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Effect of inhibitors of cell wall synthesis on phospholipid composition and metabolism in Bacillus stearothermophilus

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THE EFFECT OF INHIBITORS OF CELL WALL SYNTHESIS ON PHOSPHOLIPID COMPOSITION AND METABOLISM IN *BACILLUS STEAROTHERMOPHILUS*

by

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The effect of cell wall inhibitors on phospholipid composition and metabolism in Bacillus stearothermophilus. (75 pp.)

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The effect of various inhibitors of peptidoglycan synthesis on the composition and patterns of metabolism of membrane phospholipid was studied in whole cells and isolated membrane preparations. Bacitracin, vancomycin, and ristocetin, all of which block steps of cell wall synthesis involving the bactoprenol lipid carrier cycle, caused a marked elevation of the cardiolipin concentration with a corresponding decrease in phosphatidylglycerol. Pulse labeling studies revealed that phospholipid turnover was unaffected, however, an as yet unidentified lipid (unknown A) was seen to accumulate. Bactoprenol intermediates were extracted from washed membranes, and on comparison it was found that the unknown A corresponded to the second of three major bands. Further characterisation of this unknown lipid by mild acid hydrolysis (in order to remove the end group from bactoprenol) yielded a product that was soluble in chloroform and chromatographed on SG 81 papers apparently as a phospholipid. Subsequent deacylation of this acid hydrolysis product revealed a glycerol phosphate ester, whose chromatographic properties were similar to the unknown described by Card (Card, G.L.,J. Bacteriol. 114: 1125-1137, 1973.) Cardiolipin accumulation in vancomycin treated cells was shown to occur after a delayed response. Time dependent studies of the bactoprenol intermediates isolated from membranes treated with vancomycin showed that as the unknown A decreased with respect to time, there was an apparent increase in bactoprenol-PP. This also corresponded with the increase in cardiolipin content. These results were interpreted as evidence for the coupling of phospholipid metabolism with cell wall synthesis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Fig.12. The effect of time on vancomycin treated cells as revealed by two dimensional chromatography on SG 81 papers of the total lipid fraction, and by thin layer chromatography of bactoprenol intermediates isolated from washed membranes.

Reality has no obligation to be interesting; only our hypotheses about it...

Jorge Luis Borges " Death and the Compass "

INTRODUCTION

The concept of the lipid bilayer as the principal mode of packing for lipids in biological membranes is now 50 years old, and in recent years this has been developed into the generally accepted Fluid Mosaic Model (110). There is now compelling evidence that membranes are vectorial structures; that is, there is an asymmetric distribution of not only their protein constituents but also their phospholipid components (4, 8, 9, 82, 100, 101). The physiological relevance of phospholipid asymmetry is unknown. Asymmetric lipid compositions could possibly regulate differential membrane fluidity, or act as bilayer couples; here, they would differentially respond to perturbation (8). One clearly established fact is that there is no universal preference with regards to the bilayer distribution of a single phospholipid species (8, 82, 100).

Any model dealing with membrane topography must allow for the membrane's remarkable ability to maintain it's integrity. By changing it's phospholipid composition, an organism has the means to withstand the pleiotrophic changes within it's environment. A ready interpretation in certain cases could be the maintenance of a suitable anionic surface charge. This can be readily achieved by an alteration of the distribution of the acidic and basic phospholipids within the membrane. The question of how an organism can adapt so readily remains unresolved. Phospholipids have an extremely high rate of lateral diffusion (62), whereas trans-bilayer interchange (flip-flop), which is thermodynamically unfavorable, proceeds very slowly (8, 89). Rothman and Kennedy (99) when studying Bacillus megaterium, determined that the principal phospholipid, phosphatidylethanolamine (PE), is synthesised in an internal pool with subsequent rapid translocation to the extra-cytoplasmic side of the mem-

brane. They suggest that this rapid translocation - on the order of three minutes - is due to catalysis by a specific membrane protein.

Membranes are composed primarily of phospholipid and protein. Phospholipids are amphipathic molecules that account for 20-40% of the dry weight of bacterial membranes; the remainder representing the protein content. The hydrophylic and hydrophobic ends arise due to the unique structure of phospholipids. All phospholipids contain a glycerol backbone, to which fatty acids are usually esterified in the 1 and 2 positions. The polar head group is joined by a phosphodiester linkage to the 3 position of this diglyceride molecule. The distribution of the polar head groups determines the composition of the membrane. In reality, each individual phospholipid species can change within itself. This is achieved by changing it's fatty acids. Changes resulting from this type of molecular rearrangement tend to affect membrane fluidity (25). The study of the biosynthesis of the individual phospholipid species, as determined by the polar head group, has come under considerable attention in procaryotic organisms during the past two decades, and it is this aspect that is the primary concern of this present study.

Phospholipid metabolism as determined for Escherichia coli.

The vast majority of this reseach has dealt with the Gram negative bacterium Escherichia coli. The phospholipid composition of E.coli wild-type cells is PE, phosphatidylglycerol (PG), and cardiolipin (CL), in the approximate ratios 70:20:10 respectively. Kennedy and his coworkers (89) have been able to elucidate the biosynthetic pathway from either glycerol or dihydroxyacetone to each of the individual phospholipid species. The scheme presented in Fig. 1 has been verified by

three different experimental approaches. These are:

1. Classical demonstration of enzymes in crude extracts capable of catalysing these reactions;
2. Radioactive labeling to demonstrate the existance and turnover of such intermediates;
3. The isolation and lipid composition of mutants defective in particular enzymes in Fig. 1.

Beginning with the acylation of sn-glycerol-3-P, all of the reactions occur on the cytoplasmic membrane (6, 127). Phosphatidic acid (PA) formed from this initial acylation is next converted to a liponucleotide, CDP-diglyceride, by it's reaction with CTP. CDP-diglyceride is located at the biosynthetic branchpoint. From here, phosphatidyl transferase reactions donate the phosphatidyl moiety to either the hydroxyl group of L-serine or to the hydroxyl group at the 1 position of sn-glycerol-3-P to form phosphatidylserine (PS) and phosphatidylglycerolphosphate (PGP) respectively. PS is rapidly decarboxylated to form PE: PGP is dephosphorylated to PG. PG however, is further metabolised by a condensation reaction to form CL. This latter reaction requires no metabolic energy. It has long since been recognised that PG is a rapidly metabolising molecule with approximately 1/3 turning over in a single generation in E.coli (103).

All of the proposed enzymes in Fig. 1 have been at least partially purified and characterised to some extent. The lone exception is cardiolipin synthetase which we have found to be resistant to extraction by the use of detergents (unpublished observation). The common pathway proceeds with the synthesis of PA. Membrane bound acyltransferases ester-

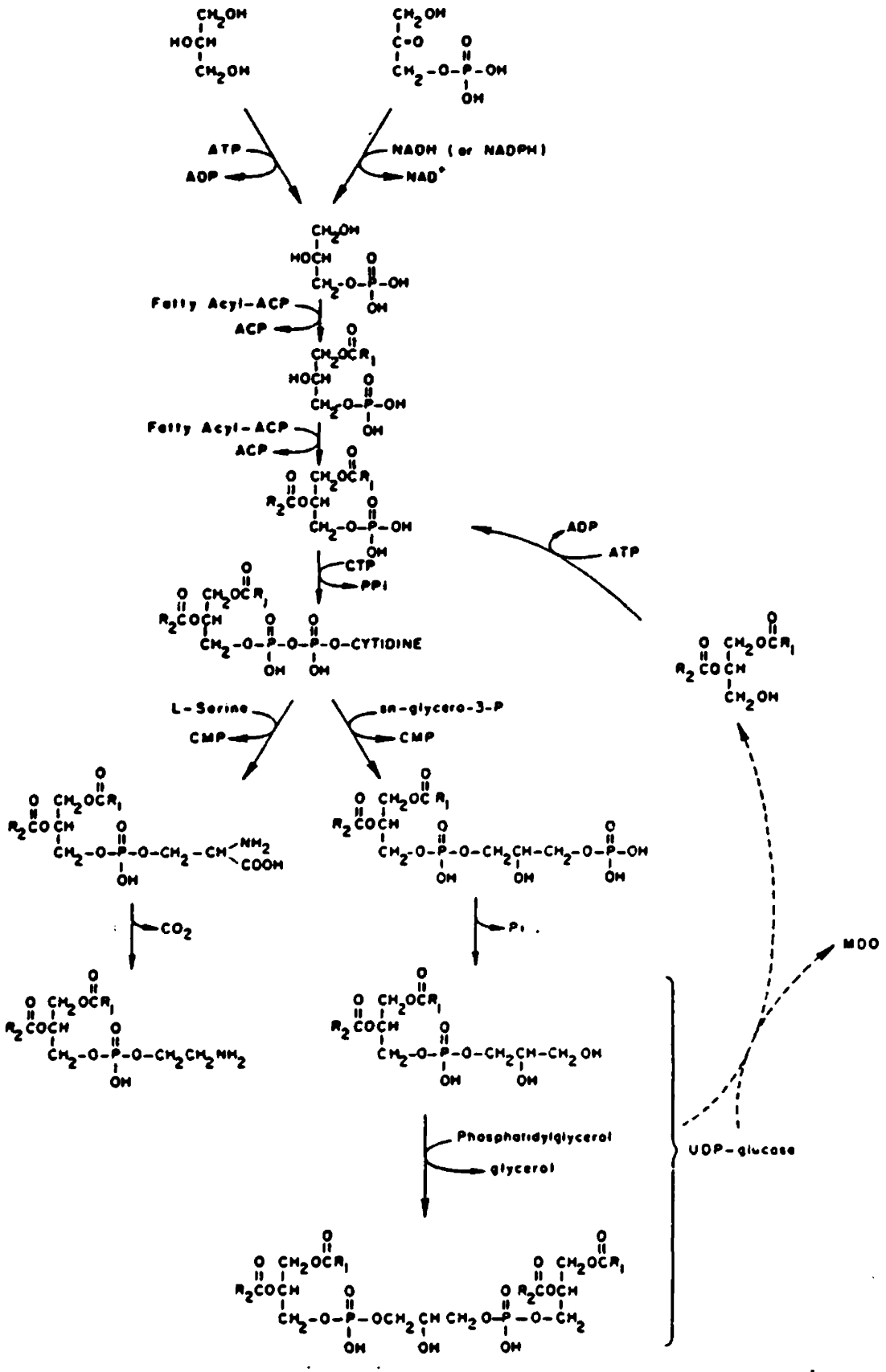

Fig.1. The pathway of phospholipid metabolism as determined for Escherichia coli (89).

ify positions 1 and 2 of sn-glycerol-3-P. Kito's group (55, 56) has found that these reactions are activated by PG. The acyltransferases appear to be the key regulatory enzymes in response to temperature fluctuations (25). Studies with a glycerol auxotroph under glycerol starvation, have revealed that the synthesis of the phospholipids and fatty acids are co-ordinately controlled. During glycerol depletion, free fatty acid accumulates in the cytoplasm of the mutant (76). CDP-diglyceride synthetase is the enzyme that converts PA into the lipo-nucleotide. This enzyme, unlike others in the pathway, appears to have a specific requirement for an unsaturated fatty acid esterified to position 2 (89). Recently, Ganong and Raetz (37) have isolated a conditionally lethal CDP-diglyceride synthetase mutant in which there is a sharp elevation in PA content, with a corresponding decrease in PE and PG. The authors claim that the results "constitute definitive physiological proof that CDP-diglyceride is an obligatory precursor for over 90% of PE and PG in E.coli."

From CDP-diglyceride we find two different enzymes, phosphatidylserine synthetase (PSS) and phosphatidylglycerolphosphate synthetase (PGPS), that ostensibly catalyse similar reactions; the transfer of the phosphatidyl group from CDP-diglyceride to an alcohol.

In eucaryotic systems, the synthesis of PE involves a head group exchange reaction of L-serine and PE, followed by irreversible decarboxylation (12, 27). However, in procaryotes, no such mechanism is known to occur. Also, PSS derived from bacteria could well be the one exception to the general observation that all the enzymes involved in the latter stages of phospholipid biosynthesis are membrane bound. In vitro

ribosomal association has been documented on numerous occasions (30, 58, 92). Dowhan's group (67) suggests that ribosomal association does occur in vitro, however they found that PSS is easily dissociated from this complex by lipid substrates and they argue that this would indicate that the enzyme would not be closely associated with ribosomes during actual catalysis within close proximetry of the membrane. When studying the kinetic parameters of the enzyme, Ishinaga (54) also noted the effect of various phospholipids. He found that CL and PE activated the enzyme, in contrast to PG's inhibitory effect. Phospholipid compositions of E.coli mutants for the PSS gene have shown a decrease in PE with a concomitant rise in CL when compared to wild-type (45, 78, 79). Raetz (91) has also shown that this rise in CL occurs in both the inner and outer membranes of the PSS mutant. It is interesting to note that the PE content in any of the above mutants never falls below 25%.

Homogeneous PSS not only transfers the phosphatidyl group from CDP-diglyceride to L-serine, but also exchanges free CMP with the CMP moiety of CDP-diglyceride, and L-serine with PS (64, 93). These exchange reactions suggest the possibility that there may well be a ping-pong type of reaction with the formation of an intermediary phosphatidyl complex (18, 93). K_m values show that PSS has a very low affinity for L-serine (93). Utilising a mixed micellar system of Triton X-100 and CDP-diglyceride, it was found that increasing the concentration of the detergent maximally stimulated the the PSS activity. This is consistent with "substrate dilution kinetics" in which an equilibrium ordered binding of the enzyme to a surface occurs prior to catalysis (18). Consequently, the stability of the phosphatidyl-enzyme complex may be important in controlling the

ratio of the zwitterionic to acidic phospholipids if in fact PSS is only membrane bound during catalysis.

