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METABOLISM OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE IN BACILLUS
SUBTILIS

City University of New York

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METABOLISM OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE IN BACILLUS
SUBTILIS

by

DAVID A. KLEIN

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

1981

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This manuscript has been read and accepted by the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

The phosphonate isostere of L-glycerol 3-phosphate, 3,4,-dihydroxybutyl-1-phosphonate, (DHBP), was found to inhibit the growth of both the glycerol phosphate (168) and ribitol phosphate strain (W23) teichoic acid strains of Bacillus subtilis. The inhibition was bacteriostatic for the 168 strain, but bactericidal for strain W23. The growth inhibition could be prevented by inclusion of phosphate, glucose, or glycerol in the growth medium.

Both strains incorporated intact [3-³H]-3,4-dihydroxybutyl-1-phosphonate, ([³H]-DHBP), into a polar lipid, a non-dialyzable fraction found in the aqueous phase of a phenol extract, and the cell wall.

Lipids labeled in vivo with [³H]-DHBP were fractionated on a DEAE-cellulose column with an ammonium acetate gradient. The structure of the major tritium-labeled lipid, Fraction IV, (89-95% of the labeled lipids) was determined by degradation with phospholipase C and alkaline phosphatase. Fraction IV was identified as the phosphonate analogue of phosphatidylglycerol phosphate, (1,2-di-acyl)-sn-glyceryl D-phosphoryloxy-3'-hydroxy-1-phosphonate.

Both strains of B. subtilis covalently incorporated DHBP into the walls in vivo. The 168 strain had 5.4 times as much DHBP as the W23 strain, 57 and 11 mmole DHBP/mole of wall hexosamine, respectively.

The route by which DHBP became incorporated into B. subtilis cell walls was determined by demonstrating that DHBP is a substrate for CDP-glycerol pyrophosphorylase, forming the phosphonic acid analogue of CDP-glycerol, CMP-DHBP. CMP-DHBP, in turn, can participate in an in vitro teichoic acid synthesizing assay. The means by which DHBP can replace glycerol 3-phosphate in teichoic acid synthesis was thus demonstrated.

For CDP-glycerol pyrophosphorylase, the apparent K_m of DHBP is 36 mM Σ -DHBP and the apparent K_i is 6.5 mM Σ -DHBP. The natural substrate, L-glycerol 3-phosphate, had an apparent K_m of .18 mM. The apparent V_{max} was 1.8 mmole CMP-DHBP/min-mg which was only one-third the rate obtained for L-glycerol 3-phosphate. These values suggest that DHBP can inhibit because it may be actively transported to large intracellular concentrations.

Explanations for the differential effect of DHBP between the two strains of B. subtilis are offered.

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Dr. Robert Hickey deserves my great thanks for many irreverant and irrelevant discussions.

My error must be conceded to those who, despite contrary evidence believed this would be done: Mom, Dad, and Jean.

A verse by Walt Kelly is apt:

A song not for now, you need not put stay...
A tune for the was can be sung for today...
The notes of the does not will sound as the does...
Today you can sing of the will-be that was.

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Introduction

Phosphonic acid analogues of naturally occurring compounds have been used to probe glycerol dissimilation in *Escherichia coli* (1-8). The most extensively studied analogue, 3,4-dihydroxybutyl-1-phosphonate (DHBP), the phosphonate isostere of glycerol phosphate, was found to be an effective inhibitor of bacterial growth (1).

The glycerol 3-phosphate transport system is responsible for DHBP uptake in *E. coli* (2). Inhibitory effects of DHBP can be offset by high internal pools of glycerol phosphate (3). Although both glycerol 3-phosphate and its phosphonate analogue inhibit the growth of *E. coli* strains lacking the aerobic glycerol 3-phosphate dehydrogenase (1,9), the modes of action differ. High concentrations of phosphate or the presence of glucose in the medium reduce the inhibition due to glycerol 3-phosphate, but not to DHBP (1).

The primary effect of glycerol 3-phosphate inhibition in *E. coli* is the reduction of RNA synthesis. In contrast, the primary action of DHBP is the preferential inhibition of lipid synthesis (1). Addition of DHBP to the growth medium causes a rapid decrease in phosphatidylglycerol synthesis, followed shortly (20minutes later) by an equally pronounced decrease in phosphatidylethanolamine synthesis. The synthesis of cardiolipin is slightly affected (4,5). The pathway for the synthesis of phospholipids in bacteria is shown in Fig. 1.

DHBP is a competitive inhibitor and substrate for the CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase and glycerol 3-phosphate synthase. It is not recognized by the aerobic glycerol 3-phosphate dehydrogenase or the acyl-CoA:sn-glycerol 3-phosphate acyltransferase. As expected, CDP-diglyceride:serine phosphatidyl transferase is not inhibited by DHBP.

When DHBP is present in the growth medium, a polar lipid is formed in vivo (3,5,6) and in vitro (3,6) by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase. The lipid has been identified as the phosphonic acid analogue of phosphatidylglycerol phosphate, (1,2-diacyl)-sn-glyceryl D-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate (3) (See Fig. 2). Because the phosphonate linkage cannot be hydrolyzed enzymatically, the phosphatidylglycerol phosphate analogue cannot be converted to phosphatidylglycerol. Turnover of phosphatidylglycerol to cardiolipin is unaffected by DHBP (4,5). Thus the net phosphatidylglycerol content of the phospholipids decreases during DHBP inhibition because of constant depletion and reduced synthesis. The V_{max} of DHBP as a substrate for the CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase explains the observed inhibition of phosphatidylglycerol synthesis (3).

L-Glyceraldehyde phosphate may also be considered an analogue of glycerol 3-phosphate. In aqueous solution, glyceraldehyde 3-phosphate exists primarily as the gem diol (the hydrated form of the aldehyde) (10). On a molar basis,

96% of the compound exists as the gem diol with only 4% as the free aldehyde. Viewed from this perspective, glyceraldehyde and glyceraldehyde 3-phosphate are analogues of glycerol and glycerol 3-phosphate, respectively. Tang, et al., investigated the effects of glyceraldehyde phosphate, its phosphonate analogue, 3-hydroxy-4-oxobutyl-1-phosphonate, and glyceraldehyde on E. coli. Glyceraldehyde phosphate is bactericidal, while the glyceraldehyde phosphate analogue is bacteriostatic. Glyceraldehyde inhibition is ascribed to its phosphorylation to glyceraldehyde 3-phosphate by glycerol kinase (8). Glyceraldehyde phosphate inhibition is due to the L-enantiomer. No specific macromolecular process is preferentially inhibited by glyceraldehyde phosphate or its analogue in E. coli. Both glyceraldehyde phosphate and its analogue are competitive inhibitors, but not substrates for acyl-CoA:sn-glycerol 3-phosphate acyltransferase and cytidine 5'-diphosphate-diglyceride:sn-glycerol 3-phosphate phosphatidyltransferase neither had an effect on acyl-CoA:lysophosphatidate acyltransferase activity (8).

The effect of DHPP on Bacillus subtilis was investigated to determine whether DHBP could inhibit gram-positive bacteria as well as gram-negative bacteria such as E. coli. The principal difference between gram-positive and gram-negative bacteria lies in their cellular envelopes. The envelope of the gram-positive organism consists of a plasma membrane surrounded by peptidoglycan. Teichoic acids

are frequently associated with the latter. The cell wall of gram negative bacteria is sandwiched between an inner plasma membrane and an outer membrane containing lipopolysaccharide. It is speculated that the lipopolysaccharide of gram-negative bacteria and the lipoteichoic acids of gram-positive bacteria have common functions (11).

B. subtilis is an especially attractive gram-positive organism in which to study DHBP inhibition because there are two strains of B. subtilis, distinguished primarily by the composition of their teichoic acids. The 168 (Marburg) strain contains a glycerol phosphate teichoic acid polymer attached to its cell wall. In the W23 strain, the teichoic acid consists of ribitol phosphate. Other differences between the strains relate to transformability and the composition of their teichuronic acids (12,13).

The lipids of B. subtilis are similar to those found in E. coli. B. subtilis, however, contains an additional species, lysylphosphatidylglycerol (14,15) and a novel phosphoglycolipid obtained from a W23 derivative (16) (Fig. 2).

The regulation of glycerol phosphate metabolism in B. subtilis was studied by Lindgren (17). Genotypes of B. subtilis exist with lesions analogous to the glpD, glpK, glpT and glpF mutants of E. coli. A comparison of the genetic control of glycerol dissimilation by B. subtilis and E. coli reveals major differences: 1) the glycerol kinase found in B. subtilis is not inducible whereas the E.

coli enzyme is; 2) the glp system in B. subtilis is under the positive control of the regulatory gene product (GlpP), with the gene product acting as an inducer, whereas the E. coli regulatory gene, glpR produces a repressor which regulates the E. coli glp system (18); and 3) the diglycerides of B. subtilis turnover, presumably into glucosyldiglycerides (19) while the diglycerides of wild-type E. coli are much less metabolically active (20). The diglycerides of E. coli generated when phosphatidylglycerol loses sn-glycerol 1-phosphate during the synthesis of membrane derived oligosaccharides, are converted to phosphatidic acid by a membrane bound diglyceride kinase (21).

Teichoic Acids

Teichoic acids occupy a unique position in the annals of biochemistry. Following the identification of CDP-ribitol and CDP-glycerol it was predicted that bacteria would use these nucleotides to synthesize polymers of glycerol phosphate and ribitol phosphate. CDP-glycerol and CDP-ribitol were postulated to participate in a polymerization process analogous to that by which cellulose and glycogen are formed from UDP-glucose and chitin from UDP-N-acetylglucosamine (UDP-GlcNAc) (22).

The predicted polymers were first isolated from the trichloroacetic acid extracts of the cell walls of Lactobacillus arabinosus. Further studies suggested that teichoic acids were a general feature of gram-positive bacteria (see references 23-27 for reviews).

The term teichoic acid originally designated polymers of either glycerol phosphate or ribitol phosphate in which the subunits were linked by phosphodiester bonds and the residue substituted with various glycosyl and D-alanine ester groups. The use of the term has been extended to include other polymers found in gram-positive bacteria with properties, biosynthesis, and probable functions similar to those of the teichoic acids.

While the chemical composition of the teichoic acids found in the TCA extracts of the cell walls of different bacteria vary, the teichoic acids extracted from bacterial membranes are invariably glycerol phosphate teichoic acids. The terms wall teichoic acid and membrane teichoic acid were used to designate the polymers released from these sites by TCA extraction.

Often, when cell walls were digested with lysozyme a glycerol phosphate teichoic acid could be found in the medium. In the belief that these teichoic acids were located between the membrane and the wall and released when the wall was destroyed, these polymers were termed "intracellular teichoic acids". "Intracellular teichoic acids" were subsequently shown to be an artifact dependent on the magnesium ion concentration (23,28).

There are major distinctions between the teichoic acids associated with the wall and those associated with the membrane:

- 1) While membrane teichoic acids are an invariant

biochemical marker for gram-positive bacteria, the wall teichoic acids are not found in all gram-positive bacteria and their presence is dependent on growth conditions (13).

- 2) Wall teichoic acids are covalently linked to the peptidoglycan and vary widely in their chemical composition. Membrane teichoic acids, on the other hand, are covalently linked to a plasma membrane glycolipid and are always composed of glycerol phosphate (13).

Wicken and Knox (24) proposed the term "lipoteichoic acid" (LTA) for the glycolipid associated teichoic acids. Another term, lipoteichoic acid carrier (LTC) was introduced to designate a Triton X-100 extractable teichoic acid from *E. subtilis* membranes onto which teichoic acid could be polymerized (29,30). LTC is assayed by its stimulatory effect on the enzyme which polymerizes teichoic acid (29,31).

Structure

The polymeric structure of the teichoic acids found in *E. subtilis* are illustrated in Fig. 3. The glycerol phosphate teichoic acids may be substituted at the 2-position with D-alanine or an α -D-glucospyranosyl residue (23). In the ribitol teichoic acids derived from the wall of strain W23, the residue are joined by phosphodiester bonds between the 1 and 5 position of adjacent ribitols. The 2 and 4 positions may be substituted with D-alanine and

β -D-glucopyranosyl residues, respectively (21).

The cell wall teichoic acids of both strains are mixtures of glucosylated and unglucosylated chains (32,33).

Although the structure of the LTA of *B. subtilis* has not been elucidated, it probably shares the features common to the LTAs of other gram-positive bacteria (27, 34). LTAs consist of a polymer of 25-30 glycerol phosphate residue connected to a glycolipid through a phosphodiester bond between a sugar hydroxyl of the glycolipid and the phosphate terminal glycerol phosphate residue of the teichoic acid chain. In some bacteria the glycosyl terminal glycerol phosphate or the glycosyl sugars are reported to be acylated with fatty acids (27). Recent investigations have not confirmed this (34).

Lambert, et. al. found a difference between the teichoic acid species isolated from the aqueous layer of phenol extracted membranes of *Staphylococcus aureus* (LTA) and the Triton X-100 extractable teichoic acid polymer from the same source (LTC) (36). The teichoic acid isolated in the phenol extraction had one less fatty acid residue and a greater number of glycerol phosphate residues than the Triton X-100 extractable polymer. Chiu and co-workers found two types of LTA polymers in *Streptococcus sanguis*. Initially differentiated by solubility, these two polymers differ by the presence or absence of fatty acids (37).

Acid hydrolysates of LTC contained glycerol, glucosamine, amino acids and an ether soluble fraction. The

amino acids could not be released by pronase treatment (38). The evidence suggested that LTA and LTC shared common structural features, but the biosynthetic relationship was unknown.

Fischer and co-workers have recently shown that differences between LTC and LTA are artifactual. The ability of the extracted teichoic acid polymer to serve as a substrate for the teichoic acid polymerases, i.e., to have "LTC-activity", is dependent upon the degree to which the teichoic acid polymer is substituted. Fischer showed that native LTA, i.e., LTA extracted at low pH to preserve the alanyl esters, has no ability to function as an acceptor in the teichoic acid polymerase assay (35). LTAs with as few as 40% of the glycerol phosphate residues substituted are inactive as substrates for poly(ribitol phosphate) polymerase (35).

The lability of the alanyl esters on teichoic acids had long been known (23), but it was only this recent work which showed that "LTC-activity" is due to the isolation procedure decreasing the alanine substitution of the LTA, and not the presence of a different species of teichoic acid (35). Thus LTC was shown to be a less substituted form of LTA.

Teichoic Acid Biosynthesis

Despite similarities between the teichoic acids of various bacteria, differences exist. The discussion of teichoic acid biosynthesis which follows will offer an overview bearing in mind that determinations made with one

organism might not be applicable to another.

In vivo studies of LTA biosynthesis have been conducted with S. aureus and S. sanguis. The wall teichoic acid of S. sanguis is composed of a polymer of D-glucose and L-rhamnose (39) while the polymer found in S. aureus is composed of poly(N-acetylglucosaminy l ribitol phosphate) (40). Phosphatidylglycerol is the donor of the glycerol phosphate residues found in the LTA of these bacteria. In vitro confirmation of the in vivo observations have been made with S. sanguis (37,41) and Streptococcus faecium (42). It should be noted that a glycerol phosphate residue donated from phosphatidylglycerol will yield polymers of sn-glycerol 1-phosphate, whereas polymers made from CDP-glycerol would be composed of sn-glycerol 3-phosphate residues.

Although the structure of B. subtilis LTA has not been determined, the most likely candidate for the hydrophobic anchor is β -gentiobiosyldiacylglycerol (16) (Fig. 2). The pathway by which this lipid is synthesized is not known. It is likely that the diacylglycerol portion is derived from dephosphorylation of phosphatidic acid (19) or from phosphatidyl glycerol through the loss of sn-glycerol 1-phosphate. The similarities between the acyl substitution of the membrane lipids of B. subtilis and the acyl substituents of the sn-glycero-1-phospho- β -gentiobiosyldiacylglycerol suggest such a precursor-product relationship (43,44). The glucose in the gentiobiose moiety is probably donated sequentially from UDP-glucose in the same

manner as other glycolipids are made (45).

Further support for the phosphoglycolipids serving as LTA precursors comes from the study of a phosphoglucomutase deficient mutant of Bacillus licheniformis (46). The mutation renders the bacteria unable to convert glucose-6-phosphate into glucose-1-phosphate and thus unable to make UDP-glucose. The LTA isolated from the mutant lacked the glucose residues found in wild type LTA. The authors offered two explanations: 1) phosphatidylglycerol or diglyceride can substitute for the gentiobiosyldiglyceride as the receptor onto which the glycerol phosphate residues are polymerized or 2) the usual synthetic route for LTA in B. licheniformis involves the polymerization of glycerol phosphate onto phosphatidylglycerol or diglyceride prior to transfer of the polymer to the usual acceptor, presumably the glycerophosphoglycolipid (Fig. 2).

The work of Deutsch, et. al., with B. subtilis supports the involvement of phosphatidylglycerol in LTA and phosphoglycolipid synthesis (47). The synthesis of both the phosphoglycolipid and LTA are drastically inhibited in B. subtilis grown in the presence of DHBP. DHBP is known to preferentially inhibit the synthesis of phosphatidylglycerol in B. subtilis (47).

The polymerization of teichoic acid has been studied most intensively in vitro in S. aureus. Although the bulk of the investigations were directed toward elucidating the function of LTC (29-31,36,48-50), the findings are still

useful in understanding the mechanism of teichoic acid synthesis.

The teichoic acid polymerases extracted from membranes were found to have an absolute requirement for an acceptor (which at the time was believed to be LTC) and phospholipids (29). It was found that LTC from one bacteria could act as a substrate for the polymerizing enzyme(s) obtained from a different bacteria. Thus the LTC obtained from a bacteria producing glycerol phosphate teichoic acid can serve as an acceptor of ribitol subunits (49). These results are compatible with more recent work indicating that the true acceptor is the linkage unit (34,35). The LTC and the linkage unit being unsubstituted glycerol phosphate polymers could very well appear similar to the polymerase.

The mechanism by which the chain length is determined is unknown. It was found that LTC from *S. aureus* would make a polymer of finite size (30 residues) when loaded by the homologous polymerase, but produced a larger polymer when used as a substrate for a poly(glycerol phosphate) producing enzyme (49).

Electrophoretic separations of the contents of the in vitro poly(ribitol phosphate) polymerizing assay show two polymeric species, LTC and LTC with a full complement of 30 ribitol phosphate residues attached. Intermediates of LTC with fewer than 30 residues are not found. By altering the in vitro assay conditions for *S. aureus* poly(ribitol phosphate) polymerase, Glaser and co-workers were able to

obtain an intermediate sized LTC to which an average of 12 ribitol phosphate residue were linked (31). If these intermediate sized polymers are used in place of LTC as the acceptor under the usual assay conditions, the polymerase recognizes the intermediate as a substrate and extends the length of the ribitol phosphate chain to 30 residues. The rate at which the partially loaded LTC is extended is only one-ten thousandth as great as the rate at which the ribitol phosphate residues are added to unloaded LTC (31). Although LTC is not the physiological acceptor, these findings suggest that intermediate sized teichoic acid polymers are not released into solution during normal biosynthesis.

The polymerization of the glycerol phosphate and ribitol phosphate residues from CDP-glycerol and CDP-ribitol, respectively, was found to proceed by stepwise addition onto the hydroxyl terminus of the growing polymer (51-53).

Modification of Teichoic Acids

Modification of the teichoic acid polymer by glucosylation (50) and esterification with D-alanine (54) is performed by membrane associated enzymes. The synchrony of teichoic acid polymerization and modification has not been determined. Indirect evidence suggests that modification of the main chain occurs before the chain is attached to the cell wall (33,55). Teichoic acids extracted from *B. subtilis* walls or membranes can be completely glucosylated in vitro (33). If walls containing teichoic acid are used as the substrate, however, the extent of glucosylation is

far less (33). The apparent difficulties involved in the in vitro glucosylation of teichoic acid bound to the cell wall argues strongly that the glucosylation occurs before the polymer is attached to the wall.

Analysis of wall derived polymers by immunological methods shows that wall bound teichoic acids are a mixture of unsubstituted and completely glucosylated polymers in both strains of *B. subtilis* (32,33,55). The means by which two polymers can be synthesized is unknown.

The glucosylating enzyme extracted from the ribitol teichoic acid strain does not glucosylate the glycerol phosphate teichoic acid polymer. Similarly, the glucosylating enzyme from the glycerol phosphate strain will not accept the ribitol phosphate teichoic acid as a substrate. Although both glucosylating enzymes transfer the glucose from UDP-glucose, the glucose is carried as the β -D-glucopyranosyl residue in strain W23, but as the α -D-glucopyranosyl substituent in the teichoic acids of strain 168 (23).

Teichoic acid biosynthesis is currently viewed as follows (Fig. 4) (56):

The linkage unit which will serve to connect the main teichoic acid polymer to the muramic acid of the peptidoglycan is assembled on a polyprenol phosphate carrier (56-60). By a sequential process, first GlcNAc (from UDP-GlcNAc) and then three glycerol phosphate residues (from CDP-glycerol) are added in succession (61,62). The teichoic acid polymer may be polymerized directly onto Lipid IV. The

linkage unit-teichoic acid polymer is subsequently joined through a phosphodiester bond between the C-1 of the GlcNAc of the linkage unit and the C-6 hydroxyl of the muramic acid of the glycan chain (63,64). Alternatively, the teichoic acid may be polymerized directly onto the linker region which had previously attached to the peptidoglycan (58).

The synthesis of Lipid I (Fig. 4) is sensitive to tunicamycin inhibition (23).

Despite similarities in the teichoic acid synthesizing pathways, the linker region of *B. subtilis* W23 differs from that of both *Micrococcus varians* and *S. aureus* (56,63). In the latter bacteria the first glycerol phosphate residue of the linker region forms a phosphodiester bond to the C-4 hydroxyl of the N-acetylglucosamine, whereas the bond is to the C-6 hydroxyl in *B. subtilis* W23 (Fig. 5). The linkage unit of strain 168 does not lend itself to the elegant degradative efforts of Baddiley and co-workers, but similar investigation of the linkage unit of strain 168 indicates the presence of glucosamine (56).

The attachment of the linker region-teichoic acid polymer to the cell wall differs from a similar step in cell wall synthesis. In wall synthesis, the undecaprenyl wall precursor, undecaprenylpyrophosphate-N-acetyl-muramyl-pentapeptide- β (4 $\rightarrow$ 1)-N-acetylglucosamine transfers the disaccharide pentapeptide moiety to the peptidoglycan acceptor releasing the undecaprenyl pyrophosphate which is subsequently dephosphorylated. In teichoic acid synthesis,

Lipid IV (Fig. 4) releases the polyprenol phosphate (56).

Regulation of Teichoic Acid Synthesis

Although newly synthesized teichoic acid is linked to newly synthesized peptidoglycan (65) and the ratio between teichoic acid and peptidoglycan is constant during logarithmic growth (66) and germination (67), the syntheses do not appear to be coupled in vivo. Peptidoglycan synthesis proceeds at a normal rate when teichoic acid synthesis is inhibited (68). When *B. subtilis* cell wall synthesis is selectively inhibited in vivo, the rate of teichoic acid synthesis appears unaffected. The teichoic acid synthesized during cell wall inhibition is attached to previously synthesized sections of wall (68,69).

Reports of joint inhibition of peptidoglycan and teichoic acid synthesis by bacitracin in vitro can be attributed to the antibiotic depleting the pool of undecaprenyl phosphate available for teichoic acid synthesis. Bacitracin inhibits conversion of undecaprenylpyrophosphate to the phosphate. Thus no undecaprenylphosphate would be available for teichoic acid synthesis.

Transferring *B. subtilis* from culture conditions in which teichoic acids are being incorporated into cell walls to low phosphate medium results in the selective removal of teichoic acids from the walls and their replacement with uronic acid polymers known as teichuronic acids. Low phosphate concentration of itself is not sufficient to instigate the change as the additional presence of high salt

concentrations or low pH conditions in the growth medium can prevent the conversion (For a review see reference 13).

The transfer of teichoic acid synthesizing *B. subtilis* into low phosphate medium does not affect the rate of synthesis of LTA or phospholipids or reduce the pool size of undecaprenyl phosphate (70). Although there is a decrease in CDP-glycerol pyrophosphorylase activity, the loss in activity lags behind the cessation of teichoic acid synthesis (70). Such findings would be expected if the teichuronic acid synthesizing system used CDP-glycerol pyrophosphorylase to make CDP-glycerol for a linker region or if the site of control for conversion were at a different step in the synthetic pathway. In addition to the aforementioned results, Glaser and Loewy found that cells grown under low phosphate conditions have a reduced ability to synthesize Lipid I (Fig. 4) (70). *De novo* protein synthesis is required following a shift in medium to inhibit incorporation of teichoic acid into cell wall (Fig. 6). It should be kept in mind that a metabolite other than phosphate may activate the regulatory mechanism (13,69,70).

Grant showed quite clearly that the teichoic acid lost under phosphate limitation is not excreted into the medium (71). Enzymes have been identified which, acting in consort, provide a means to recycle teichoic acid during phosphate limitation. A membrane bound alkaline phosphatase and a phosphodiesterase, presumed to be located in the periplasmic space, are simultaneously derepressed under low

phosphate conditions (72). The two enzymes appear to be under the same regulatory control and are required for growth of *B. subtilis* during teichoic acid to teichuronic acid conversion (72). A teichoicase capable of degrading wall-bound teichoic acid has been isolated from sporulating cells (73). Whether the teichoicase is actually related to the phosphodiesterase is not known. The two enzymes could be derepressed by either low phosphate or sporulation. Acting on wall-bound teichoic acid, these two enzymes allow the recovery of the phosphate and the glycerol in the teichoic acid.

CDP-glycerol pyrophosphatase, an enzyme which hydrolyzes CDP-glycerol to CMP and glycerol phosphate, has a high K_m for CDP-glycerol suggesting that it may serve to scavenge CMP and glycerol phosphate when CDP-glycerol levels accumulate (74). This might happen when teichoic acid synthesis is turned off during conversion to a teichuronic acid synthesizing system. The expectation is reinforced by the finding that the activity of CDP-glycerol pyrophosphorylase, the CDP-glycerol synthesizing enzyme, does not decrease as rapidly as the cessation in teichoic acid synthesis (70).

Function of Teichoic Acids

The observation that teichoic acids can be replaced by another polymer raises questions as to the function and necessity of teichoic acids. One aspect of teichoic acids which has been studied extensively but which remains open to

considerable speculation is the ability of teichoic acids to serve as antigens (reviewed in reference 13) and as attachment sites for bacteriophage (75). Neither property is conducive to the survival of the cell although the exchange of genetic material during infection may yield evolutionary dividends.

Teichoic acids must be glucosylated (76-78) and be bound to the peptidoglycan matrix (26,79) to serve as phage binding sites. High ionic strength buffers inhibit the binding of phage to teichoic acid (80). The explanation undoubtedly lies in the salt induced change of wall teichoic acid from a rigid extended configuration to a random coil (81).

The chemical excision of teichoic acids from isolated cell walls causes the walls to agglutinate, indicating that the maintenance of an array of negative charges on the cell surface helps keep the cells apart (80). The extensive regular pattern of negatively charged phosphate groups in the teichoic acid polymer led to the suggestion that teichoic acids serve to sequester or aid in the transport of cations, especially magnesium (82). This position is supported by *in vitro* (83) and physical chemical studies (84-86), and the structural analysis of teichoic acids synthesized by bacteria grown in high salt media (86,87).

In vitro teichoic acid synthesis by membrane preparations lacking "membrane teichoic acid" (LTA) show a marked dependence on exogenous magnesium ion. In contrast,

membranes possessing "membrane teichoic acids" do not require the addition of magnesium ions to synthesize teichoic acids in vitro (83). The same study showed that in vitro teichoic acid synthesis by wall/membrane preparations which were washed after preincubation with magnesium ions is independent of exogenous magnesium ions. These observations demonstrate the ability of teichoic acid polymers, whether bound to the wall or as LTA, to bind magnesium ions.

Careful physical chemistry studies have found that wall bound teichoic acid and teichuronic acid bind magnesium ions equally well (84-86). The magnesium ions appear to be bound between adjacent chains (84). Alanine substitutions of teichoic acid stoichiometrically decreases the amount of magnesium bound (85, 86). This dovetails with the observation that there is a decrease in alanyl substitution of the teichoic acids produced by cells cultured at high sodium or potassium concentrations (84,87). Phosphate limitation alone is not sufficient to cause teichoic acid to teichuronic acid synthesis. The added constraints of high monovalent salts (which can compete with magnesium ion for the teichoic acid phosphate) or low pH force the cell to retain and continue to synthesize teichoic acids even when phosphate is growth limiting (24,26).

Although the magnesium binding constants of teichoic acids and teichuronic acids were found to be the same (84), the teichoic acids may be more efficient at higher ionic strength or lower pH because the K_a of phosphate is lower

than that of the the uronic acid carboxyl group. An alternative explanation is that there is an as yet unknown function of teichoic acid for which teichuronic acid cannot substitute. Teichoic acids are found to be synthesized in limited quantities even under conditions where only teichuronic acids were expected (88).

Another role proposed for teichoic acids is involvement in cell wall growth and turnover through interaction with an autolytic enzyme, N-acetyl muramyl-L-alanine amidase. New cell wall and teichoic acids are randomly inserted over the surface of *B. subtilis* as old wall is excised and released into the growth medium (reviewed in reference 89). The rounded ends or caps of the capsid are less susceptible to autolysis than the rest of the cell surface and newly synthesized wall is immune from turnover for one-half to one generation. This is the same time frame for translocation of the teichoic acid from deposition on the inner surface of the wall to exposure on the outside (88). Other data suggests that the autolytic enzyme, tightly bound to teichoic acid, is transported with the teichoic acid into the cell wall (90). The pattern of autolytic activity appears directed by wall bound teichoic acid (91,92). The assumption underlying the hypothesis is that as successive layers of old wall is excised and sloughed off revealing new layers, the new microenvironment permits the enzyme to function thus enabling the cell to maintain it's shape. Those areas not actively turning over (caps) would expand

little, while those areas with rapid turnover would grow considerably (lateral walls). In this respect, the shape of phosphoglucomutase mutants supports this hypothesis. These mutants cannot make the gentiobiose needed for LTA and are round rather than rod shaped (46). Other mutants lacking teichoic acids show morphological aberrations (93,94).

Rationale of Approach

The primary objective in studying the effect of DHBP on B. subtilis metabolism was to determine if DHBP would affect teichoic acid metabolism. The similarity of lipid metabolism between E. coli and B. subtilis suggested that the perturbation of B. subtilis lipids would be similar to that of E. coli (17,21). Thus it was expected that DHBP would substitute for glycerol phosphate in phosphatidyl-glycerol synthesis resulting in the accumulation of a phosphonate analogue of phosphatidyl glycerol phosphate (3). This would be expected to depress phosphatidylglycerol synthesis. The participation of phosphatidylglycerol in the synthesis of LTA predicted that the synthesis of this teichoic acid polymer would be inhibited.

It was hoped that DHBP would become incorporated into the wall polymer, the linker region, or both. The pathway by which such an incorporation would occur would be investigated by using DHEP in place of glycerol phosphate in vitro in reactions involved in the synthesis of teichoic acids (see references in 31).

Materials and Methods

Chemicals: rac-3,4-dihydroxybutyl-1-phosphonate (DHBP) (95) rac-3,4-dihydroxy-[3-³H]-butyl-1-phosphonate ([³H]-DHBP) (96), and rac-3-hydroxy-4-oxobutyl-1-phosphonic acid (diethyl acetal, dilithium salt) (97) were prepared as previously described. D-Glyceraldehyde 3-phosphoric acid (diethyl acetal, dicyclohexyl ammonium salt) was a product of Boehringer Mannheim Corp., N.Y., N.Y.

The glyceraldehyde 3-phosphoric acid and 3-hydroxyl-4-oxobutyl-1-phosphonic acid were generated from the corresponding acetals using the method recommended by the Sigma Chemical Company: 0.5 mmole of acetal dissolved in 6.0 ml of distilled water was mixed with 3.0 gm of Dowex-50 (H+). The solution was placed in a boiling water bath for 3.0 minutes and chilled immediately on ice. By placing a spatula in the test tube and shaking the tube with a gyrotory motion during the heating, the suspension was kept in constant agitation. The Dowex beads were washed twice with 2.0 ml of distilled water. The combined supernatants containing 50 mM aldehyde were stored frozen until used.

The following were obtained from Sigma Chemical Co., St. Louis, Mo.: *Bacillus cereus* phospholipase C (Type III); tris-(hydroxymethyl)aminomethane (Tris); Triton X-100 (octylphenoxypolyethoxyethanol); casein hydrolysate (enzymatic digest); glucosamine-HCl; trypsin (type III); ribo-

nuclease-A (type 1-A); deoxyribonuclease (type DN-25); lysozyme (Grade III); α -chymotrypsin (type 1-S); and protease from Streptomyces griseus (type VI) (pronase).

Eugon agar and Eugon broth are products of Difco Laboratories, Detroit, Michigan. All other chemicals were of reagent grade.

Bacteria: B. subtilis 168 strains BD-170 (thr, trp) and 42 (gly c, glp D, ind, his) and B. subtilis strain 1005 (met), a W23 strain mutant were gifts of Drs. L. Mindich and P. Dubnau of the Public Research Institute of the City of New York. Strains were allowed to sporulate on Eugon agar slants at 37C and stored in the cold until needed. Unless otherwise noted, these strains were cultured in casein hydrolysate broth, casein hydrolysate (10 gm) and NaCl (5 gm) per liter of medium with the pH adjusted to 7.0 with NaOH. Cells were grown in a gyrotory shaker at 37C and 150-160 rpm in unbaffled nephelo flasks.

Determination of the effect of DHBP on the growth and viability of B. subtilis: Overnight cultures of B. subtilis were started directly from spores. Experimental cultures were inoculated with 0.3-0.5 ml of a stationary overnight culture diluted into 19.5 ml of fresh casein hydrolysate broth in 250 ml nephelo flasks. Growth was monitored turbidometrically using a Klett-Summerson colorimeter fitted with a 660 nm filter. When cultures reached 20 Klett units (ca. 3×10^7 cells/ml) DHBP (0.5 ml) was added.

