ANTIBIOTIC INHIBITORS OF BACTERIAL CELL WALL BIOSYNTHESIS

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ANTIBIOTIC INHIBITORS OF BACTERIAL CELL WALL BIOSYNTHESIS

SECTION EDITOR

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DEDICATION

This volume is dedicated to DR. JACK L. STROMINGER *whose intelligence, profound biochemical insight, enthusiasm, and unfailing ability to ask the right questions has been largely responsible for the major advances made in understanding the mode of action of inhibitors of bacterial cell wall peptidoglycan biosynthesis over the past twenty-five years.*

PREFACE

Antibiotic Inhibitors of Bacterial Cell Wall Biosynthesis: the present and some thoughts on future trends

Epidemic bacterial infectious diseases, such as cholera and plague, have played such a prominent part in the history of the 'Western' world that their horrors still echo in our memories. Although apparently laid to rest by modern medicine, they are still capable of ravaging less fortunate societies. The spectre stirred again in the United States in 1976 with the recognition of Legionella as a 'new', frequently lethal, bacterial epidemic disease. It now looms menacingly in the form of AIDS, a viral disese whose mechanisms could not have been understood as recently as twenty years ago. Such is the pace of modern biological research, however, that there is hope that this disease, undoubtedly the most serious challenge to preventative medicine and therapy of infectious disease ever recognized by man, can be contained by a massive research effort to pinpoint its mechanisms and weaknesses. Fear is widespread; panic may not be far behind.

Nevertheless, it is undoubtedly true that the development of sanitation and other public health measures, dating back at least to the Minoan civilization, by thwarting the food and water-borne routes of transmission of infectious diseases, did more to improve the longevity of Urban man than all of modern microbiology. Moreover, vaccination, developed out of an intuitive response to scientific observation long before infectious agents were identified, conquered smallpox and still provides the best chance of holding in check the pandemic viral and bacterial infections such as influenza, measles, mumps and TB that debilitate, maim, and sometimes kill. It was the establishment of the science of microbiology in the nineteenth century, however, that was the necessary precursor to one of the major success stories of twentieth century medicine—the development of effective antimicrobial chemotherapy.

From the beginning, this was an enterprise carried on the crest of the wave of developing science—microbiology, genetics, organic chemistry, then biochemistry and, more recently, molecular biology and genetics. Slow to develop, it came of age with the introduction of penicillin during the second World War. This volume represents an overview of the field initiated with that event: The mechanisms of action and some major mechanisms of resistance to chemotherapeutic agents that interfere with bacterial cell wall biosynthesis. These agents, and the β-lactam antibiotics in particular, still provide some of the best weapons for treatment of many bacterial infectious diseases, in spite of the spread of resistance to these agents in many genera as a consequence of selection. Diseases such as pneumococcal pneumonia, whose pathogenic agents remain for the most part susceptible, are no longer feared random killers of the relatively healthy. The major challenges for chemotherapy include the increasing population of individuals with severely impaired immunity, the broad range of pathogens to which they are susceptible, including some which are intrinsically resistant to most chemotherapeutic agents, and the variants of common pathogens which have acquired resistance to the commonly used agents and sometimes even to their latest derivatives.

Ward's chapter on the biosynthesis of peptidoglycan and the points of attack by wall inhibitors sets the stage for this field. The chapters by Neuhaus and Hammes on alanine analogues, by Toscano and Storm on Bacitracin, and by Perkins on Vancomycin and related antibiotics, follow in the sequence of their targets in the pathway of peptidoglycan biosynthesis. My own chapter on the mode of action of β-lactam antibiotics, inhibitors of the final stages of cell wall biosynthesis, provides further background for the final chapters on modern β-lactam antibiotics (Sykes *et al.*), the role of permeability barriers in resistance to these agents (Nikaido), and the role of β-lactamase inhibitors in chemotherapy (Neu).