The final step in converting CDP-diglyceride to PE occurs by a rapid decarboxylation of PS. Treatment of wild-type cells with hydroxylamine (an inhibitor of the decarboxylase enzyme) results in the accumulation of large amounts of PS. This shows that in E.coli decarboxylation represents the major biosynthetic route for the formation of PE in vivo.

The synthesis of PGP represents the first committed step towards the formation of PG and CL. PGPS catalyses the esterification of the 1-hydroxyl group of sn-glycerol-3-P by the transfer of the phosphatidyl moiety from CDP-diglyceride (21). Consequently, the non-acylated glycerol of PGP is bound to the phosphatidyl group by a sn-glycerol-1-phosphodiester linkage. The occurance of this particular enantiomeric form of a glycerol phosphate bond is exceedingly rare (sn-glycerol-3-P being by far the most common in nature). PGPS is able to catalyse the reverse reaction. However, due to the highly irreversible nature of the dephosphorylation step that forms PG, the reaction is pulled in the forward direction (49). The homogeneous enzyme is specific for sn-glycerol-3-P but it's mode of action appears to differ from that of PSS. PGPS is unable to catalyse the exchange reactions between CMP and CDP-diglyceride, and sn-glycerol-3-P with PGP. Reversal of the reaction occurs by incubating PGP with CMP (21, 49).

PGP has never been isolated from wild-type cells due to the rapid dephosphorylation by phosphatidylglycerolphosphate phosphatase (22). The phosphatase has a relatively high affinity for PGP and partially

purified fractions do not hydrolyse sn-glycerol-3-P at any significant rate (22).

Available evidence suggests that CL is undoubtedly formed by the stoichiometric condensation of two PG molecules to form CL and free glycerol as shown by the following equation:

$$2 \text{ PG} \longrightarrow \text{CL} + \text{glycerol}.$$

The reaction requires no metabolic energy, and is supported by in vivo evidence for the release of free glycerol from specifically labeled PG molecules (3, 17, 41, 50, 70). This reaction was further substantiated by in vitro CL synthesis using a particulate enzyme preparation from Micrococcus lysodeikticus (28). Characterisation of an E.coli mutant deficient in the enzyme cardiolipin synthetase resulted in 15 fold less CL and a 5 fold reduction in PG turnover (86). Definitive evidence for the above reaction came from two independent studies. Utilising Staphylococcus aureus, Short and White (106) showed that the fatty acids and the phosphorus groups from two PG molecules could be quantitatively recovered in CL. Likewise, Hirschberg and Kennedy (50) demonstrated that when PG is doubly labeled with ^{32}P and ^{3}H in the non-acylated glycerol of PG, that the ratio of $^{32}P/^{3}H$ recovered in CL was two times greater than in PG. This would be compatible with the above equation.

Both PG and CL are further metabolised in E.coli to a soluble extra-cytoplasmic product. Membrane-derived-oligosaccharide (MDO) consists of sn-glycerol-1-P moieties from both PG and CL joined to carbon 6 of glucose residues which are present in a glucose polymer (123). The formation of MDO and CL accounts for 75% of PG turnover in E.coli (89). The presence of a diglyceride kinase, which recycles sn-1,2-diglyceride

back to PA within the membrane, helps further to support the idea that MDO is derived from PG and CL (53, 95, 104). A double mutant, defective in both the kinase and MDO formation, caused a striking accumulation of diglyceride within the membrane (96). The presence of the kinase is interesting because it is well established (89) that acylation of sn-glycerol-3-P is the major de novo route of PA synthesis in vivo. The availability of ATP on the inside of the membrane suggests a cytoplasmic location for the kinase (96). If this were the case then it would necessitate the translocation of MDO across the membrane. An interesting property of the enzyme is the strong effect exerted by CL; both stimulatory and in the stabilisation of the enzyme itself (104).

Regulation of phospholipid synthesis.

Clarification of our knowledge as to the co-ordination of membrane lipid, membrane protein, and macromolecular synthesis in growing cells would be greatly enhanced by the elucidation of the controls of phospholipid metabolism. Early studies of phospholipid mutants demonstrated that genetic manipulation was possible (47). With the advent of Raetz's selection procedure (87) for mutants defective in phospholipid metabolism we have seen a great accumulation of genetic information (88). As the greatest expense of energy within phospholipid biosynthesis is due to the synthesis of the fatty acids, one would therefore expect a certain degree of control to be exerted upon fatty acid availability. Indeed, there appears to be a tight coupling of phospholipid and fatty acid syntheses in bacteria (76). Additional regulation could occur at areas in the metabolic pathway where common intermediates such as PA or CDP-diglyceride are formed. As yet, the control of CDP-diglyceride synthe-

sis is ill defined. However, several mutants have been isolated for E.coli that have defective genes for PSS and PGPS (36, 78, 80, 81, 88, 94, 113). An important fact that appears to emerge from the above studies is that "the degree of alteration of the phospholipid composition brought about by an increase in enzyme concentration is not in the order that would be expected if fluctuations in enzyme levels in vivo were an important regulatory mechanism in phospholipid metabolism (81)". It should be noted that drastic results do occur by decreasing the concentration of the phospholipid enzymes. Control would then appear to be mediated either by modulating the enzymatic activity or by controlling the availability of necessary substrates. Studies with a trans-acting regulatory mutant of E.coli for the over production of PS revealed a regulatory locus distinct from the structural gene that it controlled (113). A similar method of control was also recognized with mutants defective in the gene coding for diglyceride kinase (90). Sparrow and Raetz (113) proposed that the control of PSS production is by "set-point" regulation. This is neither classical induction nor repression. Here the gene product is maintained at all times at a certain level by the action of a regulator.

Phospholipid metabolism in Gram positive organisms.

We find that there are many striking similarities with regard to phospholipid metabolism in Gram positive organisms as those found in E.coli. Early on it was recognised in both Bacilli and S.aureus that all the enzymes that are needed for the biosynthesis of PE, PG, and CL are present (73, 83, 106) and that the biosynthesis of each phospholipid species proceeds via the CDP-diglyceride intermediate (83). Character-

isation of PSS (31) has demonstrated that CDP-diglyceride can act as a substrate for PS synthesis, and that the enzyme behaves in a similar fashion as that that was determined for E.coli's PGP. In vitro levels of PS decarboxylase were also reported. Comparable results have also been demonstrated for several other Gram positive organisms (19, 24, 63). The glycerophosphatide sequence had been elegantly demonstrated by Short and White (106).

Phospholipid turnover in Gram positive bacteria also appears to bear some similarity to that found in E.coli. Although MDO has not been found in Gram positives, the turnover of PG shows some resemblance in that the glycerophosphate groups of lipoteichoic acid appear to be derived from this molecule (17, 32, 33, 61, 74).

Nevertheless, several pieces of evidence have surfaced (based primarily from in vivo labeling studies) that suggest that an alternative pathway may exist in conjunction with the classic Kennedy scheme. Langley (63) has reported that PE was much more slowly labeled than the corresponding uptake by PG. He found that at early times in the pulse, PG sequestered 60 times more label than did PE (the latter's rate of labeling was comparable to that demonstrated by CL). He also found an elevated rate of PG turnover. Further pulse labeling studies with B.megaterium (65, 66) showed there to be two distinct metabolic pools of PG. The use of tritium showed that the label was recovered in diglyceride on utilising a short pulse, and that rapid turnover of the non-acylated glycerol of PG occurred with the liberation of the label in free glycerol. To account for this, Lombardi proposed the possibility of two intermediary complexes that would involve PG and two different acceptors to

provide:

1. Glycerophosphoryl groups for lipoteichoic acid with the release of diglyceride:

$$PG + X \longrightarrow \text{glycerophosphoryl-X} + \text{diglceride}$$

2. Phosphatidyl groups for PE synthesis with the liberation of free glycerol:

$$PG + Y \longrightarrow \text{phosphatidyl-Y} + \text{glycerol}$$

Similar pulse chase studies with Bacillus stearothermophilus also revealed biphasic patterns of turnover for both PG and CL. These results would suggest the existance of two pools for each lipid; one metabolically active and one that was stable (17). From the same study it was also shown that turnover of CL and the phosphatidyl group of PG caused the label to appear in lipoteichoic acid. To account for this Card further modified Lombardi's model in order to include CL synthesis:

$$PG + Y \longrightarrow \text{phosphatidyl-Y} + \text{glycerol}$$

$$\text{phosphatidyl-Y} + PG \longrightarrow CL + Y$$

The glycerol so released could then be recovered in lipoteichoic acid with a recycling of the initial acceptor. It was interest with regard to this proposed intermediary complex that prompted the present study.

Phospholipid metabolism in relation to other cellular metabolic processes

The regulation of macromolecular synthesis has come under a great deal of scrutiny during the past 20 years (20, 26, 39, 43, 69, 73, 77). In all these studies it was clearly understood that regulatory mechanisms must couple membrane metabolism with other macromolecular processes in order that balanced growth can be achieved. The molecular evidence in support of this conclusion is particularily scant. With the utilisation of synchronous cultures, several authors reported a transient

increase in phospholipid synthesis immediately prior to cell division (26, 43, 69). It has also been determined that phospholipid synthesis is coupled to DNA, RNA, and protein syntheses (20, 39, 40, 77). Studies with various systems have shown that the synthesis of membrane-associated protein is dependent upon phospholipid metabolism proceeding at a normal rate (20, 40, 77). The initial observation by Card that bacitracin (an inhibitor to cell wall biosynthesis) caused an increase in the CL content of exponentially growing cells, led us to believe that a possible direct correlation may exist between phospholipid metabolism and cell wall biosynthesis in B.stearothermophilus. Also, scattered references within the literature have often alluded to a correlation between changes in phospholipid composition accompanying antibiotic resistance by a microorganism (2, 10, 29, 52, 122). This is especially interesting when the primary inhibitory mode of the antibiotic is directed at some target other than the cytoplasmic membrane. It appears that Gram positive organisms are more prone to this phenomenom than their Gram negative counterparts (10, 29, 52).

Bacterial cell wall biosynthesis.

Bacterial cell wall biosynthesis and the study of antibiotic action are synonymous (35). From Card's initial observation with bacitracin, it appeared to us that attention should be focused primarily upon inhibitors of cell wall biosynthesis, with a special emphasis with regard to the inhibitors of the bactoprenol lipid carrier cycle. The pioneering work from Strominger's laboratory (107) revealed the nature of this unique procaryotic carrier system, and this work is summarised in Fig. 2. Their work unequivocably demonstrated that the antimicrobial effect

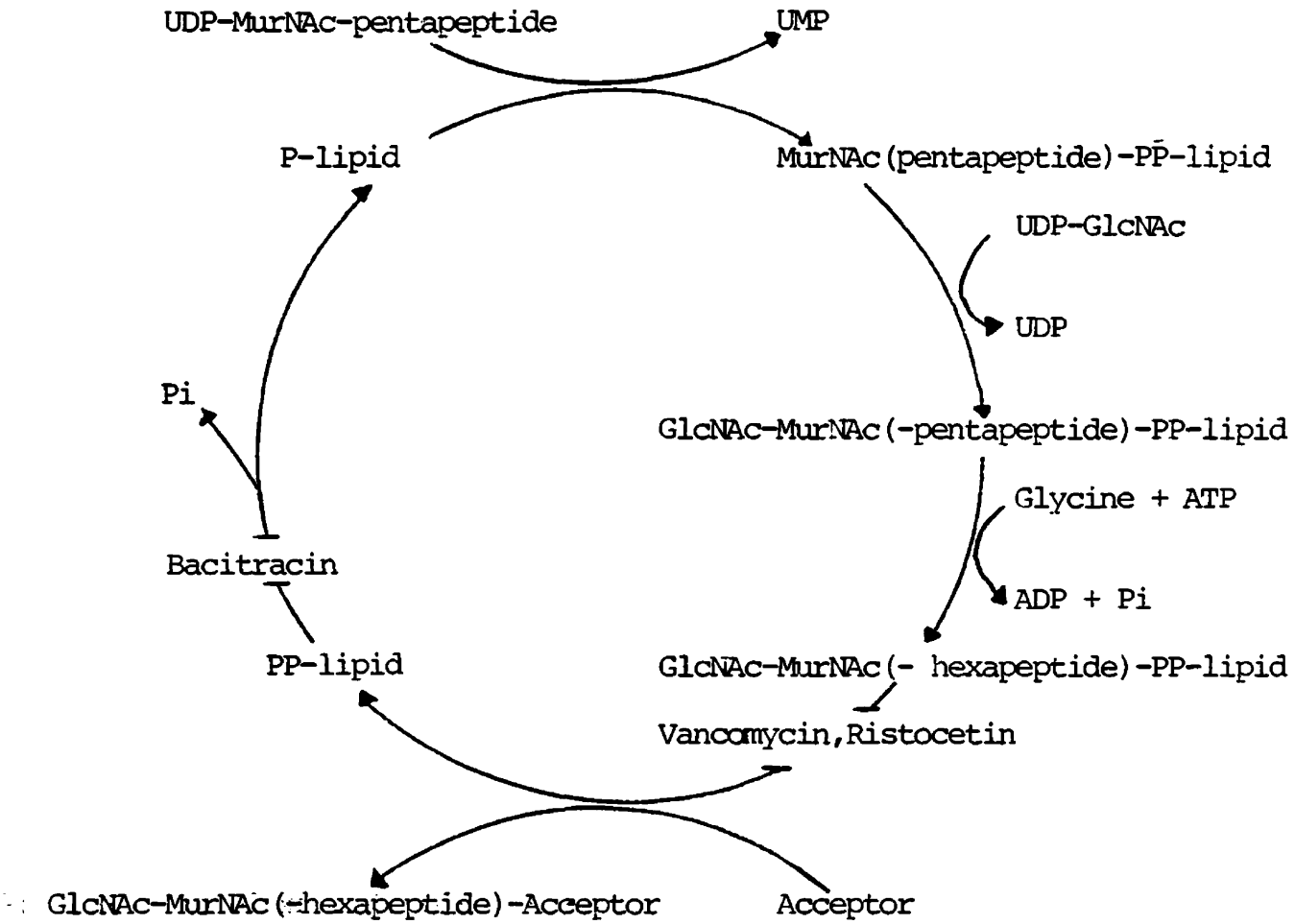

Fig.2. Reaction cycle of peptidoglycan synthesis in Micrococcus lysodeikticus.(107)

of bacitracin, a peptide antibiotic, to be due to the prevention of a crucial dephosphorylation step that is integral to cell wall synthesis. This occurred due to the formation of a complex between bacitracin, a divalent cation, and bactoprenol-PP (107, 116, 117, 118, 119).