In those experiments in which the viability of the bacteria was determined, 0.1 ml aliquots of culture were withdrawn at hourly intervals after addition of the DHBP, diluted in Spizizens's minimal salts medium at room temperature (98) and plated on Eugon agar plates. Colonies could be counted after 14 hours of incubation at 37C.

Incorporation of [³H]-DHBP into whole cells: When the cell density in culture reached 20 Klett units, [³H]-DHBP was added to a final concentration of 30 uM and 1 uCi/ml. the incubation was continued for 2 hours. Cells were harvested by centrifugation at 5,000 x g for 10 minutes, washed once with ice cold 0.9% NaCl, resuspended, and a portion removed for lipid extraction, and the remainder disrupted by sonic oscillation. The chilled suspension was treated with three 30 sec. bursts using a Heat Systems-Ultrasoncs, Inc. (Plainview, N.Y.) Model W140 Sonifier Cell Disruptor at a power setting of 9. Half of the sonicate was mixed with an equal volume of distilled 88% phenol and stirred in an ice bath for 1 hour. The phases were separated by centrifugation, back washed, and the combined aqueous phases washed twice with ether. The samples were dialyzed against 16 l of distilled water at 4C for 16 hours. The distilled water was changed once during the dialysis. The level of radioactivity in the dialyzing water was monitored to insure that the dialysis was complete.

The cell pellet from an 80 ml culture was resuspended in

13 ml of distilled water, 1 or 2 ml of suspension removed for lipid extraction and the remainder divided for recovery of cell walls or to obtain the phenol extract

Cell walls were collected from the other half of the sonicate by centrifugation at $24,000 \times g$ for 15 minutes. The cell wall pellet was suspended in 5.0 ml of 2.5% sodium dodecyl sulfate and heated for 30 minutes at 80-90°C to destroy autolytic activity. Detergent was removed from the walls with repeated washings with 5.0 ml portions of distilled water. Four washings were usually sufficient to remove all traces of foaming.

Lipids were extracted using the Ames (99) modification of the Bligh and Dyer method (100). During extraction, care was taken not to disturb the interface between the chloroform and aqueous layers as the aqueous layers were being aspirated off. The chloroform layer was washed successively once with 2.5 ml of 2 M KCl, twice with 2.5 ml of 1 mM DHBP, and lastly, once with 2.5 ml of distilled water. After the last wash sufficient methanol was added to allow the chloroform and aqueous layer to form a single phase. For a lipid extraction of a 2.0 ml suspension of cells or sonicate (corresponding to ca. 12 ml of original culture), 2.5 ml of methanol was required. The insoluble debris was removed by filtration through glass wool.

Radioisotopic measurements:

a) Aqueous samples: Aqueous samples were placed in plastic scintillation vials, water or acid added, if

necessary, to bring the aqueous volume to 1.0 ml, and 10 ml of Patterson-Greene scintillation fluid (101) added. Chromatographic samples were allowed to soak for 10 minutes in 1.0 ml of water before addition of the scintillation fluid.

b) Lipid samples: Samples of lipid in organic solvents were placed in plastic scintillation vials, dried under a gentle stream of warm air and counted as previously described (3).

Radioactive measurements were made with a Beckman LS 200 or a Searle Isocap 300 liquid scintillation counter.

DEAE-cellulose column chromatography of [³H]-DHP-
labeled lipids: DEAE-cellulose (Whatman DE-52) was prepared according to the manufacturer's instructions to remove fines. DE-52 directly from the bottle was suspended in a graduated cylinder in a volume of water (expressed in milliliters) equal to six times the weight (in grams) of the ion exchanger. The cylinder was inverted several times until the suspension was uniform. The exchanger was allowed to settle for the time (in minutes) equal to the height of the suspension (in centimeters) multiplied by 1.3. At the end of the time period, the "wet settled volume", the volume of the precipitated DEAE-cellulose was noted, and the supernatant aspirated off so that the final volume of the DEAE-cellulose and the supernatant left in the cylinder was equal to the wet settled volume plus 20%.

In preparing the DEAE-cellulose, 40 gram of DE-52

suspended in 240 ml of water in a 250 ml graduated cylinder had a height of 31 cm. The settling time was 40 minutes and the wet settled volume was 97 ml.

The settled DEAE-cellulose was suspended in glacial acetic acid to convert the exchanger to the acetate form. The cellulose was then washed free of acid in a coarse sintered glass filter with chloroform-methanol-water (2:3:1, v/v/v). The exchanger was equilibrated in the same solvent for 1 hour at 4C. The DE-52 was suspended in enough solvent to produce a thick uniform slurry and poured into a 25 ml burette fitted with a teflon stopcock and some glass wool. Several additions were required to achieve the desired 30 cm bed height.

To insure that the column would be uniformly packed, the following procedure was employed: When the bed had packed sufficiently to permit the addition of more slurry, the solvent was drawn off the top, more slurry added, the stopcock closed, the top of the column sealed with aluminum foil, and the column inverted several times until the suspension above the packed bed was homogeneous. The column was then allowed to settle by gravity with the stopcock open. The column was filled approximately 5 cm higher than the desired bed height. Solvent was passed through the column at operating pressure until a constant bed volume was obtained. Excess column packing was removed with a pipette.

A gradient former, constructed from two 250 ml erlenmeyer flasks joined by a stopcock with teflon fittings,

was connected to the column with teflon tubing. The teflon tubing extending from the low salt reservoir was shrinkfitted to a small section of glass tubing. The glass tubing was inserted through a 14/20 standard taper teflon adapter which would serve to connect the gradient former to the column. The erlenmeyer flasks rested on a block of wood so that the bottoms of the two flasks lay in the same plane and insuring that there would be no movement through the stopcock when equal volumes of liquid were placed in each flask. The low salt reservoir was equipped with a magnetic stirring bar and positioned over a magnetic stirrer.

Column chromatography was performed at 4°C. Lipid samples contained in 1 ml or less of chloroform-methanol-water (2:3:1, v/v/v) were layered over the top of the column. After the sample was absorbed into the column, the sample was washed in with three 0.5 ml portions of the same solvent. Several milliliters of the solvent were placed above the column bed to insure adequate mixing of the gradient. The gradient former was connected to the column, the teflon thermometer adapter-column junction wrapped with Parafilm, 150 ml of chloroform-methanol-water poured into the mixing (low salt) flask, and an equal volume of the same solvent containing .10 M ammonium acetate added to the high salt reservoir. After equalizing the fluid levels in the two flasks, the stopcocks were opened and the solvents allowed to flow. Fractions of 231 drops (approximately 4 ml) were collected. The gradient apparatus produced a 300

ml linear gradient of ammonium acetate (0-0.1 M) in chloroform-methanol-water. Salt concentrations were measured using a Beckman model RC 16B2 conductivity bridge with a K-1 probe. The conductivity was determined by immersing the probe in the sample and compared with standards prepared in the original solvent.

Following elution, the fractions were located by radioactive measurement, the desired fractions pooled, reduced in vacuo, and the residues extracted by the method of Bligh and Dyer (100). The column was regenerated by elution with chloroform-methanol-water-glacial acetic acid (2:3:1:1, v/v/v/v) until the acetic acid could be detected in the eluate by litmus. The column was then washed free of the acetic acid with chloroform-methanol-water.

Hydrolysis of lipids with phospholipase C and alkaline phosphatase: Lipids isolated from the DEAF-cellulose column were hydrolyzed with phospholipase C in a two-phase system similar to that of DeHaas, *et. al.* (102). Samples of lipid dissolved in chloroform were placed in screw capped test tubes and the solvent removed under a stream of nitrogen. Diethyl ether (1.0 ml), 0.2 ml of B. cereus phospholipase C solution (10 U/ml in 100 mM Tris-HCl buffer, pH 7.2), and 20 ul of 1 mM zinc chloride were added. If the water soluble extracts were to be examined, 100 mM ammonium acetate and 1 mM zinc acetate were substituted for the Tris-HCl and the zinc chloride respectively. The substitution was made to allow removal of the volatile salts

in subsequent steps. Samples containing the phosphatidylglycerol phosphate analogue (fraction IV) had 100 ug of crude unlabeled bacterial lipids added as carrier to the hydrolysis mixture. The tubes were tightly capped and shaken overnight on a Buchler Evapomix at 25C for complete hydrolysis or for 2 hours when the water soluble products were to be examined. At the end of treatment, the ether was removed under a stream of nitrogen and the water and chloroform soluble products separated as previously described (3,100). The chloroform layer was backwashed twice with 0.5 ml portions of distilled water. The aqueous layer was backwashed with two 0.5 ml portions of chloroform. To determine the loss of chloroform soluble label, the organic phases were combined and the radioactivity determined as previously described (3). When the water soluble products were studied, the aqueous phases were combined and boiled for 5 minutes to destroy alkaline phosphatase activity.

The aqueous extract was divided and each part dried under reduced pressure at 25-30C. One part was redissolved in a minimum volume of distilled water and treated with Dowex 50(H⁺) before chromatography. The other part, dissolved in 0.2 ml of 50 mM sodium bicarbonate buffer, pH 8.1 containing 0.25 U of *E. coli* alkaline phosphatase was incubated for one hour at 25C. After heating the reaction mixture in a boiling water bath for 5 minutes to inactivate the enzyme, the reaction mixture was filtered through a

Millipore filter, the water removed in vacuo and the residue examined chromatographically.

Incorporation of [³H]-DHBP into cell walls in vivo:
Cell walls were prepared as described in this section except that the cells were cultured in 80 ml of casein hydrolysate broth in a 500 ml baffled nephelo flask incubated at 37C and 200 rpm. [³H]-DHBP (33 mCi/mmol) was added to a final concentration of 30 μ M. Cells were allowed to incubate in the presence of the drug for one hour.

Enzymatic treatment of [³H]-DHBP-labeled cell walls:
[³H]-DHBP-labeled cell wall suspensions (50 μ l) containing $1.5-8 \times 10^3$ cpm were incubated at 37C for 5 hours as follows:

a) Trypsin: 0.01 ml of a trypsin solution (15 mg/ml in 1 mM HCl, 700 U/mg), 1.5 ml of 200 mM potassium phosphate buffer, pH 7.6, and 1.4 ml of water.

b) Pronase: 0.9 ml of a S. griseus protease suspension (1 mg/ml of water, 3 U/mg), 2.2 ml of 92 mM potassium phosphate buffer, pH 7.1, and 0.9 ml of water.

c) Lysozyme: 1.0 ml of a lysozyme solution (1 mg/ml of water, 40,000 U/mg), 1.3 ml of a 200 mM potassium phosphate buffer, pH 7.6, and 1.7 ml of water.

d) RNase and DNase: 0.2 ml of RNase (0.2 mg/ml of water, 65 Kunitz U/mg), 0.2 ml of DNase (0.2 mg/ml of water, 115 Kunitz U/mg), 2.0 ml of 200 mM Tris, pH 8.0, 1.0 ml of 40 mM magnesium chloride, and 0.6 ml of water.

e) α -Chymotrypsin: 0.5 ml of an α -chymotrypsin

solution (0.1 mg/ml of water, 42 U/mg), 2.5 ml of 200 mM potassium phosphate buffer, pH 7.6, and 2.0 ml of water.

At the end of the incubation the mixtures were centrifuged at 23,000 x g. A 1.0 ml sample of the supernatant was tested for radioactivity.

Chemical hydrolysis of [³H]-DHBP-labeled cell walls:

[³H]-DHBP-labeled cell wall suspensions containing 1.2-6 x 10³ cpm were treated as follows:

a) Complete acid hydrolysis: Wall suspensions were hydrolyzed overnight in 2 N HCl in sealed screw capped test tubes at 100C. After cooling, the solvent was removed under reduced pressure at 25-30C. The residue was dissolved in distilled water and chromatographed.

b) Partial acid hydrolysis: The excision of teichoic acids and the linker region from cell walls by gentle acid hydrolysis is based on the work of Coley, et. al. (56) and Hughes and Tanner (103). The phosphodiester bond joining the N-acetylglucosaminyl residue of the linkage unit to the C-6 hydroxyl of the muramic acid residue of the glycan chain is cleaved to leave the phosphate attached to the muramic acid (Fig. 5). Wall suspensions were hydrolyzed in 0.1 N HCl for 10 minutes in a boiling water bath, cooled immediately in ice and the walls collected as previously described. This mild acid treatment is reported to cleave 90% of the susceptible bonds (104). After a sample of the supernatant was removed for radioactive counting, the remainder of the supernatant was aspirated off and the cell

wall pellet washed several times with distilled water. The washed pellet was resuspended in distilled water and a portion tested for radioactivity.

c) Partial alkaline hydrolysis: The phosphodiester bond between the first glycerol phosphate of the linkage unit and the N-acetylglucosaminy residue is susceptible to basic hydrolysis due to the neighboring group participation of the β -hydroxyl groups (Fig. 5). Basic hydrolysis is reported to liberate 93 (104) to 100% (103) of the glycerol phosphate in the linker region.

1) Following the method of Hughes and Tanner (103), cell wall suspensions were incubated for 5 hours in 1% sodium borohydride (w/v) at 35C. An equal volume of 2% sodium borohydride (w/v) in 0.2 N NaOH was added and the incubation continued for 8 hours for BD-170 cell walls and 48 hours for walls isolated from strain 1005. It was found that the longer time was required to get the same effective release of wall phosphate from the walls of strain W23 (103). Because walls are known to contain free reducing ends and alkaline hydrolysis of polysacharides proceeds from free reducing terminals, sodium borohydride was included in the reaction mixture.

2) Following the method of Coley, et. al. (56), cell wall suspensions were incubated at room temperature (22C) for 4 hours in the presence of

0.5 N NaOH.

After alkaline hydrolysis the walls were pelleted and samples of the supernatant and residual walls obtained as described above. Samples exposed to sodium borohydride were suspended in or made acidic with 0.5 N HCl before being tested for radioactivity. Addition of acid was necessary to eliminate chemiluminescence.

Quantitation of the hexosamine content of [³H]-DHBP-labeled cell walls: The method of Levvy and McAllen (105) was modified to allow determination of the submicromolar amounts of hexosamine in the cell wall preparations.

A sample of cell wall suspension containing up to 1 umole of hexosamine (the equivalent of 10 ml of *E. subtilis* culture at a cell density of 50-60 Klett) was lyophilized in a 12 ml screw capped glass conical centrifuge tube and hydrolyzed with 100 ul of standardized 3 N HCl in the tightly capped tube at 95C for 4 hours. The tubes were agitated several times during the hydrolysis to insure that the samples clinging to the sides of the tube would be hydrolyzed. After cooling to room temperature, the acid was neutralized by the addition of 150 ul of NaOH containing the same number of equivalents as the HCl employed for the hydrolysis. The sample was agitated on a vortex mixer and centrifuged. A 200 ul sample of the neutralized hydrolysate was transferred to a conical centrifuge tube and treated with 50 ul of a freshly prepared solution of 3% acetic

anhydride in acetone. Less than 15 minutes after the addition of the acetic anhydride reagent, 170 μ l of a 0.7 M potassium borate buffer, pH 9.1¹ was added, the tubes agitated on a vortex mixer and allowed to stand at room temperature for 10 minutes. The tubes were capped with a marble, heated in a boiling water bath for 3 ($\pm$ 0.06) minutes and immediately cooled on ice. The tubes were removed from the ice. When the tubes had warmed to room temperature, 2.0 ml of Morgan-Elson reagent² was added, and the tubes incubated for 20 minutes in a 37C water bath. Full color development required 18-20 minutes, persisting for 3-5 minutes before slowly fading. The absorbance was measured at 585 nm on a Gilford 250 spectrophotometer. A calibration curve using glucosamine-HCl standards was used as a reference. One-tenth mole of glucosamine had an absorbance of 0.130. Because the absorbances were low, it was necessary to correct for differences between individual cuvettes.

Preparation of CDP-glycerol pyrophosphorylase: CDP-glycerol pyrophosphorylase was extracted from *B. subtilis* by modifications of the methods of Glaser (74) and Shaw (107). Two or three drops of a rich broth culture of *B. subtilis* were used to inoculate 1.2 l of Eugon broth. Six liters of *B. subtilis* were grown 16 hours at 37C at 200 rpm in erlenmeyer flasks 60% filled with broth. Some preparations were terminated when the turbidity reached 200 Klett units. Cells were harvested in the cold at 7,000 x g

in a GSA rotor, resuspended and washed, first, in 0.9% saline and then in 200 ml of 50 mM Tris-HCl with 10 mM magnesium chloride and 1 mM EDTA buffer adjusted to pH 7.5 at 4C. Cells (31 gm wet weight) were resuspended in 600 ml of 0.6 M sucrose buffered with 50 mM NaCl, pH 7.5 that had been prewarmed to 37C. After adding lysozyme (700 mg), the cell suspension was stirred for 15-20 minutes with a magnetic stirrer without forming a vortex.³ Spheroplasts were collected by centrifugation at 15,000 x g for 30 minutes, carefully drained, and allowed to lyse at 37C for 15 minutes in 80 ml of 50 mM Tris-HCl, pH 7.5, containing 10 mM magnesium chloride and 1 mM EDTA in the presence of 5 mg each of RNase and DNase. Whole cells and debris were removed by centrifugation at 15,000 x g for 10 minutes. Membranes containing teichoic acid synthetase were pelleted by centrifuging the supernatant at 19,000 rpm (36,000 x g) in a Beckman SW 25.1 rotor for 15 minutes. The remaining supernatant was centrifuged at 24,000 rpm (60,000 x g) in the same rotor for 60 minutes. The resulting supernatant (90 ml) was treated with ammonium sulfate (24.9 gm) to bring the solution to 45% saturation (277 gm/l) (108). The addition of the ammonium sulfate did not change the pH of the supernatant by more than 0.2 pH units and no attempt was made to adjust the pH. After stirring for 20 minutes, the precipitated proteins were removed at 9,000 rpm (10,000 x g) in a Sorval SS 34 rotor. The resulting supernatant (97 ml) was treated with small portions of 9.7 gm of ammonium

sulfate with the pH adjusted as necessary with additions of dilute ammonia to bring the supernatant to 60% saturation (390 gm/l) and maintain the pH at 7.5. The 45-60% saturated precipitate was collected at 10,000 x g.

If a crude preparation of CDP-glycerol pyrophosphorylase were desired, the 45-60% saturated precipitate was suspended in several milliliters (ca. 3-5 ml) of 5 mM Tris-HCl adjusted to pH 7.5 at 0C and dialyzed against two changes of 16 l of 5 mM Tris, pH 7.5 at 0C for 16 hours. Samples of the dialyzed enzyme were frozen and stored at -20C. Samples retained activity for three years if kept frozen. Once defrosted, the sample was not used again. In a typical preparation, 6 l of cells yielded 1.3 gm of protein in the lysis supernatant and 680 mg of dialyzed enzyme preparation.

More purified preparations of CDP-glycerol pyrophosphorylase were prepared by dissolving the 45-60% saturated ammonium sulfate precipitate in a minimum volume of 50 mM Tris-HCl, pH 7.5 with 10 mM magnesium chloride and 1 mM EDTA and passing 3.0 ml through a 2.5 x 50 cm column of Sephadex G-100 equilibrated in the same buffer. Fractions of 96 drops (approximately 3 ml) were collected. The fractions containing the CDP-glycerol pyrophosphorylase were identified by assaying the individual fractions as described below (Fig. 7). Fractions were pooled and stored frozen at -20C. The frozen enzyme retained activity for 3 years. A typical preparation yielded 70 mg of gel-purified enzyme.

CDP-glycerol pyrophosphorylase assay: CDP-glycerol

pyrophosphorylase was assayed by the method of Glaser (74), which is based on the conversion of ^{14}C -labeled glycerol phosphate into a charcoal absorbable form. Each assay contained 10 umole of Tris-HCl, pH 7.5, 2 umole of magnesium chloride, 0.2 umole of CTP (absorbance at 271 nm = 1.8), enzyme, and substrates in a final volume of 0.7 ml. The Sephadex G-100 fractions were assayed using 0.1 umole of L-glycerol 3-phosphate.

Assay tubes were prepared in advance without CTP or enzyme and stored in the cold or frozen. In a timed assay, CTP was added to the assay mixture, incubated for 4 minutes at 25C and the reactions initiated by the addition of enzyme. Reactions were stopped with the addition of 0.7 ml of ice cold 2% TCA in a 0.15 M sodium acetate buffer, pH 4.2 (TCA/OAc solution). The precipitated protein was pelleted using a desk top centrifuge. Without disturbing the precipitate, a sample of the supernatant was transferred to a screw capped test tube. The largest sample which could be conveniently transferred was 1.15 ml. Fifty mg of charcoal suspended in 0.5 ml of the TCA/OAc solution was added to the transferred supernatant. The suspension was mixed on a vortex mixer and allowed to stand in the cold for at least 20 minutes. The charcoal was pelleted by centrifugation in a desk top centrifuge and the supernatant aspirated off. The charcoal was washed three times with 3 ml portions of the TCA/OAc solution and once with the same volume of 1 mM HCl. The washed charcoal was treated with 1.3 ml of

ammoniacal ethanol (ethanol:water:conc. ammonia, 50:50:0.09) in a tightly capped test tube for 1 hour at 70C. When the radioactive label was [^{14}C]-glycerol 3-phosphate, the ammoniacal ethanol solution contained 0.77 mM α -glycerol 3-phosphate. If [^3H]-DHBP was the substrate, 1 mM unlabeled DHBP was substituted for the glycerol phosphate. After the charcoal was pelleted by centrifugation, 1.0 ml of the supernatant was tested for radioactivity with 10 ml of Patterson-Greene scintillation fluid (101). Recovery of the radioactive nucleotides from charcoal was assumed to be 80% (74).

Identification of the enzymatic product when [^3H]-DHBP is used as a substrate for CDP-glycerol pyrophosphorylase: Using 20 mM [^3H]-DHBP (sp. act.= 1 uCi/umole), the enzyme assay in 0.7 ml was performed as previously described using a dialyzed CDP-glycerol pyrophosphorylase preparation from strain 42 with a 30 minute incubation. The product was eluted from the charcoal after the dilute HCl wash by treating the charcoal for 20 minutes at room temperature with 1 ml of ammoniacal ethanol (ethanol:conc. ammonia: water, 50:2:48, v/v/v). The charcoal was removed by centrifugation and the supernatant transferred to other test tubes and dried under a stream of air. The degradation of CDP-glycerol under similar conditions is reported to be less than 3% (107).

One sample of the material removed from the charcoal was hydrolyzed with 1 N HCl at 100C for 2 hours in a tightly

capped test tube, the hydrolysate dried under a stream of warm air, the residue taken up in a small volume of distilled water, and chromatographed. Another portion of the product was dried under a stream of air, taken up in a small volume of distilled water and chromatographed without further treatment.

Two samples of the untreated material were spotted side by side. After chromatography one lane of the untreated sample was cut up for radioactive counting. The area corresponding to the phosphonate analogue of CDP-glycerol was cut out of the other lane, rinsed in 80% ethanol containing 1% ammonia, eluted, the eluate reduced in volume, hydrolyzed with HCl as described above, and rechromatographed. All chromatography was performed on Whatman 1MM paper using 95% ethanol:0.575 M ammonium acetate, pH 3.8 (79:29).

Teichoic acid synthesis assay: The synthesis of teichoic acids by crude membrane preparations was based on the method of Burger and Glaser (110). The same preparation is capable of synthesizing the linker region (31).

The membranes used in the teichoic acid synthesis assay were obtained during the preparation of CDP-glycerol pyrophosphorylase. These membranes were the 36,000 x g pellet from the 15,000 x g supernatant of the spheroplast lysate. The pellet was washed once with 50 mM Tris-HCl, pH 7.5 at 0C containing 10 mM magnesium chloride and 1 mM EDTA and stored frozen at -20C until use. The membranes were resuspended in 10 mM Tris-HCl with 25 mM magnesium chloride,

pH 7.5 at 37C before being added to the assay. Each assay contained 10 umole of Tris-HCl, pH 7.5 at 37C, 25 umoles of magnesium chloride, washed B. subtilis membranes and the appropriate nucleotides in a final volume of 0.5 ml.

Forty mg of W23 membrane or 8 mg of 168 membranes were used with either 69,400 cpm of CDP-[¹⁴C]-glycerol or 640,000 cpm of CMP-[³H]-DHBP. See Table 7 for additional details. The complete reactions mixture was incubated at 37C for 30 minutes and the assay terminated with the addition of 80 ul of 3 N perchloric acid. The membranes were collected at 10,000 x g for 10 minutes, washed three times with 3 ml portions of .15 N perchloric acid. Samples of the washed membranes were suspended in 1.5 ml of 0.3 N ammonia and placed in a boiling water bath for 2 minutes to hydrolyze the polymerized material. The samples were acidified with HCl and the radioactivity measured.

Preparation of nucleotide substrates for the teichoic acid synthetase assay: CDP-glycerol and the phosphonate analogue, 3,4-dihydroxybutyl-1-phosphonyl-O-(cytidine 5'-phosphate), (CMP-DHBP) (Fig. 8), were prepared by the method of Roseman, et. al., (111) as well as by a modification of a microscale modification of the same method by Elbein (112). In the method of Roseman, et. al., (111), CMP-morpholidate (0.5 mmole) was dissolved in 15 ml of anhydrous pyridine and the pyridine removed under vacuum at room temperature. The morpholidate was rendered anhydrous by three additional evaporations of anhydrous pyridine.

After each evaporation, the vacuum was released by admission of dry air.

Glycerol 3-phosphate or DHBP (1.5 mmole) was dissolved in water and converted to the free acid with Dowex-50 (H^+). The supernatant was removed and the Dowex beads washed several times with distilled water. The supernatant, combined with the washings was concentrated to 10-15 ml under reduced pressure at 30C. The solution was made basic to pH indicator paper by addition of pyridine and concentrated to 5 ml under reduced pressure. A solution of tri-n-octylamine (1.5 mmole, 0.66 ml)* in pyridine was added and the solution rendered anhydrous by 3 evaporations of anhydrous pyridine. The dried residue was a viscous white slurry. The residue was suspended in fresh anhydrous pyridine, added to the anhydrous CMP-morpholidate, and the combined mixture dried with four more evaporations of anhydrous pyridine. After the final evaporation, 10 ml of anhydrous pyridine was added, the flask fitted with a drying tube, and allowed to stand with stirring at room temperature for 5 days.

The solvent was removed under vacuum and the residue stirred with 5 ml each of ether and water containing sodium acetate (2.4 mmole) equal to 120% of the combined number of moles of tri-n-octylamine and CMP-morpholidate used. The layers were separated, backwashed, and the combined aqueous fractions concentrated under reduced pressure at room temperature.

The reaction mixture was fractionated on a water-packed

1.5 x 20 cm Dowex-1X8 (chloride) (20-50 mesh) column eluted with a 2 l linear gradient of 0-100 mM LiCl in 3 mM HCl. Fifteen ml fractions were collected. Peaks were located by absorbance at 271 nm (Fig. 9). The product was found in the second peak (fractions 27-50) which eluted at 25 mM LiCl. Pooled fractions were adjusted to pH 7.0 with LiOH before being evaporated to dryness. The white residue was treated with 55 ml of an acetone:methanol (10:1, v/v) mixture for several minutes. Ether (5 ml) was added and the precipitate collected on a sintered glass filter. The precipitate was washed several times in this fashion until the eluate tested free of chloride ions (silver nitrate test). The product contained some CDP.

The product was purified further by elution from a 2 x 20 cm DEAE-cellulose (bicarbonate) column with a 3 l 5-200 mM triethylamine bicarbonate, pH 7.5 gradient. Buffer was prepared by adding dry ice to an aqueous solution of triethylamine until the proper pH and volume were obtained. A 2 l suction flask fitted with an outlet at the base and a 2 l separatory funnel were used to form the gradient maker. The top of the column was sealed with a rubber septum. An air-tight connection between the outlet of the suction flask and the column was made with rubber tubing which ended in a needle inserted through the septum. The side arm of the suction flask was sealed. After adding 1.5 l of 5 mM buffer and a stirring bar to the suction flask, the separatory funnel was joined to the suction flask through a one-hole

stopper. After adding the high salt buffer (400 mM triethylamine bicarbonate) to the separatory funnel the top of the separatory funnel was closed with a one-hole stopper through which a glass tube had been inserted. The glass tube allowed the separatory funnel to be used as a Marriot flask to control the flow rate.

Fractions of 13.5 ml were collected (Fig. 10) and the products identified by descending paper chromatography. CDP-glycerol eluted at 53 mM buffer while the analogue eluted somewhat earlier at 40 mM salt. The desired fractions (peak II) were pooled and the water removed under reduced pressure at 30°C. The triethylamine bicarbonate was removed from the residue with four evaporations of methanol under reduced pressure at 30°C. The product was converted to either the calcium (111) or potassium salt (113). The calcium salt was prepared by dissolving the product in 5 ml of methanol with 250 μ l of a 1 M (saturated) ethanolic calcium chloride solution. The calcium salt was precipitated by addition of 25 ml of acetone. The white precipitate was washed free of chloride ions with repeated treatment with 5 ml of ethanol followed by 30 ml of acetone. The potassium salt was made by dissolving the product in methanol, adding 1 ml of a 1 M KI in acetone and washing the precipitated product with an acetone:ether (5:1, v/v) mixture. The halide-free product was dried under a stream of air.

The separation afforded by the DEAE-cellulose column was sufficient to eliminate the Dowex-1 column from the syn-

theses of each nucleotide. Nucleotides which had been stored longer than two months showed degradation to CMP. These samples were repurified using a 1 x 14 cm DEAE-cellulose (bicarbonate) column with a 10-150 mM triethylamine gradient in a total of 250 ml.

The method of Elbein (112) was used to synthesize the radioactive nucleotides, [^{14}C]-CDP-glycerol and [^3H]-CMP-DHBP and as an alternative method for the synthesis of the non-radioactive nucleotides. Because of the cost of [^{14}C]-glycerol 3-phosphate and [^3H]-DHBP, their proportions with respect to the CMP-morpholidate were reversed, i.e., the morpholidate was used in three-fold molar excess with respect to the glycerol 3-phosphate or its analogue. For the synthesis of [^{14}C]-CDP-glycerol, 2 umole of [^{14}C]-glycerol 3-phosphate (sp. act. 6.6 Ci/mole), 6 umole CMP-morpholidate, and 2.4 umoles of tri-n-octylamine (equivalent to 1.05 ul neat) were used. Synthesis of [^3H]-CMP-DHBP used 16.6 umoles of purified [^3H]-DHBP (sp. act. 367 Ci/mole), 20 umoles of tri-n-octylamine (equivalent to 8.7 ul neat), and 49.9 umoles of the morpholidate. Glycerol 3-phosphate or DHBP was converted to the free acid, concentrated, made basic with pyridine, and rendered anhydrous as previously described (111). The appropriate weight of crystalline CMP-morpholidate was added to the anhydrous glycerol 3-phosphate or DHBP in a 50 ml round bottom flask, 5-10 ml of anhydrous pyridine added and the mixture dried with four evaporations of anhydrous pyridine. After the final drying,

the mixture was suspended in 10-15 ml of anhydrous pyridine, fitted with a drying tube and heated overnight at 55-60C.

After cooling the reaction mixture was treated as previously described except the radioactive products were isolated by descending paper chromatography. Bands were located under ultra violet light, cut out, rinsed once with 8% ethanol containing 1% ammonia to remove traces of salt, eluted with water, concentrated by lyophilization or under reduced pressure at 3C, and rechromatographed (Fig. 11).

Descending paper chromatography was performed with Whatman 3MM paper with abs. ethanol :.5 M ammonium acetate, pH 3.8 (5:2, v/v) or 95% ethanol:.575 M ammonium acetate, pH 3.8 (79:29, v/v) as the developing solvent. The following Rf values were observed: 5'CMP, .29; CMP-morpholidate, .48; CDP-glycerol, .18; and CMP-DHBP, .21. It was found to be more convenient to calculate the mobilities of the products with respect to a 5'CMP or morpholidate standard. This allowed the solvent to drip off the end of the strip to obtain a greater separation between CMP and the product.

Purification of [^{3} H]-DHBP: Stored solutions of [^{3} H]-DHBP were found to contain three radioactive components. These were separated by eluting aqueous solutions of [^{3} H]-DHBP from a 1.0 x 12 cm DEAE-cellulose (bicarbonate) column equilibrated and packed in water with a 0-100 mM ammonium bicarbonate, pH 7.0 gradient in 200 ml (Fig. 13). The major peak eluting between 27 and 45 mM buffer was identified as DHBP by comparison with standards on ascending

paper chromatography. On Whatman 1MM paper using 95% ethanol:1 M ammonium acetate, pH 7.0 (1:1, v/v), DHBP had an Rf of 0.69. In n-propanol:conc. ammonia:water (6:3:1, v/v/v), DHBP had an Rf of 0.22. The fractions comprising the major peak were pooled, the water removed under reduced pressure at 30°C, the buffer salts removed by several evaporations with alcohol, and the purified [³H]-DHBP stored at -20°C in 20% ethanol.

Although the [³H]-DHBP sample applied to the column was colorless, a colored band developed at the top of the column. The band was not apparent when the sample was absorbed into the column, but became increasingly visible as the gradient increased, first as a pale yellow which gradually darkened to a deep red. The band never moved from the top of the column. No attempt was made to characterize the colored material.

Footnotes

1. Potassium borate buffer, pH 9.1 was prepared by adding KOH to a suspension of 43.3 gm of boric acid until the solution reached pH 9.1. The volume was adjusted to 1 liter with distilled water. Potassium tetraborate obtained from Fisher Scientific Co., Springfield, N.J. was not sufficiently soluble to yield a 0.7 M solution at pH 9.1.
2. Morgan-Elson stock solution was prepared by dissolving 1 gm of dimethylaminobenzaldehyde in 8.75 ml of glacial acetic acid, 1.04 ml of 12 N HCl, and 0.21 ml of water. The stock solution was stored at 4C for less than a month (106). The stock solution was diluted with 9 volumes of glacial acetic acid on the day of use.
3. Sphereoplasts are more stable if they are not exposed to air (109).
4. The literature (104) called for 2-3 equivalents of tri-n-octylamine with respect to the phosphomonoester rather than the morpholidate. The decrease in tri-n-octylamine added undoubtedly lowered the solubility of the glycerol 3-phosphate and the DHBP resulting in yields lower than those reported in the literature: 20-30% vs. 65% (111).