It is worth noting that the more interesting and valuable recent developments in the

chemotherapy of bacterial infectious disease have come from the rational application of the tools of modern organic chemistry to the design and synthesis of new derivatives of natural antibiotics, such as the monobactams and the β-lactam containing β-lactamase inhibitors, and to the production of completely synthetic agents such as alafosfalin. It is quite possible that the next significant advances in this field will come from two directions: First, the developing, detailed insight into the mode of action of known antibiotic inhibitors of transpeptidases andother peptidoglycan biosynthetic enzymes, based on the X-Ray crystallographic structural analysis of these targets, should lead to more rational and less empirical methods for the design of better inhibitors. These may either be variants of known antibiotics or completely new types of compound. Second, the rational design of inhibitors of other potential targets in wall biosynthesis for which no adequate natural or synthetic inhibitor currently exists, remains an open challenge. These targets include muramic acid biosynthesis and the production of D-glutamate. Phosphonomycin is an excellent inhibitor of muramic acid synthesis. Although resistance is easily acquired in the laboratory due to loss of the uptake pathways, this apparently does not impair its clinical utility, as shown by experience in Japan.. D-Glutamate production must be as carefully regulated as D-alanine production, but it has not been extensively studied. The mechanisms controlling cell wall autolysis, if sufficiently general, could also be specific targets for selective disruption. They are now known to be triggered in a species- and inhibitor-specific fashion by many of the agents discussed in this volume. Inhibitors of outer membrane assembly, particularly of lipopolysaccharide biosynthesis, might be effective agents specific for Gram-negative bacteria. At least they might disrupt the outer membrane permeability barrier sufficiently to act synergistically with β-lactam or other antibiotics. The recent reports concerning dipeptide derivatives of inhibitors of keto-deoxyoctanate biosynthesis are encouraging. They are highly effective against Gram-negative organisms *in vitro*, though possibly susceptible to hydrolysis by dipeptidase *in vivo*. It also seems possible that the recent application of the the techniques of modern molecular genetics to investigation of the mechanisms of pathogenesis of faculative intracellular parasites, such as *Salmonella, Yersinia* and *Legionella*, will lead to new strategies for treatment involving a dual attack on the bacteria themselves (conventional antibiotics) and on their mechanisms for avoiding intracellular killing mechanisms and surviving in this antibiotic-protected niche. Any such combined strategy may have to be tailored to specifically diagnosed infections. Analysis of the pathogenic mechanisms of several major extracellular bacterial pathogens, such as *Neisseria, Staphylococci, Campylobacter*, and enteropathogenic *E. coli* is also advancing rapidly, and should yield important clues for improvement in methods of prophylaxis and treatment. One aspect of antibacterial chemotherapy seems predictable: it will evolve and become more complex as our knowledge increases and as the pathogens adapt to our best efforts by mutation and plasmid acquisition.

Many of the recent advances listed and suggested above have been and will be pursued, appropriately, in the laboratories of the pharmaceutical industry. As previously implied, however, this enterprise is built on accumulated knowledge in the fields of organic chemistry, biochemistry, microbial physiology, and molecular biology and genetics. These subdisciplines of biology have named themselves as reflections of the aspirations of their practitioners. In turn, these names reflect the history of increasing sophistication in our drive to understand the life process. This intellectual evolution superbly illustrates the benefits to be derived from basic research, research aimed first at understanding, and only later at practical issues. Such basic research must be publicly funded. Applications come when the utility of tools developed by basic research become obvious. It is easy for the controllers of the public purse to identify the targets for such use. It is far more difficult for them to understand that the vitality and progression of the entire field of medicine is dependent on maintenance of an adequate level of funding for basic research and research training. We must continue to attract our ablest minds to this enterprise by demonstrating a firm political commitment to its support. The only argument should concern the appropriate fraction of our national resources to be devoted to this essential activity.