The first step in the cycle involves a phospho-N-Acetyl-Muramyl-pentapeptide translocase (46). This is the first of several membrane bound enzymes. The reaction involves the transfer of N-Acetyl-Muramyl-pentapeptide from a soluble nucleotide diphosphate precursor to the mono-phosphorylated lipid carrier molecule to form the bactoprenol-PP-N-Acetyl-Muramyl-pentapeptide intermediate (121). From here, a second sugar residue is added, also utilising a nucleotide diphosphate derivative, and the nascent cell wall component is completed by the addition of the cross-linking amino acids (35). It was determined that several of these enzymes involved in this initial series of events had complex lipid requirements (115, 121). The final translocation step of the completed disaccharide-pentapeptide moiety to an acceptor on the existing cell wall was determined by utilising the inhibitory action of the two aglycone antibiotics vancomycin and ristocetin (71, 75, 84, 85, 98). Due to their interaction with acyl-peptides that terminate with the sequence D-Ala-D-Ala, which are the final two amino acids in the cross-linking peptide (84), these antibiotics prevented the polymerisation of the cell wall resulting in the accumulation of the bactoprenol-PP-disaccharide-pentapeptide intermediate (119). From here, the bacitracin sensitive step regenerates the initial bactoprenol-P acceptor due to the action of the enzyme bactoprenolpyrophosphate phosphatase and the cycle is complete (42).

When assaying particulate enzyme preparations obtained from S.aureus (48), it was found that ATP had a marked stimulatory effect on peptidoglycan synthesis. It was subsequently determined that 90% of the bactoprenol in S.aureus existed in the alcohol form (47). This and other data (38, 47, 48, 102, 129) have made the proposal that a possible regulatory mechanism may be occuring by the combined action of two enzymes, a phosphokinase and a phosphatase, that mediate bactoprenol-P availability appear most favorable. An unusual property of the phosphatase is that it has an optimal pH of 5. This is in contrast to the other enzymes in the cycle that operate around neutrality. Consequently, it is conceivable that pH gradients across the membrane are thereby a means for the cell to regulate peptidoglycan synthesis.

Further inhibitory action ascribed to bacitracin, vancomycin, and ristocetin.

In addition to these primary sites of action for the three antibiotics mentioned, numerous reports have appeared as to possible secondary effects that may also be attributable to them. For example, bacitracin has been shown to effect phospholipid metabolism in several microorganisms by decreasing the ability to incorporate (^{14}C)-glycerol into the lipid fraction of Halobacterium cutirubrum (5); the excretion of cellular lipids from a number of Gram positive organisms into the medium (51); or in the case of S.aureus, to cause an increase in CL content with a proportional decrease in PG (59). Consistent with bacitracin's inhibitory mode in procaryotes, the peptide has been a powerful tool in the study of glycolipid metabolism in eucaryotes. Studies utilising yeast membranes showed that bacitracin inhibited the transfer

of N-Acetyl-glucosamine from UDP-N-Acetyl-Glucosamine to the isoprenoid carrier dolichyl-P; utilising porcine aorta microsomes, bacitracin appeared to bind to the dolichyl-P itself (114); rat liver microsomes, showed bacitracin inhibition to be directed at the formation of dolichyl-P from endogenous precursor (60). In fact, bacitracin may well inhibit all processes that involve polyisoprenoid intermediates as postulated by Stone and Strominger (118).

Early reports of the inhibitory effects of vancomycin and ristocetin often alluded to possible secondary interaction with the cytoplasmic membrane (44, 57, 98, 105, 132). Experiments designed to locate the site of action of the two antibiotics revealed that adsorption to the membrane occured coincidently with binding to the cell wall (111). An iodinated vancomycin preparation showed that the affinity for the membrane was greater in whole cell cultures than for isolated protoplasts (85). Labeling studies with ^{32}P and (^{14}C)-glycerol in Gram positive bacteria (57, 132) revealed that there was a marked decrease in the uptake into the lipid fraction on incubation with vancomycin. Vancomycin also appears to have a selective effect on the synthesis of polyglycero- and polyribitolphosphate (13, 14). Using large concentrations of vancomycin (but not with ristocetin) caused a 60% decrease in the synthesis of teichoic acid. Consequently, the use of inhibitors to cell wall synthesis in persuing gainful information with regard to the study of phospholipid metabolism appears to already have some precedence within the literature.

The proposed objectives of this study.

The objective of this study was to investigate the perturbation of phospholipid metabolism caused by bacitracin, and determine whether in fact that cell wall synthesis and phospholipid metabolism are intimately linked. The specific aims were:

1. Identification of the lipid that accumulates on bacitracin treatment;
2. Quantification of the changes in lipid composition induced by the antibiotic;
3. An analysis of the changes in phospholipid synthesis and subsequent turnover that are induced by bacitracin;
4. The determination of the effect of other inhibitors to cell wall synthesis on the phospholipid composition and metabolism;
5. The isolation of bacitracin resistant mutants and an analysis of their phospholipid composition and metabolism.

MATERIALS AND METHODS

Growth conditions.

Bacillus stearothermophilus NCA 2184 was grown in a complex medium (TYE) consisting of 2% Trypticase (BBL Microbiology Systems) and 1% yeast extract (Difco Laboratories). The cells were grown at 59 C in a shaking water bath. The larger culture volumes required for the isolation of washed membranes necessitated the use of a gyratory incubator shaker at the indicated temperature. Growth was monitored by the measurement of optical density at 600 nm in a Coleman Junior II spectrophotometer.

Mutant isolation.

B.stearothermophilus 2184 was mutagenised by N-methyl-N'-nitro-N-nitrosoguanidine by a slight modification of the procedure as outlined by Miller (72). At an optical density of 0.3, nitrosoguanidine (50μg/ml) was added to 50 ml of an exponentially growing culture and incubation was continued for a further 30 min at 59 C. The cells were then harvested on ice and washed with 20 ml of TYE medium. The pellet from this washing step was diluted 1:10 and resuspended in 50 ml of prewarmed medium and further incubated until an optical density of 0.4 was reached. Portions of the culture (0.1 ml) were pipetted onto bacitracin selection plates consisting of Trypticase Soy Agar (BBL Microbiology Systems) with varying concentrations of bacitracin. Two individual colonies were picked from the plates containing 10μg/ml of bacitracin. Growth in liquid culture was in a medium that consisted of the complex medium previously described to which was added bacitracin (10μg/ml) and 0.5% glucose. The mutants were lyophilised and stored at -15 C.

Lipid analysis.

The distribution of ^{32}P into the various lipid fractions was obtained by growing the cells in the TYE medium containing 2μCi of $H_3{}^{32}PO_4$ per ml. Relative specific activities (in counts per min per nmol of phosphate) for each phospholipid were determined.

Cells were either continuously labeled (for approximately five cell doublings) or pulse labeled for a specific period of time when the culture had reached an optical density of 0.24. Growth was terminated by pouring the cells over ice and removing the label by centrifugation at 10,000 rpm.

Extraction of phospholipids.

Labeled cells were extracted by a modified Bligh and Dyer procedure (11). Cells from 10 ml samples were collected by centrifugation, resuspended in 0.8 ml of 0.3% NaCl and transferred to a screw-capped test tube. Three ml of chloroform/methanol (1:2) were added to this screw-capped tube and the single phase system was centrifuged using a clinical bench top centrifuge in order to pellet the particulate matter. The supernatent was removed, further adjusted by the addition of 1 ml of chloroform and 1 ml of 0.3% NaCl that resulted in a two phase system with a chloroform/methanol/water ratio of 2:2:1.8. The chloroform phase was removed with a capillary pipette. A single drop of 5N HCl was then added and the upper aqueous phase was reextracted twice with 1 ml of chloroform. The chloroform extracts were then pooled.

Card (15) had previously noted that lysozyme treatment of cells increased the CL content of the lipid extract. Consequently, the delipidated residue was resuspended in 0.8 ml of 50 mM Tris HCl and 5 mM

$MgCl_2$ containing 200 µg/ml lysozyme and incubated at 25 C for 30 min. The reaction was stopped by the addition of 3 ml of chloroform/methanol (2:1) and the tubes were vigorously shaken at 60 C for 10 min. Further shaking for 30 min at room temperature succeeded this heating step. A second Bligh and Dyer extraction as described above was then performed. The choloform phases from both extractions were pooled and taken to dryness.

The lipids were quantified either by chromatography in two dimensions on Whatman SG 81 papers or by deacylation by methanolysis and separation of the phosphate esters. The following solvent systems were used for chromatography on SG 81 papers: chloroform/methanol/2,6-dimethyl-4-heptanone/acetic acid/water in the ratio 35:10:50:25:4 in the first direction and chloroform/methanol/2,6-dimethyl-4-heptanone/pyridine/0.5 M NH_4Cl pH 9.8 in the ratio 60:30:45:70:12 in the second direction. Mild alkaline hydrolysis was performed as described by White (128). The lipid extract was suspended in 0.5 ml of chloroform and an equal volume of 0.2 M KOH in methanol was added. After incubation for two hours at 4 C, the solution was neutralised by repeated passage over Bio-rex 70 beads that had been prewashed with 2N HCl. At approximately pH 7, one ml of chloroform and one ml of 0.3% NaCl were added and the solution was then subsequently vortexed for two minuts. The aqueous phase was removed and the lower organic phase was reextracted twice more with 0.5 ml of water. The aqueous extracts were pooled and taken to dryness. Separation of the phosphate esters was by two dimensional chromatography on acid washed Whatman #1 papers. These were developed in the first dimension by butanol/acetic acid/water in the ratio of 5:3:1, and in the second

by Phenol/0.1 N NH_4OH (100:38 w/v).

Autoradiography using Kodak XAR-5 film or the procedure of Vaskovsky and Kotetsky (124) revealed the specific loci of each lipid or lipid derivative. The spots were then either cut out and counted in a Beckman LS-7500 programmed for dual label ^{3}H-^{32}P counting using Aquasol 2 (New England Nuclear Corp) as scintillation fluor, or in the case of certain SG 81 separations, the phospholipids were eluted from the papers and then subjected to deacylation and rechromatographed as the phosphate esters.

Preparation of washed membranes.

Membranes were prepared by the procedure as detailed by Sone et al. (112). The cells were grown in 200 ml of TYE medium to an optical density of 0.24, at which time they were harvested on ice and centrifuged for 30 min at 2500 rpm. The cells were then twice washed with 20 ml of 0.15 M NaCl-50 mM Tris HCl (pH 7.8) -10 mM $MgCl_2$. The washed cells were then treated with lysozyme (150 μg/ml in the above buffer) for 20 min, after which time they were centrifuged at 10,000 rpm for 10 min. The pellet was then resuspended in 20 ml of 50 mM Tris HCl (pH 7.8), to which was added 0.25 ml of DNase I (Sigma) (2 mg/ml in 50 mM Tris HCl (pH 7.8) -10 mM $MgCl_2$). This was then incubated for 10 min at 25 C. The bright red membrane pellet was obtained by centrifugation at 10,000 rpm for 30 min. This was then washed once with 20 ml of 0.2 M NaCl-50 mM Tris HCl (pH 7.8)-10 mM $MgCl_2$, subsequently thrice washed with 50 mM Tris HCl (pH 7.8)-10 mM $MgCl_2$. Protein concentration was determined by the method of Lowery et al. (68), using bovine serum albumin as the standard. An average amount of membrane protein obtained from a 200 ml culture was in

the order of 2 mg.

Extraction procedure for isolating bactoprenol intermediates.

The method used was that described by Ward (125) for small scale extractions from prepared membranes. All glassware was silanised and all solutions were kept at 4 C due to the unstable nature of bactoprenol. Two milligrams of ^{32}P labeled washed membranes were suspended in 1 ml of distilled water, to which was added an equal volume of butanol/6 M pyridinium acetate pH 4.2 (2:1 v/v). The system was separated into two phases and the upper butanol layer was removed by capillary pipette. The aqueous phase was reextracted two more times with 0.5 ml of water-saturated butanol. The butanol extracts were combined and taken to dryness.

Further purification of this extracted lipid fraction was achieved by subjecting the lipid extract to mild alkaline methanolysis as previously described. The material that remained soluble in the organic phase was chromatographed on Whatman LPH-K plates using 2,6-dimethyl-4-heptanone/acetic acid/water in the ratio 8:5:1 as the solvent. Autoradiography revealed the presence of the bactoprenol intermediates.

Cardiolipin synthetase assay.

Cardiolipin synthetase was assayed by the following protocol: the incubation mixture contained 40 μl of ^{32}P labeled membrane, 100 μl of freshly prepared membrane, 180 μl of 0.2 M acetate buffer (pH 5.6) containing 10 mM EDTA, and 80 μl aliquots of the aqueous antibiotic solutions to give a final total volume of 0.4 ml. This was then incubated for 5 min at 60 C. The reaction was terminated by the addition of 1.5 ml of chloroform/methanol/1 N HCl (1:2:0.03). The controls were incu-

bated at 0 C and 60 C without the presence of the specific antibiotics. The phospholipids were isolated from this assay mixture by adding 1 ml of 0.3% NaCl and 1 ml of chloroform. A two phase system resulted and the chloroform layer was removed. The aqueous phase was reextracted two more times with 1 ml of chloroform. The chloroform extracts were pooled, taken to dryness, and chromatographed on SG 81 papers.