Results and Discussion

Effect of DHBP on Growth and Viability

DHBP was found to inhibit the growth of both strains of B. subtilis grown in casein hydrolysate broth (Fig. 13), with the inhibition attributable to the L-enantiomer (Fig. 14). Figure 14 shows that 75 μ M L-DHBP caused the same inhibition as 150 μ M of D-DHBP.

Although growth inhibition of both strains appeared similar, the viability of the two strains cultured in the presence of 2.5 mM DHBP differed dramatically (Fig. 15). DHBP was found to be bacteriostatic for the glycerol phosphate strain, BD-170, but bactericidal for strain 1005, the ribitol phosphate teichoic acid strain.

Teichoic acid to teichuronic acid conversion in B. subtilis occurs below 0.4 mM phosphate (65). The inorganic phosphate concentration of casein hydrolysate broth is 1 mM (Deutsch, private communication). Casein hydrolysate medium was supplemented with phosphate to raise the inorganic phosphate concentration to 6 mM. The additional 5 mM phosphate in the culture medium made no difference in the response of B. subtilis to 2 mM DHBP (data not shown).

To determine if additions to the growth medium could alleviate DHBP inhibition, cells were cultured in the presence of 100 mM phosphate (Fig. 16), glycerol (Fig. 17), or 0.5% (28 mM) glucose (Fig. 18) (see figures for

details). In unsupplemented casein hydrolysate medium, growth inhibition can be observed two-thirds of a doubling time after addition of 0.15-0.20 mM DHBP. Inclusion of 100 mM phosphate in the medium completely prevents DHBP inhibition of strain BD-170 although the result is not so clear cut with strain 1005 (Fig. 16). In strain 1005, the onset of inhibition is delayed to one and one-half doubling times after the addition of DHBP. *E. coli* cultured in high phosphate minimal medium (62 mM) derived no protection against DHBP inhibition from the presence of the phosphate (1).

Phosphate is thought to reduce the entry of DHBP into *B. subtilis* by competing with DHBP for the same transport binding site. If this is so, transport studies utilizing [^3H]-DHBP should find slower uptake of label in phosphate supplemented medium compared with unsupplemented medium.

Both glucose and glycerol reduce DHBP inhibition of *B. subtilis* (Figs. 17 and 18). The protection is probably due to elevation of glycerol phosphate pools. Glycerol phosphate, by being a more effective substrate than DHBP, can reduce DHBP inhibition competitively. Glycerol phosphate could be formed from glycerol by glycerol kinase or from glucose through glycolysis. Catabolite repression of DHBP transport by glucose may also explain the decrease in DHBP inhibition in the glucose supplemented medium. Mutants are available to examine these assumptions (17).

Effect of Other Glycerol Phosphate Analogues

Other analogues of glycerol and glycerol phosphate, glyceraldehyde, glyceraldehyde phosphate, and its phosphonate analogue, 3-hydroxy-4-oxobutyl-1-phosphonate when added to the growth medium inhibit the growth of both strains of *B. subtilis* (Fig. 19). The biologically active form of glyceraldehyde is the L-enantiomer as the D-enantiomer has no effect on the growth of *B. subtilis* (Fig. 19). Figure 19 suggests that in *B. subtilis* as with *E. coli*, glyceraldehyde phosphate is bactericidal while the glyceraldehyde phosphate analogue is bacteriostatic (8). The mode of action of these compounds in *B. subtilis* was not studied further. The effect of L-glyceraldehyde in *E. coli* was found to be dependent on phosphorylation to glyceraldehyde phosphate (7). Glyceraldehyde 3-phosphate and its phosphonate analogue were found to be competitive inhibitors, but not substrates for acyl-CoA:sn-glycerol 3-phosphate acyltransferase and cytidine 5'-diphosphate-di-acyl-glycerol:sn-glycerol 3-phosphate phosphatidyltransferase, but had no effect on acyl-CoA:lysophosphatidate acyltransferase activity (8).

Incorporation of DHBP In Vivo

Lipids

B. subtilis cultured in casein hydrolysate broth containing 3 μ M [^{3} H]-DHBP incorporated the tritium label into lipids, cell wall, and a non-dialyzable product which appeared in the aqueous phase of phenol extracts of sonical-

ly disrupted cells (Table 1). Table 1 shows that the bulk of the tritium label is found in the lipids or the phenol extracted product.

Incorporation of DHBP into the lipid fraction was expected in light of a similar finding with *E. coli* (3,6). Fractionation of the [³H]-DHBP-labeled lipids on a DEAE-cellulose column as described in Materials and Methods section revealed four tritium-labeled components (Fig. 20). Fraction IV, a very polar lipid eluting at 45 mM ammonium acetate contained 89-95% of the tritium-labeled lipids. The similarity of Figure 20 to the elution profile of the [³H]-DHBP-labeled lipids extracted from *E. coli* under similar conditions (6) suggested that the major tritium-labeled lipid, fraction IV was the phosphonic acid analogue of phosphatidylglycerol phosphate, (1,2-diacyl)-sn-glycerol D-phosphoryloxy-3'-hydroxyl-1-phosphonate (Fig. 2). Eighty-nine to ninety-five percent of the tritium label in the lipids was found in fraction IV.

The structure of fraction IV was determined using selective enzymatic hydrolysis to recover intact DHBP. Phospholipase C cleaves phospholipids to leave diglycerides. Tritium associated with the diglyceride portion of the phospholipid will be found in the chloroform soluble products of the phospholipase C hydrolysis. Tritium incorporated into the polar head group of the phospholipid will be cleaved from the hydrophobic part of the molecule and be recovered as a water soluble product.

Fractions I, II, and III were pooled and treated together. Table 2 shows that the tritium label in fraction IV is located in the distal portion of the lipid. Greater than 97% of the tritium label appeared in the diglycerides after phospholipase C treatment of the pooled samples, while less than 2% of the label of fraction IV remained soluble in chloroform. The pooled fractions were clearly different from the phosphatidylglycerol phosphate analogue. Similar results were found in *E. coli* (3). The tritium label found in the minor tritium-labeled peaks extracted from *E. coli* cultured in the presence of [^3H]-DHBP was found in glycerol of the glycerol phosphate backbone and did not represent incorporation of intact DHBP (3).

The release of DHBP as the water soluble product in the phospholipase C hydrolysis indicated that DHBP was being used by the cell to synthesize the phosphonate analogue of phosphatidylglycerol phosphate. The structure of the water soluble products of the phospholipase C treatment were investigated to confirm the structure.

If fraction IV were the phosphonate analogue of phosphatidylglycerol phosphate, phospholipase C treatment would be expected to liberate 4-phospho-3-hydroxybutyl-1-phosphonate as a water soluble product. Figure 21 shows that the principle water soluble product had the chromatographic behavior of 4-phospho-3-hydroxybutyl-1-phosphonate. The minor product migrated as DHBP (3). The identity of the major product of the phospholipase C hydrolysis was confirmed

by the alkaline phosphatase treatment. Alkaline phosphatase cleaves the phosphate from 4-phospho-3-hydroxybutyl-1-phosphonate to yield DHBP and inorganic phosphate. The only tritium labeled product found after alkaline phosphatase treatment of the water soluble phospholipase C products of fraction IV was DHBP. Fraction IV was, as expected, (1,2-diacyl)-sn-glycerol 2-phosphoryloxy-3'-hydroxyl-1'-phosphonate. Although the data presented here is for strain 1005, the same results were obtained for strain BD-170. DHBP appeared as a minor product in the water soluble phospholipase C hydrolysis products because of alkaline phosphatase contamination of the phospholipase C preparation (6).

The synthesis of the phosphonate analogue of phosphatidylglycerol phosphate indicates that DHBP serves as a substrate for the CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase in both strains of *B. subtilis*. Phosphatidylglycerol phosphate, the lipid normally formed by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase, is subsequently dephosphorylated. Because the phosphonate cannot be similarly cleaved from the phosphatidylglycerol phosphate analogue, the phosphatidylglycerol phosphate analogue accumulates. This was known to be the case with *E. coli* (6) where DHBP inhibits the synthesis of phosphatidylglycerol. Deutsch, et. al. subsequently found that DHBP inhibited synthesis of phosphatidylglycerol in *B. subtilis* (47).

Walls and Phenol Extract

The other [^3H]-DHBP-labeled cellular fractions, the walls and the phenol extract, were also found to contain intact DHBP. The walls and phenol extract were hydrolyzed with HCl and chromatographed as described in Materials and Methods and the legend to Fig. 22. At least 85% of the tritium migrated as DHBP ($R_f=0.22$) while the remainder had an R_f of 0.51. Subjecting DHBP to the same HCl hydrolysis as the walls and the phenol extract produced the same two chromatographic components seen in the chromatograms of wall and phenol extract hydrolysates. The unidentified compound which was not studied further is probably a cyclic ester of DHBP. Untreated DHBP gave a single spot with an R_f value of .22. Glycerol had an R_f of 0.68 in this solvent system.

The incorporation of DHBP into the phenol extract is an intriguing and as yet unexplained observation. It is tempting to believe that DHBP is incorporated into LTA, but the current understanding of LTA biosynthesis precludes this. Because the lipid anchor of LTA is probably derived from pre-existing lipids (43,44) and because DHBP is not found in the diglyceride moiety of the phospholipids, DHBP should not be found in the lipid portion of LTA (16,46). The studies of LTA synthesis in *S. aureus* (40), *S. sanguis* (39,41), and *S. faecium* (42) found phosphatidylglycerol to be the donor or the polymeric glycerol phosphate residues in LTA. Again, this route is excluded as a means by which DHBP can enter LTA. It is possible that the tritium labeled substance

represents an as yet uncharacterized intermediate in LTA synthesis. While such speculation should await further experimentation, it is noteworthy that the incorporation of intact DHBP into a water soluble non-dialyzable product occurred to a significant extent in both strains of B. subtilis.

The greater level of incorporation of DHBP into the walls of strain BD-170 indicated that DHBP was being used in place of glycerol phosphate in the main teichoic acid chain and the linker region, while the incorporation of DHBP was probably limited to the teichoic acid linker region of strain 1005.

The nature of the association of the DHBP with the cell wall was investigated by subjecting the [^3H]-DHBP-labeled cell walls to different enzymatic and chemical hydrolyses as described in Materials and Methods. At the conclusion of the treatment, the walls were collected by centrifugation and the supernatant sampled for radioactivity. The tritium appearing in the supernatant was taken as a measure of the solubilization of the wall.

The data in Table 3 demonstrates quite clearly that the DHBP is covalently attached to the wall. Only those enzymes capable of degrading bacterial walls (lysozyme and pronase) solubilize the label. The other enzymes cannot use the wall as a substrate and the label remained with the wall after enzymatic treatment. Chloroform-methanol extraction to remove lipids did not extract any tritium from the wall

preparations. Thus none of the tritium label associated with the wall was due to contamination with [^3H]-DHBP-labeled lipids. The mild acid and basic hydrolyses were designed to cleave the teichoic acid linker region at the sites indicated in Fig. 5. Unsubstituted glycerol phosphate polymers are susceptible to basic hydrolysis in the same fashion as RNA. The data is consistent with DHBP substituting for glycerol phosphate in the wall of both strains of *B. subtilis*.

The amount of DHBP incorporated into strain BD-170 was 5.4 times greater than that in strain 1005 when normalized with respect to the hexosamine content of the walls (Table 4). The greater incorporation of DHBP in the glycerol teichoic acid strain would be expected if the DHBP replaced glycerol phosphate in both the teichoic acid main chain and the linker region of strain BD-170. The incorporation of DHBP is probably limited to the teichoic acid linker region in strain 1005. In either case, the vector by which DHBP would be incorporated should be the phosphonate analogue of CDP-glycerol.

It is not known whether insertion of DHBP terminates a growing teichoic acid chain, be it the linker region or the main chain. The presence of DHBP as an internal residue of a teichoic acid chain could be demonstrated by treating [^3H]-DHBP-labeled cell walls with periodate and sodium borohydride followed by acid hydrolysis. Recovery of intact DHBP would indicate that DHBP had been an internal residue,

protected from degradation. Terminal residues would be recovered as 3-hydroxy-propyl-1-phosphonate (Fig. 23).

In Vitro Incorporation of DHBP

The Kinetics of CDP-glycerol Pyrophosphorylase with DHBP

The incorporation of DHBP into the linker region as well as the main teichoic acid chain of the 168 strain is predicated upon CDP-glycerol synthesizing enzyme, CDP-glycerol pyrophosphorylase, accepting DHBP as a substrate to synthesize the CDP-glycerol analogue, 3,4-dihydroxybutyl-1-phosphonyl-O-(cytidine 5'-phosphate), (CMP-DHBP) (Fig. 8). The teichoic acid synthesizing enzyme(s) would then incorporate the DHBP into the wall in place of glycerol phosphate.

The ability of *B. subtilis* to synthesize CMP-DHBP from CTP and DHBP was investigated using a dialyzed preparation of CDP-glycerol pyrophosphorylase from strain 42, a 168 strain of *B. subtilis*. Using CTP and DHBP in the assay described in Materials and Methods, the products of the enzymatic reaction were adsorbed onto charcoal, the charcoal washed several times, and the products desorbed from the charcoal into ammoniacal ethanol at room temperature. The sample was divided and each part dried under a stream of air. One portion was hydrolyzed in HCl before being chromatographed. The other portion was chromatographed without further treatment.

The untreated sample was spotted as two samples in adjacent lanes on a descending paper chromatogram. One lane

of the untreated sample was used for radioactive counting. The area corresponding to CMP-DHBP was eluted from the other lane, hydrolyzed with HCl and rechromatographed. The chromatograms are shown in Fig. 24. The two tritium labeled products desorbed from the charcoal had mobilities corresponding to those of CMP-DHBP and DHBP. The only tritium labeled compound found in the chromatograms of the acid hydrolysates was DHBP. This confirms the ability of CDP-glycerol pyrophosphorylase to synthesize CMP-DHBP from DHBP and CTP.

Fifty-six percent of the tritium label in the untreated products desorbed from the charcoal migrated as DHBP. Only 3% degradation of CDP-glycerol was reported by similar treatment (105). The DHBP found in the chromatogram does not necessarily indicate extensive degradation of the CMP-DHBP. Control reactions, assay mixtures lacking only CTP, released tritium labeled material from the charcoal which migrated as DHBP (data not shown). The amount of label released from the charcoal in control reactions was approximately 0.1% of the label added to the assay (Table 5). It was for this reason that a control assay was included for each concentration in the kinetic assays of CDP-glycerol pyrophosphorylase. Similar difficulties were reported with high background levels of ^{32}P in an analogous charcoal assay (105).

Only the CDP-glycerol pyrophosphorylase obtained from the 168 strain was used for kinetic measurements because the

level of enzymatic activity in the W23 strain was approximately 1200-fold lower than that found in the 168 strain. A comparison of dialyzed extracts of strain 42 (a glycerol teichoic acid strain) and 1005 (a ribitol teichoic acid strain) found the rate of CDP-glycerol synthesis to be 2.3 nmole/min-mg and 1.9 pmole/min-mg, respectively. The assays contained 0.1 umole β -glycerol 3-phosphate in 0.7 ml of the standard assay mixture described in Materials and Methods. The lower level of CDP-glycerol pyrophosphorylase activity in strain W23 compared with strain 168 may also explain the lower incorporation of DHBP into the walls of strain W23 when the two strains were incubated in the presence of DHBP (Table 4).

The data obtained from the CDP-glycerol pyrophosphorylase extracted from the 168 strain of *E. subtilis* is shown in Figs. 25 A-D and summarized in Table 6. DHBP was found to be a substrate for CDP-glycerol pyrophosphorylase (apparent K_m =36 mM β -DHBP) and a competitive inhibitor of glycerol 3-phosphate (average K_i =6.5 mM β -DHBP). The V_{max} of DHBP was comparable to that of the natural substrate, glycerol 3-phosphate, 1.8 and 5.3 nmole/min-mg, respectively. The apparent K_m of DHBP is 200-fold greater than that for glycerol phosphate. Thus the intracellular concentration would have to far exceed that of glycerol phosphate for DHBP to be an effective inhibitor of CDP-glycerol synthesis or for syntheses of significant amounts of CMP-DHBP *in vivo*.

The appearance of [^3H]-DHBP in the walls of cells cultured in medium containing 15 μM Σ -DHBP suggests that the DHBP was indeed concentrated to an intracellular level sufficient to allow synthesis of CMP-DHBP in amounts great enough to compete with endogenous CDP-glycerol in teichoic acid synthesis (Table 1). DHBP is found to be actively transported by *E. coli* from a nominal medium concentration of 0.3 mM DHBP to an intracellular concentration of 53 mM (2). The formation of CMP-DHBP could be explained if the ability of *B. subtilis* to transport DHBP were similar to that of *E. coli*.

The apparent K_m value for L -glycerol 3-phosphate reported here (0.18 mM) is approximately one-third the value reported in the literature, .64 mM (70). The difference may be due to differences between strains or because the published value was obtained from assays performed in the presence of ammonium sulfate (70).

Glyceraldehyde 3-phosphate did not inhibit CDP-glycerol pyrophosphorylase (data not shown).

In Vitro Teichoic Acid Synthesis with CMP-DHBP

Having shown *B. subtilis* capable of making CMP-DHBP, CMP-DHBP was substituted for CDP-glycerol in an *in vitro* teichoic acid synthesis assay to determine if CMP-DHBP is the donor of the DHBP which appeared in *B. subtilis* cell walls (Table 1). CDP-glycerol is the source of both the glycerol phosphate residues in the linkage unit (62) and glycerol phosphate wall teichoic acid (51).

The membranes used in the teichoic acid synthesizing reaction had been prepared in the course of extracting the CDP-glycerol pyrophosphorylase as described in Materials and Methods. The buffered membrane preparations were incubated in the presence of teichoic acid precursors, washed by precipitation, treated with ammonia, and a portion of the membrane suspension sampled for radioactivity as described in Materials and Methods. The radioactivity associated with the washed membranes was taken as a measure of the incorporation of the labeled compound into teichoic acid polymers (110).

Table 7 shows that glycerol phosphate and DHBP were incorporated into teichoic acids from their respective nucleotides by membranes of both *B. subtilis* strains. The inhibition of [^3H]-DHBP incorporation in the presence of CDP-glycerol indicated that both nucleotides compete for the same enzyme(s). Although the concentration of CMP-DHBP was 3 times greater than that of CDP-glycerol, the DHBP incorporation was reduced 83% (Expts. IIa and b). The 1% reduction in glycerol phosphate incorporation in the presence of 24-fold excess CMP-DHBP (Expts. Ia and b) may represent experimental error as the amount of product formed in Expts. Ia and b represents a third of the added substrate.

The incorporation of glycerol phosphate and DHBP into the membrane of strain 1005 undoubtedly represents their presence in the linker region. Here as with CDP-glycerol pyrophosphorylase, it can be seen that the phosphonate ana-

logue is a decidedly poorer substrate than the natural compound. The polymerase activity in BD-170 membranes, the glycerol phosphate strain, was found to be more than 200-fold greater than in the membranes of strain 1005, 48 and .22 pmole/min-mg, respectively. The ratio between the activities of the membranes toward CMP-DHBP as a substrate was not as great. BD-170 membranes were 20-fold more active toward CMP-DHBP than the 1005 membranes. Because CDP-glycerol and CMP-DHBP were used at different concentrations, the only statement which can be made from the data is that of the two substrates, CDP-glycerol is much preferred over CMP-DHBP.

The DHBP incorporation with the boiled enzyme control may represent DHBP molecules trapped in the flocculant boiled membrane. The corresponding observation was not made when [^{14}C]-CDP-glycerol was used as a substrate because the amount of radioactivity added as [^{14}C]-CDP-glycerol was 9-fold lower than that added as [^3H]-DHBP, 69,400 and 640,000 cpm, respectively.

Discussion

DHBP may exert its differential effect on the two strains of *E. subtilis* through its inhibition of CDP-glycerol synthesis. DHBP is thought to inhibit the growth of *E. coli* through perturbation of lipid synthesis (4,5). Similar effects were found on *E. subtilis* lipid metabolism (47). In addition, 1-phospho- β -gentiobiosyldiacylglycerol and LFA synthesis are inhibited, but no difference was noted

between the two strains (47). Thus the reason DHBP is bactericidal for strain 1005 and bacteriostatic for strain BD-170 may lie elsewhere than inhibition of lipid and LTA synthesis.

The CDP-glycerol pyrophosphorylase activity in BD-170 was approximately 1200-fold greater than in strain 1005. If strain 1005 normally makes small basal quantities of CDP-glycerol for synthesizing the linker region, inhibition of CDP-glycerol synthesis in 1005, but not BD-170 might lower the intracellular CDP-glycerol concentration below a critical minimum. CDP-glycerol synthesis and teichoic acid to teichuronic acid conversion may not be coupled.

In *B. subtilis*, the first site of inhibition of teichoic acid synthesis during conversion to teichuronic acid synthesis is the inhibition of the synthesis of Lipid I (70). The fall off in CDP-glycerol synthesis trails rather than proceeds the teichoic acid to teichuronic acid conversion (70). The intracellular concentration of CDP-glycerol would be expected to rise after the conversion starts, consistent with the high K_m of CDP-glycerol found for CDP-glycerol pyrophosphatase, the enzyme which degrades CDP-glycerol to CMP and glycerol phosphate (74).

If the bacteria has no means of recognizing a shortage of CDP-glycerol, the bacteria would attempt to synthesize teichoic acids, be unable to do so, but not shift to making teichuronic acids because it had not received the correct biochemical signal. Although teichoic acids may not be an

absolute requirement for cell survival, the absence of both teichoic acid and teichuronic acid may be conditionally lethal for cells grown in casein hydrolysate broth.

There are several ways of examining these conjectures. Cells maintained in chemostatic cultures under teichuronic acid synthesizing conditions (24) may not require CDP-glycerol or by requiring less, exhibit only those effects caused by inhibition of lipid and LTA synthesis. Under these conditions, DHBP may be bacteriostatic for both strains of *B. subtilis*.

Alternatively, the bactericidal effect may be due to autolysin induced lysis. If LTA acts as an inhibitor of autolysin, then, DHBP, by inhibiting LTA synthesis (47) may activate or prevent the inactivation of autolysin with lethal results. This autolytic enzyme appears to be inactivated by lowering the pH of the medium (115). Thus culturing the cells in a medium with a low pH may alter the susceptibility of strain 1005 to DHBP.

DHBP may provide a useful probe with which to examine the control of teichoic acid synthesis. For example, phage absorption to cell walls isolated from DHBP treated cells decreased relative to controls although the phosphate content of the DHBP treated and control walls were the same (Deutsch, private communication). It would seem that under the experimental conditions, synthesis of glucosylated teichoic acid, or the glucosylation of teichoic acids was selectively inhibited. If these results stand, the *in vitro*

glucosylation assay could be examined using mixtures of membranes and homogenates of untreated and DHBP-treated cells in a fashion similar to that by which Glaser and Loewy investigated the control of teichoic acid synthesis under phosphate limitation (70). If cells incubated in the presence of DHPP have less glucosylating enzyme in the membrane, then mixing membranes from treated and control cultures should show the glucosylating ability equal to the sum of the added membrane preparations. If, on the other hand, an inhibitor is produced, the activity of the mixed homogenates would be less than the sum of the added membranes. It is possible that the inhibition is due to the presence of the phosphonate analogue of phosphatidylglycerol phosphate in the membranes. In this case artificial membranes constructed from lipids extracted from control and experimental cultures could be used.

Cells grown in the presence of DHBP may be forced to switch from making teichoic acids to synthesizing teichuronic acids. This might be most easily examined by measuring the uronic acid content of DHBP-treated and control cultures. It would be interesting to see if growth in media containing low levels of DHBP altered the morphology of the cells. This might be related to the proposal that LTA serves as an inhibitor of autolysin which in turn, by its involvement in cell division, maintains the cell's shape (89).

Fundamental questions regarding teichoic acids remain

unanswered. The regulation of LTA metabolism is unexplored. The relationship of teichuronic acids and an N-acetylglucosamine polymer (68,69) found in the walls of *B. subtilis* is still unknown. The mechanisms regulating the modifications of the teichoic acids are all but unexplored. If teichoic acid synthesis is permitted *in vivo* under conditions where wall synthesis is inhibited, the newly synthesized teichoic acids are attached to the old wall. Whether the new teichoic acid chain is inserted onto a glycan segment already containing a teichoic acid chain or on a different segment is unknown.

It is not known if inhibition of wall synthesis changes the pattern of substitution on the chains made during wall inhibition.

Thus, despite a quarter of a century of investigation, basic questions concerning teichoic acids remain to be answered.

Table 1. Metabolic fate of DHBP in B. subtilis. B. subtilis was cultured in the presence of 30 uM DHBP (sp. act.= 16.7 uCi/umole) (1 uCi/ml of culture), harvested by centrifugation, disrupted by sonic oscillation, and portions of the sonicate used to obtain cell walls, lipids, or the non-dialyzable phenol extract as described in Materials and Methods.

Table 1. Metabolic Fate of DHEP in *B. subtilis*

Trial	Strain	Radioactive Incorporation (cpm $\times 10^{-3}$ /40 ml cells)			Ratios	
		Lipid	Phenol	Walls	Lipid/Phenol	Wall/Phenol
I	1005	215	144	3.7	1.5	.026
	BD-170	117	657	60	.17	.091
	BD-170/1005	.54	4.5	16	.12	3.5
II	1005	280	144	3.5	1.9	.024
	BD-170	137	723	80	.19	.11
	BD-170/1005	.49	5.0	23	.10	4.6

Table 2. Percent of tritium label of [^3H]-DHEP labeled phospholipids remaining soluble in chloroform after phospholipase C hydrolysis. B. subtilis phospholipids which had been labeled in vivo with [^3H]-DHEP were fractionated on a DEAE-cellulose column (Fig. 20). Fractions I,II, and III (Fig. 20) were pooled and treated together. Lipids were hydrolyzed with phospholipase C overnight and the products separated by the method of Bligh and Dyer (100). The amount of label recovered in the chloroform layer was determined by counting a dried sample of the chloroform layer with a toluene based scintillation fluid (3). Details are presented in Materials and Methods. The label recovered in the chloroform layer represents tritium incorporated into the diglyceride portion of the phospholipid(s). Loss of label from the chloroform layer indicates that the tritium label was located in the polar head group portion of the molecule.

Table 2. Tritium-labeled Lipid Remaining Soluble in Chloroform
After Phospholipase C Hydrolysis

Strain	Fraction	Label Added (³ H cpm)	CHCl ₃ Soluble Label (³ H cpm)	% Label Recovered in CHCl ₃
BD-170	I-III	1,753	1,708	97
	IV	19,093	442	2.3
1005	I-III	2,652	2,633	99
	IV	12,161	157	1.3

Table 3. Percent of [^3H]-DHBP-labeled cell wall solubilized

Cell walls labeled with [^3H]-DHBP as described in Materials and Methods. After enzymatic treatment, undegraded walls were collected by centrifugation and the radioactivity of the supernatant was determined using Patterson-Greene scintillation fluid (95). The tritium label recovered in the supernatant represents the degradation of the wall in the enzymatic and 2 N HCl hydrolysis. In other cases, the solubilized label indicated degradation of the teichoic acid and the linker region.

Table 3

The Percent of Cell Wall-Associated Tritium Solubilized

Treatment	BD-170	1005
Lysozyme	100	100
Pronase	100	100
α -Chymotrypsin	2	4
Trypsin	5	4
DNase + RNase ¹	7	9
2 N HCl (100C-overnight) (53)	100	100
0.1 N HCl (100C-10 min) (53) ²	84	79
0.1 N NaOH/1% NaPH (35C) (97) ³	99	96
0.5 N NaOH (22C-4 hr) (53)	100	100

1. DNase, Deoxyribonuclease; RNase, ribonuclease
2. This should be compared with a literature value of 90% (53).
3. Labeled walls from BD-170 were incubated for 8 hr. Walls from 1005 were incubated for 48 hr according to directions (97).

Table 4. Incorporation of [^3H]-DHBP into cell wall *in vivo*.
Cell walls were obtained from *B. subtilis* cultured in the presence of 30 μM DHBP (33 $\mu\text{Ci}/\mu\text{mole}$) as described in Materials and Methods. Hexosamine content was determined by a micromodification of Levvy and McAllen (105) as described in Materials and Methods.

Table 4

Incorporation of [³H]-DHBP Into Cell Walls In Vivo

Trial	Strain		Ratio BD-170/1005
	BD-170 (mmole DHBP/mole hexosamine)	1005	
I	61.8	11.8	5.4
II	54.1	10.1	5.4

Table 5. Data for the determination of the K_m of DHBP for CDP-glycerol pyrophosphorylase. DHBP (sp. act. = .286 $\mu\text{Ci}/\mu\text{mole}$) was used in the standard CDP-glycerol pyrophosphorylase assay as described in Materials and Methods. In the assay 1.15 ml of the TCA/OAc supernatant was treated with charcoal. Of the 1.5 ml of ammoniacal ethanol used to treat the charcoal, 1.0 ml was sampled for radioactivity. Thus for the 10 mM DHBP sample, the actual cpm measured for the experimental and control samples were 2525 and 2503, and 304 and 275, respectively. The average of these values was multiplied by the dilution factor ($1.3 \times 1.5/1.0 \times 1.15$) to obtain the values presented in the table. The values in the parentheses are the value of the control reaction (complete reaction lacking CTP) expressed as a percentage of the [^3H]-DHBP added to the assay. The counting efficiency was 15.5%.

Data for the Determination of the K_m of DHBP

DHBP Conc. (mM)	Label Added (cpm $\times 10^{-5}$)	Label Desorbed from Charcoal (cpm)		Control Reaction as % of Product
		+CTP	-CTP	
2.0	1.36	869	260 (0.19%)	30
2.5	1.70	1091	222 (0.13)	20
3.35	2.28	1561	350 (0.15)	22
5.0	3.41	2406	335 (0.10)	14
6.7	4.57	3018	442 (0.10)	15
10	6.81	4274	493 (0.07)	12
20	13.63	7487	644 (0.05)	9

Table 6. Kinetic data for CDP-glycerol pyrophosphorylase extracted from *B. subtilis* 168. See Fig. 25 and Materials and Methods for details of the assay.

Table 6

Kinetic Data for CDP-glycerol Pyrophosphorylase

Strain	Enzyme Preparation	Km (mM)		Ki (mM)		Vmax (mmole/min-mg)	
		L-G3P	L-DHBP	L-DHBP	L-G3P	L-DHBP	
42	Dialyzed	0.19	36	6.8	5.3 ¹	1.8	
BD-170	Gel-purified	0.17	-	6.1	19.7 ¹	-	

1. The increase in specific activity of the enzyme derived from passage through the Sephadex column is much greater than indicated here. Column fractions containing similar activity (low, medium, or high) were pooled. The pool with the lowest activity was used. The highest activity pool was approximately 15-fold higher than the enzyme preparation used here.

Table 7. In vitro teichoic acid synthesis. The assay is based on conversion of a nucleotide precursor of teichoic acid into a membrane associated form. Membranes (8 mg of BD-170 membranes or 40 mg of 1005 membranes), CDP-[^{14}C]-glycerol (1.45 $\mu\text{Ci}/\mu\text{mole}$) or CMP-[^3H]-DHBP (1.45 $\mu\text{Ci}/\mu\text{mole}$), and other additions as indicated were incubated together in a buffered solution. Details are presented in Materials and Methods. For the boiled enzyme control, the membranes were suspended in the buffer placed in a boiling water bath for two minutes and cooled on ice before the labeled nucleotide was added. In all other experiments the reaction was initiated by addition of the membrane preparation. Experiments contained 69,400 ^{14}C -cpm or 640,000 ^3H -cpm as indicated.