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CONTENTS

CHAPTER 1

BIOSYNTHESIS OF PEPTIDOGLYCAN: POINTS OF ATTACK BY WALL INHIBITORS

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1. INTRODUCTION

The bacterial wall is of major importance not only to the organism itself, but to man as the focus of interaction between the host, the bacterium and antimicrobial agents. It is now thirty years since Park (1952a,b,c) reported the isolation of the first nucleotide linked precursor of peptidoglycan from *Staphylococcus aureus* grown in the presence of benzylpenicillin. Since that time studies of the biosynthesis of peptidoglycan have been essential in furthering our knowledge not only of the complexities of the biosynthetic mechanisms but also of the mode of action of inhibitors of this process. These now include a wide range of compounds whose sites of action cover the whole biosynthetic pathway.

2. STRUCTURE OF PEPTIDOGLYCAN

The walls of all bacteria, with the exception of the Archaebacteria, contain peptidoglycan as their main structural component. In Gram-positive organisms this polymer alone may account for as much as 80% of the total weight of the wall, although values of 40–50% are more commonly found. In Gram-negative organisms peptidoglycan represents only a minor fraction of the wall. As its name implies, it consists of glycan chains with peptide substituents that are cross-linked. Thus, the organism is enclosed in a network; the bag-shaped macromolecule of Weidel and Pelzer (1964), which is responsible for the shape of the bacterium.

The glycan chains consist of alternating residues of glucosamine and muramic acid, the 3-*O*-D-lactyl ether derivative of glucosamine. In most bacteria the amino groups of these hexosamines are *N*-acetylated although in certain organisms including Bacilli, Clostridia and Rhodopseudomonads, glucosamine is largely unsubstituted. Other minor modifications have been reported (for review see Rogers *et al.*, 1980). Mycobacteria, *Nocardia kirovani* and Micromonospora contain *N*-glycoyl- and rather than *N*-acetylmuramic acid and *O*-acetylation of *N*-acetylmuramic acid occurs in *Staph. aureus* and *Neisseria gonorrhoea*. The biosynthetic aspects of these modifications are discussed elsewhere in this review.

The glycan chains vary considerably in length from several hundred to ten to twenty disaccharide repeating units (Ward, 1973). They appear to be biosynthesized as extremely long chains which are then subject to cleavage by autolytic glycosidases. Fractionation of the glycans isolated from *Bacillus subtilis* walls revealed a heterogeneous population, the largest size class having an average chain length of 500 disaccharide units. This would be approximately 500 nm long (Fox *et al.*, 1977). Even much shorter chains would be too long to be arranged radially in the peptidoglycan and there is now microscopic and physical evidence that the glycan chains of *Escherichia coli* lie more or less perpendicular to the length of the cell (Verwer *et al.*, 1978, 1980).

The carboxyl groups of the muramic acid residues are, with the exception of some residues in *Micrococcus luteus*, substituted with short peptides, a proportion of which are

cross-linked, either directly or through a second short peptide. It is this cross-linkage which joins the glycan chains to form the macromolecular network of high tensile strength and rigidity. The configuration of the amino acids in the primary peptide side chain extending from muramic acid are L (or glycine) D, L, D and D. Linkages are α-peptide bonds except for the one between D-glutamic acid and the third residue which is formed by the γ-carboxyl group of the glutamyl residue. In Gram-negative organisms and Bacillus spp. the majority of cross-links are direct between the penultimate D-alanine residue of one peptide side chain and the free amino group of the meso-diaminopimelic acid residue of a second peptide (Fig. 1).

However, recent studies (Glauner and Schwarz, 1983) using high-pressure liquid chromatography of muramidase-solubilized sacculi of *E. coli* demonstrated that the classical monomers and dimers accounted for only some 70% of the total digest. The remaining 30% was made up of other disaccharide-peptides including dimers in which an additional D,D-diaminopimelic acid residue was present. About 20% of the dimers present had this unusual structure, which was associated with the linkage of lipoprotein to the peptidoglycan. Some 30–40% of the peptidoglycan-bound lipoprotein was attached to the D,D-diaminopimelic acid containing dimers and not to those which had direct cross-linkage.

