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# The phosphonic acid analog of phosphatidylglycerol phosphate: Influence on *Escherichia coli* growth and physiology

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# THE PHOSPHONIC ACID ANALOG OF PHOSPHATIDYLGLYCEROL PHOSPHATE: INFLUENCE ON *ESCHERICHIA COLI* GROWTH AND PHYSIOLOGY.

by

Lei Ke

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**ABSTRACT**

The phosphonic acid analog of phosphatidylglycerol phosphate: Influence on *Escherichia Coli* growth and physiology.

by

Lei Ke

Adviser: Professor Burton Tropp

The growth of *Escherichia coli* is only slightly inhibited when the cells are incubated with 20 μM 3,4-dihydroxybutyl-1-phosphonate (DBP)[1]. However, the cells lose viability when exposed to a combination of 20 μM *rac*-DBP and 20 mM magnesium or calcium ions. Magnesium ions stimulate the incorporation of DBP into (1,2-diacyl)-*sn*-glycerol-*D*-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate, the phosphonate analog of phosphatidylglycerol phosphate.

Much higher DBP concentrations are needed to block the growth of a *pgsA3* mutant than to block the growth of an

isogenic wild-type strain. The DBP-treated *pgsA* mutant also has a much higher survival rate when stored in the cold than does the DBP-treated wild-type strain. Furthermore, the *pgsA3* mutant grows normally in the presence of DBP and magnesium chloride. Treatment with DBP and magnesium ions does not appear to disrupt the cell's inner or outer membranes. The combination of DBP and magnesium chloride does not have serious metabolic consequences for the *pgsA3* mutant strain but inhibits macromolecular, phosphoglyceride and ATP synthesis in wild-type strains. A combination of 20 μM *rac*-DBP and 0.5 mM spermidine or 0.125 mM spermine is bacteriostatic. These studies suggest that the PGP analog contributes to DBP's bacteriostatic effect when the growth medium contains low concentrations of magnesium or calcium ions and is responsible for its bactericidal effect when the medium contains high concentrations of these ions.

**Dedicated to my family for all their encouragement:**

**To my parents,**

**to my husband,**

**and**

**to Kevin, my son,**

**for all the happiness he has brought us.**

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## INTRODUCTION

The *Escherichia coli* cell envelope has three distinct regions (1). The inner or cytoplasmic membrane is a typical glycerophospholipid bilayer containing a variety of polypeptides (1, 2). The functions of inner membrane include transport, energy production, biosynthesis, and excretion (1). Membrane lipid synthesis occurs at the cytoplasmic surface of the inner membrane (1, 2). The region between the outer and inner membranes is called the periplasmic space (1). It contains proteins, membrane-derived oligosaccharides and peptidoglycan (2). Periplasmic proteins control the traffic and processing of molecules entering or leaving the cell and peptidoglycan determines cell shape and osmotic stability (1). The outer membrane is a fully asymmetric bilayer (2). Its inner leaflet is composed of glycerophospholipid, whereas its outer leaflet consists of lipid A (2, 3). Lipid A, a 2,3-diacylglucosamine-based lipid, is a unique molecule in the outer membrane of most gram-negative bacteria. It plays an important role in outer membrane assembly and function (3). Lipid A is the membrane anchor of so called lipopoly-saccharide (LPS) (3). The basic LPS structure comprises four regions, the lipid A, inner core, outer core and O-

antigen regions (3). Most of the biological activities of LPS are carried by lipid A (3). The asymmetric distribution and the chemical characteristics of LPS give the outer membrane many of its unique barrier properties.

One of the most significant functions of the outer membrane is to exclude a variety of environmental molecules such as phospholipases and hydrolytic enzymes, and large hydrophilic molecules but to permit selective uptake of other molecules by porin and nonporin pathways (1, 4). The selective permeability of the outer membrane allows cells to export several different classes of molecules (1, 4). The outer membrane is responsible for the interaction with the environment and contains receptors for binding specific nutrients, conjugative pili and bacteriophages. Furthermore, the outer membrane helps to determine the shape of gram-negative bacteria and serve as the anchoring points for certain external structures (1, 4).

The major glycerophospholipids in *E. coli* are phosphatidylethanolamine (PE)[1], phosphatidylglycerol (PG)[1] and cardiolipin (CL)[1] (2, 5). PE accounts for 75% of total phosphoglycerides, PG for 20%, and CL for 5% (2, 5, 6). A number of minor phospholipids, as biosynthetic intermediates, are present in the range of 0.01-0.1% of the total membrane lipid (6). The metabolic pathway for the biosynthesis of glycerophospholipids is shown in Fig. 1.

*sn*-Glycerol-3-phosphate (G3P)[1] is a precursor for phosphoglyceride synthesis (5). When *E. coli* is cultured in medium without glycerol, G3P can be generated from a direct reduction of dihydroxyacetone phosphate (7). The *gpsA* gene product, G3P synthase, catalyzes this reaction (8). Mutants with lesions in *gpsA* have been isolated and used to study phosphoglyceride synthesis and the influence that phosphoglyceride synthesis has on other cellular functions (9). Inhibition of phosphoglyceride synthesis is not complete in these mutants after starvation for G3P. A second glycerol-3-phosphate auxotroph, *plsB*, produces a G3P acyltransferase with a very high $K_m$ for G3P (10, 11). The G3P requirement is only observed when the cells have a second mutation *plsX* (11). The function of the *plsX* gene product is unknown. However, the *plsB plsX* double mutant retains the ability to synthesize phosphoglyceride, albeit less than wild-type (9). A triple mutant (*gpsA20 plsB26 plsX50*) has been constructed (12). This mutant shows complete inhibition of phosphoglyceride synthesis in the absence of G3P, which indicates that acylation of G3P is required for *de novo* phosphoglyceride synthesis (5). *E. coli* stops growing when the phosphoglyceride level is reduced by one-third. This suggests that phosphoglycerides are essential (2).

G3P acyltransferase catalyzes the first committed step

in phosphoglyceride synthesis in *E. coli*, the acylation of G3P to form 1-acylglycerol-3-phosphate (13). The *plsB* gene has been cloned and sequenced. Overproduction of G3P acyltransferase results in the formation of intracellular membrane tubules consisting of ordered arrays of enzyme (14). An *E. coli* strain with a mutation in 1-acylglycerol-3-phosphate acyltransferase, catalyzing the conversion of 1-acylglycerol-3-phosphate to phosphatidic acid (PA)[1], has been isolated (5). The studies of the mutant suggest that G3P acyltransferase and 1-acylglycerol-3-phosphate acyltransferase interact in the native membrane (5). The recent development of a direct nonchromatographic assay for 1-acylglycerol-3-phosphate acyltransferase will allow the further genetic and biochemical characterization of this enzyme (15).

The conversion of PA to activated intermediate CDP-diacylglycerol is catalyzed by CDP-diacylglycerol synthase, the *cds* gene product (16, 17). The three major phospholipid species in *E. coli* are synthesized from CDP-diacylglycerol (2, 5). Phosphatidylserine synthase (*pss* gene product) catalyzes the synthesis of phosphatidylserine (PS) from CDP-diacylglycerol and serine (18). As an intermediate, PS does not accumulate in the cell and is decarboxylated to phosphatidylethanolamine by PS decarboxylase (*psd* gene product) (18, 19). CDP-

diacylglycerol can also be used to synthesize various anionic phosphoglycerides. The reaction of G3P with CDP-diacylglycerol to form PGP, catalyzed by PGP synthase, is at an important branchpoint in bacterial phosphoglyceride synthesis (2, 5). PGP synthase is specified by *pgsA*, an essential *E. coli* gene (20). The phosphate group on PGP is then rapidly cleaved by PGP phosphatases (21). Two enzymes encoded by *pgpA* and *pgpB* have been found to convert PGP to PG (21, 22). However, recent studies indicated that both gene products are nonessential for PG synthesis (22). The last step in the synthetic pathway is the formation of cardiolipin by the action of the *cls* gene product, CL synthase (23). Mutants affected in each of steps in this pathway have been isolated except for the one defective in PGP phosphatase which is essential for PG synthesis (22). Most of these structural genes are also cloned and sequenced.

The *pss* gene product, phosphatidylserine synthase, catalyzes the committed step to PE biosynthesis from CDP-diacylglycerol (18). Temperature-sensitive *pss* mutants have been isolated and studied (24, 25). PS synthase is required under normal growth conditions (26). At permissive temperature, the *pss* mutant contains more anionic phosphoglycerides and less PE than does the wild-type parent (27). Under nonpermissive conditions, the *pss*

mutant contains even less PE and has increases in PG and CL levels (26, 27). Furthermore, the growth rate of the mutant gradually declines and cells lose viabilities (26, 27). This growth inhibition at nonpermissive temperatures is partially suppressed by addition of magnesium ions to the growth medium (26). Since these temperature-sensitive mutants still contain significant levels of PE, the nature of the PE requirement by *E. coli* could not be determined. Recently, DeChavigry *et al.* constructed a strain with an interrupted *pss* gene. The mutant stopped growing when the level of PE declined to 30 mol% of the total phospholipid (28). An increase in PG and CL synthesis compensates for the absence of PE synthesis (28). Again, divalent metal ions, such as $Ca^{2+}$, $Mg^{2+}$, $Sr^{2+}$ suppressed the growth inhibition of the mutant with undetectable phosphatidylserine synthase activity and trace amounts of PE (28). The replacement of PE by divalent metal ions suggests that the major physiological role of PE is to maintain an optimal physical state of the membrane.

The *psd* gene product, PS decarboxylase, which is required for PE biosynthesis from PS, is also essential under normal growth condition (29, 30). Under nonpermissive conditions, temperature-sensitive *psd* mutants do not grow and accumulate large amounts of PS (31). Divalent cations allow temperature-sensitive *psd* mutants to

grow at nonpermissive temperatures (29, 30). The growth defect in the *psd* mutant appears to be the result of the accumulation of PS at toxic levels rather than the lack of PE (30). However, the growth defect in *pss* mutant is due both to membrane instability in the absence of PE and possibly to the accumulation of anionic phosphoglycerides (20).

Phosphatidylglycerol phosphate (PGP)[1] is a direct precursor of phosphatidylglycerol, one of three major phosphoglycerides in the *Escherichia coli* cell envelope (2, 5). In addition to being a metabolic intermediate, PGP may help to regulate phosphoglyceride metabolism (6). The first PGP synthase mutant was isolated by colony autoradiography (32). Although the mutants had less than 5% of the normal PGP synthase activity, the cells grew normally and had only slightly reduced levels of PG. These mutants, designated *pgsA*, map at minute 42 (33). A second-step temperature-sensitive mutation *lpxB* (originally called *pgsB*), distant from *pgsA*, was isolated from the *pgsA* mutant (33, 34). The double mutants were unable to synthesize PG and did not grow at nonpermissive temperature. Furthermore, these mutants accumulated two novel glycolipid precursors of lipid A at nonpermissive temperature (34). The *lpxB* gene has been found to code for a tetraacyldisaccharide-1-phosphate synthetase (35). It is

the accumulation of these glycolipid precursors that results in a terminal phenotype in the *lpxB pgsA* double mutant but not in either of the single mutants (34). Therefore, these mutants have not been very useful in manipulating PG levels.

Studies with cloned *pgsA* showed that the carboxyl-terminal coding region of *pgsA* shares some nucleotide sequences with the transcriptional regulatory element of *glyW* (36). Overlap also exists between *pgsA* and its nearest neighbor *uvrC* on the other side (36).

A tighter point mutation, *pgsA3*, has been isolated by Miyazaki *et al* (37). This mutant had undetectable levels of PGP synthase *in vitro* and its PG content was less than 1% of total phosphoglycerides (37). However, the mutant could grow with a slightly longer doubling time than the wild-type parent (37). Subsequent study suggested that the genetic background in the *pgsA3* mutant has a defect in lipoprotein synthesis, a mutation in *lpp* gene, which allows a low steady state level of PG and CL in the cells (38, 39). Such a low level of PG is sufficient to serve as a substrate for synthesis of membrane-derived oligosaccharide and murein lipoprotein in the mutants (38, 39).

Recently, a null allele, *pgsA30*, was constructed by Heacock and Dowhan to establish whether or not PG is essential to *E. coli* for functions other than maintenance

of membrane structure and integrity (20). A strain with inactive chromosomal *pgsA (pgsA30)* was obtained by homologous recombination with an extrachromosomal copy of *pgsA::kan* allele (20). After this null allele was transferred to cells harboring a temperature-sensitive plasmid with a *pgsA* gene, the growth of these cells became temperature dependent. At 30°C, these cells grow normally; whereas at 42°C , they stop growing after 7-8 generations due to the loss of functional *pgsA* on the plasmid (20). At restrictive temperature, arrested cells contain 3% of the PG synthase activities of wild-type cells with one copy of *pgsA* gene and the level of PG is less than 2% of the total phosphoglycerides (20). Since *pgsA* is the sole *E. coli* gene on the plasmid, the growth dependence of the strain on *pgsA* suggests that *pgsA* is essential for *E. coli* (20). In general, mutants stop growing when the PG level is 1-2% of the total phosphoglycerides except in cells with a nonfunctional *lpp* gene. However, insertional inactivation of the *pgsA* gene is lethal even in an *lpp* mutant (20). An *lpp* mutation acts as a suppressor in *pgsA* mutant, which diminishes but does not eliminate the PG requirement (20).