Table 7. Teichoic Acid Synthesis in vitro

Source of Membranes	Additions		Products			Synthesis Activity of Membranes (pmole/min-mg)
	CDP-glycerol (uM)	CMP-DHBP (mM)	nmoles	cpm	As % of Reactants	
I. Label in ¹⁴ C -glycerol						
a. BD-170	69.6	-	11.6	22,900 (100%)	33	48
b. BD-170	69.6	1.67	10.6	20,900 (91%)	31	-
c. BD-170 (boiled)	69.6	-	0	0	0	-
d. 1005	69.6	-	0.26	531	0.77	0.22
II. Label in CMP ³ H -DHBP						
a. BD-170	-	1.67	2.22	1,700 (100%)	0.27	9.3
b. BD-170	54.6	1.67	0.38	288 (17%)	0.045	-
c. BD-170 (boiled)	-	1.67	0.13	96	0.016	-
d. 1005	-	1.67	0.57	439	0.068	0.48

**Fig. 1. Biosynthetic pathway of bacterial phospholipids
(after reference 21).**

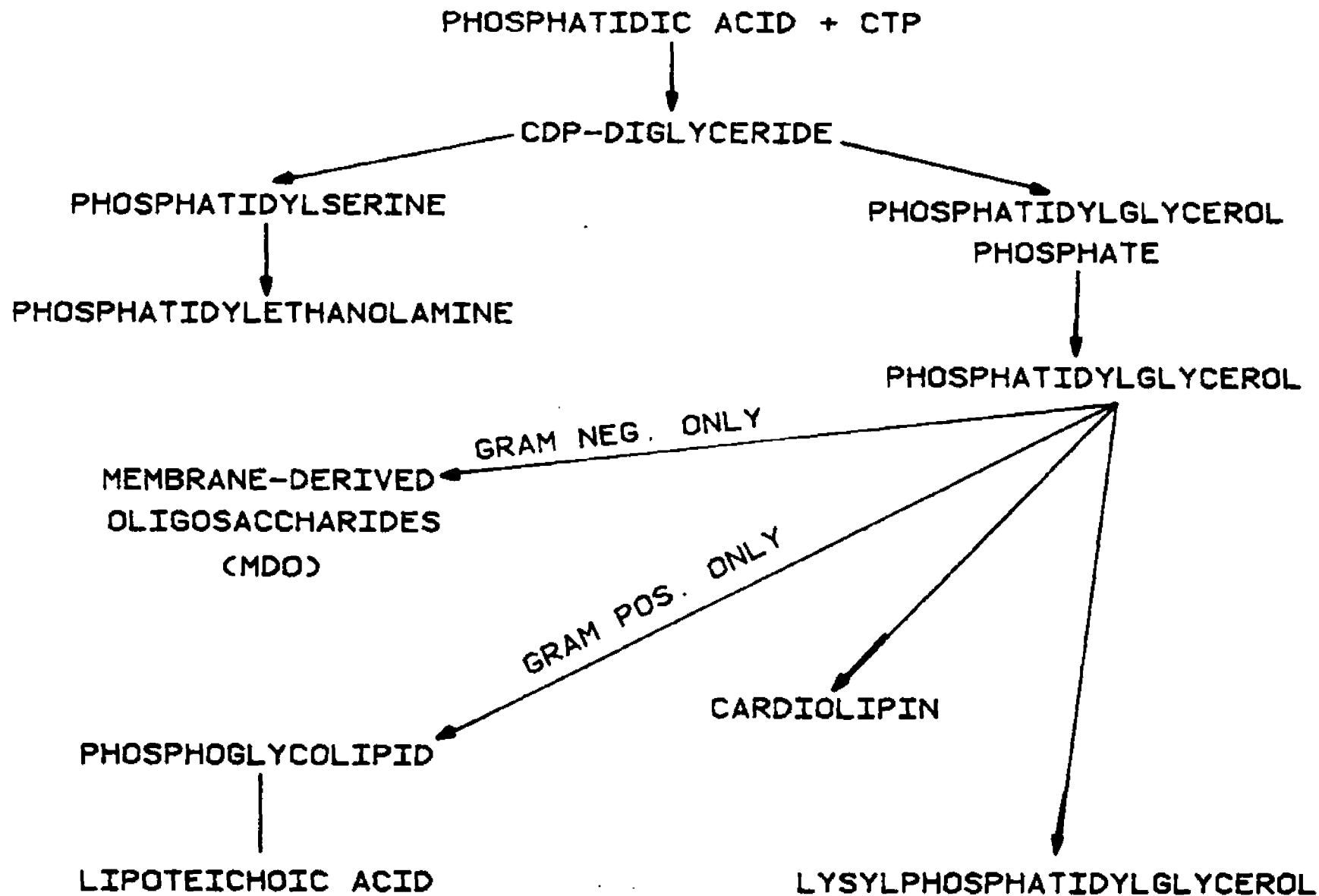


Fig. 2. (A), (1,2-Diacyl)-sn-glyceryl D-phosphoryloxy-3'-hydroxyl-1'-phosphonate. (B), β -Gentiobiosyl-diglyceride. (C), Phosphoglycolipid (This has not been reported in B. subtilis). (D), Glycerophosphoglycolipid from strain W23 (16).

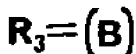
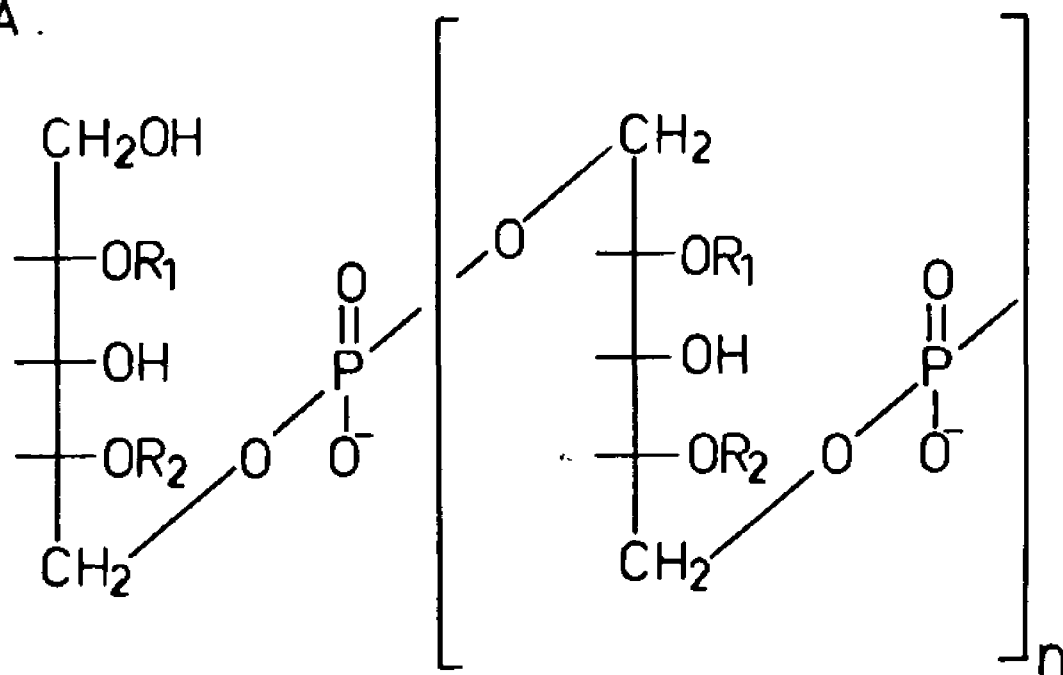


Fig. 3. Polymeric structures of teichoic acids. (A), Ribitol teichoic acid (strain W23). $R_1 =$ D-alanyl. $R_2 = \beta$ -D-glucopyranosyl. (B), Glycerol teichoic acid. $R =$ H, D-alanyl, or α -D-glucopyranosyl.

A.



B.

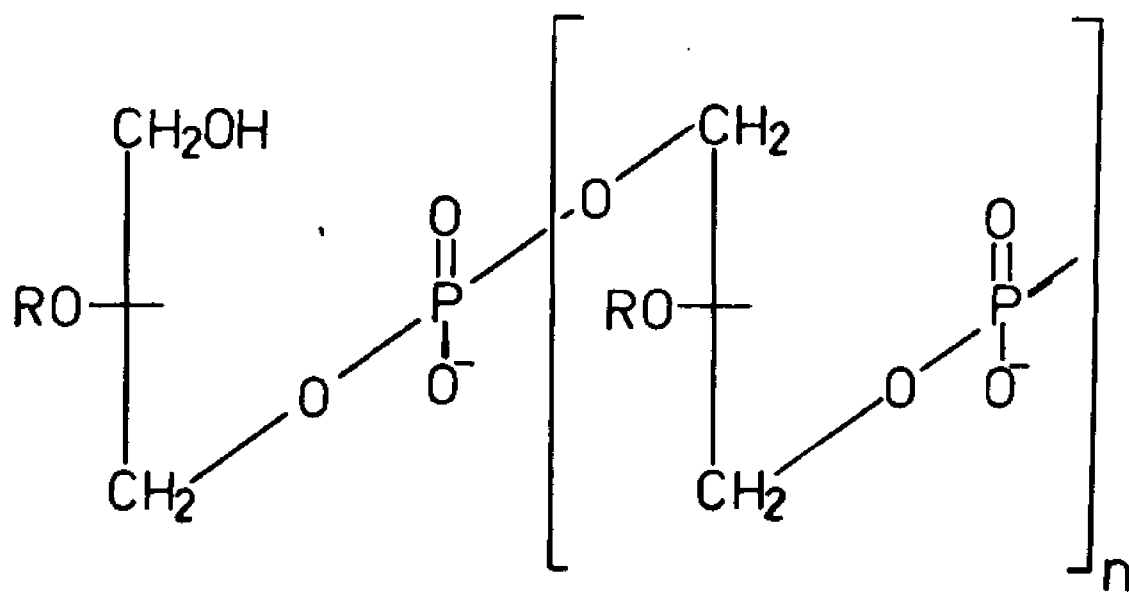


Fig. 4. Teichoic acid biosynthesis (after references 58-60). Abbreviations: PRENOL-P = uncharacterized polyisoprenoid carrier, GlcNAc = N-acetyl-glucosamine, GP = glycerol 3-phosphate, RP = ribitol 5-phosphate.

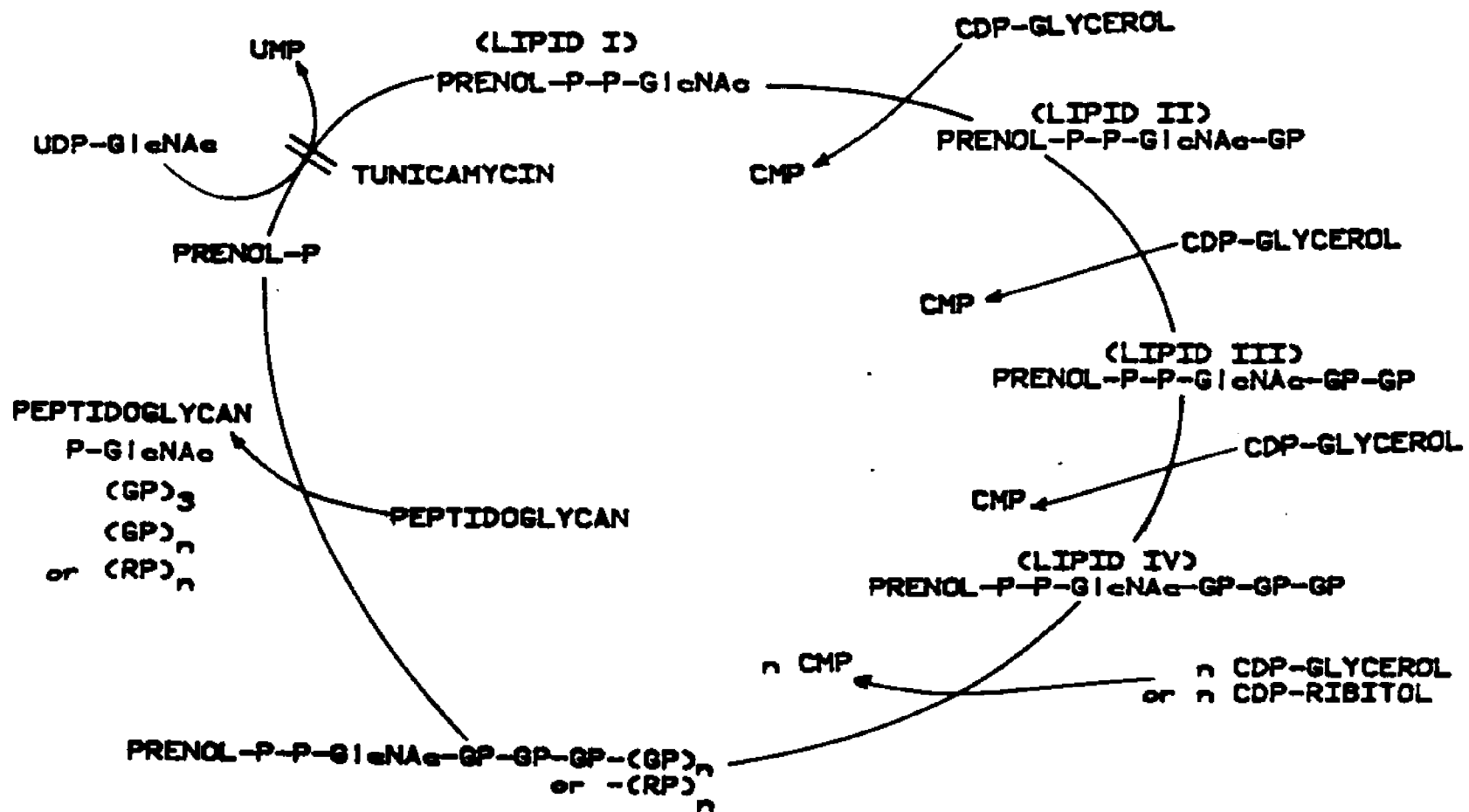


Fig. 5. Linker region of B. subtilis W23 (after reference 56). The linker region of B. subtilis W23 showing the sites of hydrolysis by (A), 0.1 N HCl for 10 minutes at 100C (53) and (B), 0.1 N NaOH with sodium borohydride at 35C (103) or 0.5 N NaOH at 22C. See Materials and Methods for details.

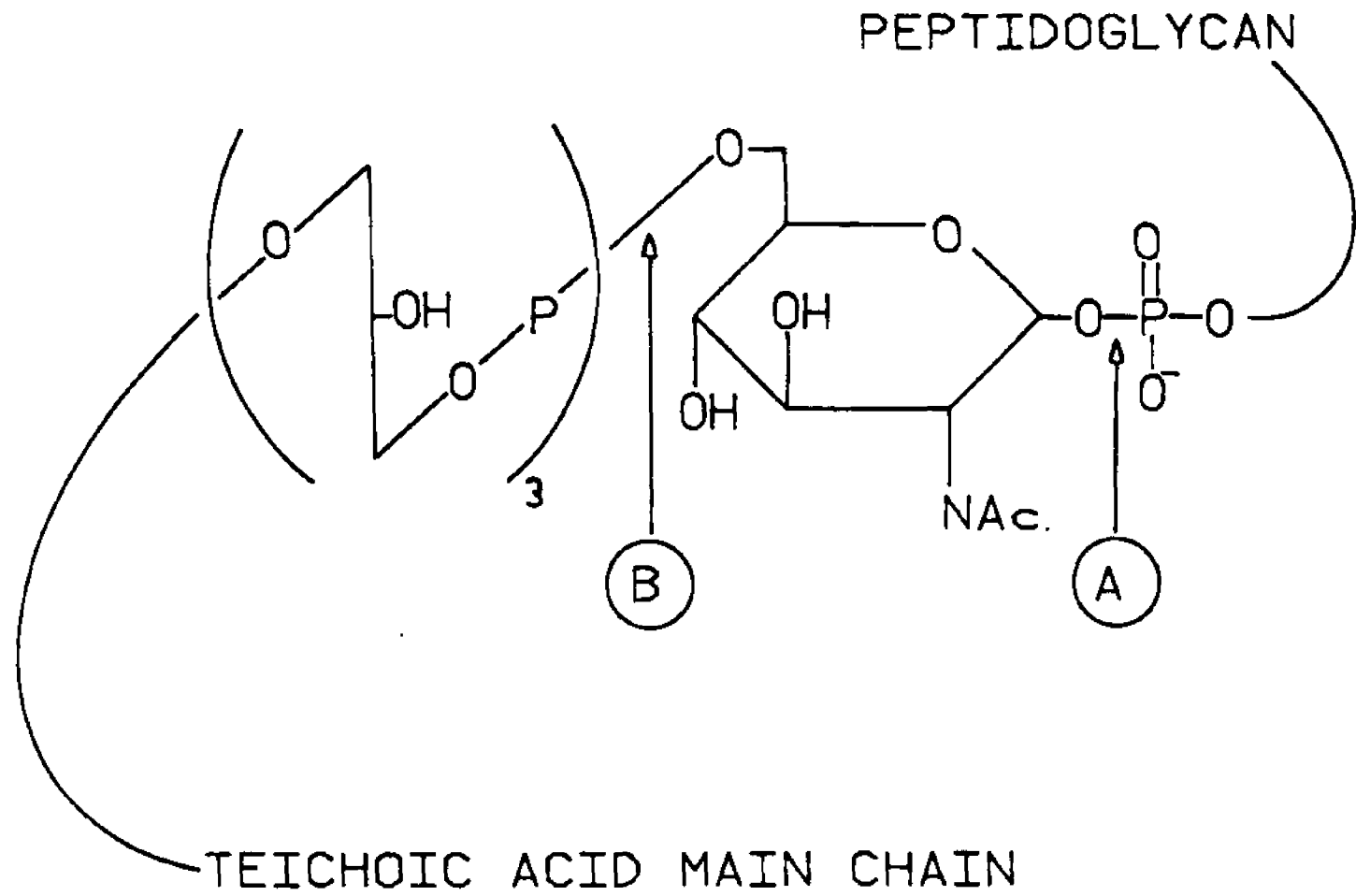
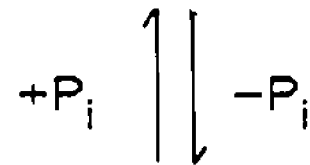


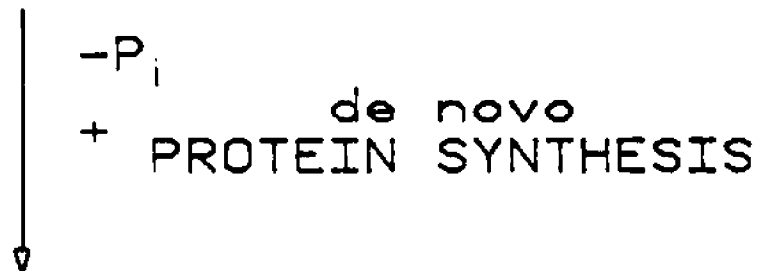
Fig. 6. Proposed scheme for the regulation of teichoic acid synthesis (after reference 68).

REGULATION OF TEICHOIC ACID SYNTHESIS (AFTER REFERENCE 68)

TEICHOIC ACID SYNTHESIZING SYSTEM



INHIBITED TEICHOIC ACID SYNTHESIZING SYSTEM



INACTIVE TEICHOIC ACID SYNTHESIZING SYSTEM

Fig. 7. Elution profile of the 45-60% saturated ammonium sulfate precipitate from the 2.5 x 50 cm Sephadex G-100 column. A Sephadex G-100 column was used to prepare CDP-glycerol pyrophosphorylase as described in Materials and Methods (74). CDP-glycerol pyrophosphorylase activity was detected by the amount of [^{14}C]-glycerol 3-phosphate which could be converted to a charcoal absorbable form in the standard assay as described in Materials and Methods (74). Symbols: $\square$, [^{14}C]-cpm released from charcoal, $\odot$, Absorbance at 280nm.

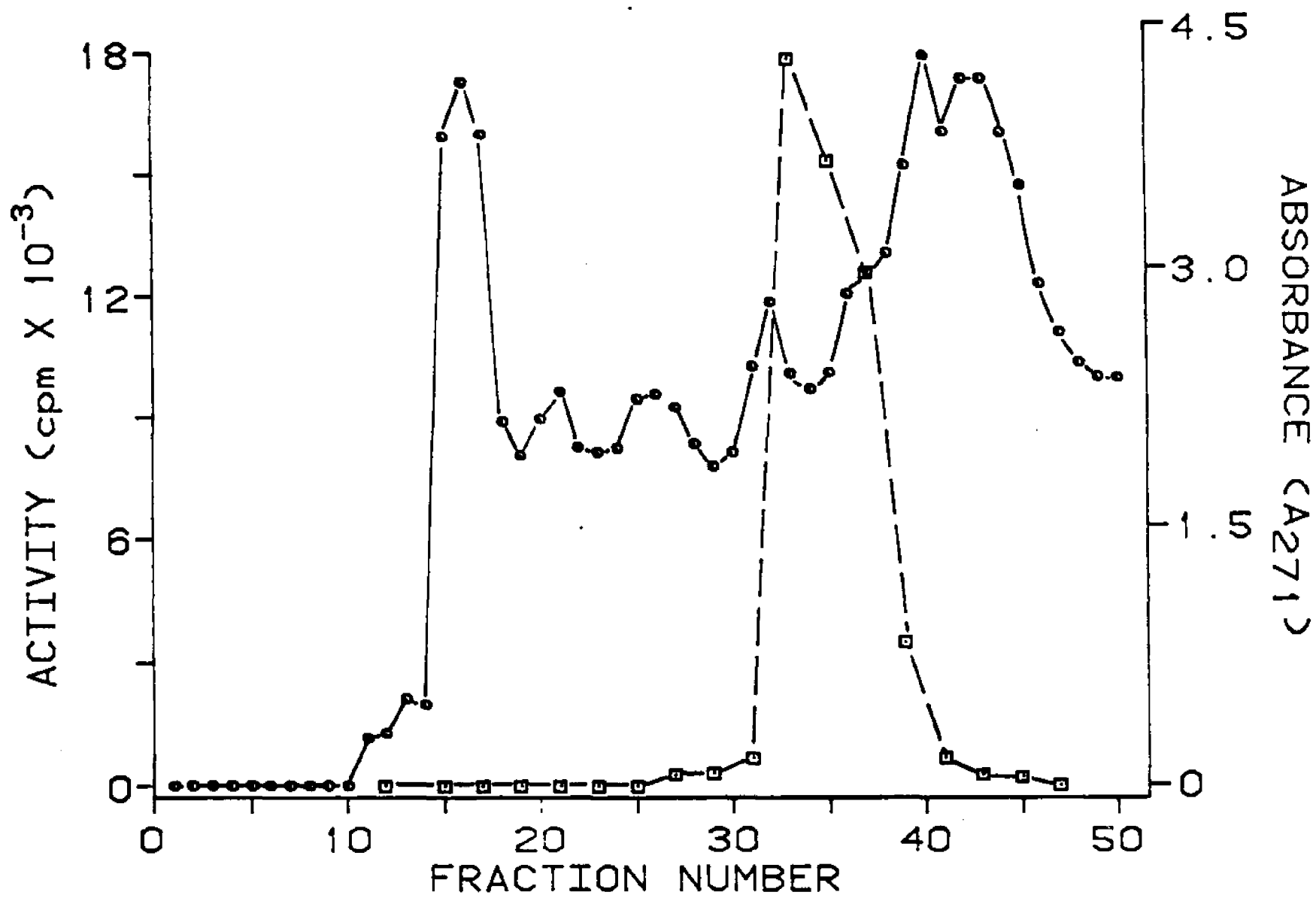


Fig. 8. Structures of (A), Cytidine 5'-diphosphate glycerol. (B), 3,4-Dihydroxybutyl-1-phosphonyl-O-(cytidine 5'-phosphate).

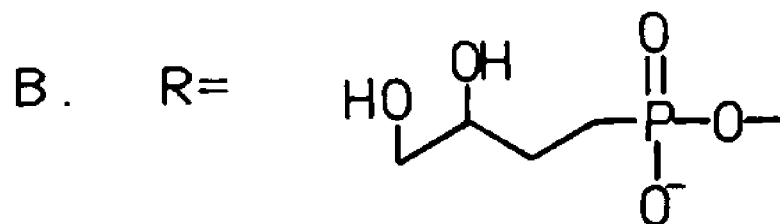
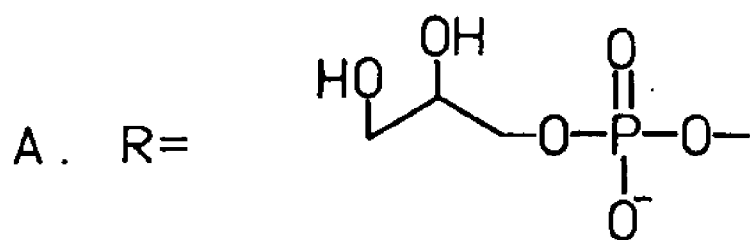
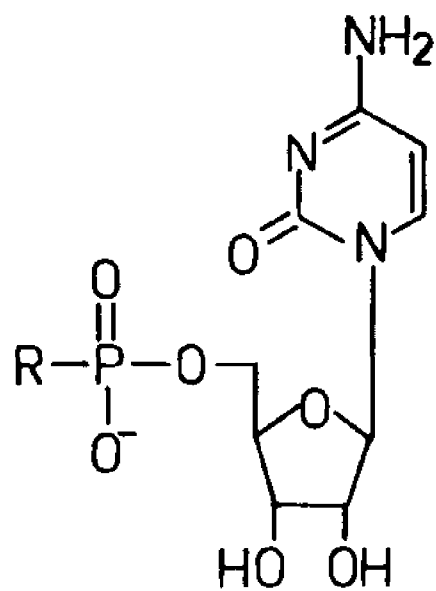


Fig. 9. Elution profile of UV-absorbing material in the products of the chemical synthesis of CDP-glycerol on Dowex-1. The reaction mixture from the chemical synthesis of CDP-glycerol was worked up, applied to the column, eluted with a 0-50 mM LiCl gradient in 3 mM HCl as described in Materials and Methods. Symbols: Δ , Absorbance at 271 nm, — , LiCl concentration.

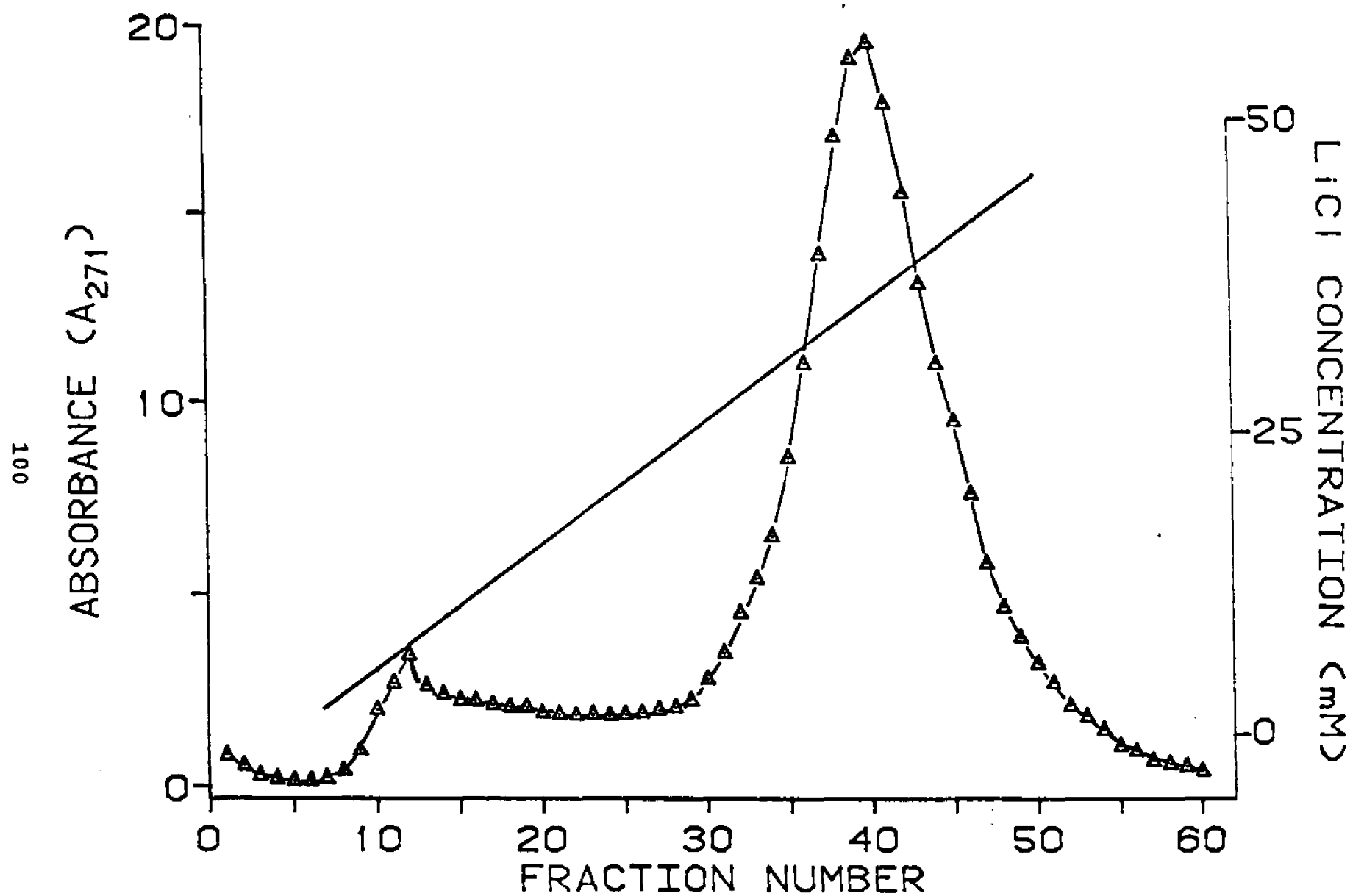


Fig. 10. Elution profile from a DEAE-cellulose column of nucleotides used in the teichoic acid synthesis assay. Nucleotides which had been recovered from a Dowex-1 column were eluted from a DEAE-cellulose (bicarbonate) column with a 5-200 mM triethylamine bicarbonate buffer as described in the Materials and Methods section. (A), CDP-glycerol. (B), CMP-DHBP. Symbols: Δ , Absorbance at 271 nm, —, gradient concentration, $(Et)_3NHCO_3$ = triethylamine bicarbonate. Peaks I, II, III, and IV correspond to cytosine, cytidine 5'-monophosphate, the nucleotide product, and cytidine 5'-diphosphate, respectively.

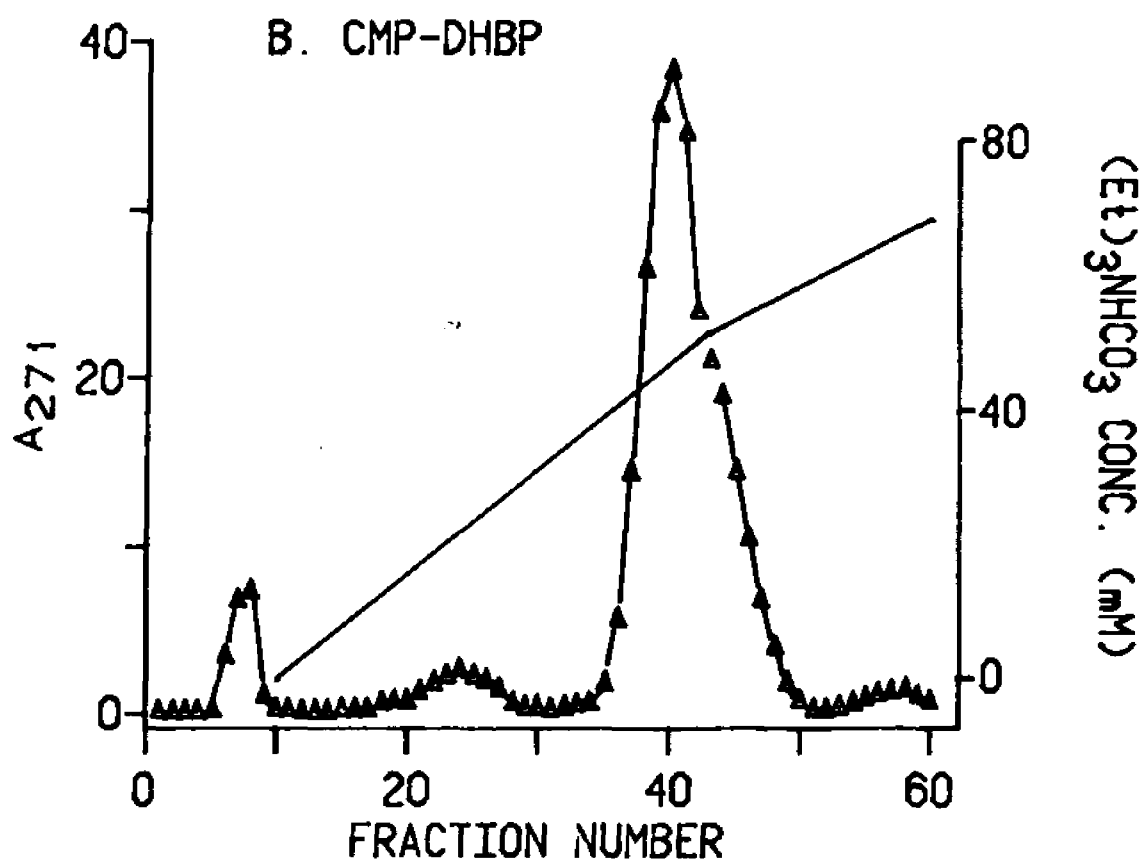
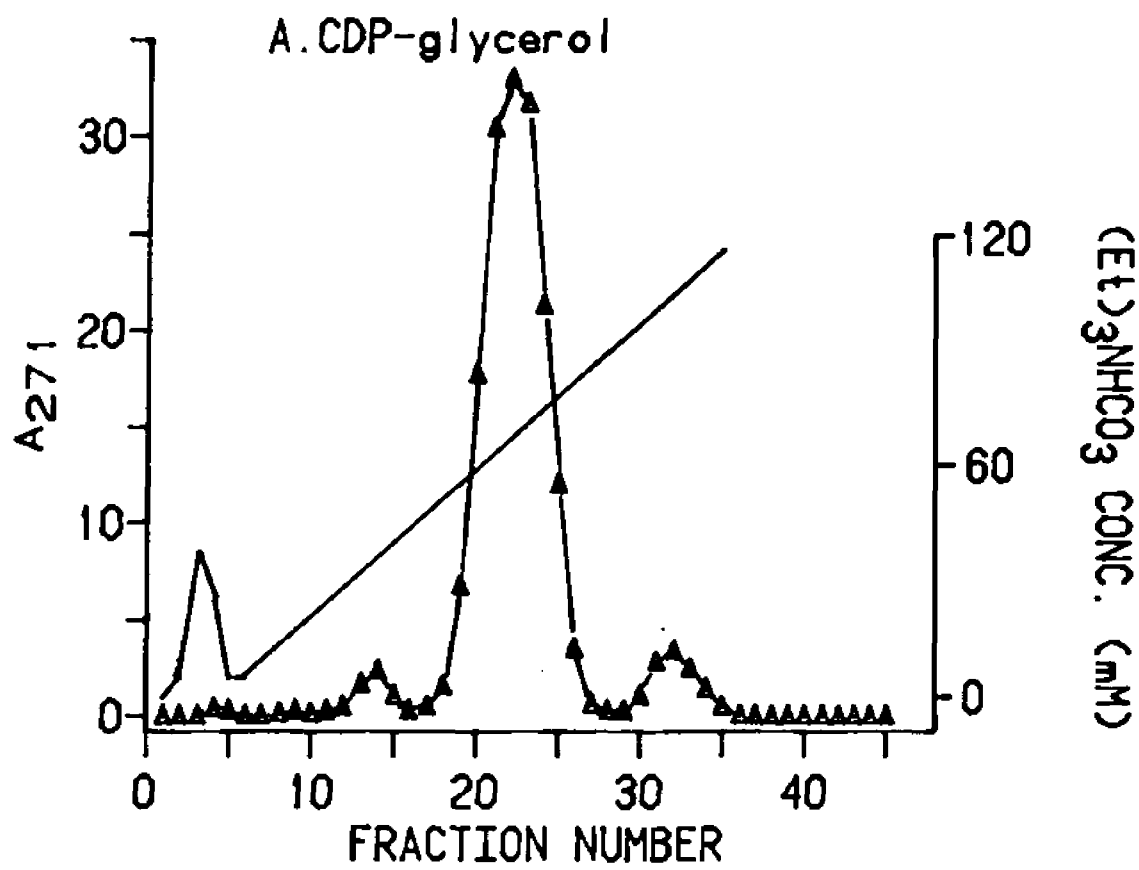
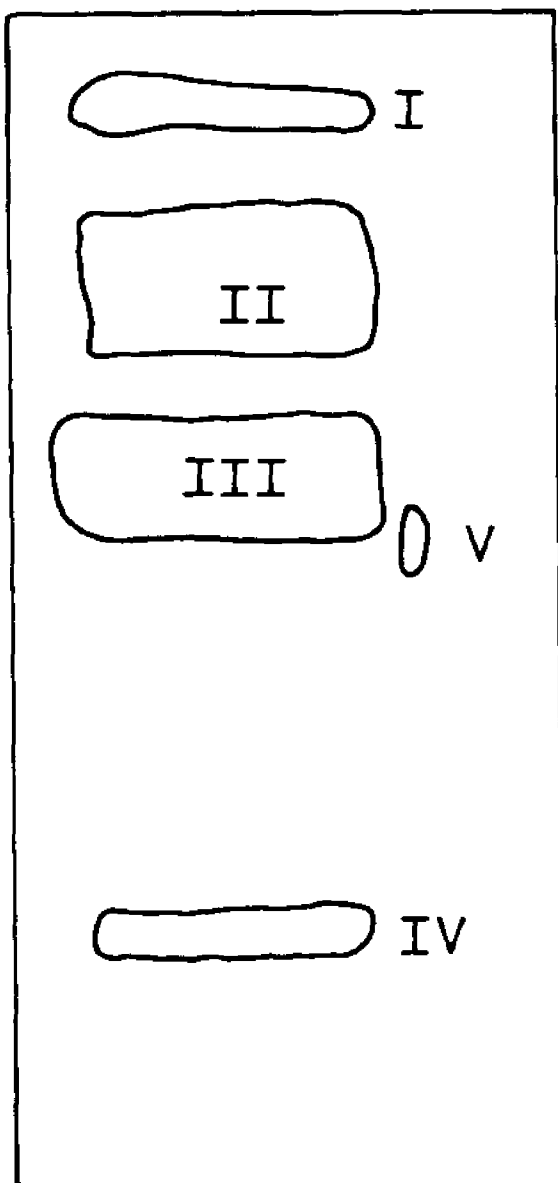
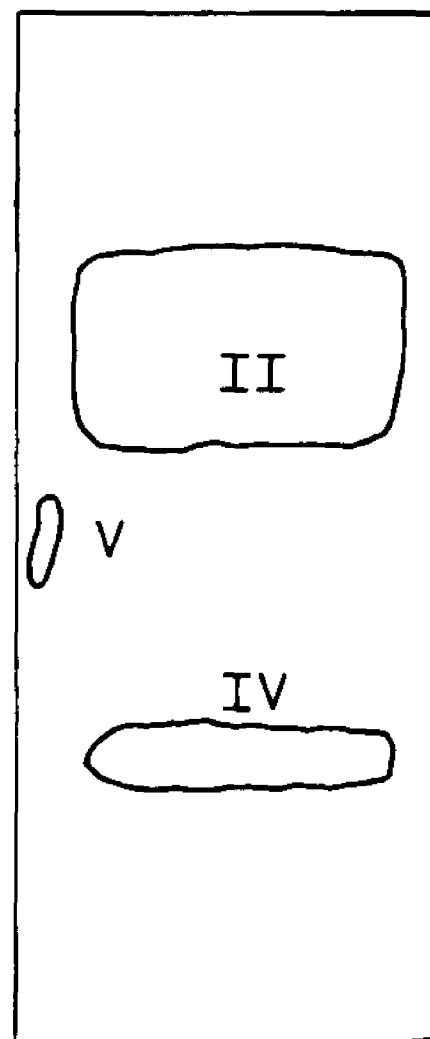


Fig. 11. Paper chromatography of CDP-[^{14}C]-glycerol and CMP-[^3H]-DHBP. Descending paper chromatograms on Whatman 3 MM paper using 95% ethanol:.575 M ammonium acetate, pH 3.8 (79:26) as the developing solvent. The location of the nucleotides were determined with a UV-lamp. The area corresponding to the desired nucleotide (III) was eluted from the first chromatogram, (A) or (C), and re-chromatographed as shown, (B) and (D), respectively. (A) and (B), CDP-[^{14}C]-glycerol. (C) and (D), CMP-[^3H]-DHBP. I, II, III, and IV correspond to CDP, the product, CMP, and cytosine, respectively. V is a CMP-morpholidate marker.

