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An examination of the relationship between the structure and topology of peptidoglycan synthesis in bacterial membranes and enzyme activity

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AN EXAMINATION OF THE RELATIONSHIP BETWEEN THE STRUCTURE AND TOPOLOGY OF PEPTIDOGLYCAN SYNTHESIS IN BACTERIAL MEMBRANES AND ENZYME ACTIVITY

by

MAUREEN KELLY TALBOT

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

An Examination of the Relationship between the Structure and Topology of Peptidoglycan Synthesis in Bacterial Membranes and Enzyme Activity

by

Maureen Kelly Talbot

Adviser: Professor Frank Margolis

The peptidoglycan biosynthetic pathway proceeds from nucleotide-linked precursors in the cytoplasm, through lipid-linked intermediates associated with the cytoplasmic membrane, to an insoluble, cross-linked, polymeric product outside the membrane. In this work, right-side out (RSO) and inside-out (ISO) *E. coli* membrane vesicles were used to study the membrane-associated steps. Vesicles of both orientations incorporated label from UDP-[^{14}C]N-acetylglucosamine (UDP-[^{14}C]GlcNAc) in the presence of UDP-N-acetylmuramyl-L-ala-D-glu-*meso*-DAP-D-ala-D-ala (UDP-MurNAc-pentapeptide), and from UDP-MurNAc-[^{3}H]pentapeptide in the presence and absence of UDP-GlcNAc. The activity seen in RSOs was surprising because the synthetic enzymes, phospho-N-acetylmuramyl-pentapeptide transferase (translocase) and N-acetylglucosamine transferase, were presumed to be localized on the inner surface of the cytoplasmic membrane. Since the phosphorylated substrates cannot cross the membrane, the enzymes measured must be accessible from both sides of the membrane. Differential effects of several proteases eliminated the possibility

that the enzymes are randomly oriented in the membrane. In addition, EDTA-treated and whole, untreated *E. coli* cells were shown to incorporate label from the nucleotide-linked precursors into SDS-insoluble peptidoglycan.

Inhibitors of specific steps of peptidoglycan synthesis revealed that, in addition to the translocase and the N-acetylglucosamine transferase, the undecaprenyl pyrophosphate phosphatase and at least the transglycosylase domain of the penicillin-binding proteins are active in vesicles of both orientations.

The products of incorporation of UDP-MurNAc-[^{3}H] pentapeptide and UDP-[^{14}C]GlcNAc by RSOs and ISOs was examined by TLC. Vesicles of both orientations synthesized a rapidly migrating labelled material that comigrated with authentic [^{14}C]-labelled lipid-linked disaccharide on TLC. A significant amount of polymer was produced by ISOs; RSOs produced very little of this product.

A model of a membrane-spanning multi-enzyme complex is proposed to correlate the activity in vesicles of both orientations with the physical and biochemical observations made in other laboratories.

"The most beautiful experience we can have is the mysterious. It is the fundamental emotion that stands at the cradle of true art and true science. Whoever does not know it and can no longer wonder, no longer marvel, is as good as dead." - Einstein

DEDICATION

This thesis is dedicated to my best friend, Walt Talbot

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I would first of all like to express my eternal gratitude to Dr. James Christenson, my friend and mentor, who started me along the path of higher education and stood behind me all the way. His scientific knowledge and insight have never let me down. He has spent many hours in discussions dedicated to opening my mind, allowing knowledge to enter. I hope he deems his efforts successful.

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Perhaps most important of all to the completion of this thesis has been the constant encouragement and friendship of Felicity Schaefer. Felicity listened while my ideas were forming and often made suggestions and criticisms which became part of this work. She is also responsible for the beautiful PBP gels shown within. I will always be grateful for her help.

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ABBREVIATIONS

UDP-GlcNAc - Uridine-5'-diphospho-N-acetylglucosamine

UDP-MurNAc-pentapeptide - Uridine-5'-diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine

MurNAc - N-acetylmuramyl

RSO - right-side out membrane vesicles

ISO - inside-out membrane vesicles

DAP - diaminopimelic acid

IC_{50}- concentration which produces 50% inhibition

tempyo- 2,2,5,5-tetramethyl-N-oxylpyrroline-3-carbonyl

R_{max} - maximum exchange rate

C_{55} lipid - undecaprenol

LPS - lipopolysaccharide

PBPs - penicillin-binding proteins

TLC - thin layer chromatography

diS-C_3-(5), cyanine dye - 3,3'-diisopropylthiodicarbocyanine

BSA - bovine serum albumin

undecaprenyl-PP-monosaccharide - undecaprenyl-diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine

$\Delta\mu_{H^+}$ - electrochemical gradient of protons

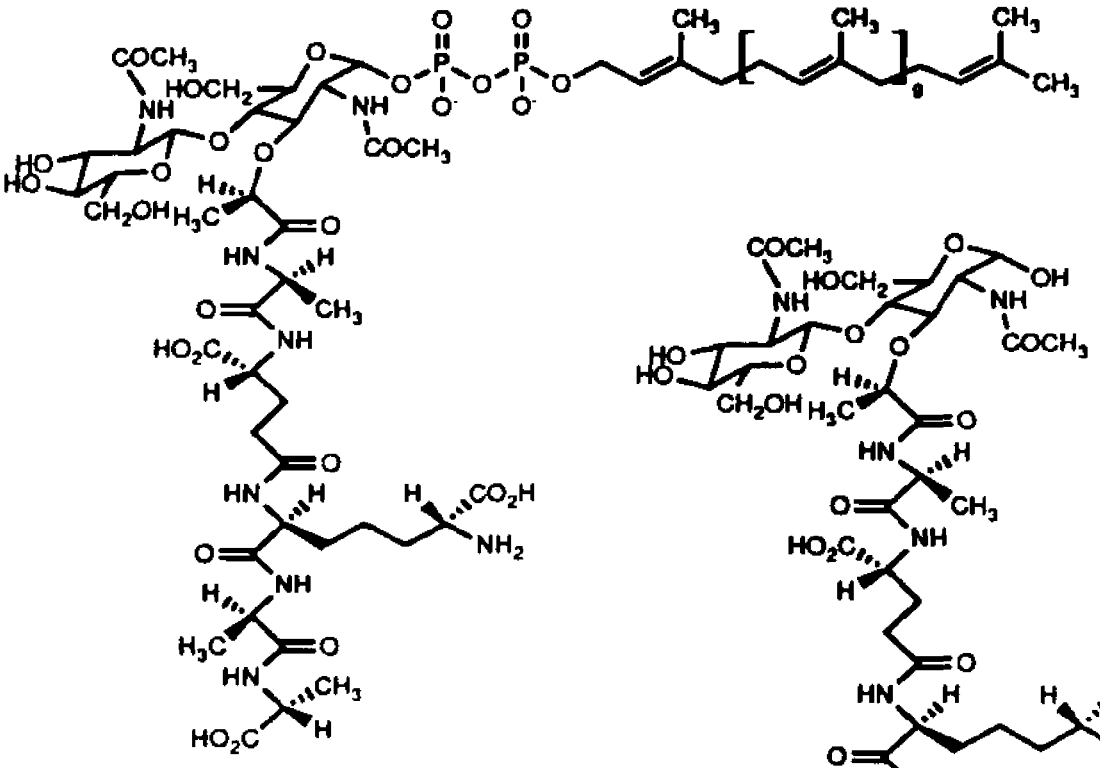
ΔpH - pH gradient

$\Delta\psi$ - electrical potential

PTS - phosphotransferase system

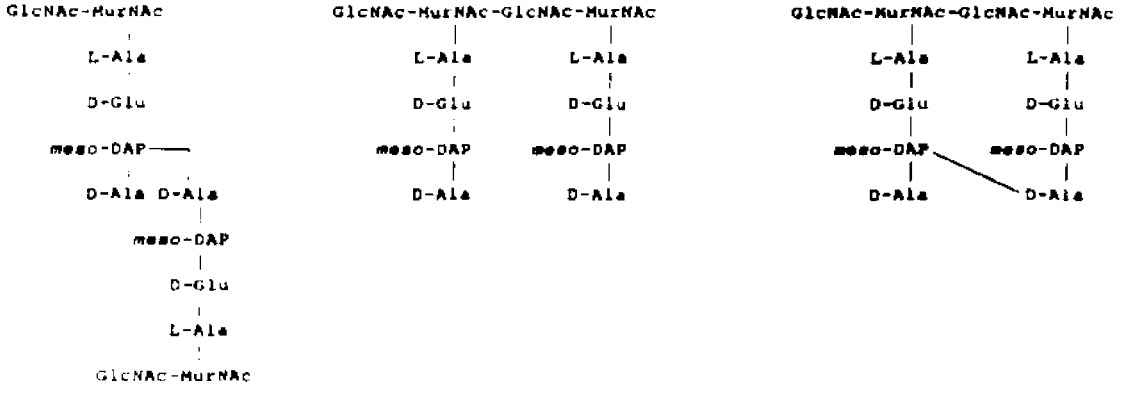
ETB - ether-treated bacteria

Chemical Structures

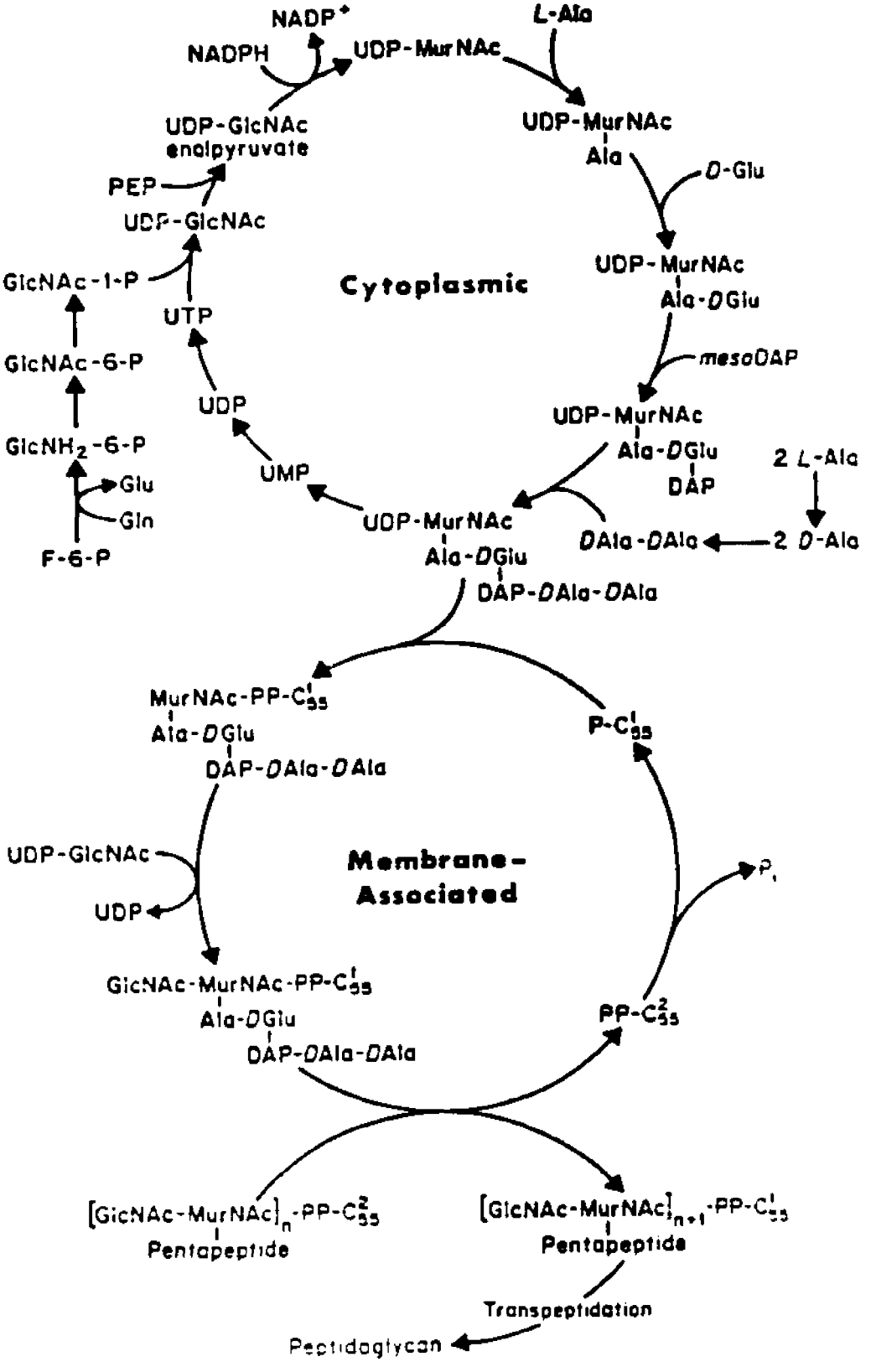


A. Structure of the lipid-linked intermediate of peptidoglycan synthesis, undecaprenyl-diphospho-N-acetylmuramyl-(L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine)-N-acetylglucosamine.

B. Chemical structure of the monomeric *E. coli* peptidoglycan lysozyme fragment. The amino acid side chain is either a tetrapeptide as shown, or in some cases a tripeptide. The dimer lysozyme fragment consists of two monomer units joined by glycosidic linkage and/or through the peptide side chain of the muramic acid. (See below.)



Peptidoglycan Biosynthetic Pathway in *E. coli*



Introduction

The main architectural feature of the cell wall of nearly all bacteria is the peptidoglycan or murein. Weidel and Pelzer (1964) coined the term "sacculus" to describe this bag-shaped macromolecule which maintains and defines the shape of bacteria. Although variations exist, all peptidoglycan may be described as strands of alternating N-acetylglucosamine and N-acetylmuramyl-peptide units joined together through the amino acid side chains of the N-acetylmuramyl-peptide. The extent to which adjacent strands are joined together or "cross-linked" varies greatly and is characteristic of individual organisms. The peptidoglycan thus forms a rigid net immediately exterior to the cytoplasmic membrane. This structure allows bacteria to maintain a characteristic shape and insures the integrity of the membrane at various osmotic pressures. Protoplasts, which are bacteria from which the peptidoglycan has been removed, burst unless maintained in isotonic media. Intact macromolecules of peptidoglycan can be separated from all other cellular constituents and these "ghosts" maintain the shape of the bacteria.

INTRICACIES OF PEPTIDOGLYCAN SYNTHESIS

Several complex problems are inherent in the synthesis

of peptidoglycan. First, the peptidoglycan is rigid, but the volume of the enclosed cell doubles with every generation. In order to allow the cells to increase in size, the peptidoglycan must be a dynamic structure into which new polymer can easily be added. Second, the peptidoglycan of rod-shaped bacteria is synthesized into an "unnatural" (i.e. energetically unfavorable) shape, presumably by a variety of enzymes. The peptidoglycan of the long axis of the rod may have subtle differences which differentiate it from that of the polar caps. How the correct enzymes deposit the correct type of polymer in the appropriate location remains unknown. Third, and the most pertinent to this thesis, the UDP-linked precursors of peptidoglycan are synthesized in the cytoplasm and are impermeant, while the enzymes which catalyze the initial reactions are wholly within the membrane. In addition, the final polymerized product is deposited outside the cytoplasmic membrane. The biosynthetic pathway essentially proceeds from water soluble substrates, to lipid soluble intermediates, to insoluble product. Any study of peptidoglycan synthesis is necessarily complicated by these interrelated, complex problems.

The biosynthesis of peptidoglycan appears to be vectorial in nature, the precursor units being synthesized in the cytoplasm and the polymerized product ultimately deposited exterior to the cell membrane. The problem

chosen for examination in this thesis is the mechanism by which the precursors are covalently joined and passed through the membrane. The actual processes by which membrane-bound enzymes catalyze the reactions and transfer the subunits to the periplasmic space have not been clearly defined. The cytoplasmic source of substrates has led to the assumption that the synthetic enzymes are located near the cytoplasmic face of the membrane although no experimental evidence supports this assumption. The peptidoglycan polymerases or penicillin-binding proteins are known to be membrane-bound, with the bulk of the protein, including the catalytic site for transpeptidation, in the periplasm. The division of enzymes which catalyze succeeding steps in a pathway on opposite sides of a membrane presented an interesting problem.

An appropriate test system to examine the topology of these proteins seemed to be membrane vesicles of opposite orientation. Membrane vesicles allow the study of membrane-bound enzymes <u>in vitro</u> with the architectural framework found <u>in vivo</u> but without the complication of active metabolism of living organisms. Vesicles are a well-characterized system and the preparations of right-side out (RSO) and inside-out (ISO) vesicles are distinct and specific.

The biochemistry of the pathway of peptidoglycan synthesis has been studied for a significantly long period

of time, in part because penicillin, the "magic bullet" of bacterial infectious disease, as well as other antibacterial agents such as bacitracin and vancomycin, inhibit peptidoglycan synthesis. Investigation of the mechanisms of action of these inhibitors has helped to elucidate the pathway.

THREE STAGES OF PEPTIDOGLYCAN SYNTHESIS

1. Cytoplasmic Synthesis of Precursors.

The reaction sequences of peptidoglycan synthesis may be divided into cytoplasmic, membrane-associated, and periplasmic steps. The UDP-linked precursors, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide) are synthesized in the cytoplasm. In *E. coli*, a set of six enzymes catalyzes the formation of the UDP-MurNAc-pentapeptide from UDP-GlcNAc, phosphoenolpyruvate, L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP), and D-alanyl-D-alanine. D-alanyl-D-alanine is produced by two additional cytoplasmic enzymes (D. Mengin-Lecreulx et al., 1982). These enzymes have been isolated and characterized.

2. Membrane-Associated Steps

An Overview

The membrane-associated steps of peptidoglycan synthesis begin with the incorporation of UDP-MurNAc-pentapeptide into a lipid intermediate. This reaction was first demonstrated in a cell-free system by Chatterjee and Park (1964). Using the particulate membrane fraction of Staphylococcus aureus as the source of enzymes, they found that label from UDP-MurNAc-[^{14}C]pentapeptide was incorporated into peptidoglycan. Anderson et al. (1965) further elucidated the membrane associated steps in both S. aureus and Micrococcus lysodeikticus. The use of [^{32}P]-, [^{14}C]- and [^{3}H]-labelled UDP-MurNAc-pentapeptide and [^{32}P]- and [^{14}C]-labelled UDP-GlcNAc enabled them to follow the fate of each part of each molecule. The membrane-associated intermediate in the pathway was determined to be lipid in nature by its solubility in organic solvents. In the absence of UDP-GlcNAc the lipid intermediate accumulated. The addition of UMP drove the reaction backward, depleting the lipid-linked compound. Label from UDP-GlcNAc only entered the lipid fraction in the presence of UDP-MurNAc-pentapeptide. Hydrolysis of the intermediate indicated that both sugars were linked to the same lipid.

These results indicated that the initial membrane-associated step of peptidoglycan synthesis is the transfer of phospho-N-acetylmuramylpentapeptide from UDP-MurNAc-pentapeptide to a lipid carrier molecule within the membrane with the release of UMP. This is followed by the

transfer of GlcNAc from UDP-GlcNAc to the lipid intermediate and the formation of UDP. Finally the disaccharide is transferred to an acceptor, pre-existing peptidoglycan or nascent glycan strands, and the lipid carrier is released.

Characteristics of the lipid intermediate

The nature of the lipid intermediates attracted the intense interest of several laboratories. Dietrich et al. (1965) and Struve et al. (1966) established that the linkage between MurNAc-pentapeptide and acceptor is a pyrophosphate. Both the lipid-linked mono- and disaccharides were isolated by Anderson et al. (1967) from both S. aureus and M. lysodeikticus. The lipid-linked monosaccharide, in the presence of UDP-GlcNAc, was a substrate for the particulate fractions of both organisms. The lipid-linked disaccharide alone also served as a substrate. The product of both reactions was uncrosslinked peptidoglycan.

Identification of the lipid carrier

The lipid carrier molecule itself was identified as a C_{55}-isoprenoid alcohol, or undecaprenol, by Higashi et al., (1967) from M. lysodeikticus and later found to be identical to the analogous molecule isolated from S. aureus (Higashi et al. 1970). It was determined by NMR that the

alcohol contained two internal trans double bonds. The phosphorylated form of the lipid, undecaprenyl-phosphate, serves as the carrier molecule for peptidoglycan synthesis. This same lipid acceptor had previously been shown to participate in O-antigen synthesis in Salmonella (Wright et al., 1967; Osborn and Tse-Yuen 1968)

Regeneration of undecaprenyl-phosphate

The final membrane-associated step of peptidoglycan synthesis proposed by Anderson et al. (1965) is the transfer of the disaccharide-peptide from the lipid carrier molecule to an extracellular acceptor. The lipid carrier is left as a pyrophosphate which must be dephosphorylated in order to be utilized again. Stone and Strominger (1972) found that several organisms contained the pyrophosphate, but at exceedingly low concentrations, perhaps indicative of efficient dephosphorylation. Sufficient quantities of the undecaprenyl pyrophosphate were finally isolated from M. lysodeikticus grown in the presence of bacitracin which specifically inhibits the recycling of the lipid-pyrophosphate. The enzyme which was shown to catalyze the dephosphorylation, C_{55}-isoprenylpyrophosphate phosphatase, was purified from M. lysodeikticus by Goldman and Strominger (1972). The discovery of this enzyme completed the cycle of the membrane associated steps of peptidoglycan synthesis.

Characterisation of the initial membrane-associated step

The reversal of the initial membrane-associated reaction by added UMP reported by Anderson et al. (1965) was further investigated by Struve and Neuhaus (1965), who proposed a reaction mechanism to account for this result:

UDP-MurNAc-pentapeptide + acceptor <———>

acceptor-P-MurNAc-pentapeptide + UMP

This mechanism suggested that the "exchange" of free UMP with the UMP moiety of UDP-MurNAc-pentapeptide could be measured. This was accomplished using [^{3}H]UMP and UDP-MurNAc-pentapeptide in the presence of particulate membrane fractions from S. aureus. This reaction:

UMP* + UDP-MurNAc-pentapeptide <———>

UDP*-MurNAc-pentapeptide + UMP

has subsequently become known as the "exchange" reaction, the completed reaction being the "transfer" reaction. It is possible to assay the two reactions separately, using the transfer of label from [^{3}H]UMP to UDP-MurNAc-pentapeptide as a measure of the exchange activity and the incorporation of label from UDP-MurNAc-[^{14}C]pentapeptide into lipid-linked intermediates or into peptidoglycan as a measure of transfer activity.

These reactions have been measured in bacterial membranes by using radiolabelled UMP and monitoring for the appearance of radiolabelled UDP-MurNAc-pentapeptide, or vice versa (Struve et al., 1966; Stickgold and Neuhaus,

1967; Anderson et al., 1967; Pless and Neuhaus, 1973; Geis and Plapp, 1978). The transfer activity of the enzyme has also been determined by measuring the production of undecaprenyl-diphospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide labelled in the pentapeptide side chain. Both activities have been measured in membrane fragments from S. aureus (Anderson et al., 1967; Weppner and Neuhaus, 1967; Heydanek et al., 1969; Hammes and Neuhaus, 1974; Anderson and Strominger, 1974; Pless and Neuhaus, 1973), M. lysodeikticus, (Anderson and Strominger, 1965; Anderson et al., 1967) Gaffkya homari (Manteuffel and Neuhaus, 1987), Bacillus megaterium (Tanaka et al., 1982, Taku and Fan, 1979) and E. coli (Taku et al., 1980; Geis and Plapp, 1978).

Mechanism of the translocase

The reaction sequence was later amended by Pless and Neuhaus (1973), based upon the stimulation of the exchange reaction by the addition of either the lipid substrate, undecaprenyl phosphate, or the lipid product, undecaprenyl diphospho-MurNAc-pentapeptide. UDP-MurNAc-pentapeptide: undecaprenylphosphate-phospho-MurNAc-pentapeptide transferase (EC 2.7.8.13), the enzyme that catalyzes this reaction, was extracted from membranes of S. aureus with Triton X-100. The enzyme preparation was largely depleted of both the lipid substrate and product and had less than

5% of the original exchange activity. Exchange activity could be restored to the enzyme by the addition of a phosphatide. Activity was greatly stimulated by the addition of either undecaprenyl phosphate or undecaprenyl diphospho-N-acetylmuramyl-pentapeptide. The original reaction mechanism did not require the presence of either lipid species for the exchange reaction to proceed. These new observations implied that either lipid species must be bound to the enzyme before the first product, UMP, was released. The mechanism proposed on the basis of these observations may be summarized as:

$$E+C_{55}P \rightleftharpoons E\text{-}C_{55}P \quad (1)$$

$$E\text{-}C_{55}P+UMPPMurNAc \rightleftharpoons UMPPMurNAc\text{-}E\text{-}C_{55}P \quad (2)$$

$$UMPPMurNAc\text{-}E\text{-}C_{55}P \rightleftharpoons UMP\text{-}E\text{-}C_{55}PP\text{-}MurNAc \quad (3)$$

$$\begin{matrix} \| & & \| \\ \| & & \| \end{matrix}$$

$$UMP\text{-}E\text{-}C_{55}P$$

$$P\text{-}MurNAc$$

where E is the enzyme, UDP-MurNAc is UDP-MurNAc-pentapeptide and $C_{55}P$ is the phosphorylated lipid carrier

molecule. This mechanism also provided a rationale for the higher rate of the exchange reaction than the transfer reaction. Once either lipid species was bound to the enzyme, the exchange reaction could proceed freely. The transfer reaction, however, required both the binding of the lipid substrate and the release of the lipid product, both possibly rate-limiting steps. Implied in the proposed mechanism is the expectation that conditions which altered the interaction between the lipid substrate or lipid product and the enzyme should differentially affect the rates of the exchange and transfer reactions. Heydanek et al. (1960) had previously reported such a differential effect by various detergents.

Characterization of the Translocase

Exchange and transfer activities

The enzyme that catalyzes the exchange and transfer reactions, UDP-MurNAc-pentapeptide: undecaprenylphosphate-phospho-MurNAc-pentapeptide transferase, is commonly called translocase, translocation being the transfer of a hydrophilic substrate into the hydrophobic environment of the membrane (Neuhaus, 1971). This feature of the enzyme, having essentially two measurable activities, has allowed a considerable amount of research into the characteristics of the enzyme, despite the fact that it has never been

completely purified.

Kinetics

Struve et al. (1966) calculated kinetic constants for the transfer reaction of S. aureus by measuring the reverse reaction. The particulate membrane fraction was incubated with the lipid-linked phospho-MurNAc-pentapeptide and UMP and the formation of UDP-MurNAc-pentapeptide was measured. The K_ms for UDP-MurNAc-pentapeptide and UMP are 1.8 X 10^{-6} M and 2.7 X 10^{-5} M respectively and the equilibrium constant for the reaction is 0.25.

Activity with substrate analogs

Hammes and Neuhaus (1974) examined the specificity of the P-MurNAc-pentapeptide transferase for the peptide side chain for both the exchange and transfer reactions. Both amino acid substitutions and shortened peptides were tested and the K_m and V_{max} (or R_{max}, the maximum rate of exchange reaction) were determined. UDP-MurNAc-pentapeptide and UDP-MurNAc-tripeptide had the same V_{max} in the transfer reaction, but the K_m for the transfer reaction was 80-fold higher for the tripeptide. Ratios of V_{max}/K_m in the transfer reaction correlated well with R_{max}/K_m in the exchange reaction for the different substrates. The individual parameters, however, showed no correlation between the two activities. The authors suggest that this

difference is due to the proposed reaction sequence. This model requires the association of the lipid carrier and dissociation of the lipid-linked product for the transfer reaction. Only the association of either lipid species is required for the exchange reaction to proceed.

The enzyme did not appreciably differentiate between the normal UDP-MurNAc-pentapeptide and the analogous molecule with a glycine in the terminal position. Similarly, the third amino acid, lysine, could be substituted with diaminopimelic acid (DAP) with little effect on the kinetic parameters.

Stickgold and Neuhaus (1967) found that 5-fluorouridine-5'-diphospho-MurNAc-pentapeptide did not substitute for UDP-MurNAc-pentapeptide as a substrate for the phospho-N-acetylmuramyl-pentapeptide transferase. F-UMP acted as a competitive inhibitor of UDP-MurNAc-pentapeptide and caused loss of label from lipid-phospho-N-acetylmuramyl pentapeptide, but to a lesser degree than UMP itself. Since F-UMP is a very poor substrate for the exchange reaction, this result is difficult to explain.

Effects of monovalent cations

The translocase of *S. aureus*, in addition to having an absolute requirement for Mg^{++}, also showed increased activity in the presence of monovalent cations, especially K^+ (Heydanek et al. 1970). K^+ stimulated the exchange

reaction 20-fold and the transfer reaction 2-fold. The translocase of B. megaterium, however, was inhibited rather than stimulated by the presence of monovalent cations (Tanaka et al., 1982). The requirement for Mg^{++} appears to be universal.

Effects of inhibitors

Several laboratories tested the effects of known inhibitors of peptidoglycan synthesis in both transfer and exchange assays. Anderson et al. (1965) reported IC_{50}s for peptidoglycan synthesis of 12 μg/ml and 6 μg/ml for ristocetin and vancomycin, respectively. However, at 20 μg/ml, neither inhibited the exchange reaction and actually stimulated lipid-intermediate formation. At 10-fold higher concentrations, some inhibition of lipid-intermediate formation was seen. Struve and Neuhaus (1965) also reported stimulation of the transfer reaction at 50 μg/ml for ristocetin and vancomycin. Ristocetin also stimulated the transfer reaction at 500 μg/ml, while vancomycin at that concentration produced significant inhibition. Struve et al. (1966) later reported that ristocetin increased production of lipid intermediates and decreased exchange activity and peptidoglycan synthesis. Both antibiotics bind to the terminal D-ala-D-ala of the UDP-MurNAc-pentapeptide. Peptidoglycan synthesis is inhibited because the pentapeptide side chain with free D-ala-D-ala is

necessary for polymerization. These results suggest that it is also necessary for the exchange reaction.

The peptide antibiotic, amphomycin, has been shown to be a specific, non-competitive inhibitor of the transfer reaction of Bacillus megaterium (Tanaka et al., 1982). Tunicamycin, which is thought to be a transition state analog of the enzyme-substrate complex (Heifetz et al., 1979) also inhibits the transfer reaction. Anderson et al. (1967) found that penicillin, an inhibitor of the final, crosslinking reaction of peptidoglycan synthesis, had no effect on either activity.

Activity of UDP-MurNAc-pentapeptide-linked probes

UDP-MurNAc-pentapeptide bearing a radioactive and/or fluorescent label has been used to examine the location of the activity of phospho-N-acetylmuramyl transferase. In addition, spin-labelled stearic acids have allowed precise measurements of membrane fluidity while the activity of the enzyme was being measured. The results of these experiments have created a three dimensional picture of enzyme-substrate interactions within the membrane.

Dansylated UDP-MurNAc-pentapeptide

Weppner and Neuhaus (1977) prepared UDP-MurNAc-pentapeptide with a dansylated lysine at the third position of the side chain which served as a substrate for both the

transfer and exchange reactions in S. aureus. The fluorescent substrate had a higher V_{max} and V_{max}/K_m (3.8 and 1.6 times) for the transfer reaction than the natural substrate and a higher R_{max} but lower R_{max}/K_m (1.8 and 0.78 times) for the exchange reaction. The equilibrium constant for the transfer reaction was higher for the dansylated substrate than for the natural substrate (5.9 v 1.1). The authors concluded that the enzyme had decreased affinity for the lipid-linked MurNAc pentapeptide, perhaps because of a greater association of the fluorescent substrate with a hydrophobic area in the membrane.

The dansylated substrate was much less effective in producing polymerized peptidoglycan than the natural substrate (V_{max} = 0.1 times and V_{max}/K_m = 0.05 times the values for the natural substrate). This may also be a function of the association of the lipid-linked fluorescent molecule with the hydrophobic area of the membrane. Alternatively, the decrease in affinity of the lipid-linked MurNAc-pentapeptide, indicated by the increase in K_m, may cause it to be released from the enzyme rather than being channeled to the next active site.

Since the dansylated UDP-MurNAc-pentapeptide was an effective substrate for the enzyme, it became possible to produce lipid-linked monosaccharide bearing a fluorescent label. Weppner and Neuhaus (1978) found changes in fluoresence spectrum as the result of the transfer of the

P-MurNAc-pentapeptide to the undecaprenyl phosphate by the translocase. The emission maximum, quantum yield and fluorescence lifetime of the lipid-linked phospho-MurNAc-(dansyl)pentapeptide indicated the absence of mobile polar molecules within the microenvironment of the fluorophore. The authors concluded that the dansyl moiety was in a hydrophobic environment such as found within the matrix of the lipid bilayer.

Quenching of fluorescence was used to determine the location of the molecule (Weppner and Neuhaus, 1978). Quenchers which intercalate within the hydrocarbon regions of the membrane indicated that the dansyl was within 4 to 6 Å of this environment. Twenty-three percent of the fluorescent lipid-linked molecule was effected by an aqueous quencher, suggesting that two populations of fluorophores exist. The population of fluorophores responsive to the aqueous quencher must be located close to the surface of the membrane.

Quenching by lipid-associated compounds decreased with increasing temperature, indicating that the quencher was being removed from the proximity of the fluorophor (Weppner and Neuhaus, 1978) . This may be due to lateral phase separation of lipid from protein which occurs with increasing temperature. The decrease in quenching suggested that the lipid-linked fluorescent phospho-MurNAc-pentapeptide remained associated with the protein. In

addition, high anisotropy and rotational relaxation times for the lipid-linked fluorescent substrate indicated that it was immobilized within the membrane. These observations led the authors to suggest that the lipid-linked species is associated within a synthetic complex of one or more proteins within the lipid bilayer of the membrane.

Weppner and Neuhaus (1978) estimated the fluidity of the membrane by measuring the fluorescence anisotropy of perylene in paraffin oil of known microviscosities intercalated into membranes. Arrhenius plots of fluidity of the lipid matrix, fluorescence intensity of the lipid-linked-P-MurNAc-pentapeptide, and activity of the phospho-N-acetylmuramyl transferase had very similar breakpoints; 20° and 30°C and 18° and 30°C and 22°C and 30°C respectively. However, the anisotropy of the fluorescent lipid-linked-P-MurNAc-pentapeptide was temperature-independent.

Lee et al. (1980) examined the effect of changing the microviscosity of the membrane on the fluorescence-labelled UDP-MurNAc-pentapeptide by adding n-butanol to reaction mixtures. Both transfer and exchange activities were measured in presence of either UDP-MurNAc-pentapeptide or the dansylated substrate. Activity increased up to 0.18 M butanol, but sharply decreased at higher concentrations. Increasing concentrations of up to 0.18 M butanol gradually decreased the fluorescence intensity of the dansylated UDP-

MurNAc-pentapeptide incorporated into the lipid. Higher concentrations sharply decreased the intensity. In the presence of butanol, Arrhenius plots of fluorescence intensity showed only one discontinuity at 22°C rather than the two at 19° and 30°C seen in the control. The activity of the exchange reaction also showed only one slope change in the presence of 0.12 M n-butanol, at 20°C rather than two discontinuities, at 22° and 30°C, as in the control. These observations suggest that butanol decreased the characteristic immobility of lipids associated with membrane proteins. Thus, the increase in activity of the enzyme was due to the increased fluidity of the lipid bilayer, rather than any direct effect on the lipid-linked monosaccharide.

Relationship between membrane viscosity and translocase activity

The Arrhenius plots of translocase activity and membrane fluidity and the stimulation of activity by butanol indicated that the physical state of the membrane played a significant role in the activity of the enzyme. Weppner and Neuhaus (1979) used both spin-labelled and fluorescent probes to measure the physical characteristics of membranes from S. aureus during the synthesis of lipid-linked phospho-MurNAc-pentapeptide. Electron spin resonance (ESR) spectroscopy was used to monitor the effect

of temperature variation on the physical state of the membranes. A pair of spin-labelled stearic acids were intercalated in the membranes and τ or $2T_{\parallel}$, the ESR spectra parameters, which are a measure of the motion of the probes, were determined. The activity of P-MurNAc-pentapeptide transferase was monitored simultaneously using UDP-MurNAc-[^{14}C]pentapeptide to measure transfer activity or [^{3}H]-UMP to measure exchange activity. Arrhenius plots showed two discontinuities in the slopes of both the exchange and transfer activities at 22° and 30°C. The motion parameters of the spin-labelled probes also showed two discontinuities at 16° and 30°, or 22°C and 30°C. These results confirm that the changes in the physical state of the lipid bilayer are reflected in changes in the activity of the translocase.

The participation a lipid-linked intermediate complicates the interpretation of experiments designed to determine the effect of altering the physical state of the membrane on the activity of the enzyme. Conditions which affect the largely lipid membrane might also affect the several lipid-linked species involved in the biosynthesis of peptidoglycan. The proposed reaction mechanism of Pless and Neuhaus (1973) predicts that conditions which alter the interaction between the lipid substrate or lipid product and the enzyme will have differential effects on the exchange and transfer reactions. Weppner and Neuhaus

(1979) concluded that, because the effects of temperature on the exchange and transfer reaction were identical, the effects of temperature on the hydrophobic substrate were minimal. If the effect of temperature on the lipid-linked substrate were significant, the two activities should show significantly different responses to temperature variation.

Data from this entire series of experiments confirmed that conditions which increased the fluidity of the membrane (e.g., increased temperature or addition of butanol) stimulated the activity of the phospho-MurNAc-pentapeptide transferase. However, more extreme disruption of protein-membrane interactions, such as caused by high concentrations of butanol, sharply inhibited enzyme activity.

Dansylated UDP-MurNAc-[^{14}C]pentapeptide

Manteuffel and Neuhaus (1987) prepared a double-labelled probe, dansylated UDP-MurNAc-[^{14}C]pentapeptide to separately follow the membrane associated steps of peptidoglycan synthesis. Membranes of Gaffkya homari were incubated with the dansylated UDP-MurNAc-[^{14}C]pentapeptide and the incorporation of label into the lipid-linked monosaccharide, disaccharide and peptidoglycan was measured. The probe was also used to follow the disappearance of the lipid-linked disaccharide and the appearance of peptidoglycan. The fluorescence intensity of

the dansyl was followed through peptidoglycan synthesis by the stepwise addition of substrates. Intensity increased when the lipid-linked monosaccharide was formed and when the lipid-linked disaccharide was formed, but decreased when peptidoglycan was synthesized.

The location of the fluorescent group within the membrane was measured by using doxyl-labelled stearic acid derivatives, with the label at different positions along the chain, as quenchers. Maximum quenching of the lipid-linked monosaccharide was seen with 7-doxylstearate indicating that the fluorophor was 8Å from the surface. After the addition of UDP-GlcNAc, the quenching became bimodal with a peak at 16-doxystearate and 5-doxystearate. These correspond to distances of 20 and 4Å from the surface. The disaccharide was then "chased" into peptidoglycan by the addition of unlabelled UDP-MurNAc-pentapeptide. Quenching by the 16-doxystearate decreased with time as did the quenching at the other major peak by 5-doxystearate, although to a lesser degree (30% v 67%). The two peaks indicate that the fluorophor is at two locations or perhaps the existence of two conformations of the lipid intermediate. The more rapid decrease in the quenching of the deeper lipid linked disaccharide may indicate that this moiety is used for peptidoglycan synthesis at a higher rate than that at the shallower location.

Spin-labelled UDP-MurNAc-pentapeptide

UDP-MurNAc-pentapeptide spin-labelled on the lysine with 2,2,5,5-tetramethyl-N-oxylpyrroline-3-carbonyl (tempyo) was prepared by Johnston and Neuhaus (1975). This compound served as a substrate for the translocase, resulting in the synthesis of spin-labelled lipid-linked UDP-MurNAc-pentapeptide. Membranes containing this compound were shown (by ESR) to bind both ristocetin and vancomycin. These antibiotics specifically bind to the terminal D-ala-D-ala of the pentapeptide side chain of UDP-MurNAc-pentapeptide. These results indicate that the terminal D-ala-D-ala of the lipid-linked P-MurNAc-pentapeptide must lie near the surface of the membrane since it was accessible to bind to these compounds. The association constants were lower for the lipid-linked phospho-MurNAc-(tempyo)-pentapeptide than for the unreacted compound. This may be attributable to the barrier imposed by the membrane.

Taken together, the results of the fluorescent and spin-labelled probe experiments indicate that: 1.) At least the third amino acid of the pentapeptide side chain of lipid-linked-P-MurNAc-pentapeptide is in contact with a hydrophobic environment, presumably the lipid bilayer. 2.) The terminal D-alanyl-D-alanine of the pentapeptide side

chain is accessible to the external reagents, specifically vancomycin and ristocetin. 3.) The lipid-linked intermediate is immobilized within the membrane in close association with a protein, perhaps a multi-enzyme complex. And 4.) the location of the probe changes at the addition of N-acetylglucosamine to what appear to be two pools of lipid-linked disaccharide, differing either in location or conformation.

Solubilization of the translocase

Numerous attempts have been made to isolate and purify the translocase from bacterial membranes. Heydanek and Neuhaus (1969) found that any one of several reagents (sodium lauroyl sarcosinate, KOH or 10 M urea) allowed the solubilization of the activity of the translocase from membranes of S. aureus. The soluble enzyme, however, behaved in a peculiar manner when further purifications were attempted. Gel-filtration yielded at least two peaks of activity, probably due to aggregation of the hydrophobic protein.

Alternatively, the translocase was rendered lipid-free (and inactive) by organic extraction of S. aureus membranes (Heydanek et al., 1969). Activity could be reconstituted upon addition of detergents, but the effects of detergents on the exchange and transfer activities of the transferase varied. Heydanek et al. (1969) also found that

deoxycholate stimulated transfer activity but was an effective inhibitor of exchange activity. Cutscum (isooctylphenoxypoly-(oxyethylene)ethanol) stimulated the activity of both reactions. Addition of crude lipid-acceptor (essentially that which had been extracted from the membranes) stimulated both exchange and transfer activities and increased the activity seen in the presence of both detergents.

Geis and Plapp (1978) also found that various detergents had opposite effects on the exchange and transfer reactions in a partially purified preparation of the translocase from E. coli. Those with high hydrophilic-lipophilic balance numbers increased the exchange rate but inhibited the transfer rate. Those which inhibited the exchange rate (e.g., Triton X-100) had no effect on the transfer rate. The addition of an aqueous dispersion of crude lipids reversed the inhibition of these detergents on the exchange reaction. The inhibitory effects of Triton and Span on the exchange reaction may be due to the extraction of essential phospholipids. Enhancement of activity by other detergents was attributed to the increased solubilization of the enzymes.

