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EFFECT OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON PHOSPHOGLYCERIDE AND LIPOTEICHOIC ACID METABOLISM IN BACILLUS SUBTILIS

City University of New York PH.D. 1980

EFFECT OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON PHOSPHOGLYCERIDE AND LIPOTEICHOIC ACID METABOLISM IN BACILLUS SUBTILIS

ROBERT M. DEUTSCH

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

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

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ABSTRACT

3,4-Dihydroxybutyl-1-phosphonate ($CH_2CHOHCH_2OHCH_2PO_3H$), an analog of glycerol 3-phosphate preferentially inhibits the rate of synthesis and accumulation of phosphatidylglycerol in Bacillus subtilis 1005 and BD170. The rate of phosphatidylethanolamine synthesis is only slightly inhibited whereas that of lysylphosphatidylglycerol is somewhat stimulated. As expected, decreased phosphatidylglycerol synthesis results in the inhibition of the formation of the putative lipoteichoic acid precursor, sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol and of lipoteichoic acid itself in strain 1005. Turnover of phospholipids was not affected by drug. Accumulation of phosphatidylglycerol and lysylphosphatidylglycerol was depressed in the presence of drug which follows from synthesis and turnover studies. Neutral lipids accumulated in the presence of drug. Upon removal of drug a striking enhancement of the synthesis of phosphatidylglycerol and lysylphosphatidylglycerol as compared to synthesis in untreated cultures occured. Phosphatidylethanolamine synthesis remained normal. The 'stimulatory' effect on lipid synthesis that occurred after removing the drug was greater in strain 1005 than in strain BD170. On the other hand, all areas of synthetic activity in the presence of drug were inhibited to a greater extent in

Strain BD170 than in Strain 1005. The ability of bacteriophage 025 to bind to its cell wall receptor in Strain BD170 is decreased dramatically after a 60 minute incubation with drug.

Dedicated to the scientists behind the Iron Curtain who are denied the right to engage in their profession and are refused the right to emigrate.

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EFFECTS OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON PHOSPHOGLYCERIDE AND LIPOTEICHOIC ACID METABOLISM*.

INTRODUCTION

Phosphoglycerides are a major component of the cell membrane Metabolism of phospholipids have been extensively studied and numerous reviews published (1-6). Phosphoglycerides have a structural role in the bacterial cell. They serve to maintain the integrity of the cell and play a role in transport (7), oxidative phosphorylation (8), and cell wall synthesis (9). In addition phosphatidylglycerol, a class of phospholipid present in all bacterial cells, plays a very active metabolic role in bacterial organisms.

Synthesis of phosphatidylglycerol, starting with the common bacterial phospholipid precursor CDP-diacylglycerol, occurs in two steps (Fig. 1 and Fig. 2). A synthetase catalyzes formation of phosphatidylglycerolphosphate from CDP-diacylglycerol and sn-glycerol-3-phosphate in the first step. The reaction results in the release of CMP. To

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*A portion of the work described in this manuscript has been published in the Journal of Biological Chemistry 255,1521-1525 (1980).

date all procaryotic and eucaryotic systems examined contain this synthetase (10-14) . The E. coli synthetase has been purified to homogeneity (15). The enzyme appears to bind a large amount of detergent (15). Triton X-100 and Mg++ greatly stimulate activity (15). ADP-diacylglycerol and UDP-diacylglycerol can act as substrates for the enzyme (15). Serine, glycerol, sn-glycerol-2-phosphate, and sn-glycerol-1-phosphate are not recognized by the synthetase enzyme(15). However DHBP*, the phosphonic acid analog of glycerol-3 phosphate, can replace the natural metabolite (16).

Unlike the normal lipid, the phosphatidylglycerolphosphate analog that is produced in the presence of DHBP is not dephosphorylated (17). Consequently, the anomalous lipid accumulates in vivo and perturbs growth and cellular metabolism (17). The natural lipid, phosphatidylglycerolphosphate, does not accumulate under normal physiological conditions because the lipid is immediately dephosphorylated by a phosphatidylglycerolphosphate specific phosphatase. This represents the second step in the synthesis of phosphatidylglycerol. The enzyme catalyzing this reaction requires Mg++ for activity and is stimulated by Triton X-100 (15). The same synthetic pathway is believed to be involved in the

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*3,4-dihydroxybutyl-1-phosphonate

formation of phosphatidylglycerol in Gram-positive organisms(Fig. 2). For instance, in Bacillus lichenformis, Lasson et al. detected phosphatidylglycerol synthetase and phosphatidylglycerol phosphatase activities and resolved their activities chromatographically (11).

In contrast to Gram-negative microorganisms, Gram-positive cells (19) contain glycosyl diglycerides (Fig. 3). Synthesis of glycolipid starts with diglyceride. The metabolic pool of diglyceride in Bacillus lichenformis is actively turning over (20) unlike the small metabolically less active pool found in E. coli (21). This difference is probably related to the utilization of diglyceride for production of glycosyl diglyceride in Gram-positive bacteria. The pathway for phospholipid synthesis in E. coli is outlined in figure 1. There is a great deal of genetic and chemical support for this pathway (1,4). Recently, Kennedy et al. demonstrated that the synthetic pathway for the phospholipid in Bacillus megaterium, a Gram-positive organism, is similar to the one established for E. coli (22). As seen from Figure 1 and Figure 2, phosphatidylglycerol is an active metabolic precursor in both classes of bacterial organisms.

De novo biosynthetic reactions involving phosphatidylglycerol utilize either the phosphatidyl or glycerol

phosphate moiety. The first demonstration of phosphatidylglycerol as a phosphatidyl donor was in the conversion of phosphatidylglycerol to diphosphatidylglycerol (cardiolipin) in E. coli (23) and Staphylococcus aureus (24). When cells prelabeled with ^{32}Pi, were placed in medium without ^{32}Pi, the amount of label in phosphatidylglycerol decreased and that in diphosphatidylglycerol increased. The increase of label in diphosphatidylglycerol could be correlated with the loss in phosphatidylglycerol (24). In the biosynthesis of phosphoglycolipid by Streptococcus faecalis (faecium) (25) and Pseudomonas diminuta, (26). phosphatidylglycerol functions as a phosphatidyl donor. Phosphatidylmonoglucosyldiacylglycerol and glycerol are the end products in a transphosphatidylation reaction between phosphatidylglycerol and diglucosyl diacylglycerol in Pseudomonas diminuta (26). When phosphatidylglycerol was labeled with either [^{14}C]glycerol or ^{32}Pi, radioactivity was incorporated into phosphoglycolipid.

Kennedy and coworkers have demonstrated incorporation of radioactivity from lipids labeled with [3-^{3}H]glycerol-3-[^{32}P]phosphate into water soluble oligosaccharides in E. coli cells (27). These novel oligosaccharides contain glycerol phosphate in the sn-1 configuration (28). Phosphoglycolipids, a class of lipid found in many Gram-positive bacteria contain

sn-glycerol-1-phosphate (29,30). Phosphatidylglycerol is the probable sn-glycerol-1-phosphate donor for phosphoglycolipid formation and it appears to possess a similar role in the synthesis of lipoteichoic acid (31-35).

Lipoteichoic acid, the membrane-associated teichoic acid, usually has a linear backbone of polyglycerol phosphate covalently bound to glycolipid. There are structural variations among lipoteichoic acids isolated from different gram-positive bacteria. These variations include differences in the amount of glucose substituted at position-2 of glycerol (36), the presence or absence of D-alanine ester residues (36), and the type of glycolipid attached to the backbone (37). The chemistry and biochemistry of this membrane associated amphipathic molecule of Gram-positive bacteria has been reviewed extensively (36-39).

A correlation between the turnover of phosphatidylglycerol and the appearance of lipoteichoic acid has been demonstrated in Staphylococcus aureus (34) and Streptococcus sanguis (35). Pulse-chase experiments demonstrated that radioactivity was rapidly transferred from phosphatidylglycerol to a water-soluble high molecular weight compound, subsequently identified as membrane-derived lipoteichoic acid (34,35). The glycer-

ol-3-phosphate monomer units in the polyglycerophosphate chain of lipoteichoic acid appear to be derived from the distal sn-glycerol-1-phosphate of phosphatidylglycerol. In vitro support for this hypothesis has been provided by studies with crude membrane preparations of S. sanguis and Streptococcus faecalis (31-33).

Fischer and coworkers have isolated and identified several glycerophosphoglycolipids that may be lipoteichoic acid precursors (29). A precursor-product relationship is suggested by the structural similarity between the membrane phosphoglycolipid and the glycolipid portion of the lipoteichoic acid isolated from a given bacterial strain (29). sn-Glycerol-1-phospho-B-gentiobiosyldiacylglycerol, the phosphoglycolipid of B. subtilis is synthesized in several steps that may begin with either the dephosphorylation of phosphatidic acid (20) or the loss of sn-glycerol-1-phosphate from phosphatidylglycerol. The diacylglycerol reacts with two molecules of UDP-glucose to form gentiobiosyldiacylglycerol. Transfer of sugar residues from a nucleotide derivative to diglyceride has been demonstrated in several Gram-positive organisms (40-44). Phosphatidylglycerol may serve as the sn-glycerol- 1-phosphate donor in the last step to yield the phosphoglycolipid.

A precursor-product relationship between glycolipid and lipoteichoic acid was supported in studies of a mutant of

Bacillus lichenformis lacking phosphoglucomutase (45). This mutant has a normal phosphoglyceride composition but lacks the glycolipid gentiobiosyldiacylglycerol which is present in the wild-type parent (45). The lipoteichoic acid isolated from the mutant lacked glucose groups normally associated with the glycolipid portion of lipoteichoic acid. Two explanations have been offered for these results: (i) The enzymes that function in lipoteichoic acid synthesis can recognize diacylglycerol as an alternative substrate, or (ii) Polymerization of the glycerol-3-phosphate monomer occurs on diacylglycerol and the polymer is subsequently transferred to gentiobiosyldiacylglycerol.

DHBP, an analog of glycerol-3-phosphate, is an inhibitor of phosphatidylglycerol synthesis (40). As such it should affect the synthesis of those substances for which phosphatidylglycerol is a precursor. To date, little is known about the effect of DHBP on Gram-positive microorganisms since most of the initial effort has been directed towards understanding its effects on the metabolism of Escherichia coli (16,17,46-48).

At 2.5 mM, the drug is bacteriostatic to Bacillus subtilis strain BD170 and E. coli strain 8 and bactericidal

to Bacillus subtilis strain 1005 (46,49). These bacteria accumulate the analog of phosphatidylglycerophosphate* when treated with a low concentration of DHBP (60uM) (17,49). DHBP is a competitive substrate for the CDP-diacylglycerol:sn-glycerol-3-phosphate phosphatidyl transferase of E. coli (16). Escherichia coli treated with 15 uM (S)-DHBP exhibit an immediate inhibition of the rate of phosphatidylglycerol synthesis and a delayed but almost equally pronounced inhibition of the rate of phosphatidylethanolamine synthesis (46). The former effect has recently been exploited to support the hypothesis that phosphatidylglycerol is the source of glycerol in the lipoprotein of E. coli (48). A similar rationale of approach is employed in the present study to demonstrate that the inhibition of phosphatidylglycerol synthesis in B. subtilis results in blocking the formation of the putative lipoteichoic acid precursor, sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol, (29,30) and in decreasing the rate of synthesis of lipoteichoic acid itself.

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*(1,2-diacyl)-sn-glyceryl-S-phosphoryloxy-3'-hydroxybutyl-1-'-phosphonate

MATERIAL AND METHODS

CHEMICALS: rac-3,4-Dihydroxybutyl-1-phosphonate rac-3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate were prepared as previously described (50). Tris (hydroxymethyl) aminomethane (Tris), Sepharose 6B, Dowex-1, sodium dodecyl sulfate, alkaline phosphatase, and casein hydrolysate were obtained from the Sigma Chemical Co., St. Louis, Mo. L-[^{3}H]leucine, [^{14}C]acetate and [2-^{3}H]glycerol were purchased from New England Nuclear Corp., Boston, Mass. Carrier-free [^{32}P]phosphate was obtained from ICN Corp. Irvine, CA.. Anasil G thin-layer plates were products of Analabs, Inc., New Haven, Conn.. Bacterial phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin used as chromatographic standards were purchased from Supelco, Inc., Bellefonte, Pa.. Penassay broth and Bacto agar were products of Difco Laboratories, Detroit Michigan. Kodak no-screen medical X-ray film, Kodak liquid X-ray developer and Kodak rapid fixer were obtained from Eastman Kodak Co. All other chemicals were of reagent grade.

BACTERIAL AND VIRAL STRAINS AND CULTURE CONDITIONS: B. subtilis BD170 (thr,trp), a derivative of strain 168 (Marburg) and B. subtilis 1005 (met), a derivative of

strain W23 were kindly provided by Dr. L. Mindich and Dr. D. Dubnau of the Public Health Research Institute of the City of New York. The former contains polyglycerol phosphate and the latter polyribitol phosphate cell wall teichoic acid (51). The cells were cultured in 1% casein hydrolysate and 0.5% NaCl (adjusted to pH 7 with NaOH) at 37 C. Cells were shaken in a New Brunswick metabolite shaker at 150rpm. Cell growth was monitored on the Klett-Summerson colorimeter as previously described (46,49). Bacteriophage 025 was generously provided by Dr. R.J. Boylan of the New York University Dental School. This phage requires the glucose moiety of polyribitol teichoic acid for attachment to the cell and causes lysis in strain BD170 (52). Penassay broth was used to prepare phage from B. subtilis BD170 and to incubate phage 025 with cell wall. Plaque forming units were determined on petri dishes containing penassay broth and 1.5% bacto agar. Top agar contained penassay broth and 0.7% bacto agar.

RATE OF MACROMOLECULAR SYNTHESIS: Cultures of B. subtilis at a turbidity of 20 Klett units were treated with the indicated concentration of rac-DHBP. The rate of DNA, RNA, and phosphoglyceride synthesis were determined by removing 1 ml of cells at the indicated times and incubating with 20-30 uC_i of [^{32}P]phosphate for eight

minutes. These samples were treated with an equal volume of ice cold 10% trichloroacetic acid, and 1 ml of sonic extract (Sonic extract was derived from overnight cultures at 220-240 Klett. Cells were collected by centrifugation, suspended in 4 ml of medium, and sonicated for five minutes). Precipitates were spun down in 15 ml Corex centrifuge tubes at 5000xg on a Sorvall model RC-2B centrifuge equipped with an SS34 rotor washed twice with cold 5% trichloroacetic acid and suspended in 5 ml of chloroform:methanol:water (5:5:1). After one hour at room temperature, the samples were centrifuged and supernatant fluid containing phosphoglycerides was removed. A 1.6 ml portion of water was added making the solution biphasic. The chloroform layer was washed three times with 2M potassium chloride and once with water. Equal portions were removed from the chloroform layer evaporated and counted in 10 ml of toluene scintillation fluid. The remainder was saved for subsequent phospholipid analysis. Pellets that remained after the treatment with chloroform:methanol:water (5:5:1) were washed again with this solvent system, and were then suspended in 2 ml of 0.5N potassium hydroxide. These suspensions were kept in the warm room overnight to hydrolyze RNA after which they were chilled in an ice bath. Next 2 ml of ice cold 1M perchloric acid was added to the samples and they were centrifuged for 10 minutes at 8000xg to sediment the DNA. A 1 ml aliquot of the supernatant fluid containing hydrolyzed

RNA was counted in 10 ml of Patterson-Greene scintillation fluid. The DNA containing pellets were redissolved in 2 ml of 1N potassium hydroxide, neutralized with hydrochloric acid, and precipitated with 12% trichloroacetic acid. Pellets were washed once with 5% trichloroacetic acid, dissolved in 1 ml of 0.2N potassium hydroxide and counted in 10 ml of Patterson-Greene Scintillation fluid(53).

RATE OF PROTEIN SYNTHESIS: Cultures of B. subtilis at a cell density of 20 Klett units were treated with .06 mM DHBP or .12mM lithium chloride. At various time intervals thereafter, 2 ml samples were removed and incubated with 2.5 uC_i of [^{3}H]leucine. After eight minutes an equal volume of 10% TCA was added and the samples were incubated at 0 ^oC for 30 minutes prior to filtration and collection of the precipitate on Whatman #3mm filter papers. The filters were washed twice with 2 ml of ice cold 5% trichloroacetic acid. The filters were also washed twice with 2 ml of ethanol:ether (1:1) maintained at 37 ^oC and twice with diethyl ether at room temperature. The filters were air dried and counted in toluene scintillation fluid as described earlier.

ACCUMULATION AND TURNOVER OF PHOSPHOGLYCERIDES:

B. subtilis at a turbidity of 8-10 Klett units were cultivated in the presence of 10-16 uC_i [^{32}P]phosphate. When a turbidity of 15-25 Klett units was reached the cultures were used to study phosphoglyceride accumulation or turnover. In the former case either 60 uM rac-DHBP or 120 uM lithium chloride was added to the cultures and samples removed at the indicated times. In the latter case the cells were collected on Millipore filters (HA 0.45u), washed twice with minimal Tris-HCl buffered growth medium described by Garen and Levinthal (54), and resuspended in unlabeled preconditioned casein hydrolysate medium. The resuspended cultures were treated with either 60 uM rac-DHBP or 120 uM lithium chloride. Samples were removed at the indicated times. The phosphoglycerides were extracted by the method of Bligh and Dyer except that the chloroform layer was washed three times with 2M potassium chloride and once with distilled water. A portion of the chloroform layer was evaporated and counted as previously described. The remainder was retained for subsequent analysis of phospholipids.

RECOVERY FROM DHBP TREATMENT: When the turbidity of a culture of B. subtilis reached 15 Klett units 60 uM rac-DHBP was added. After a 35 minute incubation the cultures were filtered on Millipore Filters (HA 0.45u), washed once with 5 ml of 0.1 M Tris buffer, pH 7.5

at 25 C, once with 5 ml preconditioned casein hydrolysate medium, and then resuspended to a turbidity of 10 Klett units in preconditioned casein hydrolysate medium lacking 3,4-dihydroxybutyl-1-phosphonate. DHBP treated and untreated cultures had similar growth properties after resuspension (Fig. 6). The rate of phosphoglyceride synthesis during recovery from drug treatment was determined as described above except that the lipids were extracted directly from the cultures using chloroform and methanol (1:2) without prior trichloroacetic acid treatment. The procedure for this extraction has been described (46). A parallel experiment performed with 60 uM rac-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (0.5 uC_i per ml) indicated that most of the DHBP was removed after the cultures were washed and resuspended since no further incorporation into phospholipids was observed in the preconditioned medium lacking DHBP (Fig. 18).

INCORPORATION OF [3-^{3}H]DHBP INTO LIPIDS IN VIVO: B. subtilis cells were cultured in 30 ml of medium. At a turbidity of 15 Klett units, 15 uC_i of [^{3}H]DHBP was added to a final concentration of 60uM. Samples were withdrawn at the indicated time points and lipids were extracted by the Bligh and Dyer

procedure described earlier with the following modifications: (i) B. subtilis sonic extract was prepared from overnight cultures grown in the presence of 30uM DHBP, (ii). the chloroform layer was washed once with 1mM DHBP in place of a water wash (iii) throughout the wash procedure, care was taken not to perturb the interface. One hour after drug addition, cultures were filtered on Millipore filters (HA 0.45u) washed once with 5 ml of 0.1M Tris (pH,7.5; 0 C), and once with 5 ml of pre-conditioned medium, then resuspended in preconditioned medium lacking phosphonate. Samples were removed at various time points thereafter and lipids extracted. A portion of the lipid extract was evaporated and counted as described.

ANALYSIS OF PHOSPHOGLYCERIDES: The lipid extracts obtained as described above were analyzed by thin-layer chromatography on silica gel G plates. The solvent system (solventA) usually used was the diisobutylketone:acetic acid:water (8:5:1) system of Marinetti (56). Solvent system B was used occasionally and it consisted of chloroform:acetic acid:methanol:water (80:18:12:5). Autoradiography of chromatograms containing [^{32}P]phosphate labeled lipids revealed four fractions (Fig.4). Cardiolipin did not appear on the autoradiograms because of the low content of this lipid and the relatively short exposure

times. Phospholipids were usually visualized by exposure of the plates to iodine vapors. The sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol was also detected by the a-Naphthol/sulfuric acid spray reagent* (58). Phosphatidylethanolamine and lysylphosphatidylglycerol could be seen following treatment of the plates with ninhydrin spray. The identification of the latter lipid was based upon comparison with published R_f values and by the release of lysine upon mild basic hydrolysis. The identification of each of the other phosphoglycerides was established by simultaneously chromatographing with authentic standards. The two solvent systems used for verification of the phosphoglycolipid were: chloroform:acetone:methanol:acetic acid:water (50:25:25:10:5) and chloroform:methanol:ammonia:water (50:35:5:5). In the former solvent system, the R_f value was 0.67, in the latter the R_f value was 0.51. The R_f value of phosphatidylethanolamine, phosphatidylglycerol, lysylphosphatidylglycerol, and sn-glycero-1-phospho-B-gentiobiosyl diacylglycerol were 0.42, 0.33, 0.18, and .08 respectively in solvent A and .057, .38, .22, and .05 respectively in solvent B. R_f values have been

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*This reagent was prepared by dissolving 0.5 grams of a-Naphthol in 100 ml of methanol:water (1:1). It was sprayed on chromatograms until damp. The chromatograms were allowed to air dry and were then sprayed lightly with a sulfuric acid spray containing 5% water by volume. Color was developed by heating in an oven at 120 °C for several minutes.

reported for the first three lipids in solvent A and are in agreement with the values reported here (58).