In other Gram-positive bacteria a great variety of peptides are found although the peptidoglycan from any one species still contains no more than four or five amino acids (Fig. 1). Many of these additional amino acids are present in peptides forming a cross-bridge linking two peptide side chains. One such example, in an organism on which much of the earlier biosynthetic work was carried out, is the pentaglycine cross-bridge in *Staph. aureus*. Two major attempts have been made at clasifying peptidoglycans on the basis of their chemical structures (Ghuysen, 1968; Schleifer and Kandler, 1972). In each the type of cross-linking present was regarded as being of major importance.

The extent to which cross-linkage occurs also varies considerably from one organism to another. In Gram-negative organisms and Bacilli about 30–50% of the peptides are cross-linked as dimers, whereas in *Staph. aureus* cross-linkage is almost complete and a high proportion of the peptides may be isolated as oligomers containing an average of five to eight repeating units (Tipper and Berman, 1969). However, recent observations (discussed in detail in a later section) have shown that *Staph. aureus* can grow apparently normally with much reduced cross-linkage (Wyke *et al.*, 1981).

There is no evidence available which suggests any specific location of uncross-linked peptides and they are probably interspersed throughout peptidoglycan. Peptides which do not become involved in cross-linkage often lose either one or both of their terminal D-alanine residues by the action of carboxypeptidases. Hence, peptidoglycan isolated from walls of many bacteria contained an average one or less D-alanine residue per glutamic acid residue.

3. BIOSYNTHESIS OF PEPTIDOGLYCAN

The synthetic pathway has now been studied in many organisms including *Staph. aureus*, *E. coli*, several Bacilli and *M. luteus*. Despite wide differences in bacterial wall structure, from both a chemical and morphological point of view, peptidoglycan synthesis shows an overall similarity in these organisms which confirms the fundamental nature of the process.

Three stages in the synthetic pathway can be recognized, each occurring at a different cellular site. The first stage, catalyzed by soluble enzymes in the cytoplasm, is the formation of the nucleotide-linked precursors. In the second stage, which is presumed to occur at the interface of the cytoplasm and membrane, the precursor subunits are transferred to a lipophilic carrier, undecaprenyl phosphate, and further modified. These reactions are catalyzed by membrane-bound enzymes while the disaccharide pentapeptide subunit remains attached to the lipid carrier. Modifications including the addition of *N*-acetyl-glucosamine, amidation and the addition of the cross-bridge amino acids may occur at this

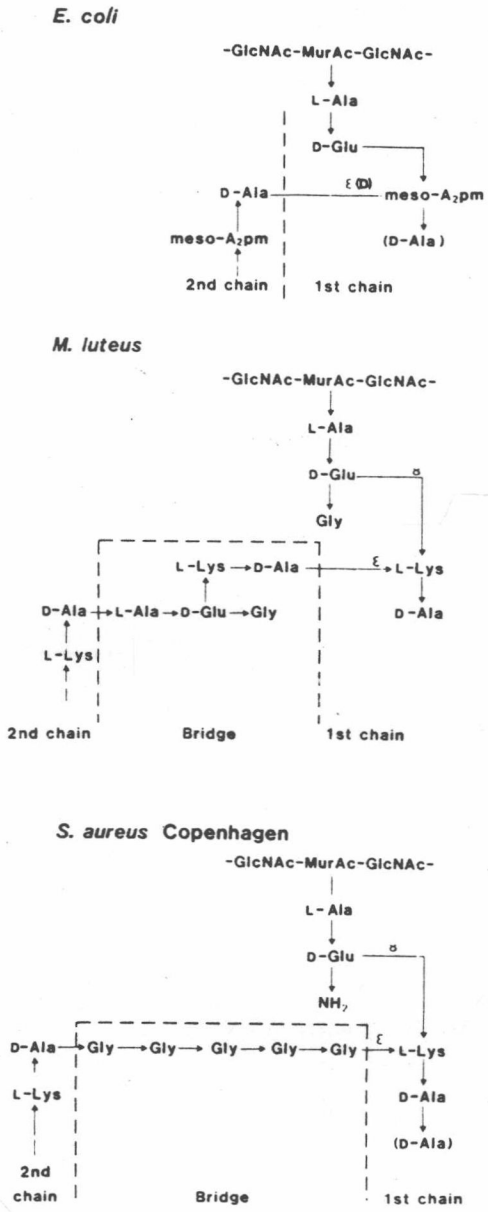

FIG. 1. Examples of peptidoglycan structure.