Treatment of S. aureus membranes with Triton X-100 also allowed the solubilization of translocase activity, as measured by the exchange reaction (Pless and Neuhaus, 1978). Using ion-exchange chromatography, the detergent

was removed and the enzyme was freed of 60% of the undecaprenyl phosphate and 90% of the undecaprenyl pyrophosphate found in the membranes. This treatment also left the enzyme with very little activity. Addition of a S. aureus lipid extract in the presence of sodium lauroyl sarcosinate restored 75% of the exchange activity to the translocase. Fractionation of the lipid extract revealed that the activating components of the extract were undecaprenyl phosphate and undecaprenyl pyrophosphate-MurNAc-pentapeptide. This stimulation of the exchange reaction by the purified undecaprenyl-phosphates was increased to a significant degree by the addition of a phosphatide. The most active phosphatide was phosphatidylcholine which stimulated the exchange reaction in the absence of added lipid-linked product or substrate. This activity probably indicated the presence of endogenous undecaprenyl-phosphate or undecaprenyl-pyrophosphate-MurNAc-pentapeptide associated with the solubilized enzyme.

Similar experiments using membrane fragments from M. lysodeikticus (Umbreit and Strominger, 1972) showed that the exchange activity of the translocase could be restored by phosphatidylethanolamine, phosphatidylinositol or phosphatidylglycerol. Interestingly, a neutral lipid and undecaprenyl-phosphate, but not phosphatides, were required for reactivation of the transfer reaction.

The translocase of E. coli was extracted from

membranes by repeated freezing and thawing (Geis and Plapp, 1978). After centrifugation at 200,000 X g, 6% of the membrane-bound exchange activity was found in the supernatant. No transfer activity or lipid carrier could be detected. Adding lipids in the presence of detergent did not restore transfer activity as reported for S. aureus or Micrococcus. Activity was restored by adding an aqueous lipid dispersion. It was shown by sucrose gradient centrifugation that the translocase associated with liposomes formed by the lipid dispersion. This suggested that the lipids required for reconstitution of activity served a structural, rather than catalytic purpose in the reaction.

Geis and Plapp (1978) detected no lipid carrier associated with the S200 fraction but could measure exchange activity. They concluded that the lipid-enzyme complex proposed by Pless and Neuhaus was not required for the exchange reaction and suggested that their data supported the original reaction scheme proposed by Neuhaus. This scheme described the formation of an enzyme-UDP-MurNAc-pentapeptide complex followed by the release of UMP. Phospho-MurNAc-pentapeptide remained on the enzyme to be transferred to the lipid. This reaction mechanism was later rejected by Neuhaus and replaced by the one diagrammed on page 10.

Identification of the gene encoding phospho-N-acetylmuramyl-pentapeptide transferase

The genes which are involved in peptidoglycan synthesis in E. coli are clustered together on the chromosome, in five discrete regions: *mra, mrb, mrc, mrd, mre* (Matsuhashi et al., 1990). A previously unidentified open reading frame in the *mra* cluster, now called *mraY*, has recently been shown to encode phospho-N-acetylmuramyl-pentapeptide transferase (Ikeda et al., 1991). The presence of the gene on a plasmid amplified the activity of the chromosomal enzyme, measured as production of undecaprenyl-PP-monosaccharide or -disaccharide and by the exchange reaction. The activity was inhibited by tunicamycin, a specific inhibitor of the enzyme.

The predicted amino acid sequence of the enzyme was compared to the dolichyl-phosphate-dependent GlcNAc-1-phosphate transferase from Saccharomyces cerevisiae, an enzyme with similar activity. Optimal alignment of the two proteins showed 20.7% homology. Hydropathy profiles of the two enzymes were similar and suggested that the functions were similar. The repeated sequences of hydrophobic and hydrophilic domains of the phospho-N-acetylmuramyl transferase suggested that the enzyme spans the membrane several times.

N-Acetylglucosamine Transferase

The second membrane-associated step of peptidoglycan synthesis is the transfer of N-acetylglucosamine from UDP-GlcNAc to the undecaprenyl-pyrophosphate-MurNAc-pentapeptide with the release of UDP (Anderson et al., 1967). N-Acetylglucosamine is linked in β-(1->4) linkage to the N-acetylmuramylpentapeptide. The activity of N-acetylglucosamine transferase is measured indirectly in all assays of peptidoglycan synthesis. Taku and Fan (1976) identified a protein extracted from B. megaterium with LiCl, as N-acetylglucosamine transferase. This protein could restore peptidoglycan synthesizing activity to membranes of toluene-treated cells which had been depleted of that activity by LiCl extraction (Taku and Fan, 1976). Membranes of these depleted cells could incorporate label from UDP-[^{14}C]GlcNAc into peptidoglycan only in the presence of this factor. The identity of the factor was confirmed to be the N-acetylglucosamine transferase by incubating extracted membranes with lipid-linked MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc and measuring the appearance of lipid-linked disaccharide or peptidoglycan. The gene encoding the N-acetylglucosamine transferase has also recently been identified (Mengin-Lecreulx et al., 1991). The gene, *murG*, like *mraY*, lies within the *mura* cluster of genes encoding for proteins involved in peptidoglycan synthesis. Temperature-sensitive mutants in *murG* lysed when shifted to the non-permissive temperature.

Overproduction of the protein did not increase the rate of peptidoglycan synthesis, indicating that *murG* gene product is not rate-limiting. The gene sequence encodes a protein with a predicted amino acid sequence of 355 residues and a molecular weight of 37,771.

Reconstitution of the Membrane-Associated Enzymes of Peptidoglycan Synthesis

As evidence accumulated that classical methods of purification and isolation of integral membrane proteins lead to inactivation of enzyme function, alternatives to these methods were attempted. One procedure to simplify the enzyme-membrane complex was to separate proteins from the membrane, selectively discard some of the proteins, and reconstitute the enzymes of interest back into the membranes.

Bacillus megaterium

Taku and Fan (1979) reconstituted membranes capable of synthesizing peptidoglycan from B. megaterium membranes solubilized with sodium cholate. The membranes were reconstituted by dialysis in the presence of Mg^{++}. The solubilized membranes were fractionated on a Bio-Gel column and fractions from three regions pooled and reconstituted in various combinations. One of these pools could synthesize peptidoglycan and lipid-linked monosaccharide

alone or in combination with either of the other two. N-Acetylglucosamine transferase, extracted from membranes with LiCl, was added to the solubilized membranes, and the cholate dialyzed away. This complex synthesized lipid-linked disaccharide and a small amount (5%) of peptidoglycan. An additional fraction of the solubilized membranes eluted from a hydroxylapatite column stimulated this reconstituted enzyme complex to synthesize peptidoglycan. The authors called this component PG-II and assume it to be a peptidoglycan polymerase. The PG-II-mediated peptidoglycan synthesis was inhibited by vancomycin and bacitracin, but not by penicillin. The effect of bacitracin was limited; even very high concentrations did not inhibit the final 35% of activity. The residual activity may be a measure of the lipid available in the multi-enzyme complex since inhibition of this enzyme by bacitracin prevents the recycling of the lipid carrier. The effect of inhibitors suggests that the complexes formed after dialysis of solubilized membranes include undecaprenyl pyrophosphatase as well as phospho-MurNAc-pentapeptide transferase, N-acetylglucosamine transferase, and PG-II and which is probably a PBP.

E. coli

Essentially the same procedure was used by Taku et al. (1979) to solubilize and reconstitute the membrane proteins

of E. coli. Of three pools constructed from the Bio-Gel column, two were necessary for peptidoglycan synthesis. One, which could be replaced by crude lipids from E. coli membranes, appeared to be mainly phospholipids. Apparently all the enzymes of peptidoglycan synthesis (both transferases and the polymerase) remained together, unlike the situation in B. megaterium. The polymerase, which is most likely one or more PBPs, was separated from the two transferases by hydroxylapatite chromatography. As in B. megaterium, the synthetic activity of the enzyme complex was sensitive to bacitracin, indicating the presence of the pyrophosphatase. This characteristic of the membrane-bound enzymes of peptidoglycan synthesis to remain together as a complex is significant. The fluorescent and spin-labelled probes used by Neuhaus indicated that the lipid-linked mono-and di-saccharide intermediates were associated with a protein complex.

Other Systems to Measure Membrane-Associated Peptidoglycan Synthesis

Measurement of peptidoglycan synthesis has also been useful as a target of antibacterial agents or as a measure of cell metabolism. Neither of these objectives requires purified enzymes or even well-characterized systems. For these purposes, assays of peptidoglycan synthesis have been developed which measure end-product formation in the

presence of non-limiting substrate concentrations.

Ether-permeabilized cells

Several laboratories have successfully measured the membrane-associated steps of peptidoglycan synthesis (up to and including transpeptidation) using bacterial cells briefly treated with ether. This method was introduced by Vosberg and Hoffmann-Berling (1971) to measure DNA synthesis. Mirelman et al. (1976) used ether-treated E. coli and later Pseudomonas aeruginosa, to study the final steps of peptidoglycan synthesis (Mirelman and Nuchamowitz, 1979). Ether-treatment purportedly causes the cytoplasmic membranes to become permeable to the nucleotide-linked precursors, UDP-MurNAc-pentapeptide and UDP-GlcNAc. This system has the advantage of preserving the architectural and regulatory features of the intact cell. It has proved useful in separating the individual reactions of the series which results in polymerized product. In addition, the effect of the solvent on enzymes being measured is uncertain. As shown by Lee et al. (1980) solvents which affect the nature of the membrane also affect the activity of the integral membrane enzymes.

Partially autolysed cells

Harrington and Baddiley (1983) found that B. subtilis cells which had been allowed to autolyse for 20 min synthesized uncrosslinked peptidoglycan from UDP-[^{14}C]-

GlcNAc and UDP-MurNAc-pentapeptide. A considerable amount of incorporation also occurred in the absence of UDP-MurNAc-pentapeptide. UDP-MurNAc-pentapeptide-dependent incorporation was sensitive to bacitracin, tunicamycin and vancomycin but not to penicillin, indicating the presence of the translocase and undecaprenyl-pyrophosphatase. The polymer produced in the presence of UDP-MurNAc-pentapeptide was digested by lysozyme; that produced in the absence of UDP-MurNAc-pentapeptide was not. Incorporation was inhibited by incubating the autolyzed cells with trypsin or p-chloromecuribenzene-sulfonic acid (pCMBS). The autolyzed cells could also catalyze the exchange reaction and formation of UDP-GlcNAc from UTP and GlcNAc-1-phosphate. Autolyzed cells did not take up either UTP or UMP.

B. subtilis cells which were not autolyzed also incorporated label from UDP-[^{14}C]GlcNAc. This synthesis was much more dependent on UDP-MurNAc-pentapeptide. Only about half of the peptidoglycan produced in both whole and autolyzed cells remained associated with the cells when filtered. Protoplasts formed from lysozyme-EDTA treatment of B. subtilis were also tested and were found to synthesize the lipid-linked disaccharide precursor of peptidoglycan but no polymer. This synthesis was sensitive to bacitracin, vancomycin and tunicamycin, and trypsin and pCMBS. The lack of polymer formation in protoplasts was interpreted as a result of lysozyme remaining in the preps.

Both autolyzed cells and protoplasts were shown to be intact by (1) the lack of DNA leakage into the supernatant fluid, and (2) by the loss of activity upon sonication.

Mechanism of Translocation

The original meaning of translocation used to describe the reactions of peptidoglycan synthesis was: "the transfer of a hydrophilic substrate into the hydrophobic environment of the membrane" (Neuhaus, 1971). Thus, the initial membrane-associated reaction of peptidoglycan synthesis may be considered a translocation reaction. Subsequently, the term has more generally been used to describe the movement of the lipid-linked mono- and disaccharide through the membrane. For example, Weppner and Neuhaus (1979) describe the membrane-associated steps in which "cytoplasmic precursors are transferred to the membrane where intermediates covalently linked to this prenyl phosphate are assembled and translocated across the membrane to the sites of cell wall assembly."

Translocation in lipopolysaccharide synthesis

This second meaning of translocation is also applied to the passage of precursors of lipopolysaccharide (LPS) through the inner and outer membranes Gram-negative bacteria. The polysaccharide side chain (O-antigen) of LPS is assembled in the inner membrane using the same

undecaprenyl phosphate used in peptidoglycan synthesis (Umbreit and Strominger, 1972). O-Antigen synthesis has also been shown to be inhibited by bacitracin (Osborne, 1969). Nascent O-antigen chains attached to the undecaprenyl phosphate have been localized on the outer surface of the inner membrane using ferritin-labelled antibodies (Mulford and Osborn, 1983). The mechanism of this translocation is unknown, but Marino et al. (1991) have shown that it is not energy dependent. O-Antigen synthesis is profoundly inhibited by 2,4-dinitrophenol (DNP), an uncoupler. The mechanism of the inhibition was not, however, inhibition of translocation but rather inhibition of the initial membrane-associated reaction. The expected consequence of the inhibition by DNP was the accumulation of undecaprenyl-PP-oligosaccharides. Instead, the authors found a complete absence of undecaprenyl-phosphate-linked saccharides of any length, indicating that formation of the lipid-linked monomer, undecaprenyl-PP-galactose, was inhibited. This effect was observed only in intact cells; DNP had no effect on the synthesis of undecaprenyl-PP-galactose in isolated membranes. The authors concluded that the inhibitory effect of DNP was specifically due to the dissipation of $\Delta\mu_H^+$, and suggested that the target could be the undecaprenyl-pyrophosphate cycle.

The synthesis and assembly of the other components of

LPS, Lipid A and the core oligosaccharide, also occur in the cytoplasmic membrane. Lipid A serves as the lipid-carrier molecule for the addition of the various saccharides but, unlike undecaprenyl-phosphate, is part of the completed LPS. Final assembly of the LPS, the joining of O-antigen and core lipopolysaccharide, occurs on the outer surface of the inner membrane. The completed LPS is then rapidly translocated to the outer surface of the outer membrane. The process of translocation of the core lipopolysaccharide through the inner membrane has been shown to be energy-dependent (McGrath et al., 1991). The translocation of the completed lipopolysaccharide from the inner membrane to the outer membrane is also dependent on $\Delta\mu_{H^+}$.

N-Glycosylation of proteins in eukaryotes

An analogous system is found in eukaryotic cells in the dolichyl phosphate pathway for protein N-glycosylation occurring in the rough endoplasmic recticulum. The lipid carrier in this case is dolichol, a polyprenol consisting of 16 to 22 isoprene units. The oligosaccharide (Glc_3 $Man_9GlcNAc_2$), as well as several mono-and polysaccharide intermediates, are attached to the lipid through a pyrophosphate linkage. This process is certainly more complicated than the synthesis of either LPS or peptidoglycan, but has in common the passage of lipid-

linked sugars through membrane barriers. Vesicles prepared from rough endoplasmic reticulum (RER) membranes have been used to localize the position of various intermediates in the pathway to either the lumenal or cytoplasmic side of the membrane. These results indicate that lipid-linked-$Man_5GlcNAc_2$ passes through the membrane (Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1990). This process has been described as a "flip-flop" and is believed to be protein-mediated because dolichol-linked saccharides do not "flip-flop" in artificial vesicles (Hanover and Lennarz, 1982; McCloskey and Troy, 1980). Accumulation of UDP-GlcNAc and UDP-glucose in the lumen of these vesicles indicates that these compounds are transported through the membrane. However, GDP-mannose does not accumulate and dolichol-linked mannose may also "flip-flop" through the membrane to serve as substrate for the lumen-oriented mannose-adding enzyme. The mechanism of protein-mediated "flip-flop" is unknown.

Translocation of lipid-linked saccharides in peptidoglycan synthesis

Initially, the lipid-mediated steps of peptidoglycan synthesis were interpreted as a transport process by which subunits of the polymer, formed and modified at the cytoplasmic face of the membrane, were delivered to the exterior of the membrane for polymerization (Anderson et

al., 1967). As the membrane-associated steps continued to be elucidated, the mechanism of translocation remained unknown and largely uninvestigated.

These membrane-associated steps have proved to be the most challenging to study. It has been widely assumed that the enzymes which catalyze these reactions lie on the interior face of the cytoplasmic membrane, adjacent to the supply of substrates (Tipper, 1987). The lipid-linked disaccharide is then "translocated" or "flipped" through the hydrophobic interior of the membrane, allowing the synthetic sequence to continue in the periplasmic space.

As discussed above, Harrington and Baddiley (1983) showed that protoplasts and partially autolyzed B. subtilis could synthesize uncrosslinked peptidoglycan from externally supplied UDP-MurNAc-pentapeptide and UDP-GlcNAc. Bacitracin inhibited synthesis indicating that recycing of undecaprenyl pyrophosphate was occurring during synthesis. Four hypotheses were offered to explain the utilization of the externally supplied substrates: 1.) Active sites on both sides of the membrane; 2.) Transfer of precursors across the membrane to active sites on the outside of the membrane; 3.) Rotation or reorientation of the synthetic enzyme complexes so that the active sites are transiently available from the outside of the membrane; and 4.) Partial release of the enzyme complexes by autolysis. The authors concluded that either rotation of the wall-synthesizing

complexes or displacement of the tightly associated complexes from their usual location could account for their results *in vitro*. Only rotation of the complexes, however, could account for the translocation which occurs *in vivo*.

The hypothetical rotation of enzyme complexes requires an energy source which Harrington and Baddiley (1984) suggest could be supplied by the electrochemical proton gradient ($\Delta\mu_H^+$). The effect of $\Delta\mu_H^+$ on peptidoglycan synthesis was investigated in whole cells, protoplasts and partially autolyzed *B. subtilis* in the presence and absence of ionophores and uncouplers. Synthesis in whole cells was inhibited by valinomycin, which dissipates the $\Delta\psi$ portion of $\Delta\mu_H^+$. In addition, valinomycin inhibited the uptake of label from [^{14}C]-GlcNAc by whole cells by 40%. UDP-MurNAc-pentapeptide accumulated in the presence of valinomycin whereas, in the control, UDP-GlcNAc accumulates. Valinomycin had very little effect on incorporation in the *in vitro* test systems, i.e., partially autolyzed cells or protoplasts. The authors conclude that the *in vitro* results indicate that valinomycin has no direct effect on the synthetic enzyme complexes. It was suggested that *in vivo*, valinomycin inhibited peptidoglycan synthesis by dissipating $\Delta\mu_H^+$ at pH 7.5, and that living *B. subtilis* require maintenance of $\Delta\mu_H^+$ to enable rotation or reorientation during translocation. This rotation or reorientation could occur by conformational changes of the

synthetic enzyme complexes.

3. Polymerisation of Peptidoglycan

The final steps of peptidoglycan synthesis are those which form the polymerized product. The enzymes which catalyze these reactions are localized in the periplasmic space of E. coli and have been intensely studied. This set of enzymes is commonly known as the penicillin-binding proteins (PBPs) or penicillin-sensitive enzymes. The initial observation that penicillin binds irreversibly to bacterial membranes (Cooper, 1956) was followed many years later by the determination that a covalent interaction occurred between several membrane-bound proteins and penicillin. Studies of this phenomenon followed two paths: 1) isolation and identification of the proteins which irreversibly bound penicillin and 2) the biosynthetic activities necessary to produce the peptidoglycan and their sensitivity to penicillin. A number of laboratories, most notably that of Strominger, were able to assign specific enzyme activities to these proteins and characterize their interaction with penicillin. This early work has been reviewed by Blumberg and Strominger (1974). Spratt and Pardee (1975) introduced a relatively simple assay to evaluate the ability of other ß-lactam antibiotics to compete with penicillin to bind PBPs. Membranes of E. coli were incubated with [^{14}C]penicillin and unlabelled

antibiotics and the proteins were fractionated on a SDS-polyacrylamide slab gel. The resulting pattern that appeared on autoradiograms of the gels revealed that penicillin not only had multiple targets, but other ß-lactams could prevent the binding of penicillin to some or all of the PBPs. It had been previously noted, that while all ß-lactam antibiotics inhibited peptidoglycan synthesis, classes of inhibition could be differentiated by their effect on the morphology of E. coli. These effects included the inhibition of cell division resulting in filamentous cells, bulge formation, rapid cell lysis and the formation of osmotically stable ovoid cells. Spratt (1975) showed that the effect of a ß-lactam on the morphology of E. coli could be correlated with its affinity for a particular PBP. This result confirmed that the multiplicity of PBPs corresponded to different enzyme activities (Blumberg and Strominger, 1972). Subsequently, PBPs 1A and 1Bs, 2 and 3, the high molecular weight PBPs, have been identified as bifunctional enzymes which catalyze both transglycosylation and transpeptidation (Ishino et al, 1980; Nakagawa et al., 1984; Ishino et al., 1986). PBPs 5 and 6 function as D,D-carboxypeptidases (DePedro and Schwarz, 1981). PBP4 has been variously described as a transpeptidase, a D,D-carboxypeptidase and a D,D-endopeptidase (Tamura et al., 1976; Matsuhashi et al., 1979).

The association of enzyme activity of the PBPs and the morphological consequences of the inhibition of specific PBPs has led to speculation that particular PBPs may be localized at specific locations in the cytoplasmic membranes. For example, the inhibition of PBP3 by cephalexin results in filamentous cells suggesting that the function of this PBP is the synthesis of the peptidoglycan that forms the septum of cells (Waxman and Strominger, 1983). Since septum formation occurs at the midline of the rod-shaped cell, PBP3, by inference, may be located at that site in each cell. The mechanism by which these enzymes might be "anchored" in one particular site in the membrane is unknown. However, as discussed above, Taku et al. (Taku and Fan, 1979; Taku et al., 1979) were unable (in E. coli) to separate the activities of phospho-N-acetylmuramyl transferase, N-acetylglucosamine transferase, undecaprenyl pyrophosphatase, and the polymerase, which is probably one or more PBPs. This suggests that, at least in E. coli, PBPs are part of a membrane-associated protein complex.

MEMBRANE VESICLES

Vesicles have been used to study biochemical membrane phenomena since the early 1960's. Preparation of vesicles has been essentially standardized (Kaback, 1971), as have methods of measuring transport in these vesicles (Kaback, 1974). As a result of the intense interest in transport in

vesicles and its relationship to electrochemical ion gradients, the system has been extremely well characterized. The homogeneous orientation of vesicles prepared by osmotic lysis is key to the interpretation of results of transport experiments. Biochemical and microscopic evidence presented by Stroobant and Kaback (1975) indicates that these vesicles have the same orientation as live cells. In addition, Owen and Kaback (1979a) have used crossed immunoelectrophoresis to identify a series of immunogens associated with the membranes and establish the distribution of the antigens on the inner or outer surface of the membrane (Owen and Kaback, 1979b). In this way they were able to show that over 95% of the vesicles retained the configuration of the original cell.

Membrane vesicles have also been prepared by passage of cells through a French pressure cell at low pressure (Hertzberg and Hinkle, 1974; Rosen and McClees 1974). Vesicles prepared by this method are much smaller and have the opposite orientation from those prepared by osmotic lysis or whole cells (Futai, 1974; Altendorf and Stachelin, 1974). Both these inside-out and the right-side out vesicles have been shown to generate $\Delta\mu_H^+$ in the presence of an electron donor (Reenstra et al., 1980). When $\Delta\mu_H^+$ is present, vesicles of opposite orientation generate opposite electrochemical gradients, i.e., the interior of right-side out vesicles (and whole cells) is alkaline and negative;

the interior of inside-out vesicles is acid and positive. This difference can be detected by following the partitioning of fluorescent dyes into (or out of) vesicles in response to $\Delta\mu_H^+$ (Waggoner, 1979). Inside-out vesicles have been used by Reenstra et al. (1980) together with right-side out vesicles to examine the relationship between Δ pH and $\Delta\psi$, the components of $\Delta\mu_H^+$.

Our experience with permeabilized E. coli indicated that at least some of the membrane-associated steps of peptidoglycan synthesis could be supported in vesicles. The simplicity of membrane vesicles, in addition to the availability of techniques which produce a preponderance of either right-side out or inside-out vesicles, induced us to investigate peptidoglycan synthesis in this system.

Materials and Methods

Materials:

Uridine-5'-diphospho-N-acetyl-D-[U-^{14}C]glucosamine (UDP-[^{14}C]GlcNAc, ca. 300 mCi/mmol); 2,6-diamino[1,7-^{14}C]-pimelic acid (mixed D,D-, L,L- and meso-isomers, 281 mCi/mmol); and [^{3}H]-benzylpenicillin (17.2 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. [U-^{14}C]Uridine-5'-monophosphate (450 mCi/mmol) and (meso)2,6-Diamino[3,4,5-^{3}H]pimelic acid (23 Ci/mmol) were purchased from Research Products International, Mount Prospect, Ill. Omnifluor and En^{3}Hance scintillation media are products of Dupont, NEN Research Products, Boston, MA. FiltronX was from National Diagnostics. N-Acetylmuramidase from *Streptomyces globisporis* was from Miles Scientific, Div., Miles Laboratories, Inc., Naperville, Ill. Plates for thin-layer chromotography (TLC) were plastic-backed Polygram Sil G, 0.25-mm-thick silica gel (Brinkmann Instruments, Inc., Westbury, N.Y.). Nucleotides, valinomycin, tunicamycin, lysozyme, deoxyribonuclease I, alkaline phosphatase (*E. coli*, Type III-N), sarkosyl and sodium dodecyl sulfate (SDS) were from Sigma Chemical Co., St. Louis, Mo. 3,3'-diisopropylthiodicarbocyanine [diS-C_3-(5)] and oxonol VI were from Molecular Probes.

Penicillin G was from Eli Lilly & Co., Indianapolis, Ind.; ampicillin, from Wyeth Laboratories; prasinomycin

from E.R. Squibb & Sons. Moenomycin was a gift from Professor Seibert, Hoechst. Nigericin was a gift from Dr. C-M. Liu. Membrane filters were from Millipore, DE81 anion exchange filters were from Whatman.

Bacterial strains. E. coli B ATCC 23226, Bacillus cereus ATCC 11778 , and S. aureus ATCC 29213 were obtained from the American Type Culture Collection, Rockville, Md.

Methods:

Preparation of UDP-MurNAc-peptides. Unlabelled uridine-5'-diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide) was prepared from B. cereus ATCC 11778 by the procedure of Moore et al. (1979). The concentration was determined by an assay for N-acetylamino sugar esters based on that of Strominger (1957) and by the UDP concentration which was determined spectrophotometrically (ϵ_{260} = 1.0 X 10^4). In addition, several preparations were characterized by high-pressure liquid chromotography and amino acid analysis as described in Talbot et al. (1989). Uridine-5'-diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-[^{3}H]-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-[^{3}H]-pentapeptide) was prepared essentially as described by Tanaka et al. (1982) using B. cereus ATCC 11778, instead of B. megaterium KM. Briefly, cells were grown in medium containing lysine and meso-diaminopimelic acid (DAP) to A_{660} = ~0.6. Cells were harvested, washed twice with 0.01M

potassium phosphate buffer, pH 7.0 with 1 mM $MgCl_2$ and 0.8% NaCl and resuspended at a concentration of 100 mg/ml in a medium containing glucose, uracil, L-alanine, L-glutamic acid, L-lysine, chloramphenicol, vancomycin and [^{3}H]DAP. Incubation was continued for 35 min. Bacteria were harvested and cells were resuspended in cold 5% trichloroacetic acid (TCA). UDP-MurNAc-[^{3}H]pentapeptide was isolated from the TCA supernatant by the same procedure used for the cold UDP-MurNAc-pentapeptide as described by Moore et al. (1979). Concentration of the final product was determined by UV-absorbance (ϵ_{260} for uridine = 1.0 X 10^4). The small amount of product precluded use of the N-acetylamino sugar assay. The specific activity for each batch was determined by counting an aliquot and calculating μCi/μM. Subsequent TLC and autoradiography (see below for procedure) of UDP-MurNAc-[^{3}H]pentapeptide, however, revealed two radioactive bands in all of the preparations. One of the bands coincided exactly with unlabelled UDP-MurNAc-pentapeptide located by UV absorbance (R_f = 0.15). The percentage of radioactivity in each peak was estimated using a computing densitometer (Molecular Dynamics, Model 300A). The concentration of each batch was corrected for this contamination. The second radioactive band had a higher mobility than the UDP-MurNAc-pentapeptide and may be UDP-MurNAc-L-ala-D-glu-meso-DAP.

Uridine-5'-diphospho-N-acetylmuramyl-L-alanyl-D-

glutamyl-L-lysyl-D-alanyl-D-alanine from **Staphylococcus aureus** ATCC 29213 was prepared by modifications of the procedures of Flouret et al. (1981), Park and Chatterjee (1966) and Moore et al. (1979). S. aureus ATCC 29213 was grown in medium containing 0.5% each of peptone and yeast extract, 0.3% K_2HPO_4 and 0.2% glucose. Cells were harvested by centrifugation and resuspended in resting medium (Park and Chatterjee, 1966), containing glycine, L-glutamic acid, L-alanine and L-lysine. Following a 90 min incubation, cells were harvested and precipitated with 5% TCA. UDP-MurNAc-pentapeptide was isolated from the supernatant by the same procedure used for the cold UDP-MurNAc-pentapeptide from E. coli as described by Moore et al. (1979). Concentration of the final product was determined by UV-absorbance and N-acetylaminosugar content as described above. Amino acid analysis found the ratio of glu:lys:ala to be 1.05:1.0:2.9. The expected ratio of amino acids in S. aureus UDP-MurNAc pentapeptide is glu:lys:ala = 1:1:3.

UDP-MurNAc-L-alanyl-D-glutamate was prepared from S. aureus ATCC 29213 using the same procedure used to prepare the UDP-MurNAc-pentapeptide from that organism, except the resting medium did not contain lysine. This results in the accumulation of the UDP-MurNAc-dipeptide which was isolated and purified as above.

Preparation of inside-out membrane vesicles from E. coli

B. ISOs were prepared essentially by the method of Hertzberg and Hinkle (1974) and modified by Reenstra et al. (1980). E. coli B was grown in Trypticase Soy Broth (BBL) until $A_{550} \approx 0.75$. Cells were harvested by centrifugation and resuspended in 100 ml of cold 50 mM potassium phosphate buffer, pH 7.5, with 10 mM $MgSO_4$. The resuspended cells were passed through a continous-flow French pressure cell (Amico) at 4000 psi. Deoxyribonuclease I (Sigma) was added and the mixture incubated at 37°C for 5 min. This was then centrifuged at 10,000 rpm for 10 min. The supernatant was removed and centrifuged at 100,000 x g overnight. The supernatant was discarded and the pellet resuspended in 50 mM potassium phosphate buffer, pH 7.5, with 10 mM $MgSO_4$ and centrifuged again at 100,000 x g for 2 hours. The resulting pellet was resuspended in the same buffer. Protein content was determined and adjusted to 10 mg/ml with the same buffer. The vesicles were aliquoted and fast-frozen in liquid nitrogen.

Preparation of right-side out membrane vesicles. RSOs were prepared by osmotic lysis of lysozyme-EDTA-treated cells as described by Kaback (1971). Vesicles were resuspended in a small volume of 50 mM potassium phosphate buffer, pH 6.6, and frozen in liquid nitrogen. Protein content was determined after the vesicles were frozen and usually ranged from 5 to 7 mg/ml.

Preparation of ethylenediaminetetraacetic acid (EDTA)-

treated cells. E. coli B was grown in Trypticase Soy Broth (BBL) until log phase. Cells were harvested by centrifugation and the pellet washed once with 0.01 M Tris hydrochloride (pH 8.8). Cells were suspended in 0.05 M Tris hydrochloride (pH 8.8) and 1 M K_2EDTA added to give a final concentration of 1 mM. Cells were incubated at 37°C for 2 min and a 10-fold excess of 0.01 M Tris hydrochloride (pH 8.8) was added. Cells were harvested by centrifugation and suspended in Basic Medium which is composed of 80 mM KCl, 40 mM Tris hydrochloride (pH 7.4), 7 mM $MgCl_2$, 2 mM [ethylene-bis(oxoethylenenitrilo)]tetraacetic acid, 0.4 mM spermidine · 3HCl, and 0.5 M sucrose (Mirelman et al., 1976). EDTA-treated cells were assayed for activity immediately. Protein content was determined after cells were assayed.

Preparation of whole cells for incorporation assay. E. coli B cells were grown and havested as for EDTA-treated cells. The cells were suspended in T-2 buffer which contains per liter: 4 g NaCl, 5 g K_2PO_4, 1.5 g KH_2PO_4, 2 g Na_2PO_4, 120 mg $MgSO_4$ and 10 mg gelatin (Meynell and Meynell, 1970). Cells were washed once and then suspended in a small volume of T-2 buffer. Cells were assayed immediately and the protein content determined later.

Assays for enzyme activity. Activity of the membrane-associated enzymes of the peptidoglycan synthetic pathway was determined using several different assays. In most

cases results were quantitated by scintillation counting. All samples were prepared in duplicate; protease-treated samples were prepared in triplicate. Variation of replicates was rarely more than 10%.

1. Incorporation assays. Vesicles were incubated in reaction mixtures containing 50 mM Tris·HCl, pH 8.8, 25 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 10 mM disodium ATP, and either UDP-[^{14}C]GlcNAc or UDP-MurNAc-[^{3}H]pentapeptide. UDP-[^{14}C]GlcNAc was present at ~2 μM together with UDP-MurNAc-pentapeptide at 75μM. The concentration of UDP-MurNAc-[^{3}H]pentapeptide was usually about 5 μM and was assayed with and without 75 μM cold UDP-GlcNAc. Reactions were incubated at 30°C for 15 min and terminated by the addition of 1 ml of 2.5 mM acetic acid. Samples were filtered through 0.22 μm membrane filters, washed five times with water and dissolved and counted in Filtron X scintillation fluid.

Assays of EDTA-treated cells, whole cells and ether-treated cells were terminated by boiling in 5% SDS and/or precipitation with 10% TCA.

2. Assay of phospho-N-acetylmuramyl-pentapeptide transferase activity. This was an adaptation of an assay described by Tanaka et al. (1982). Vesicles (~100 μg of protein) were incubated in a reaction mixture containing 50 mM Tris hydrochloride (pH 8.8), 25 mM $MgCl_2$, 1 mM dithiothreitol, UDP-MurNAc-[^{3}H]pentapeptide or UDP-

[^{14}C]GlcNAc and unlabelled UDP-MurNAc-pentapeptide in a total volume of 30 µl. Samples were incubated for 15 min at 30°C. Reactions were terminated by the addition of 20 µl of 6 M pyridinium acetate, pH 4.2. Water-saturated 1-butanol (100 µl) was added and the reaction mixture were vortexed for several minutes. Butanol and aqueous phases were separated by centrifugation at 2000 rpm for 3 min. The butanol phase was removed and the aqueous phase was extracted again with water-saturated butanol. Butanol phases were pooled and backwashed twice with an equal volume of butanol-saturated water. The final butanol extracts were placed in scintillation vials and evaporated to dryness. Five ml of Omnifluor scintillation fluid was added to vials and the samples were counted.

3. Assay of the exchange reaction. The assay procedure was adapted from Geis and Plapp (1978). Reaction mixtures contained, in a final volume of 20 µl: 50 mM Tris·HCl, pH 8.8; 25 mM $MgCl_2$; 4.8 µM [^{14}C]UMP (43 nCi); 91.2 µM unlabelled UMP; and 20 µg of protein from vesicles. Reaction mixtures were incubated at 37°C for 15 min. Reactions were terminated by boiling for 1 min. Twenty µl of alkaline phosphatase (0.6 units) was added to the cooled tubes and samples incubated at 37°C for 15 min. Samples were again boiled for 1 min and the entire reaction mixtures were spotted onto Whatman DE81 filter paper discs. Filters were placed on a filter manifold and washed 5 times

with 4 ml of water. The filters were dried under a heat lamp and counted in 5 ml of Omnifluor scintillation fluid. Alkaline phosphatase from E. coli (Type III-N) was prepared in 0.1 M Tris, pH 10.0, with 1 mM $MgCl_2$.

4. TLC anaylsis. Reaction mixtures of each assay were also analyzed by TLC. For incorporation assays and the assay for the exchange reaction, reacted vesicles were sedimented by centrifugation in an Eppendorf microfuge after addition of acetic acid. The supernatant was removed and the pellets suspended in a small volume (20 - 40 μl) of the chromatography solvent, isobutyric acid-1 N ammonia (5:3). Butanol extracts of the products of the assay for the phospho-N-acetylmuramyl-pentapeptide transferase were evaporated and dissolved in 20 - 40 μl of chloroform/methanol (1:1). The aqueous layers were evaporated to dryness in a Speed-Vac evaporator and suspended in 20-40 μl of isobutyric acid - 1 N ammonia (5:3). In some cases, the entire reaction mixture stopped with 5 μl glacial acetic acid was spotted.

All samples were spotted as bands onto preactivated, plastic silica gel TLC plates, together with appropriate authentic standards. The plates were developed twice in isobutyric acid -1 N ammonia (5:3) (Martin and Gmeiner,1979). Plates were air dried, and sprayed with En^{3}Hance and placed in contact with Amersham Hyperfilm-ECL film for 7 days. Results were visualized by

autoradiography. Areas corresponding to bands on the autoradiographs were cut from the TLC plate and counted in Filtron-X. Alternatively, results were quantitated by computer densitometry using a Molecular Dynamics Model 300 A densitometer. Data calculated by both methods were comparable.

For the purpose of preparative separation and analysis of [^{14}C]-labelled assay products, direct autoradiography of TLC plates was done using Amersham ß-Max film. In this case, plates were not sprayed with En^{3}Hance, but were placed directly in contact with the film. Film was developed manually according to the manufacturer's instructions.

Muramidase digestion of assay products. Products of the incorporation assay were sedimented and the pellets incubated with 500 µg/ml of N-acetylmuramidase from S. globisporis in 25 mM sodium phosphate buffer (pH 6.5) with 0.1 mM $MgCl_2$. Samples were incubated at 37°C for at least 5 hours. The entire samples were then spotted on TLC plates and developed and autoradiographed as above.

In some cases plates were spotted with cold UDP-MurNAc-pentapeptide as a standard. The portion of the plate with this standard was cut off and sprayed with 10% triethylamine in methylene chloride, then 0.05% fluorescamine (Roche), then again with 10% triethylamine in methylene chloride. Primary amines fluoresce under UV

light.

Determination of the penicillin-binding protein pattern in vesicles. An adaptation of the method of Spratt (1977) was used to visualize the PBP pattern of ISOs and RSOs. Samples were prepared by incubating various amounts of vesicles with [^{3}H]benzylpenicillin (10 μCi, 0.58 nmoles) for 10 min at 30°C. The reaction was terminated by the addition of 5 μl of 10% sarkosyl and incubation at room temperature for 5 min. Cold acetone was added and the samples incubated at 0°C for 5 min. Precipitates were collected by centrifugation. Pellets were air dried, suspended in sample buffer and boiled for 3 min prior to loading. Difficulties encountered with ISO samples led to several variations of this procedure. Alternatively, the sarkosyl supernatants were mixed with sample buffer without acetone precipitation. In some experiments, incubation of ISOs with [^{3}H]benzylpenicillin was carried out in a sonicating waterbath.

Samples were fractionated on polyacrylamide gels as described by Then and Kohl (1985). Electrophoresis was performed using the discontinuous buffer system of Laemmli and Favre (1973). Gels were prepared for autoradiography as described by Talbot et al. (1989). Gels were placed in contact with Amersham Hyperfilm-ECL film at -80°C for at least 2 weeks.

Preparation of ^{14}C-labelled monomer and dimer fragments.

Purified monomer and dimer fragments were prepared as described by Talbot et al. (1989).

Ether-treated E. coli B. Ether-treated E. coli B were prepared and used to confirm the identity of UDP-MurNAc-[^{3}H]-pentapeptide. These methods were described in Talbot et al. (1989).

Preparation of ^{14}C-labelled lipid-disaccharide. This procedure was adapted from an assay of phospho-N-acetylmuramyl-pentapeptide transferase activity described by Tanaka et al. (1982). ETB (100 μg of protein) were incubated in a reaction mixture containing 50 mM Tris·HCl (pH 8.8), 25 mM $MgCl_2$, 1 mM dithiothreitol, 125 μM UDP-MurNAc-pentapeptide, 13.8 μM UDP-[^{14}C]GlcNAc (1.25 μCi), and 100 μg/ml moenomycin (to prevent transglycosylation) in a total volume of 300 μl. After a 5 min incubation at 30°C, 200 μl of 6 M pyridinium acetate, pH 4.2, was added to the reaction mixture followed by 1.0 ml of 1-butanol. The reaction mixture was vortexed and the phases separated by centrifugation at 2000 rpm for 3 min. The butanol phase was removed and the aqueous phase extracted again with butanol. Butanol phases were pooled and backwashed three times with an equal volume of water. The final butanol extract was evaporated to dryness and suspended in chloroform:methanol (1:1) and kept at -25°C. The pooled aqueous phases were also evaporated to dryness and suspended in a small volume of water. Samples of the

butanol extract and aqueous layers were spotted together with UDP-[^{14}C]GlcNAc onto silica gel TLC plates and developed as above. Autoradiography revealed a single major spot ($R_f \approx 0.47$) in the butanol extract. Aqueous samples contained only UDP-GlcNAc.

Other analytical procedures. Sodium dodecyl sulfate-polyacrylamide electrophoresis was performed in 8-18% polyacrylamide gels with 5% polyacrylamide stacking gels. Electrophoresis was performed at a constant current of 30 mA by the method of Laemmli and Favre (1973). Gels were fixed, stained with Coomassie blue (Biorad) and destained by the usual procedures.

Total phosphate of vesicle preparations was determined using the method of Ames and Dubin (1960).

Vesicle orientation was characterized by fluorescence quenching of diS-C_3-(5) and oxonol VI in a Perkin-Elmer MPF-4 spectrophoto-fluorimeter. Excitation of diS-C_3-(5) was at 622 nm, emission at 670 nm; excitation of oxonol VI was at 588, emission at 616. Samples contained 100 μg of protein from vesicles, 50 mM potassium phosphate buffer (pH 7.5), 0.5 μM diS-C_3-(5) or 2 μM oxonol VI in a total volume of 2.0 ml. These reaction mixtures were placed in cuvettes and allowed to equilibrate while the flourescence was observed. An electron donor was then added (ubiquinol-1 or lactate) to generate $\Delta\mu_{H^+}$ and the resulting quenching of fluorescence recorded. When ubiquinol-1 was used it was

necessary to add dithiothreitol to maintain it in the reduced state. Valinomycin was added to dissipate $\Delta\mu_{H^+}$ and the resulting increase in fluorescence recorded. In some experiments, reactions were observed in the reaction mixture used for the incorporation assays. No attempt was made to quantitate this data.