RATE OF LIPOTEICHOIC ACID FORMATION: The recovery of lipoteichoic acid was variable. It was therefore necessary to utilize an internal standard. This was accomplished by adding 500 uC_i of [2-^{3}H]glycerol (sp. act. 250 uC_i/umole) to a 30 ml culture of B. subtilis 1005 at a turbidity of 20 Klett units After a 20 minute incubation at 37 °C and 150 rpm, the cells were collected by filtration and suspended in 2 ml distilled water. Experimental cultures were treated in a similar fashion. When a 30 ml culture reached a turbidity of 20 Klett units it was treated with the indicated concentration of rac-DHBP for 30 minutes prior to the addition of 10 uC_i [^{32}P]$_{phosphate}$ per ml for a 15 minute pulse. The cells were collected on a Millipore filter (HA 0.45u), washed once with 5 ml of medium, and resuspended in 1.5 ml of water. To each [^{32}P]phosphate cell suspension, 1 ml of [2-^{3}H]glycerol labeled cells was added followed by 2.5 ml of 90% phenol. The samples were kept at 65-70 °C for 30 minutes with constant agitation (61). The phases were separated by centrifugation and the phenol phase reextracted with 2 ml of water. The combined aqueous phases were dialyzed at 0 °C against 2 liters of distilled water with one change of water after 24 hrs. After 48 hours, the dialyzed mater-

ial was made 50 mM in Tris-HCl, pH 7.4, 1 mM in EDTA, and 1 mM in NaCl and incubated with 25 ug per ml of RNase at 37 ^{o}C for twelve hours. The solution was then made 20 mM in MgCl2 and 25 ug per ml of DNase added. After a two hour incubation at 37 C, 1 mg of trypsin was added to each sample and incubated for two hours. The samples were treated with an equal volume of 90% phenol and dialyzed as above. The lipoteichoic acid was recovered from the dialyzed material and purified by passing 1-2 ml through a 37x2.6 cm Sepharose 6B column (Appendix A) (62,63). The column was eluted with a 0.05 M Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide at a rate of 15-16 ml per hour and 3.5 ml fractions were collected. Following elution, 0.5 ml samples were removed and ^{3}H and ^{32}P content determined in Patterson-Greene scintillation fluid. Typical elution patterns are shown in Fig. 5 and Fig. 6. The second peak does not contain [^{3}H]glycerol and probably represents undegraded nucleic acid (62). The lipoteichoic acid fractions of each experiment were pooled, reduced in vacuo at 30 C, and dialyzed against distilled water overnight. A 1 ml sample of the pooled material was counted in 10 ml of Patterson-Greene scintillation fluid (Appendix B) All values for the DHBP inhibition of lipoteichoic acid formation were corrected for non-specific losses in [^{3}H]glycerol and compared with untreated samples that were prepared at the same time.

CHEMICAL ANALYSIS OF LIPOTEICHOIC ACID: Cultures of B. subtilis 1005 in 1 liter of casein hydrolysate medium and at a turbidity of 5 Klett were incubated with 0.01 uC_i of [2-^{3}H]glycerol per ml. When cell density reached 25 Klett units, labeled lipoteichoic acid was extracted and purified as described. After purification, lipoteichoic acid was hydrolyzed by adding hydrochloric acid to a final concentration of 2N and heating for three hours at 100 ^oC under nitrogen. Acid was removed in vacuo over potassium hydroxide pellets. The pellet remaining after the solvent evaporation was extracted twice with ether to remove fatty acids and then suspended in 2 ml of ammonium carbonate, pH 8.9 and 0.05M. Alkaline phosphomonoesterase (.01%) was added to the solution for a 16 hour incubation at 37 C. Ammonium carbonate was removed by evaporation under vacuum at 60 C. The sample was divided into several portions. One portion was used directly for phosphate analysis (65). Another portion was eluted through a Dowex-1 column (13cm x 8mm; OH cycle) with distilled water. Roughly 4% of the contents remained bound to the column and were not further characterized. Appropriate fractions were collected and pooled. A portion was utilized for analysis of glycerol by the procedure of Hanahhan and Olley (66). In quantitative terms, the ratio of phosphate to glycerol was 1.07:1.00. This ratio takes into account the material presumed to be glycerol phosphate that remained bound to the Dowex column.

Fatty acid analysis of lipoteichoic acid revealed 5 peaks. Fischer and co-workers have reported the existence of 9 fatty acid in the lipoteichoic acid of B. subtilis(29). The discrepancy may be attributed to the small sample size of lipoteichoic acid taken for fatty acid analysis.

PREPARATION AND PURIFICATION OF BACTERIOPHAGE 025: B. subtilis BD170 were cultured in 20 ml of Difco antibiotic medium 3 supplemented with thymine (50 ug per ml) and tryptophan (50 ug per ml). At a cell density of 80 Klett, $5x10^8$ virions were added. Four to six hours were allowed for lysis of bacterial cells. The resulting clarified solution was centrifuged for 20 min at 8000xg in a Sorvall model RC-2B centrifuge, equipped with an SS-34 rotor. After centrifugation the supernatant was removed and filtered through a sterile Millipore filter (HA 0.45u). The phage was stored at 0-5 C.

BACTERIOPHAGE ADSORBTION TO CELL WALL: B. subtilis BD170 were cultured in 320 ml of casein hydrolysate medium containing 1% casein hydrolysate and 0.5% sodium chloride. At a cell density of 25 Klett units, 20 umoles DHBP or 40 umoles lithium chloride was added. After 60 minutes incubation period, the cells were collected by centrifugation at 5000xg for

20 minutes and resuspended in 10 ml of 0.9% saline solution. Five passes were made with a coarse ground glass homogenizer to initially resuspended the cells. Afterwards, the sample was placed in a rosette cell surrounded by an ice-salt-water bath and sonicated for 5 minutes. Sonication was followed by centrifugation for 15 minutes at 5000xg to bring down unbroken cells and centrifugation of supernatant at 12000xg for 30 minutes to spin down cell walls. The resultant pellet was incubated with 8 ml of a 1% SDS solution for 30 minutes at 80-90 C. Each pellet was washed four times with distilled water following the SDS treatment. To determine the weight of the cell wall in each sample, a portion was removed placed on a previously weighed dessicated microwatchglass and weighed on a Mettler model H51 balance. After determining the weight in each sample, the concentration was adjusted to 500 ug cell wall per ml by the addition of water. The concentration of inorganic phosphate per mg cell wall as measured by the method of Chen (65) was found to be the same for untreated and treated samples.

DETERMINATION OF PLAQUE-FORMING UNITS: Cell wall suspensions, containing 5-35 ug of cell wall per ml penassay broth, was incubated with 0.1 ml of phage suspension ($5x10^8$ plaque-forming units per ml) for 10 minutes at 37 ^{o}C under bubbling aeration. A 0.1 ml portion was

removed diluted with 10 ml of penassay broth and centrifuged for 15 minutes at 8000 rpm in an SS34 rotor. After centrifugation, 0.1 ml of supernatant was mixed together with 0.2 ml of B. subtilis BD170 cells (30 Klett) in a sterile test tube and allowed to stand for ten minutes at 37 C. A 1 ml portion of top agar maintained at a temperature of 46 °C was added. The mix was vortexed and immediately poured onto a petri dish containing a solid layer of agar. The top agar was spread homogeneously throughout the agar and allowed to solidify. Petri dishes were placed in the warm room overnight and resultant plaques counted the following day.

AUTORADIOGRAPHY: Chromatograms containing radioactive material were placed on top of individually wrapped Kodak no-screen X-ray film. Exposure times ranged from one to seven days depending upon the isotope used and on its intensity. The film was removed from its wrapper in total darkness and placed in x-ray developer solution for approximately five minutes. To stop the reaction, the film was dipped in a stop bath solution, consisting of 3% acetic acid, for 30 seconds. Next the film was soaked in fixer solution for ten minutes. All solutions were prepared according to manufacturers directions. Traces of chemicals remaining on the film were removed by running cold water over the film for thirty minutes.

GAS-LIQUID CHROMATOGRAPHY: Fatty acids obtained from lipoteichoic acid were converted to their methyl esters by boron triflouride/methanol treatment (29). They were analyzed on a Varian gas chromatograph Series 1200 equipped with a column containing 3% SE-30 silicon on Chromosorb G AW,DMCS (28"x1/4"). Fatty acid methyl esters were separated on the column at a programmed temperature rise of 2 °C per minute from 150 °C to 210 °C and were detected by a flame ionization apparatus.

ASSAY FOR GLYCEROL DETERMINATION: A 2 ml aliquot was mixed together with 0.1 ml 10N sulfuric acid and 0.5 ml 0.1M sodium periodate. After allowing the mix to stand for 5 minutes, 0.5 ml of 10% sodium bisulfite was added, followed by 1 ml of chromotropic acid reagent (1.0 gram chromotropic acid, 100 ml water, The mix was heated in a boiling water bath for thirty minutes and then cooled in an ice bath. When the sample reached room temperature, 0.5 ml of half saturated thiourea was added. Five minutes later the absorbance was read at 520nm.

RESULTS

The effect of various concentrations of rac-DHBP upon the growth of B. subtilis 1005 and B. subtilis BD170 is depicted in Fig. 7 and Fig. 8 respectively. At 60 uM rac-DHBP, the concentration at which most of the metabolic studies were performed, there is a slight inhibition of growth (Figs. 7&8). A similar effect upon the increase of the viable cell count was observed (Fig. 9). This concentration of drug caused a marked inhibition in the rate of phospholipid synthesis but had considerably less affect upon the rate of protein, DNA, and RNA synthesis (Fig. 10A). Strain BD170 was somewhat more sensitive to DHBP than strain 1005 in all categories of macromolecular synthesis (Fig. 11A).

Analysis of the drugs effect upon the individual phospholipids revealed a stimulation in the rate of lysylphosphatidylglycerolphosphate synthesis and an inhibition in the rate of phosphatidylethanolamine and phosphatidylglycerol synthesis(Fig. 10B and Fig. 11B). A 35-45% inhibition of phosphatidylglycerol synthesis was observed in strain 1005, and an even greater inhibition, 47-54%, was found in strain BD170 (Fig. 10B and Fig. 11B). In untreated cells, the rate of synthesis of

phosphatidylglycerol is approximately 4.5-5.0 times that of lysylphosphatidylglycerol. On a molar basis, there is at least seven times more inhibition in the rate of synthesis of phosphatidylglycerol than can be explained by the stimulation in the rate of lysylphosphatidylglycerolphosphate for strain 1005. Consistent with its greater sensitivity to drug, strain BD170 has a value for phosphatidylglycerol inhibition nine times greater than the stimulation observed in its rate of lysylphosphatidylglycerolphosphate synthesis. The rate of turnover of prelabeled phospholipids was not affected by the presence of 60 uM rac-DHBP (Fig. 12 and Fig. 13). Therefore one might expect drug treated cells to exhibit a slight increase in the accumulation of lysylphosphatidylglycerol, a slight decrease in the accumulation of phosphatidylethanolamine, and a marked decrease in the accumulation of phosphatidylglycerol. This was in fact observed (Fig. 14 and Fig. 15).

A comparison between Fig. 14 and Fig. 15 shows that the effect of 60 uM rac-3,4-dihydroxybutyl-1-phosphonate had upon the accumulation of phosphatidylglycerol was more pronounced in strain BD170. Accumulation of the phosphatidylglycerolphosphate analog into a chloroform extractable fraction in the presence of DHBP was 30% greater in strain 1005 than in strain BD170 (Fig. 16) (16). On the other hand, Klein et al. have reported

that the amount of incorporation of [3-^{3}H]DHBP into the cell wall fraction is five fold higher in strain BD170 than in 1005 (49)

The ability of cell walls isolated from untreated and DHBP treated B. subtilis BD170 to inactivate bacteriophage 025 was compared by an assay devised by Boylan et al. (52). Boylan's assay is based upon phage adsorption to the glucosylated wall teichoic acid. The assay provides an index for the presence of normal glycosylated wall teichoic acid. After incubation of the bacteria for one hour, cell wall was isolated. The same weight of wall material isolated from treated cells was 75% as effective in adsorbing 025 bacteriophage as that from untreated cells.

Chromatographic mobility of the phosphatidylglycerol-phosphate analog was determined by autoradiography of the tritium labeled compound. The radioactive analog was obtained by the incubation of B. subtilis BD170 cells with high levels of [3-^{3}H]DHBP followed by chloroform extraction of cellular lipids. Interestingly, a small percentage of the tritium label was found in positions corresponding to the other B. subtilis lipids (Table 3). A similar phenomena occurs with lipids from treated E. coli cells (17). In both B. subtilis

strains the anionic lipid did not turnover (Fig. 16).

Two other components of the phospholipid fraction were detected by thin layer chromatography. The first of these, comprising less than 1% of the total phospholipid, was identified as cardiolipin. DHBP slightly affected the rate of its synthesis (Table 1). The second component was approximately 3% of the total phospholipid. The a-Naphthol/sulfuric acid spray reagent caused a positive reaction suggesting a glycophospholipid. Fischer et al. (29) identified sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol as the glycophospholipid present in B. subtilis (30). The second component had chromatographic properties that were identical to those of an authentic standard most generously provided by Prof. Werner Fisher. As evident by comparing the data in Table 1 with that presented in Fig. 10 and Fig. 11, 60 uM rac-DHBP had an even greater inhibitory effect upon the rate of formation of the glycerophosphoglycolipid than it did upon that of phosphatidylglycerol. As is the case with phosphatidylglycerol synthesis, the inhibitory effect is stronger in the BD170 strain than in the 1005 strain. It would follow that if phosphatidylglycerol served as a donor of sn-glycero-1-phosphate for diglucosyldiacylglycerol, an increase in the accumulation of diglucosyldiacylglycerol would occur in the presence

of DHBP. A small increase in accumulation was observed (Table 2).

One method for examining regulatory mechanisms in the synthesis of phospholipids is to subject cells to an agent that will perturb phospholipid composition, then measure synthesis after removal of that agent. In our study, cells cultured in the presence of 60 uM rac-DHBP for 35 minutes were washed free of the drug and resuspended* in preconditioned medium as described in the Materials and Methods section. These cells had an abnormal phospholipid composition. After a lag of 60-80 minutes, the cells previously exposed to DHBP exhibited a greater rate of lysylphosphatidylglycerol and phosphatidylglycerol synthesis than did those from a washed untreated control culture in both strains (Figs. 20A&20B). The rate of enhancement of phospholipid production during recovery was less pronounced in strain BD170 than in strain 1005 (Fig. 20A and 20B). Strain BD170 was expected to exhibit a greater stimulation in the rate of phospholipid synthesis to compensate for the stronger inhibition by DHBP on phospholipid synthesis in this strain. No stimulation of the

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*A preliminary experiment revealed a decline in phospholipid synthesis after cells reached a turbidity of 120-140 Klett (Fig. 19). The recovery experiment was set up so that all the time points were taken prior to this Klett value. This was achieved by resuspending a fraction of the cells that had previously incubated with drug into cell-free preconditioned medium.

rate of phosphatidylethanolamine was evident in either strain BD170 or strain 1005 (Fig. 30). Both DHBP treated and untreated cultures had similar doubling times after suspension in preconditioned medium (Fig. 6).

Fig. 21 reveals that increasing concentrations of rac-DHBP have the most profound effect upon the rates of phospholipids and lipoteichoic acid synthesis in strain 1005. The rates of DNA, RNA, and protein synthesis are considerably less affected. An analysis of the pattern of inhibition among the three major phospholipids clearly shows that phosphatidylglycerol is the only species that is markedly inhibited (Fig. 21B). When cells are treated with 240 uM rac-DHBP the rate of phosphatidylglycerol synthesis drops to about 22% of normal whereas that of phosphatidylethanolamine is close to 82% of normal (Fig. 21B). In contrast similar studies of E. coli revealed that DHBP causes a delayed but very pronounced inhibition in the rate of phosphatidylethanolamine synthesis (46).

DISCUSSION

A fundamental difference between strains 1005 and BD170 lies in the composition of their wall teichoic acid, the former strain contains a polyribitol phosphate and the latter a polyglycerol phosphate backbone. This variation may have a direct bearing on the differential effects of DHBP on cellular metabolism. Incorporation of drug into phosphatidylglycerolphosphate analog and cell wall material occurs in both strains (17,49). However accumulation of the phosphatidylglycerolphosphate analog in the presence of DHBP was 30% greater in 1005 than in BD170 (Fig.16). In the absence of turnover, this indicates a faster rate of synthesis of this novel phospholipid in strain 1005. This was expected since incorporation of radioactive DHBP into cell wall material is five fold greater in strain BD170 than in strain 1005 and therefore more of the intracellular DHBP of strain BD170 is diverted from the metabolic pool toward cell wall production than in strain 1005.

The increase in lysylphosphatidylglycerol synthesis in DHBP treated cells evident in rate and accumulation studies may reflect a regulatory process that is necessary to compensate for the acidic nature of the

phosphatidylglycerolphosphate analog that accumulates under these conditions (69-71). On the other hand, the increase in lysylphosphatidylglycerol observed in "recovery studies" (Fig.20) may simply reflect the increased availability of phosphatidylglycerol precursor. This is corroborated by a comparison of phospholipid stimulation in the two strains. Synthesis of both phosphatidylglycerol and lysylphosphatidylglycerol were more highly stimulated in strain 1005 than in BD170 (Fig. 20). The differential stimulation of lipid synthesis between the two strains may be related to the greater perturbation of membrane in strain 1005 due to the higher content of acidic phospholipid analog in this strain.

The continued presence of the phosphatidylglycerolphosphate analog in the "recovery studies" may have some relation to the lag period prior to the stimulation of lipid synthesis. It should be noted that this lipid does not turnover. (Fig.16). The lag was probably not due to the continued presence of intracellular DHBP in drug treated cells because [^{3}H]DHBP incorporation into the phosphatidylglycerolphosphate analog was completely absent following washing and resuspension. (Fig.16)

It has been previously reported that 3,4-dihydroxybutyl-1-phosphonate at a concentration of 2.5 mM is bacteriostatic

toward strain BD170 and bactericidal toward strain 1005 (49). From the results in Figures 10 and 11 one can not account for the bactericidal effect upon strain 1005 since lipid and macromoleculer synthesis is less inhibited in this strain than it is when the drug is bacteriostatic. However if the degree of stimulation of lipid synthesis after brief treatment with drug in the recovery studies can be viewed as an index of cellular perturbation, it is clear that strain 1005 is affected more than strain BD170 (Fig. 20). The specific mechanism that causes loss of cell viability requires further study.

The probable pathway for formation of phosphoglycolipid involves conversion from diacylglycerol. Diacylglycerol can be formed by the dephosphorylation of phosphatidic acid or as a consequence of the transfer of sn-glycerol-1-phosphate from phosphatidylglycerol to either sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol or LTA. Lennarz et al. have provided support for the former pathway (20). They demonstrated the presence of phosphatidic acid phosphatase in B. subtilis and also showed that the diglyceride pool is actively turning over. Addition of DHBP should not affect the dephosphorylation of phosphatidic acid but will, because of its effect upon phosphatidylglycerol synthesis, inhibit conversion of glycolipid to phosphoglycolipid. One would predict that sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol will

accumulate during DHBP treatment. A very small increase in the accumulation of the lipid sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol was observed (Table 2). In view of the strong inhibitory effect on phosphoglycolipid synthesis by DHBP, a much larger increase in accumulation was expected. The fact that this was not the case may be attributed to a decrease in phosphatidic acid phosphatase activity and/or to a diminished contribution of phosphatidylglycerol to the diglyceride pool. Further studies on the rate of synthesis and turnover of sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol should be performed to elucidate the preferred pathway.

One of the biological effects caused by DHBP is the inhibition of phage adsorption to walls of drug-treated cells (Fig. 17). Biochemical determinations have shown the site of phage attachment to be the glucosylated wall teichoic acid (72). Our studies indicated this attachment is inhibited by 25% (Fig.17). A 25% effect is quite significant if one considers that the time of drug exposure allows only one doubling and that cells initially have the usual complement of normal teichoic acid. Among the explanations offered to account for this effect are the following: (i)poly DHBP is formed replacing poly glycerol phosphate in the wall and cannot be glucosylated (ii) poly

DHBP even when glucosylated is not a receptor (iii) the amount of glucosylated polymer of either type is diminished. Chemical characterization of the cell wall from drug-treated cultures would be helpful in differentiating from among these possibilities.

Studies of Escherichia coli strain 8 revealed that DHBP causes a delayed but very pronounced inhibition in the rate of phosphatidylethanolamine synthesis (46). In B. subtilis cells treated with 240 uM rac-DHBP, the rate of phosphatidylglycerol synthesis drops to about 22% of normal whereas that of phosphatidylethanolamine is close to 82% of normal (Fig. 21). The present studies indicate that at least in B. subtilis the synthesis of phosphatidylethanolamine can be uncoupled from phosphatidylglycerol synthesis. The reason for DHBP's affect on phosphatidylethanolamine synthesis in E. coli remains to be explained. A comparison of enzyme activities of E. coli and B. subtilis may prove helpful in examining this question.