stage. In addition, recent evidence (discussed in detail below) has suggested that polymerization of subunits may occur while the growing glycan chain remains linked to the lipid carrier. Stage three of peptidoglycan biosynthesis involves the translocation of the newly-synthesized subunit(s) through the cytoplasmic membrane to the outer surface, where polymerization and transpeptidation occur. In this way the nascent peptidoglycan is incorporated into the pre-existing wall.

Thus peptidoglycan synthesis is a vectorial process leading from intracellular soluble precursors to an extramembranal insoluble cross-linked polymer capable of withstanding a wide range of unfavorable environments. Similar mechanisms are involved in the biosynthesis of other wall polymers in both Gram-positive and Gram-negative bacteria, e.g. lipopolysaccharide and teichoic acids. In this article I will discuss the biosynthesis of peptidoglycan with particular reference to points of attack by cell wall inhibitors. I will briefly mention related aspects of the biosynthesis of other wall polymers.

3.1. SYNTHESIS OF NUCLEOTIDE-LINKED PRECURSORS

The initial reaction of the complex series (Fig. 2) leading to the synthesis of UDP-*N*-acetylmuramoyl-pentapeptide is the formation of UDP-*N*-acetylglucosamine from UTP and *N*-acetylglucosamine-1-phosphate. The pyrophosphorylase catalyzing this reaction is analogous to those involved in the synthesis of UDP-glucose and other sugar nucleotides of this type. Since *N*-acetylglucosamine is a common constituent of wall polymers in both Gram-positive and Gram-negative bacteria, this reaction is not specific to peptidoglycan synthesis. However, muramic acid is a unique constituent of peptidoglycan. Hence, the following reactions involving the transfer of enolpyruvate to UDP-*N*-acetylglucosamine and the subsequent reduction of the enolpyruvyl ether to give UDP-*N*-acetylmuramic acid, are the first specific steps in peptidoglycan biosynthesis.

The two enzymes catalyzing these reactions have been purified from *Enterobacter cloacae* (Zemell and Anwar, 1975) and *E. coli* (Anwar and Vlaovic, 1979) and partially purified from *Staph. epidermidis* (Wickus *et al.*, 1973). The UDP-*N*-acetylglucosamine:enolpyruvate transferases were found to be subject to feed-back inhibition by either UDP-*N*-acetylmuramic acid or UDP-*N*-acetylmuramoyl-peptides (Wickus and Strominger, 1973; Zemell and Anwar, 1975). The possible role of such inhibition in the regulation of peptidoglycan biosynthesis is discussed in a later section.

Phosphoenolpyruvate:UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyl transferase is competitively inhibited by phosphonomycin (fosfomycin). This antibiotic acts as an analog of phosphoenolpyruvate and reacts covalently with a cysteine residue in the active site of the

