$orn = 5.6 mM; K_mGTP (activator) = < 1 μM; $K_m$orn = 0.28 mM (+ GTP)		Applebaum (1972)
Ornithine decarboxylase	Rat liver	100,000	Pyridoxal phosphate	None detected	K_m = 0.13 mM; K_mPLP = 25 μM	8–30 min	Ono *et al.* (1972); Obenrader and Prouty (1977a,b); Russell and Snyder (1969)
Arginine decarboxylase (biosynthetic)	*Escherichia coli*	296,000	Pyridoxal phosphate	Mg^{2+} (positive)	$K_m$arg = 0.03 mM; K_mPLP = 0.6 μM		Wu and Morris (1973a,b)
S-Adenosylmethionine decarboxylase	*Escherichia coli*	113,000	Covalent pyruvate; Mg^{2+} activated		K_mSAM = 0.09 mM		Wickner *et al.* (1970)
S-Adenosylmethionine decarboxylase	Rat liver	68,000	Covalent; pyruvate	Putrescine (positive)	$K_m$put = 5 μM 33 μM	20–60 min	Hannonen *et al.* (1972); Pegg (1974, 1977a, b)
Spermidine synthase	*Escherichia coli*	73,000	None		$K_m$put = 12 μM; K_md.SAM = 2.2 μM		Bowman *et al.* (1973)
Spermidine synthase	Rat liver				$K_m$put = 0.1 mM	Long	Hannonen *et al.* (1972)

PLP = Pyridoxal phosphate; dSAM = decarboxylated SAM.

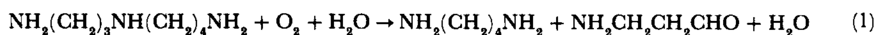
enzyme half-lives (Goldberg and St John, 1976; Jänne *et al.*, 1976; Litwack and Rosenfield, 1973). Putrescine production is regulated by the amount of ornithine decarboxylase produced. Regulation of the amount of enzyme probably occurs at the post-transcriptional level (Kay and Lindsay, 1973b; Clark and Fuller, 1975; Pösö, 1977). Using an immunological method, Hölttä (1975) demonstrated that, in rat liver, the level of ornithine decarboxylase activity is regulated by *de novo* synthesis and degradation of the enzyme itself. More recently, Obenrader and Prouty (1977a) have shown that multiple forms of ornithine decarboxylase exist in rat liver. Two forms of ornithine decarboxylase exist in 3T3 cells differing in the K_m value for pyridoxal phosphate, but the two forms do not account for the fluctuations that occur in ornithine decarboxylase activity under different growth conditions (Clark and Fuller, 1976). There is also some evidence that a protein which inhibits ornithine decarboxylase is produced in response to growth of hepatoma cells in culture in the presence of putrescine (Fong *et al.*, 1976; McCann *et al.*, 1977; Heller *et al.*, 1977) and that this protein binds ornithine decarboxylase but can be separated from the enzyme with 0.25 M sodium chloride. The main properties of the biosynthetic enzymes from *E. coli* and rat liver are summarized in Table 3.

F. OLIGOAMINE METABOLISM AND TURNOVER

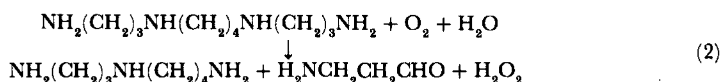
Catabolism of oligoamines and their elimination from cells has been studied far less extensively than their biosynthesis. In *E. coli* growing exponentially, no detectable turnover of oligoamines has been found, and labelled putrescine taken up from the medium is found quantitatively conserved in the intracellular putrescine and spermidine (Tabor and Dobbs, 1970). Towards the end of the exponential phase of growth, most of the intracellular spermidine is converted to glutathionylspermidine (Tabor and Tabor, 1975) but, if stationary-phase cultures are added to fresh medium, glutathionylspermidine is converted back to free spermidine. Tabor and Tabor (1976b) have found no evidence for a glutathionyl derivative of putrescine. The intracellular concentration of putrescine, but not that of spermidine, is greatly decreased when *E. coli* is transferred from medium of low osmolarity to one of high osmolarity, and this is brought about by excretion of putrescine into the medium rather than by its catabolism (Munro *et al.*, 1972). Under certain conditions, putrescine and spermidine may become acetylated and this may be

another means of lowering the free intracellular oligoamine concentration. Thus, in bacteria as exemplified by *E. coli*, oligoamine concentrations are regulated mainly by control of biosynthesis, conjugation and excretion, and catabolism seems to be virtually absent during normal growth.

In animal tissues, there is some evidence for relatively slow turnover of spermidine and spermine, though the catabolism of oligoamines has not been extensively studied. Using C^{14}-ornithine as precursor, Russell *et al.* (1970) and Siimes (1967) have shown that the half-life of spermidine in regenerating liver is about four days and that of spermine seems to be considerably longer. In the other tissue which has been studied, that is rat brain, turnover of spermidine is slower than in regenerating liver having a half-life of 16–19 days (Shaskan and Snyder, 1973). Until very recently, efforts to detect enzymes which catabolize oligoamines in rat liver have been unsuccessful. But Hölttä (1977) has now isolated and purified a polyamine oxidase which oxidizes spermidine and spermine at the secondary amino groups respectivevly according to the following equations:



$$NH_2(CH_2)_3NH(CH_2)_4NH_2 + O_2 + H_2O \rightarrow NH_2(CH_2)_4NH_2 + NH_2CH_2CH_2CHO + H_2O \quad (1)$$



$$\begin{array}{c} NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2 + O_2 + H_2O \\ \downarrow \\ NH_2(CH_2)_3NH(CH_2)_4NH_2 + H_2NCH_2CH_2CHO + H_2O_2 \end{array} \quad (2)$$

The enzyme is mainly, possibly exclusively, located in peroxisomes. The significance of this enzyme has still to be established, but it could be concerned with regulation of intracellular oligoamine levels; the levels of activity detected are more than adequate for it to have this physiological role.

Turnover of oligoamines has not been studied in plant tissues although a number of amine oxidases have been detected (Smith, 1975, 1977b). Polyamine oxidases, which oxidize spermine and spermidine, have been found with high activities in barley leaves and other plant tissues (Smith, 1974; Smith and Bickley, 1974). In contrast with the rat-liver polyamine oxidase, the barley enzyme oxidizes spermine and spermidine to give 1-pyrroline and 1-(3-aminopropyl) pyrroline, respectively. A large number of other amine oxidases have been described from plant, animal and bacterial sources (see Tabor and Tabor, 1972; Bachrach, 1973; Smith, 1975) but many of these are not specific for spermidine, spermine or putrescine, and they are probably not primarily concerned with regulation of intracellular oligoamine levels.

G. RELATIONSHIP BETWEEN OLIGOAMINE BIOSYNTHESIS AND GROWTH

Oligoamine synthesis occurs most rapidly in proliferating cells and tissues. In cells undergoing a change from a non-dividing to a dividing state, an increase in oligoamine synthesis is usually one of the earliest changes detectable. The generality of the correlation between oligoamine synthesis and growth was well established in the 1960s. Raina and Cohen (1966) showed that, in exponentially growing *E. coli*, oligoamine synthesis was rapid and that the ratio of oligoamine nitrogen to RNA phosphorus varied only between narrow limits (0.21–0.23). In rat liver regenerating after partial hepatectomy, it was shown that there is a stimulation of oligoamine synthesis in parallel with the increase in RNA synthesis (Raina *et al.*, 1966; Dykstra and Herbst, 1965) and the earliest reported change in enzyme activity during liver regeneration was that of ornithine decarboxylase (Jänne and Raina, 1968; Russell and Snyder, 1968). Similarly, growth hormone was shown to stimulate liver growth and also oligoamine synthesis (Jänne *et al.*, 1968; Jänne and Raina, 1969). Another early finding was that developing chick embryos also have levels of oligoamines which can be related to their growth rate (Caldarera *et al.*, 1965). Since 1970, many other examples of the correlation between oligoamine synthesis and growth have been found, e.g. germinating bacterial (Setlow, 1974a, b) and fungal spores (Mitchell and Rusch, 1973; Stevens *et al.*, 1976), hormone-induced growth in target tissues (Kaye *et al.*, 1971; Stastny and Cohen, 1972), insect metamorphosis (Wyatt *et al.*, 1973) and frog metamorphosis (Fischer and Cohen, 1974).

In order to try to establish at a more molecular level the nature of the relationship between oligoamine synthesis and growth, and in particular whether it is related to (a) RNA synthesis, (b) DNA synthesis or (c) a particular sequence of events in the cell cycle, more detailed studies have been made using cell systems which divide synchronously and ones in which growth rate may readily be changed by external factors. In Fig. 3, we have summarized the sequence of events which occur during synchronous growth of a number of different types of cell. In the case of regenerating liver, rat hepatoma, mouse fibroblasts, human lymphocytes and Don C cells, synchrony was induced, whereas in Chinese hampster ovary fibroblasts cell synchrony was selected (Mitchison, 1971). In all six examples, the increase in ornithine decarboxylase is biphasic but the time

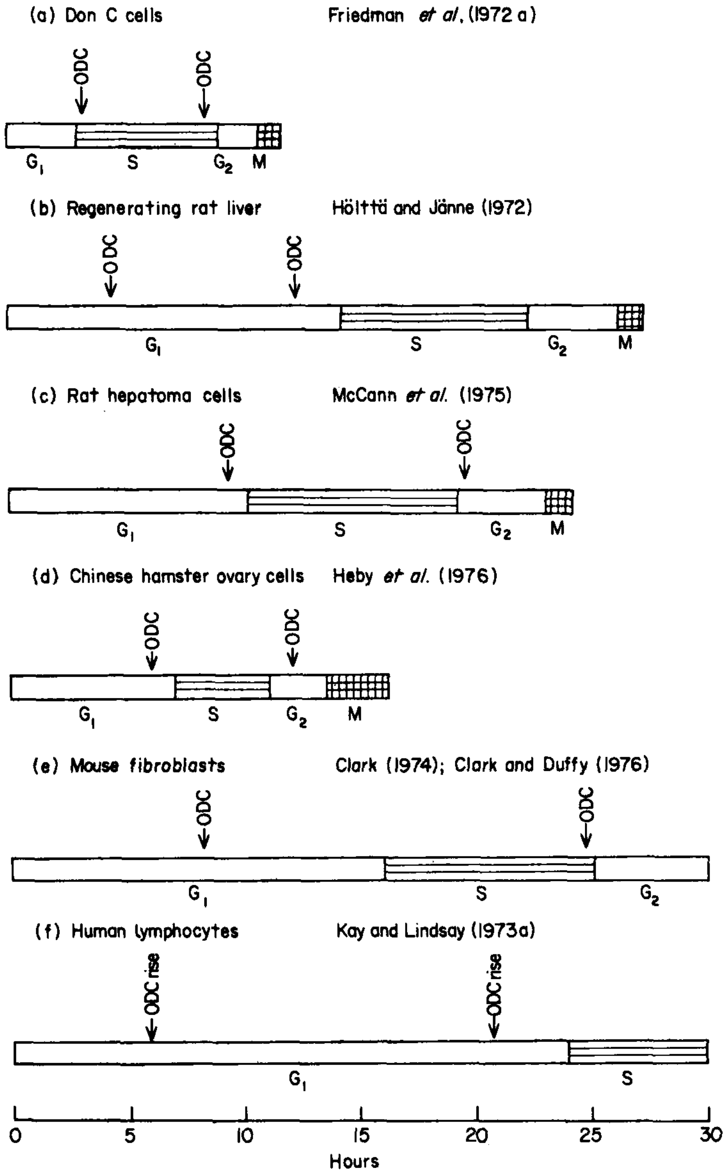


FIG. 3. Ornithine decarboxylase in relation to the cell cycle. ODC indicates the time peak of ornithine decarboxylase activity.

of the peak activity is somewhat variable between systems. In most cases there is at least one ornithine decarboxylase peak in the presynthetic phase (G_1) and the second peak often occurs at the end of the synthetic phase or in the postsynthetic phase. Although DNA synthesis occurs discontinuously throughout the cell cycle, RNA synthesis often occurs continuously. In three of the cell systems, namely regenerating liver (Raina *et al.*, 1966), mouse fibroblasts (Clark and Duffy, 1976) and human lymphocytes (Kay and Cooke, 1971; Kay and Lindsay, 1973a), the most rapid synthesis of oligoamines and RNA occurs concurrently. When RNA synthesis is blocked by low concentrations of actinomycin D, the increase in ornithine decarboxylase activity which normally occurs when cells are stimulated to divide is suppressed. However, when oligoamine synthesis is inhibited by α-methylornithine (Mamont *et al.*, 1976), diaminopropane (Pösö and Jänne, 1976; Sunkara *et al.*, 1977) or methylglyoxal bis(amidinohydrazone) (Kay and Pegg, 1973), although DNA synthesis is inhibited or delayed, RNA synthesis is relatively unaffected. Thus, although oligoamine synthesis often occurs in parallel with RNA synthesis, their rates may be uncoupled from each other; on the other hand, it may be essential for some oligoamine synthesis to precede DNA synthesis. A change in oligoamine:RNA ratio in a putrescine-requiring auxotroph of *Aspergillus nidulans* is discussed in Section V (p. 123).

Little is known about how the rapid increase in ornithine decarboxylase is brought about at the onset of growth, but there are indications that extracellular stimuli may be mediated via cyclic AMP (Beck *et al.*, 1972; Byus and Russell, 1975) and also that the integrity of the microtubule system is necessary for a response to occur (Chen *et al.*, 1976).

III. Oligoamines in Fungi

A. OLIGOAMINE ESTIMATION

The simplicity of the structure of oligoamines makes estimation of their concentrations in tissue and cell extracts a complex process. The only reactive groups which oligoamines possess are those of primary and secondary amino groups. Since many other compounds present in cell extracts also contain amino groups, the latter cannot be used to estimate oligoamines without prior fractionation of the extracts. Most procedures for oligoamine estimation involve (a) a method for separating

TABLE 4. Oligoamine distribution in fungi

Species	Class[e]	Putrescine	Spermidine	Spermine	Other oligoamines	Molar ratio putrescine: spermidine: spermine	Method of estimation	References
Dictyostelium discoideum	Acrasiomycete	49.6[a]	13.8[a]	trace	1,3 diamino-propane 29.9[a]	3.6:1:-	*f,g*	North and Turner (1977)
								Turner and North (1977)
Physarum polycephalum	Myxomycete	89[b]	38[b]	trace		2.3:1:-	*f*	Mitchell and Rusch (1973)
		7 mM	3 mM	7 mM		2.3:1:2.3	—	Atmar *et al.* (1976)
Blastocladiella emersonii	Chytridiomycete	75[b]	25[b]	—		3:1-	*g*	Mennucci *et al.* (1975)
Pythium ultimum	Oomycete	+	+	—			*g*	Nickerson *et al.* (1977a)
Phythium debaryanum	Oomycete	+	+	-			*g*	Nickerson *et al.* (1977a)
Rhizopus stolonifer	Zygomycete	0.98[a]	3.13[a]			0.31:1:-	*g*	Nickerson *et al.* (1977b)
		6.9[a]	42[a]	10.8[a]		0.16:1:0.26	*f*	Hart *et al.* (1978)
Mucor hiemalis	Zygomycete	+	—	—			*g*	Nickerson *et al.* (1977a)
		6.9[a]	74[a]	6.3[a]		0.09:1:0.09	*f*	Hart *et al.* (1978)
Saccharomyces cerevisiae	Hemiascomycete	0.03[d]	0.140[d]	0.09[d]		0.21:1:0.64	*f*	Pösö *et al.* (1976)
			0.18[a]	0.28[a]		-:1:1.6	*f*	Castelli and Rossoni (1968)
		1.54[b]	14.98[b]	3.65[b]	1,3-diamino-propane		*h*	Herbst *et al.* (1958)
								Whitney *et al.* (1978)
Hansenula subpelliculosa	Hemiascomycete	+	+	+			*g*	Nickerson *et al.* (1977a)
Debaryomyces globosus	Hemiascomycete	+	+	+			*g*	Nickerson *et al.* (1977a)
Geotrichum candidum	Hemiascomycete		+	+			*h*	Herbst *et al.* (1958)
Aspergillus nidulans	Plectomycete	0.4[a]	22.0[a]	6.0[a]		0.018:1:0.27	*f,g*	Winther and Stevens (1976)
			20[c]	1.2[c]		-:1:0.06	*g*	Bushell and Bull (1974)
Aspergillus niger	Plectomycete	7.4[a]	38[a]	10.2[a]		0.19:1:0.27	*f*	Hart *et al.* (1978)
Aspergillus oryzae	Plectomycete	7.4[a]	61[a]	15.0[a]	Cadavarine	0.12:1:0.25	*f,h*	Hart *et al.* (1978)
								Umezu and Tochio (1962)

Penicillium expansum	Plectomycete	5.2[a]	17[a]	7.3[a]		0.31:1:0.43	*f*	Hart *et al.* (1978)
Penicillium fumiculosum	Plectomycete	8.3[a]	23[a]	4.5[a]		0.36:1:0.20	*f*	Hart *et al.* (1978)
Penicillium nigricans	Plectomycete	8.0[a]	46[a]	10.0[a]		0.17:1:0.22	*f*	Hart *et al.* (1978)
Neurospora crassa	Pyrenomycete	0.22[c]	40[c]	0.6[a]		0.006:1:0.015	*f*	Viotti *et al.* (1971)
		0.6[c]	21[c]	0.2[c]		0.03:1:0.009		Bowman and Davis (1977a)
		3.0[a]	44[a]	2.9[c]		0.07:1:0.07	*f*	Hart *et al.* (1978)
		+	+	—	1,3-diamino-propane		*g*	Nickerson *et al.* (1977a) Herbst *et al.* (1958)
Neocosomospora vasinfecta	Pyrenomycete	+	+	—			*g*	Nickerson *et al.* (1977a)
Sclerotina sp.	Discomycete	+	+	—			*g*	Nickerson *et al.* (1977a)
Ustilago sphaerogena	Hemibasidiomycete	+	+	—			*g*	Nickerson *et al.* (1977a)
Ustilago maydis	Hemibasidiomycete	+	+				*h*	List and Wagner (1963)
Coprinus atramentarius	Hemibasidiomycete	+			cadavarine		*h*	List and Reith (1960)
Fusarium moniliforme	Hypomycete	+	+	—			*g*	Nickerson *et al.* (1977a)
Alternaria alternata	Hypomycete	2.2[a]	51[a]	4.1[a]		0.04:1:0.08	*f*	Hart *et al.* (1978)
Trichoderma viride	Hypomycete	3.5[a]	31[a]	9.7[a]		0.11:1:0.31	*f*	Hart *et al.* (1978)
Helminthosporum maydis	Hypomycete	+	+				*g*	Nickerson *et al.* (1977a)
Periconia circinata	Hypomycete	+	+				*g*	Nickerson *et al.* (1977a)
Rhizoctonia solani	Hypomycete	+	+				*g*	Nickerson *et al.* (1977a)
Macrophomina phaesoli	Coelomycete	+	+				*g*	Nickerson *et al.* (1977a)
Botryodiplodia theobromae	Coelomycete	—	2.7[a]	—			*g*	Nickerson *et al.* (1977b)

[a] Indicates μmole/mg DNA.
[b] Indicates μmole/g protein.
[c] Indicates μmole/g dry weight.
[d] Indicates μmole/g wet weight.
[e] Indicates classifications are those given by Ainsworth (1966).
[f] Indicates paper electrophoretic method (Raina, 1963).
[g] Indicates t.l.c. separation of dansyl amines (Seiler and Wiechmann, 1967).
[h] Indicates other methods.

oligoamines from other low molecular-weight compounds, followed by (b) a method for separating the individual amines, and (c) a method for estimating the separated oligoamines. The extent to which separation of oligoamines from other amino compounds is necessary depends on the concentrations of the latter in a particular cell extract and whether they interfere with subsequent oligoamine separation and estimation. Two methods of oligoamine estimation are most widely used, namely (i) the electrophoretic method devised by Raina (1963) and (ii) the separation of dansyl oligoamines by t.l.c. devised by Seiler and Wiechmann (1967). Both methods extract the amines using either trichloroacetic acid or perchloric acid. In Raina's method, the amines are then separated from most of the amino acids in the extract by alkaline extraction into *n*-butanol. The individual oligoamines are then separated by paper electrophoresis in citrate buffer (pH 3.6). They are usually estimated by their reaction with ninhydrin. Acylated amino groups can only be estimated after hydrolysis. This method proves very satisfactory for many tissues.

In some tissues, however, a substantial amount of ninhydrin-positive material other than oligoamines is also extracted with *n*-butanol and, although these migrate more slowly than oligoamines, they can, if present in large amounts, interfere with the spermine band. A modification introduced by Inoue and Mizutani (1973) largely overcomes this problem. It involves a preliminary separation of perchlorate extracts on Dowex 50 using a phosphate buffer wash followed by a wash with hydrochloric acid. It is our experience with fungal extracts, particularly if the fungi have been grown on complex media, that this modification is desirable. Electrophoretic separation at pH 3.6 in 0.1 M citrate buffer does not separate 1,3-diaminopropane from putrescine, but these amines can be separated from each other by 0.065 M sulphosalicylic acid buffer (pH 3.1) (Raina, 1963). 1,3-Diaminopropane is not generally regarded as being widely distributed, but its presence in *Dictyostelium discoideum* which has recently been detected (North and Turner, 1977) suggests that separations should also be carried out in 0.065 M sulphosalicylic acid buffer to check for the prescence of 1,3-diaminopropane. Cadavarine also appears to be present in certain species (Table 4). Although it does not separate well from putrescine in the sulphosalicylic acid-buffer electrophoresis, it does give a more intensely purple colour when reacted with ninhydrin.

The second method involves dansylating a perchlorate extract of the

tissues and then extracting the dansyl derivatives into benzene. This extraction procedure separates most of the dansylated oligoamines from the dansylated amino acids. The subsequent separation by thin-layer chromatography separates individual oligoamines. A number of different solvent systems have been used to optimize the separation of particular amines (see Seiler and Wiechmann, 1970). The amines are estimated by the fluorescence emission of their dansyl derivatives. This method is more sensitive than the electrophoretic method but, to ensure good reproducibility for each plate, standards must be run and quantitated. With some extracts it may be desirable to carry out a preliminary separation on Dowex 50 prior to dansylation. This decreases background fluorescence which may be significant, for example, with fungi extracted from a large amount of mycelia in order to detect the presence of a small quantity of oligoamines. For unambiguous identification of amines, particularly unusual amines, it is insufficient to rely on their mobility in a single solvent system; a number of solvent systems should be tested or, more satisfactorily, identification should be confirmed by mass spectrometry (Seiler, 1971).

B. OLIGOAMINE DISTRIBUTION IN FUNGI

The concentrations of various oligoamines have been measured in a number of fungi and the findings are summarized in Table 4. However, it is difficult to make detailed comparisons of the values obtained by different workers since estimations have been carried out under a variety of conditions, and they are expressed on different bases. The concentrations of oligoamines in fungi vary with the state of growth; for example, putrescine is readily measurable in *A. nidulans* 6–8 hours after germination, but it is more difficult to detect at other times. The concentrations also depend to some extent on the medium in which they are grown.

One factor is clear from Table 4, namely that spermidine is the predominant oligoamine in most fungi so far investigated. Another generalization made by Nickerson *et al.* (1977a) is that, whereas filamentous fungi lack spermine, it is present in yeasts. Their claim has not however been substantiated by other workers who have deteeted spermine in *A. nidulans* (Bushell and Bull, 1974; Winther and Stevens, 1976) and in *N. crassa* (Viotti *et al.*, 1971) and, furthermore, incorporation of putrescine into spermine has been demonstrated in a

variety of fungi (Hart *et al.*, 1978). Thus, it appears that, while some filamentous fungi may either lack spermine or contain low contents of it, it is also clear that others are capable of synthesizing spermine.

Most fungi contain putrescine in low content compared with other amines, but there are three exceptions; in *D. discoideum, P. polycephalum* and *B. emersonii* putrescine contents are high and, furthermore, the putrescine:spermidine ratios are comparable with those found in *E. coli.* It is perhaps relevant that both *D. discoideum* and *P. polycephalum* are Mycetozoa and their taxonomic relationship with other fungi is doubtful. They show many similarities with protozoa. High contents of putrescine often occur in species in which their *S*-adenosylmethionine decarboxylase is not putrescine activated. This situation has been found in both *P. polycephalum* (Mitchell and Rusch, 1973) and *D. discoideum* (Turner and North, 1977), whereas, in the other fungi so far investigated, the *S*-adenosylmethionine decarboxylases are putrescine activated (Hart *et al.*, 1978). *S*-Adenosylmethionine decarboxylase has not yet been measured in *B. emersonii.*

C. BIOSYNTHESIS OF OLIGOAMINES IN FUNGI

1. *Biosynthetic Pathway*

Enzymes involved in oligoamine biosynthesis have been studied in only a limited number of fungi, and they have often been studied with different aims in mind. Those organisms in which one or more enzymes have been measured are: *Saccharomyces cerevisiae, Aspergillus nidulans, Neurospora crassa, Physarum polycephalum, Dictyostelium discoideum, Blastocladiella emersonii* and *Panus tigrinus. Saccharomyces cerevisiae* has been studied primarily because it can be easily obtained in large quantities and it is thus a good source from which to purify oligoamine biosynthetic enzymes. *Aspergillus nidulans* has been studied mainly from a developmental standpoint; in particular, the control of ornithine decarboxylase activity during conidial germination has been studied. The particular interest in *P. polycephalum*, apart from developmental aspects, is in the multiple forms of ornithine decarboxylase present in this organism (Mitchell and Carter, 1977). Oligoamine synthesis during differentiation, but in the absence of cell division, has been studied in *D. discoideum* (Turner and North, 1977).

As mentioned previously (Section IIE, p. 73), oligoamine biosynthesis has been most thoroughly investigated in *E. coli*, rat liver and rat prostate and thus, in examining oligoamine synthesis in other

organisms, comparison is usually made with these organisms. There is no evidence that putrescine is synthesized in fungi by more than one route. The occurrence of seven allelic putrescine-requiring auxotrophs of *A. nidulans* (C. Herman and J. Clutterbuck, unpublished observations) suggests that a single enzyme is responsible for putrescine biosynthesis. *Aspergillus nidulans* puA_1 has been shown to be deficient in ornithine decarboxylase and no arginine decarboxylase is detectable in the wild type (Stevens, 1975). Two putrescine-requiring auxotrophs of *N. crassa* have also been found which are probably allelic (K. J. McDougall, unpublished observations). *Neurospora crassa, A. nidulans* and *Sacch. cerevisiae* all have high arginase activity and, thus, an arginine decarboxylase would seem to be redundant for putrescine production. *Dictyostelium discoideum* has very little arginase activity and also has undetectable arginine decarboxylase activity (M. J. North and R. Turner, unpublished observations). Arginine decarboxylase has, however, been detected and partially purified from the fruiting body of the basidiomycete *Panus tigrinus* (Boldt *et al.*, 1971; Uhlemann and Reinbothe, 1977), but the enzyme is probably not primarily concerned with oligoamine production but more with arcain and arginine catabolism. Its properties are rather different from those of the *E. coli* enzyme; e.g. it has a pH optimum of 5.2 and K_m value for arginine of 0.66 mM compared with a pH optimum of 8.4 and K_m value for arginine of 0.03 mM in the enzyme from *E. coli*.

2. *Ornithine Decarboxylase—General Properties*

Ornithine decarboxylases have been measured in *P. polycephalum* (Mitchell and Rusch, 1973), *D. discoideum* (Turner and North, 1977), *B. emersonii* (Mennucci *et al.*, 1975), *A. nidulans* (Stevens *et al.*, 1976) and *N. crassa* (Deters *et al.*, 1974; Weiss and Davis, 1973). Some of their properties are summarized in Table 5. The enzymes from different sources have a number of common characteristics. All have slightly alkaline pH optima, low K_m values for ornithine, and a requirement for pyridoxal phosphate (to obtain maximum activity in extracts pyridoxal phosphate is required). Putrescine is a weak inhibitor of ornithine decarboxylase and, generally, spermidine is even weaker. In common with the mammalian ornithine decarboxylases, the enzymes from *A. nidulans* and *D. discoideum* are strongly stabilized in extracts by dithiothreitol but, surprisingly, the enzyme from *P. polycephalum* is

TABLE 5. Fungal ornithine decarboxylases

Species	pH optimum	K_m Value for ornithine (mM)	Pyridoxal phosphate requirement	Effect of putrescine	Effect of dithio-threitol	Half-life (min)	Reference
Physarum polycephalum	8.3	0.11	+	inhibits	inhibits	14.0	Mitchell and Rusch (1973)
Dictyostelium discoideum	8.2	0.67	+	inhibits	stabilizes	—	R. Turner and M. J. North (unpublished observations)
Blastocladiella emersonii	7–8	0.6–0.8	+	—	—	—	Mennucci *et al.* (1975)
Aspergillus nidulans	7.9	0.06	+	inhibits	stabilizes	35.0	Stevens *et al.* (1976); Stevens and McKinnon (1977)
Neurospora crassa		0.04	?	inhibits	—	—	Weiss and Davis (1973); Sikora and McDougall (1977)

inhibited by 5 mM dithiothreitol or 2-mercaptoethanol. The half-lives of ornithine decarboxylases from *P. polycephalum* and *A. nidulans* are 14 min and 35 min, respectively, and the *D. discoideum* enzyme is very unstable although its half-life has not been measured. There is no evidence for any of the ornithine decarboxylases being particle-bound; activity is found in high-speed supernatants.

3. *Ornithine Decarboxylase in* Aspergillus nidulans

Highest ornithine decarboxylase activity is found in germinating conidia of *A. nidulans* at the time of most rapid oligoamine synthesis (Stevens *et al.*, 1976). Enzyme activity is measured in the protein fraction precipitated by 50% saturated ammonium sulphate from a 25,000*g* supernatant. Maximum activity is found after dialysis. An inhibitor, which is not excluded from Sephadex G15, is detectable in the diffusate. The inhibitor has not yet been identified, and so it is not possible to say whether it is a physiological inhibitor or whether it is an artefact arising during the isolation procedure.

Putrescine is a competitive inhibitor of the enzyme, but it is unlikely to control ornithine decarboxylation *in vivo* by competitive inhibition, for the following reason. The intracellular concentration of putrescine in *A. nidulans*, growing on minimal medium, does not rise above 0.2 mM, whereas the intracellular ornithine concentration measured at the same time in the growth cycle is about 2.8 mM (M. D. Winther, unpublished observations). The values for both $K_{I\,putrescine}$ and $K_{m\,ornithine}$ are 60 μM. Thus, intracellular putrescine could not bring about more than about 5% decrease in ornithine decarboxylation when the organism is growing on minimal medium. However, when putrescine is added to the growth medium, it is responsible for a substantial decrease in the ornithine decarboxylase activity measurable in dialysed extracts. Thus, putrescine is probably able to control production of ornithine decarboxylase at the level of transcription or translation.

Ornithine decarboxylase turnover is rapid in *A. nidulans* and its activity is probably controlled by regulation of both its synthesis and degradation. A little non-specific proteinase activity has been detected in extracts from 16-hour germinated conidia, which is the time when rapid decrease in ornithine decarboxylase activity occurs. Increased activities of ornithine decarboxylase are detected in dialysed extracts of conidia which have been grown in medium in which the putrescine analogue, 1,4-

diaminobutanone, has been included (Stevens *et al.*, 1977; Stevens and McKinnon, 1977). This analogue increases the half-life of the enzyme from 35 min to 5 hours, and almost certainly this is achieved by protecting the enzyme from proteolysis; it has been shown that diaminobutanone protects ornithine decarboxylase from inactivation by chymotrypsin (Stevens and McKinnon, 1977).

4. *Ornithine Decarboxylase in* Physarum polycephalum

Ornithine decarboxylase activity in *P. polycephalum* fluctuates during synchronous growth of the plasmodium (Sedory and Mitchell, 1977) and, although the enzyme has a short half-life (see Table 5), control by regulation of synthesis and degradation does not seem to be the only method of control. Mitchell and Carter (1977) have evidence that the enzyme exists in two forms, which differ in their affinities for pyridoxal phosphate. When crude homogenates are passed down Sephadex G-200 columns, at least two clear peaks of activity emerge corresponding to molecular weights of 80,000 and 160,000 and possibly higher (Mitchell *et al.*, 1976). The relative proportions of the two peaks depend on the state of the organism at harvest and also on the buffer in which the homogenate is equilibrated. In 0.05 M Hepes, tris or phosphate buffers, a single 80,000 molecular weight peak emerges whereas, with 0.005 M phosphate or 0.05 M borate buffer, the higher molecular weight forms are evident. However, over and above the size distributions of the enzyme, the amounts of activity detectable depend on the pyridoxal phosphate concentration used in the assay. Lineweaver-Burk plots suggest that there are two different ornithine decarboxylase activities having K_m values for pyridoxal phosphate equal to 33 μM and 0.13 μM, respectively. The former, having the lower affinity for pyridoxal phosphate, has a greater tendency to polymerize to higher molecular-weight forms. The exact nature of the differences between the two forms is not clear, but Mitchell and Carter (1977) suggest that the 80,000 and and 160,000 molecular-weight peaks represent monomer: dimer equilibrium, and that the different activities are probably due to conformational changes of, or to covalent modification of, the same basic protein subunit.

Although the enzyme has been purified 500-fold, it has not been possible to separate the different activities from each other. Adding cycloheximide to growing microplasmodia induces changes in the

ornithine decarboxylase activity (Mitchell and Sedory, 1974). In the presence of 200 μM pyridoxal phosphate (detecting both low and high affinity forms), there is a slight increase followed by a slight fall in activity whereas, if assayed in the presence of 1 μM pyridoxal phosphate (detecting only high affinity), there is a sharp rise followed by a dramatic fall. These changes can be explained as interconversions of the two forms of the enzyme. Both forms have identical values for $K_{m\,ornithine}$. At physiological concentrations of pyridoxal phosphate, the low-affinity form would have severely decreased activity. Mitchell and Carter (1977) suggest that interconversion of the two forms may act as an initial control mechanism, possibly backed up by proteinases which may have preferential ability to degrade one form of the enzyme. There is also evidence for two forms of ornithine decarboxylase in cultured mouse fibroblasts (Clark and Fuller, 1976) which differ in their affinities for pyridoxal phosphate but, in this case, rapid stimulation of ornithine decarboxylase activity at the onset of growth cannot be accounted for by interconversion of the two forms.

In hepatoma cells, there is evidence that ornithine decarboxylase activity is regulated by a protein inhibitor called an "antienzyme", which is capable of binding to the enzyme and rendering it inactive (Fong *et al.*, 1976). No evidence has been obtained for an antienzyme in *P. polycephalum.*

5. *Ornithine Decarboxylase in Other Fungi*

Other fungal ornithine decarboxylases have been less extensively investigated. High activity has been detected in myxamoebal extracts of *D. discoideum*, but the enzyme is very unstable and extracts have to be prepared in the presence of 35% glycerol (Turner and North, 1977). When amoebae are induced by starvation to differentiate, their ornithine decarboxylase activity falls, as do the contents of oligoamines and RNA. In *N. crassa*, ornithine decarboxylase activity increases during germination and, like the enzyme from *A. nidulans*, activity is decreased by growing it on putrescine-containing medium (K. J. McDougall, unpublished observations). But, unlike *A. nidulans*, higher activities are detected when growth is in the presence of arginine. There is also some evidence for possibly more than one ornithine decarboxylase, i.e. a constitutive and an inducible enzyme (Sikora and McDougall, 1977).

6. S-*Adenosylmethionine Decarboxylase in Fungi*

S-Adenosylmethionine decarboxylase has been measured in a number of fungi, but only in *Sacch. cerevisiae* has it been purified and studied in detail. As with ornithine decarboxylase, the *S*-adenosylmethionine decarboxylase from *E. coli* and the similar enzyme from rat liver and prostate are often considered to be typical prokaryote and eukaryote enzymes, respectively, though, as more *S*-adenosylmethionine decarboxylases are studied, the pattern seems to be more complex. The major difference between the enzyme from *E. coli* and rat liver is that the former requires Mg^{2+} for maximum activity and is insensitive to putrescine, whereas the latter is strongly activated by putrescine and unaffected by Mg^{2+}. At one time it was thought also that the cofactor requirements differed, since covalently-bound pyruvate had been shown to be present in purified *S*-adenosylmethionine decarboxylase from *E. coli* (Wickner *et al.*, 1970), whereas pyridoxal phosphate was thought to be a possible cofactor for the mammalian enzymes. However, Pegg (1977a) has recently purified rat-liver *S*-adenosylmethionine decarboxylase and been unable to detect either the presence of, or indeed a requirement for pyridoxal phosphate. Coupled with activation of rat-liver *S*-adenosylmethionine decarboxylase by putrescine is its sensitivity to the inhibitor methylglyoxal bis(amidino hydrazone) ($H_2N.C(:NH).NH.N:C(CH_3).CH:N.NH.C(NH).NH_2$(MGBA). This inhibitor, at 1.5 μM, causes inhibition of purified rat-prostate *S*-adenosylmethionine decarboxylase, whereas the *E. coli* enzyme is approximately two orders of magnitude less sensitive (Williams-Ashman and Schenone, 1972).

S-Adenosylmethionine decarboxylase from *Sacch. cerevisiae* has been purified 1100-fold by affinity chromatography using Sepharose with covalently bound MGBA (Pösö *et al.*, 1975). The properties of the enzyme from *Sacch. cerevisiae* are similar to those from rat liver. Activity is present in the high-speed supernatant. The enzyme is strongly activated by putrescine (K_a value for putrescine is 0.012 mM); other diamines such as cadavarine and 1,3-diaminopropane stimulate it but to a lesser degree. The K_m value for *S*-adenosylmethionine is dependent on putrescine concentration, i.e. 0.09 mM with saturating putrescine, and five times as high in the absence of putrescine. The maximum velocity is, however, virtually unchanged in the absence of putrescine. Decarboxylated *S*-adenosylmethionine is a powerful product inhibitor (K_i value about 1

μM). The enzyme is strongly inhibited by MGBA. The inhibition is reversible, and affects both the shape and intercept of a Lineweaver-Burk plot. Methylglyoxal bis(aminoamidinohydrazone):

$H_2N.NH.C(:NH).NH.N:C(CH_3).CH:N.NH.C(:NH).NH.NH_2$

is also a very powerful irreversible inhibitor of *S*-adenosylmethionine decarboxylase for both *Sacch. cerevisiae* and rat liver (Pegg and Conover, 1976). Cohn *et al.* (1977) have shown that the cofactor for *S*-adenosylmethionine decarboxylase from *Sacch. cerevisiae* is covalently bound pyruvate and not pyridoxal phosphate. Regulation of *S*-adenosylmethionine decarboxylase has been little studied in *Sacch. cerevisiae*, although it has been shown that a decrease in activity occurs on sporulation and a rapid increase occurs on ascospore germination (Shapiro and Ferro, 1977). In *A. nidulans*, highest *S*-adenosylmethionine decarboxylase activity is found in germinating conidia (Stevens *et al.*, 1976). The enzyme is stimulated by putrescine, to a lesser extent by the putrescine analogue, 1,4-diaminobutanone, and slightly by cadavarine and 1,6-diaminohexane (Stevens *et al.*, 1977). It is inhibited by MGBA, 50 μM of the compound causing 50% inhibition in crude extracts (Winther and Stevens, 1976). Its sensitivity to MGBA thus appears to lie between that of the enzymes from *E. coli* and *Sacch. cerevisiae*, and this is probably due in part to the fact that only crude extracts from *A. nidulans* have been used and so much of the inhibitor could be non-specifically absorbed onto other proteins.

Therefore, on the whole, *S*-adenosylmethionine decarboxylase from both *Sacch. cerevisae* and *A. nidulans* resemble the enzyme from rat liver. Two other eukaryote species in which *S*-adenosylmethionine decarboxylase has been investigated show different behaviour. The enzyme from *P. polycephalum* is insensitive to putrescine, but is sensitive to inhibition by MGBA (Mitchell and Rusch, 1973). Pyridoxal phosphate does not appear necessary for activity. *Dictyostelium discoideum* has low *S*-adenosylmethionine decarboxylase activity, and the enzyme is not activated by putrescine or Mg^{2+} and is highly sensitive to 10 μM MGBA which decreases activity to 20% of the controls (R. Turner, unpublished observations). Both *P. polycephalum* and *D. discoideum* show fungal and protozoan characteristics, and their evolutionary relationships remain uncertain. In both the distribution of their oligoamines and in their possession of putrescine-insensitive *S*-adenosylmethionine decarboxylases, they resemble bacteria and the protozoan *Tetrahymena*

pyriformis (Pösö *et al.*, 1976). In several species of *Aspergillus* and *Penicillium*, in *Mucor hiemalis, Rhizopus stolonifer, N. crassa, Trichoderma viridie* and *Alternaria alternata, S*-adenosylmethionine decarboxylases are all activated by putrescine (Hart *et al.*, 1978). It seems probable that, with the exception of the Mycetozoans, most fungi will be found to have putrescine-activated *S*-adenosylmethionine decarboxylases like those of mammals.

7. *Other Enzymes Concerned with Oligoamine Metabolism in Fungi*

Very little is known about possible spermidine and spermine synthases in fungi. The assays for these enzymes are complex and time-consuming, and one of the substrates, decarboxylated *S*-adenosylmethionine, is not commercially available but has to be synthesized enzymically using partially purified *S*-adenosylmethionine decarboxylase from *E. coli* (Pösö *et al.*, 1976). The more rapid assays recently devised by Raina *et al.* (1976) and by Hibasami and Pegg (1978) may encourage further studies of these enzymes. Spermidine synthase has been partially separated from *S*-adenosylmethionine decarboxylase in *Sacch. cerevisae* (Jänne *et al.*, 1971). There is approximately a six-fold excess of spermidine synthase over *S*-adenosylmethionine decarboxylase in crude sonicated extracts. Spermidine synthase has been measured in *A. nidulans*, and there is a rapid increase in activity with the onset of germination (L. Stevens, unpublished observations).

There is no evidence that oligoamines become acetylated in fungi, although putrescine has been found to be carbamoylated in *Sacch. cerevisiae* and in *A. nidulans*; the physiological role of this reaction is unclear (Friedman, 1957).

IV. Ornithine, Arginine and Methionine Metabolism in Relation to Oligoamine Metabolism in Fungi

A. INTRODUCTION

Although oligoamines are present in moderate concentrations in fungi, especially when compared with other intracellular cations such as magnesium, they represent less than 5% of the total cellular nitrogen. Many fungi are able to use a wide variety of nitrogen sources, e.g. nitrate, ammonia, amino acids, urea, amines and amides, and from these they synthesize all of the intracellular nitrogenous components. They thus

have a wide range of biosynthetic capabilities. Many of the anabolic pathways involve common precursors and intermediates, and the flux through any given pathway may depend on the nutritional and physiological state of the organism. Oligoamines are synthesized from either ornithine or from arginine and methionine, and these amino acids are used extensively in other metabolic pathways. Arginine is a good source of nitrogen in fungi, and a number of fungi can grow using arginine as the sole source of nitrogen. It is therefore important to understand the extent to which arginine, ornithine and methionine are used on other metabolic pathways, and how these pathways compete with the requirements for oligoamine synthesis.

Nitrogen metabolism in fungi has been most extensively studied in *Sacch. cerevisiae, N. crassa* and *A. nidulans*, and the work described in this section will be largely confined to results obtained using these organisms. The metabolic pathways of arginine and ornithine metabolism in these organisms have been worked out by a combination of biochemical and genetic methods. Control of nitrogen metabolism is a complex process. All three organisms exhibit nitrogen repression, a process which is similar to that of carbon catabolite repression, well known in bacteria (see Pateman and Kinghorn, 1976; Kinghorn and Pateman, 1977). In addition to regulation of *de novo* enzyme synthesis, and allosteric mechanisms, there is an additional control mechanism to be considered, namely compartmentation of the enzymes and substrates. This aspect has been explored by R. H. Davis's group using *N. crassa* (Davis, 1975); it has been less extensively studied in *Sacch. cerevisiae* but not at all in *A. nidulans*. Here we aim to discuss those aspects of arginine, ornithine and methionine metabolism which have a bearing on oligoamine metabolism in fungi.

B. METABOLIC PATHWAYS OF ARGININE AND ORNITHINE IN FUNGI

Metabolism of arginine and ornithine in fungi is outlined in Fig. 4 in such a way as to emphasize the main routes of ornithine production and ornithine utilization. There is either biochemical or genetic evidence for the existence of the steps indicated in *Sacch. cerevisiae* (De Deken, 1962, 1963), *N. crassa* (Weiss and Davis, 1973; Cybis and Davis, 1975; Castaneda *et al.*, 1967) and *A. nidulans* (Cybis *et al.*, 1972a). Although the pathway is similar in all three organisms, there are minor differences.

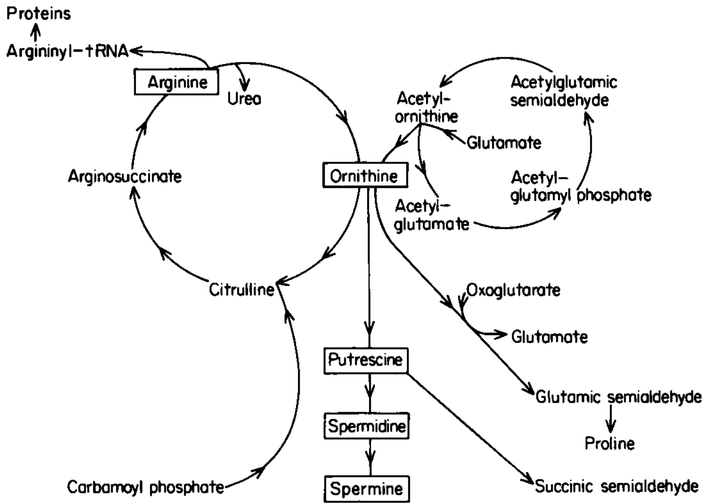
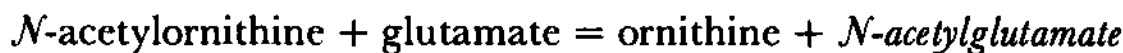


FIG. 4. Metabolic relationships between arginine, ornithine and oligoamines in fungi.

Both Sacch. cerivisiae and *N. crassa* convert *N*-acetylornithine to ornithine using acetylornithine transactylase:



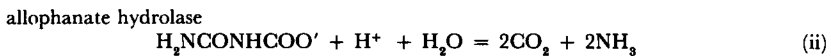
N-acetylornithine + glutamate = ornithine + *N-acetylglutamate*

whereas *A. nidulans* possesses an *N*-acetylornithase and, in this respect, resembles *E. coli* (Vogel and Vogel, 1974).

Another difference lies in the conversion of urea to carbon dioxide and ammonia. Both *A. nidulans* and *N. crassa* possess an active urease (Scazzocchio and Darlington, 1968; Kolmark, 1969; Srb and Horowitz, 1944) whereas, in *Sacch. cerevisiae*, two enzymes are necessary for the conversion (Whitney *et al.*, 1973):

urea carboxylase

$$\text{urea} + \text{ATP} + \text{HCO}_3' = \text{H}_2\text{NCONHCOO}' + \text{ADP} + \text{Pi} \qquad \text{(i)}$$



allophanate hydrolase

$$\text{H}_2\text{NCONHCOO}' + \text{H}^+ + \text{H}_2\text{O} = 2\text{CO}_2 + 2\text{NH}_3 \qquad \text{(ii)}$$

In both *Sacch. cerevisiae* and *N. crassa* there is evidence for two separate carbamoyl phosphate synthetases, the function of one being to provide carbamoyl phosphate for arginine synthesis and of the other for pyrimidine biosynthesis (Lacroute *et al.*, 1965; Davis, 1972), but it is only in *N. crassa* that the products of the two enzymes are separated and not available for synthesis of both arginine and pyrimidines. The two

enzymes are inhibited by their end products, namely arginine and uracil (Thuriaux *et al.*, 1972).

Ornithine is synthesized both from arginine via the arginase reaction and from glutamate via *N*-acetylornithine. Ornithine is utilized in reactions catalysed by ornithine carbamoyl transferase, ornithine decarboxylase and transaminase.

The reaction catalysed by ornithine transaminase is reversible, the equilibrium constant favouring conversion of ornithine to glutamic semialdehyde (Strecker, 1965):

$$K_{eq} = \frac{[\text{glutamic-}\alpha\text{-semialdehyde}]\ [\text{glutamate}]}{[\text{L-ornithine}]\ [\text{2-oxoglutarate}]} = 70$$

However, since glutamic semialdehyde spontaneously cyclizes to form Δ '-pyrroline-5-carboxylate, its reversibility under physiological conditions is limited. The same does not hold for *N*-acetylornithine transaminase where the amino group is protected. Ornithine transaminase may, however, contribute in a minor way to biosynthesis of ornithine since double mutants of *A. nidulans* lacking arginase and blocked in ornithine biosynthesis still show residual ornithine biosynthesis, whereas triple mutants (aga A^- orn C^- orn A^-), also lacking ornithine transaminase, show complete ornithine auxotrophy (Arst, 1977). Ornithine transaminase-less mutants of *N. crassa* are prototrophic (Davis, 1968). In the species investigated, i.e. *Sacch. cerevisiae* (Middlehoven, 1964), *A. nidulans* (Cybis and Weglenski, 1969) and *N. crassa* (Davies *et al.*, 1970), higher levels of arginase and ornithine transaminase are induced by growth on media containing arginine. Induction of ornithine transaminase does not depend on arginase activity as it occurs in arginase-less mutants of *N. crassa* (Davis *et al.*, 1970). Arginine is also thought to be the co-inducer of ornithine transaminase in *A. nidulans* (Bartnik *et al.*, 1973). Ornithine will also act as an inducer of ornithine transaminase and of arginase, but it is less effective than arginine (Middlehoven, 1964; Davis *et al.*, 1970; Stevens and Heaton 1973).

Arginase has not been detected in bacteria such as *E. coli* (Morris *et al.*, 1970), *K. aerogenes* or *B. stearothermophilus* (L. Stevens, unpublished observations), but fungi, such as *A. nidulans, N. crassa* and *Sacch. cerevisiae,* have appreciable activities. The role of arginase in fungi differs from that in mammalian liver. In the latter it is primarily concerned with mechanisms for removing excess nitrogen from the organism whereas, in fungi, arginine can act as a good source of nitrogen and arginase serves to

TABLE 6. Arginine- and ornithine-metabolizing enzymes in *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae*

Enzyme	Activity	Effect of presence of arginine in the medium	Effect of presence of ornithine in the medium	Subject to ammonia repression	Intracellular location	References
Aspergillus nidulans						
Arginase EC.3.5.3.1	100	induction	no effect	+		Cybis and Weglenski (1969); Bartnik *et al.* (1973)
Ornithine transaminase EC.2.6.1.13	10.0	induction	induction	+	cytosol	Cybis and Weglenski (1969); Bartnik *et al.* (1973); Stevens and Heaton (1973)
N-Acetylornithine transminase EC.2.6.1.11	6.0					Cybis *et al.* (1972a)
Ornithine carbamoyl transferase EC.2.1.3.3	230	depression when arginine pool depleted	induction			Cybis *et al.* (1972a,b)
Ornithine decarboxylase EC.4.1.1.17	0.07	no effect	no effect		cytosol	Stevens *et al.* (1976)
Acetylornithinase EC.3.5.1.16	4000					Cybis *et al.* (1972a)
Neurospora crassa						
Arginase EC.3.5.3.1	1200	induction	induction	+	cytosol	Mora *et al.* (1972); Weiss and Davis (1973); Davis *et al.* (1970)

Ornithine transaminase EC.2.6.1.13	22.0	induction	induction	+	cytosol	Weiss and Davis (1973); Davis *et al.* (1970)
N-Acetylornithine transaminase EC.2.6.1.11	14.7				mitochondria	Cybis and Davis (1975)
Ornithine carbamoyl transferase EC.2.1.3.3	250				mitochondria	Weiss and Davis (1973)
Ornithine decarboxylase EC.4.1.1.17	0.051	induction			cytosol	Weiss and Davis (1973); Sikora and McDougall (1977)
Acetylornithine transacetylase EC.2.3.1.35	14.6				mitochondria	Weiss and Davis (1973); Cybis and Davis (1975)
Saccharomyces cerevisiae						
Arginase EC.3.5.3.1	233	induction	induction	+	cytosol	Middlehoven (1964); Penninckx and Wiame (1976)
Ornithine transaminase EC.2.6.1.13	3.3	induction	induction	+		Middlehoven (1964); Penninckx and Wiame (1976)
N-Acetylornithine transaminase EC.2.6.1.11	38.9				mitochondria	Wipf and Leisinger (1977)
Ornithine carbamoyl transferase EC.2.1.3.3	1004				cytosol	Bechet *et al.* (1962); Messenguy *et al.* (1971); Ramos *et al.* (1970); Urrestarazu *et al.* (1977); Wipf and Leisinger (1977)
Acetylornithine transacetylase EC.2.3.1.35	46.4				mitochondria	Wipf and Leisinger (1977)

Enzyme activities in minimal medium are expressed as nmoles product/min/mg protein.

convert arginine into other amino acids for further metabolism. The urea formed may be degraded further to ammonia and carbon dioxide, and the ammonia incorporated into amino acids via the glutamate dehydrogenase reaction. Both arginase and ornithine transaminase are subject to repression by ammonia (Middlehoven, 1964; Bartnik *et al.*, 1973). Nitrogen starvation increases arginase and ornithine transaminase activities by depression (Middlehoven, 1970). These increases in activity, caused by nitrogen starvation, probably enable intracellular arginine reserves to be used as sources of other amino acids.

Unlike arginase and ornithine transaminase, synthesis of ornithine carbamoyl transferase is suppressed when organisms are grown in the presence of arginine (Bechet *et al.*, 1962). In *Sacch. cerevisiae*, activity of ornithine carbamoyl transferase is also inhibited by ornithine and arginine by what appears to be a unique mechanism. Arginase binds tightly to ornithine carbamoyl transferase in the presence of ornithine and arginine. This binding completely inhibits ornithine carbamoyl transferase activity while leaving arginase activity unmodified (Messenguy *et al.*, 1971).

The rate-limiting steps in biosynthesis of arginine from glutamate via ornithine in *Sacch. cerevisiae* and *N. crassa* have been shown to be carbamoyl phosphate synthase and acetylglutamate kinase (Cybis and Davis, 1975; Hilger *et al.*, 1973). Arginine inhibits the activities of both enzymes (Lacroute *et al.*, 1965; Cybis and Davis, 1974). The properties of enzymes connected with ornithine metabolism are summarised in Table 6.

C. BIOSYNTHESIS OF METHIONINE AND *S*-ADENOSYLMETHIONINE IN FUNGI

Besides ornithine, the other amino acid directly involved in synthesis of oligoamines is methionine, which after adenylation to *S*-adenosylmethionine provides a single propylamine group for spermidine synthesis and two propylamine groups for spermine synthesis. This role in which it is involved in transfer of the propylamine moiety seems to be an unusual one for *S*-adenosylmethionine, which is more often thought of as catalysing transfer of methyl groups. Biosynthesis of methionine in fungi has been studied mainly in three species, *Sacch. cerevisiae, N. crassa* and *A. nidulans.* In all three species, synthesis occurs from aspartate via homoserine and *O*-acetylhomoserine as shown in Fig. 5. The sulphur

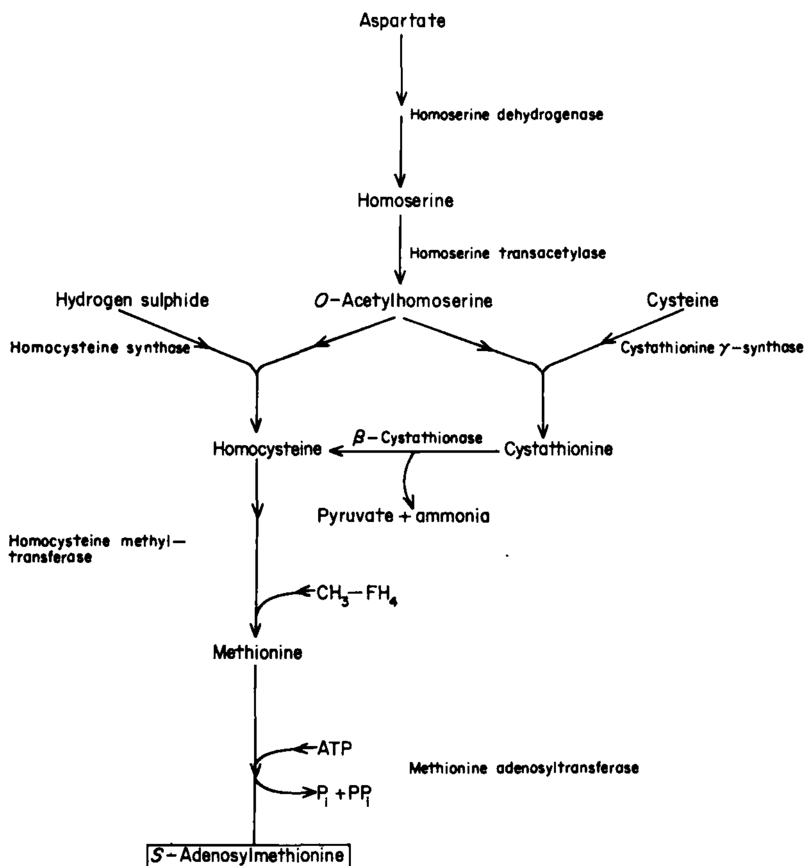


FIG. 5. Biosynthesis of *S*-adenosylmethionine in fungi.

atom is derived from inorganic sulphate by steps similar to those occurring in bacteria (Umbarger and Davis, 1962). There are two different routes from acetylhomoserine to homocysteine, either by direct sulphurylation of acetylhomoserine with hydrogen sulphide, or by a combination of acetylhomoserine with cysteine to yield cystathionine which is then hydrolysed to homocysteine. The relative importance of these two routes varies in different fungi, and is discussed by De Robichon-Szulmajster and Surdin-Kerjan (1971). In *Sacch. cerevisiae*, direct sulphurylation seems to be the favoured route (Cherest *et al.*, 1969), whereas the cystathionine route is the main pathway in *N. crassa* (Kerr and Flavin, 1970) and *A. nidulans* (Paszewski and Grabski, 1975).

Methionine is converted to *S*-adenosylmethionine by methionine adenosyl transferase. The presence of either methionine or *S*-adenosylmethionine in the growth medium causes repression of biosynthesis of methionine. Further, the presence of methionine in the growth medium for *Sacch, cerevisiae* represses homoserine dehydrogenase, homoserine *O*-transacetylase, ATP sulphurylase, homocysteine synthetase and methionine-adenosyl transferase but, at the same time, there are increases in intracellular methionine and *S*-adenosylmethionine concentrations (Cherest *et al.*, 1969; Mertz and Spence, 1972; Holcomb and Shapiro, 1975). Growth in the presence of *S*-adenosylmethionine represses synthesis of methionine-synthesizing enzymes such as ATP sulphurylase, sulphate reductase, homocysteine synthetase and methyladenosyl transferase (Cherest *et al.*, 1973; Spence *et al.*, 1977). The intracellular *S*-adenosylmethionine concentration is also dependent on the glucose concentration in the growth medium and there is some evidence that *S*-adenosylmethionine may regulate RNA synthesis in *Sacch. cerevisiae* (Law and Ferro, 1977). The intracellular pool of *S*-adenosylmethionine may vary between about 0.3 mM, when growth is on a minimal medium, to about 5 mM when the medium is supplemented with methionine (Cherest *et al.*, 1973). Much of this *S*-adenosylmethionine may be concentrated in the vacuole in *Sacch. cerevisiae* (Schwencke and De Robichon-Szulmajster, 1976) but there is still likely to be sufficient in the cytosol to saturate the *S*-adenosylmethionine decarboxylase which has an apparent K_m value of 0.09 mM in the presence of saturating putrescine (Pösö *et al.*, 1975).

D. TRANSPORT AND INTRACELLULAR COMPARTMENTATION OF ARGININE, ORNITHINE, METHIONINE AND *S*-ADENOSYLMETHIONINE IN FUNGI

The amounts of ornithine and *S*-adenosylmethionine available for oligoamine synthesis and for other reactions in fungi not only depend upon metabolic interconversions taking place within the cell but also on the rate of uptake into the cells of arginine, ornithine, methionine and *S*-adenosylmethionine from the growth media and their subsequent distribution within the cells. Amino-acid transport has been most studied in *Sacch. cerevisiae, N. crassa, A. nidulans* and *Penicillium chrysogenum* and has been reviewed by Pateman and Kinghorn (1976). A number of permeases have been discovered having different ranges of specificity. One of the difficulties in studying permeases is that it is the transport

TABLE 7. Sizes of intracellular amino-acid pools in *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae*

Organism	Arginine (mM)	Ornithine (mM)	Methionine (mM)	*S*-Adenosyl-methionine (mM)	Conditions	References
Aspergillus nidulans	7.18	2.8	0.7		Minimal medium	Robinson *et al.* (1973); L. Stevens and M. Winther (unpublished observations)
Neurospora crassa	13.0[a]	6.9[a]			Minimal medium	Schmidt and Brody (1975);
	8	12			Minimal medium	Weiss and Davis (1973)
Saccharomyces cerevisiae				0.1	Minimal medium	Holcomb and Shapiro (1975);
			0.5[a]	0.3[a]	Minimal medium	Cherest *et al.* (1973)

[a] Calculated from the data given in the papers cited assuming that one gram dry weight equivalent of cells contains 3 ml cell water (Pall, 1970a).

function that is usually measured, and it has not yet been possible in any fungi to identify the proteins involved.