Several lines of in vitro (31-33) and in vivo (34,35) evidence indicate that phosphatidylglycerol is the precursor of the polyglycerophosphate chain present in lipoteichoic acid. The results from the present investigation show that DHBP inhibits phosphatidyl-

glycerol synthesis and as might be expected also blocks the synthesis of the putative LTA precursor sn-glycerol-1-phospho-B-gentiobiosyldiacylglycerol and LTA. Several functions have been proposed for lipoteichoic acid. Among these are the suggestions that it may be involved in cell wall teichoic acid formation (73-75) and in the control of autolysin activity (76,77). There have also been suggestions that lipoteichoic acid may play some role in disease mechanisms as have been proposed for lipopolysaccharide (78). The existence of a drug able to block lipoteichoic acid formation may have application to these and related questions.

FIGURE 1: Pathway for the biosynthesis of phospholipids in Gram-negative bacteria. The pathway, adapted from Raetz(1), indicates the most prevalent lipids synthesized in this class of microorganism. It includes membrane-derived oligosaccharides (MDO), a non-lipid substance, since a lipid serves as its precursor.

FIGURE 1

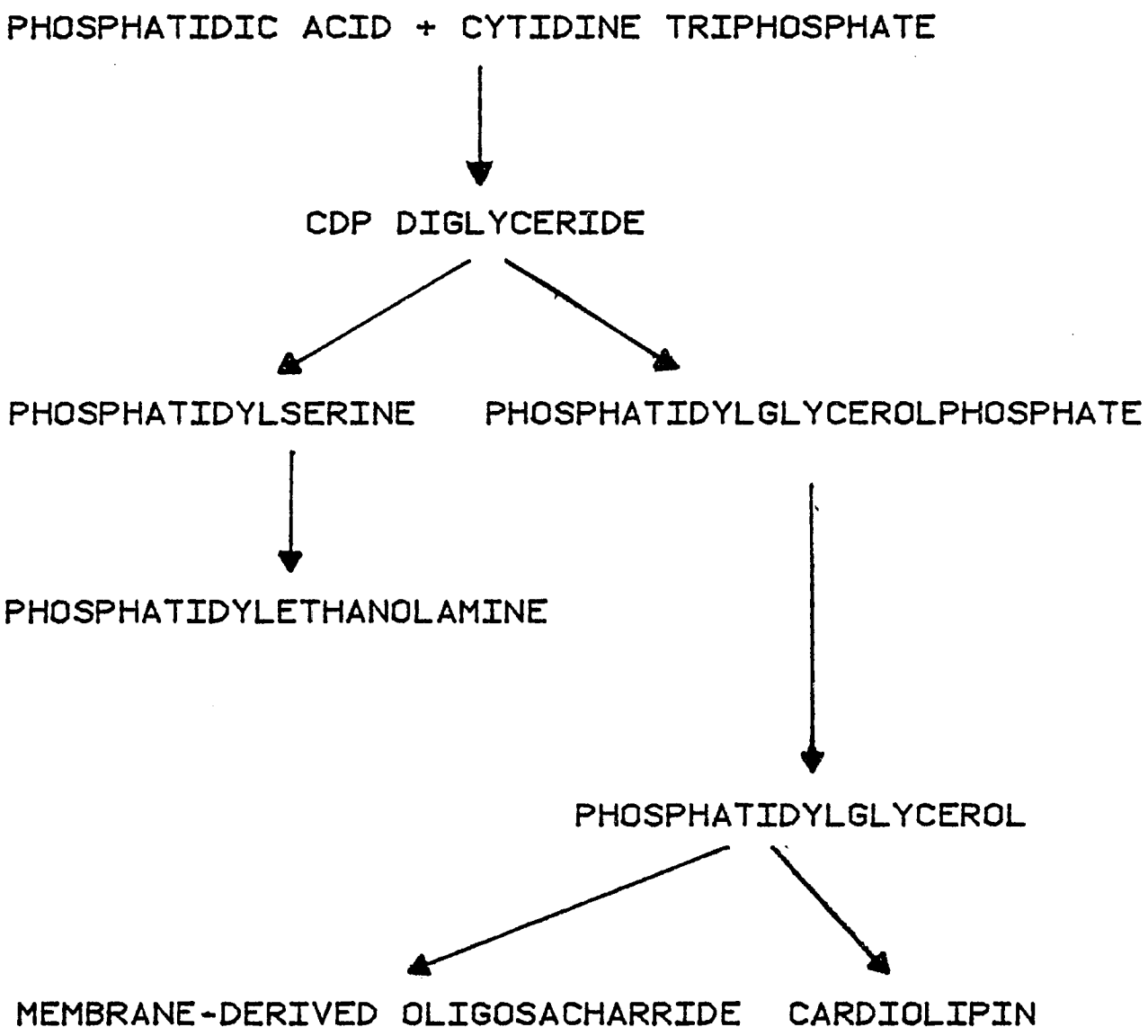
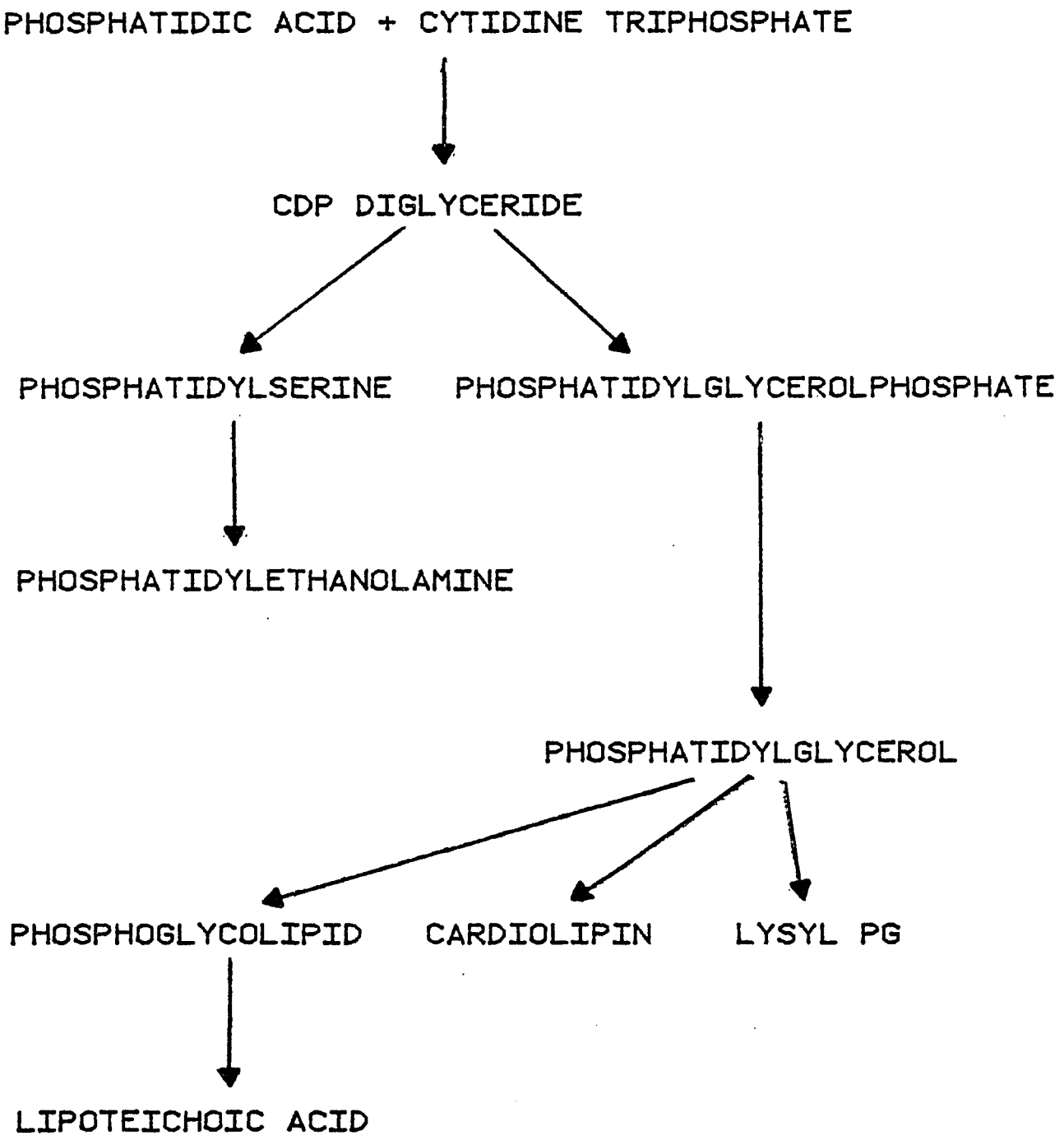

PHOSPHATIDIC ACID + CYTIDINE TRIPHOSPHATE
CDP DIGLYCERIDE
PHOSPHATIDYLSERINE
PHOSPHATIDYLGLYCEROLPHOSPHATE
PHOSPHATIDYLETHANOLAMINE
PHOSPHATIDYLGLYCEROL
MEMBRANE-DERIVED OLIGOSACHARRIDE
CARDIOLIPIN

FIGURE 2: Pathway for the biosynthesis of phospholipids in Gram-positive bacteria. The pathway, adapted from Kennedy (22), indicates the most prevalent lipids synthesized in this class of microorganism. Lipoteichoic acid, does not derive its lipid moiety from phosphatidylglycerol. Phosphatidylglycerol is believed to be the donor of glycerol phosphate to lipoteichoic acid.

FIGURE 2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CDP DIGLYCERIDE
PHOSPHATIDYLSERINE
PHOSPHATIDYLGLYCEROLPHOSPHATE
PHOSPHATIDYLETHANOLAMINE
PHOSPHATIDYLGLYCEROL
PHOSPHOGLYCOLIPID
CARDIOLIPIN
LYSYL PG
LIPOTEICHOIC ACID

FIGURE 3: Structures of common lipids present in Gram-positive microorganisms.

(A), monoglucosyldiacylglycerol

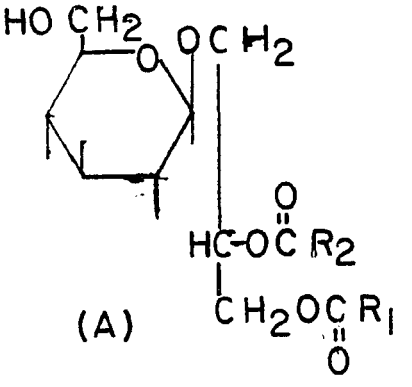
(B), B-gentiobiosyldiacylglycerol

(C), sn-glycero-3-phospho-B-gentiobiosyldiacylglycerol;

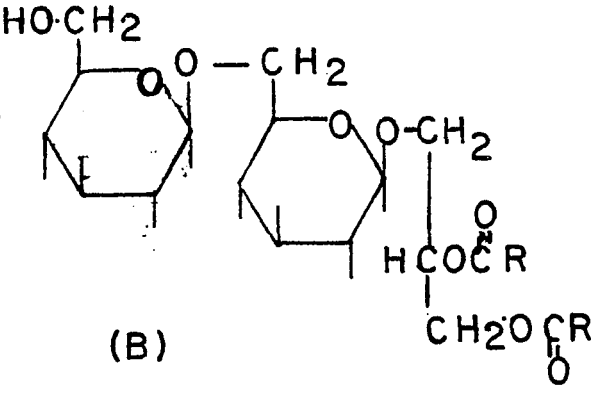
(D), lysylphosphatidylglycerol.

Lipids A and B are neutral lipids, lipids C and D are polar lipids.

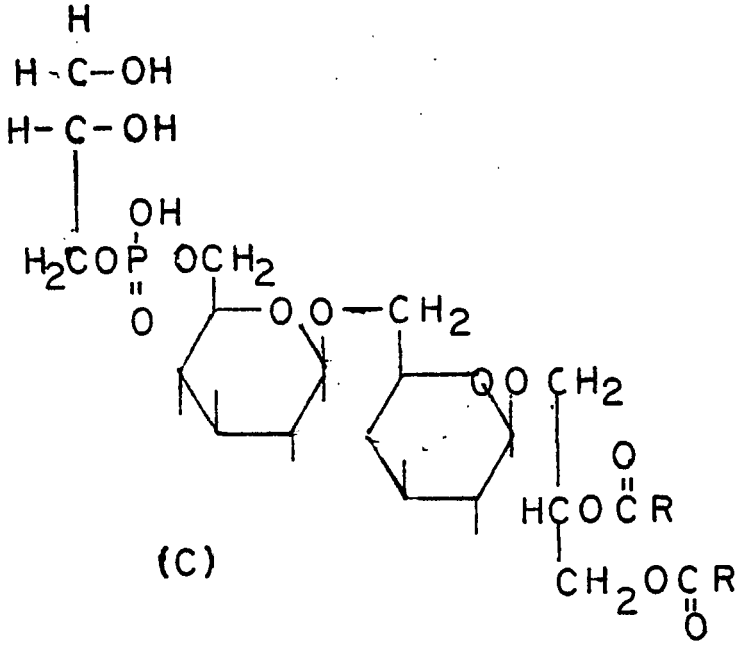
FIGURE 3



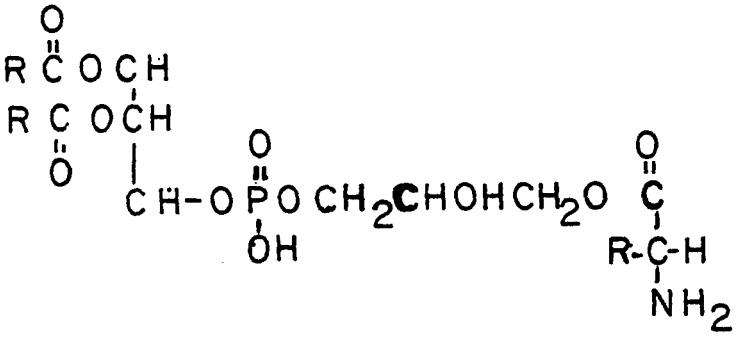
(A)



(B)



(C)



(D)

FIGURE 4: Separation of phospholipids by thin-layer chromatography. Cultures were grown to log phase on casein hydrolysate media. An aliquot was removed from the culture and incubated with [^{32}P]phosphate. Lipids were extracted as described in the text, spotted on Brinkman Silica gel G thin layer sheets, and developed in two dimensions. Diisobutylketone:acetic acid:water (8:5:1) was used in the first dimension, chloroform:acetic acid:methanol:water (80:18:12:5) in the second dimension. Lipids were visualized by developing a negative previously exposed to the radioactive chromatogram.

FIGURE 4

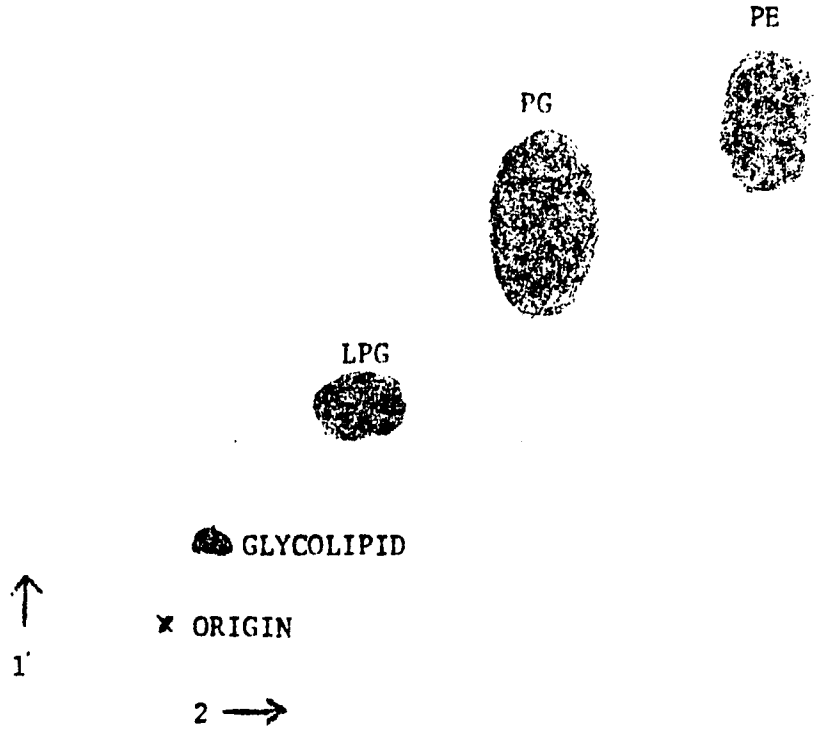


FIGURE 5: Purification of lipoteichoic acid from B. subtilis 1005 on a Sepharose 6B column. Cultures were grown as described in the Materials and Methods section. At a cell density of 20 Klett, 240 uM lithium chloride (Fig. 5A) or 240 uM DHBP (Fig. 5B) was added. After 30 minutes the cultures were incubated with 10 uC_i [^{32}P]phosphate for 15 min. The cells were collected on a millipore filter(HA 0.45u), washed once with 5 ml of medium, and resuspended in 1.5 ml of water. To this suspension 1 ml of [^{3}H]glycerol labeled cells were added, followed by 2.5 ml of 90% phenol. The lipoteichoic acid was removed from the aqueous phase after centrifugation and purified on a Sepharose 6B column as described in the Materials and Methods section. Fraction 27-38 were collected, reduced in vacuo, and counted as previously described.

Symbols: ■, [^{32}P]; ▲, [^{3}H].

FIGURE 5A

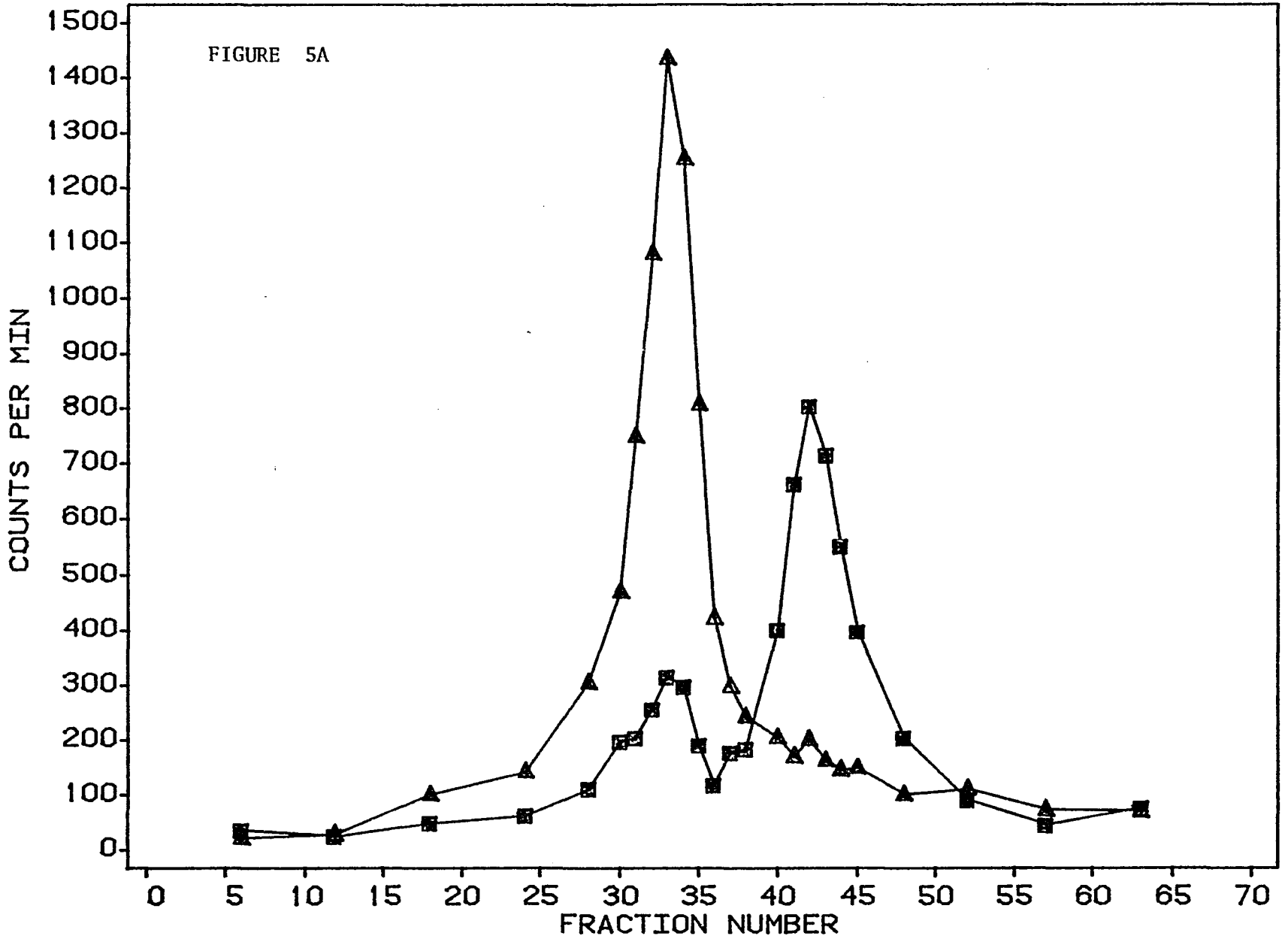
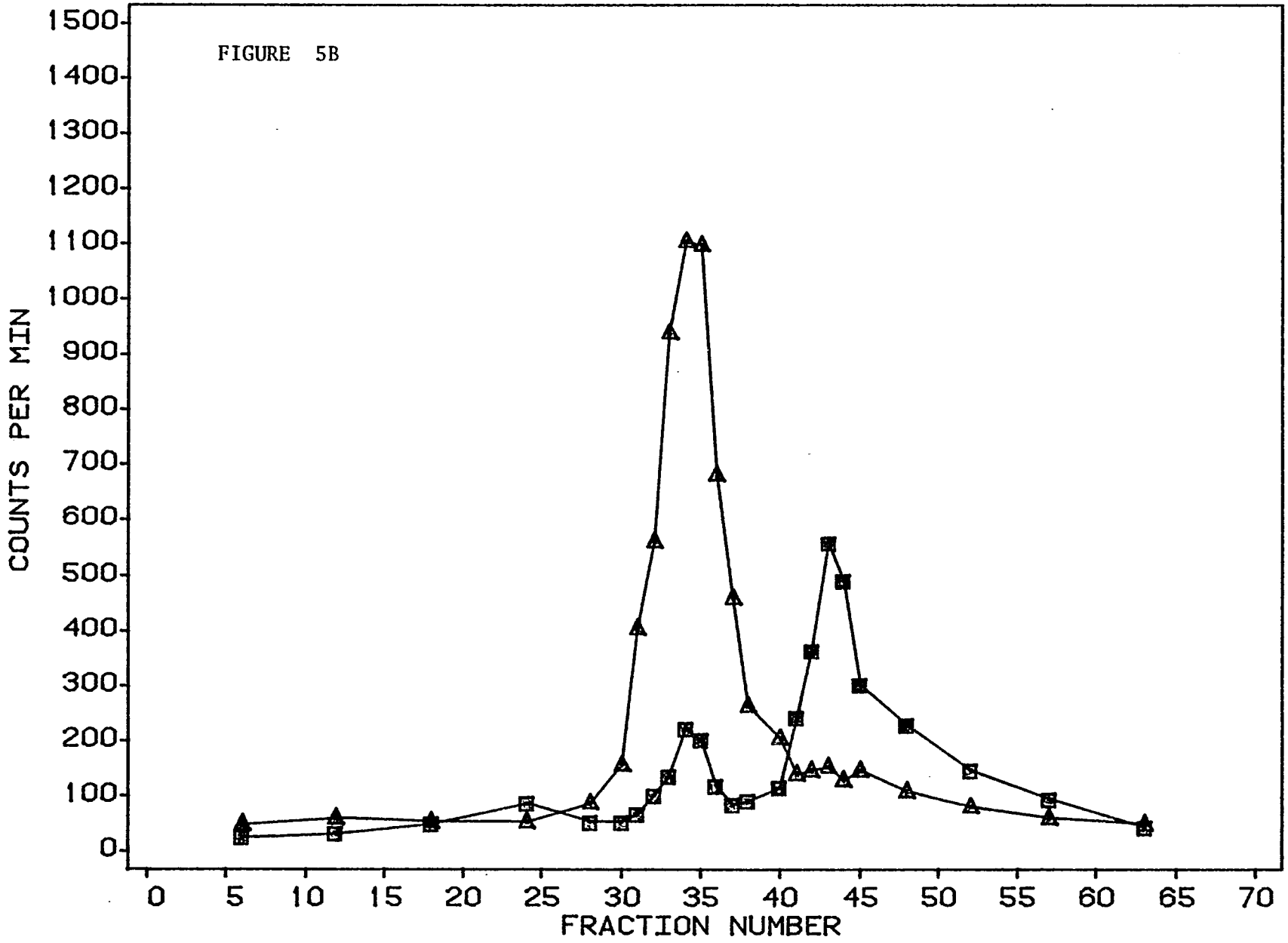