Further study of the regulation of *pgsA* gene expression was carried out using a strain with the *pgsA* gene fused to the chromosomal copy of the *lacOP* system (40). The results indicated that the expression of *pgsA*,

the synthesis of PG, and the cell growth depend on the level of isopropyl-β-D-thiogalactoside (IPTG)[1] in the growth medium which further confirm the essential role of PG in *E. coli* (40). Taken together, it seems that PG does more than just maintain the structural integrity of the membrane (2, 20). It is the donor of glycerol and glycerophosphate residues to several outer membrane lipoproteins including the major murein lipoprotein (41, 42), and membrane-derived oligosaccharides (43). It also plays a crucial role in protein translocation across the inner membrane (44) and initiation of DNA replication (45).

At least two enzymes catalyze the dephosphorylation of PGP to form PG (21, 22). They are encoded by *pgpA* and *pgpB* which map near minutes 18 and 28, respectively (21, 46). The *pgpA* product is quite specific while the *pgpB* product has a multiple substrate specificity and can also dephosphorylate PA and lysophosphatidic acid (LPA)[1] (21, 47). The *pgpB* product has alternative subcellular locations and appears to have a different substrate specificity when the enzyme is localized in the outer and cytoplasmic membranes (47). Its PGP phosphatase activity is higher when the enzyme is localized in cytoplasmic membrane. However, its PA and LPA phosphatase activities are higher in the outer membrane (47). The molecular basis for this is unknown. The point mutations of *pgpA* and *pgpB*

were both introduced into a single strain. The resulting double mutant appears to grow normally and continue to synthesize PG. However, it accumulates an unusually high level of PGP (21). For example, PGP accounts for about 5% of the total lipids when the double mutants are in stationary phase. The comparable value for wild-type cells is fifty times lower (21). Maintenance of a normal level of PG in the double mutant is probably due to the existence of other phosphatases (21, 22).

Recently Funk *et al.* reexamined the *pgpA*, *pgpB* gene, and their products (22). More stringent mutations in the *pgpA* and *pgpB* loci were constructed by insertional inactivation of these genes by drug markers at known positions (22). Mutants carrying the disrupted genes appear to have the same growth and biochemical properties as the point mutants (22). This excludes the possibility of the residual phosphatase activities from partial function of the mutated genes. Therefore, other unknown gene products are responsible for the residual phosphatase activities which account for 50% of the total activity in wild-type cells (22). Since mutations in either or both genes have no effect on growth or PG synthesis, neither gene product is essential for cell viability and PG synthesis *in vivo* (22). The physiological roles of the PGP A and PGP B phosphatase activities are still unknown.

These phosphatase activities may contribute to nonessential cell functions (22). Thus far, PGP phosphatase is the only enzyme in the phosphoglyceride biosynthetic pathway which has not been identified.

In *E. coli*, two molecules of PG combine to form CL and glycerol in a reaction catalyzed by CL synthase (48). An *E. coli* mutant defective in CL synthesis was first isolated by examining the lipid extracts of mutagenized temperature-sensitive cells (49). The *cls* gene, which appears to be the structural gene for CL synthase, maps at minute 27. Further genetic study suggested that *cls* lesion was unrelated to the temperature-sensitive phenotype (49). A *cls* mutant with CL level less than 1% of total phosphoglycerides has a slightly longer doubling time under ordinary growth conditions (50). It is more resistant to 3,4-dihydroxybutyl-1-phosphonate (DBP)[1], a G3P analog (51) and unable to grow at high pH (unpublished data, this laboratory). Mutants with a *cls* null allele have been isolated (38). These mutants lack CL synthase activity, but retain a small amount of CL in their membranes (38). This small amount of CL in *cls* null mutants indicates an alternate pathway in *E. coli* to synthesize CL rather than the leakiness of original *cls* mutation. Further study is required to determine the physiological role of CL. The residual level of CL may be made by the wild-type PS

synthase which transfers a phosphatidyl residue to PG since PS synthase has been shown to contribute a phosphatidyl residue to alternative acceptor molecules (38, 52).

The phosphoglyceride composition in wild-type *E. coli* is not strikingly altered by changes in culture condition. A decrease in the ratio of unsaturated to saturated fatty acids is observed at high temperature and an increase in level of CL, at the expense of PG, is observed in the stationary phase (35). The cloned *pss*, *pgsA*, and *cls* gene were used to study the phenotypic consequences of their amplifications. Amplification of PS synthase was proportional to gene dosage (53). A ten-fold overproduction of PS synthase in a wild-type strain harboring multicopy plasmid with the *pss* gene resulted in only a slight increase in PE level (53). Similarly, intracellular PGP synthase activity is directly proportional to gene dosage (54). High gene dosage produces only a modest increase in cellular PG content (54). Studies with cloned *cls* gene reveal that overproduction of the enzyme has no significant effect on cellular phosphoglyceride composition (55). This suggests that enzymatic regulation occurs in *E. coli* to control the overproduction of each phosphoglyceride. However, the possibility that the regulation occurs at genetic level can not be ruled out. A *trans*-acting regulatory mutation in

the *pssR* gene was isolated and the mutation affected the expression of the structural gene for PS synthase (56). The regulation of the *cls* gene expression was studied using strains carrying operon and protein fusion between *cls* and *lac* genes (57). The results indicated that the *cls* gene expression increases when cells are deprived of oxygen and when they enter the stationary phase. The control of the *cls* gene expression appears to be at the transcriptional level (57).

Since PS synthase and PGP synthase compete for a common pool of CDP-diacylglycerol, the inability of wild-type strains with overproduction of one or the other of the enzymes of the branchpoint to significantly alter the ratio of polar head-groups indicates that *E. coli* has regulatory mechanisms which maintain a balanced membrane phosphoglyceride composition. Jackson *et al.* studied the regulatory mechanisms using the phosphoglycerol transferase I model substrate arbutin to remove PG from cells continuously and specifically (58). The removal of PG from arbutin-treated cells could stimulate the PGP synthase activity. The study showed that PS synthase activity remained unchanged while PGP synthase activity was stimulated (58). Their results supported independent feedback regulation of PS synthase and PGP synthase instead of the coordinate regulation of the two enzymes (58).

Since a ten-fold or greater modulation of the activities of these two enzymes results in a change in 10% or less in the phosphoglyceride composition, this feedback mechanism is very effective (58). Moreover, the study indicated that the variations in the amount of a minor lipid seem to be more important in regulation of the phosphoglyceride composition than variations in a major lipid content (58). Therefore, they suggested that the true regulator might be a minor phosphoglyceride intermediate such as PGP (58).

The physiological role of PGP can be investigated using a strain with mutations in PGP phosphatases. However, as stated above, the ability of the *pgpA pgpB* double mutant to synthesize PG is probably due to the presence of at least one additional phosphatase. Until mutants for the additional phosphatase(s) are isolated, it will not be possible to determine the metabolic consequences of a complete blockage of the conversion of PGP to PG. However, some clues may be obtained by studying the physiological properties of cells that accumulate a phosphonate analog of PGP.

3,4-Dihydroxybutyl-1-phosphonate (DBP)[1], an analog of glycerol-3-phosphate, has been used to study its effect on cell growth and phosphoglyceride metabolism in this laboratory. The methylene group in DBP is substituted for the esteric oxygen. When cultured in the presence of

DBP, *E. coli* synthesize (1,2-diacyl)-*sn*-glycerol-*D*-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate, the phosphonate analog of PGP (59). A $CH_2$-P bond replaces the O-P bond normally found in PGP and G3P. The phosphatases that normally convert PGP to PG cannot hydrolyze the PGP analog.

DBP is transported into *E. coli* by the GlpT, Ugp, and Uhp transport systems (60). Low DBP concentrations perturb bacterial phosphoglyceride metabolism but have little effect on cell growth (61, 62). Phosphatidylglycerol synthesis is particularly sensitive to DBP (61). Neither the intracellular nucleoside triphosphate pool size nor the rate of macromolecular synthesis are markedly affected by the presence of low concentrations of DBP (61, 62, 63). High DBP concentrations inhibit *E. coli* growth (64).

Enzyme studies indicate that DBP is an inhibitor of G3P synthase (the *gpsA* gene product) and a competitive substrate of PGP synthase (65). However, it is not recognized by either the catabolic G3P dehydrogenase (the *glpD* gene product) or G3P acyltransferase (the *plsB* gene product) (65).

*E. coli* growth inhibition caused by DBP depends upon both the genetic background and the culture medium. The *glpD* and *cls* genes influence sensitivity to DBP. Strain 8 (*glpR glpD*) is much less sensitive to DBP than is strain 7

(*glpR*) (unpublished data, this laboratory). This is probably due to lower intracellular G3P concentrations in strain 7. A mutant with a *cls* lesion is also less sensitive to DBP than is its wild-type parent (51). The reason for this is not yet known. Strain 8 is much more sensitive to DBP when cultured in low-phosphate minimal medium than when cultured in casein hydrolysate medium (unpublished data, this laboratory). In addition, both strains 7 and 8 are much more sensitive to DBP when grown in the presence of high concentrations of magnesium or calcium ions (51). The *cls* gene does not appear to influence this divalent cation effect (51).

*E. coli* cultured in minimal medium containing glycerol are sensitive to growth inhibition by DBP (64). This suggests that G3P synthase is probably not the primary target of drug action since an alternative pathway is available to synthesize G3P. PGP synthase appears to be a more likely target for DBP action. However, until recently there was no way to distinguish between effects due to inhibition of PG synthesis and those due to the appearance of the PGP analog. The isolation of *pgsA* mutants permits such a distinction to be made. The present studies were performed to determine whether the PGP analog is responsible for the growth inhibitory effect and to examine the role of divalent cations on this process.

## MATERIALS AND METHODS

**Chemicals.** Carrier-free [$^{32}$P]phosphate, [U-$^{14}$C]glycerol-3-phosphate, sodium [1-$^{14}$C]acetate, and L-[U-$^{14}$C]leucine were purchased from ICN Biomedical Inc., Irvine, CA. *rac*-3,4-dihydroxy[3-$^{3}$H]butyl-1-phosphonate ([3-$^{3}$H]DBP), *rac*-3,4-dihydroxy[1,2-$^{3}$H]butyl-1-phosphonate ([1,2-$^{3}$H]DBP), and the unlabeled *rac*-DBP were prepared as described previously (66, 67). [3-$^{3}$H]DBP and [1,2-$^{3}$]DBP were purified by colum chromatography. A 1 x 6 cm colum of DE-52 (bicarbonate form) was prepared. [$^{3}$H]DBP was applied and eluted with 200 mL of a linear gradient of ammonium bicarbonate (0 - 0.2 M, pH 8.5). Fractions of 2 mL were collected. Fractions containing pure [$^{3}$]H were pooled, reduced *in vacuo*, and the residue redissolved in a measured amount of water. The recovery of labeled material was calculated by counting a dilution of the recovered material. Casein hydrolysate, spermine (tetrahydrochloride), spermidine (trihydrochloride), *DL*-glycerol-3-phosphate, Triton X-100, tris(hydroxymethyl)aminomethane (Tris), chloramphenicol, ATP, isopropyl-$\beta$-*D*-thiogalactoside (IPTG)[1], *o*-nitrophenyl-$\beta$-*D*-galactoside (ONPG)[1], cephalosporin C, ampicillin, and tetracycline were obtained from the Sigma Chemical Co., St.Louis, MO. CDP-dipalmitin was purchased from Serdary

Research Lab., London, ON, Canada. β-Mercaptoethanol was purchased from Matheson Coleman & Bell, Los Angeles, CA and Ecoscint A from National Diagnostics, Manville, NJ. Polygram Sil Gel G and Cellulose MN 300 (PEI) thin-layer chromatography plates were products of Brinkmann Instruments Inc., Westbury, NY. Membrane filters(type HAWP, pore size 0.45 μm) were purchased from Millipore Corp., Bedford, MA. All other chemicals were reagent grade. Protein concentrations were determined by the enhanced Lowry method, using bovine serum albumin as a standard (68).

**Growth of bacteria.** CH medium contained 10 g casein hydrolysate and 5 g NaCl per liter at pH 7.4 and CHG medium contained CH medium plus 10 μg/mL glycerol. Growth at 37°C was monitored turbidimetrically in a Klett-Summerson colorimeter fitted with a 660 nm filter as previously described (64). Viability studies were performed by diluting cultures in CH broth and quickly plating on CH agar. In some viability studies, LB agar or LB agar plus glucose were used in place of CH agar. No viability differences were observed when these rich media were used.

**Construction of bacterial strains.** The genotypes of all strains used are listed in Table 1. SD12 and HD3122/pHD102 were kindly provided by Dr. William Dowhan (U. of Texas Medical School, Houston, Texas). Strain HD3122 was

obtained by streaking HD3122/pHD102 on LB plates at 42°C and testing for chloramphenicol sensitivity. The *glpD*$^+$ gene was transferred from strain 7 to SD12 and HD3122 by $P1_{vir}$ transduction to produce LK8 and LK9, respectively. The *pyrD*$^+$ gene was transferred from strain 7 to LK8 and LK9 by $P1_{vir}$ transduction to produce LK88 and LK99, respectively (See Table 1). Phosphoglyceride analyses indicate that LK99 retains the *pgsA3* lesion (see Results).