Phospholipase D assay.

Authentic phosphatidic acid was obtained from either PG or PE by the method of Yang (130). PG and PE were eluted from SG 81 papers and the samples were taken to dryness. They were then resuspended in water by the means of ultrasonification and in this micellar form they were used as substrates for the reaction. The assay mixture was as follows: 50 μl of the resuspended lipid, 200 μl of 0.2 M acetate buffer (pH 5.6), 100 μl of the 0.4 M $CaCl_2$, and 10 μl phospholipase D (10 mg/ml)(Sigma). The volume was adjusted to 0.4 ml with the buffer, to which 0.5 ml of diethyl ether was overlaid. The mixture was incubated overnight on a wrist action shaker. The ether was then evaporated and 0.5 ml of chloroform and 1 ml of methanol were added to give a single phase system. The solution was adjusted to a chloroform/methanol/water ratio of 2:2:1.8 and the resulting chloroform phase was removed. The aqueous phase was reextracted two more times with 1 ml of chloroform. All the chloroform extracts were pooled, taken to dryness and chromatographed on SG 81 papers.

Phosphorus assays.

Total inorganic phosphorus was determined by the ashing procedure described by Ames (1). The phosphorus content from SG 81 papers was obtained by dissolving the paper in 1 ml of 70% perchloric acid. This

was then digested for four hours at 160 C. After cooling, 5 ml of deionised water were added, followed by 0.5 ml of 2.5% ammonium molybdate and 0.5 ml of Fiske-Subba Row reagent in that order. The tubes were heated for 7½ minutes at 95 C, centrifuged to remove the insoluble silica acid and read at 820 nm in a Coleman Junior II spectrophotometer. Phosphorus was determined by comparison with known standards.

RESULTS

The effect of various inhibitors of cell wall metabolism on phospholipid composition.

The major phospholipids of B.stearothermophilus are PG, PE, and CL which constitute about 45, 35, and 20% respectively, of the total lipid fraction of exponentially growing cells at 60 C (16). As shown in Table I, exposing exponentially cells to 10 μg/ml of bacitracin, vancomycin, and ristocetin all caused an elevation of CL from 20% to approximately 45%, with a corresponding decrease in the amout of PG present. In contrast, the phospholipid composition of cells incubated in the presence of penicillin and D-cycloserine were similar to control cultures where no antibiotic was present. The effect of antibiotic concentration on the amount of CL synthesis is shown in Fig. 3. Increasing the concentration of bacitracin up to 250 μg/ml (at this point the cells were beginning to lyse) resulted in a linear increase of CL. A comparable effect was observed with low conentrations of both vancomycin and ristocetin (higher concentrations tended to cause the cells to revert back towards wild-type phospholipid composition). Penicillin and D-cycloserine did not alter the phospholipid compositions at any concentration of the antibiotics.

A similar experiment to that described in Fig. 3 was performed with a mutant resistant to low concentrations of bacitracin and the results are depicted in Fig. 4. At low concentrations of bacitracin there appeared to be no effect on the amount of CL synthesis: at high concentrations the level was similar to that found using wild-type cells.

Table I. Phospholipid composition of B.stearothermophilus in the presence of bacterial cell wall inhibitors.[a]

	PE%	PG%	CL%
control[b]	38	39	23
bacitracin	34	20	46
vancomycin	31	25	43
ristocetin	34	24	42
penicillin[c]	35	39	25
cycloserine	34	38	28

[a] The antibiotics were added to an exponentially growing culture at 59 C at a final concentration of 10 μg/ml for 20 minutes.

[b] The control was identical in all respects except that no antibiotics were present.

[c] In this experiment, the final concentration of penicillin was 5 μg/ml due to cell lysis.

Fig. 3. The effect of antibiotic concentration on the amount of cardiolipin synthesis in wild-type cells.

A culture of B.stearothermophilus was grown at 59 C and was continuously labeled with ^{32}P until an optical density of 0.24. 10 ml samples were then pipetted into flasks containing the aqueous solutions of the antibiotics and the cells were further incubated for 20 min. The reaction was stopped by pouring the cells over ice and they were then centrifuged at 10,000 rpm for 10 min in order to remove the label. The phospholipids were extracted from the pellet by a modified Bligh and Dyer procedure (11). The total lipid fraction was chromatographed in two dimensions on Whatman SG 81 papers utilising the following solvent systems: chloroform/methanol/2,6-dimethyl-4-heptanone/acetic acid/water in the ratio 35:10:50:25:4 in the first dimension, and chloroform/methanol/2,6-dimethyl--4-heptanone/pyridine/0.5 M NH_4Cl pH 9.8 in the ratio 60:30:45:70:12 in the second. Autoradiography revealed the presence of the lipids, which were then cut out from the papers and counted in a Beckman LS-7500.

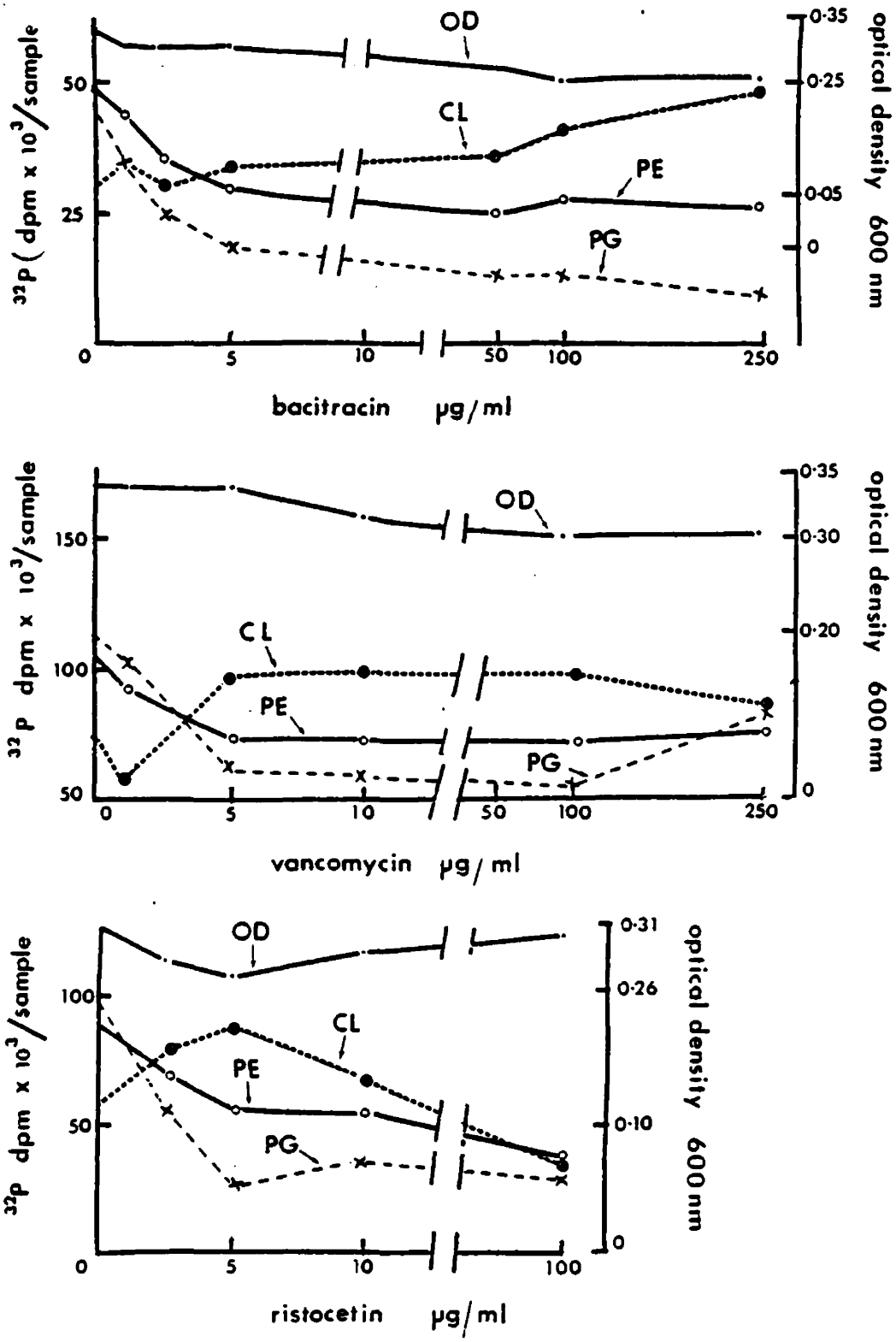
OD
CL
PE
PG
^{32}P (dpm x 10^3/sample
optical density 600 nm
50
25
0
0·35
0·25
0·05
0
5
10
50
100
250
bacitracin µg/ml
OD
CL
PE
PG
^{32}P dpm x 10^3/sample
optical density 600 nm
150
100
50
0·35
0·30
0·20
0
0
5
10
50
100
250
vancomycin µg/ml
OD
CL
PE
PG
^{32}P dpm x 10^3/sample
optical density 600 nm
100
50
0
0·31
0·26
0·10
0
0
5
10
100
ristocetin µg/ml

Fig.3

Fig.4. The effect of antibiotic concentration on the amount of cardiolipin synthesis for a mutant resistant to low levels of bacitracin.

B.stearothermophilus was mutagenised by nitrosoguanidine (50 µg/ml) and a single colony was isolated from bacitracin selection plates (10 µg/ml). The mutant was then grown in liquid culture in TYE medium to which was added bacitracin (10 µg/ml) and 0.5% glucose under identical conditions as described in Fig.3.

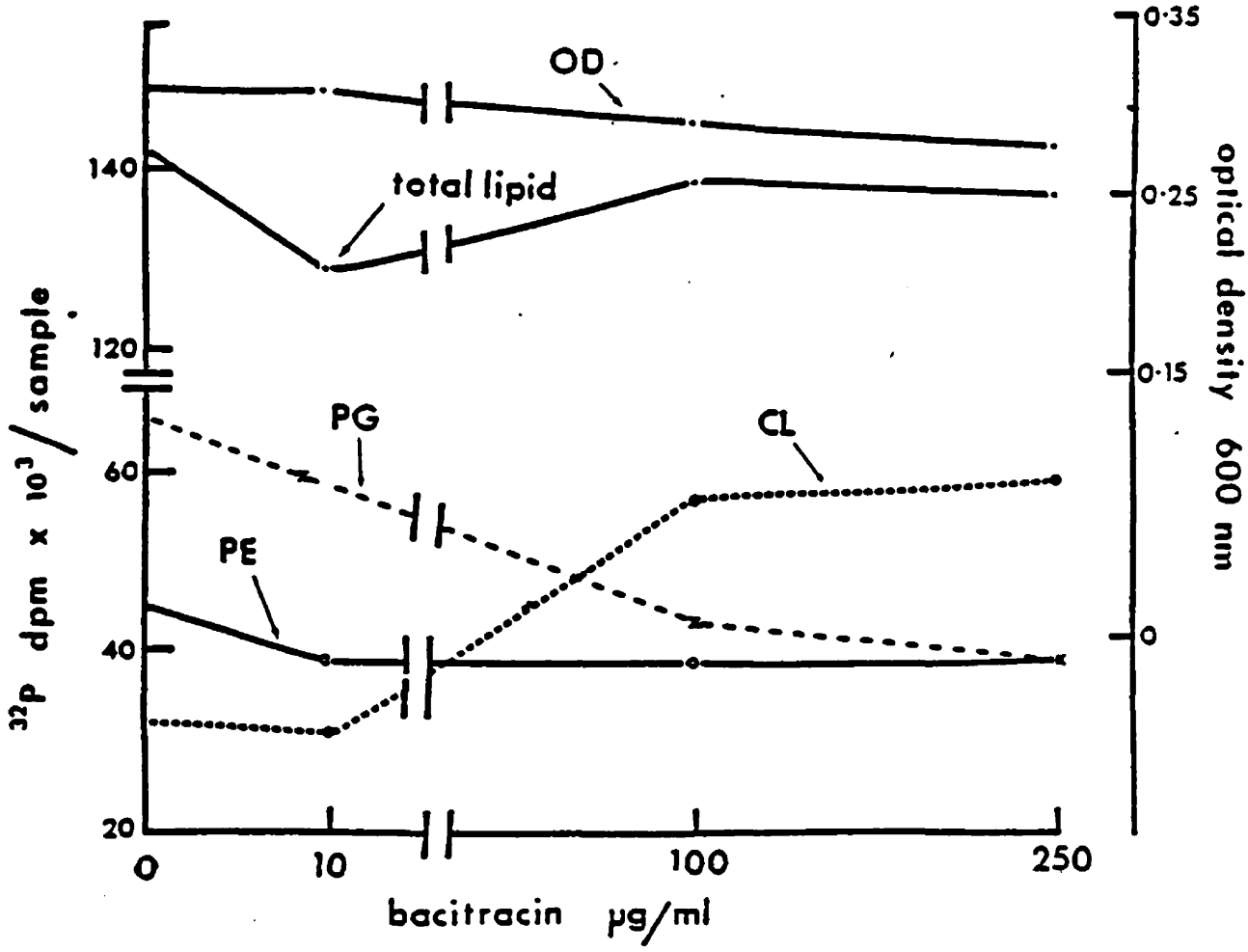
OD
total lipid
PG
CL
PE
140
120
60
40
20
0
10
100
250
bacitracin µg/ml
^{32}P dpm x 10^3 / sample
optical density 600 nm
0·35
0·25
0·15
0

Fig. 4

Turnover patterns of cells labeled with ^{32}P and exposed to bacitracin.