Permeases are able to concentrate amino acids within the cell against concentration gradients and, with many amino acids, the transport is energy dependent. Some have also been shown to be ammonia repressible. Much of the evidence for specific permeases depends upon isolation of mutants defective in the transport of a particular amino acid. The intracellular concentrations of some amino acids are high in fungi (Table 7), especially when compared with those in bacteria.

In *N. crassa*, Pall (1969, 1970a, b) has shown the existence of four major transport systems responsible for uptake of acidic, neutral and basic amino acids, and similar transport systems for groups of amino acids have been identified in *A. nidulans* (Robinson *et al.*, 1973). In addition, transport systems for specific amino acids have been demonstrated. The arginine permease in *Sacch. cerevisiae* transports arginine at a rate which is unaffected by the presence of most other amino acids in the medium. *Saccharomyces cerevisiae* and *N. crassa* mutants, resistant to the arginine analogue canavanine, have impaired arginine uptake (Grenson *et al.*, 1966; Sanchez *et al.*, 1972). This arginine permease may transport other basic amino acids at a slower rate, but there is also evidence for a separate lysine permease (Grenson, 1966). The basic amino-acid permease in *A. nidulans* transports arginine preferentially to lysine and ornithine, and methionine seems to be transported by a less specific permease for neutral amino acids (Piotrowska *et al.*, 1976). *Saccharomyces cerevisiae*, in contrast has two methionine permeases, one high-affinity permease ($K_{m\ methionine} = 12\ \mu M$) and a low-affinity permease ($K_{m\ methionine} = 0.77\ mM$) (Gits and Grenson, 1967). *S*-Adenosylmethionine is actively taken up from the medium and concentrated in the vacuoles of *Sacch. cerevisiae* by means of a single highly specific permease, $K_m = 2\ \mu M$ (Spence *et al.*, 1977; Robichon-Szulmajster *et al.*, 1977). The ability of conidia to transport amino acids increases during the early stages of conidial germination in *N. crassa* (Tisdale and Debusk, 1970).

The distribution of amino acids in the cytoplasm of fungal cells is not uniform. This applies both to amino acids entering the cytoplasm from the growth medium and to amino acids synthesized endogenously. Both yeasts (Wiemkin and Durr, 1974; Wiemkin and Nurse, 1973) and *N. crassa* (Subramanian *et al.*, 1973) have vacuoles capable of storing amino acids, particularly arginine and ornithine. The intracellular distribution of arginine and ornithine has been studied in *N. crassa* by Davis's group

TABLE 8. Michaelis constants for ornithine- and arginine-metabolizing enzymes from *Aspergillus nidulans* and *Neurospora crassa*

Enzyme	Michaelis constant (mM) *Neurospora crassa*	*Aspergillus nidulans*	Reference
Arginase	5	—	Weiss and Davis (1973)
Ornithine transaminase	2	7	Vogel and Kopac (1960); Stevens and Heaton (1973)
Arginyl-tRNA synthetase	0.02	—	Nazario (1967)
Ornithine decarboxylase	0.4	0.06	Weiss and Davis (1973); Stevens *et al.* (1976)

(Bowman and Davis, 1977a, b; Weiss, 1976; Weiss and Davis, 1977). The enzymes involved in oligoamine synthesis, together with arginyl-tRNA synthetase, arginase, urease, argininosuccinate synthetase and ornithine transaminase, are present in the cytosol, whereas ornithine carbamoyl transferase and *N*-acetylornithine transaminase are present in the mitochondria (Weiss and Davis, 1973; Cybis and Davis, 1975). Over 90% of the intracellular arginine and ornithine in *N. crassa* growing in minimal medium is present in the vesicular pool (Subramanian *et al.*, 1973). Endogenous ornithine is synthesized in mitochondria and then transported through the cytosol to the vesicle. Endogenous arginine is synthesized from citrulline which is formed in mitochondria and then converted to arginine in the cytosol. As with ornithine, most of of arginine is taken up into the vesicle.

When *N. crassa* is grown in minimal medium, the small amount of arginine and ornithine present in the cytosol is used mainly for protein synthesis and for oligoamine synthesis, and there is little catabolism of ornithine and arginine. From the relevant Michaelis constants (Table 8), it will be seen that arginine-tRNA synthetase and ornithine decarboxylase have higher affinities for cytosolic arginine and ornithine than do arginase and ornithine transaminase. Since most of the cell's arginine and ornithine is stored in vesicles, there is little wasteful catabolism of these amino acids when cells are grown on minimal medium. When the medium is supplemented with arginine, the cytosolic arginine pool expands greatly although the total amount of intracellular arginine increases by a much smaller proportion (Weiss, 1976), and arginine and ornithine catabolism is increased. This method for controlling arginine and ornithine metabolism differs from that in yeast, described earlier, in which ornithine carbamoyl transferase and

arginase combine in the presence of arginine and ornithine with the result that the activity of ornithine carbamoyl transferase is inhibited.

From a comparison of ornithine decarboxylase and ornithine transaminase in both *N. crassa* and *A. nidulans*, it is clear that, under most physiological conditions, ornithine decarboxylase must be saturated with ornithine and its rate must then be independent of any fluctuations in intracellular ornithine concentration, whereas ornithine transaminase must be operating in the proportional region and the rate must therefore vary with the intracellular concentration.

V. Oligoamines and Fungal Development

A. INTRODUCTION

In Section III (p. 86) we have seen that, in general, fungi synthesize oligoamines in a manner similar to that of higher eukaryotes rather than to that of prokaryotes. This applies both to the proportions present and also to the characteristics of the biosynthetic enzymes. However, the amounts of putrescine, spermidine and spermine in cells of higher eukaryotes are not constant but vary with the stage of growth or development of the particular organism or tissue concerned, and this also is the case in the fungi which have so far been investigated. In this section, we briefly review the relevant morphological changes and biochemistry of different stages of fungal growth and development in order to put in perspective oligoamine metabolism and possible function. We do this under three headings: (i) germination, (ii) vegetative growth and hyphal differentiation, and (iii) sporulation and reproductive development. There are a number of aspects of fungal development in which oligoamine metabolism has not been studied, and we attempt to show areas in which it may be profitable to make more detailed studies.

B. GERMINATION

Germination of fungal spores has been been a favourite system for pursuing physiological and biochemical studies of fungal development. The reasons for this are numerous and include such aspects as the simplicity of the development, especially in unicellular uninucleate spores, the ability to obtain uniform and synchronous changes in a homogeneous population of cells, and the universality of the dormant phase in the life cycle capable of resuming active growth when a suitable

change in the environment occurs. There are many similarities in the underlying molecular changes between the onset of growth in fungal spores and other tissues or cells about to undergo rapid growth, for example the development of eggs, and the onset of cell proliferation in tissue regeneration and tumour growth.

Spore germination often results in a near synchronous cell division, at least in the early stages, and this has lead to its use as a system for studying changes during the cell cycle. However, it is important when so doing to distinguish between events which are peculiar to germination as opposed to those which occur regularly during each cell division.

Four distinct but overlapping stages can be discerned during germination of most fungal spores: (i) hydration and swelling of the spore, (ii) physiological and morphological changes within the intact spore which depend on metabolism of endogenous or exogenous energy sources, (iii) protrusion of the germ tube through the cell wall, and the polarization of growth (this is often used as a visual criterion of germination), and (iv) germ tube elongation and apical growth.

1. *Inhibitors and Activators of Dormancy*

By entering a dormant state, many fungal spores are capable of remaining viable for long periods of time in unfavourable environmental conditions. Dormancy can be due to one of two factors. Where the resting period is simply a response to unfavourable environmental conditions, this is termed "exogenous dormancy". In some spores, a metabolic block occurs or a self inhibitor is present and, in these cases, the dormancy is known as "constitutive dormancy". Only a few self inhibitors have so far been identified, including *N*, *N*-dimethylguanosine in *D. discoideum* (Bacon *et al.*, 1973; Bacon and Sussman, 1973), 5-isobutyroxy-β-ionone in *Peronospora tabacina* (Leppik *et al.*, 1972) and methyl cis-3,4-dimethoxycinnamate and methyl cis-ferulate in rust fungi (Hess *et al.*, 1975). The mode of action of these self-inhibitors is not yet clear, but some appear to be inhibitors of protein synthesis (Leppik *et al.*, 1972; Lingappa *et al.*, 1973; Adelman and Lovett, 1974) and one group is thought to inhibit digestion of the germ-pore plug (Hess *et al.*, 1975). Since many of the self inhibitors act at early stages of germination, their study may lead to a greater understanding of germination-specific events.

As spores may sometimes be activated by exposure to extremes of

temperature and moisture, or by variations in lighting and chemical treatment (Sussman, 1976), it is possible that investigations on the underlying mechanisms may also help in understanding germination.

2. *Morphological Changes During Germination*

One of the first detectable changes to occur is swelling of the spore. The degree of swelling is variable, but involves volume increases of 2- to 30-fold. The initial swelling is a simple rehydration process, and this is followed by a metabolic swelling, or spherical growth. Spherical growth normally ceases at the start of germ-tube emergence, when growth becomes polarized, incorporation is limited to one or a few areas of the transition to polarized growth may be retarded or prevented in *Aspergillus niger* by growing it at elevated temperatures, thereby allowing prolonged spherical growth to occur.

Marked changes in morphology of the spore wall occur in early germination. In *A. nidulans*, two new layers become apparent soon after hydration (Florence *et al.*, 1972), and synthesis of new cell wall material during spherical growth has been detected by autoradiography in *Mucor rouxii* (Bartnicki-Garcia, 1973). During spherical growth, new cell wall material is deposited uniformly around the spore but, once growth becomes polarized, incorporation is limited to one or a few areas of the spore wall from which the germ-tube will arise. In most fungi, the wall of the germ tube is continuous with the new inner spore-wall layer. Enzymic digestion is thought to weaken the cell wall in the region of germ-tube emergence, but the role of hydrolases in this process has been studied in only a few species (Leighton and Stock, 1970).

The morphological changes already described represent the general pattern of development which occurs when most fungal spores germinate. There are a number of departures from this pattern. Cell-wall growth in budding yeasts is comparable to the spherical growth phase (Cortat *et al.*, 1972), but it is not followed by a polarized growth phase. The germination process in zoospores of, for example, *Blastocladiella emersonii* (Leaver and Lovett, 1976) and *Allomyces* sp. (Turian, 1975), has many unique features associated with their motility and also with development of a rhizoidal system on germination. In the myxomycetes, when a spore germinates, the wall splits and an amoeba emerges leaving the spore wall behind (Aldrich and Blackwell, 1976).

3. *Organelle Changes*

Dormant spores of fungi have a number of subcellular structural features in common. Vacuoles are few or absent, endoplasmic reticulum is sparse and mitochondria are often large and irregularly shaped. Those spores not requiring an exogenous energy source for germination have visible food reserves. As spores resume active growth, there are major changes in the number and morphology of most subcellular organelles. The organelles may aggregate around the site of the emergent germ tube and remain at the growing point until the establishment of apical growth (Bracker, 1971). There is an increase in nuclear volume followed by nuclear division, usually before or during germ-tube emergence (Van Etten *et al.*, 1976).

Certain spores have special features not universally found. For example, ribosomes in zoospores of *Blastocladiella* sp. are contained in a membrane-bound structure or nuclear cap which fragments at the round cell II stage. Also, another membrane-bound organelle, or "gamma particle", is present in zoospores and may be involved in wall synthesis since it has been shown to contain chitin synthetase, and it breaks up prior to wall biosynthesis (Myers and Cantino, 1971).

4. *Carbohydrate and Lipid Metabolism During Germination*

There is a large increase in oxygen consumption during germination accompanying the increase in metabolic rate. Some spores require exogenous nutrients in order to germinate, but others possess endogenous reserves and can often germinate without added nutrients. These reserves are varied. Carbohydrates are present in some spores, e.g. trehalose in *Neurospora* sp. (Lingappa and Sussman, 1959) and *Phycomyces* sp. (Rudolph and Ochsen, 1969) and glycogen in *Blastocladiella* sp. (Suberkropp and Cantino, 1972). The lipid content of dormant spores may vary from 1 to 30% of the dry weight (Steiner, 1957). The spores of *Neurospora* sp. and of rust fungi can germinate independently of an external supply of energy. They oxidize the fatty-acyl residues of their triglyceride reserve by β-oxidation. In many species, both carbohydrates and fats are metabolized at the onset of spore germination (Daly *et al.*, 1967) and the activities of respiratory enzymes undergo increases of up to 210-fold during germination (Ohmori and Gottlieb, 1965). Not only do lipids serve as energy reserves, but they may also, for example, undergo conversion to carbohydrates which are necessary for synthesis of cell-wall

polysaccharides (Reisener *et al.*, 1961; Weber and Hess, 1974), and they may also be used in phospholipid synthesis (Jackson and Frear, 1967). Unlike prokaryotes, fungi contain sterols, and these compounds have been found to stimulate germination and possibly sporulation (Weete, 1973; Hendrix, 1965). Sterols also function as hormones in sexual reproduction (Gooday, 1974; Raper, 1950).

5. *Protein Synthesis*

Protein synthesis has been found to occur during germination of all spores investigated to date. Furthermore, on the basis of studies using inhibitors of protein synthesis, there appears to be a universal requirement for some protein synthesis before complete germ-tube emergence (Lovett, 1976), although "activation" and encystment of aquatic zoospores may be an exception (Lovett, 1968; Soll and Sonneborn, 1971). Although synthesis of proteins occurs during germination, net accumulation may not; for example, germination of obligate parasites may result in small decreases in both total protein and RNA but, at the same time, extensive turnover of these macromolecules occurs (Trocha and Daly, 1970). Consequently it appears that new proteins required for germination may be synthesized at the expense of other non-essential proteins.

Although a wide range of proteins are synthesized by germinating spores (Van Etten *et al.*, 1972; Silverman *et al.*, 1974) only a small number may be essential and rate-limiting for further development. So far little progress has been made in identifying these essential proteins and the stages in which they become essential.

Protein synthesis is either undetectable or occurs at very low rates in dormant spores although, in some cases, many of the components necessary for protein synthesis, such as tRNAs, probably mRNA, ribosomes and amino acids, are present (see Lovett, 1976). The lack of protein synthesis in dormant spores may be due to one of a number of the following factors: (i) absence of initiation and elongation factors, (ii) the presence of endogenous inhibitors, and (iii) the low water content of the spores. In both *N. crassa* (Mirkes, 1974) and *B. emersonii* (Leaver and Lovett, 1974), the dormant spores appear to contain mainly monosomes and subunits but, within 20 min after initiation of germination, about 50% of the ribosomes are present as polysomes. The rapidity of this conversion suggests that preformed stored mRNA is used, especially as actinomycin D fails to affect this early occurrence of polysome formation

in *B. emersonii* (Leaver and Lovett, 1974). Polysome formation in conidia of *N. crassa* may be limited by the presence of oxidized glutathione. This latter compound can dissociate mRNA from ribosomes, and this leads to breakdown of polysomes (Kosower *et al.*, 1972). During germination, there is a rapid reduction of oxidized glutathione (Fahey *et al.*, 1975) and this might be responsible for removing the restriction on protein synthesis.

As mentioned earlier, protein turnover may be more evident than protein accumulation during germination. In the early stages of germination of *B. emersonii*, polysomes are present and radioactive precursors are incorporated into proteins, yet there is no net increase in protein. This is a consequence of an increase in proteolysis which maintains a steady state. Proteolysis must also be essential as a source of amino acids in those spores not requiring exogenous nutrient for germination, e.g. those species of *Uromyces* and *Peronospora* (Hollomo, 1971; Trocha and Daly, 1970). This is further supported by the significant role of proteolysis in *Microsporum gypseum* (Leighton and Stock, 1970) when phenylmethylsulphonylfluoride, an inhibitor of certain proteases, inhibits macroconidial germination.

6. *DNA Synthesis*

The fungal genome displays many of the characteristics of typical eukaryotes. Although there was initially some controversy, it now seems established that histone-like proteins are present in fungi (Morris, 1976; N. R. Morris *et al.*, 1977; Wintersberger *et al.*, 1973; Hsiang and Cole, 1973; Horgen *et al.*, 1973), and that the chromatin contains RNA and non-histone proteins and has the characteristic nucleosome structure (Morris, 1976; Noll, 1976). Measurements of the size of the fungal genome indicate a range of two to eleven times that of *E. coli* (Storck, 1974; Ojha *et al.*, 1977). The fungal genome comprises a number of chromosomes. Hybridization analysis has shown that fungi have 5–25% of their DNA present as re-iterated transcripts, and these can only be partly accounted for by re-iterated rRNA and tRNA cistrons (Christiansen *et al.*, 1971; Dutta, 1973; Ojha *et al.*, 1977). Part of fungal DNA is extranuclear; from 1–20% may be mitochondrial DNA and additional species have also been found in some fungi (Myers and Cantino, 1971; Sinclair *et al.*, 1967).

Synthesis of DNA and nuclear division occur during the germination

of many fungal spores. This has been established by chemical determinations of DNA, by cytology and by measurement of incorporation of radioactive precursors into DNA (Van Etten *et al.*, 1976). There is, however, an important difference between DNA synthesis in some multinucleate spores when compared with uninucleate spores. Uninucleate spores contain the haploid genome and so must be duplicated before nuclear division can occur. In the dormant state, they are in the G_1 or G_0 phase. On the other hand, multinucleate spores appear to contain nuclei in both G_1 and G_2 phases of the cell cycle. Those in G_2 are capable of nuclear division without DNA synthesis. An increase in the number of nuclei in each spore prior to a significant incorporation of radioactive precursors into DNA was detected in *Phycomyces blakesleeanus* (Van Assche and Carlier, 1973) and, in *N. crassa*, 30% of the nuclei in the conidia can divide when DNA synthesis is completely inhibited (Loo, 1976). As both uni- and multi-nucleate spores may be produced in the life-cycle of a single species, it is important to be aware of this consideration. Thus, in the case of multinucleate macroconidia of *N. crassa*, about 40% of the conidia can germinate in the absence of DNA synthesis (Loo, 1976). However, data from uninucleate microconidia of *N. crassa* did not reveal whether or not DNA synthesis is essential for germination.

It is clear that, in most fungal spores, DNA synthesis occurs subsequent to early transcription and other pregermination changes, but the sequence of the later changes is not so clear, in particular whether DNA synthesis is necessary for germ-tube emergence. Some experiments using inhibitors of DNA synthesis which have been designed to assess the role of DNA synthesis in germination have proved inconclusive since they have not demonstrated whether the inhibitor penetrates the spores in question or the specificity of inhibition.

An interesting observation in *Uromyces* sp. is that nuclear division occurs only in germ tubes which are induced to form "infection structures" (appressoria) and not in those which continue apical extension (Maheshwari *et al.*, 1967). Thus nuclear division could be a necessary prerequisite for appressoria differentiation.

The DNA polymerase activity of a number of fungi has now been investigated, and the work of Gong *et al.* (1973) showed that the purified enzymes of both dormant and germinated spores have nearly identical properties, though the polymerase molecules appear not to bind as tightly to DNA in dormant spores. It has been suggested that, in general, the

DNA synthetic capacity of an organism is not controlled directly by levels of DNA polymerase (Rosenberg and Cavalieri, 1965).

A number of temperature-sensitive mutants of *A. nidulans*, defective in their mitotic apparatus, have been isolated (Morris, 1976; Orr and Rosenberger, 1976). These mutants are capable of germination at the restrictive temperature, but conidial development is abnormal in that they remain uninucleate. This shows that mitosis is not, in itself, a prerequisite for germ-tube emergence. However, there is a doubling of DNA content prior to germ-tube emergence, but whether or not this DNA synthesis is necessary is uncertain. Both synthesis of DNA and germ-tube emergence may simply be responses to a common inducer.

7. *Ribonucleic Acid Synthesis*

Synthesis of RNA nearly always occurs during the germination of fungal spores, although its timing and the amounts of RNA made are quite variable. The synthesis can begin before, during, or after initiation of protein synthesis depending on the species (Van Etten *et al.*, 1976). In those fungi where protein synthesis preceeds RNA synthesis, at least some mRNA must be present in the dormant spore. Indeed, a presumptive mRNA fraction has been found in uredospores of *Uromyces phaseoli* (Ramakrishnan and Staples, 1970) and in conidia of *N. crassa* (Bhagwat and Mahadevan, 1970). Similarly the presence of a small number of polysomes in dormant conidia of *N. crassa* (Mirkes, 1974) is good evidence for the existence of mRNA in the spore.

Though fungal spores may synthesize rRNA during germination, this does not establish that such synthesis is essential for germ-tube emergence. The most frequent means of attempting to establish whether or not such synthesis is essential is using specific metabolic inhibitors. Unfortunately, most of the commonly used inhibitors do not have sufficient specificity to be used with confidence. Not only is it necessary to establish that RNA synthesis is being diminished in the presence of the inhibitor, but it is also necessary to monitor incorporation into DNA and protein as well to establish whether or not they are affected by the inhibitor. In those experiments where this has not been done, only tentative conclusions can be drawn.

Another possible way to determine whether germination requires nucleic acid synthesis is using auxotrophic mutants. Many adenine-requiring auxotrophs of *N. crassa* have been found to germinate in the

absence of adenine (Meaden and Wellman, 1967), but this is not in itself proof that RNA synthesis is not needed; turnover of endogenous RNA could provide sufficient precursors for new mRNA to be synthesized. It is tentatively concluded that many spores require some RNA synthesis for germination to take place (Van Etten *et al.*, 1976), but that others may not, for example *Sacch. cerevisiae* ascopores (Rousseau and Halvorson, 1973) and *Botryodiplodia theobromae* pycnidiospores (Brambl and Van Etten, 1970).

The overall means of control of RNA synthesis in fungi is largely unknown. On the basis of studies showing sequential timing of rRNA, tRNA and mRNA synthesis during germination, as in *Aspergillus oryzae* (Ono *et al.*, 1966). *Peronspora tabacina* (Hollomo, 1970) and *Botryodiplodia theobromae* (Knight and Van Etten, 1976), it seems that fungi are capable of independently controlling the rates of synthesis of various RNA species. As fungi have multiple RNA polymerase activities (Gong and Van Etten, 1972; Van Etten *et al.*, 1976), it is possible that variation in these activities could be a controlling factor in RNA synthesis. Three RNA polymerase activities have been detected in *A. nidulans* and separated by chromatography on phosphocellulose (M. D. Winther and L. Stevens, unpublished observations). The proportions of these activities do not change significantly during germination, but there is a four-fold increase in specific activity of each during germination. Thus, synthesis of RNA polymerase may be important in the early stages of germination.

8. *Oligoamine Synthesis*

Dramatic increases in synthesis of oligoamines have been found in many organisms and tissues at the onset of proliferative growth. Although fungal germination has not been examined comprehensively from this standpoint, the studies which have been made to date in this area show a similar relationship (see Table 9). The fungi so far studied are *Puccinia graminis titrici* (Kim, 1971), *B. emersonii* (Mennucci *et al.*, 1975), *A. nidulans* (Stevens *et al.*, 1976; Winther and Stevens, 1976), *N. crassa* (McDougall *et al.*, 1977), *Rhizopus stolonifer* (Nickerson *et al.*, 1977b), *Botryodiplodia theobromae* (Nickerson *et al.*, 1977b), *Sacch. cerevisiae* (Shapiro and Ferro, 1977) and *P. polycephalum* (Mitchell and Rusch, 1973). However, the same parameters have not been studied in each of the organisms and this sometimes makes comparisons difficult.

TABLE 9. Changes in contents of oligoamines, protein and nucleic acids, and in enzyme activities during germination

Organism	Increases during germination[a]					References
	Oligoamines	Enzyme[b]	Protein	RNA	DNA	
Physarum polycephalum microcysts	3-5 putrescine 1.0 spermidine	>10 Ornithine decarboxylase >20 *S*-adenosylmethionine decarboxylase	1.0	—	—	Mitchell and Rusch (1973)
Blastocladiella emersonii zoospores	1.3 spermidine	>50 Ornithine decarboxylase	1.0[b]	1.2	—	Mennucci *et al.* (1975); Lovett (1968)
Rhizopus stolonifer sporangiospores	100 putrescine 6.2 spermidine	—	4.6	8.2	3.1	Nickerson *et al.* (1977b)
Aspergillus nidulans conidia	7.1 spermidine 4.9 spermine	>50 Ornithine decarboxylase >50 *S*-adenosylmethionine decarboxylase	2.6	7.7	2.0	Winther and Stevens (1976); Bainbridge (1971); Stevens *et al.* (1976)
Saccharomyces cerevisiae ascospores	—	>50 *S*-adenosylmethionine decarboxylase	2.6	1.6	1.6	Choih *et al.* (1977); Rousseau and Halvorson (1973)
Puccinia gramminis uredospores	1.1 spermidine	—	1.0[b]	1.0[b]	1.0	Kim (1971); Trocha and Daly (1970)
Botryodiplodia theobromae pycnidiospores	2.0 spermidine	—	1.4	2.8	1.8	Nickerson *et al.* (1977b)

[a] All amounts are expressed relative to that found in ungerminated spores.
[b] Turnover of macromolecule occurs.

In the first of these studies Kim (1971) showed that, in germinating uredospores of *Puccinia graminis tritici*, there is a small increase in the concentrations of spermidine and of another unidentified amine at a time when there was no net accumulation of RNA and protein. However, there is considerable turnover of protein during germination of spores of *Puccinia* sp. and of other rust fungi so that active protein synthesis is presumably occurring. In *B. emersonii* there is an increase in the intracellular spermidine level, but little change in the putrescine level during germination (Mennucci *et al.*, 1975). At the same time there is a 50-fold increase in ornithine decarboxylase activity and this is presumably necessary for production of putrescine which thus enables more spermidine biosynthesis. As with *Puccinia* sp., no net increase in the protein content of each cell occurs until after germ-tube emergence (Lovett, 1968). The 50-fold increase in ornithine decarboxylase illustrates how a substantial increase in the activity of one enzyme protein can occur without an increase in total protein, as long as turnover is taking place.

Ornithine decarboxylase is also synthesized very early in germination of conidia of *A. nidulans*, increasing by at least 100-fold in specific activity eight hours after the initiation of germination (Stevens *et al.*, 1976). This increase in activity is followed by an almost equally dramatic fall in activity by 20 hours after initiation of germination. This peak activity seems to occur at a fixed point in the conidial germination programme since, if a low concentration of cycloheximide is used to delay germ-tube emergence by two hours, there is an equal delay in the peak ornithine decarboxylase activity. Other amino acid-metabolizing enzymes, such as ornithine transaminase (Stevens *et al.*, 1976) and arginase (L. Stevens, unpublished observations), continue to increase in activity for at least 24 hours after initiation of germination. The increase in *S*-adenosylmethionine decarboxylase activity parallels that of ornithine decarboxylase (Stevens *et al.*, 1976) and so also does spermidine synthase (L. Stevens, unpublished observations).

Rapid increases in enzyme activities are accompanied by rapid increases in the intracellular concentrations of spermidine and spermine (Winther and Stevens, 1976). Putrescine concentrations rise only slightly at germination and then fall, suggesting a primary role for putrescine as a precursor for spermidine synthesis. The increase in spermidine concentration parallels an increase in the amount of RNA in germinating conidia (Stevens, 1975) and precedes the increase in DNA concentration

(Stevens *et al.*, 1976). These relationships have also been examined using mitotic mutants of *A. nidulans*. Winther and Stevens (1977) also measured the concentrations of oligoamines, RNA and DNA in temperature-sensitive mutants (Orr and Rosenberger, 1976) which are unable to enter mitosis when grown above the restrictive temperature. Spermine and spermidine accumulate at the time of germ-tube emergence in parallel with an accumulation of RNA and protein, whereas the oligoamine: DNA ratio changes during germination.

An essentially similar pattern, namely that the spermidine:RNA ratio is nearly constant during germination, has been found in both *Rhizopus stolonifer* and *Botryodiplodia theobromae* (Nickerson *et al.*, 1977b). Again, the increase in putrescine concentration during germination of *Rhizopus stolonifer* appears to be transient. In *B. theobromae*, accumulation of spermidine and macromolecules is very slow, with RNA only doubling by the time of germ-tube emergence (Nickerson *et al.*, 1977b).

In *Sacch. cerevisiae*, *S*-adenosylmethionine decarboxylase is among the first enzymes to increase in activity during germination of the ascopores (Choih *et al.*, 1977). In this organism, protein synthesis precedes increases in contents of RNA and DNA, but all three types of macromolecule are actively synthesized during later stages of germination (Rousseau and Halvorson, 1973). Intracellular oligoamine concentrations in ascospores have not yet been reported. The fungal spores discussed in this section have a variety of different patterns of germination; nevertheless, there appears to be a good correlation between increasing amounts of spermidine and macromolecular synthesis in general, the correlation being closest with RNA.

Evidence of a more direct nature for the involvement of oligoamines in fungal growth comes from putrescine-requiring auxotrophs of *A. nidulans* (Sneath, 1955) and *N. crassa* (Deters *et al.*, 1974). There is an absolute requirement for putrescine or spermidine for germination of the *A. nidulans* mutant (J. A. Hope, unpublished observations; Stevens, 1975) showing that at least one putrescine-requiring function is essential for germ-tube emergence. It is difficult to starve the putrescine-requiring auxotroph of *A. nidulans* after it has germinated in liquid culture; if putrescine is removed from a liquid culture after germination has occurred, endogenous oligoamines in the conidia enable it to continue growth for some time. However, the requirement for oligoamines in all stages of growth is evident (see Section V.C.3, p. 121). When conidia of the putrescine-requiring auxotroph are grown in the absence of

putrescine, a small amount of RNA and protein synthesis occurs but not sufficient to allow germination (M. D. Winther, unpublished observations).

Somewhat in contrast to the finding that exogenous oligoamines are required for germination of some fungal spores are reports of spermine, and to a lesser extent of spermidine, inhibiting germination of *Penicillium digitatum* (Eckert *et al.*, 1972), *P. expansum* and *N. crassa* (Brown and Woodcock, 1973). The levels of spermine that prevent 50% of the conidia from germinating are very low, namely from 0.8 μM to 24 μM, so a specific effect is observed, especially when compared to the lack of effects of other oligoamines on germination. This demonstrates the need for an organism to regulate carefully its oligoamine levels by avoiding excesses as well as deficiencies. The mechanism of this inhibitory ability of spermine certainly warrants further study, and may aid in determining the roles played by these cations in the fungal cell.

C. VEGETATIVE GROWTH

1. *Morphological Aspects*

Although fungi exhibit a variety of vegetative forms of which the majority of units are composed of relatively undifferentiated hyphae, it is the disposition of hyphae into mycelial aggregates of greatly varying size and shape that give rise to the range of vegetative structures. In addition, hyphae can develop to form either sexual or asexual spore-bearing structures. Apical growth is the principal mode of vegetative growth in the majority of fungi, exceptions being the yeasts, some chytrids and the Myxomycota.

a. *Hyphal Apex and Apical Growth.* The overall shape of fungal hyphae is governed by the rigid outer cell wall. This wall is a dynamic structure comprising a number of chemically distinct layers and also possessing enzyme activities (Bartnicki-Garcia, 1968; Hunsley and Burnett, 1970). Different fungal developmental patterns, e.g. apical extension, fission, hyphal fusion and sporulation, must involve changes in the cell wall and thus the wall is an important determinant in fungal growth. The wall is composed of interwoven polysaccharide microfibrils embedded in an amorphous matrix of polysaccharides and proteins in an ordered layered structure. The detailed structures and mechanisms of growth have been reviewed by Smith and Berry (1974) and Bartnicki-Garcia (1973).

Growth of hyphae usually occurs at the hyphal apex. The mechanism

of this apical extension is not well understood in molecular terms. Electron microscopic studies have revealed that there is progressive linear differentiation along the length of the hyphae from the tip (see Grove and Bracker, 1970; Beckett *et al.*, 1974). For example, in *Pythium ultimum*, the extreme apical zone is predominantly filled with cytoplasmic vesicles (Grove *et al.*, 1970). Behind this zone, the subapical zone is rich in mitochondria, nuclei, ribosomes and microtubules, and it merges into a third zone which contains vacuoles with a high lipid content. This arrangement, with minor variations, occurs in many taxonomic groups.

Hyphae grow by synthesis and deposition of new wall material at the apex (Robertson, 1965; Marchant and Smith, 1968). Maximum incorporation of wall precursors, such as *N*-acetylglucosamine, occurs within 1 μM of the apex (Gooday, 1971). Wall synthesis also occurs at the sites of septa formation, lateral branching, wall thickening and formation of reproductive structures. Both synthetic and lytic enzymes appear to be involved in wall synthesis, and Bartnicki-Garcia (1973) has proposed a model for wall growth involving synthesis and degradation regulated in balance with one another.

b. *Growth by Fission and Budding.* Mycelial growth is not the sole means by which fungi grow. There are also unicellular yeasts in both the ascomycetes and basidiomycetes. Yeasts which reproduce by fission grow terminally and, upon reaching a given size, form a new wall dividing the cell in two cells of equal size. Division by budding produces two cells of unequal size, the mother and the daughter cells, the latter being formed as a protuberance from the side of the mother cell which is then separated by wall formation.

Some species of fungi exhibit dimorphism in that they can grow in either a yeast-like or mycelial fashion, depending on environmental conditions. The transition is usually brought about by changes in temperature, nutrients or oxygen or carbon dioxide tensions. The occurrence of mycelial and yeast-like growth forms clearly involves varying interactions between the rates of cell-wall growth, general cell growth and cell division. Bartnicki-Garcia (1968) has suggested that the crux of the difference between the two types of growth lies in the extent of growth polarization, apical growth requiring restriction of wall growth to one small region whereas yeast-like growth requires relatively little polarization.

c. *The Cell Cycle.* Further important aspects of vegative growth are the processes of cell and nuclear division. In some fungi, cell and nuclear

divisions alternate at the same rate giving rise to a uninucleate cell population but, in others, cell division occurs less frequently resulting in multinucleate or coenocytic cells. In coenocytic fungi, the number of nuclei per compartment is controlled to maintain a constant ratio of genome to hyphal volume under a variety of conditions (Clutterbuck, 1969).

In order to study biochemical changes during the cell cycle or during nuclear division, a synchronously dividing population of cells is required. Synchrony can be induced artificially (Mitchison, 1973), but naturally occurring synchronously-dividing populations are preferable, since the induction process may produce artifacts. One of the best naturally occurring synchronously-dividing cell populations for studies of the nuclear cycle is the giant multinucleate plasmodium of *Physarum* sp.

A variety of cellular programmes occur during the cell cycle. Mitchison (1973) suggests that the principal ones are: (a) a "growth cycle" during which the cell mass increases continuously; and (b) a "DNA division cycle" in which DNA is synthesized and the chromosomes separate periodically. Under most conditions, these will occur concurrently but their rates may be altered experimentally or they may change naturally during fungal development.

Using suitable fungi, there is thus the possibility of studying oligoamine metabolism during developmental stages which are almost exclusive to fungi, e.g. (a) when the pattern of growth is changed, as in dimorphism, which can be induced in the laboratory; (b) when nuclear division and cell division are not synchronous, as occurs in coenocytic organisms; (c) where rapid elongation of the stipe occurs in the absence of nuclear division (Smith and Berry, 1974); and (d) during the extensive formation of membranes as in zoospore production in *Blastocladiella* sp. and related organisms (see Gay and Greenwood, 1966).

2. *Growth Conditions and Colony Formation*

Under laboratory conditions, vegetative growth is usually obtained on solid media, in static or shaking liquid cultures or in a chemostat. Each method produces cultures having different degrees of homogeneity, and also having different growth kinetics. This subject has been thoroughly reviewed (Bull and Trinci, 1977; Bull and Bushell, 1976; Burnett, 1968) and we only highlight some of the differences in growth pattern.

Culture on solid media is useful for many types of experiment, and

growth is very easy to assess simply by measurement of the radius of a colony. This type of growth results in zones of different physiological and morphological development. There is a central non-growing zone, a transition zone and a narrow growing zone on the periphery which extends radial growth (Trinci, 1970). Growth on solid media is largely two dimensional. Generally it leads to a greater degree of hyphal differentiation than growth in submerged culture. Asexual or sexual fruiting bodies may be formed above the mycelial mass, and rhizoids may develop into the agar below.

When filamentous fungi are grown by shaking in submerged liquid culture, the hyphae often clump together and form pellets. Large pellets are not uniform; they usually comprise a growing zone on the periphery where apical extension is occurring, a zone of hyphal branching behind the apex, and a central non-growing zone in which some autolysis occurs. In general, there is less physiological variation in pellets grown in submerged culture than in radial colonies on solid media, and this is an advantage for biochemical studies. Moreover, both asexual and sexual differentiation can be induced in submerged culture by changes in growth media and other physiological factors (Vezena and Singh, 1975; Saxena and Sinha, 1977).

Growth in continuous culture is less satisfactory for filamentous fungi than for bacteria, and this accounts for the less widespread use of the chemostat for fungal studies (Bull and Trinci, 1977). The main difficulty stems from the hyphal form of growth in filamentous fungi which causes them to form pellets in the chemostat and thus deposit on the walls of the fermenter. The maximum growth rate achieved in continuous culture is lower than that achieved in submerged batch culture. The extent of hyphal differentiation depends on the dilution rate of the cultures. Low dilution rates lead to increased differentiation and formation of fruiting bodies.

3. *Oligoamines during Vegetative Growth*

A number of studies on oligoamine synthesis during vegetative growth in fungi have been made (for examples, see Table 10). By careful control of culture conditions, it is possible to maintain fungi in steady-state growth, during which very little hyphal differentiation occurs. During this more uniform growth phase, it is possible to study the effect of various treatments on growth rate and in particular to study oligoamine synthesis

TABLE 10. Oligoamines and vegative growth in fungi

Organism	Growth conditions	Effects	References
Neurospora crassa	Shaken culture; Mg^{2+} limitation	Oligoamine synthesis stimulated to increase the oligoamine:RNA ratio	Viotti *et al.* (1971)
Aspergillus nidulans	Continuous culture; carbon limitation	Oligoamine:RNA ratio increases with growth rate	Bushell and Bull (1974)
Aspergillus nidulans puA$_1$	Submerged culture; putrescine starvation	Growth-limited; low rates of macromolecular synthesis	Winther and Stevens (1978)
Aspergillus nidulans puA$_1$	Submerged culture; putrescine-supplemented	Rapid increase in RNA and protein synthesis	
Saccharomyces cerevisiae	Spermine-supplemented (3 mg/ml)	Decreased lag phase and faster growth rates in log phase	Castelli and Rossoni (1968)
Various yeast species	Spermine- and spermidine-supplemented	Toxic effect on certain strains	Sakurado and Matsumura (1964); Razin *et al.* (1958)
Physarum polycephalum	Synchronous cell division of plasmodium	Ornithine decarboxylase has peak activities in S and G_2 phases	Sedory and Mitchell (1977)
Puccinia graminis	Induction of infection structures	Nuclear division occurs; 50% increase in spermidine	Kim (1971)

when the amounts of protein and RNA are increasing at a regular rate.

Viotti *et al.* (1971) measured oligoamines and RNA in *N. crassa* which was grown in shaken batch cultures in the presence of different concentrations of Mg^{2+}. When the cultures are magnesium-limited, the intracellular magnesium concentrations fall during mid-exponential growth; at the same time, intracellular spermidine and spermine concentrations rise as does the RNA content. With prolonged limitation, both RNA and DNA contents decline. Therefore, it appears that oligoamines can substitute for magnesium in the early stages of magnesium limitation but not in the later stages. This may be because there are functions in the cell which either magnesium or oligoamines can fulfil and others which require magnesium specifically. Viotti *et al.* (1971) suggest that, since magnesium can exchange with oligoamines in ribosomes *in vitro*, they may also do so *in vivo*.

Bushell and Bull (1974) have carried out similar experiments using *A. nidulans* grown in continuous culture. In these experiments, growth was controlled by carbon limitation and dilution rate. Similarly, they found a reciprocal relationship between intracellular magnesium and oligoamine contents and, at all but the slowest growth rate, a fairly constant ratio of (Mg^{2+} + oligoamine):RNA. At the fastest growth rate, the intracellular Mg^{2+} concentration fell and that of oligoamines rose. The higher ratio of spermidine:RNA may optimize conditions in the cell to allow more rapid growth. At the slowest growth rate, hyphal differentiation and conidiation occur.

As mentioned in Section VB (p. 117), when nuclear division is prevented, as in the case of mitotic mutants of *A. nidulans* grown above the permissive temperature, intracellular concentrations of oligoamines still closely parallel those of RNA (Winther and Stevens, 1977).

Thus, whether growth rates are peturbed by Mg^{2+} limitation, carbon limitation or by inhibition of mitosis, a relationship between oligoamines and RNA can be discerned. It would be interesting to determine the distribution of oligoamines in different regions of the hyphae at various stages of differentiation in vegetative cultures, since the main growth occurs in the apical regions and this is also the region in which ribosomes are most concentrated, and which might be expected to contain most oligoamines. Another type of experiment which has been carried out to discover the relationships between oligoamines and vegetative growth is that in which intracellular concentrations of oligoamines are limited and the effects of this on other parameters are studied. There exists auxotrophic

mutants of *A. nidulans* (Sneath, 1955), *N. crassa* (Deters *et al.*, 1974) and *Sacch. cerevisiae* (Whitney *et al.*, 1978) which require putrescine for growth. Hope and Stevens (1976) found that spermidine and spermine would substitute for putrescine and thus permit growth of *A. nidulans* puA_1 though at 10 and 100 times the minimum concentration of putrescine required, respectively. The higher concentrations of spermine and spermidine are required as they are not as readily taken up by the fungus as is putrescine. A mutant derived from *A. nidulans* puA_2, designated spsA1, has an increased ability to utilize and grow on spermidine (N. Spathas and J. Clutterbuck, unpublished observations), although, at higher concentrations, both spermidine and spermine become toxic. If *A. nidulans* puA_1 is inoculated on solid medium lacking putrescine, very slow and limited residual growth is observed. Microscopic examination of the hyphae grown under these conditions show them to be short, highly swollen and contorted (J. A. Hope, unpublished observations) and a similar abnormal morphology has been observed for putrescine-starved *N. crassa* (Davis *et al.*, 1970).

Arst and Scazzocchio (1972) studied the control of nucleic acid synthesis in *A. nidulans* using auxotrophs requiring leucine, uridine and putrescine. In the wild type, RNA synthesis is stringent during amino-acid starvation. Arst and Scazzochio (1972) were unable to find a relationship between putrescine supplementation or starvation and control of RNA synthesis. However, their experiments compared incorporation into RNA relative to incorporation into protein. Thus, if oligoamines were involved in synthesis of both types of macromolecule, this would not have been detected. By starving *A. nidulans* puA_1 of putrescine at an early stage of growth, i.e. during pre-exponential growth, Winther and Stevens (1978) were able to show that oligoamines are required for maximum rates of both protein and nucleic acid synthesis. Five- to ten-fold increases in rates of macromolecule synthesis were induced by subsequent addition of putrescine to starved cultures, while the total protein and RNA contents increased by 50–70% over two hours. The largest increase occurs with ribosomal RNA synthesis. The addition of putrescine to starved cultures also increases the number and size of the polysomes which can be isolated. Results from experiments using inhibitors suggest that stimulations of protein, RNA and DNA synthesis are independent of each other. During putrescine-limited growth of *A. nidulans* puA_1, there was no longer a constant ratio of spermidine:RNA, but there was a correlation between total oligoamine

content and the rates of nucleic acid and protein synthesis (M. D. Winther, unpublished observations). Castelli and Rossoni (1968) found that the amounts of oligoamines increase rapidly in the early exponential growth of yeast. Addition of spermine (3 mg/ml) decreased the length of the lag phase and stimulated the final growth rates. Thus, synthesis of oligoamines may be a limiting factor in achieving more rapid growth rates under these conditions.

Somewhat in contrast to the findings that oligoamines are required for growth are the reports that spermine and, to a lesser extent, spermidine are toxic to growth of some species of yeasts. Razin *et al.* (1958) found that the sensitivity of yeasts varied but that inhibition could be caused by as low as 15 μg spermine and 30 μg spermidine/ml, depending on pH value and ionic composition of the media. Significantly, the antimycotic effect required an active metabolism. This could be due to oxidation of spermine to a toxic derivative. Similarly, Sakurado and Matsumura (1964) were able to subculture strains of *Sacch. cerevisiae* resistant to 10 mM spermine which is normally toxic to this species. Resistant strains had retarded multiplication but also lower respiratory activity than the original strain.

Studies on oligoamines and growth in *Physarum polycephalum* have been primarily concerned with the relationship between oligoamines and the cell cycle. Multinucleate plasmodia of *P. polycephalum* are particularly suitable for studying cell division because of the occurrence of a precise

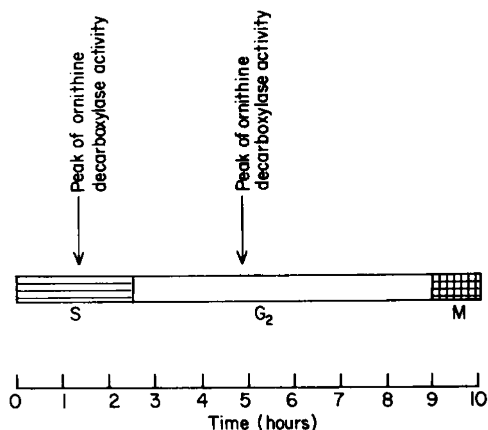


FIG. 6. Activity of ornithine decarboxylase in relation to the cell cycle in *Physarum polycephalum*. From Sedory and Mitchell (1977). Ornithine decarboxylase was assayed in the presence of 1μM pyridoxal phosphate.

naturally-occurring synchronous mitotic cycle. Mitchell and Carter (1977) have shown that ornithine decarboxylase from *P. polycephalum* exists in two different forms (see Section III C4, p. 90) and that the proportions of these two forms of the enzyme vary throughout the mitotic cycle (Sedory and Mitchell, 1977). Total ornithine decarboxylase activity fluctuates throughout the mitotic cycle with peak activities in the S and G_2 phases (Fig. 6). *Physarum polycephalum* has no G_1 phase, and Sedory and Mitchell, 1977). suggest that the pattern observed is comparable with that which occurs in Don C cells, hepatoma cells, and Chinese hampster ovary cells (see Fig. 3, p. 80) where the two peaks occur in late G_1 and in G_2. *S*-Adenosylmethionine decarboxylase activity also varies during the mitotic cycle (Mitchell and Rusch, 1973). Sedory and Mitchell (1977) were unable to detect any elevations in oligoamine concentrations corresponding with these changes in enzyme activities. Fluctuations of *S*-adenosylmethionine decarboxylase activity also occur in synchronously-dividing cultures of *Sacch. cerevisiae* (Shapiro and Ferro, 1977).

After germination of uredospores of *Puccinia graminis*, apical growth can continue or infection structures may be induced by simple manipulation of the extracellular environment (Maheshwari *et al.*, 1967). Nuclear division occurs only in those hyphae induced to form infection structures (appressoria) and may be a necessary prerequisite for this development. Levels of spermidine increase by over 50% in those hyphae induced to differentiate (Kim, 1971).

D. SPORULATION AND REPRODUCTIVE DEVELOPMENT

The third phase in the life cycle of a fungus is spore formation, and development of the associated reproductive structures. In contrast to germination, sporulation more usually occurs when environmental conditions become adverse and the supply of nutrients limited. This phase involves varying degrees of hyphal differentiation depending on the species and on the type of spores formed. At its simplest, it may only entail fragmentation of vegetative hyphae followed by development of a new encasing spore walls as, for example, in chlamydospores. At the other extreme, it may entail development of large and complex reproductive structures such as basidia.

Reproductive development and sporulation are generally the most difficult phase of the life cycle to study at a molecular level. In many

instances, it is difficult to bring about synchronous sporulation in the laboratory. Reproductive development usually occurs at the same time as continuing vegetative growth, the latter being gradually phased out whilst reproductive development is initiated. It is therefore necessary to distinguish between processes associated with the two phases. Since sporulation usually occurs when the nutrient supply is becoming depleted, it is more difficult to incorporate radioactive precursors at this stage. There are, however, a few instances where it has been possible to study sporulation and the preceding differentiation, e.g. in *Sacch. cerevisiae, D. discoideum, P. polycephalum* and *B. emersonii*. In each of these organisms, removal of the source of nutrient induces a well characterized series of changes culminating in formation of an ascus, fruiting body or sporangium (see Ashworth and Dee, 1975; Leaver and Lovett, 1976). The fact that spores generally have a lower oligoamine content than rapidly growing mycelia (see Section V.C.3, p. 118) suggests that oligoamine contents must fall in the developing spore. It seems possible that the spore, once separated from the cytoplasm of the reproductive structure, may degrade some of the remaining oligoamines.

1. *Asexual Reproduction*

The principal types of asexually produced spores are chlamydospores, sporangiospores, zoospores and conidia. Chlamydospores are formed from terminal or intercalary portions of vegetative hyphae by becoming enclosed in a thick cell wall. Sporangiospores are born within spore-bearing structures, known as sporangia, the latter being formed by differentiation of vegetative hyphae. If the sporangiospores are motile, they are known as zoospores. Conidia are spores which are born externally on specially differentiated hyphal structures known as conidiophores.

Chlamydospores are not generally easy to study biochemically since they usually develop slowly. However, Cochrane and Cochrane (1971) have been able to manipulate macrospores of *Fusarium solani* by using acidic media to induce rapid formation of chlamydospores which can be isolated in good yield and purity. Chlamydospore formation from ungerminated macrospores requires both RNA and protein synthesis, but formation from germinated macrospores occurs in the absence of RNA and protein synthesis (Cochrane and Cochrane, 1970).

Sporangiospores and zoospores are formed in a variety of different ways. In *Saprolegnia* sp., for example, sporangia develop at the hyphal tips. The sporangium swells and fills with a multinucleate mass of cytoplasm. A cross wall then develops separating the sporangium from the main mycelial mass. In *Blastocladiella* sp., a rhizoidal system develops penetrating the substrate matrix, whilst the whole of the upper part of the organism develops into a multinucleate cell which transforms into a sporangium. In the latter, extensive nuclear division and membrane formation occurs but, at the same time, there is no wall synthesis.

Blastocladiella emersonii produces zoospores or naked flagellated cells, which are capable of developing into different types of sporangia depending on the environmental conditions (Leaver and Lovett, 1976). In *B. emersonii*, RNA synthesis ceases during differentiation of the sporangia. In contrast, if young rhizoid plants of another water mould, *Achlya* sp., are induced to form sporangia, RNA and protein synthesis is required, but there is no such requirement if older plants are induced to differentiate after they have reached a hyphal growth stage (Burke *et al.*, 1972).

Conidiophores of conidia-producing fungi vary considerably in their complexity, from being only slightly modified hyphae as in *Neurospora* sp. to the complex structures present in species of *Aspergillus* and *Penicillium*. An indication of the complexity of organization of the conidiophore in *Aspergillus* sp. comes from genetic studies in which it has been found that between 45 and 150 loci affect conidiogenesis (Martinelli and Clutterbuck, 1971). Several mutants have been isolated which are able to initiate conidiophore development, but which are blocked before formation of conidia (Clutterbuck, 1969, 1977). One of the difficulties in studying the biochemistry of conidiogenesis is that of obtaining cultures which undergo sporulation synchronously. Manipulation of growth conditions can induce a partially synchronous conidiogenesis in *A. nidulans* (Saxena and Sinha, 1973; Martinelli, 1976), but perhaps the best system in which to study conidiogenesis is the microcycle conidiation which can be induced in *Aspergillus niger* (Anderson and Smith, 1972; Smith *et al.*, 1977). By suitable changes in temperature, a complete growth cycle(microcycle) from conidial germination to condiogenesis takes place in 48 hours, and this includes a synchronous conidiogenesis. It would be a suitable system in which to study the role of oligoamines in conidiogenesis, particularly if putrescine-requiring auxotrophs could be successfully produced.

2. *Sexual Reproduction*

Sexual reproduction in fungi is even more difficult to study at a molecular level than that of asexual reproduction since, in addition to requiring synchronous development of gametangia, it also requires synchronous fusion of the gametes and maturation of the zygote if the whole sequence of sexual reproduction is to be studied. It entails three main sequential steps, namely plasmogamy, karyogamy and meiosis, that is fusion of the conjugant cells, fusion of the nuclei, and then a reduction division. The time between each step is quite variable in one species as compared with others; for example, in certain yeasts and smut fungi, the dikaryon persists for a large part of the life cycle whereas, in others, it has only a transient existence. It would be valuable to be able to study oligoamine metabolism during sexual reproduction of fungi with the aim of discovering whether oligoamines play an important role in meiosis. Oligoamine metabolism has been studied throughout the mitotic cycle and it appears to be important in S phase. If oligoamines are important in nucleotide—nucleotide interactions, they could be involved in crossing over which occurs during meiosis. Some success has been achieved in artificially inducing synchronous meiosis in *Schizophyllum commune* (Carmi *et al.*, 1977) and meiosis is naturally synchronous in *Coprinus lagopas* (Raju and Lu, 1970). These might prove suitable organisms in which to study oligoamine synthesis during meiosis.

As hormones are likely to have an important role in controlling fungal morphogenesis (Gruen, 1963; Gooday, 1974), and oligoamine levels may be altered by hormonal stimulation in higher organisms (see Tabor and Tabor, 1976a), it may be useful to examine this aspect of oligoamines in fungal development.

3. *Oligoamine Metabolism during Development in* Dictyostelium discoideum, Physarum polycephalum, Blastocladiella emersonii *and* Saccharomyces cerevisiae

a. *Dictyostelium discoideum.* When myxamoebae of the cellular slime mould, *D. discoideum,* are starved, they aggregate to form a multicellular grex which then differentiates into a fruiting body comprising two main types of cell, namely spore cells and stalk cells. This process of differentiation involves a synchronous development during which no cell division takes place. During this developmental phase, there is a rapid loss of RNA, replacement of ribosomal subunits, alterations in the types of

RNA transcripts, and loss of protein. Turner and North (1977) have found that, during this developmental phase, there is a fall in the amount of putrescine and spermidine per cell to about 20% of the amounts present in the amoeba. The oligoamines are largely degraded during this phase.

The amounts of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase in each cell also decrease substantially during the first few hours of starvation, but then rise again during the later stages of development. Separate measurements of contents of oligoamines in the spore and stalk cells showed that the distribution between these cells was uniform. *Dictyostelium discoideum* was also found to contain high concentrations of 1,3-diaminopropane (North and Turner, 1977). Both putrescine and 1,3-diaminopropane are taken up from the medium by amoebae and, except at high concentrations, they do not affect the growth of amoebae. The function of 1,3-diaminopropane is unclear. In a related cellular slime mould, *Polysphondylium pallidum*, (2–4 mM) putrescine induces microcyst formation. Spermidine caused a decrease in the induction of microcyst formation by 50 mM putrescine (Lonski and Pesut, 1977). The mechanism of these effects is unknown.

b. *Physarum polycephalum*. The acellular slime mould, *P. polycephalum*, has a life cycle which is similar to that of the cellular slime moulds. In particular, when the microplasmodium is starved it undergoes differentiation into dormant spherules after about 36 hours. During this period of differentiation, the amounts of protein and RNA in the cells fall to between 20 and 30% of the initial levels. Mitchell and Rusch (1973) have found that the amounts of putrescine and spermidine fall as rapidly as that of protein but more slowly than that of RNA. The activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase decrease more rapidly than the bulk of the proteins; their specific activities are lowered by over 90% in 12 hours.

c. *Blastocladiella emersonii*. The water mould *B. emersonii*, although belonging to the Eumycota, has features in common with both the cellular and acellular slime moulds. During its growth period, there is intense nuclear and cell proliferation leading to formation of the sporangium. When the supply of nutrient is sparse, differentiation occurs within the sporangium with formation of zoospores. During the sporulation phase, there is no net increase in protein, RNA or DNA, but intensive turnover of both protein and RNA occurs, presumably to enable synthesis of new proteins required by the zoospores (Murphy and

Lovett, 1966; Lodi and Sonneborn, 1974). After formation of zoospores within the sporangia, they are released through special structures called papilli. During formation of papillated cells, there is a dramatic rise in ornithine decarboxylase activity and an increase in intracellular putrescine concentrations (Mennucci *et al.*, 1975). Though sporulation is a non-proliferative process, increasing oligoamine synthesis may be associated with an increasing macromolecule synthesis as shown by the extensive turnover that occurs at this time.

d. *Saccharomyces cerevisiae.* Ascospore formation in *Sacch. cerevisiae* is also induced by nutrient deprivation and can result in a near synchronous differentiation. There is a very extensive protein turnover at this time (Halvorson, 1958). The amount of RNA increases immediately after transfer to sporulation medium, but then falls throughout development (Sando and Miyake, 1971; Fast, 1973). As *S*-adenosylmethionine decarboxylase activity rapidly drops to nearly undetectable levels during starvation (Shapiro and Ferro, 1977), it is clear that oligoamines are not actively synthesized in sporulation.

VI. Possible Functions of Oligoamines

In the previous section, we considered changes in oligoamine metabolism which occur at various stages during the fungal life cycle, and related them to other processes occurring concurrently. In this final section, possible molecular mechanisms by which oligoamines might affect, in particular, rates of nucleic acid and protein synthesis are discussed. In the first half we summarize evidence that oligoamines affect nucleic acid and protein synthesis when measured *in vitro*, and in the second we propose a hypothesis for oligoamine functions taking into account both the effects of oligoamines observed *in vitro*, and also changes in oligoamine, protein and nucleic acid synthesis occurring *in vivo*.

The principal conclusions which may be drawn from the work on oligoamines described in the previous section are listed below. (i) In most fungal spores, protein and RNA synthesis occurs at a very early stage in germination and in advance of DNA synthesis. Oligoamine synthesis occurs rapidly and in parallel with RNA and protein synthesis. (ii) During vegetative growth, there is a correlation between the amounts of oligoamines and magnesium present within the hyphae and the amounts of RNA. Limitation of intracellular Mg^{2+} or of oligoamines limits RNA synthesis and the rate of growth. (iii) During differentiation in the

absence of cell proliferation, bearing in mind the limited range of organisms in which this has been studied so far, there appears to be loss of oligoamines and oligoamine-synthesizing capacity occurring concurrently with a loss of RNA and protein from the cells.

These conclusions are in general agreement with the results of studies on oligoamines in other organisms. However, an additional conclusion resulting from much recent work in higher eukaryotes has been that oligoamines are concerned with DNA synthesis. If oligoamine synthesis is suppressed by use of specific inhibitors, there results an inhibition of DNA synthesis though RNA synthesis is less affected. Therefore it seems clear that, in order to locate more precisely the sites of action of oligoamines in the cell, it will be necessary to carry out *in vitro* studies, so that the effects of oligoamines on the synthesis of protein, RNA and DNA can be investigated independently of each other.

A. *IN VITRO* STUDIES OF OLIGOAMINE FUNCTION

A major problem with *in vitro* studies is to ascertain which of the observed effects also occurs *in vivo*. Since many of the effects of oligoamines observed *in vitro* are sensitive to changes in concentrations of other cations, it is important that extrapolations to the living organism make adequate allowance for this. Such extrapolations are difficult to make since the concentrations of free intracellular cations are not known accurately, and neither for that matter is the intracellular distribution of oligoamines. On the other hand, it is possible to find out which enzyme reactions are affected by oligoamines, and this has been attempted in the case of enzymes catalysing nucleic acid and protein synthesis.

1. *Aminoacyl tRNA Synthetases*

Several studies have shown that oligoamines stimulate aminoacyl tRNA formation when the reactions are measured in the presence of suboptimal concentrations of Mg^{2+}, but there has been controversy as to the mechanism of the reaction (Santi and Webster, 1975; Takeda and Ohnishi, 1975; Carr *et al.*, 1975) and also therefore as to the mechanism by which oligoamine stimulates. Approximately equivalent stimulations are brought about by 1 mM spermine, 5 mM spermidine or 20 mM putrescine (Igarashi *et al.*, 1974a). Stimulation by these oligoamines also depends on the concentrations of monovalent ions (Takeda and Igareshi,

1970). Stimulation by spermine still occurs in the presence of 0.2–0.4 M sodium chloride and it may therefore be considered to occur at intracellular ionic strengths.

2. *Protein Synthesis*

In vitro studies have indicated that oligoamines are also involved in protein synthesis. For example, isolated ribosomes, even after washing in buffers of moderate ionic strength, contain oligoamines (Konecki *et al.*, 1975). Furthermore, to obtain maximum rates of protein synthesis in such washed ribosomes, oligoamines have to be present. Spermine is the most effective oligoamine for stimulating protein synthesis but, at high concentrations, it becomes inhibitory and inhibition by spermine may well occur at physiological concentrations. Spermidine and putrescine are only inhibitory at much higher concentrations which are above the normal physiological range. Binding of tRNA to ribosomes and to mRNA (Takeda, 1969; Tanner, 1967) increases in the presence of oligoamines, and stimulation of protein synthesis is thought to result from this increased binding (Igarashi *et al.*, 1974b) rather than from increased rates of peptide-bond formation or translocation. In addition, there is evidence which suggests that oligoamines cause a differential stimulation of protein synthesis. Atkins *et al.* (1975) have shown that the pattern of proteins synthesized *in vitro*, in the presence of oligoamines, more closely resembles the pattern occurring *in vivo* than the pattern obtained in *in vitro* synthesis in the absence of oligoamines. Oligoamines also preferentially increase the synthesis of larger polypeptides (Atkins *et al.*, 1975; Igarashi and Hirose, 1978).