*o*-Nitrophenyl-*β*-*D*-galactoside (ONPG) permeation studies were performed in a derivative of HW55 with a *lacY* lesion. This strain was constructed as follows. The *proC*::Tn*5* mutation was transferred from SVS111 to HW55 by P1 transduction. One of the Kan$^r$, Pro$^-$ transductants was converted to LacY$^-$ with a $P1_{vir}$ lysate from LE392 to produce LK35 (Pro$^+$ Kan$^s$ LacY$^-$). HW55/pBR322 was obtained by introducing pBR322 into competent HW55 and selecting for tetracycline and ampicillin resistance.

**[$^3$H]DBP transport assay.** The transport assay was a slight modification of that described by Leifer *et al.*(69). In the present study, the strains of LK88 and LK99 were cultured in 10 mL of CHG medium to 100 Klett Units. Cells were centrifuged and resuspended in 2 mL of 10 mM potassium phosphate, pH 7.0, containing 0.85% sodium chloride and 40 μg of chloramphenicol. Uptake was initiated by adding one volume of cell suspension to a vial containing 5 volumes of

10 mM potassium phosphate (pH 7.0), 0.85% sodium chloride, and 0.25 mM *rac*-[3-$^3$H]DBP (sp. act. 10 $\mu$Ci/ $\mu$mol). Where indicated, magnesium chloride was maintained at 20 mM in both the culture and assay media. After a 2 min incubation at 30°C, cells (0.1 mL) were collected on a membrane filter (0.45-$\mu$m pore size and 25-mm diameter) and washed with 1 mL 0.85% sodium chloride at 30°C or with distilled water at 5°C. Dried filters were placed in vials containing Ecoscint A scintillation fluid and counted in an Isocap 300 liquid scintillation counter.

**[$^3$H]DBP incorporation into lipid *in vivo*.** HW55 was cultured in CH medium until a turbidity of 15 Klett units was reached. Samples of 1 mL were then removed and incubated with 1 $\mu$Ci of *rac*-[3-$^3$H]DBP (sp. act. 50 $\mu$Ci/$\mu$mol) in the presence or absence of 20 mM magnesium chloride for the indicated times. Samples were then removed and analyzed as described previously (51) or by a filter paper assay (70). In the former case, the total $CHCl_3$ phase was dried and counted in 5 mL of Ecoscint A scintillation fluid. In the latter case, 100 $\mu$L of the reaction mixture was removed and deposited on a Whatman No. 3 MM filter paper square (2.25 cm$^2$). The paper was washed with four changes of ice-cold trichloroacetic acid (TCA) (10 mL/filter). TCA concentrations in successive washes were 10%, 5%, 5%, and 1%. The filters were dried and counted as

described above. When [1,2-$^{3}$H]DBP was used as a radioactive tracer, 1 μCi of *rac*-[1,2-$^{3}$H]DBP (sp. act. 50.5 μCi/μmol) was added to 1-mL culture. The same procedure was used to follow [3-$^{3}$H]DBP incorporation into LK88 and LK99. However, these strains were cultured in CHG medium.

**PGP synthase assay.** PGP synthase was isolated and assayed as described previously (65). The final concentrations of radioactive *rac*-[U-$^{14}$C]G3P (sp. act. 26 μCi/μmole) and *rac*-[3-$^{3}$H]DBP (sp. act. 31 μCi/ μmole) were 0.8 mM and 30 μM, respectively. The reaction was initiated by the addition of the enzyme and the reaction mixture incubated at 37°C for 20 min. Labeled phosphoglyceride fraction was extracted into chloroform as described previously (65).

Authentic PGP analog was prepared enzymatically from CDP-diacylglycerol and [3-$^{3}$H]DBP. PGP synthase was isolated from strain JA200/pPGL2019 and assayed as described above. The reaction mixture contained 0.35 μCi of [3-$^{3}$H]DBP (sp. act. 31 μCi/ μmole). The reaction mixture was incubated at 37°C for 30 min.

**ONPG permeation assay.** *E. coli* inner membrane integrity was evaluated by measuring the rate of permeation and subsequent hydrolysis of ONPG, the chromogenic substrate for β-galactosidase (71, 72). LK35 (*lacY*) was cultured in CH medium containing 1 mM IPTG. When the cultures reached

a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added where indicated. After a further 1 h incubation, cells were centrifuged and resuspended in 10 mL of Z-buffer (73). Z-buffer (pH 7.0) contained 0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$ and 0.05 M β-mercaptoethanol. A portion of the suspension was used to assay ONPG permeation into whole cells. A second portion was sonicated to determine total β-galactosidase activity. The β-galactosidase assay was that described by Miller (73). A tenth mL of treated cells or sonicates were used. $A_{420}$ was followed for 10 min at 25°C.

**β-Lactamase assay.** *E. coli* outer membrane integrity was evaluated by measuring β-lactamase permeation across the membrane (71, 74). β-Lactamase activity was determined by measuring cephalosporin C hydrolysis (74). HW55/pBR322 was cultured in CH medium containing 100 μg/mL of ampicillin and 5 mM magnesium chloride. The magnesium chloride was included to prevent β-lactamase leakage before treatment. When the cells reached a turbidity of 15 Klett units, the magnesium chloride concentration was increased to 20 mM and DBP was added to 20 μM where indicated. Samples were removed after a 90 min incubation and either centrifuged or sonicated. Five mL of cells were sonicated using a Branson Model W140D with a microtip. The sonifier was set at a power level of 8. The cells were exposed to five 30 sec

bursts with 1 min pauses between each burst. β-Lactamase activity in the supernatant of a centrifuged sample provides a measure of the enzyme that leaked out of the cells and β-lactamase activity in the sonicate provides a measure of total β-lactamase activity. DBP, magnesium chloride, or a combination of the two was added to each sample before assaying for β-lactamase so that all reaction mixtures contained the same final concentrations of these compounds. The assay mixture contained, in a final volume of 1 mL: 0.25 μmol cephalosporin C, and 8.5 μmol phosphate buffer (pH 7.0). The assay was initiated by adding 100 μL of cell culture supernatant or sonicate. Cephalosporin C hydrolysis was monitored by following the decrease in $A_{260}$ for 20 min at 25°C.

**Determination of the rates of DNA, RNA, and phosphoglyceride syntheses.** The rates of RNA, DNA and phosphoglyceride syntheses were determined by pulse labeling with [$^{32}$P]phosphate as described previously (61). Both LK88 and LK99 were cultured in CHG medium. When they reached a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added to the indicated cultures. A 2-mL sample was immediately removed and incubated with 20 μCi [$^{32}$P]phosphate for 10 min. After 1 h treatment with DBP and magnesium chloride, a second 2-mL sample was removed and incubated with 20 μCi [$^{32}$P]phosphate for 10 min. These

samples were then analyzed for labeled DNA, RNA, and phosphoglycerides (61). To each cultural sample, 2 mL of 10% cold TCA and 40 μL of carrier cells were added. After centrifugation, pellets were washed twice with 2 mL of 5% cold TCA and resuspended in 5.5 mL of MeOH:$CHCl_3$:$H_2O$ (5:5:1). Both the supernatant and pellet, collected by centrifugation, were used for further studies.

The pellets were washed once more with 2 mL of the above solvent, resuspended in 2 mL of 0.5 N KOH and incubated overnight at 37°C. Suspensions were chilled, treated with 2 mL of 1 M perchloric acid and then centrifuged. One mL sample of the supernatants were counted as RNA. The DNA pellets were dissolved in 2 mL of 1 N KOH, neutralized with HCl, and precipitated with 12% of cold TCA to give final concentration of TCA 5%. The pellets were washed with 5% cold TCA and dissolved in 1 mL of 0.2 N KOH. A half mL of the suspension was counted as DNA.

The supernatants containing phosphoglycerides were washed twice with 2 mL of 2 M KCl and once with 2 mL of $H_2O$. The chloroform phases were passed through glasswool and 0.1 mL of each of these fractions was counted.

**Determination of macromolecular and phosphoglyceride accumulation.** When 10 mL cultures of HW55 in CH medium reached turbidities of 15 Klett units, 20 μCi of carrier-

free [$^{32}$P]phosphate were added. Then DBP, magnesium chloride, or both were added where indicated. Samples of 1.0 mL were removed at the indicated times and analyzed for labeled DNA, RNA, and phosphoglycerides as described above. Phosphoglyceride synthesis was also measured by labeling 10 mL cultures with 10 $\mu$Ci of [1-$^{14}$C]acetate (sp. act. 500 $\mu$Ci/ mmole).

Protein synthesis was followed by measuring the conversion of L-[U-$^{14}$C]leucine into TCA-insoluble material. When HW55 in CH medium reached a turbidity of 15 Klett units, 1 mL samples were removed and incubated with 0.9 $\mu$Ci L-[U-$^{14}$C]leucine (sp. act. 0.27 $\mu$Ci/ mmole). Then DBP, magnesium chloride, or both were added where indicated. Samples (0.1 mL) of the culture were removed at the indicated times and deposited on Whatman No.3 MM filter paper squares which were immediately immersed in 5% TCA. The filters were washed with three changes of fresh 5% TCA and then with two changes of acetone. Radioactivity retained on the dry filters was measured in a Beckman LS7500 liquid scintillation counter.

**Phosphoglyceride analysis.** Phosphoglycerides were analyzed by Polygram Sil G thin-layer chromatography. The plates were developed either with $CHCl_3$:MeOH:HAc (65:25:8) (solvent system 1) or with $CHCl_3$:MeOH:HAc:$H_2O$ (25:15:4:2) (solvent system 2). The latter system was used to resolve

PGP analog from other phosphoglycerides (20). Radioactive phosphoglycerides were detected by autoradiography. Authentic PGP analog, prepared enzymatically from CDP-diacylglycerol and [3-$^{3}$H]DBP (see above), was used as a chromatographic standard.

**Turnover of DNA, RNA, and phosphoglyceride.** DNA, RNA, and phosphoglyceride turnover were monitored after culturing LK88 for about five generations in CHG medium containing 2 $\mu$Ci/mL of [$^{32}$P]phosphate. When cells reached a turbidity of 15 Klett units, they were collected by centrifugation, washed with CHG medium, and resuspended in the original volume of unlabeled culture medium containing 1 mM $NaH_2PO_4$. Samples of 2 mL were transferred to test tubes. DBP, magnesium chloride, or both were added where indicated. After another 2 h incubation, radioactive DNA, RNA and phosphoglycerides were determined as described above. Portions of phosphoglyceride fractions were analyzed by thin-layer chromatography in solvent system 1 as previously described.

**[$^{32}$P]Phosphate incorporation into ATP.** Intracellular and extracellular ATP concentrations were determined by the procedure of Cashel *et al.* (75). Strain HW55 was cultured in CH medium. When cultures reached turbidities of 15 Klett units, 2-mL samples were removed and placed in tubes containing 200 $\mu$Ci [$^{32}$P]phosphate. DBP, or magnesium

chloride, or a combination of both were added where indicated and the cultures were incubated for 30 or 60 min as indicated. A 1-mL sample was removed and centrifuged in an Eppendorf microfuge at maximum speed for 30 sec. Both the supernatant and pellet were used to test for ATP. The supernatant (300 μL) was mixed with 100 μL of 4 N formic acid. The pellet was resuspended in 50 μL of cold distilled water, and an equal volume of ice-cold 2 N formic acid containing 22 μmole of ATP was added to each centrifuge tube. Both mixtures were left on ice for at least 15 min and clarified by centrifugation for 1 min. The ATP levels were determined by spotting 10 μL of the soluble acidic supernatants on cellulose MN300 PEI plates and developing the plates with 0.85 M potassium phosphate buffer (pH 3.4). ATP spots were visualized with an ultraviolet light.

## RESULTS

**Growth and viability studies:** HW55 is only slightly inhibited by 20 μM *rac*-DBP when cultured in CH medium (62). However, this same DBP concentration completely blocks cell growth when the medium also contains 20 mM magnesium chloride (Fig. 2A). Viability studies reveal that a combination of 20 μM *rac*-DBP and 20 mM magnesium chloride is bactericidal (Fig. 2B). Growth inhibition and viability loss are also observed when DBP-treated cells are cultured in the presence of 20 mM calcium chloride (Figs. 3 and 5). The divalent metal cations, by themselves, have very little effect on cell growth. Furthermore, the synergistic effect is cation concentration dependent. For example, in the presence of 5 mM magnesium ion or 2.5 mM calcium ion, DBP is bacteriostatic rather than bactericidal (Figs. 4 and 5).

**PGP analog synthesis:** Previous studies showed that DBP is a precursor of (1,2-diacyl)-*sn*-glycerol-*D*-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate, the phosphonate analog of PGP (59). [3-$^{3}$H]DBP is also converted to the PGP analog when HW55 are cultured in the presence of 20 mM magnesium chloride (Fig. 6). Identical results were obtained when [1,2-$^{3}$H]DBP incorporation was followed (Fig.

7). Therefore, the radioactive label in the PGP analog is due to incorporation of intact DBP rather than to tritium transfer from DBP to G3P or some other metabolite. Furthermore, the incorporation studies showed that after 1 h, PGP analog synthesis is greater when 20 mM magnesium chloride is present in the medium than when it is not (Figs. 6 and 7). Sodium azide completely blocks PGP analog synthesis (Fig. 8), which indicates that energy is needed for DBP transport, PGP analog formation, or both.