Protein was assayed by the method of Lowry et al. (1951) and by the Pierce BCA protein assay using BSA as the standard.

Mild acid hydrolysis of butanol extracts was performed by the method of Pless and Neuhaus (1973). The evaporated sample was dissolved in 0.5 ml of 0.01 M HCl in 50% methanol and boiled for 20 min. The cooled sample was spotted onto TLC plates and developed as above.

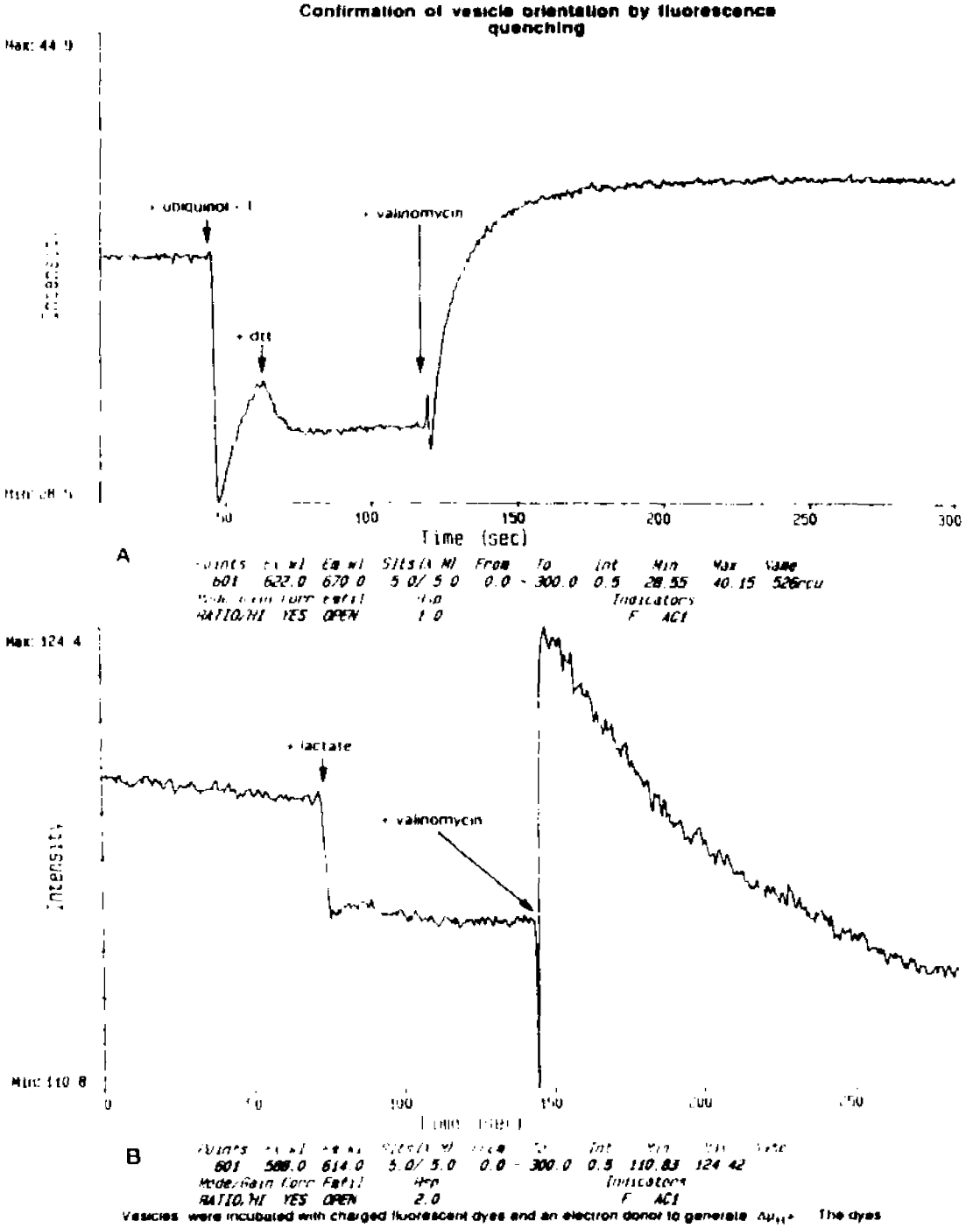
Results

Vesicles of both orientations were found to incorporate label from UDP-[^{14}C]GlcNAc in a reaction mixture which supports peptidoglycan synthesis. This incorporation was dependent on the presence of UDP-MurNAc-pentapeptide and Mg^{++}. This result was suprising because the phosphorylated substrates provided should not penetrate the membranes and it is widely presumed that the membrane-bound enzymes which link these precursors to the undecaprenyl phosphate carrier molecule are located on the cytoplasmic side of the membrane. This would seem to preclude any interaction between the substrates provided externally and the target enzymes in RSOs. This initial observation suggested that the phospho-N-acetylmuramyl-pentapeptide transferase and the N-acetylglucosamine transferase are accessible from both sides of the membrane. Subsequent experiments were designed to test this hypothesis.

Confirmation of Vesicle Orientation

Cytoplasmic membrane vesicles were prepared in both the right-side out (RSO) and inside-out (ISO) orientation. The orientation of vesicle preparations was confirmed by fluorescence spectroscopy using a cyanine dye (Figure 1A) for RSOs or an oxonol dye (Figure 1B) for ISOs. In either

Figure 1



Vesicles were incubated with charged fluorescent dyes and an electron donor to generate $\Delta\mu_{H^+}$. The dyes preferentially accumulate in the interior of the vesicles in response to charge separation and the fluorescent intensity is reduced. Valinomycin dissipates $\Delta\mu_{H^+}$ allowing the dye to exit the vesicles and the fluorescent intensity to increase. A. RSOs were incubated in 100 mM potassium phosphate buffer, pH 8.6, with 0.5 µM diisopropylrhodacarbocyanine. Ubiquinol-1 was added followed by dithiothreitol to maintain the ubiquinol in the reduced state. Valinomycin was added as indicated. B. ISOs were incubated in 50 mM potassium phosphate buffer, pH 7.5, with 2 µM Oxonol VI. D-Lactate was added as indicated followed by valinomycin.

case, the dye, which freely passes through the membrane (Waggoner, 1979a; Waggoner, 1979b), was allowed to equilibrate with the vesicles in potassium phosphate buffer. An electron donor --ubiquinol-1 (with dithiothreitol to maintain it in the reduced state; (Stroobant and Kaback, 1975) for RSOs or D-lactate for ISOs (Reenstra et al., 1980) -- was then added to the vesicles to generate $\Delta\mu_H+$. The interior of RSO vesicles in the presence of an electron donor becomes alkaline and negative (Altendorf et al., 1975; Hirata et al., 1973; Schuldiner et al., 1975). The positively charged cyanine dye accumulates in the interior of the vesicles and as a result, the fluorescence intensity decreases (Figure 1A). The addition of valinomycin, an ionophore which allows free passage of K^+ through the membrane, collapses the electrical component of $\Delta\mu_H+$, $\Delta\psi$, causing the dye to re-equilibrate and the fluorescence intensity to increase. For ISOs, the addition of D-lactate results in the development of $\Delta\mu_H+$ with the interior of the vesicle positive and acidic. The negatively charged dye oxonol responds to the separation of charge by moving to the interior of the vesicle (Matsushita et al., 1984; Bashford et al., 1979), which is also seen as a decrease in fluorescence intensity (Figure 1B). The effect is reversed by the addition of valinomycin. Increased quenching seen after the addition of valinomycin may be due to the re-establishment of $\Delta\mu_H+$ through the

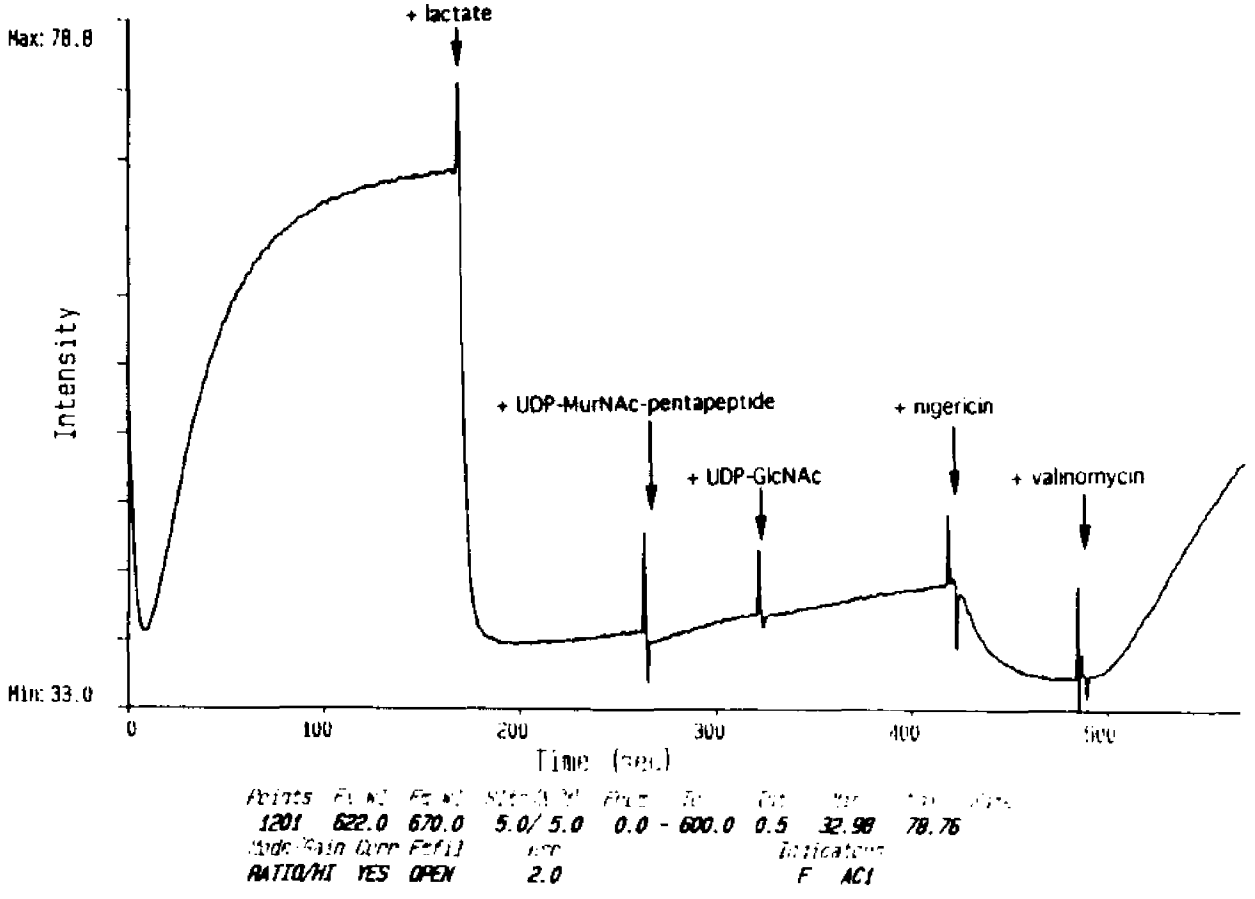
component.

It also was demonstrated in RSOs, using D-lactate as an electron donor, that $\Delta\mu_H^+$ can be generated and maintained in the reaction mixture used to measure peptidoglycan synthesis. As shown in Figure 2, sequential addition of the substrates after $\Delta\mu_H^+$ was established did not substantially alter the fluorescence intensity. Subsequent measurement of incorporation of [^{14}C]GlcNAc in the presence and absence of D-lactate showed no effect of $\Delta\mu_H^+$ on peptidoglycan synthesis. (See Table 1). Neither nigericin or valinomycin produced any substantial inhibition of incorporation of UDP-[^{14}C]GlcNAc by ISOs or RSOs. As seen in Table 1, incorporation by ISOs was inhibited 20% by valinomycin; nigericin, an ionophore which exchanges H^+ and K^+ thus dissipating ΔpH, inhibited by 5 %. In comparison, addition of lactate inhibited incorporation by ISOs by 14%. Two preparations of RSOs were tested. One was stored in the usual 0.05 mM potassium phosphate buffer, pH 6.6, the second was prepared as usual, but resuspended and stored in Tris buffer, pH 8.3. The addition of lactate and nigericin or valinomycin had essentially no effect on either of the RSO preparations. Variations seen are well within the limits of experimental error.

This series of experiments served two purposes: First, since the generation of an electrochemical gradient of protons can only be accomplished with sealed membranes,

Figure 2

Fluorescence quenching of cyanine dye in the presence of peptidoglycan substrates and RSOs



RSOs were incubated with 0.5 mM diisopropylthiodicarbocyanine in 50 mM Tris, pH 8.8, 25 mM $MgCl_2$, 0.5 mM mercaptoethanol, 10 mM ATP and 5 mM potassium phosphate buffer, pH 6.6. D-Lactate was added to generate $\Delta\mu_{H^+}$, followed by UDP-MurNAc-pentapeptide to 75 µM and UDP-GlcNAc to 2 µM. Nigericin, which dissipates ΔpH, was added to demonstrate that ΔpH was not present. Valinomycin was added as indicated.

Table 1

Incorporation of [^{14}C]GlcNAc in the presence of lactate, nigericin and valinomycin

(% of Control Incorporation)[a]

	ISOs	RSOs stored in: potassium phosphate buffer pH 6.6	RSOs stored in: Tris buffer pH 8.3
+ 20 mM lactate	86	102	98
+ 0.025 μM nigericin	95	102	102
+ 1 μM valinomycin	80	95	101

[a] - Control incorporation (pmoles/mg protein):
ISOs, 90
RSOs in potassium phosphate, 173
RSOs in Tris, 202
Specific activity = 405 dpm/pmole

the fact that these vesicles exhibited $\Delta\mu_H^+$ established their integrity; and second, the behavior of the dye molecules in the presence of vesicles of the appropriate orientation confirmed that orientation.

Characteristics of Incorporation of UDP-[^{14}C]GlcNAc

In reaction mixtures terminated by dilution and filtration, 7 to 14% of the available label was incorporated into vesicle-associated material (i.e., material which remains with vesicles after filtration) using RSOs, slightly less in ISOs. ISOs consistently had less activity per milligram of protein than RSOs. TCA precipitates of these reaction mixtures varied somewhat, showing from 30 to 100% of the incorporation seen in the diluted samples. SDS-boiled reactions contained only background levels of radioactivity, indicating that no authentic, SDS-insoluble peptidoglycan is formed in vesicles. This was not an unexpected finding since newly synthesized peptidoglycan is thought to become insoluble in SDS upon its crosslinking to the pre-exsisting polymer (Mirelman et al., 1976), very little of which is expected to be present in vesicles (Kaback, 1971). Incorporation was completely dependent upon the presence of Mg^{++}, strongly dependent on the unlabelled substrate, UDP-MurNAc-pentapeptide, and slightly dependent on ATP (Table 2). ATP is not required for the steps of peptidoglycan synthesis

Table 2

Effects of omissions on incorporation of [^{14}C]GlcNAc

Condition	% of Control Incorporation[a] ISOs	RSOs
- Mg^{++}	7.7	5.0
- mercaptoethanol	116	104
- UDP-MurNAc-pentapeptide	11	32
- ATP	80	87

[a] - Control incorporation (pmoles; protein concentration not determined):
ISOs, 13.5
RSOs, 19.1 (Mg^{++}, ATP, and mercaptoethanol)
12.3 (UDP-MurNAc-pentapeptide)
Specific activity = 405 dpm/pmole

which appear to be measured in vesicles. Some assays using particulate enzyme (i.e. membrane fragments) from S. aureus to measure peptidoglycan synthesis have been reported to be stimulated by ATP. Higashi et al. (1970) concluded that the stimulation was due to synthesis of the C_{55}-phosphate which does require ATP. We have continued to include ATP in the reaction mixture because a precipitate, possibly potassium magnesium phosphate, forms in its absence.

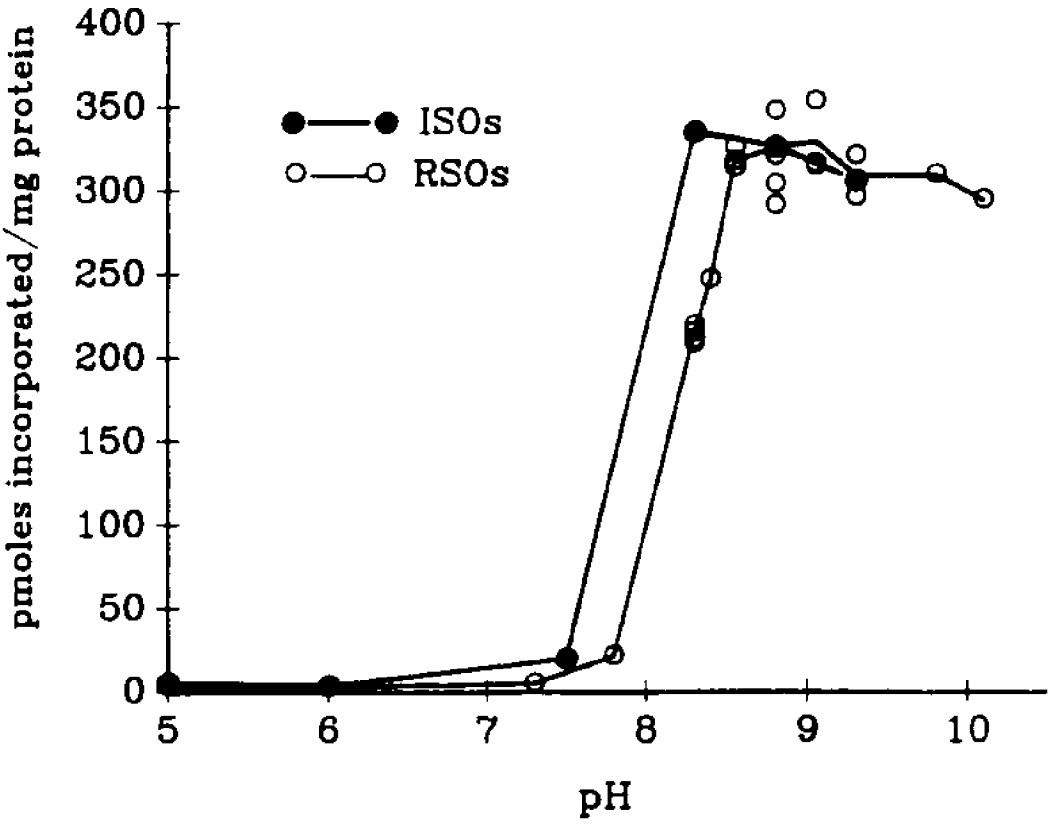
The conditions used in this assay are very different from those found to be optimal for transport assays which are usually done in phosphate buffer at pH 5.5 to pH 7.5. The incorporation into both RSO and ISO vesicles was found to have a very high pH optimum (Figure 3). The rate of incorporation in 50 mM Tris increased sharply with pH from pH 8 to pH 8.55, and then was relatively constant up to at least pH 10.1. As a result, pH 8.8 was chosen for the standard reaction conditions. The observed difference between the ISO and RSO response to the pH of the buffer may be due to the difference in the storage buffer used for ISOs (pH 7.5) and RSOs (pH 6.6).

Incorporation decreased sharply below pH 7 to essentially zero at pH 5. Subsequently, reactions were terminated by the addition of 1 ml of cold 2.5 mM acetic acid which decreased the pH to <5.0.

Several buffers at pH 8.8 at 50 and 25 mM were

Figure 3

Effect of pH on incorporation of label from UDP-[^{14}C]GlcNAc by ISO and RSO vesicles



Vesicles were incubated for 40 min at 30°C with 0.05M Tris buffer at the indicated pH. Reactions were terminated by the addition of 1ml of 500µM UDP-GlcNAc.

compared to Tris at pH 8.8 using RSOs (Table 3). Incorporation in Tricine was almost equal to Tris, glycine did not allow any incorporation, and the results in borate were intermediate. The effect of buffer concentration was tested in RSOs using Tris, (Figure 4) and the concentration used in the assay, 50mM, proved to be the optimum. Incorporation in RSOs is dependent on the nature of the buffer (Tris > Tricine > borate > phosphate > glycine) as well as its concentration, with an optimum at 50 mM.

The time course of incorporation was followed in both ISOs and RSOs (Figure 5). Both showed an immediate, high rate of incorporation, followed by a slower, steadily decreasing rate.

Effects of Inhibitors on Incorporation

Several inhibitors of known steps of peptidoglycan synthesis were used to characterize incorporation in ISOs and RSOs. Inhibition of incorporation by tunicamycin, a specific inhibitor of phospho-N-acetylmuramyl-pentapeptide transferase; bacitracin, which inhibits the recycling of the undecaprenol-pyrophosphate; prasinomycin, a transglycosylase inhibitor; and ampicillin, a transpeptidase inhibitor, was measured.

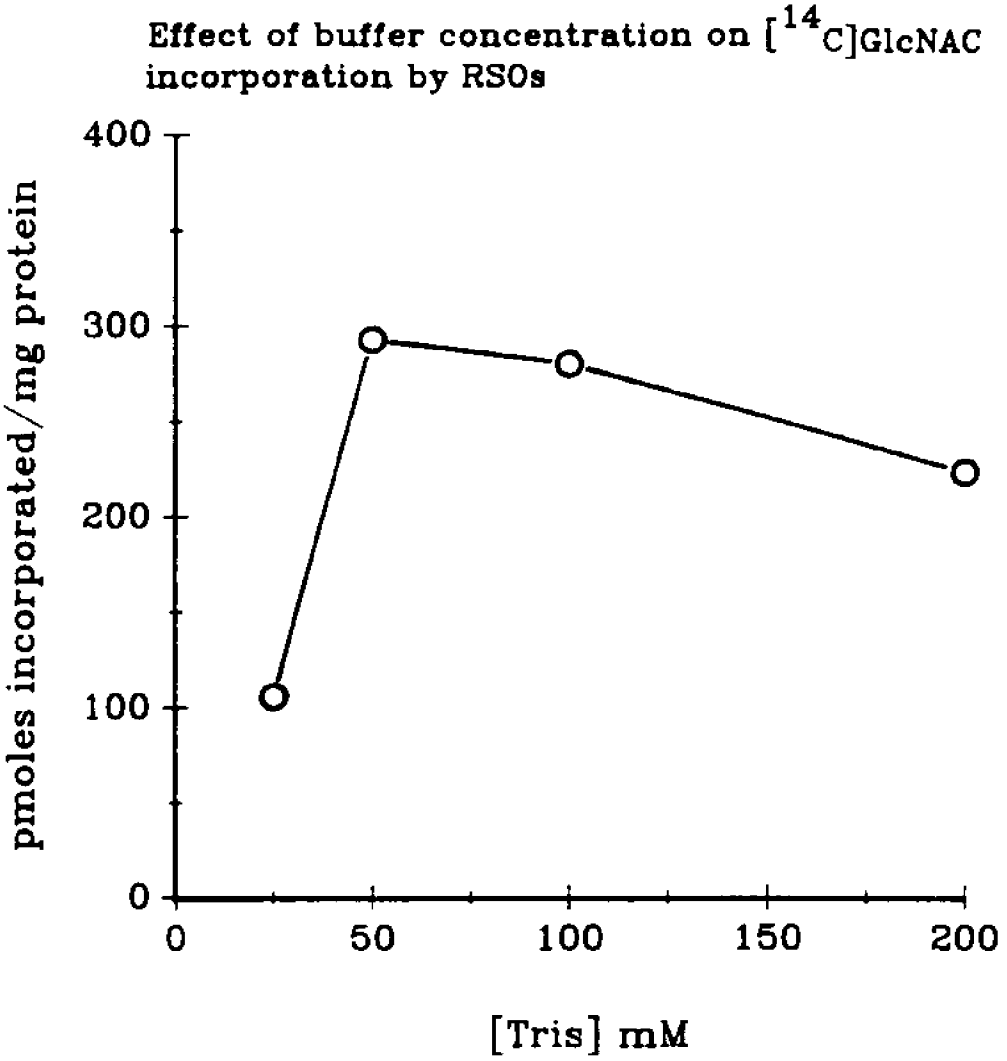
Inhibitors were also tested in the presence and absence of D-lactate to determine the effect of $\Delta\mu_H^+$ on incorporation and its inhibition. As shown in Table 4, no difference was seen in the effects of the inhibitors when

Table 3

Effects of buffer on incorporation of [^{14}C]GlcNAc (pmoles incorporated/mg protein)

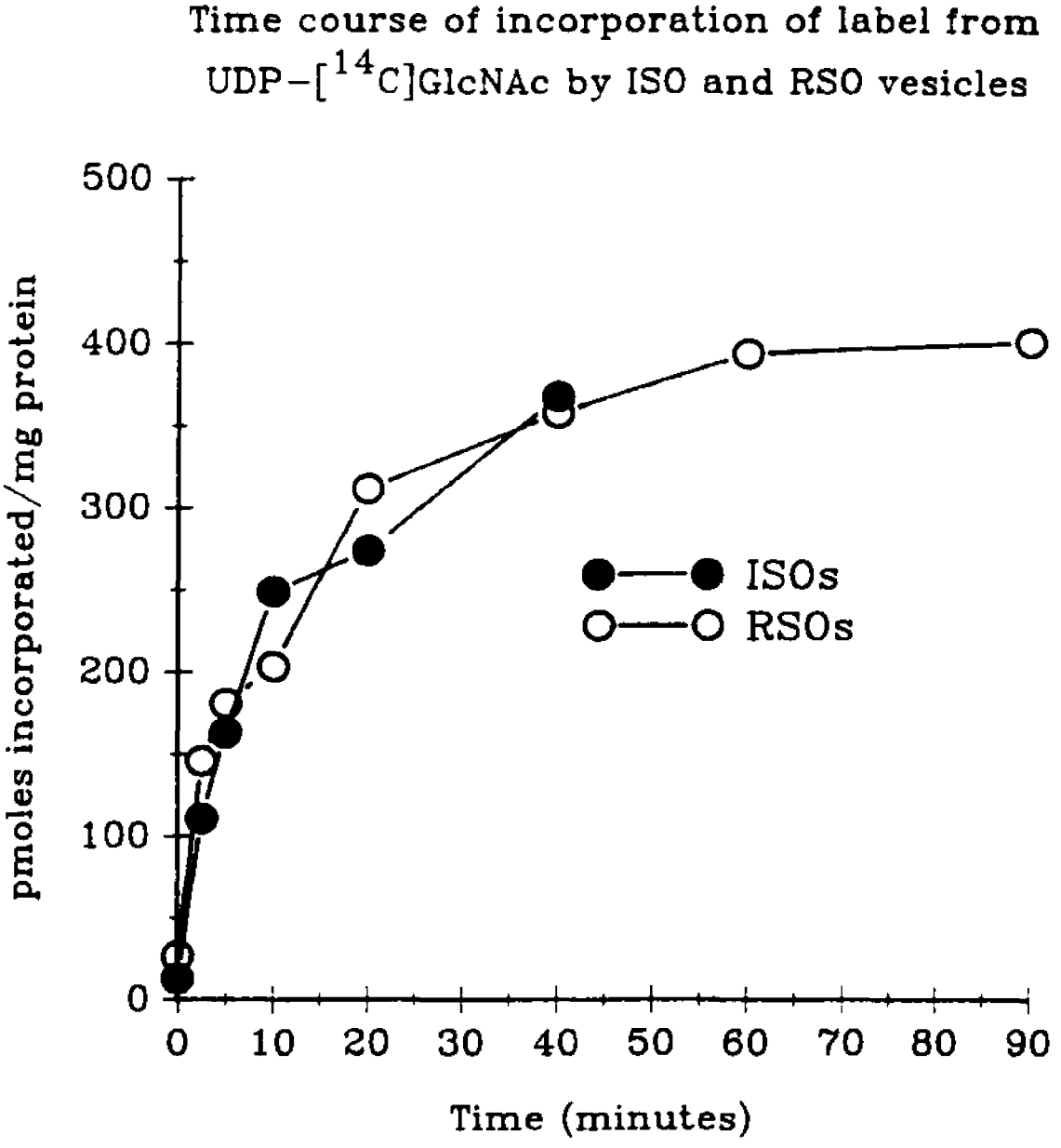
Buffer pH 8.8	25 mM	50 mM
Tris	9.8	32.1
Glycine	0.69	0.68
Tricine	9.2	27.4
Borate	3.9	13.9

Figure 4



RSO vesicles were incubated at 30°C for 40 min with both substrates and Tris buffer pH 8.8 at the indicated concentration.

Figure 5



Vesicles were incubated with substrates at 30°C. Duplicate 200 µl aliquots were removed at the indicated time and added to 1 ml of cold 500 µM UDP-GlcNAc. Samples were filtered and the filters counted.

Table 4

Effects of inhibitors on [^{14}C]GlcNAc incorporation in ISO and RSO vesicles

(% of Control Incorporation)[a]

Inhibitor	ISO		RSO	
	- Lactate	+ Lactate	- Lactate	+ Lactate
tunicamycin 100 µg/ml	5.3	5.3	13	10
bacitracin 1000 µg/ml	70	72	98	83
ampicillin 1000 µg/ml	84	98	93	96
prasinomycin 100 µg/ml	61	63	173	168

[a] - Control incorporation (pmoles/mg protein):
ISOs, 83
ISOs + lactate, 77
Specific activity = 405 dpm/pmole
RSOs, 135
RSOs + lactate, 143
Specific activity = 270 dpm/pmole

tested in the presence of $\Delta\mu_{H^+}$. Tunicamycin was an excellent inhibitor of incorporation in all the systems tested. Bacitracin had a small inhibitory effect although the amount was quite variable - from 2.8 to 30.3%. Incorporation appeared to be insensitive to ampicillin. This very high concentration of a ß-lactam antibiotic (1 mg/ml) is sufficient to completely inhibit peptidoglycan synthesis in other test systems. The most interesting effect of the inhibitors tested was that of prasinomycin, which inhibited incorporation to a moderate degree in ISOs, but significantly stimulated incorporation in both of the RSO preparations tested.

Is the Incorporation by RSOs Artifactual or Actual?

The incorporation of label from UDP-[^{14}C]GlcNAc by RSO vesicles was an unexpected result. The active sites of the membrane- associated enzymes which catalyze the incorporation of UDP-MurNAc-pentapeptide and UDP-GlcNAc were presumed to be on the interior surface of the cytoplasmic membrane and thus inside of RSOs. Before continuing to study the topology of these enzymes, it was necessary to eliminate possible trivial explanations for the observation. These included: 1) contamination of RSO preparations with ISOs. 2) non-enzyme-mediated entrapment of the labelled substrate within the RSOs; 3) rearrangement of the target enzymes in the membrane during

vesicle preparation. Investigation of this question raised the possibility that 4) the nucleotide-linked substrate, UDP-[^{14}C]GlcNAc, could be degraded to N-acetyl[1^{4C}]glucosamine and transported to the inside of the cell via the phosphotransferase system (PTS). Then, as N-acetyl[^{14}C]glucosamine-6-phosphate, the label could re-enter the pathway as a precursor to UDP-[^{14}C]GlcNAc and serve as a substrate for the enzymes on the inner surface of the membrane. Each of these possible explanations for the UDP-[^{14}C]GlcNAc incorporation observed in RSO vesicles was investigated.

1. Examination of the Uniformity of ISO and RSO Preparations.

The unexpected finding of peptidoglycan-synthesizing activity in vesicles of both orientations caused us to suspect that the RSO preparations might be contaminated with ISOs which were solely responsible for the activity. Two methods were used to answer this question.

Fluorescence Spectroscopy

The charged, fluorescent dyes employed above to probe ISOs and RSOs were used to test vesicles of the opposite orientation. In the presence of $\Delta\mu_H^+$, the interior of RSOs becomes alkaline and negative. Negatively charged oxonol dye, the usual probe for ISOs, would not be expected to remain within the vesicles, so no quenching of the

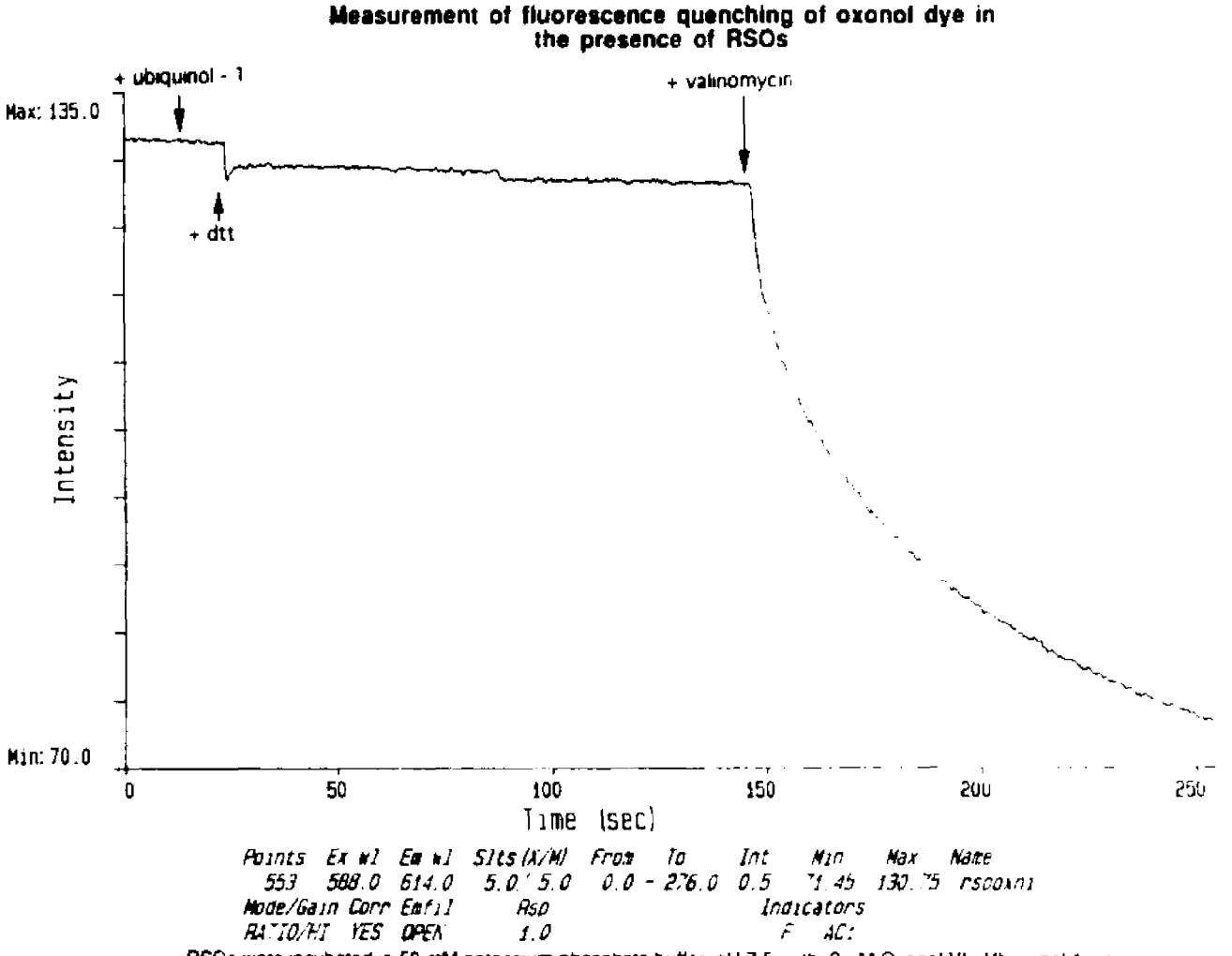
fluorescence would be seen. Any quenching would necessarily be due to the presence of contaminating ISO vesicles, which in the presence of $\Delta\mu_H^+$ become acidic and positive within. As seen in Figure 6, when the RSO preparation was tested in the presence of the oxonol dye, the fluorescence intensity did not decrease significantly in the presence of $\Delta\mu_H^+$, indicating that oxonol did not accumulate within the vesicles. The ISO preparation was similarly tested for the presence of contaminating RSOs using the positively charged cyanine dye (Figure 7). These results also indicated that the ISO preparation contains very few (if any) contaminating RSO vesicles.

These results confirm that the different methods used to prepare RSO and ISO vesicles are specific and result in vesicles of the preferred orientation.

Sucrose Gradient Centrifugation of Reacted Vesicles

An additional physical difference between ISO and RSO vesicles is in size and interior volume of the vesicles. RSOs are formed by osmotic lysis of spheroplasts produced by lysozyme-EDTA-treated cells. This procedure causes the rapid opening and resealing of the membrane resulting in vesicles which are approximately the same size as the spheroplasts. ISO vesicles are produced from pieces of the membrane formed from cells broken by pressure in a French press. The small size of these membrane pieces is thought to be the reason for the inside-out orientation of the

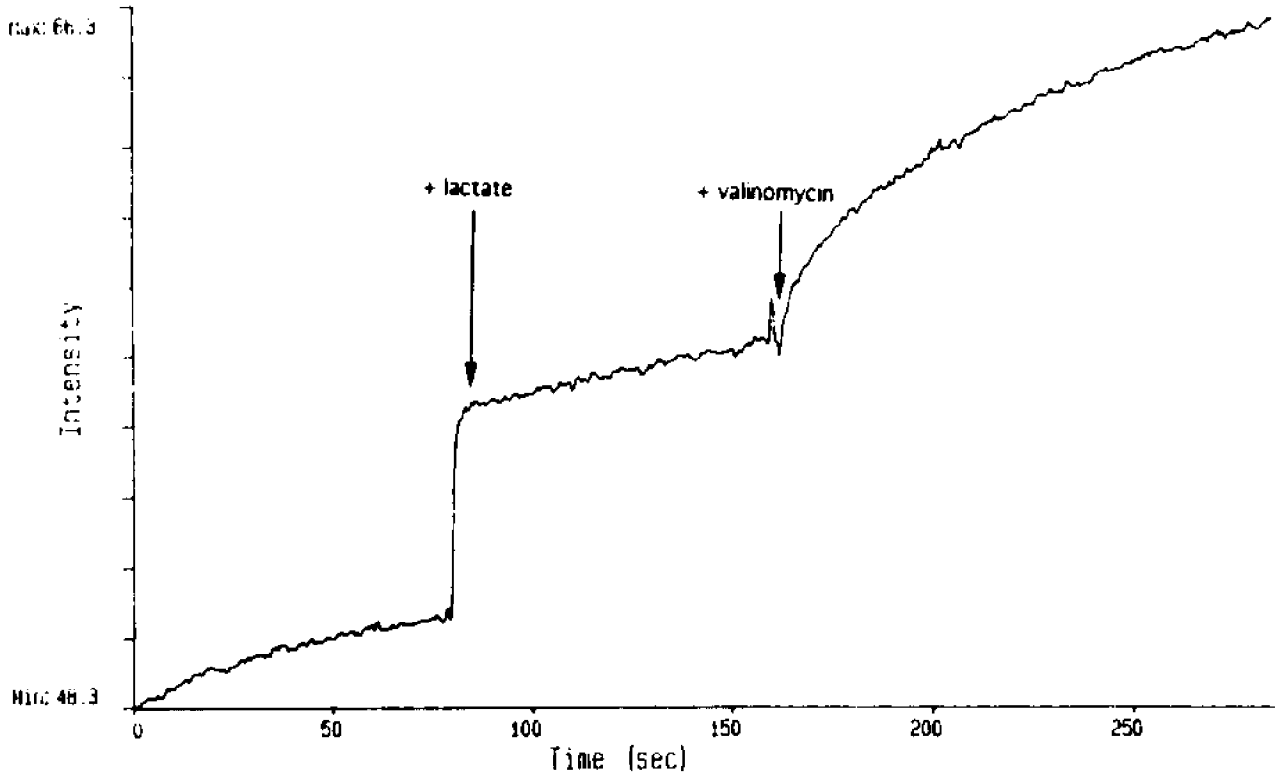
Figure 6



RSOs were incubated in 50 mM potassium phosphate buffer, pH 7.5, with 2 µM Oxonol VI. Ubiquinol-1 was added to generate $\Delta\mu_{H^+}$, followed by dithiothreitol to maintain the ubiquinol-1 in the reduced state. Little fluorescence quenching was observed in response to the charge separation. This was expected because the interior of RSOs becomes negatively charged upon generation of $\Delta\mu_{H^+}$ and Oxonol VI is a negatively charged dye. Valinomycin was added as indicated.