3. *Ribonucleic Acid and DNA Polymerases*

In both prokaryotes and eukaryotes, DNA-dependent RNA polymerases are stimulated by addition of oligoamines in the presence of suboptimal Mg^{2+} (Fuchs *et al.*, 1967; Abraham, 1968; Moruzzi *et al.*, 1975; Stirpe and Novello, 1970). In eukaryotes, the multiple RNA polymerases which have different functions also show differing sensitivities to spermine (Jacob and Rose, 1976). In addition, these sensitivities are also affected by the salt concentrations in which they are assayed. It is thus possible that variation in intracellular spermine concentrations may be a means by which the extent of activation or inhibition of RNA polymerases is controlled. Deoxyribonucleic acid polymerases are also stimulated by

spermine and spermidine, the extent of stimulation depending on the salt concentration of the medium and also on the nature of the template used (Yoshida *et al.*, 1976; Brewer and Rusch, 1966). Oligoamines also affect the activities of various nucleases (Schlenk and Dainko, 1966; Levy *et al.*, 1974; Kumagai *et al.*, 1977) usually by stimulation of activity, but also by altering the specificity of the enzymes for certain polynucleotides.

B. HYPOTHESIS FOR OLIGOAMINE FUNCTIONS

Although there are not yet sufficient data to define precisely the functions of oligoamines, in this section we put forward a hypothesis which may help to focus on the area where further experimentation is needed. We suggest that oligoamines do not perform a single cellular function, but rather a number of related functions, probably all connected with their interactions with nucleic acids. These roles may be divided into two basic types: (i) those which involve a *transient* stabilization of nucleotide–nucleotide interactions such as are necessary in the processes of replication, transcription and translation; and (ii) the more static roles of structure stabilization; for example, stabilization of condensed chromosomes, ribosomes, and possibly tRNA. Important examples in the transient category would be stabilization of codon-anticodon interactions, and the interaction of nucleoside triphosphates with polynucleotide templates during DNA and RNA synthesis. In these processes, nucleotide–nucleotide interactions have to be stabilized for very short periods of time, e.g. in exponentially growing bacteria, peptide elongation occurs at a rate of 10–20 amino acids per second and RNA elongation at a rate of 30 to 47 nucleotides per second (Morris and Hansen, 1973; Lacroute and Stent, 1968; Forchhammer and Lindahl, 1971), and thus stabilization for from 10^{-2} to 10^{-1} seconds would be required, whereas the stabilization times required in the second category would be much longer. It is further suggested that the relative importance of the two types of interaction in a given tissue or organism would depend on its growth rate at a given time. A particular cell would require a certain fixed amount of oligoamines to stabilize its chromosomes and ribosomes, but the amount it would require for the more transient type of stabilization would vary depending on its rate of accumulation of new cell material at a particular time. There is an important difference here between eukaryotes and prokaryotes since, in proliferating higher eukaryote cells, cell division may occur about once

every 24 hours whereas, in prokaryotes and lower eukaryotes such as fungi, doubling time is much shorter, for example, between 20 min and six hours.

If oligoamines are involved in stabilization of codon–anticodon and nucleoside triphosphate–polynucleotide interactions in the way we suggest, it is necessary to envisage that the interactions be of a transient nature only, as over-stabilization, particularly in the case of codon-anticodon interactions, would be detrimental and retard the rate of protein synthesis. In the process of DNA and RNA synthesis, stabilization of nucleotide–nucleotide interactions between substrate and template would not be necessary once the phosphodiester link had formed.

In this role of transient stabilization, it is further envisaged that any one of the oligoamines, putrescine, spermidine or spermine, might serve, but that usually spermidine would give the optimum degree of stabilization. Putrescine would be required at higher concentrations to give comparable stabilization. Too high concentrations of spermine would be inhibitory as had been shown in *in vitro* studies. This may be due to over-stabilization. However, the relative efficiency of the oligoamines would also vary with the ionic conditions, and it is important to bear in mind that the latter may vary in different parts of the same cell, e.g. nucleus and cytosol.

For the transient type of stabilization, it is also envisaged that oligoamines would be initially necessary to optimize the efficiency of the system concerned, e.g. a cell undergoing a rapid increase in protein or RNA synthesis at the onset of proliferative growth. The cell may already possess the macromolecular components for protein and RNA synthesis but to function optimally would also require the presence of certain cations. The latter requirement might be fulfilled by oligoamines, Mg^{2+} or other cations. The cell may be able to synthesize oligoamines endogenously at a more rapid rate than it could take up Mg^{2+} from the external environment. Magnesium ions may not be readily available in the extracellular environment. Hence, the rapid increases in ornithine decarboxylase observed at the onset of proliferation in many cells may enable the fulfilment of this requirement. Subsequently the cell may be able to adjust its cation content by means other than oligoamine synthesis. However, it should be emphasized that there must be some functions in the cell for which Mg^{2+} ions are essential, some for which oligoamines are essential, and some in which either can serve.

Over and above the cell's requirement for oligoamines for this transient

type of stabilization, certain amounts of oligoamines may be required as structural components of, for example, chromosomes. This requirement would then double every time the nucleus divides. Much recent evidence suggests that, in a number of higher eukaryotes, inhibition of oligoamine synthesis inhibits DNA synthesis rather than RNA and protein synthesis (Pösö and Jänne, 1976; D. R. Morris *et al.*, 1977). These studies have been made using cells which were growing slowly (doubling times about 24 hours) as compared with fungi and bacteria, and in which oligoamine depletion took several hours. In these slow-growing cells, oligoamine synthesis may be necessary for the structural role whereas the increased rate of RNA and protein synthesis may be met without an obligatory requirement for further oligoamine biosynthesis. In contrast, in *E. coli* (Young and Srinivasan, 1972) and *A. nidulans*, which are capable of rapid growth, protein and RNA synthesis are the first processes to respond to a change in oligoamine concentration.

In a number of tissues and organisms the increase in ornithine decarboxylase activity during the transition from a non-dividing to a proliferating state is more dramatic than the total increase in oligoamine levels. The latter may be quite small in many instances. If much of a resting cells's oligoamines are bound to nucleic acids, then it seems likely that, at the start of proliferative activity, in order to produce the significant increase in free oligoamines required for the transient stabilization, a rapid increase in biosynthetic capacity is also required, although only a small increase in total oligoamines may be observed.

In conclusion, in order to test this hypothesis further, more information will be required about the binding of oligoamines to various components of the cell, the rates at which they can exchange on and off these components and also the sites of synthesis of oligoamines in the cell.

As regards the distribution of oligoamines in fungi it is difficult at present to see whether it has any evolutionary significance. The ability to synthesize spermine in many fungi might be indicative that those species possess a specific spermine synthase and thus have a biosynthetic ability not known to be present in prokaryotes. Such a conclusion would be premature. Spermine and spermidine synthase have only been partially purified in two eukaryote tissues so far. Propylamine transferase from *E. coli* has been purified to homogeneity, and has been shown to be capable of spermidine biosynthesis and also spermine biosynthesis at a lower rate. Several fungi have been shown capable of synthesizing spermine from ^{14}C-putrescine. In some cases, fungi contain small or undetectable levels

of spermine. It is possible that a single enzyme is capable of catalysing synthesis of both spermine and spermidine, and that its specificity may be modulated by changes in the intracellular environment. The different proportions of putrescine, spermidine and spermine in Eumycota, at least, may simply reflect the growth state and intracellular ionic conditions within the organism.

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Note added in proof: Auxotrophic mutants of *Sacch. cerevisiae* deficient in ornithine decarboxylase (spe_1), *S*-adenosylmethionine decarboxylase (spe_2) and propylamine transferase (spe_3) have been isolated (Cohn *et al.*, 1978a; Whitney and Morris, 1978). The spe_2 mutants grown on minimal medium contain no detectable spermine or spermidine, have a much slower growth rate, are defective in meiotic sporulation and do not permit the replication of a killer RNA plasmid (Cohn *et al.*, 1978b). *S*-Adenosylmethionine decarboxylase mutants have also been isolated from *E. coli* (Tabor *et al.*, 1978). Lövgren *et al.* (1978) discuss the steps involved in formation of aminoacyl-tRNA. They suggest a dual cation requirement (Mg^{2+} and oligoamines) for the overall process to account for the *in vivo* rate when taking into consideration the physiological concentrations of these cations.

Physiology of Thermophilic Bacteria

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I. Introduction

Micro-organisms are able to grow and reproduce at temperatures ranging from slightly below the freezing point to the boiling point of water (Morita, 1975; Brock and Darland, 1970). In other words, microbial life seems to be restricted to the availability of liquid water. This is not to say that a microbial species is capable of growth over the entire temperature range from below 0°C to 100°C. The temperature for growth of each microbial species is quite limited, and seldom extends over a span of 30°C. This has led to the development of arbitrary classification systems, according to which micro-organisms are divided into at least three groups depending on their cardinal temperatures, that is their minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) growth temperatures. Stanier *et al.* (1970) in *The Microbial World* list bacteria with growth spans from −5°C to +22°C as "psychrophiles," from 10°C to 47°C as "mesophiles," and between 40°C to 80°C as "thermophiles". It is the physiology of the last group, limited to prokaryotic micro-organisms but expanded to include bacteria growing above 80°C, which is the subject of this review. However, strict adherence to this division is not possible, nor desirable.

II. General Comments about Thermophilic Micro-organisms

A. CLASSIFICATION OF THERMOPHILES

Considering cardinal growth temperatures of micro-organisms, it immediately becomes evident that their division into psychrophiles, mesophiles and thermophiles is not satisfactory. Many micro-organisms would fall on a border line and may be classified, for instance, as either a mesophile or a thermophile depending on an investigator's point of view. The cardinal temperature may also vary with the growth conditions. Furthermore, thermophiles cover a growth span between 40°C and 100°C, and thermophiles in the lower temperature range have been considered different from those in the upper temperature range. Consequently, several attempts have been made to subdivide the thermophiles. Some of these attempts are listed in Table 1, which shows different terms used to name thermophiles and their definitions. The latest classification, that of Williams (1975), suggests the name "caldo-active," which was originally proposed by Heinen and Heinen (1972) for bacteria growing over 70°C. This group is also often referred to as

TABLE 1. Classification systems for thermophilic bacteria

Reference	Term	Definition
Miehe (1907)	Thermophiles	$T_{min} > 25°C$
	Orthothermophiles	$T_{max} > 60–70°C$
	Thermotolerants	$T_{max} \simeq 50–55°C$
Robertson (1927)	Thermoduric mesophiles	Grow between 20 and 57°C and survive pasteurization
Imsenechi and Solnzeva (1945)	True thermophiles	$T_{opt} \simeq 55–60°C$
	Stenothermal thermophiles	$T_{min} > 28°C$, $T_{max} < 60°C$
	Eurithermal thermophiles	Grow at 60°C and at 28–30°C
Farrell and Campbell (1969)	Strict or obligate thermophiles	T_{opt}, 65–70°C, $T_{min} > 40–42°C$
	Facultative thermophiles	T_{max}, 50–65°C, T_{min}, room temp.
	Thermotolerant organisms	T_{max}, 45–50°C
Williams (1975)	Caldoactive bacteria	$T_{max} > 90°C$, $T_{opt} > 65°C$, $T_{min} > 40°C$
	Thermophilic bacteria	$T_{max} > 60°C$, $T_{opt} > 50°C$, $T_{min} > 30°C$

"extreme thermophilies," which is preferred by this reviewer. Any arbitrary classification system is, of necessity, of limited value.

B. ORIGIN AND DEVELOPMENT OF THERMOPHILES

The existence of micro-organisms which grow at, and actually require, temperatures far above the upper limit of most life has stimulated speculation and research as to how these organisms evolved and what makes them capable of functioning at high temperatures. Arrhenius (1927) theorized that thermophiles originate from the planet Venus, and that they travel to Earth with the help of rays of the sun. Such an exotic origin and explanation does not fit established facts, which instead strongly indicate that mesophiles and thermophiles have a common origin. Thus, species of thermophilic micro-organisms are found in many prokaryotic groups and genera including blue-green algae, photosynthetic bacteria, spore-formers (both aerobic bacilli and anaerobic clostridia), actinomycetes, sulphur-oxidizing and -reducing bacteria, methane-oxidizing and -producing bacteria and Gram-negative aerobes. Thermophilic eukaryotic organisms also exist among protozoa, algae and fungi. Tansey and Brock (1978) have compiled an excellent and extensive list of well characterized, thermophilic, prokaryotic and eukaryotic micro-organisms, and have quoted their cardinal growth temperatures. The diversity of thermophilic micro-organisms, and the fact that their structures and metabolic processes are very similar to those of mesophiles, indicate a common origin. What is more convincing, however, are the findings that many enzymes and enzyme systems, like ribosomes and ATPase, are much alike in mesophiles and thermophiles. These proteins and structures reflect the composition of DNA, which thus has been preserved to a great extent during the differentiation of mesophiles and thermophiles. This observation is certainly compatible with the common origin of the two types of micro-organism. The similarities between homologous proteins from thermophiles and mesophiles have been discussed in reviews by Singleton and Amelunxen (1973), Williams (1975), Ljungdahl and Sherod (1976) and Amelunxen and Murdock (1977).

Several theories have been advanced to explain how mesophilic bacteria have adapted to become thermophilic. It has been suggested that thermophiles first developed, and that they adapted to grow at lower temperatures. These theories have been discussed in some detail in the review by Gaughran (1947), and only a few comments may be

appropriate here.

Kluyver and Baars (1932) expressed the belief that thermophilcs are the result of spontaneous mutation, i.e. occurring in one step. This is most unlikely. Enzymes and proteins in thermophiles are very similar to homologous proteins in mesophiles, except that the former often have much higher thermal stabilities. It has been noticed by several investigators (see reviews by Singleton and Amelunxen, 1973; and Ljungdahl and Sherod, 1976) that the higher thermal stabilities of many proteins from thermophiles are intrinsic, and that they depend on subtle differences in amino-acid composition and sequence between homologous proteins. Thus, a single mutation may change the thermal stability of a protein. However, to convert a mesophilic organism to a thermophilic one requires mutations affecting several, if not most, of the enzymes needed for survival. That this will occur in a single step seems impossible. If a single mutation would convert a mesophile to a thermophile, it implies the existence of a thermophilic factor which would impose thermal stability on most enzymes and structures in the cell. Such a factor has not beeen characterized. Furthermore, this author is not aware of any successful attempt to convert a mesophilic organism to a thermophilic one using mutagenic agents.

The suggestion of Golikowa (1926) and others (see the reviews by Gaughran, 1947, and Brock, 1967) that thermophilic micro-organisms evolved before mesophiles is intriguing. Assuming a reduced and hot environment when bacterial life began to develop, it is plausible that anaerobic and thermophilic bacteria preceeded mesophilic and aerobic bacteria. Aerobic thermophiles may then have developed either directly from anaerobic thermophiles or, more likely, from aerobic mesophiles. It is likely that the sequence of development of different types of bacteria soon will be answered. Homologous proteins, such as ferredoxins and cytochromes, are being isolated from more and more bacterial species. These proteins are being sequenced and, from the homology, the line of evolution may be deduced (Vogel *et al.*, 1977; Schwartz and Dayhoff, 1978). Furthermore, the cataloguing of ribosomal RNAs has been found to be extremely useful in determining lines of phylogeny (Woese *et al.*, 1976; Balch *et al.*, 1977a).

C. MOLECULAR BASIS OF THERMOPHILICITY

Early theories about how thermophilic micro-organisms are able to grow and reproduce at high temperatures are abundant. They include the

composition of the medium, protoplasmic organization, protective coating, low water or moisture content, changed nature of the cell membrane, heat stability of the enzymes and the enzyme complexes, and a dynamic theory which states that the rate of destruction is not significant, if the rate of replacement is faster (Allen, 1950, 1953; Gaughran, 1947). More recently, Ljunger (1970, 1973) suggested that the heat stability of thermophilic bacteria is attributable to active transport of calcium from the environment into the cell. Notwithstanding, the prevalent theory for thermophilicity is that macromolecules from thermophiles have high thermal stability. It has also been demonstrated that the nature of the cell membrane is important for thermophilic growth. Evidence for and against these theories is discussed in some detail throughout this review.

III. Description and Properties of Some Thermophiles

The list of known thermophilic micro-organisms is long (Tansey and Brock, 1978). Only a few of them have been the subjects of extensive research and, before 1969, work leading to an understanding of thermophilicity was almost exclusively done using *Bacillus stearothermophilus*. However, the discovery of extreme thermophilic bacteria, and the realization that thermophiles have macromolecules of high thermal stability, have spurred interest in thermophilicity not only amongst microbiologists but also biochemists. What follows is a short description of some of the bacteria which have received the attention of investigators interested in thermophilicity.

A. *BACILLUS STEAROTHERMOPHILUS*

Bacillus stearothermophilus is the *Escherichia coli* of the thermophiles; that is, it is the most studied thermophile, and many strains have been isolated. Furthermore, many unclassified bacilli used in studies of thermophilicity seem to be related to *B. stearothermophilus* and will, therefore, be discussed in this section. The type strain of *B. stearothermophilus* was described by Donk (1920). It grows optimally between 50°C and 65°C. Its T_{max} value is slightly above 70°C, and it does not grow at 28°C. *Bacillus stearothermophilus* is found in soil, mud and water. Both Gaughran (1947) and Allen (1953) in their reviews list references to earlier papers dealing with the distribution of this micro-organism. Recently, it was isolated from soil taken from Hawaii (Harris and Fields, 1972), Egypt (Elwan *et*

al., 1972) and Iceland (Fields and Lee, 1974), and from water obtained from hot springs in Colorado, discharge from Indiana University Physical Plant (Ramaley and Bitzinger, 1975) and from a thermal spring in Tadzhik, S.S.R. (Pozmogova, 1975).

Bacillus stearothermophilus is easy to grow, which probably is one reason for its popularity. Cells for inoculum may be stored lyophilized or on Difco nutrient-agar slants. Many investigators use a relatively complex medium such as the one described by Amelunxen (1966). This medium contains nutrient broth (Difco), trypticase (BBL), glucose, potassium phosphate and the chloride salts of ammonium, sodium, magnesium, calcium and iron (Fe^{3+}). Cells grown in this medium at 60°C in a shaker with vigorous aeration reach the end of the logarithmic phase of growth within 2.5 hours.

Defined and minimal media for *B. stearothermophilus* have been described by Welker and Campbell (1963) using strain 1503-4 and Rowe *et al.* (1975) with strain N.C.A. 1503 (A.T.C.C. 7954). Welker and Campbell's (1963) minimum medium, adjusted to pH 7.3, contains arginine, methionine, valine, thiamin, nicotinic acid, biotin, potassium phosphate, the chloride salts of ammonium, sodium, iron, magnesium and calcium, potassium acetate and a carbohydrate. The last of these constituents could be glycerol, glucose, sucrose, maltose or starch. Although this medium supports growth and production of extracellular α-amylase, growth and α-amylase production are increased substantially by including casein hydrolysate in the medium (0.1% w/v). Interestingly, fructose can serve as a carbon source in the fortified, but not in the minimal, medium. Gluconate, succinate, and acetate do not serve as carbon sources in either of the two media.

Rowe *et al.* (1975) found that the medium of Welker and Campbell (1963) fails to support growth in agar and that, in the liquid form after several 10% transfers, growth is unsatisfactory and only reaches 10^8 cells per ml. A newly defined medium was developed. It contains 20 different amino acids, vitamins (biotin, thiamin and nicotinic acid), chloride salts (calcium, iron, manganese, magnesium, ammonium and sodium), zinc sulphate, potassium phosphate and a carbon source. The pH value of the medium is 7.3. Of the carbon sources tested, glucose, fructose, sucrose, glycerol and starch support growth, whereas lactate, acetate, citrate, α-oxoglutarate, succinate, fumarate and malate do not. A similarly defined agar-containing medium was established. The most important difference between Welker and Campbell's (1963) medium and that of Rowe *et al.*

(1975) is the inclusion of manganese and zinc in the latter. Manganese has a dramatic effect on growth yield of *B. stearothermophilus*. Rowe *et al.* (1975) also observed that phosphate concentrations higher than 5×10^{-3} M are inhibitory in agar-containing medium but not in liquid medium.

A prototrophic, thermophilic *Bacillus* species, by some investigators called "var. *nondiastaticus*", has been isolated from manure by Epstein and Grossowicz (1969a). It is very similar to *B. stearothermophilus* but is unable to hydrolyse starch. It grows optimally between 55°C and 58°C, the T_{max} value is 65°C, and it does not grow at 37°C. When grown in nutrient broth, acids are produced from glucose, fructose, sorbose, raffinose, rhammose, ribose, glycerol, mannitol and sorbitol, but not from galactose, lactose, arabinose or xylose. The bacterium also grows over its entire temperature range in a mineral medium containing ammonium ions, mineral salts, and with glucose, succinate and acetate as carbon sources. Alanine, aspartate and glutamate can also serve as single carbon and nitrogen sources.

An interesting *Bacillus* species related to *B. stearothermophilus* has been isolated from a thermal spring in Kirgiz, S.S.R. by Pozmogova (1971). Its cardinal temperatures are T_{min}, 45°C, T_{opt}, between 55°C and 65°C, and T_{max}, 77°C. It is able to grow on an agar medium containing tap water, ammonium and potassium phosphates, magnesium sulphate, and with paraffin (n-alkanes from C_{12} to C_{24}) as a carbon source which could be replaced by either maltose or sucrose. With maltose, but not with paraffin, it is able to grow in a liquid culture. Klug and Markovetz (1967) and Mateles *et al.* (1967) have also reported that thermophilic bacilli related to *B. stearothermophilus* are able to grow on hydrocarbons.

Buswell (1974, 1975) isolated from industrial sediments in Scotland a strain of *B. stearothermophilus* named "PH 24". It grows with either phenol, *o*-cresol, *m*-cresol, *p*-cresol, catechol, 3-methylcatechol or 4-methylcatechol as the carbon source in a medium containing yeast extract (Oxoid), casamino acids (Difco) and mineral salts. Strain PH 24 must be induced to grow on the phenolic substrates. Non-induced cells use either succinate or fumarate as substrate. Golovacheva and Oreshkin (1975) found that 11 out of 26 strains of *B. stearothermophilus* isolated from gas thermal wells, which contain tars and phenols, are able to grow on phenol in media containing yeast extract (Difco) and mineral salts. The maximum concentration of phenol at which the bacteria are able to grow is 0.3% (w/v). There was no growth when the phenol concentration was

increased to 0.35% (w/v). The pathway for oxidation of phenolic compounds has been studied by Buswell and Clark (1976). A homoprotocatechuate 2,3-dioxygenase has been isolated from *B. stearothermophilus* (Jamaluddin, 1977).

Methyl-α-D-glucoside is an analogue of glucose, and it has been used extensively in studies of glucose transport in micro-organisms (Kaback, 1970). It enters the cell and is phosphorylated, but is generally not further metabolized. However, Harris and Kornberg (1972), Harris and Miller (1976) and Reizer *et al.* (1976) have reported that the glucoside is metabolized by the strain of *B. stearothermophilus* isolated by Epstein and Grossowicz (1969a). Uptake of the glucoside and its metabolism will be dealt with later (p. 214).

B. EXTREMELY THERMOPHILIC BACILLI

Heinen and Heinen (1972) have isolated three spore-forming, obligate, aerobic Gram-variable rods from the slightly alkaline hot springs of Yellowstone National Park. They proposed the names: "*Bacillus caldolyticus* YT-P", "*B. caldovelox* YT-F" and "*B. caldotenax* YT-G". The prefix "caldo" was derived from the latin adjective *caldus* meaning hot. *Bacillus caldotenax* has a pH optimum range from 7.5 to 8.5, a T_{opt} value of 80°C, and a T_{max} value of 85°C. *Bacillus caldovelox* grows well between pH 6.3 and 8.5, and has a T_{opt} value between 60°C and 70°C and a T_{max} value of 76°C. *Bacillus caldolyticus* grows in the pH range from 6.0 to 8.0 and has a T_{opt} value of 72°C and a T_{max} value about 82°C. In addition to a carbon source, the basic growth medium for these bacteria contains brain–heart infusion, ammonium nitrate, potassium phosphate, magnesium and iron sulphate, calcium chloride, and the following trace elements: Mn, Zn, Cu, Mo, Co and B. As a carbon source, *B. caldotenex* can use pyruvate, glucose, succinate, acetate and, less efficiently, glycerol (Heinen, 1971). Interestingly, with pyruvate, growth is obtained at a temperature as high as 86°C whereas, with the other carbon sources, growth stops at 70°C. With the use of [^{14}C] glucose, it was established that glucose is taken up by the cells at 80°C and converted to a compound chromatographically identified as α-glycerophosphate. Such behaviour may indicate that certain enzymes needed for metabolism of glucose, succinate and acetate are inactivated above 70°C. *Bacillus caldolyticus* grows on glucose, pyruvate and succinate whereas *B. caldovelox* prefers succinate. Extracellular alkaline phosphatase, protease and α-amylase are formed by

B. caldolyticus. These enzymes have very high thermal stabilities.

Tairo Oshima (unpublished results) has isolated several thermophilic, spore-forming aerobic bacteria from hot springs in Japan. Some of their properties have been described by Kagawa (1976). Of particular interest is strain PS3, which has been used very successfully in studies on transport of nutrients and on ATPase, as will be discussed later in this review (p. 170). Strain PS 3 has a T_{opt} value of 65°C and a T_{max} value of 80°C. It has been cultured in a medium containing 0.8% polypeptone, 0.4% yeast extract and 0.3% sodium chloride at a temperature between 65°C and 74°C with vigorous aeration. After 12 hours, the yield was 6 kg of cells per 1000 litres of culture (Yoshida *et al.*, 1975).

C. *THERMUS* AND *THERMOMICROBIUM* SPECIES

Brock and Freeze (1969) were the first to isolate a Gram-negative, non-sporulating, aerobic, extremely thermophilic rod-shaped micro-organism for which they chose the name *Thermus aquaticus*. It was found in a variety of thermal springs in Yellowstone National Park, but similar organisms have also been isolated from hot-water heaters in Madison, Wisconsin (Brock and Boylen, 1973) and in The London Hospital, London, England (Pask-Hughes and Williams, 1975). Several species of the genus *Thermus* have now been isolated. They include *Thermus thermophilus* (first described as *Flavobacterium thermophilum*; Oshima and Imahori, 1971, 1974), *Thermus flavus* (Saiki *et al.*, 1972), *Thermus* X-1 (Ramaley and Hixson, 1970) and a pink isolate, K-2 (Ramaley *et al.*, 1975). The properties of these micro-organisms are listed in Table 2 together with the properties of the extremely thermophilic bacterium *Thermomicrobium roseum* (Jackson *et al.*, 1973a). In addition to these *Thermus* species, a micro-organism with a bright red pigment has been isolated from hot springs in Kamchatka by Loginova *et al.* (1975) and Loginova and Egorova (1975). They have named this isolate *Thermus ruber*. It is a rod, 3–6 μm in length, 0.5–0.8 μm in diameter, and it grows optimally around pH 8 at about 55°C.

Nutritionally, members of the genus *Thermus* seem to be similar. *Thermus aquaticus* needs a basal salts medium. Good growth is then obtained in a medium containing tryptone and yeast extract or casein hydrolysate. Glutamic acid alone seems to serve both as a carbon and nitrogen source. In the presence of a low concentration of glutamate, acetate, sucrose, glycerol, mannitol, citrate, succinate or glucose also

TABLE 2. Properties of *Thermus* species and *Thermomicrobium roseum*

	Thermus aquaticus[a]	*Thermus flavus*[b]	*Thermus thermophilus*[c]	*Thermus* X-1[d,e]	K-2[f]	*Thermus roseum*[f]
Rod size						
diameter (μm)	0.5–0.8	0.5–0.7	0.5		0.7–0.8	1.3–1.8
length (μm)	5–10	2–5	3		5–8	3–6
Colony colour	yellow-orange	yellow-brown	yellow-brown	cream	pink	pink
DNA composition (GC-content %)	65.4–67.4	69–71	69	64	64.4	64.3
Value for:						
T_{max} (°C)	79	81	85	80	80	85
T_{opt} (°C)	70–72	70–75	65–72	69–71	60	70–75
T_{min} (°C)	40	40	47	40		
pH_{opt}	7.5–7.8	7–7.5	7		7.6–7.8	8.2–8.5
pH_{range}	6–9.5	6–9	5.1–9.6	7.5–10	7.5–10	6–9.4

[a] Brock and Freeze (1969).
[b] Saiki *et al.* (1972).
[c] Oshima and Imahori (1974).
[d] Ramaley and Hixson (1970).
[e] Ramaley *et al.* (1975).
[f] Jackson *et al.* (1973a).

serve as carbon sources (Brock and Freeze, 1969; Jackson *et al.*, 1973a). *Thermus flavus* grows well on a rather complex medium such as polypeptone or bouillon. More defined media require several amino acids and vitamins (Saiki *et al.*, 1972). *Thermus thermophilus* can be cultured on polypeptone and yeast extract, and it also grows on bovine serum albumin or milk casein. Acids are produced from glucose and galactose, but these sugars will not support growth in a synthetic medium with ammonia as a nitrogen source (Oshima and Imahori, 1974). *Thermus roseum* grows well when up to 0.5% (w/v) of yeast extract and an equal amount of tryptone are added to a basal salt medium (Jackson *et al.*, 1973a). All species seem to grow best when the medium is relatively low in organic material, while concentrations of yeast extract or tryptone (above about 1–2%, w/v) seem to inhibit growth.

A bacteriophage (ϕYS40) which is infectious to *T. thermophilus* has been isolated by Sakaki and Oshima (1975). The phage grows over a temperature range of 56–78°C. It has a burst size of 80 at 65°C. The DNA is double-stranded, and it has a molecular weight of 1.36×10^8. The GC content is 35% and the T_m value is 83.5°C.

D. THERMO-ACIDOPHILIC MICRO-ORGANISMS

Thermo-acidophiles are a group of micro-organisms which grow at low pH values and at high temperatures. One of the first reports about thermo-acidophiles is that by Uchino and Doi (1967). They isolated three strains belonging to the genus *Bacillus*, which grow well between pH 2 and 5 with a T_{opt} value of 65°C. Several thermo-acidophiles have now been described. They are listed together with some of their properties in Table 3. It is a rather diverse group which includes a spore-former, *Bacillus acidocaldarius*, the *Sulfolobus* group comprised also of species of *Ferrolobus* and *Caldariella*, and *Thermoplasma acidophilum*. Morphologically, they differ in that *B. acidocaldarius* has a cell wall and is rod-shaped, whereas the members of the *Sulfolobus* group are spherical with probably a flexible coat. Finally, *Thermoplasma* lacks a cell wall.

Bacillus acidocaldarius grows with either glucose, galactose, sucrose, gluconate, starch or glycerol as a carbon source in a basal medium containing ammonium and magnesium sulphates, calcium chloride, and phosphate at a pH value between 2 and 5 (Darland and Brock, 1971). New isolates (Oshima *et al.*, 1977) of *B. acidocaldarius* found in acidic

TABLE 3. Properties of thermo-acidophilic micro-organisms

	Bacillus Acidocaldarius[a]	*Sulfolobus acidocaldarius*[b]	*Ferrolobus*[c]	*Caldariella*[d]	*Thermoplasma acidophilum*[e]
Shape	Rod	Sphere with lobes	Sphere with lobes	Sphere with lobes	Sphere, varied sizes
Diameter	0.5–1 μm	0.8–1 μm	1–1.5 μm	1–1.5 μm	0.1–10 μm
Spores	yes	no	no	no	no
DNA composition (GC content %)	59–62.2	60–68	54–60	39–47	24–29
Values for:					
T_{max} (°C)	70–75	75–85	70	80–89	65
T_{opt} (°C)	65	70–75	60	75–87	59
T_{min} (°C)	40	55	45	50–63	37
pH_{opt}	2–3	2–3	2	3–4.5	1–2
pH_{range}	2–5.5	1–5.9	—	1.5–5	0.5–4.5

[a]Darland and Brock (1971); Oshima *et al.* (1977).
[b]Brock *et al.* (1972).
[c]Brierley and Brierley (1973).
[d]De Rosa *et al.* (1974a, 1975a).
[e]Darland *et al.* (1970); Brock *et al.* (1972); Belly *et al.* (1973).

Japanese springs use ammoniun ions as a nitrogen source and either glucose, maltose, galactose or malate as an energy and carbon source. They also grow in media containing albumin or casein. Oshima *et al.* (1977) found that enzymes and DNA are thermostable. The latter has a T_{max} value of 93.2°C. They also found that glyceraldehyde 3-phosphate dehydrogenase and DNA are rapidly denaturated at low pH values, which indicates that the internal pH value is much higher than in the environment. It was subsequently shown that the internal pH value is about 6.

Sulfolobus acidocaldarius, Ferrolobus sp. and *Caldariella* sp. clearly have much in common and should perhaps be classified in the same genus. The main difference between *Caldariella* sp. and the other two species seems to be the GC content in the DNA. All three species grow autotrophically with sulphur as the electron acceptor and energy source. Iron also is oxidized, but only by species *Ferrolobus* and *Caldariella* (Brock *et al.*,1972; Brierly and Brierly, 1973; De Rosa *et al.*, 1975a). *Sulfolobus acidocaldarius* is able to grow heterotrophically using amino acids and some sugars as carbon sources (Brock *et al.*, 1972). Also, *Caldariella* is able to grow heterotrophically, but grows best with sugars as the carbon source (De Rosa *et al.*, 1975a). Species of *Ferrolobus* seem to be obligate autotrophs and there is no growth unless either sulphur or iron is present in the medium (Brierly and Brierly, 1973). Oxidation of sulphur to sulphuric acid by *S. acidocaldarius* has been studied by Shivvers and Brock (1973). It is possible that this organism can grow by reducing ferric iron with elemental sulphur as the energy source (Brock and Gustafson, 1976). *Sulfolobus acidocaldarius* has been found in springs in Yellowstone National Park in the United States of America, Italy, Dominica and El Salvador. A similar species has also been isolated from hot springs in New Zealand (Bohlool, 1975). The morphology of *Caldariella* sp. has been studied utilizing electron microscopy (Millonig *et al.*, 1975).

Thermus acidophilum was isolated from a coal-refuse pile which had undergone self-heating (Darland *et al.*, 1970). It grows heterotrophically on either yeast extract alone or on a low concentation (0.025%, w/v) of yeast extract complemented with either sucrose, glucose, galactose, mannose or fructose as a carbon source. It does not grow on casamino acids, peptone or glucose alone (Belly *et al.*, 1973). The requirement for yeast extract became obvious when Smith *et al.* (1975) discovered that peptides consisting of 8 to 10 amino-acid residues, which are present in yeast extract, are required for growth. Although *T. acidophilum* grows best

at pH 1–2, it seems to have an internal pH value of about 5.5 (Searcy, 1976) or between 6.4 and 6.9 (Hsung and Haug, 1975).

The thermo-acidophiles have to withstand both an acidic environment and high temperature. They have developed unusual membranes, the features of which are discussed later in this review (p. 175).

E. THERMOPHILIC THIOBACILLI AND SULPHATE-REDUCING BACTERIA

Egorova and Deryugina (1963) have described *Thiobacillus thermophilica*, which they isolated from hot sulphur springs in North Caucasus. It has a T_{min} value of 40°C, a T_{max} value of 80°C, and a T_{opt} value between 55°C and 60°C. It uses ammonium salts or nitrates as nitrogen sources, and oxidizes sulphide, thiosulphate and sulphite to sulphate. Organic compounds do not promote growth and indeed are inhibitory Thus, *T. thermophilica* is an obligate autotroph. It forms spores which are very heat-resistant and withstand autoclaving.

More recently, Williams and Hoare (1972) isolated a facultative, autotrophic, thermophilic *Thiobacillus* sp. from hot springs in Yellowstone National Park. It grows between 35°C and 55°C with a T_{opt} value of about 50°C at pH values between 4.8 and 8. It grows autotrophically and oxidizes thiosulphate tetrathionate and, more slowly, sulphite and sulphur, whereas thiocyanate is not used. In the presence of thiosulphate, the growth yield is increased by including either yeast extract, acetate, malate, aspartate or glutamate in the medium. Either ammonium salts, nitrates, glutamate, aspartate or urea serves as a nitrogen source. Activities of sulphite oxidase (AMP-independent), thiosulphate oxidase, rhodanase and ribulose bisphosphate carboxylase were determined in extracts of cells grown autotrophically and with 2 mM acetate in the medium. Activities of the first three enzymes were decreased in the presence of acetate, but the ribulose bisphosphate carboxylase activity was not affected.

With the use of [^{14}C]acetate, Williams and Hoare (1972) demonstrated that *Thiobacillus* sp. incorporates acetate into cell material during growth. Autotrophically-grown cells also assimilate acetate, which is partly oxidized to carbon dioxide. This oxidation is prevented by addition of thiosulphate to the suspension. Although acetate is oxidized, this does not occur via the tricarboxylic acid cycle since *Thiobacillus* sp. lacks α-oxoglutarate dehydrogenase. Acetate seems to be exclusively

assimilated via the glyoxylate cycle. The levels of the enzymes of this cycle are much higher in cells grown on thiosulphate in the presence of acetate than in those grown on thiosulphate alone.

Two micro-organisms that carry out dissimilatory sulphate reduction have been described. *Desulfotomaculum nigrificans* is a spore-former, which grows on media containing lactate or pyruvate together with sulphate which is reduced to hydrogen sulphide. Acetate and formate are not utilized. It grows between 30°C and 70°C with a T_{opt} value of 55°C (Campbell and Postgate, 1965). *Desulfovibrio thermophilus* was isolated by Rozanova and Khudyakova (1974). It does not form spores. It is an obligate anaerobe and grows between 45°C and 85°C with a T_{opt} value at 65°C. Lactate, pyruvate, formate and acetate or hydrogen and acetate are used as carbon sources, whereas sulphate, sulphite and thiosulphate may serve as electron acceptors.

F. THERMOPHILIC CLOSTRIDIA

Several strictly anaerobic spore-forming bacteria exist that are thermophilic. They have not had the same attention by investigators of thermophilicity as aerobic bacteria. This is probably due to the disadvantage of having to work under anaerobic conditions. However, anaerobic techniques have been developed, which make work with anaerobes almost as easy as with aerobes (Hungate, 1969; Bryant and Robinson, 1961). Furthermore, most enzymes and other cell components in anaerobic micro-organisms are not sensitive to oxygen and can be investigated without any extra precautions.

Thermophilic, anaerobic spore-forming micro-organisms of the genus *Clostridium* are considered a nuisance by the food and, especially, the canning industry since they survive normal heat treatments, including boiling. However, a great interest has recently developed in thermophilic clostridia. They have the capacity to ferment different organic matter, including cellulose, to simple organic acids and alcohols, which are now manufactured from petroleum or natural gas. Thus, the possibility is being considered of using these bacteria for production of feedstock chemicals for industry. As raw material, municipal and farm waste may be used, as well as cellulose directly produced for such a purpose. Thermophilic clostridial fermentations may also be coupled with thermophilic methane-producers in mixed cultures which would directly yield methane from cellulose, as has been demonstrated by Weimer and

Zeikus (1977) and Varel *et al.* (1977). The first authors mixed pure cultures of *Clostridium thermocellum* and *Methanobacterium thermoautotrophicum*, whereas the latter authors used the bacteria naturally occurring in cattle waste.

Several thermophilic, saccharolytic clostridia have been described. Among them are *Clostridium thermoaceticum* (Fontaine *et al.*, 1942), *Clostridium thermosaccharolyticum* (McClung, 1935), *Clostridium tartarivorum* (Mercer and Vaughn, 1951) and *Clostridium thermohydrosulphuricum* (Klaushofer and Parkkinen, 1965). The last three of these clostridia have in common that they produce a mixture of products including lactate, acetate, ethanol, carbon dioxide and hydrogen. The cell-wall structures of these three clostridia have been extensively characterized by Hollaus and Sleytr (1972), Sleytr and Thorne (1976) and Sleytr and Glauert (1976). The outer layer of the cell wall of *C. thermohydrosulphuricum* has hexagonally-arranged particles, whereas *C. tartarivorum* and *C. thermosaccharolyticum* have cell-wall particles arranged in rectangular patterns.

Clostridium thermohydrosulphuricum grows between 37°C and 76°C. In addition to fermenting sugars, it reduces sulphite and thiosulphate but not sulphate to sulphide.

Recently, in the author's laboratory, some strains with properties similar to *C. thermohydrosulphuricum* were isolated from soil collected from the vicinity of Athens, Georgia, U.S.A. The products of the fermentation are ethanol, acetate, lactate, carbon dioxide and hydrogen gas. The fermentation proceeds via the Embden-Meyerhof glycolytic pathway as evidenced by using differently-labelled glucose.

Clostridium thermosaccharolyticum grows between 37°C and 65°C. It fermentation proceeds via the Embden-Meyerhof glycolytic pathway as evidenced by using differently-labelled glucose.
(Sjolander, 1937):

$$8 \text{ glucose} + 4\ H_2O \rightarrow 14\ CO_2 + 18\ H_2 + 4 \text{ acetate} + 5 \text{ butyrate} + 2 \text{ lactate}$$

In addition to these products, some strains of *C. thermosaccharolyticum* produce ethanol. The fermentation proceeds via the glycolytic pathway for which all enzymes (including the pyruvate-ferredoxin reductase system) have been demonstrated (Lee and Ordal, 1967). Low activities of glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase were also found. The enzymes of the glycolytic pathway

seem to be repressed by growing cells on pyruvate instead of on glucose. All of these enzymes except for pyruvate kinase are more thermostable in both *C. thermosaccharolyticum* and *C. tartarivorum* than are corresponding enzymes in the mesophile *Clostridium pasteurianum* (Howell *et al.*, 1969). *Clostridium tartarivorum* seems to differ from *C. thermosaccharolyticum* only in that it is able to ferment tartrate in addition to the carbohydrates fermented by the latter. Tartrate is fermented to hydrogen, carbon dioxide, acetate and butyrate (Mercer and Vaughan, 1951). *Clostridium tartarivorum* grows between 37°C and 67°C.

Fructose-bisphophate aldolase (Class II) (Barnes *et al.*, 1971), triosephosphate isomers (Shing *et al.*, 1975), ribosomes, polyribosomes and DNA (Irwin *et al.*, 1973) have been obtained from *C. thermosaccharolyticum, C. tartarivorum* and *C. pasteurianum*. The corresponding enzymes and structures from the three clostridia appear to be very similar except for the higher thermostabilities exhibited by the enzymes and ribosomes from the thermophiles. Ferredoxins have been isolated from *C. thermosaccharolyticom* and *C. tartarivorom* (Devanathan *et al.*, 1969). These have been sequenced (Tanaka *et al.*, 1971, 1973).

Clostridium thermoaceticum which grows between 45°C and 65°C differs from other saccharolytic, thermophilic clostridia. It ferments glucose, fructose, xylose and pyruvate to acetate, which is the only product (Fontaine *et al.*, 1942; Andreesen *et al.*, 1973). This interesting fermentation, which involves the synthesis of acetate from carbon dioxide, will be discussed later in this review.

McBee (1950) isolated *Clostridium thermocellum* and Enebo (1954) obtained *Clostridium thermocellulaseum*. These bacteria ferment cellulose to hydrogen, carbon dioxide, ethanol, formate, acetate, lactate and, to a lesser degree, dicarboxylic acids. The difference between the two cellulose fermenters was that *C. thermocellum* only ferments cellulose, cellobiose, xylose and hemicelluloses, whereas *C. thermocellulaseum* also ferments glucose, fructose and arabinose (Enebo, 1954). However, Patni and Alexander (1971a, b) found that, after induction, *C. thermocellum* also grows on glucose, fructose and mannitol. Therefore, it is possible that the two bacteria are different strains of the same species. All enzymes of the glycolytic pathway have been detected in *C. thermocellum* (Patni and Alexander, 1971a). This bacterium also contains phosphoenolpyruvate: fructose phosphotransferase, fructose 1-phosphate kinase, phosphoenol-pyruvate: mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase, which are all needed for fermentation of fructose and

mannitol (Patni and Alexander, 1971b). A cellobiose phosphorylase has also been characterized from *C. thermocellum* by Alexander (1968).

Recently, new isolates of *C. thermocellum* have been obtained by Lee and Blackburn (1975) and Ng *et al.* (1977). These isolates differ in the same way as described earlier for *C. thermocellum* and *C. thermocellulaseum*. Thus, the Lee and Blackburn (1975) isolate also ferments, in addition to cellulose, a variety of sugars, whereas the Ng *et al.* (1977) isolate ferments only cellulose and its derivatives. When these bacteria ferment cellobiose or sugars, cellulase activity is completely depressed and cannot be demonstrated in the medium. The products of extracellular cellulase activity are glucose and cellobiose.

G. THERMOPHILIC METHANE-PRODUCING AND METHANE-UTILIZING BACTERIA

Zeikus and Wolfe (1972) isolated an autotrophic methane-producing bacterium, *Methanobacterium thermoautotrophicum*, from sewage sludge. It is anaerobic and grows on a mineral medium in an atmosphere of hydrogen and carbon dioxide, which is reduced to methane and also serves as the carbon source. The energy required for growth must be produced in reduction of carbon dioxide with hydrogen. How transport of electrons from hydrogen to carbon dioxide is coupled with phosphorylation is a major problem. Recent progress with *M. thermoautotrophicum* indicates the involvement of a new electron acceptor, namely Factor_{420} (Eirich *et al.*, 1978) and a membrane-bound ATP synthetase (Doddema *et al.*, 1978).

Methanobacterium thermoautotrophicum has a T_{opt} value between 65°C and 70°C and a temperature range of 40–75°C. The optimum pH value is between 7.2 and 7.6, and there is no growth below pH 6.0 or above pH 8.8. The GC content of the DNA is 52%. The ribosomes are stable at 80°C. Methane is not produced when hydrogen is replaced with methanol, ethanol, formate, pyruvate or acetate, and neither acetate nor pyruvate, in the presence of hydrogen and carbon dioxide, stimulates methane production. Thus, *M. thermoautotrophicum*, on all substrates tested, utilizes only carbon dioxide as a carbon source and hydrogen as a source of electrons. Cell-free extracts catalyse methane formation from carbon dioxide and hydrogen at 65°C indicating that the enzymes required have high thermostability. Methane formation in cell-free extracts is enhanced substantially by coenzyme M which has been demonstrated to be involved in methane formation in mesophilic, methane-producing

bacteria (Taylor and Wolfe, 1974). The fine structure of *M. thermoautotrophicum* has been studied by Zeikus and Wolfe (1973). In this bacterium, several oxido reductases have been found that may be involved in synthesis of cell constituents (Zeikus *et al.*, 1977).

It is generally accepted that acetate is a substrate for microbial methane formation. The simple cleavage of acetate to form methane and carbon dioxide will not give enough energy to sustain bacterial growth. If acetate, on the other hand, is completely reduced to two moles of methane with hydrogen as a source of electrons, the energy available would be sufficient (Zeikus *et al.*, 1975). As already discussed, methane production by *M. thermoautotrophicum* isolated by Zeikus and Wolfe (1972) was not stimulated by acetate. Recently, Zeikus *et al.* (1975), using a new isolate of *M. thermoautotrophicum*, found that both carbons of [^{14}C]acetate are converted to methane in the presence of hydrogen. They also state that methane production from acetate is greatly enhanced by carbon dioxide. Although their results definitely show a hydrogen-dependent conversion of both acetate carbons to methane, a careful examination of their data reveals that most methane produced must have been derived from either an endogenous source or carbon dioxide and not from acetate. Furthermore, stimulation by carbon dioxide of methane formation from acetate was small, indeed. In a later paper, Weimer and Zeikus (1977) reported that, in mixed fermentations of cellulose using *C. thermocellum* and *C. thermoautotrophicum*, methane was derived from carbon dioxide reduction rather than from acetate.

A thermophilic, obligate, methane-oxidizing bacterium has been isolated by Malashenko *et al.* (1975). It grows in the range of 37–62°C with a T_{opt} value between 50°C and 56°C. As a nitrogen source, it uses nitrite, nitrate and ammonia. The GC content of the DNA is 63.3%. The name *Methylococcus thermophilus* has been given to the organism.

IV. Properties of Macromolecules and Structures in Thermophiles

A. THERMOSTABILITY OF PROTEINS

Massive evidence now indicates that thermophilic micro-organisms have macromolecules which are stable at the temperatures of growth of these micro-organisms. This is discussed in reviews by Koffler (1957), Farrell and Rose (1967), Campbell and Pace (1968), Farrell and Campbell (1969) and Welker (1976a). Other reviews by Singleton and Amelunxen

(1973), Ljungdahl and Sherod (1976) and Amelunxen and Murdock (1977) deal especially with enzymes and other proteins from thermophiles.

Well over 100 enzymes and proteins have, up to this date, been purified to homogeneity from thermophilic micro-organisms. From *Bacillus stearothermophilus* alone, over 75 proteins have been isolated. They all share the common property of being more thermostable than their corresponding proteins from mesophiles. The amino-acid sequences in some of these proteins have been determined, and it has been conclusively proven that most of them are not stabilized by non-proteinaceous material. It must, therefore, be concluded that the higher thermostability of proteins from thermophiles is an intrinsic property which must depend on the composition and the sequence of the amino-acid residues. Examples of proteins from thermophiles with known sequences are glyceraldehyde 3-phosphate dehydrogenase (Biesecker *et al.*, 1977), 6-phosphofructokinase (Hengartner *et al.*, 1976) and ferredoxin (Hase *et al.*, 1976), all from *B. stearothermophilus*, ferredoxins from *Clostridium tartarivorum* (Tanaka *et al.*, 1971) and from *C. thermosaccharolyticum* (Tanaka *et al.*, 1973), and cytochrome *c* from the thermophilic fungus *Humicola lanuginosa* (Morgan *et al.*, 1972).

The discovery that many proteins from thermophilic micro-organisms have higher thermostability than homologous proteins from mesophiles has, of course, inspired a search for the basis of this higher thermostability. Brandts (1967), discussing the factors affecting thermal stability of proteins, lists hydrogen bonds, hydrophobic bonds and electrostatic interactions (including ion interactions) as stabilizing and conformational entropy as destabilizing structural features in proteins. The stabilizing and destabilizing contributions are nearly in balance, and the free energy of denaturation of a protein is generally small. A minor change in the amino-acid composition, giving one or a few additional hydrogen bonds, hydrophobic bonds or ionic interactions, would account for a change in thermostability. This is what has been found with most enzymes from thermophiles (including the examples listed above) when compared with their counterparts from mesophiles (Singleton, 1976). Direct evidence also exists that a single amino-acid substitution dramatically changes the thermal stability of a protein as demonstrated for β-galactosidase and tryptophan synthetase (Langridge, 1968; Yutani *et al.*, 1977).

Structures more complex than proteins also have higher thermo-

stability in thermophiles than in mesophiles. This has been shown for the ribosomes in *B. stearothermophilus* (Stenesh and Yang, 1967; Pace and Campbell, 1967; Friedman, 1971; Nomura *et al.*, 1968; Altenburg and Saunders, 1971), *Thermus aquaticus* (Zeikus *et al.*, 1970), *Thermus thermophilus* (Ohno-Iwashita *et al.*, 1975a), *C. tartarivorum* and *C. thermosaccharolyticum* (Irwin *et al.*, 1973). The higher thermostabilities of ribosomes from thermophiles reflect the thermostability of their protein components. The ribosome of *B. stearothermophilus*, which consists of 30S and 50S ribosomal subunits, has been reconstituted (Nomura and Held, 1974). Recently, Cohlberg and Nomura (1976) purified 27 protein components from the 50S ribosomal subunit, and showed that these proteins combine with 23S RNA and 5S RNA to form active 50S subunits. Reconstitution was affected by incubation for four hours at 60°C. The *E. coli* ribosome has also been reconstituted, but by using a more complex procedure (Dohme and Nierhaus, 1976). It appears to be easier to work with the ribosomal system of *B. stearothermophilus* than with the homologous system from *E. coli*. This may, to a large extent, depend on the fact that, in the former system the components, especially the proteins, have high thermostability.

A second complex system studied in thermophiles is ATPase. Militzer and Tuttle (1952) and Marsh and Militzer (1956a) were the first to report that ATPase in *B. stearothermophilus* has a high thermal stability, which was confirmed by Hachimori *et al.* (1970) and Hachimori and Nosoh (1977). Sone *et al.* (1977a, b) and Yoshida *et al.* (1977), working with a thermophilic bacillus, PS3, have obtained a stable ATPase (TF_0F_1) composed of eight protein subunits which, with phospholipids, can be reconstituted into vesicles capable of energy-transfer reactions. The system works up to a temperature of 70°C, but is most efficient between 45°C and 50°C. The reconstitution is temperature-dependent, and this dependence is governed by the types of phospholipids used in the reconstitution. Thus, with phospholipids from soybeans containing unsaturated fatty acids, reconstitution was best at 30°C and, with phospholipids from the thermophilic bacterium PS3, it was best at 45°C. The PS3 lipids contain only branched-chain saturated fatty-acyl groups. As with the ribosomal system, reconstitution of the energy-transforming systems appears to be facilitated by using thermostable components from a thermophilic bacterium.

It should be evident from this discussion that, in thermophiles, many of the enzymes and complex functional systems are stable at "thermophilic"

growth temperatures. However, all enzymes and other proteins from the same organism do not have similar thermostability. Some enzymes from thermophiles are denatured *in vitro* at the temperature at which the bacterium is grown and such enzymes often have higher thermostability in the presence of substrate, metals, activators, inhibitors or other factors. This is often also true for proteins from mesophiles. Thus, in the absence of the stabilizing factors, proteins from thermophiles have higher thermostability than the corresponding proteins from mesophiles.

Good examples of proteins from thermophiles, which are stabilized by metals, are thermolysin, α-amylase and aminopeptidases. Thermolysin is an endopeptidase produced by *B. thermoproteolyticus.* Its three-dimensional structure is known (Matthews *et al.*, 1972a, b), and it contains four calcium atoms per mole. The presence of calcium in the protein increases its thermostability to about 40–50°C and, without the calcium, autolysis occurs at temperatures above 50°C. The role of calcium in the thermal stability of thermolysin has recently been studied by Voordouw and Roche (1975) and Dahlquist *et al.* (1976). Yutani (1976) has investigated the role of calcium ions in the thermostability of α-amylase from *B. stearothermophilus* and *B. subtilis*. Both enzymes bind four g-atoms of calcium per mole, and the free energy changes for binding of the four Ca^{2+} by the thermophilic and mesophilic α-amylases are −31.6 and 30.9 kcal per mole of enzyme, respectively. Thus, both enzymes are stabilized by calcium to the same extent, but with different temperature ranges.

Bacillus stearothermophilus contains three aminopeptidases (API, APII and APIII) (Roncari and Zuber, 1969). Aminopeptidase I, when isolated from cells grown in a medium containing 10^{-3} M Co^{2+}, contains 20.4 g-atoms of cobalt and 2.5 g-atoms of zinc. From the apoenzyme, either the cobalt or the zinc enzyme can be prepared. The cobalt-enzyme is the most active and the zinc-enzyme has only 5% of the activity of the cobalt-enzyme. The metals affect the heat stability of API. Thus, when the cobalt-enzyme is heated to 80°C in 10^{-3} M Co^{2+} at a pH value between 7 and 8, the enzyme is stable for five hours whereas, in the absence of cobalt ions, it loses 65% of the activity during one hour. The apoenzyme is completely destroyed at 80°C (Roncari *et al.*, 1972). Aminopeptidase II contains four cobalt atoms per mole; however, these do not increase the thermostability. On the contrary, in the presence of EDTA, the enzyme seems to be more stable (Stoll *et al.*, 1976).

Although many proteins that have been isolated from thermophiles

have been shown to have thermal stability, there is at least one interesting exception. Crabb *et al.* (1975, 1977) reported that glyceraldehyde 3-phosphate dehydrogenase, isolated from the facultative thermophile *Bacillus coagulans* KU grown at 37°C or 55°C, was inactivated by heat well below the thermophilic growth temperature. They found, however, that the enzyme from cells grown at either temperature is stabilized by including 0.61 M $(NH_4)_2$ SO_4 or 1.7 M NaCl in the enzyme solution (Amelunxen and Singleton, 1976). Based on these observations, Crabb *et al.* (1975, 1977) and Amelunxen and Murdock (1977) have suggested that, in facultative thermophiles, proteins may be stabilized by a highly charged macromolecular environment. However, as pointed out by Amelunxen and Murdock (1977), further experimental documentation is needed to prove such a mechansism.

B. MEMBRANES AND LIPIDS

It is now generally accepted that the microbial plasma membrane consists of a phospholipid bilayer containing peripheral proteins, which are probably loosely bound to the membrane, and integeral proteins which are embedded in the membrane (Singer and Nicolson, 1972; Welker, 1976a, b). Studies of membrane-bound NADH oxidase from *B. stearothermophilus* by Wisdom and Welker (1973) have shown that the membrane influences the stability of proteins associated with it. This may affect the cardinal temperatures of a micro-organism. However, these temperatures may be influenced more directly by the stability of the lipid bilayer itself (McElhaney, 1976; Esser and Souza, 1976), and it has been suggested that the thermostability of the cell membrane is a factor determining thermophilic growth (Brock, 1967).

Many investigators have found that the fatty-acyl composition of cellular lipids in micro-organisms varies with changes in environmental temperature and with the composition of the growth medium (McElhaney, 1976). An altered fatty-acyl composition of the cell is reflected by the fatty-acyl content of the lipid bilayer. An important feature of the lipid bilayer is its ability to undergo a reversible phase transition from a thermotropic gel to a liquid-crystalline phase. The temperature of this phase transition is dependent to a large extent on the fatty-acyl composition of the bilayer. Straight-chain saturated fatty acids have, as a class, the highest melting points, which increases with the length of the carbon chain. An extra methyl group at the penultimate

carbon atom of the carbon chain yields acids (*iso*-branched acids) with only slightly lower melting temperatures than corresponding straight-chain acids. If a methyl group is present on other carbon atoms the melting temperatures of the acids (*ante-iso*-branched acids) are decreased considerably. Finally, acids with *cis*-double bonds have the lowest melting points of all the acids (McElhaney, 1976). It follows that membranes with lipid bilayers containing straight-chain and *iso*-branched acids have high phase-transition temperatures, whereas membranes with *ante-iso*-branched acids and *cis*-double bond acids have low phase-transition temperatures. Mixtures of the different types of acids yield membranes with intermediate phase-transition temperatures. However, the transition temperature is not sharp due to the heterogeneity in the composition of the membrane, and domains of gel and liquid-crystalline phases exist simultaneously.

The fatty-acyl composition of members of the genus *Thermus* has been determined by Heinen *et al.* (1970), Ray *et al.* (1971), Jackson *et al.* (1973b) and Oshima and Miyagawa (1974). The general finding is that *iso*-C_{17}- and *iso*C_{15}-fatty acids are the most abundant, followed by *ante-iso*-C_{17}- and *ante-iso*-C_{15}-acids. Very few straight-chain, saturated, fatty acids are present. Heinen *et al.* (1970) reported the presence of a relatively large proportion of C_{15}-acid residues, but Jackson *et al.* (1973b) suggested that this may have been a misinterpretation. Both Heinen *et al.* (1970) and Oshima and Miyagawa (1974) found that the composition of the fatty-acyl residues varied with growth temperature. At a higher growth temperature, the ratio of C_{17}:C_{15} increased indicating a chain elongation. Ray *et al.* (1971) observed an increase in the lipid content on raising the growth temperature. Oshima and Yamakawa (1972, 1974) and Oshima and Ariga (1976) have isolated and determined the structure of a novel galactofuranose-containing glycolipid from *T. thermophilus* and *T. flavus*. The structure is: Gal (f) ($1\overset{\beta}{-}2$) Gal (p) ($1\overset{\alpha}{-}6$) GlcN (15-methylhexadecanoyl ($1\overset{\beta}{-}2$) Glc(p)$\overset{\alpha}{-}$2,3 diglyceride, in which "Gal(f)" indicates a galactofuranose residue and "Glc(p)" a glucopyranose residue. This complex lipid constitutes 70% of the total lipids in the two extreme thermophiles. Its content increases with the culture temperature.

Card *et al.* (1969) and Card (1973) have reported that the lipids of *B. stearothermophilus* strain 2184 consist of 30–40% neutral lipids and 60–70% phospholipids. The latter consist of diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. They found that

91% of the total phospholipids of the whole cell is in the membrane fraction. The fatty-acyl content of several bacteria, including *B. stearothermophilus*, was analysed by Cho and Salton (1966), who found that the thermophile has a substantial amount of C_{15}- and C_{17}-, branched-chain (mostly *iso*) fatty-acyl residues. The effect of growth temperature on the fatty-acyl and lipid composition of *B. stearothermophilus* has been investigated by Daron (1970) and Oo and Lee (1971). The latter reported that the total lipid content decreases with increased growth temperature (from 5.5% dry weight at 37°C to 4.3% at 55°C); however, an increase in phospholipid content occurs at higher temperatures. The most abundant fatty-acyl residues are *iso*-C_{16}, n-C_{16}, and *ante-iso*-C_{17}; there is a very low level of unsaturated fatty-acyl residues. A shift of the content of *iso*-acids to C_{16}-acids was noticed with increased growth temperature. Similarly, Daron (1970) observed a shift in the *iso*-acids to the C_{16}-acid by using glucose instead of acetate as a carbon source.