[$^{32}$P]Phosphate studies confirmed the synthesis of the PGP analog and provided information about the contribution that this analog makes to the total phosphoglyceride pool. HW55 was cultured in the presence of carrier-free [$^{32}$P]phosphate (2 $\mu$Ci/mL) until a turbidity of 15 Klett units was reached. DBP, magnesium chloride, or both were then added where indicated and the incubation continued for another hour. The phosphoglyceride fraction was extracted and analyzed by thin-layer chromatography in solvent system 2. The PGP analog, clearly resolved from the other phosphoglycerides, accounts for 13.9% of the phosphoglycerides in cultures treated with DBP and magnesium chloride and 7.4% in those treated with DBP alone (Table 2). Total phosphoglyceride concentrations, as judged by [$^{32}$P]phosphate incorporation, were identical in cultures treated with DBP and DBP plus magnesium chloride.

Results in Table 2 are consistent with those shown in Figs. 6, 7 and 8. All results indicate that cells treated with DBP and magnesium chloride for 1 h synthesize nearly twice as much PGP analog as do cells that are treated with just DBP.

**The influence of a *pgsA* mutation on growth and viability:** Mutants with a defective PGP synthase provide a means for determining whether the combined effects of DBP and magnesium chloride are due to the appearance of the PGP analog or the disappearance of PG. Unfortunately, the lesion responsible for this defect, *pgsA3*, can only be transferred to cells with an *lpp* mutation (20). This excludes the possibility of direct gene transfer to HW55. An alternate approach was therefore taken. HD3122 (*pgsA3*) and its parent SD12 ($pgsA^+$) were converted to LK99 and LK88, respectively, by two successive transductions (see Materials and Methods). Phosphoglyceride composition was analyzed in both strains. PG accounts for about 1.8% of the total phosphoglycerides in LK99 and about 16% in LK88, indicating that each of these strains retains the *pgsA* genotype of its parent. LK99 has a more rapid rate of DBP uptake than does LK88, both in the presence and absence of magnesium chloride (Table 3). The reason for this difference is not known. Both strains release intracellular DBP when washed with cold distilled water

(Table 3).

As shown in Fig. 9, LK88 (*pgsA*$^+$) is considerably more sensitive to *rac*-DBP than LK99 (*pgsA3*). When tested for sensitivity to the combined effects of DBP and magnesium chloride, LK88 was shown to be sensitive and LK99 to be resistant (Fig. 10). Furthermore, viability studies revealed that LK88 treated with DBP and magnesium chloride for 5 hr contains $9.67 \times 10^4$ viable cells per mL culture while one untreated contains $3.74 \times 10^9$ viable cells per mL culture. This suggests that a combination of DBP and magnesium chloride is bactericidal to LK88 just as it is to HW55. When 20 mM calcium chloride was used in place of magnesium chloride, LK88 growth was inhibited and LK99 growth was not (Fig. 11). Viability studies also showed that LK88 treated with DBP and calcium chloride for 5 hr contains $3.85 \times 10^4$ viable cells per mL culture while one untreated contains $3.86 \times 10^9$ viable cells per mL culture. Viability of LK99 was not determined since LK99 continued to grow in the presence of DBP and magnesium or calcium chloride (Figs. 10 and 11). Therefore, a combination of DBP and magnesium ions or calcium ions has similar effects on viability and growth of a strain with wild-type *pgsA* gene. Radioactive tracer experiments show that [$^3$H]DBP is incorporated into LK88 lipids but very little is incorporated into LK99 lipids (Table 4). Furthermore, magnesium ions cause an increase

in PGP analog formation in LK88 just as they do in HW55.

Previous studies showed that cells treated with 100 μM *rac*-DBP lose viability when stored in the cold (51). This viability loss was attributed to changes in the phosphoglyceride composition of the cell membrane. However, these experiments did not distinguish between the contributions made by the loss of PG from those made by the accumulation of the PGP analog. This question was reexamined by studying the viabilities of DBP-treated LK88 and LK99 after storage in the cold (Fig. 12). The viability of DBP-treated LK88 drops considerably during storage at 5°C. Although untreated LK99 is somewhat more sensitive to cold storage than untreated LK88, no significant difference was observed between DBP-treated LK99 and untreated LK99. The cold storage effect therefore appears to be due to the accumulation of PGP analog.

**Inner and outer membrane integrity:** One possible explanation for the viability loss is that the PGP analog causes the inner or outer membranes to become permeable when exposed to the divalent metal cations. The permeabilities of the two cell membranes were therefore examined. Inner membrane permeability changes were measured by following the permeation of ONPG in strain LK35 (*lacY*) (see Materials and Methods). ONPG permeation was

measured in untreated cells and in cells that had been treated with DBP and magnesium chloride. No differences were observed after 1 h incubation (Table 5). Outer membrane permeability changes were followed by measuring the release of β-lactamase (MW 31.5 KDa), a periplasmic enzyme (see Materials and Methods). β-Lactamase activity in the supernatant of cells treated with DBP and magnesium chloride was the same as that in untreated cells (Table 6).

**Macromolecular and phosphoglyceride synthesis:** At 20 μM, *rac*-DBP blocks phosphoglyceride and macromolecular synthesis (Fig. 13). Cells regain the ability to synthesize macromolecules and phosphoglycerides by the third hour of incubation (Fig. 13). Both macromolecular and phosphoglyceride synthesis also come to a halt during the first hour of incubation in the presence of DBP and magnesium chloride (Fig. 13). However, synthesis does not resume by the third hour of incubation. A combination of DBP and magnesium chloride also inhibited phosphoglyceride synthesis when [$^{14}$C]acetate was used in place of [$^{32}$P]phosphate (Fig. 14). The acetate studies eliminate the possibility of an artifact related to the use of [$^{32}$P]phosphate as the radioactive tracer.

Previous studies have shown that DBP alone blocks the initial rate of phosphoglyceride synthesis to a greater extent than it does that of DNA, RNA, or protein synthesis

(61). It is difficult to see this preferential inhibition of phosphoglyceride synthesis in Fig. 13 because the scale used tends to obscure the differences observed at 30 min. However, these differences are readily apparent when incorporation is compared after a short time interval. As indicated in Table 7, either DBP alone, or together with magnesium chloride, has a much greater inhibitory effect on phosphoglyceride synthesis than on either DNA, RNA, or protein synthesis.

The rates of DNA, RNA, and phosphoglyceride syntheses in LK88 were compared with those in LK99 using pulse-labeling method. DBP, magnesium chloride, or both were added to cultures of LK88 and LK99 when they reached a turbidity of 15 Klett units. A 2-mL sample was immediately removed from each culture and incubated with 20 μCi of carrier-free [$^{32}$P]phosphate for 10 min. The cultures were then incubated for an additional hour. A second 2-mL sample was removed and incubated with 20 μCi of [$^{32}$P]phosphate for 10 min. As indicated in Tables 8 and 9, a combination of DBP and magnesium chloride inhibits DNA, RNA, and phosphoglyceride synthesis in LK88 but not in LK99. At early times, phosphoglyceride synthesis in LK88 is much more sensitive to both DBP alone and to DBP and magnesium chloride than is either DNA or RNA synthesis. These results agree with those from HW55 shown in Table 7.

**DNA, RNA and phosphoglycerides turnover:** A combination of DBP and magnesium chloride might influence DNA, RNA, or phosphoglyceride turnover. This possibility was examined by monitoring DNA, RNA, and phosphoglyceride turnover in LK88 as described in Materials and Methods. Neither DBP nor a combination of DBP and magnesium chloride had any effect on DNA, RNA, or total phosphoglyceride turnover (Table 10). Therefore, both DBP and the combination of DBP and magnesium chloride influence the rates of DNA, RNA, and phosphoglyceride syntheses but not their rates of turnover. When the turnover of individual phosphoglyceride species was examined, two differences were observed : (1) Cells treated with DBP and magnesium chloride had considerably less [$^{32}$P]PG than untreated cells. (2) Cells treated with DBP and magnesium chloride had considerably more [$^{32}$P]CL than untreated cells (Table 11).

**ATP synthesis:** The ability of DBP and magnesium chloride to completely block macromolecular and phosphoglyceride biosynthesis might be explained by the inhibition of ATP synthesis or by the loss of ATP from the cells. These possibilities were tested by measuring intracellular and extracellular ATP levels in treated and untreated cells. When cells are treated with just DBP for 30 min, their intracellular ATP level is about 61% of that in untreated cells (Table 12). When cells are treated with

both DBP and magnesium chloride for 30 min, their intracellular ATP level is about 76% of that in untreated cells (Table 12). However, after a 60 min incubation, the intracellular ATP level in DBP-treated cells increases, albeit more slowly than the level in untreated cells. In contrast, intracellular ATP level continues to decrease in cells treated with a combination of DBP and magnesium chloride (Table 12). The ATP released into the growth medium was also monitored. The extracellular [$^{32}$P]ATP levels were virtually the same in all the cultures (about 6000 cpm/mL culture). This supports the notion that the inner cell membrane remains intact.

**Polyamine influence on DBP-treated cells:** A possible explanation for the synergistic bactericidal effect is that DBP treatment causes changes in the phosphoglyceride content of the cell membranes due to the accumulation of PGP analog and that these changes make cells much more sensitive to divalent cations. If this were true, one might predict that DBP would also make the cells more sensitive to organic cations. DBP-treated HW55 cells stop growing when incubated with either 0.5 mM spermidine or 0.125 mM spermine (Fig. 15A), but do not lose viability (Fig. 15B).

**Role of magnesium ions in the synergistic bactericidal effect:** Magnesium ions stimulate PGP analog synthesis

(Figs. 6, 7 and 8) and probably interact with the PGP analog. The role of magnesium ions in the synergistic bactericidal effect could be determined by studing a strain with a high copy number plasmid bearing a *pgsA* gene. An HW55 carrying pPGL2019 was constructed. The experiment was designed to compare strain HW55/pPGL2019 treated with 20 μM *rac*-DBP with strain HW55 treated with a combination of DBP and magnesium chloride. In the presence of 20 μM *rac*-DBP, HW55/pPGL2019 accumulats the same amount of PGP analog as HW55 treated with a combination of DBP and magnesium chloride. In 1-mL culture, both strains incorporated $4.8 \times 10^4$ cpm of [3-$^3$H]DBP after 1 hr treatment. After 5 hr treatment, viability studies showed that HW55/pPGL2019 from this culture contains $3.2 \times 10^8$ viable cells, whereas HW55 contains $7.1 \times 10^4$ viable cells. Although HW55/pPGL2019 accumulates a high level of the PGP analog when treated with *rac*-DBP alone it does not lose viability.

**Chloramphenicol's influence on DBP-treated cells:** When added along with DBP and magnesium chloride, chloramphenicol decreases the rate of cell viability loss (Fig. 16A). The sparing effect is less evident when chloramphenicol is added 0.5 h or 1 h after exposure to DBP and magnesium chloride. While it is tempting to propose that these observations indicate that continued protein synthesis is necessary for the synergistic bactericidal

effect, there are two reasons for caution. First, chloramphenicol has a slight inhibitory effect on PGP analog formation (Fig. 16B). Second, a combination of DBP and magnesium chloride blocks protein synthesis (Fig. 16C). However, it should be noted that cultures containing DBP and magnesium chloride synthesize more protein during the first hour than do those that also contain chloramphenicol.

## DISCUSSION

A combination of 20 μM *rac*-DBP and 20 mM magnesium chloride is bactericidal to *E. coli* HW55 (Fig. 2). A similar effect is observed when calcium chloride replaces magnesium chloride (Figs. 3 and 5). The bactericidal effect is cation concentration dependent. DBP is bacteriostatic rather than bactericidal when the medium contains less than 5 mM magnesium ion or 2.5 mM calcium ion (Figs. 4 and 5).

The loss of viability in the presence of calcium ions is especially significant. Silver has proposed that calcium ions, unlike magnesium ions, are not normally transported into *E. coli* and that the intracellular level of calcium ions is very low (76). Consequently, he concluded that calcium ions probably exert their effect by acting on the outer membrane or the outer surface of the inner membrane (76). This postulation has been confirmed by Chang *et al.* (77). They found that cellular magnesium ion concentration is approximately 114 mM and is uniformly distributed within the bacterial cell, while a high concentration of calcium ion is localized in the cell envelope (77). In the case of the synergistic effect of DBP and calcium chloride, calcium ions could act at the

outer membrane or the outer-leaflet of inner membrane if the permeability of inner membrane to calcium ion does not change due to the accumulation of PGP analog. The same may be true for the magnesium ions. This agrees with the assumption from DeChavigny *et al.* that divalent metal ions are required for growth of a *pss* mutant which interact with acidic phosphoglycerides to compensate for the lack of PE (28).