A.



B.



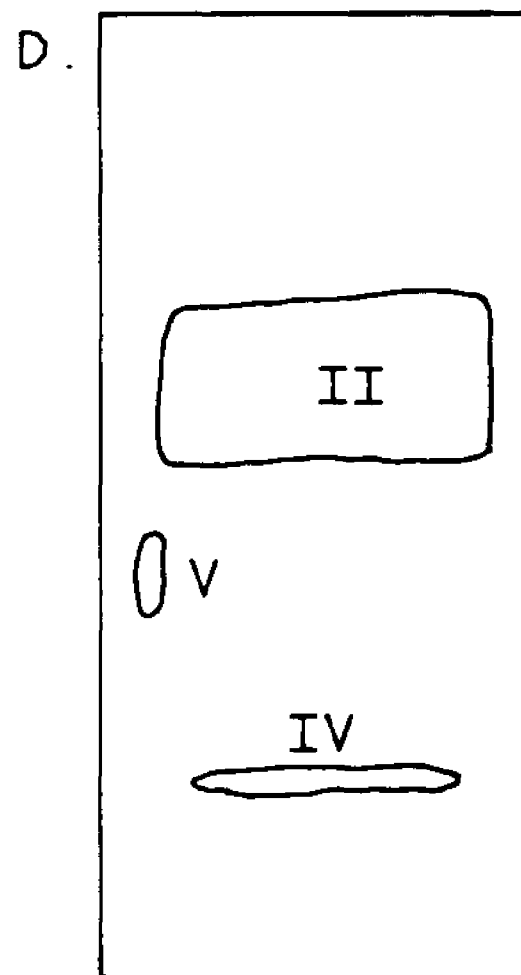
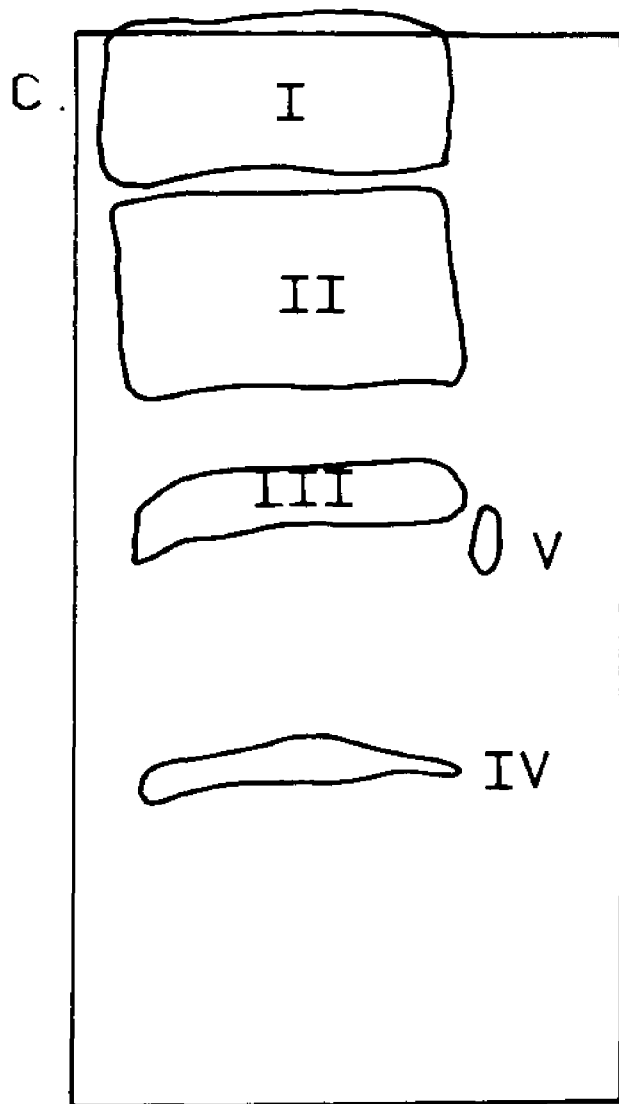


Fig. 12. Purification of [^3H]-DHBP on DEAE-cellulose column. [^3H]-DHBP was chromatographed on a 1.0 x 12 cm Whatman DE-52 DEAE-cellulose (bicarbonate) column with a 0-100 mM ammonium bicarbonate gradient as described in Materials and Methods. The radioactivity in each fraction was determined by counting a sample of each fraction with Patterson-Greene scintillation fluid (101) as described in Materials and Methods.

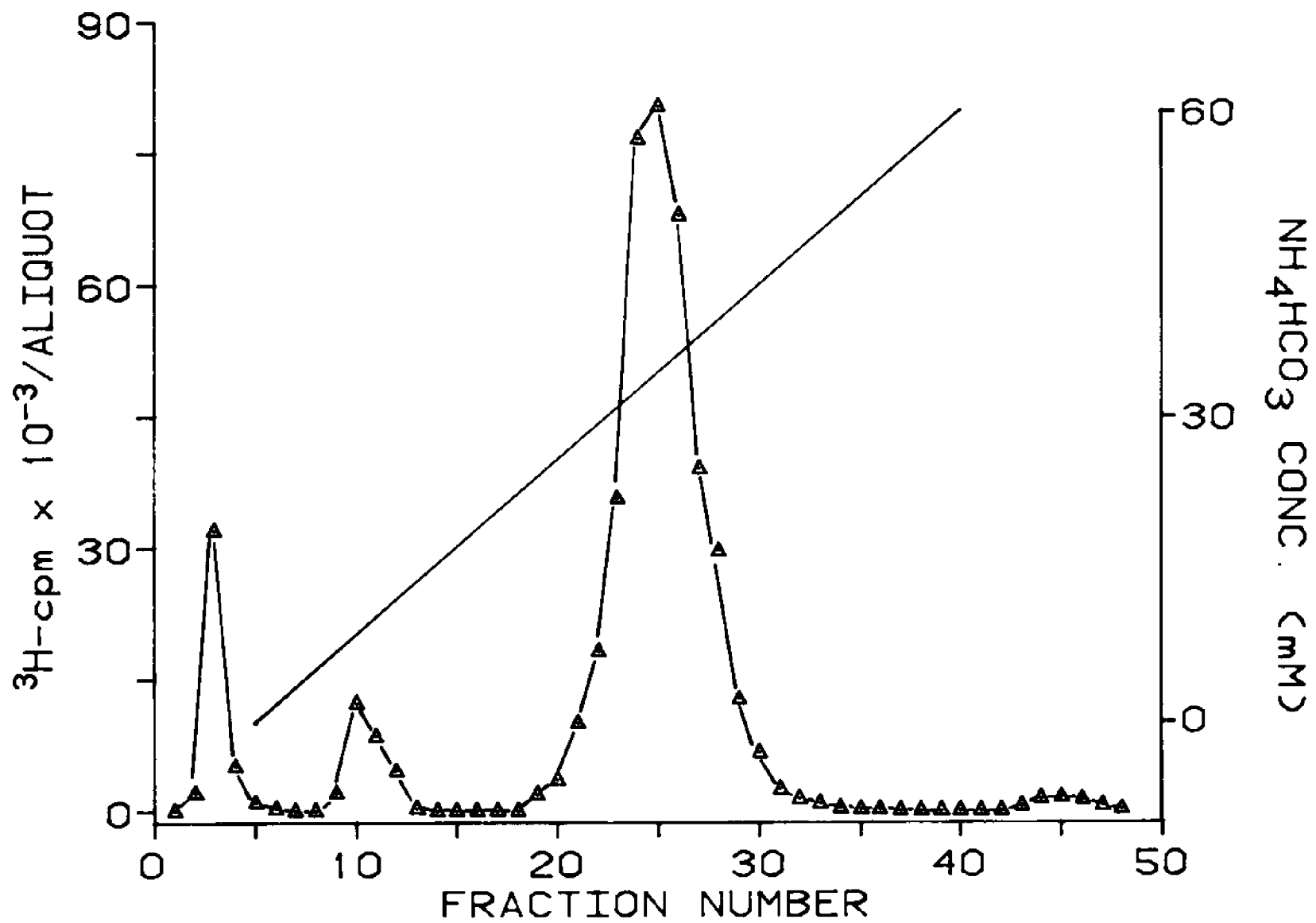


Fig. 13. Effect of different concentrations of DHBP on the growth of *B. subtilis*. *B. subtilis* was cultured in casein hydrolysate broth as described in Materials and Methods. (A), Strain BD-170. (B), Strain 1005. At the time indicated by the arrow λ g-DHBP was added to the following final concentrations: Δ , .50 μ M, $\square$, 100 μ M, $\odot$, 200 μ M, $\bullet$, 2 mM DHBP, and 4 mM LiCl, $\diamond$.

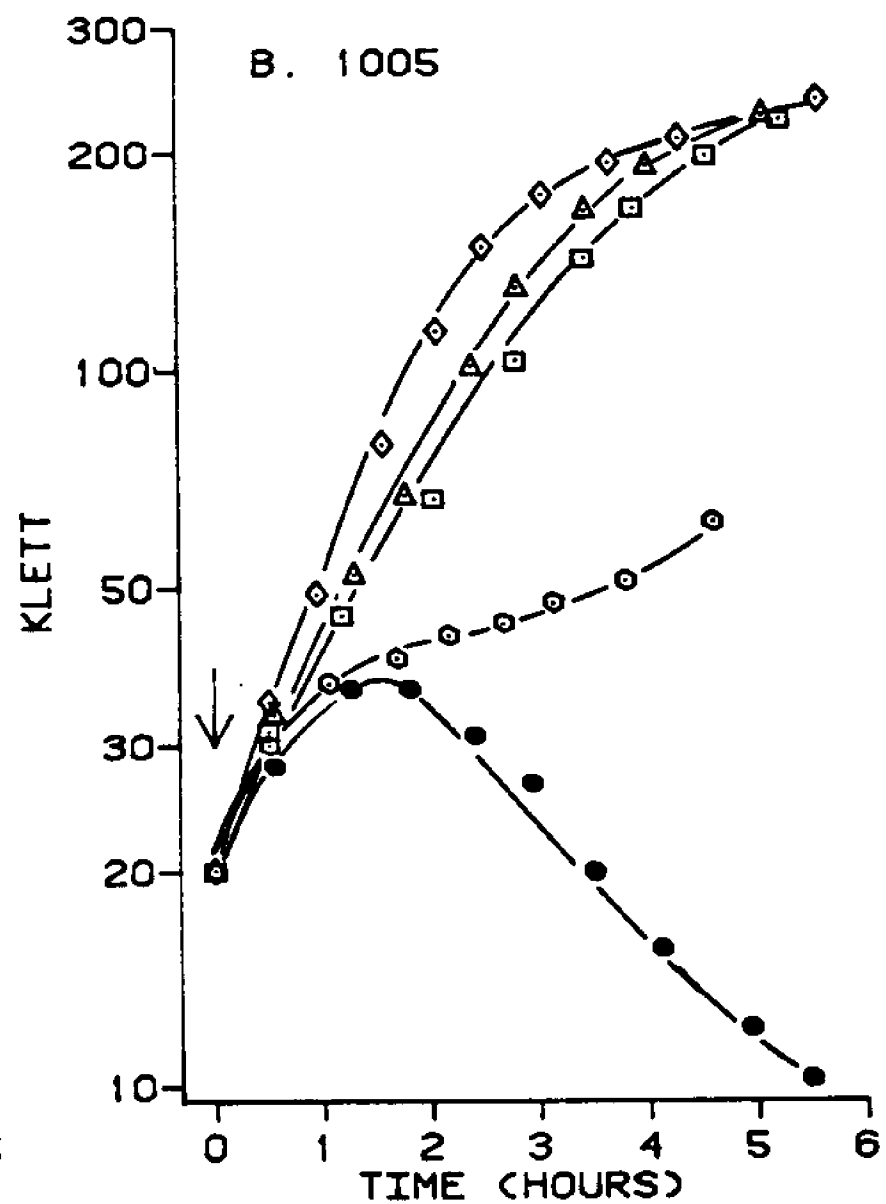
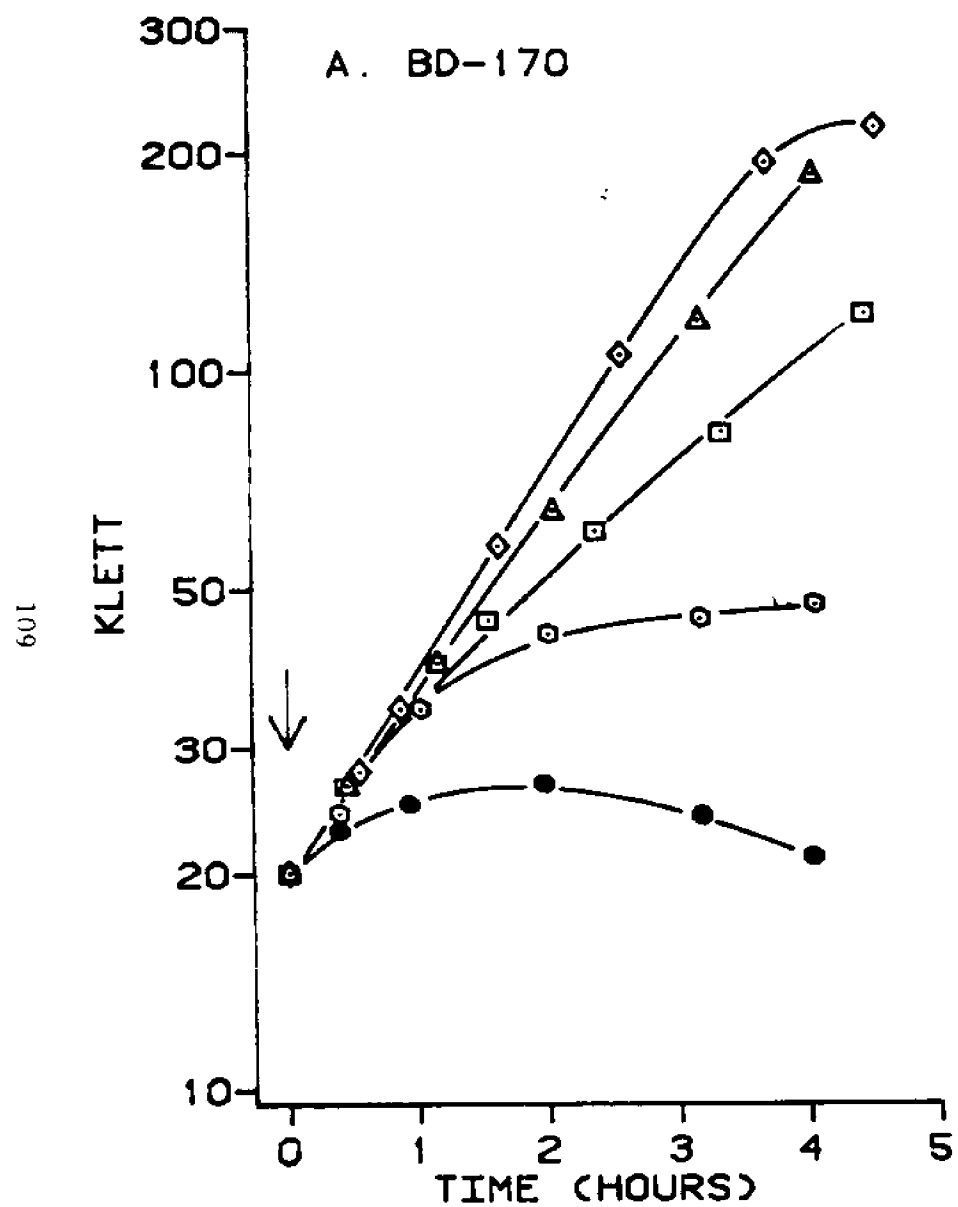


Fig. 14. Comparison of rac- and §-DHBP on the growth of B. subtilis. Cells were cultured in casein hydrolysate broth as described in Materials and Methods. At the time indicated by the arrow, additions were made to the following final concentrations: Δ , 75 μ M §-DHBP, $\square$, 150 μ M rac-DHBP, and 300 μ M LiCl, $\diamond$.

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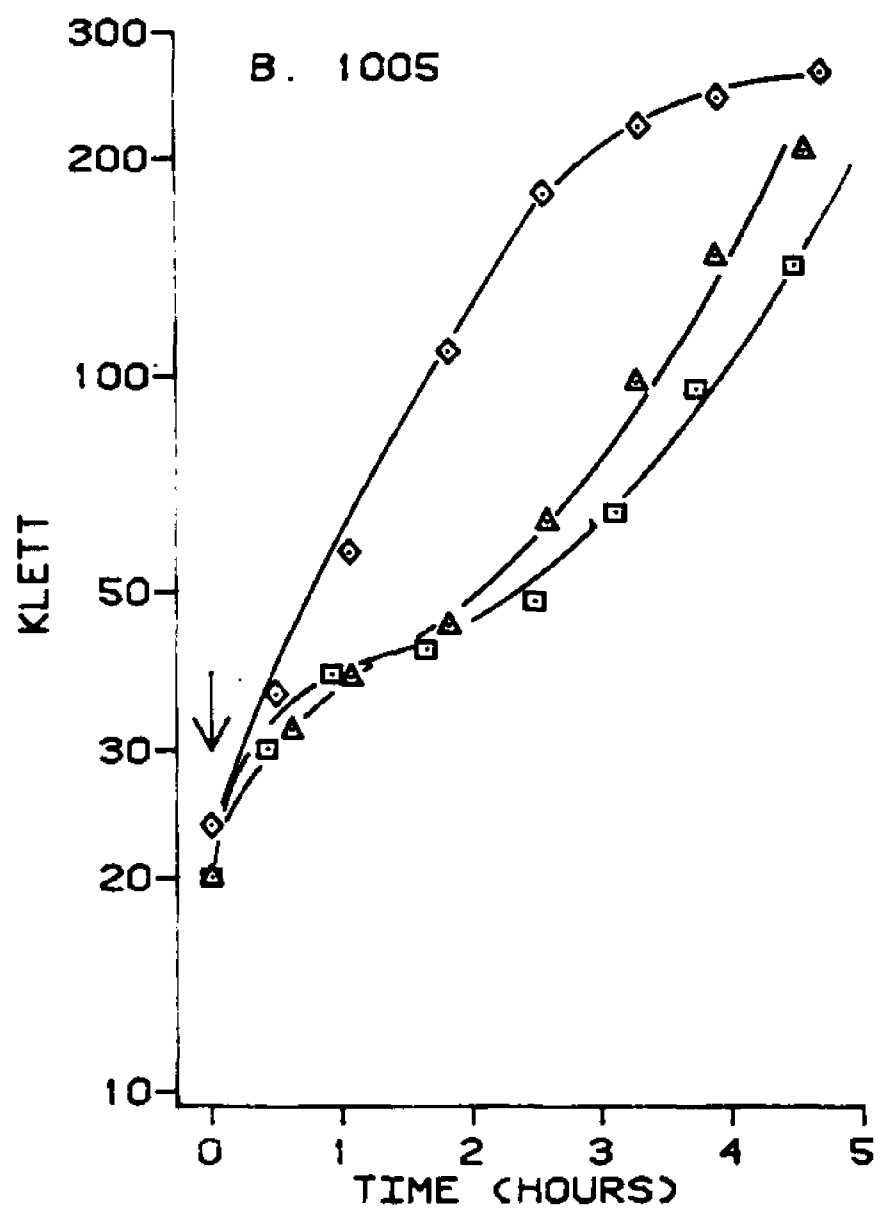
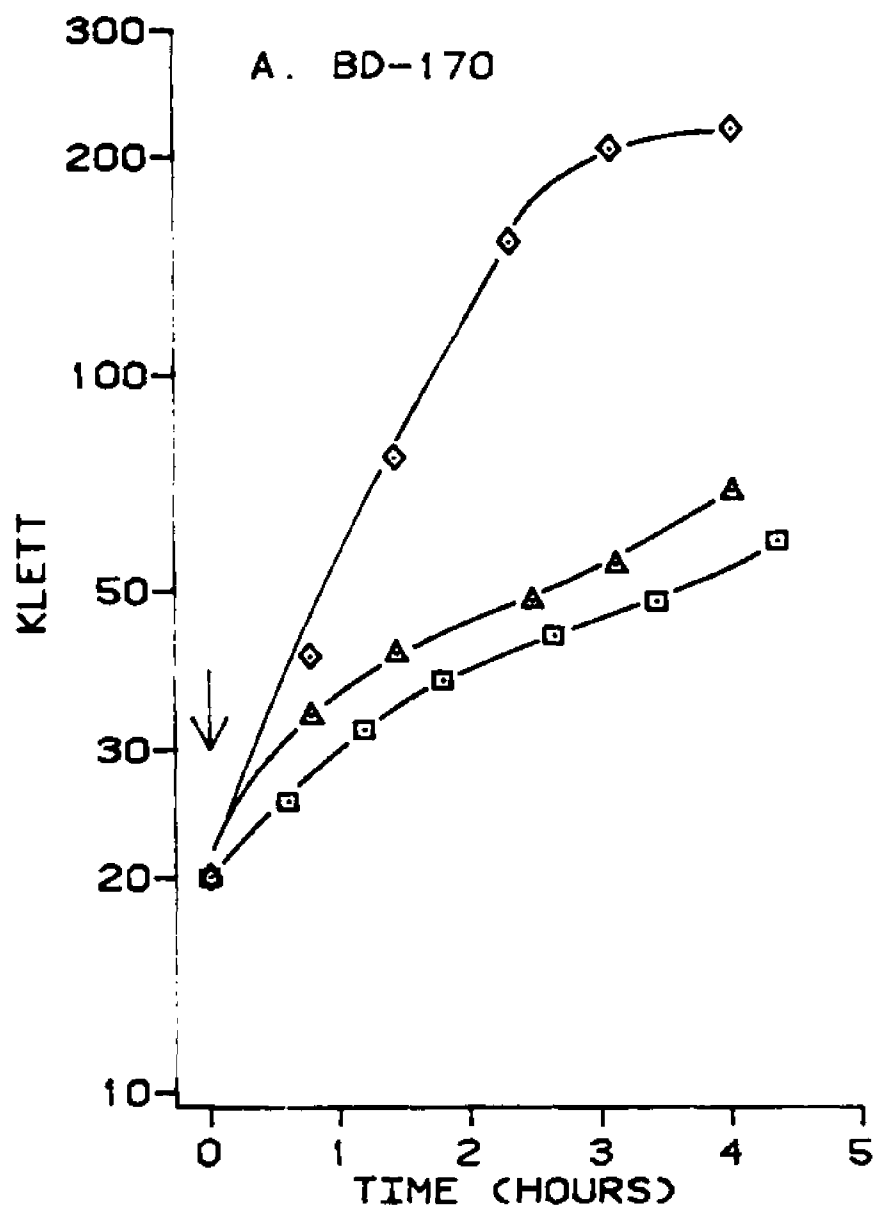
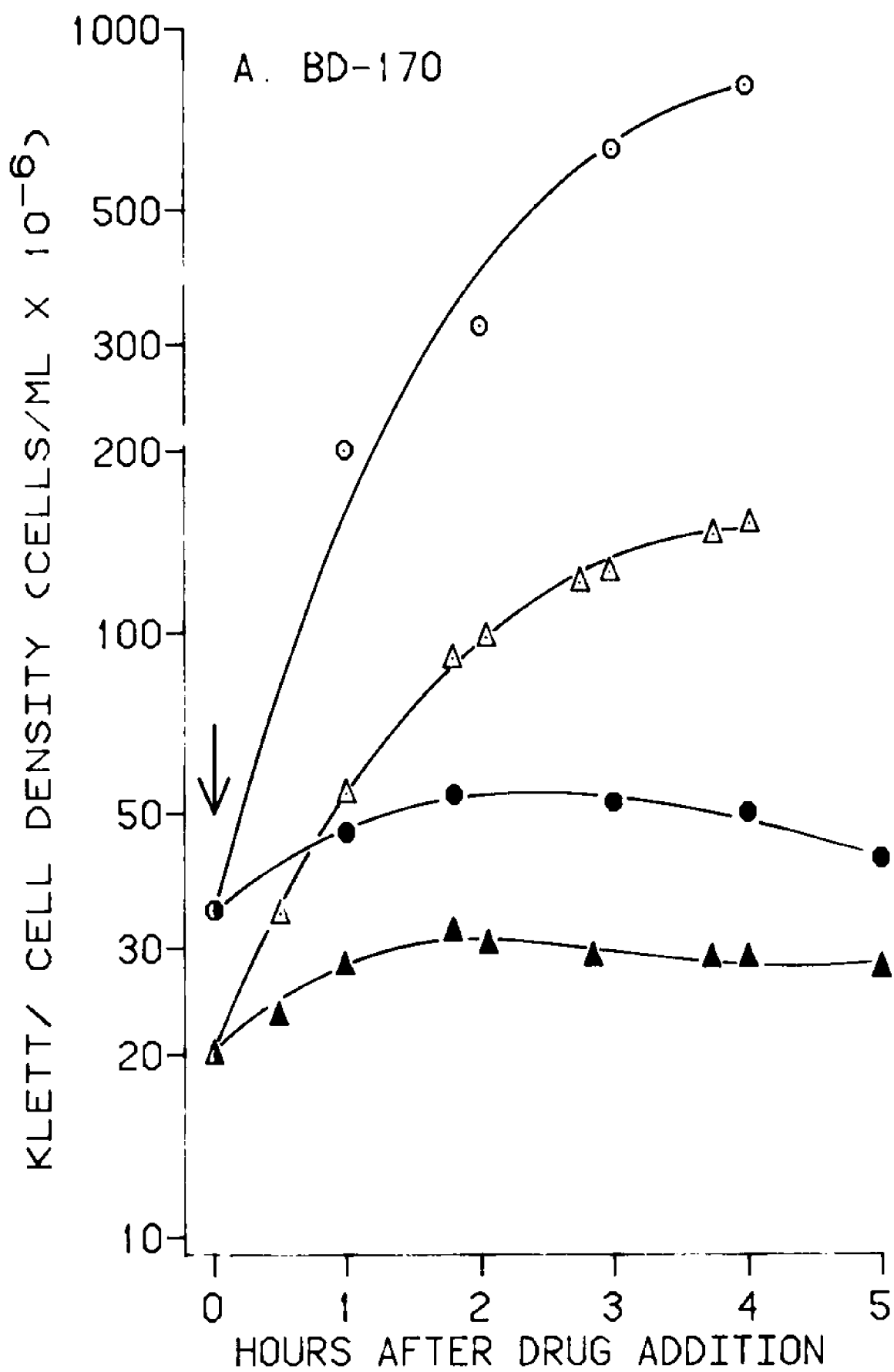


Fig. 15. The effect of DHBP on the viability of *B. subtilis*. Cell viability was determined as described in the Materials and Methods section. Symbols: Δ , $\blacktriangle$, turbidity, $\odot$, $\bullet$, cell density (cells/ml). Open symbols treated with 4 mM LiCl, closed symbols treated with 2 mM rac-DHBP. (A) Strain BD-170. (B). Strain 1005.



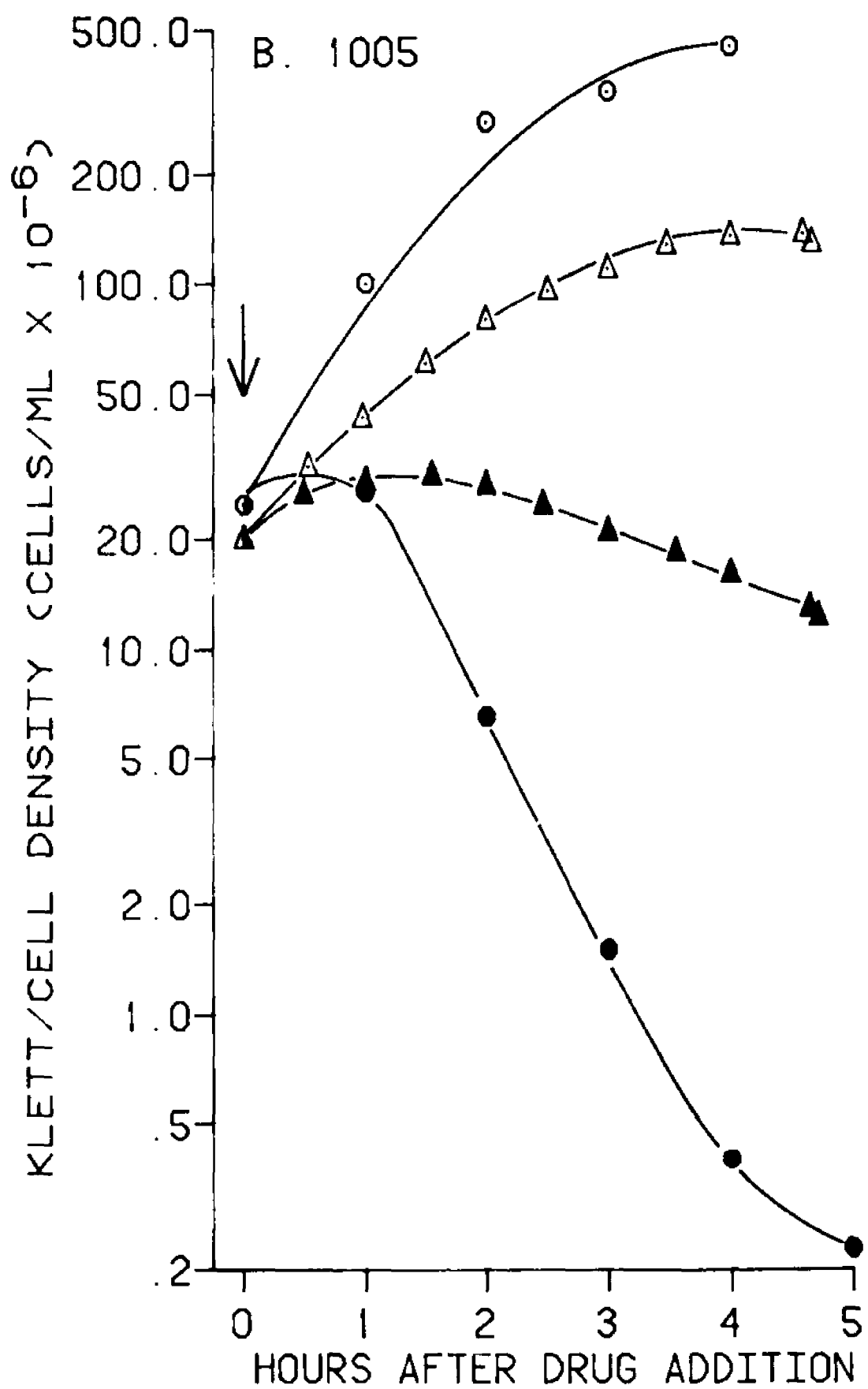


Fig. 16. The effect of additions to the medium on the DHBP inhibition of *B. subtilis*. As described in Materials and Methods cells were cultured in casein hydrolysate broth augmented with 100 mM phosphate, pH 7.0. (A), Strain BD-170. (B), Strain 1005. Symbols: $\odot$, $\bullet$, supplemented cultures and $\triangle$, $\blacktriangle$, unsupplemented cultures. Closed symbols represent cultures treated with 0.2 mM rac-DHPP. Open symbols represent control cultures treated with LiCl.