The fatty-acyl composition of the extreme thermophiles, *Bacillus caldolyticus* and *Bacillus caldotenax*, grown at 60°C, 70°C and 80°C, has been examined by Weerkamp and Heinen (1972). At all temperatures, the *iso*-acids (mainly *iso*-C_{15},*iso*-C_{16}, and *iso*-C_{17}) are the major constituents. With an increase in temperature, a shift is seen from *iso*-C_{15}- to *iso*-C_{17}- and also from *iso*-C_{16}- to n-C_{16}-fatty acids. A thermophilic, spore-forming bacterium, PS3, contains only saturated fatty-acyl residues and mainly *iso*-C_{15}-, *iso*-C_{16}- and *iso*-C_{17}-fatty-acyl residues (Kagawa and Ariga, 1977). The phospholipids in this species are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The distribution of the fatty-acyl residues between positions 1 and 2 of the glycerol moiety was also determined, and position 2 is mostly occupied by *iso*-C_{15}-acid.

The thermophilic species of *Bacillus* already discussed contain saturated fatty-acyl residues almost exclusively and these are generally of the *iso*-type. The precursors of the *iso*-acids, in addition to acetyl-CoA, are isobutyryl-CoA, isovaleryl-CoA and α-methylbutyryl-CoA. These coenzyme-A esters may be formed by oxidative decarboxylation of the α-oxo acids corresponding to valine, leucine and isoleucine. Daron (1973) has grown, at 50°C, a thermophilic *Bacillus* species on acetate in media containing isobutyrate, isovalarate, α-methylbutyrate, leucine and isoleucine. Valine, when added to a similar medium, seems to prevent growth of the bacterium. The fatty-acyl compositions of the cells

varied considerably. When grown on isobutyrate, the main fatty-acyl residue (76%) was *iso*-C_{16}; on leucine, the dominant acids were *iso*-C_{15} (58%) and *iso*-C_{17} (29%); and on isoleucine it was *ante-iso*-C_{17} (72%). The wide variation in the fatty-acyl composition ought to be reflected in the properties of the membranes. One would expect that bacteria grown in the presence of isoleucine should have a less stable membrane since *ante-iso*-acids have lower melting points (McElhaney, 1976) than the *iso*-acids. However, the organism tolerates the variation in fatty-acyl composition.

The fatty-acyl composition of lipids from the thermophiles *Clostridium tartarivorum* and *Clostridium thermosaccharolyticum*, the mesophilic *Clostridium pasteurianum*, and a psychrophilic *Clostridium* species have been determined by Chan *et al.* (1971). The thermophilic clostridia have a much lower content of unsaturated fatty-acyl residues than the mesophilic and the psychrophilic counterparts. The main residues in the thermophiles are *iso*-C_{15}, n-C_{14} and n-C_{16}. *Iso*-acids are almost absent from the mesophile and from the psychrophile, and both contain mono-unsaturated acids. All four clostridia have an unusual unsaturated cyclopropane fatty-acyl residue (12,13-methylene-9-tetradecanoic acid). It is more prominent in the psychrophile than in the thermophiles.

Unusual lipids are present in a group of micro-organisms called "thermo-acidophiles" which include *Bacillus acidocaldarius* (Darland and Brock, 1971), *Sulfolobus acidocaldarius* (Brock *et al.*, 1972; Brierley and Brierley, 1973), the *Caldariella* species described by De Rosa *et al.* (1974a, 1975a) and *Thermoplasma acidophilum* (Darland *et al.*, 1970; Belly *et al.*, 1973). The cell membranes of the thermo-acidophiles must be constructed in such a way that they can withstand both very acid pH values and high temperatures. This probably explains the presence of unusual lipids in these micro-organisms.

Of the thermo-acidophiles, *B. acidocaldarius* is unique in that it predominantly contains the C_{17}- and C_{19}-ω-cyclohexyl fatty-acyl residues, 11-cyclohexylundecanoic acid and 13-cyclohexyltridecanoic acid (De Rosa *et al.*, 1971a, b; 1974a). The cyclohexyl ring in the residues is probably synthesized from glucose with shikimate and cyclohexylcarboxyl-CoA as intermediates (De Rosa *et al.*, 1972; Oshima and Ariga, 1975). The shikimate pathway is the normal route for synthesis of aromatic amino acids. The cyclohexyl fatty-acyl residues are either ester- or amide-linked in the novel glycolipids (*N*-acylglucosaminylpentacyclic tetrol and glucosyl-*N*-acylglucosaminyl-diglyceride), the structures of which have been elucidated by

Langworthy *et al.* (1976). They also found the following lipids: sulphonylglycosyl diglyceride, diphosphatidylglycerol, and phosphatidylglycerol in *B. acidocaldarius*. The pentacyclic triterpene was detected in this organism by De Rosa *et al.* (1971a). Similarly, Langworthy and Mayberry (1976) have identified 1,2,3,4-tetrahydroxypentane-substituted pentacyclic triterpene in *B. acidocaldarius*. This polyol exists both in the free state and as an aglycone.

The membranes of the other thermo-acidophiles differ dramatically from that of *B. acidocaldarius*. Thus, both *T. acidophilum* and the micro-organisms of the *Sulfolobus* group (this group includes *Sulfolobus acidocaldarius*, the organism described by Brierley and Brierley, 1973, and the *Caldariella* strains reported by De Rosa *et al.*, 1974a) seem to lack, or have low levels of, fatty-acyl residues. Instead, they have long-chain, C_{40}-*iso*-prenoid, branched, alkyl diols, the hydroxyl groups of which are both terminal (Langworthy *et al.*, 1972, 1974; De Rosa *et al.*, 1974a, c, 1976; Ruwart and Haug, 1975). In addition to the alkyl diols, the last authors found some fatty-acyl esters (2.1% by weight of total lipids) in *T. acidophilum* membranes indicating that this organism may not be completely devoid of them.

It was first concluded that the alkyl diols formed glycerol diethers, which would be the principal constituents of the membrane bilayer. However, Langworthy (1977a) recently established that the C_{40}-alkyl diols formed diglycerol tetra-ethers. These are structurally composed of two glycerol moieties which are joined together by two C_{40}-alkyl diols. These structures suggest a possible occurrence of a new type of membrane assembly in micro-organisms composed of a monolayer of diglycerol tetra-ethers. De Rosa *et al.* (1977) have confirmed the tetra-ether structure.

Sulfolobus acidocaldarius contains about 2.5% total lipid, based on dry weight. The lipid fraction which contains the diglycerol tetra-ether is composed of 10.5% neutral lipid, 67.6% glycolipid and 21.7% polar lipid. The structures of these lipids have been studied by Langworthy *et al.* (1974). In a later paper, Langworthy (1977b) reported that *S. acidocaldarius* and the organism which was isolated by Brierley and Brierley (1973) and often referred to as "ferrolobus", when grown autotrophically, have similar lipids consisting of 15% neutral lipids, 35% glycolipids and 50% acidic lipids. Heterotrophically grown *S. acidocaldarius* had a similar lipid composition except that one of the acidic lipids was absent. In *C. acidophila*, De Rosa *et al.* (1975b) have identified a

terpenoid 4,7-thianaphthene-quinone. This compound is related to vitamin K_2, and it may have a function in electron transport.

Thermoplasma acidophilum lacks a cell wall. Its membrane, which must be stable at low pH values and high temperatures, has been the subject of several investigations. Belly and Brock (1972) reported that *T. acidophilum* is much more resistant to lysis in distilled water by surface-active agents and heating compared with that of mesophilic mycoplasma. However, it lyses at alkaline pH values. Similarly, Smith *et al.* (1973) found that the osmolarity of the environment plays a minor role in the stability of *T. acidophilum*. However, they reported that intact cells and membranes disaggregate below pH 1 and above pH 5, which is a narrower range of stability than that reported by Belly and Brock (1972). The membrane fraction contains several proteins, which contain a low proportion of free amino and carboxyl groups. The low proportion of these groups seems to be related to the sensitivity of the membrane to pH values over 5. Thus, removal of potential negative charges either by reacting the carboxyl groups with glycine methylester or by increasing the density of positive charges extends the stability of the membrane to, at least, pH 8 (Smith *et al.*, 1973). Ruwart and Haug (1975) have prepared pure membranes from *T. acidophilum* by lysis at pH 9.3.

The lipid composition of *T. acidophilum*, which contains 3% total lipid based on the dry weight, has been determined by Langworthy *et al.* (1972). A lipopolysaccharide polymer with a molecular weight of 1,200,000 is present in *T. acidophilum* (Mayberry-Carson *et al.*, 1974). It consists of monomers, with a molecular weight of about 5300, which are composed of carbohydrate, glycerol and the C_{40}-alkyl diols. The monomer contains 24 mannose, one glucose, one glycerol and two alkane residues. The composition of this monomer as given seems to differ from what can be predicted if it contains tetra-ethers, in which case it should contain two glycerol residues. Electron microscope examination of the polymer revealed a ribbon-like structure with some branching (Mayberry-Carson *et al.*, 1975).

Thomas A. Langworthy (personal communication) has summarized the lipid content of thermo-acidophiles, as follows: *T. acidophilum* contains diglycerol tetra-ethers, phosphoglycolipids, and cyclized hydrocarbons; *Sulfolobus* sp., diglycerol tetra-ethers, phosphoglycolipids, sulpholipids and cyclized hydrocarbons; and *B. acidocaldarius*, ω-cyclohexyl fatty acid esters, glucosamidyl glycolipids, sulphonolipids and cyclized hydrocarbons.

It may now be concluded that thermophilic micro-organisms have membranes composed of fatty-acyl residues with higher melting temperatures than the residues from mesophilic organisms. Furthermore, they have the capacity to change the fatty-acyl composition with temperature, which indicates a thermal adaption. The unusual composition of the membrane lipids from thermo-acidophiles, which allows them to grow under environmental conditions of extreme acidity and temperature, testifies further to the adaptability of micro-organisms. The strong evidence of a monolayered lipid membrane in the thermo-acidophiles is certainly exciting. However, it was recently discovered that *Methanobacterium thermoautotrophicum, Methanobacterium formicum* and *Methanospirillum hungatii* contain dialkyl glyceryl ethers as well as diglycerol tetra-ethers. The latter are similar to those found in the thermo-acidophiles (Makula and Singer, 1978). Thus, the occurrence of diglycerol tetra-ethers is not restricted to thermo-acidophiles.

As already discussed, an important feature of the membrane is its ability to undergo a reversible phase transition from a thermotropic gel to a liquid-crystalline phase. The temperature of this phase transition has been investigated using spin-labelling techniques and differential thermal analysis. The general view is that membranes must be in the liquid-crystalline state to function in transport and for activity of membrane-associated enzymes. Assuming that this is correct, then it is easy to imagine that the minimum and, perhaps, the maximum growth temperatures may be directly dependent on the phase-transition temperature of the membrane.

McElhaney (1974, 1976) has used differential thermal analysis in studying the phase-transition of the membrane of *Acholeplasma laidlawii*. In this organism, the fatty-acyl composition of the membrane lipids can be dramatically changed in a controllable manner by including fatty acids in the growth medium. Thus, membranes with different transition temperatures, which are dependent on the fatty-acyl composition, can be obtained. The transition temperature of the membrane lipids may then be correlated with T_{min} and T_{max} of the bacterium. It should be noted that the phase transition of a membrane is not sudden but occurs over a 15–25°C range in temperature, during which the membrane has both gel and liquid-crystalline phases simultaneously. McElhaney (1974, 1976) did not find a relationship between T_{max} and the phase-transition temperature of the membrane. However, he found an effect on the T_{min} value. Thus, the T_{min} value of the cells corresponded with the

temperature at which 10% of the membrane lipids still existed in a liquid-crystalline state. However, when the temperature at which 10% of the membrane was in a liquid-crystalline state was below 8°C, the cells stopped growing at 8°C. Thus, at 8°C, something other than the phase-transition temperature determined the T_{min} value. The state of the membrane also has an effect on the growth rate. When the phase-transition midpoint temperature was below the T_{min} value, Arrhenius plots of growth rates were linear with the slopes corresponding to a growth characteristic (μ) of 16–18 kcal per mole. When the midpoint temperature of the phase-transition was above the T_{max} value of the cells, a linear Arrhenius plot was also observed, but the μ value was about 45 kcal per mole indicating a very long generation time. Finally, when the phase-transition mid-point fell between the T_{min} and T_{max} values of the cells, a break in the Arrhenius plot occurred giving a μ value of about 45 kcal per mole at low temperatures and 16–18 kcal per mole at high growth temperatures.

Souza *et al.* (1974) isolated a thermophilic bacterium, which was characterized as a strain of *Bacillus stearothermophilus* YTG-2 with a T_{min} value of 37°C and a T_{max} value of 71°C. When grown at different temperatures (42°C, 58°C and 65°C), the proportion of fatty-acyl residues with high melting points (>55°C) increased from 42% to 64% and 69%, respectively, which indicates a temperature-dependent regulation of the fatty-acyl composition. A mutant (TS-13) obtained from this strain failed to grow above 58°C. It exhibited an apparent loss of membrane stability at high temperatures. The mutant failed to regulate its fatty-acyl composition in the manner of the parent strain. It was suggested that the mutant had a defect in lipid metabolism at high temperatures and also that changes in fatty-acyl composition observed at increased growth temperatures may be an essential feature of thermophilicity. In following papers, Esser and Souza (1974, 1976), using spin-label techniques, established that *B. stearothermophilus* YTG-2 has a typical bilayer membrane and also that the cells varied their lipid composition in response to growth temperature. They further observed that cells grown at different temperatures produced membranes with the same constant fluidity at the temperature of growth. Such a phenomenon has been called "homeoviscous adaptation" by Sinensky (1974). The mutant did not exhibit homeoviscous adaptation, and its fatty-acyl composition was similar when grown at two different temperatures. They interpreted their data to indicate that the maximal and minimal growth

temperatures of thermophiles are regulated by the onset and conclusion of the phase transition of the lipid bilayer.

The suggestion by Esser and Souza (1974, 1976) that T_{min} and T_{max} values of *B. stearothermophilus* are regulated by the phase separation of the lipid bilayer is not completely in agreement with the data presented by McElhaney (1974, 1976), studying *A. laidlawii*, and already discussed. McElhaney found that this organism is able to grow well above the upper boundary of the gel to liquid-crystalline membrane lipid phase transistion. He also found that *A. laidlawii* stopped growing at 8°C, although the membrane was still at least partly in the liquid-crystalline state. Recently, McElhaney and Souza (1976) re-examined the effect of temperature on membranes from *B. stearothermophilus* YTG-2 and the mutant strain TS-13, using differential thermal analysis. They confirmed the earlier studies which showed that *B. stearothermophilus* changes its fatty-acyl composition and fluidity and the physical state of its membrane lipids with a change in the growth temperature. The organism thus has an effective and sensitive homeoviscous adaptation mechanism which maintains similar membrane fluidity over a wide range of growth temperatures. They also found that the mutant lost the ability for homeoviscous adaptation. However, it was also clear that *B. stearothermophilus* prefers to grow at or slightly above the upper boundary of membrane-lipid phase transition at which state all of the membrane lipids exist essentially in the liquid-crystalline state. Furthermore, the lower boundary for the gel to liquid-crystalline phase transition is well below 37°C which is the T_{min} value for this micro-organism. This indicates that the T_{min} value is not determined by the state of the membrane lipids in *B. stearothermophilus*. The mutant TS-13, grows well above the upper boundary of the phase transition, which may mean that the lipid phase transition does not directly determine the maximum growth temperature. However, growth ceases abruptly when the environmental growth temperature exceeds the upper boundary of the phase transition by about 18–19°C, which implies an upper limit on membrane lipid fluidity which is compatible with cell growth. The authors suggested that the electron paramagnetic resonance technique used in previous work is, in fact, not monitoring a solid-fluid lateral phase transition of the bulk of the membrane lipids but lateral phase separation.

Spin-labelling studies of membranes of a facultative, thermophilic *Bacillus* species, strain T1, have been reported by Chan *et al.* (1973). They used a nitroxide stearate as a spin-label which was incorporated into the

membrane fraction in cells grown at 37°C and 55°C. The results indicated that cells grown at 55°C have a more rigid membrane than those grown at 37°C. Spin-labelling was also used by Smith *et al.* (1974) in studies of the phase transition of membranes from *T. acidophilum*. The lipid region in the membrane is highly rigid, and this rigidity is dependent on temperature. The membrane undergoes two lipid phase transitions, at 45°C and 60°C, which are close to the T_{min} (45°C) and T_{max} (62°C) values of this organism (Darland *et al.*, 1970), which suggests a relationship.

Sphaeroplasts have been prepared from *B. stearothermophilus* (Bodman and Welker, 1969; Golovacheva *et al.*, 1969). They are very stable and insensitive to changes in osmotic pressure. Sphaeroplasts from cells grown at temperatures between 55°C and 70°C have different thermostability. Those from cells grown at 55°C rupture at 65°C while those from 70°C cells rupture at 80°C (Wisdom and Welker, 1973). Divalent cations improved the stability of sphaeroplasts. The stability of NADH oxidase activity, which is membrane-bound, is also affected by growth temperature. Incubation for one hour at 75°C completely destroyed this activity in cells grown at 55°C whereas, in those grown at 70°C, the activity is completely stable during this treatment.

Kiszkiss and Downey (1972b) found that sodium dodecyl sulphate (SDS) treatment disrupts membranes from *B. stearothermophilus* 2184D. The SDS-treatment leads to a loss of NADH-nitrate reductase and cyanide-sensitive activities whereas reduced methyl viologen-nitrate reductase and NADH-menadione activities are unaffected. The lost activities are restored by dilution in the presence of divalent cations of which Mg^{2+} seems to be best. The reconstitution is pH-dependent with an optimum at pH 7.4.

Halverson *et al.* (1978), studying *B. stearothermophilus* sphaeroplasts with freeze-fracture techniques, has obtained results indicating that membrane proteins are randomly dispersed above temperatures of the upper boundary of the membrane-lipid phase transition, but that they form network-like patterns at lower temperatures. Below the temperature of the lower boundary of the phase transition, large areas of the membrane are almost free of proteins. These results demonstrate a temperature-dependent interaction between membrane lipids and membrane proteins.

Hashizume *et al.* (1976) studied the effect of temperature on the viability of *B. stearothermophilus* A.T.C.C. 7953. They determined the

survival of bacteria grown at 55°C after they were transferred to 30°C or 4°C. About 80% of the population died over a period of four hours at the lower temperatures. The decline in incorporation of ^{14}C-amino acids and ^{14}C-uracil, assayed at 55°C in cells incubated at 30°C or 4°C, closely followed the death curve at respective temperatures. Similarly, NADH oxidase activity was also lost. With the progress of death, enzymes (such as fructose 1,6-diphosphate aldolase) and ribonucleoproteins leaked out of the organisms. The authors suggested that changes in the state of the membrane were the possible cause of death. Electron microscope studies on intact cells, protoplasts and membranes from *B. stearothermophilus* have been published by Abram (1965).

Ljunger (1970) noticed that cells of *B. stearothermophilus* survive longer when suspended in 0.1 M tris buffer (pH 7.2) if calcium ions and a source of energy are supplied. In a later paper, Ståhl and Ljunger (1976) suggested that calcium ions are actively taken up by cells. However, their data do not distinguish between absorption of calcium on the cell and its entry into the cell. Mosley *et al.* (1976) also observed that calcium prevents lysis of cells suspended in 0.1 M tris buffer. However, they also noticed that cells incubated under anaerobic conditions for 20 min before being harvested and suspended in tris buffer were stable in the absence of calcium. An increase in membrane cardiolipin content occurred during anaerobic incubation. Mosley *et al.* (1976) suggested that the effect of calcium was to increase membrane stability. That *B. stearothermophilus* cells easily lyse when suspended in buffer is in contrast to observations that sphaeroplasts are very stable and insensitive to changes in osmotic pressure (Bodman and Welker, 1969; Golovacheva *et al.*, 1969).

C. SYNTHESIS OF NUCLEOTIDES, DNA, RNA AND PROTEIN

Pyrimidine-nucleoside: orthophosphate ribosyltransferase (EC 2.4.2.2) has been purified by Saunders *et al.* (1969) from *B. stearothermophilus*. The enzyme is active with the nucleosides uridine, thymidine and deoxyuridine. In the reverse reaction, with uracil as the acceptor, both ribose 1-phosphate and deoxyribose 1-phosphate serve as substrates. The specificity of the enzyme is broader than that of corresponding enzymes from other sources. Synthesis of the enzyme appears to be induced by either uridine or thymidine.

A thermostable pyrimidine ribonucleoside kinase (EC 2.7.1.48) has been purified from *B. stearothermophilus* by Orengo and Saunders (1972). The Arrhenius plot is biphasic, and the sharp break in the line is at 46°C. At 60°C, the optimum growth temperature of the micro-organism, the presence of ATP induces sigmoidal kinetics. However, at lower temperatures, the saturation curves show both positive and negative co-operativity. The enzyme is also regulated by CTP. This regulation is most strongly expressed at the thermophilic growth temperature whereas, at lower temperatures, the enzyme is less sensitive to regulation by CTP.

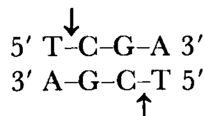
An enzyme which catalyses phosphorylation of deoxyuridine and deoxythymidine, using either ATP or dATP as the phosphate donor, has been purified more than 1000-fold from *B. stearothermophilus* by Kobayashi *et al.* (1974). This enzyme is highly thermostable and, during incubation at 100°C for 30 min, it loses only 40% of its activity. It is active as a dimer (molecular weight, 54,000) but it also exists as a tetramer. The enzyme requires magnesium ions for activity.

Adenosylcobalamin-dependent ribonucleotide reductase (EC 1.17.4.2) has been demonstrated in *Thermus aquaticus* YT-1 (Gleason and Hogenkamp, 1972) and in *Thermus* X-1 (Sando and Hogenkamp, 1973). The enzyme in the latter organism is present during logarithmic growth but is virtually absent from stationary-phase cells. The enzyme has been partially purified. It has a molecular weight of about 80,000 and is stable at 70°C. The enzyme catalyses reduction of GTP, CTP, ITP, UTP and ATP to the corresponding deoxyribonucleoside triphosphates, and the activity is variously affected by deoxyribonucleotides. The effect seems to be allosteric and is more pronounced at high temperatures.

Deoxyribonucleic acid polymerases (EC 2.7.7.7) from *B. stearothermophilus* and the mesophile, *Bacillus licheniformis* have been compared by Stenesh and Roe (1972a, b) and Stenesh and McGowan (1977). The first paper deals with purification of the enzymes which was achieved using ammonium sulphate fractionation and DNA-cellulose chromatography. The two enzymes have almost identical properties except that the *B. stearothermophilus* enzyme has a temperature optimum about 20°C higher and requires a higher magnesium ion concentration for maximum activity than the *B. licheniformis* enzyme. The other two papers deal with the lack of fidelity during replication at 37°C, 45°C and 55°C of DNA from the two bacilli and calf thymus and of poly(dA-dT) ·poly (dA-dT) and poly (dC) ·poly(dG) as templates. For the enzymes from both micro-organisms, the error rate for dCTP and dGTP was independent of

temperature whereas, for dATP and dTTP, it increased significantly with temperature. These results and others regarding information transfer in *B. stearothermophilus* have been discussed at length by Stenesh (1976). Deoxyribonucleic acid polymerase has also been obtained from *T. aquaticus* YT-1 (Chien *et al.*, 1976). This enzyme has a temperature optimum of 80°C. Its molecular weight is about 65,000, and it requires all four deoxynucleoside triphosphates, template DNA and Mg^{2+} for activity.

A thermostable restriction endonuclease (EndoR.Bst 1503) has been isolated from *B. stearothermophilus* (Catterall and Welker, 1977). Endonucleases recognize and cleave specific base pairs in DNA, and aid in studies of the structure and function of DNA. They are also of interest because of their specificity in the interaction with DNA. There are two types of endonucleases. Type I requires ATP and Mg^{2+} for activity and either requires or is stimulated by *S*-adenosylmethionine. Type II requires Mg^{2+} and produces characteristic limit digests of DNA. The endonuclease, EndoR.Bst 1503, is of Type II. It is stable for two hours at 70°C and is active as a dimer (molecular weight, 96,000) and a tetramer. Its specificity toward some phage DNAs is described. An endonuclease, Taq I, has also been obtained from *T. aquaticus* YT-1. It is active at temperatures up to 70°C and cleaves DNA of the following sequence (Sato *et al.*, 1977):



Remold-O'Donnell and Zillig (1969) have obtained a DNA-dependent RNA-polymerase from *B. stearothermophilus*. The enzyme was purified 234-fold. Its activity is dependent on Mg^{2+}, DNA and the nucleotides ATP, CTP, UTP and GTP. The enzyme has a high thermal stability and exhibits a biphasic Arrhenius plot with the transition at 33°C. Deoxyribonucleic acid-dependent RNA-polymerases have been purified by Air and Harris (1974) from both *B. stearothermophilus* and *T. aquaticus*. Both enzymes consist of four subunits of different molecular weights which for the *B. stearothermophilus* enzyme are about 148,000, 140,000, 95,000 and 40,000 and, for *T. aquaticus*, 170,000, 160,000, 99,000 and 45,000. The enzyme from *T. aquaticus* has also been studied by Fábry *et al.* (1976) who obtained essentially the same results as Air and Harris (1974) except that, instead of the 160,000 subunit, they found a 130,000 subunit.

The polymerase from *T. aquaticus* has a temperature optimum around 65°C.

Two RNA-polymerases, A and B, have been isolated from *T. thermophilus* HB8 (Date *et al.*, 1975a, b). Polymerase A utilizes various DNAs as templates whereas enzyme B is only active with either an alternating copolymer of deoxyadenylic and deoxythymidylic acids or a mixture of homopolymers of deoxyadenylic acid or deoxythymidylic acid. As with other RNA polymerases, enzyme A consists of four subunits with molecular weights as follows: I-42,000, II-58,000, III-140,000, and IV-180,000. The subunit composition for enzyme A is: I_2-II-III-IV and, for enzyme B, I_2-III-IV. Enzyme B is partly associated (about 25%) with an extra polypeptide (X) having a molecular weight of 100,000 which seems to substitute for subunit III giving the composition: I_2-X-IV (Tsuji *et al.*, 1976). The two B-forms have not been separated, and they crystallize together. Enzyme A is considered a holo-enzyme whereas enzyme B is a core enzyme. That enzymes A and B have different catalytic properties allows the conclusion that the subunits play different roles in the overall reaction catalysed by RNA polymerase. The *T. aquaticus* enzymes are stable for three hours at 70°C.

Deoxyribonucleic acid-dependent RNA polymerase has also been obtained from two strains of a thermo-acidophile named *Caldariella acidophila* (a relative to *Sulfolobus* species) which grow at 75°C and 87°C around pH 2 (Cacace *et al.*, 1976a). The enzyme from MT-4, which is the strain that grows at 87°C, has been studied in some detail (Cacace *et al.*, 1976b). It seems to consist of five different subunits with molecular weights of 38,000, 65,000, 72,000, 120,000 and 127,000. Optimal activity is obtained in the presence of 250 mM KCl and with either 0.5 mM Mn^{2+} or 5 mM Mg^{2+}. The enzyme is remarkably thermostable with a temperature optimum around 80°C. The pH optimum is between 7.5 and 8.0 which is much higher than the pH value of the culture medium (3.0). This may indicate that the internal pH value of the thermo-acidophilic bacteria is close to neutrality. Such bacteria may have an energy-dependent, proton-exclusion mechanism to maintain internal pH (Yamazaki *et al.*, 1973).

Amino-acyl-tRNA synthetases catalyse the following reaction:

$$aa_x + ATP + tRNA_x \xrightarrow{\text{aa-tRNA synthetase}_x,\ Mg^{2+}} aa\text{-}tRNA_x + AMP + PP_i$$

In vivo, this reaction is specific and, for each amino acid, there is a specific transfer ribonucleic acid (tRNA) and an amino-acyl-tRNA synthetase.

The reaction activates the amino acid and couples it with the correct tRNA which is recognized and allows the amino acid to be incorporated at the right place during protein synthesis. Several tRNAs have been isolated from thermophilic micro-organisms. They include tRNA for phenylalanine ($tRNA^{Phe}$; Guerrier-Takada *et al.*, 1975) and $tRNA_2^{Val}$ (Takada-Guerrier *et al.*, 1976), which are both from *B. stearothermophilus*, and $tRNA^{Met}$ from *T. thermophilus* (Watanabe *et al.*, 1976a). The structures of these have been determined and compared with the homologous tRNAs from mesophiles. Some differences are noted which may be of importance, thermophilically; however, structures among tRNAs are very similar, regardless of source. The $tRNA^{Val}$ and $tRNA^{Phe}$ from *B. stearothermophilus* react with the valine- and phenylalanine-tRNA synthetases from *E. coli* and yeast (Takada-Guerrier *et al.*, 1976; Giegé *et al.*, 1974). The structure, sequence and properties of tRNAs were recently reviewed by Rich and RajBhandary (1976).

All tRNAs so far sequenced have a common structure incorporated into a cloverleaf pattern consisting of a stem and four loops. Furthermore, some of the nucleotide sequences seem to be common to all tRNAs. Among these is the TΨC loop which contains the sequence: guanidylate-thymidylate-pseudouridylate-cytidylate. The sequence is invariant, especially in tRNAs from prokaryotes. However, Watanabe *et al.* (1974, 1976b) noticed that tRNA from *T. thermophilus* contained the modified nucleotide, 5-methyl-2-thiouridylate, and also that the content of this nucleotide and thymidylate varied with changes in growth temperature in such a way that the sum of the two was always one for each tRNA molecule. They subsequently showed that the 5-methyl-2-thiouridylate replaces thymidylate in the G-T-Ψ-C sequence to form a new sequence, G-M^5s^2U-Ψ-C, and that the extent of this replacement depends on growth temperature. Thus, the ratio of m^5s^2U over T is 27.8/72.2 in cells grown at 50°C whereas it is 57.4/42.6 in cells grown at 80°C. The melting temperature of the tRNA also increases with growth temperature from 83.3°C at 50°C to 86.4°C at 80°C. This increase in melting temperature is attributed to the content of 5-methyl-2-thiouridine in the tRNA and constitutes a mechanism of thermo-adaptation.

Several amino-acyl-tRNA synthetases have been obtained from *B. stearothermophilus*. The activities of isoleucyl- and leucyl-tRNA synthetases were reported to be retained above 70°C (Arcà *et al.*, 1964). These authors also found that isoleucyl-tRNA synthetases and tRNAs from *B. stearothermophilus* and *E. coli* are interchangeable; however, they vary in

their temperature ranges. Purifications of *B. stearothermophilus* amino-acyl-tRNA synthetases have been achieved as follows: leucyl-tRNA synthetase (Vanhumbeeck and Lurquin, 1969), arginyl-tRNA synthetase (Godeau, 1976), isoleucyl-tRNA synthetase (Charlier and Grosjean, 1972), valyl-tRNA synthetase (Wilkinson and Knowles, 1974), tyrosyl-tRNA synthetase (Koch, 1974) and methionyl-tRNA synthetase (Mulvey and Fersht, 1976). In the summary of an extensive study by Grosjean *et al.* (1976) are listed 20 amino-acyl-tRNA synthetases from *B. stearothermophilus* which have been purified to varying degrees. They list the thermostabilities, molecular weights and subunit structures of these enzymes which are also compared with the equivalent synthetases from *E. coli*. The similarities between the synthetases from the two bacteria are striking; however, the enzymes from *B. stearothermophilus* have higher thermal stabilities than the corresponding enzymes from *E. coli*.

Zeikus and Brock (1971) studied formation of phenylalanyl- and isoleucyl-tRNA using synthetases partly purified from *T. aquaticus*. The temperature optimum is about 70°C whereas, with the enzyme from *E. coli*, it is 45°C. As was demonstrated with *B. stearothermophilus* (Arcà *et al.*, 1964), the tRNA and synthetases from *E. coli* are interchangeable with the corresponding structures from *T. aquaticus*. The optimum temperature with *T. aquaticus* tRNA and *E. coli* synthetase is 35–45°C whereas, with *E. coli* tRNA and *T. aquaticus* synthetase, it is 65°C. These results clearly reflect the temperature stabilities of the respective synthetases. The isoleucyl-tRNA from *T. thermophilus* HB8 has been partly purified, and its properties are very similar to the corresponding enzymes from *B. stearothermophilus* and *E. coli* except for its very high temperature optimum of 80°C (Wakagi *et al.*, 1975).

Protein synthesis in prokaryotic micro-organisms requires ribosomes, mRNA, amino-acyl-tRNAs, elongation factors, Mg^{2+} and GTP. It is also stimulated by polyamines. Ribosomes in prokaryotic micro-organisms have a molecular weight of approximately 2.7×10^6 and a sedimentation coefficient of 70S. The complete 70S ribosome consists of a 30S and a 50S ribosomal subunit. The 30S subunit contains 16S rRNA and 21–23 proteins whereas the 50S subunit contains 23S rRNA, 5S rRNA, and 27–34 proteins. It is impossible here to discuss in detail the properties of the prokaryotic ribosome, and the reader is referred to articles in the monograph edited by Nomura *et al.* (1974). However, a few properties and some recent articles will be mentioned.

It has already been pointed out in this review that ribosomes from thermophiles have higher thermostabilities than those from mesophiles (also see review by Friedman, 1968). The thermostabilities of 30S and 50S ribosomal subunits and of 16S, 5S and 23S ribosomal RNAs of thermophiles have also been compared with the same structures from mesophiles. Although results reported from different laboratories differ as to the T_m values, they all show that the structures from thermophiles are, in general, more thermostable (Friedman, 1971; Zeikus *et al.*, 1970; Oshima and Imahori, 1971; Varricchio and Marotta, 1976). This has also been shown for DNA and total RNA (Stenesh *et al.*, 1968).

A comparison has been made of the sequences in 16S ribosomal RNAs from the mesophiles, *B. subtilis* and *B. pumilus*, and from the thermophile, *B. stearothermophilus* (Woese *et al.*, 1976). The 16S rRNAs from the three bacilli were digested by T1 ribonuclease, and the catalogues of oligomers produced were compared. The results signify primary structural homology between the 16S rRNAs which contain highly conserved and important regions. However, there is a slight elevation of the GC content in the 16S rRNA from *B. stearothermophilus.* It is suggested that changes observed in the thermophile are largely in double-stranded regions. The structure of 16S rRNA has been discussed by Fellner (1974).

The structure of 5S rRNA has been reviewed by Monier (1974). The sequences in 5S rRNA from *B. stearothermophilus* have been elucidated by Marotta *et al.* (1976) and from *T. aquaticus* by Nazar and Matheson (1977). These sequences were compared with sequences in 5S rRNA from mesophilic sources which included human KB cells. The *T. aquaticus* 5S rRNA has the greatest sequence homology with the 5S rRNA from *B. stearothermophilus* (69%). But the homology with 5S rRNAs from *E. coli* (68%) and human KB cells (56%) is also great. The 5S rRNAs from the two thermophiles contain unique nucleotides compared with the 5S rRNAs from mesophiles. Of particular interest are ten residues common to the thermophilic 5S rRNAs. These residues may well be of importance in connection with the higher thermal stability of 5S rRNAs from the thermophiles and also for the stability of the intact ribosomes.

The 5S rRNA is an essential component of the ribosome as indicated by the finding that the 50S subunit, reconstituted but lacking 5S rRNA, has a greatly diminished activity (Erdmann *et al.*, 1971). Evidence has been presented indicating that the 5S rRNA functions in binding tRNA to ribosomes (Erdmann *et al.*, 1973). It, together with two 50S ribosomal proteins from *B. stearothermophilus*, B-L15 and B-L22, also catalyses

hydrolysis of GTP and ATP. Adenosine triphosphate acts as noncompetitive inhibitor of GTP (Horne and Erdmann, 1973).

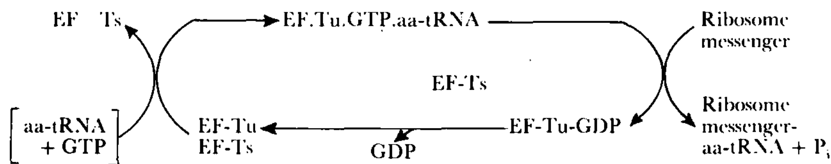
The protein fraction of the 50S ribosomal subunit from *B. stearothermophilus* has been separated into 27 components which were purified to homogeneity (Cohlberg and Nomura, 1976). The molecular weight of each was determined. The 50S ribosome was reconstituted with 5S rRNA, 23S rRNA and the 27 proteins to form active 50S subunits. The activities of reconstituted 50S subunits in the absence of single proteins were also examined. Different activities were obtained. The results demonstrate that the 50S ribosome has an almost absolute requirement for some of the proteins whereas, in the absence of other proteins, the ribosome is still functional but generally with decreased activity.

Purification and characterization of 23 30S ribosomal proteins from *B. stearothermophilus* have been carried out by Isono and Isono (1975). Their molecular weights and amino-acid compositions were determined. The *N*-terminal sequences of many of the 30S ribosomal proteins from *E. coli* and *B. stearothermophilus* have been compared (Yaguchi *et al.*, 1974). The results indicate that there is an essentially one-to-one structural correspondence between proteins from the two bacteria. This is, of course, in agreement with an earlier finding by Higo *et al.* (1973) who established a functional correspondence between 30S ribosomal proteins from *E. coli* and *B. stearothermophilus*.

Although the similarity between ribosomes from *E. coli* and *B. stearothermophilus* is now well established, the ribosomes show different specificities in recognizing mRNA. Bacteriophage R17 or f2 has three cistrons which direct synthesis of a maturation protein, a coat protein and a synthetase. The *E. coli* ribosomes are able to synthesize all three proteins. However, the *B. stearothermophilus* ribosomes are able to synthesize only the maturation protein (Lodish, 1969; Goldberg and Steitz, 1974).

To study the interaction between 30S and 50S ribosomal subunits from *B. stearothermophilus*, Miller *et al.* (1976) investigated the surface topography of the *B. stearothermophilus* ribosome using a procedure involving iodination of tyrosine residues. The results indicate that some of the tyrosine residues are less reactive in the intact ribosome than in the separated 30S and 50S subunits. A comparison was made, and similar results obtained, with the *E. coli* ribosome, and it is possible that some of the homologous proteins may be positioned at different loci in the ribosomes.

Elongation factors from *B. stearothermophilus*, EF-Tu and EF-Ts, have been studied by Beaud and Lengyel (1971) and Wittinghofer and Leberman (1976). The reactions in which elongation factors participate involve amino-acyl-tRNA, GTP and the ribosome messenger unit as follows:



EF-Tu and EF-Ts are proteins with molecular weights of 49,000 and 35,500, respectively. They have been purified to homogeneity, and EF-Tu has been crystallized (Wittinghofer and Leberman, 1976).

The polyamines, spermine and spermidine, are synthesized by *B. stearothermophilus* and are associated with ribosomes (Stevens and Morrison, 1968). Garciá-Patrone *et al.* (1975) found an association factor in *B. stearothermophilus* which contains both spermidine and spermine. Spermidine or the factor aids in association of the ribosomal subunits, especially at high temperatures and at low Mg^{2+} concentrations. Similarly, *T. thermophilus* contains a polyamine named "thermine" (1,11-diamino-4,8-diazaundecane, $NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2NHCH_2CH_2CH_2NH_2$; Oshima, 1975). Thermine and spermine increase the rate and saturation level of phenylalanine incorporation by ribosomes from *T. thermophilus* directed by polyuridylic acid at high temperatures and at low Mg^{2+} concentrations (Ohno-Iwashita *et al.*, 1975b, 1976). The ternary complex containing amino-acyl-tRNA, polyuridylic acid and a ribosome is formed when the components are incubated together at low temperature. It is capable of initiating polyphenylalanine synthesis at high temperatures. When the same components are added together and incubated at high temperature, a ternary complex is also formed but it is inactive. However, when thermine is present during the incubation at high temperatures (50–80°C), an active ternary complex is formed. The results indicate that thermine stimulates protein synthesis by inducing formation of an active ternary complex. It is quite possible that thermine is of significance in protein synthesis in *T. thermophilus*.

Protein and nucleic acid turnover rates have been determined with *B. stearothermophilus* with controversial results. Bubela and Holdsworth (1966) found a very rapid turnover of these macromolecules whereas

Epstein and Grossowicz (1969b), with their prototrophic strain, found a protein turnover and breakdown rate of 6.5% per hour which is similar to that of resting cells of *E. coli.* Coultate *et al.* (1975) re-examined the turnover using both the prototrophic strain as well as strain A.T.C.C. 12980 of *B. stearothermophilus.* They found turnover of protein during exponential growth in a minimum salts medium with glucose or succinate to be 4% per hour and, in a rich nutrient broth medium, 23% per hour. Turnover of non-mRNA was 1% per hour and 9% per hour in respective media. These turnover rates are modest and not as massive as found by Bubela and Holdsworth (1966). Coultate *et al.* (1975) confirmed their results by also studying the decay of specific enzymes in intact cells at thermophilic growth temperatures. It seems safe to conclude that turnover rates in thermophiles (at least *B. stearothermophilus*) are similar to those of mesophiles.

V. Metabolism and Enzymes of Thermophilic, Aerobic Micro-organisms

Interest in thermophilic micro-organisms has been, and still is, centered on the question—why are they able to grow at high temperatures?—whereas very few studies have been reported on their intermediary metabolism. However, many enzymes have now been purified from thermophiles, especially from *B. stearothermophilus.* In this section, an attempt will be made to summarize the enzyme studies in order to assess the metabolism of thermophiles. Figure 1 shows the enzymes of the glycolytic pathway and the pentose cycle, which have been detected in *B. stearothermophilus.* Similarly, Fig. 2 shows enzymes of the tricarboxylic acid and glyoxylate cycles and amino-acid metabolism studied in *B. stearothermophilus.* Table 4 lists those enzymes which have been obtained from other aerobic thermophiles.

A. GLYCOLYTIC AND PENTOSE-CYCLE ENZYMES

Bacillus stearothermophilus metabolizes sugars via the glycolytic pathway as shown in Fig. 1. Most enzymes (numbered in Fig. 1) on this pathway have now been isolated and many of their properties are known. The only enzyme which appears to have not been demonstrated is phosphoglycerate phosphomutase, (EC 5.4.2.1). The presence of pyruvate kinase (EC 2.7.1.40) has been shown by Amelunxen and Lins

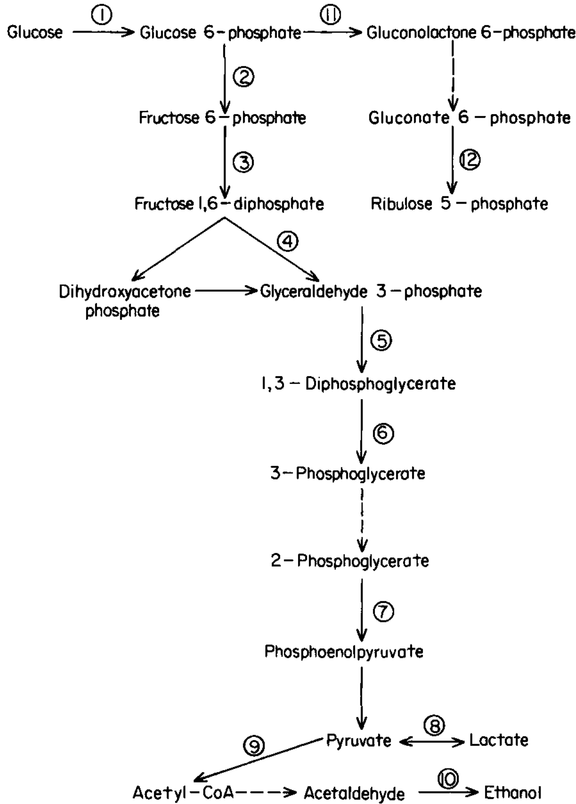


FIG. 1. Glycolytic enzymes in *Bacillus stearothermophilus*. Numbered reactions refer to enzymes whose properties have been studied. References are as follows: *1*. Glucokinase (EC 2.7.1.2); Haberstich and Zuber (1974), Hengartner and Zuber (1973). *2*. Glucosephosphate isomerase (EC 5.3.1.9); Haberstich and Zuber (1974), Muramatsu and Nosoh (1971). *3*. 6-Phosphofructokinase (EC 2.7.1.11); Comer *et al.* (1975), Hengartner *et al.* (1976). *4*. Fructose-diphosphate aldolase (EC 4.1.2.13); Hill *et al.* (1976), Howard and Becker (1972), Sugimoto and Nosoh (1971), Thompson *et al.* (1958), Thompson and Thompson (1962). *5*. Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12); Allen and Harris (1975, 1976), Amelunxen (1966, 1967, 1975), Amelunxen and Clark (1970), Amelunxen and Singleton (1976), Amelunxen *et al.* (1970, 1974), Biesecker *et al.* (1977), Bridgen *et al.* (1972), Comer *et al.* (1975), Haberstich and Zuber (1974), Jung *et al.* (1974), Sauvan *et al.* (1972), Singleton *et al.* (1969), Suzuki and Harris (1971), Suzuki and Imahori (1973). *6*. Phosphoglycerate kinase (EC 2.7.2.3); Suzuki and Imahori (1974). *7*. Enolase (EC 4.2.1.11); Boccù *et al.* (1976). *8*. Lactate dehydrogenase (EC 1.1.1.27); Frank *et al.* (1976). *9*. Pyruvate dehydrogenase complex, Militzer and Burns (1954). *10*. Alcohol dehydrogenase (EC 1.1.1.1); Comer *et al.* (1975), Jung *et al.* (1974). *11*. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49); Haberstich and Zuber (1974), Jung *et al.* (1974). *12*. Phosphogluconate dehydrogenase (decarboxylating; EC 1.1.1.44); Fontana *et al.* (1975), Pearse and Harris (1973), Veronese *et al.* (1974, 1975, 1976a, b).

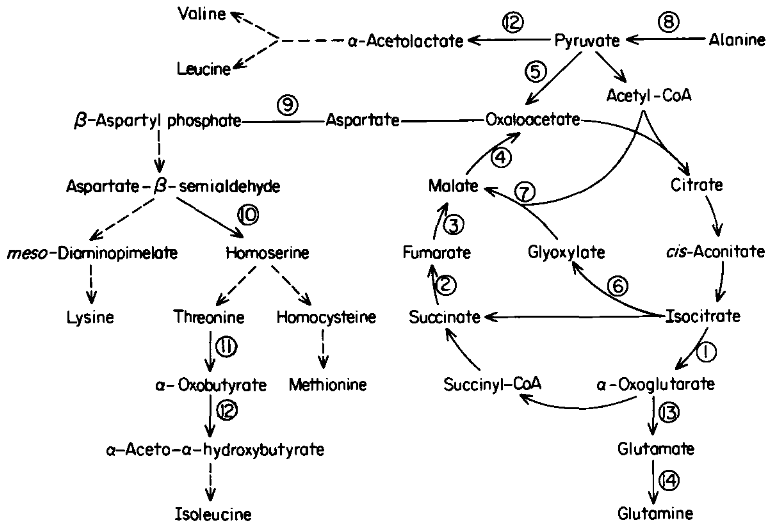


FIG. 2. Enzymes of the tricarboxylic acid cycle and amino-acid metabolism in *Bacillus stearothermophilus*. Numbered reactions refer to enzymes which have been studied in some detail. References are as follows: *1.* Isocitrate dehydrogenase ($NADP^+$; EC 1.1.1.42); Haberstich and Zuber (1974), Hibino *et al.* (1974), Howard and Becker (1970), Jung *et al.* (1974). *2.* Succinate dehydrogenase (EC 1.3.99.1); Jung *et al.* (1974). *3.* Fumarate hydratase (EC 4.2.1.2); Cook and Ramaley (1976), Della Valle *et al.* (1962). *4.* Malate dehydrogenase (EC 1.1.1.37); Marsh and Militzer (1952), Murphey *et al.* (1967). *5.* Pyruvate carboxylase (EC 6.4.1.1); Cazzulo *et al.* (1969, 1970a, b, 1971), Libor *et al.* (1975), Sundaram (1973), Sundaram *et al.* (1969, 1971, 1976). *6.* Isocitrate lyase (EC 4.1.3.1); Daron (1967), Griffiths and Sundaram (1973), Sundaram *et al.* (1976). *7.* Malate synthase (EC 4.1.3.2); Sundaram *et al.* (1976). *8.* Alanine dehydrogenase (EC 1.4.1.1); Epstein and Grossowicz (1976). *9.* Aspartate kinase (EC 2.7.2.4); Cavari *et al.* (1972), Kuramitsu (1968, 1970), Kuramitsu and Yoshimura (1971, 1972). *10.* Homoserine dehydrogenase (EC 1.1.1.3); Cavari and Grossowicz (1973). *11.* Threonine dehydratase (deaminase) (EC 4.2.1.16); Muramatsu and Nosoh (1976) Thomas and Kuramitsu (1971). *12.* Acetolactate synthase (EC 4.1.3.18); Chin and Trela (1973). *13.* Glutamate dehydrogenase (EC 1.4.1.4); Epstein and Grossowicz (1975). *14.* Glutamine synthetase (EC 6.3.1.2); Hachimori *et al.* (1974, 1975b), Matsunaga and Nosoh (1974), Wedler (1974), Wedler and Hoffmann (1974a, b), Wedler *et al.* (1976a, b).

(1968) and triosephosphate isomerase (EC 5.3.1.1) by Fahey *et al.* (1971). *Bacillus stearothermophilus* also appears to have the pentose cycle although only glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase have been subjects for investigations. The pentose cycle is, of course, needed for synthesis of pentoses and generation of NADPH. It may also function in metabolism of several pentoses which are used by *B. stearothermophilus* as carbon substrates. Lactate is used as a

TABLE 4. Enzymes of glycolysis, citrate and glyoxylate cycles, and amino-acid metabolism studied in members of the genus *Thermus* and some thermophilic bacilli

Enzyme	Bacterium	Reference
Phosphoglucomutase	*Thermus thermophilus*	Yoshizaki *et al.* (1971)
6-Phosphofructokinase	*Thermus thermophilus*	Yoshida (1972)
	Thermus X	Cass and Stellwagen (1975)
Hexose diphosphatase	*Thermus thermophilus*	Yoshida *et al.* (1973)
Fructose diphosphate aldolase	*Thermus aquaticus*	Freeze and Brock (1970)
Glyceraldehyde phosphate dehydrogenase	*Thermus aquaticus*	Hocking and Harris (1976)
	Thermus thermophilus	Fujita *et al.* (1976)
Enolase	*Thermus aquaticus*	Stellwagen *et al.* (1973)
	Thermus X	Barnes and Stellwagen (1973)
Lactate dehydrogenase	*Bacterium caldolyticus*	Weerkamp and MacElroy (1973)
	Bacterium caldotenax	Frank *et al.* (1976)
Ribose phosphate isomerase	*Bacterium caldolyticus*	Middaugh and MacElroy (1976)
Phosphoenolpyruvate carboxylase	*Thermus aquaticus*	Bridger and Sundaram (1976)
Isocitrate dehydrogenase ($NADP^+$)	*Thermus aquaticus*	Ramaley and Hudock (1973)
Fumarate hydratase	*Thermus* X-1	Cook and Ramaley (1976)
Malate dehydrogenase	*Thermus aquaticus*	Biffen and Williams (1976)
Aspartate kinase	*Thermus flavus*	Saiki and Arima (1970)
Homoserine dehydrogenase	*Thermus flavus*	Saiki *et al.* (1973)
Threonine dehydratase (deaminase)	*Thermus* X	Higa and Ramaley (1973)
Acetolactate synthase	*Thermus aquaticus*	Chin and Trela (1973)

carbon source, at least by some strains of *B. stearothermophilus*. This requires the presence of lactate dehydrogenase. This enzyme and alcohol dehydrogenase may be of importance during anaerobic growth for regeneration of oxidized nicotinamide adenine dinucleotide needed in the fermentation of sugars. From the list of enzymes in Table 4, it is evident that thermophiles of the genus *Thermus* (like *B. stearothermophilus*) use the glycolytic pathway to metabolize sugars.

Phosphoglucomutase (EC 2.7.5.1) has been purified from *T. thermophilus* (formerly *F. thermophilum*) by Yoshizaki *et al* (1971). It has a temperature optimum of 75°C but is only slowly denatured at temperatures below 95°C. The pH optimum is between 8.5 and 9.0, and glucose 1,6-diphosphate and Mg^{2+} are required for activity. A sulphydryl compound, such as dithiothreitol, is stimulatory. Sodium ethylene diaminetetra-acetate and *trans*-1,2-diamino-cyclohexanetetra-acetate are strong inhibitors. Kinetic data indicate a "ping-pong" mechanism.

Glucokinase has been purified from *B. stearothermophilus* N.C.L.B. 8924 (Hengartner and Zuber, 1973). The pure enzyme, which is stable at 65°C, has a specific activity of 304 μmoles $min^{-1}mg^{-1}$ at 37°C. It has a molecular weight of 67,000 and consists of two, most likely identical, subunits. The enzyme phosphorylates glucose and *N*-acetylglucosamine but not D-mannose, D-galactose, D-fructose, 2-deoxy-D-glucose, glucosamine hydrochloride or D-xylose. The specificity for the nucleotide triphosphate is broad, and ATP, ITP, GTP, UTP and CTP serve as substrates. However, ATP with a K_m value of 0.06 mM, is the best. The K_m value for glucose is 0.25 mM. Haberstich and Zuber (1974) have shown that glucokinase in *B. stearothermophilus* grown at 55°C has a higher thermostability than in cells grown at 37°C. This phenomenon will be discussed in the section on thermo-adaptation (p. 224).

Glucose phosphate isomerase has been purified to homogeneity by Muramatsu and Nosoh (1971) from strain N.C.A. 2184 of *B. stearothermophilus* grown at 65°C. The molecular weight is 172,000. The enzyme is stable at 50°C and, at 65°C, it is stabilized by glucose 6-phosphate or 6-phosphogluconate. The enzyme may undergo a structural change at 55°C. The K_m value for glucose 6-phosphate at 65°C is 3.68×10^{-4} M, the V_{max} value is 4.64 μmoles $min^{-1}mg^{-1}$, and the optimum pH value is between 7 and 8. With glucose 6-phosphate as substrate, inorganic phosphate and 6-phosphogluconate behave as competitive inhibitors. Cells of *B. stearothermophilus* N.C.L.B. 8924 grown

at 37°C or 55°C have the same level of glucose 6-phosphate isomerase (0.24 units mg^{-1} in extracts). However, the enzyme is more thermostable in cells grown at 55°C than at 37°C (Haberstich and Zuber, 1974).

6-Phosphofructokinase has been obtained in crystallized form from *B. stearothermophilus* N.C.A 1503 by Hengartner *et al.* (1976). It has a molecular weight of 130,000 and consists of four identical subunits. The amino-acid sequence has been determined. The kinetic and regulatory properties of the enzyme have not yet been reported. Comer *et al.* (1975) have purified the enzyme using affinity chromatography, which was the method also used by Hengartner *et al.* (1976).

Phosphofructokinase from *T. thermophilus* is an allosteric enzyme (Yoshida *et al.*, 1971; Yoshida, 1972) as is the enzyme in *Thermus* X-1 (Cass and Stellwagen, 1975). With both enzymes, phosphoenolpyruvate functions as a negative effector and ADP as a positive effector. The allosteric properties are present over the temperature range of 20–80°C in *Thermus* X-1 and 30–75°C in *T. thermophilus*. The *Thermus* X-1 enzyme has been purified to homogeneity. It consists of four subunits and has a molecular weight of 132,000. The amino-acid composition is similar to that of the enzymes from *E. coli* and *C. pasteurianum*. Phosphofructokinase has also been purified from *T. aquaticus* (Hengartner *et al.*, 1976). Like the other phosphofructokinases, it has a molecular weight around 130,000 and consists of four subunits. It has a very high thermal stability and retains full activity after 24 hours at 80°C.

Hexose diphosphatase (EC 3.1.3.11) is present in *T. thermophilus* (Yoshida and Oshima, 1971; Yoshida *et al.*, 1973). The enzyme specifically hydrolyses the C-1 phosphate-ester bond of fructose 1,6-diphosphate and there is no activity with other sugar phosphates. Magnesium, manganese or zinc satisfies the metal requirement. The enzyme is allosteric with phosphoenolpyruvate as an activator and AMP as an inhibitor. The AMP inhibition is reversed to some extent by phosphoenolpyruvate. The enzyme is stable up to 80°C. Activation by phosphoenolpyruvate is most pronounced at 65°C. The presence of allosteric phosphofructokinase and hexose diphosphatase in *T. thermophilus* indicates that both glycolysis and gluconeogenesis are regulated in this bacterium, as has been proposed by Yoshida (1972).

Fructose diphosphate aldolases are of two classes. Class I, typified by rabbit muscle aldolase, appears to have a lysine residue in the active site. Class II, typified by the yeast enzyme, is a metallo-enzyme, and it has

been suggested that the metal functions at the active site. Thompson and Thompson (1962) reported that the aldolase in *B. stearothermophilus* does not require a metal for activity, and that it is not inhibited by the chelating reagents 8-hydroxyquinoline and 2,2′-dipyridyl. However, Sugimoto and Nosoh (1971) obtained homogenous preparations of the enzyme, and found that it contains two atoms of Zn and that the activity is strongly inhibited by EDTA. Later, Hill *et al.* (1976) prepared the apo-enzyme, which is activated by either Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} or Cd^{2+} but not by Ni^{2+}, Mg^{2+} or Cu^{2+}. The cobalt and manganese enzymes are stable for three hours at 64°C and pH 7.5 whereas the zinc and the cadmium enzymes are less stable. The aldolase from *B. stearothermophilus* is clearly of the Class II type. Howard and Becker (1972) discovered that the enzyme is inactivated at 60°C in the presence of the substrates in phosphate, triethanolamine or TES buffers, but not in tris or glycylglycine buffers. Ethylenediaminetetra-acetate, dithiothreitol and manganese ions prevent inactivation, which may involve a conformational change. A conformational change is also suggested from plots of log (1/Km) and log V_{max} against 1/T, which reveal broken lines with the transition temperature at 50°C (Sugimoto and Nosoh, 1971; Hill *et al.*, 1976). The aldolase has a molecular weight of 60,600 and consists of two subunits. It seems to have an ordered mechanism with dihydroxy-acetone phosphate as the last ligand to leave (Hill *et al.*, 1976).

Aldolase from *T. aquaticus* also is of Class II (Freeze and Brock, 1970). However, it differs from the aldolase of *B. stearothermophilus* in that it exists as a tetramer (molecular weight about 140,000), as well as a dimer. It also has a significantly higher thermal stability and can be heated for two hours at 97°C. Furthermore, it has almost no activity below 60°C. Cysteine, which strongly activates the enzyme, also causes its labilization. Similarly, as with the *B. stearothermophilus* enzyme, substrates labilize the *T. aquaticus* enzyme. Manganese, nickel, zinc, iron and cobalt activate the enzyme, which is strongly inhibited by EDTA.

Glyceraldehyde phosphate dehydrogenase is one of the most investigated enzymes (see reviews by Harris and Waters, 1976, and Amelunxen and Murdock, 1977). The enzyme from all known sources is a tetramer with a molecular weight of 145,000, which binds four moles of NAD^+. The NAD^+ binding is co-operative, and the enzyme has been the subject for postulating models of allosteric action (Monod *et al.*, 1965; Koshland *et al.*, 1966). The enzyme from *B. stearothermophilus* has been

extensively researched in the laboratories of Amelunxen and Harris. It was the first intracellular enzyme from a thermophile to be crystallized (Amelunxen, 1966).

Glyceraldehyde 3-phosphate dehydrogenases from *B. stearothermophilus* and lobster muscles have been compared. Recently, the complete sequence of the 333 amino-acid residues of the enzyme from the thermophile was reported together with the structure as determined from an electron-density map of 2.7 Å resolution (Biesecker *et al.*, 1977). The structure of the lobster enzyme had been previously elucidated (Buehner *et al.*, 1973, 1974; Moras *et al.*, 1975). This is the first time that it has been possible to compare the sequences and structures of two, similar, multisubunit enzymes from both a thermophile and a mesophile (Biesecker *et al.*, 1977). The complete sequence of amino-acid residues in the enzyme from the thermophile is highly homologous with that of the lobster enzyme. The overall structure of the subunits, the secondary and tertiary features, and the NAD^+-binding region are similar in the two enzymes. However, it is difficult to identify directly the source of the higher thermostability of the enzyme from *B. stearothermophilus*, but several possibilities are recognized. Thus, three salt bridges made by each subunit to the others may contribute to the thermostability of the tetramer. A preliminary report about the coenzyme-binding site and co-operativity in glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* has been published by Biesecker and Wonacott (1977).

The amino-acid sequence of glyceraldehyde 3-phosphate dehydrogenase from *T. aquaticus* has been reported (Hocking and Harris, 1976). The enzyme was purified using NAD-Sepharose affinity chromatography (Hocking and Harris, 1973). It is stable at 90°C and remarkably stable in denaturing solvents like urea, guanidine hydrochloride and sodium dodecyl sulphate. Other properties are very similar to those of glyceraldehyde 3-phosphate dehydrogenases from other sources. The enzyme has also been purified from *T. thermophilus* (Fujita *et al.*, 1976). The *T. thermophilus* enzyme is very similar to the *T. aquaticus* enzyme, including its stability to heat and denaturing agents. It also exists as a tetramer, but its molecular weight is slightly lower (133,000) than other glyceraldehyde 3-phosphate dehydrogenases. The *T. thermophilus* enzyme is activated by ammonium ions and some alcohols.