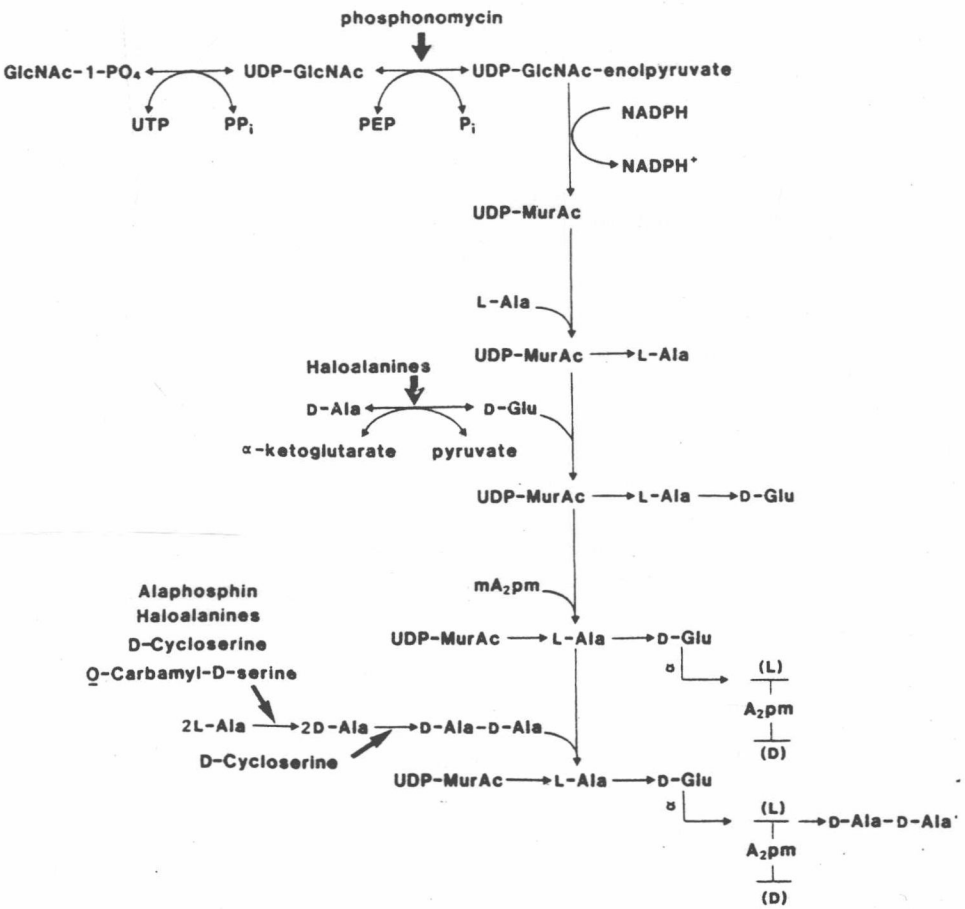

FIG. 2. The reactions involved in the biosynthesis of UDP-*N*-acetylmuramoyl-pentapeptide and their inhibition by wall antibiotics. The reactions shown are those for the biosynthesis diaminopimelic acid containing UDP-*N*-acetylmuramoyl-pentapeptide which is present in most Gram-negative bacteria, Bacilli and certain Clostridia. As described in the text, the ligases require either Mn^{2+} or Mg^{2+} for activity and the hydrolysis of ATP.

transferase. Pre-incubation of the enzyme with phosphonomycin leads to irreversible inactivation and 2-*S*-L-cysteinyl-L-hydroxypropylphosphate has been isolated from proteolytic digests of such inactive enzyme (Kahan *et al.*, 1974). UDP-*N*-acetylglucosamine is an obligatory cofactor for inhibition.

Since the enolpyruvyl transferase is a cytoplasmic enzyme inhibition by phosphonomycin requires that the antibiotic be transported into the organism. In sensitive bacteria this occurs predominantly via the L-α-glycerophosphate transport system (Hayashi *et al.*, 1964, Kahan *et al.*, 1974). Alternatively, uptake can occur via the inducible hexose monophosphate system (Kadner and Winkler, 1973) and it is the activation of this mechanism that explains the increased sensitivity of several bacteria, including those known to lack the L-α-glycerophosphate transport system, when blood is present in the growth medium. Resistance to phosphonomycin occurs quite readily by loss of either of the above transport systems. In addition, a temperature-sensitive mutant of *E. coli* has been described in which resistance appears to be associated with decreased affinity of the transferase for both phosphonomycin and the natural substrate, phosphoenolpyruvate (Venkateswaran and Wu, 1972).