COUNTS PER MIN
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
FRACTION NUMBER

FIGURE 5B


COUNTS PER MIN
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1300
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1100
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600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
FRACTION NUMBER

FIGURE 6: Effect of removal of DHBP upon the growth of B. subtilis 1005. The culture medium was 1% casein hydrolysate, pH 7.0, and 0.5% sodium chloride. Cells were cultured in 30 ml of medium in a 250 ml side arm flask and incubated in a water bath shaker set at 150 rpm and 37 C. When a culture reached 20 Klett, DHBP was added to a final concentration of 60 uM. After 30 minutes, cells were collected on Millipore filters(HA 0.45u), washed with 0.1M Tris buffer (0 C), and a portion resuspended in preconditioned medium. Cell density was followed turbidimetrically in a Klett-Summerson colorimeter with a 600 nm filter.

Symbols: ■, untreated; ▲, 60 uM DHBP

FIGURE 6

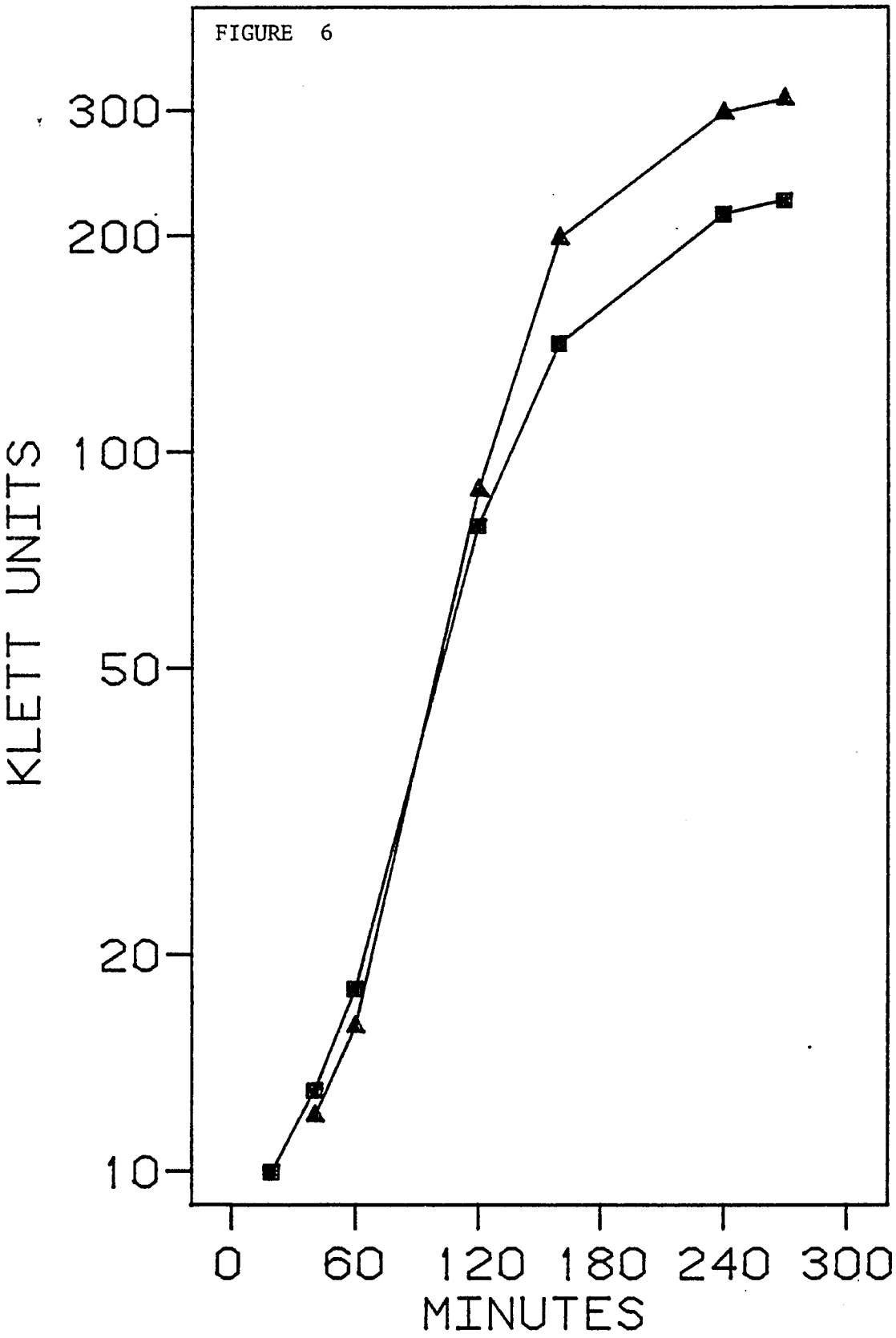

FIGURE 7: Effect of DHBP upon the growth of B. subtilis 1005. The culture medium was 1% casein hydrolysate, pH 7.0, and 0.5% sodium chloride. Cells were cultured in 30 ml of medium in a 250 ml side arm flask and incubated in a water bath shaker set at 150 rpm and 37 C. Cell density was followed turbidimetrically in a Klett-Summerson colorimeter with a 600 nm filter.

Symbols: ■, untreated cells; ●, 30 uM DHBP; ▲, 60 uM DHBP; ⬟, 120 uM DHBP; ◆, 240 uM DHBP

FIGURE 7

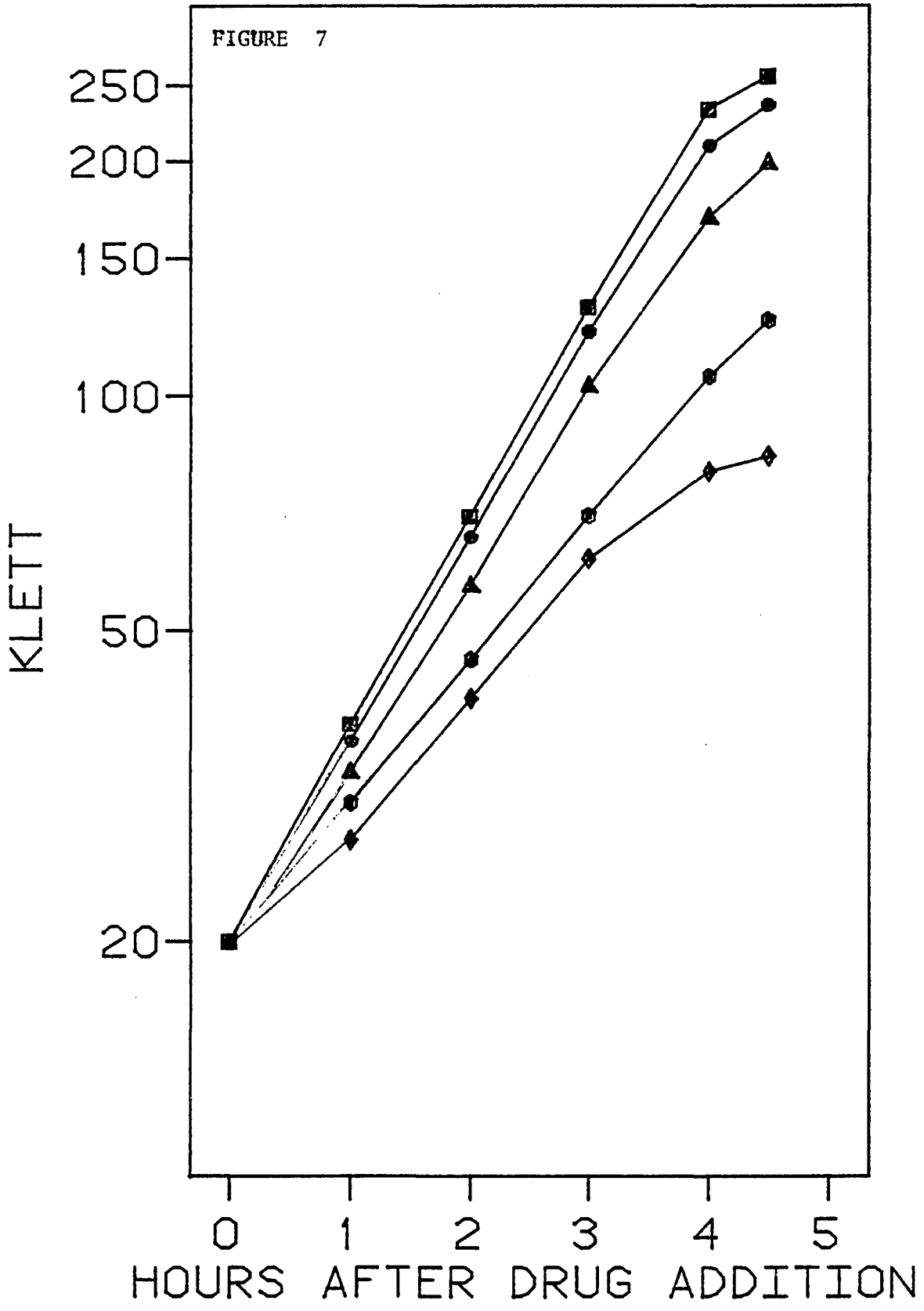

FIGURE 8: Effect of DHBP upon the growth of B. subtilis BD170. The culture medium was 1% casein hydrolysate, pH 7.0, and 0.5% sodium chloride. Cells were cultured in 30 ml of medium in a 250 ml side arm flask and incubated in a water bath shaker set at 150 rpm and 37 C. Cell density was followed turbidimetrically in a Klett-Summerson colorimeter with a 600 nm filter.

Symbols: ■, untreated cells; ●, 30 uM DHBP; ▲, 60 uM DHBP; ⬢, 120 uM DHBP; ♦, 240 uM DHBP

FIGURE 8

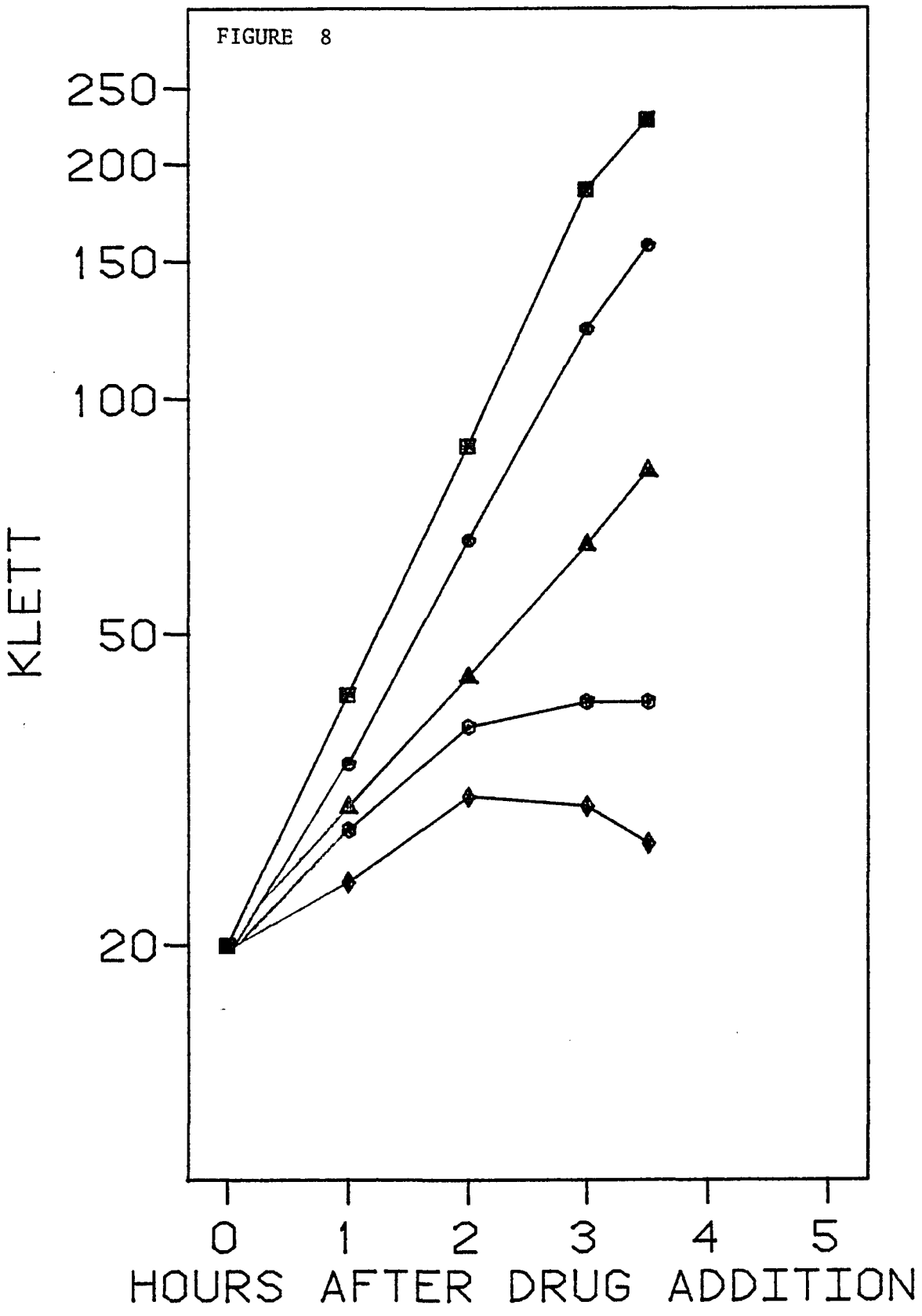

FIGURE 9: Effect of DHBP on the turbidity and cell number of a growing culture. B. subtilis were cultured as described in figure 7. DHBP was added at 20 Klett. At the indicated times thereafter cell density was monitored in a Klett-Summerson colorimeter and a sample of culture was removed. These samples were diluted with G&L minimal salts medium and plated out on petri dishes containing Eugon Agar. Colonies appearing on the plates after an overnight incubation at 37 ^{o}C were counted (Fig. 9C and Fig. 9D represent cell number; Fig. 9A and 9B indicate their corresponding klett value.

Symbols: ■,untreated cells; ▲, 60uM DHBP

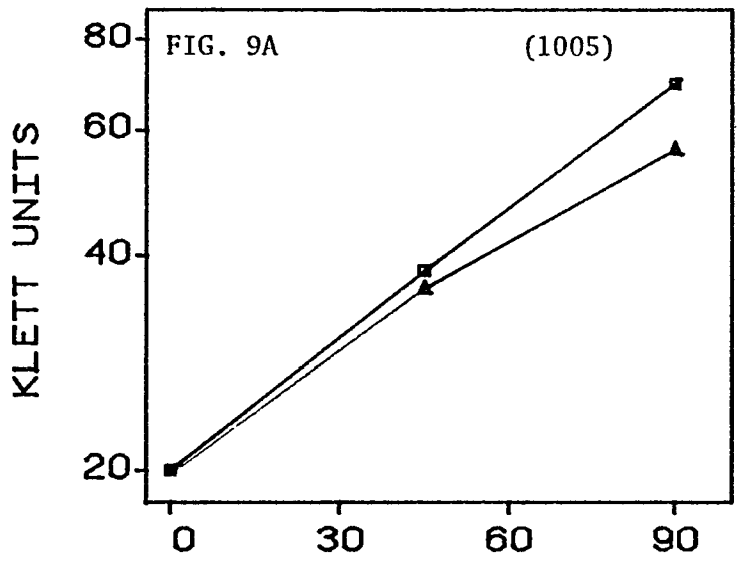
FIG. 9A
(1005)
KLETT UNITS
80
60
40
20
0
30
60
90

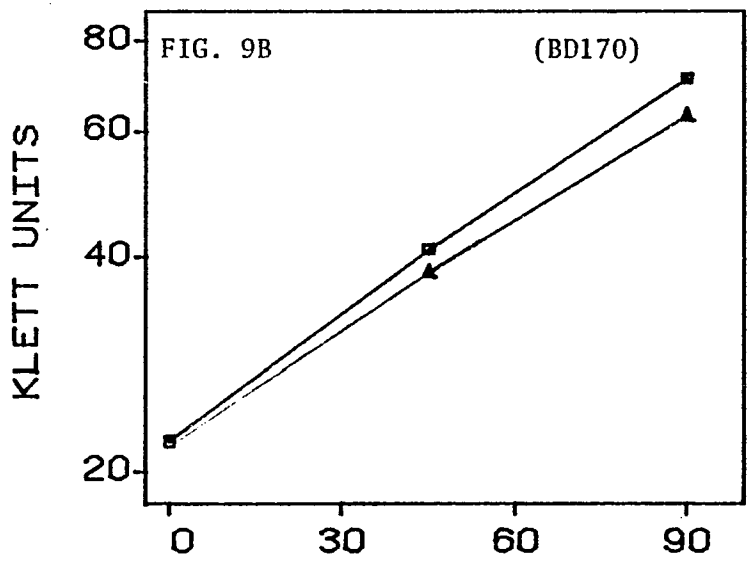
FIG. 9B
(BD170)
KLETT UNITS
80
60
40
20
0
30
60
90

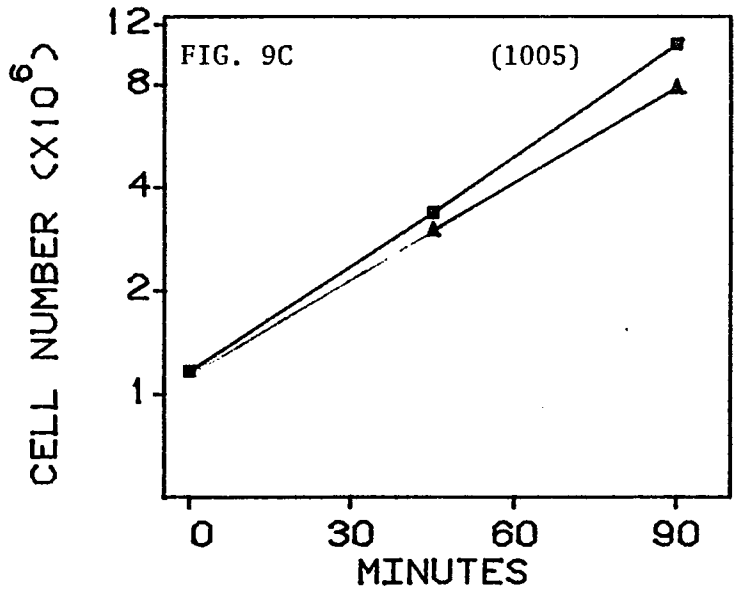
FIG. 9C
(1005)
CELL NUMBER (X10^6)
12
8
4
2
1
0
30
60
90
MINUTES