As described previously, DBP is a competitive substrate for the *E. coli* PGP synthase; it blocks PG synthesis and is incorporated into the phosphonate analog of PGP (59, 65). The present study indicates that HW55 cultured in the presence of DBP and magnesium chloride also synthesizes the same PGP analog (Figs. 6 and 7). Either kind of phosphoglyceride perturbation could be responsible for the bacteriostatic effect observed when *E. coli* are incubated with DBP alone and the bactericidal effect observed when cells are incubated with a combination of DBP and magnesium chloride. Strain LK99 (*pgsA3*) synthesizes very low levels of PG and cannot form PGP analog. It can therefore be used to determine whether the bacteriostatic effects of DBP and bactericidal effects of DBP and magnesium chloride are due to the appearance of the PGP analog or the disappearance of PG.

LK99 has a more rapid rate of DBP uptake than does

wild-type LK88 (Table 3). The reason for this is not known. LK99 does not incorporate DBP into the PGP analog when cultured in the presence or absence of magnesium chloride (Table 4). As shown in Fig. 9, LK99 is more resistant to DBP than LK88. Furthermore, LK99 grows in the presence of DBP and magnesium chloride while LK88 does not (Fig. 10). Similar results are observed when calcium chloride replaces magnesium chloride (Fig. 11, also see Results). A combination of DBP and magnesium chloride inhibits DNA, RNA, and total phosphoglyceride syntheses in LK88 but not in LK99 (Tables 8 and 9). This combination has no effect on DNA, RNA, or phosphoglyceride turnover in LK88 (Tables 10 and 11).

LK88 and LK99 studies suggest that growth inhibition and viability loss are due to the accumulation of PGP analog rather than to the inability to synthesize PG. Therefore, PGP analog appears to play an important role in the bacteriostatic effect of DBP alone and the bactericidal effect of the combination of DBP and magnesium ions. Additional factors may also be involved. Chloramphenicol blocks the ability of DBP and magnesium chloride to cause a loss in cell viability (Fig. 16A). However, chloramphenicol-treated cells continue to synthesize the PGP analog (Fig. 16B). These observations suggest that continued synthesis of some specific protein(s) may be

required for viability loss.

After incubation with 100 μM *rac*-DBP for 1 hour, LK88 loses viability when stored in the cold but LK99 does not (Fig. 12). This observation suggests that the PGP analog is also responsible for the cold sensitivity of DBP-treated cells (51). LK88 has a higher survival rate in cold storage than LK99. This is probably due to the difference in phosphoglyceride composition between these two strains.

Several possible explanations can be offered for the loss of viability when HW55 is treated with a combination of 20 μM *rac*-DBP and 20 mM magnesium chloride. Perturbation of inner or outer membrane structure seems the most likely. Magnesium chloride causes a two-fold increase in cellular PGP analog levels (Figs. 6, 7 and 8). Furthermore, The interaction between phospholipids and metal cations, particularly calcium ions has received considerable attention and has important physiological consequences (78). Binding of calcium ions to model membranes can cause changes in lipid bilayer structure (78, 79, 80). When added to many acidic and neutral lipid mixtures, calcium ions cause a lateral phase separation in which anionic phosphoglycerides are sequestered into separate bilayer domains (81, 82). Calcium ions can also promote bilayer to hexagonal ($H_{II}$) phase transitions (78). Fluorescence studies suggest that the PGP analog has a

significant effect on the physical state and fluidity of model membranes below transition temperature and the addition of calcium or magnesium ions to the system probably leads to further perturbations ( M. Glazman, personal communication). These perturbations may cause changes in membrane structure or function that will result in the observed synergistic effect.

If divalent metal cations influence cell growth and viability by interacting with PGP analog in the inner or outer membrane, then a polyamine such as spermidine or spermine might have a similar effect. DBP-treated HW55 cells stop growing when incubated with either 0.5 mM spermidine or 0.125 mM spermine (Fig. 15A), but remain viable (Fig. 15B). The observation that DBP-treated cells lose viability when cultured in the presence of divalent metal cations but not in the presence of the polyamines probably does not reflect a difference in mechanism of action of the two types of cations. The polyamine concentrations used might have been too low to produce a viability loss. Higher polyamine concentrations cannot be used because, at these higher concentrations, polyamines alone block cell growth (83).

Magnesium ions can stimulate PGP analog synthesis and interact with the PGP analog. To determine which of these two actions of magnesium ions is responsible for the

synergistic bactericidal effect, an HW55 with a high copy number plasmid bearing a *pgsA* gene was constructed. In the presence of 20 μM *rac*-DBP, this strain can accumulate as much PGP analog as HW55 treated with a combination of DBP and magnesium chloride. However, no viability loss was observed when the plasmid bearing strain was incubated with *rac*-DBP alone (See results). This result suggests that the interaction between divalent metal ions and PGP analog plays the primary role in growth inhibition, cell death, and the observed physiological effects in wild-type *E. coli*.

The interaction of the PGP analog and divalent cations might cause the outer or inner membrane to become permeable. However, the present study suggests that growth in the presence of 20 μM *rac*-DBP and 20 mM magnesium chloride does not lead to a loss of either inner or outer bacterial membrane integrity. As shown in Fig. 2A, the culture turbidity drops only slightly during the period of viability loss. A much greater turbidity decrease would be expected for lysing cells. Furthermore, when permeabilities of the two cell membranes were examined, the inner membrane remains a permeability barrier to ONPG and [$^{32}$P]ATP, and the outer membrane remains a permeability barrier to β-lactamase after cells have been incubated in the presence of DBP and magnesium chloride. Although the

inner membrane appears to retain its integrity, it may be altered in a more subtle fashion. The influence of colicin K on membrane permeability is instructive in this regard. After treatment with colicin K, the inner membrane remains a permeability barrier to ONPG but not to potassium ions (84, 85). The inner membrane of cells that have been treated with DBP and magnesium chloride may also lose the ability to serve as a permeability barrier to potassium or some other essential ion. Such leakiness may be responsible for the loss of cell viability. Further studies are required to evaluate this possibility.

Previous studies showed that DBP alters phosphoglyceride metabolism and blocks macromolecular synthesis (61). The experiments performed in this study show that a combination of DBP and magnesium chloride has similar effects (Fig. 13). However, one important difference was observed. Cells treated with DBP alone recover the ability to synthesize macromolecules and phosphoglycerides by the third hour of incubation, whereas those treated with DBP and magnesium chloride do not. Both DBP alone and a combination of DBP and magnesium chloride act primarily on phosphoglyceride synthesis (Table 7).

Previous studies showed that low concentrations of DBP do not block ATP synthesis (63). Because of the broad inhibitory effects of DBP and magnesium chloride, acting

together, DBP's effect on ATP synthesis was reexamined. As shown in Table 12, both DBP alone and a combination of DBP and magnesium chloride inhibit ATP synthesis to the same extent after 30 min treatment. The difference in results between the present and previous studies may be due to a variation in genetic background or culture conditions. After an initial drop, intracellular ATP concentrations in DBP-treated cells increase. In contrast, intracellular ATP concentrations continue to decrease when cells treated with DBP and magnesium chloride. The formation of a large amount of PGP analog also attributes to the decrease in intracellular ATP concentration. Rapid incorporation of DBP into PGP analog may help to deplete the pool of CDP-diacylglycerol and increase the CMP level. This results in the consumption of intracellular ATP. A decrease in ATP level with a concomitant increase in ADP and AMP levels would lower the adenylate energy charge (86). Taken together, the inability of cells to synthesize ATP in the presence of DBP and magnesium chloride may have an inhibitory effect on macromolecular synthesis and cause viability loss (85, 86).

The studies with the PGP analog may have significance for untreated cells since PGP is a normal metabolic intermediate, and PGP and its analog are very similar. The stimulation of PGP analog synthesis by magnesium ions

suggests that magnesium ions may also influence the rate of synthesis of the normal metabolic intermediate PGP. Furthermore, an *E. coli* mutant that accumulates high levels of PGP may have serious physiological problems. Such a mutant will probably be very sensitive to cold and divalent cations. PGP has been proposed to regulate phosphoglyceride composition (58). This effect may be influenced by magnesium ions, calcium ions, spermidine or other cations. Finally, interactions between PGP and divalent cations may affect other aspects of bacterial metabolism.

**TABLE 1**

**Strain list**

| Strains | Relevant characteristics | Source or reference |
|---|---|---|
| SD12 | F⁻ *glpD3 glpR3 glpK glpKp phoA8 pyrD34 his-68 galk35 xyl-7 mtl-2 rpsL118* | 37 |
| HD3122 | SD12 *pgsA3 uvrC279*::Tn*10* | W. Dowhan (20) |
| LK8 | SD12 *glpD*⁺ | This study |
| LK9 | HD3122 *glpD*⁺ | This study |
| LK88 | SD12 *glpD*⁺ *pyrD34*⁺ | This study |
| LK99 | HD3112 *glpD*⁺ *pyrD34*⁺ | This study |
| HW55 | HfrC *glpR cls-1* | 51 |
| LK35 | HW55 *lacY* | This study |
| LE392 | F⁻ *supE supF hsdR galK trpR metB lacY tonA* | 87 |
| SVS111 | W3110 *bglR551 proC*::Tn*5* | T.J.Silhavy |
| Strain 7 | HfrC *glpR* | 88 |
| JA200 | F⁺ *pgsA*⁺ delt(*trpE5*) *recA thr leu thi* | 89 |
| **Plasmids** | | |
| pPHD102 | *pgsA*⁺ *cam* (Temperature Sensitive) | W. Dowhan (20) |
| pPBR322 | *bla tet* | 90 |
| pPGL2019 | *pgsA*⁺ *glyW*⁺ Amp$^r$ Kan$^s$ | 89 |

**TABLE 2**

**Effect of DBP and $MgCl_2$ on phosphoglyceride distribution of HW55**

| | Total cpm per mL culture (% composition) | | | |
|---|---|---|---|---|
| | PGP analog | PE+PG | CL | Others |
| untreated | 222(0.6)* | 32831(91.3) | 485(1.4) | 2439(6.8) |
| +DBP | 1407(7.4) | 16385(86.2) | 358(1.9) | 864(4.5) |
| +$MgCl_2$ | 232(0.8)* | 25614(92.0) | 349(1.3) | 1642(5.9) |
| +DBP+$MgCl_2$ | 2641(13.9) | 15141(79.5) | 550(2.9) | 724(3.8) |

HW55 was cultured in CH medium containing carrier-free [$^{32}$P]phosphate (2 $\mu$Ci/mL). When the cultures reached a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added where indicated. After a further one hour incubation, phosphoglyceride fraction was extracted and analyzed by thin-layer chromatography with $CHCl_3$:MeOH:HAc:$H_2O$ (25:15:4:2) as described in Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively.

* Cells not treated with DBP accumulate a trace quantity of radioactive product that has the same $R_f$ as the PGP analog. Although this labeled material was not characterized, it can not be the PGP analog.

**TABLE 3**

**[3-$^3$H]DBP transport into strain LK88 and LK99**

| Wash Medium | DBP transport (cpm) after 2 min | | | |
|---|---|---|---|---|
| | LK88 | | LK99 | |
| | $-Mg^{2+}$ | $+Mg^{2+}$ | $-Mg^{2+}$ | $+Mg^{2+}$ |
| 0.85% NaCl | 1,778 | 1,508 | 3,190 | 2,646 |
| Cold $H_2O$ | 292 | 306 | 574 | 640 |

Transport assays were performed with LK88 and LK99 that had been cultured in CHG medium. Where indicated, magnesium chloride was maintained at 20 mM in both the culture and assay media. After a 2 min incubation with *rac*-[3-$^3$H]DBP at 30°C, cells (0.1 mL) were collected on a membrane filter ( 0.45-$\mu$m pore size and 25-mm diameter) and washed with 1 mL 0.85% sodium chloride at 30°C or with distilled water at 5°C. Radioactivity was determined as described in Materials and Methods.

**TABLE 4**

**[3-$^3$H]DBP incorporation into the phosphoglyceride fractions of LK88 and LK99**

| Time (hr.) | cpm per mL of culture | | | |
|---|---|---|---|---|
| | LK88 | | LK99 | |
| | $-Mg^{2+}$ | $+Mg^{2+}$ | $-Mg^{2+}$ | $+Mg^{2+}$ |
| 0 | 100 | 46 | 88 | 54 |
| 1 | 2,188 | 5,742 | 74 | 202 |

Strain LK88 and LK99 were assayed for *rac*-[3-$^3$H]DBP incorporation into the phosphoglyceride fraction in the presence or absence of magnesium chloride. Phosphoglyceride fractions were extracted into chloroform as described in Materials and Methods. The final concentration of magnesium chloride was 20 mM.

**TABLE 5**

**Effect of DBP and $MgCl_2$ on ONPG permeation**

| | $\Delta A_{420}$/min mL culture | | | |
|---|---|---|---|---|
| | **untreated** | **+DBP** | **+$MgCl_2$** | **+DBP+$MgCl_2$** |
| cell suspension | 0.064 | 0.047 | 0.072 | 0.058 |
| sonicate | 1.095 | 1.018 | 1.074 | 0.999 |

LK35 (*lacY*) was cultured in CH medium containing 1 mM IPTG. When the cultures reached a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added where indicated. After a further 1 h incubation, ONPG permeation was examined for cell suspensions and sonicates as described in Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM magnesium chloride, respectively. Data shown are changes in $A_{420}$ per min of 1-mL culture.