Figure 7

Measurement of fluorescence quenching of cyanine dye in the presence of ISOs



Points	Ex wl	Em wl	Slits (X/M)	From	To	In:	Min	Max	Name
601	622.0	670.0	5.0/ 5.0	0.0 -	300.0	0.5	48.27	66.29	
Mode	Gain	Corr	Cal'd	HSD			Indicators		
RATIO/HI	YES		OPEN	2.0			F	AC1	

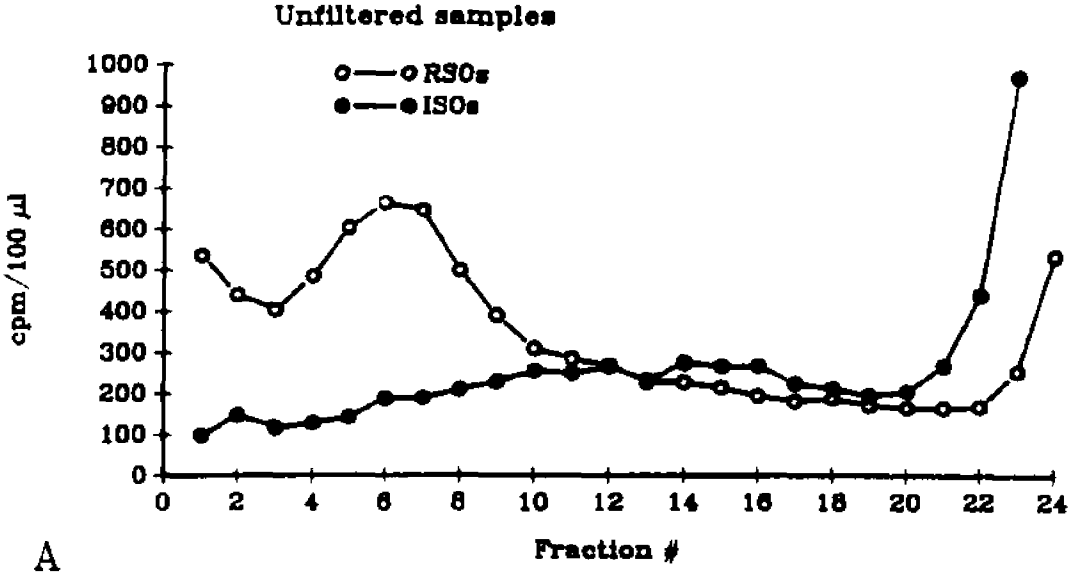
ISOs were incubated in 100 mM potassium phosphate buffer, pH 6.6 ,with 0.5 µM diisopropylthiodicarbocyanine. D-Lactate was added to generate $\Delta\mu_{H^+}$. Little fluorescence quenching was observed in response to the charge separation. This was expected because the interior of ISOs becomes positively charged upon generation of $\Delta\mu_{H^+}$ and diisopropylthiodicarbocyanine is a positively charged dye Valinomycin was added as indicated.

resulting vesicles. This size difference allows separation of ISO and RSO vesicles by physical methods, such as sucrose gradient centrifugation. ISOs and RSOs were incubated with substrates in the reaction mixture used to measure peptidoglycan synthesis. After incubation, the total reaction mixture was layered on top of 20 to 60% sucrose gradients which were then centrifuged at 65000xg overnight. The gradients were fractionated by pumping from the bottom of each tube and collecting 10 drop fractions. 100 μl aliquots of each fraction were counted. The remainder of each fraction was filtered through 0.22 μm filters and the filters counted.

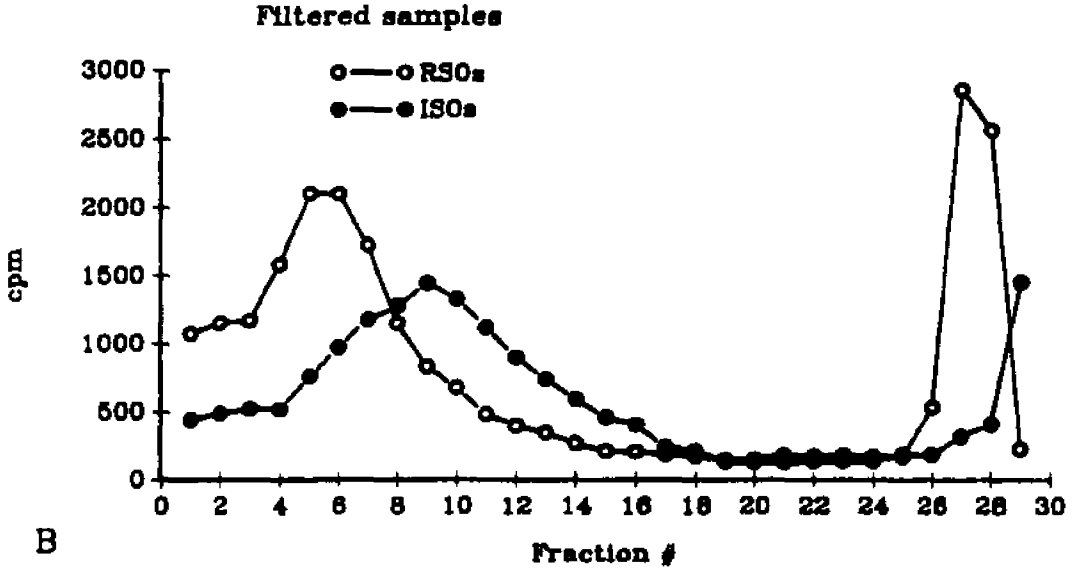
As seen in Figure 8A, it is difficult to determine the location of labelled ISO vesicles by counting unfiltered aliquots. The original reaction mixture contains a large amount of label which remains unincorporated. This label appears as the peak at the very top of the sucrose gradient, and may also contaminate other fractions. When filtered, however, the peaks are clearly seen (Figure 8B). As expected, the RSO vesicles were found farther down the gradient than the ISO vesicles. It is important to note that almost all of the label found in RSOs coincided with the only visible band found after centrifugation. The ISOs produced no visible band after centrifugation. The peak of radioactivity was broader than that seen in RSOs, but is clearly at a different location than the RSO peak.

Figure 8

Distribution of radioactivity after sucrose gradient fractionation of reacted vesicles



A



B

Vesicles were incubated in the normal reaction mixture for 40 min at 30°C. One ml of each reaction mixture was layered on top of a 20-60% sucrose gradient prepared in 100 mM potassium phosphate buffer pH 6.6 (RSO's) or 50 mM potassium phosphate buffer pH 7.5 (ISO's). Gradients were centrifuged overnight at 65000 x g at 4°C. Ten drop fractions were collected by pumping each gradient from the bottom. One hundred µl aliquots of each fraction were counted in scintillation fluid. The remainder of each fraction was filtered through a 0.22µm filter and the filters were counted.

2. Non-enzymatic Association of UDP-[^{14}C]GlcNAc with Vesicles

A second possible artifactual basis for the activity seen in RSO vesicles, non-enzymatic association of labelled substrate within the vesicles was also investigated. Reaction mixtures at a pH below 7 do not incorporate label, although RSOs are able to generate $\Delta\mu_{H^+}$ at pH 6.6. In addition, incorporation of label from UDP-[^{14}C]GlcNAc was completely inhibited in the absence of Mg^{++}, which is known to be necessary for activity of the phospho-N-acetylmuramyl-pentapeptide transferase (Struve et al., 1966). However, this result was determined by filtration of the reacted vesicles and extensive washing of the filter. Trapped UDP-[^{14}C]GlcNAc might have been lost in response to the concentration gradient. To test this, reaction mixtures containing either ISOs or RSOs with and without Mg^{++} were incubated for 15 min and the reactions terminated with a very small volume of 100 mM acetic acid. Reaction mixtures were layered atop 40 μM cold UDP-GlcNAc in 30% sucrose and centrifuged at 16000Xg for 30 min. Tubes were immediately frozen in dry ice and the tip of each tube cut off and the contents counted in scintillation fluid. Results seen in Table 5 show that UDP-[^{14}C]GlcNAc did not associate with vesicles of either orientation in the absence of enzymatic activity.

Table 5

Non-specific binding of UDP-[^{14}C]GlcNAc to vesicles

	pmoles Incorporated			
	ISOs		RSOs	
	Filtered	Spun	Filtered	Spun
Control	6.0	2.9	48.5	19.5
-Mg^{++}	0.41	0.83	2.7	2.0

3. Comparison of RSOs with Permeabilized and Whole, Untreated Cells

An alternative explanation for the incorporation measured in RSO vesicles is rearrangement of the enzymes in the membrane, allowing the phosphorylated substrates to interact with the newly exposed active sites. Such a rearrangement would be an artifact of vesicle preparation and would not reflect the in vivo situation. To examine this possibility, incorporation was measured in whole cells which had been briefly treated with EDTA. EDTA slightly disrupts the outer membrane of E. coli, while leaving the inner membrane unharmed. The result of this disruption is an increase in the permeability of the outer membrane to previously excluded molecules (Leive et al., 1965). E. coli B cells were treated with EDTA for 2 min and tested in the reaction mixture used to measure incorporation in vesicles. EDTA-treated cells incorporated label from UDP-[^{14}C]GlcNAc into SDS-insoluble material, indicating that the product of the assay was mature peptidoglycan. The amount of incorporation varied between preparations from 4 to 15 pmole/mg of protein, averaging 10 pmole/mg of protein. E. coli cells permeabilized with ether (ETB) incorporate about 35 pmole/mg of protein in this assay. This incorporation was inhibited by typical inhibitors of peptidoglycan synthesis, including ß-lactams. (The results for these and the following experiments are listed in Table

6 together with comparable experiments done using ETB and vesicles.)

It was possible, however, that treatment with EDTA in some way deranged the inner membrane of these cells. To eliminate this possibility, whole, untreated E. coli B cells were harvested, resuspended in T2 buffer (an osmotically stabilizing buffer used for viable counts; Meynell and Meynell, 1970),and assayed as were the EDTA-treated cells and vesicles. These cells also incorporated label, 4.6 pmoles/ml of protein, into SDS-insoluble material and this incorporation was inhibited by ampicillin.

4. Is the Phosphotranferase System Responsible for Incorporation by RSOs ?

These results, in essentially intact cells, led to speculation that the phosphorylated substrate, UDP-[^{14}C]-GlcNAc, was being enzymatically degraded to N-acetylglucosamine and transported to the interior of the cell via the phosphotransferase system. N-Acetylglucosamine has been shown to be transported by this route (Mortimer-Jones and Kornberg, 1980). If label from UDP-[^{14}C]GlcNAc was also entering the cells via this mechanism, the presence of unlabelled N-acetylglucosamine should decrease incorporation of label. However, when tested in the presence of a 100-fold excess of cold N-acetylglucosamine, incorporation into SDS-insoluble

Table 6.

% of Control Incorporation[a]

	EDTA-treated Cells	Whole Cells	Compare to: ETB ISOs RSOs		
Reaction terminated with:	SDS	SDS	SDS	HOAc	HOAc
Inhibitor					
tunicamycin 100 μg/ml	19	NT	3	5	13
bacitracin 1000 μg/ml	77	NT	63	70	97
ampicillin 1000 μg/ml	1	7	5	84	93
prasinomycin 100 μg/ml	3	NT	0.5	91	173

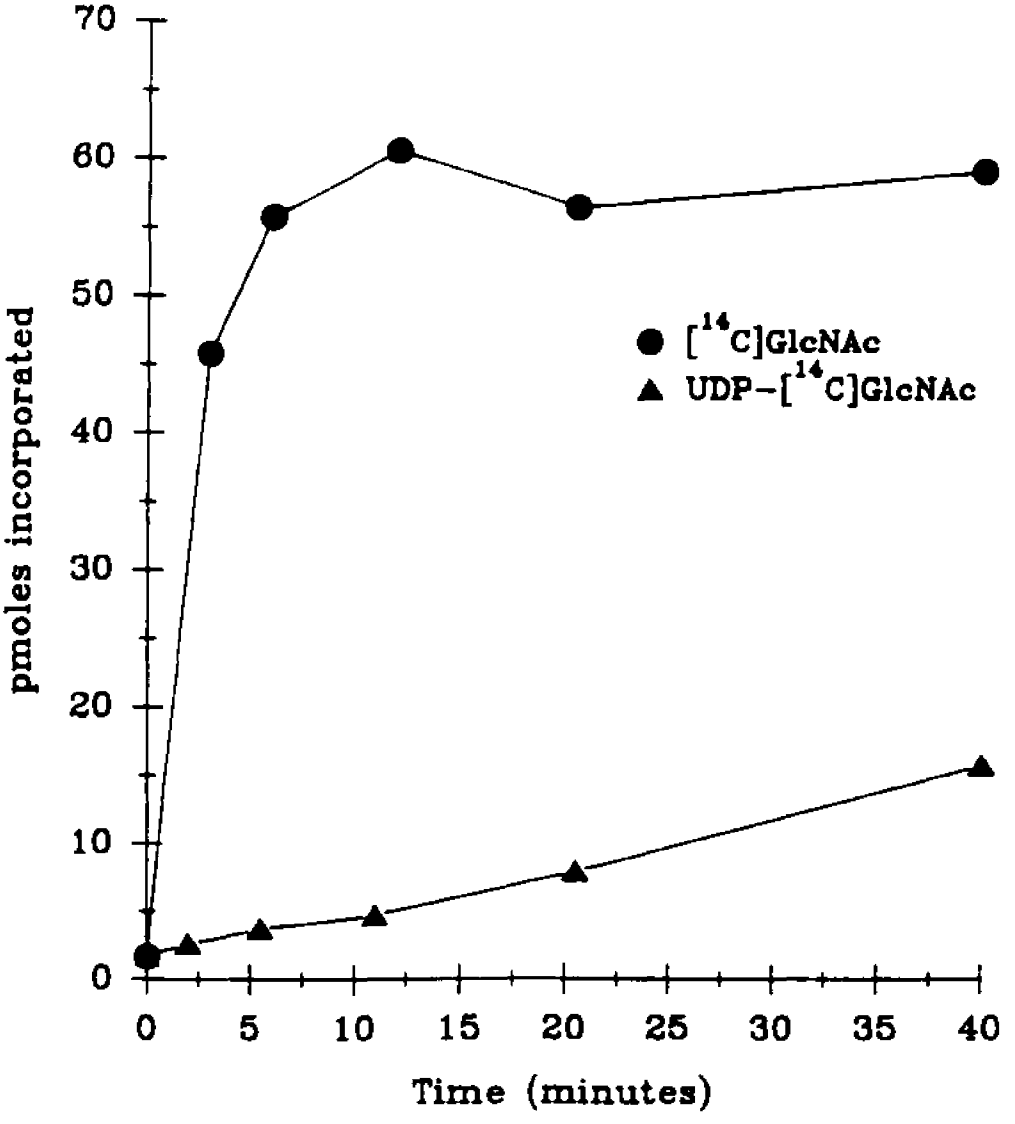
NT - not tested

[a] - Control incorporation
EDTA-treated cells, 81.6 pmoles/mg protein
Whole cells, 23.1 pmoles/mg protein
Specific activity = 252 dpm/pmole
ISOs, 83 pmoles/mg protein
Specific activity = 405 dpm/pmole
RSOs, 135 pmoles/mg protein
Specific activity = 270 dpm/pmole

material by EDTA-treated cells was only slightly decreased. When repeated with N-acetyl-[^{14}C]glucosamine, incorporation was almost completely inhibited by cold N-acetylglucosamine. This activity was further examined by following the time course of incorporation of label from either UDP-[^{14}C]GlcNAc or N-acetyl-[^{14}C]glucosamine. EDTA-treated cells incubated with UDP-N-acetylmuramyl-pentapeptide, Mg^{++} and either labelled substrate, incorporated label into SDS-insoluble material, indicating that the product is mature peptidoglycan. However, as seen in Figure 9, the time course of incorporation appeared very different for the two substrates. EDTA-treated cells incubated with N-acetyl-[^{14}C]glucosamine showed a rapid burst of incorporation within the first 5 min of incubation after which incorporation essentially stopped. Label from UDP-[^{14}C]N-acetylglucosamine, was incorporated at a slower rate, which increased slightly but consistently over the 40 min course of the experiment. This difference in the rate of incorporation of label from the two substrates was suprising. The very rapid initial rate of incorporation of N-acetyl-[^{14}C]glucosamine may be characteristic of a substrate which is actively transported. Further evidence that these two substrates are incorporated into peptidoglycan by different means was seen when the time course was repeated for each labelled substrate in the presence of a 100-fold excess of the other, cold substrate.

Figure 9

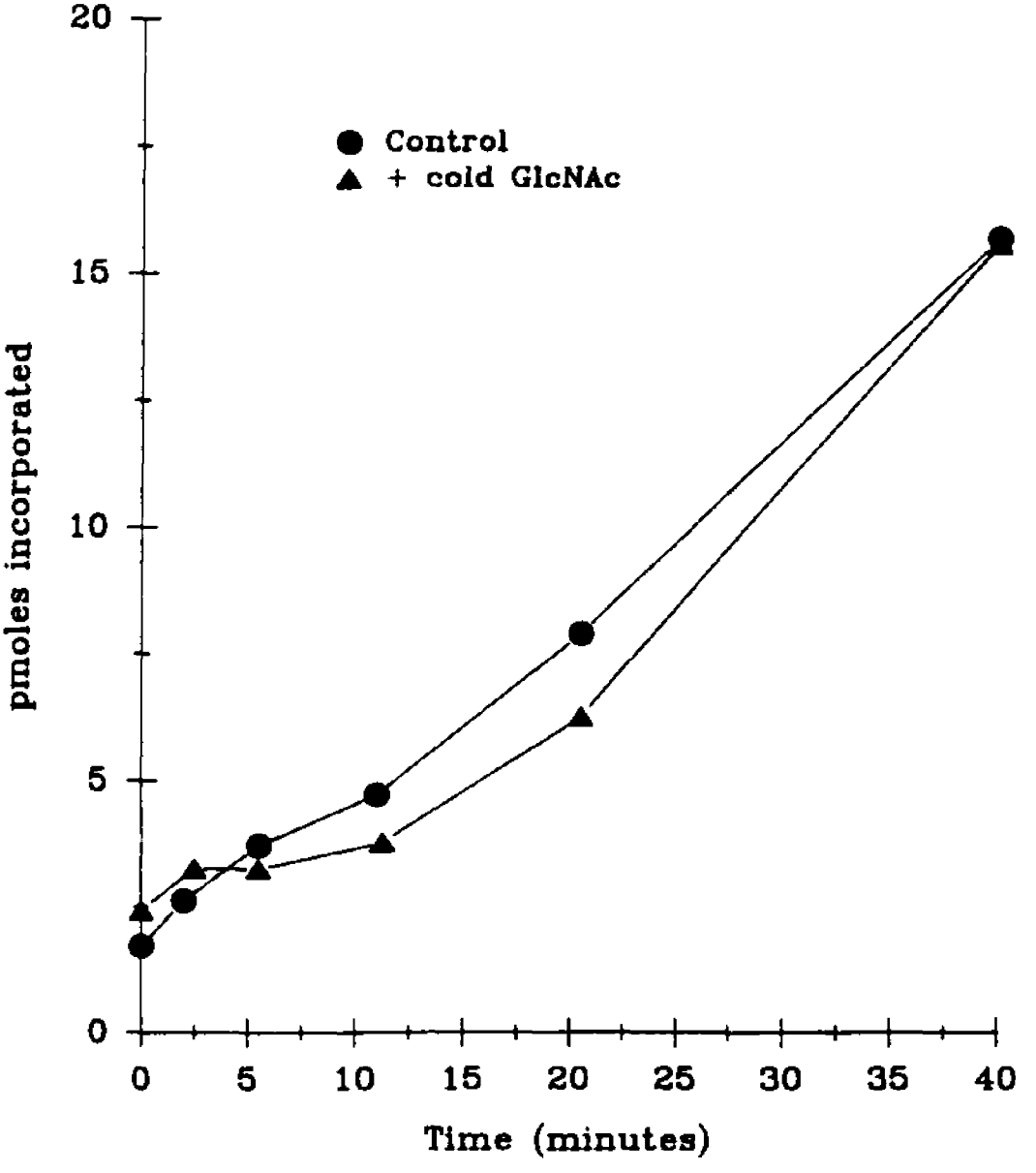
Incorporation of label from [^{14}C]GlcNAc and UDP-[^{14}C]GlcNAc by EDTA-treated *E. coli*



Exponentially growing *E. coli* cells were washed and briefly treated with EDTA. Centrifuged cells were suspended in basic media and assayed in the presence of either [^{14}C]N-acetyl-glucosamine or UDP-[^{14}C]N-acetylglucosamine. Duplicate aliquots were removed at the indicated times, added to 4% SDS and boiled. Samples were filtered and the filters counted.

Figure 10

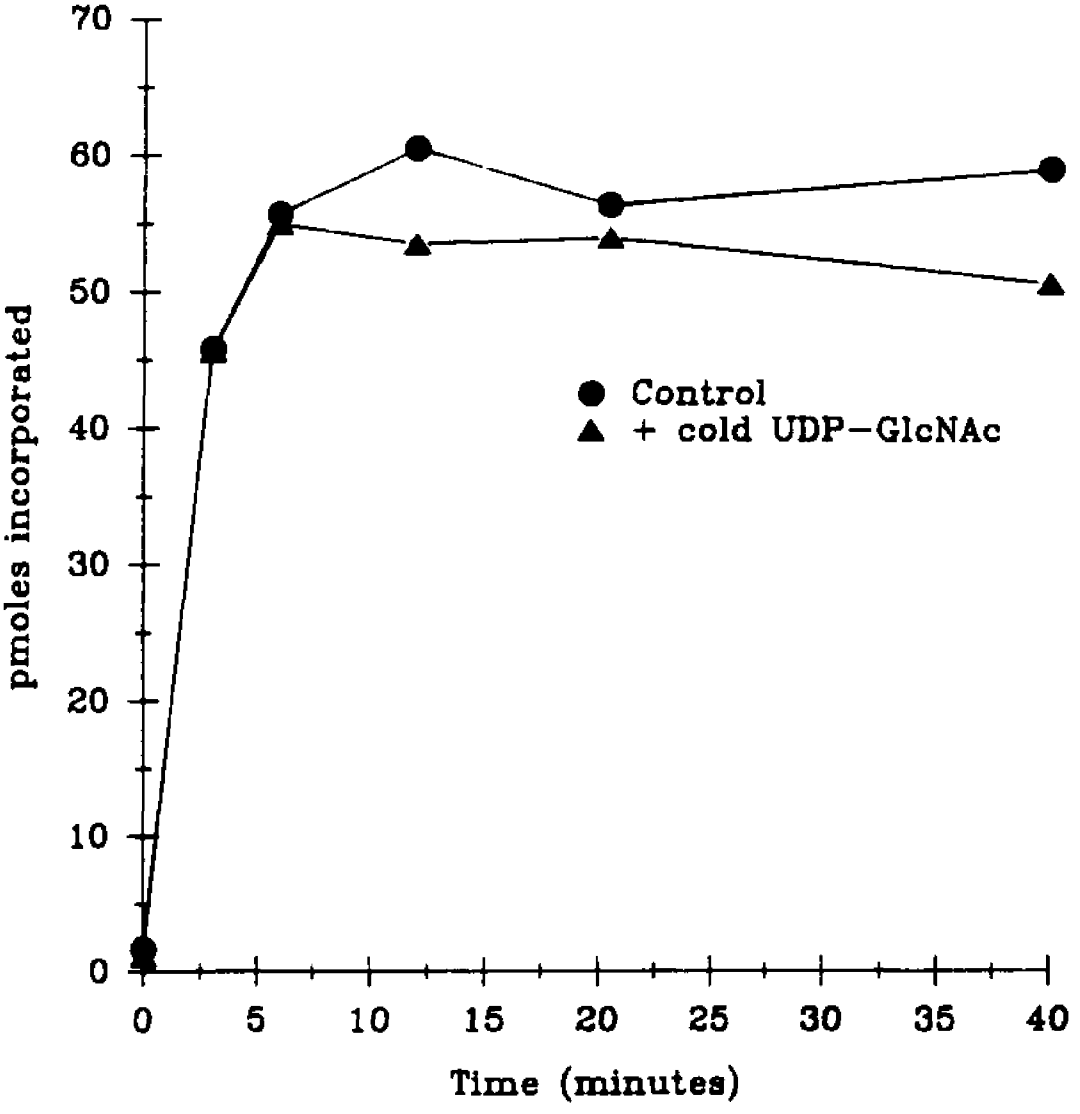
Incorporation of label from UDP-[^{14}C]GlcNAc by EDTA-treated *E. coli* in the presence of cold GlcNAc



EDTA-treated *E. coli* cells were incubated with UDP-[^{14}C]GlcNAc at 2.07 μM and unlabelled GlcNAc at 207 μM. Duplicate aliquots were removed at the indicated times, added to 4% SDS and boiled. Samples were filtered and the filters counted.

Figure 11

Incorporation of label from [^{14}C]GlcNAc by EDTA-treated *E. coli* in the presence of UDP-GlcNAc



EDTA-treated *E. coli* cells were incubated with [^{14}C]-GlcNAc at 2.07 μM and unlabelled UDP-GlcNAc at 207 μM. Duplicate aliquots were removed at the indicated times, added to 4% SDS and boiled. Samples were filtered and the filters counted.

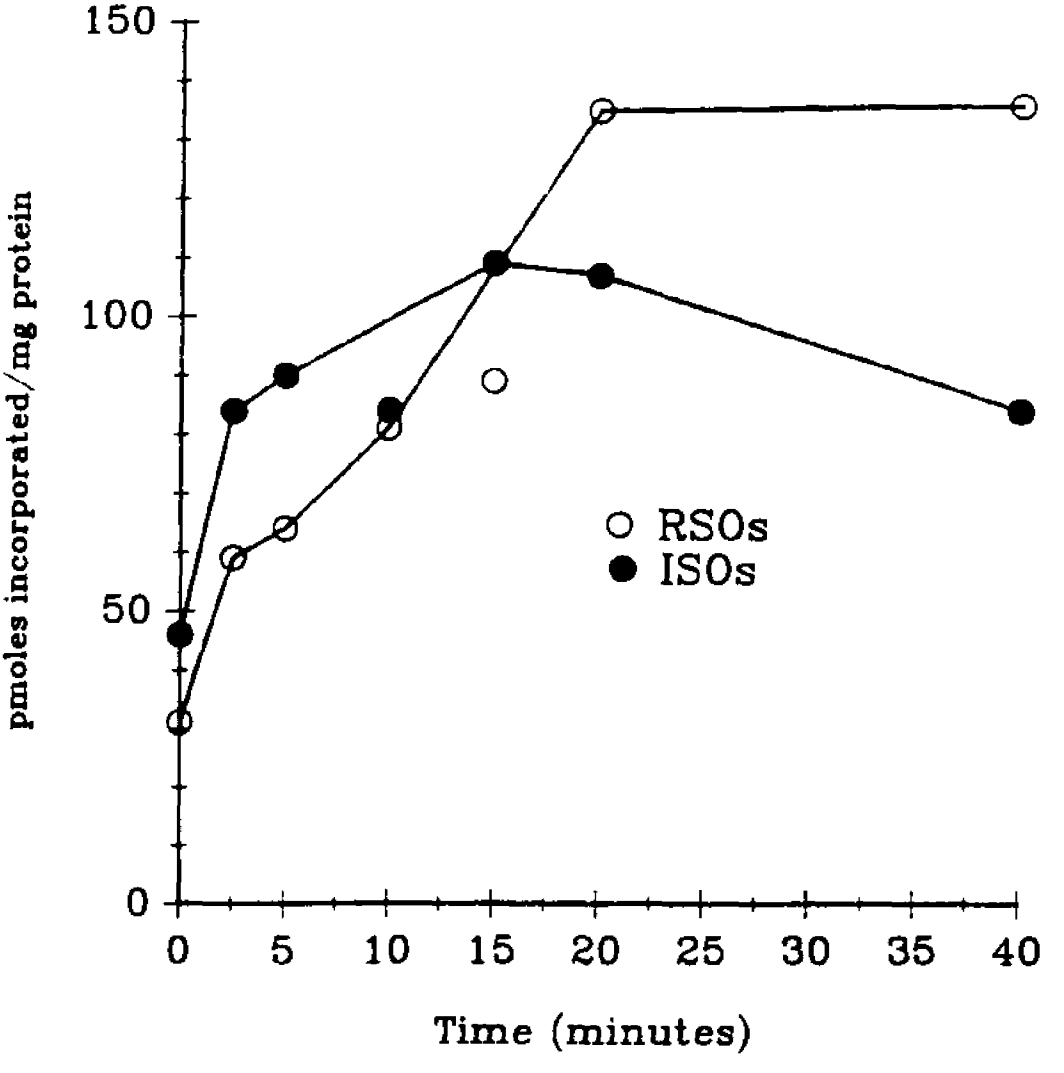
incorporation reaction was measured as radioactivity retained on 0.22 μm filters. Incorporation was measured in the same reaction mixture used to measure the incorporation of UDP-[^{14}C]GlcNAc with or without cold UDP-GlcNAc. Vesicles and substrates were incubated at 30°C and the reaction terminated by pH drop, i.e., addition of sufficient cold 2.5 mM acetic acid to reduce the pH below 7. Vesicles of both orientations incorporated UDP-MurNAc-[^{3}H]pentapeptide into vesicle-associated material. This incorporation was time-dependent to about 15 or 20 minutes (Figure 12). Fifteen-minute incubations were routinely used for all further incorporation reactions. As shown in Figure 13, incorporation was greatly stimulated by the addition of UDP-GlcNAc, the substrate of the next reaction of peptidoglycan synthesis. Table 7 summarizes the results of incorporation assays using UDP-MurNAc-[^{3}H]pentapeptide. Incorporation was completely dependent upon Mg^{++}. In the absence of UDP-GlcNAc incorporation was somewhat dependent upon ATP. The dependence on ATP was increased in the presence of UDP-GlcNAc, more so in RSOs than in ISOs.

Effect of inhibitors on incorporation of UDP-[^{3}H]MurNAc-pentapeptide

Inhibition by tunicamycin, bacitracin, penicillin, and prasinomycin was measured using UDP-MurNAc-[^{3}H]pentapeptide with and without UDP-GlcNAc. As shown in

Figure 12

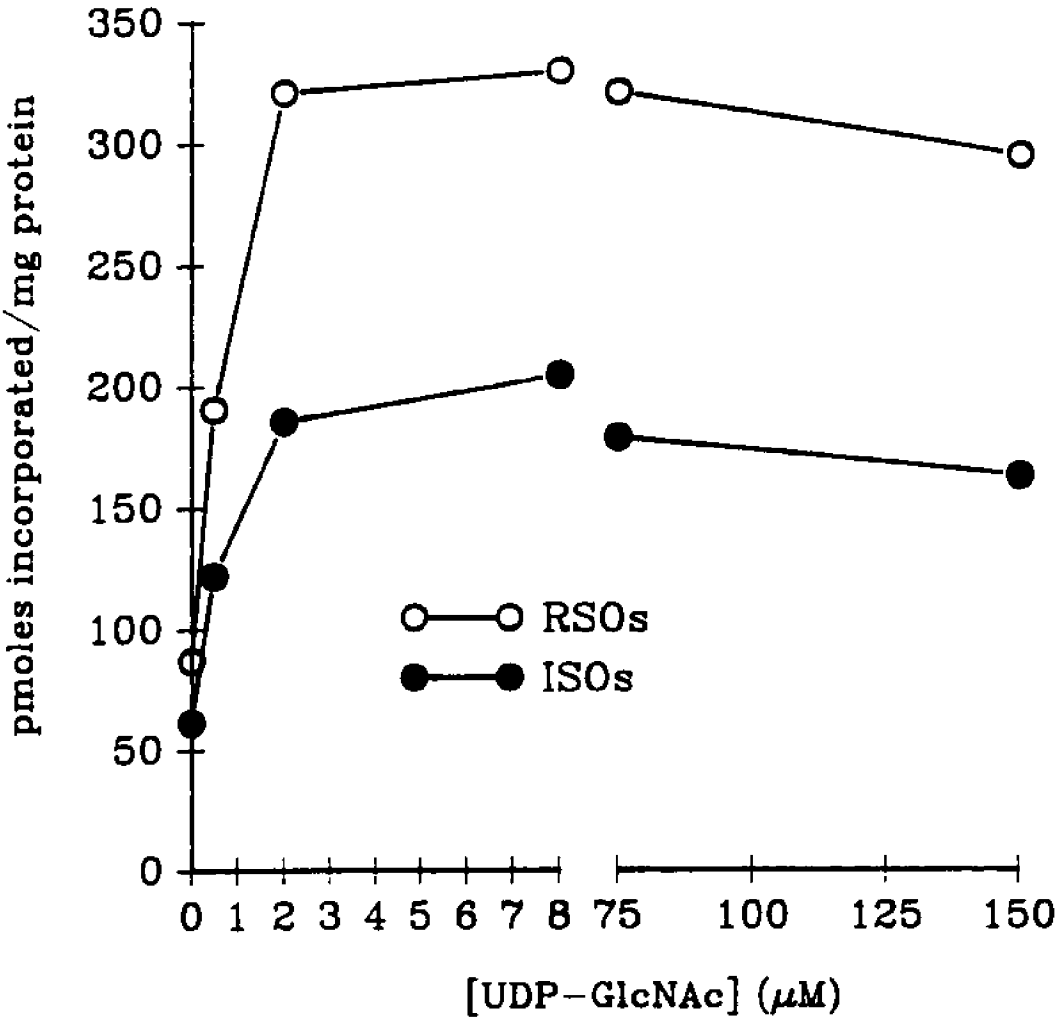
Time course of incorporation of label from UDP-MurNAc-[^{3}H]pentapeptide by vesicles



Vesicles were incubated with 6.5 μM UDP-MurNAc-[^{3}H]pentapeptide. Duplicate 100 μl aliquots were removed at the indicated time and added to 1 ml of cold 2.5mM acetic acid. Samples were filtered and the filters counted.

Figure 13

Effect of UDP-GlcNAc on incorporation of label from UDP-MurNAc-[^{3}H]pentapeptide



Vesicles were incubated for 15 min with 8.33 μM UDP-MurNAc-[^{3}H]pentapeptide and UDP-GlcNAc at the indicated concentration.

Table 7

Effects of omissions on incorporation of label from UDP-[^{3}H]MurNAc-pentapeptide by ISO and RSO vesicles

	pmoles incorporated/mg protein	
	ISOs	RSOs
Control	209	364
- UDP-GlcNAc	70	60
- ATP	141	103
Assayed in the absence of UDP-GlcNAc		
- Mg^{++}	13*	25*
- ATP	50	32

* Dependency on Mg^{++} is probably greater than indicated by this result. Zero time samples in this experiment had approximately the same amount of incorporation as reaction mixtures incubated without Mg^{++}.
Specific activity = 186 dpm/pmole

Table 8, incorporation of label from UDP-MurNAc-[^{3}H]-pentapeptide was inhibited by tunicamycin in the presence and absence of UDP-GlcNAc. Bacitracin, however, did not inhibit incorporation in the absence of UDP-GlcNAc. In its presence, incorporation was inhibited by 44% in ISOs, by 17% in RSOs. Penicillin had little effect on incorporation as seen previously for ß-lactams. Prasinomycin also had the same interesting effect reported previously: that of inhibiting to a small degree in ISOs but stimulating incorporation in RSOs.

Tunicamycin is thought to be a substrate-product transition state analog (Heifetz et al., 1979) and as such should compete with the substrate for the active site of the enzyme. In the presence of UDP-GlcNAc, tunicamycin inhibits nearly completely in both RSOs and ISOs. Without UDP-GlcNAc, inhibition is somewhat greater in ISOs than RSOs. This difference in the effect of tunicamycin in reactions lacking UDP-GlcNAc could be a function of the topological arrangement of the enzyme in the membrane.

The effect of bacitracin suggests that low incorporation of UDP-MurNAc-[^{3}H]pentapeptide in the absence of UDP-GlcNAc is not due to the saturation of lipid-carrier molecules. Incorporation in the presence of a high concentration of bacitracin and UDP-GlcNAc effectively titrates the concentration of lipid carrier: 36 pmoles in

Table 8

Effect of inhibitors on incorporation of UDP-MurNAc-[^{3}H]pentapeptide in the presence and absence of UDP-GlcNAc

% of Control Incorporation

	ISOs		RSOs	
	+ UDP-GlcNAc	- UDP-GlcNAc	+ UDP-GlcNAc	- UDP-GlcNAc
tunicamycin 100 μg/ml	15 (176)*	12 (112)*	8 (450)*	32 (66)*
bacitracin 1000 μg/ml	56 (161)*	100 (40)*	83 (284)*	100 (72)*
penicillin G 1000 μg/ml	100 (391)*	NT	94 (955)*	NT
prasinomycin 100 μg/ml	79 (436)*	NT	137 (1312)*	NT

*control incorporation (pmole/mg protein)
Two different batches of UDP-MurNAc-pentapeptide were used: Tunicamycin and bacitracin were tested with a batch having a specific activity of 186 dpm/pmole; penicillin and prasinomycin with a batch having a specific activity of 15dpm/pmole.

RSOs, 9 pmoles in ISOs. In the absence of UDP-GlcNAc, assuming all the label from UDP-MurNAc-[^{3}H]pentapeptide ends up in the form of lipid-linked saccharide, less than half of the available lipid carrier molecules are utilized.

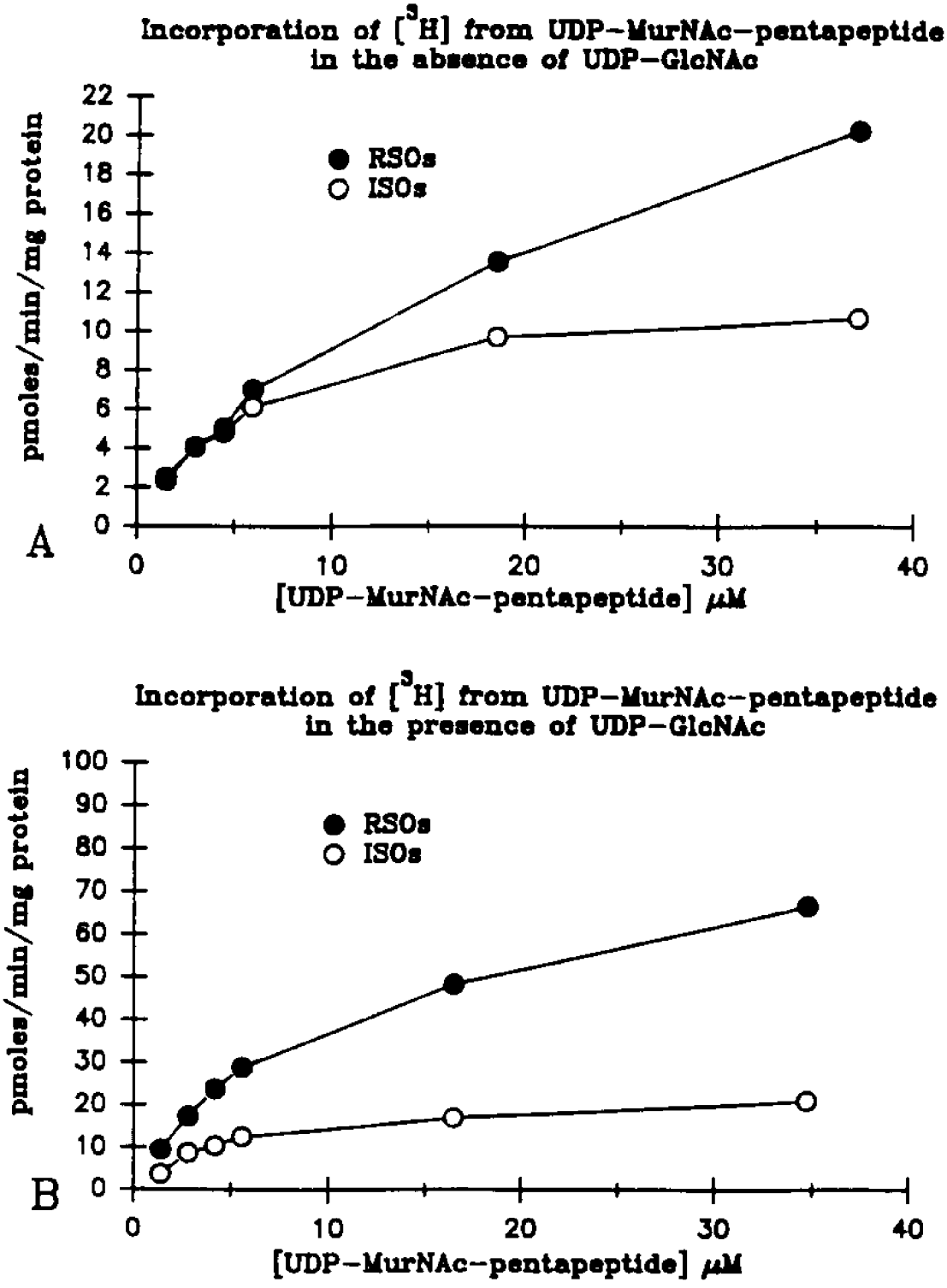
Prasinomysin and penicillin were tested only in the presence of both UDP-MurNAc-[^{3}H]pentapeptide and UDP-GlcNAc, since their point of inhibition is the joining together of the disaccharide units.

Kinetics of incorporation

UDP-MurNAc-[^{3}H]pentapeptide

Apparent kinetic constants of phospho-N-acetylmuramyl pentapeptide transferase for UDP-MurNAc-[^{3}H]pentapeptide in the presence and absence of UDP-GlcNAc, were calculated for both RSO and ISO vesicles. Incorporation followed Michaelis-Menten kinetics surprisingly well when incubation was carried out for 15 min with concentrations of 1.0 to 50 μM UDP-MurNAc-[^{3}H]pentapeptide. Figure 14 shows the incorporation at varying concentrations of UDP-MurNAc-pentapeptide with or without UDP-GlcNAc. Figure 15 shows the Lineweaver-Burk plots. UDP-GlcNAc was present in a non-limiting concentration (75 μM), or completely absent. K_m and V_{max} values for ISOs and RSOs are listed in Table 9. K_m and V_{max} values were consistently lower for ISOs than for RSOs, both with and without UDP-GlcNAc. The V_{max} values in the absence of UDP-GlcNAc were lower for both RSOs and ISOs.