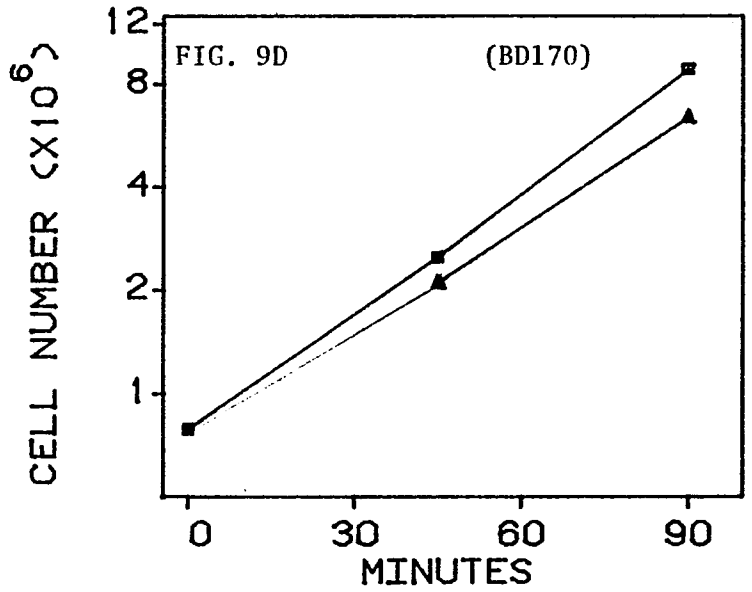
FIG. 9D
(BD170)
CELL NUMBER (X10^6)
12
8
4
2
1
0
30
60
90
MINUTES

FIGURE 10A: Effect of 3,4 dihydroxybutyl-1-phosphonate on the rate of protein, DNA,RNA, and phospholipid synthesis. B. subtilis 1005 was cultured in casein hydrolysate media as described in Fig. 7. At a cell density of 20 Klett, DHBP was added to a final concentration of 60 uM and the rate of macromolecular synthesis determined by removing 1 ml of cells at the indicated time points and incubating with 20-30 uC_i of [^{32}P]phosphate. After 8 min, samples were treated with an equal volume of ice cold 10% trichloroacetic acid (TCA) and 1 ml of sonic extract.Precipitates were spun down at 5000g, washed 2x with ice cold 5% TCA, and suspended in 5 ml of chloroform/methanol/water (5:5:1). One hour later, the sample was centrifuged. Lipids were extracted from the supernatant by adding 4.5 ml water to it to make it biphasic. After removing the aqueous phase, the chloroform layer was washed 3x with 2M KCl and once with water. Pellets were suspended in 0.5N potassium hydroxide for an overnight incubation at 37 C. After acidification with perchloric acid and centrifugation an aliquot was removed to determine RNA. The remaining DNA pellet was washed as described, dissolved in 0.2N potassium hydroxide, and counted in Patterson Greene scintillation fluid. The value, % of untreated culture, in the ordinate was obtained by dividing the incorporation observed in DHBP treated cells by that in untreated cells and

then correcting for cell density. The values for the rate of protein, DNA, RNA, and phospholipids for untreated cells at the 40 minute time point are 2,725 cpm, 8,230 cpm, 160,000 cpm, and 10,092 cpm per ml of culture respectively.

Symbols: ■, protein; ▲, DNA; ♦, RNA; ●, phospholipid.

FIGURE 10B: Effect of DHBP upon the rate of synthesis of phosphatidylglycerol, lysylphosphatidylglycerol, and phosphatidylethanolamine of B. subtilis 1005. Phospholipids were obtained as described in Fig.10A. They were chromatographed on Silica gel G thin layer plates. Diisobutylketone:acetic acid:water (8:5:1) was utilized to develop the chromatogram. Individual spots, visualized by iodine vapors, were scraped into scintillation vials and counted. The counts were corrected for cell density. The ordinate, % of untreated culture, was described in Fig. 10A . The values for the rate of synthesis of lysylphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol for untreated cells at the 40 minute time point are 825 cpm, 1,892 cpm, and 6,625 cpm per ml of culture respectively.

Symbols: ♦, lysylphosphatidylglycerol; ▲, phosphatidylethanolamine; ■, phosphatidylglycerol.

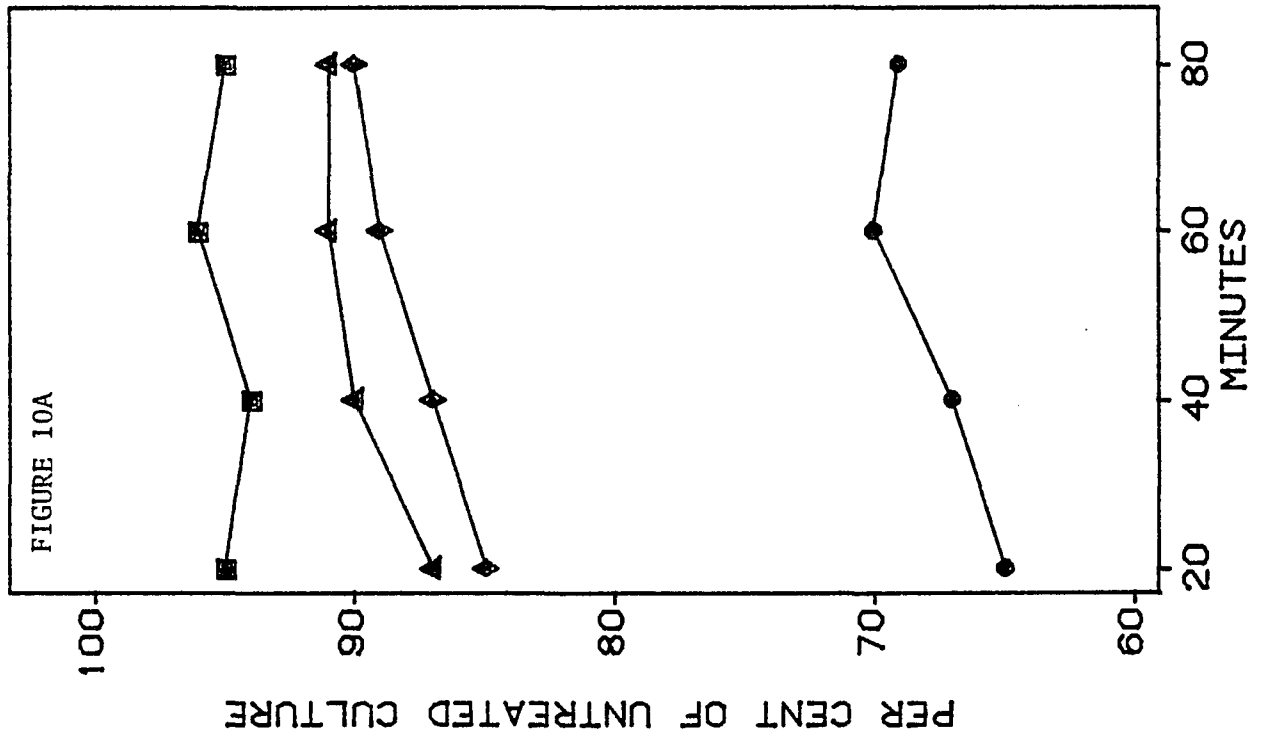
FIGURE 10A
PER CENT OF UNTREATED CULTURE
100
90
80
70
60
20
40
60
80
MINUTES

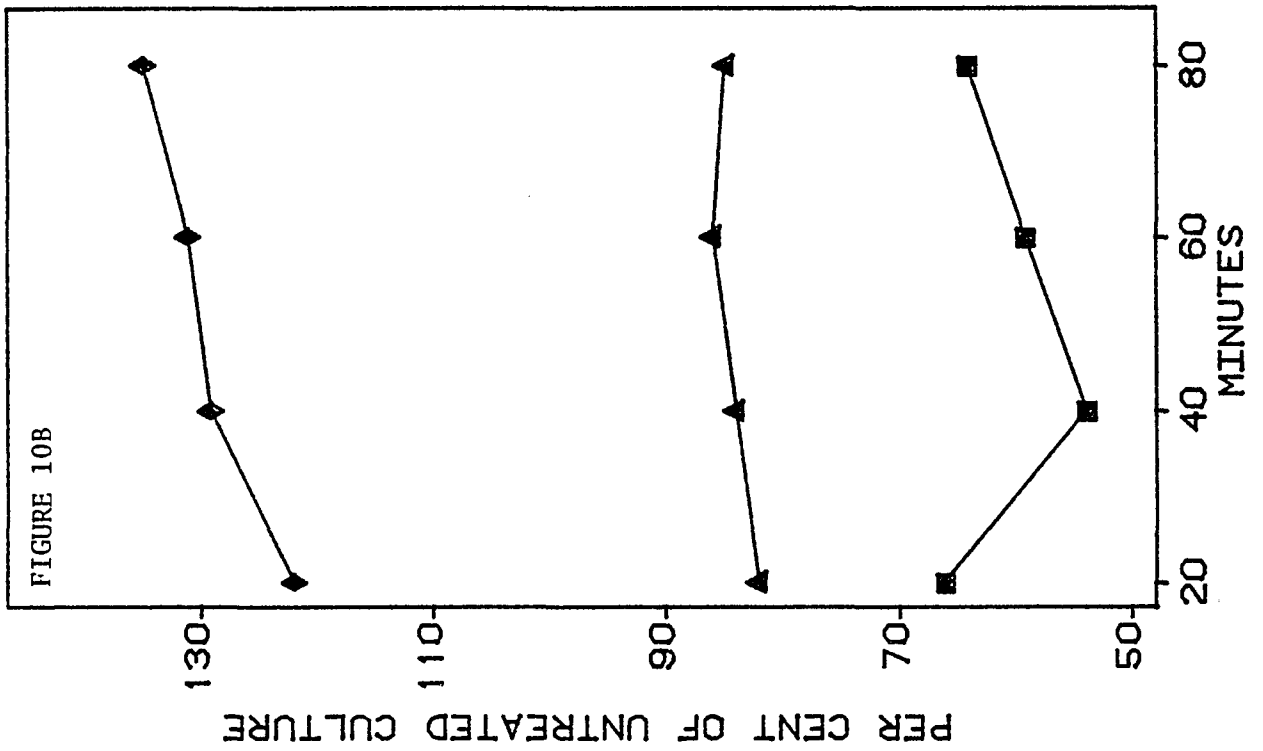
FIGURE 10B
PER CENT OF UNTREATED CULTURE
130
110
90
70
50
20
40
60
80
MINUTES

FIGURE 11A: Effect of 3,4 dihydroxybutyl-1-phosphonate on the rate of protein, DNA,RNA, and phospholipid synthesis. B. subtilis ED170 were cultivated in casein hydrolysate media as described in Fig. 7. At a cell density of 20 Klett, DHBP was added to a final concentration of 60 uM and the rate of macromolecular synthesis determined by removing 1 ml of cells at the indicated times and incubating with 20-30 uC_i of [^{32}P]phosphate. After 8 min, samples were treated with an equal volume of ice cold 10% trichloroacetic acid (TCA) and 1 ml of sonic extract. Precipitates were spun down at 5000 g, washed 2x with ice cold 5% TCA, and suspended in 5 ml of chloroform/methanol/water (5:5:1). One hour later, the sample was centrifuged. Lipids were extracted from the supernatant as described in Fig. 10A. Pellets were suspended in 0.5N potassium hydroxide for an overnight incubation at 37 C. After acidification with perchloric acid and centrifugation an aliquot was removed to determine RNA. The remaining DNA pellet was washed as described, dissolved in 0.2N potassium hydroxide, and counted in Patterson Greene scintillation fluid. The value, % of untreated culture, in the ordinate was obtained by dividing the incorporation observed in DHBP treated cells by that in untreated cells and then correcting for cell density. The values for the rate of protein, DNA, RNA, and phospholipids for untreated cells at the 40 minute time

point are 1,816 cpm, 6,680 cmp,146,8940 cpm, and 9,012 cpm per ml of culture respectively.

Symbols: ■, protein; ♦, DNA; ▲, RNA; ●, phospholipid.

FIGURE 11B: Effect of DHBP upon the rate of synthesis of phosphatidylglycerol, lysylphosphatidylglycerol, and phosphatidylethanolamine of B. subtilis BD170. Phospholipids were obtained as described in Fig.10A. They were chromatographed on thin layer Silica gel G plates. Diisobutylketone:acetic acid:water (8:5:1) was utilized to develop the chromatogram.

Individual spots, visualized by iodine vapors, were scraped into scintillation vials and counted. The counts were corrected for cell density. The ordinate, % of untreated culture, was described in Fig. 10A . The values for the rate of synthesis of lysylphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol for untreated cells at the 40 minute time point are 1,068 cpm, 1,730 cpm, and 6,217 cpm per ml of culture respectively

Symbols: ♦, lysylphosphatidylglycerol; ▲, phosphatidylethanolamine; ■, phosphatidylglycerol

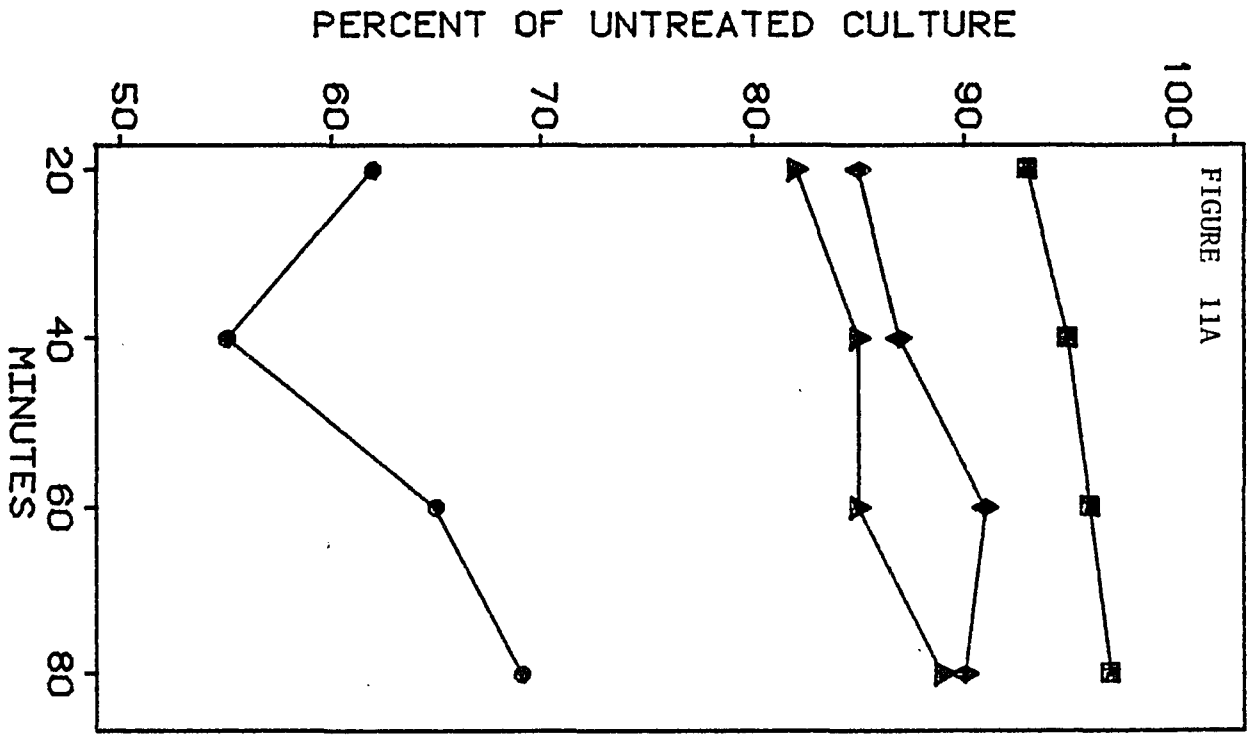

FIGURE 11A

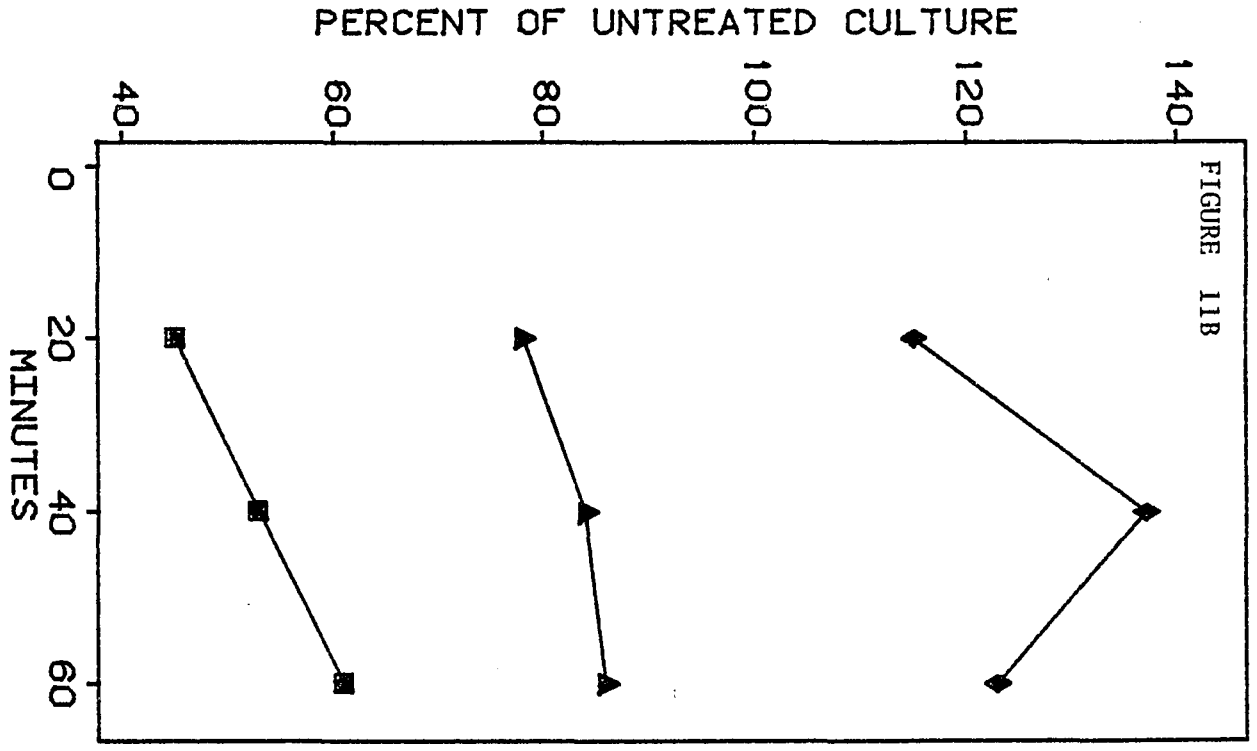

FIGURE 11B

FIGURE 12: Turnover of phospholipids in the presence of DHBP. Strain 1005 was cultured as described in fig. 7. The culture was started at a turbidity of 5 Klett units. At 10 Klett units, [^{32}P]phosphate was added. At a density of 20-25 Klett units, cells were collected on Millipore filters(HA 0.45u), washed with minimal salts medium, and resuspended in pre-conditioned medium. One culture was made 60 uM DHBP, the other 120 uM lithium chloride. Lipids were extracted at various time points and counted as described under "Materials and Methods".