Conversion of UDP-*N*-acetylmuramic acid to form UDP-*N*-acetylmuramoyl-pentapeptide precursor then occurs by sequential addition of three amino acids and the pre-formed dipeptide, D-alanyl-D-alanine. In each case the addition is catalyzed by a specific ligase which requires a divalent cation (Mg^{2+} or Mn^{2+}) and the hydrolysis of ATP for activity. Unlike protein synthesis where the addition of amino acids is directed by a nucleic acid template, the ordered formation of UDP-*N*-acetylmuramoyl-pentapeptide is dependent upon the substrate specificities of the ligases. For example the L-lysine ligase of *Staph. aureus* fails to ligate *meso*-diaminopimelic acid to UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamic acid and the converse result has been obtained with *m*-diaminopimelic acid ligase from *E. coli* (Ito and Strominger, 1962, 1966, 1973).

D-Glutamic acid, one of the D-amino acids characteristic of peptidoglycan, is produced either by the transamination of D-alanine and α-ketoglutarate (Martinez-Carrion and Jenkins, 1965) or by glutamic acid racemase (Diven, 1969). The transaminase from *B. sphaericus* is inhibited by β-halo (i.e. chloro- and fluoro-)-D-amino acids and by D-cycloserine (Soper and Manning, 1981). Inhibition by the halo alanines results in inactivation of the enzyme by a β-elimination reaction in which an α-aminoacrylate Schiffs base with pyridoxal phosphate is a key intermediate. Inhibition of the transaminase by D-cycloserine was reversed by dialysis of the inactive enzyme against pyridoxal phosphate at pH 6.5 to 7.0. At higher pHs (8.0 to 8.5) reactivation did not occur. DL-gabaculine (5-amino-1,3-cyclohexyldienyl carboxylic acid) also inhibited the transaminase (Soper and Manning, 1981, 1982). In this case the enzyme was protected from inhibition by D-alanine; α-ketoglutarate was less effective.

As described above the formation of UDP-*N*-acetylmuramoyl-pentapeptide is completed by the addition of D-alanyl-D-alanine. The synthesis of this dipeptide from L-alanine and its addition to UDP-*N*-acetylmuramoyl-tripeptide has been studied in detail by Neuhaus and his colleagues. The formation of the dipeptide is unique to prokaryotes and is of particular interest since a number of inhibitors have been described. These aspects are discussed in a recent review (Neuhaus and Hammes, 1981) hence the following description will deal primarily with biosynthesis and addition of the dipeptide. The conversion of L- to D-alanine is catalyzed by alanine racemase. The reaction, which has now been studied in numerous organisms, appears to require pyridoxal phosphate as a cofactor. Alanine racemase is competitively and irreversibly inhibited by cycloserine, the halo alanines and *O*-carbamyl-D-serine (Wang and Walsh, 1978). In addition L-1-aminoethylphosphonic acid inhibits the racemase of Gram-negative organisms competitively and reversibly and that of Gram-positive bacteria irreversibly in a time-dependent manner (Lambert and Neuhaus, 1972). In recent studies the antibiotic was incorporated into a peptide, L-alanyl-L-1-aminoethylphosphonic acid being most effective. The peptide, after being transported into the bacteria, was hydrolysed intracellularly to yield the antibacterial agent (Allen *et al.*, 1978, 1979; Atherton *et al.*, 1979).