As shown in Fig. 5, the turnover patterns of bacitracin treated cells and control were similar. In both cases, PG was rapidly metabolised, whereas PE and CL were relatively stable whether the cells were chased in the presence of bacitracin or not.

The effect of bacitracin, vancomycin, and ristocetin on cardiolipin synthetase activity.

The effect on cardiolipin synthetase activity in washed membrane suspensions when assayed in the presence of the antibiotics is shown in Table II. As can be seen, there is little difference with the suspensions including the antibiotics to the 60 C control.

Characterisation of the unknown X.

Throughout the course of this study we noted the presence of an as yet unidentified lipid in the total lipid fraction (Fig. 6B). In wild-type cells this lipid accounted for less than 1% of the total lipid present. In exponentially growing cells subjected to 50 μg/ml of bacitracin for 5 minutes, this particular component increased to 5% of the total lipid in the extract. This lipid was recovered from SG 81 papers by a conventional Bligh and Dyer extraction, and after mild alkaline methanolysis yielded a single phosphate ester which we initially thought could be glycerol phosphate (Fig. 6C).

In order to ascertain whether the deacylated form of X originated from phosphatidic acid (as suggested by the above results), we chromatographed authentic PA with the unknown X on SG 81 papers. As can be be seen in Fig. 7, the acylated forms of X and PA appear to be two distinct molecules. Further confirmation of this fact was obtained by

Fig.5. The effect of bacitracin on the phospholipid turnover of B.stearothermophilus.

A culture was continuously labeled with ^{32}P until an optical density of 0.24 was reached. The label was removed by centrifugation and the cells were resuspended in TYE medium containing bacitracin (0.8 μg/ml). The culture was then further incubated for 20 minutes.

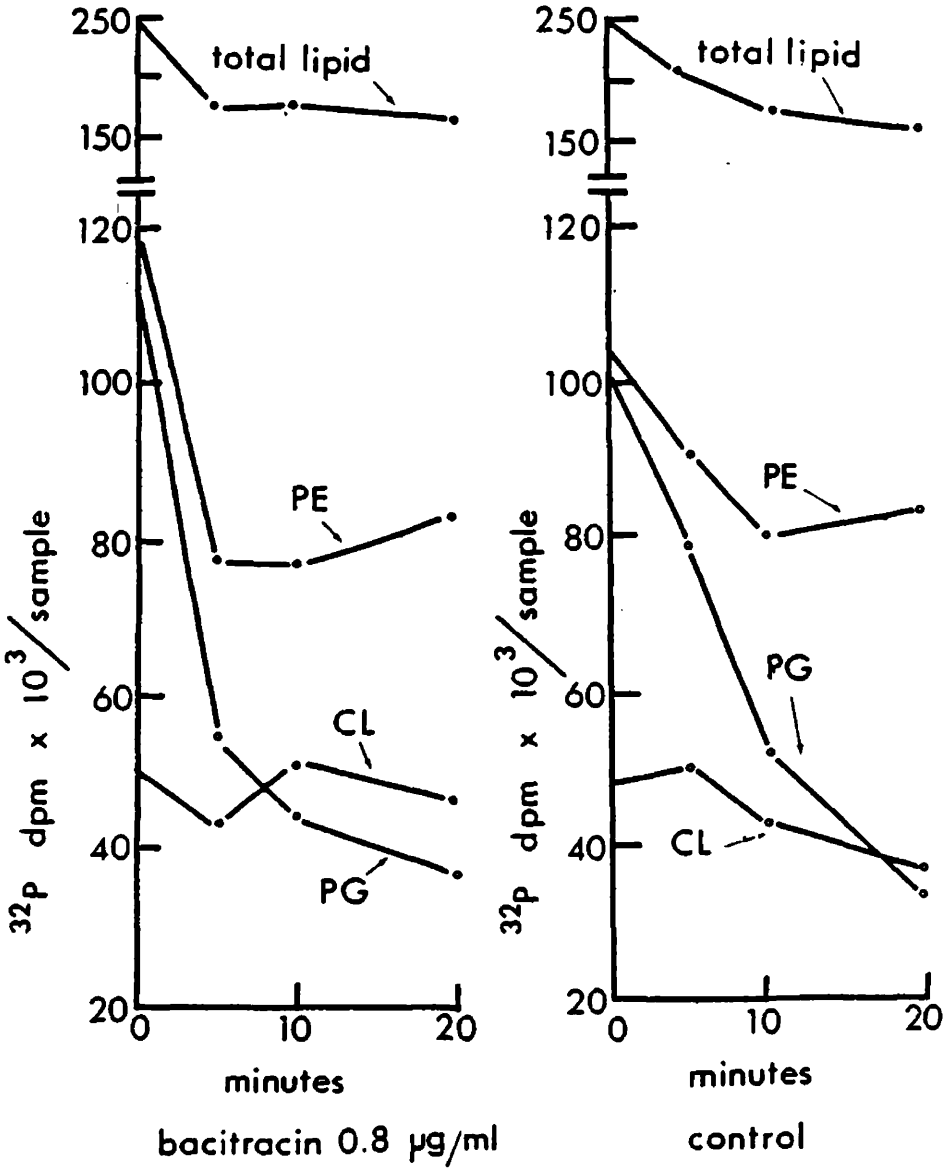
250
150
120
100
80
60
40
20
total lipid
PE
CL
PG
^{32}P dpm x 10^3/sample
0
10
20
minutes
bacitracin 0.8 µg/ml
control

Fig.5

Table II. The effect of bacterial cell wall inhibitors on the *in vitro* assay of cardiolipin synthetase.[a]

	PE%	PG%	CL%
control 0 C[b]	32	46	23
control 60 C[c]	29	10	61
bacitracin	25	12	63
vancomycin	26	13	61
ristocetin	29	10	61

[a] The reaction was performed for 5 min with the incubation buffer containing the antibiotics to a final concentration of 10 μg/ml.

[b] Identical assay on ice in the absence of antibiotics.

[c] Identical assay at 60 C in the absence of antibiotics.

Fig.6. Two dimensional chromatographic characterisation of the unknown X: chromatography on SG 81 papers and chromatography of the phosphate ester derived from X.

The total lipid fraction was extracted as described in Fig.3.

Fig.6A. two dimensional chromatography on SG 81 papers of the total lipid fraction.

Fig.6B. Two dimensional chromatography on SG 81 papers of the total lipid fraction from cells incubated in the presence of bacitracin (50 μg/ml for 20 min) revealing the position of of the unknown X. The unknown X was subsequently eluted from the SG 81 papers and was then subjected to deacylation by mild alkaline methanolysis.

Fig.6C. Two dimensional chromatography of the phosphate ester derived from X using Whatman # 1 papers developed with butanol/acetic acid/water in the ratio 5:3:1 in the first direction and phenol/0.1 N NH_4OH (100:38 w/v) in the second direction.

Fig.6D. Two dimensional chromatography on Whatman # 1 papers of the deacylated total lipid fraction.

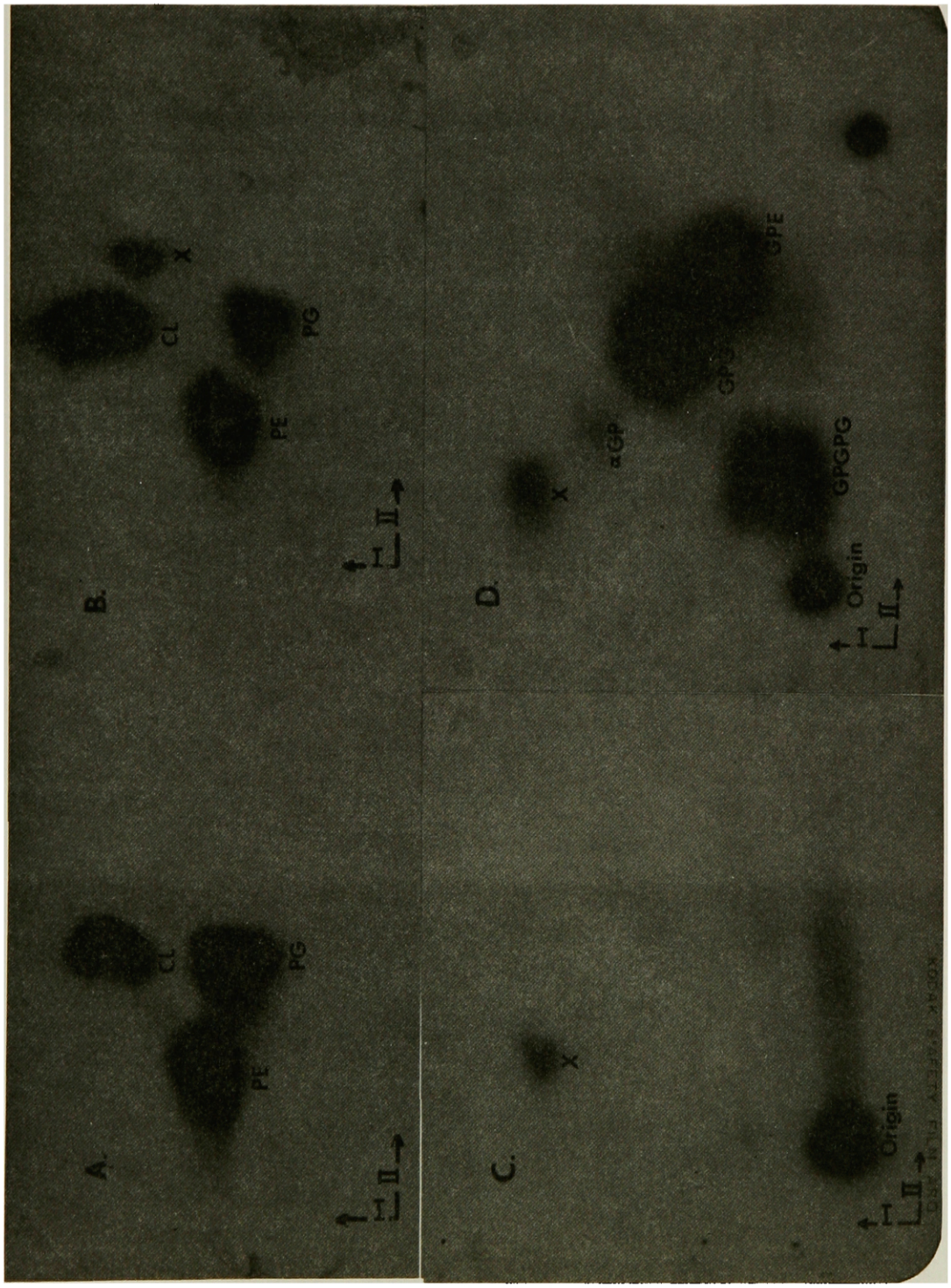
A.
CL
PG
PE
I
II
B.
X
CL
PG
PE
I
II
C.
X
Origin
I
II
D.
X
αGP
GPE
GPG
GPGPG
Origin
I
II

Fig.6

Fig.7. Two dimensional chromatography on SG 81 papers comparing the unknown X with authentic phosphatidic acid.

Autoradiographs of two dimensional separations using SG 81 papers:

Fig.7A. X isolated from Fig.6B and rechromatographed.

Fig.7B. PA obtained by the action of phospholipase D using PG as substrate.

Fig.7C. X and PA (obtained by enzymatic action on PE) chromatographed together revealing two distinct molecules.

Fig.7D. Total lipid fraction serving as control.

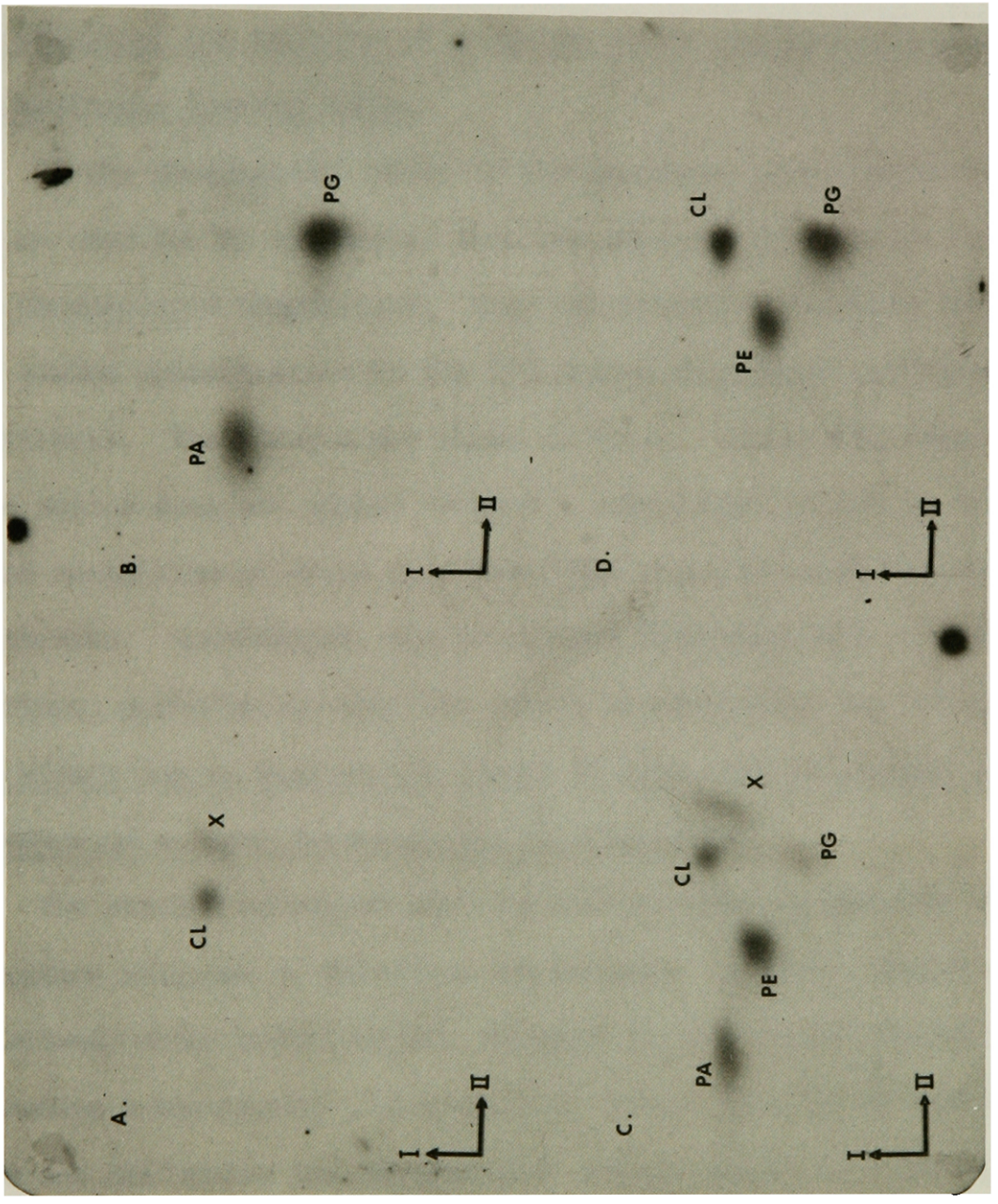

Fig.7

co-chromatographing the glycerol phosphate esters of each lipid, and likewise, autoradiography revealed the presence of two spots (data not shown).