Symbols: ■, untreated; ▲, 60 uM DHBP

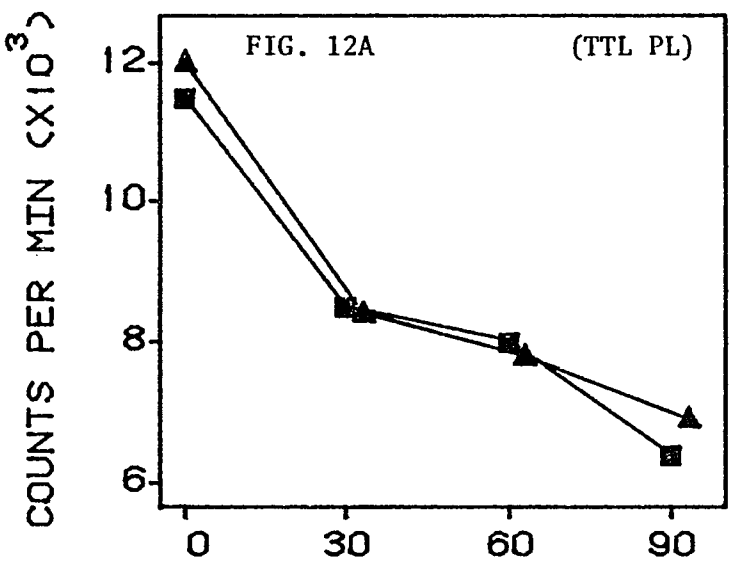
FIG. 12A
(TTL PL)
COUNTS PER MIN (X10^3)
12
10
8
6
0
30
60
90

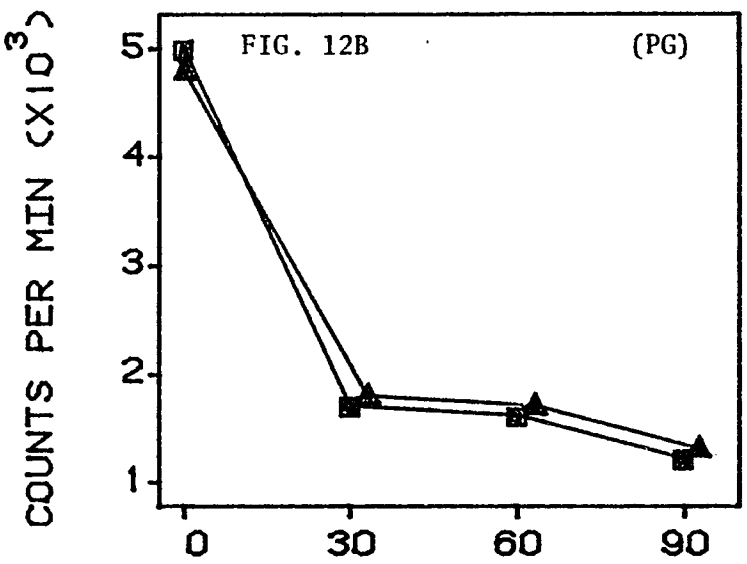
FIG. 12B
(PG)
COUNTS PER MIN (X10^3)
5
4
3
2
1
0
30
60
90

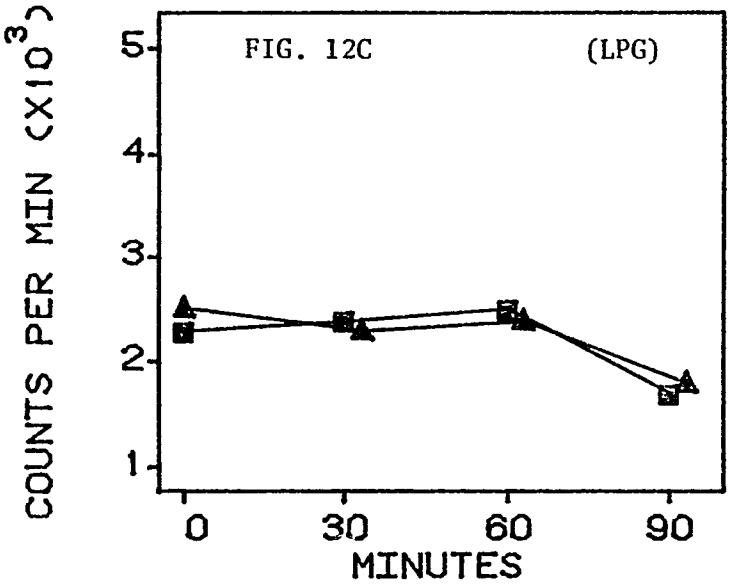
FIG. 12C
(LPG)
COUNTS PER MIN (X10^3)
5
4
3
2
1
0
30
60
90
MINUTES

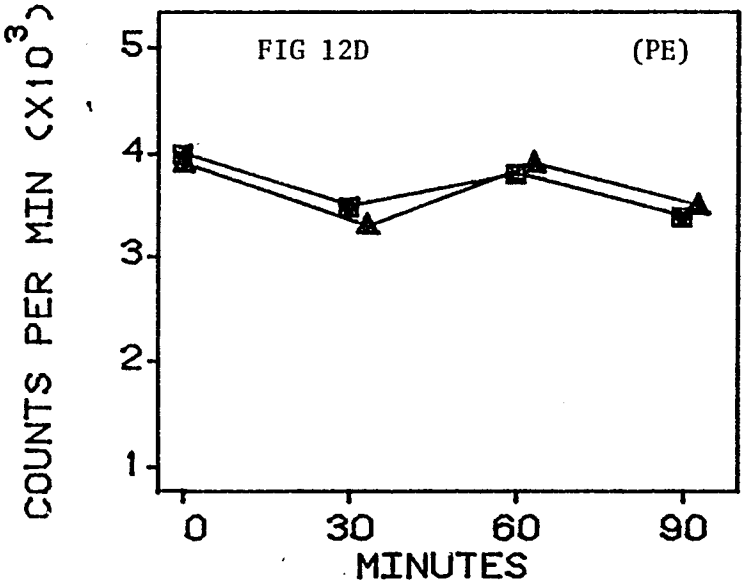
FIG 12D
(PE)
COUNTS PER MIN (X10^3)
5
4
3
2
1
0
30
60
90
MINUTES

FIGURE 13: Turnover of phospholipids in the presence of DHBP. Strain BD170 was cultured as described in fig. 7. The culture was started at a turbidity of 5 Klett units. At 10 Klett units, [^{32}P]phosphate was added. At a density of 20-25 Klett units, cells were collected on Millipore filters(HA 0.45u), washed with minimal salts medium, and resuspended in pre-conditioned medium. One culture was made 60 uM DHBP, the other 120 uM lithium chloride. Lipids were extracted at various time points and counted as described under "Materials and Methods".

Symbols: ■, untreated: ▲, 60 uM DHBP

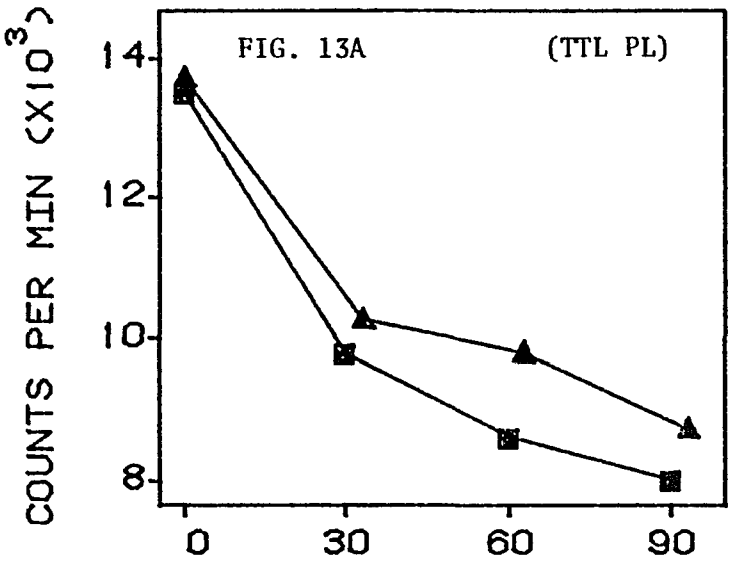
FIG. 13A
(TTL PL)
COUNTS PER MIN (X10^3)
14
12
10
8
0
30
60
90

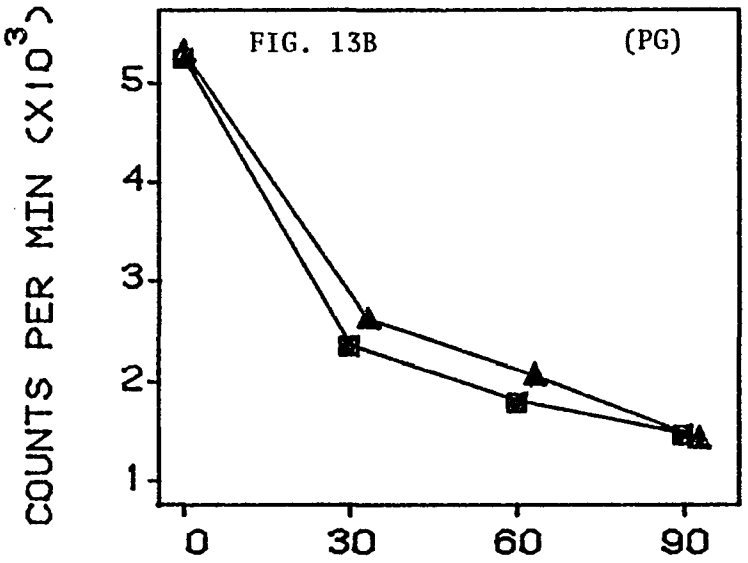
FIG. 13B
(PG)
COUNTS PER MIN (X10^3)
5
4
3
2
1
0
30
60
90

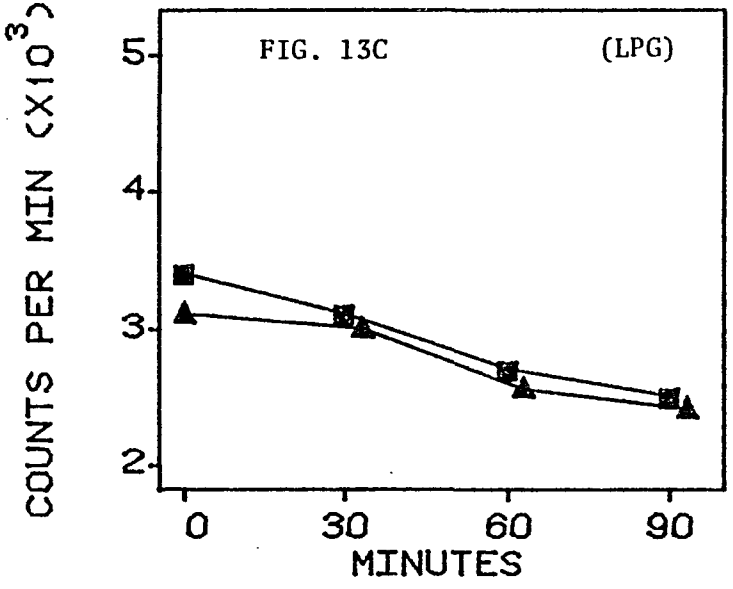
FIG. 13C
(LPG)
COUNTS PER MIN (X10^3)
5
4
3
2
0
30
60
90
MINUTES

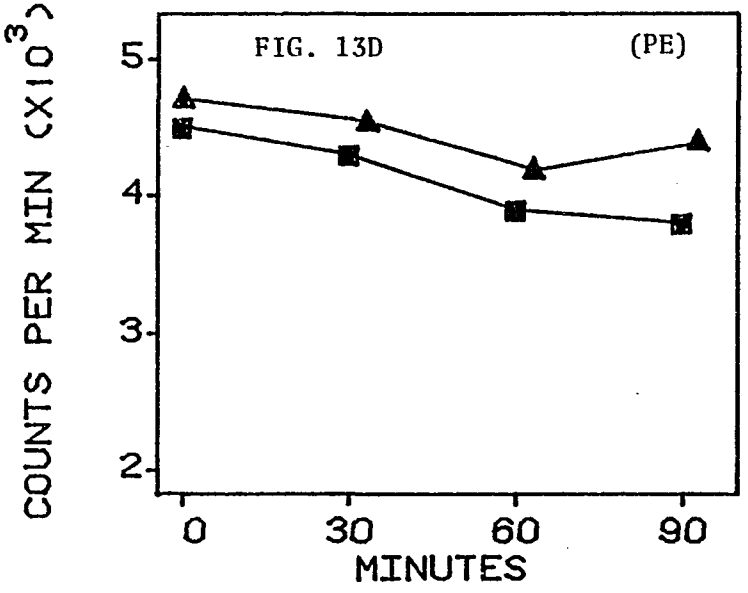
FIG. 13D
(PE)
COUNTS PER MIN (X10^3)
5
4
3
2
0
30
60
90
MINUTES

FIGURE 14: Accumulation of phospholipids during treatment with DHBP in B. subtilis 1005. Cells were cultured in 30 ml of medium as described in Fig. 7. At a cell density of 10 Klett [^{32}P]phosphate was added to the cultures. After a 1 hour incubation with label, DHBP was added. Samples were removed at the indicated times thereafter. Lipids were extracted and an aliquot taken for counting in toluene scintillation fluid . The amount of each individual lipid present was determined after separating the lipids on thin layer chromatography plates, scraping the individual lipid into scintillation vials and counting

Symbols: ■, untreated; ▲, 60 uM DHBP.

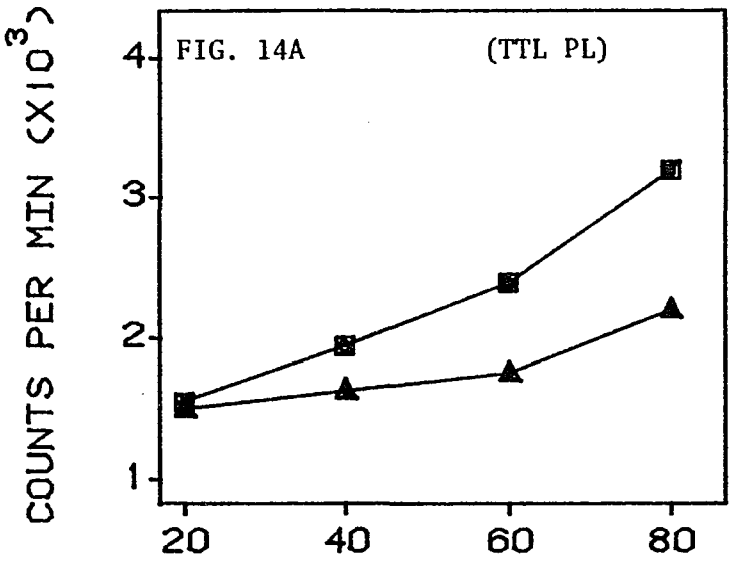
FIG. 14A
(TTL PL)
COUNTS PER MIN (X10^3)
4
3
2
1
20
40
60
80

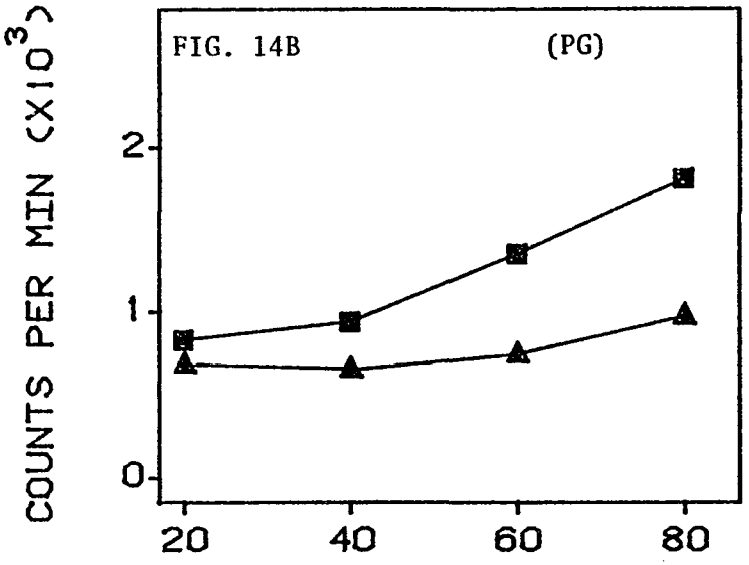
FIG. 14B
(PG)
COUNTS PER MIN (X10^3)
2
1
0
20
40
60
80

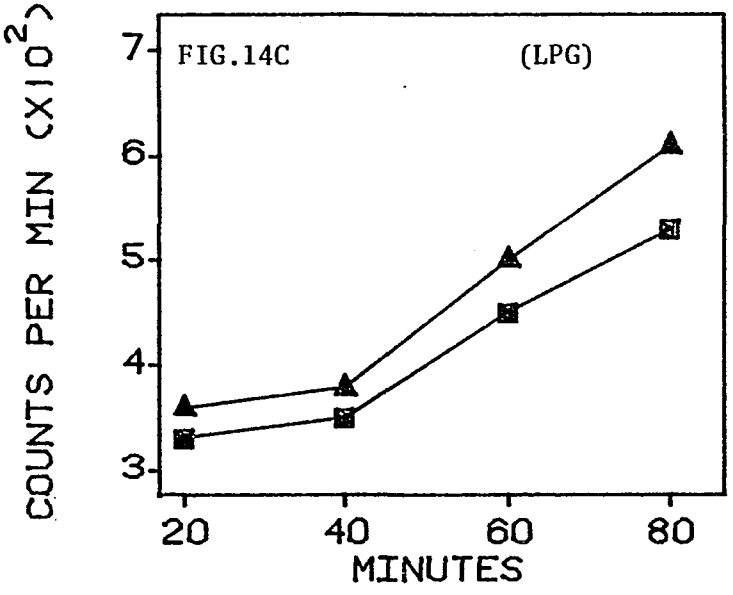
FIG.14C
(LPG)
COUNTS PER MIN (X10^2)
7
6
5
4
3
20
40
60
80
MINUTES

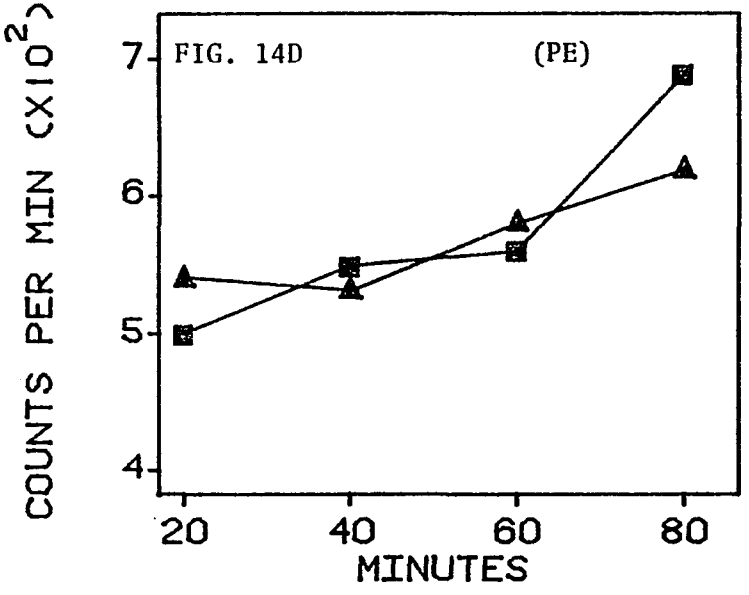
FIG. 14D
(PE)
COUNTS PER MIN (X10^2)
7
6
5
4
20
40
60
80
MINUTES

FIGURE 15: Accumulation of phospholipids during treatment with DHBP in B. subtilis BD170. Cells were cultured in 30 ml of medium as described in Fig. 7. At a cell density of 10 Klett [^{32}P]phosphate was added to the cultures. After a 1 hour incubation with label, DHBP was added. Samples were removed at the indicated times thereafter. Lipids were extracted and an aliquot taken for counting in toluene scintillation fluid . The amount of each individual lipid present was determined after separating the lipids on thin layer chromatography plates, scraping the individual lipid into scintillation vials and counting

Symbols: ■, untreated; ▲, 60 uM DHBP.