D-alanyl-D-alanine synthetase (D-alanine:D-alanine ligase [ADP]) has been purified from *S. faecalis* (Neuhaus, 1962a,b) and *Staph. aureus* (Ito and Strominger, 1962). Enzyme activity requires K^+, Mg^{2+} (or Mn^{2+}) and the hydrolysis of ATP. In *Staph. aureus* an additional unidentified heat-stable cofactor is required. The synthetase has two binding sites for D-alanine to which residues bind sequentially as the donor (N-terminal) and acceptor (C-terminal). In *S. faecalis* K_m values determined for the binding of D-alanine to the donor and acceptor sites were 0.66 and 10 mM respectively (Neuhaus, 1962b). The synthetase showed an absolute specificity for D-amino acids or glycine. D-aminobutyric acid was the only D-alanine analog binding to the donor site. Incorporation of other D-amino acids at the acceptor site occurred only in the presence of D-alanine to produce mixed dipeptides with N-terminal D-alanine. Addition of the dipeptide to UDP-*N*-acetylmuramoyl-tripeptide shows the opposite specificity: i.e. D-alanine is required as the C-terminal residue with a low specificity for the N-terminal residue. Thus, acting in concert, the two enzymes ensure that D-alanyl-D-alanine is preferentially synthesized and added to the tripeptide to form the complete nucleotide precursor, UDP-*N*-acetylmuramoyl-pentapeptide.

3.1.1. *Regulation of UDP-*N*-Acetylmuramoyl-Pentapeptide Synthesis*

The role of feed-back inhibition in the control of UDP-*N*-acetylmuramoyl-pentapeptide synthesis remains unclear. As discussed above UDP-*N*-acetylglucosamine:enolpyruvate transferases from *E. cloacae*, *B. cereus* and *E. coli* were inhibited by UDP-*N*-acetylmuramoyl-pentapeptide and -tripeptide (Taku *et al.*, 1970). On the other hand the same enzyme from *Staph. epidermidis* was unaffected by these precursors and significant inhibition was obtained only with UDP-*N*-acetylmuramic acid (Wickus and Strominger 1973, Wickus *et al.*, 1973). In *Staph. aureus* UDP-*N*-acetylmuramoyl-L-alanine and -L-alanyl-D-glutamic acid both inhibit the L-alanine ligase as does UDP-*N*-acetylmuramoyl-pentapeptide (Tipper and Wright, 1980). These observations may explain the accumulation of UDP *N*-acetylmuramic acid and UDP-*N*-acetylmuramoyl-L-alanine in addition to pentapeptide when Staphylococci are treated with β-lactam antibiotics. In contrast similar antibiotic treatment of *E. coli* fails to cause the accumulation of either UDP-*N*-acetylmuramoyl-pentapeptide or earlier precursors. This finding suggests that in *E. coli* UDP-*N*-acetylmuramoyl-pentapeptide might control its own biosynthesis by feedback inhibition. Clearly this type of regulation is not apparent in *Staph. aureus*. Moreover, treatment of *E. coli* with D-cycloserine results in significant accumulation of UDP-*N*-acetylmuramoyl-tripeptide. Thus regulation by product inhibition in *E. coli* appears to be limited to the pentapeptide although, as discussed above, both pentapeptide and tripeptide precursors inhibit the UDP-*N*-acetylglucosamine:enolpyruvate transferase.

The amount of D-alanyl-D-alanine synthesized also appears to be carefully regulated. The synthetase from *S. faecalis* was inhibited by D-alanyl-D-alanine as well as by D-cycloserine and certain analogs of the dipeptide (Neuhaus and Lynch, 1964). Since alanine racemase has an equilibrium constant of approximately one and the synthetase may be regarded as being physiologically irreversible because of the concomitant hydrolysis of ATP, regulation of the synthetase by product inhibition is necessary to prevent all the L-alanine pool of the organism being converted to D-alanyl-D-alanine.

3.1.2. *Mutants in the Early Stage of Peptidoglycan Synthesis*

Osmotically fragile, temperature sensitive mutants of *E. coli*, *Staph. aureus* and *B. subtilis*, impaired in various steps of UDP-*N*-acetylmuramoyl-pentapeptide synthesis, have been isolated and characterized. Selection of these mutants was based on their ability to grow at the restrictive temperature only in the presence of an osmotic stabilizer such as sucrose or NaCl. Identification of the temperature-sensitive lesions was, in general, made by characterization of the nucleotide-linked precursor which accumulated and was confirmed by assay of the actual enzyme involved. In this way mutants of *E. coli* with