**TABLE 6**

**Effect of DBP and $MgCl_2$ on β-lactamase leakage**

| | $\Delta A_{260}$/min mL culture | |
|---|---|---|
| | +$MgCl_2$ | +DBP+$MgCl_2$ |
| supernatant | 0.004 | 0.004 |
| sonicate | 0.095 | 0.039 |

HW55/pBR322 was cultured in CH medium containing 100 μg/mL ampicillin and 5 mM magnesium chloride. When the cultures reached a turbidity of 15 Klett units, additional magnesium chloride or with *rac*-DBP were added to 20 mM and 20 μM, respectively. After a further 90 min incubation, samples of the cultures were either centrifuged or sonicated. β-lactamase activity in the supernatants and sonicates were determined as described in Materials and Methods. Data shown are changes in $A_{260}$ per min of 1-mL culture.

**TABLE 7**

**Effects of DBP and $MgCl_2$ on macromolecular and phosphoglyceride synthesis in HW55**

| | % of CPM of untreated cultures | | | |
|---|---|---|---|---|
| | **DNA** | **RNA** | **lipid** | **protein** |
| untreated | 100(261) | 100(11609) | 100(3563) | 100(5760) |
| +DBP | 70 | 79 | 61 | 85 |
| +$MgCl_2$ | 105 | 122 | 104 | 96 |
| +DBP+$MgCl_2$ | 87 | 94 | 42 | 78 |

HW55 was cultured in CH medium. [$^{32}$P]phosphate or [$^{14}$C]leucine was added when the cultures reached a turbidity of 15 Klett units. Cells were either untreated, or treated with DBP and magnesium chloride, DBP, or magnesium chloride. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. Incorporation of [$^{32}$P]phosphate into DNA, RNA, phosphoglycerides, and [$^{14}$C]leucine into protein was followed as described in Materials and Methods. Data shown were obtained by comparing [$^{32}$P]phosphate or [$^{14}$C]leucine incorporation of treated HW55 to that of untreated cells at 30 min. The numbers given in parenthesis represent cpm/mL culture.

**Table 8.** LK88 was cultured in CHG medium. When the cultures reached a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added where indicated. A 2-mL sample was immediately removed from each culture and incubated with 20 $\mu$Ci of [$^{32}$P]phosphate for 10 min. After the cultures were incubated for an additional hour, a second 2-mL sample was removed and incubated with 20 $\mu$Ci of [$^{32}$P]phosphate for 10 min. Labeled DNA, RNA and phosphoglycerides were determined as described under Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. Data shown were obtained by comparing [$^{32}$P]phosphate incorporation of treated LK88 to that of untreated LK88. The numbers given in parenthesis represent cpm/mL culture.

**TABLE 8**

**Effects of DBP and $MgCl_2$ on the rates of DNA, RNA and phosphoglyceride syntheses in LK88**

| | 10 Minutes | | |
|---|---|---|---|
| | %DNA | %RNA | %Phosphoglyceride |
| untreated | 100 (1201) | 100 (39318) | 100 (4862) |
| +DBP | 95.5 | 106 | 46.5 |
| +$MgCl_2$ | 168 | 110 | 101 |
| +DBP+$MgCl_2$ | 110 | 121 | 50.1 |
| | **70 Minutes** | | |
| | %DNA | %RNA | %Phosphoglyceride |
| untreated | 100 (2230) | 100 (66968) | 100 (8212) |
| +DBP | 23.4 | 19.6 | 35.3 |
| +$MgCl_2$ | 59.5 | 98.0 | 76.1 |
| +DBP+$MgCl_2$ | 3.8 | 4.9 | 9.3 |

**Table 9.** LK99 was cultured in CHG medium. When the cultures reached a turbidity of 15 Klett units, DBP, magnesium chloride, or both were added where indicated. A 2-mL sample was immediately removed from each culture and incubated with 20 $\mu$Ci of [$^{32}$P]phosphate for 10 min. After the cultures were incubated for an additional hour, a second 2-mL sample was removed and incubated with 20 $\mu$Ci of [$^{32}$P]phosphate for 10 min. Labeled DNA, RNA and phosphoglycerides were determined as described under Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. Data shown were obtained by comparing [$^{32}$P]phosphate incorporation of treated LK99 to that of untreated LK99. The numbers given in parenthesis represent cpm/mL culture.

**TABLE 9**

**Effects of DBP and $MgCl_2$ on the rates of DNA, RNA, and phosphoglyceride syntheses in LK99**

| | 10 Minutes | | |
|---|---|---|---|
| | %DNA | %RNA | %Phosphoglyceride |
| untreated | 100 (562) | 100 (36932) | 100 (4562) |
| +DBP | 76.8 | 82.4 | 84.9 |
| +$MgCl_2$ | 108 | 123 | 101 |
| +DBP+$MgCl_2$ | 104 | 111 | 86.0 |
| | **70 Minutes** | | |
| | %DNA | %RNA | %Phosphoglyceride |
| untreated | 100 (1485) | 100 (65836) | 100 (8925) |
| +DBP | 114 | 105 | 101 |
| +$MgCl_2$ | 142 | 141 | 147 |
| +DBP+$MgCl_2$ | 120 | 139 | 127 |

**TABLE 10**

**Effect of DBP and $MgCl_2$ on DNA, RNA and phosphoglycerides turnover in LK88**

| | Total cpm per mL of culture | | |
|---|---|---|---|
| | DNA | RNA | Phosphoglycerides |
| untreated | 5,676 | 50,610 | 12,294 |
| +DBP | 6,340 | 57,323 | 10,138 |
| +$MgCl_2$ | 6,074 | 56,305 | 13,103 |
| +DBP+$MgCl_2$ | 5,812 | 56,328 | 12,526 |

LK88 was cultured in CHG medium containing [$^{32}$P]phosphate (2 $\mu$Ci/mL) for five generations. At 15 Klett units, cells were washed and resuspended in fresh CHG medium containing 1 mM $NaH_2PO_4$. Samples of 2- mL were removed and incubated for an additional 2 h or treated with DBP, magnesium chloride, or both and then incubated for an additional 2 h. Labeled DNA, RNA and phosphoglycerides were determined as described under Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride are 20 $\mu$M and 20 mM, respectively.

**TABLE 11**

**Effect of DBP and $MgCl_2$ on each phosphoglyceride turnover in LK88**

| | Total cpm per mL of culture | | |
|---|---|---|---|
| | PE | PG | CL |
| untreated | 10,475 | 1,339 | 480 |
| +DBP | 8,646 | 779 | 712 |
| +$MgCl_2$ | 11,106 | 1,506 | 491 |
| +DBP+$MgCl_2$ | 9,482 | 407 | 2,636 |

LK88 was cultured in CHG medium containing [$^{32}$P]phosphate (2 $\mu$Ci/mL) for five generations. At 15 Klett units, cells were washed and resuspended in fresh CHG medium containing 1 mM $NaH_2PO_4$. Samples of 2 mL were removed and incubated for an additional 2 h or treated with DBP, magnesium chloride, or both and then incubated for an additional 2 h. Labeled phosphoglycerides were extracted as described under Materials and Methods. Phosphoglyceride fractions were further analyzed by thin-layer chromatography in solvent system 1 as described in Materials and Methods. The final concentrations of *rac*-DBP and magnesium chloride are 20 $\mu$M and 20 mM, respectively.

**TABLE 12**

**Effect of DBP and $MgCl_2$ on the intracellular levels of ATP in HW55**

| Time(min) | $^{32}P$ incorporated into ATP(cpm/ mL culture) | | | |
|---|---|---|---|---|
| | untreated | +DBP | +$MgCl_2$ | +DBP+$MgCl_2$ |
| 30 | 13,080 | 8,020 | 15,540 | 9,970 |
| 60 | 26,850 | 13,300 | 19,060 | 8,440 |

HW55 was cultured in CH medium. When the cultures reached a turbidity of 15 Klett units, cells were labeled with [$^{32}P$]phosphate (100 $\mu$Ci/mL) and treated with DBP, magnesium chloride, or both where indicated. [$^{32}P$]ATP was extracted and analyzed as described in Material and Methods. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively.

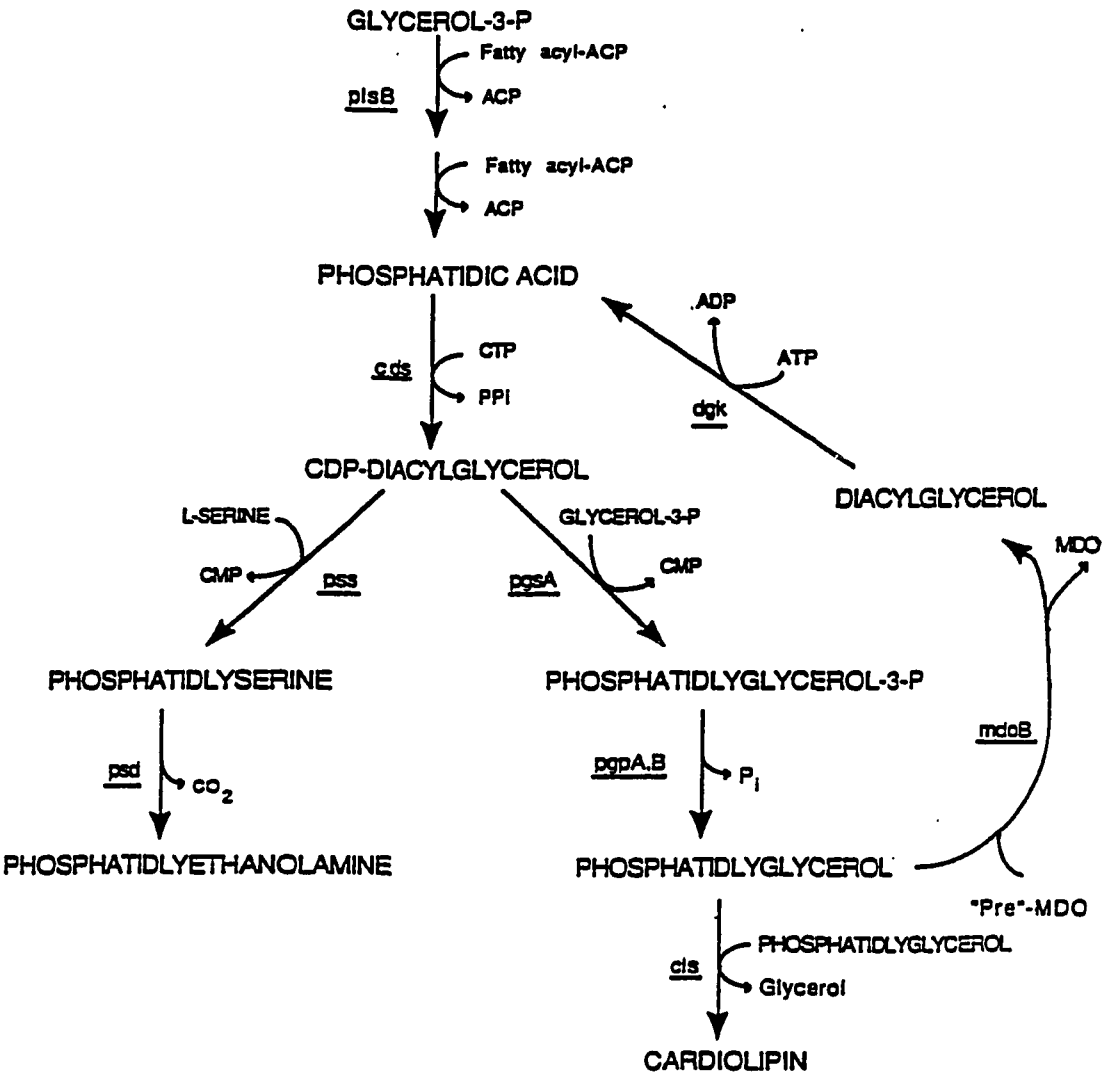



**Fig. 1.** Phosphoglyceride biosynthesis in *E. coli*.

**Fig. 2.** Effects of DBP and magnesium chloride on the (A) growth and (B) viability of HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP, magnesium chloride, or both were added where indicated. The final concentrations of *rac*-DBP, and magnesium chloride were 20 $\mu$M and 20 mM, respectively. **Symbols:** DBP+$MgCl_2$ (○); $MgCl_2$ (●); DBP (▲); and untreated (■).