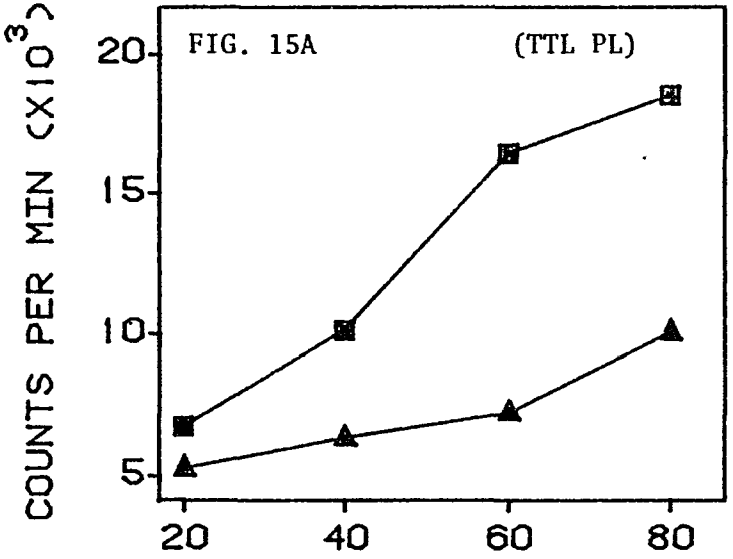
FIG. 15A
(TTL PL)
COUNTS PER MIN (X10^3)
20
15
10
5
20
40
60
80

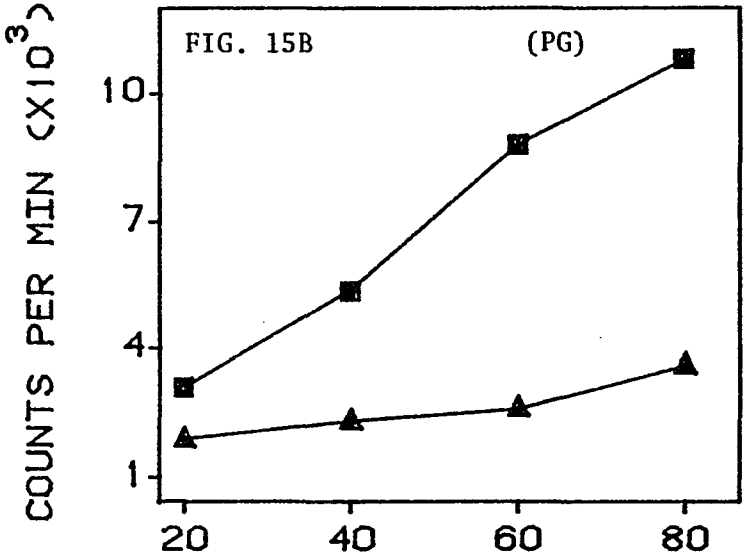
FIG. 15B
(PG)
COUNTS PER MIN (X10^3)
10
7
4
1
20
40
60
80

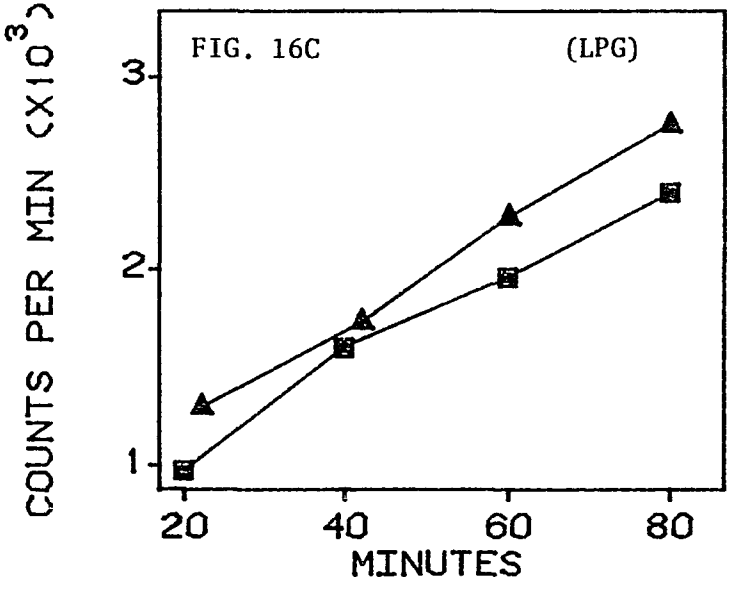
FIG. 16C
(LPG)
COUNTS PER MIN (X10^3)
3
2
1
20
40
60
80
MINUTES

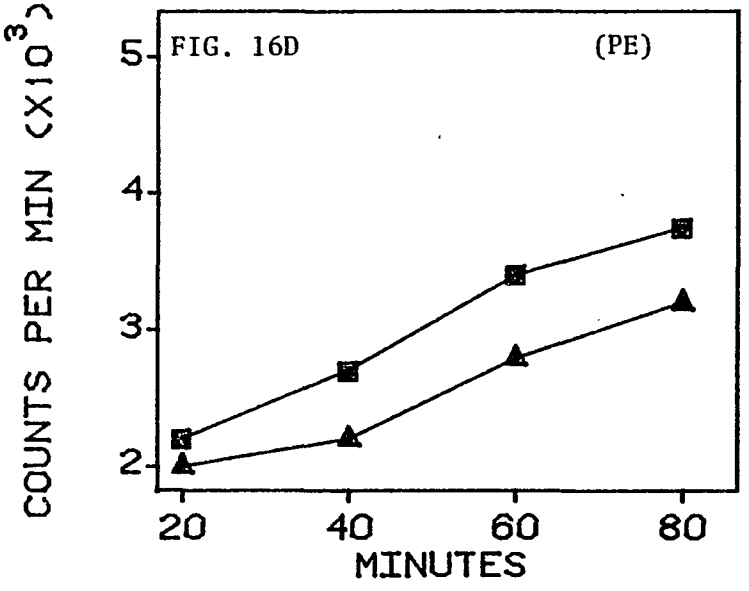
FIG. 16D
(PE)
COUNTS PER MIN (X10^3)
5
4
3
2
20
40
60
80
MINUTES

FIGURE 16: Formation and turnover of (1,2-diacyl)-sn-glyceryl-S-phosphoroloxy-3-hydroxylbuyy-1-1- phosphonate. Cells were cultured as described in Fig. 7. At a cell density of 15 Klett units, [2-^{3}H]DHBP (1.0 uC_i per ml; .06mM) was added to the culture for a 60 minute incubation period. The labeled precursor was removed by collecting cells on Millipore filters(HA 0.45u) and washing with 0.1M Tris buffer (0 C) at the time indicated by the arrow. Cells were resuspended in preconditioned medium. Samples of culture were taken at time points before and after removing label. Lipid was extracted and counted as described under "Materials and Methods". The cpm values were converted to micromoles per ml.

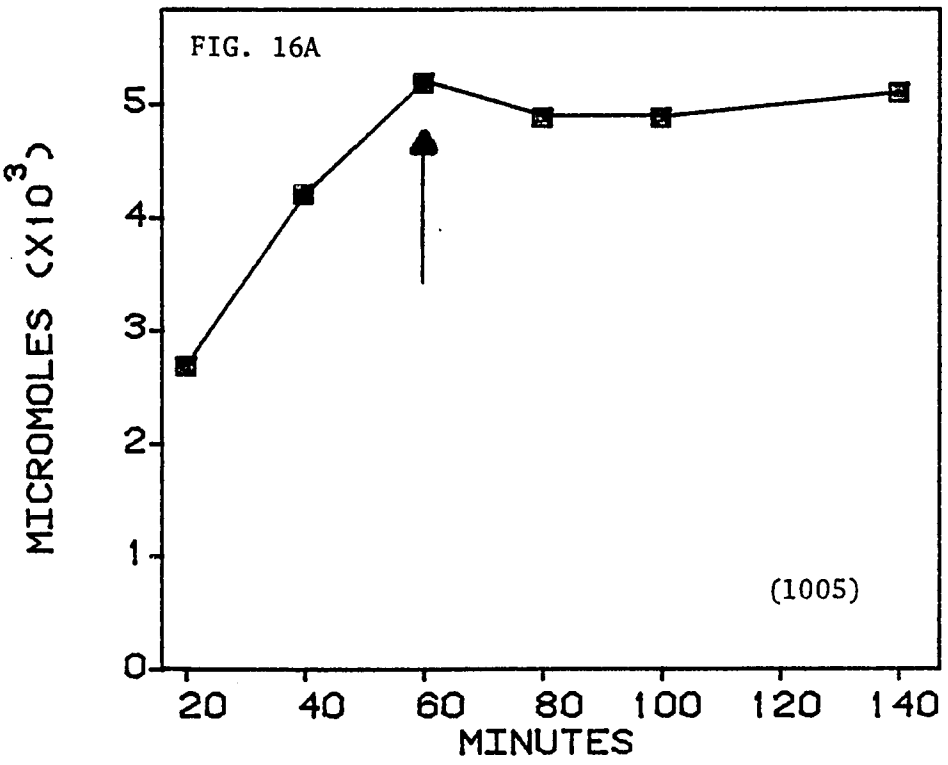
FIG. 16A
MICROMOLES (X10^3)
0
1
2
3
4
5
20
40
60
80
100
120
140
MINUTES
(1005)

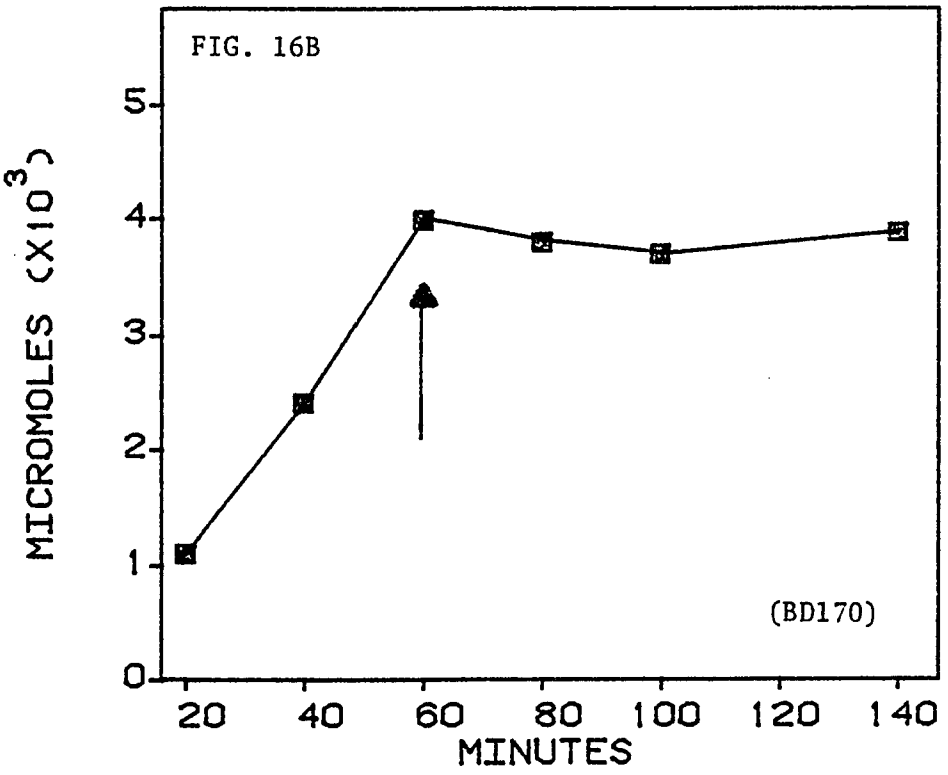
FIG. 16B
MICROMOLES (X10^3)
0
1
2
3
4
5
20
40
60
80
100
120
140
MINUTES
(BD170)

FIGURE 17: Effect of DHBP on bacteriophage 025 adsorbtion to cell walls of B. subtilis. Cultures were grown in 320 ml of casein hydrolysate medium. At a turbidity of 25 Klett units 20 umoles of DHBP or 40 umoles of lithium chloride were added. After 60 minutes, cells were collected by centrifugation. the walls were extracted by sonicating whole cells, then sequentially separating unbroken cells from whole cells by centrifugation. Varying concentrations of cell wall were incubated with bacteriophage 025 for 10 minutes at 30 °C under bubbling aeration. The mix was centrifuged and a portion of the supernatant was taken for incubation with B. subtilis cells. After 10 minutes at 37 C, top agar was added. The whole gemisch was poured onto a petri dish and spread homogeneously. Plauqes were counted after an overnight incubation at 37 C.

Symbols: ■,untreated; ▲,60uM DHBP

FIGURE 17

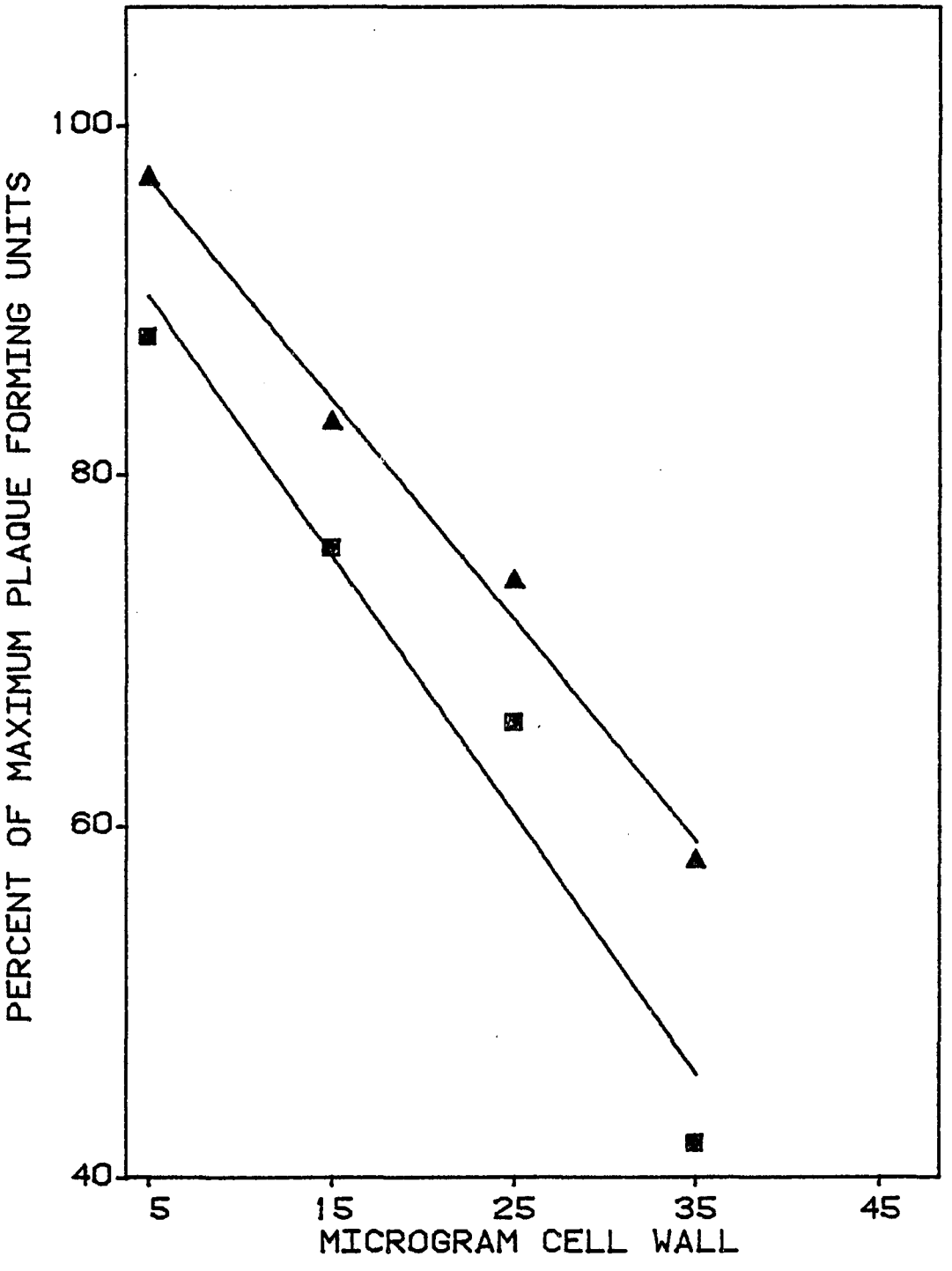
PERCENT OF MAXIMUM PLAQUE FORMING UNITS
100
80
60
40
5
15
25
35
45
MICROGRAM CELL WALL

FIGURE 18: Separation of phospholipids by thin-layer chromatography. Cultures were grown to log phase on casein hydrolysate media. Lipids were extracted and mixed together with lipids extracted from *B. subtilis* 168 cells obtained commercially. Lipids were spotted on thin layer plates. After developing with the indicated solvent system, lipids were visualized with a phosphorous-sensitive reagent (79). Spots were marked off and a tracing made of the positions occupied by the various phospholipids.

FIGURE 18

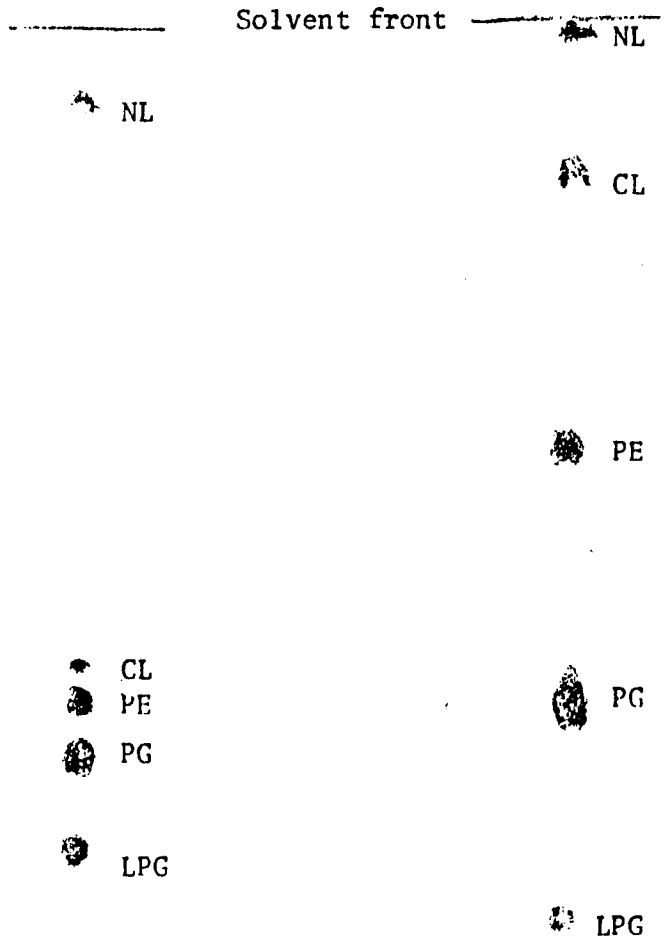
Solvent front
NL
NL
CL
PE
CL
PE
PG
PG
LPG
LPG
origin
origin
Diisobutylkeytone/HAc/H_2O
(8:5:1)
$CHCl_3$:HAc:CH_3OH:H_2O
(80:18:12:5)

FIGURE 19: Rate of phospholipid synthesis as a function of cell turbidity. Cultures were grown as described in Fig. 7. Samples were removed at various cell densities and pulsed with [^{32}P]phosphate for 8 minutes. Phospholipids were extracted from whole cells and counted as was described for accumulation and turnover studies in the Materials and Methods section.

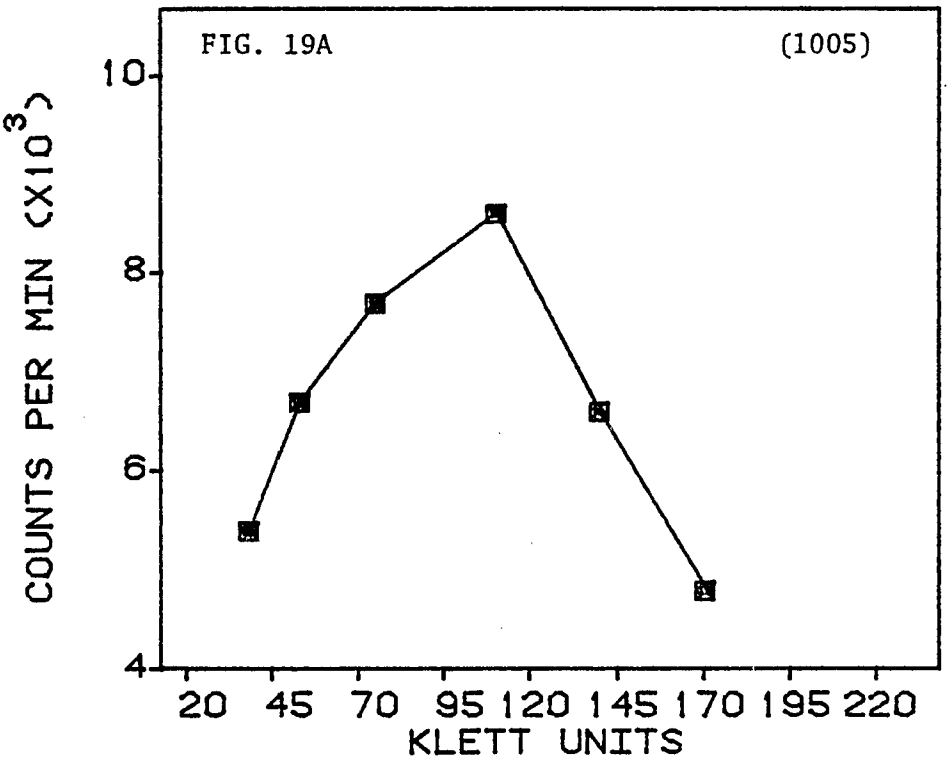
FIG. 19A
(1005)
COUNTS PER MIN (X10^3)
10
8
6
4
20 45 70 95 120 145 170 195 220
KLETT UNITS

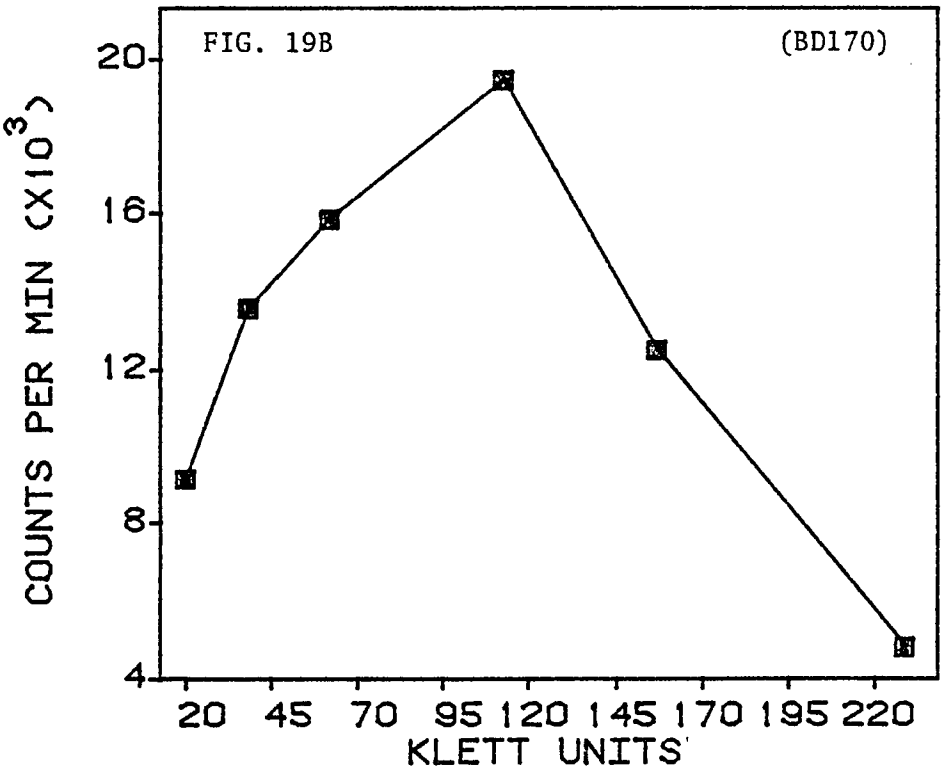
FIG. 19B
(BD170)
COUNTS PER MIN (X10^3)
20
16
12
8
4
20 45 70 95 120 145 170 195 220
KLETT UNITS

FIGURE 20: Rate of synthesis of different phospholipid classes after removal of drug from B. subtilis. When a culture reached 20 Klett, DHBP was added to a final concentration of 60 uM. After 30 minutes, cells were collected on Millipore filters(HA 0.45u), washed with 0.1M Tris buffer (0 C), and suspended in preconditioned medium. Samples were removed at various time points thereafter. Lipids were extracted as described. The amount of each individual lipid present was determined after separating the lipids on thin layer chromatography plates, scraping into scintillation vials, and counting. The data was treated as described in Fig. 10A.