The effect of the addition of L-serine on the phospholipid composition of bacitracin treated cells.

If the phosphatidyl group of the suggested phosphatidyl intermediate can be used for PE synthesis, then the addition of L-serine might effect the phospholipid composition. This possibility was tested by increasing the serine concentration in the TYE medium at a fixed concentration of bacitracin. The results are shown in Fig.8. From these data, we conclude that serine does not appear to have a significant effect on the phospholipid metabolism of cells that have been simultaneously inhibited with bacitracin. Nonetheless, the PE content responded with a slight, but distinct, perturbation when the serine concentration was increased.

The effect due to varying the length of time that cells were in the presence of a fixed concentration of bacitracin.

The results presented thus far relied entirely upon the extraction procedure outlined in Materials and Methods. However, employing two relatively minor modifications designed to enhance the possibility of isolating a bactoprenol intermediate; namely, the use of silanised glassware and performing the phospholipid extraction at 4 C, for all the steps other than the lysozyme treatment and the 60 C heating step, revealed the presence of several other unknown lipid species (Fig.9). Exposing the cells to 50 μg/ml of bacitracin for 5 minutes caused the accumulation of a previously unseen lipid designated A. Performing the same experiment but substituting vancomycin in place of bacitracin, also caused the

Fig.8. The effect of L-serine on the phospholipid composition of cells treated simultaneously with a fixed concentration of bacitracin.

The same experiment was performed as described in Fig.3 except that bacitracin (50 μg/ml) and L-serine were added together.

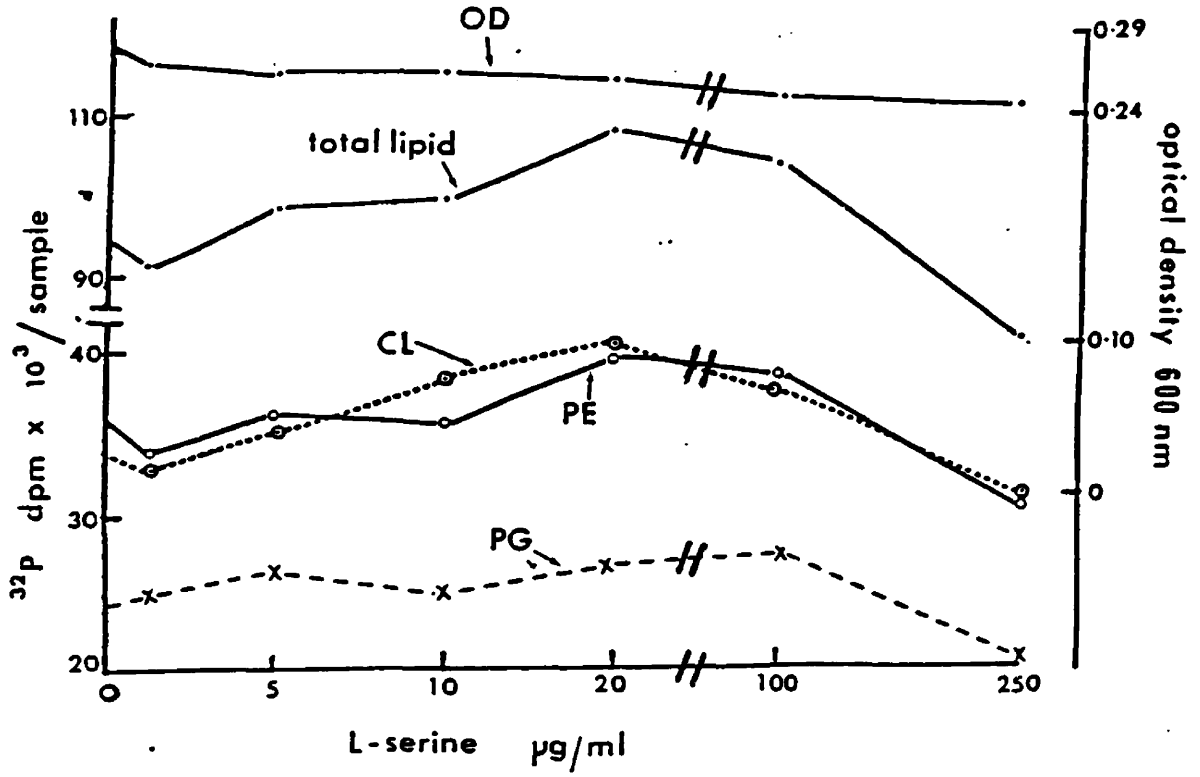
OD
total lipid
CL
PE
PG
110
90
40
30
20
0
5
10
20
100
250
L-serine µg/ml
^{32}P dpm x 10^3/sample
0·29
0·24
0·10
0
optical density 600 nm

Fig.8

Fig.9. The time dependent effect of bacitracin on the phospholipid composition as revealed by two dimensional chromatography on SG 81 papers.

The same experiment was performed as described in Fig.3 except that the phospholipid extraction procedure was slightly modified by using silanised glassware and performing the extraction at 4 C, in order to accomadate the isolation of bactoprenol intermediates. The autoradiographs show samples taken at 0 min, 5 min, 10 min, and 20 min, and reveal the presence of three lipids that were not previously isolated. These are designated as the unknowns A, B, and C.

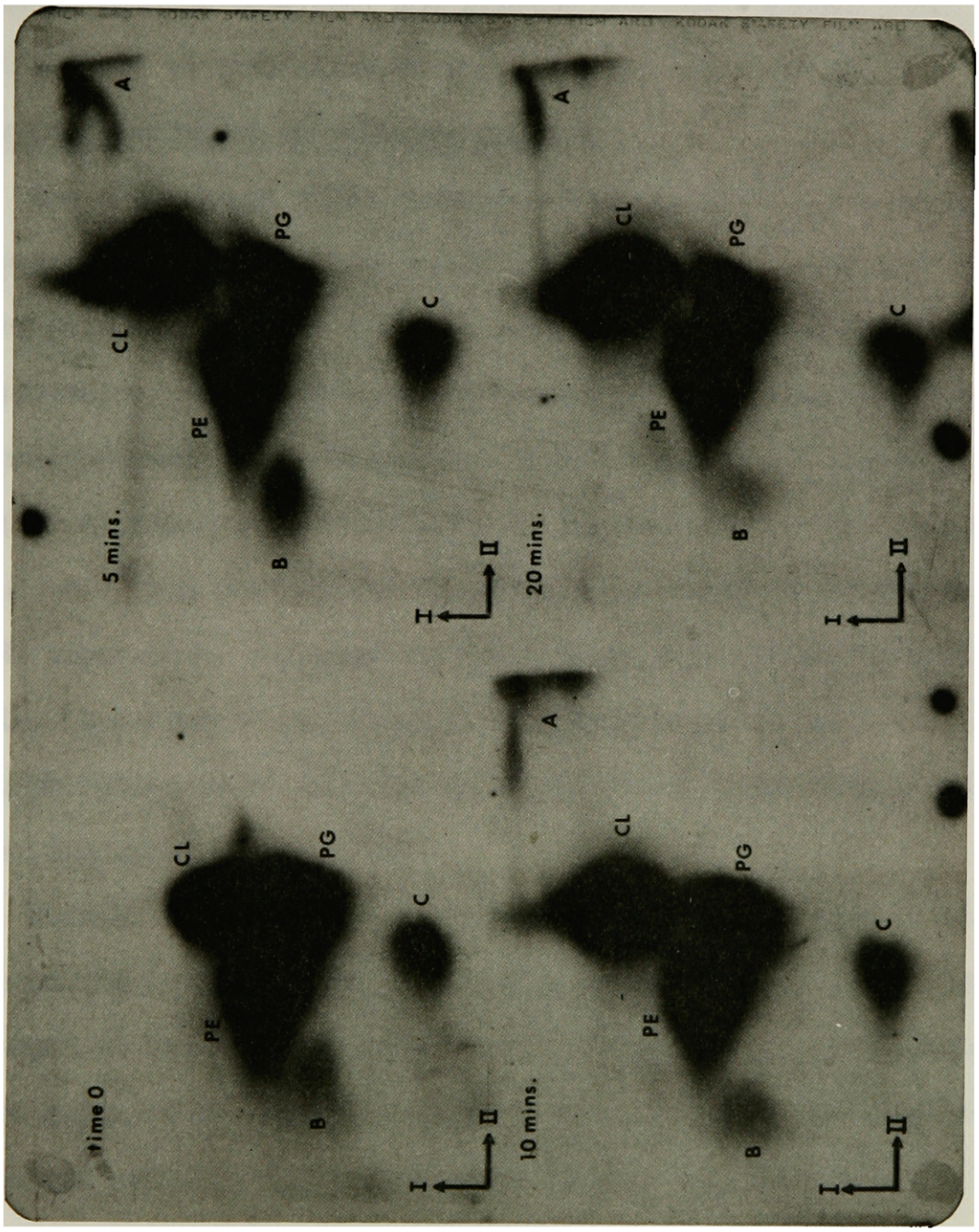

Fig.9

accumulation of this particular lipid (data not shown). Further inspection of Fig.9 showed that the unknown lipids B and C (tentatively identified as lyso-CL (Fig.10D) and lyso-PE (Fig.10C) respectively), decreased with respect to time after an initial transient increase.

Characterisation of the unknown lipid A.

The accumulated lipid (unknown A) was eluted from SG 81 papers by a conventional Bligh and Dyer procedure. After a brief period of acid hydrolysis (2 N HCl for 5 min at 100 C) and yet another Bligh and Dyer purification, the lipid that was remaining in the organic phase was rechromatographed for a second time on SG 81 papers. A molecule with very similar migratory characteristics as demonstrated by the unknown X (Fig.7B) resulted. This acid hydrolysis product was recovered from this second SG 81 separation, subjected to deacylation and rechromatographed yet again, this time as the glycerol phosphate ester as shown in Fig.10B. This phosphate ester appeared to correspond to a previously unidentified ester described by Card (15).

Further characterisation consisted of comparing the unknown lipid with bactoprenol intermediates isolated from prepared washed membranes. As shown in Fig.11, thin layer chromatography revealed the presence of three major bands for all membrane preparations. Intact unknown A (isolated prior to acid hydrolysis) appeared to correspond to the second of these major bands. Previous evidence (117) and the exacting nature of the extraction procedure, would clearly correlate all three bands with bactoprenol. If that is so, and that the unknown A does in fact correspond to the second band, then we conclude that our accumulated phospholipid due to bacitracin inhibition is composed in part with the

Fig.10. Characterisation of the unknowns A, B, and C by two dimensional chromatography of their phosphate esters.

The three lipids were eluted from the SG 81 chromatographs shown in Fig.9. Unknown A was then subjected to mild acid hydrolysis (2 N HCl for 5 min at 100 C) in order to remove the end group from bactoprenol. The acid hydrolysis product, and the unknowns B and C were then subjected to mild alkaline methanolysis and chromatographed as described in Fig.6. The phosphate ester of C was tentatively identified glycerol phosphate ethanolamine (Fig.10C); the phosphate ester of B was tentatively identified as glycerylphosphoglycerylphosphoglycerol, the phosphate ester resulting from CL (Fig.10D); A still remains unknown (Fig.10B); Fig.10A is the total lipid control.