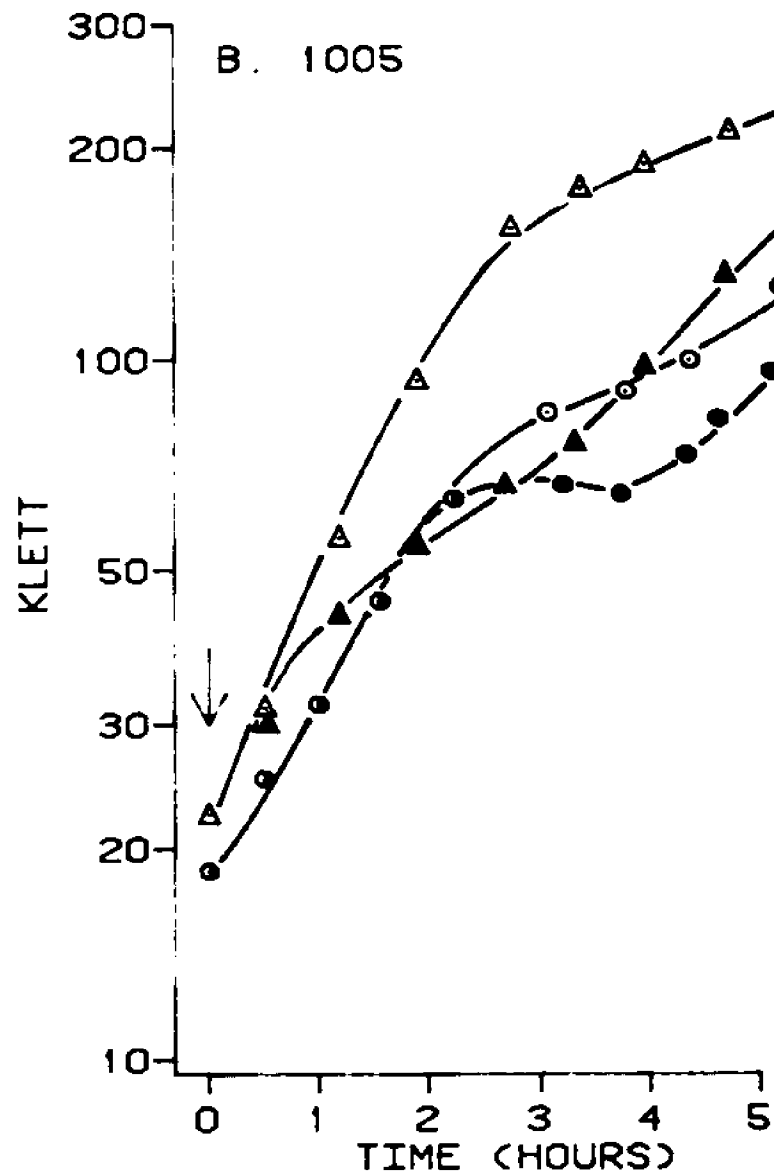
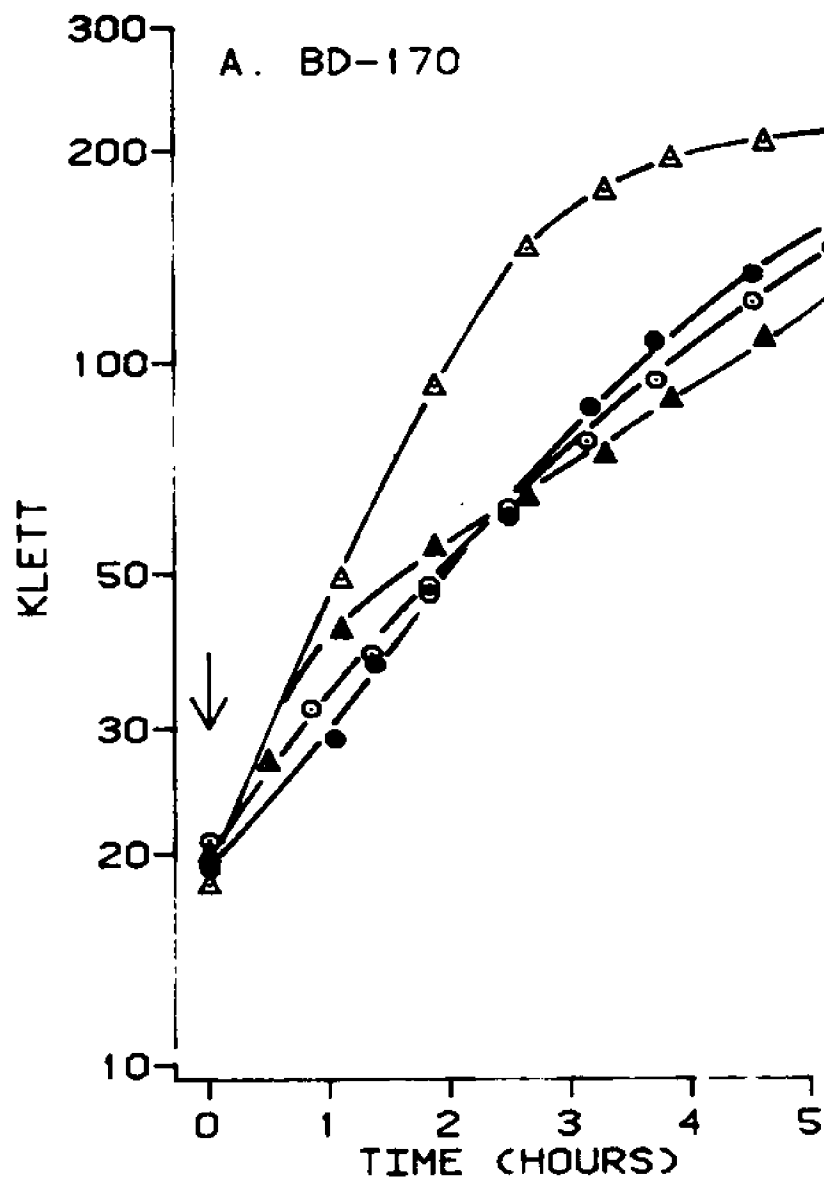
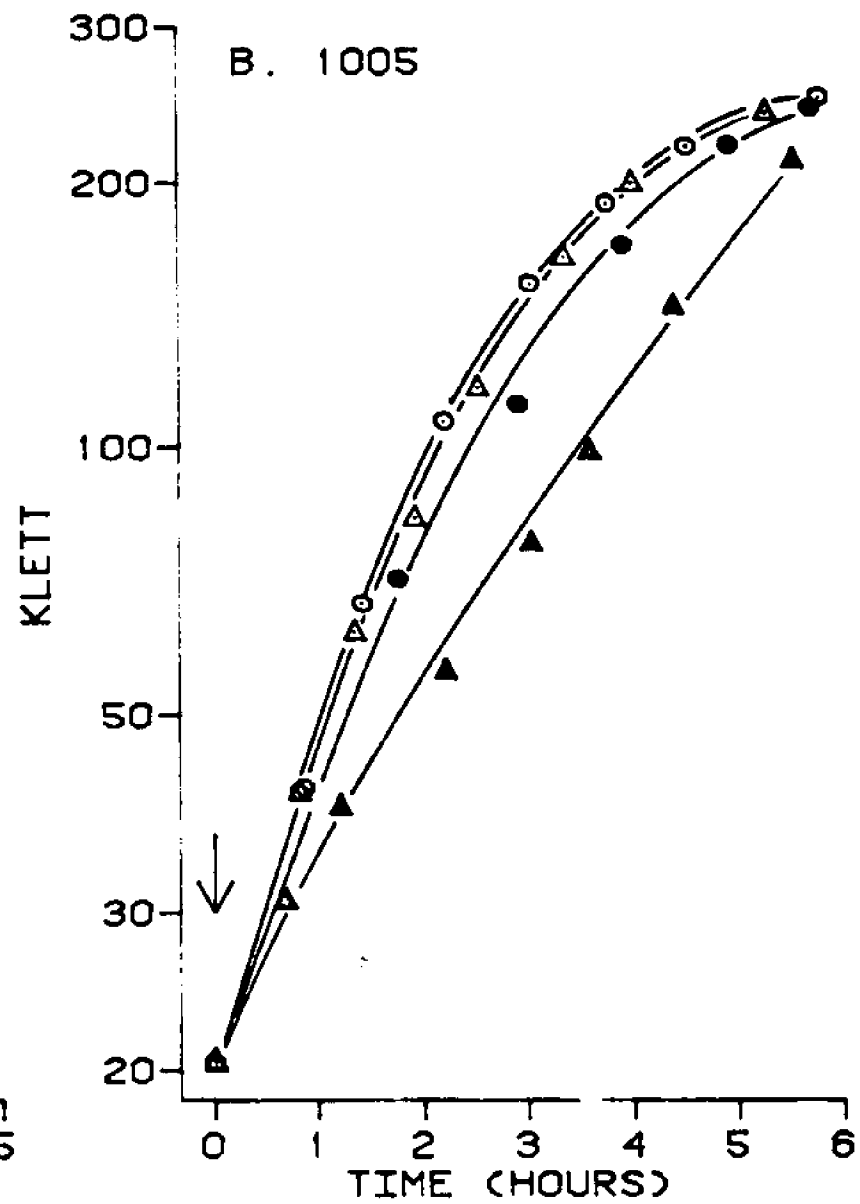
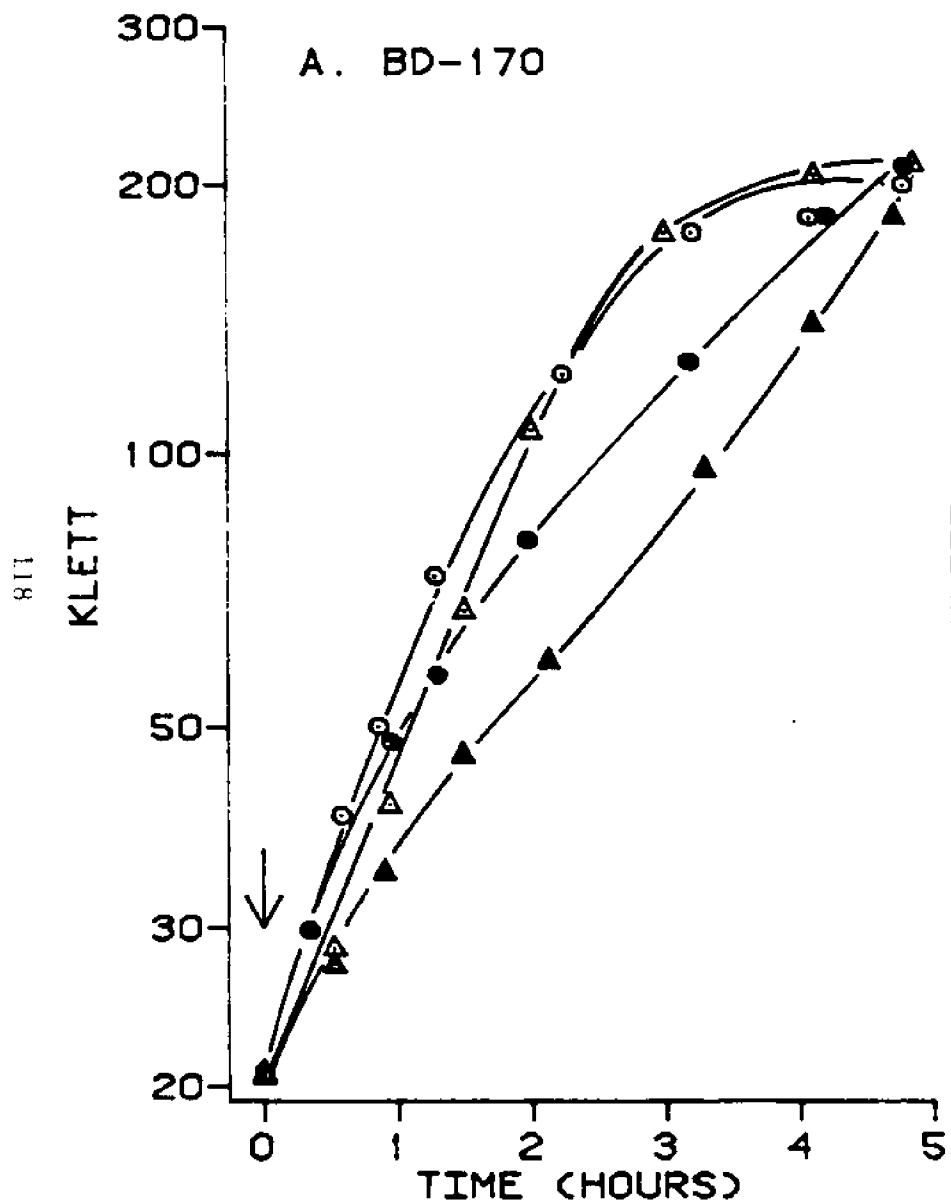


Fig. 17. The effect of additions to the medium on the DHBP inhibition of *B. subtilis*. As described in Materials and Methods, cells were cultured in casein hydrolysate broth augmented with 1 mM glycerol, (A) and (B), or 10 mM glycerol, (C) and (D). (A) and (C), Strain BD-170. (B) and (D), Strain 1005. At the time indicated by the arrow, DHBP was added to a final concentration of 0.2 mM. Symbols:  ,  , supplemented cultures and  ,  , unsupplemented cultures. Closed symbols are DHBP treated cultures. Open symbols represent control cultures treated with LiCl.



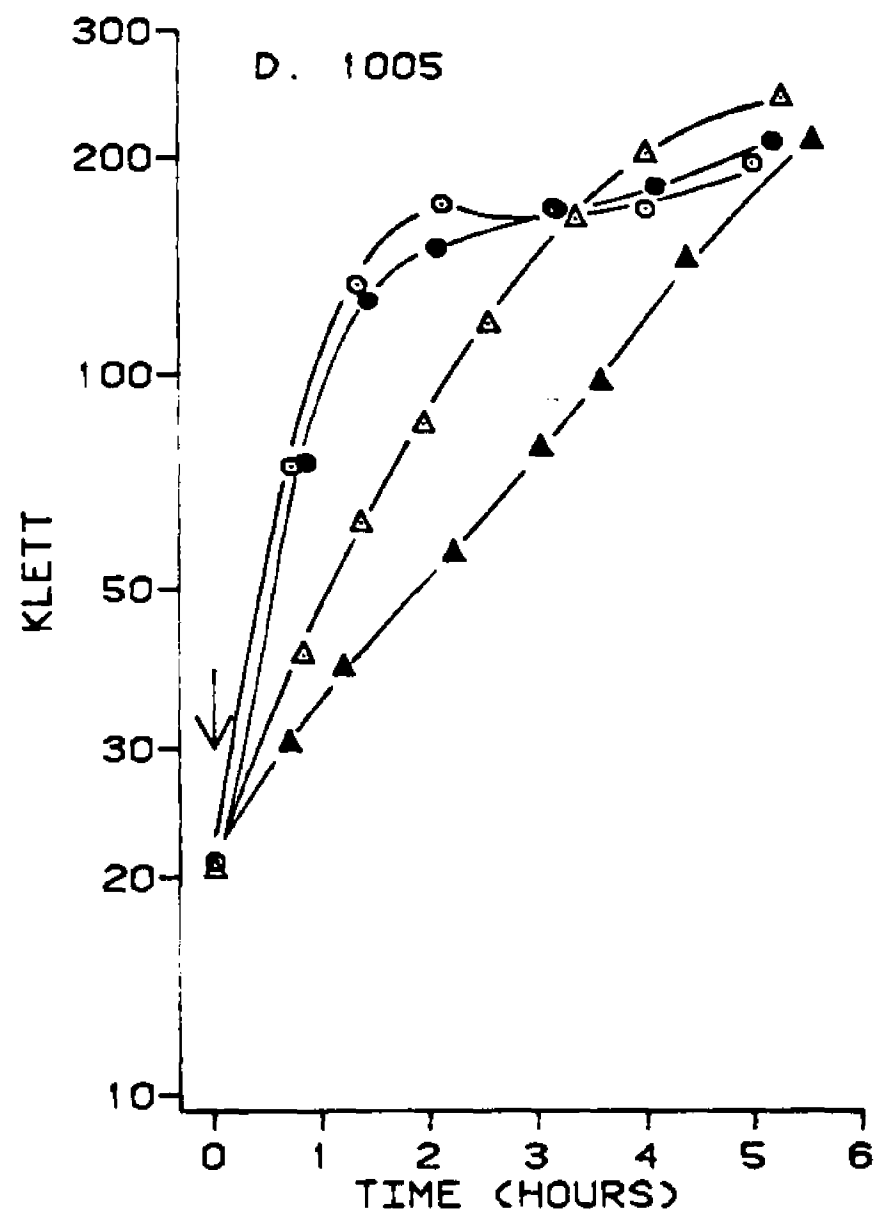
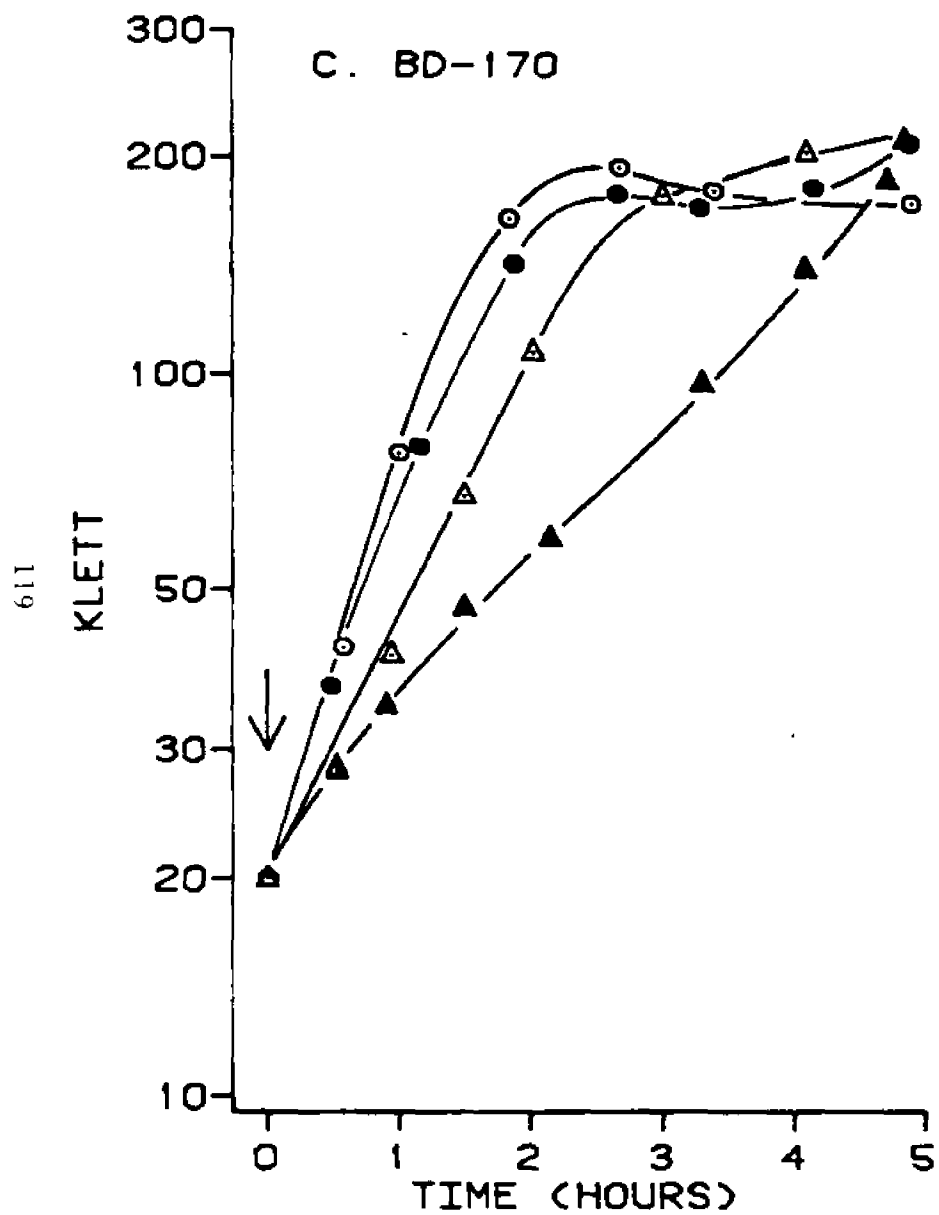


Fig. 18. The effect of additions to the medium on the DHBP inhibition of *B. subtilis*. As described in Materials and Methods, cells were cultured in casein hydrolysate broth augmented with 0.5% (28 mM) glucose. DHBP was added to a final concentration of 0.2 mM at the time indicated by the arrow. (A), Strain BD-170. (B), Strain 1005. Symbols: $\odot$, $\bullet$, supplemented cultures and $\triangle$, $\blacktriangle$, unsupplemented cultures. Closed symbols are DHBP treated cultures. Open symbols represent control cultures treated with LiCl.

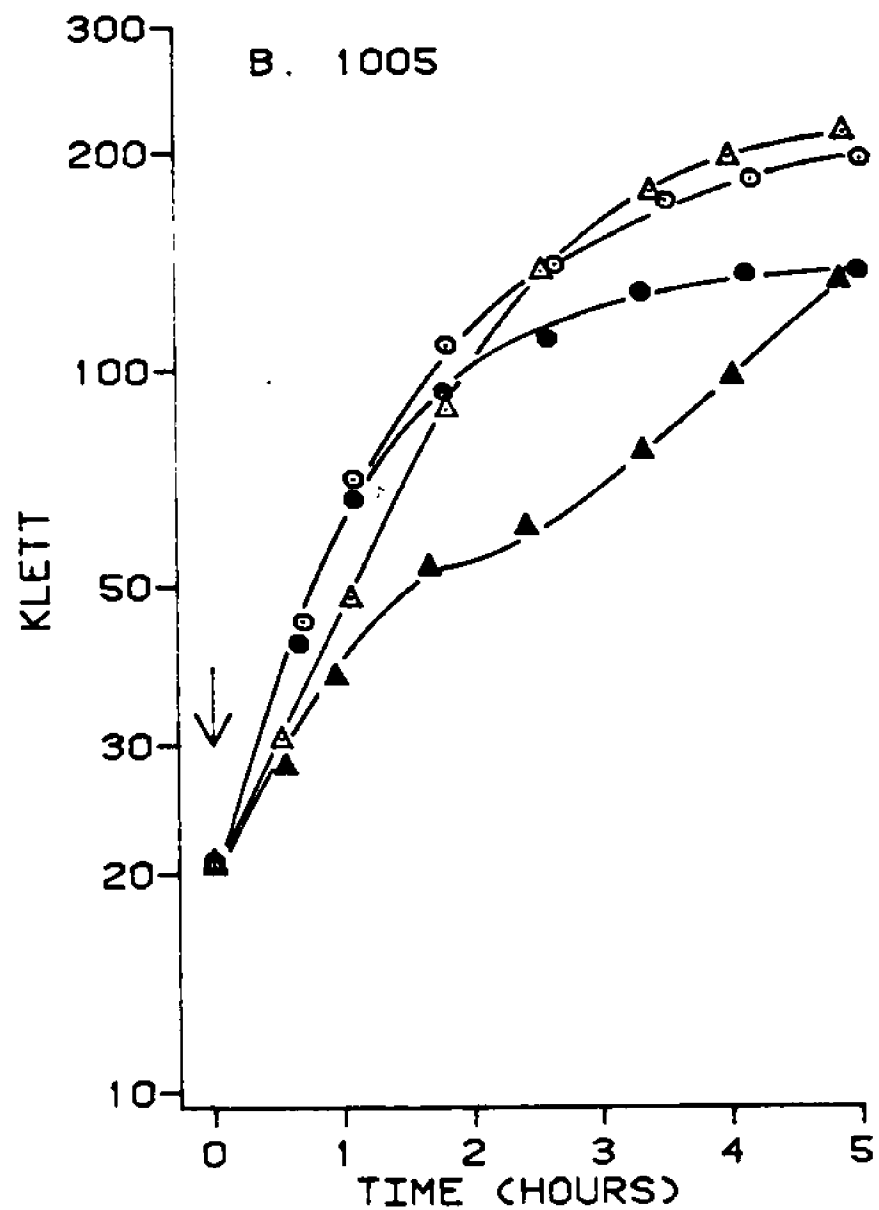
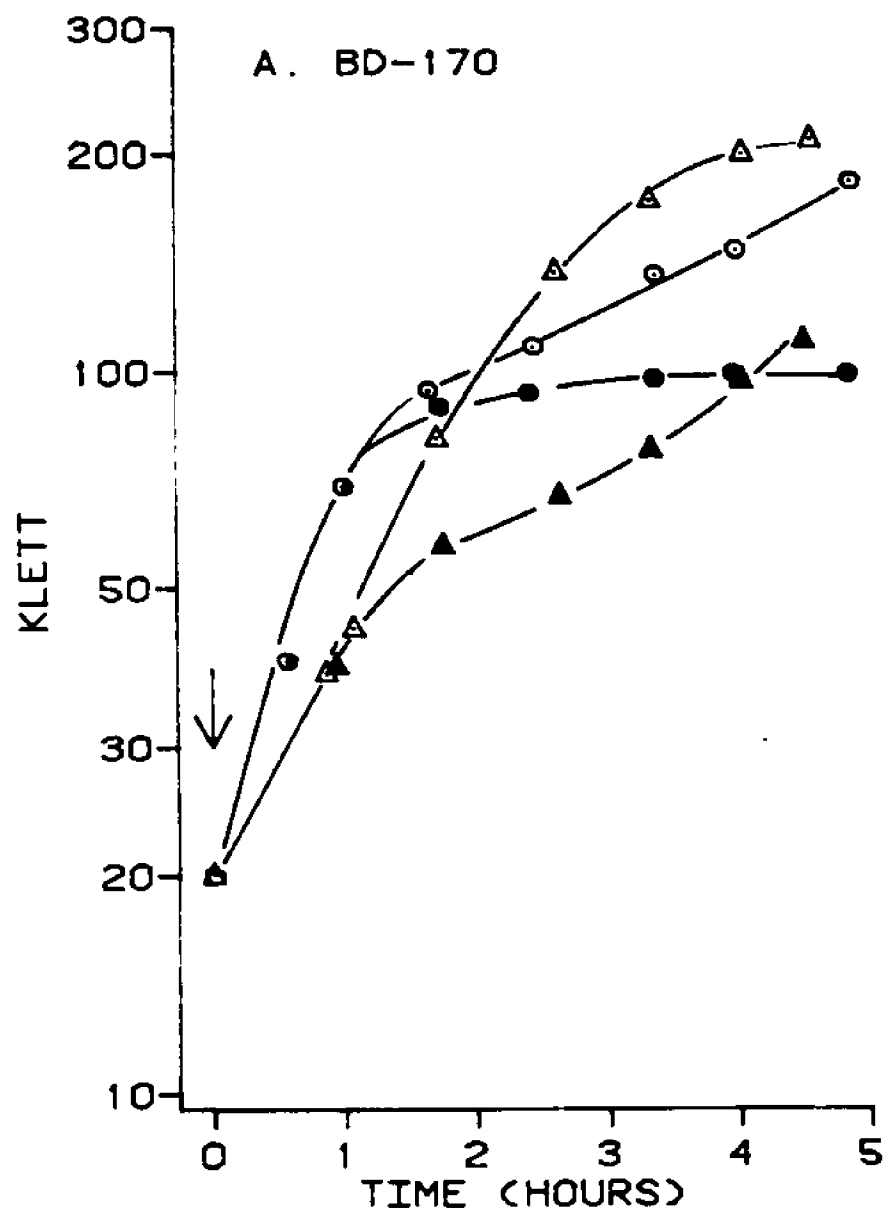


Fig. 19. The effect of glyceraldehyde, glyceraldehyde 3-phosphate, and 3-hydroxy-4-oxobutyl-1-phosphonate on the growth of *B. subtilis*. Cells were cultured as described in Materials and Methods. (A) Strain BD-170. (B) Strain 1005. At the time indicated by the arrow, additions were made to the following final concentrations: Δ , 1.3 mM D-glyceraldehyde, $\odot$, 1.1 mM rac-3-hydroxy-4-oxobutyl-1-phosphonate, $\square$, 1.3 mM L-glyceraldehyde, $\bullet$, 1.1 mM rac-glyceraldehyde 3-phosphate, and $\diamond$, water.