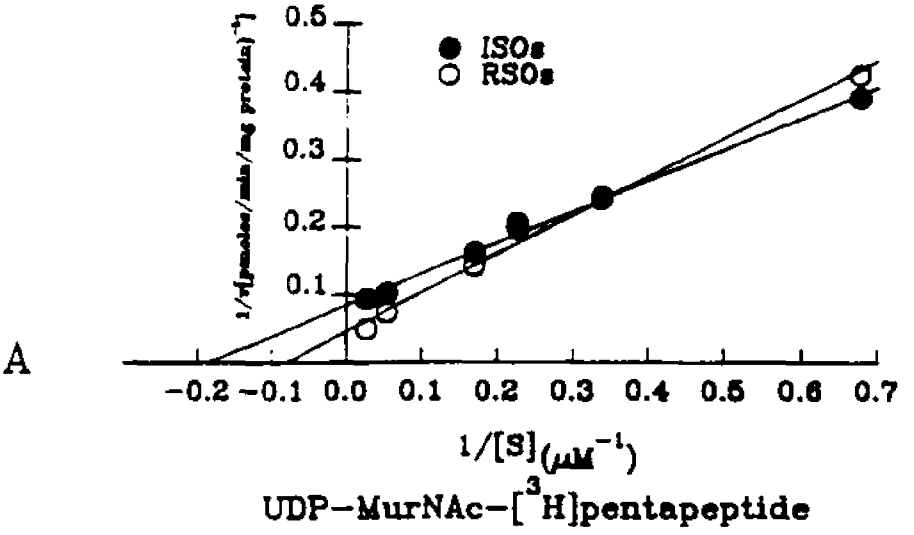
Figure 14



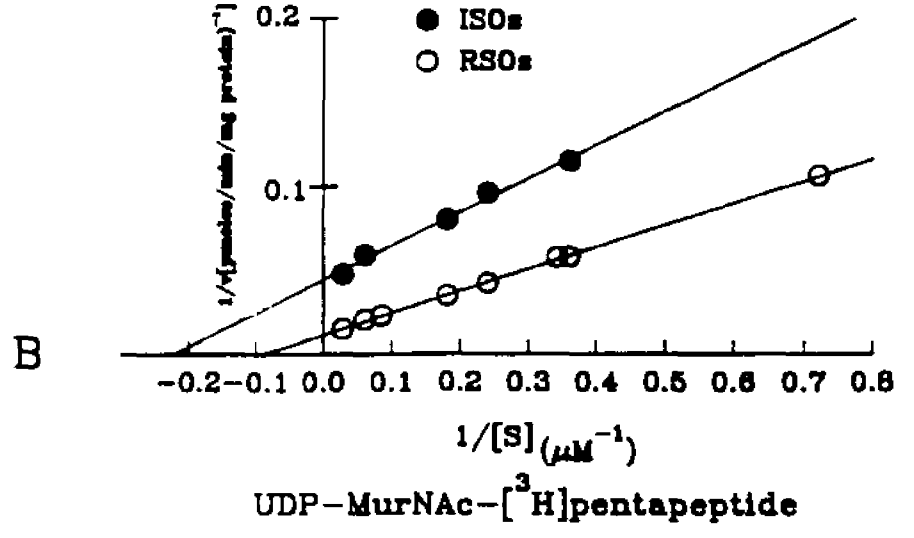
Vesicles were incubated with UDP-MurNAc-[^{3}H]pentapeptide alone (A) or with 75 μM UDP-GlcNAc (B). Reactions were terminated by addition of acetic acid and filtration.

Figure 15

Incorporation of [^{3}H] from UDP-MurNAc-pentapeptide in the absence of UDP-GlcNAc



Incorporation of [^{3}H] from UDP-MurNAc-pentapeptide in the presence of UDP-GlcNAc



Lineweaver-Burk plots for UDP-MurNAc-pentapeptide alone (A) and in the presence of 75 μM UDP-GlcNAc(B). The rate of incorporation was measured for 15 minute incubations in the presence of varying concentrations of UDP-MurNAc-[^{3}H]pentapeptide.

Table 9

Kinetic constants

Apparent K_m and V_{max} values

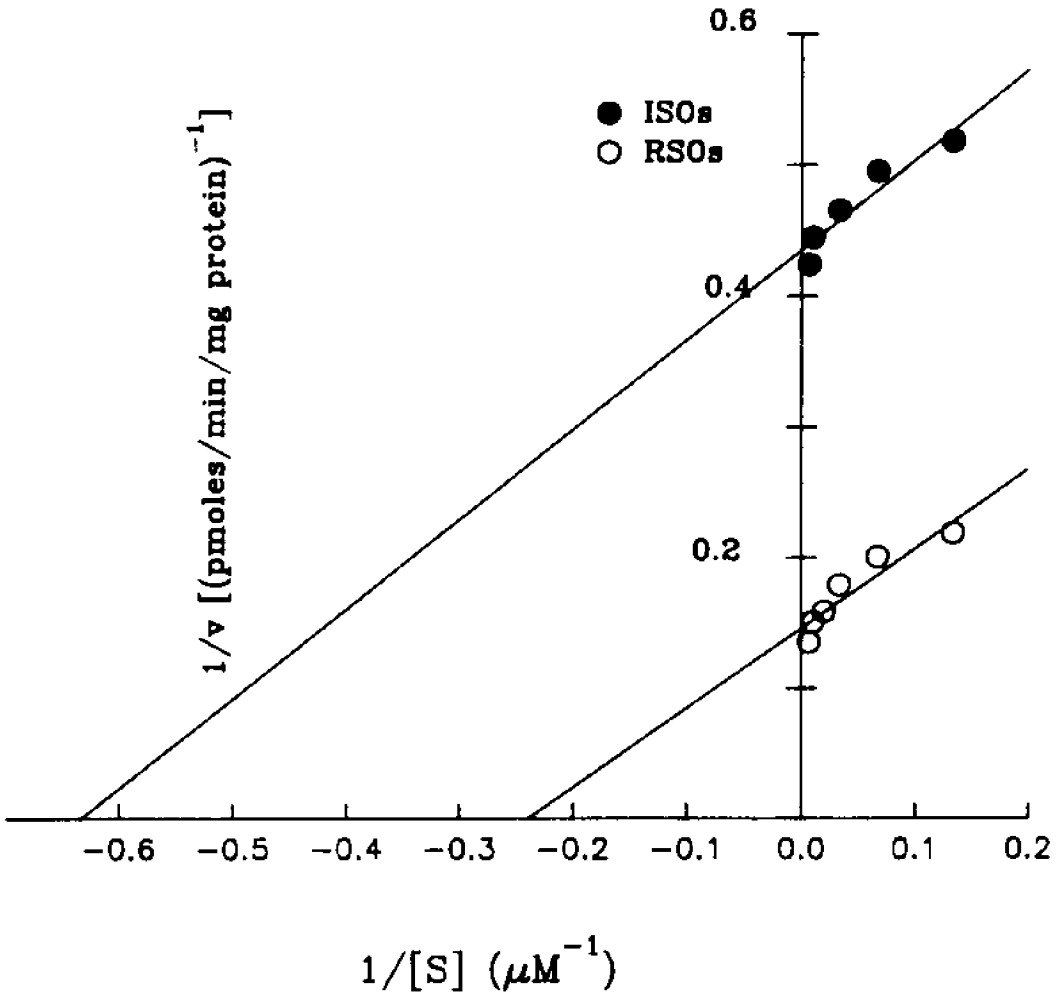
	ISOs		RSOs	
	K_m (μM)	V_{max} pmoles/min/ mg protein)	K_m (μM)	V_{max} (pmoles/min/ mg protein)
For:				
UDP-MurNAc-pentapeptide	5.4	12	12	22
UDP-MurNAc-pentapeptide in the presence of UDP-GlcNAc	4.5	22	11	85
UDP-MurNAc-pentapeptide (measured by incorporation of UDP-[^{14}C]GlcNAc)	1.6	23	4.2	69
UDP-GlcNAc	0.8	17	1.5	31

TLC analysis of the UDP-MurNAc-[^{3}H]pentapeptide showed it to be contaminated with a second component, most likely UDP-MurNAc-tripeptide. Densitometric analysis of autoradiograms of each batch has allowed quantitation of the contamination. The concentration of UDP-MurNAc-[^{3}H]pentapeptide was corrected for the contamination and the kinetic constants calculated using the corrected values. These are the values listed in Table 9. These values cannot be presumed to be exact; Hammes and Neuhaus (1974) reported that the translocase from S. aureus was able to use the UDP-MurNAc-tripeptide as a substrate. However, the K_m for the tripeptide was 80-fold higher than for the pentapeptide. Thus, the presence of the tripeptide may have had some effect on the translocase. Apparent kinetic constants for UDP-MurNAc-pentapeptide were also determined indirectly by the incorporation of label from UDP-[^{14}C]GlcNAc in the presence of varying concentrations of cold UDP-MurNAc-pentapeptide. Lineweaver-Burke plots are shown in Figure 16. K_m and V_{max} values for ISOs and RSOs are listed in Table 9. K_m values determined using the unlabeled UDP-MurNAc-pentapeptide were lower than those determined directly, although V_{max} values were comparable. This may indicate that the contaminant in the UDP-MurNAc-[^{3}H]pentapeptide preparation was acting as a competitive inhibitor of the translocase.

A possible explanation for the lower rate of

Figure 16

Incorporation of label from UDP-[^{14}C]GlcNAc in the presence of varying concentrations of UDP-MurNAc-pentapeptide



Lineweaver-Burk plots for UDP-MurNAc-pentapeptide. Rates were determined indirectly by measuring the incorporation of [^{14}C]N-acetylglucosamine for 15 minute incubations in the presence of varying concentrations of UDP-MurNAc-pentapeptide.

incorporation in the absence of UDP-GlcNAc is the dominance of the "exchange reaction" when the second substrate for the continued synthesis of peptidoglycan is missing. This reaction is defined as the exchange of free UMP with UMP released from UDP-N-acetylmuramyl pentapeptide by the transferase. To test this possibility, the reaction was measured with UDP-[^{14}C]GlcNAc in ISOs and RSOs in the presence of a sufficient quantity of alkaline phosphatase to remove any UMP generated during the reaction. No enhancement of incorporation was seen (Table 10).

UDP-[^{14}C]GlcNAc

The kinetics of incorporation of [^{14}C]GlcNAc in the presence of 10 μM UDP-MurNAc-pentapeptide were also measured. Lineweaver-Burk plots are shown in Figure 17. The apparent K_m and V_{max} values are shown in Table 9.

Specificity of phospho-N-acetylmuramyl-pentapeptide transferase

The specificity of the transferase was assessed by measuring incorporation of label from UDP-[^{14}C]GlcNAc by both RSOs and ISOs in the presence of analogs of UDP-MurNAc-pentapeptide. UDP-MurNAc-L-ala-D-glu, the dipeptide precursor of UDP-MurNAc-pentapeptide and UDP-MurNAc-pentapeptide from Staphylococcus aureus, which has lysine in place of diaminopimelic acid in the third position of the pentapeptide side chain were tested. The

Table 10

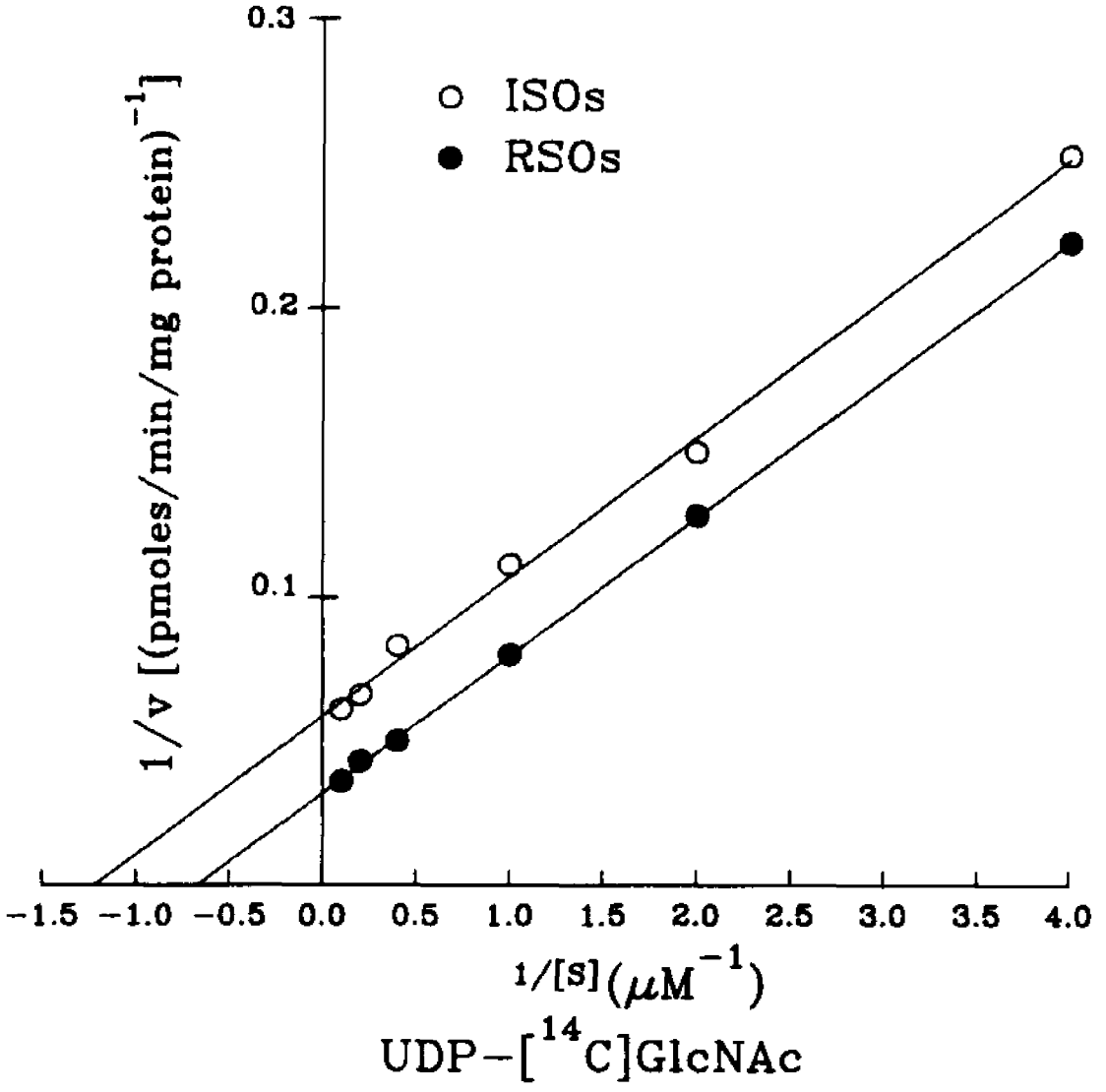
Incorporation of [^{14}C]GlcNAc in the presence of alkaline phosphatase

(pmoles incorporated)

	ISOs	RSOs
- Alkaline phosphatase	6.2	34
+ Alkaline phosphatase	6.5	33

Figure 17

Incorporation of label from UDP–[^{14}C]GlcNAc



Lineweaver–Burk plots for UDP–[^{14}C]GlcNAc. Rates were determined for 15 minute incubations. UDP–MurNAc–pentapeptide was present at 75 μM.

S. aureus pentapeptide supported peptidoglycan syntheis by both RSOs and ISOs at least as well as the natural substrate (Table 11). The UDP-MurNAc-dipeptide, however, supported only a very low level of incorporation. Subsequent experiments measured activity of ISOs and RSOs in the presence of varying concentrations of the S. aureus pentapeptide. As seen in Figure 18, the dependence of velocity on substrate concentration was very similar to that of the natural substrate but at 10-fold higher concentrations. Incorporation appeared to follow Michaelis-Menten kinetics; Lineweaver-Burk plots are shown in Figure 19. Apparent kinetic constants were determined for the lysine-containing substrate, and as expected the K_m values of both ISOs and RSOs were higher than for the natural substrate. For ISOs, the K_m was 6 μM, the V_{max} was 3.5 pmoles/min/mg of protein. For RSOs the K_m was 17 μM, the V_{max} was 39 pmoles/min/mg of protein. The K_m values for ISOs and RSOs for the (unlabelled) natural substrate were 1.6 μM and 4.2 μM, respectively. The V_{max} for ISOs was 23 pmoles/min/mg of protein; for RSOs, 69 pmoles/min/mg of protein.

Effects of Proteases on Incorporation in RSOs and ISOs

Vesicles of both orientations catalyze the incorporation of label from UDP-[^{14}C]GlcNAc and UDP-MurNAc-[^{3}H]pentapeptide, suggesting that the enzymes being

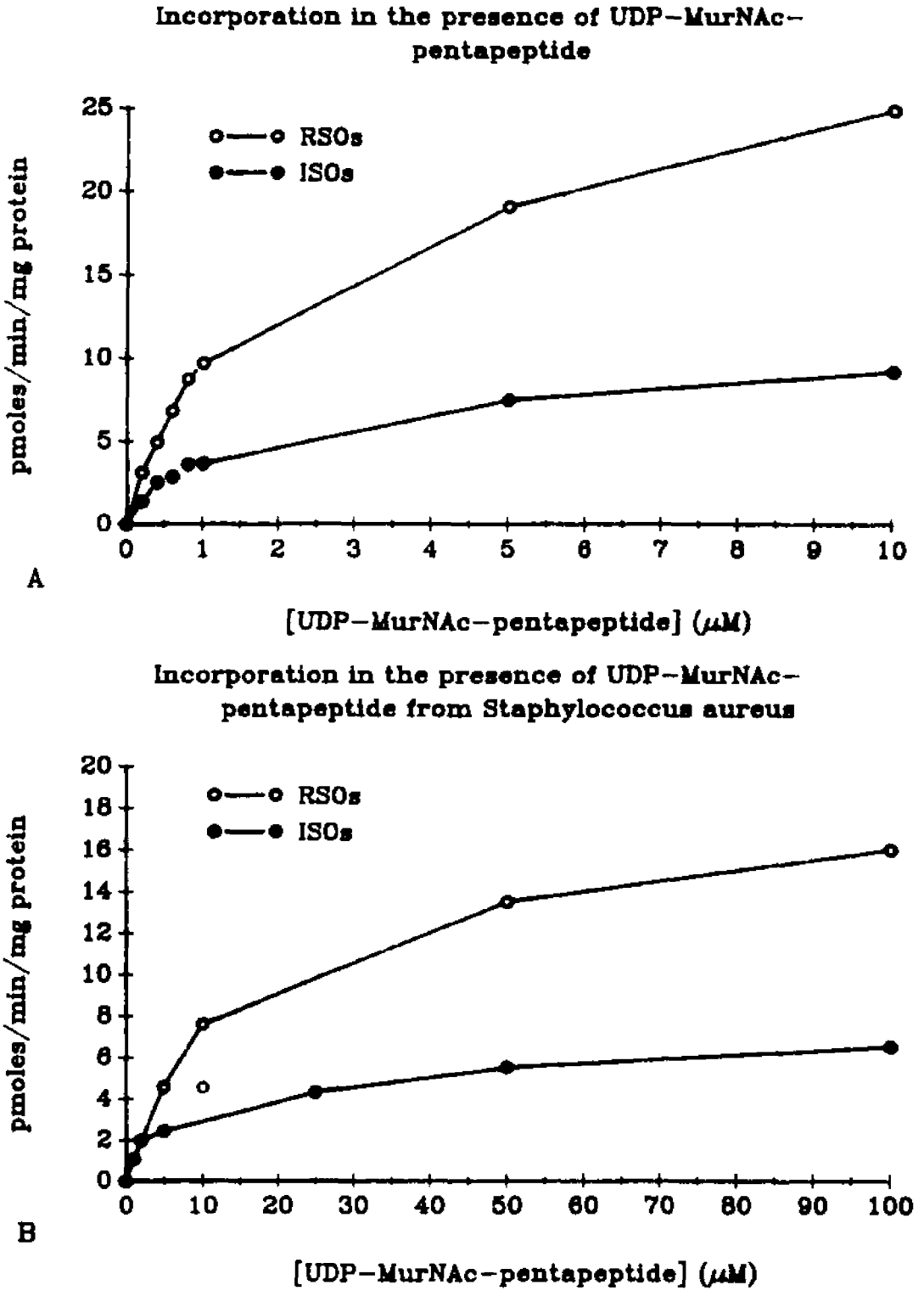
Table 11

Effect of the species of UDP-N-acetyl-peptide on [^{14}C]GlcNAc incorporation in ISOs and RSOs

[^{14}C]GlcNAc Incorporated

	ISOs		RSOs	
	pmoles/ mg protein	% of control	pmoles/ mg protein	% of control
Control (1.5 mM UDP-MurNAc-pentapeptide)	164	100	429	100
+2 mM UDP-MurNAc-dipeptide	20	12	94	22
+2.5 mM UDP-MurNAc-pentapeptide from S. aureus	224	137	571	133

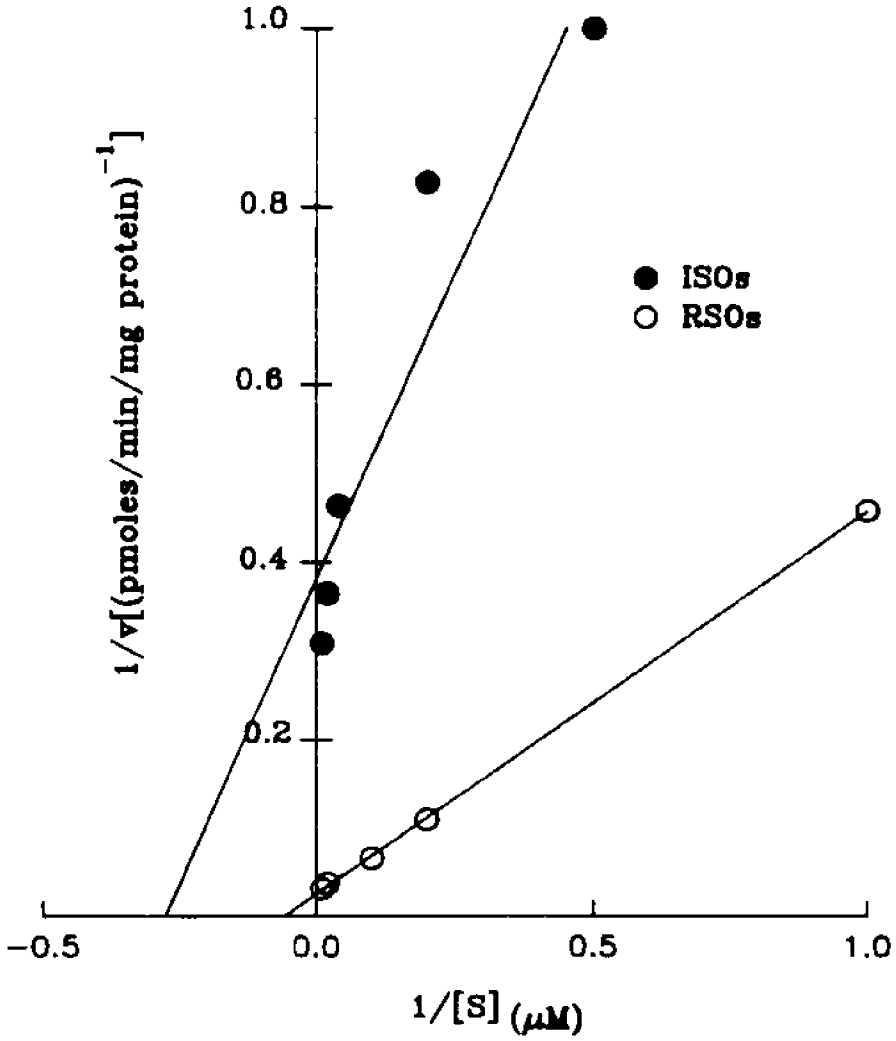
Figure 18



Vesicles were incubated for 15 min with the indicated concentration of DAP-containing UDP-MurNAc-pentapeptide from *E. coli* (A), or the lysine-containing UDP-MurNAc-pentapeptide from *S. aureus* (B).

Figure 19

Incorporation of label from UDP-[^{14}C]GlcNAc in presence of varying concentrations of *S. aureus* UDP-MurNAc-pentapeptide



S. aureus UDP-MurNAc-pentapeptide

Lineweaver-Burk plots for the lysine-containing UDP-MurNAc-pentapeptide prepared in *S. aureus*. Rates were determined indirectly by measuring incorporation of [^{14}C]GlcNAc for 15 minute incubations. UDP-GlcNAc was present at 10 μM.

measured, phospho-N-acetylmuramyl pentapeptide transferase and UDP-N-acetylglucosamine transferase, are accessible to the substrates from either side of the membrane. Possible explanations for this effect include random orientation of the enzymes in the membrane or rapid reorientation of the enzyme through the plane of the membrane. In each case, the active site of each individual molecule of enzyme would be available at any moment to only one side of the membrane, but the activity of the enzyme would be measurable on both sides. If so, protease treatment of RSOs and ISOs should result in essentially equal digestion of the enzymes and thus similar effects on measured activity in vesicles of either orientation. An asymmetric arrangement of the enzymes should be reflected in a differential effect of protease digestion on ISOs and RSOs. To test this hypothesis, ISOs and RSOs were incubated with trypsin, chymotrypsin and papain and then assayed. Controls were vesicles incubated without protease and non-incubated vesicles.

Vesicles were incubated with each protease for 15 min at 25°C. The enzyme was then washed away and the vesicles assayed for the ability to incorporate label from UDP-[^{14}C] GlcNAc into vesicle-associated material. As seen in Table 12, protease digestion affected the activity of ISOs and RSOs very differently. Both trypsin and chymotrypsin greatly decreased incorporation by ISOs, but papain had

Table 12

Effect of proteases on incorporation of [^{14}C]GlcNAc

% of Control Incorporation

	ISOs	RSOs
papain (508 μg/ml)	86 (27)*	31 (36)*
trypsin (82 μg/ml)	6 (27)*	40 (37)*
chymotrypsin (82 μg/ml)	21 (27)*	73 (52)*

* control incorporation (pmoles). Initial protein concentration for ISO reaction mixtures was 2 mg/ml; for RSOs was 1 mg/ml (papain and trypsin) or 1.6 mg/ml (chymotrypsin). Specific activity of UDP-[^{14}C]GlcNAc was 604 dpm/pmole.

very little effect. The activity of RSOs was greatly decreased by papain and typsin but not chymotrypsin. This differential effect of proteases on ISOs and RSOs indicated that the non-random orientation of the enzymes results in a definite "sidedness" to the membrane.

Characterization of Product

The observed differences between the activity of ISO and RSO vesicles have been the effects of proteases, the difference in specific activity (pmoles incorporated/mg of protein) and the effect of the transglycosylase inhibitor, prasinomycin. This latter observation, a stimulation of incorporation by RSOs and a slight inhibition in ISOs, was intriguing, but could not be explained by data obtainable from the incorporation assay. It seemed likely that more than one product resulted from the incorporation of UDP-[1^{4C}]GlcNAc and an examination of these products would be helpful.

Thin-Layer Chromatography of the Products of the Incorporation Assay

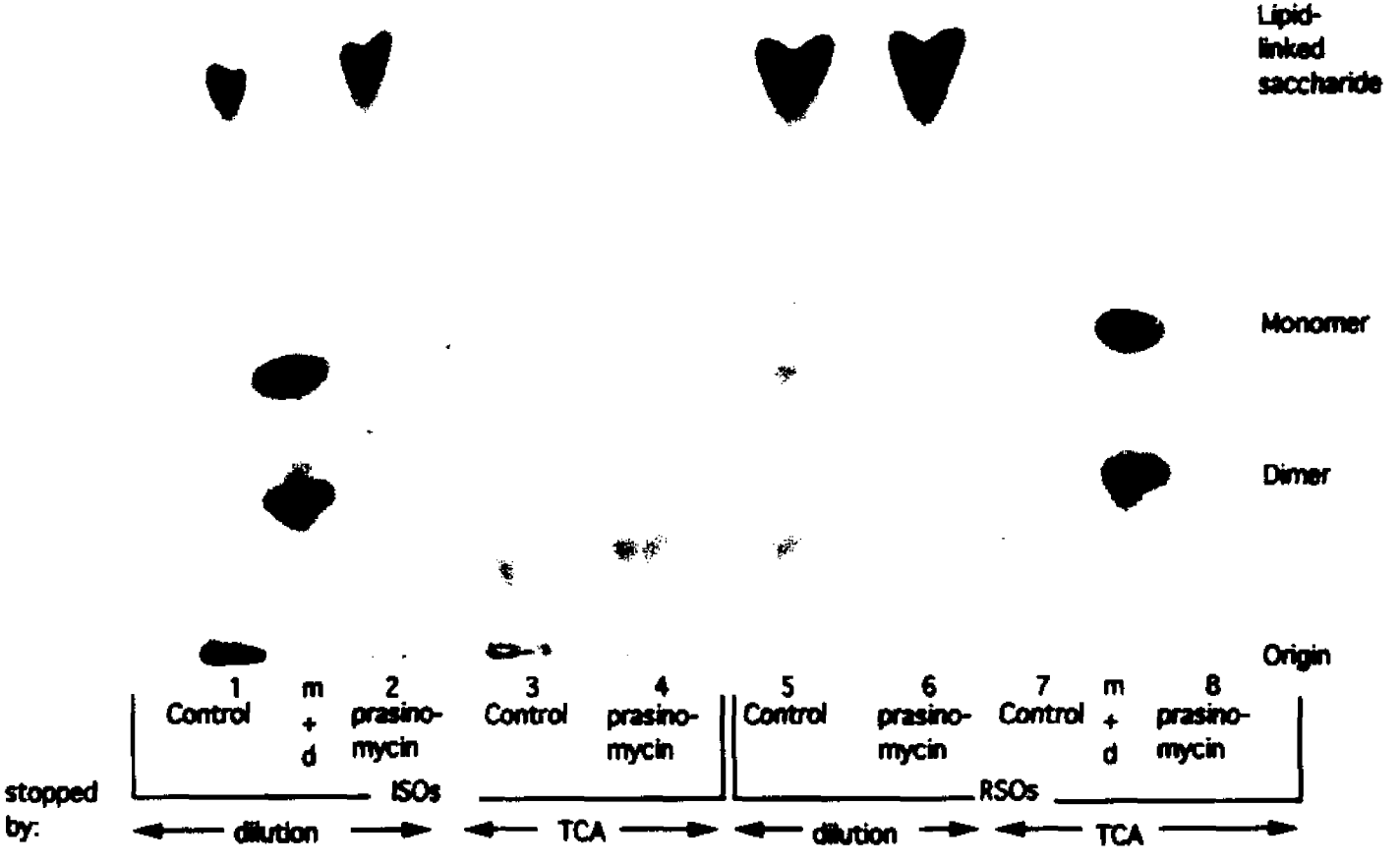
The labelled product of RSOs and ISOs incubated in the presence and absence of prasinomycin was examined by a silica gel TLC system which has been used to separate the lysozyme digestion products of peptidoglycan. Intact peptidoglycan does not migrate in this solvent system, but

the disaccharide and bis-disaccharide lysozyme fragments of peptidoglycan (monomers and dimers) have characteristic mobilities. Labelled products were located on the developed plate by autoradiography. An autoradiogram is shown in Figure 20. Labelled areas were located on the plate using the autoradiogram, cut out and counted in Filtron X scintillation fluid. Figures 21 and 22 show the quantitated results in cpm for the reaction mixtures terminated by dilution and TCA precipitation, respectively.

The major observable difference between ISOs (Figure 20, Lanes 1 and 3-5) and RSOs (Lanes 6-8 and 10) was the considerable amount of material seen at the origin in the ISO samples, but which was completely missing in the RSO samples. Comparing Lane 1 (control) to Lane 2 (prasinomycin), it was this polymerized product which appears to be the main target of inhibition by the drug in ISOs. Comparing Lane 5 (control) to Lane 6 (prasinomycin), the main effect of prasinomycin in RSOs was to increase the amount of the major product. This product has been identified as the lipid-linked disaccharide (vide infra). Thus, the effect of prasinomycin in ISOs was to inhibit the formation of the polymerized product and increase the amount of its immediate precursor, the lipid-linked disaccharide (Figure 21, Fraction 6). This result is consistent with the fact that prasinomycin inhibits the first polymerization step of peptidoglycan synthesis,

Figure 20

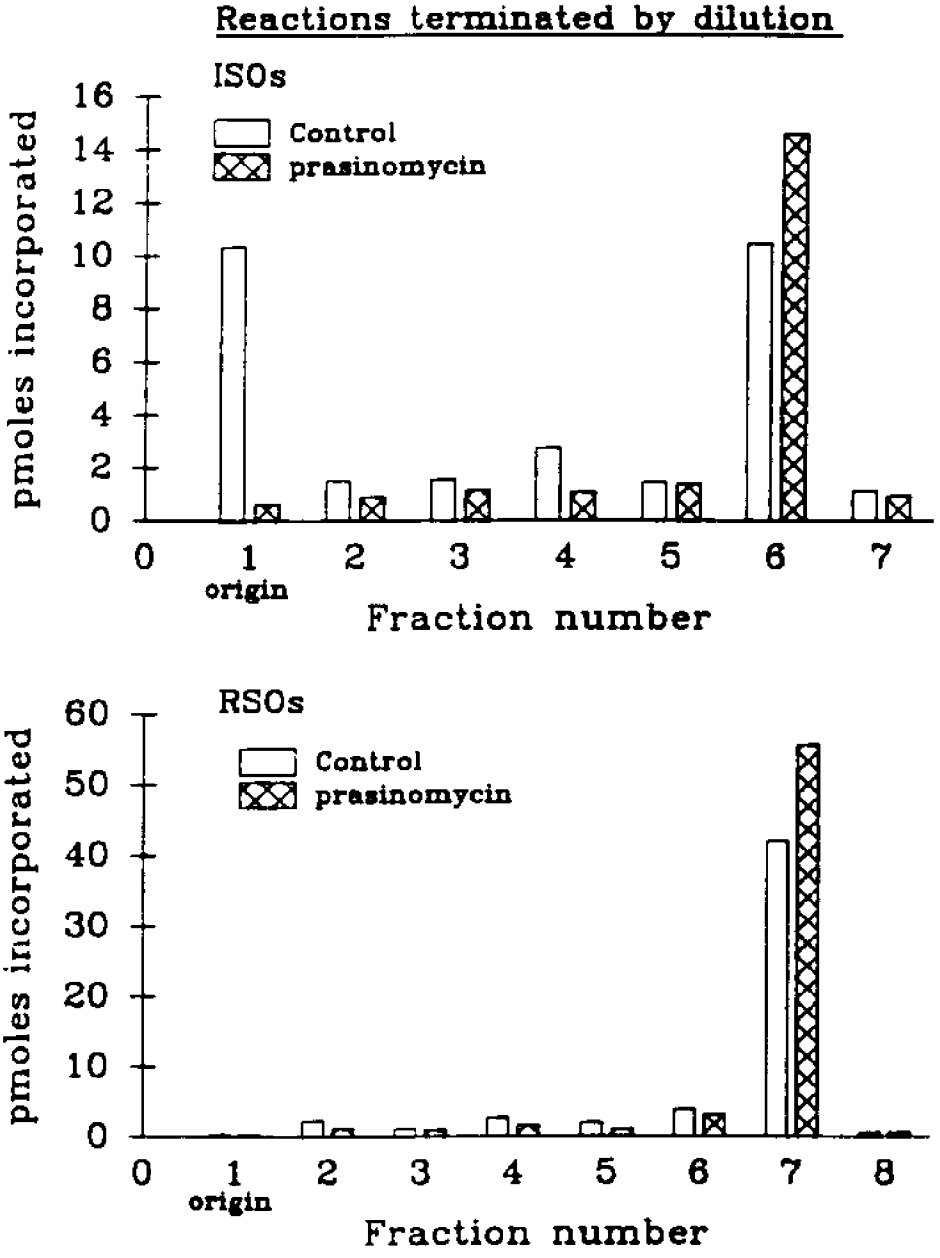
TLC analysis of the product of incorporation of label from UDP-[^{14}C]GlcNAc by ISO and RSO vesicles



Vesicles were incubated with substrates and with or without prasinomycin for 30 min. Reactions were terminated by the addition of an excess of cold UDP-GlcNAc (dilution) or precipitated with TCA. Samples were centrifuged, the pellets suspended in isobutyric acid : 1 N ammonia (5:3) and and spotted onto silica gel TLC plates. Plates were developed and autoradiographed as in Materials and Methods. (m+d, [^{14}C]-labelled monomer and dimer fragments.)

Figure 21

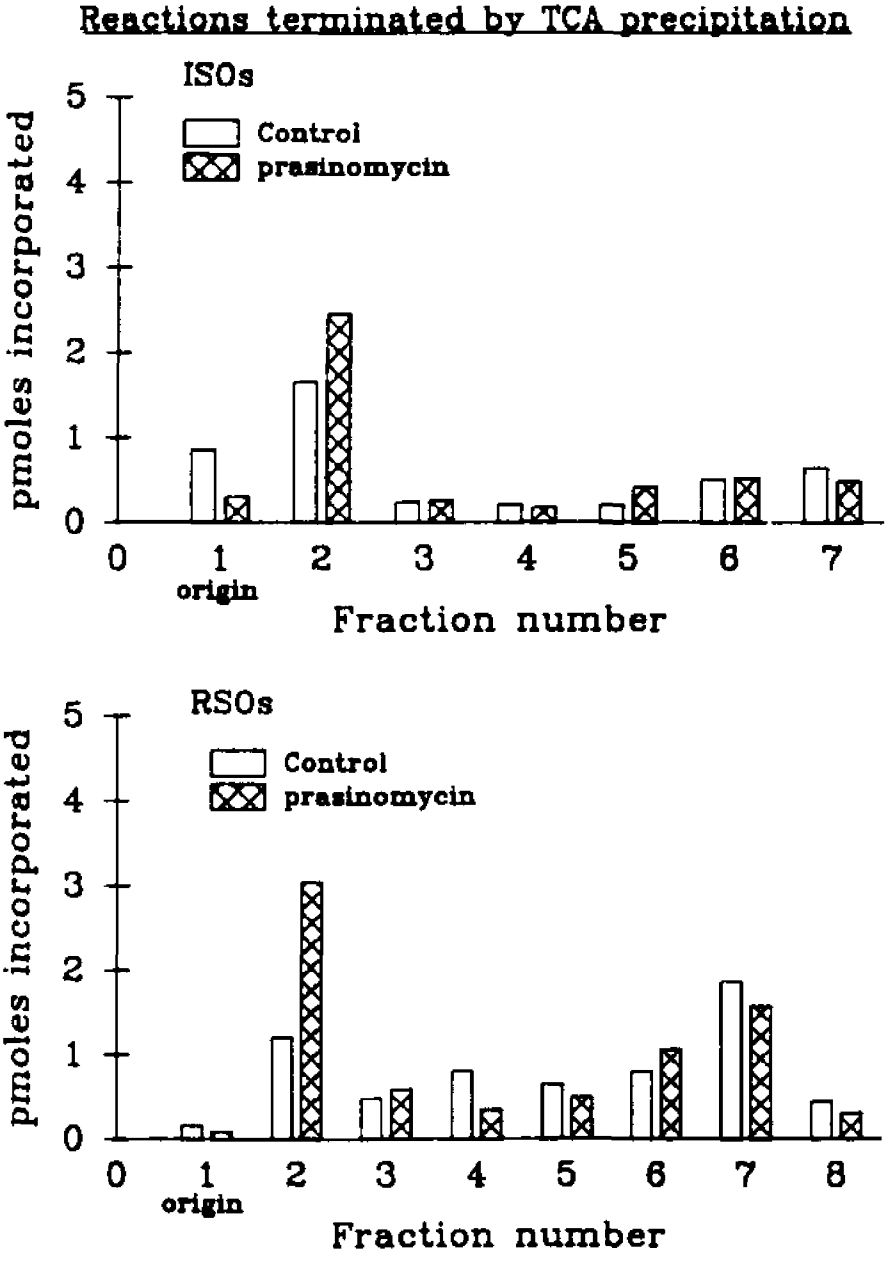
Quantitation of TLC analysis of the products of incorporation of [^{14}C]GlcNAc by ISOs and RSOs



Areas corresponding to bands on the autoradiogram shown in Figure 20 were cut from the TLC plate and counted in scintillation fluid. Reactions were terminated by addition of an excess of cold UDP-GlcNAc. Samples correspond to lanes 1 and 2 (ISOs) and lanes 5 and 6 (RSOs) in Figure 20.

Figure 22

Quantitation of TLC analysis of the products of incorporation of [^{14}C]GlcNAc by ISOs and RSOs



Areas corresponding to bands on the autoradiogram shown in Figure 20 were cut from the TLC plate and counted in scintillation fluid. Reactions were terminated by addition of 1 ml of 20% TCA. Samples correspond to lanes 3 and 4 (ISOs) and lanes 7 and 8 (RSOs) in Figure 20.

transglycosylation (Van Heijenoort and Van Heijenoort, 1980). In RSOs, this polymer was not found. The transglycosylase in RSOs must be active, however, since the addition of prasinomycin did have an effect on incorporation, the increase of the amount of lipid-linked disaccharide (Figure 21, Fraction 7). This result may be explained by assuming that the transglycosylase did remove the disaccharide from the lipid carrier molecule, but either transferred it to water because no acceptor peptidoglycan was available or transferred it to an acceptor molecule which was washed away from the vesicle during the course of the incubation or centrifugation. In either case, the product of the transglycosylase could not be measured in the assay, but the effect of prasinomycin - the inhibition of this loss of label to the solvent - would be seen as the accumulation of the lipid-linked disaccharide.

Lanes 3 and 4 are identical to Lanes 1 and 2 and Lanes 7 and 8 are identical to Lanes and 6 except that the reaction was terminated by the addition of 10% TCA. The most obvious effect of TCA was the diminution of label in the product. Once again the ISO samples contained material at the origin, but the RSO samples did not. The tentatively identified lipid-linked disaccharide did not appear in the ISO lanes, but as shown in Figure 22, some counts were found at that location when the plate was cut

and counted (Fraction 6). The majority of the label found in the ISO product in both the control and treated samples was in Fraction 2, a slow-moving fraction just above the origin. The increase seen in this fraction in the presence of prasinomycin was similar, in proportion, to the increase in the lipid-linked disaccharide (Fraction 6) seen in the dilution-terminated samples (Figure 21). In fact, this fraction may be the product of acid hydrolysis of the lipid-linked disaccharide formed in the presence of TCA. A similar pattern was seen in the RSO samples; Fraction 2 increased in the presence of prasinomycin, much as the lipid-linked disaccharide increased in the presence of prasinomycin in the dilution terminated samples.

Preparation of the lipid-linked disaccharide

As described above, a major product of incorporation of label from UDP-[^{14}C]GlcNAc was tentatively identified as lipid-linked disaccharide. This identification was based on the observation that the R_f of a major band, seen after TLC and autoradiography of reacted vesicles, approximately coincided with values reported in the literature for lipid-linked precursors of peptidoglycan. We sought to confirm the identity of this product by preparing the authentic lipid-linked precursors, following published procedures, to run as standards together with reacted vesicles. This method was originally used by Strominger, among others, to

isolate and purify lipid-linked peptidoglycan precursors from whole cells.