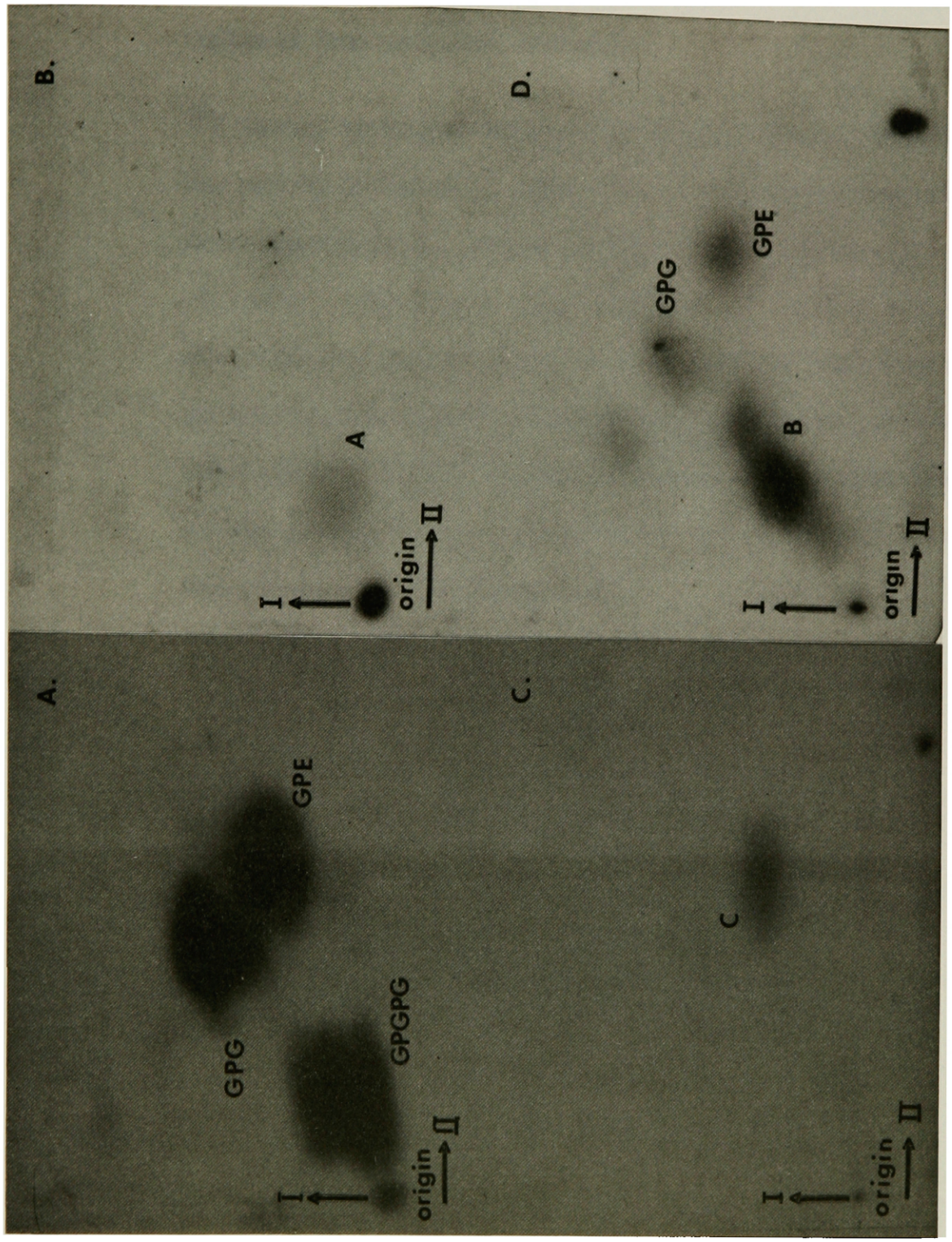
A.
GPG
GPE
GPGPG
I
origin
II
B.
A
I
origin
II
C.
C
I
origin
II
D.
GPG
GPE
B
I
origin
II

Fig.10

Fig.11. Thin layer chromatography of bactoprenol intermediates isolated from prepared membranes.

^{32}P washed membranes were prepared from 200 ml cultures by the method detailed by Sone (112). Bactoprenol was extracted into butanol using butanol/6 M pyridinium acetate pH 4.2 (2:1 v/v). This total lipid extract was further purified by mild alkaline methanolysis and the material that remained soluble in the organic phase was then separated on Whatman LPH-K plates using 2,6-dimethyl-4-heptanone/acetic acid/water in the ratio 8:5:1 as the solvent. Autoradiography revealed the presence of the bactoprenol.

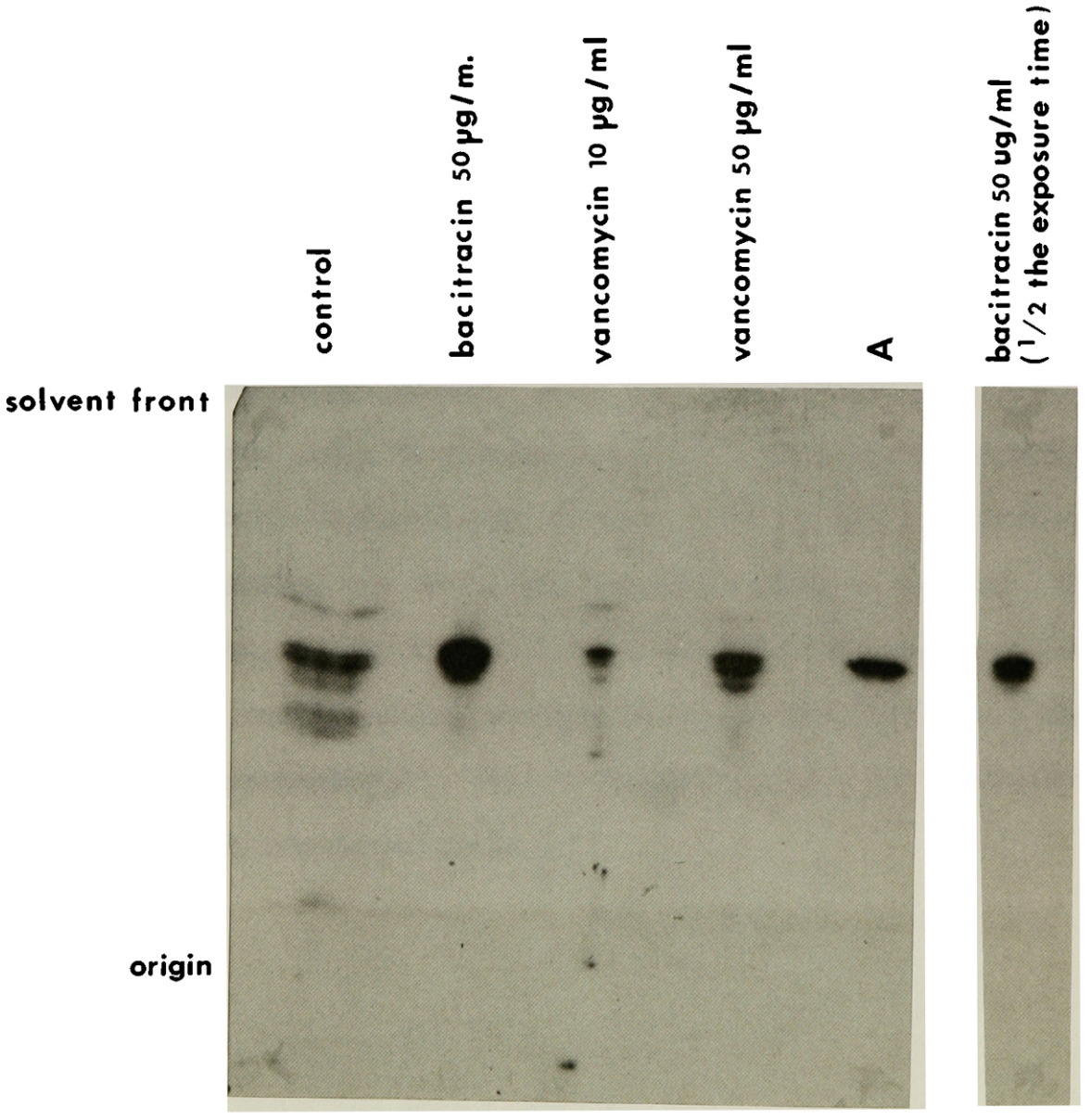
control
bacitracin 50 µg/m.
vancomycin 10 µg/ml
vancomycin 50 µg/ml
A
bacitracin 50 ug/ml
(1/2 the exposure time)
solvent front
origin

Fig.11

C_{55}-isoprenoid complex.

The time dependent effect of vancomycin on the lipid composition of washed membranes.

Exponentially growing cells were inhibited with 50 µg/ml of vancomycin and samples were taken at various times thereafter. The harvested cells were then extracted for either phospholipid or bactoprenol. Fig.12A shows that the elevation of CL occurs sometime after 10 minutes from the start of the inhibitory period. This is in contrast to bacitracin where this rise occured prior to 5 minutes (Fig.7). Thin layer chromatography of the bactoprenol intermediates isolated from the cells harvested at various times during the inhibitory period is shown in Fig.12B. Upon close inspection, it appeared that the first band increased with respect to time with a corresponding decrease in the second band (our postulated phospholipid intermediate). This coordinated effect also appeared to correspond with the elevation of CL.

Fig.12. The effect of time on vancomycin treated cells as revealed by two dimensional chromatography on SG 81 papers of the total lipid fraction, and by thin layer chromatography of bactoprenol intermediates isolated from washed membranes.

Fig.12A demonstrates the delayed elevation of the CL content in an analogous experiment as to the one described in Fig.9. Fig.12B shows the separation of the bactoprenol intermediates isolated from washed membrane samples as described in Fig.11 that were taken at various times during the inhibitory period.

All antibiotic concentrations were 50 μg/ml.