Phosphoglycerate kinase has been purified to homogeneity from *B. stearothermophilus* N.C.A. 1503 by Suzuki and Imahori (1974). It consists of a single peptide chain with a molecular weight of 42,000. Thus, it

differs from other glycolytic enzymes which have two or more subunits. The pure enzyme, assayed at pH 7.5 and 25°C, has a specific activity of 560 units mg^{-1}, which is lower than that for mesophilic sources (650–1000 units mg^{-1}). However, the Q_{10} value for the enzyme is about 1.5 and, at 60°C (the physiological temperature), it should have an activity close to 2000 units mg^{-1}. The enzyme is specific for D-3-phosphoglycerate. The best phosphate donor is ATP, but GTP and ITP also serve as substrates. Purine nucleotide diphosphates are strong inhibitors whereas nicotinamide nucleotides are less so. The enzyme requires Mg^{2+}, which can be replaced by Mn^{2+}, for activity. The enzyme is fully active at pH 5.5–8.5. The K_m value for ATP is 2.9 mM and, for D-3-phosphoglycerate, 2.2 mM (both determined at 25° C). The amino-acid composition has been determined. The most striking observation is that the enzyme contains only one cysteine residue, which seems to be buried in the interior of the enzyme. Phosphoglycerate kinases from animal sources have high contents of cysteine (8–11 residues), and Suzuki and Imahori (1974) have suggested that two classes of the enzyme exist, Class I being the mammalian type and having a high sulphydryl content, and Class II representing the yeast or the bacterial type with a single sulphydryl group.

Enolase has recently been purified from *B. stearothermophilus* N.C.A. 1503 by Boccù *et al.* (1976). It has a molecular weight of 376,000 and consists of eight apparently identical subunits. In this respect, it resembles the enzymes from *T. aquaticus* and *Thermus* X-1, which also are octamers (Stellwagen *et al.*, 1973; Barnes and Stellwagen, 1973). Enolases from mesophiles are dimers with molecular weights of about 88,000. Such differences between homologous enzymes from mesophiles and thermophiles are rare and seem to indicate a specific thermophilic property. However, the properties of the subunits of enolases from different sources are very similar, as are their kinetic properties. Therefore, it has been suggested that enolases from different sources are closely related (Stellwagen and Barnes, 1976). Purified enolase from *B. stearothermophilus* has a specific activity of 130 (μmoles $min^{-1}mg^{-1}$) at 23°C. The pH optimum is 7.8, and the optimum concentration of Mg^{2+} is 1mM. The K_m value for 2-phosphoglycerate is 0.1 mM. The enzyme is moderately thermostable; however, it is stabilized by magnesium ions. The enzymes from *Thermus* X-1 and *T. aquaticus* have temperature optima at 70°C and 89°C, respectively (Stellwagen and Barnes, 1976).

Pyruvate kinase and the pyruvate dehydrogenase enzyme complex

have not been studied in detail. Militzer and Burns (1954) reported that the pyruvate dehydrogenase enzyme complex is rapidly inactivated at 60°C *in vitro* but not *in vivo*. However, *in vivo*, it is stabilized by substrates.

Lactate dehydrogenase has been purified from *B. stearothermophilus* grown at both 37°C and 55°C and from *B. caldotenax* grown at 37°C and 77°C (Frank *et al.*, 1976). The enzymes from cells grown at 37°C have much lower thermostabilities than those from cells grown at 55°C and 77°C, respectively. It is evident that the two bacilli are producing lactate dehydrogenases with different thermostabilities when grown at different temperatures. The mesophilic enzyme from *B. stearothermophilus* and the thermophilic enzymes from both bacilli were compared with regard to physical properties, amino-acid composition and partial amino-acid sequence. All of the enzymes are tetramers and have molecular weights of about 140,000. The amino-acid compositions are very similar. Comparison of the *N*-terminal sequences reveals a high homology between the enzymes. However, some differences occur, most of which can be explained by single-base exchanges. Whether these changes affect the thermostability of the enzymes is hard to evaluate. This work is still in progress (Schär *et al.*, 1976), as are X-ray crystallography studies (Jansonius *et al.*, 1976). When completed, this work will allow a comparison to be made of a mesophilic and a thermophilic enzyme isolated from the same bacterium. It should be of great interest from the standpoint of protein structure, as well as of thermo-adaption.

Lactate dehydrogenase from *B. caldolyticus* appears to be different when compared with the enzymes from *B. stearothermophilus* and *B. caldotenax*. Weerkamp and MacElroy (1972) found a molecular weight of 70,000 for the *B. caldolyticus* enzyme which is only half of the molecular weight of lactate dehydrogenases from the other bacilli. The *B. caldolyticus* enzyme showed sigmoidal kinetics at temperatures above 50°C which may indicate co-operative properties at physiological growth temperatures. Phosphoenolpyruvate and ADP are negative effectors. Interestingly, the enzyme from cells grown in minimal medium has less thermal stability than that from cells grown in the same medium fortified with brain-heart infusion.

Alcohol dehydrogenase has been obtained from *B. stearothermophilus* (Bridgen *et al.*, 1975; Comer *et al.*, 1975). The level of this enzyme is high in cells grown at 55°C whereas it is absent from cells grown at 37°C (Jung *et al.*, 1974). This is in agreement with *B. stearothermophilus*' production of alcohol during anaerobic growth at 55°C or above, but not at lower

temperatures. Alcohol production from carbohydrates using this organism has been considered (Atkinson *et al.*, 1975).

Phosphogluconate dehydrogenase appears to be the only enzyme of the pentose cycle which has been purified from *B. stearothermophilus* (Pearse and Harris, 1973; Veronese *et al.*, 1974). The enzyme has a specific activity of 20 units mg^{-1} at 25°C (Pearse and Harris, 1973) and 87 units mg^{-1} at 43°C (Veronese *et al.*, 1974). It was crystallised in the presence of $NADP^+$ by Pearse and Harris (1973). Both groups report a molecular weight of about 100,000 and a two-subunit structure. The enzyme is stable at 60°C. Veronese *et al.* (1974) found a pH optimum at about 8 and a K_m value at 43°C of 25×10^{-5} M for $NADP^+$ and 20×10^{-5} M for 6-phosphogluconate. The enzyme exhibits a broken Arrhenius plot with the transition temperature at 50°C. In a series of papers, Veronese *et al.* (1975, 1976a, b) and Fontana *et al.* (1975) compared the structures of phosphogluconate dehydrogenase from *B. stearothermophilus* and *E. coli*. The two enzymes are very similar with regard to physical parameters, amino-acid composition, kinetic properties and in reactions with inhibitors. However, the enzyme from *B. stearothermophilus* has a higher thermal stability and is more stable toward denaturing agents like urea. The conclusion is that the mesophilic and the thermophilic enzymes are very similar, and that the difference in thermal stabilities of the enzymes depends on a small difference in structure.

Ribose phosphate isomerase (EC 5.3.1.6) has been studied by Middaugh and MacElroy (1976). They purified the enzyme from the mesophile, *Thiobacillus thioparus*, and the thermophile, *B. caldolyticus*. The enzymes have very similar properties except that the *B. caldolyticus* enzyme is more thermostable. The molecular weight is about 40,000. At equilibrium, the ratio of ribulose 5-phosphate to ribose 5-phosphate is 0.28 at 45°C. Both enzymes have increased thermal stabilities in the presence of $(NH_4)_2SO_4$, NaCl, KCl and LiCl, but lowered thermal stabilities in LiBr, $CaCl_2$ and some alcohols. Plots of log K_m and log V_{max} against 1/T show sharp changes in the slopes which indicates temperature-dependent alterations of the catalytic properties of the enzymes.

B. ENZYMES OF THE TRICARBOXYLIC ACID-CYCLE AND AMINO-ACID METABOLISM

Strains of *B. stearothermophilus* are able to use acetate, lactate, fumarate, succinate, alanine, aspartate and glutamate as sources of carbon. These

substrates are metabolized in the tricarboxylic acid cycle, and the ability to grow on acetate requires operation of the glyoxylate cycle. Glucose is also oxidized in the tricarboxylic acid cycle which is indicated by formation of $^{14}CO_2$ from [6-^{14}C]glucose (Coultate and Sundaram, 1975). These investigators also obtained growth yields ($Y_{glucose}$) of 55 to 83 grams of dry cells per mole of glucose. Similar growth yields have also been obtained for *B. stearothermophilus* by Pozmogova and Mal'yan (1976). Such high molar growth yields are indicative of oxidation of glucose carbons in the tricarboxylic acid cycle concomitant with electron-transport phosphorylation. A strict fermentation would yield much lower $Y_{glucose}$ values (Payne, 1970; Stouthamer, 1973). Both Coultate and Sundaram (1975) and Pozmogova and Mal'yan (1976) observed that, as the temperature increased, the value of $Y_{glucose}$ decreased. This suggests a less efficient use of glucose at higher temperatures. Coultate and Sundaram (1975) found more acetate formation at higher temperatures which indicates incomplete utilization of glucose, but they also suggested uncoupling of energy production from respiration as a possible explanation. They eliminated the lack of oxygen since they found that the respiratory system has a very high affinity for oxygen.

All enzymes of the tricarboxylic acid cycle have been demonstrated in *B. stearothermophilus*; however, the levels of the enzymes are apparently low (Rowe *et al.*, 1973, 1976). Perhaps, this is the reason why only a few of the enzymes have been studied in detail. Rowe *et al.* (1973, 1976) obtained four mutants of *B. stearothermophilus* 1503 which lack ability to synthesize fumarase, aconitase and alcohol dehydrogenase. Under conditions of efficient spore formation by the wild type, these mutants are either unable or have difficulty in forming spores. This suggests an important role for the tricarboxylic acid cycle in sporulation in *B. stearothermophilus*.

An outline of the citric acid cycle and amino-acid metabolism in *B. stearothermophilus* is given in Fig. 2 (p. 193). The reactions which are numbered are catalysed by enzymes which have been studied in some detail. They will be considered here. Table 4 (p. 194) shows the enzymes which have been isolated from *Thermus* species.

1. *Tricarboxylic Acid Cycle*

Isocitrate dehydrogenase ($NADP^+$) is present in *B. stearothermophilus*

grown at 37°C and 55°C. The thermostability of the enzyme in the cells grown at 55°C is greater than in the cells grown at 37°C (Jung *et al.*, 1974; Haberstich and Zuber, 1974). Pure isocitrate dehydrogenase has been obtained from *B. stearothermophilus* N.C.A. 2184 (Howard and Becker, 1970; Hibino *et al.*, 1974). The molecular weight is about 92,500 and the enzyme consists of two subunits. The K_m values for $NADP^+$ and isocitrate at pH 7.5 at 50°C are 4.5 μM and 6 μM, respectively. The enzyme is specific for $NADP^+$, and NAD^+ is completely inactive. The approximately 1000-fold purified enzyme has a specific activity of 38 units mg^{-1} at 35°C and about 750 units mg^{-1} at 66°C. The enzyme loses 30% of its activity when stored at 63°C for three hours. Above 65°C, it is rapidly inactivated. The enzyme requires Mg^{2+} or Mn^{2+} for activity and the combination of Mg^{2+} together with isocitrate protects the enzyme from thermal inactivation.

Thermus aquaticus (Ramaley and Hudock, 1973) and *T. flavus* (Saiki and Arima, 1975; Saiki *et al.*, 1976) contain isocitrate dehydrogenase ($NADP^+$). Both enzymes from the *Thermus* species have very high thermostabilities, and are inhibited by glyoxylate and oxaloacetate in a concerted manner. The *T. flavus* enzyme seems to differ from that from *T. aquaticus* in that the inhibitors are less efficient above 60°C whereas the enzyme from the latter is still inhibited at 70°C. The *T. flavus* enzyme also gives a broken Arrhenius plot with the transition at 60°C. This behaviour is not manifested by the *T. aquaticus* enzyme. The inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate is also seen in other bacteria and indicates a regulatory mechanism between the citrate and the glyoxylate cycles.

Succinate dehydrogenase was demonstrated in *B. stearothermophilus* by Amelunxen and Lins (1968) who found it to be stable for 10 min at temperatures below 80°C. Jung *et al.* (1974) assayed the enzyme in extracts from cells grown at 37°C or 55°C. They found that the level of the enzyme in cells grown at 37°C is higher (about 3.5 times) than in cells grown at 55°C. This is in agreement with the observation that *B. stearothermophilus* prefers to grow aerobically at 37°C while, at higher temperatures, it grows equally well aerobically and anaerobically.

Fumarate hydrolase in *B. stearothermophilus* was studied by Della Valle *et al.* (1962) and was purified to homogeneity by Cook and Ramaley (1976). The enzyme has a specific activity of about 46.5 units mg^{-1}. The molecular weight, as determined by gel filtration, is 180,000. The T_{opt} value is about 81°C at pH 7.6. The enzyme is active between pH 7 and 10

with an optimum around 8. Cook and Ramaley (1976) also purified the enzyme from *Thermus* X-1, and this enzyme has properties similar to that of the enzyme from *B. stearothermophilus*. They compared these enzymes with fumarate hydrolases from mesophilic organisms and concluded that, with the exception of thermostability, the properties of all fumarases are similar.

Malate dehydrogenase in *B. stearothermophilus* was found by Marsh and Militzer (1952) to have high thermostability. About 6000-fold purification of the enzyme to a specific activity of 210 units mg^{-1} at 25°C at pH 7.5 was achieved by Murphey *et al.* (1967). The molecular weight is about 115,000, and it consists of four probably identical subunits. Murphey *et al.* (1967) also purified the enzyme from *E. coli* and *B. subtilis*. The molecular weights are 61,000 and 117,000, respectively. The *E. coli* enzyme consists of two subunits whereas the *B. subtilis* enzyme has four. Calculations of the molecular size of the subunits and similarities in chemical properties suggest that the tetrameric enzymes are related to the dimeric form. The *B. stearothermophilus* enzyme has a much higher thermal stability than the enzymes from other sources, and it is stable at 70°C for about 20 min. A comparison of specific activities reveals that, at 25°C, the enzyme from *B. stearothermophilus* is only one-tenth as active as those from mesophilic bacteria. However, at the physiological working temperature (about 55°C), its activity is similar to that of the other enzymes.

Malate dehydrogenase has been purified to homogeneity from *T. aquaticus* (Biffen and Williams, 1976). The pure enzyme has a specific activity of 690 (μmoles $min^{-1}mg^{-1}$) at 45°C and pH 11. Its molecular weight is about 73,000, which differs from that of the enzyme from *B. stearothermophilus* but is close to that for the enzyme from *E. coli*. The enzyme is stable at 70°C and has high stability toward denaturing agents such as guanidine.

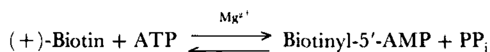
2. *Conversion from C_3 Compounds to Dicarboxylic Acids*

Bacillus stearothermophilus, growing on glucose, replenishes intermediates of the tricarboxylic acid cycle by fixation of carbon dioxide with pyruvate as the acceptor in a reaction catalysed by pyruvate carboxylase (Sundaram *et al.*, 1969). The enzyme, which is inhibited by avidin, requires acetyl-CoA and Mg^{2+} or Mn^{2+} for activity. Biotin, when added to the growth medium, sharply enhances activity. The enzyme in the prototrophic strain of *B. stearothermophilus* isolated by Epstein and

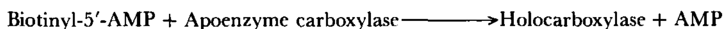
Grossowicz (1969a) has been studied extensively. Cazzulo *et al.* (1969) purified the enzyme 409-fold to a specific activity of 31.5 units mg^{-1} at 30°C from cells grown on acetate with 0.1 mg of biotin ml^{-1}. Libor *et al.* (1975) published an improved method of purification yielding 40 units mg^{-1}.

The purified enzyme, although more stable than corresponding enzymes from mesophiles, is not particularly thermostable when compared with other enzymes from thermophiles. It loses about half of its activity after 40 min at 50°C in tris-HCl buffer (pH 7.6). However, it is protected against thermal inactivation by acetyl-CoA and L-aspartate which are both allosteric effectors of the enzyme. This action is synergistic. The enzyme has an almost absolute requirement for acetyl-CoA, action of which is antagonized by L-aspartate. A strong inhibitor is ADP, and this inhibition is competitive with respect to ATP. This inhibition may be of physiological significance (Cazzulo *et al.* (1906).

Bacillus stearothermophilus (prototrophic strain), when grown in biotin-deficient medium, produces apopyruvate carboxylase. Cell-free extracts, in the presence of biotin, convert apo-enzyme to holo-enzyme in a reaction completely dependent on acetyl-CoA, and which is inhibited by L-aspartate (Cazzulo *et al.*, 1969). Further studies revealed the presence of a holopyruvate carboxylase synthetase in the extracts (Cazzulo *et al.*, 1970a, 1971; Sundaram *et al.*, 1971). This enzyme catalyses the following reactions:



$$(+)\text{-Biotin} + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{Biotinyl-5'-AMP} + \text{PP}_i$$



$$\text{Biotinyl-5'-AMP} + \text{Apoenzyme carboxylase} \longrightarrow \text{Holocarboxylase} + \text{AMP}$$

Both the apopyruvate carboxylase and the holo-enzyme synthetase have been partially purified. Requirements for the reactions are ATP (K_m value, 3.3×10^{-7} M), (+)-biotin (K_m value, 7.5×10^{-8} M. Mg^{2+} and acetyl-CoA. The last compound acts as an allosteric effector where action is counteracted by aspartate. Both effectors seem to act on the apopyruvate carboxylase, apparently by modifying it as an acceptor of the biotinyl moiety. Thus, acetyl-CoA and aspartate serve a dual function in pyruvate carboxylase in *B. stearothermophilus*. They regulate both synthesis of the holo-enzyme from apo-enzyme and the catalytic properties of the holopyruvate carboxylase itself. This dual role appears to be unique for *B. stearothermophilus* since an effect of acetyl-CoA on synthesis of the holo-enzyme in mesophilic systems has not been observed.

Otherwise similar results to those referred to have also been found in mesophilic micro-organisms.

The physiological role of pyruvate carboxylase in *B. stearothermophilus* has been studied by Sundaram (1973) and Sundaram *et al.* (1976) using a mutant (PC2) of the prototrophic strain. This mutant lacks pyruvate carboxylase. The wild type is able to grow on acetate almost as well in the absence, as in the presence, of biotin. However, on lactate, growth is very sluggish in the absence of biotin. The mutant (PC2) is incapable of growing on both lactate and glucose; however, it does grow on succinate and acetate. With acetate, but not with succinate, growth of the mutant is inhibited by glucose or lactate. This appears to be due to a depressed isocitrate lyase, which was shown by isolation of a secondary mutant (PC2 NG35) of mutant PC2. This new mutant still lacks pyruvate carboxylase, but is able to grow on acetate. The results clearly demonstrated the importance of pyruvate carboxylase as an anaplerotic enzyme in *B. stearothermophilus.*

Thermus aquaticus differs from *B. stearothermophilus* in that it uses phosphoenolpyruvate carboxylase (EC 6.4.1.1) instead of pyruvate carboxylase to convert C_3 metabolites into oxaloacetate (Bridger and Sundaram, 1976). The enzyme requires Mg^{2+} and acetyl-CoA for activity. That the latter activates the enzyme indicates a metabolic control mechanism which has not yet been elucidated.

3. *Glyoxylate Cycle*

Isocitrate lyase and malate synthase are enzymes of the glyoxylate cycle which is important in bacteria that utilize acetate as a carbon source. Daron (1967) isolated a bacterium which is similar to *B. stearothermophilus* in most respects except that it is not capable of hydrolysing starch. It grows on glucose or acetate. In extracts from glucose-grown cells, the isocitrate lyase activity is 0.0013 units ml^{-1} but, in acetate-grown cells, 7.52 units ml^{-1}, indicating that the enzyme is inducible.

Sundaram *et al.* (1976) purified isocitrate lyase from a prototrophic strain of *B. stearothermophilus* (Epstein and Grossowicz, 1969a) and from a mutant (PC2 NG35) which is devoid of pyruvate carboxylase and depressed for isocitrate lyase (Sundaram, 1973). Pure enzymes from the wild type and the mutant have the same properties and the same activity of 2.9 units mg^{-1}. The enzyme has a molecular weight of about 180,000 and consists of four subunits. The optimum pH value is 8 and the K_m

value for isocitrate at pH 6.8 is 0.021 mM. The enzyme requires Mg^{2+} for activity which is increased about three-fold by 0.4 M NaCl or KCl (Griffiths and Sundaram, 1973). Salts significantly increase the thermostability of the enzyme.

Malate synthase has been isolated by Sundaram *et al.* (1976) from the same strain of *B. stearothermophilus* as they used for isolating isocitrate lyase. Malate synthase was purified to an activity of about 25 units mg^{-1} as assayed at 30°C at pH 8. The enzyme appears to be a monomer with a molecular weight of about 60,000. It has high thermostability and loses only 5% of its activity in one hour at 60°C. The pH optimum is 8.6 and the K_m values at pH 8 are 0.088 mM for glyoxylate and 0.008 mM for acetyl-CoA.

4. *Amino-Acid Metabolism*

Alanine dehydrogenase has been partially purified from *B. stearothermophilus* (Epstein and Grossowicz, 1976). At 55°C, reductive amination of pyruvate is maximal at pH 8.0–8.4 whereas oxidative deamination of L-alanine is optimal at pH 9.8–10.2. The enzyme is stable for three hours at 70°C but is rapidly inactivated at 80°C. It is specific for NAD^+ whereas, with $NADP^+$, there is no activity. The K_m values (M) at 55°C are as follows: NH_4^+, 4.0 $\times 10^{-2}$; pyruvate, 5.0 $\times$ 10^{-4}; NADH, 6 $\times 10^{-5}$ Lalanine, 3.1 $\times$ 10^{-3}; and NAD^+, 2.0 $\times$ 10^{-4}. The V_{max} value for reductive amination of pyruvate is ten times higher than for deamination. Synthesis of alanine dehydrogenase is under metabolic control. Cells grown on glucose or succinate almost lack activity, whereas cells grown on pyruvate have a detectable activity. L-Alanine, D-alanine and L-serine induce synthesis of the enzyme when added to cultures growing on succinate but not on glucose in the presence of ammonia. However, synthesis of the enzyme is induced in cells growing on glucose when ammonia is excluded from the medium. The role of alanine dehydrogenase in metabolism of *B. stearothermophilus* was considered, and Epstein and Grossowicz (1976) suggested that it normally serves a catabolic function (formation of pyruvate). However, under certain conditions as when cells are growing on pyruvate, the enzyme may have an anabolic role.

Aspartate in *B. stearothermophilus* is formed from oxaloacetate which is removed from the tricarboxylic-acid cycle. The enzyme involved is glutamate oxaloacetate transaminase which is present in this bacterium

(Amelunxen and Lins, 1968). Aspartic acid is the precursor of threonine and methionine which are synthesized via homoserine, and of dipicolinate and lysine which are formed via diaminopimelate (Fig. 2, p. 193). The first reaction common to the two pathways is catalysed by aspartate kinase, which converts aspartate to β-aspartyl phosphate. This enzyme is under allosteric control by the end products of the two pathways. Furthermore, different types of aspartate kinase have been found in the same organisms, each regulated by a single amino-acid end product (see review by Umbarger, 1969). *Bacillus stearothermophilus* 1503-4R contains two different aspartate kinases; one is regulated by threonine and lysine (Kuramitsu, 1968, 1970) and the second by diaminopimelate (Kuramitsu and Yoshimura, 1971, 1972). The diaminopimelate-sensitive enzyme is elevated during sporulation, which appears logical since both dipicolinate and diaminopimelate are important in bacterial spore formation. The two aspartate kinases have been partially purified and separated from each other. The properties of the threonine-lysine-sensitive enzyme have been investigated by Kuramitsu (1970) and those of the diaminopimelate-sensitive enzyme by Kuramitsu and Yoshimura (1971, 1972).

Threonine-lysine-sensitive aspartate kinase has a molecular weight of about 110,000. It has a pH optimum of 8.0 and requires both Mg^{2+} and a monovalent ion for maximum activity. At 70°C, it is completely inactivated after 5 min; however, in the presence of both lysine (0.5 mM) and threonine (2.5 mM), the enzyme is completely stable during this treatment. Lysine and threonine act singly as feedback inhibitors and, with both present, the action is concerted. The enzyme does not show a homotropic interaction with regard to substrates, but homotropic interaction is observed with respect to threonine. The sensitivity of the enzyme to feedback inhibition decreased with increasing temperature; but, even at 55°C, the sensitivity of the converted feedback inhibition is comparable with what is found at lower temperatures for mesophilic bacteria.

The *meso*-diaminopimelate-sensitive aspartate kinase (DAP-aspartate kinase) is of particular importance during sporulation. The level of the enzyme is increased when sporangia become visible and when dipicolinate appears in the developing spores. Addition of glucose, chloramphenicol and inhibitors of RNA-synthesis (which all block sporogenesis) prevent the increase of DAP-aspartate kinase. The enzyme is almost completely inhibited by *meso*-diaminopimelate. The molecular

weight of DAP-aspartate kinase is about 110,000, which is similar to the threonine-lysine aspartate kinase. The enzyme is stable at 55°C, and it is also partially protected by its feedback inhibitor from inactivation at 70°C. The mode of inhibition by *meso*-diaminopimelate changes with temperature. At 37°C, the inhibition curve is hyperbolic but, at 55°C, it is sigmoidal. This may suggest multiple and, perhaps, co-operative inhibitor sites on the enzyme. Antibodies against the threonine-lysine aspartate kinase do not react with the DAP-aspartate kinase, and it is suggested that two aspartate kinases in *B. stearothermophilus* are coded by separate cistrons.

Cavari *et al.* (1972) have also investigated aspartate kinase. They used the strain of *B. stearothermophilus* which was isolated by Epstein and Grossowicz (1969a). They established the presence of only the threonine-lysine aspartate kinase. The results obtained were similar to those of Kuramitsu (1970), already discussed. However, some differences are noteworthy. The enzyme of the Epstein and Grossowicz strain shows higher inhibition by threonine than by lysine, and there is no decrease in sensitivity to feedback inhibition with an increase in temperature. Cavari *et al.* (1972) observed that feedback inhibition is reversed by several amino acids of which alanine and valine are the most effective. Furthermore, they observed that L-threonine added to the growth medium prevents growth. This inhibition is reversed by either L-methionine, L-homoserine or L-isoleucine. It is possible that addition of L-threonine to the medium inhibited the aspartate kinase and thus prevented synthesis of methionine and homoserine.

Regulation of aspartate kinase in *T. flavus* involves threonine and lysine which act synergistically (Saiki and Arima, 1970). With both inhibitors present, the enzyme is almost completely inhibited. The *T. flavus* enzyme is stable up to 80°C.

Homoserine dehydrogenase catalyses reduction of L-aspartate-β-semialdehyde with NADH or NADPH to yield homoserine. Homoserine is the branch point for synthesis of threonine, isoleucine and methionine (Fig. 2, p. 193). The enzyme in *B. stearothermophilus* has been investigated by Cavari and Grossowicz (1973). They found that L-threonine is a feedback inhibitor of the enzyme, and that this inhibition is not affected by temperature (up to 70°C). Furthermore, L-threonine and L-methionine are both repressors of synthesis of the enzyme, and their effects are additive. The enzyme is most active with $NADP^+$; however, activity is also observed with NAD^+. The enzyme is highly thermostable

and has a temperature optimum of about 70°C. A monovalent ion is essential for activity.

Homoserine dehydrogenase in *T. flavus* is also activated by potassium or sodium ions whereas other monovalent cations are less effective (Saiki *et al.*, 1973). This enzyme is highly thermostable. In contrast to the *B. stearothermophilus* enzyme, that from *T. flavus* does not appear to be regulated by either L-threonine or L-methionine or any other amino acid. However, it is inhibited by a rather high concentration of cysteine (10 mM causes 85% inhibition). The kinetics with respect to L-homoserine are complex, and curves show intermediate plateaux perhaps indicating both negative and positive co-operativity. This behaviour, which is seen only at temperatures above 50°C, may be related to a conformational change at 50°C as is indicated by a broken Arrhenius plot. The transition temperature occurs at 50°C.

Threonine dehydrase, also referred to as threonine deaminase, catalyses conversion of threonine to α-oxobutyrate. It is the first reaction in a series which leads to synthesis of isoleucine from threonine (Fig. 2, p. 193). The enzyme is allosteric with isoleucine as a feedback inhibitor. Thomas and Kuramitsu (1971) partly purified the enzyme from *B. stearothermophilus* 1503-4R, and Muramatsu and Nosoh (1976) obtained highly purified enzymes from strain N.C.A. 2184. Both groups report very similar results. The molecular weight is about 200,000; however, at temperatures below 40°C, the enzyme aggregates to form complexes of molecular weights of $1.5–5 \times 10^6$ (Muramatsu and Nosoh, 1976). It probably consists of four subunits. The enzyme is active at 65°C and its pH optimum is between 9.2 and 9.6. L-Isoleucine (above 0.5 mM) completely inhibits the enzyme, both at 40°C and at 65°C. At lower concentrations, the inhibition is more effective at the lower temperature. The substrate-saturation curve is hyperbolic for threonine at all temperatures, but it changes to sigmoidal in the presence of isoleucine. Inhibition by isoleucine is antagonized by valine. The inhibition curve with isoleucine is also sigmoidal. Thus, the kinetic data suggest an enzyme with multiple binding sites for both the substrate and isoleucine.

Threonine dehydratase from *Thermus* X-1 has been studied by Higa and Ramaley (1973). They reported that the enzyme in this bacterium is not under allosteric or feedback control. The enzyme from *Thermus* X-1 differs from that in *B. stearothermophilus* by also having a lower molecular weight (about 110,000). However, when crude extracts are separated on Sephadex G 200 columns in the presence of pyridoxal 5′-phosphate and

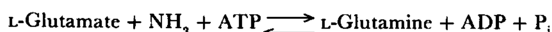
mercapto-ethanol, a 200,000 molecular-weight species is detected. Purified enzyme is always of the lower molecular weight. The enzyme is quite stable at 70°C.

Acetolactate synthase catalyses formation of α-acetolactate from two moles of pyruvate. This reaction is the first step in the synthesis of valine and leucine. The enzyme also catalyses synthesis of α-aceto-α-hydroxybutyrate, which is an intermediate in the biosynthesis of isoleucine. Chin and Trela (1973) have compared acetolactate synthases from *T. aquaticus* and a thermophilic *Bacillus* species. Both bacteria were grown on defined mineral salt media with glutamate as the carbon and nitrogen source. The enzymes from both bacteria are highly thermostable with temperature optima over 70°C. Valine serves as a feedback inhibitor for both enzymes but, interestingly, only at temperatures over 60°C. Thus, the enzyme is under feedback control only at thermophilic temperatures. The pH optima are 6 and 8, respectively, for the enzymes from *Bacillus* species and *T. aquaticus*. Both enzymes require Mg^{2+}, thiamin pyrophosphate and FAD^+ for maximum activity. Inhibition by valine in relation to pyruvate is competitive in the *Bacillus* species but noncompetitive in *T. aquaticus*. In addition to valine, isoleucine and leucine were tested as inhibitors. Only isoleucine was inhibitory and far less so than valine. The three branched-chain amino acids, when added together to the growth medium, appear to repress synthesis of the enzyme in both bacteria. Alone, they did not seem to be effective.

An important link between the tricarboxylic-acid cycle and amino-acid metabolism is the reversible reductive amination of α-oxoglutarate to glutamate, which is catalysed by glutamate dehydrogenase. Three types of the enzyme are recognized: a NAD^+-specific, a $NADP^+$-specific and one which uses both coenzymes. The enzyme in *B. stearothermophilus* (prototrophic strain; Epstein and Grossowicz, 1969a) is $NADP^+$-dependent. It has been purified about 300-fold to a specific activity of 46.2 units mg^{-1} at 55°C and pH 8.4 (Epstein and Grossowicz, 1975). The molecular weight is high (about 2×10^6) which is similar to the enzyme from mesophilic bacteria. It is homogeneous according to several criteria but several protein bands, all having activity, are observed on polyacrylamide gel electrophoresis. The enzyme is stable for 60 min at 85°C at pH values between 6.2 and 7.6 and, at 75°C, in a pH range of 5.5–8.3. Conversion of α-oxoglutarate to glutamate is maximal at pH 7.2 whereas the reverse reaction has a pH optimum at 8.4. The enzyme

follows Michaelis-Menten kinetics and the K_m values at 55°C at pH 8.2 are for glutamate, 1.1×10^{-2} M; NADP+, 3×10^{-4} M; ammonium, 2.1 $\times 10^{-2}$ M; α-oxoglutarate, 1.3×10^{-3} M; and NADPH, 5.2×10^{-3} M. In consideration of the K_m values, it was suggested that the enzyme serves an anabolic function by catalysing formation of glutamate in *B. stearothermophilus*.

Glutamine synthetase catalyses the reaction:



$$\text{L-Glutamate} + NH_3 + \text{ATP} \rightleftharpoons \text{L-Glutamine} + \text{ADP} + P_i$$

This reaction is very important in nitrogen metabolism because glutamine serves as the immediate source of nitrogen in biosynthesis of many amino acids and other nitrogenous compounds. Glutamine synthetase from *E. coli* has been extensively studied (see review by Shapiro and Stadtman, 1970). Its regulatory mechanisms include repression by ammonia and amino acids, substrate and product inhibition, response to divalent metal ions, feedback inhibition, and covalently bound ligands (adenylylation of the enzyme).

Glutamine synthetase has been purified from *B. stearothermophilus* strain 4S (Wedler and Hoffmann, 1974a) and strain N.C.A 2184 (Hachimori *et al.*, 1974). It has also been obtained from *B. caldolyticus* which has two distinguishable glutamine synthetases (Wedler *et al.*, 1976a, 1978. Like all glutamine synthetases from mesophilic bacteria, the enzymes from the three thermophiles have molecular weights of about 600,000 and consist of 12 subunits of equal molecular weight. Furthermore, the enzymes are regulated at thermophilic temperatures by complex mechanisms which, however, are modified for each individual enzyme. This follows the statement by Shapiro and Stadtman (1970) in their review: "The regulatory function is more constant than the regulatory mechanism".

All of the glutamine synthetases from thermophiles have high thermostability which is further augumented especially by glutamate or glutamine or, still better, by pairing ammonia and glutamate, ATP and ammonia, or ATP and glutamate. The enzymes require a divalent metal ion for activity; either Mg^{2+} or Mn^{2+} satisfies this requirement. The diversity between thermophilic glutamine synthetases is expressed, among other things, in their different responses toward the two metal ions. Thus, the enzyme isolated by Hachimori *et al.* (1974) prefers Mg^{2+} over Mn^{2+} whereas the enzyme from strain 4S is more active with Mn^{2+} (Wedler and Hoffman, 1974a). The Mg^{2+}-containing glutamine

synthetase and the Mn^{2+}-containing glutamine synthetase may respond differently in their interactions with substrates and effectors.

Alanine, glycine, serine, tryptophan, histidine, glutamine, cysteine, 18 different nucleotides, glucosamine 6-phosphate and carbamoyl phosphate have been found in varying degrees and in different manners to modify thermophilic glutamine synthetases. The modifiers also influence each other's actions. Thus, the interaction between two modifiers may be synergistic, additive, cumulative or antagonistic. Obviously, a detailed review of all possible interactions between the modifiers and the different glutamine synthetases is not possible here, and the interested reader is referred to the original publications.

Histidinol dehydrogenase (EC 1.1.1.23), which catalyses the terminal reaction in formation of histidine, has been purified from *Bacillus psychrophilus, B. subtilis, B. stearothermophilus* and *B. caldolyticus* (Lindsay and Creaser, 1977). The enzymes from all of the bacilli have similar molecular weights (around 29,200) and other physical and kinetic properties. However, they differ in thermostabilities and temperature optima. The latter are 25°C, 45°C and 85–92°C for the psychrophiles, the mesophiles, and the thermophiles, respectively. It was concluded that the differences in thermostabilities are related to intrinsic properties of the enzymes.

C. ADDITIONAL ENZYMES OF CARBOHYDRATE AND ENERGY METABOLISM

β-Galactosidase (EC 3.2.1.23) has been found by Goodman and Pederson (1976) in five out of eight strains of *B. stearothermophilus* which were isolated from hot springs in the San Bernardino Mountains in California, U.S.A. This finding is a little surprising since several reports state that lactose is not a substrate for *B. stearothermophilus*. The enzyme appears to be constitutive, and the level of it in the organisms is not affected by the presence of either lactose or isopropyl-β-D-thiogalactopyranoside in the medium. The enzyme has a pH optimum between 6.0 and 6.4 and a T_{opt} value around 65°C. The Arrhenius plot is biphasic with the break at 47°C. The K_m value (0.11 M) for lactose is high, and the enzyme is inhibited by galactose (the K_i value being 25×10^{-3} M). The high K_m value for lactose indicates that it may not be a normal substrate. It was suggested that other galactosides, such as D-galactans or galactolipids, could be natural substrates.

An inducible β-galactosidase has been partly purified from an extreme thermophile resembling *T. aquaticus* (Ulrich *et al.*, 1972). The enzyme has almost no activity below 40°C but is active at 80°C. Its synthesis is induced by lactose, galactose, melibiose and isopropyl-thio-β-D-galactopyranoside. Cells grown on glucose lack the enzyme. The enzyme, which has a molecular weight about 57,000, is similar to the enzyme from *E. coli* except for its thermostability.

Uptake of glucose and methyl-α-D-glucoside has been studied by Harris and Kornberg (1972), Harris and Miller (1976) and Reizer *et al.* (1976) using the *B. stearothermophilus* strain isolated by Epstein and Grossowicz (1969a). Glucose uptake is dependent on a phosphoenolpyruvate-sugar phosphotransferase system similar to that described for several mesophilic bacteria (see review by Anderson and Wood, 1969). *Bacillus stearothermophilus* grown on succinate or acetate takes up glucose at less than one-twentieth of the rate of cells grown on glucose. However, when cells growing on either succinate or acetate are exposed to glucose, they rapidly develop the capability to assimilate glucose and also methyl-α-glucoside. Glucose is then preferentially used as the carbon source. Protein synthesis, which is inhibited by chloramphenicol, is required before cells grown on acids are capable of using glucose (Harris and Kornberg, 1972). Glucose uptake is prevented by fructose in *B. stearothermophilus* and, when cells are exposed to a mixture of fructose and glucose, fructose is preferentially used before glucose uptake starts (Harris and Kornberg, 1972). Interestingly, another thermophile, *Clostridium thermoaceticum*, also preferentially utilizes fructose over glucose. Results with this bacterium indicate that fructose prevents formation of the glucose-transport system (Andreesen *et al.*, 1973).

Methyl-α-D-glucoside is used to induce glucose transport. Normally, it will enter the cell and become phosphorylated, but it is not further metabolized. Induction of the glucose-transport system may thus be studied separately from general metabolism of the cell. However, *B. stearothermophilus* grows on methyl-α-glucoside and other α-glucosides like maltose, sucrose, trehalose and turanose (Harris and Miller, 1976; Reizer *et al.*, 1976). Methyl-α-glucoside uptake and phosphorylation are induced by methyl-α-glucoside and glucose. However, cells grown on glucose grow on methyl-α-glucoside only after a long lag period. In contrast, cells grown on maltose grow on methyl-α-glucoside, essentially without a lag. Furthermore, washed-cell suspensions of glucose-grown

cells do not metabolize methyl-α-glucoside, in contrast to cells grown on the glucoside. The results indicate that methyl-α-glucoside transport system induced by the glucoside is different from that induced by the glucose. Furthermore, methyl-α-glucoside-hydrolysing activity appears to be induced by methyl-α-glucoside and maltose but not by glucose.

Uptake of ^{14}C-labelled amino acids and [2-^{14}C]uracil by *B. stearothermophilus* has been investigated by Bubela and Holdsworth (1966). Very little of the radioactive compounds is incorporated into cell material at temperatures below 35°C. Above 35°C, incorporation increases rapidly with the temperature reaching a maximum at about 60°C. It was suggested that the reason why *B. stearothermophilus* does not grow at mesophilic temperatures is the very low rate of uptake of amino acids and uracil. Uptake of [^{14}C]leucine is inhibited by 2,4-dinitrophenol, which suggests that the amino-acid uptake is energy-dependent. More recently, Reizer and Grossowicz (1974) reported that α-aminoisobutyrate (AIB) is concentrated about 300-fold over the concentration (2×10^{-5} M) in the medium by *B. stearothermophilus*. Uptake is inhibited by cyanide, azide, dinitrophenol or by lack of an energy source, clearly showing the need for energy during uptake. Several competition experiments showed that uptake of L- and D-alanine, L- and D-serine and glycine is by the same transport system as AIB. Kinetic data on uptake of AIB reveal biphasic, double-reciprocal curves, which indicate at least two distinct components in the transport system.

Considerable progress in understanding transport of alanine has been achieved by Hirata *et al.* (1976a, b) and Kagawa (1976). They successfully isolated an alanine-carrier protein and ATPase from the thermophilic *Bacillus* PS3, and were able to reconstitute proteoliposomes capable of alanine transport and ATP-dependent proton translocation. The ATPase was discussed earlier in section IV A (p. 170). Alanine transport into vesicles has a temperature optimum between 45°C and 60°C. It can be driven either by oxidation of NADH or ascorbate-phenazine methosulphate, by potassium-ion efflux from potassium-loaded vesicles, or by a pH gradient. Results were obtained suggesting that protons and alanine molecules are transported simultaneously in a stoicheiometric ratio of 1:1.

Adenosine diphosphate glucose: glucan-α-1,4-glucosyltransferase (EC 2.4.1.21) activity has been found in extracts of a spontaneous mutant of *B. stearothermophilus* 1503-4R grown at 65°C (Goldemberg and Algranati, 1969). Only ADP-[^{14}C]glucose, not UDP-glucose, GDP-glucose nor

glucose 1-phosphate, is incorporated into a glycogen-like material (Goldemberg, 1972). The extract contains an endogenous acceptor, but rabbit-liver glycogen also acts as an acceptor.

Inorganic pyrophosphatase (EC 3.6.1.1) is present in *B. stearothermophilus* (Marsh and Militzer, 1956b). The enzyme requires Mg^{2+} or Co^{2+} for activity. It has been purified to homogeneity from *B. stearothermophilus* N.C.A. 2184 by Hachimori *et al.* (1975a). The purified enzyme has a specific activity of 1,800 units mg^{-1} at pH 8.5 at 65°C. The molecular weight is about 122,000 and there may be two subunits. The enzyme is inhibited by ATP and AMP but not by ADP. Inhibition by ATP shows sigmoidal kinetics, which may be of importance in regulation of the enzyme. Brown *et al.* (1957) noticed that the enzyme from *B. stearothermophilus*, grown at different temperatures, has different thermal stabilities. Morita and Mathemeier (1964) found that moderately high pressure (up to 700 atm) seems to increase both the thermal stability and also enzyme activity.

A repressible alkaline phosphate is present in *T. aquaticus* (Yeh and Trela, 1976). The enzyme is produced when low concentrations (4×10^{-5} M) of inorganic phosphate or glycerol phosphate are present in the growth medium. The enzyme exists as polymers (molecular weights from 143,000 to about 520,000) of a polypeptide with the molecular weight of 51,000. The enzyme liberates orthophosphate from *p*-nitrophenyl phosphate, α- and β-glycerol phosphate glucose 1- and 6-phosphates, pyridoxal 5-phosphate, ATP and ADP. It has no activity with pyrophosphate. A later paper (Smile *et al.*, 1977) mentions that the enzyme has a low level of phosphodiesterase activity.

A carboxylic ester hydrolase (EC 3.1.1.1) has been purified from *B. stearothermophilus* N.C.A. 2184 (Matsunaga *et al.*, 1974). It hydrolyses *p*-nitrophenyl and *m*-carboxyphenyl esters of n-fatty acids. The molecular weight is 47,000. The enzyme seems to undergo a conformational change at 55°C. The presence in the active site of a serine residue and a sulphydryl group is indicated.

An inducible amidase has been demonstrated in *B. stearothermophilus* by Thalenfeld *et al.* (1977). Synthesis of the enzyme seems to be controlled, not only by induction but also by catabolite repression. The latter control seems to be dominant and is expressed especially in dilute cultures. The enzyme has not been purified.

Catalase (Nakamura, 1960) and superoxide dismutase (Bridgen *et al.*, 1975; Brock *et al.*, 1976) are present in *B. stearothermophilus*. The catalase

has a high thermostability which is further augmented by a catalase-suppressing factor (s-factor) found in boiled extracts. Superoxide dismutase is of the prokaryotic manganese-containing type. It is a dimer and has a molecular weight of 40,000. The metal-free apoenzyme can be prepared by treatment with 8 M urea and can be reconstituted. A tetrameric Mn^{2+} superoxide dismutase has been isolated from *T. aquaticus* (Sato and Harris, 1977). Amino-acid sequence studies indicate that the dimeric and the tetrameric enzymes closely resemble each other.

Electron-transport particles with high thermostability were first reported by Militzer *et al.* (1950). The particles are capable of oxidizing succinate, malate, NADH, *p*-phenylenediamine and hydroquinone. They contain cytochromes a_3, b and c, coenzyme Q, and a naphthaquinone (Downey, 1962, Downey *et al.*, 1962). Downey (1966) found that anaerobic growth of *B. stearothermophilus* requires nitrate which serves as an electron acceptor. Nitrate and nitrite reductases appear to be induced sequentially on addition of nitrate to the growth medium in the presence of low concentrations of dissolved oxygen. Synthesis of the enzymes is repressed by oxygen, and aeration causes rapid degradation of existing nitrate reductase (Downey *et al.*, 1969). Oxygen also seems to inactivate reversibly nitrate reductase, and some evidence of an allosteric effect has been obtained by Downey and Stambaugh (1976). Nitrate reductase is localized in the cell membrane and can be solubilized by detergents like sodium dodecyl sulphate, Triton 114 and Triton X-100 (Kiszkiss and Downey, 1972a). The solubilized nitrate reductase does not oxidize NADH, but is still able to use reduced methyl viologen (Kiszkiss and Downey, 1972b). Dilution of the detergent causes re-aggregation of the membranes with partial restoration of both NADH-nitrate reductase and NADH-oxidase activities. Divalent cations promote the reconstitution with Mg^{2+}, Ca^{2+} and Mn^{2+} being the most effective. A shift from aerobic to anaerobic conditions, and to the use of nitrate instead of oxygen as the electron acceptor, causes changes in the level of cytochromes in the membrane (Downey and Kiszkiss, 1969). Thus, increases were observed in the contents of cytochromes b and c_1, whereas the level of cytochrome a_3 decreased. Induction of nitrate reductase also requires cell division, which is not so surprising since the electron-transport system is membrane-bound. Increases in cytochromes b and c indicate participation of these electron-transfer components in the NADH-nitrate reductase system.

The presence of cytochromes *a*, *b*, and *c* in an extreme thermophile

resembling *T. aquaticus* has been demonstrated by McFeters and Ulrich (1972). The rate of oxygen uptake in this bacterium is three times greater at 70°C than at 50°C; however, this is not accompanied by an increase in the content of cytochromes when the temperature is raised. Cytochromes a, a_3, c_1, c_2 and o have been found in the thermophile, *B. coagulans* A.T.C.C. 11369 (Frade and Chaix, 1973). Lowering the pH value of the growth medium from 6.5 to 5.5 increases the respiratory rate four-fold in this organism. This is accompanied by an increase in the level of the components of the respiratory system, particularly cytochrome oxidase a_3.

The electron-transport system in *B. stearothermophilus* is coupled with phosphorylation as is evidenced, among other things, by high growth yields when glucose is the substrate (Coultate and Sundaram, 1975; Pozmogova and Mal'yan, 1976). As discussed in Section IV A (p. 170), ATPase in *B. stearothermophilus* has a high thermostability (Hachimori *et al.*, 1970) as has the ATPase in the thermophilic *Bacillus* PS3 (Yoshida *et al.*, 1975; Sone *et al.*, 1975). The ATPase from strain PS3 has been extensively characterized and its components have been purified. Reconstitution of the ATPase system with formation of vesicles capable of synthesis of ATP by reverse H^+-transport and other energy-transferring reactions has been achieved (Sone *et al.*, 1976; Kagawa *et al.*, 1976; Sone *et al.*, 1977a, b; Yoshida *et al.*, 1977; Okamoto *et al.*, 1977). The role of ATPase in bacterial respiration has recently been reviewed by Haddock and Jones (1977).

Ferredoxin, which is involved in electron-transfer reactions at low redox potentials, has been purified from *B. stearothermophilus* and thoroughly characterized (Mullinger *et al.*, 1975; Hase *et al.*, 1976). The ferredoxin, which has only one $[Fe_4S_4]^*$ cluster, is rather thermostable. The molecular weight is 9,120. The amino-acid sequence shows homology with other bacterial ferredoxins. However, it has only four cysteine residues, but the number of glutamic-acid residues is high which may be related to the high thermostability.

VI. Metabolism in *Clostridium thermoaceticum*

Clostridium thermoaceticum ferments sugars with acetate as the only product and is said to carry out a "homoacetate fermentation" (Ljungdahl and Wood, 1969). The fermentation has been earlier summarized as follows:

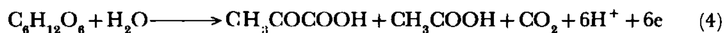
$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 8H^+ + 8e \quad (1)$$

$$2CO_2 + 8H^+ + 8e \longrightarrow CH_3COOH + 2H_2O \quad (2)$$

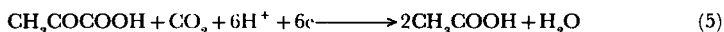
$$\text{Sum: } C_6H_{12}O_6 \longrightarrow 3CH_3COOH \quad (3)$$

According to these reactions, carbon dioxide is the acceptor of electrons generated during the fermentation. Reactions 1 and 2 were proposed when it was found that, with the use of $^{14}CO_2$, $^{13}CO_2$ and mass spectrometry, one third of the acetate was totally synthesized from carbon dioxide in *C. thermoaceticum* (Wood, 1952a). However, it has now been shown with *C. thermoceticum* that a total synthesis of acetate does not occur. Instead, it was found that the methyl group of acetate is formed from carbon dioxide while the carboxyl group is derived from the carboxyl group of pyruvate in an apparent transcarboxylation reaction (Schulman *et al.*, 1973). Incorporation of carbon dioxide into the carboxyl group occurs because *C. thermoaceticum* catalyses a rapid exchange between carbon dioxide and the carboxyl group of pyruvate and, during studies with $^{14}CO_2$ or $^{13}CO_2$, an isotope equilibrium is rapidly reached between carbon dioxide and the pyruvate carboxyl group.

The finding that pyruvate is the carboxyl donor of acetate dismissed the idea of a total synthesis of acetate from carbon dioxide. However, the concept that carbon dioxide is the ultimate electron acceptor in the homoacetate fermentation is still valid, and the fermentation as it occurs in *C. thermoaceticum* may now be summarized as follows:



$$C_6H_{12}O_6 + H_2O \longrightarrow CH_3COCOOH + CH_3COOH + CO_2 + 6H^+ + 6e \quad (4)$$



$$CH_3COCOOH + CO_2 + 6H^+ + 6e \longrightarrow 2CH_3COOH + H_2O \quad (5)$$

$$\text{Sum: } C_6H_{12}O_6 \longrightarrow 3CH_3COOH \quad (3)$$

In reaction 4, the hexose is fermented via the Embden-Meyerhof glycolytic pathway to two moles of pyruvate of which one is further metabolized to acetate and carbon dioxide (Wood, 1952b). The six equivalents of electrons formed in reaction 4 are used to reduce carbon dioxide in reaction 5 to a methyl group, which yields acetate in the "transcarboxylation" reaction with pyruvate (Schulman *et al.*, 1973). The net result is the formation of three moles of acetate per mole of hexose.

The pathway for synthesis of acetate from carbon dioxide and

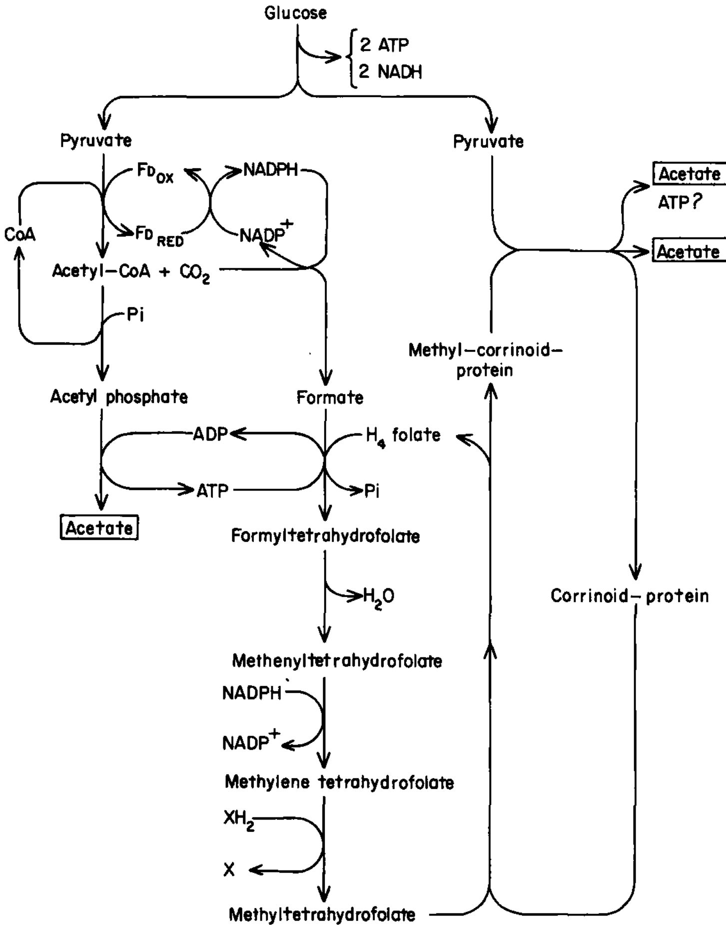


FIG. 3. Proposed pathway for synthesis of acetate from carbon dioxide in *Clostridium thermoaceticum*.

pyruvate has been established with *C. thermoaceticum* and is shown in Fig. 3. Carbon dioxide is first reduced to formate (Thauer, 1972; Andreesen and Ljundahl, 1974) by a $NADP^+$ -dependent formate dehydrogenase which appears to be a tungsten-selenium enzyme (Andreesen and Ljungdahl, 1973; Ljungdahl and Andreesen, 1975). Synthesis of formyltetrahydrofolate from formate, ATP and tetrahydrofolate is the next step. This is catalysed by formyltetrahydrofolate synthetase. Formyltetrahydrofolate is then converted to methyltetrahydrofolate in a sequence of three reactions which are catalysed by methenyltetrahydro-

folate cyclohydrolase, methylene tetrahydrofolate dehydrogenase and methylene tetrahydrofolate reductase, respectively. These three enzymes have been demonstrated in *C. thermoaceticum* (Andreesen *et al.*, 1973).

Methyltetrahydrofolate, methylcobalamin and other methylcorrinoids are excellent precursors for the methyl group of acetate (Poston *et al.*, 1966; Ghambeer *et al.*, 1971). During pulse-labelling experiments with $^{14}CO_2$, 5-methyltetrahydrofolate mono-, di- and triglutamates are formed labelled in the methyl group with high specific radioactivity (Parker *et al.*, 1971). Also, in pulse-labelling experiments, ^{14}C-methylcobyric acid and (5-methoxybenzimidazolyl)-^{14}C-methylcobamide are formed (Ljungdahl *et al.*, 1965). These and other results are good evidence supporting the postulate that methyltetrahydrofolate and methylcorrinoid are intermediates in synthesis of acetate from carbon dioxide.

Formyltetrahydrofolate synthetase has been purified from *C. thermoaceticum* (Ljungdahl *et al.*, 1970; Brewer *et al.*, 1970) and compared with the enzyme from *C. formicoaceticum* and other clostridial sources (O'Brien *et al.*, 1976). The formyltetrahydrofolate synthetases from all clostridial sources are very similar except for the higher thermostability of the enzyme from *C. thermoaceticum.* Similarly, methylene tetrahydrofolate dehydrogenase has been purified from *C. thermoaceticum (O'Brien et al.*, 1973) and compared with the enzyme from *C. formicoaceticum* (Ljungdahl *et al.*, 1976). Again, the similarities are striking except for thermostability and the cofactor requirement. The enzyme from *C. thermoaceticum* is $NADP^+$-dependent, whereas that from *C. formicoaceticum* is active only with NAD^+ (Moore *et al.*, 1974).

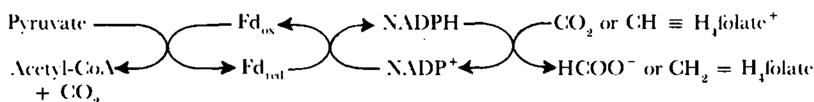
Exceptionally high growth yields are obtained with *C. thermoaceticum* when it is grown on glucose, fructose or xylose (Andreesen *et al.*, 1973). The reactions shown in Fig. 3 yield a maximum of three ATP moles. However, the growth yields indicate that as much as five moles of ATP may be produced during the fermentation. This observation, together with the findings of a cytochrome *b* and other electron carriers in *C. thermoaceticum* (Gottwald *et al.*, 1975), points toward the possibility that electron-transport phosphorylation may occur in this micro-organism. For discussions of growth yields and their relation to ATP, see reviews by Stouthamer (1973) and Payne (1970).

The scheme presented in Fig. 3 shows the pathway of the carbon atoms in the homoacetate fermentation, but it does not give the full account of electron-transfer reactions which must occur. Perhaps, one of the most

fundamental characteristics of the homoacetate fermentation is the fact that carbon dioxide and the folate derivatives are the acceptors of electrons generated during glycolysis.

It is evident that electrons generated in the glyceraldehyde phosphate dehydrogenase reaction are accepted by NAD^+, while those generated by oxidation of pyruvate are accepted by ferredoxin. The immediate electron donor is NADPH in reduction of carbon dioxide to formate and in the methylene tetrahydrofolate dehydrogenase reaction. The likely electron donor with methylene tetrahydrofolate reductase is $FADH_2$. Thus, it is obvious that some kind of electron transport must occur to couple the electron-generating reactions yielding NADH and reduced ferredoxin with the electron-accepting reactions using NADPH and reduced flavin as electron donors.

Attempts to demonstrate a direct transhydrogenation between NADH and $NADP^+$ have failed (Shiow-Shong Yang, personal communication). However, *C. thermoaceticum* contains a thermostable ferredoxin, which couples oxidation of pyruvate with reduction of $NADP^+$ as follows:



Reduced nicotinamide adenine dinucleotide phosphate may be used in either the formate dehydrogenase or in the methylene tetrahydrofolate dehydrogenase reactions. However, ferredoxin-mediated reduction of $NADP^+$ explains only the transfer of two electrons. It remains to establish transfer of electrons from $NADP^+$ and FAD. The *C. thermoaceticum* ferredoxin has been characterized (Yang *et al.*, 1977). It is an unusual clostridial ferredoxin in that it contains only one [Fe_4S_4] cluster, instead of two such clusters as in all other clostridial ferredoxins that have been isolated.

The pathway illustrated in Fig. 3 is not unique for *C. thermoaceticum.* Similar reactions are catalysed by the mesophiles *C. formicoaceticum* (Andreesen *et al.*, 1970; O'Brien and Ljungdahl, 1972) and perhaps also by *Acetobacter woodii* (Balch *et al.*, 1977; Tanner *et al.*, 1978). The latter organism grows either heterotrophically on fructose or autotrophically with carbon dioxide as a carbon source and hydrogen gas as a source of electrons. The fact that it can grow autotrophically and generate energy by reducing carbon dioxide to acetate, indicates a new type of autotrophic carbon dioxide fixation.

VII. Thermoadaption, Maximum and Minimum Temperatures, and Genetic Transfer of Thermophilicity

There is only one theory of those proposed to explain thermophilicity for which substantial experimental evidence has been obtained. According to this theory, thermophilic micro-organisms produce proteins, complex structures and membranes that are stable and functional at thermophilic growth temperatures. Accordingly, throughout this review, it has been pointed out that proteins and structures such as ribosomes, ATPase and proteins of membrane-transport systems in thermophiles, in general, have higher thermostability than corresponding structures in mesophiles. Actually, there has not been a single report about a pure protein which is not either stable intrinsically or stabilized by some known factor at the growth temperature of the micro-organism it was isolated from. Similarly, DNA and RNA have melting temperatures at least as high as the T_{max} value of their microbial sources. It should also be noted that proteins in thermophiles are very similar—physically, chemically and kinetically—to the corresponding proteins from mesophiles. Enzymes from thermophiles, like those from mesophiles, also have allosteric properties, and metabolism in thermophiles is regulated in a way similar to that in mesophiles. Thus, the main difference between corresponding enzymes or proteins from thermophiles and mesophiles is the higher thermostability of the proteins from the former. In most proteins from thermophiles, this stability is intrinsic and the result of subtle changes in the amino-acid composition or sequence. It has been shown that a single amino-acid substitution may dramatically change the thermostability of a protein (Langridge, 1968; Yutani *et al.*, 1977).

Normally, only one type of enyzme is found in a micro-organism. However, results have been presented which show that some thermophiles have the capacity to produce at least two types of the same enzyme which differ in thermal stability. Thus, *B. stearothermophilus* grown at 37°C seems to produce an α-amylase which has lower thermostability than α-amylase produced in cells grown at 55°C (Campbell, 1955; Isono, 1970; Yutani *et al.*, 1973). Similarly, Brown *et al.* (1957) reported that the heat stability of pyrophosphatase in *B. stearothermophilus* varies with the growth temperature of the organism. The results indicate the existence of two types of pyrophosphatase with different thermostabilities. Neutral proteases with different thermostabilities are also produced by *B. stearothermophilus* grown at 40°C and 50°C (Sidler and Zuber, 1972).