The ^{14}C-labelled lipid-linked disaccharide (labelled in the glucose moiety of the N-acetylglucosamine) was prepared by incubating ether-permeabilized E. coli with cold UDP-MurNAc-pentapeptide, UDP-[^{14}C]GlcNAc and a transglycosylase inhibitor to prevent polymer formation. The radioactive product was extracted with butanol-pyridinium acetate. When examined by TLC, the butanol-extracted product gave an R_f = 0.61, which is consistent with that of Anderson et al. (1967) who identified the lipid-linked intermediate as having an R_f = 0.65. The lipid component was detected with iodine vapor and corresponded exactly with the radioactive band. The [^{14}C]-labelled, lipid-linked standard was stored in chloroform: methanol at -25° C and spotted together with samples in subsequent experiments. The standard band coincided with the presumed lipid-linked product of vesicles. ^{3}H-labelled, lipid-linked disaccharide synthesized in the presence of cold UDP-GlcNAc and UDP-MurNAc-[^{3}H]penta-peptide, was isolated using the above procedure. Prolonged exposure times were required for visualization by autoradiography and a single faint band (R_f = 0.66) was found. Attempts to similarly isolate the lipid-linked monosaccharide from ether-treated E. coli incubated with UDP-MurNAc-[^{3}H]pentapeptide alone were unsuccessful. A

labelled product, presumably the lipid-linked monosaccharide, was detected in the butanol-extracts of these reaction mixtures by scintillation counting. However, it was impossible to detect any labelled product by TLC and autoradiography of the extracts, even with extremely long exposure times (>4 months). These results suggest that a very small quantity of the lipid-linked monosaccharide was synthesized in the absence of UDP-GlcNAc, an insufficient quantity to be detected by TLC and autoradiography.

Butanol extraction of incorporated label

Additional evidence that a major product of incorporation of labelled substrates was lipid-linked was obtained by extraction of reacted vesicles with butanol-pyridinium acetate. An assay for the activity of phospho-N-acetylmuramyl-pentapeptide transferase based on this method was adapted from Tanaka et al. (1982). Vesicles of both orientations were incubated with the labelled substrates and the reaction was terminated by the addition of the butanol-pyridinium acetate. The final backwashed butanol layer was evaporated to dryness and counted in scintillation fluid. Subsequent TLC showed that unincorporated label was completely removed from the butanol by the backwash procedure.

A considerable portion of label that was incorporated

into vesicles from UDP-[^{14}C]GlcNAc was extracted into butanol (Table 13), and so was presumably lipid-linked. For TLC analysis, the product of the assay was evaporated to dryness and dissolved in chloroform:methanol (1:1). The aqueous extracts were also evaporated to dryness, dissolved in water and spotted together with the butanol extracts and duplicate, unextracted reaction mixtures. Labelled components were located by autoradiography. Representative autoradiograms are shown in Figure 23. The aqueous layer gave a labelled band with the same mobility as UDP-[^{14}C]-GlcNAc and in some preparations, bands which corresponded to monomer and dimer peptidoglycan fragments. The butanol extracts gave a labelled band having an R_f = 0.61 and, in some preparations, monomer and dimer bands.

This method also confirmed the differential effect of prasinomycin on ISOs and RSOs. Prasinomycin had been found to increase incorporation in RSOs by increasing the amount of lipid-linked labelled product seen by TLC while slightly inhibiting incorporation in ISOs. As seen in Table 13, butanol-extracted reaction mixtures of RSOs treated with moenomycin, another transglycosylase inhibitor (Van Heijenoort et al., 1978), contained a large increase in radioactivity while ISOs similarly treated had less radioactivity in the butanol-extract than control reaction mixtures. These results correlate exactly with results for identical reactions which were filtered as usual for the

Table 13

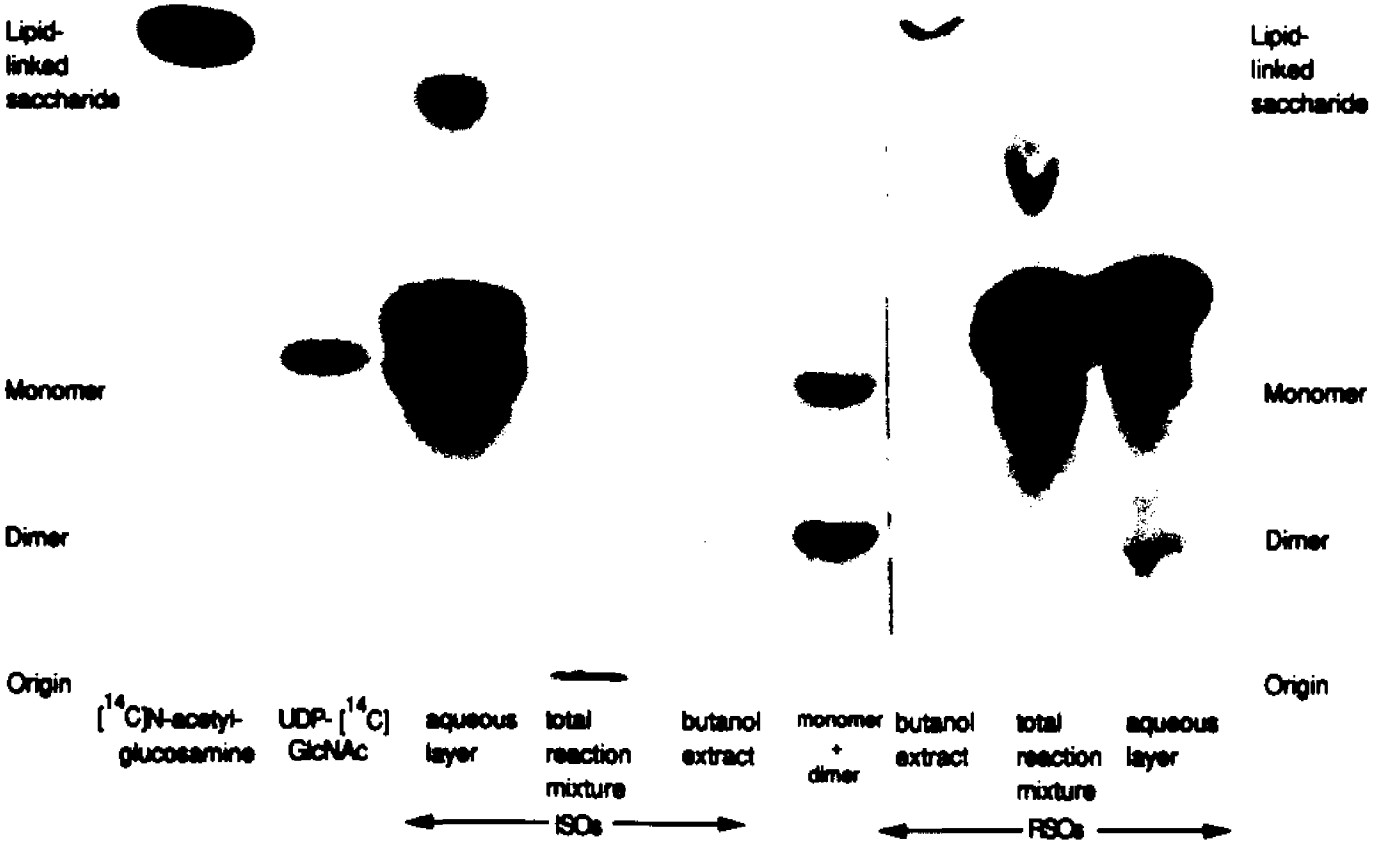
Effect of moenomycin on incorporation of [^{14}C]GlcNAc

	pmoles of [^{14}C]GlcNAc incorporated into:			
	Butanol-extractable material		Vesicle-associated material	
	ISOs	RSOs	ISOs	RSOs
Control	5.66	27.6	9.21	43.7
moenomycin (100 μg/ml)	5.07 (90)*	36.2 (131)*	7.58 (82)*	87.8 (201)*

* % of control

Figure 23

TLC of butanol extracts of reacted ISOs and RSOs incubated with UDP-[^{14}C]GlcNAc



Duplicate reaction mixtures were terminated by the addition of pyridinium acetate or 5 µl of acetic acid. The entire acetic acid terminated reaction mixtures were spotted onto silica gel TLC plates. The duplicate reaction mixtures were extracted three times with water-saturated butanol and back-washed with butanol-saturated water. Both butanol and aqueous layers were evaporated to dryness, suspended in isobutyric acid:1 N ammonia (5:3), and spotted with the above samples. Plates were developed and autoradiographed as in Materials and Methods.

incorporation assay.

Butanol-extraction of UDP-MurNAc-[^{3}H]pentapeptide labelled ETB

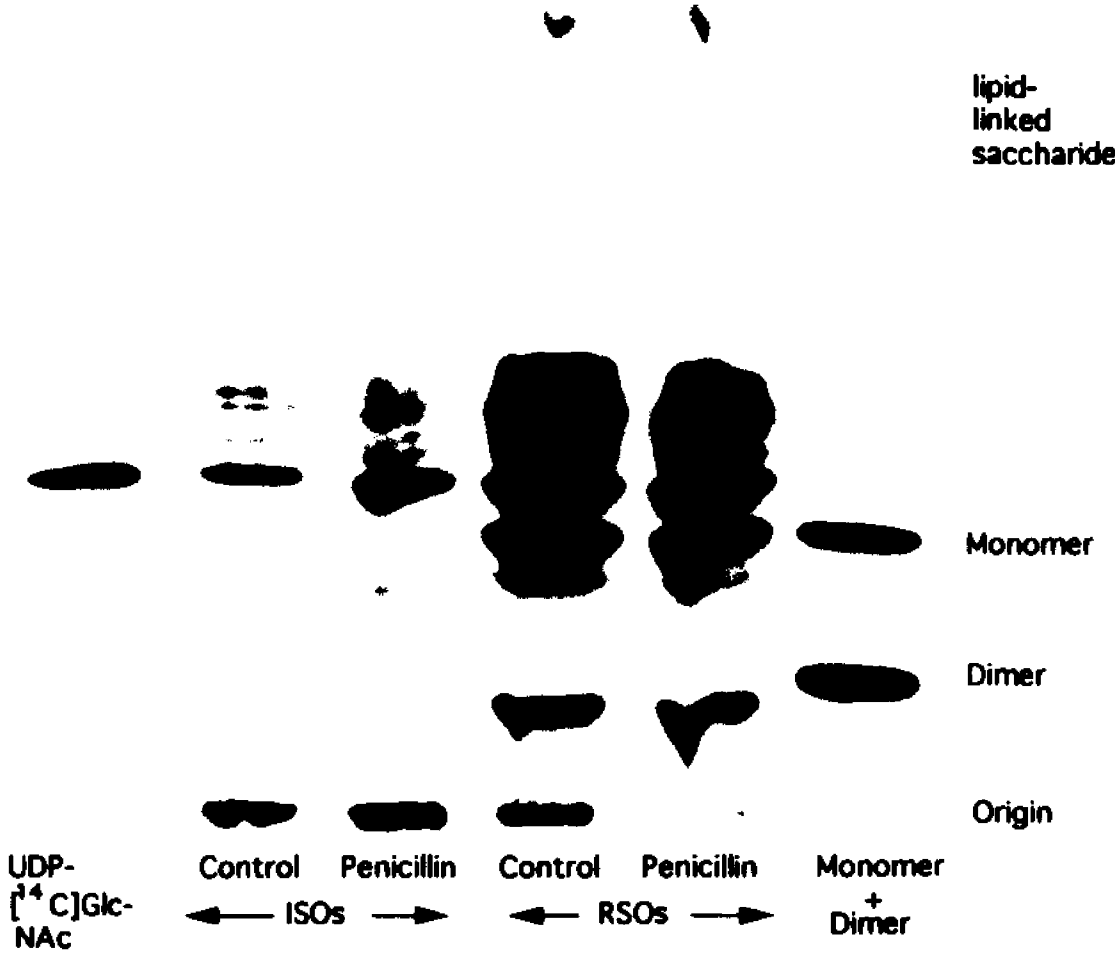
Butanol-pyridinium acetate extraction was also used to demonstrate that UDP-MurNAc-[^{3}H]pentapeptide was incorporated into butanol-extractable material by ether-treated E. coli B. This result indicates that the product was the same found upon butanol extraction of ETB incubated with UDP-MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc, namely the lipid-linked disaccharide. The synthesis of this product was greatly stimulated by the presence of UDP-GlcNAc (127 pmoles/mg of protein incorporated in the presence of UDP-GlcNAc, 63 pmoles/mg of protein in the absence of UDP-GlcNAc). In the absence of UDP-GlcNAc, the product was presumed to be the lipid-linked monosaccharide. Butanol extracts of the UDP-MurNAc-[^{3}H]pentapeptide-labelled material were not examined by TLC, however, because of the small amount of UDP-MurNAc-[^{3}H]pentapeptide available.

Product formation in the presence of penicillin

The product of the incorporation reaction in the presence of additional specific inhibitors was also examined by TLC and autoradiography (Figure 24) and the results quantitated by cutting areas from the plate and

Figure 24

TLC analysis of the product of ISOs and RSOs in the presence and absence of penicillin



Vesicles were incubated with UDP-[^{14}C]GlcNAc with and without 100 µg/ml of penicillin. Reactions were terminated by the addition of acetic acid and centrifuged. Pellets were suspended in isobutyric:ammonia (5:3) and spotted with [^{14}C]-labelled monomer and dimer fragments. Polymerized product remains at the origin.

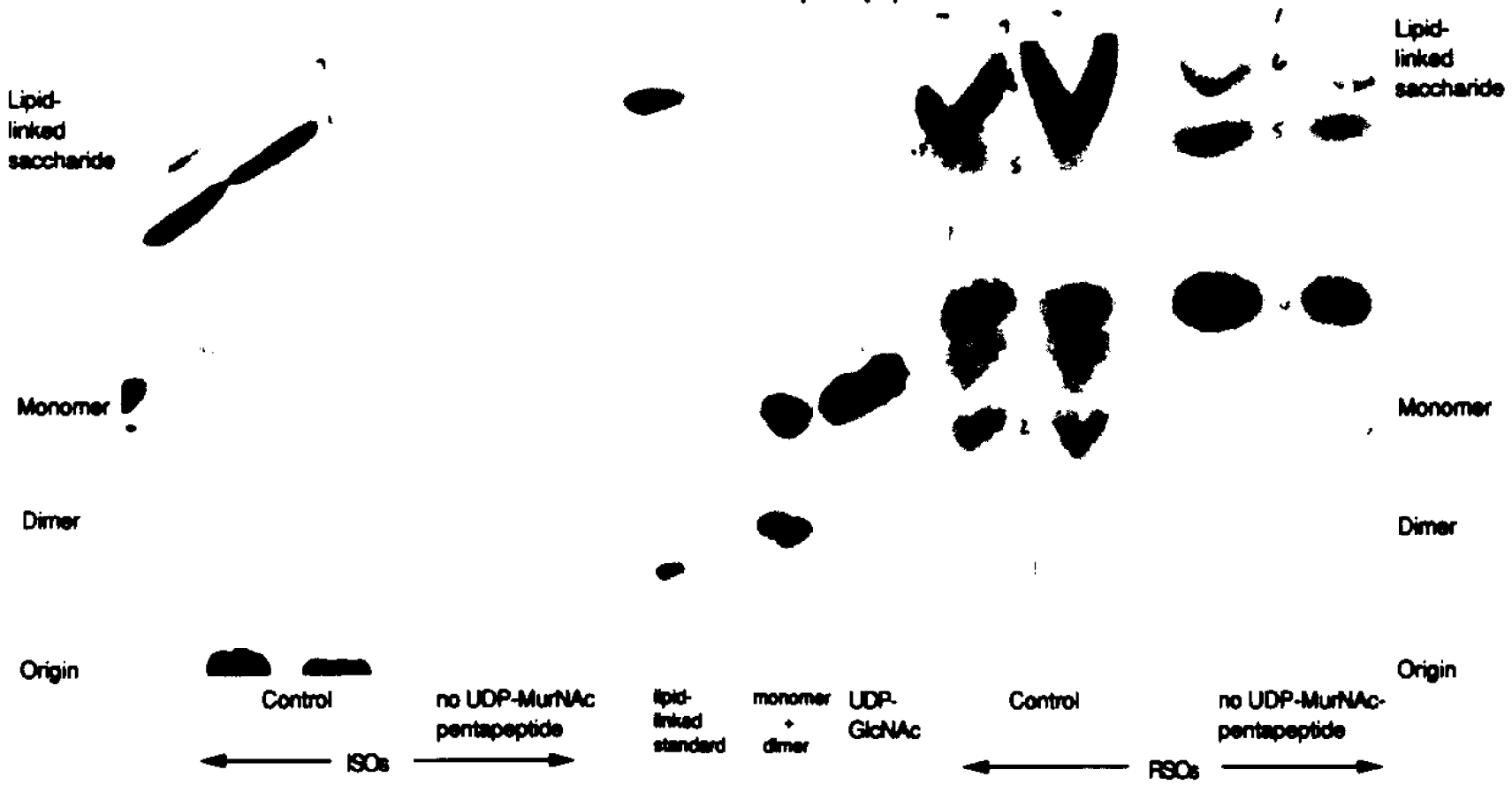
scintillation counting. Penicillin, as reported above, did not significantly inhibit incorporation from UDP-[^{14}C]-GlcNAc, in either RSOs or ISOs. One of the minor products of RSO vesicles, the immobile product, (presumably peptidoglycan) was reduced by 62% in the presence of penicillin. The products of ISOs were, in general, unaffected by penicillin, with the exception of the product remaining at the origin which increased by 30%.

Muramidase digestion of the product of incorporation in vesicles

To further investigate the polymerized product of RSOs and ISOs, the product of reacted vesicles incubated with UDP-[^{14}C]GlcNAc with and without cold UDP-MurNAc-pentapeptide was examined by TLC. In addition, duplicates of these samples were first incubated with the muramidase from S. globisporus. The results were quite puzzling. Several bands appeared on autoradiograms of reactions incubated without UDP-MurNAc-pentapeptide (Figure 25). These bands corresponded to those previously identified in reaction mixtures containing UDP-MurNAc-pentapeptide as "lipid-linked", i.e., having a $R_f \approx 0.6$, including a band corresponding to the authentic ^{14}C-labelled lipid-disaccharide. In ISOs, these bands were quite faint compared with the control incubated with UDP-MurNAc-pentapeptide, but in RSOs they were quite dark, two even

Figure 25

TLC of the product of ISOs and RSOs incubated with UDP-[^{14}C]GlcNAc with and without UDP-MurNAc-pentapeptide



Vesicles were incubated for 15 min at 30°C with UDP-[^{14}C]GlcNAc with and without UDP-MurNAc-pentapeptide. Reactions were terminated by the addition of acetic acid and samples were centrifuged at 15000 x G for 5 min and washed with water. Pellets were suspended in the TLC solvent and spotted onto plates together with authentic standards. Plates were developed and autoradiographed as in Materials and Methods.

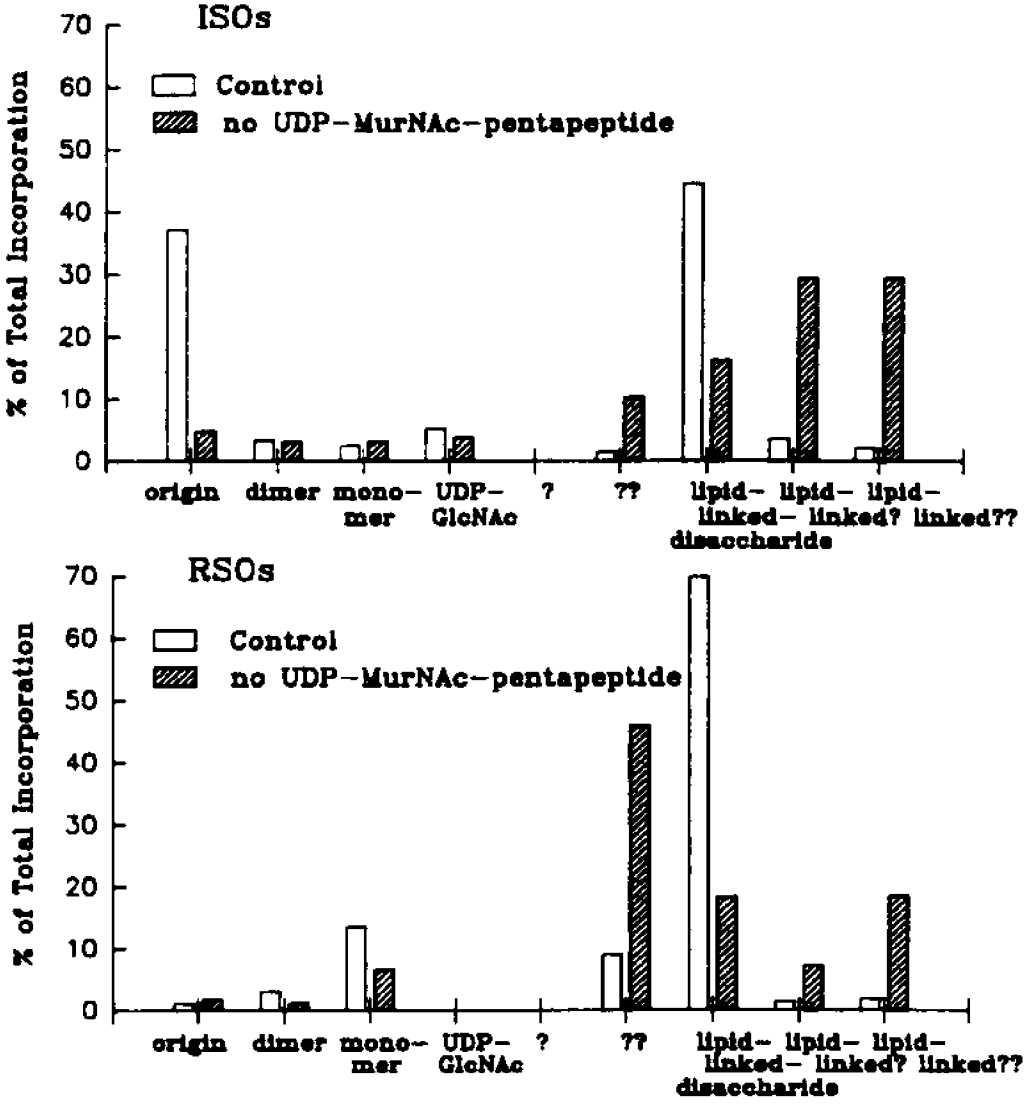
more so than the control. Bands were cut from TLC plates and counted. These results are shown in Figure 26.

Even more puzzling was the pattern of bands seen on the autoradiograms of muramidase-digested reactions (Figure 27). Quantitation of these results is shown in Figure 28. Results for ISOs were essentially as expected; the polymerized product was greatly reduced in the digested samples and new bands appeared which were not seen in the non-digested sample. In addition, the band corresponding to authentic dimer increased somewhat and the band corresponding to monomer increased significantly. Since the quantity of dimer formed by muramidase digestion is an indication of the degree of crosslinking of peptidoglycan, this result indicates that the polymerized product of ISOs has a very low degree of crosslinking. The muramidase appeared to have no effect on the product of ISO reactions incubated without UDP-MurNAc-pentapeptide (Figure 27).

In RSOs, muramidase treatment produced a large increase in immobile material in reactions with and without UDP-MurNAc-pentapeptide (compare Figure 25 to 27, Figure 26 to 28). Since this material is neither dependent on UDP-MurNAc-pentapeptide nor digested by muramidase into peptidoglycan subunits, it cannot be either peptidoglycan or a peptidoglycan precursor. The "lipid-linked" bands, including one corresponding to authentic lipid-linked disaccharide, were greatly diminished in digested reaction

Figure 26

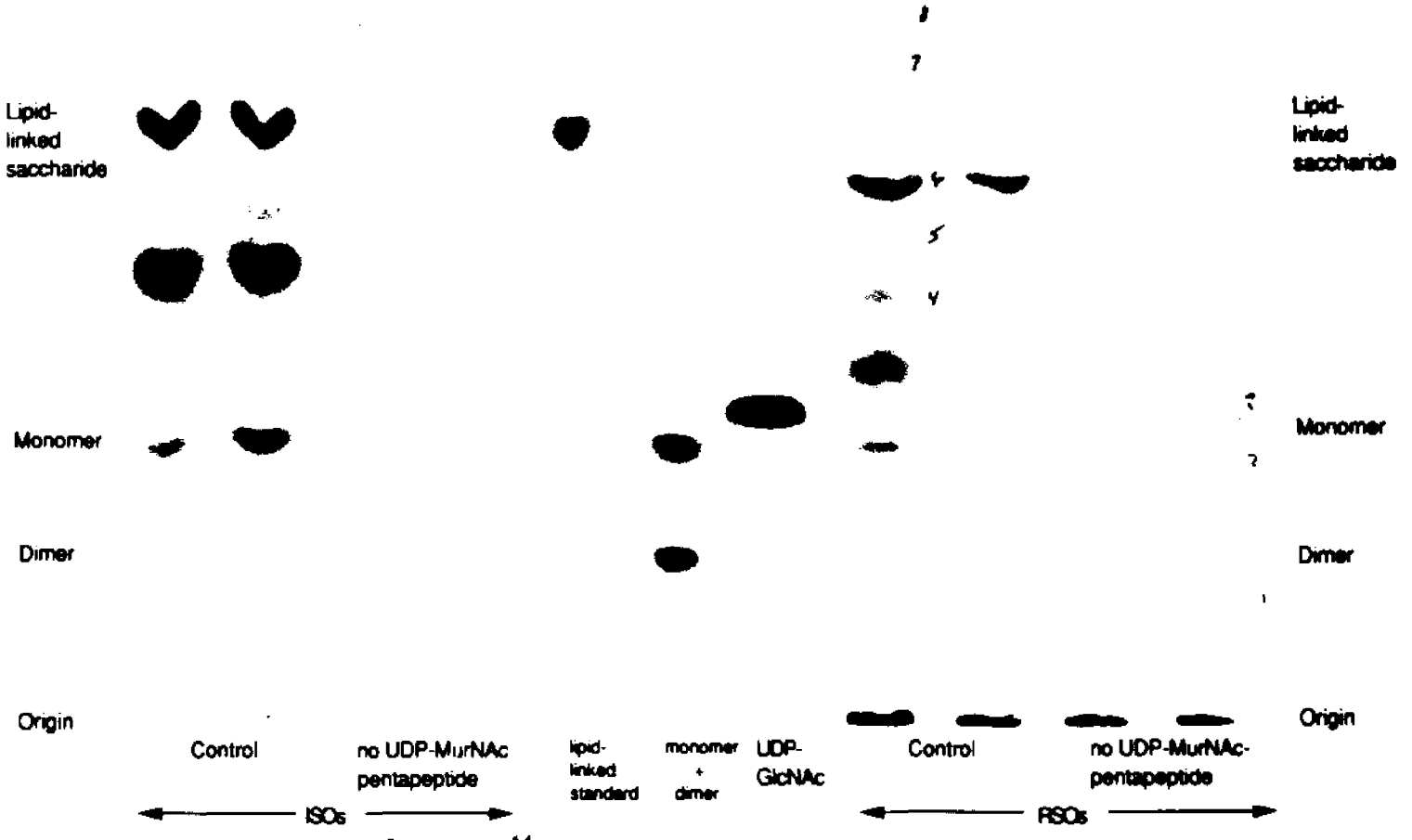
Quantitation of TLC analysis of the product of ISOs and RSOS incubated with and without UDP-MurNAc-pentapeptide



Areas corresponding to bands on the autoradiograms shown in Figure 25 were cut from the TLC plates and counted in scintillation fluid. Results are expressed as percent of total radioactivity incorporated (dpm per band/total dpm in sample x 100). Bands running ahead of the lipid-linked disaccharide appear to be lipid in nature, but were not identified. Other unidentified bands may be breakdown products of the lipid-linked disaccharide. (See Discussion)

Figure 27

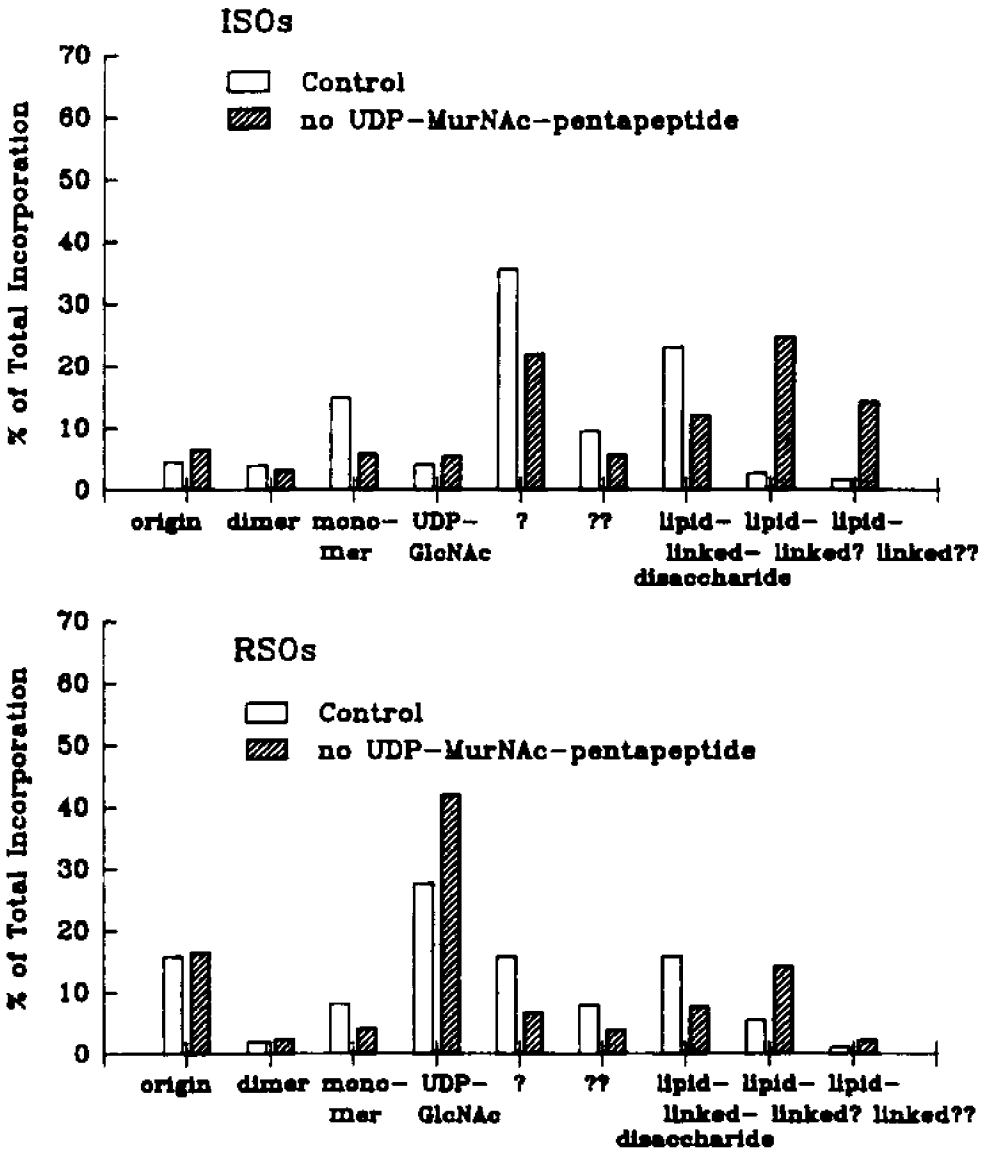
TLC of the product of ISOs and RSOs incubated with UDP-[^{14}C]GlcNAc with and without UDP-MurNAc-pentapeptide and digested with muramidase



Vesicles were incubated for 15 min at 30°C with UDP-[^{14}C]GlcNAc with and without UDP-MurNAc-pentapeptide. Reactions were terminated by the addition of acetic acid and samples were centrifuged at 15000 x g for 5 min and washed with water. Pellets were suspended in 20 µl of 25 mM sodium phosphate buffer pH 6.5 with 0.1 mM $MgCl_2$ containing 500 µg/ml *S. globisporus* muramidase. Samples were incubated at 37°C for 5 hours. Samples were spotted directly onto TLC plates together with authentic standards. Plates were developed and autoradiographed as in Materials and Methods.

Figure 28

Quantitation of TLC analysis of the product of ISOs and RSOs after muramidase digestion



Areas corresponding to bands on the autoradiograms shown in Figure 27 were cut from the TLC plates and counted in scintillation fluid. Results are expressed as percent of total radioactivity incorporated (dpm per band/total dpm in sample x 100). Bands running ahead of the lipid-linked disaccharide appear to be lipid in nature, but were not identified. Other unidentified bands may be breakdown products of the lipid-linked disaccharide. (See Discussion)

mixtures. This suggests that these bands were the source of the immobile material appearing after muramidase treatment. These results indicate that some incorporation by RSOs, particularly into lipid-linked material, was not associated with peptidoglycan synthesis.

Incorporation in the absence of UDP-MurNAc-pentapeptide was examined in the incorporation assay in the presence of tunicamycin. Tunicamycin inhibits many reactions in which undecaprenyl phosphate participates (Gale et al., 1981) , which includes several steps of lipolysaccharide synthesis (Rick, 1987), as well as the initial step of peptidoglycan synthesis catalyzed by the translocase. As seen in Table 14, tunicamycin inhibits this incorporation by 16% in ISOs and by 75% in RSOs. This data supports the assumption that some steps of lipopolysaccharide synthesis occurred in vesicles. It also is consistant with the observation that more of this "non-peptidoglycan" material is produced in RSOs than ISOs.

Product analysis of reacted vesicles incubated with UDP-MurNAc-[^{3}H]pentapeptide with prasinomycin and with or without cold UDP-GlcNAc was both simpler and more tedious. The relatively low specific activity of the substrate and the low energy of tritium led to extremely long exposure times (3 months). Autoradiograms of these experiments were simpler to interpret, however, due to the few bands which appear. Figure 29 is a representative autoradiogram.

Table 14

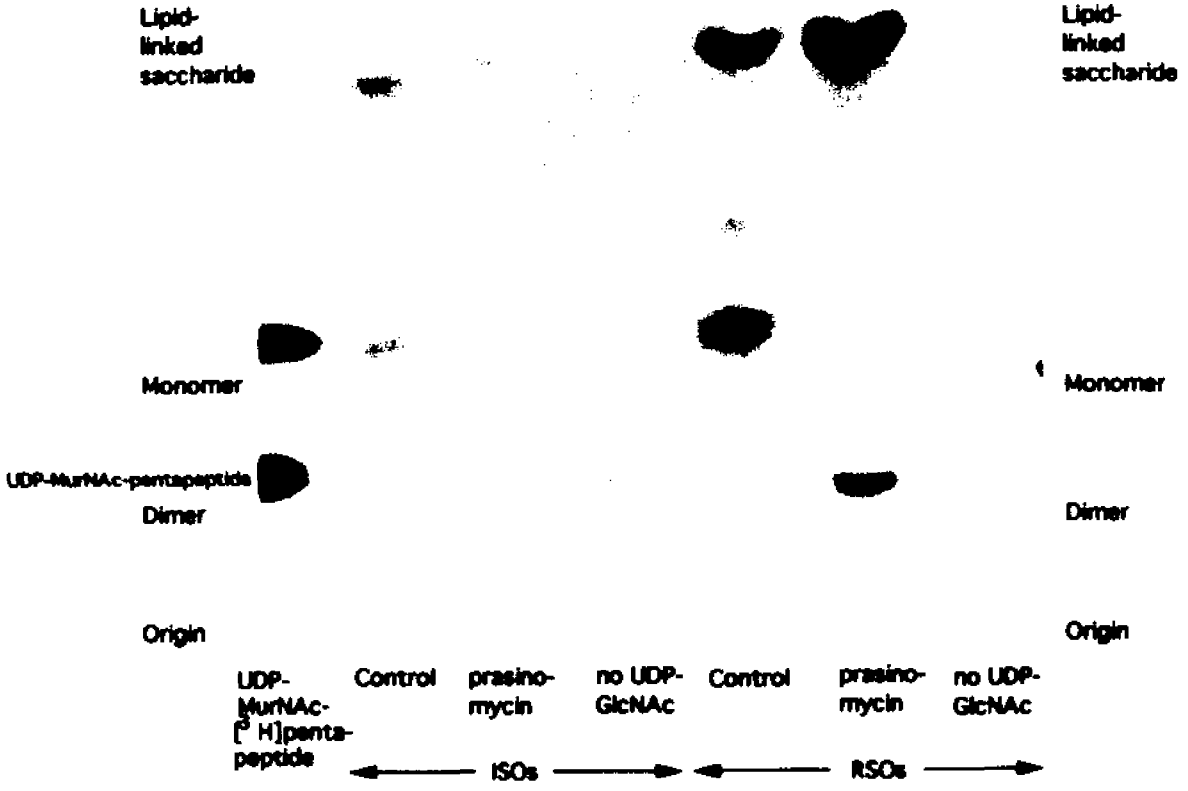
UDP-MurNAc-pentapeptide-independent incorporation of [^{14}C]GlcNAc by ISOs and RSOs

(pmoles incorporated/mg protein)

	ISOs	RSOs
Control (with UDP-MurNAc-pentapeptide)	5.0	47
- UDP-MurNAc-pentapeptide	2.5	22
+ tunicamycin	2.1	5.5

Figure 29

TLC analysis of the product of incorporation of label from UDP-MurNAc-[^{3}H]pentapeptide by ISOs and RSOs



Vesicles were incubated for 15 min with UDP-MurNAc-[^{3}H]pentapeptide alone, with UDP-GlcNAc, and with UDP-GlcNAc and prasinomycin. Reactions were terminated by addition of acetic acid and centrifugation. Pellets were suspended in TLC solvent and spotted onto TLC plates with authentic standards. Plates were developed and autoradiographed as in Materials and Methods.

Reactions incubated without UDP-GlcNAc have only two faint bands, which may correspond to the two bands of the UDP-MurNAc-[3H]pentapeptide present in the reaction mixture. ISOs incubated with UDP-GlcNAc had a polymerized product at the origin (which is absent in prasinomycin treated reactions), a "lipid-linked" product and two bands which could be monomer and dimer moieties or contamination by the substrate. RSOs incubated with UDP-GlcNAc had a "lipid-linked" band (which increased significantly in the presence of prasinomycin), two bands not seen in ISO reactions (which disappeared in the presence of prasinomycin) and the same monomer and dimer bands seen in ISOs. No immobile, polymerized product was seen in RSOs. The band which may correspond to dimer did appear to increase in density in the presence of prasinomycin.

Thus, product analysis of vesicles incubated with UDP-MurNAc-[^{3}H]pentapeptide and cold UDP-GlcNAc revealed a simple pattern of bands. UDP-MurNAc-pentapeptide has only one known fate in the bacterial cell, as part of the peptidoglycan pathway. Bands labelled with UDP-[1^{4C}]GlcNAc, but not UDP-MurNAc-[^{3}H]pentapeptide, are probably products of reactions outside of the peptidoglycan pathway.

Rechromatography of the Lipid-linked Product

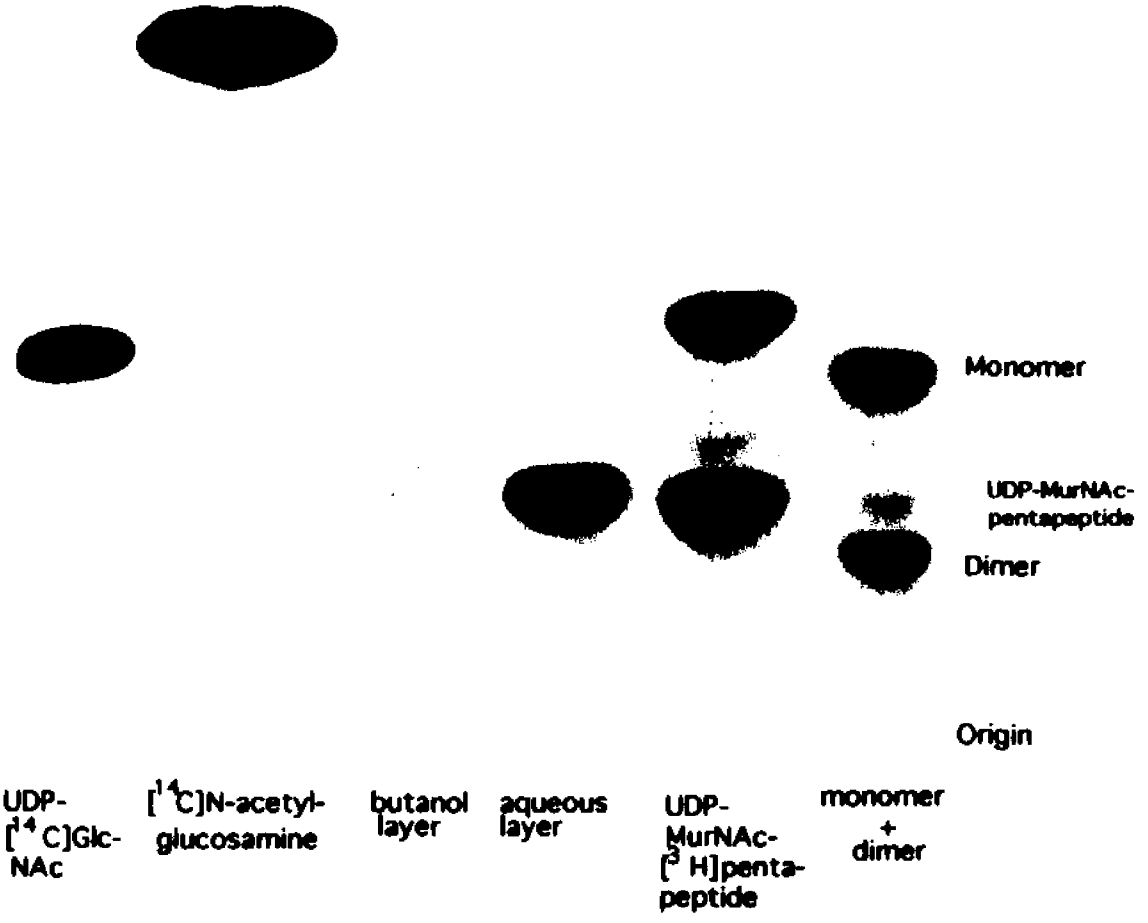
Pless and Neuhaus (1973) described TLC analysis of the

lipid-linked disaccharide (R_f=0.56) and the release of free C_{55}-phosphate by mild acid hydrolysis. An adaptation of this method was used to further characterize the putative lipid-linked disaccharide product of vesicles.