Symbols: ■, lysylphosphatidylglycerol; ▲, phosphatidylplycerol; ◆, phosphatidylethanolamine.

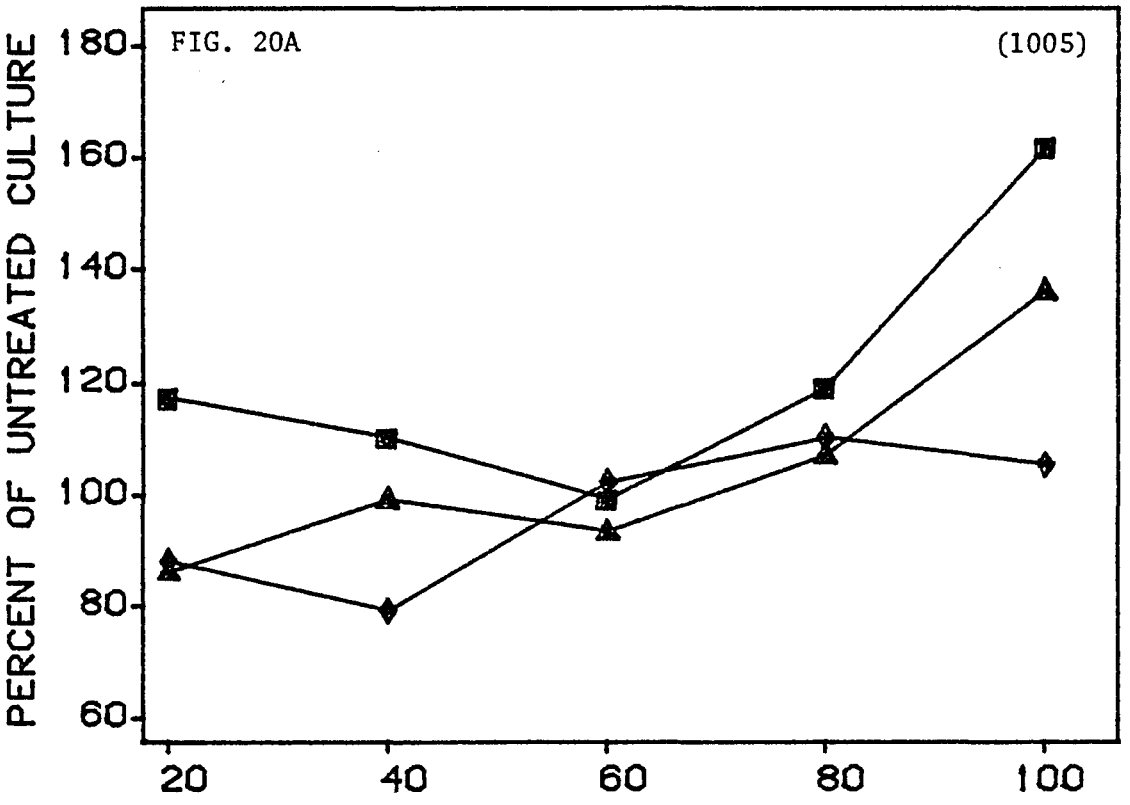
FIG. 20A
(1005)
PERCENT OF UNTREATED CULTURE
180
160
140
120
100
80
60
20
40
60
80
100

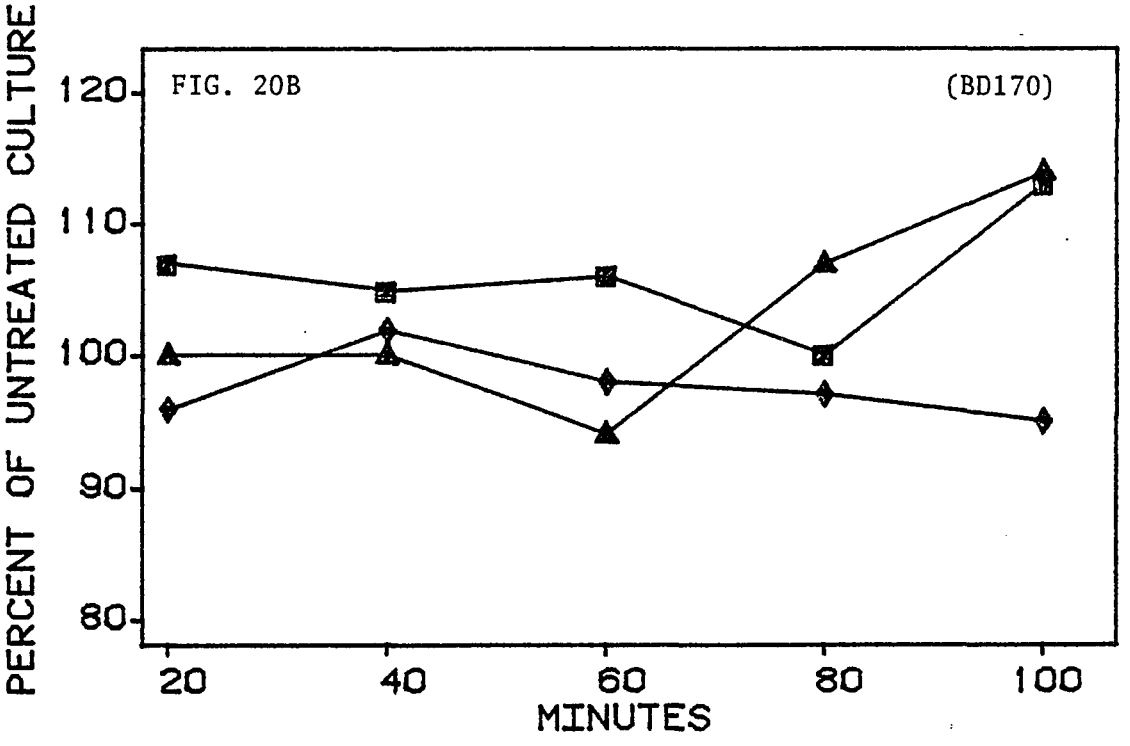
FIG. 20B
(BD170)
PERCENT OF UNTREATED CULTURE
120
110
100
90
80
20
40
60
80
100
MINUTES

FIGURE 21A: The rate of protein, DNA, RNA, phospholipid, and lipoteichoic acid synthesis of B. subtilis 1005 as a function of DHBP concentration. At a cell density of 20 Klett units, varying amounts of drug were added to each culture. After 30 minutes, samples were removed from each culture and the rate of synthesis determined as described in the Materials and Methods section. The determination of lipotheichoic acid synthesis is described in Fig. 5. The values for the rate of synthesis of protein, DNA, RNA, and phospholipid for untreated cells are 3,755 cpm, 4,563 cpm, 83,910 cpm, and 4,893 cpm per ml of culture respectively.

Symbols: ■, protein; ◆, RNA; ▲, DNA; ●, lipoteichoic acid; ⬢, phospholipid.

FIGURE 21B: The rate of synthesis of different phospholipid classes of B. subtilis 1005 as a function of DHBP concentration. Phospholipids were extracted and chromatographed as described in the Materials and Methods section. Individual spots visualized by iodine vapors were scraped into scintillation vials and counted. The ordinate, % of untreated culture, was described in Fig. 10A. The values for the rate of synthesis of lysylphosphatidylglycerol, phosphatidylethanolamine, and

phosphatidylglycerol for untreated cells are 510 cpm, 1,015 cpm, 3,367 cpm per ml of culture respectively.

Symbols: ♦ ,lysylphosphatidylglycerol; ▲,phosphatidylethanolamine; ■, phosphatidylglycerol.

FIG. 21A

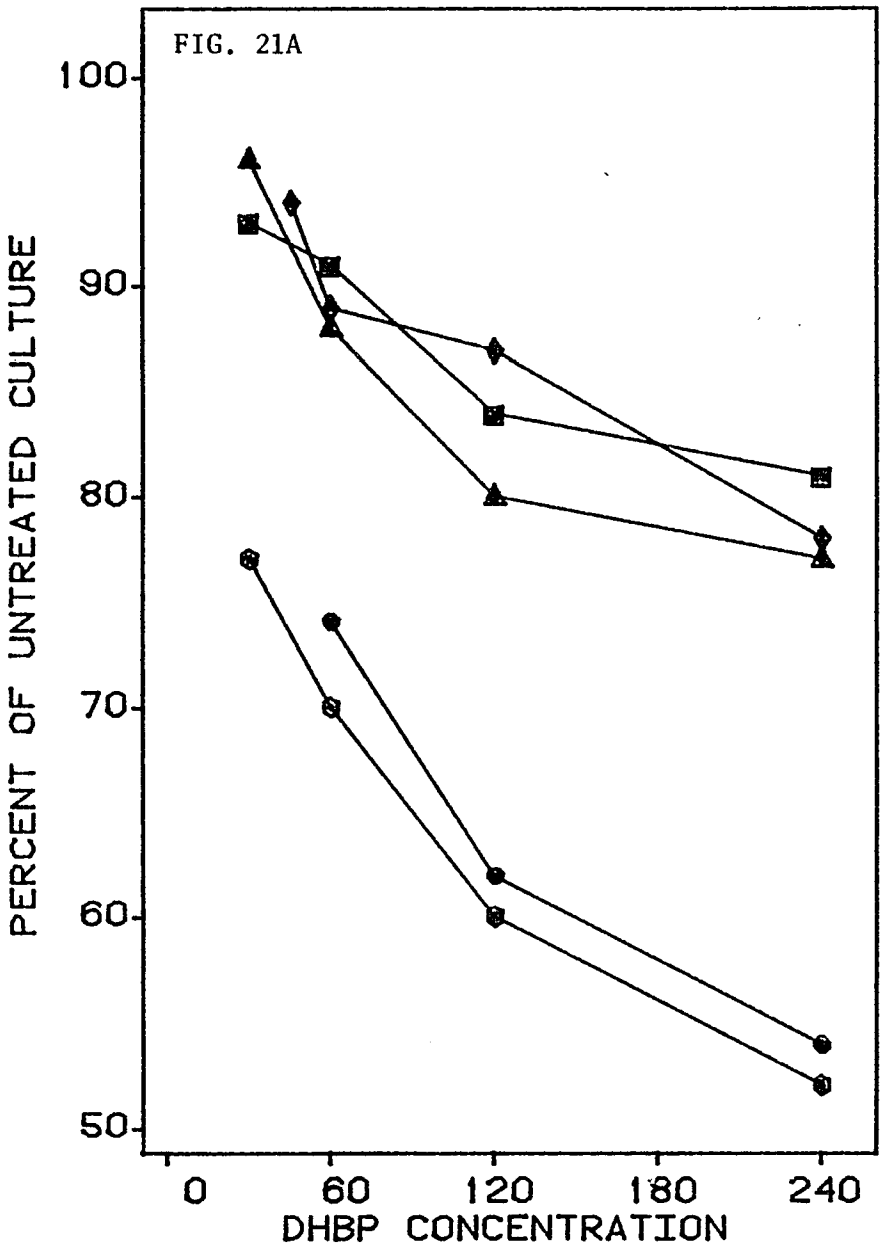

FIG. 21B

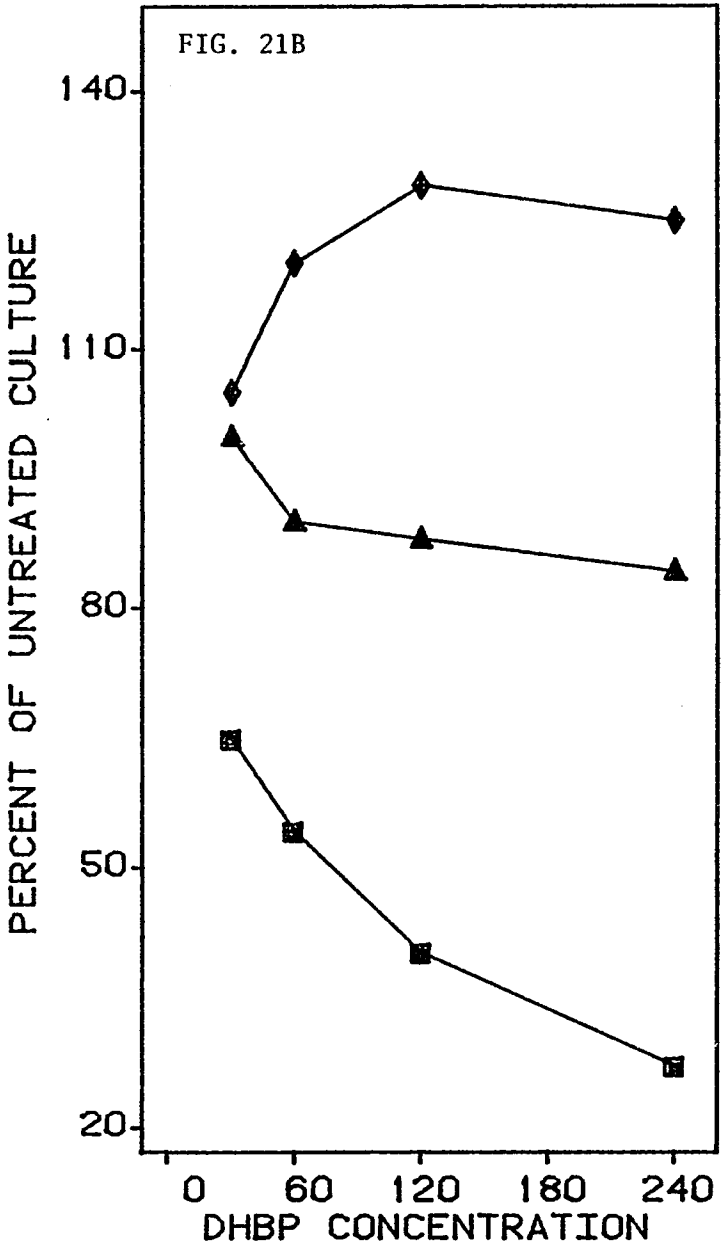

TABLE 1. Effect of DHBP on phosphoglycolipid and cardiolipin synthesis. Cultures were grown as described in Fig. 7. Cultures at 20 Klett units were treated with either 60 uM DHBP or 120 uM LiCl. After 30 minutes, a 2 ml aliquot was incubated together with 0.5 uC_i [^{32}P]phosphate for 10 minutes. Phospholipids were extracted and chromotographed as described in the Materials and Methods section.

sn-Glycero-1-phospho-B-gentiobiosydiacylglycerol was identified by its migration properties on Silica gel G developed in the following solvent systems:

1. Chloroform:Acetone:Methanol:AceticAcid:Water (50:25:25:10:5)
2. Chlororoform:Methanol:Ammonia:Water (50:35:5:5)
3. Chloroform:Acetone:Methanol:Acetic Acid:Water (50:20:10:10:5)
4. Chloroform:Methanol:Ammonia:Water (65:35:4:4)

In each case the glycolipid migrated in the same fashion as authentic standard. The R_f values in solvents 1 and 2 are 0.67 and 0.51 respectively. Fischer et. al. (29) have provided the relevent information for solvent 3 (6,7). Solvent 4 involves multiple development and therefore no R_f value is reported.

TABLE 1. Effect of DHBP on phosphoglycolipid and cardiolipin synthesis

	cpm per ml of culture		
	untreated	60 uM rac-DHBP	% Inhibition
STRAIN 1005			
Phosphoglycolipid	1531	663	57
Total lipid	61240	40980	33
Cardiolipin	533	458	14
Total lipid	55341	38239	31
STRAIN BD170			
Phosphoglycolipid	1964	732	63
Total lipid	63270	41152	35
Cardiolipin	647	459	29
Total lipid	66579	41805	38

TABLE 2. Effect of DHBP on lipid accumulation. At a cell density of 20 Klett, the culture was adjusted to 60 uM DHBP. Immediately thereafter 20 uC_i of [^{14}C]acetate (2.24uC_i/umole) was added. After a 30 minute incubation lipids were extracted from the cells as described for accumulation studies in the Materials and Methods section. The lipids were separated by two dimensional chromatography on Silica gel G. Spots were visualized with Iodine vapor and scraped off. Radioactivity was determined by counting in aquasol. Phosphorous spray was used for identification of phospholipids. The a-Naphthol spray reagent was used for identifying glucose containing lipids. The positions of the lipids on the chromatograms were similar to the positions of the lipids on chromatograms developed by Fischer et al. (29) who characterized each individual lipid.

Solvent systems used to develop chromatogram:

First dimension:

chloroform/methanol/ammonia/water (65:20:2:2)

Second dimension:

chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5)

TABLE 2. Effect of DHBP on lipid accumulation in a 30 ml culture of casein hydrolysate.

	Untreated	60 uM rac-DHBP	% of untreated
PDGDG	266	213	80
LPG	3367	4620	137
PG	10854	8144	75
PE	6490	6995	107
MGDG	132	104	78
DGDG	620	897	145
TGDG	89	61	69
NL	354	1974	557

Abbreviations Used:

PDGDG- sn-glycero-1-phospho-B-gentiobiosyldiacylglycerol

LPG- lysylphosphatidylglycerol

PG- phosphatidylglycerol

PE- phosphatidylethanolamine

MGDG- monoglucosyldiacylglycerol

DGDG- diglucosyldiacylglycerol

TGDG- triglucosyldiacylglycerol

NL- neutral lipid

TABLE 3. Radioactive lipids derived from labeled phosphonate. Cultures were grown as described in the Materials and Methods section. At twenty Klett, 10 ml of cells were incubated with 150 uC_i [^{3}H]DHBP (.06 mM) for one hour. Cells were centrifuged down at 3000 *g* for 10 minutes. Two ml of water was added to resuspend the cells followed by 7.5 ml chloroform:methanol (1:2). After standing 60 minutes at room temperature, 2.5 ml of chloroform and 2.5 ml of water was added to the suspension making it biphasic. It was then passed through a Whatman 1PS filter to separate the phases. The chloroform phase was concentrated under a stream of nitrogen and spotted on a Brinkman silica gel G sheet. The sheet was developed in solvent systems A and B. A negative was exposed to the dry chromatograms for three months, and developed as described in the materials and methods section. The negative was used as a marker for locating lipids. The radioactive PGP analogue, which was located at the origin in both solvent systems, along with the indicated lipids were scraped off. One ml of a 10% glacial acetic acid solution in absolute ethanol was added to the silica gel followed by 10 ml of toluene scintillation fluid. The samples were then counted.

TABLE 3. Radioactive lipids derived from labeled phosphonate.

Lipid	Solvent A	Solvent B
Phosphatidylethanolamine	9400	8641
Phosphatidylglycerol	11615	10180
Lysylphosphatidylglycerol	515	297
Phosphatidylglycerolphosphate	179430	161890

Solvent system A: Diisobutylketone:Acetic Acid:Water (8:5:1)

Solvent System B: Chloroform:AceticAcid:Methanol:Water (80:18:12:5)

APPENDIX A

SEPHAROSE 6B COLUMN CHROMATOGRAPHY: Sepharose beads were swelled overnight in roughly four times their volume of distilled water. The suspension was deaerated for 15 minutes under a line vacuum at 30 C. Deaerated beads were gently poured into a beaker and allowed to settle (1.5 hours). Excess water was aspirated off leaving two parts sepharose beads to one part water. The beads were gently mixed to avoid formation of clumps. The column was secured and leveled vertically and horizontally. Air bubbles were removed from the lower column bed and from the tubing connected to the bottom of the column and replaced with water. A special packing extension adaptor was attached to the top of the column to serve as a resevoir for the suspended beads during column preparation. After gently mixing the beads in a beaker at room temperature, a stirring rod was placed across the beaker, and the beads poured slowly along the side of the adaptor. After 20 min the screw clamp closing the lower column tubing was opened slghtly. Liquid began flowing through the column, speeding up the settling process. The flow rate was gradually increased to the rate employed for LTA purification. Twice the bed volume of the column was allowed to drain through for equilibration using the same eluant as in the LTA run. To determine void volume, a 2 ml

sample of 0.2% Dextran Blue was applied. After washing the Dextran Blue out, the column was ready for use.

APPENDIX B

DOUBLE LABEL COUNTING: In double label experiments involving ^{32}P and ^{3}H, samples were counted in Patterson Greene scintillation fluid using an Isocap 300 scintillation counter. Two channels were required to determine the radioactivity of each isotope. Channel 11B encompassed the entire energy spectrum and was employed to measure the combined total of ^{3}H and ^{32}P cpm. Channel 10B was used to count ^{32}P over ^{3}H. The discriminator setting on channel 10B was adjusted to reject ^{3}H cpm. This was verified with a ^{3}H standard provided by the manufacturer. Under the conditions employed for counting, ^{32}P was counted with 94% efficiency in channel 10B and 100% in 11B. Since channel 11B registered the combined total of ^{32}P and ^{3}H cpm, the ^{3}H cpm was calculated as the difference between this channel and channel 10B after taking into account the differences in ^{32}P counting efficiencies.

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