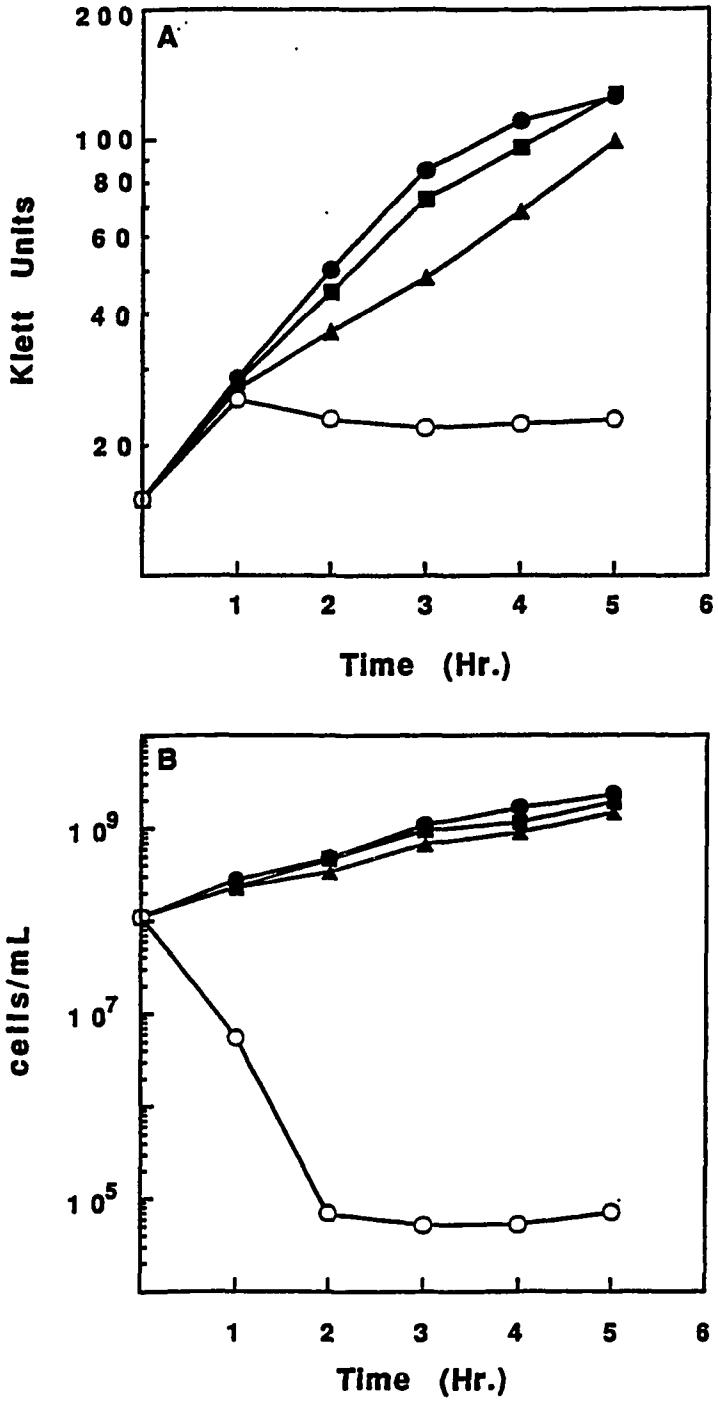

A
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)
B
$10^9$
$10^7$
$10^5$
cells/mL
1
2
3
4
5
6
Time (Hr.)

Fig. 2.

**Fig. 3.** The effect of DBP and calcium chloride on the growth of HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP, calcium chloride, or both were added where indicated. The final concentrations of *rac*-DBP, and calcium chloride were 20 μM and 20 mM, respectively. **Symbols:** DBP+$CaCl_2$ (○); $CaCl_2$ (●); DBP (▲); and untreated (■).

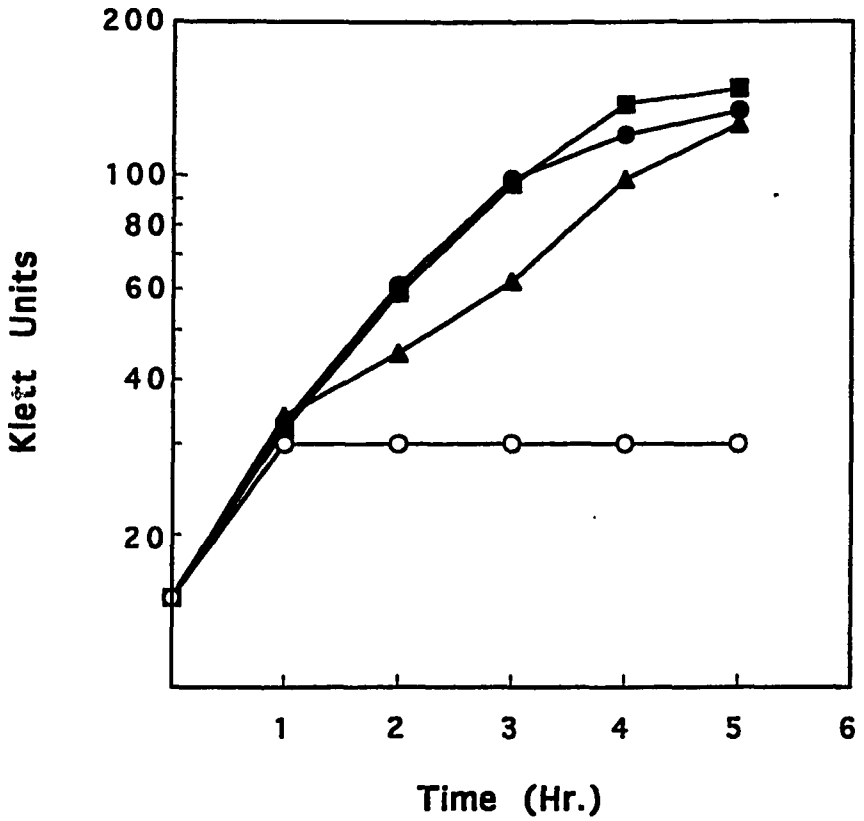



Fig. 3.

**Fig. 4.** Effects of magnesium or calcium ion concentrations on the growth of DBP-treated HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP was added. Then (A) magnesium chloride or (B) calcium chloride was added to the following final concentrations in mM : 0 (■), 2.5 (▲), 5 (●), 10 (○), 15 (◊). The final concentration of *rac*-DBP was 20 μM. Cultures treated with 20 μM *rac*-DBP plus 15 mM magnesium or calcium chloride behaved identically to cultures treated with 20 μM *rac*-DBP plus 20 mM magnesium or calcium chloride.

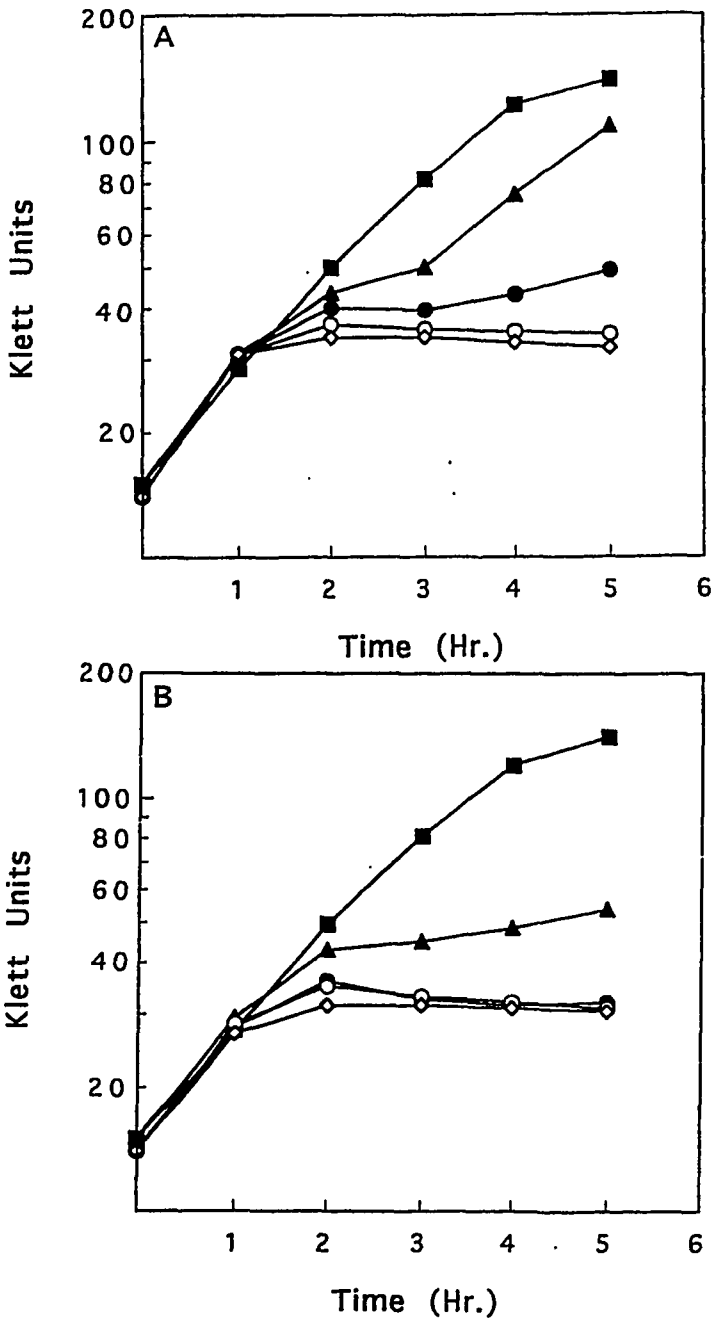

A
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)
B
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)

**Fig. 4.**

**Fig. 5.** Effects of magnesium or calcium ion concentrations on the viability of DBP-treated HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units, *rac*-DBP was added. Then magnesium (▲) or calcium chloride (■) at various concentrations was added. Viability studies were carried out at the end of 4 hr treatment. The final concentration of *rac*-DBP was 20 μM.

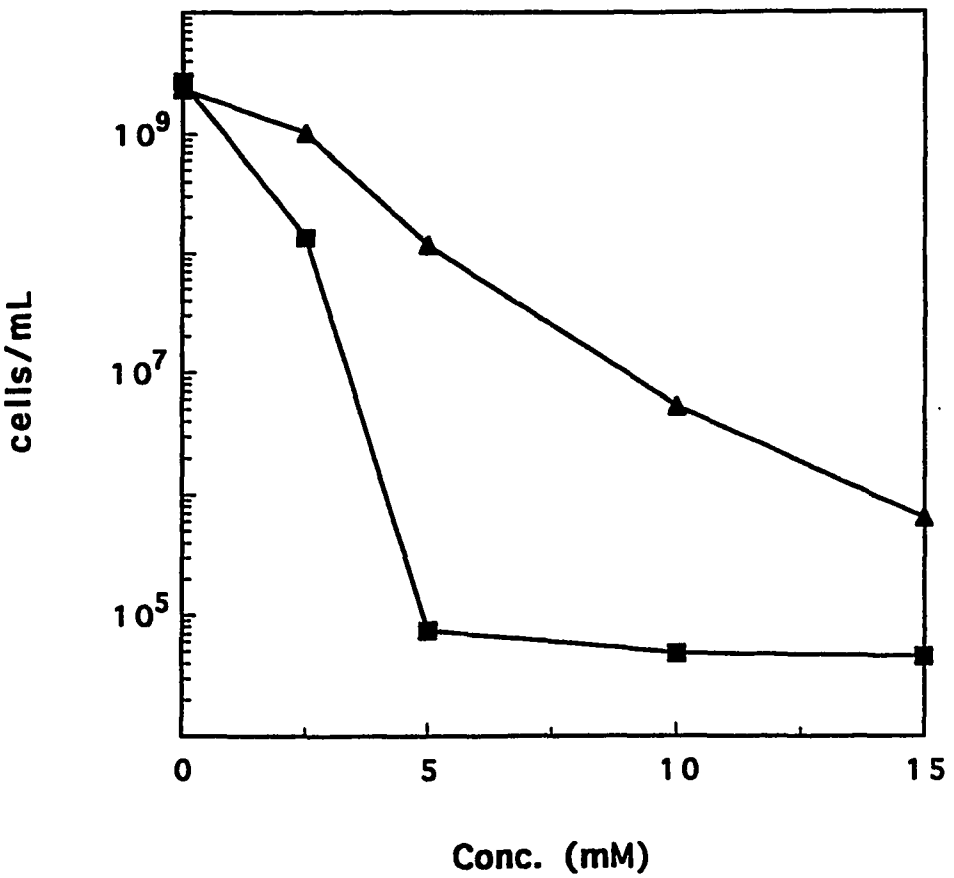



Fig. 5.

**Fig. 6.** The effect of magnesium chloride on [3-$^3$H]DBP incorporation into phosphoglycerides of HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units, (A) *rac*-[3-$^3$H]DBP (sp. act. 50 $\mu$Ci/$\mu$mole, 1 $\mu$Ci) or (B) *rac*-[3-$^3$H]DBP plus magnesium chloride were added (see Materials and Methods). After an hour of incubation, phosphoglycerides were extracted with chloroform and analyzed on TLC plates with $CHCl_3$:MeOH:HAc:$H_2O$ (25:15:4:2) as described in Materials and Methods. After drying, the plates were cut at 1 cm intervals and the radioactivity in each piece was determined. The location of PGP analog is indicated.