Bands corresponding to the lipid-linked product of RSOs were scraped from TLC plates and extracted with chloroform:methanol:water (2:1:0.8). The phases were separated by centrifugation and evaporated to dryness. The residues were dissolved in 0.1 N HCl and aliquots counted. No radioactivity was detectable in the chloroform:methanol. TLC analysis (Figure 30) revealed that the aqueous layer contained a major band which coincided with a minor band present in the dimer preparation and UDP-MurNAc-pentapeptide. No pathway is known which might account for the presence of [^{14}C]-UDP-MurNAc-pentapeptide and therefore it was assumed that the band consisted of a species of dimer. Minor bands which corresponded to the position of the lipid-linked disaccharide on the original plate and the monomer standard were also present. The latter band also appeared in the chloroform:methanol layer. The plate had been sprayed with En^{3}Hance, which contains acetic acid, for fluorography and stored at -80°C for 36 days. This treatment was apparently sufficient to hydrolyze the undecaprenyl pyrophosphate from the disaccharide since an appropriate amount of radioactivity was present, but very little as the original lipid-linked disaccharide.

Figure 30

Rechromatography of the lipid-linked product of RSOs isolated by TLC



Bands identified as the lipid-linked product of RSOs were scraped from a TLC plate and extracted with chloroform:methanol:water (2:1:0.8). Layers were separated and evaporated to dryness. Samples were suspended in 0.1 N HCl (see text) and spotted together with authentic standards. Plates were developed and autoradiographed as in Materials and Methods.

The major band was scraped from the plate and the sample eluted from the silica gel into 25 mM sodium phosphate buffer, pH 6.5. Muramidase from S. globisporis was added to give a final concentration of 500 µg/ml and the samples were incubated at 37°C for 5 hours. This enzyme cleaves the same ß,1-4 linkage as lysozyme, digesting the peptidoglycan polymers into disaccharide (monomer) units. The enzyme has no effect on the crossbridges which join the peptide side chains of mature peptidoglycan. Thus, digests of crosslinked peptidoglycan contain monomer units as well as monomers joined to one or two other monomer units through peptide crossbridges.

The digested sample was evaporated to dryness, resuspended in the TLC solvent and spotted onto a TLC plate. An autoradiogram (Figure 31) of the TLC plate revealed a single band of radioactivity corresponding to the same mobility as the sample before digestion. This R_f does not correspond exactly to any standard. The closest match is a minor band in the dimer standard which may be a species of dimer having a pentapeptide side chain attached to a tetrapeptide side chain.

Mild acid hydrolysis was repeated on samples of reacted RSOs, incubated with or without moenomycin, and extracted with butanol-pyridinium acetate. An aliquot of each butanol layer was spotted on a TLC plate before hydrolysis. Samples were boiled for 20 min in 0.01 M HCl

Figure 31

Rechromatography of the lipid-linked product of RSOs isolated by TLC and digested with muramidase

Origin

[^{14}C]N-acetyl-glucosamine | UDP-[^{14}C]Glc-NAc | Monomer | Digested Sample | Dimer

The major band appearing in the aqueous extract seen in Figure 30 was scraped from that plate and eluted with 25 mM sodium phosphate buffer, 0.1mM $MgCl_2$, pH 6.5. Muramidase from *S. globosporus* was added to 500 µg/ml and the sample incubated at 37^{o}C for 5 hr. The sample was evaporated and suspended in TLC solvent and spotted together with authentic standards. Plate was developed and autoradiographed as in Materials and Methods.

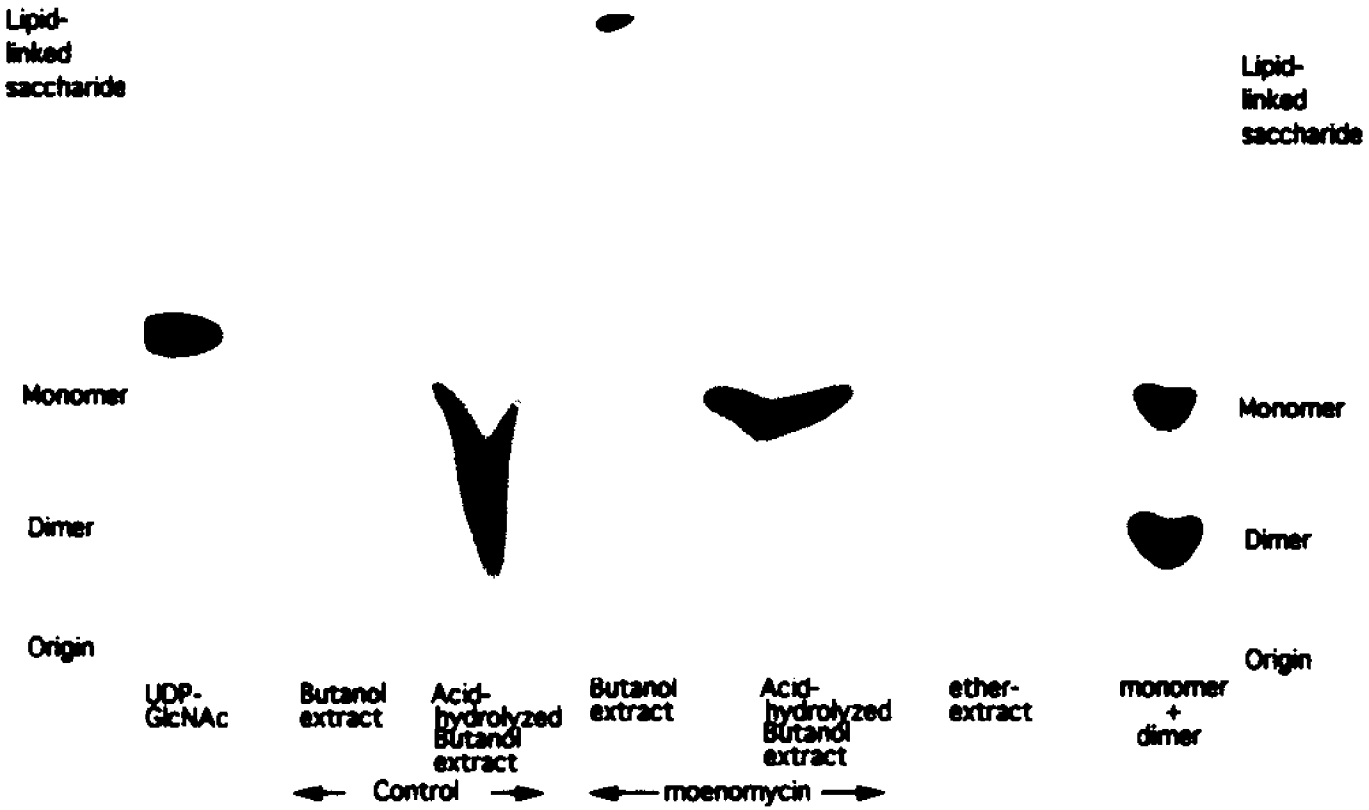
in 50% methanol. Aliquots were spotted together with the previous samples and monomer and dimer standards.

After development, radioactive bands were located by direct autoradiography on Amersham Hyperfilm ß-Max to avoid the use of fluorography enhancer. These are shown in Figure 32. Butanol extracts contained a major band corresponding to the lipid-linked disaccharide (R_f=0.60). After acid hydrolysis, the control reaction mixture contained a major band which spread from the location of the monomer to the dimer. It was not possible to tell if the band consisted of one or two separate species, although the mobility of the band more closely resembled the dimer standard. The moenomycin-treated sample contained a major band corresponding to the monomer standard and a very faint band corresponding to the dimer standard.

The single band on the control plate and the two bands on the moenomycin plate were then scraped from the plates and incubated with the muramidase from S. globisporis. The digested samples were then rechromatographed as above. An autoradiogram (Figure 33) of the control sample and the low mobility (monomer) band of the moenomycin sample produced bands corresponding to the monomer standard. The faint band in the moenomycin sample corresponding to the dimer standard produced no visible band.

Figure 32

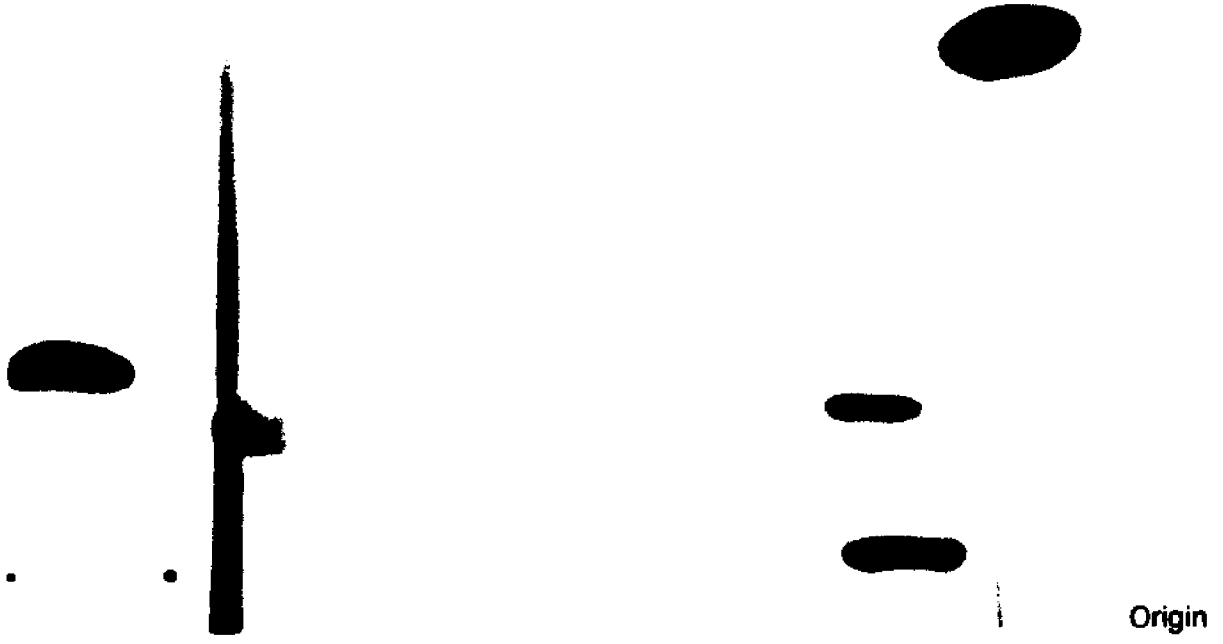
TLC analysis of butanol extracts and acid-hydrolyzed butanol extracts of reacted RSOs



RSO vesicles, with and without moenomycin, were incubated with UDP-[^{14}C]GlcNAc and extracted with butanol. Butanol extracts were spotted directly and after mild acid hydrolysis. One-half of each hydrolyzed sample was extracted with ether to remove the lipid and the ether layer spotted. Lipid was visualized with iodine vapor.

Figure 33

Rechromatography of the lipid-linked product of RSOs after acid hydrolysis and digestion with muramidase



Samples were eluted from the plates shown in Figure 32 and incubated with the muramidase from *S. globisporis* for 6 hours. The digested samples were evaporated to dryness and suspended in isobutyric acid: 1N ammonia (5:3) and applied to a silica gel TLC plate. The plate was developed and autoradiographed as detailed in Materials and Methods. Sample 1 &2; entire control spot; Sample 3, moenomycin-treated, "monomer " spot; Sample 4; moenomycin-treated , "dimer"spot.

Pulse-Chase Experiments

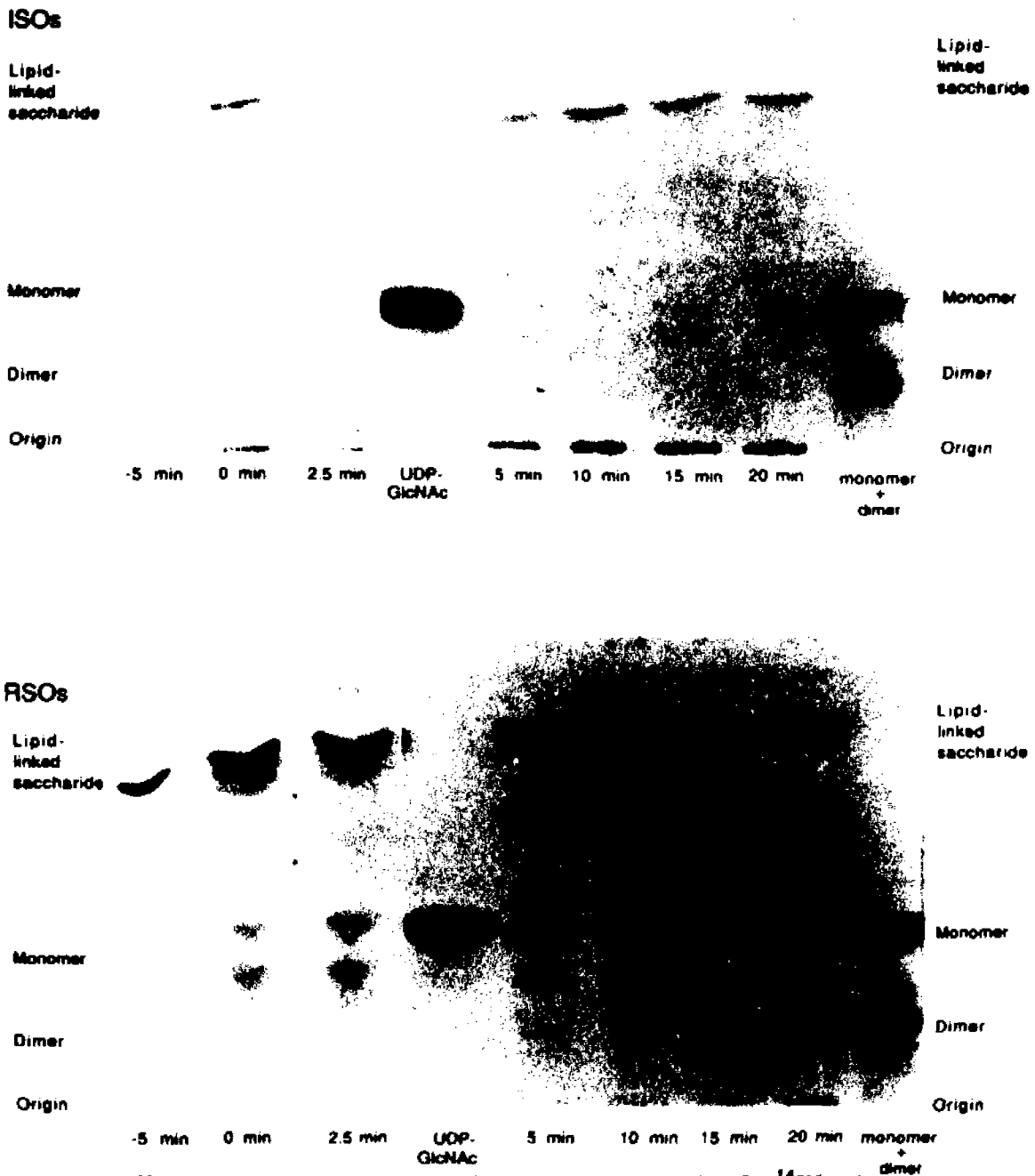
Identification of the products of the incorporation of UDP-[^{14}C]GlcNAc by vesicles was facilitated by an examination of the time-dependent relationship between them. The presumed sequence is that seen in the intact organism: lipid-linked monosaccharide to lipid-linked disaccharide which becomes polymerized product. However, in vesicles, the products include *non*-lipid linked, *non*-polymerized moieties. These products may be artifacts but may also be the result of the limitations of the system. Vesicles may be partially dysfunctional and release products which do not exist *in vivo*.

To examine the time-course of product formation, vesicles of both orientations were incubated in the routine reaction mixture containing UDP-[^{14}C]GlcNAc. After 5 min an amount of unlabelled UDP-GlcNAc approximately equal to the labelled amount was added. Duplicate aliquots were removed at appropriate time points to 20 min, one to be filtered and counted and the second to be analyzed by TLC.

Radioactivity in the filtered samples increased slightly over time in ISOs; RSO samples showed the opposite trend and decreased slightly with time. Autoradiograms of TLC plates (Figure 34) were quantititated by densitometry. Figure 35 shows the distribution of radioactivity (in densitometer units) for each time sample. Areas for measurement were chosen empirically to allow

Figure 34

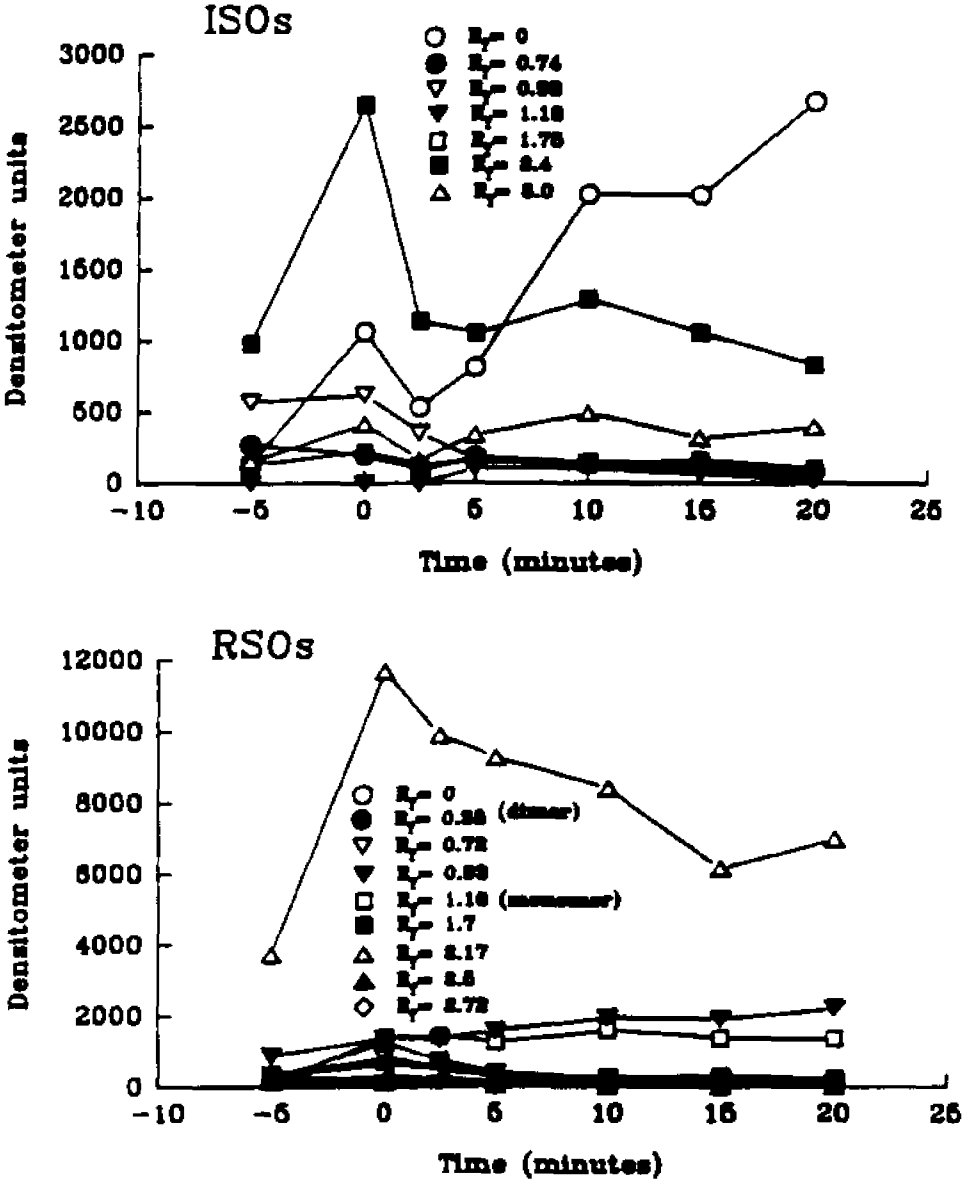
Pulse-chase of UDP-GlcNAc in ISOs and RSOs



Vesicles were incubated in the incorporation reaction mixture containing UDP-[^{14}C]GlcNAc for 5 min. Cold UDP-GlcNAc was added at zero time and incubation continued for 20 min. Aliquots were removed and filtered at the indicated times.

Figure 35

Quantitation of TLC analysis of the ^{14}C-labelled products of ISOs and RSOs after pulse-chase with UDP-GlcNAc



Autoradiograms shown in Figure 34 were quantitated by computerized densitometry. Results were calculated by digitalization of optical density and integration of the volume of each band after subtraction of background. Results are represented as the value at each R_f at each sampling time. Cold UDP-GlcNAc was added at 0 min. R_f is the mobility of the sample compared to the mobility of the monomer standard.

quantitation of every band appearing in any sample. RSO samples displayed one more band than ISOs, the most distant from the origin. Vesicles of both orientations appear to incorporate label from UDP-[^{14}C]GlcNAc directly into lipid-linked material. The earliest sample times showed the greatest amount of incorporation of label into this fraction. The addition of cold UDP-GlcNAc appears to "push" the label into this band for both RSOs and ISOs. With increasing time, ISOs showed an increase in label at the origin with a corresponding decrease in label in the lipid-linked fraction. RSOs showed a similar decrease in labelled lipid-linked disaccharide but no increase at all in the polymerized product. No other bands showed any increase which would identify them as the product of the disappearance of the lipid-linked disaccharide. The decrease in that band was much less dramatic, both in time and amount, than seen in ISOs, indicating that the lipid-linked peptidoglycan precursor was more long-lived in RSOs.

The optical density of the other bands did not vary significantly during the course of the experiment, with the possible exception of a band which approximately corresponded to the monomer standard. The correspondence was essentially exact in RSOs, but the concentration remains static after the initial 5 min of incubation. The identification of this band is less certain in ISOs. The concentration of this component was low at the start of the

experiment and decreased slightly over time.

The Effect of Uridine Monophosphate on Incorporation of [^{14}C]-N-Acetylglucosamine by RSO and ISO Membrane Vesicles

The first membrane-associated step of peptidoglycan synthesis in E. coli, the transfer of phospho-N-acetylmuramyl pentapeptide to the undecaprenyl phosphate, can be reversed by high concentrations of UMP (Anderson al., 1965). To confirm that the incorporation of [^{14}C]GlcNAc by membrane vesicles was due to the activity of the translocase (and the following reaction catalyzed by UDP-N-acetylglucosamine transferase), ISOs and RSOs were assayed in the routine reaction mixture with added UMP. UMP was tested at a concentration equal to that of UDP-MurNAc-pentapeptide, 75 μM, and in 10-fold excess. Reaction mixtures were incubated for 40 min; no attempt was made to characterize the kinetics of inhibition. Inhibition of incorporation (Table 15) was greater in RSOs than in ISOs at both concentrations of UMP. UMP at 75 μM decreased incorporation in ISOs by only 17%, but the same concentration decreased incorporation by 81% in RSOs. At the higher concentration of UMP, results were nearly identical; 89% inhibition in ISOs, 95% inhibition in RSOs.

Direct Measurement of the exchange reaction in vesicles

The reaction catalyzed by phospho-N-acetylmuramyl-

Table 15

Effect of UMP on incorporation of [^{14}C]GlcNAc or MurNAc-[^{3}H]pentapeptide by ISOs and RSOs

	ISOs		RSOs	
	pmoles incorporated/ mg of protein	% of control	pmoles incorporated/ mg of protein	% of control
Labelled with UDP-[^{14}C]GlcNAc				
Exp't #	1 2		1 2	
Control	234 164	-	404 429	-
+75 μM UMP	194 -	83	76 -	19
+750 μM UMP	- 18	11	- 21	4.8
Labelled with [^{3}H]-UDP-MurNAc-pentapeptide				
- UDP-GlcNAc				
Control	46		55	
+750 μM UMP	31	67	32	58
+ UDP-GlcNAc				
Control	153		306	
+750 μM UMP	33	22	25	8.2

pentapeptide transferase can be summarized:

(1) UMP^{*}+ UDP-MurNAc-pentapeptide <-> UDP^{*}-MurNAc-pentapeptide + UMP

and

(2) UDP^{*}-MurNAc-pentapeptide + C_{55}-isoprenylphosphate <-> C_{55}-isoprenylphosphate-P-P-MurNAc-pentapeptide + UMP^{*}

The incorporation assay has measured the end product of the reaction, the lipid linked monosaccharide, when vesicles were incubated with UDP-MurNAc-[^{3}H]pentapeptide but without UDP-GlcNAc . This reaction was indirectly measured when the labelled substrate was UDP-[^{14}C] GlcNAc.

The second activity of the enzyme (Reaction 1 above), termed the "exchange reaction" has also been measured using radiolabelled UMP and following the production of labelled UDP-MurNAc-pentapeptide (Struve et al., 1966). This activity was measured directly in both RSOs and ISOs.

The method used was suggested by Geis and Plapp (1978). Alkaline phosphatase was added to reacted vesicles to dephosphorylate "unexchanged" [^{14}C]UMP to [^{14}C]uridine. Samples were then applied to anion exchange filters which

were extensively washed to remove the [^{14}C]uridine, leaving the newly labelled [^{14}C]UDP-MurNAc-pentapeptide to be measured by scintillation counting.

Characteristics of the exchange reaction in vesicles

Similar activity was measured in vesicles of both orientations. ISOs had less activity per milligram of protein than RSOs (Table 16), a phenomenon reported previously for the incorporation assay. The rate of the reaction was dependent upon the enzyme concentration and the UMP and UDP-MurNAc-pentapeptide concentrations. The reaction was inhibited by tunicamycin, as has previously been reported for crude preparations of the enzyme (Harrington and Baddiley, 1983; Ward, 1977). Inhibition by tunicamycin was complete in ISOs; exchange was inhibited by 88% in RSOs. As seen in Figure 36, the rate of the reaction was approximately linear for 10 minutes in RSOs; in ISOs the rate was linear for the entire 30 minutes measured. The change in the rate of the reaction after 10 min in RSOs may be due to depletion of the substrate. The substrate concentration was not increased further to conserve the labelled UMP.

The phospho-N-acetylmuramyl pentapeptide transferase is known to be dependent upon the presence of Mg^{++} (Heydanek et al. 1970), but we could not demonstrate this dependence using this method. In fact, incorporation of label appeared to be higher in the absence of Mg^{++}. TLC

Table 16

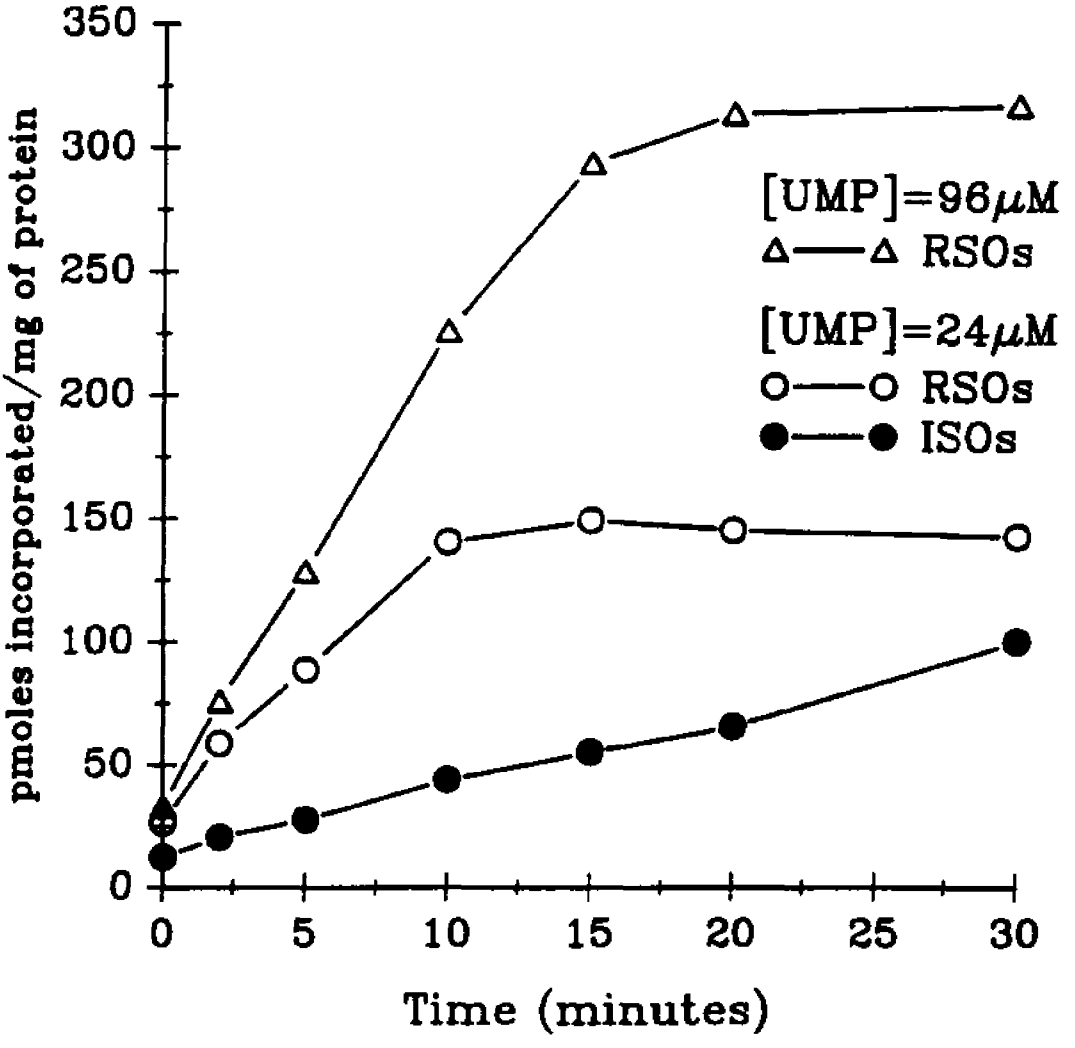
Measurement of the exchange reaction in RSO and ISO vesicles

(pmoles of UMP exchanged)

	ISOs	RSOs
1 mg/ml protein	18.5	28.6
3.75 mg/ml protein	35.0	39.0

Figure 36

Time course of the exchange reaction



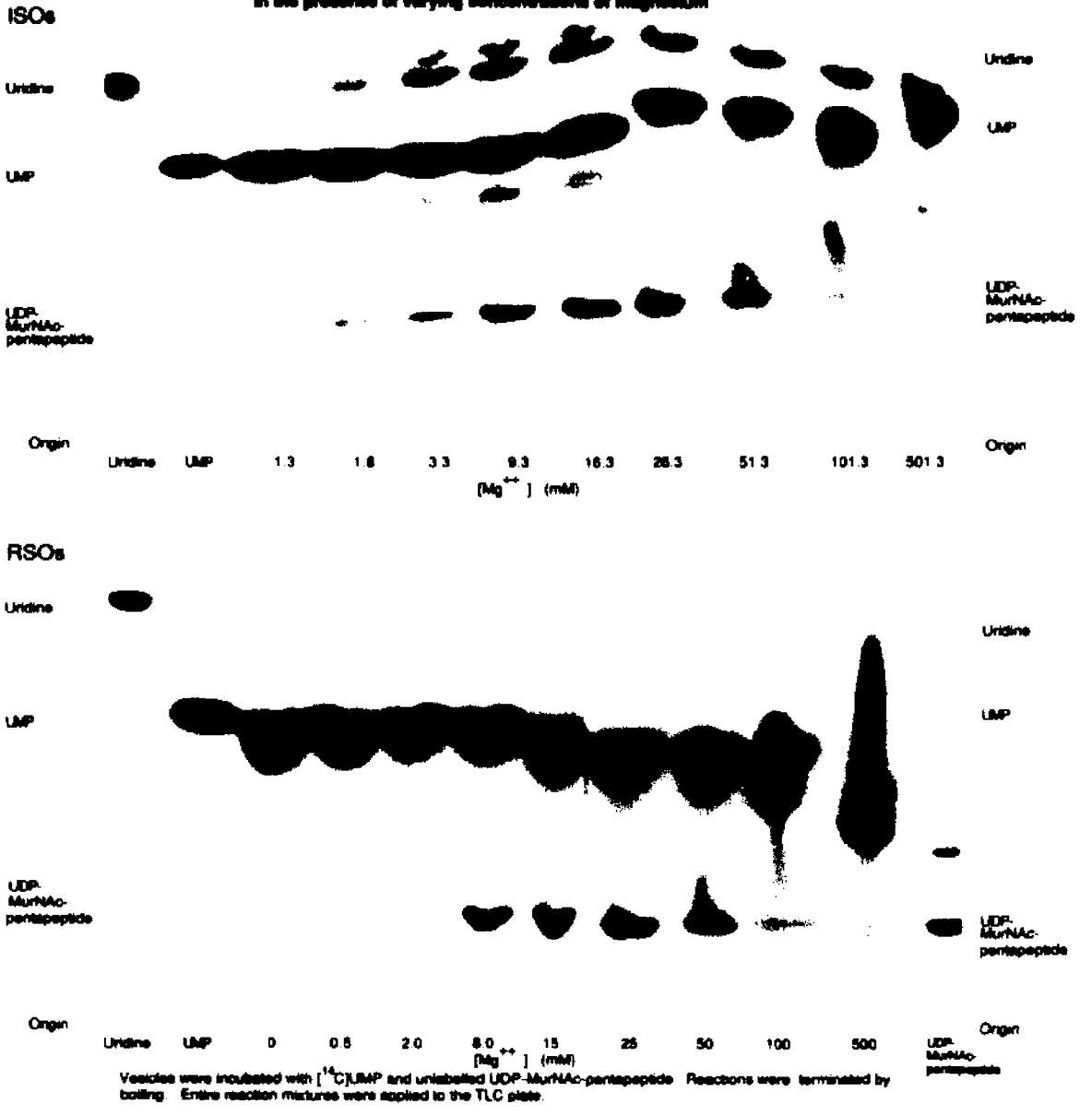
Vesicles (1 mg/ml of protein) were incubated at 37°C for 10 min with 75 μM UDP-MurNAc-pentapeptide and [^{14}C]UMP at 24 or 96 μM for the indicated time. Reaction mixtures were boiled for 1 min, and cooled. Twenty μl of alkaline phosphatase (25 units/ml) was added to each reaction mixture and all were incubated for 5 min. Reaction mixtures were spotted onto DE81 filters. Each filter was washed 5 times with 4 ml of water, dried and counted.

analysis of reaction mixtures incubated with and without Mg^{++} , however, indicated dependence on Mg^{++}. The isobutyric acid:ammonia TLC system separates [^{14}C]UMP, the substrate of the reaction, from [^{14}C]UDP-MurNAc-pentapeptide, the product of the reaction, and separates both from [^{14}C]uridine, the product of alkaline phosphatase. Reaction mixtures were examined, without incubation with alkaline phosphatase, together with authentic standards of [^{14}C]UMP, [^{14}C]uridine and UDP-MurNAc-pentapeptide.

In the presence of Mg^{++}, both RSOs and ISOs catalyzed the exchange of label from [^{14}C]UMP to UDP-MurNAc-pentapeptide, which was the only major band found by autoradiography (Figure 37). In the absence of Mg^{++}, the only band in RSO reaction mixtures coincides with UMP. ISO vesicles contain Mg^{++} in the storage buffer and so the assay is actually done in the absence of <u>added</u> Mg^{++}. The actual concentration of Mg^{++} in those reaction mixtures is 1.3 mM. The major labelled band from ISO reaction mixtures is UMP with a greatly reduced yield of [^{14}C]UDP-MurNAc-pentapeptide. These results confirmed the dependence of the exchange reaction on the presence of Mg^{++} and indicate that the increase in activity seen in the absence of Mg^{++} in the filter binding assay was due to decreased alkaline phosphatase activity in the absence of Mg^{++}. The presence of [^{14}C]UMP in reaction mixtures lacking Mg^{++} indicated

Figure 37

TLC analysis of the products of the exchange reaction in ISOs and RSOs in the presence of varying concentrations of magnesium



Vesicles were incubated with [^{14}C]UMP and unlabelled UDP-MurNAc-pentapeptide. Reactions were terminated by boiling. Entire reaction mixtures were applied to the TLC plate.

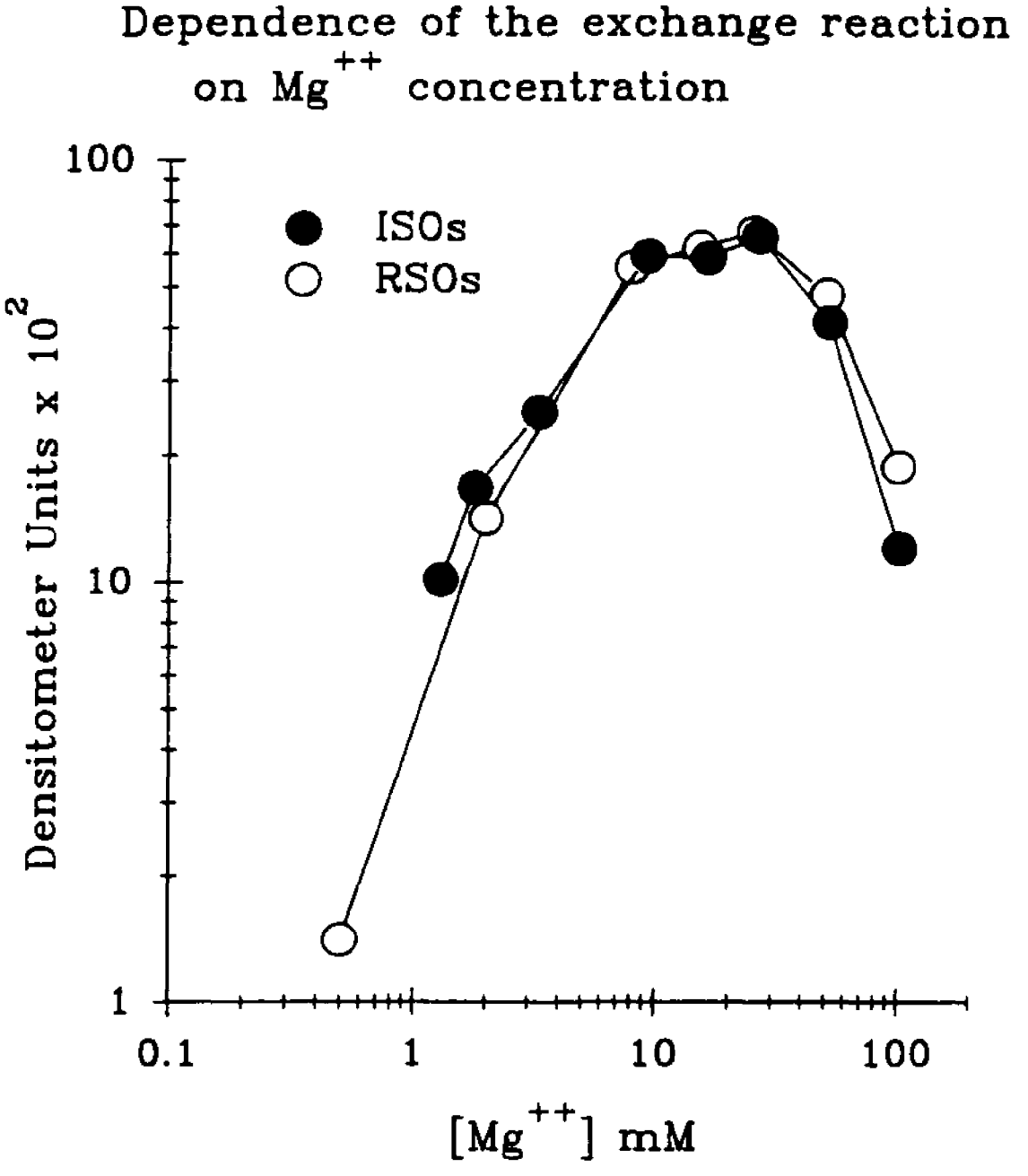
that the alkaline phosphatase was inactive under these conditions. Optimum Mg^{++} concentration was determined in ISOs and RSOs using TLC of the reaction mixtures. The results were quantitated by densitometry of the UDP-MurNAc-pentapeptide bands and are shown in Figure 38. Vesicles of both orientations appeared to have maximum activity at a magnesium concentration of 25 mM.

Specificity of the phospho-N-acetylmuramyl pentapeptide transferase for the exchange reaction

As reported above, the incorporation reaction in vesicles of either orientation can utilize UDP-MurNAc-pentapeptide from S. aureus contain which contains L-lysine in the place of the meso-DAP present in E. coli. The K_m for this substrate was higher than for the natural substrate, but the rates of the reaction in RSOs and ISOs were not very different from those for the natural substrate. The lysine- containing UDP-MurNAc-pentapeptide was also tested as a substrate in the exchange reaction. The results (Table 17) were again nearly identical to those measured using the DAP-containing substrate.

These results indicate that the phospho-N-acetylmuramyl pentapeptide transferase has little specificity for the third residue of the pentapeptide side chain. Hammes and Neuhaus (1974) have reported essentially the same result for the phospho-N-acetylmuramyl

Figure 38



Bands corresponding to UDP-MurNAc-pentapeptide on the autoradiogram seen in Figure 37 were quantitated by computer densitometry. Results were calculated by digitalization of optical density and integration of the volume of each band. Background OD was subtracted before integration.

Table 17

Exchange of label from [^{14}C]UMP to UDP-MurNAc-pentapeptide from *S. aureus* or UDP-MurNAc-dipeptide

	% of Control[a]	
	ISOs	RSOs
S. aureus UDP-MurNAc-pentapeptide	100	80
UDP-MurNAc-dipeptide	0	34

[a] Control incorporation was measured with the natural substrate of the *E. coli* enzyme, DAP-containing UDP-MurNAc-pentapeptide, isolated from *B. cereus*.

pentapeptide transferase from *S. aureus* using the DAP-containing pentapeptide.

The UDP-MurNAc-dipeptide has been tested in vesicles of both orientations in both the exchange assay and the incorporation reaction. The dipeptide was essentially inactive in the exchange reaction using ISOs and only slightly active (34% of control) using RSOs (Table 17).

These results suggest that the identity of the third amino acid residue of the pentapeptide side chain is not critical for recognition by the enzyme in either the exchange or transfer reaction.

Disparity of specific activity of RSOs and ISOs

The consistent difference in specific activity of vesicles of opposite orientation, measured as activity per milligram of protein, proved puzzling. ISO preparations diluted to 10 mg/ml of protein appear as translucent solutions. It was rarely possible, however, to resuspend the final pellet of the RSO preparations in a small enough volume of buffer to achieve a concentration of 10 mg of protein/ml. The usual final concentration of an RSO preparation was 7 or 8 mg of protein/ml. These preparations are opaque in appearance and somewhat viscous. Despite their higher protein concentration and homogeneous appearance, the specific activity of ISOs is invariably lower than RSOs.