Bausum and Matney (1965), studying a facultative thermophile, noticed that *Bacillus licheniformis* growing at 37°C is rapidly killed when the growth temperature is suddenly elevated to 55°C. However, if cells are allowed to adapt at an intermediate temperature for a limited time before being exposed to 55°C, death is not observed. When cells lose their viability, those which survive recover only after a long lag period. Incubation for a period at 49°C prevents this loss. All of these observations indicate thermoadaption.

Jung *et al.* (1974), Haberstich and Zuber (1974) and Frank *et al.* (1976) have studied thermoadaption by determining activities and thermostabilities of enzymes from *B. stearothermophilus* and *B. caldotenax*. They first established that cultures of *B. stearothermophilus* could not be directly transferred from 37°C to 55°C without loss of viability. To obtain growth at the higher temperature of a culture previously incubated at 37°C, the culture had to undergo an adaption incubation at 46°C. The levels of alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase were assayed in cells grown at 37°C and 55°C. It was found that alcohol dehydrogenase is present only in cells grown at 55°C, and that these cells have a very high level of glyceraldehyde 3-phosphate dehydrogenase compared with cells grown at 37°C. The activities of the tricarboxylic acid-cycle enzymes in cells grown at 37°C are about 3.5 times higher than those in cells grown at 55°C. Cells grown at 37°C seem almost exclusively to have an aerobic type of metabolism. At 55°C, the cells were growing well, both aerobically and anaerobically. The results suggested that *B. stearothermophilus* has different types of metabolism at low and high temperatures. Selection of a mesophilic contaminant during these experiments was considered, but several criteria seem to rule out the possibility.

The thermal stabilities of glucokinase, glucose 6-phosphate isomerase and dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and isocitrate dehydrogenase were determined in extracts from *B. stearothermophilus* N.C.I.B. 8924 and *B. caldotenax* YT-G. The former was grown at 46°C, and cells from a single colony were subsequently grown at 37°C and 55°C in BBL brain heart infusion medium. *Bacillus caldotenax* was cultivated at temperatures between 30°C and 70°C at 5°C intervals. All of the enzymes investigated in the extract of *B. stearothermophilus* grown

at 55°C were more thermostable than those from cells grown at 37°C. Enzymes from cells grown at 46°C have intermediate stability which could indicate a mixture of 55°C and 37°C enzymes or an enzyme type of intermediate stability. When 37°C-grown cultures of *B. caldotenax* are transferred to temperatures between 30°C and 50°C, growth starts within one to two hours whereas, at 55°C or higher, a long lag period is observed. The rate of growth, once started, is similar at all temperatures. The lag period is reflected on the enzyme level. Glucose 6-phosphate isomerase from cultures grown between 30°C and 50°C has the same low thermostability (completely inactivated after 30 min at 60°C), whereas the enzyme in cultures grown at 60–70°C has the same high thermostability (completely inactivated after 30 min at 90°C). The results indicate that two distinct variants, a mesophilic and a thermophilic, of glucose 6-phosphate isomerase are formed. The longer lag period observed going from a 37°C culture to cultures grown at 60°C and over indicates thermoadaption and new protein synthesis. Similar results were obtained with isocitrate dehydrogenase. In accordance with these results, when *B. caldotenax* is cultured at 70°C and then transferred to temperatures between 30°C and 70°C, a pronounced lag period is seen only in the lower temperature range. Haberstich and Zuber (1974) raise the following questions: (1) Is the genetic information for the two forms of enzymes transcribed from different genes as a function of temperature?; or (2) is there one gene and does the thermoadaption occur at a later stage in protein synthesis?; or (3) are the finished enzymes modified? Presently, it is not possible to say whether or not the phenomenon of thermoadaption, expressed in the production of two variants of the same enzyme differing in thermostability, is a common property of microorganisms.

The composition of lipids, in contrast to proteins, is apparently changed in response to the growth temperature in most, if not all, microorganisms. It is clear from the discussion of lipids and membranes (Section IV B, p. 172) that the microbe always tries to produce a membrane of the same fluidity regardless of the growth temperature (homeoviscous adaptation). This is accomplished by changing the fatty-acyl composition and the contents of more complex lipids. It is likely that these changes occur at the enzyme level. One could easily imagine that the enzymes involved in fatty acid and lipid synthesis may alter their catalytic properties in response to changes in temperature in such a way

that they synthesize fatty acids of a shorter chain length at a lower temperature.

Although it is generally accepted that DNA and RNA are relatively stable components of the microbial cell, there is at least one example showing that these molecules may also change in response to growth temperature. As was discussed in Section IV C (p. 186). Watanabe *et al.* (1976a) found that 5-methyl-2-thiouridylate replaces a ribothymidylate residue in $tRNA_f^{met}$ in *T. thermophilus*, and that the extent of this replacement is dependent on growth temperature. Incorporation of 5-methyl-2-thiouridylate increases the thermostability of the $tRNA_f^{met}$.

Micro-organisms lose their ability to grow at a certain maximum temperature, T_{max}, as well as at a certain minimum temperature, T_{min}. Thus, they grow and reproduce within a limited temperature range. It has been suggested that this range is controlled by properties of the membrane or macromolecules which must be stable and also function within the limits of T_{min} and T_{max} values.

Many investigators have suggested that the membrane determines the cardinal growth temperatures. Obviously, the membrane must be functional. However, the microbe seems to control the state of the membrane by homeoviscous adaptation. Furthermore, data obtained by McElhaney and Souza (1976) using differential thermal analysis, convincingly show that a *B. stearothermophilus* mutant is capable of growing 18–19°C over the upper boundary for the phase transition of the membrane. McElhaney (1974, 1976) also showed that *Acholeplasma laidlawii* is able to grow when only 10% of the membrane exists in the liquid-crystalline state. Thus, the capacity of the cell to regulate the composition of the cell membrane, in combination with the membrane's functionality over a relatively wide temperature range, suggests that the membrane, in general, does not determine the cardinal growth temperatures. Of course, it is self-evident that, if a cell has lost its capacity for homeoviscous adaptation, the cell membrane may very well determine either the T_{min} value or the T_{max} value or both.

If the membrane is not a factor in determining T_{min} or T_{max} values, enzymes and other proteins are. Many enzymes from thermophilic micro-organisms are, as discussed in this review, rapidly denatured just above the T_{max} value of the micro-organism. Clearly, if an enzyme which is important for survival of the cell is denatured at a certain temperature, the cell's T_{max} value may closely coincide with that temperature. That

enzymes also determine the T_{min} value is more difficult to demonstrate.

Babel *et al.* (1972) suggest the following possible causes for T_{min} values: (1) accumulation of toxic metabolic products owing to an imbalance of enzymes with different temperature characteristics (activation energies); (2) feedback inhibition owing to the same cause or to changed allosteric properties of a key enzyme; (3) repression of enzyme synthesis; (4) inhibition of membrane transport of nutrients; (5) defective energy supply; (6) changed physical state of cell constituents particularly of the cell membrane, possibly caused by solidification of lipids; and (7) translation errors in protein synthesis with consequent production of ineffective enzymes.

These possibilities were evaluated by Babel *et al.* (1972) who used *B. stearothermophilus* A.T.C.C. 12980. This organism has a T_{min} value of 37°C, a T_{opt} value of 63°C, and T_{max} value of 72°C. In shift-down experiments from 65°C to temperatures below T_{min} (25–35°C), they observed an immediate cessation of cell multiplication which, after three hours of incubation at the lower temperature, immediately resumed at the previous rate after a shift-up to 65°C. These results indicate that long time-dependent causes (such as accumulation of toxic products and repression of, or faulty, enzyme synthesis) are eliminated as possible causes for the T_{min} value. In addition, Babel *et al.* (1972) found that respiration continues far below the T_{min} value, which demonstrated to them that neither membrane transport nor energy supply are limiting factors. They suggested that a conformational change of one essential enzyme or enzyme group determines the T_{min} value. Such a change may involve loss of an allosteric property or catalytic capacity due to a more rigid enzyme which no longer is capable of induced fit.

The possibility that the T_{min} value is related to a conformational change in a protein was discussed by Ljungdahl and Sherod (1976). They compiled a list of enzymes which exhibit broken Arrhenius plots. This list can now be expanded by including several of the enzymes discussed in this review. Activation energies for these enzymes, with one exception, are much higher at low than at high temperatures. Thus, these enzymes are less efficient catalysts at low temperatures. It was concluded that this would lead to a slowing down of metabolism which, in turn, would stop growth of the micro-organisms. Furthermore, Ljungdahl and Sherod (1976) pointed out that some allosteric enzymes from thermophilic micro-organisms lose their allosteric properties at low temperatures,

which seems to be in agreement with the conclusion of Babel *et al.* (1972). The idea that variations in apparent activation energies of enzymes may affect T_{min} values was already formulated by Della Valle *et al.* (1962).

Genetic transfer of the ability to grow at a high temperature has been reported. McDonald and Matney (1963) used a streptomycin-resistant strain of *B. subtilis* (168 S^r) which fails to grow above 50°C and *B. subtilis* 16S which grows at 55°C and is streptomycin-sensitive. When 168 S^r cells were incubated with DNA obtained from 16S, the ability to grow at 55°C was transformed at a frequence of 10^{-4}. The S^s loci was also transferred to 90% of the cells capable of growing at 55°C. Lindsay and Creaser (1975) succeeded in transforming *B. subtilis* with an optimum growth at 37°C to growth at 70°C by using DNA from *B. caldolyticus*. The results indicated that more than one gene confers high-temperature growth ability. Histidinol dehydrogenase was isolated from the original strain of *B. subtilis* and from the transformed strain. The enzyme from the original strain is inactive at 70°C, whereas from the transformed strain it is stable at 100°C. However, histidinol dehydrogenase from the transformed strain was not identical with the enzyme from *B. caldolyticus* (Lindsay and Creaser, 1977). Several hypotheses to explain the results have been advanced (Lindsay and Creaser, 1975, 1976). Mojica *et al.* (1977) used DNA from *B. caldolyticus* and *B. stearothermophilus* to transform the ability to grow at 65°C to the mesophile, *B. subtilis*. They found that the recipient maintained auxotrophic markers, possessed DNA which transformed other strains of *B. subtilis*, grew in a defined medium sufficient for *B. subtilis* but not for the thermophilic donors, and was capable of reverting to 37°C. Investigations of the ribosomes revealed that the genes of almost all ribosomal proteins had been transformed. At the present time, it has not been established whether a plasmid or chromosomal DNA was transformed (S. Marvin Friedman, personal communication).

Successful conversion of a mesophilic bacterium to a thermophilic bacterium is hard to comprehend. All evidence indicates that thermophiles have, and actually must have, a complete set of thermostable proteins and structures. Assuming this is correct, in order to transform a truly mesophilic bacterium into a thermophile, genetic material corresponding to a complete new set of thermostable enzymes must be introduced. That during transformation all of the necessary genetic material is transferred is very unlikely. Nevertheless, the results

by McDonald and Matney (1963), Lindsay and Creaser (1975) and Mojica *et al.* (1977) are impressive. A possible explanation for these results may be as follows. The results regarding thermoadaption by Jung *et al.* (1974), Haberstich and Zuber (1974) and Frank *et al.* (1976) indicate the possibility that *B. stearothermophilus* and *B. caldotenax* have two sets of complete genes which are expressed at different temperatures. It is also conceivable that *B. subtilis* used in the transformation experiments has two sets of genes, but that it is unable to express the set which it needs to function at high temperature. Thus, *B. subtilis* will only grow at mesophilic temperatures. However, if in the transformation experiments a small piece of the gene is transferred (which allows expression of the second set of genes), then it will grow at thermophilic temperatures. The results with histidinol dehydrogenase, which demonstrated that this enzyme from the transformed *B. subtilis* differs from both the original *B. subtilis* and from the donor, *B. caldolyticus*, indicate that it is coded by three different genes in three bacteria (Lindsay and Creaser, 1977). This is in agreement with the above proposal.

Finally, it should be mentioned that an excellent discussion of genetic regulation of temperature responses in micro-organisms has been presented by Ingraham (1973). This review deals particularly with temperature-sensitive mutants.

VIII. Conclusions

Thermophilic micro-organisms have all of the properties normally found in mesophilic micro-organisms. These include metabolic pathways, regulatory mechanisms such as allosteric or feedback control, repression and induction of protein synthesis, growth yields and metabolic rates. The main difference between thermophiles and mesophiles is the former's capacity to grow at high temperatures. The basis for this capacity is the thermophile's capability to synthesize proteins, complex structures and membranes that are stable or are stabilized and functional at thermophilic temperatures. It is proposed that the maximum and minimum growth temperatures are normally determined by properties associated with proteins, and that the membrane plays a lesser role in determining these temperatures. Enzymes and other proteins from thermophiles, except for having higher thermostability, are very similar

to corresponding proteins from mesophiles. The higher thermostability is generally dependent on subtle changes in the composition and sequence of the amino acids and rarely dependent on non-proteinaceous factors. Although over 100 proteins have been purified from thermophiles and compared with corresponding proteins from mesophiles, the exact nature of the higher thermostability has yet to be determined in a protein from a thermophile.

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Bacterial Transformation

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I. Introduction

The purpose of this article is to review the literature on bacterial transformation from approximately 1973 onwards, with special emphasis on the mechanism of uptake of transforming molecules and their incorporation in the genome of the transformable cell. Occasionally, older literature will be cited when this is believed to contribute to a coherent presentation of the subjects to be treated. The earlier literature on bacterial transformation has previously been reviewed extensively by

Tomasz (1969), who emphasized competence, Hotchkiss and Gabor (1970) specially referring to recombination in transformation, and Notani and Setlow (1974) covering bacterial transformation and transfection in considerable detail.

Bacterial transformation is the displacement of a segment of the bacterial chromosome by a homologous, or partly homologous, segment of free deoxyribonucleic acid (DNA) present in the environment of the cell. For the displacement to occur, the DNA molecule has to enter the cell. The uptake of the molecule is believed to be complete if it is no longer susceptible to the action of exogenously added deoxyribonuclease (DNAase). The physiological condition in which the cell is able to bind and to take up the DNA is denoted as competence. After uptake, the transforming DNA molecule interacts with the recipient chromosome in a specific way, such that part of the recipient DNA is replaced by part of the molecule that has entered the cell. Thus, bacterial transformation represents, in principle, one of the simplest recombination systems, in which single donor DNA molecules interact with single cell genomes. After expression of the genetic information contained in the newly incorporated DNA, the cell has acquired a new characteristic which is usually identical with that of the cell from which the donor DNA was derived.

II. Competence

A. RECAPITULATION

Competence, the property of transformable bacteria to bind and to take up DNA in a form resistant to exogenously added DNAase, is a complex and poorly understood phenomenon. Young, logarithmically growing cultures of *Diplococcus pneumoniae, Streptococcus sanguis, Bacillus subtilis* and *Haemophilus influenzae*, the four bacterial species in which competence has been studied most extensively, show little, if any, competence. Usually, competence develops at the point where the logarithmic phase of growth changes over into the stationary phase. In all four systems, exposure of the physiologically non-competent cells to the supernatants of fully competent cultures results in precocious onset of competence development. This phenomenon, often denoted competence-induction, is attributed to the production by the competent cells of physicochemically ill-defined substances. These substances, which are largely species-specific, pass under various names: activator,

competence-(inducing) factor (or substance), competence-stimulating activity. For convenience, it will here be denoted as competence factor (CF). CF's from various sources differ considerably. Thus the CF active in competence development of *D. pneumoniae* has a molecular weight of approximately 10,000 and a net positive charge at pH 7.8; it is heat-labile and is inactivated by proteolytic enzymes such as trypsin, subtilisin and pronase (Tomasz, 1973a). In contrast, CF of *B. subtilis* is of low molecular weight (approximately 700), as could be shown by means of gel- and ultrafiltration techniques (H. Joenje, L. M. F. H. de Ley, J. A. Mulder and G. Venema, unpublished findings). In addition, the CF from *B. subtilis* is resistant to heat and to proteolytic enzymes (Joenje *et al.*, 1972). In these respects the factor resembles that demonstrated in culture fluids of *H. influenzae* (Barnhart, 1967). Competence factor of *S. sanguis*, in certain of its properties, seems to be somewhat intermediate between that of *D. pneumoniae* and that of *B. subtilis*. According to Leonard and Cole (1972) the factor is a small, dialysable, highly basic substance which is destroyed by trypsin treatment. On the basis of its high iso-electric point (higher than pH 11.0), the authors suggest that it may be a protamine or a polymer of basic amino acids. Dobrzański and his colleagues (see Dobrzański, 1972) have estimated its molecular weight to be approximately 5000 and found the factor to be relatively thermostable. In addition, Dobrzański and co-workers (see Dobrzański, 1972) obtained evidence that a second, presumably cell-surface bound factor, iF, obtainable by alkaline cell extraction, is involved in the establishment of competence in *S. sanguis*. The preparations of iF appeared to have DNAase activity.

Competence is primarily a surface phenomenon, involving the binding of DNA molecules and their transport across the cellular envelope. In the four different transformation systems mentioned above, induction of competence by CF has been studied in most detail in the *D. pneumoniae* system by Tomasz and his colleagues. Tomasz (1973a) envisaged the following surface components to be specifically involved in competence: (1) membrane-bound receptors for the CF, (2) a competence antigen, recognizable by means of specific antibodies in serum of rabbits vaccinated with formalin-killed competent pneumococci, preventing binding of radioactive DNA to competent cells, (3) an agglutinin, presumably a protein synthesized following the binding of CF to the CF receptor sites, which may be located between the cell wall and the cellular membrane, and (4) a functioning cell-envelope growth zone.

The appearance of the competence antigen and the agglutinin is believed to be initiated by the binding of CF to its receptor site. Tomasz (1973a) further speculated that the protein synthesis-requiring step, following binding of CF, either produces an as yet unidentified protein involved in DNA uptake or stabilizes a local membrane configuration produced by the binding of the CF. Alternatively, he visualized the essential protein synthesis step producing new autolysin molecules that unmask pre-existing membrane-bound DNA transport sites. One interpretation of the requirement for cell envelope growth zones in the establishment of competence is that the various components engaged in DNA binding and uptake have the proper mutual spatial relationship only in such regions.

B. CONDITIONS AFFECTING COMPETENCE

Competence development is readily affected by a large number of substances; both inhibitory and stimulating substances are known. The same applies to the uptake of native DNA from the medium. In the overwhelming majority of cases, the physiochemical basis is either totally unknown or, at best, surmised. Tables 1 and 2 in the review by Notani and Setlow (1974) list the effects of chemicals on competence development and uptake of DNA, respectively. In addition to environmental conditions, the level of transformation is also dependent on a number of physicochemical properties of the donor DNA, notably DNA-strandedness and length of the donor DNA molecules (see Notani and Setlow. 1974). This section will add a number of recently reported conditions, affecting transformation, to those already compiled.

Bacillus subtilis growing in chemostat culture can acquire competence, but the maximal level of competence, observed at a growth rate corresponding to a doubling time of 2.5 h, was approximately ten-fold less than that observed in batch cultures (López *et al.*, 1975; Portolés *et al.*, 1977). Possibly, the competent cells, which are biosynthetically latent in this organism, are preferentially washed away. Alternatively, CF may be diluted to suboptimal concentrations. Depending on the rate of multiplication, L-arginine appears either to inhibit competence for transfection by phage SPP1 DNA or to be slightly stimulatory. The inhibiting effect seems to be exerted on the level of binding and/or uptake of the DNA (Portolés *et al.*, 1977), and is reversible by Mn^{2+} ions (Espinoza *et al.*, 1976b). Of antibiotics acting on the cell envelope, penicillin and cephalosporin stimulated CF-induced competence in *B.*

subtilis, and polymyxin appeared to inhibit (López *et al.*, 1974). Doses of streptomycin, gentamycin and lincomycin subinhibitory to cell viability also inhibited competence development (Portolés *et al.*, 1974). The effect on competence of adenosine-3′,5′-cyclic monophosphate was also determined in the same studies. This compound appeared to yield ambiguous results, inhibiting CF-induced competence, but stimulating normal competence development. A number of other nucleotides also seem to stimulate uninduced competence development (Pérez Ureña *et al.*, 1976). Germinating spores of *B. subtilis*, growing on agar plates, develop competence only if the genetically required amino acids are present (Tanooka, 1973). *Bacillus subtilis* DNA, which is made available by gently lysing stable L-forms or protoplasts and is presumably of very high molecular weight, was found to be three to ten-fold more efficient in transformation than DNA isolated by conventional methods. With this DNA, widely separated markers could be co-transformed (Bettinger and Young, 1975). High transforming activity of autolysate-DNA of *S. sanguis* had previously been reported by Ranhand and Cole (1971).

A chemically defined medium has been developed in which *Staphylococcus aureus* is transformable. The optimal pH value for transformation was in the range of 6.75–7.0 and the optimal temperature was 30°C. For transformation to occur, a high concentration of $CaCl_2$ is required (0.1M). Transformation in this bacterial species is peculiar, in that there is a strict requirement for lysogeny of the recipient for ϕ11, or ϕ83A. Plasmid and chromosomal markers transform with the same frequency; some variation is found for chromosomal markers. *Nuc* mutants, which, unlike the wild-type organism, do not excrete nuclease into the medium external to the cells, show two additional waves of competence, but transform with approximately the same frequency as the wild-type organism. This frequency is low (maximally approximately 10^{-5}) (Rudin *et al.*, 1974).

Superinfection with ϕ11 of ϕ11 lysogens markedly enhances transfection. *Staph. aureus* lysogenic for a different phage (ϕ12) is not transformable, but can be made competent in supernatants of cells carrying ϕ11 prophage. The competence-stimulating activity in this case, however, has to be attributed to free ϕ11, since anti-ϕ11 serum inactivates the competence-inducing activity (Sjöström *et al.*, 1973). With the aid of temperature-sensitive derivatives of ϕ11, Sjöström and Philipson (1974) were able to show that a particular allele, probably an early gene in the lytic cycle of the phage, controls competence in *Staph.*

aureus. DNA and protein synthesis are required for its expression. The lysogeny-requirement for competence for transformation in *Staph. aureus* is in sharp contrast to the effect of lysogeny on transformation in *B. subtilis* and *S. sanguis*; in both species, transformation is severely decreased if lysogenic recipient strains are used (Peterson and Rutberg, 1969; Yasbin and Young, 1972; Yasbin *et al*., 1973a; Garro and Law, 1974; Parsons *et al*., 1973). In subsequent studies (Garro and Law, 1974; Yasbin *et al*., 1975a) it has been concluded that the low level of transformation of lysogens is probably caused by induction of lytic phage replication in competent cells. The selective elimination of potential transformants is preventable by inhibiting protein synthesis (Yasbin *et al*., 1975a). In addition, and contrary to DNA taken up by non-lysogenic cells, DNA taken up by lysogenic cells is almost completely released into the medium.

Since mutants of ϕ105 and SP02 resistant to spontaneous induction of prophage are fairly well transformable, it has been suggested that competence in *B. subtilis* promotes prophage derepression, leading to cell death and the loss of potential transformants (Garro and Law, 1974). Based on these observations, and previous reports by Harris and Barr (1969, 1971a, b), that a significant proportion of the DNA of competent *B. subtilis* is present in the single-stranded form, Yasbin *et al*. (1975a) proposed a model in which they postulated that during competence development a, possibly multifunctional, enzyme is induced, creating single-strand gaps in the chromosome. This damage would induce both a repair system and prophage, and ultimately repress the gap-creating enzyme. Yasbin *et al*. (1975b) have also related this presumed enzyme to the observations that lysogenic *B. subtilis* strains produce more bacteriophage upon transfection than non-lysogenic strains and that superinfection marker rescue also increases the frequency of transfection. Because superinfection marker rescue is most effective when the cells are just beginning to become competent (little gap-creating enzyme would then be present) and prophage induction would lead to repression of the degrading enzyme thus protecting the transfecting DNA (which is actually observed), it is postulated that the enzyme producing single-strand gaps may be responsible for inactivating transfecting DNA. Sedgwick *et al*. (1975) have also reported that a thymidine-requiring, minicell-producing strain of *H. influenzae* produces many defective phages in response to the competence regime.

In *Neisseria gonorrhoeae*, type 1 and type 2 appear to be highly

transformable, but not type 3 and type 4 (Sparling, 1966). Type 1 is unstable when grown in broth, a large proportion of the cells changing to the non-transformable types 3 and 4. In a chemically-defined medium (La Scolea and Young, 1974) type 1 is stable, and this medium also seems to stimulate, or to maintain, competence. Also, in the chemically-defined medium, the apparent transformation frequency increases substantially, but whether this is real or, for instance, results from cell clumping remains to be investigated (La Scolea *et al.*, 1975). Sparling *et al.* (1977) have determined the fraction of competent gonococcal cells in a culture grown in the chemically defined medium devised by Catlin (1973). It amounted to approximately 35%; grown in proteose-peptone medium, nearly 100% of the culture consisted of competent cells. The authors further determined optimal conditions for competence in this organism. Transformation is dependent on the availability of cations (any of several was sufficient). The optimal pH value was found to be 7.5 and the best temperature was between 35°C and 37°C.

The discovery that treatment with Ca^{2+} ions renders *Escherichia coli* transformable by plasmid-carried markers (Cohen *et al.*, 1972) and chromosomal DNA (Oishi and Cosloy, 1972) has proved to be of significant importance. Transformation is indispensable for the cloning of artificially constructed recombinant DNA molecules in this organism. The current procedure for provoking competence by $CaCl_2$ treatment is approximately the same as that originally employed by Mandel and Higa (1970) to make *E. coli* competent for transfection with phage P2 and λ-DNA. Two stages in the treatment with $CaCl_2$ are distinguished. First, treatment with 10 mM $CaCl_2$ at 0°C, followed by exposing the cells to donor DNA at 42°C in the presence of 30 mM $CaCl_2$. DNA of 10-30 × 10^6 molecular weight appears to have maximal transforming activity. The 42°C step is essential (Cosloy and Oishi, 1973a). Transformation with plasmid DNA of *S. typhimurium* changed in lipopolysaccharide (LPS) composition, is enhanced by pretreatment of the cells with $MgCl_2$ (0.1 M) prior to exposure to 0.1 M $CaCl_2$ (Lederberg and Cohen, 1974). Mutants whose lipopolysaccharide cores have an intermediate degree of incompleteness, seem to be especially efficiently transfectable with phage P22 DNA upon $CaCl_2$ treatment (Bursztyn *et al.*, 1975). An observation resembling the favourable effect of lysogeny on transfection in *B. subtilis* has been made in *E. coli;* transfection with linear phage λ-DNA of *E. coli* K12 lysogenized with λ^{1434} appeared to be approximately 100-fold more efficient than transfection of nonlysogenic strains (Drabkina *et al.*, 1976).

The $CaCl_2$ treatment for making competent bacterial species which are otherwise non-transformable, seems to be widely applicable. With this method, *Pseudomonas putida* also proved transformable with plasmid DNA, although the optimal $CaCl_2$ concentration was found to be higher than that required for *E. coli* (Chakrabarty *et al.*, 1975). Cells remain competent for transfection in chilled 0.05 M $CaCl_2$ for at least one week, and Ca^{2+} treatment is also effective in provoking competence for transfection in *Aerobacter aerogenes* and *Salmonella typhimurium* (Taketo, 1972a). Competence for transfection in *E. coli* can also be obtained by growing the cells in the presence of glycine, which gives rise to sphaeroplasts (Taketo, 1972b), and by incubating a temperature-sensitive cell-wall mutant at the restrictive temperature (Taketo *et al.*, 1972). Treatment with $CaCl_2$ seems to induce preferential uptake of double-stranded DNA. This, and the finding that native calf-thymus DNA strongly inhibits transformation with double-stranded DNA, seems to suggest that double-stranded DNA penetrates in a specific way (Taketo, 1974). Contrary to Ca^{2+}-provoked competence for transformation in *E. coli*, in which the higher temperature step is essential (Cosloy and Oishi, 1973a), the capacity to take up transfecting RFϕA-DNA is destroyed at 37°C, whereas infection at 0°C is readily accomplished (Taketo and Kuno, 1974). Barium ions appear to be even more effective in provoking competence for transfection than Ca^{2+} (Taketo, 1975).

Late growth-phase *E. coli* cells seem to be less transformable than early exponential phase cells (Irbe, quoted by Oishi and Irbe, 1977). This was substantiated by Oishi and Irbe (1977), who showed that the transformability of cells grown in a synthetic medium after resuspension in the same medium plus 0.1% casamino acids showed a sharp optimum after four hours and then dropped precipitously. The optimum was found to be much higher if *tonB* cells were subjected to the same procedure. Since the optimum correlated well with the extent of killing by Ca^{2+}, it was suggested that sensitivity of the recipient cell to Ca^{2+} is involved in provoking competence.

Both the Ca^{2+} and lysozyme-EDTA (Guthrie and Sinsheimer, 1960) methods of rendering cells permeable to transfecting DNA have the drawback of interfering with cell viability. No cell death occurs when *E. coli* is treated with sucrose and albumin, but the frequency of transfection is, in these conditions, approximately 20-fold less than when EDTA and lysozyme are also present (Osowiecki and Skalińska, 1974). These

authors also found that DNA concentrations in excess of 0.2 μg DNA/ml inhibited transfection with λ-DNA and that a 100-fold increase in transfection efficiency could be obtained by addition of protamine sulphate. The effect of basic polymers on the transfecting activity of a variety of DNAs was also investigated by Benzinger (1977). Reasonably high frequencies of transfection could be obtained by treatment of *E. coli* with protamine sulphate and spermine $(HCl)_4$. Transformation of basic polymer-treated cells was unsuccessful, possibly because of sensitivity to plating of the fraction of cells responding to the treatment.

Recently, two reports have been published concerning transformability of *Azotobacter vinelandii* (Page and Sadoff, 1976a, b). Like many other transformable species, *A. vinelandii* appears to be transformable towards the end of the exponential phase of growth. The frequency of transformation is several orders of magnitude less than that regularly obtained in species such as *B. subtilis, D. pneumoniae* and *H. influenzae.* The best results are obtained by exposing the cells to donor DNA on plates; transformation in liquid medium is particularly poor. An unusual feature of this system of transformation is that purified DNA has almost no transforming activity. Calcium ions, by stimulating extracellular polymer and capsule production, affected transformation in a deleterious way. The capsule is presumably a barrier in transformation, because non-encapsulated strains seemed to be optimally (but still relatively poorly) transformable.

C. PROPERTIES OF COMPETENT CELLS

It has been assumed for a relatively long time that, in *D. pneumoniae, S. sanguis* and *H. influenzae*, competent cells do not differ profoundly in physiology from non-competent cells. This is in contrast with *B. subtilis*, where the number of differences is impressive (for references to the older literature: see Tomasz, 1969; Notani and Setlow, 1974, and Venema *et al.*, 1977).

The implication of autolysins in the development of competence in various transformation systems was recently further substantiated. Based on the observation that glycine (approximately 0.03 M final concentration) can induce full competence in *Diplococcus pneumoniae*, Kohoutová (1973a) hypothesized that the competent cell may be considered as a temporary sphaeroplast. More direct evidence that

autolysins are involved in the acquisition of the competent state in this organism was provided by Lacks and Neuberger (1975), who observed that competent cells are rapidly converted to sphaeroplasts in concentrated sugar solutions. In fact, sphaeroplasts formed slower at 37°C than at 30°C in accord with the observation that competence at 37°C is approximately ten-fold less than at 30°C (Lacks and Greenberg, 1973). However, mutants lacking the major lytic enzyme are normally transformable. This observation, apparently conflicting with the idea that autolysin is needed for the acquisition of competence may, according to the authors, be accounted for if the mutant is still endowed with residual activity of the major enzyme, or when a second wall lytic enzyme is present in *D. pneumoniae.* They also observed that some genetically incompetent mutants are slower in sphaeroplasting, but other such mutants are, in their autolytic capacity, very similar to transformable cells. In the second class of mutants, the competence-defect may relate to a requirement different from autolytic potentiality for competence.

Autolysins have been associated with the competent state in *Streptococcus sanguis.* Ranhand (1973) examined seventeen strains of this organism for their ability to develop competence and their ability to autolyse in an alkaline buffer solution. Competence was either spontaneous, or induced with CF obtained from strain Challis. Autolysis was found to be restricted to competent cells, cells that passed their peak of competence and potentially transformable cells (a lysogenic non-transformable strain, transformable after prophage curing). It had been suggested earlier (Schlegel and Slade, 1972) that lysis of competent *S. sanguis* might be due to bacteriocin production. The authors reported later (Schlegel and Slade, 1973) that the streptocin was separable from the CF and had no CF-activity. In a later study, Ranhand (1974a) observed that chemicals combining with sulphydryl groups (mercuric chloride and *N*-ethylmaleimide) both inhibited CF-induced competence and the functioning of autolysin in *S. sanguis.* Both events could be reversed by 2-mercaptoethanol. These results were interpreted as evidence in favour of the need for local, limited autolysis for competence to develop. In addition, Ranhand *et al.* (1970) reported that competence is associated with the synthesis of a new protein and that the chemicals inhibited the biosynthesis of this cell-associated protein of unknown function (Ranhand, 1974a). The synthesis of this protein could not be confirmed by Fuchs *et al.* (1973). As in *D. pneumoniae*, exposure of *S. sanguis* to CF seems to result in reduction of peptidoglycan and RNA

synthesis; if CF-induced competence is prevented, these changes were not observed (Horne and Perry, 1974).

It is conceivable that reduction in peptidoglycan synthesis is related to change in autolytic enzyme activity. The fairly strong evidence for the involvement of autolysin action in the establishment of competence should be evaluated with caution, because it has been reported that incubation of competent *S. sanguis* with trypsin does not result in any appreciable reduction of transformability, whereas susceptibility to autolysis was substantially decreased (Leonard, 1973). Also, Fein and Rogers (1976) have recently isolated *B. subtilis* mutants that are at least 90–95% deficient in the autolytic enzymes *N*-acetylmuramyl-alanine amidase and endo-β-*N*-acetylglucosamidase. However, the mutants were, within the limits tested, apparently normally transformable.

In *Neisseria gonorrhoeae*, in which highly competent types (1 and 2) and very poorly competent types (3 and 4) can be distinguished (Sparling, 1966; Biswas *et al.*, 1977; Sparling *et al.*, 1977), autolytic activity is high, but similar in the two types of cells. However, an interesting difference exists between the two types: the competent types are piliated, the others not (Biswas *et al.*, 1977). The possible role of the pili in competence is not known; purified pili did not bind DNA (Sparling *et al.*, 1977). An interesting parallel concerning the piliated nature of competent cells of *N. gonorrhoeae* is found in *Moraxella nonliquefaciens*, *M. bovis* and *M. kingii*. The fimbriated forms seem to be transformable with much higher frequency than are the non-fimbriated types (Frøholm and Bøvre, 1973). The operation of CF in the gonococcal system of transformation, which is different from many other systems in that the cells are transformable in all stages of growth of the culture (Sparling, 1966), has been suggested (Sidiqui and Goldberg, 1975) but was neither confirmed by Biswas *et al.* (1977) nor by Young *et al.* (1977). In mixed broth cultures of antibiotic-sensitive and resistant strains of *N. gonorrhoeae*, resistance markers are transferred, in all probability by transformation since no transfer was detected in the presence of DNAase (Sarubbi and Sparling, 1974).

Because *N. gonorrhoeae* is highly transformable over the entire growth phase, produces infrequently pseudodouble transformants by congression (independent transformation for two markers on separate DNA molecules) which facilitates genetic analysis, and can be stored at low temperature (permitting genetic analysis on the same sample of cells), the determination of genes that influence the pathogenicity of this organism is expected to be facilitated (Young *et al.*, 1977). Routine

diagnostic testing by means of transformation appears to be feasible (Janik *et al.*, 1976), also for *Moraxella osloensis* (Juni, 1974) and *M. urethralis* (Juni, 1977). Genetic analysis of *N. gonorrhoea* has been started (Sarubbi *et al.*, 1974; Sparling *et al.*, 1975; Maier *et al.*, 1975; Biswas *et al.*, 1976) and a partial map of a number of antibiotic-resistant genes has been constructed (Sparling *et al.*, 1975; Sparling *et al.*, 1977). Transformation for auxotrophic markers, first reported by Catlin (1974), has also become possible owing to the development of chemically defined media (Catlin, 1973), and linkage between a number of auxotrophic markers has been established (Young *et al.*, 1977).

The hypothesis that autolysin action is of major importance in the acquisition of competence has been elaborated in a detailed way by Seto and Tomasz (1975a). They found that exposure to CF leads, in *D. pneumoniae*, to leakage of β-galactosidase, DNAase, autolysin and haemolysin if the exposed cells are subsequently incubated in buffered salt solution containing divalent cations, glucose and albumin, and to protoplast formation if subjected to nutritional- and temperature-shift-up conditions in high concentrations of sucrose. These CF-induced effects are not observed if the bacteria are kept in conditions permitting full growth. It is assumed that the CF changes, or damages, the cell membrane in a subtle way and that this change is amplified by the conditions after exposure. Leakage or transport of (limited amounts) of autolysin would unmask the DNA-binding sites, otherwise hidden by the cell wall. Thus the role of CF would be that of a highly specialized permeability-causing agent. Increased permeability of the membrane of competent cells may also be a property of other transformation systems. In *S. sanguis*, competence appears to be strictly related not only to the capacity to produce CF but also to the presence of a different factor that inactivates transforming DNA (iF). This was established with the aid of streptococcal strains Blackburn, producing CF but no iF ($CF^{+}iF^{-}$), Wicky (CF^{-}, iF^{+}) and the $CF^{+}iF^{+}$ wild type (Cegłowski *et al.*, 1974; Cegłowski and Dobrzański, 1974). The apparently cell surface-located iF activity, obtainable from cells by alkaline washing, was found to be strictly correlated with competence development in the Challis strain and seems to contain two out of three nucleases, all of which are not surface-located in stationary phase non-competent Challis strains.

Consequently, it was suggested that development of competence relates to changes in membrane permeability, permitting two of the nucleases to appear at the outer layers of the cell membrane or in the periplasmic

space (Starościak *et al.*, 1975). Evidence for the increased permeability of the cell envelope of competent cells of *B. subtilis* is provided by the release of DNA into the medium external to the cells when competence develops. The DNA excreted is enriched in markers which are also enriched in cell wall-DNA and membrane-DNA complexes. Certain markers are preferentially more available to the external medium as competence proceeds (Crabb *et al.*, 1977). These markers are close to the replication origin and terminus. The bulk of the cell wall-associated DNA is inactive in transformation (Streips *et al.*, 1977). These findings suggest that the *B. subtilis* chromosome is associated with the external cell layers, which may participate in chromosome replication and segregation during cell division (Streips *et al.*, 1977). Mutants that do not excrete DNA are apparently defective in transformation (Sinha and Iyer, 1971). Interestingly, but unexplained so far, the excreted DNA is not comparable in all respects to DNA obtained by conventional methods, such as extraction with the aid of phenol. This was concluded from the existence of a mutant that transforms perfectly well with conventionally isolated DNA, but not with excreted DNA. Since the mutant also transforms with DNA in lysates, it was additionally concluded that DNA excretion by competent *B. subtilis* cells is not due to lysis of a small part of the competent culture (Streips and Young, 1974).

The fraction of competent cells in competent cultures of *B. subtilis* is relatively low (usually not exceeding 20%); this is in contrast to cultures of *D. pneumoniae, S. sanguis* and *H. influenzae*. This complicates the study of competence, and the determination of properties of competent cells of *B. subtilis*. However, using the renographin centrifugation procedures developed by Cahn and Fox (1968) and Hadden and Nester (1968), the competent cells can be separated from the incompetent cells, thus facilitating the examination of the competent fraction. A large-scale fractionation technique for the separation of competent and non-competent cells has been developed (Joenje *et al.*, 1975). Haseltine and Fox (1971) and Joenje and Venema (1975) have reported that the two classes of cells in competent cultures of *B. subtilis* interact differently with transforming DNA. The non-competent fraction of cells inactivates the unadsorbed fraction of DNA, presumably by double-stranded endonucleolytic scission of the DNA molecules when interacting with the cells in a transient way. Haseltine and Fox (1971) observed that the decrease in length of the transforming molecules external to the cells cannot account completely for the decrease in transforming activity. In

addition to endonucleolytic breakdown, competent cells degrade bound DNA exonucleolytically (Joenje and Venema, 1975). This property is specific for the competent state.

Based on the products generated from DNA interacting with membrane vesicles obtained from competent and non-competent cells, it has been postulated that thymidine phosphorylase is almost completely absent in the vesicles from non-competent cells. Also related to the competent state in *B. subtilis* is the property of membrane vesicles prepared from competent cells of possessing binding sites with high affinity for transforming DNA. Although membrane vesicles from non-competent cells are also capable of binding DNA, the high affinity sites are either absent or substantially less abundant (Joenje *et al.*, 1975).

Furthermore, certain ultrastructural features of *B. subtilis* seem to be subject to change during competence development. It has been reported that the number and frequency of mesosomes that extend into the nuclear region is increased in competent cells as compared to non-competent cells. This was established by means of electron microscope autoradiography of cultures in which the competent cells had been specifically labelled with tritiated labelled donor DNA (Vermeulen and Venema, 1974a). Archer (1973) has shown that competent and pre-competent cells are more heat-sensitive than non-competent cells. One may speculate whether this property of competent cells of *B. subtilis* is related to their generally recognized biosynthetic latency (see Tomasz, 1969) and, possibly, also to their increased permeability.

Apart from the observation that competent cells of *H. influenzae* repair a smaller fraction of ultraviolet-irradiated phage than do the same cells in exponential growth (Setlow and Boling, 1972), little information was available concerning specific properties of competent cells in this bacterium. Recently, LeClerc and Setlow (1974, 1975) have found that, unlike cells in the exponential phase of growth, competent cells contain a special type of DNA containing single stranded regions, as judged from alkaline sucrose gradient sedimentation, benzoylated naphthoylated DEAE cellulose fractionation and digestion with a nuclease specific for single stranded regions in DNA (endonuclease S1 from *Aspergillus oryzae*). The single-stranded regions are not present in a transformation-deficient mutant incapable of associating donor DNA with the recipient chromosome (*rec*2), but are present in *rec*1 mutants, which are able to associate donor and recipient DNA molecules, but are ultimately unable to show the transformed character. These results are interpreted as

indicating that single-stranded regions in DNA of competent cells are important for an early step in the integration of donor DNA into the recipient chromosome. Previously, it had been reported that single-stranded regions also exist in DNA of competent *B. subtilis* cells (Harris and Barr, 1969; Harris and Barr, 1971a, b). Recently, Piechowska *et al.* (1977) have reported that protein is firmly bound to the single stranded regions in DNA of *B. subtilis*, presumably protecting it against nucleolytic attack. The amount of single-stranded DNA showed a tendency to increase in conditions favouring competence development. However, both competent and non-competent cells (separated by renographin-centrifugation) contained approximately the same amount of single stranded DNA, as judged from the extent of DNA digestible with nuclease S1.

Attention has recently also focussed on the composition of the cell envelope of competent and non-competent *H. influenzae* cells. Competent cells of this bacterium appear to have elevated levels of lipopolysaccharide (LPS) as compared to exponentially growing non-competent cells. The change in the amount of LPS requires protein synthesis. Increase is also observed in the total and specific activities of membrane bound succinate- and D-lactate dehydrogenases (Zoon and Scocca, 1975). Cyclic AMP is capable of stimulating competence development (Wise *et al.*, 1973; Zoon *et al.*, 1975). The latter authors have observed that cyclic AMP-induced competence amounts to frequencies of transformation of only 1–5% of those possible by conventional methods. Although, upon induction with cyclic AMP, D-lactate dehydrogenase activity is increased, no change in LPS seems to occur. The protein compositions of cell envelopes of competent and non-competent *H. influenzae* were also examined. Six newly synthesized polypeptides were found to be associated with the development of competence. Two additional ones are synthesized during growth in both competent and non-competent cells but appear specifically in the cell envelope of competent cells (Zoon *et al.*, 1976). It is conceivable that in *H. influenzae* also, increased permeability of the competent cell may account for the incorporation of these polypeptides in the cellular envelope.

D. RECEPTOR SITES FOR COMPETENCE FACTOR

A number of phytohaemagglutinins severely depress the induction of competence by CF in *D. pneumoniae*, presumably by binding to cellular re-

ceptor sites for CF (Kohoutová, 1973b). On the basis of the findings that a number of phytohaemagglutinins (notably those obtained from *Pisum sativum* L. and *Lathyrus sativus* L.) and CF have similar binding activities to the cell surface of *D. pneumoniae*, and that the induction of competence in non-competent cells by CF is inhibited in the presence of high concentrations of D-galactosamine (being the most effective inhibitor), *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, Kohoutová and Kocourek (1973) favoured the idea that the receptor site for CF consists (partly) of hexosamines located in the cell wall. In a further extension of their observations, the authors demonstrated that the inhibitory effect of D-glucosamine and D-galactosamine (but only sometimes, and to a markedly lesser extent *N*-acetyl-D-galactosamine as well) was caused by binding of the hexosamines to the CF and not to the CF receptor sites. This was inferred from the observation that, after having been exposed to the aminosugars, the cells remained completely inducible to competence when fresh CF was added. From these observations, it was concluded that D-glucosamine and D-galactosamine, in their non-acetylated forms, are an integral part of the cell surface receptors for CF (Kohoutová and Kocourek, 1974). The two sugars also prevent spontaneous competence (not experimentally induced by means of CF) in *D. pneumoniae* and prevent the natural agglutination, which normally occurs to some extent in competent cultures of this organism (Kohoutová, 1975a). This observation led Kohoutová (1975a) to assume that the spontaneous agglutination is caused by binding of the CF to the cellular receptor sites. This conclusion is, perhaps, not completely in agreement with the earlier findings (Tomasz and Zanati, 1971; Tomasz, 1973a) that agglutination is dependent on protein synthesis subsequent to CF attachment to the receptor sites. Since protein synthesis was not inhibited in Kohoutová's experiments, it may be doubted whether her conclusion is correct, in the sense that the interaction of CF is primarily responsible for natural agglutination.

Pneumococcal teichoic acid also inhibits induction of competence in physiologically non-competent *D. pneumoniae* cells by virtue of its binding to CF (Rejholcová *et al.*, 1974); however non-*N*-acetylated amino sugars could not be demonstrated in the authors' preparations of the teichoic acid.

The conclusion that amino sugars in the cell wall constitute (part of) the receptor sites for CF is in apparent conflict with the conclusion that the receptor sites are presumably located on the plasma membrane

(Ziegler and Tomasz, 1970; Horne *et al.*, 1977). This conclusion was drawn from a number of observations; for example, *D. pneumoniae* cells are capable of reversible inactivation of CF by binding the CF, and bound CF can be quantitatively recovered by brief heating in a salt solution containing mercaptoethanol. The specificity of the binding is indicated by the observation that CF does not bind to membrane preparations of heterologous systems (*B. subtilis* and group A streptococci). The inhibiting substance could be isolated from the membranes, and was fairly extensively purified and partly characterized. The substance appears to lack proteolytic and nucleolytic activity. Recovery of CF, inactivated by (bound to) formalin-killed cells, was highly specific; CF could only be liberated from the homologous cells. In cell fractions (both from homologous and heterologous sources) only the plasma and mesosomal membranes were capable of binding the CF. Pneumococcal cell walls and teichoic acid preparations were found to be poor inhibitors of CF, whereas some heterologous materials (polyglycerophosphate type lipoteichoic acid from *Lactobacillus fermenti*, and gangliosides) appeared to have a relatively high inhibitory (binding) power. Neither of these substances seems to be present in *D. pneumoniae.*

On the basis of these and similar findings, Horne *et al.* (1977) have questioned the correctness of the conclusion that non-acetylated amino sugars in the cell wall constitute receptor sites for CF (Kohoutová and Kocourek, 1973, 1974). They argue that many substances, alien to *D. pneumoniae* are capable of inactivating (binding) CF, and envisage the possibility that the CF is first captured in a non-specific way by some cell-wall structures (possibly by ionic binding to wall teichoic acids) and that amino compounds may compete with the CF in this binding. The interaction of CF with the membrane-located receptor sites, however, would constitute the biologically important step, *viz.* that initiating the cellular processes leading to the establishment of competence. The binding of CF to the cellular surface seems to proceed quickly; maximal binding was observed after three min of contact of the cells with the factor at 37°C. Within a short period (less than 10 min) the CF is released from the cells. Complete release appears to have occurred at the time of maximal induced competence, which would indicate that CF is not required for maintenance of the competent state (Kohoutová, 1975b). Since the experiments were not aimed at distinguishing between the originally adsorbed and newly synthesized CF (by the induced culture), it may be questioned whether the released quantity of CF completely

consists of the original CF. This question seems reasonable, since in the *S. sanguis* system of competence the production of CF seems to be temporarily inseparable from the concomitant release of free CF into the medium. Contrary to the *D. pneumoniae* system, cell-adsorbed CF seems not to be quickly released into the medium (Leonard, 1973). The possible production of new CF in CF-induced cultures has also been suggested to explain waves of competence observed in *B. subtilis* (Espinoza *et al.*, 1976a). Unless procedures are developed to distinguish between both, the joint presence of original and newly produced CF will continue to impede the interpretation of experiments aimed at determining release, adsorption and, possibly, uptake of CF.

Competence factor (CF) produced by strain Challis of *S. sanguis* binds to sphaeroplasts and isolated membrane fraction of cells inducible to competence by CF, but not to non-CF-inducible strains. This seems to indicate that the receptors for CF in this organism are also membrane-located. On the basis of the sensitivity of the receptors to a variety of chemicals and enzymes, it has been suggested that the receptors are composed of lipid, carbohydrate and protein (Perry, 1974).

E. DNA BINDING AND DNA BINDING SITES

In an indirect way, some properties of the binding sites for DNA in transformation can be deduced from experiments which examine the effects on the level of transformation of addition of nucleic acids to preparations of transforming DNA. The rationale for this approach is the known fact that the presence of homologous, non-transforming DNA (carrying the same genetic markers as the recipient) decreases the level of transformation for markers situated on the transforming DNA if the total concentration of DNA (transforming and non-transforming) saturates the transformation system. The decrease is proportional to the size of the fraction of competing homologous, non-transforming DNA in the DNA mixture.

Such studies have recently indicated that, in the bacterial species studied, the binding sites recognize the DNA in a fairly specific way. Thus it has been found that phage T6 DNA, which is normally glucosylated, is a poor competitor in the *B. subtilis* system of transformation, whereas non-glucosylated T6 DNA competes as strongly as homologous DNA. Evidently the presence of glucose residues on the DNA lowers the affinity of the DNA for the binding sites (Soltyk *et al.*, 1975). Exactly the same is found in *S. sanguis* (strain Challis): glucoslyated DNA from Phage T4 and T6 are poor

competitors (Cegłowski *et al.*, 1975). Also in *D. pneumoniae*, glucosylated T4-DNA does not compete with T7-DNA (Lacks, 1977). However, it has previously been reported that T4-DNA is bound to competent *D. pneumoniae* (Tomasz, 1973b). This discrepancy remains to be resolved.

In *B. subtilis*, interaction of nucleic acids with the cellular surface seems to be limited to DNA-duplexes; double-stranded RNA poorly competes with the binding of transforming DNA. Very little duplex RNA (phage f2 RF DNA and double-stranded RNA from mycophage of *Penicillium chrysogenum*) is bound by recipient cells and, contrary to homologous DNA-binding, the amount bound is independent of temperature and time of exposure to the cells. The lack of competing activity of double-stranded RNA appears not to be due to decrease of the molecular weight of the RNA species upon exposure to competent cells (Soltyk *et al.*, 1976; Sòltyk *et al.*, 1977). In *D. pneumoniae* it was noted that even RNA:DNA hybrid duplexes do not bind to the cells (Lacks, 1977). The highest degree of specificity of DNA binding seems to be exhibited by *H. influenzae*. Scocca *et al.* (1974) have observed that, of a variety of DNAs tested, only that of *H. parainfluenzae* is capable of competitive inhibition. Either the base sequence, or a DNA modification pattern characteristic of members of the genus *Haemophilus* are envisaged as determinants for successful interaction with the competent cell.

From the observation that heating at 48°C of the Challis strain of *S. sanguis* results in a decrease of competition between DNA molecules, it was assumed that some competition takes place in a step after entry of the DNA (Ravin and Ma, 1972). However, it was not entirely excluded that heating does not result in an increased capacity of the cells to adsorb DNA, which might also result in decreased competing ability of the non-transforming DNA. In *B. subtilis*, a binding step was recognized by Dubnau and Cirigliano (1972b), who reported that after initial attachment to competent cells the DNA is sensitive to shearing forces and acquires resistance to shear while still being sensitive to the action of exogenously added DNAase. In the initially attached DNA, obtained from the cell-DNA complexes avoiding shear, a component indistinguishable from the original donor DNA was present, presumably representing terminally attached, shear-sensitive DNA. In the shear-resistant, but DNAase sensitive form, the DNA had suffered double-stranded scissions, giving rise to double-stranded fragments (DSF) of 9.5×10^6 molecular weight. These were produced from DNA having a molecular weight of 90.1×10^6 (Dubnau and Cirigliano, 1972b). More

recent results suggest that the DSF generated from donor DNA of 125×10^6 molecular weight have a molecular weight of approximately 2.1×10^7 (Dubnau, 1976). It was postulated that the DSF were produced by a cell-surface located endonuclease. Scher and Dubnau (1973, 1976) have reported that an endonuclease can be obtained from material released into the medium during protoplasting of *B. subtilis*. The enzyme is stimulated by manganese or calcium ions and introduces a limited number of both double- and single-stranded breaks into DNA, generally generating segments of DNA of 1×10^6 daltons or more in size. It is speculated that this endonuclease may be responsible for the endonucleolytic activity associated with the intact cells. However, it has been suggested that double-stranded breakage may be effected by a presumably different enzyme, which is dependent on Mn^{2+} or Mg^{2+} ions (McCarthy and Nester, 1969; Burke and Spizizen, 1977). Upon re-extraction of donor DNA from the transforming cells, in addition to DSF, single-stranded fragments (SSF) of rather low molecular weight were also detected. These were also thought to be accessible to an externally added mixture of micrococcal nuclease and spleen phosphodiesterase (Dubnau and Cirigliano, 1972a).

Transforming DNA molecules bound to *D. pneumoniae*, still being sensitive to externally added DNAase, can be released from the cells by NaOH or guanidine-HCl, and, similar to *B. subtilis*, contain double-stranded scissions (Morrison and Guild, 1973a). The transforming activity of DNA released by guanidine-HCl treatment, and the DNAase sensitive fraction not releasable by this treatment, was essentially the same as that of DNA mechanically disrupted to the same size as that bound to the cells (Morrison and Guild, 1973b).

The inability to separate DNA-binding from entry of DNA has long hampered further characterization of the binding process, but the observation of Morrison (1971) that EDTA prevents entry of donor DNA in *B. subtilis* without interfering with binding has recently accelerated increase in our knowledge of the DNA-binding step in transformation. Thus it has been reported (Seto *et al.*, 1975b) that, upon binding of DNA to the cellular surface of *D. pneumoniae* in the presence of EDTA, which also prevents entry of DNA in this organism (Seto and Tomasz, 1974), half of the amount of DNA (and probably much more) remains bound to the protoplasts after protoplast formation by pneumococcal autolysin if, in addition to EDTA, glucose was present during the loading of the cells with DNA and treatment with autolysin was also carried out in the

presence of glucose. If glucose was omitted from the lysis mixture, the amount of DNA bound to the protoplasts was substantially diminished. On the basis of these observations, Seto *et al.* (1975b) have suggested that the membrane-located binding sites are stabilizable by glucose and DNA. Because isolated *D. pneumoniae* cell walls and protoplasts bind little DNA, whereas isolated wall-membrane complexes are capable of binding DNA to approximately the same extent as living cells, it was further concluded that the binding sites are largely lost during protoplasting. Stabilization of the binding sites in live cells is believed to be effected by cell wall polysaccharide. This idea is in accord with the conclusion of Ranhand (1974b) who, from studying the effect of sodium metaperiodate on both binding and entry in *S. sanguis*, concluded that the binding sites contain amino acids and are possibly attached to carbohydrate. Cell walls of *S. sanguis* deproteinized with proteolytic enzymes seem to be incapable of binding DNA (Deddish and Slade, 1971).

The molecular weight of DNA does not change as a consequence of binding to pneumococcal cells in the presence of EDTA. The surface location of the transforming DNA is indicated by the observation that the number of potential transformants decreases if the DNA-loaded cells are exposed to polycations and antisera against double-stranded DNA (Seto and Tomasz, 1974).

Similar use of EDTA to dissect the DNA adsorption process into its components has also made it clear that the competitive inhibition of transformation by non-transforming DNA is exerted at the level of binding rather than of entry of DNA, because competition is also observed in the presence of EDTA (Seto and Tomasz, 1974). Deoxyribonucleic acid bound to competent cells in the absence of glucose is sensitive to shear and autolytic enzyme treatment, but in the presence of glucose most of the bound DNA resists release by moderate shear or autolysin treatment. This observation has led to the idea that a superficial binding step (not requiring an energy source) is followed by a step in which the DNA penetrates deeper into the cellular surface (Seto *et al.*, 1975b). The binding of DNA is sensitive to heat, proteolytic enzymes and formaldehyde, which is consistent with the idea that the sites are proteinaceous in nature, or contain protein. With the aid of several methods (such as exposing the cells to conditions inducing leakage of cellular components, and osmotic shock) a DNA-binding factor can be released from the surface of competent pneumococci, which results in loss

of the capacity of the treated bacteria to bind DNA. The factor is not specific for competent cells for it is present in wash fluids of cell wall-membrane complexes of both competent and non-competent cells. Release from intact cells, however, is limited to competent cells. The factor is apparently a protein. Several properties of the protein correspond to properties of the DNA-binding sites of live, competent cells (Seto and Tomasz, 1975b).

As in *D. pneumoniae* and *S. sanguis*, so also in *B. subtilis* does DNA not bind to isolated cell walls (Joenje *et al.*, 1974). These authors also examined the DNA binding properties of membrane vesicles obtained from competent cultures of this bacterium and, from the dose-response relationship between DNA concentration and the extent of binding, they have suggested that binding sites with different affinities for DNA exist on the cellular membrane. The sites with the highest affinities seem to saturate with approximately 1 μg DNA/ml, which is similar to the *in vivo* system. Although part of the DNA attached to the membrane vesicles was resistant to removal by DNAase, the DNA apparently does not penetrate the vesicles, because removal-resistant DNA is degraded by added DNAase. Upon addition of Mg^{2+} (reversing EDTA-imposed inhibition of membrane-located nuclease) double-stranded in addition to single-stranded breaks are introduced in the adsorbed DNA. The DNA-binding capacity is sensitive to formaldehyde. In a subsequent study, Joenje *et al.* (1975) observed that the DNA binding capacity of membrane vesicles isolated from competent cells is substantially higher than that of vesicles derived from non-competent cells. The high affinity sites seem to be especially enriched in the vesicles of competent cells. This may suggest that in *B. subtilis* the DNA-binding sites are not, or not entirely, pre-existant, as is assumed in the unmasking model for competence. Alternatively, it may be that the sites in competent cells are less vulnerable to loss or inactivation by protoplasting, which is a necessary step in the preparation of membrane vesicles.

To determine the topographical location of the binding sites on the cellular surface, autoradiographic techniques and a combination of electron microscopy and biochemical methods have been used. By means of light microscope autoradiography of competent *D. pneumoniae* cells exposed to ^{3}H-DNA, Javor and Tomasz (1968) have established that the sites of uptake of DNA (and possibly of binding as well) are predominantly located in the middle cell region and the cell poles. With the aid of electron microscope autoradiography of cells exposed for a brief

period to ^{3}H-donor DNA, Vermeulen and Venema (1974b) obtained evidence that transforming DNA in *B. subtilis* is primarily attached to mesosomal membrane structures, residing both in the middle regions and tips of the cells. Kinetic analysis of the displacement of the label revealed that, during its migration, the DNA remains associated with the mesosomal structure. The DNA originally located in the cell-tip mesosomes which, unlike the centrally located mesosomes, usually do not extend into the nuclear regions, seems to be first transported peripherally to the cellular equator and from there, via the mesosomes extending into the nuclear region, to the nucleoids. The assumption that the mesosomes, which may be less conspicuous structures *in vivo* than would appear from electron microscope examination of cells stained with osmium tetroxide (Nanninga, 1973), are the primary binding sites of transforming DNA in *B. subtilis* has recently been substantiated by the observations that mesosomal membranes exhibit a higher rate and final level of binding than cytoplasmic (non-mesosomal) membranes and that the dose-response curve of binding to membranes versus DNA concentration is steeper for mesosomal membranes than for cytoplasmic membranes (Hampton and Archer, 1976). A possible interpretation of these results is that the mesosomal membranes contain the high affinity sites for DNA.

Mesosomes are preferentially found in middle regions of *B. subtilis* cells, where cross walls are being formed, and near the tips of the cells. These are, perhaps, the regions associated with synthesis of new cell-wall material. This hypothesis is in accord with observations of Mendelson and Reeve (1973) and Mendelson *et al.* (1975) that the swollen regions, forming at the restrictive temperature in *B. subtilis* carrying a temperature-sensitive rod-mutation and minicell-producing mutation, are preferentially observed near the cell tips and in the central cell regions. Such swollen regions possibly represent the cellular growth zone and the sites where cross walls will arise.