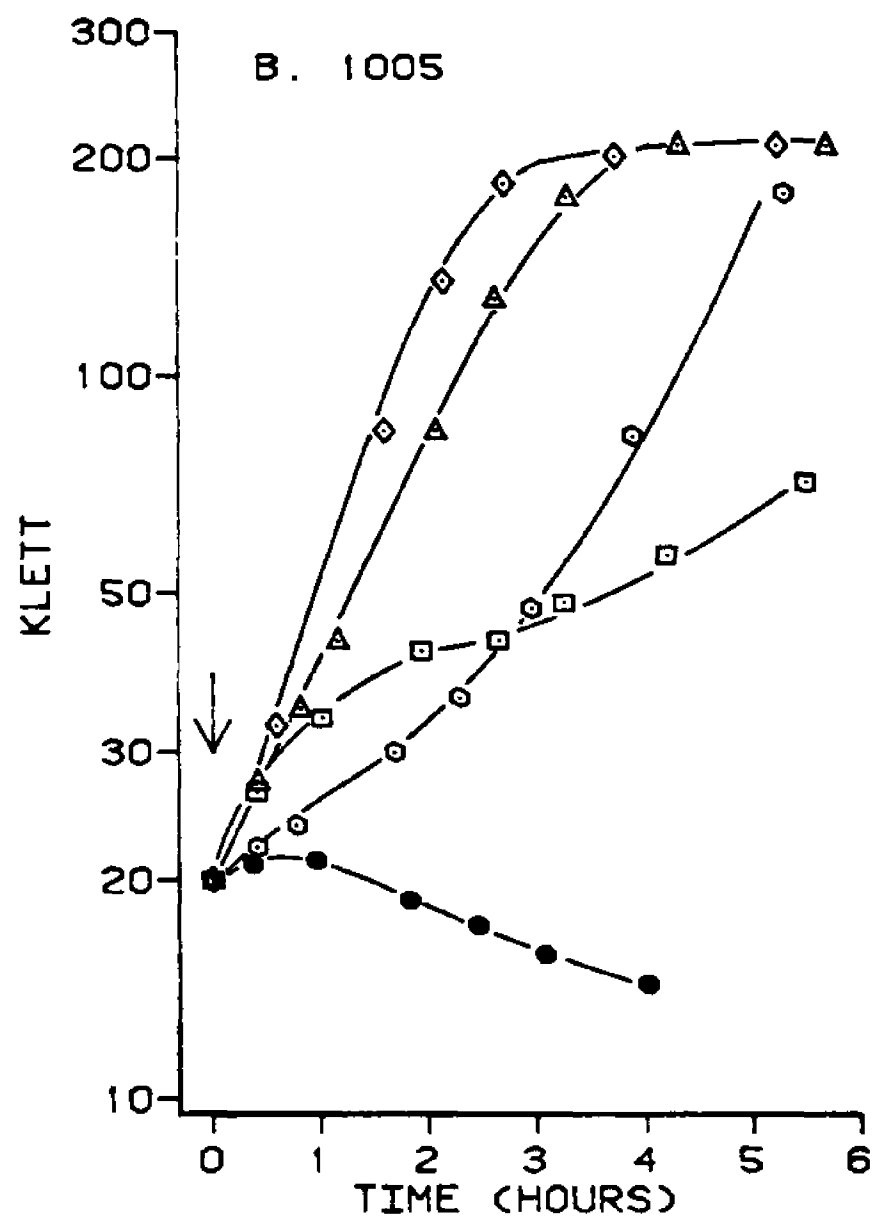
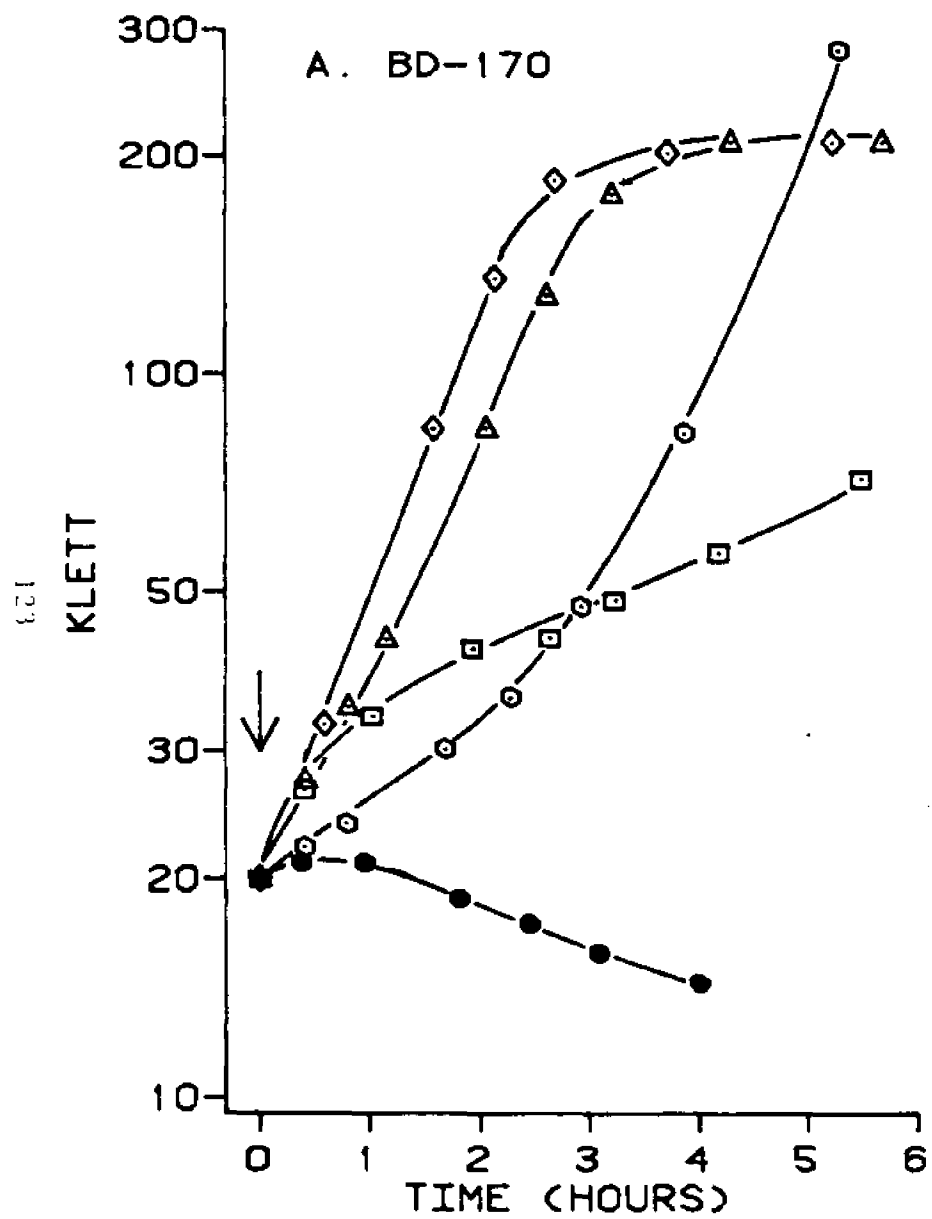


Fig. 20. Fractionation of [^3H]-DHBP-labeled phospholipids. Lipids radioactively labeled in vivo with [^3H]-DHBP were chromatographed on a 1 x 30 cm DEAF-cellulose column as described in Materials and Methods. The radioactivity of each fraction was determined by counting a sample from each with toluene scintillation fluid (3) as described in Materials and Methods. (A) Strain BD-170. (B) Strain 1005. Symbols: Δ , [^3H]-cpm, NH_4Ac = ammonium acetate.

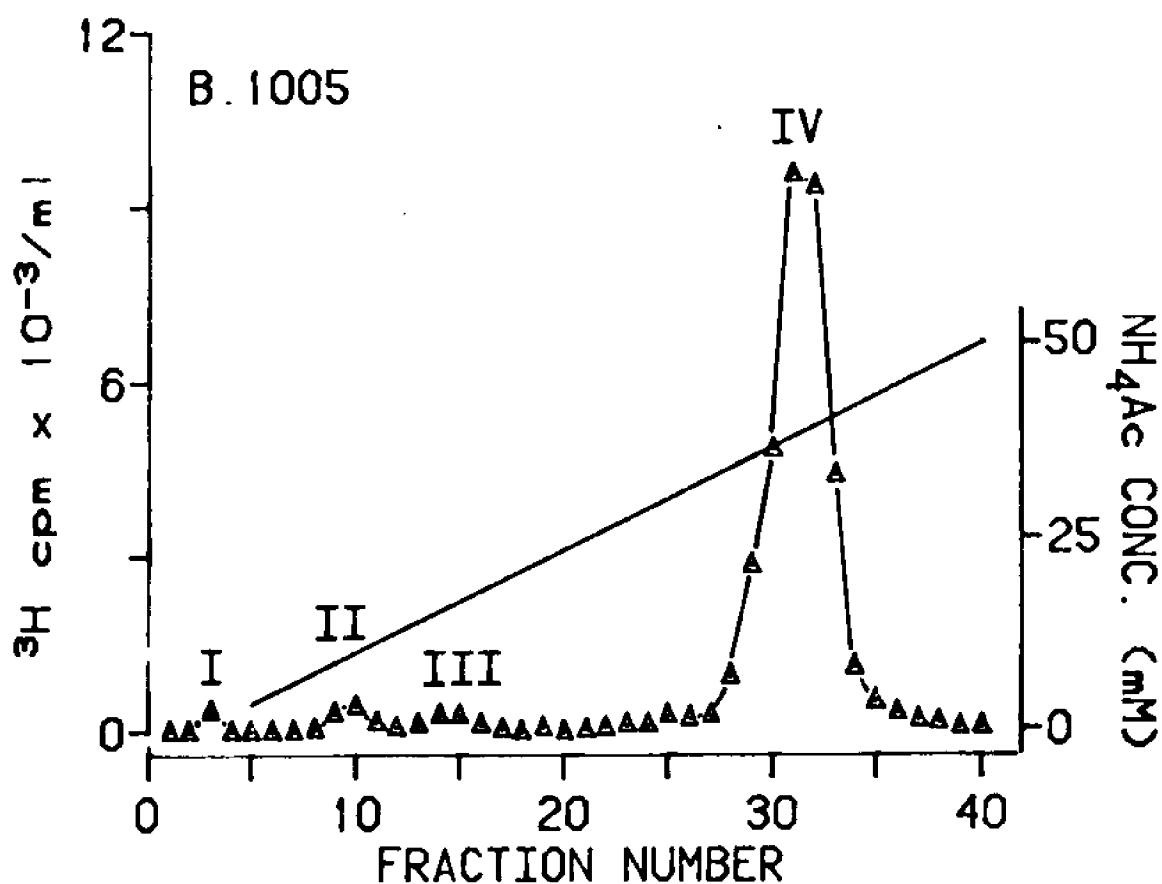
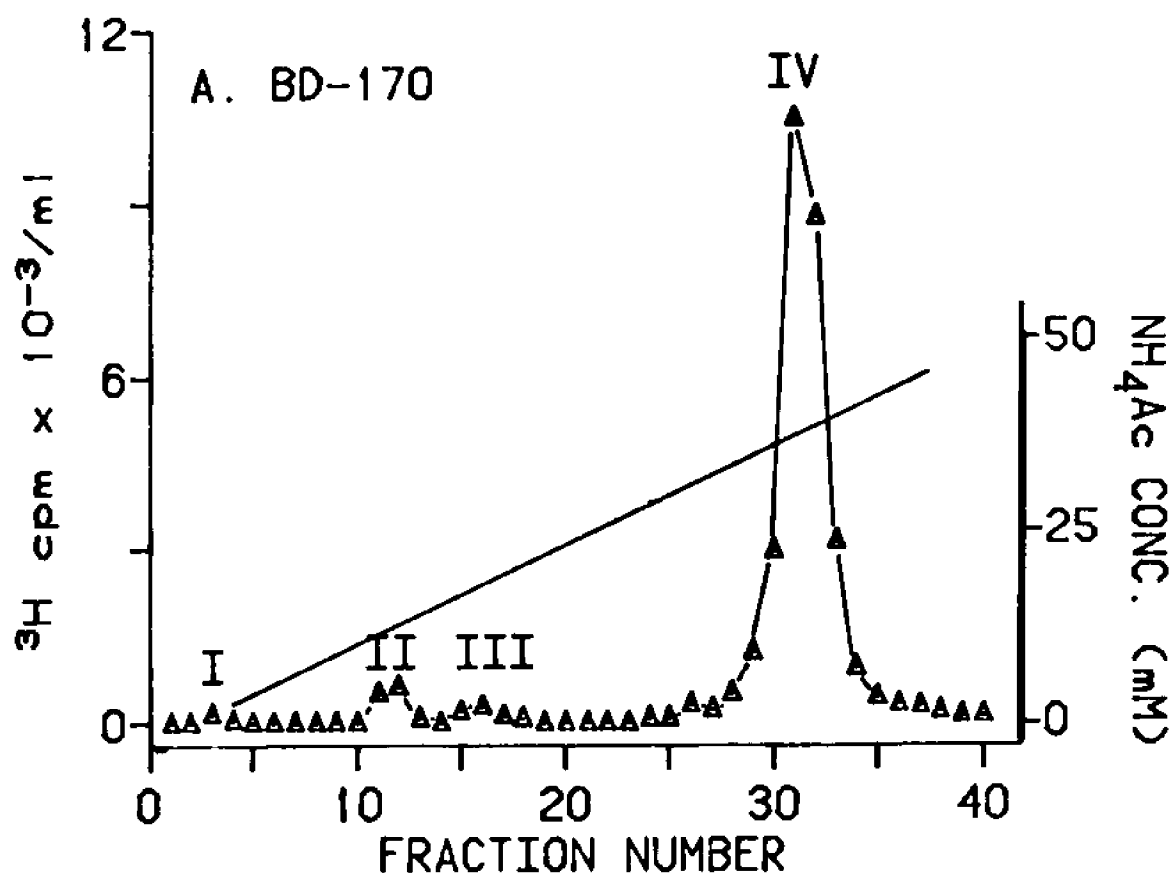


Fig. 21. Chromatographic characterization of water soluble phospholipase C hydrolysis products. A sample of fraction IV isolated as in Fig. 20 was treated with phospholipase C to obtain the water soluble products as described in Materials and Methods. The water soluble products were recovered using the method of Bligh and Dyer (100). A portion of the material was chromatographed on CEL 300 PEI thin layer sheets before (A) and after alkaline phosphatase treatment, (B), using 1 M LiCl:1 M formic acid (1:1) as the solvent system. After development, each lane was cut into 1 cm sections beginning 0.5 cm below the origin, and each section counted in 0.1 N HCl and 10 ml of Patterson-Greene scintillation fluid (101). DHBP has an Rf of 0.80. The literature gives an Rf value of 0.4 for 4-phospho-3-hydroxybutyl-1-phosphonate (3).

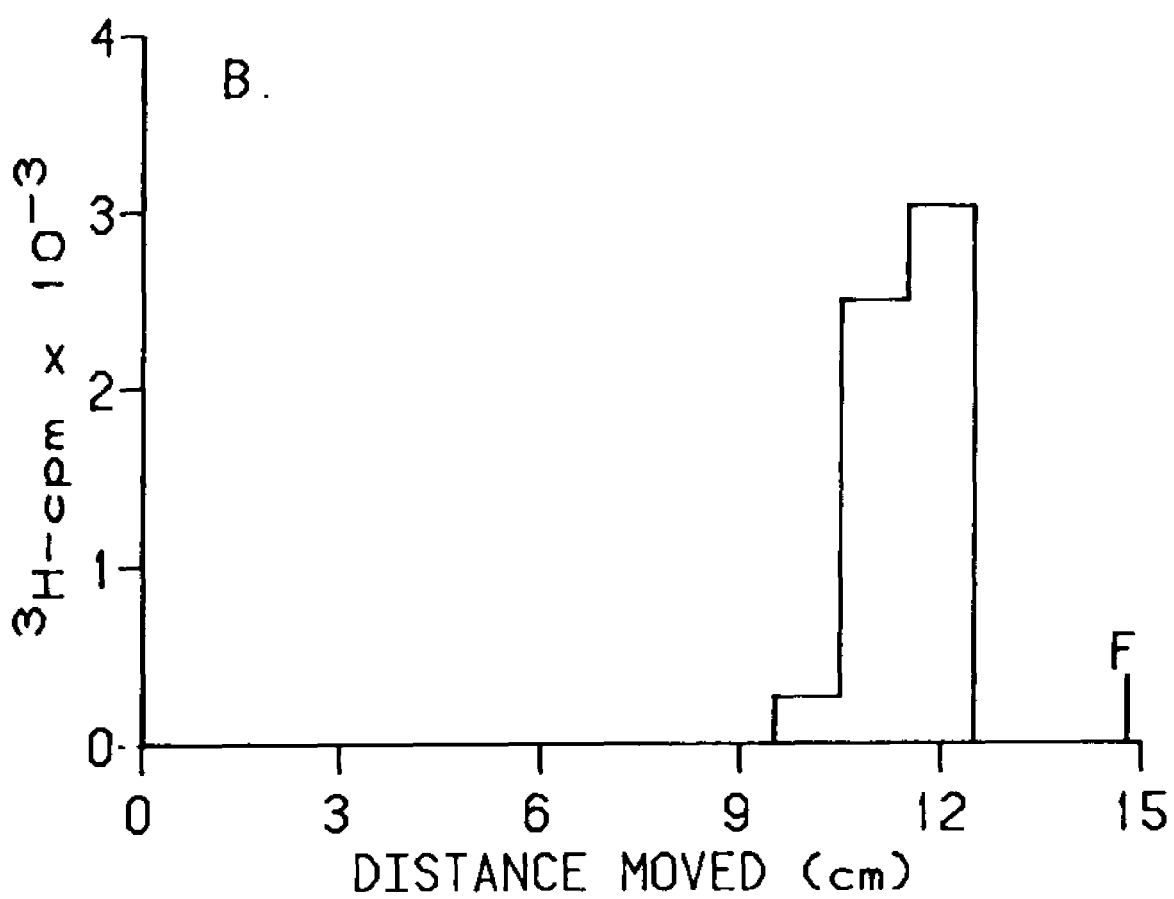
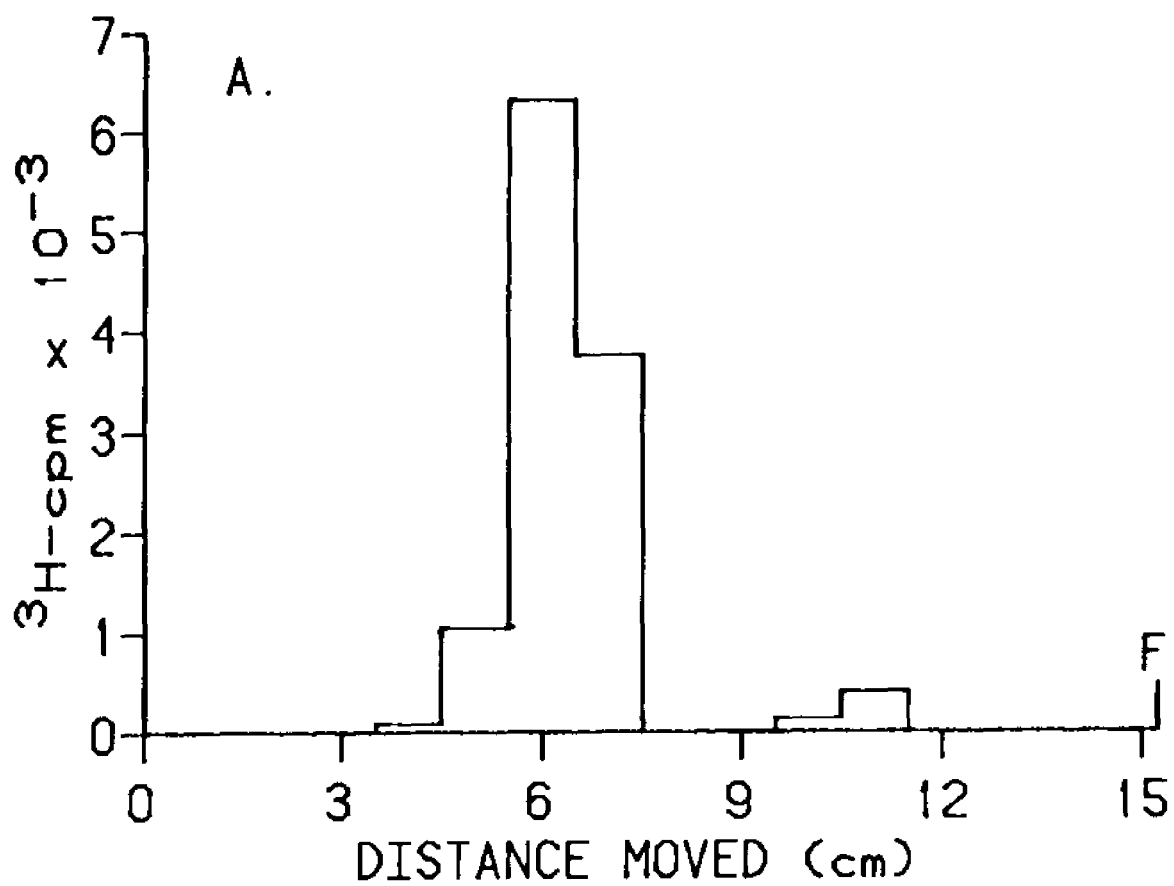
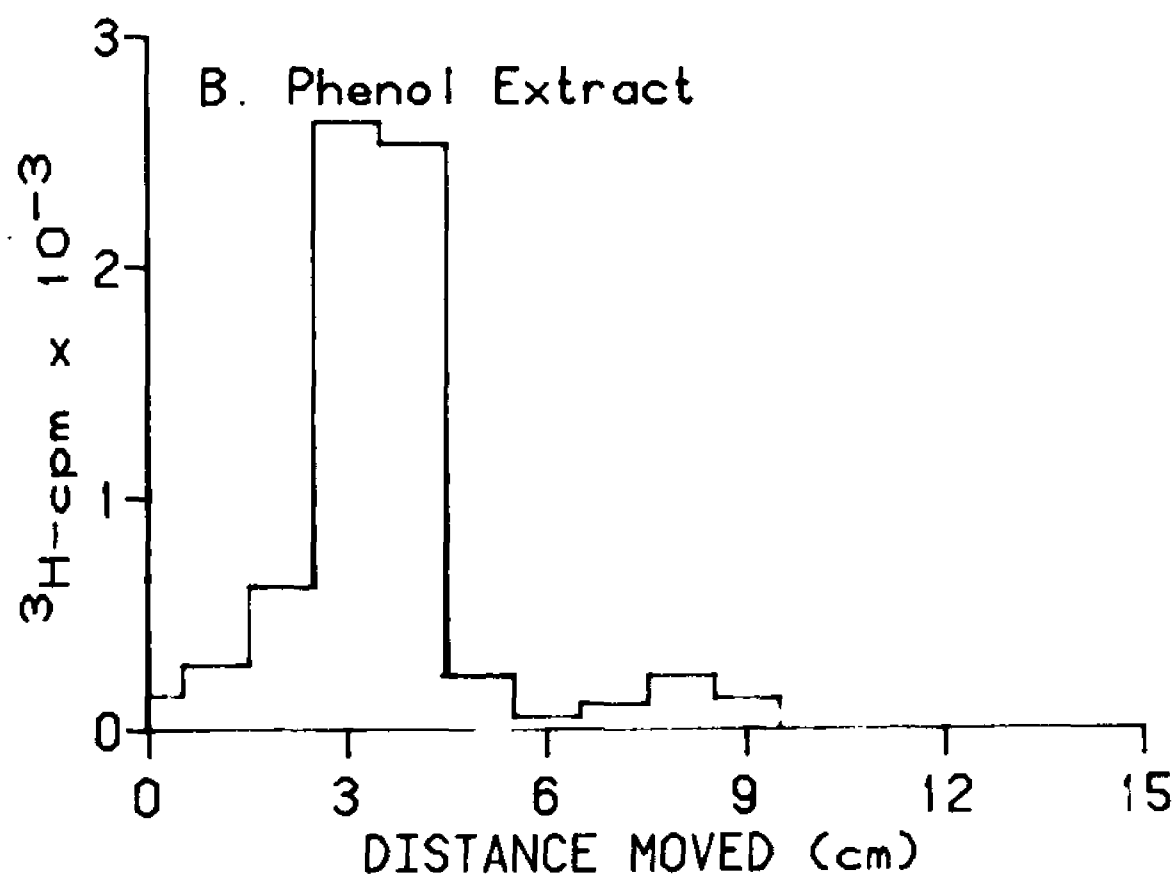
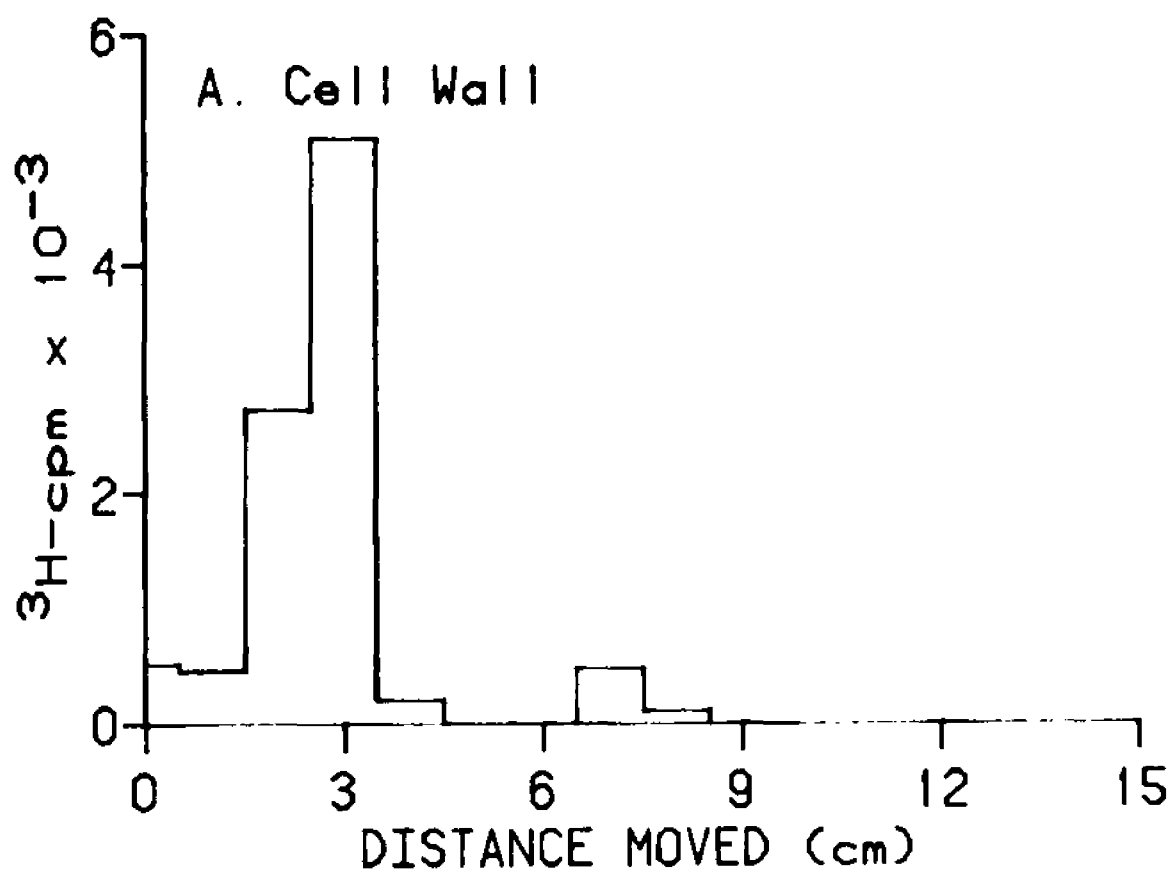


Fig. 22. Chromatographic characterization of acid hydrolysates of [^3H]-DHBP-labeled cell walls and phenol extracts. Walls labeled ~~in vivo~~ with [^3H]-DHBP were completely hydrolyzed with HCl as described in Materials and Methods. The products were chromatographed on Whatman 1MM paper with n-propanol:conc. ammonia:water (6:3:1) as the solvent. Glycerol and DHBP migrate with Rf values of 0.60 and 0.22 respectively. The unidentified component in the hydrolysate had an Rf value of 0.51. (A) Cell wall hydrolysates. (B) Phenol extract hydrolysates. (C) Untreated DHBP. (D) DHBP which had been subjected to the same hydrolytic treatment as the cell walls and phenol extract preparations.



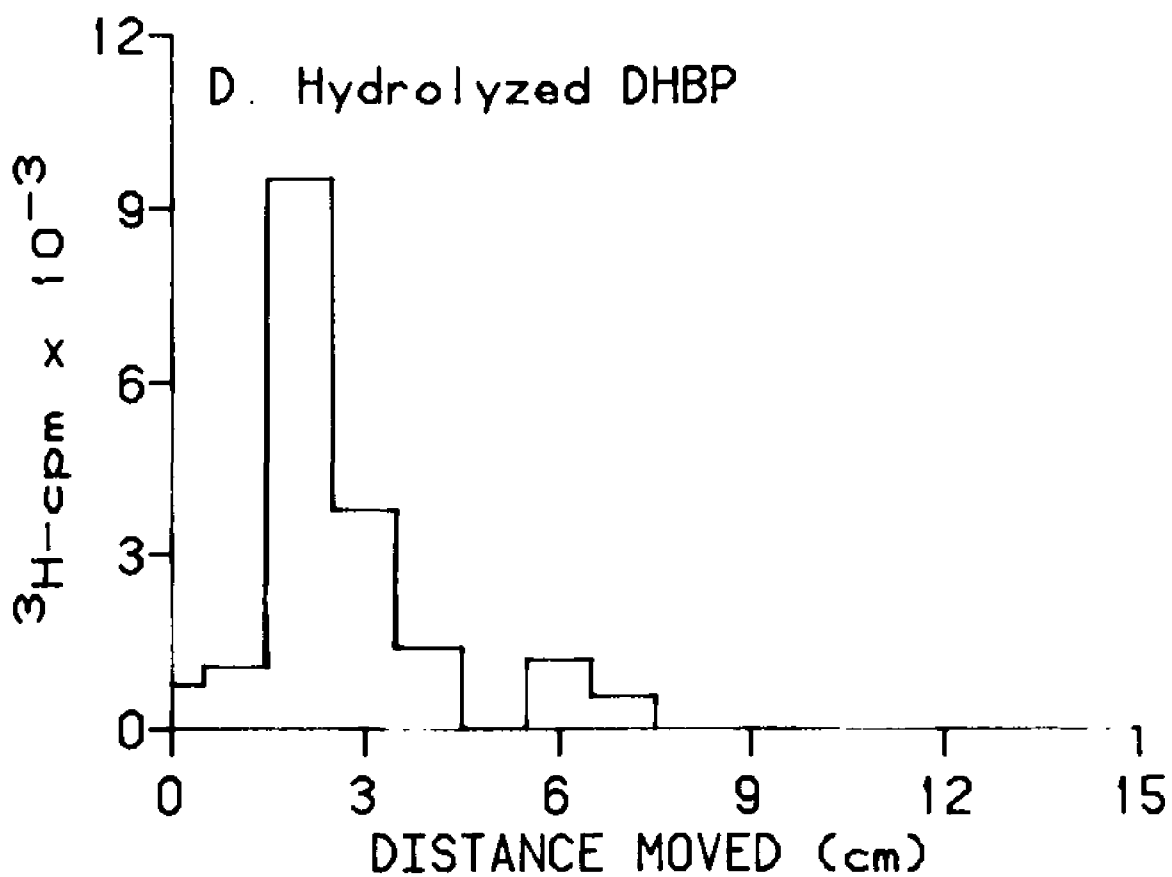
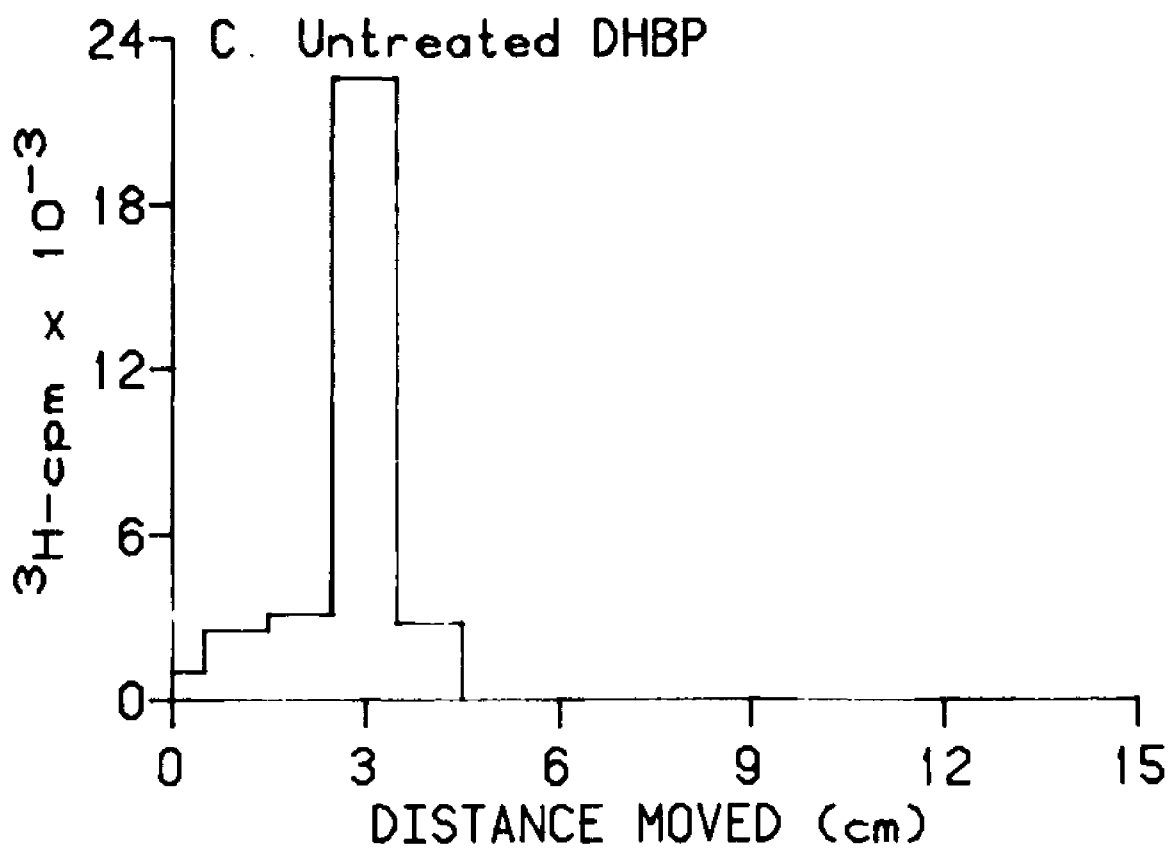


Fig. 23. Structure of 3-hydroxypropyl-1-phosphonate.

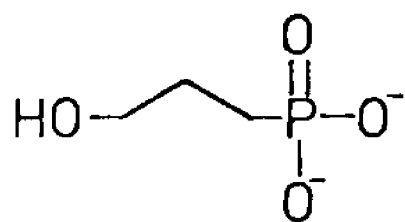


Fig. 24. Chromatographic characterization of the products formed by CDP-glycerol pyrophosphorylase using [^3H]-DHPP as a substrate. 20 mM ~~rac~~-[^3H]-DHBP (1 $\mu\text{Ci}/\mu\text{mole}$) was used as a substrate for a dialyzed preparation of CDP-glycerol pyrophosphorylase extracted from strain 42 (23 μg) in the standard enzyme assay as described in Materials and Methods. Products were desorbed from charcoal into ammoniacal ethanol, the solvent removed, and the products chromatographed on Whatman 3MM paper using 95% ethanol:0.575 ammonium acetate, pH 3.8 (79:29) as the developing solvent (A) without further treatment or (B) after HCl hydrolysis. The component of the untreated sample having the mobility of CMP-DHBP was eluted, hydrolyzed with HCl, and rechromatographed. The chromatogram of the acid hydrolyzed CMP-DHBP was identical to (E).