Phospholipid content of several different ISO and RSO

preparations, as measured by total phosphate, was determined as a possible alternative to protein concentration for as a basis for calculating specific activity. As seen in Table 18, ISO preparations were at least 5-fold higher in phosphate concentration than RSO preparations. Protein-to-phosphate ratios appeared to be characteristic for each orientation, but have not been useful to relate the activity of ISOs and RSOs.

It has been noted in the literature that ISO preparations contain a considerable amount of outer membrane proteins (Ames et al., 1989). To determine the extent of contaminating outer membranes present in both ISO and RSO preparations, the inner- and outer-membrane fractions of ISOs and RSOs were separated by their differential solubilities in sarkosyl (Filip et al., 1973). SDS-PAGE gels of the separated fractions revealed differences in the patterns of the bands in both the inner and outer membranes of ISOs and RSOs. Outer membrane fractions of ISOs contained a considerable number of other bands, absent or undetectable in outer membrane fractions of RSOs.

To quantitate the outer membrane protein contamination present in the vesicle preparations, protein concentrations of sarkosyl-soluble and insoluble fractions were determined RSOs were found to contain a higher concentration of protein in the sarkosyl-insoluble fraction, indicating a greater amount of contamination with outer membrane

Table 18

Protein and phosphate concentrations of ISOS and RSOs

Prep#	[Protein] (mg/ml)	[Phosphate] (mM)	Protein/Phosphate
ISOs			
1	6.1	22.2	0.272
2	5.4	25.7	0.209
3	5.0	19.7	0.255
4	42	169	0.251
5	2.7	9.5	0.285
6	5.5	56.6	0.097
RSOs			
1	8.1	5.3	1.5
2	4.7	2.0	2.3

proteins. The source of the difference in specific activity between ISOs and RSOs remains uncertain.

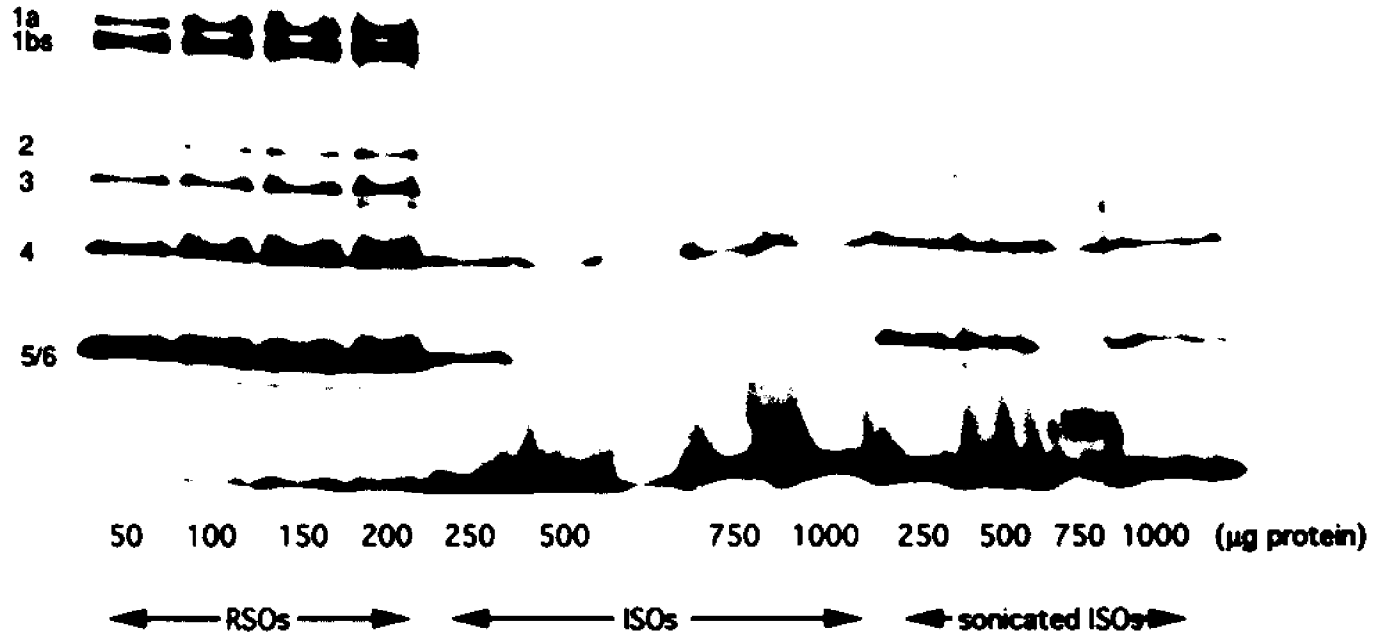
Penicillin-binding proteins in vesicles

The penicillin-binding protein pattern of RSOs and ISOs is shown in Figure 39. The RSO pattern (lanes 1 - 4) was typical of E. coli and the same as seen in ether-treated E. coli B (Talbot et al., 1989). The ISO pattern (lanes 5 -8), however, appeared very different. The ISOs were tested at much higher protein concentrations than RSOs (250 to 1000 μg vs. 50 to 200 μg) because of initial difficulties detecting any pattern of PBPs. The high-molecular weight PBPs, 1,2, and 3, appear in only lane 5, the lowest protein concentration tested. This effect is probably an artifact, since a large dark band appears below all the PBPs in all ISO lanes, but this is not a satisfactory explanation for the very low levels of PBPs seen in ISOs.

The inhibition of incorporation and polymer formation by moenomycin and prasinomycin indicated that at least the transglycosylase activity of the high-molecular weight PBPs is functioning in ISO vesicles. However, there is little strong evidence of active crosslinking since penicillin had no effect on polymer formation in ISOs. It is unlikely, however, that one of the two activities of these bifunctional enzymes could be completely destroyed while

Figure 39

Penicillin - Binding Proteins of ISOs and RSOs



For each sample, the indicated amount of protein of ISO or RSO vesicles was incubated with 10 µCi (581 pmoles) of [^{3}H]benzylpenicillin. One set of ISO vesicles was incubated in a sonicating water bath to disrupt the vesicles allowing the [^{3}H]benzylpenicillin access to the ISO PBPs. Sample preparation, electrophoresis, and autoradiography continued as in Materials and Methods.

the other activity was unaffected.

The apparent absence of PBPs and inhibitory effects of penicillin in ISOs could be due to the inability of penicillin to penetrate the plasma membrane. No definitive evidence exists that penicillin does reach the cytoplasm in living cells. The PBPs in ISOs are on the opposite side of the membrane from the penicillin and may be safely beyond its inhibitory effects.

To test this, reaction mixtures containing ISOs and [^{3}H]-penicillin were incubated in a sonicating water bath for the normal 10 minute incubation. These samples are seen in lanes 9 - 12 of Figure 39. This treatment appears to result in a slightly more distinctive pattern of PBPs than the unsonicated ISOs, but the pattern remains significantly different from that of RSOs and is not at all the familiar pattern of E. coli PBPs (Spratt, 1977).

Discussion

The membrane-associated steps of peptidoglycan synthesis, especially that catalyzed by phospho-N-acetylmuramyl-pentapeptide transferase (the so-called translocase), were the subject of intensive study during the 1970's. The activity of the translocase was found to be dependent upon the presence of at least some of the components of the membrane and also on the physical state of the membrane. In addition, the kinetics of the translocase reaction and the specificity of the enzyme for the peptide side chain of the substrate were determined. The actual amino acid sequence of the enzyme, its arrangement in the membrane, and the relationship between the phospho-N-acetylmuramyl-pentapeptide transferase and the N-acetylglucosamine transferase were unknown. The active sites of both enzymes were presumed to be at or near the cytoplasmic face of the inner membrane, but no experimental evidence supported this belief. It was the purpose of this thesis to examine the proposed topological location of these enzymes utilizing as a model system, cytoplasmic membrane vesicles of opposite orientations. Data presented here indicate that the active sites of both phospho-N-acetylmuramyl pentapeptide transferase and N-acetylglucosamine transferase are accessible from both sides of the membrane.

Vesicles of both orientations incorporate peptidoglycan precursors.

The initial observation, that vesicles of both right-side out and inside-out orientations incorporated label from UDP-[^{14}C]GlcNAc, into vesicle-associated material and TCA-precipitable material, immediately called into question the presumed location of the enzymes. If the active sites of these enzymes did face the cytoplasmic source of the substrates, RSOs would be expected to be unable to synthesize any intermediates in the pathway. The nature of the substrates, nucleotide-linked sugars, precluded the possibility that they could cross through the lipid-bilayer to active sites on the interior of the RSOs.

Trivial explanations for these results were evaluated. The truly surprising result was the activity seen in RSOs. If this activity were low, the results might be equivocal, due perhaps to the presence of some ISO contamination. In fact, every RSO preparation was more active per mg of protein than every ISO preparation. No evidence was found from fluorescence spectroscopy and sucrose gradient centrifugation of any significant contamination of RSO vesicles with ISOs (or the opposite). Considering the lower level of activity found in ISOs, only extensive contamination could account for the high activity seen in RSOs. Peptidoglycan synthesis by whole and EDTA-treated cells from externally supplied substrates confirmed the

activity seen in RSOs. Thus, incorporation by RSOs was shown not to be due to contamination with ISOs and not to be an artifact caused by the procedures used to prepare RSOs.

Initially, enzyme activity in vesicles was measured by incorporation of UDP-[^{14}C]GlcNAc which is commercially available. The activity of the phospho-N-acetylmuramyl pentapeptide transferase in vesicles of both orientations was confirmed by incorporation of label from UDP-MurNAc-[^{3}H]pentapeptide, in the presence and absence of UDP-GlcNAc, demonstrated in both RSOs and ISOs. In addition, the second activity of the translocase, the exchange reaction, was also measurable in vesicles of both orientations. Clearly, the active sites of both the translocase and the transferase were accessible to substrates provided from either side of the membrane.

Harrington and Baddiley (1983) also observed incorporation of label from the nucleotide-linked precursors into uncrosslinked peptidoglycan, using partially autolyzed B. subtilis. The model proposed to explain this activity was based upon reorientation or rotation of an enzyme complex through the plane of the membrane (Harrington and Baddiley, 1984). The energy required for the movement of the enzyme-substrate complex was proposed to be provided by the electrochemical proton gradient, $\Delta\mu_{H^+}$. Dissipation of $\Delta\mu_{H^+}$ by valinomycin caused

a decrease in the polymeric product, but also increased a product soluble in TCA. This product was identified as UDP-MurNAc-pentapeptide, but the mechanism of this effect of valinomycin was not discussed.

Effect of $\Delta\mu_H+$ on incorporation of peptidoglycan substrates by vesicles

Incorporation of label from UDP-[^{14}C]GlcNAc by vesicles of either orientation appeared to be only slightly affected by lactate, added to generate $\Delta\mu_H+$, or the ionophores nigericin and valinomycin, added to dissipate $\Delta\mu_H+$. Measurements of $\Delta\mu_H+$ are usually taken between pH 5.5 and pH 7.5, but little or no peptidoglycan-synthesizing activity is seen in vesicles below pH 8.0. It was shown by fluorescence spectroscopy, that $\Delta\mu_H+$ could be generated in RSOs at pH 8.8 in the normal reaction mixture for peptidoglycan synthesis in the presence of lactate. The addition of lactate resulted in the appropriate pattern of fluorescence quenching. Quenching was unperturbed by the addition of nigericin indicating that no ΔpH was present. Valinomycin did disrupt the fluorescence quenching, thus confirming the presence of the $\Delta\psi$ component of $\Delta\mu_H+$. Incorporation of label from UDP-[^{14}C]GlcNAc by RSOs was not increased in the presence of lactate and was not decreased in the presence of nigericin. Valinomycin inhibited incorporation by < 5% in RSOs prepared and tested in phosphate buffer and not at all in RSOs prepared in Tris

and tested in phosphate. In ISOs, addition of valinomycin, lactate or nigericin decreased incorporation somewhat. Valinomycin inhibited the most, by 20%, but the concentration used, 1 μM, is sufficient to completely dissipate $\Delta\mu_H+$ when measured by fluorescence spectroscopy. Addition of lactate inhibited by 14%, nigericin by 5%. These results indicate that, in E. coli, energy-requiring rotation or reorientation was not necessary for the membrane-associated steps of peptidoglycan synthesis. Thus it appears that incorporation of label from UDP-[^{14}C]GlcNAc into peptidoglycan precursors is not dependent upon $\Delta\mu_H+$ in vesicles of either orientation.

Several steps of lipopolysaccharide synthesis are analogous to peptidoglycan synthesis. Precursors are cytoplasmic in origin, become linked to undecaprenyl pyrophosphate and cross through the inner membrane. McGrath and Osborn (1991) and Marino et al. (1991) have recently published results indicating that, in vivo, the initial step of O-antigen synthesis and the final transfer of O-antigen to core lipopolysaccharide are both dependent on $\Delta\mu_H+$. It was shown that the uncoupler 2,4-dinitrophenol inhibited the initial formation of lipid-linked saccharide and the final transfer of the completed O-antigen from the lipid phosphate. However, in peptidoglycan synthesis 1 mM dinitrophenol, the concentration used by McGrath et al., had only a slight

effect on incorporation of label from UDP-[^{14}C]GlcNAc in vesicles of either orientation. Incorporation was increased by 6.9% in ISOs and 6.7% in RSOs. This may be due to an indirect effect of dinitrophenol on membrane fluidity.

Protease treatment of vesicles

In light of the significant volume of data suggesting that ISOs and RSOs are strikingly alike with respect to membrane-associated peptidoglycan synthesis, the results of pre-treatment of vesicles with proteases provided evidence of definite differences. Not only did the different proteases inhibit incorporation to different degrees, but each protease inhibited ISOs and RSOs to different degrees. Thus, chymotrypsin inhibited strongly in ISOs (79%) and weakly in RSOs (27%), while papain inhibited weakly in ISOs (14%) and strongly in RSOs (69%). Even without interpretation of the significance of inhibition by particular proteases, these differences suggest that different amino acid sequences are exposed on each side of the membrane. Interpretation of these results must be tempered by the fact that activity was measured by incorporation of UDP-[^{14}C]GlcNAc rather than with UDP-MurNAc-[^{3}H]pentapeptide. The unknown effect of the proteases on the N-acetylglucosamine transferase makes appraisal of the results difficult.

Ikeda et al. (1991) recently identified the gene

encoding the phospho-N-acetylmuramyl-pentapeptide transferase in E. coli. The deduced amino acid sequence and the hydropathy profile of the protein were also published. The protein is very hydrophobic, having only three areas of significant length which have hydrophilic properties. These regions would be most likely the areas which extend outside the membrane, on either side of the membrane, and thus be susceptible to digestion by proteases. Cleavage sites for all three proteases are found within the three areas. One of the regions contains 7 cleavage sites for trypsin, which inhibited incorporation by ISOs by 94%. This same region contains 6 chymotrypsin sites and this protease inhibited incorporation by ISOs by 79%. This may indicate that this region lies on the inside of the membrane, corresponding to the exterior surface of ISOs. Both of the other hydrophilic regions contain cleavage sites for trypsin, suggesting that at least one of these regions may also form a loop on the inside of the membrane. These results may be helpful in postulating a three dimensional configuration of the phospho-N-acetylmuramyl-pentapeptide transferase within the membrane.

Effects of inhibitors

The effects on incorporation of specific inhibitors of most of the membrane-associated steps of peptidoglycan synthesis allowed assessment of the activity of each enzyme. Incorporation by vesicles of both orientations was

inhibited by tunicamycin, prasinomycin (and moenomycin) and bacitracin. These results indicate that the phospho-N-acetylmuramyl-pentapeptide transferase (the target of tunicamycin), the transglycosylase, (the target of prasinomycin and moenomycin) and the C_{55}-isoprenylpyrophosphate phosphatase (which is inhibited by bacitracin) are all active in vesicles of both orientations. No specific inhibitor of N-acetylglucosamine transferase was tested, but the nature of the assay, the measurement of incorporation of label from UDP-[^{14}C]GlcNAc, is dependent on the activity of this enzyme.

A model of membrane-associated peptidoglycan synthesis

These results are consistent with with those of Harrington and Baddiley (1983) who found all of these activities present in partially autolyzed B. subtilis cells. As discussed above, these authors proposed a model based on a multi-enzyme complex, consisting of the above enzymes, which rotates through the membrane. The cytoplasmic precursors are in this way transported through the membrane, utilizing $\Delta\mu_{H^+}$ as the energy source (Harrington and Baddiley, 1984).

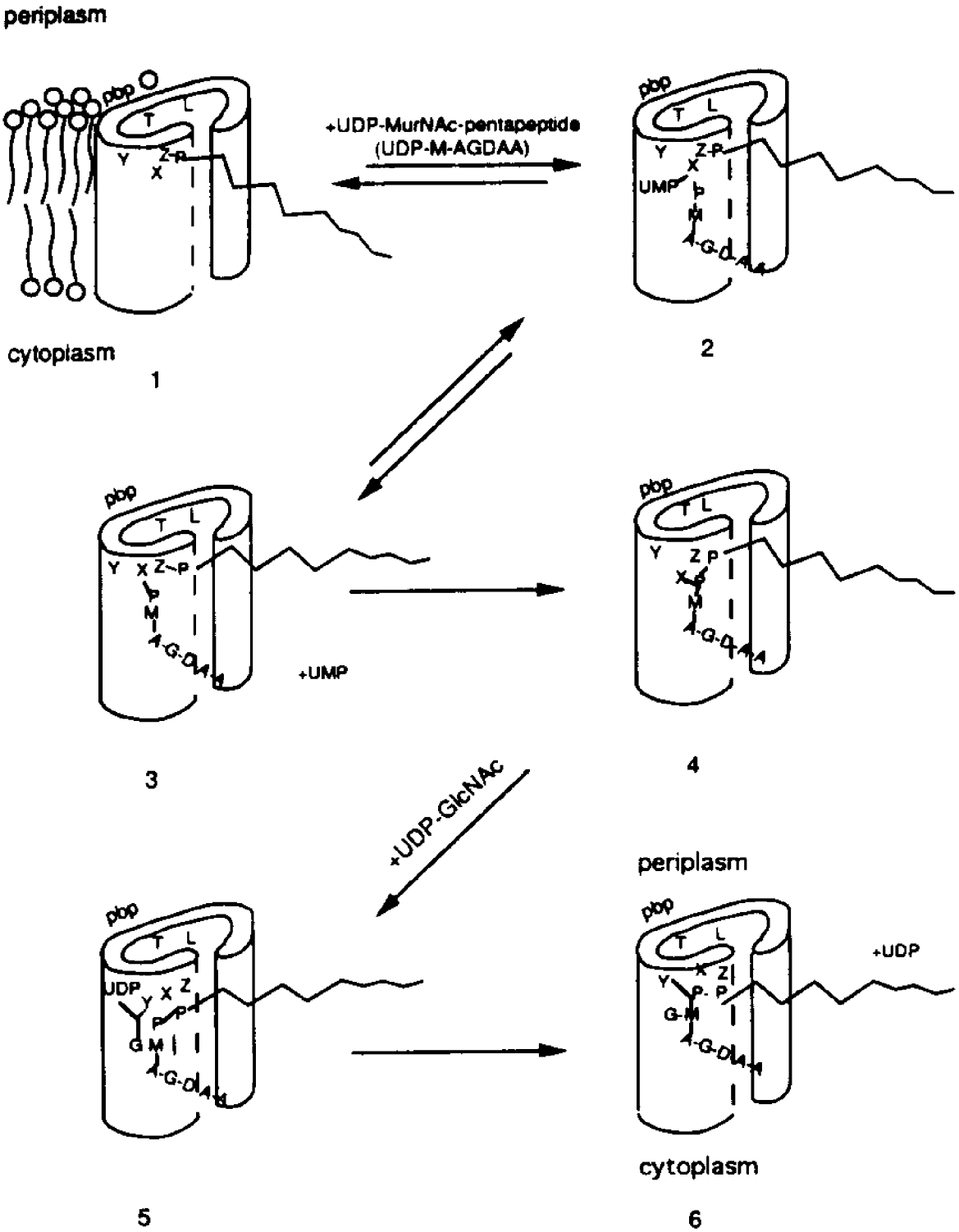
The model supported by our data is also based on a multi-enzyme complex consisting of phospho-N-acetylmuramyl-pentapeptide transferase, N-acetylglucosamine transferase, C_{55}-isoprenylpyrophosphate phosphatase, and at least one of the multiple, high molecular weight penicillin-binding

proteins. At least one molecule of C_{55}-isoprenylphosphate is also associated with each enzyme complex. Our results do not support the need for $\Delta\mu_{H^+}$ as an energy source for peptidoglycan synthesis. As reviewed above, neither the addition of an energy source nor an ionophore or uncoupler, had more than a small effect on activity of vesicles of either orientation. In addition, the results of protease digestion suggest that different parts of the enzymes are exposed on each side of the membrane, so no rotation or reorientation of the enzyme complex appears to occur in vesicles.

Figure 40 illustrates the proposed model. The translocase, transferase and C_{55}-isoprenylpyrophosphate phosphatase, form a membrane-spanning groove. The C_{55}-isoprenylphosphate lies in the plane of the membrane, with the phosphate head group within the interior of the membrane complex and the hydrophobic tail within the membrane. The active sites of the enzymes lie within the interior of the groove. The transglycosylase domain of the PBP lies near the exterior end of the catalytic groove and the transpeptidase domain is in the periplasmic space. Either end of the groove is open, allowing access to the active sites from either side of the membrane. The interior of the groove is lined with hydrophilic residues, to allow the entrance of the charged substrates.

Figure 40

Membrane-bound steps of peptidoglycan synthesis



LEGEND TO FIGURE 40

The enzyme complex consists of phospho-N-acetylmuramyl-pentapeptide transferase, N-acetylglucosamine transferase and undecaprenyl-pyrophosphate phosphatase. A penicillin-binding protein is also associated with the complex at the interface with the periplasmic space.

X = binding site for UDP-MurNAc-pentapeptide on the translocase
Y = binding site for UDP-GlcNAc on the transferase
Z = binding site for the undecaprenyl phosphate on the translocase
T = active site of the transglycosylase domain of the PBP
L = Active site of the undecaprenyl pyrophosphate phosphatase

1. Undecaprenyl phosphate bound to translocase within the enzyme complex with the hydrophobic tail extending into the lipid matrix of the membrane through the opening in the protein complex.

2. Binding of UDP-MurNAc-pentapeptide (UDP-M-AGDAA) to active site, amino acid side chain lies near the opening adjacent to the hydrophobic environment of the membrane.

3. Release of UMP. 1 - 3 are reversible and constitute the exchange reaction.

4. Covalent attachment of phospho-N-acetylmuramyl-pentapeptide to the undecaprenyl phosphate. Formation of the lipid-linked monosaccharide.

5. Binding of UDP-GlcNAc to the active site of the transferase. This binding is independent of the translocase and can occur before 1 - 4.

6. Covalent attachment of N-acetylglucosamine to the lipid-linked monosaccharide with release of UDP.

Subsequently, the disaccharide pentapeptide is transferred to nacent peptidoglycan by the transglycosylase domain of the PBP, undecaprenyl pyrophosphate is released and dephosphorylated by the undecaprenyl pyrophosphate phosphatase.

This model is based upon our data and observations of other laboratories. The existence of a multi-enzyme complex has been suggested by results from several laboratories . Taku and Fan (1980) found that only polymerase activity (probably the PBP) could be separately extracted from the membranes of E. coli. All of the other enzyme activities remained together. Anderson et al. (1972) proposed peptidoglycan and teichoic acid synthetic complexes as a rationale for the organized use of the C_{55}-lipid carrier molecule in Bacillus sp., which is used in both pathways.

Our data also suggests that the membrane-associated enzymes of peptidoglycan synthesis exist in a multi-enzyme complex. The stimulation of incorporation of label from UDP-MurNAc-[^{3}H]pentapeptide by the addition of UDP-GlcNAc suggests a close association of these two enzymes. The addition of the substrate of the second enzyme might not be expected to stimulate the activity of the first, if the two proteins were free to laterally diffuse in the membrane, depending on chance collision for catalysis.

The association of a molecule of undecaprenyl phosphate with each enzyme complex was suggested by Anderson et al. (1972). They proposed that a single molecule might be shared by the teichoic acid and peptidoglycan synthetic complexes, and that only in the presence of the appropriate nucleotide precursors could the enzymes bind the

phospholipid. Taku and Fan (1980), however, found that "lipid-depleted membranes" of E. coli could still synthesize the lipid-linked intermediate, indicating the continued association of the C_{55}-isoprenylphosphate even after ammonium sulfate precipitation and cholate extraction. In addition, it has been shown that, although the C_{55}-isoprenylphosphate is used as a carrier in both lipopolysaccharide and peptidoglycan synthesis, separate pools exist for each synthetic activity (Rundell and Shuster, 1975). These observations in all support the concept of C_{55}-isoprenylphosphate which is tightly associated with the synthetic complex. Free C_{55}-isoprenylphosphate may actually be very rare, the only common species being that bound to the enzyme complex.

The opening in the protein complex to the lipid bilayer extends from one side of the membrane to the other allowing the lipid carrier molecule to maintain the hydrophobic tail within the membrane and the charged head group bound to an active site within the synthetic complex. This arrangement allows for the C_{55}-isoprenylphosphate, with an extended length of 54 Å (Weppner and Neuhaus, 1978; V. Madison, personal communication) to reside within the lipid bilayer, with an estimated thickness of 30-40 Å. This model is also consistent with the data of Weppner and Neuhaus (1978) which indicated that the lipid-linked monosaccharide, dansylated at the third position of the

pentapeptide side chain, was immobilized within the membrane, with the dansyl reporter group within 4 to 6 Å of the lipid environment. This observation may correspond to the lipid-linked monosaccharide bound to the active site of the translocase (or transferase) with the hydrophobic tail of the lipid extending out through the opening and the third position of the pentapeptide side chain close to the opening, away from the active site. Thus the dansyl reporter group is immobilized, being bound to the enzyme complex, but still lies close to the hydrophobic environment of the membrane.

The relative lack of specificity of the translocase for the third amino acid of the pentapeptide side chain is also consistent with this hypothesis. The K_m for the UDP-MurNAc-pentapeptide from S. aureus was 3.5-4.0-fold higher than the natural substrate. Hammes and Neuhaus (1974) found that the K_m of the S. aureus translocase for the UDP-MurNAc-pentapeptide from E. coli was only 1.1 times higher than that for the natural substrate. These two species of UDP-MurNAc-pentapeptide differ in the amino acid residue in the third position of the side chain, DAP in E. coli, lysine in S. aureus. The K_m values for both substrates in both organisms are similar enough to indicate that recognition by the enzyme of the third position of the side chain is not crucial.

The membrane spanning feature of this model accounts

for the observed ability of vesicles to utilize peptidoglycan precursors provided on either side of the membrane. The complex does not act as a non-specific pore or transport mechanism, however. The model presumes the enzyme complex to have complete specificity for the substrates and products of the catalyzed reactions. UDP-MurNAc-pentapeptide and UDP-GlcNAc do not accumulate within the lumen of vesicles of either orientation indicating that merely pass through. The flow of substrates through the enzyme complex is channelled by the nature of the environment (hydrophilic substrates within the hydrophilic enzyme complex surrounded by the hydrophobic lipid bilayer) and the high affinity each enzyme has for its substrate. Thus a substrate molecule can reach its binding site from either side of the membrane, but cannot pass by an occupied site.

The K_m values calculated for the two substrates are very similar for ISOs and RSOs, but are consistently higher for RSOs. Since the same enzymes are being measured in each case, this difference may indicate a difference in the access of the substrates to the active sites in vesicles of each orientation, with the active sites in ISOs being slightly more accessible. Assuming that the enzymes are more accessible to the inside of the cell and therefore to external substrates in ISOs, a simplified reaction mechanism in ISOs would be:

$E\text{-}C_{55}\text{-}P$ + UDP-MurNAc-pentapeptide <=====>

$E\text{-}C_{55}\text{-}P\cdot$UDP-MurNAc-pentapeptide <=====>

E + UMP + C_{55}-PP-MurNAc-pentapeptide

This reaction mechanism in RSOs would have the additional step of the substrate reaching the active site:

UDP-MurNAc-pentapeptide (outside) <=====>

$E\text{-}C_{55}\text{-}P$ + UDP-MurNAc-pentapeptide (within channel)

The rate of this additional step would presumably be less than in ISOs, and the K_m for RSOs would appear higher.

The appearance of some Lineweaver-Burk plots of ISO and RSO rates are striking. Figures 16 and 17 show essentially parallel lines for vesicles of opposite orientations. The ratio of V_{max} to K_m in each case is very similar for RSOs and ISOs. Interpretation of these observations is problematic because of the complexity of the system. Incorporation from UDP-[^{14}C]GlcNAc provides an indirect measurement of the translocase; measurement of the N-acetylglucosamine tranferase is dependent on the activity of the translocase. In addition, product analysis showed that polymerized product was also formed, and therefore transglycosylase activity was also being measured.

The movement of substrates and intermediates through the enzyme complex is facilitated by the proximity of the active sites of the different enzymes. The location of the intermediates in the pathway within the membrane was

determined by Manteuffel and Neuhaus (1987). The dansylated undecaprenyl-phospho-N-acetylmuramyl-pentapeptide was found to lie 8 Å from the surface of the membrane in G. homari. The lipid-linked disaccharide was found in two positions, 4 and 20 Å from the membrane surface. The deeper position was shown to be used as a substrate for the polymerase more rapidly than the more shallow position. In our model, these two populations could be bound to sites corresponding to the active sites of the N-acetylglucosamine transferase and the transglycosylase. The orientation of the membranes used by Manteuffel and Neuhaus was not stated. Membranes were prepared by mechanical disruption with plastic beads, followed by differential centrifugation. This method would probably result in small membrane fragments yielding ISOs, but an unknown amount of contamination with RSOs could exist. Thus the deeper site, 20 Å from the surface of ISOs, could actually be closer to the exterior surface and the active site of the transglycosylase.

Weppner and Neuhaus (1978) also reported two populations of fluorescently labelled lipid-linked monosaccharide in membranes from S. aureus. One, making up 23% of the total, was quenched by an aqueous quencher, indicating it was close to the surface of the membrane. The second population was unaffected. These results may also be due to distribution of the lipid-linked

monosaccharide between the binding site for the translocase and the binding site for the N-acetylglucosamine transferase. If these two binding sites are near each other, the substrates and intermediates may be "channelled" through the multi-enzyme complex. This effect has been described for several steps of pyrimidine biosynthesis as the passage of a reaction intermediate from one enzyme-active site to the next without liberating it into solution (Traut and Jones, 1977; Christopherson and Jones, 1980). The model proposed here for peptidoglycan synthesis provides for the compartmentalization of substrates and intermediates in a hydrophilic environment within the membrane, isolated from solution. Close proximity of active sites would increase the efficiency of the pathway and take complete advantage being part of a multi-enzyme complex.

The active site of the transglycosylase domain of the PBP may also be within a short distance. The transglycosylase itself is close to the surface of the membrane, a position mandated by the periplasmic location of the transpeptidase domain of this bifunctional enzyme. The location of the transglycosylase, at the membrane-solvent interface, is responsible for the vectorial flow of substrates and intermediates through the multi-enzyme complex, both <u>in vivo</u> and in vesicles of both orientations. The polymerized product of the transglycosylase is the

substrate for the periplasmic transpeptidase. Thus, the series of active sites of the multi-enzyme complex extends from the interior of the membrane to the surface and out into the periplasmic space. This mechanism does not require the rotation of the enzyme complex or the expenditure of energy.

The disaccharide unit is released from the lipid-carrier molecule by the transglycosylase, leaving the C_{55}-isoprenyl pyrophosphate. The phosphatase, which is also part of the multi-enzyme groove, has an active site close to that of the transglycosylase. Thus, the lipid-carrier can quickly be regenerated and recycled. Breaking of the phosphate bond by the phosphatase may provide energy for conformational change needed to return the C_{55}-isoprenylphosphate to the active site of the phospho-N-acetylmuramyl-pentapeptide transferase.

Differences between ISOs and RSOs

One of the major differences observed in the activity of RSOs and ISOs is the nature of the product formed when provided with the nucleotide-linked precursors of peptidoglycan synthesis. One of the two main products of ISOs is a polymer of N-acetylglucosamine and N-acetylmuramyl-pentapeptide. Very little of this product is found in RSOs. The primary product of RSOs is a lipid-linked saccharide, mainly consisting of one and two

disaccharide units. This product is the substrate for the transglycosylase and thus precursor of the polymer. This lipid-linked species is also found in ISOs. In time course experiments, it was shown that in ISOs the lipid-linked product was "chased" into polymer, presumably utilized as substrate by the transglycosylase. In RSOs, the lipid-linked product also disappeared in a time-dependent manner, but no other product appeared. Active transglycosylase was present in RSOs, as indicated by the activity of prasinomycin, a specific inhibitor of the enzyme, and by the time-dependent disappearance of the lipid-linked product in the pulse-chase experiments. Inhibition of the transglycosylase by prasinomycin caused an increase in the amount of lipid-linked product.

Together these results suggest that the difference in the product of RSOs and ISOs is due solely to the orientation of the vesicles and not to any intrinsic difference in enzyme activity. The unidirectional polymerization, from inside to outside of the cytoplasmic membrane, appears to be due to the exclusive location of the PBPs on the exterior surface of the inner membrane. Since this corresponds to the interior of the ISOs, the product of the transglycosylase is deposited within the vesicles. This same sequence of events results in the polymer being extruded from the surface of RSOs into the bulk solvent. The methods normally used to detect activity

in the assays, filtration or centrifugation, apparently do not detect the polymerized product of RSOs. The glycan strands may be very short and not retained on filters or brought down by the usual centrifugation. However, prolonged centrifugation and ultracentrifugation of RSO reaction mixtures resulted in a larger proportion of polymerized product. In light of the low R_f of the dimer, immobile polymer may include product consisting of only three or four disaccharide units.

The distribution of label in the product of ISOs and especially RSOs also appears different when the labelled substrate is UDP-MurNAc-[^{3}H]pentapeptide than when [^{14}C]-UDP-GlcNAc is used. Many more [^{14}C]-labelled bands appear than [^{3}H]-labelled bands. The difference in specific activity of the two substrates is probably not sufficient to account for this difference. The product of incorporation of UDP-[^{14}C]GlcNAc in the absence of UDP-MurNAc-pentapeptide, however, revealed that this substrate is also utilized by enzymes which are not part of the peptidoglycan biosynthetic pathway. RSOs incorporated label from UDP-[^{14}C]GlcNAc in the absence of UDP-MurNAc-pentapeptide much more than ISOs. These products may be involved in lipopolysaccharide synthesis, for which UDP-[^{14}C]GlcNAc is also a substrate. The enzymes which synthesize lipopolysaccharide are also membrane-bound. It is possible that RSO reaction products which are found in

the absence of UDP-MurNAc-pentapeptide and which have R_fs similar to peptidoglycan precursors, are actually Lipid A-core oligosaccharide precursors.

Interestingly, it is this "lipid-linked" species formed in the absence of UDP-MurNAc-pentapeptide which decreased after muramidase treatment, accompanied by the inexplicable increase in immobile polymer in RSOs. This effect was observed in the presence and absence of UDP-MurNAc-pentapeptide, suggesting that this is a product of some secondary that synthesis occurs concomitant with, but independent of, peptidoglycan synthesis in RSOs. Several reports have appeared in the literature on the effect of lysozyme on lipopolysaccharide. The effect was measured in vivo as the loss of toxicity of LPS after lysozyme treatment (Caselli and Callerio, 1960; Michaels and Eagon, 1970). More recently, Ohno and Morrison (1989) reported the binding of lysozyme to lipopolysaccharide which results in the inactivation of the enzyme. Binding occurs at hydrophobic regions of lysozyme, near the active site, suggesting interaction with the lipid A region of the LPS. Although the muramidase used in our experiments has not been as well characterized as egg white lysozyme, it may also have this characteristic. The polymerized product which appeared after treatment with this enzyme could be [^{14}C]GlcNAc-labelled LPS, bound to the muramidase. This binding could account for the change in mobility of the

labelled product. Alternatively, cleavage of some portion of [^{14}C]GlcNAc-labelled LPS from the rest of the oligosaccharide core-lipid A molecule by the muramidase could result in a labelled polymer large enough or polar enough to be immobile in this TLC system.

Harrington and Baddiley (1983) also reported the UDP-MurNAc-pentapeptide-independent incorporation of UDP-[^{14}C] GlcNAc by partially autolyzed B. subtilis. Incorporation was not inhibited by tunicamycin, leading the authors to conclude that the product was not synthesized through the lipid-linked intermediate. Our results are different, in that the product formed appears to be lipid-linked. Tunicamycin inhibited in RSOs by 74%, consistent with formation of the lipid-linked product. In ISOs, the amount of UDP-MurNAc-pentapeptide-independent incorporation was much less than in RSOs and tunicamycin inhibited by only 14%. Although the product formed in ISOs had the high mobility by TLC that is characteristic of lipid-linked species, it did not coincide with the lipid-linked band found in the presence of UDP-MurNAc-pentapeptide, or with the major bands formed by RSOs in the absence of UDP-MurNAc-pentapeptide. These results may indicate that the enzymes of LPS synthesis, unlike peptidoglycan synthesis, are accessible from only one side of the membrane, the outside.

Identification of reaction products

To avoid confusion caused by UDP-MurNAc-pentapeptide-independent incorporation, the products of vesicles of both orientations were identified from experiments using UDP-MurNAc-[^{3}H]-pentapeptide and cold UDP-GlcNAc. The bands corresponded to: 1) lipid-linked saccharide; 2) N-acetylmuramyl-pentapeptide; 3) N-acetylglucosamine-N-acetylmuramyl-peptide (disaccharide or monomer); 4) phospho-N-acetylmuramyl-pentapeptide; 5) (N-acetylglucosamine-N-acetylmuramyl-peptide)$_2$ (bisdisaccharide or dimer) and, 6) polymer.

All of the above were found in RSO reaction mixtures. All but 2 and 4 were found in ISOs. Heydanek et al. (1969) found phospho-N-acetylmuramyl-pentapeptide in reaction mixtures measuring the activity of the translocase. They suggested that this compound could result from the simultaneous release of UMP and phospho-N-acetylmuramyl-pentapeptide by the enzyme. The dephosphorylated product was formed when alkaline phosphatase was included in the reaction mixture. In our assays, the acid conditions used to terminate the reactions may produce the N-acetylmuramyl-pentapeptide. Neither of these compounds has any known significance *in vivo*.

Identification of monomer and dimer bands was difficult. The UDP-MurNAc-[^{3}H]pentapeptide used in the assay and as a chromatography standard is contaminated with a second compound, probably UDP-MurNAc-tripeptide. These

two bands are very close in R_fs to the monomer and dimer standards. The identification was based upon the effect of prasinomycin on the density of the bands, as well as mobility on TLC. If the bands were due to contamination with substrate, the same amount of tripeptide should always be present. In fact the intensity of the band varied from lane to lane. Again, these two products are probably not of any importance *in vivo*. Their formation may be due to the inefficient activity of the transglycosylase or the acid lability of the lipid-linked product.

As previously discussed, very little polymer was found in RSOs, while it was the major product of ISOs. Digestion of ^{14}C-labelled product with muramidase followed by TLC and autoradiography revealed that, in ISOs, this product was only slightly crosslinked. The major product of the digested samples corresponded to monomer, indicating that few peptide cross bridges were present in the polymer. Only a faint band corresponding to dimer appeared in the digested samples.

PBPs of ISOs and RSOs

It is difficult to assess the difference in PBP patterns of RSOs and ISOs. The PBPs of RSOs appear to be identical to the pattern found in many different strains of *E. coli* as reported in the literature. In addition, this laboratory has used this strain of *E. coli* for many years and we have compared the PBPs of ether-treated cells with

the pattern found using isolated membranes prepared using a French pressure cell. In each case the pattern appeared to be identical to the RSO pattern. Despite repeated attempts using up to 5 times the protein content of RSO assays, the PBP pattern seen in ISOs is very faint and contains bands with abnormally low molecular weights. That the transglycosylase activity of the PBPs is functioning in ISO vesicles is indicated by polymer formation. Since the major product of muramidase digestion of the polymerized product of ISO vesicles coincides with monomer, there is little strong evidence of active crosslinking, the putative target of inhibition by penicillin. It is unlikely, however, that one of the two activities of these bifunctional enzymes could be completely destroyed while the other activity was unaffected.

Alternatively, the apparent sparseness of PBPs and lack of inhibitory effects of penicillin in ISOs could be due to the inability of penicillin to penetrate the plasma membrane. No definitive evidence exists that penicillin does reach the cytoplasm in living cells. The PBPs in ISOs are on the opposite side of the membrane from the penicillin and may be safely beyond its inhibitory effects. However, attempts to disrupt ISOs by sonication and allow [^{14}C]-penicillin access to the PBPs did not increase binding.

An additional, and possibly related, question is the

difference in specific activity between ISOs and RSOs. The low ratio of activity to protein content of ISOs is not due to contamination with outer membranes and their proteins. In fact, RSO preps contain more contaminating outer-membrane proteins than ISOs. An explanation may lie in the model of the multi-enzyme complex. If close association between the enzymes of the complex is necessary for activity, the methods used for ISO preparation may be sufficiently harsh to disrupt that association. ISOs are formed from small fragments of broken membranes. The forces exerted by the French pressure cell may also break apart some of the peptidoglycan-synthesizing complexes. The enzymes may be present in the ISOs but not in complexes or perhaps in incomplete complexes, in either case inactive.

Exchange reaction in vesicles

It is difficult to assess the significance of the exchange reaction in vivo. Synthesis of the UDP-MurNAc-pentapeptide in E. coli has been reported to be under metabolic control (Lugtenberg et al., 1972); precursors do not accumulate to any great extent if peptidoglycan synthesis is inhibited by antibiotics or by mutation. The kinetics of incorporation of UDP-MurNAc-pentapeptide and UDP-GlcNAc also indicate that the exchange reaction is unlikely to occur in vivo. Rates of synthesis of the lipid-linked disaccharide by both RSOs and ISOs were 2- to

4-fold higher the rates of synthesis of the monosaccharide (See Table 9). Thus, in the presence of UDP-GlcNAc, the forward reaction would almost certainly predominate. In the absence of UDP-GlcNAc, the synthesis of UDP-MurNAc-pentapeptide would be inhibited.

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