Evidence that DNA binding takes place at the site of cell wall growth had previously been obtained by Tomasz *et al.* (1971), who observed that incorporation of ethanolamine into newly synthesized cell walls strongly inhibited competence (ethanolamine is an analogue of choline, which is required for cell-wall synthesis in *D. pneumoniae*). By exposing ethanolamine-grown cells for a brief period to choline, which restores competence, and subsequent digestion of the newly synthesized cell wall with pneumococcal autolysin, it could be shown that the newly

synthesized cell wall material was located at the cellular equator. Thus, it appears that the DNA binding activity of the cells is intimately associated with the cellular growth zone and, therefore, that functional DNA binding sites are presumably located in these zones.

Thanks to the isolation by Lacks *et al.* (1974) of pneumococcal mutants (*noz*) that lack the major, membrane-located, endonuclease, a significant step forward has been made in the separate analysis of DNA binding and entry of DNA. The DNA in *noz* mutants cannot enter the cell, but the mutants are able to bind the DNA. The bound DNA is double-stranded, but has suffered single-strand breakage (one or other strand). The breaks are separated by 2×10^6 daltons of single-stranded DNA. Breakage also occurs in the presence of EDTA and is a consequence of attachment of the DNA molecules to the cells, which is also observed in endonuclease-proficient cells. The binding requires an energy source, but not divalent cations. Lacks and Greenberg (1976) have proposed an attractive model in which binding protein nicks the DNA and binds to it covalently, possibly at the 5′ end. It is conceivable, as Lacks (1977) has suggested, that the binding protein partly characterized by Seto and Tomasz (1975b) may play a role in the nicking of the transforming DNA.

F. MECHANISM OF ENTRY OF TRANSFORMING DNA

Thanks to the discovery that EDTA prevents the entry of DNA molecules into competent cells of *B. subtilis* (Morrison, 1971) and *D. pneumoniae* (Seto and Tomasz, 1974), and the isolation of mutants of *D. pneumoniae* that are capable of binding, but not taking up DNA (Lacks *et al.*, 1974), substantial progress has recently been made in analysis of the entry step in transformation. The physiological and genetic isolation of the entry step promises understanding of the physicochemical details of the process.

Competent cells of *D. pneumoniae* are both able to take up DNA and to degrade donor DNA to acid-soluble fragments that are released into the medium external to the cells. Because the culture supernatants do not degrade DNA, the nuclease activity is apparently located at the cellular surface (Lacks and Greenberg, 1973). These authors have reported that sugar and Mg^{2+} are essential for both the nucleolytic breakdown of donor DNA and its uptake. In a number of *D. pneumoniae* strains tested, the amount of DNA which entered was equal to the amount of DNA degraded and released into the medium. Similar observations had been reported earlier (Morrison and Guild, 1973a) and are in agreement with

an old idea of Lacks (1962) that, while one strand of the double helix is pulled in by the competent cell, the other is degraded.

In studying the effect of EDTA on binding and entry of donor DNA in *D. pneumoniae* it was observed that EDTA blocks entry and breakdown of DNA to small molecular weight products. Both processes started again upon addition of Ca^{2+} and Mg^{2+} (Seto and Tomasz, 1974; Seto *et al.*, 1975a). These authors further observed that large fragments of donor DNA appeared in the medium upon permitting DNA to enter the cells. The native DNA molecules liberated from the cell surface had undergone molecular weight reduction and, though still endowed with transforming activity, were considerably less active than those of the original donor DNA preparation. Virtually all of the DNA added to competent cultures appeared to be subject to degradation and only competent cells were found to be capable of interacting with donor DNA in this way. The authors also reported that treatment of DNA with nitrous acid and irradiation of DNA with ultraviolet radiation, agents known to induce interstrand crosslinks in duplex DNA, inhibited entry but not degradation of such DNA. This seems to indicate that the two processes are not necessarily interdependent. Based on these observations it was postulated that, in normal transformation of *D. pneumoniae*, DNA molecules attach to the competent cell and, as the result of surface-located nuclease activity, are released into the medium external to the cell. The released molecules may attach again and may be recycled many times in this dynamic process.

Similarities, but also differences, seem to exist between the systems of transformation of *D. pneumoniae* and *B. subtilis*. In agreement with what was observed in the pneumococcal system is the observation that the non-adsorbed fraction of DNA added to competent *B. subtilis* cells, separated from the non-competent fraction by renographin gradient centrifugation, had suffered endonucleolytic scissions across both strands of the DNA. The difference relates to the interaction of the non-competent part of the culture with transforming DNA. Thus, it has been reported (Haseltine and Fox, 1971; Joenje and Venema, 1975) that DNA exposed to the non-competent fraction also suffers double-strand breakage. Apparently, DNA is capable of interacting in a transient way both with competent and non-competent cells of *B. subtilis*, whereas in *D. pneumoniae* this type of interaction is restricted to competent cells only. However, mechanical or enzymic damage to the cell wall or a change in the ionic conditions can induce DNA binding and surface-located nuclease activity also in non-

competent *D. pneumoniae* (Seto *et al.*, 1975b). In addition to double-stranded breakage, DNA interacting with competent *B. subtilis* cells suffers exonucleolytic degradation (Dubnau and Cirigliano, 1972a; Joenje and Venema, 1975). The amount of acid-soluble breakdown products vastly exceeds the quantity of DNA entering the cells (Joenje and Venema, 1975). It seems, therefore, that only a minor fraction of DNA escapes total degradation and enters into the cell or, conversely, that this DNA escapes destruction because it enters the competent cell. Entry of donor DNA into *S. sanguis* also seems to be accompanied by the production of acid-soluble DNA fragments (Ranhand, 1976).

Strong evidence that nuclease activity is required for entry of transforming DNA in *D. pneumoniae* was obtained by Lacks *et al.* (1974). Lacks and his co-workers succeeded in the isolation of mutants (*noz*) from a mutant already substantially deficient in nuclease activity. (The designation *noz* refers to the inability of the mutant colonies to form a colourless zone on DNA-methyl green plates.) The level of transformation in the *noz* mutants is strongly decreased (mostly between 0.1–1% of the parental strain) and their capacity to take up DNA is correlated with this decrease. However, the mutants are perfectly able to bind DNA. The mutants lack the major endonuclease, or very nearly so, whereas their exonuclease activity is, relative to the parental strain, only slightly diminished. This strongly indicates that the major endonuclease is required for entry of donor DNA into *D. pneumoniae*. In addition, the acid-soluble breakdown products of DNA exposed to the enzyme are very similar to those produced during entry of DNA; they consist of oligonucleotides containing 1–10 residues. The nuclease is presumably membrane located (Lacks and Neuberger, 1975). In addition to oligonucleotides, the wild type and those mutant endonucleases endowed with residual activity produce double- and single-stranded breaks in DNA *in vitro*. Both types of enzymes are dependent on the presence of divalent cations; Mn^{2+} can replace Mg^{2+}, but Ca^{2+} is not effective, and RNA inhibits their activity. The residual activity of the endonuclease in *noz* mutants has to be zero, or very nearly so, in order to endow the mutants with the DNA-entry deficient phenotype. Even if the residual activity of the endonuclease is as low as 1.4%, the mutants are still nearly normally transformable. Genetic analysis has indicated that the *noz* mutations in all probability reside in the locus specifying the major endonuclease (Lacks, 1974; Lacks *et al.*, 1975).

The cation requirement for entry of DNA into *D. pneumoniae* seems

somewhat controversial at the moment. Seto and Tomasz (1976) have reported that Ca^{2+} ions are required and that Ca^{2+} can not be replaced by Mg^{2+}. The latter ions would rather stimulate loss of attached DNA to the external medium, which seems to be limited in the presence of Ca^{2+} alone. On the other hand, Lacks (1977) has reported that entry of DNA in the presence of Ca^{2+} is only 1% of that possible in the presence of Mg^{2+}. As Lacks (1977) has pointed out, this controversy may be due to differences in the composition of the washing fluids used prior to testing the effect of various cations on entry of DNA. If possible, preferential retention, either of Ca^{2+} or of Mg^{2+}, is prevented, and, when care is taken to grow the cells in micro-crystal-free medium, it appears that an absolute requirement exists for Ca^{2+}, Mg^{2+} and K^{+}. Also an energy source seems to be required (Lacks and Greenberg, 1973).

On the basis of the properties of the *noz* mutants referred to earlier, Lacks and Greenberg (1976) have proposed an attractive model for entry of DNA into competent cells, which accounts for a number of features of the *D. pneumoniae* transformation system and may extend to that of *B. subtilis* as well. The model postulates that the endonuclease, situated adjacent to the binding protein, cuts the strand opposite to the break produced as a consequence of binding. The bound DNA strand can then enter the cell, while the immobile endonuclease digests the opposite strand to produce oligonucleotides which are liberated into the medium external to the cell. The motive force for entry may be sequential attachment and hydrolysis of the strand with polarity opposite to the one entering and, if the DNA molecules are attached to the cell surface at several sites such that the extremities of the molecule are loose, their ends may be excluded, provided that entry is initiated in the adjacent segment of DNA.

III. Intracellular Fate of Transforming DNA

A. RECAPITULATION

Intracellular transforming DNA is defined as transforming DNA which has become resistant to removal from the cell-surface by exogenously added pancreatic DNAase. Whether the newly entered DNA is really intracellular, in the sense that it has lost association with the cellular membrane, is uncertain. The definition of intracellularity is therefore an operational one.

The fate of transforming DNA can be followed in two ways; namely

physicochemically and genetically, which are frequently combined. Physicochemical methods often involve the following basic procedure: competent cultures are exposed for a certain period to donor DNA labeled with a heavy and a radioactive isotope. Further uptake of DNA by the cells is prevented by addition of DNAase, which is subsequently removed by extensive washing in the cold. The transforming cells are then lysed and the structure of the entered donor DNA is examined by means of isopycnic centrifugation. This enables one to distinguish whether the DNA is present in the double-stranded or single-stranded form, or whether association of the DNA with other cellular constituents has occurred. The size of the transforming DNA in the lysate is usually estimated by means of sucrose-gradient sedimentation analysis. By measuring the transforming activity of donor markers in lysates obtained at various intervals after addition of DNAase, supplementary information can be obtained concerning the state of the donor DNA after entry into competent cells. If the donor DNA and the recipient are suitably marked genetically (a linked marker pair a^+b^- on the transforming DNA and an a^-b^+ pair on the recipient genome of a transformable auxotrophic bacterium), the production in the recipient cells of recombinant-type molecules (a^+b^+) as a function of time can be followed by assaying joint a^+b^+ activity in the lysates on an a^-b^- second recipient. By concerted use of the physicochemical and the biological methods of assay of the lysates from transforming cells, it has been concluded, and verified on several occasions, that in the process of genetic recombination one strand of the transforming DNA ultimately replaces a homologous part of one of the strands of the recipient chromosome. The recombinant chromosome is, therefore, a heteroduplex molecule, containing the recipient's genetic information in one strand and the donor genetic information in the complementary strand of DNA. A heteroduplex molecule of DNA as the end product of genetic recombination has been identified in *D. pneumoniae, H. influenzae* and *B. subtilis*, the transformable species in which the fate of transforming DNA has been investigated most extensively. Also, the kinetics of recombinant molecule formation in the three species are closely similar; integration of donor DNA is completed within approximately 30 min at 37°C. In the interval between entry of transforming DNA and formation of heteroduplex molecules, the transforming DNA is re-obtainable from *D. pneumoniae* cells as high molecular weight, single-stranded DNA. At the time of completion of Notani and Setlow's review (1974), the state of the

donor DNA after entry in *B. subtilis* was still controversial; both double-stranded and single-stranded DNA had been identified after entry of the transforming DNA. After entry, donor DNA re-extracted from *H. influenzae* had, on several occasions, been shown to exist in the double-stranded form.

The double-strandedness of intracellular transforming DNA in *H. influenzae* corresponds well with the observation that, immediately after uptake following a short period of exposure to donor DNA, the biological activity of the DNA has not suffered substantially. This is in sharp contrast to the situation in *D. pneumoniae* and *B. subtilis*, in which transforming activity of re-extracted donor DNA is initially almost absent and recovers as a function of time of re-extraction. This transient loss of transforming activity is known as the eclipse of donor DNA.

In addition to physicochemical examination and assay of the biological activity of re-extracted donor DNA in transformation-proficient cells, mutants defective in transformation have also been used to identify the various steps leading to production of heteroduplex recombinant DNA molecules. Mutants which are capable of taking up DNA but which are nevertheless deficient in transformation should be useful for this purpose. By the use of such mutants the existence of at least three steps in the process of transformation subsequent to the entry of DNA have been presumptively identified: (1) step(s) leading to association of the donor DNA and the recipient chromosome; (2) step(s) completing the integration of the donor DNA; and (3) replication and/or expression of the donor DNA (Notani and Setlow, 1974).

B. MACROMOLECULAR STATE OF TRANSFORMING DNA SHORTLY AFTER ENTRY

Characterization of the physical state of transforming DNA shortly after uptake by competent cells of *D. pneumoniae* proved relatively straightforward. By means of methylated albumin kieselguhr column chromatography, Lacks concluded, as early as 1962, that re-extracted transforming DNA was present as single-stranded DNA in lysates of transforming cells, and subsequent investigations (Fox and Allen, 1964; Lacks *et al.*, 1967; Ghei and Lacks, 1967) have confirmed this conclusion. In accord with this conclusion is that the donor marker activity eclipses in *D. pneumoniae* (Fox, 1960; Lacks, 1962).

The question whether donor DNA in *B. subtilis* is single- or double-

stranded just after entry yielded different answers. Thus, it has been reported that duplex and single-stranded DNA could be recovered from transforming cells (Bodmer and Ganesan, 1964) or duplex DNA only (Pène and Romig, 1964; Ayad and Barker, 1969; Arwert and Venema, 1973a). One report claimed the recovery of single-stranded donor DNA (Buc, 1965), and Harris and Barr (1971b) observed single-stranded DNA only once out of eight repeats.

In a series of investigations aimed at resolving this controversy and to provide further information concerning the mechanisms of integration of transforming DNA in *B. subtilis*, Dubnau and Davidoff-Abelson (1971) observed, in sucrose gradients of lysates of transforming cells which had been exposed for a short time to donor DNA and quickly lysed thereafter (transformation had *not* been stopped by DNAase), that two slowly sedimenting bands of donor origin were present. The slowly sedimenting bands contained only 1–2% of the original donor DNA activity. In further characterizing the nature of the donor DNA in these bands (transformation was stopped in these experiments by an excess of salmon sperm DNA), the slowest sedimenting band appeared to consist of low-molecular-weight single-stranded DNA fragments (SSF, ranging between 9.6×10^4–2.5×10^5 daltons), whereas the faster sedimenting material consisted of native donor DNA of 9.5×10^6 daltons molecular weight in neutral, and 3.1×10^6 daltons in alkaline sucrose gradients. Experiments aimed at resolving the question whether the low molecular-weight single-stranded DNA originated from the degradation of a high molecular-weight single-stranded precursor during lysis or from manipulation of the lysates tended to exclude both of these possibilities (Dubnau and Cirigliano, 1972a).

The first clear evidence that transforming DNA after entry into *B. subtilis* can be recovered as high molecular-weight single-stranded DNA was obtained by Piechowska and Fox (1971) who observed, upon isopycnic gradient centrifugation of lysates of transforming cells when DNA uptake had been terminated with exogenous DNAase, that the donor DNA banded broadly from the position of single-stranded DNA up to the meniscus of the gradient, indicating that donor DNA is complexed to a cellular constituent. Heating of the lysates to 70°C in 4M sodium chloride and treatment with sarcosyl dissociated the complex, and a distinct band of single-stranded DNA was observed in casesium chloride gradients. Sedimentation gradient analysis showed that the single-stranded DNA in the complex was not associated with the recipient chromosome. It further

appeared that, in reconstruction experiments involving addition of denatured DNA to lysing cells, the denatured DNA acquired buoyant densities similar to those of the complex obtained from the transforming cells. Apparently, cellular components exist with high affinity for single-stranded DNA, which may mask its specific density. In the experiments of Piechowska and Fox, no evidence was obtained for the presence of double-stranded DNA, suggesting that double-stranded fragments (DSF) are generated from transforming DNA prior to entry. This inference is in accord with conclusions reached by Davidoff-Abelson and Dubnau (1973a, b) and Buitenwerf and Venema (1977a). Davidoff-Abelson and Dubnau (1973a) have reported that breakdown of high-molecular weight single-stranded DNA is prevented if certain basic proteins, such as lysozyme or cytochrome *c* are present when the cells are lysed. Under such conditions the molecular weight of the single-stranded DNA ranges from 2—5 × 10^6. Kinetic analysis of the products of transforming DNA at 37°C indicated that one to two minutes after formation of DSF, DSF are converted to single-stranded DNA which is an intermediate in *B. subtilis* transformation (Davidoff-Abelson and Dubnau, 1973b). Subsequent to entry, heterologous *E. coli* DNA (Lacks *et al.*, 1967) and non-glucosylated phage T6 DNA (Piechowska *et al.*, 1975) are also present as single-stranded DNA.

Complexing of intracellular donor DNA with a cellular constituent, probably (a) cell membrane component(s), has also been reported by Harris and Barr (1971b) and Dooley and Nester (1973). Harris and Barr (1971b) found that shortly after entry 30–35% of the total donor DNA was associated with the membrane and that the donor DNA preferentially associated with interphase material of lysates from transforming cells treated with phenol. Arwert and Venema (1973a) observed that donor radioactivity in lysates of transforming cells was initially located close to the meniscus of caesium chloride gradients, also indicating that cellular constituents are complexed with donor DNA.

The experimental results cited above constitute convincing evidence that transforming DNA in *B. subtilis* becomes single-stranded upon entry and is complexed to (a) cellular, probably membranous component(s). Based on the observation that, unlike *E. coli* and non-glucosylated phage T6 DNA, glucosylated T6 DNA is poorly taken up by competent cells of *B. subtilis* and that, in reconstitution experiments, single-stranded glucosylated T6 DNA is unable to complex with the cellular constituent(s) in lysates of competent cultures, Soltyk *et al.* (1975)

speculated that the constituent(s) might function in uptake of DNA. A similar suggestion has been made by Eisenstadt *et al.* (1975), who found a protein activity in soluble extracts of competent *B. subtilis* which protects denatured DNA from solubilization by nuclease. This activity was not found in physiologically non-competent cells and in a non-competent mutant. Recently, Pieniazek *et al.* (1977) examined the physical state of the entered donor DNA and reached the conclusion that the single-stranded DNA, when complexed to cellular constituents, is partially resistant to DNAase. The constituents adhering to the single-stranded DNA are believed to originate both from the cell wall and the plasma membrane, and are endowed with autolytic and endonucleolytic activity. An artificial complex between single-stranded DNA and cellular constituents, which is formed by adding single-stranded DNA to cells prior to lysis, does not show the two types of enzymic activity, possibly indicating that the two activities are relevant to the processes of penetration and uptake of donor DNA. Also, in *D. pneumoniae*, a complex between single-stranded DNA and undetermined cellular constituents (eclipse complex) has been demonstrated (Morrison, 1977). The complex has a lower affinity for DEAE-cellulose and hydroxypatite than does single-stranded DNA, shows increased sedimentation in sucrose gradients, and has a lower buoyant density in caesium sulphate gradients. Various treatments dissociate the complex, but it is resistant to ribonuclease.

So far, only one report has dealt with the fate of transforming DNA in *S. sanguis* (Raina and Ravin, 1977). It was observed that the transforming activity of donor DNA, like that in *D. pneumoniae* and *B. subtilis*, eclipses and that the unintegrated DNA is bound to some cellular constituent in a form resistant to exogenous DNAase. Like the complex formed between SSF and the cellular constituent in *B. subtilis*, the re-extracted *S. sanguis* donor DNA strongly trails to the lighter positions in caesium chloride gradients and, although no single-stranded DNA could be detected, it would be of interest to examine the state of the donor DNA in the complex by subjecting the lysates of transforming cells to the procedure used by Piechowska and Fox (1971).

No truly single-stranded intermediate is formed in *H. influenzae* as a consequence of entry of transforming DNA (Stuy, 1965; Notani and Goodgal, 1966). In accord with the double-stranded nature of entered DNA, its biological activity is not subject to eclipse; instead, the biological activity of the donor DNA decreases to approximately 25% as a

function of time of re-extraction (Voll and Goodgal, 1965; Kooistra and Venema, 1971; Sedgwick and Setlow, 1976). Based on the observations: (1) that integration of donor DNA in *H. influenzae* is blocked at 17°C (Voll and Goodgal, 1965; Kooistra and Venema, 1971) but that the inactivation of donor markers continues; and (2) that during the inactivation mononucleotides are generated from the entered DNA and that the formation of these breakdown products preceeds the integration of the donor DNA, Kooistra and Venema (1971) have proposed that single-stranded regions are introduced into the donor DNA prior to its integration. Evidence for this hypothesis was provided by Sedgwick and Setlow (1976), who showed that approximately 15% of the donor DNA is sensitive to S1 endonuclease digestion. Apparently, the single-stranded regions are at the ends of the donor DNA molecules.

The single-strandedness decreases at 37°C to approximately 4% in 30 min, presumably by base pairing of the single-stranded ends with complementary single-stranded regions which appear to be characteristic for the chromosome of the competent *H. influenzae* cells (LeClerc and Setlow, 1974, 1975). This decrease is not observed at 17°C, when integration of DNA is blocked (Sedgwick and Setlow, 1976).

Because phage HP1 DNA in lysogenic (HP1) and non-lysogenic *H. influenzae* recipients is broken down with the same kinetics and almost to the same extent, whereas in the lysogenic recipient HP1 DNA can be integrated, Stuy (1974) does not subscribe to the proposition that breakdown of donor DNA is necessary if it is subsequently to associate with the recipient chromosome.

C. INTEGRATION OF TRANSFORMING DNA

1. *Synapsis of donor and recipient DNA*

The obvious mechanism for initiation of integration of transforming DNA is contained in the possibility of pairing of complementary base sequences of the donor DNA and the recipient chromosome. Because entered donor DNA in *D. pneumoniae* and *B. subtilis* is completely single-stranded, and seems to be partially so in *H. influenzae*, potential pairing ability with the recipient chromosome is abundantly available in the intracellular donor DNA. The exposure of unpaired base sequences in the recipient chromosome is less obvious. However, both in competent *B. subtilis* (Harris and Barr, 1969; 1971a, b; Piechowska *et al.*, 1977) and in *H. influenzae* (LeClerc and Setlow, 1974, 1975), the chromosomal DNA

seems to carry single-stranded regions, thus providing opportunities to pair with the donor DNA and to initiate integration. No rigorous proof has been presented as to whether the single-stranded regions of chromosomal DNA are functional in initiating integration of the donor DNA. However in *H. influenzae*, suggestive evidence has been obtained that such regions are involved in integration. The evidence is derived from experiments in which it was shown that the chromosome contains gaps and single-strand tails in DNA pulse-labeled during competence, and that newly integrated donor DNA is predominantly, but not exclusively, associated with this DNA. In addition, *rec*2 mutants of *H. influenzae*, which are incapable of associating the donor and recipient DNA, do not contain single-stranded regions (LeClerc and Setlow, 1974, 1975). At 17°C, no association (covalent or noncovalent) of donor DNA with the chromosome occurs, although single-stranded ends are produced in donor DNA. Under conditions permitting integration (higher temperature), the amount of single-stranded regions in the donor DNA decreases, most probably as a consequence of pairing (Sedgwick and Setlow, 1976). These authors suggested that the single-stranded regions in the donor DNA were formed as a consequence of interaction with the single-strand gaps in the recipient chromosome. This would also be the case at 17°C, but stable pairing of the donor and recipient DNA would not now occur.

Although the importance of single-stranded chromosomal regions for integration of donor DNA in *H. influenzae* seems probable, it appears that there is no need to invoke such regions as an absolute requirement for initiation of integration. Teitelbaum and Englander (1975a, b) have studied hydrogen exchange in double-helical artificial polynucleotides, and reached the conclusion that long-lived, large travelling loops exist, that are trapped by twisting of the sugar-phosphate backbone. On the basis of published hydrogen-exchange results, it appears that the same kind of open state (the large, stacked, random migrating loop) also exists in DNA. It seems that the kinetic model of DNA postulated by Teitelbaum and Englander (1975a, b) meets many of the requirements necessary to ensure initiation of integration of transforming DNA. A second mechanism for initiating integration has been suggested by Holloman *et al.* (1975), who observed that small single-stranded homologous fragments (25–80 nucleotides in length) can be assimilated into superhelical DNA of phage ϕX174. Superhelical regions of DNA seem to behave as if they were transiently single-stranded (Dean and

Lebowitz, 1971; Beard *et al.*, 1973; Beerman and Lebowitz, 1973; Kato *et al.*, 1973; Wang, 1974; Wiegand *et al.*, 1974) and have also been observed in the folded chromosome of *E. coli* (Worcel and Burgi, 1972; Worcel *et al.*, 1973; Pettijohn and Hecht, 1973). Holloman *et al.* (1975) observed that random nicking of the superhelical DNA liberates the assimilated fragments of DNA. Stabilization is thought to occur by nicking the recipient chromosome at the site of triple-strandedness and enzymic digestion of the redundant recipient strand. A series of recent papers (Beattie *et al.*, 1977; Wiegand *et al.*, 1977; Radding *et al.*, 1977) reports further properties of the system of assimilation of single-stranded DNA in superhelical phage DNA. Using relatively large molecular-weight single-stranded pieces of DNA (400 or more nucleotide residues of DNA), it was found that, as a consequence of assimilation into the superhelical RF1 DNA of phage G4, the superhelical turns had disappeared. The properties of such complexes are reconcilable with a relaxed circular molecule of closed phage DNA in which one strand is displaced for a length corresponding to the region of base-paired single-stranded homologous DNA. The association was found to be reversible, and the rate-limiting step of assimilation is probably the frequency of opening of the supercoiled helix. At physiological temperatures, the rate of association is extremely low, which suggests that, if initiation of recombination *in vivo* occurs through assimilation of single-stranded DNA into superhelical domains, conditions exist which facilitate opening of the DNA helix (Beattie *et al.*, 1977). The displacement loop was visualized by electron microscopy and the effect has been studied of a number of nucleolytic enzymes on the assimilation-complex (Wiegand *et al.*, 1977). Pancreatic DNAase (and heat) dissociated single-stranded DNA from the complex; exonuclease VII appeared to remove the non base-paired single-stranded moiety while the displacement loop remained intact, and treatment with nuclease S1 or ATP-dependent DNAase stabilized the association. Deoxyribonucleic acid ligase treatment of the nuclease S1-stabilized complex was found to link single-stranded DNA covalently to a strand of the recipient RF molecule. Relaxation of the recipient DNA in the complex by random nicking by pancreatic DNAase leads to dissociation, provided that the displacement loop is intact. Dissociation of the complex by DNAase-treatment appears to be biphasic; a fraction of the complexes dissociates in a few seconds (possibly representing complexes in which the displacement loop was close to the end of the assimilated strand), whereas the rest dissociates

considerably more slowly. This fraction may represent complexes in which the displacement loop is trapped. It was observed that at 37°C in 0.15 M sodium chloride certain complexes may survive for at least one minute (Radding *et al.*, 1977).

Indirect evidence for the existence of a stabilizable state of association of donor and recipient DNA (synaps) has been obtained in *D. pneumoniae* by Collins and Guild (1972) and Shoemaker and Guild (1972), who observed that the transforming activity of DNA of low molecular-weight taken up by cells at 37°C is partially lost when the cells are subsequently incubated at 25°C. If the cells, transforming at 25°C, are pulse-exposed to 37°C (the period required is only seconds) no loss of transforming activity could be found. It is suggested that at 25°C the donor DNA has synapsed with the recipient chromosome in a reversible state. This labile complex is thought to be rendered stable by pulse-exposure to 37°C, which would lead to initiation of further displacement of the recipient strand, preventing reversal of the association between donor and recipient DNA.

In a recent study, (Popowski and Venema, 1978) more direct evidence has been obtained for formation of a synaptic DNA structure during transformation of *B. subtilis*. The authors have argued that, in standard re-extraction experiments, unstable complexes of donor DNA and recipient DNA might dissociate during lysis and subsequent manipulation of the lysates, and escape detection for that reason; this labile state of association might be rescued if it were possible to stabilize it artificially. Two methods were tried to achieve stabilization: (1) irradiation with high doses of short-wave ultraviolet radiation in the cold of transforming cells prior to lysis; and (2) treatment with 4,5′,-8-trimethylpsoralen in conjunction with long wave ultraviolet irradiation of the lysates of transforming cells. Both agents are capable of crosslinking complementary strands of DNA. In both instances, it could be demonstrated that these treatments cause an increase in the amount of donor label banding in caesium chloride gradients at the position expected for donor-recipient complex of DNA. Since this effect was not observed if the cells had taken up *E. coli* DNA, it would appear that the artificially stabilized complex is specific. Because the complex was also formed upon long-wave ultraviolet irradiation of psoralen-treated lysates (which treatment is known to cause little DNA-protein crosslinking), it is believed that the donor moiety in the labile complex is base-paired to the recipient DNA.

In a recent study, Seto and Tomasz (1977) have observed that

treatment of transforming *D. pneumoniae* cells with a number of DNA-intercalating agents shortly after entry of donor DNA inhibits the appearance of transformed cells. The substances do not seem to prevent association between the donor and recipient DNA. It is suggested that the donor-recipient complexes contain single-stranded, not base-paired, segments of DNA.

2. *Stable intermediate complexes of donor and recipient DNA*

Stable complexes of donor and recipient DNA are defined here as complexes which resist lysis and further treatment of the lysates, such as deproteinization, exposure to moderate temperature, or fractionation procedures such as column chromatography and velocity- and density-gradient centrifugations.

After the labile association of donor and recipient DNA in *B. subtilis*, referred to in a previous section, the complex apparently passes over into a stable structure of the two components (donor-recipient complex, DRC; Dubnau and Davidoff-Abelson, 1971; Davidoff-Abelson and Dubnau, 1973b; Harris and Barr, 1971b; Piechowska and Fox, 1971; Dubnau and Cirigliano, 1973a; Arwert and Venema, 1973a). Apparently, either the extent of base-pairing between the donor and recipient moieties of DNA is such that dissociation of the complex is prevented during experimental manipulation, or the two components are held together by covalent bonds.

With respect to the physical properties of the stable complex, it is certain that at least two forms can be distinguished. Firstly, a state of the complex has been recognized with decreased biological activity (Dubnau and Davidoff-Abelson, 1971). In this state, the donor DNA can still be separated from the resident moiety during alkaline sucrose and caesium chloride gradient centrifugation (Dubnau and Cirigliano, 1973a). The presence of single-stranded nicks, separating the donor moiety from the recipient moiety, has been inferred by Arwert and Venema (1973b), who observed that incubation of re-extracted DNA with DNA-ligase increased both the donor marker and recombinant type activity of the DNA. Because DNA-ligase and DNA polymerase jointly increased these activities no more than did DNA-ligase alone, it seems that the discontinuities which separate the donor and recipient parts of the complex are predominantly nicks rather than gaps.

The absence of gaps adjacent to the inserted segment of donor DNA

seems, in general, to be supported by the ineffectiveness of inhibitors of DNA synthesis on completion of integration and the transformation proficiency of mutants defective in DNA synthesis. Thus, in *B. subtilis*, Dubnau and Cirigliano (1973b) neither observed an effect of 6-(*p*-hydroxyphenylazo)-uracil (HPU, an inhibitor of DNA polymerase III) on the amount of DRC formed nor, more importantly, on the biological activity of molecules jointly carrying a donor and a recipient marker—which was established by exposing recipients to the lysates of cells transforming in the presence of the drug. This seems to imply that no sizeable discontinuities persisted in the DNA spanning the two markers. Essentially similar observations have been made by Levin and Landman (1973). Recently, Canosi *et al.* (1976) observed a relatively small effect of HPU on the frequency of transformation to HPU resistance, using donor DNA obtained from a HPU-resistant strain, when the drug was also present in the selective agar plates. From this, the authors concluded that DNA polymerase III may be involved in a late stage of the recombination process. However, in the absence of data concerning the possible differential effect of HPU on survival of competent and noncompetent *B. subtilis*, this conclusion should be viewed with caution. The DNA repair-synthesis by DNA-polymerase I does not seem to be required for successful transformation, since *pol* A mutants are equally well transformable as wild-type cells (Laipis and Ganesan, 1972; Hadden, 1973; Villani *et al.*, 1974). Although, in general, these data do not support a requirement for DNA-synthesis in transformation, the possibility that some residual DNA-synthesis is required cannot be dismissed completely. In approaches such as those indicated above, the level of DNA synthesis escaping from inhibition by drugs, or possible slight leakiness of the mutant strains used, may suffice to allow little, but essential, DNA-synthesis.

Recently, evidence has been obtained for the existence of a novel, stable intermediate in transformation of *B. subtilis*, in which the single-stranded donor DNA is only partly base-paired to the recipient chromosome (Buitenwerf and Venema, 1977a, b, 1978). These authors observed that cells preloaded with transforming DNA at 30°C with the aid of EDTA, and subsequently transferred to 17°C under conditions permitting entry of the DNA (by adding an excess of Mg^{2+}), accumulate a stable complex of donor and recipient DNA which has little biological activity and has a buoyant density considerably higher than the heteroduplex molecules of DNA finally formed. If the cells are transferred to 30°C, a complex with

normal buoyant density and full biological activity are formed. These results suggest that the complex formed at 17°C contains single-stranded regions (Buitenwerf and Venema, 1977b, 1978). To determine whether or not the single-strandedness resided in the donor moiety, the isolated complex was incubated with nuclease S1. It was found that donor radioactivity is liberated from the complex and that incubation of the lysates with nuclease S1 also reduces the donor marker and recombinant-type activity. On the basis of these results, it has been suggested that only part of the donor DNA was base-paired to the recipient chromosome. Alkaline sucrose and CsCl gradient centrifugation showed that only a small part of the donor DNA could be displaced from the complex, which seems to indicate that the partly integrated donor DNA was already covalently bound to the recipient DNA (Buitenwerf and Venema, 1978).

Little is known about the location of the integration process, but it has been reported (Dooley and Nester, 1973) that recombinant type activity (that is, the biological activity of molecules jointly carrying a donor and a recipient marker) is enriched by a factor of 1.5–1.7 in the membrane fractions of transforming *B. subtilis* cells. This enrichment persists for at least 30 min after terminating DNA uptake, suggesting that integration occurs at, or close to, the cellular membrane.

3. *The end product of integration: heteroduplex DNA*

Both physicochemical and biological evidence indicate that the end product of integration consists of a heteroduplex molecule of DNA. It is not certain whether there exist, amongst the three best studied bacterial species, differences in the average length of the donor single-strand in the heteroduplex molecules formed, for the methods employed for measuring the size of the integrated segment give only an approximation. From the probability of a recombination event, Lacks (1966) has calculated that the average length of integrated donor DNA in *D. pneumoniae* is approximately 2000 nucleotides (approximately 7.1×10^5 daltons). Based on density shifts in CsCl gradient centrifugation of fragmented re-extracted DNA, Gurney and Fox (1968) arrived at a higher value. They estimated that the weight-average molecular weight of integrated DNA was $1.5–3.0 \times 10^6$ daltons. Also based on density displacement measurements, Notani and Goodgal (1966) have estimated, in the *H. influenzae* system of transformation, that the size of the integrated region is approximately 6×10^6 daltons. Using the same technique, Dubnau and

Cirigliano (1972c) have estimated that *B. subtilis* integrates, on the average, segments of donor DNA of 2.8×10^6 daltons. These data seem to indicate that the segments of single-stranded DNA integrated are largest in the *H. influenzae* system of transformation, which corresponds with the absence of surface-located DSF formation. Based on the consideration that the efficiency of integration of donor DNA in transformation is high, and the observation that the donor DNA molecules suffer endonucleolytic scissions at the surface of the competent *B. subtilis* cells, Dubnau and Cirigliano (1972c) have suggested that several segments of donor DNA may be integrated in a limited segment of the chromosome. Direct visual evidence for this has recently been obtained by Fornili and Fox (1977) in electron microscopic examination of heteroduplex DNA obtained from *B. subtilis* cells transformed with bromouracil-containing DNA. Bromouracil-containing DNA denatures more readily than normal DNA. From the size and the distribution of loops formed in the recombinant DNA at pH 10.8 at 20°C in the presence of 5% formaldehyde, it was concluded that the average length of the integrated segment corresponded to approximately 3.3×10^6 daltons, and that several segments of one molecule of transforming DNA can become inserted into limited sections of the recipient chromosome. This conclusion is in accord with the model of Lacks and Greenberg (1976) for binding and entry of DNA in *D. pneumoniae*, which assumes that a molecule of DNA is attached to several sites at the surface of the competent cell and that the entry of DNA is initiated at these sites. As Fornili and Fox (1977) have pointed out, the clustering of several segments of DNA in the chromosome of the recipient cell may restore the linkage of remote markers, previously separated during the conversion of high molecular weight donor DNA to double-stranded segments at the surface of the competent cell.

Since methods have become available to separate the complementary strands of transforming DNA more or less successfully by gradient elution of DNA adsorbed to methylated albumin kiezelguhr columns (Roger *et al.*, 1966; Roger, 1968; Rudner *et al.*, 1968; Strauss, 1970) and density gradient centrifugation (Guild and Robison, 1963; Chilton, 1967; Peterson and Guild, 1968), it has become feasible to determine whether both strands of a transforming duplex molecule can be integrated. Such studies, in which either the biological activity of separated strands was directly tested or that of artificially prepared heteroduplex molecules (often carrying a wild type allele on one particular strand and the mutant

allele on the complementary strand), indicated that either strand of the duplex molecule had transforming activity in *D. pneumoniae* (Guild and Robison, 1963; Gabor and Hotchkiss, 1966; Peterson and Guild, 1968). The same was found by Gurney and Fox (1968), by determining biological activity of heteroduplex DNA, containing a heavy donor DNA strand in addition to donor and recipient marker. Also in *H. influenzae* (Goodgal and Notani, 1968) and in *B. subtilis* (Chilton, 1967; Rudner *et al.*, 1968; Strauss, 1970) either strand was active in transformation. In *B. subtilis* either strand had approximately the same efficiency of transformation (Rudner *et al.*, 1968; Chilton, 1967; Strauss, 1970). Essentially similar conclusions were reached by Köhnlein (1974) using bromouracil-containing DNA obtained from a mutant of *B. subtilis* having a thymine-adenine bias in the complementary strands. Equal transforming activity of either strand of transforming DNA was also noted in *D. pneumoniae* (Peterson and Guild, 1968; Gurney and Fox, 1968) and in *H. influenzae* (Goodgal and Notani, 1968).

4. *Resolution of the heteroduplex DNA*

The obvious way of resolving the heteroduplex molecule of DNA is by replication, which should give rise to two homoduplex molecules, one of which will contain the recipient genetic information, and the other the information derived from the donor. Thus, in the absence of selection, or events at the molecular level destroying the mismatch at the site of mutation in the heteroduplex molecule prior to replication, a transformed cell harbouring only one chromosome should give rise to mixed clones, consisting of equal numbers of transformed and non-transformed progeny cells. Clonal analysis might, therefore, provide a means of examining whether the heteroduplex recombinant DNA molecule is resolved by replication, or whether additional ways of processing the structure should be considered. Several factors may complicate clonal analysis of transformed cells. These concern the possible growth in chains of competent cells, the presence of more than one nucleoid per cell and the possibility that competent cells contain partially replicated chromosomes. All of these situations will give rise to formation of mixed clones, for reasons different from that of segration by replication of heteroduplex molecules. Notwithstanding these complications, clonal analyses have been carried out. Bressler *et al.* (1968) observed that pure clones could be formed with fairly high frequency

upon transformation of *B. subtilis*, indicating that the recipient information can be lost from the heteroduplex molecules of DNA. The extent of loss of the recipient information appeared to be mutation-specific. No effect of ultraviolet irradiation on the frequency of appearance of pure clones was observed, which argues in favour of uninuclearity of competent *B. subtilis*. Although it has been reported that competence and uninuclearity are positively correlated (Singh and Pitale, 1968), direct electron microscopic observations of competent cells have indicated that approximately 50% of the competent cells contain two nucleoids (Vermeulen and Venema, 1973a). It is conceivable that the use of different competence regimens may have an influence on the extent of nuclearity in the competent cells.

Repair of heteroduplex DNA at the site of the mutation (mismatch repair) was not observed in *D. pneumoniae* (Guerrini and Fox, 1968), who used two linked markers conferring resistance to sulphanilamide. In this case, the chains of cells were broken down successfully by means of ultrasonic treatment of the competent culture. It appeared that sonication after one doubling subsequent to transformation yielded clones which were 90% pure, presumably indicating that the heteroduplex had been resolved by replication. This conclusion has been further supported by examining the kinetics of displacement of the position of the donor and recipient marker activity of heteroduplex molecules in CsCl gradients as a function of time of replication. The heteroduplex DNA contained one heavy strand. It appeared that the donor marker activity, contained in the heavy strand of DNA, shifted to the light position upon DNA-replication, and that after two rounds of replication the amount of donor marker activity was approximately the same in the hybrid and light position. One round of replication proved to be sufficient to deplete the heteroduplex to a large extent from the recipient marker. These data appear to show, therefore, that the heteroduplex molecule, at least for the markers used, segregated completely through DNA replication (Gurney and Fox, 1968).

It has been known for some time that certain single-site mutations in *D. pneumoniae* show widely different transformation efficiences. These mutations, being allele-specific rather than gene-specific, have been grouped in two classes, denoted high efficiency (HE) and low efficiency (LE) markers (Ephrussi-Taylor *et al.*, 1965). The relative transformation efficiences of HE and LE markers differ by approximately a factor of ten. Based on the relative transformation efficiences, it seems probable that

additional classes exist (Lacks, 1966; Tiraby and Sicard, 1973b).

Two hypotheses have been advanced to explain the phenomenon of different efficiency of HE and LE marker transformation; both have in common that certain base-pair mismatches between the single strand of donor DNA and the single strand of recipient chromosome, created during integration, are not tolerated in the genetically heterozygous and physically heteroduplex molecule. Ephrussi-Taylor and Gray (1966) have proposed that the mismatched bases in heteroduplex molecules involving LE markers are preferentially excized from the donor strand and replaced by bases complementary to the ones residing in the recipient single strand. With much lower frequency the excision system would remove the recipient contribution to the mismatch, which would result in effective LE marker transformation through heteroduplex formation. Two types of observations seem to support this hypothesis: earlier transmission to progeny and phenotypic expression of LE markers as compared to HE markers (Ephrussi-Taylor, 1966) and increase in random double transformants for unlinked markers when one of the markers is LE (Louarn and Sicard, 1968, 1969). The second hypothesis entertains the possibility that an endonuclease with equal probability cuts the recipient or donor strand of unsealed heteroduplex DNA at the site of the LE. Subsequent digestion by exonuclease of the recipient strand would lead to the creation of a double stranded (lethal) chromosomal break as soon as the exonuclease has advanced to the site opposite the physical discontinuity between the inserted donor strand and the recipient DNA (Tiraby and Fox, 1974a). It is further assumed that surviving LE transformants originate from cells in which the digestion has failed to reach the terminus of the inserted segment of donor DNA, and from those in which the donor DNA became covalently sealed to the recipient DNA prior to digestion of the complementary region. This hypothesis finds some support in the observation that reduction in length of DNA molecules carrying LE markers interferes relatively strongly with their biological activity in cells capable to correct mismatches. Increased sensitivity of LE markers to decrease in molecular weight of the DNA could, however, not be detected by Guild and Shoemaker (1976). The hypothesis of Tiraby and Fox (1974a) neatly explains why LE markers transform at much less than half the frequency of HE markers which are not subject to mismatch repair. In addition, this hypothesis does not require specific recognition of the excizing system of the donor strand base sequence.

The existence of an elimination mechanism of LE markers is strongly supported by the isolation of mutants (*hex*) in which DNA carrying LE markers is as effective in transformation as that carrying HE markers; apparently such mutants do not possess a functional discrimination system against LE markers (Lacks, 1970; Tiraby *et al.*, 1973; Tiraby and Sicard, 1973a, b; Tiraby and Fox, 1973). The loss of the *hex* function also results in a substantial increase in the spontaneous mutation rate, and it was suggested that the *hex* function is able to eliminate certain classes of mismatched base pairs that are formed as intermediates in both transformation and mutagenesis (Tiraby and Sicard, 1973b; Tiraby and Fox, 1973). Subsequent analysis of the effects of the *hex* function on mutagenesis showed that spontaneous mutations arising in the locus specifying sensitivity to fusidic acid in *D. pneumoniae* were predominantly of the HE class, whereas those introduced on transforming DNA treated with hydroxylamine or nitrous acid were nearly all of the LE class. From this it has been concluded that the *hex*-determined discrimination appears to be directed towards the elimination of heteroduplexes containing mismatches of at least the two transition mutation types A:C and G:T (Tiraby and Fox, 1974b).

Markers for LE might also be rescued if integration occurred at sites on the chromosome somewhat in advance of the replication fork. Passing of the replication fork over the site of mismatch would result in two homoduplex molecules (one carrying, in homozygous state, the recipient marker, the other, in the same state, the LE marker) which would, of course, not be recognized by the mismatch repair system. The previously noted observation that transformation of random doubles for unlinked markers increases if one of the markers is LE argues against the validity of this hypothesis. Further evidence against its validity is supplied by observations based on the use of CsCl gradient centrifugation, indicating that DNA carrying LE marker activity is not replicated sooner than DNA carrying HE marker activity, and that LE markers do not show increased sensitivity to inhibition of DNA synthesis by 6-(*p*-hydroxyphenylazo) uracil as compared to HE markers (Tiraby and Fox, 1974a).

It had been shown that the two strands of DNA carrying HE markers may show different efficiences in transforming activity (Gabor and Hotchkiss, 1966, 1969). Recently, Roger (1977) obtained evidence that this should be ascribed to the presence of a functional *hex* system. The strands of DNA carrying HE markers were separated and artificial

heteroduplexes were prepared carrying a drug-resistance marker on one specific strand and its wild-type allele on the other, and *vice versa*. By transforming *hex*$^+$ recipient cells with these heteroduplexes, it was shown that the activity of either of the two strands (depending on the marker considered) containing a HE marker can be greatly reduced. Since the bias in strand efficiency is eliminated when *hex*$^-$ cells are used as recipients (both strands are then approximately as effective as the HE strand), it seems clear that one strand of HE markers can be eliminated by the *hex*-controlled mismatch-repair system.

The efficiency of *hex*-controlled mismatch-repair systems appears to be affected by a number of conditions, prominent amongst which are the DNA concentration and duration of exposure to DNA of the recipient competent cells. Based on a quantitative study of these effects, Guild and Shoemaker (1974) advanced the hypothesis that the *hex*-controlled system can be easily saturated, thus allowing increased efficiency of transformation with LE marker-carrying DNA. Support for this hypothesis was obtained by examining the effect of non-isogenic pneumococcal DNA on the efficiency of transformation of LE markers carried on isogenic DNA. Since integration of non-isogenic DNA is likely to create many mismatches, which would capture one or more of the components composing the *hex* system, the mismatch introduced on the isogenic DNA might escape elimination. This prediction proved correct: the frequency of transformation of certain LE alleles could be increased to nearly the level of that of HE markers.

There seems to be general agreement that the substrate of *hex*-controlled mismatch-repair consists of heteroduplex DNA; however, it is not clear at present whether the system is acting upon non-covalently sealed integrational heteroduplexes or upon covalently sealed ones. Unsealed heteroduplex DNA is a necessary component of the model proposed by Tiraby and Fox (1974a) required to explain the extent of transformation of LE markers. Although the random strand incision assumed in this model, and the subsequent digestion of the recipient strand, neatly explains the preferential loss of potential LE transformants, a different hypothesis has been advanced in which the strand carrying the LE marker is rejected during integration by degradation accomplished by the 3′-exonuclease of polymerase III, which is additionally believed to be required for transformation (Dubnau, 1976). The attractiveness of this postulate is that, during integration, asymmetry is present in the recombining complex of donor and recipient DNA, which would provide

a basis for specific recognition (and subsequent degradation) of the donor LE marker. The problem remains unsolved and is the source of continuing controversy. Shoemaker and Guild (1974) have observed that the LE marker-transforming activity in DNA re-extracted at various time intervals after uptake by competent cultures, recovers from eclipse and then decreases substantially. These results have been interpreted as indicating that the destruction of LE markers is a relatively slow process. Since complete covalent sealing of the inserted segment of donor DNA proceeds quickly, it would seem that removal of the LE strand can continue after integration is complete. On the basis of the model proposed by Tiraby and Fox (1974a), Guild and Shoemaker (1976) have estimated that, on average, 7000–10000 nucleotides of recipient strand should be removed for the double strand break to occur. Since the size of this stretch of DNA is far in excess of that removed in other known excision processes, these authors have speculated that the *hex*-dependent enzyme is triggered by an LE mismatch and would then attack a free end (a single-strand interruption between the inserted donor DNA segment and the recipient DNA moiety) which may be at a considerable distance from the LE mismatch site.

D. MUTANTS AFFECTED IN RECOMBINATION

1. *General*

Determination of the enzyme deficiency in mutants affected in recombination should, in principle, provide information concerning the enzymic machinery that effects the integration of transforming DNA into the recipient chromosome. Many different types of transformation-deficient mutants have been isolated, and the ones most relevant to recombination are those which are still capable of taking up DNA from the surrounding medium. Several mutants of this type are known, and in a number of instances the chromosomal location has been mapped. However, apart from the conclusion that recombination is an extremely complex phenomenon, until now most studies have yielded little additional information which was not already derived from studying wild-type cells engaged in transformation. Although seldom found in practice, ideally to construct a consistent classification of the various known mutants, information concerning a large number of phenotypic properties should be available. These include: the efficiency with which the

mutant cells process the DNA to a transformant, their capacity to be transduced, both homologously and heterologously, and to be transfected with bacteriophage DNAs, both requiring recombination and requiring no recombination to produce infective centres; their sensitivity to a number of deleterious agents, such as ultraviolet radiation, X-rays, alkylating agents and mitomycin C (MC), which may provide information with respect to the effectiveness of the recombination machinery to eliminate damage inflicted by such agents upon the DNA; their capacity to be lysogenized with temperate bacteriophage and to be induced for phage production; their nuclease deficiencies and the physical and biological fate of donor DNA in the mutants. Information regarding one or more of these properties is available for individual mutants but, because of its incompleteness for all mutants, classification on the basis of such partial phenotypic characterization is an extremely hazardous enterprise. Fortunately, in several instances, mapping of mutations has been possible and has been carried out, so that a genotypic classification and, therefore, a meaningful comparison of several mutants has become possible. This classification is most advanced in *B. subtilis*.

Our knowledge of the enzymes involved in integration of transforming DNA is in a particularly rudimentary state. The starting point for acquiring an insight into the enzymology of recombination in transformation has been, almost without exception, the isolation of recombination-deficient mutants and, subsequently, efforts to determine their enzymic deficiency. Apparently, this approach has met with little success, and the question may be posed whether the approach chosen by Lacks and his colleagues (Lacks, 1970; Lacks *et al.*, 1974) for the identification of enzymes involved in uptake of transforming DNA in *D. pneumoniae* would not also result in quicker accumulation of relevant information concerning the integrating enzymes and, possibly, other proteins participating in the recombination process.

2. *Classification of mutants and fate of donor DNA*

Efforts have been made to classify transformation-deficient mutants in both *H. influenzae* and *B. subtilis*.

On the basis of DNAase-resistant binding of both native and denatured donor DNA, and the yield of streptomycin-resistant transformants in *H. influenzae*, Caster *et al.* (1970) have distinguished four classes of mutants endowed with the following properties: class 1 mutants transform well

with denaturated DNA, but do not bind significant amounts of native donor DNA; class 2 mutants neither bind native nor denatured DNA; class 3 mutants bind both native and denatured DNA, but produce fewer transformants than expected on the basis of the amount of DNA bound, and class 4 mutants transform disproportionally poorly with respect to the amount of denatured DNA bound. Like class 1 mutants, class 4 mutants do not bind native DNA. In view of their DNA-binding capacities class 1 and 2 mutants probably have little relevance to studies of recombination.

Eleven class 3 *H. influenzae* mutants have been subdivided further on the basis of their ultraviolet and X-ray sensitivities and their capacity to form transformed clones as a consequence of exposure to ultraviolet or X-ray irradiated DNA. On these criteria several phenotypically different subclasses could be distinguished within this group (Postel and Goodgal, 1972b). A further phenotypic distribution could be made within class 3 mutants by determining the physical and biological fate of the donor DNA in these mutants (Postel and Goodgal, 1972a). By determining the percentage of total donor radioactivity in re-extracted DNA cosedimenting with the recipient DNA, 15 out of 19 mutants were found to be capable of associating donor atoms with the recipient chromosome, with varying degrees of efficiency, ranging from 2.7% to approximately 50% (the latter is the wild-type value), whilst the remaining four were found to be incapable of doing so (*dad* mutants, for donor association defective). However, those mutants which are capable of associating an equal, or lesser but still substantial, number of donor DNA atoms with the recipient chromosome, do not form, or not to any appreciable level, biologically active molecules carrying both a donor and a recipient marker (*dab* mutants, for donor association biologically defective). Apparently, this indicates that the DRC was either abnormal, or was not formed as a complex between high molecular-weight donor DNA and the recipient chromosome, instead representing recipient DNA in which low molecular weight breakdown products of donor DNA had been incorporated. The biological activity of the donor marker in lysates obtained from *dad* mutants decreased as a function of time, presumably because, not being integrated, they fail to replicate. Several of the mutants of the *dab* subclass appeared to take up more DNA than the wild type. It was speculated that this hypercompetence reflects an abnormal activity of one part of the integration machinery. The fate of donor DNA was further investigated in one of the *dab* mutants, which was fairly

efficiently transfectable with phage S2-, but not with prophage DNA (Caster and Goodgal, 1972). It appeared that the buoyant density of the DRC-like material formed in this mutant was indistinguishable from that of native, normal recipient DNA (the cells had been exposed to donor DNA carrying a density label; wild type DRC bands at a heavier position than recipient DNA under these conditions). Presynaptically, the fate of the donor DNA was largely the same as in the wild-type organism. Since evidence could be found neither for the presence of single-strand nicks in the DRC-like product, nor for increased donor DNA breakdown, it was postulated that the mutant contained a hyperactive nuclease which decreases the effective size of donor DNA during the process of integration.

Mutants with phenotypes similar to the *dad* and *dab* subclass have been obtained and characterized by Setlow *et al.*, (1972a). One of the transformation-deficient mutants is the product of transformation of wild-type cells with DNA from a transformation-deficient ultraviolet-sensitive mutant (DB117). The DB117 mutant is now commonly known as *rec*1; the transformation-deficient mutant obtained by wild-type transformation with DB117 DNA has been denoted *rec*2. The *rec*1 and *rec*2 strains differ with respect to a number of phenotypic properties. The *rec*1 strain is sensitive to ultraviolet or X-rays, and to methyl methanesulfonate (MMS), and *rec*1 lysogens are not inducible. The *rec*2 strain is resistant to ultraviolet radiation, and *rec*2 lysogens are inducible and show a moderate sensitivity to X-rays and MMS. In addition, the *rec*1 strain is deficient in post-replication repair, whereas *rec*2 is not (LeClerc and Setlow, 1972a). The combination of *rec*1 and *uvr*1 is apparently lethal (LeClerc and Setlow, 1972b) and the *rec*1$^+$ allele can be transcribed in *rec*1 cells without integration (Setlow *et al.*, 1972b).

Both by treatment of lysates from transforming cells with digitonin, which leaves unintegrated DNA in the supernatant after centrifugation, and equilibrium sedimentation in CsCl, Notani *et al.* (1972) demonstrated that the *H. influenzae* strains *rec*2 and KB6 (another transformation-deficient strain) were unable to associate donor label with the recipient DNA. The *rec*1 strain is able to do so, but no recombinant-type activity was detectable. Thus, the *rec*2 and KB6 mutations are phenotypically similar to the *dad* subclass, whereas the *rec*1 mutation seems to belong to the *dab* category. The mutant strains have also been analysed with respect to formation of donor DNA species II, whose molecular weight is less than that of the original donor DNA

preparation, and which seems to arise as a consequence of DRC-formation (Notani, 1971). In contrast to the *rec*1 strain, the donor-recipient DNA association-negative *rec*2 and KB6 strains do not seem to be able to form species II DNA from the original donor DNA. Notani *et al.* (1972) have suggested several possibilities for the production, in the *rec*1 strain, of biologically inactive integrated DNA: (1) incorporation of donor material in the recipient chromosome is non-macromolecular, (2) donor DNA segments are very small, or (3) branched, biologically inactive molecules are produced.

In a mutant similar to *rec*1 (TD24), it has been shown that the amount of heavy donor DNA incorporated into the recipient chromosome is equal to that in the wild type, but the buoyant density displacement of the DRC-like material towards the heavier side in CsCl gradients was found to be considerably less than that observed in the wild type (Kooistra and Venema, 1974). Concomitantly with attempted synapsis of the donor DNA with the recipient chromosome, the donor DNA seems to lose its biological activity. These results have been interpreted to indicate that the association of donor and recipient DNA to form a DRC-like product results from local DNA synthesis at the site of association, in which the newly synthesized DNA is, to a large extent, composed of low molecular weight donor DNA breakdown products. The *rec*1 and TD24 mutations, together with another one (LB4), are all mutations in the same gene, and produce the same phenotype (Kooistra and Setlow, 1976).

In an analysis of the physical state of the recipient DNA in competent *rec*1 and *rec*2 mutants, LeClerc and Setlow (1974, 1975) found that, in contrast to the wild type and *rec*1 cells, *rec*2 mutants contain many fewer single-stranded breaks and single-stranded regions in newly synthesized DNA. Apparently, these chromosomal discontinuities, present in wild-type and *rec*1 cells, are important for association of donor and recipient DNA. Also, the physical state of the recipient DNA seems to be affected differently in wild-type and *rec*1 on the one hand, and *rec*2 cells on the other, as a consequence of exposure of cells to transforming DNA. Thus whereas in wild-type and *rec*1 cells following the entry of donor DNA lower molecular weight recipient DNA is produced which seems to contain covalently-bound donor DNA, no such effect is seen in *rec*2 cells. This covalent linkage of donor DNA and the lower molecular weight recipient DNA is also observed in cells carrying a temperature-sensitive mutation affecting DNA synthesis at the restrictive temperature. The authors deduced from this finding that in *rec*1, the association of donor

label was not caused by degradation of the transforming DNA and re-utilization of the breakdown products for recipient DNA synthesis. However, in the absence of information concerning the nature of the conditional DNA-synthesis mutation used, their conclusion that the DRC-like material did not result from DNA synthesis is, perhaps, not justified. This, in turn, also poses questions with respect to the validity of the postulate of LeClerc and Setlow (1974) that the *rec*1$^+$ gene product may be required for the removal of redundant recipient DNA from an intermediary recombinational structure in which the donor DNA has paired with a redundant tail of the recipient chromosome, so that it can function as a substrate for base pairing between the donor and recipient DNA moieties.

Both *H. influenzae rec*1 and *rec*2 cells are considerably less well transfectable than are the wild-type cells, especially at higher DNA concentrations. However, as in the case of transforming DNA, *rec*1 and *rec*2 mutants differ with respect to the processing of transfecting DNA. Firstly, *rec*1 mutants degrade bacteriophage HP1C1 DNA to a greater extent than do wild-type cells, and secondly, a fast sedimenting molecular species of phage DNA, observed in the wild type cells and generated from re-assembled fragments, is not found. *Rec*2 cells subject the phage DNA to little degradation and, although the fast-sedimenting transfectional intermediate is formed, proportionally much less is present than in the wild-type cells. On the basis of these findings it has been postulated that both fragmentation of transfecting DNA and formation of the fast-sedimenting phage DNA species by recombination are required for successful transfection in *H. influenzae* (Notani *et al.*, 1973). Since both in *rec*1 and *rec*2 mutants the probability of creating a functional phage replicon from entered bacteriophage DNA by recombination is greatly reduced, it might be expected that rescue of wild-type markers from transfecting DNA in competent *H. influenzae* cells by superinfection with temperature-sensitive phage (marker-rescue) will also be affected in *rec*1 and *rec*2 strains. This has been found to be the case (Boling and Setlow, 1974).

In *B. subtilis*, many mutants impaired in transformation, transfection or transduction have been isolated and studied by several groups (Searashi and Strauss, 1965; Okubo and Romig, 1966; Hoch *et al.*, 1967; Prozorov *et al.*, 1968; Dubnau *et al.*, 1969; Prakash and Strauss, 1970; Hoch and Anagnostopoulos, 1970; Spatz and Trautner, 1971; Davidoff-Abelson and Dubnau, 1971; Zadražil and Fučik, 1971; Chestukhin *et al.*,

1972; Hill *et al.*, 1972; Kada *et al.*, 1972; Sinha and Iyer, 1972;; Dubnau *et al.*, 1973; Hadden, 1973; Harford *et al.*, 1973; Polsinelli *et al.*, 1973; Sadaie and Kada, 1973; Yasbin *et al.*, 1973b; Dubnau and Cirigliano, 1974; Harford, 1974; Naumov *et al.*, 1974; Samojlenko *et al.*, 1974; Mazza *et al.*, 1975; Shibata and Ando, 1975; Garro *et al.*, 1976; Sadaie and Kada, 1976; Buitenwerf and Venema, 1977a). Much confusion has arisen, because individual groups of workers have used their own system of classification. An additional confusing element has been the circumstance that a number of groups focussed their attention on a number of phenotypic properties such as sensitivities to ultraviolet radiation, X-rays, ethyl methanesulfonate (EMS), MMS and MC (which are presumably of secondary importance to an understanding of the nature of the transformation or recombination-deficiency). Other workers have concentrated on more pertinent aspects of the recombination-deficiencies, such as deviations in the processing of the transforming DNA, the elucidation of the mutant's enzymic deficiency and, most importantly with respect to a proper and sensible classification, the mapping of the mutant sites. Unknowingly, the various properties were often studied in independently obtained isolates, which, as later appeared to be the case, belonged to the same linkage group.