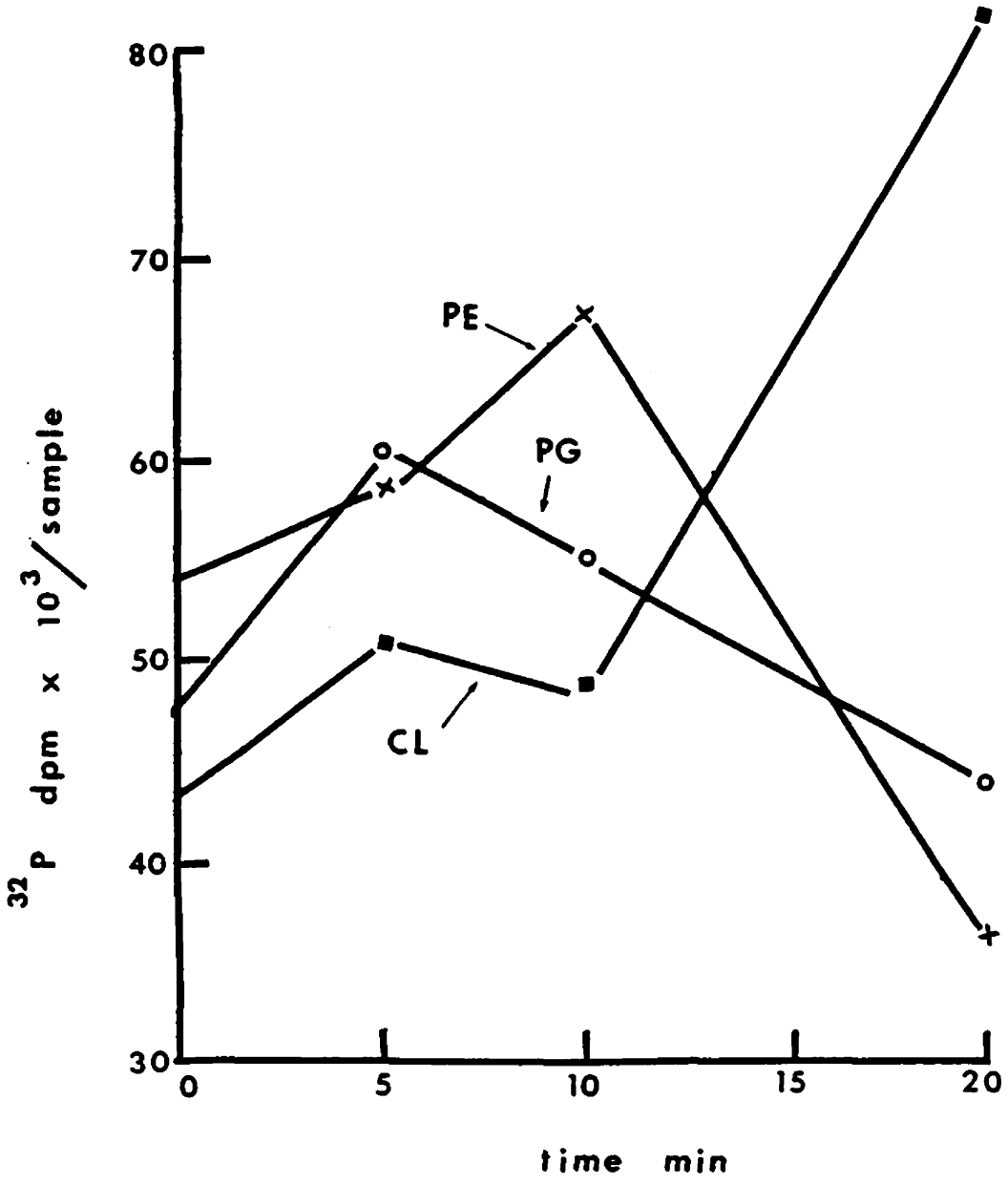

Fig.12A

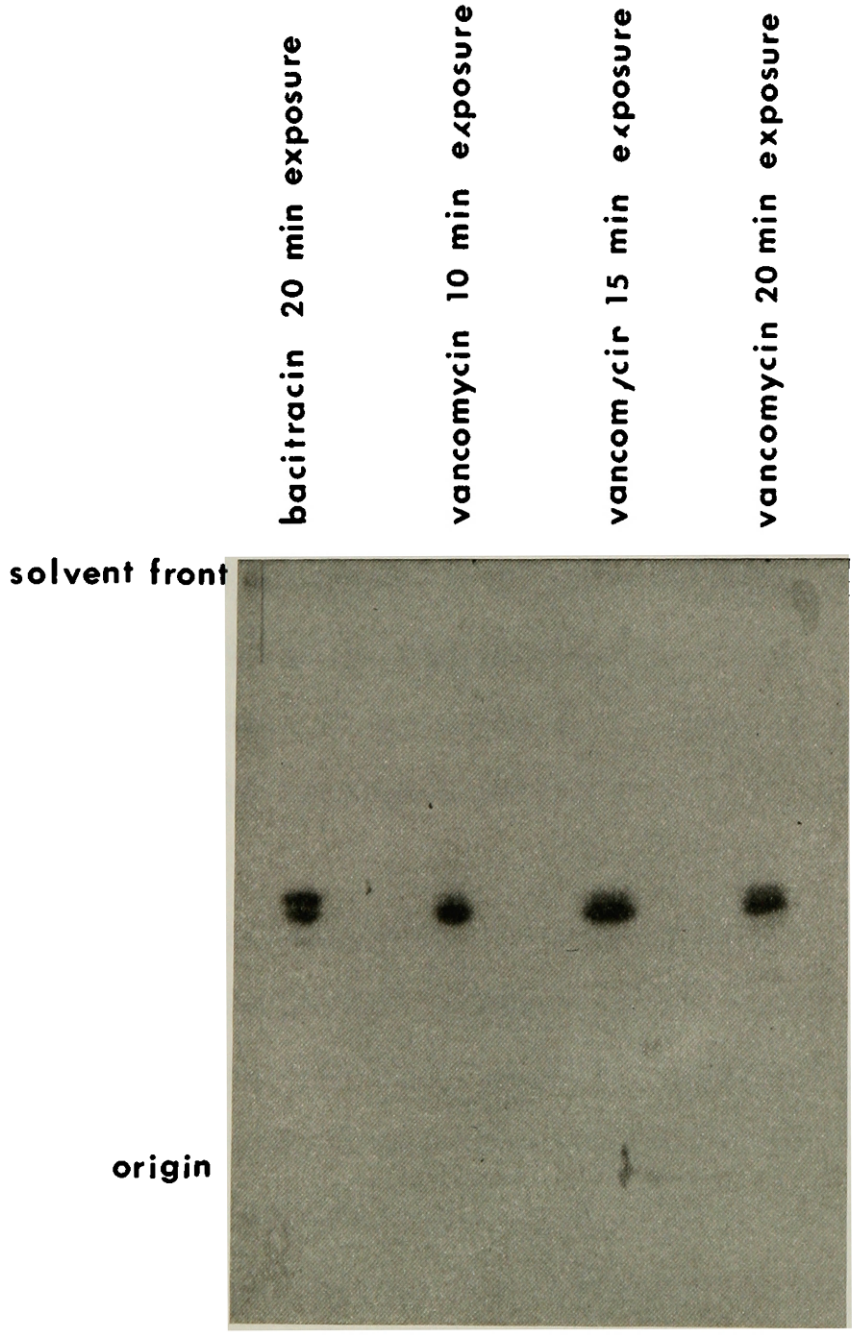
bacitracin 20 min exposure
vancomycin 10 min exposure
vancomycir 15 min exposure
vancomycin 20 min exposure
solvent front
origin

Fig.12B

DISCUSSION

The data presented in Table I and Fig.3 show that on exposing B.stearothermophilus to twenty minutes of antimicrobial action, that one can readily see that bacitracin and low concentrations of vancomycin and ristocetin all cause a sharp rise in the CL content; apparently at the expense of PG. The lack of this response with penicillin and D-cycloserine would suggest the possible involvement of the bactoprenol carrier system. A similar effect was recently documented with S.aureus (59). The results that are summarised in Table II would tend to suggest that this increase did not appear to be due to a direct effect of the antibiotics on cardiolipin synthetase activity. Also, it would appear from Fig.5 that bacitracin is not exerting an effect on phospholipid turnover, albeit that in this experiment low concentrations of bacitracin were used.

Fig.6 clearly demonstrates that an unidentified lipid (X) was seen to accumulate on bacitracin treatment and initially we thought the phosphate ester derived from X was possibly glycerol phosphate. If this were the case then this would have been consistent with Card's proposal (17) that increased CL synthesis should result in a greater number of phosphatidyl complexes. However, two dimensional chromatography of the unknown X with authentic PA clearly showed the existance of two different molecules. Also, we failed to demonstrate a significant increase in PE content with a simultaneous addition of bacitracin and L-serine to an exponentially growing culture. These two pieces of data tend to support Dowhan's contention (31) that PG is not metabolised via a phosphatidyl complex. However, in light of Kito's (131) recent exchange data of PG with PE in a serine auxotroph of E.coli, and some particularily puzzling

studies on PE synthesis in Gram positive anaerobic organisms (19, 108, 109) would serve to remind us that further clarification of this point is needed.

The hope of possibly isolating a bactoprenol intermediate led us to slightly modify the phospholipid extraction procedure. The detection of three additional lipids using this modification (Fig.9) was particularily encouraging. From a cursory visual inspection the unknown designated A appeared to bear some apparent differences from the other well-characterised phospholipids present. (This particular unknown also accumulated in the corresponding experiment using vancomycin-data not shown). Subsequent characterisation of A showed that mild acid hydrolysis (to release any end group from bactoprenol) yielded a product that was soluble in chloroform and that chromatographed as a single spot. This appeared to correspond to the position of the unknown X using SG 81 papers as shown in Fig.7B. The deacylated end group (Fig.10) turned out to be yet another glcerol phosphate ester when compared to deacylated X. With this in mind, the observation of the chromatographic properties of the end group and the unknown X would appear to suggest that though they have been shown to be different molecules they may be chemically related.

Finally, attention was focused on the isolation from washed membrane preparations of the bactoprenol intermediates themselves. It was hoped that if bactoprenol was involved with phospholipid metabolism then changes in the phospholipid composition caused by the presence of the antibiotics would be reflected at the bactoprenol level. As Fig.11 shows, the extract from each membrane preparation appeared to consist of three major bands. Previous evidence (117) documents a single major band identified as bacto-

prenol-PP, however, a second smaller, unidentified band was also detected. Chromatographing isolated intact unknown A (i.e. prior to acid hydrolysis) on thin layer plates showed that this compound migrated with the second of these major bands. We believe that this is particularily strong evidence for the involvement of bactoprenol in phospholipid metabolism. Further support of this contention is found in the results depicted in Fig.12. In this experiment we relied upon the fact that vancomycin did not cause an immediate increase in the CL content, and this was shown at the bactoprenol level by a diminishment of the phospholipid intermediate accompanying the elevation of CL. Close inspection of the bacitracin lane in Fig.12 shows that most of the label appeared to be present in the first of these major bands (evidence to date would suggest that this compound is bactoprenol-PP (117)). Not only was the diminishment of our phospholipid intermediate reflected in the rise in CL concentration but it also appeared to be in conjunction with an increase of bactoprenol-PP.

From the evidence presented thus far, it can be seen that two fundamental questions remain with regards to the accumulated phospholipid product. They are: what is the molecular nature of this particular phospholipid intermediate? and what is the role that this purported intermediate plays with regard to phospholipid metabolism?

As yet the molecular identity of the intermediate A is unknown. The evidence presented in Fig.11 would strongly implicate the presence of bactoprenol in this intermediate, but doubt still remains as to the identity of the end group. Various factors would suggest that the unknown

X and the acid hydrolysis product are derived from the same molecule. This conclusion is based upon such things as the effect of modifying the extraction procedure which resulted in the disappearance of X with the appearance of the unknown A; the sporadic isolation of X, that could be accounted for by it's attachment to bactoprenol; and the extremely similar chromatographic behaviour exhibited by both molecules. (In fact with regard to this latter point, the only difference is in the deacylated state using the solvent system butanol/acetic acid/water). The chromatographic properties exhibited by X and the acid hydrolysis product in the two systems described herein, with their apparent analogous susceptibility to mild alkaline hydrolysis would suggest the possibility of a phospholipid end group joined to bactoprenol. However, neither of the two molecules were found to correspond to PA (Figs.6, 7, and 10), yet this is by far the most plausible explanation for a bactoprenol-phospholipid complex of this nature. This would in fact correspond to a phosphatidyl intermediate. In fact neither the acylated nor deacylated products appeared to correspond to any readily identifiable lipid in either system.

Speculation as to the possible identity of the end group invariably leads one to consider PG turnover. It has long been believed that the elucidation of PG turnover would appear to solve many of the complexities we now face concerning phospholipid metabolism. Recently, Card (17) presented evidence that the turnover of the phosphatidyl group of PG in B.stearothermophilus resulted in the label being incorporated in lipoteichoic acid. Consequently, the possibility could in fact exist that the accumulated unknown phospholipid intermediate may be involved in

lipoteichoic acid synthesis. With regard to this possibility, an interesting property of both X and the acid hydrolysis product in the deacylated form is that the bulk of the label applied to the papers in Figs.6C and 10B remained at the origin. This could possibily indicate the presence of a chemically unfavorable group or the presence of a polymer of some nature that remains at the origin due to some possible physical constraints.

Turning our attention now to the possible role that our proposed bactoprenol phospholipid intermediate could play in phospholipid metabolism we find that several questions deserve further consideration.

Initially, our thoughts on the possible inhibitory effects of bacitracin were that an analogous situation to cell wall biosynthesis was occuring within phospholipid metabolism, namely, that bactoprenol-P would be acting as the possible acceptor for the phosphatidyl complex that Card proposed. However, the data from Fig.3 clearly show that upon increasing the bacitracin concentration there is a corresponding increase in CL content. It has been determined that increasing the amount of bacitracin would increase the level of bactoprenol-PP and not bactoprenol-P, so consequently, the possibility now arises in that bactoprenol-PP may itself stimulate CL synthesis by possibly acting as the initial acceptor in order to form the intermediary complex. One problem with this hypothesis is that neither vancomycin nor ristocetin has ever been associated with the accumulation of bactoprenol-PP as a consequence of their inhibitory action (35). However, the results in Fig.12 would in fact appear to support this line of reasoning. Here it appears that the phospholipid intermediate decreases with respect to time with an elevation of CL that also corresponded to an increase in bactoprenol-PP.

A possible explanation to account for this anomalous behaviour with the vancomycin treatment could be as follows. Inihibition of the translocase step, which results in the accumulation of the intermediate bactoprenol-PP-disaccharide-pentapeptide in cell wall biosynthesis, ostensibly removes bactoprenol from the system. Consequently, the cell in trying to provide bactoprenol-P (apparently the crucial acceptor in the bactoprenol lipid carrier cycle) breaks down the phospholipid intermediate and as a result of this causes an elevation of CL and the formation of bactoprenol-PP. The latter would then be subsequently dephosphorylated to bactoprenol-P. This explanation would not account for bacitracin's almost identical effect: bacitracin treated cells have an inoperable phosphatase enzyme and would be unable to produce the desired bactoprenol-P.

A further question that arises is at what particular stage does bactoprenol enter into phospholipid metabolism? The answer to this particular question is also unknown. Without knowing the identity of the end group nor the form of the initial acceptor make speculation on bactoprenol entry in phospholipid metabolism engaging but as yet unfortunately fruitless. Possible approaches as to solving this particular impasse and the one regarding the identity of the end group could be the use of dual label studies with the determination of the $^{32}P/^{3}H$ ratio in the acid hydrolysis product; in vitro assays of cardiolipin synthetase in which the incubation buffer is supplemented with either the unknown A or bactoprenol-PP and determining the relative ratios of the particular products; chemical tests to determine the exact nature of the unknown A could include the use of mass spectroscopy and nuclear magnetic resonance.

Nonetheless, the results presented here constitute particularily

strong evidence for the coupling of cell wall synthesis and phospholipid metabolism. The fact that bactoprenol should appear in a phospholipid complex should not appear too surprising. Evidence is now rapidly accumulating (23, 34, 125, 126) that would appear to suggest that the synthesis of teichoic acids also involves a bactoprenol intermediate. As teichoic acids are believed to be synthesised in part from phospholipid degradation, then the occurance of a common reaction vehicle would appear quite plausible. A critical aspect of this particular scenario would be the particularily fine control that the cell could exert on macromolecular synthesis. This could be achieved by possibly regulating the flow of bactoprenol intermediates that may be serving as acceptors for each individual reaction sequence. Strominger's group (38, 47, 48, 102, 129) believes that cell wall synthesis is regulated by the dual action of a kinase and phosphatase that maintain the appropiate levels of bactoprenol-P. An interesting property of this system is that the phosphatase enzyme has an equivalent pH optimum as the one that we have determined for cardiolipin synthetase. These two enzymes are in sharp contrast to the rest of the phospholipid and cell wall enzymes, whose pH optima are all within the range of 7-8. Consequently, the possibility exists that both phospholipid and cell wall biosynthesis may well be controlled by pH gradients across the cytoplasmic membrane.

SUMMARY

The results obtained from this study can be summarised as follows:

1. Bacitracin and low concentrations of vancomycin and ristocetin (all of which have been determined to inhibit the bactoprenol lipid carrier cycle in cell wall synthesis) all cause a marked elevation of CL apparently at the expense of PG;
2. This elevation does not appear to be due to the antibiotics interfering with phospholipid turnover nor by them exerting an effect on cardiolipin synthetase activity;
3. Depending upon the extraction procedure, two different lipid intermediates, A and X, were seen to accumulate on antibiotic treatment. They have been partially characterised and they appear to bear some similarity with each other based on their chromatographic properties. It would appear that the unknown A is composed in part by the C_{55}-isoprenoid lipid bactoprenol (based on comparative thin layer chromatography) and possibly a phospholipid moiety, the identity of which is as yet unknown;
4. When bactoprenol intermediates were specifically isolated from washed membranes, it was demonstrated that one of three major bands corresponded to the unknown A. It was also shown that our accumulated phospholipid intermediate apparently diminished with respect to time, with a concomitant increase in bactoprenol-PP when the cells were subjected to vancomycin inhibition. This was also coordinated with the elevation of CL;
5. We believe that the data presented here provides evidence for the coupling of membrane and cell wall metabolisms.

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