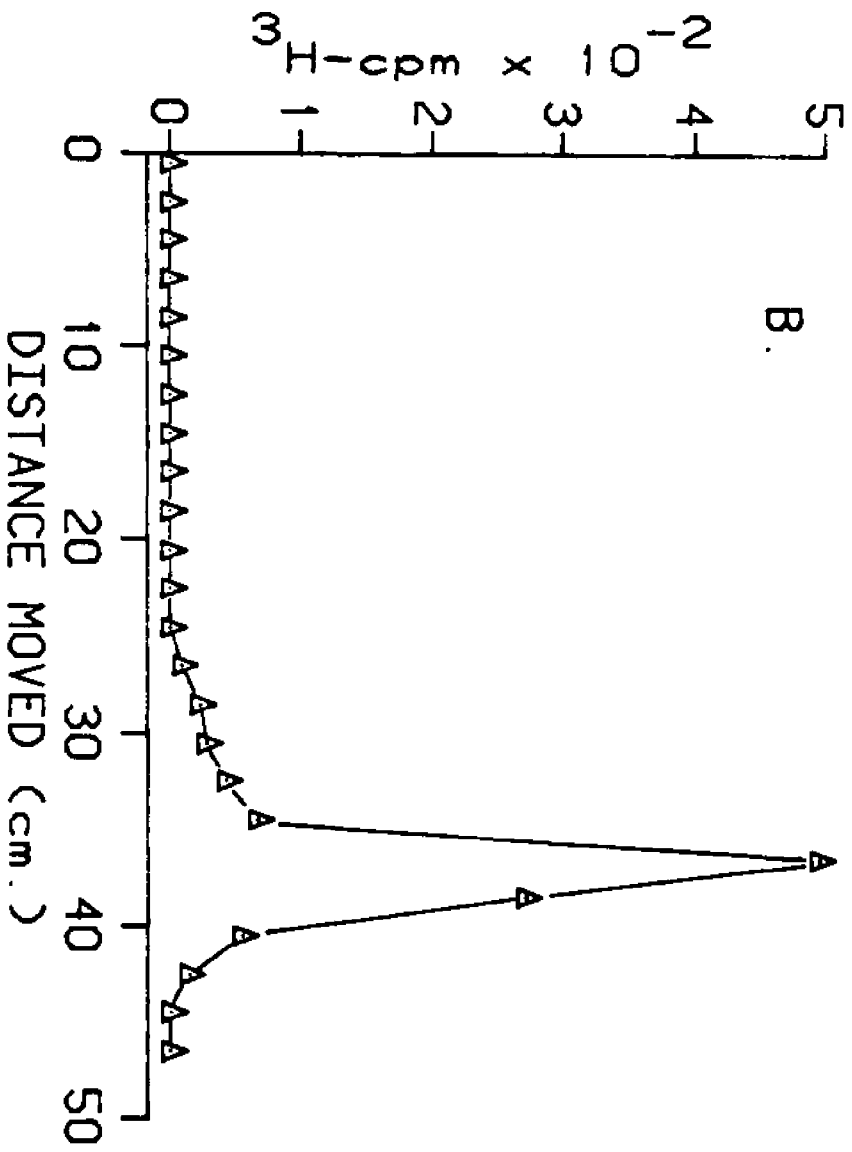
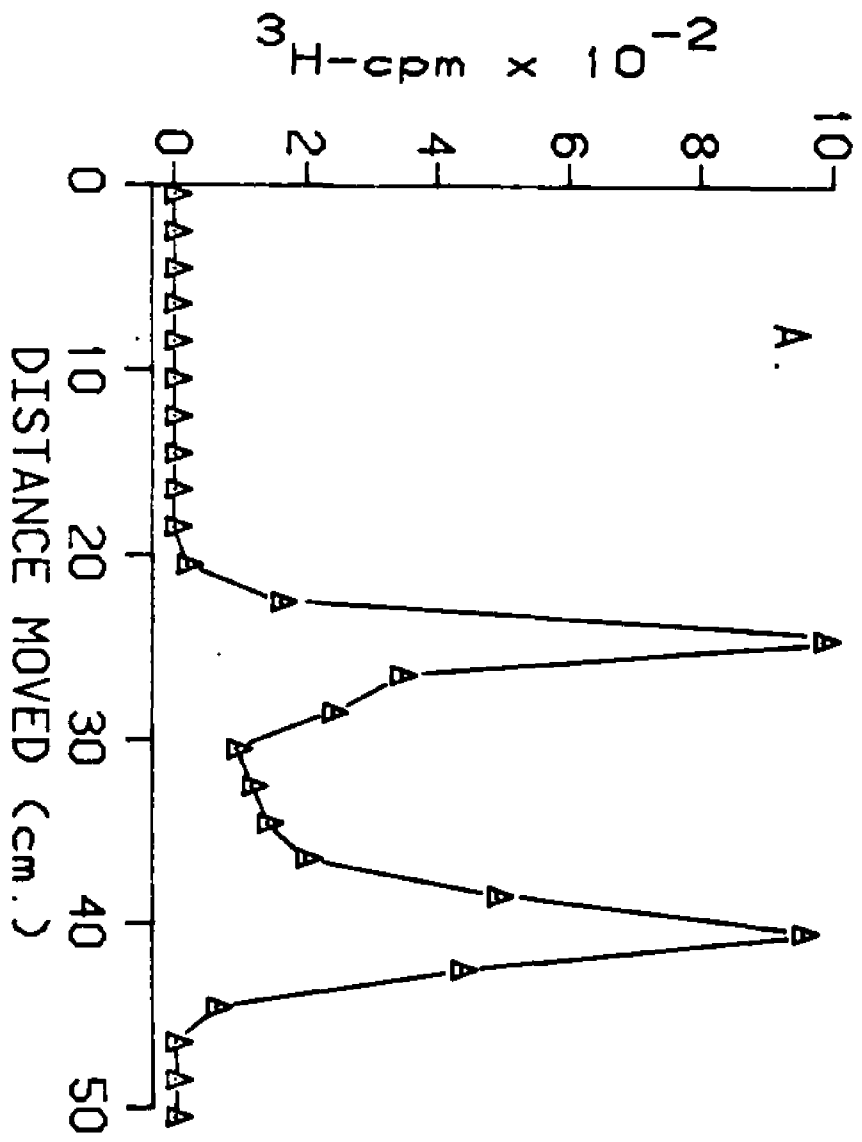
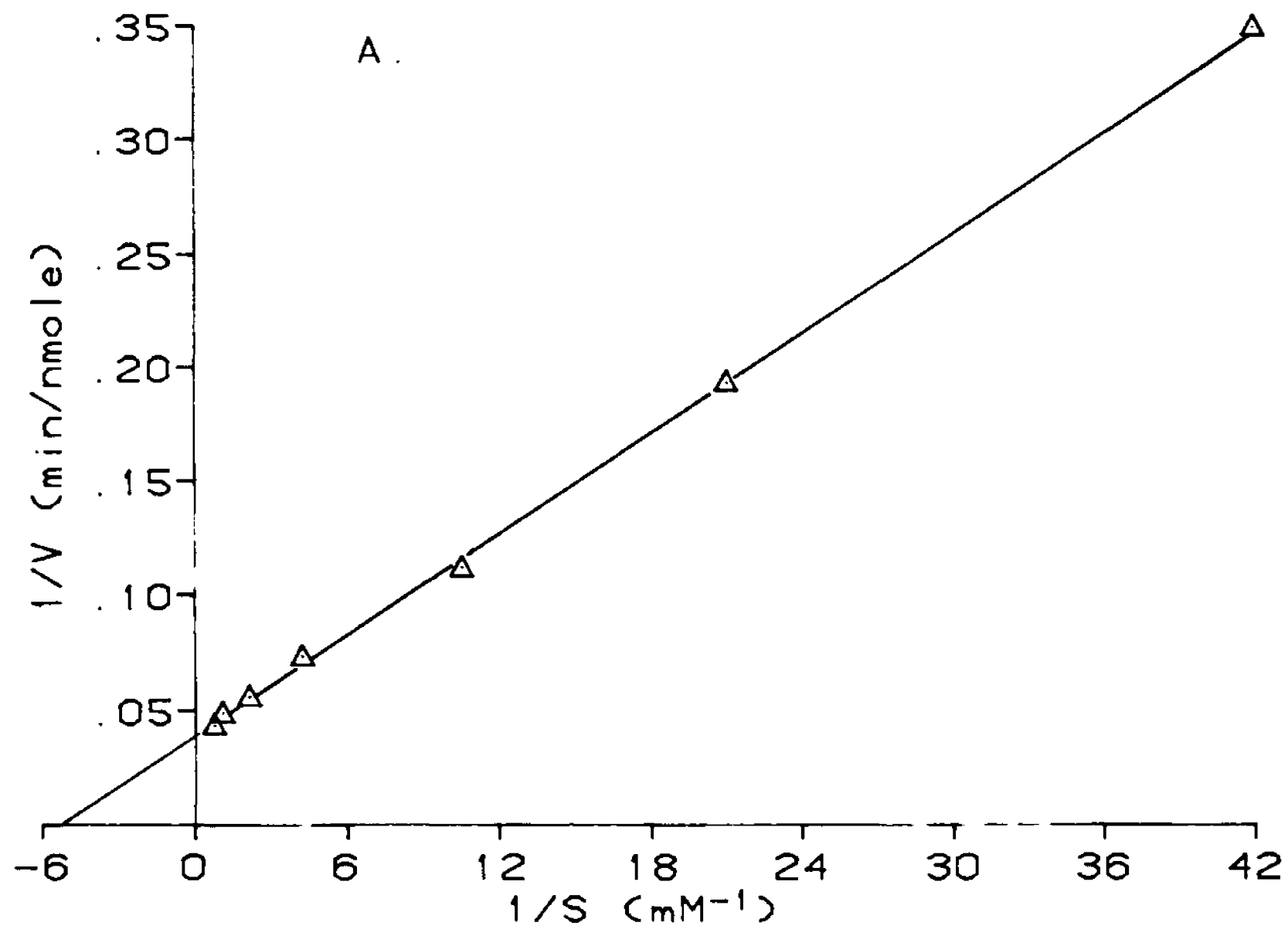
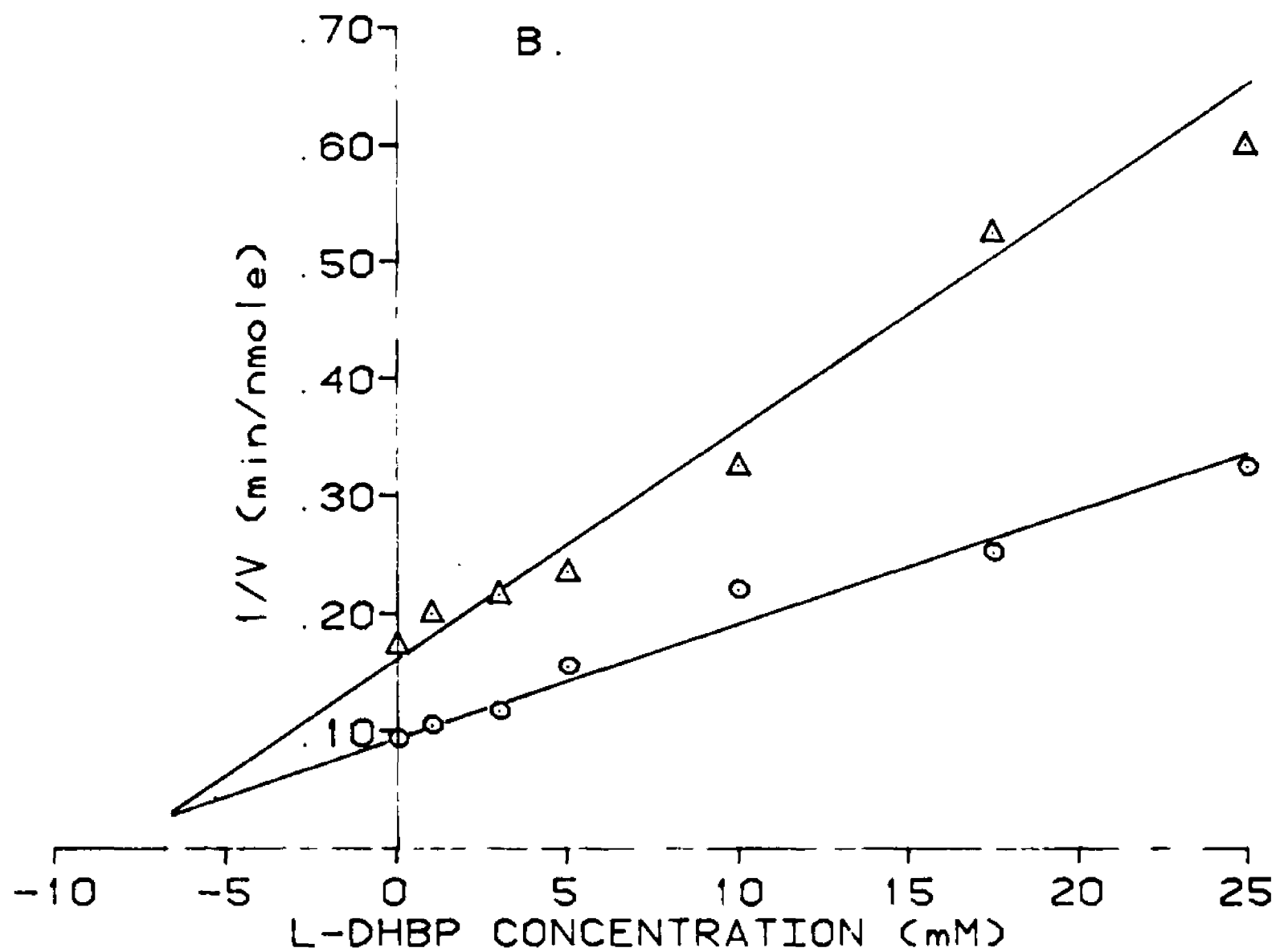
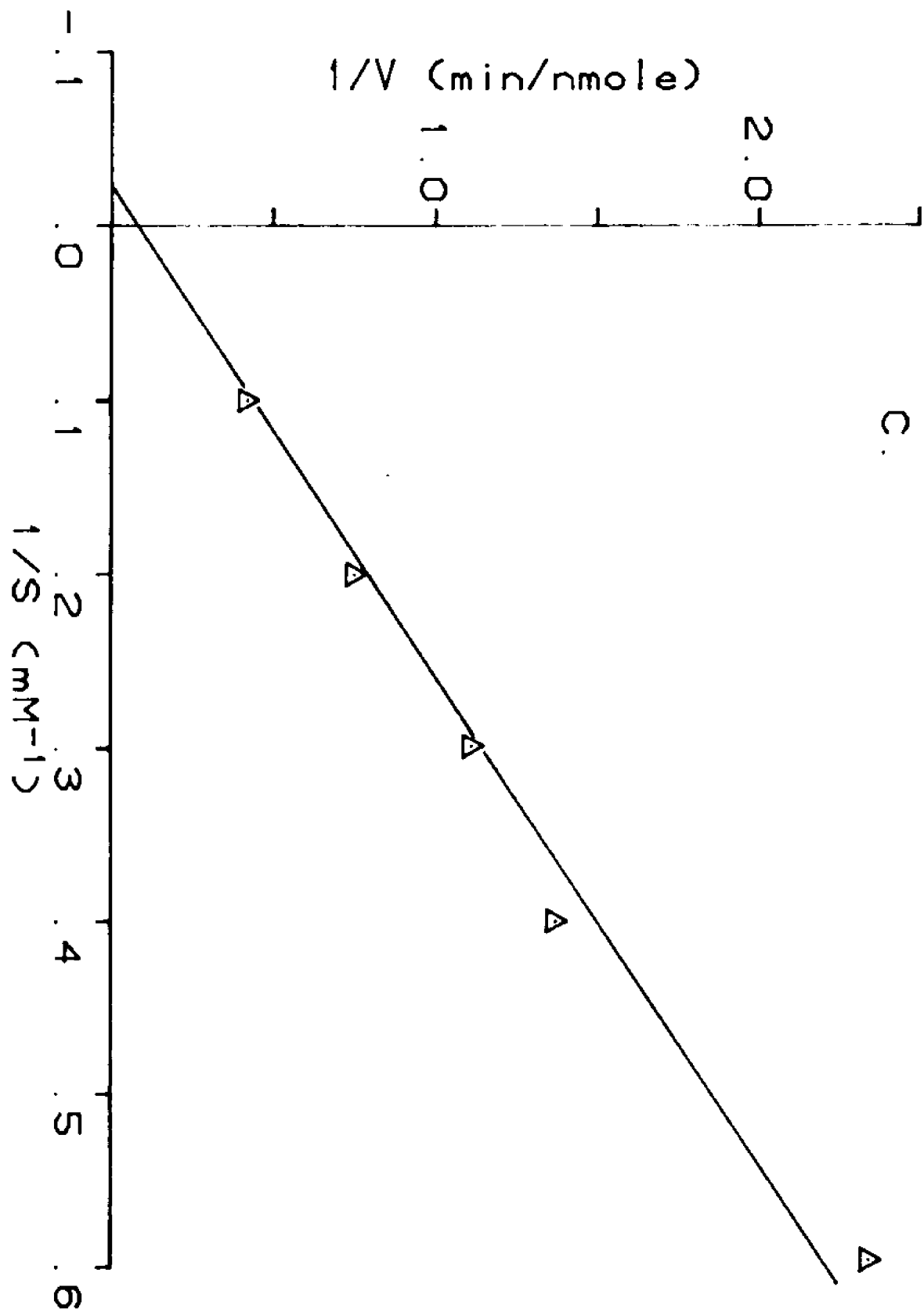
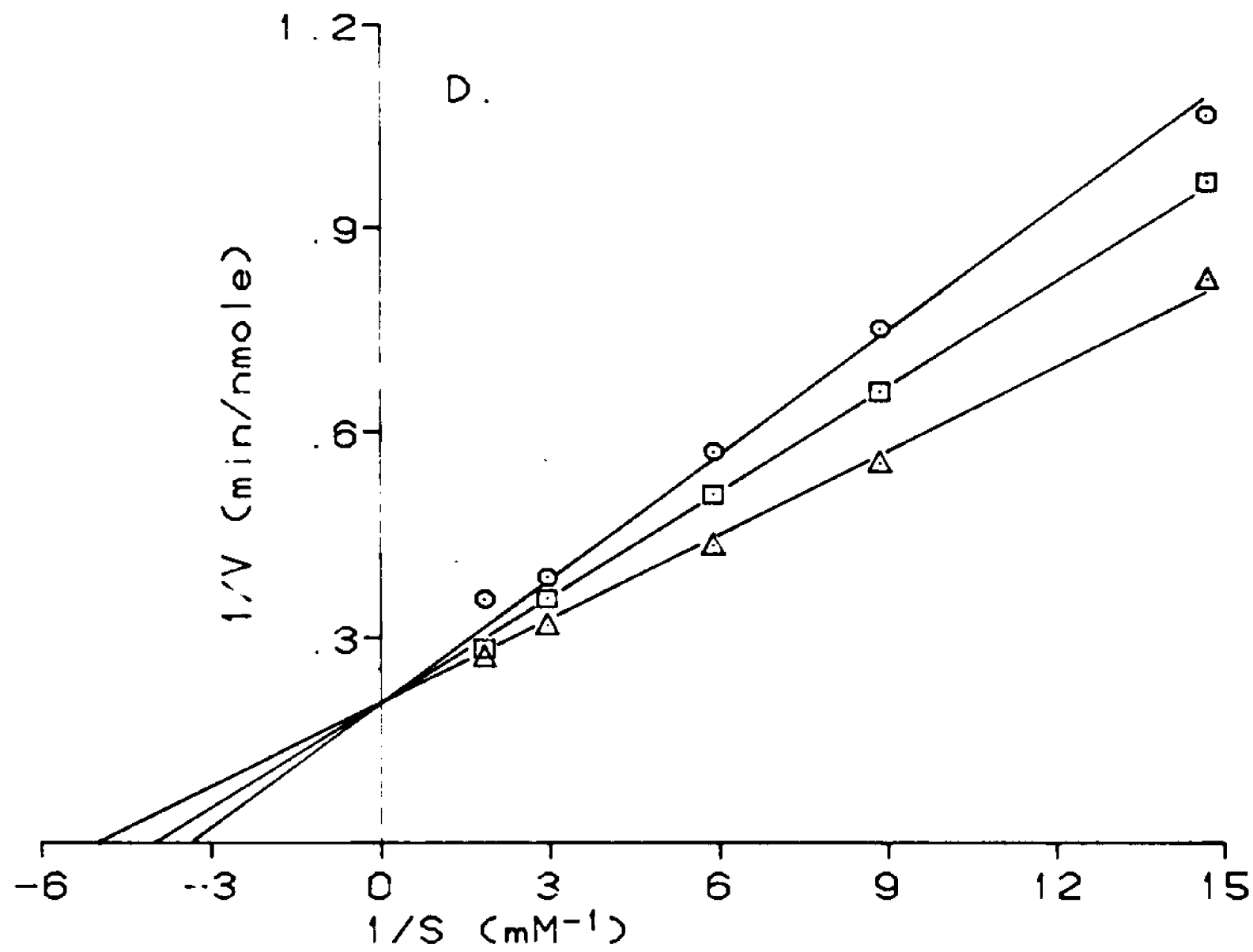


Fig. 25. Kinetic data for CDP-glycerol pyrophosphorylase. The assay is based on the conversion of [^{14}C]-glycerol 3-phosphate or [^3H]-DHBP into a charcoal absorbable form. Details are presented in Materials and Methods. (A), (B), and (C) are dialyzed enzyme preparations from strain 42. (D) Gel-purified enzyme from strain BE-170. (A) Lineweaver-Burke plot for determining the K_m of glycerol 3-phosphate. Each assay contained [^{14}C]-glycerol 3-phosphate (1 $\mu\text{Ci}/\mu\text{mole}$) and 4.7 mg of enzyme. (B), Dixon plot (116) for determining the K_i of DHBP. Each assay contained 4.7 mg of enzyme. Symbols: Δ , 47.6 μM (1.5 $\mu\text{Ci}/\mu\text{mole}$) or $\odot$, 93.2 $\mu\text{Ci}/\mu\text{M}$ (.75 $\mu\text{Ci}/\mu\text{mole}$) L-glycerol 3-phosphate. (C), Lineweaver-Burke plot for the K_m of DHBP. Each assay contained 5.7 mg of enzyme and DHPP (0.29 $\mu\text{Ci}/\mu\text{mole}$). (D), Lineweaver-Burke plot for determining the K_m of glycerol 3-phosphate and K_i of DHBP. Each assay contained L-glycerol 3-phosphate (0.5 $\mu\text{Ci}/\mu\text{mole}$) in the absence of DHBP, Δ , or the presence of 2.5 mM Σ -DHBP, $\square$, or 5.0 mM Σ -DHBP, $\odot$.









Bibliography

1. Shopsis, C.S., Engel, R., and Tropp, B.E., J. Bact., 112, 408 (1972).
2. Leiffer, S., Engel, R., and Tropp, B.E., J. Bact. 130, 968 (1977).
3. Tyhach, R.J., Engel, R., Tropp, B.E., J. Biol. Chem., 251, 6717 (1976).
4. Shopsis, C.S., Nunn, W.D., Tyhach, R.J., Goldstein, S.L., Engel, R., and Tropp, B.E., Antimicrob. Agents Chemother., 4, 467 (1973).
5. Shopsis, C.S., Engel, and Tropp, B.E., J. Biol. Chem., 249, 2473 (1974).
6. Cheng, P-J, Nunn, W.D., Tyhach, R.J., Goldstein, S.L., Engel, P., and Tropp, B.E., J. Biol. Chem., 250, 1633 (1975).
7. Tang, C-T, Engel, P., and Tropp, B.E., Antimicrob. Agents Chemother., 11, 147 (1977).
8. Tang, C-T, Ph. D. Dissertation, Queens College (CUNY), (1978).
9. Cozzarelli, N.R., Koch, J.P., Hayashi, S., and Lin, E.C.C., J. Bact., 90, 1325 (1965).
10. Trentham, D.R., McMurray, C.H., and Pogson, C.I., Biochem. J., 114, 19 (1969).
11. Sharon, N., Complex Carbohydrates, Addison-Wesley Pub. Co., Reading, Mass (1975).
12. Mindich, L., J. Mol. Biol., 42, 415 (1970).
13. Ellwood, D.C. and Tempest, D.W., Adv. Microbiol. Physiol., 7, 83 (1972).
14. Op den Kamp, J.A.F., Redai, I., van Deenan, L.L.M., J. Bact., 99, 298 (1969).
15. Bishop, D.G., Putberg, L., Samuelsson, B., Eur. J. Biochem., 2, 443 (1967).
16. Fischer, W., Laine, P.A., Nakano, M., Biochim. Biophys. Acta., 528, 298 (1978).
17. Lindgren, V. and Putberg, L., J. Bact., 127, 1047

(1976).

18. Cozzarelli, N.R., Freedberg, W.B., and Lin, E.C.C.,
J. Mol. Biol., 31, 371 (1968).
19. Krag, S.S., Robinson, M.D., and Lennarz, W.J.,
Biochim. Biophys. Acta., 337, 71 (1974).
20. Chang, Y.Y. and Kennedy, E.P., J. Lipid Res., 8, 457
(1967).
21. Raetz, C.R.H., Microbiol. Rev., 48, 614 (1978).
22. Armstrong, J.J., Baddiley, J., Buchanan, J.G., Carss,
B., and Greenberg, J., Chem. Soc., 4344 (1958).
23. Baddiley, J., Essays in Biochem., 8, 35 (1972).
24. Wicken, A.J. and Knox, K.W., J. Gen. Microbiol., 60,
293 (1970).
25. Knox, K.W. and Wicken, A.J., Act. Rev., 37, 215
(1973).
26. Archibald, F.J., Adv. Microbiol. Physiol., 11, 53
(1974).
27. Wicken, A.J. and Knox, K.W., Science, 187, 1161
(1975).
28. Van Driel, I., Wicken, A.J., Dickson, M.P., and Knox,
K.W., Ultrastruct. Res., 43, 483 (1973).
29. Fiedler, F. and Glaser, L., J. Biol. Chem., 249, 2684
(1974).
30. Fiedler, F. and Glaser, L., J. Biol. Chem., 249, 5349
(1974).
31. Bracha, P., Chang, M., Fiedler, F., and Glaser, L.,
Methods in Enz., 50, 387 (1978).
32. Burger, M.M., Proc. Nat. Acad. Sci. (USA), 56, 91
(1966).
33. Chin, I., Burger, M.M., and Glaser, Arch. Biochem.
Biophys., 116, 358 (1966).
34. Fischer, W., Koch, H.U., Rosel, P., Fiedler, F., and
Schmuck, L., J. Biol. Chem., 255, 4350 (1980).
35. Fischer, W., Koch, H.U., Rosel, P., and Fiedler, F.,
J. Biol. Chem., 255, 4557 (1980).

36. Lambert, P.F., Coley, J., and Baddiley, J., FEBS Lett., 79, 327 (1977).
37. Mancuso, D.J., Junker, D.D., Hsu, S.C., and Chiu, T-H, J. Bact., 140, 547 (1979).
38. Glaser, L., Proc. Nat. Acad. Sci. (USA), 69, 2386 (1972).
39. Emdur, L.I. and Chiu, T-H, Biochem. Biophys. Res. Comm., 59, 1137 (1974).
40. Glaser, L. and Lindsay, B., Biochem. Biophys. Res. Comm., 59, 1131 (1974).
41. Emdur, L.I. and Chiu, T-H, FEBS Lett., 55, 216 (1975).
42. Ganfield, M-C.W. and Pieringer, R.A., J. Biol. Chem., 255, 1521 (1980).
43. Laine, P.A. and Fisher, W., Biochim. Biophys. Acta., 529, 250 (1978).
44. Duckworth, M., Archibald, A.R., and Baddiley, J., FEBS Lett., 53, 176 (1975).
45. Sastry, P.S., Adv. Lipid Res., 12, 251 (1974).
46. Button, D. and Hemmings, N.L., J. Bact., 128, 149 (1976).
47. Deutsch, R.M., Engel, R., and Tropp, B.E., J. Biol. Chem., 255, 1521 (1980).
48. Fiedler, F. and Glaser, L., Carbo. Res., 37, 37 (1974).
49. Mauk, J. and Glaser, L., Proc. Nat. Acad. Sci. (USA), 69, 2386 (1972).
50. Fiedler, F. and Glaser, L., J. Biol. Chem., 249, 269 (1974).
51. Glaser, L., J. Biol. Chem., 239, 3168 (1964).
52. Ishimoto, N. and Strominger, J.L., J. Biol. Chem., 241, 639 (1966).
53. Kennedy, L.D. and Shaw, D.R.D., Biochem. Biophys. Res. Comm., 32, 861 (1968).
54. Neuhaus, F.C. Linzer, R., and Reusch Jr., V.M., Ann. N.Y. Acad. Sci., 235, 502 (1974).

55. Glaser, L., J. Biol. Chem., 239, 3178 (1964).
56. Coley, J., Tarelli, E., Archibald, A.R., and Baddiley, J., FEBS Lett., 88, 1 (1978).
57. Burger, M.M. and Glaser, L., J. Biol. Chem., 239, 3168 (1964).
58. Wyke, A.W. and Ward, J.B., J. Bact., 130, 1055 (1977).
59. Coley, J., Archibald, A.R., and Baddiley, J., FEBS Lett., 80, 405 (1977).
60. Hancock, I.C. and Baddiley, J., J. Bact., 125, 88 (1976).
61. Roberts, F.M., McArthur, H.A.I., Hancock, I.C., and Baddiley, J., FEBS Lett., 97, 211 (1979).
62. McArthur, H.A.I., Hancock, I.C., Roberts, F.M., and Baddiley, J., FEBS Lett., 111, 317 (1980).
63. Wyke, A.W. and Ward, J.B., FEBS Lett., 159 (1977).
64. Lin, T-Y. and Gotschlich, E.C., J. Biol. Chem., 242, 471 (1967).
65. Mauk, J. and Glaser, L., J. Biol. Chem., 247, 118 (1972).
66. Boylan, C.W. and Ensign, J.C., J. Bact., 96, 421 (1968).
67. Chin, T., Younger, J. and Glaser, L., J. Bact., 95, 2044 (1968).
68. Glaser, L. and Loewy, A., J. Biol. Chem., 254, 2187 (1979).
69. Rosenberger, R.F., Biochim. Biophys. Acta., 428, 516 (1976).
70. Glaser, L. and Loewy, A., J. Biol. Chem., 254, 2187 (1979).
71. Grant, W.D., J. Bact., 137, 35 (1979).
72. Grant, W.D., FEBS Microbiol. Lett., 6, 301 (1979).
73. Wise Jr., F.M., Glickman, R.S., and Teimer, E., Proc. Natl. Acad. Sci. (USA), 69, 233 (1972).
74. Glaser, L., Biochim. Biophys. Acta., 101, 6 (1965).

75. Glaser, L., Ionesco, H., and Schaeffer, P., Biochim. Biophys. Acta., 124, 415 (1966).
76. Young, F.E., Proc. Nat. Acad. Sci. (USA), 58, 2377 (1967).
77. Archibald, A.R. and Coapes, H.E., J. Gen. Microbiol., 73, 581 (1972).
78. Brooks, D., Mays, L.L., Hatefi, Y., and Young, F.E., J. Bact., 107, 223 (1971).
79. Boylan, R.J., Mendelson, N.H., Brooks, P. and Young, F.E., J. Bact., 110, 281 (1972)
80. Heptinstall, S., Archibald, A.P., and Baddiley, J., Nature, 225, 519, (1970).
81. Archibald, A.R., Armstrong, J.J., Baddiley, J., and Hay, J.F., Nature, 191, 570 (1961).
82. Doyle, R.J., McDonnell, M., Streips, U.N., Birdsell, D.C., and Young, F.E., J. Bact., 118, 606 (1974).
83. Hughes, F.H., Hancock, I.C., and Baddiley, J., Biocher. J., 132, 83 (1973).
84. Heckels, J.E., Lambert, F.A., and Baddiley, J., Biocher. J., 162, 359 (1977).
85. Lambert, F.A., Hancock, I.C., and Baddiley, J., Biocher. J., 151, 671 (1975).
86. Archibald, A.R., Baddiley, J., and Heptinstall, S., Biochim. Biophys. Acta., 291, 629 (1973).
87. Peusch Jr., V.M. and Neuhaus, F.C., J. Biol. Chem., 246, 6136 (1971).
88. Anderson, A.J., Green, R.S., Sturman, A.J., and Archibald, A.P., J. Bact., 136, 886 (1978).
89. Fiedler, F. and Glaser, L., Biochim. Biophys. Acta., 300, 467 (1973).
90. Fooley, H.P., J. Bact., 125, 1129 (1976).
91. Herbold, D.P. and Glaser, L., J. Biol. Chem., 250, 723 (1975).
92. Herbold, D.P. and Glaser, L. J. Biol. Chem., 250, 1676 (1975).

93. Forsberg, C.W., Wyrick, P.B., Ward, J.B., and Rogers, H.J., J. Bact., 113, 969 (1973).
94. Bracha, R., Davidson, R., and Mirelman, D., J. Bact., 134, 412 (1978).
95. Kaback, J., DeFillipe, L., Engel, R., and Tropp, B.E., J. Med. Chem., 17, 363 (1974).
96. Goldstein, S.L., Braksmeyer, D., Tropp, B.E., and Engel, R., J. Med. Chem., 17, 363 (1974).
97. Goldstein, S.L., Pulcrano, M., Tropp, B.E., and Engel, R., J. Med. Chem., 17, 1115 (1974).
98. Anagnostopulis, C. and Spizizen, J., J. Bact., 81, 741 (1961).
99. Ames, G., J. Bact., 25, 833 (1968).
100. Bligh, E. and Dyer, W., Can. J. Biochem., 37, 911 (1959).
101. Fatterson, M. and Greene, R., Anal. Chem., 37, 854 (1965).
102. DeHaas, G.P., Fonsen, P.P.M., and Van Deenan, L.L.M., Biochim. Biophys. Acta., 116, 114 (1966).
103. Hughes, R.C., and Tanner, P.J., Biochem. Biophys. Res. Comm., 33, 22 (1968).
104. Coley, J., Archibald, A.R., and Paddiley, J., FEBS Lett., 80, 405 (1977).
105. Levvy, G.A. and McAllen, A., Biochem. J., 73, 127 (1959).
106. Peissig, J.I., Strominger, J.L., and Leloir, L.F., J. Biol. Chem., 217, 954 (1955).
107. Shaw, D.R.D., Biochem. J., 82, 297 (1962).
108. Green, A.A., and Hughes, W.L., Methods in Enzymology, 1, 67 (1955).
109. Spizizen, J., Methods in Enzymology, 5, 122 (1966).
110. Burger, M.M. and Glaser, L., Methods in Enzymology, 8, 430 (1966).
111. Roseman, S., Distler, J.J., Moffat, J.G., and Khorana, H.G., J. Am. Chem. Soc., 83, 659 (1961).

- 112. Elbein, A.D., Methods in Enzymology, 8, 142 (1966).
- 113. Reiss, J.P. and Moffatt, J.G., J. Org. Chem., 30, 3381 (1965).
- 114. Goddell, M., Lopez, R., and Tomasz, A., Proc. Nat. Acad. Sci. (USA), 73, 3293 (1976).
- 115. Dixon, M., Biochem. J., 55, 170 (1953).