Notwithstanding these and other sources of confusion, efforts have been made to establish a uniform system of classification of the transformation (recombination)-deficient mutants described. Thus, Mazza *et al.* (1975) have proposed a system of classification based on a variety of phenotypic properties of the mutants and mapping data, in which they distinguish eight phenotypic classes of mutants and 13 (and possibly 15) different genes.

Instead of expanding on all possible data concerning phenotypic properties which may be only of secondary significance to an understanding of the deficient phenotype, attention will be primarily focussed on the mode of processing of donor DNA during the abortive transformation and the enzymatic deficiencies of the mutants.

In an extensive investigation, involving 16 isogenic recombination-deficient *B. subtilis* 168 strains, which incorporate DNA from the medium to approximately the same extent as a recombination-proficient parent, Dubnau *et al.* (1973) originally distinguished four different classes on the basis of their capacity to be transformed and to be transduced homologously (by phage PBS1 grown on *B. subtilis* strain 168) and heterologously (by PBS1 grown on *B. subtilis* strain W23). Class 1 is

deficient in transformation and heterologous transduction, but shows an almost normal level of homologous transduction; class II is deficient in transformation and both modes of transduction; class III is deficient in transformation only, and class IV is fairly well transformable, but deficient in transduction. All mutants are sensitive to ultraviolet radiation, MC and MMS, class III the least so. On the basis of DRC formation, as judged from sucrose-gradient centrifugation of lysates obtained from cells in which the DNA had entered, a fifth class could be distinguished, because class II contained a mutant (*rec*B2) which produces normal amounts of DRC (class IIa) whereas the other class II mutants produce little, if any, DRC (class IIB). The class I mutants form little DRC; whatever DRC they produce corresponds approximately to the leakiness of the strains. The mutants of this class are, therefore, (partially) deficient in DRC formation and are in this respect similar to *rec2* mutants of *H. influenzae*. Class IIa and class III mutants produce more DRC than corresponds to their transformability. Class IIa mutants fail to replicate the heteroduplex DNA (Davidoff-Abelson and Dubnau, 1971). As appeared later, the transformation deficiency of class III mutants is not due to a chromosomal mutation but rather to the presence of an integrated SP02 prophage (Garro *et al.*, 1976). Lysogenization with either of the temperate bacteriophages SP02 and ϕ105 can produce a transformation-deficient phenotype (Peterson and Rutberg, 1969; Yasbin *et al.*, 1973a; Garro and Law, 1974).

The DRC formed in the class IIa mutant appeared to be normal as judged by neutral and alkaline CsCl-gradient centrifugation. In particular, no single-strand discontinuities between the donor and recipient moieties in the DRC could be detected, nor was the DRC subject to secondary nicking and its biological activity, which should be a sensitive indicator of structural abnormalities, appeared to be normal. However, the remote possibility that the donor moiety of DNA was covalently bonded to the recipient DNA at one side only could not be excluded. The very inefficient production of DRC in the mutant classes I and IIb was not due to their failure to produce SSF, because the amount of SSF extractable from these mutants appeared to be at least 70% of that obtainable from the wild type.

Genetic analysis of this series of mutants (Dubnau and Cirigliano, 1974) permitted a more detailed classification. Class IIb mutants, which are transformation- and transduction-deficient and do not form DRC, appeared to map in two different chromosomal regions; the same applied

to class IV mutants which are deficient in phage PBS1 transduction only. The remainder of the phenotypically distinguishable classes each corresponded with a particular chromosomal location. As determined in this group of sixteen mutants, seven loci were involved in recombination in *B. subtilis*. This number should apparently be reduced to six, because of the coincidence of the group III mutant locus with that of the phage SP02 prophage site.

With respect to the number of loci that seems to be involved in recombination on *B. subtilis*, the tendency can be clearly observed that, as a function of time, this number has increased rather drastically, pointing to the complexity of the phenomenon. Starting with Hoch and Anagnostopoulos (1970), two *rec* loci were mapped, one in class I and the other in class IIa of the system of classification proposed by Dubnau and Cirigliano (1974). Thanks to subsequent mapping studies, notably those of Sinha and Iyer (1972), Polsinelli *et al.* (1973), Dubnau and Cirigliano (1974), Harford (1974) and Mazza *et al* (1975), the number of genes participating in genetic recombination has increased to thirteen (and possibly fifteen, Mazza *et al.*, 1975). Genetic analyses of recombination-deficient *B. subtilis* mutants support the view that transformation proceeds through a number of separate steps. Two such steps seem now also to be identifiable from the genetic point of view. Class I (e.g. *rec*A, both in the classification system of Dubnau and Cirigliano (1974) and that of Mazza *et al.*(1975)), is apparently blocked in the step which results in association of SSF with the recipient chromosome. Class IIa (*rec*B, in both classification systems) is blocked in a second, apparently critical, step not previously specifically recognized *viz.* the replication of the integrated DNA (Davidoff-Abelson and Dubnau, 1971). Since mutations resulting in the deficiency of production of DRC are found in three different loci, *rec*A, *rec*D and *rec*E, at least three genes appear to participate in formation of stable DRC. For two additional steps in the processing of transforming DNA in *B. subtilis*, inferred from following the physicochemical fate of transforming DNA, mutants have been isolated which are unable to carry out these steps. Zadražil and Fučik (1971) have reported that an MMS-sensitive strain accumulates a non-covalently-sealed DRC intermediate and, recently, a slightly ultraviolet-sensitive mutant has been described which is capable of binding but not taking up transforming DNA. Few if any SSFs are extractable from this mutant. As judged from velocity sedimentation, DNA bound to this mutant is converted to DSF, indicating that double-stranded fragmentation is not a sufficient condition for entry of donor

DNA (Buitenwerf and Venema, 1977a) These authors also followed the fate of transforming DNA in two other mutants. One of these forms very little DRC and, therefore, is similar to class I or class IIb mutants of Dubnau *et al.* (1973). The other mutant is capable of DRC formation. However, the DRC is initially of abnormally high buoyant density, and probably represents an intermediate partly consisting of non-base paired single-stranded donor DNA, which is also detectable during synchronous transformation at low temperature (17°C) (Buitenwerf and Venema, 1977a, b). Presumably, this mutant is delayed in the further processing of this intermediate, possibly in digestion of the redundant recipient strand of DNA during donor strand assimilation into the recipient chromosome.

3. *Enzymology of recombination in transformation*

Apart from binding and entry of transforming DNA in *D. pneumoniae*, our ignorance of the enzymes which catalyse processing of transforming DNA is almost complete and, as is often the case in such circumstances, is inversely proportional to the number of hypotheses. However, the start was promising. Vovis and Buttin (1970) isolated a γ-ray, ultraviolet-sensitive mutant of *D. pneumoniae*, deficient in ATP-dependent DNAase activity, which, on the basis of efficiency of producing transformants per amount of DNA taken up, was 5-6-fold less well transformable than the wild type. Both HE and LE markers were equally affected. A similar mutant was isolated by Tiraby *et al.* (1973). An ATP-dependent DNAase has been shown to be present in all three well-studied transformation systems: *D. pneumoniae* (Vovis and Buttin, 1970), *H. influenzae* (Friedman and Smith, 1972a, b; Smith and Friedman, 1972) and *B. subtilis* (Ohi and Sueoka, 1973; Doly *et al.*, 1974; Doly and Anagnostopoulos, 1976). The enzyme requires Mg^{2+} and ATP for optimal activity and preferentially acts, at rather high pH values, on native linear DNA. The enzyme acts both endo- and exonucleolytically and, in *H. influenzae*, appears to produce, from native DNA, rather large fragments of double-stranded DNA flanked by one or two single-stranded segments approximately 2000 nucleotides long (Friedman and Smith, 1973). The enzyme appears to be composed of different subunits (three in that of *H. influenzae* (Wilcox *et al.*, 1975); five in that of *B. subtilis* (Doly and Anagnostopoulos, 1976)). Complementation tests involving eight different mutations in ATP-

dependent DNAase have also indicated that three cistrons code for the three known subunits of the *H. influenzae* ATP-dependent nuclease (Kooistra *et al.*, 1976).

Vovis (1973) followed the fate of donor DNA in the ATP-dependent DNAase-deficient *D. pneumoniae* mutant and obtained results which indicated that the ATP-dependent DNAase is not required for any of the steps leading to physical association of the donor and recipient DNA. It was speculated that the enzyme might be necessary for the trimming of (hypothetical) overlapping recipient and/or donor polynucleotides in the DRC.

In *B. subtilis*, deficiency of ATP-dependent DNAase is also accompanied by a moderate decrease in transformability (Chestukhin *et al.*, 1972; Doly *et al.*, 1974). As in *D. pneumoniae*, the association of donor and recipient DNA in *B. subtilis* proceeds normally (C. Anagnostopoulos, personal communication), apparently suggesting that the nuclease plays a non-essential role in recombination or, if its role is essential, in a very late step of the integration process.

The majority of data concerning the role of this enzyme in *H. influenzae* agree with this conclusion, although Greth and Chevallier (1973) have concluded that the ATP-dependent DNAase is effectively required in transformation of this organism. However, following re-examination of the mutant studied by Greth and Chevallier (1973) and a number of other ATP-dependent DNAase-deficient mutants, Wilcox and Smith (1975) suggested that the enzyme is not essential to recombination. To a large extent, the apparent transformation deficiency of the mutant could be explained on the basis of the observation that a large proportion (50–90%) of the cells in culture failed to produce visible colonies when plated. Possibly a changed cellular envelope is basic to this phenomenon. Indications that ATP-dependent DNAase mutants are more permeable to MC have been obtained (Small *et al.*, 1976). Wilcox and Smith (1975) speculated that the *in vivo* function of the enzyme is to remove free hanging tails from DNA which is partly base-paired, and that strains lacking the enzyme activity may use alternative enzymic pathways to remove such tails. LeClerc and Setlow (1974) have postulated that the ATP-dependent DNAase in *H. influenzae* may be required for breakage of the redundant recipient strand during assimilation of a single strand from double-stranded donor DNA into the recipient chromosome.

The conclusion drawn by Wilcox and Smith (1975) has been further substantiated by following the fate of donor DNA in an ATP-dependent

DNAase-deficient strain of *H. influenzae*. Presynaptically, the fate of the donor DNA in the mutant was the same as in the wild type, and recombinant-type activity (activity displayed by molecules jointly carrying a donor and a recipient marker) was formed with nearly the same efficiency as in the wild type. Moreover, the rate of replication of the integrated donor marker was equal to that of recipient markers for many (approximately 40 at least) generations. From these findings it was suggested that the ATP-dependent DNAase-deficiency results in a decreased capacity of transformed mutant cells to form visible colonies on plates (Kooistra and Venema, 1976). These authors have also introduced into a *rec*1 mutant a mutation resulting in ATP-dependent DNAase-deficiency and observed that the excessive degradation of chromosomal DNA following ultraviolet irradiation, which is characteristic of *rec*1 mutants, was strongly inhibited in the double mutant, apparently indicating that the ATP-dependent DNAase is responsible for the instability of chromosomal DNA in *rec*1 mutants. Since the degradation of chromosomal DNA following exposure to ultraviolet radiation in ATP-dependent DNAase deficient strains is also considerably less than in the wild type (Wilcox and Smith, 1975; Kooistra and Venema, 1976), it would seem that, *in vivo*, ATP-dependent DNAase is participating in repair functions rather than in the integration of transforming DNA. However, since cell survival of ultraviolet irradiated wild type and ATP-dependent DNAase-deficient mutants does not greatly differ, it would seem that the participation of ATP-dependent DNAase in repair functions can be substituted by other enzymes.

It may be possible that the 3′-exonuclease activity intrinsic to DNA-polymerase III is required for assimilation of single-stranded donor DNA once initiation of integration has started. Dubnau (1976) reported that Pol III mutants of *B. subtilis* are defective in the formation of completely sealed DRC and suggested that the 3′-exonuclease activity of the enzyme may digest the redundant recipient strand from the intermediate. Polsinelli *et al.* (1973) reported that a *B. subtilis* mutant, moderately reduced in transfectability but only slightly reduced in transformability, shows greatly diminished DNA polymerase activity, presumably that of DNA polymerase I. It is doubtful whether this mutant is classifiable as a transformation-deficient mutant because, if allowance is made for the slightly decreased uptake of donor DNA, the mutant appears to process the DNA as efficiently as the wild type organism. Mazza *et al.* (1975) isolated a *B. subtilis* mutant, transforming with approximately 10% wild

type efficiency, which appeared to lack an ill-defined single-strand specific nuclease.

IV. Fate of Transforming Chromosomal DNA in *Escherichia coli*

As compared to the transformation systems referred to earlier, which usually transform with frequencies up to 1–2% and occasionally higher, the low frequency of transformation of *E. coli* has, thus far, precluded physical and biological analysis of the transforming DNA during recombination. Nevertheless, from studying the effect of the recipient's genetic constitution and the use of various types of donor DNA on the frequency of transformation, and genetic analysis of the transformants, certain inferences have been drawn with respect to the processing of donor DNA or, rather, the hazards that linear chromosomal-transforming DNA may encounter when it has entered into the recipient cell (for a recent review, see Oishi and Irbe, 1977). These hazards appear to be very considerable. Assuming that all cells of a competent culture of *E. coli* are capable of taking up DNA, it has been estimated that DNA uptake amounts to 6×10^8 daltons of donor DNA per cell, whereas the efficiency of transformation for a given marker is approximately 10^5-fold less than in the case of the easily transformable *H. influenzae* (Sabelnikov *et al.*, 1975). However, substantially lower values for uptake have also been reported (Cosloy and Oishi, 1973b).

Transformation by chromosomal DNA (linear fragments of the chromosome) is not detectable in wild type *E. coli* recipients whose primary recombination depends upon the presence of the *rec*BC gene product (ATP-dependent DNAase). However, transformation of *rec*B$^+$C$^+$ recipients is possible if one or another of two additional pathways for recombination (different from the *rec*BC pathway) is functional. These are *rec*F which becomes functional by mutation of the *sbc*B gene, and *rec*E which is active in the presence of a mutated *sbc*A gene (for details regarding the various recombination pathways in *E. coli*, see Clark, 1973). Further increase (at least five-fold) in the frequency of transformation can be obtained in recipients possessing activated *rec*E or *rec*F pathways in which the *rec*BC pathway is eliminated (Oishi and Cosloy, 1972; Cosloy and Oishi, 1973a; Wackernagel, 1973). These observations show that the *rec*BC DNAase has an adverse effect on transformation with chromosomal DNA in *E. coli*. The deleterious effect of *rec*BC proficiency on transfection by λ DNA of sphaeroplasts has also

been observed (Wackernagel, 1972). Oishi *et al.* (1974) and Basu and Oishi (1975) have examined the effect of the recipient's genotypic constitution on the relative frequencies on substitution-type of recombination, which appears to occur when chromosomal DNA is used as donor (Cosloy and Oishi, 1973b; Oishi and Irbe, 1977), and addition-type of recombination (arising as a consequence of the insertion of circular DNA by recombination). Using intact DNA from ϕ80pt (*trp* $A^+B^+C^+$), in which the *int-red* region is replaced by the *trp* operon as donor, the authors observed that recipients carrying a functioning *rec*F pathway and a non-functioning *rec*BC pathway were predominantly transformed by means of the substitution type of recombination, whereas recipients having both a functional *rec*F and *rec*BC pathway, transformed predominantly as the results of the addition-type of recombination. These results argue in favour of the hypothesis that linear DNA molecules, which would give rise to substitution-type recombination, are likely to be degraded by the exonucleolytic action of ATP-dependent DNAase. A number of observations support this view. Pilarski and Egan (1973) have observed that both linear (half DNA molecule) and circular (whole DNA molecule) provide λ gene function if introduced into *rec*BC nuclease-deficient mutants, whereas the template function of the linear molecule is lost in *rec*BC nuclease-proficient recipients. In a systematic study involving the infective capacity of phage T4, T7, λ and P22 DNA in *E. coli* sphaeroplasts, Benzinger *et al.* (1975) obtained clear evidence that the efficiency of transfection depends on the structure of the transfecting DNA and that circularizing DNA is resistant to the ATP-dependent DNAase *in vivo*.

Based on genetic analysis of transformants obtained by transformation with chromosomal DNA, it has been reported (Hoekstra *et al.*, 1976; Bergmans *et al.*, 1977) that multiple crossing-over events are very infrequent. This suggests that transformants are predominantly derived from a single piece of donor DNA. Based on linkage data in transduction of *rec*BC DNAase-proficient and deficient recipients, Bergmans *et al.* (1977) suggested that recombination in *rec*BC DNAase-proficient strains involves an increased number of crossing-over events as compared to that in *rec*BC DNAase-deficient strains. If these results may be extrapolated to transformation, the lack of multiple crossing-over events observed may be due to the absence of the *rec*BC DNAase.

V. Heterospecific Transformation

A general feature of heterospecific transformation (which is transformation by DNA from closely related species) is the very low efficiency as compared to homospecific transformation. The extent of reduction of the transforming activity is marker specific. It is believed that those markers transforming with relatively high efficiency in heterospecific transformation have not been subject to drastic change during the evolutionary divergence of the species concerned (Dubnau *et al.*, 1965a, b). In particular, resistance markers for antibiotics interacting with the functioning of prokaryotic ribosomes show relatively large transforming activities. This has been established in a fair number of heterologous transformation systems (for references, see Notani and Setlow, 1974), and is understandable if it is assumed that, prior to the divergence of the organisms concerned, the DNA base sequences coding for the various ribosomal components have been selected to ensure optimal ribosomal functioning in the common ancestor. In fact, if this hypothesis is basically correct, it would imply that the main obstacle to successful heterospecific transformation is the extent of non-homology of the donor and recipient base sequences. However, several other possibilities to account for the low efficiency of heterospecific transformation have been envisaged, prominent amongst which are decreased uptake of heterospecific DNA and the presence of restriction endonucleases that would destroy the integrity of the heterologous DNA, either before or after integration or when the markers on heteroduplex DNA (assuming single-stranded integration of the heterologous DNA) have segregated through DNA replication.

The first of these alternative possibilities does not seem to agree, in general, with experimental data (for documentation see Notani and Setlow, 1974), and the second possibility has become increasingly unattractive, since it has been shown that, in homospecific systems of transformation, DNA obtained from non-modifying recipients is equally effective in transformation of restricting and non-restricting recipients. This has been established both in the *B. subtilis* and *H. influenzae* systems of transformation. In the *B. subtilis* system, Trautner *et al.* (1974) reported that, contrary to non-modified transforming DNA, infection and transfection with non-modified phage SPP1, ϕ105 and SP02 DNA and transduction with PBS1 are greatly reduced in the restricting host carrying a type II restriction endonuclease. Similar observations for the

in vivo resistance of transforming DNA to type II restriction enzymes have been made in *H. influenzae*. With respect to the *in vivo* sensitivity of transforming DNA to type I restriction enzymes, no, or only a slight, effect could be detected (Gromkova and Goodgal, 1974; Piekarowicz *et al.*, 1975). Stuy (1976) neither found any effect of the restriction phenotype on transformation of antibiotic-resistance markers and a prophage marker in homospecific crosses, nor an effect on such markers carried on non-modified *H. parahaemolyticus* DNA in *H. influenzae*. Thus, it appears that the low efficiency of heterospecific transformation is mainly determined by a lack of homology in base-sequences. In accord with this hypothesis is the finding that heterospecific transformation with markers which are derived from heterospecifically transformed recipients (intergenotes) is much higher than transformation effected by pure heterospecific DNA, both in *Streptococcus* (Biswas and Ravin, 1971; Ravin and Chakrabarti, 1975) and *Bacillus* sp. (Wilson and Young, 1972, 1973). The heterospecific marker carried on intergenotic DNA will, on average, be flanked by DNA which is more homologous to that of the recipient than is the pure heterospecific DNA.

Studies on the fate of transforming heterologous DNA after uptake are surprisingly limited in number. Steinhart and Herriott (1968) have observed that *H. influenzae* is able to form DRC if exposed to *H. parainfluenzae* donor DNA. The complex was formed with 60% of the efficiency in the homologous system and appeared to be alkali-stable. Notani and Setlow (1972) have examined the fate of density-labelled *H. parainfluenzae* DNA in *H. influenzae* and *vice versa*. They observed that the kinetics and extent of association of *H. parainfluenzae* DNA with the recipient chromosome were closely similar to those in the homospecific system, and that the heterologous DNA associated with recipient DNA carried donor marker activity which was much more pronounced using *H. parainfluenzae* than using *H. influenzae* as second recipient. The same applied to the reciprocal situation. On the basis of these results, the authors suggested that initially the associated heterologous DNA is present as long pieces, so that there is more homology of single-stranded regions surrounding the donor marker with the donor species than with the recipient species. As a function of time of re-extraction of the heterologous DNA, in the presence of growth, the biological activity of the heterologous marker decreases when assayed on the donor species and increases on the recipient species, and more donor label than recipient label is lost. These results would seem to be in accord with the

hypothesis that incompletely integrated heterologous DNA is eliminated, or that some of the integrated stretches may contain mismatches which become susceptible to degradation, and may be replaced by recipient DNA sequences. However, part of the decrease of donor marker activity on the donor-type recipient is presumably caused by death of the heterospecifically transformed recipients. This killing effect of heterologous DNA has been observed in *H. influenzae* (Steinhart and Herriott, 1968; Beattie and Setlow, 1970) and has previously been attributed to synthesis of incompatible macromolecules coded for by the integrated heterologous DNA. This conclusion is probably incorrect, since it was found that heterologous DNA is capable of inducing defective prophage carried by the strains used. A *H. influenzae* strain which contains no inducible defective prophage appears to be resistant to killing by *H. parainfluenzae* DNA (Setlow *et al.*, 1973).

The efficiency of heterospecific transformation appears to be affected by external conditions. Ravin and Ma (1972) reported that exposing *S. sanguis* recipients for a brief period to 48°C prior to exposure to *S. pneumoniae* DNA, decreases the efficiency of the homospecific system to a larger extent than the heterospecific system. From these results it has been suggested that after heat treatment longer pieces of heterologous DNA are integrated. This hypothesis is supported by the observation that linkage of markers carried on the heterologous DNA appears to be better preserved in the heat-treated recipient than in the untreated cells; apparently the discrimination system for heterospecific transformation has become less exacting as a consequence of heating the recipient cells. The relatively favourable condition for heterospecific transformation after heat treatment decays as a function of time at 37°C (Deddish and Ravin, 1974). The decay is inhibited by a variety of inhibitors of nucleic acid synthesis but not of protein-synthesis. In particular, treatment with rifampin is capable of inducing loss of discrimination. Similarly, a *S. sanguis* mutant carrying a temperature-sensitive mutation in RNA polymerase activity was also found to be relatively less discriminating against heterologous transforming DNA. It was speculated that the RNA involvement in stabilizing coiling and folding of the bacterial chromosome (Worcel and Burgi, 1972; Worcel *et al.*, 1973; Pettijohn and Hecht, 1973) may play a role in this phenomenon (Raina and Ravin, 1976).

Because discrimination against LE markers is, relatively, also diminished in heat-treated pneumococcal cells, Tiraby and Ravin (1973)

examined whether the system recognizing LE markers also recognizes heterologous streptococcal DNA. It appeared that a *hex* strain, transforming equally well with HE and LE marked donor DNA, retained its capacity to discriminate against heterologous DNA, indicating that the two systems of discrimination may be different or, at least, partially so. However, since the number of mismatches arising as a consequence of heterospecific transformation is bound to be much larger than that arising by transformation with LE marker-carrying transforming isogenic DNA, the inefficiency of the *hex* system in heterospecific transformation may also be explained on the basis of a limiting cellular content of the *hex*-specified component(s) (Tiraby *et al.*, 1975).

Although the molecular mechanism that rejects heterologous DNA so efficiently has not yet been fully clarified, it seems that it mostly acts post-synaptically, at least when the lack of base-sequence homology between the donor and the recipient DNA is not too large. Removal of unpaired donor regions and gapfilling by resynthesis of recipient type DNA would seem a likely mechanism to account for the inherently low efficiency of heterologous systems. Although this hypothesis has not been thoroughly investigated, it seems that the absence of a functional excision-repair mechanism does not affect the efficiency of the heterologous system in *H. influenzae* (Beattie and Setlow, 1970). Nevertheless, the observation that DNA carrying a heterologous marker acquires, as a function of time, properties which make it more acceptable for the recipient type and less so for the donor type recipient (Notani and Setlow, 1972) seems compatible with the operation of an excision-repair system mainly directed towards the DNA moiety carrying the heterospecific genetic information.

VI. Merodiploidy in Transformation

Merodiploidy is the phenomenon whereby two copies of a genetic segment are carried by haploid cells in a more or less persistent way. Merodiploidy, especially if large parts of the genome are involved, is an important phenomenon because it allows dominance and complementation tests to be carried out. In fact, such studies have been performed in *B. subtilis* (Karmazyn *et al.*, 1972) or are in progress (Anagnostopoulos, 1977).

Merodiploid conditions involving short duplications have been reported by Ravin and Takahashi (1970). One shows merodiploidy in the *ery* region of *D. pneumoniae*, the other in the *str* region. The merodiploid

states are associated with particular mutations in these regions. A limited degree of merodiploidy was suggested, because the condition was transferable by a single molecule of DNA. The merodiploid state appeared to be unstable and haploid segregants were formed. Based on the different properties of segregrants, two different merodiploid states could be distinguished which appeared to be interconvertible. The duplicated region can be quite short, since joint transformation by two closely linked *ery* mutations, one of which confers the merodiploid condition on the recipient, can result in stable integration of the mutation not involved in establishing merodiploidy (Ravin and Ma, 1975). Merodiploidy in the region specifying resistance to sulphanilamide has also been reported in *D. pneumoniae* (Hotchkiss *et al.*, 1971). Both the resistance to sulphanilamide and the instability (the particular mutant segregates haploid progeny) could be transferred by a single transformation event. Transformation is rather low, but does not appear to be caused by a LE character of the locus (Ledbetter and Hotchkiss, 1975a). Indirect evidence was obtained (Ledbetter and Hotchkiss, 1975b) that production of haploid segregants is dependent on recombination. In a more direct test involving the introduction of a mutation resulting in ATP-dependent DNAase deficiency, no evidence was obtained that the haploid segregant frequencies had changed. On the basis of this finding the authors suggested that this may limit the type of recombination responsible for segregation of haploids. However, since the role of the ATP-dependent DNAase in transformation is disputable, too great a significance should not be attached to this observation. Low recovery of activity of merodiploid markers carried on artificially prepared heteroduplexes, consisting of one strand obtained from the merodiploid and the other from a haploid strain, was observed. This, and a genetic analysis of the merodiploids, have suggested that the merodiploid condition involving the region specifying sensitivity to sulphanilamide is caused by a tandem duplication (Kashmiri and Hotchkiss, 1975a, b).

A different type of merodiploidy, presumably originating in a different way from those referred to above, has been analysed extensively by Anagnostopoulos and his colleagues (for reviews, see Anagnostopoulos and Trowsdale, 1976; Anagnostopoulos, 1977). A certain mutation, *trp*E26, originally carried by *B. subtilis* strain 166 is transferable to *B. subtilis* strain 168 (the latter being the strain commonly used in the *B. subtilis* transformation system), but the frequency of transfer is very low. If

such transformants, which now carry the *trp*E26 mutation in the strain 168 genetic background, or the original strain 166 *trp*E26 are transformed to tryptophan independence by strain 166 donor DNA, unstable *trp*E$^+$/*trp*E26 merodiploids are produced, which segregate tryptophan-requiring progeny (Audit and Anagnostopoulos, 1972). Subsequent studies (Audit and Anagnostopoulos, 1973a, b) showed that the merodiploidy was extensive and that the greater part of the diploidized region was a replica of the recipient chromosome, carrying alleles identical to the recipient (homodiploidy), which had not been involved in the transformation event (Audit and Anagnostopoulos, 1975). It also appeared that the diploid region in unstable diploids was more extensive than in stable ones.

The development of a hypothesis concerning the nature of the origin and the genetic structure of these merodiploids in *B. subtilis* is intimately connected with the reorganization of a tentative genetic map of *B. subtilis* strain 168, proposed by Dubnau (1971). This reorganization was necessitated when it became clear that the chromosome of *B. subtilis* replicates symmetrically and fully bidirectionally (Gyurasits and Wake, 1973; Wake, 1973, 1974; Harford, 1975), and new linkage data by the use of PBS-1 mediated transduction became available. The revised genetic map of *B. subtilis* strain 168 (Lepesant-Kejzlarova *et al.*, 1975, 1976; Harford, 1975; Harford *et al.*, 1976; Young and Wilson, 1976) shows considerable differences from that of strains carrying the *trp*E26 mutation. These differences involve, firstly a translocation of a segment (C) which is relatively close to the presumed terminus of replication in strain 168, and secondly, a large inversion, between a third and a quarter of the chromosome, containing the origin of replication (Trowsdale and Anagnostopoulos, 1975, 1976). Of the genetic differences between strain 168 and *trp*E26 strains, translocation of the C segment especially appears to be basic to the production of merodiploids when *trp*E26-carrying strains are transformed to tryptophan independence with strain 166 DNA. The model has been proposed that strain 166 donor DNA, carrying a non-interrupted *trp* region, recombines with part of the *trp* region of one chromosome of the recipient and with the other part of the *trp* region on a second chromosome (the *trp* region has been split through the translocation characteristic for *trp*E26 strains). By a recombination between the two, via the segment of donor DNA connected chromosomes, unstable merodiploids are formed carrying, in addition to a diploid C region, a chromosomal region (B) which is adjacent to region

C (Trowsdale and Anagnostopoulos, 1975; Anagnostopoulos and Trowsdale, 1976; Anagnostopoulos, 1977). It seems that two chromosomes recombine fairly frequently, as was established in fused protoplasts of *B. subtilis* (Schaeffer *et al.*, 1976). The model would predict that merodiploids are also produced from strain 168 recipients and *trp*E26 donor DNA if markers are selected which are widely separated in strain 168, but are closely linked in *trp*E26 donors as a result of the translocation. This prediction turned out to be correct (Trowsdale and Anagnostopoulos, 1975; Anagnostopoulos and Trowsdale, 1976; Anagnostopoulos, 1977).

The unstable diploids carrying both the C and B segments of the chromosome segregate *trp*$^+$ cells. Such cells are still redundant for segment C, but have lost the adjacent segment B, possibly by recombination between zones of internal homology residing at the extremities of the B segment (Anagnostopoulos and Trowsdale, 1976; Anagnostopoulos, 1977). These class I stable merodiploids differ from another more rarely formed type (class II), in which the C segment is lost but which continue to carry the B segment in a diploid state (Anagnostopoulos, 1977).

VII. Concluding Remarks

Much progress has been made during recent years towards a better understanding of the phenomenona of competence and entry of donor DNA, and basic properties of the mechanism of integration of transforming DNA. In particular, the isolation of mutants incapable of taking up cell-surface bound DNA, and the characterization of their enzyme deficiency, have provided an extremely useful model which in turn provokes many more experiments directed at clarifying the details of the mechanism which is able to translocate the DNA from without to within the cell. It is likely however that even amongst the best studied transformable bacterial species, the model will not have general applicability because all existing evidence points to differences in the physical state of donor DNA just after entry; single-stranded in *D. pneumoniae* and *B. subtilis* but predominantly double-stranded in *H. influenzae*. It would seem, therefore, that transforming DNA enters into competent *H. influenzae* cells in a fundamentally different way than into *D. pneumoniae* and *B. subtilis*.

Tracing the physical and biological fate of transforming donor DNA

has substantially added to our knowledge of the recombination process. A fairly detailed picture of this process of fundamental biological significance has evolved, and previous uncertainties with respect to the early processing of DNA in *B. subtilis* have been eliminated. However, one feature of the recombination process, its initiation, remains largely obscure. It is forseeable that, in the near future, knowledge concerning this aspect of transformation will advance rapidly, since some basic characteristics of superhelical chromosomes and their capacity to assimilate single-stranded DNA are beginning to be understood.

Extremely little progress has been made concerning the enzymology of integration of transforming DNA into the recipient chromosome. Nevertheless, the means of progress are available. Various recombination-deficient strains are known in which, *in vivo*, the block in the processing of the DNA is rather well characterized, and methods are available for examining which enzyme (or other protein) is present in the recombination-proficient strain but is lacking in the mutant strains. Admittedly, locating the enzyme deficiencies is bound to be laborious, but will, in all likelihood, prove to be a rewarding enterprise.

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Note added in proof: Since the completion of the manuscript early in 1978, several papers have been published bearing on one or other aspect of bacterial transformation. The discovery by Ehrlich (1977), that *Bacillus subtilis* is transformable by plasmid DNA from *Staphylococcus aureus* has accelerated research in the field of plasmid transformation and molecular cloning in *B. subtilis.*

In dealing with the new information, I have adopted the subject division of p. 245, and have added a section describing the recent progress made in transformation by plasmid DNA and molecular cloning in *B. subtilis*.

II. Competence

B. CONDITIONS AFFECTING COMPETENCE

Based on an old observation by Borenstein and Ephrati-Elizur (1969), that outgrowing *B. subtilis* cells originating from a culture of spores release DNA in sequential order, Orrego *et al.* (1978) have explored the physical nature of the released DNA. The transformation assay consisted of outgrowing spores as DNA donors and multiply marked components cells as recipients. It was found that the bacterial chromosome remains attached to the cell in a form inaccessible to DNAase (up to 500 μg/ml), but is available for transformation. A tight physical contact between the outgrowing spores and the recipients is required in this mode of transformation. It is suggested that in the tight contact between the donor and the recipient cells autolysins are necessary to trigger the unmasking of the donor's chromosome. Resistance to DNAase has also been observed in presumed transformation occurring when multiply marked *B. subtilis* strains were mixed and grown in soil (Graham and Istock, 1978). Exchanges of blocks of genes were detected. Frequencies of triple transformants as high as 2.3×10^{-4} were observed after two days of growth. The frequency of the transformations was even higher when DNA was added to a single strain culture growing in soil. This report shows that fairly high levels of competence can be obtained in the natural habitat of *B. subtilis* and should be considered in an appraisal of the risk of spread of recombinant DNA cloned in *B. subtilis*.

Extremely high transformation frequencies with supercoiled plasmid DNA (up to 80%) have been reported, using *B. subtilis* protoplasts in which DNA uptake was induced by polyethylene glycol (Chang and Cohen, 1978). After uptake of DNA, the protoplasts were incubated on media facilitating the regeneration of the bacterial cell wall. This method enhanced the frequency of plasmid-mediated transformation by several orders of magnitude; the recipients were prepared from mid-log cultures which were physiologically incompetent. DNA-uptake was very rapid and the frequency of transformation was proportional to the DNA concentration. Linear and open circular plasmid DNA were also capable of transformation, although at a markedly decreased frequency as compared to closed DNA. Chromosomal transforming DNA was not active, suggesting that either some components of competence were missing in the protoplasts system, or that chromosomal DNA was degraded.

The frequency of transformation of *Streptococcus sanguis* can be enhanced (10–600%) by proteolytic enzymes (Fuchs and Dobrzanski, 1978). The presence of trypsin during cell-DNA contact caused increased release of ^{3}H-aminoacid-labeled components, an increase in binding and entry of donor DNA and a decrease of nucleolytic degredation of the transforming DNA. The first two of these observations may be explained on the basis of the unmasking of receptor sites for DNA. The latter observation may suggest that some surface nuclease(s) affect transformation in a negative way.

In addition to transformation with plasmid DNA, *Pseudomonas putida* has now also proved to be transformable with chromosomal DNA (Mylroie *et al.*, 1978). The frequency of transformation was low and treatment of the cells with $CaCl_2$ was required. Calcium was also required for transformation with chromosomal DNA of *Mycobacterium smegmatis*, especially for the DNA adsorption step (Norgard and Imaeda, 1978). Transformation was low, only observed when the recipient cells were incubated on rich agar plates, and appeared to be independent of any specific phase of growth of the recipient culture. By

freezing and thawing, tumour-inducing plasmids could be introduced into *Agrobacterium tumefaciens*; the maximal efficiency of transformation was very low, 4.5×10^{-8} (Holsters *et al.*, 1978).

Several attempts have been made to increase the competence for transformation of *Escherichia coli*, both for chromosomal and plasmid DNA. Reijnders *et al.* (1978) have reported that pretreatment with calcium ions of *rec*B *rec*C *sbc*B recipients is not required for transformation with chromosomal DNA; calcium ions added simultaneously with DNA suffice. A 100-fold increase of transformation over that obtained by the standard system was observed when the recipients were incubated with DNA in the presence of calcium ions (30 mM) and magnesium ions (26 mM). Competence for plasmid DNA-mediated transformation has been reported to increase gradually after dilution of stationary phase cultures into fresh medium (Brown *et al.*, 1978). No evidence was obtained for the involvement of either a repressor or activator substance. A favourable effect of β-lactam antibiotics on competence was noted. High frequencies of plasmid pBR322 transformation of the NIH-certified EK2 host χ1776 were obtained (in excess of 10^7 transformants/μg plasmid DNA) by Norgard *et al.* (1978). Optimal conditions for transformation included exposure to 100 mM $CaCl_2$ in 250 mM KCl + 5 mM $MgCl_2$ and 5 mM Tris, pH 7.6, before mixing with DNA. Heat shocks at 37°C or 42°C inhibited transformation, and recovery of transformants was best when small samples of cell-DNA mixtures (5 μl) were plated on pre-cooled agar plates. Growth of *E. coli* in the presence of 0.5 M sucrose and addition of 1 μg/ml of lysozyme also appear to increase transformation with plasmid DNA (Molholt and Doskočil, 1978). These authors also reported the isolation of a mutant showing a ten-fold enhanced transformation frequency. Apparently, internal factors also govern the level of competence in *E. coli*. The presence of incompatible *col*E1 replicons in the recipient decreases transformation by plasmid DNA of the same incompatability group, although some depression was also observed by a resident compatible replicon (Warren and Sherratt, 1978). Interference between two plasmids, either during uptake or plasmid establishment, has also been observed by Weston *et al.* (1978). This was displayed by both compatible and incompatible plasmid pairs.

C. PROPERTIES OF COMPETENT CELLS

It has been known for a long time that newly transformed *B. subtilis* cells do not multiply and are resistant the thymineless death (Nester and Stocker, 1963; Nester, 1964; McCarty and Nester, 1967). Evidence has been presented (McCarty and Nester, 1967; Dooley *et al.*, 1971) that relative to the non-competent fraction in competent cultures, DNA-synthesis is severely depressed in the competent fraction. Notwithstanding this, approximately 25% of bacteriophage DNA label taken up during transfection with ϕe DNA can be incorporated into the bacterial chromosome, which may be interpreted as indicating that deoxylnucleotide metabolism rather than DNA replication is blocked in competent *B. subtilis* cells (Loveday, 1978).

The inability of competent *B. subtilis* to multiply is in sharp contrast to competent *Haemophilus influenzae* cells which resume growth at once and, in addition, show synchronous division, when returned to complete growth medium. DNA-synthesis is quickly resumed, but the rate of RNA and protein synthesis remains low until the time of division. Since the decay of competence closely follows the change of RNA synthesis, an inverse relationship between the rate of RNA synthesis and the capacity to take up DNA has been suggested (Scocca and Habersat, 1978).

The role of pili in DNA-binding and/or uptake by *Neisseria gonorrhoeae* remains uncertain, especially since it has been reported that non-virulent type 4 cells of this

organism, which are not piliated, are transformable with high frequency (2%) to piliated, virulent type 1 or type 2 morphology. Competence was found to be maximal in late log-phase cultures (Baron and Saz, 1978). Transformation with the plasmid-carried *β*-lactamase gene was found to be much lower than with chromosomal DNA. This may be due to surface-located endonuclease activity, since the transformation by plasmid DNA frequently resulted in deletions and rearrangements of the plasmid (Sparling *et al.*, 1978).

D. DNA BINDING AND DNA BINDING SITES

The effect of temperature on binding of DNA has been determined in *B. subtilis*. The binding appears to be fairly constant between 25°C and 45°C, but is reduced at temperatures below 15°C. DNA attached to the recipients of 4°C can be taken up if the culture is shifted to 37°C, suggesting that DNA binds to the appropriate receptors at 4°C. It is supposed that a change in the DNA receptor sites at 4°C causes loss of affinity for DNA (Weppner and Leach, 1978). Diminution of DNA-binding has also been observed in *B. subtilis* in the presence of 10 mM EDTA (Garcia *et al.*, 1978). These authors observed that both binding and transformability are irreversibly inhibited by EDTA, which is in marked contrast with the generally accepted idea that EDTA is reversibly inhibiting the entry of DNA, both in *B. subtilis* and *Diplococcus pneumoniae*. Kubinski and Kubinski (1978) have reported that changes in the DNA caused by two carcinogenic monofunctional alkalting agents resulted in the loss of recognition of transformation-specific receptor sites. The chemically modified DNA appeared to bind to non-specific sites on the surface of both competent and noncompetent cells.

The binding of DNA to *D. pneumoniae* has been further explored in mutants blocked in the entry of DNA. Two steps have been distinguished. The first appears to be reversible, the second is associated with the nicking of DNA. The nicking of bound DNA has been quantified by using phage PM2 DNA, which is a closed circular duplex. One to two single-stranded breaks are introduced per molecule (Lacks, 1978).

Binding of DNA to *S. sanguis* seems to be influenced by contaminating protein. Protein-containing DNA binds to *S. sanguis* in the presence of chelators. Treatment of the DNA with proteolytic enzymes, precipitation with ethanol and heating causes diminution of binding in the presence of chelator, which can be restored by a number of divalent cations (Ranhand, 1978).

Homogenous preparations of DNA-binding factor have now also been obtained from *S. sanguis* (Cegłowski *et al.*, 1978). The factor was partially characterized. It appears to be thermostable, and binds *in vitro* both the double- and single-stranded DNA. Competition of DNA for the binding factor was observed, *in vitro* except for glucosylated phage DNA, which was also a poor competitor *in vivo*.

The ability of *H. influenzae* specifically to recognize and to take up homologous or closely related DNA has been further analysed by the use of the *E. coli* plasmid pBR322 in which a piece of *H. influenzae* DNA was inserted (Deich *et al.*, 1978; Goodgal and Chung, 1978). It was concluded that the specificity for binding and uptake resides in specific sequences of the cloned *Haemophilus* DNA. Deich *et al.* (1978) further showed that the *H. influenzae* genome contains approximately 800 uptake sequences, similar to that of *H. parainfluenzae, H. haemolyticus* and *H. aegypticus*. The *Haemophilus* DNA-specific binding activity, which is not present in noncompetent cells, can be released from the competent cells by 1% Triton X-100 and was partially purified. It was further observed that non-specific DNAs compete at only a level of 1–4% with *Haemophilus* DNA, that the extent of competition depends principally on the concentration of specific recognition sequences and that competition is independent of DNA length over a range of 1–50 kilobases. The number of sequence-specific receptors is limited to 4–6 per cell and appears to be used

only once. An apparently specific DNA-uptake system has very recently also been reported by Dougherty *et al.* (1978) in *N. gonorrhoaea*. Two types of receptor sites for DNA have been suggested to exist on the surface of this organism. Receptor 1 adsorbs large quantities of both homologous and heterologous DNA, but the DNA remains at a site accessible to external DNAase. Adsorbtion to receptor 2 seems to be limited to homologous DNA or DNA from taxonomically related species. A portion of this DNA enters into the recipients. It was further observed that homologous DNA molecules exclusively compete for receptor 2.

E. MECHANISM OF ENTRY OF TRANSFORMING DNA

Seto *et al.* (1978) have reported results which complicate the view expressed by Lacks *et al.* (1974) that, in *D. pneumoniae*, a surface located nuclease is required for entry of transforming DNA. Seto *et al.* (1978) found that *noz* mutants lost their incapacity to take up DNA by treatment with activator protein; this treatment did not detectably increase the endonuclease activity of the mutants. Apparently the entry deficiency of DNA was due to inhibiting factors released by the mutants. It is clear that further research is required to determine the role of the surface-located endonuclease in DNA-entry.

Chemical modification of DNA by monofunctional alkylating agents has been reported to slow down entry of donor DNA, and eventually to prevent entry of DNA in *B. subtilis* (Kubinski and Kubinski, 1978). DNA cross-linked with nitrogen mustard decreases the frequency of cotransformation of two linked markers in *B. subtilis* (Scheinbach and Rudner, 1978). Although, in principle, the unlinking event may occur in several of the steps recognized in transformation, one may suppose it to occur preferentially during the processs of entry, because cross-links may interfere with the transition of duplex DNA to single-stranded DNA during entry. Chromosomal DNA cross-linked by trimethylpsoralen binds equally efficiently to competent *B. subtilis* as does untreated DNA, but its entry is severely curtailed (G. Venema and U. Canosi, manuscript in preparation).

III. Intracellular Fate of Transforming DNA

B. MACROMOLECULAR STATE OF TRANSFORMING DNA SHORTLY AFTER ENTRY

In an extension of his previous work (Morrison, 1977), Morrison (1978) has determined the nature of the material complexed to single-stranded DNA after entry in *D. pneumoniae*. Several properties of the complex indicate that single-stranded DNA is non-covalently associated with a protein or a class of proteins with a narrow range of polypeptide sizes, syntheized during the development of competence. The determination of the macromolecular state of donor DNA has also progressed in *S. sanguis*. Raina and Ravin (1978a) have reported that DNA resistant to exogenous DNAase also exists as a complex of single-stranded DNA and recipient constituents, probably proteins, because the donor DNA can be removed from the complex by pronase digestion and treatment with phenol. The DNA in the complex is resistant to digestion with DNAase 1 and nuclease S1.

C. INTEGRATION OF TRANSFORMING DNA

In following the fate of homospecific transforming DNA in *S. sanguis*, Raina and Ravin (1978a) have observed that the complex of single-stranded DNA and protein, described above, moves as a whole to associate with the recipient chromosome. Since the formation of recombinant type DNA lags behind the physical association of the single-stranded DNA-protein complex with the recipient chromosome, they speculated that the protein moiety of the donor-recipient complex may help to stabilize synapsis. Furthermore, it was found that exposure to ethidium bromide inhibits transformation of *S. sanguis*. Since the

percent of inhibition was equal to the percent of donor DNA-protein complex not yet associated with the recipient chromosome, it was suggested that the substance prevents synapsis. The interaction of ethidium bromide with the donor DNA-protein complex apparently changes the structure of the complex, since the DNA in the complex becomes subject to endogenous nuclease activity (Raina *et al.*, 1978b).

The observation made by Tiraby and Fox (1974a) that size reduction of transforming DNA carrying LE markers diminishes their biological activity relatively greatly, was not confirmed by Guild and Shoemaker (1976), nor again by Claverys *et al.* (1978) who used both mechanical means and restriction enzymes to decrease the size of the DNA.

Grist and Butler (1978) have observed that, contrary to *hex*, in *hex*$^+$ strains of *D. pneumoniae* the frequency of mutation decreases with the onset of competence. The extent of the decrease was reduced by the addition of heterologous DNA. On the basis of these observations it is suggested that the *hex*-system is more active in competent cells than in non-competent cells.

D. MUTANTS AFFECTED IN RECOMBINATION

Three classes of transformation-deficient mutants from piliated transformable *N. gonorrhoeae* types have been isolated. One class, which is normally transformable with plasmid DNA, but not with chromosomal DNA, may be faulty in recombination. Two other classes have been recognized, one is deficient in DNAase-resistant uptake of DNA, the other is probaly blocked in a late step of uptake which affects both circular and linear DNA molecules (Sparling *et al.*, 1978).

V. Heterospecific Transformation

Further evidence has been obtained indicating that mainly base sequence inhomologous underly the low frequency of transfomation with heterologous DNA. Thus, it has been reported that DNA obtained from *B. subtilis* recipients, transformed with DNA from *B. amyloliquefaciens* (intergenotes), transforms *B. subtilis* less well than does homologous DNA, and that the efficiency of transformation with the intergenote DNA appears to be inversely related to the length of the heterologous portion in the intergenote genome. Two out of 16 host cell reactivation-deficient mutants proved to be better transformed with intergenote DNA than was the proficient recipient, suggesting that correction of the mismatched region may be carried out by functions related to excision-repair of UV-damage (Matsumoto *et al.*, 1978). In agreement with earlier reports, Harris-Warrick and Lederberg (1978a) showed that base sequence inhomologies in *B. subtilis* are the major barriers to heterospecific transformation. These authors (Harris-Warrick and Lederberg, 1978b) also showed that transformation by *B. subtilis* DNA of intergenotes obtained by previous transformation of *B. subtillis* with B. globigii DNA, was unusually sensitive to decrease below a critical size of the DNA, indicating a requirement for concurrent transformation of adjacent homologous sequences. It further appeared that at all levels of size reduction of the donor DNA 100% cotransfer was observed of a block of genes carried by the intergenote, which agrees with the hypothesis that efficient transformation of the intergenote is possible only if homology exists between the donor DNA and the sequences present on both flanks of the intergenote.

The fate of heterologous DNA in an additional heterospecific transformation system has been studied by Raina *et al.* (1978a). Similarly to homologous DNA, it was found that DNA from *D. pneumoniae* complexes with protein in *S. sanguis*. However, the rate of association of the protein-heterologous DNA complex with the recipient chromosome appeared to be markedly slower. Also, the complex was less stable than that formed in homospecific transformation and more time was required to establish genetic integration.

In contrast to the homospecific DNA complex with the recipient chromosome, which is little, if at all, degraded to acid-soluble material, 50% of the heterospecific DNA was degraded. Native heterospecific DNA is reversibly inactivated *in vitro*, probably by a protein present in lysates of competent *S. sanguis* cells. Although it has been shown that the inactivation is not due to degredation of the DNA, the nature of the inactivation remains to be elucidated. No major differences were found in the early processing of the inactivated DNA. The possibility was entertained that the donor DNA after having synapsed with the recipient chromosome is degraded either by an enzyme recognizing the inactivating protein or by the inactivating protein itself, activated following synapsis (Raina and Ravin, 1978b).

Some further observations have been made concerning the effect of RNA metabolism on the extent of transformation by heterologous DNA in *S. sanguis*. Streptolydigin appears to degrade nascent RNA in *S. sanguis*. When recipient cells are incubated with streptolydigin until nascent RNA is degraded, and then exposed to heterospecific DNA, the difference between the level of homospecific and heterospecific transformation is reduced. In this respect streptolydigin is as effective as rifampin. It is suggested that the loss of condensed recipient DNA, due to removal of nascent RNA, facilitates the integration of poorly paired heterospecific DNA (Deddish and Ravin, 1978).

Transformation by Plasmid DNA and molecular cloning in *B. subtilis*

Except for two cases, one reporting successful transformation of *S. sanguis* to erythromycin resistance by plasmid DNA from *S. faecalis* (Ceglowski and Pieniażek, 1978), and the other, reporting the construction and cloning of hybrid plasmids of *Staphylococcus aureus* in *S. aureus* (Löfdahl *et al.*, 1978b), most attention has been recently directed towards transformation by plasmid DNA of *B. subtilis* and to the development of suitable vectors for the cloning of DNA in this organism.

Prior to the discovery by Ehrlich (1977) that *B. subtilis* can be transformed by certain *S. aureus* plasmids, which have subsequently proved to be suitable vectors for cloning of foreign DNA in *B. subtilis*, DNA has been introduced into *B. subtilis* which was inserted into plasmids of *E. coli* and cloned in *E. coli*. It was shown that composite plasmids, containing the DNA coding for thymidilate synthetase of the temperature *B. subtilis* bacteriophage ϕ3T and the *E. coli* plasmid pSC101 (Ehrlich *et al.*, 1976), or pMB9 (Duncan *et al.*, 1977) transformed *thy*$^-$ *B. subtilis* to *thy*$^+$. However, clones arising from cells transformed by the composite pSC101 plasmid did not contain detectable pSC101 sequences, indicating integration of the thymidilate synthetase gene into the bacterial chromosome (Ehrlich *et al.*, 1977). Similarly, integration of *B. subtilis* leucine genes, carried by plasmid pMB9, into the *B. subtilis* chromosome has been observed by Mahler and Halvorson (1977). A composite plasmid from *E. coli* and *B. subtilis* plasmid DNA has been constructed by Horinouchi *et al.* (1977). The plasmid replicated as a biologically functional unit in *E. coli* and appeared to synthesize *B. subtils*-specific mRNA in cell-free *E. coli* extracts. The thymidilate synthetase gene from the virulent *B. subtilis* bacteriophage β22 has also been inserted into pMB9 (Duncan *et al.*, 1978). This plasmid transformed *E. coli thy*$^-$ to *thy*$^+$, but failed to do so in *B. subtilis*. However, if the gene was inserted into a plasmid containing the pMB9 DNA in addition to a region of homology to the chromosome of *B. subtilis* (ϕ3T DNA, not containing the ϕ3T thymidilate synthetase gene), *thy*$^+$ clones were obtained. Analysis of the genetic composition of these clones revealed that the entire composite plasmid had been incorporated into the *B. subtilis* chromosome. It was inferred that circular double-stranded DNA had entered the cell and had become integrated by a reciprocal cross-over event. A similar mechanism of integration has been suggested for the integration of the

*thy*A gene from *E. coli* carried on an *E. coli* plasmid in the *B. subtilis* chromosome (Rubin *et al.*, 1978).

A major breakthrough in cloning recombinant DNA in *B. subtilis* came when Erlich (1977) discovered that plasmids from *S. aureus*, coding for antibiotic resistances are able to transform *B. subtilis* with fairly high frequency and are maintained as independent replicons. The plasmids investigated (four coding for resistance to chloramphenicol, one for tetracycline resistance) were considered to be promising vectors for cloning because they are small, carry easily selectable markers and contain a small number of cleavage sites for certain restriction enzymes (those carrying resistance markers for chloramphenicol contain only one *hind III* site, the one coding for tetracycline resistance three such sites). Two further observations were made. Firstly, it appeared that the frequency of transformation with *S. auraus* plasmid DNA obtained from transformed *B. subtilis* was approximately fifty times higher than with plasmid DNA isolated from *S. aureus*, and secondly, that linearization of the plasmid DNA decreased transformation by a factor in excess of 200. *S. aureus* plasmid DNA-mediated transformation of *B. subtilis* was also observed by Löfdahl *et al.* (1978a). Berhard *et al.*, (1978) have characterized a number of plasmids carried mainly by *B. cereus*. One plasmid, coding for tetracycline-resistance, appeared to be able to transform *B. subtilis*; the plasmid was stably maintained. When it was isolated from *B. subtilis* it did not have increased transforming activity. The plasmid, pBC16, contains 2 *Eco*R1 and one *Bam*1 cleavage sites. Several *S. aureus* antibiotic resistance marker-carrying plasmids have been transformed into *B. subtilis* by Gryczan and Dubnau (1978). Five of these were reobtainable from *B. subtilis* as covalently closed circular DNA, but three were not. The plasmids studied replicated as multicopy autonomous replicons in both recombination proficient *B. subtilis* and the recombination deficient *rec*E4 mutant. Transduction of the plasmids multiplying in *B. subtilis* was possible by means of the generalized transducing phage AR9. Restriction endonuclease cleavage site maps were constructed for the plasmids pUB110 (Km^R), pSA2100 (Cm^R, Sm^R), pCM194 (Cm^R) and pSA0501 (Sm^R). In studying the effect of a large number of temperature-sensitive *dna* mutations, Shivakumar and Dubnau (1978a) observed that plasmid pUB110 continues to replicate at the non-permissive temperature in several mutants (notably *dna*A13, *dna*C30 and *dna*J102) in the absence of chromosome replication, such that approximately equal amounts of plasmid and chromosomal DNA are present. This facilitates the isolation of large quantities of covalently closed circular DNA. The copy number of several *S. aureus* plasmids is also increased when *B. subtilis* growing in the presence of deoxyadenosine and thymidine is exposed to hydroxyurea, an inhibitor of ribonucleotide reductase (Shivakumar and Dubnau, 1978b).

When the Cm^R-carrying *S. aureus* plasmid pC194 DNA, obtained from *B. subtilis*, is subjected to gel electrophoresis, eight bands can be distinguished. The nature of the DNA in these bands has been examined by means of electron microscopy and their biological activities have been determined (Canosi *et al.*, 1978). It appeared that monomeric DNA had the lowest specific biological activity and that the transforming activity of DNA from the bands containing various multimeric forms of the plasmid was approximately 1000-fold higher than that of the monomeric form. The curves relating DNA concentration and transformation frequency did not exceed the value of one for any of the various types of DNA examined, indicating that no cooperation between molecules is required to produce a transformant. The finding that the transforming activity of the plasmid preparation is almost exclusively carried by the minor subclass of oligomeric molecules may offer an alternative interpretation for the observation that *S. aureus* plasmids isolated from *B. subtilis* show a higher transforming activity than when isolated from *S. aureus*. Rather than invoking the operation of a restriction-modification system (Ehrlich, 1977), this increase

may also result from a lower frequency of oligomeric molecules present in plasmid preparations obtained from *S. aureus*.

The successful *in vitro* insertion in, and cloning of, chromosomal DNA in *B. subtilis* and the *in vitro* construction of hybrid plasmids capable of replication and expression in *B. subtilis* was reported simultaneously by Keggins *et al.* (1978a), Gryczan and Dubnau (1978) and Ehrlich (1978). Keggins *et al.* (1978a) demonstrated that chromosomal DNA fragments, carrying *trp* genes, obtained by *Eco*R1 digestion from *B. pumilus* and *B. licheniformis* DNA can be inserted in the *Eco*R1 site of the *S. aureus* plasmid pUB110 (*Neo*^{R}). The *Trp*-plasmids complemented the *trp*C_2 mutation in *B. subtilis*. Stable cloning of a *Trp* segment of *B. subtilis* DNA in *B. subtilis* was obtained in *B. subtilis* carrying the *rec*E4 mutation. Recombination between the Trp region of the DNA carried by the plasmid and the chromosome was suggested by the observation that 0.1% of the cells derived from one *Neo*^{R} Trp^{+} transformant in the *Rec*$^{+}$ background had lost their resistance to neomycin, but continued to show the Trp^{+} phenotype.

Hybrid plasmids obtained by ligase-joining of restriction enzyme-treated *S. aureus* plasmids have been obtained by Gryczan and Dubnau (1978) and Ehrlich (1978). The hybrid plasmids replicate and express their antibiotic resistance characters derived from the parental plasmids. Some of the constructed plasmids suffered a loss of DNA; the loss involved one of the two termini to join the parental plasmids (Gryczan and Dubnau, 1978). In addition to joining different *S. aureus* plasmids, Ehrlich (1978) also succeeded in constructing recombinant plasmids by joining restriction enzyme-digested plasmids from *S. aureus* and *E. coli*. These plasmids express the antibiotic resistance information carried by the *S. aureus* and *E. coli* moieties in *E. coli* cells, but in *B. subtilis* only the *S. aureus* plasmid information is expressed. Essentially similar results have been obtained by Kreft *et al.* (1978), who found that tetracycline resistance of the DNA moiety originally obtained from a *B. cereus* plasmid of a hybrid plasmid also containing *E. coli* plasmid DNA is expressed both in *B. subtilis* and *E. coli*, whereas the antibiotic resistance information carried by the *E. coli* moiety is not expressed in *B. subtilis*. Over a longer period of time the hybrid plasmids containing *B. subtilis* and *E. coli* plasmid DNA appeared to be unstable in *B. subtilis*, but not in *E. coli*.

The expression in *E. coli* of foreign DNA residing in *E. coli* replicons is not limited to antibiotic resistance information. In addition to the thymidylate synthetase genes of *B. subtilis* bacteriophage, referred to earlier, *pyr*$^{+}$ and *leu*$^{+}$ genes from *B. subtilis*, inserted into bacteriophage λ, are capable to transform pyrimidine- and leucine-requiring *E. coli* (Chi *et al.*, 1978). Similarly, leucine genes of *B. subtilis* are expressed in *E. coli* when cloned in an *E. coli* plasmid. However, the leucine genes of *E. coli* inserted into the plasmid were unable to transform *B. subtilis* auxotrophs (Nagahari and Sakaguchi, 1978). Successful transformation of *B. subtilis* auxotrophs has been obtained with leucine genes carried by a composite plasmid constructed from a *B. subtilis* (*natto*) plasmid. The *rec*E mutation appeared to be required for stable maintenance of the plasmid in *B. subtilis*. A derivative plasmid was obtained in which insertion of DNA into the *Bam*N1 site inactivates the *leu*A gene, but not the *lau*C gene, which facilitates the selective obtaining of recombinant DNA. The *rec*E gene of *B. subtilis*, which is indispensible for DRC formation in transformation with chromosomal DNA, is both required for recombination between *B. pumilus* DNA carried by a plasmid and *B. pumilus* chromosomal DNA, and for recombination between compatible plasmids containing homologous *B. pumilus* sequences (Keggins *et al.*, 1978b).

In conclusion, cloning of DNA in *B. subtilis* has become possible. Although the information available is still rather scarce, it seems that the following tentative conclusions can be drawn: (1) DNA cloned in *B. subtilis* is not readily expressed, unless the DNA

originates from taxonomically related species, (2) DNA from taxonomically unrelated species can be maintained in *B. subtilis* on plasmic replicons and can also be expressed if it is integrated into the recipient chromosome, (3) plasmid replicons carrying *B. subtilis* DNA are stably maintained in *rec*E mutants of *B. subtilis*, but not in recombination proficient recipients.

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