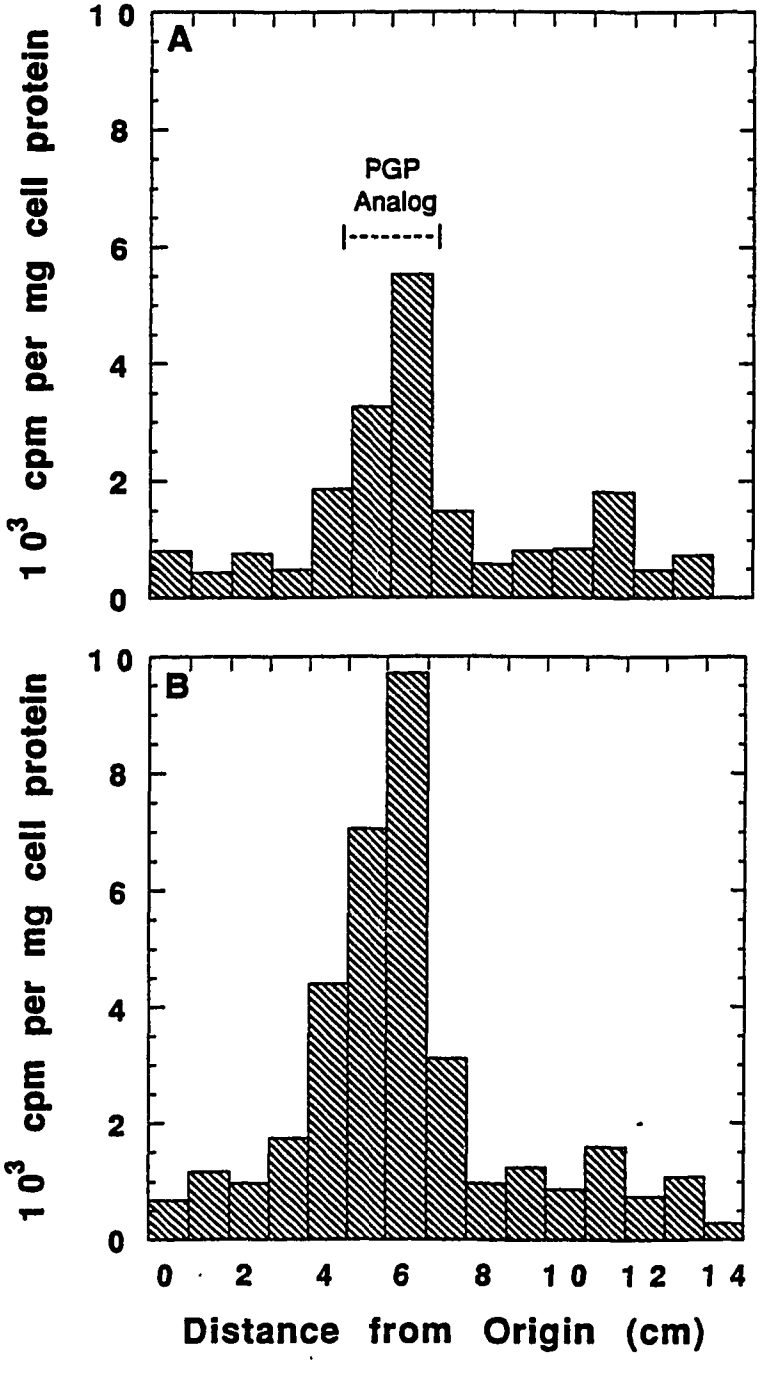

A
PGP
Analog
B
10 cpm per mg cell protein
Distance from Origin (cm)
0
2
4
6
8
10
12
14

Fig. 6.

**Fig. 7.** The effect of magnesium chloride on [1,2-$^3$H]DBP incorporation into phosphoglycerides of HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units, (A) *rac*-[1,2-$^3$H]DBP (sp. act. 50.5 $\mu$Ci/$\mu$mole, 1 $\mu$Ci) or (B) *rac*-[1,2-$^3$H]DBP plus magnesium chloride were added (see Materials and Methods). After an hour of incubation, phosphoglycerides were extracted with chloroform and analyzed on TLC plates with $CHCl_3$:MeOH:HAc:$H_2O$ (25:15:4:2) as described in Materials and Methods. After drying, the plates were cut at 1 cm intervals and the radioactivity in each piece was determined. The location of PGP analog is indicated.

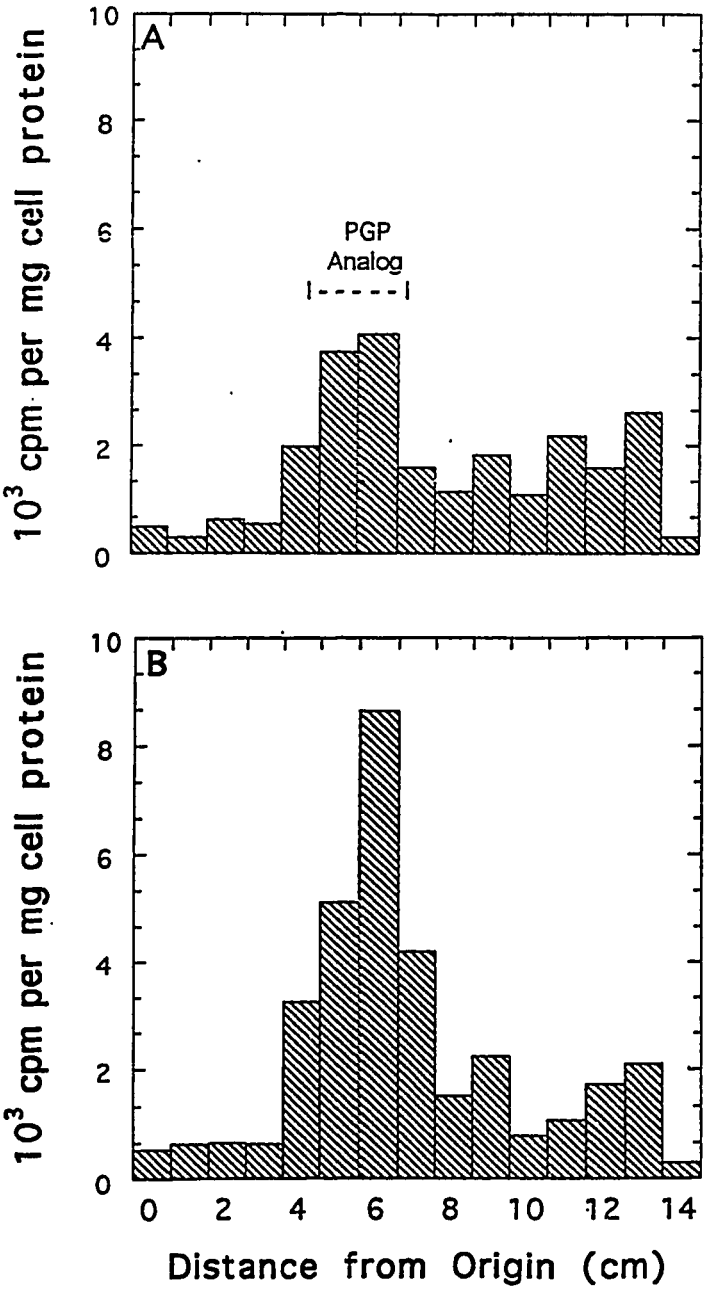

A
B
PGP
Analog
$10^3$ cpm per mg cell protein
$10^3$ cpm per mg cell protein
0
2
4
6
8
10
12
14
Distance from Origin (cm)

**Fig. 7.**

**Fig. 8.** The effect of sodium azide on [3-$^3$H]DBP incorporation into phosphoglycerides of HW55 cultured in CH medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-[3-$^3$H]DBP (sp. act. 50 $\mu$Ci/$\mu$mole, 1 $\mu$Ci) was added. Magnesium chloride, sodium azide, or both were added where indicated. The final concentrations of magnesium chloride and sodium azide were 20 mM and 10 mM, respectively. Samples were removed and phosphoglyceride fractions were extracted by the filter paper assay method as described in Materials and Methods. **Symbols:** DBP+$MgCl_2$ (●); DBP (■); DBP+$MgCl_2$+$NaN_3$ (○); and DBP+$NaN_3$ (□).

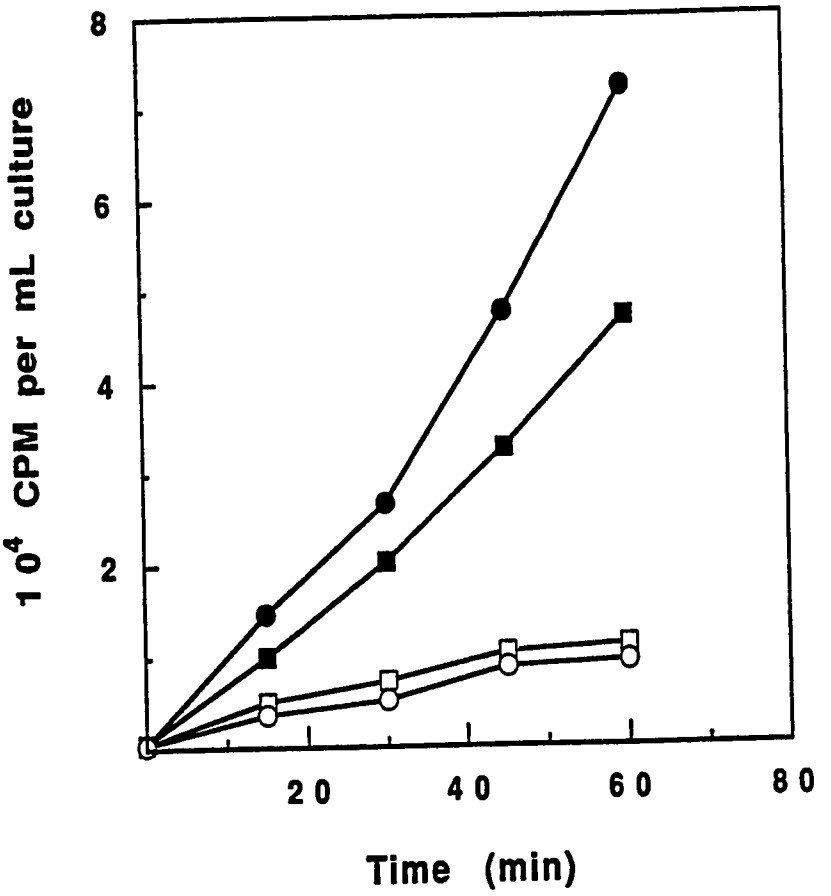
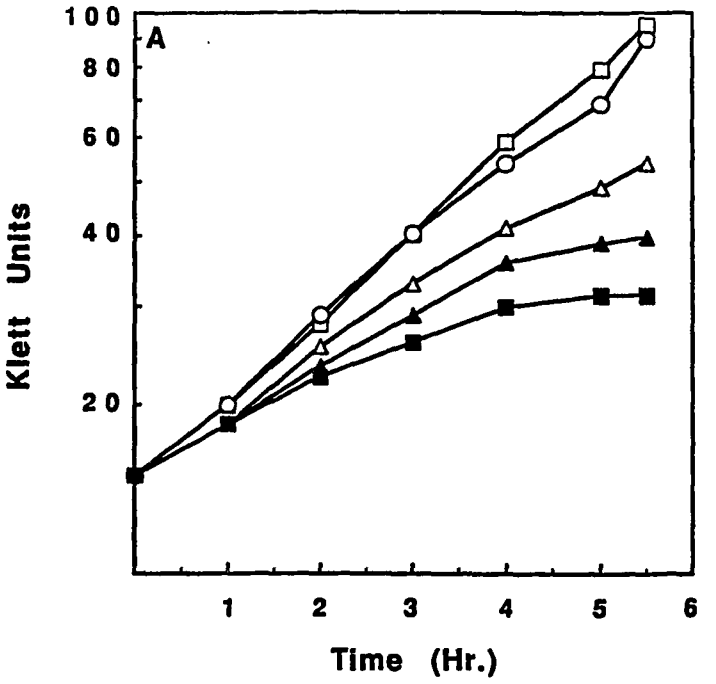



Fig. 8.

**Fig. 9.** The effect of DBP alone on the growth of (A) LK99 (*pgsA*) and (B) LK88 (*pgsA*$^+$) cultured in CHG medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP was added to the following final concentrations in $\mu$M (A): 0 (□), 100 (○), 250 (△), 500 (▲), 1000 (■); and in (B): 0 (□), 40 (△), 60 (○), 80 (■), 100 (▲).


A
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)

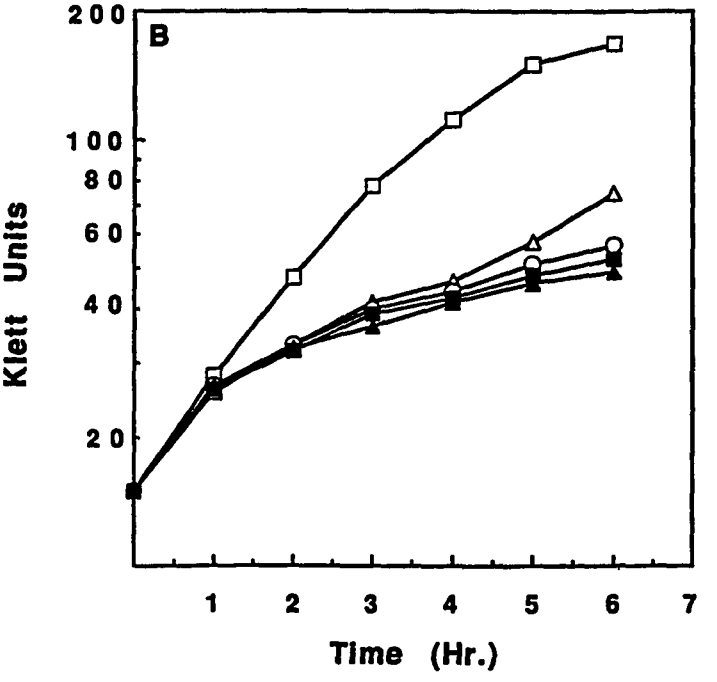
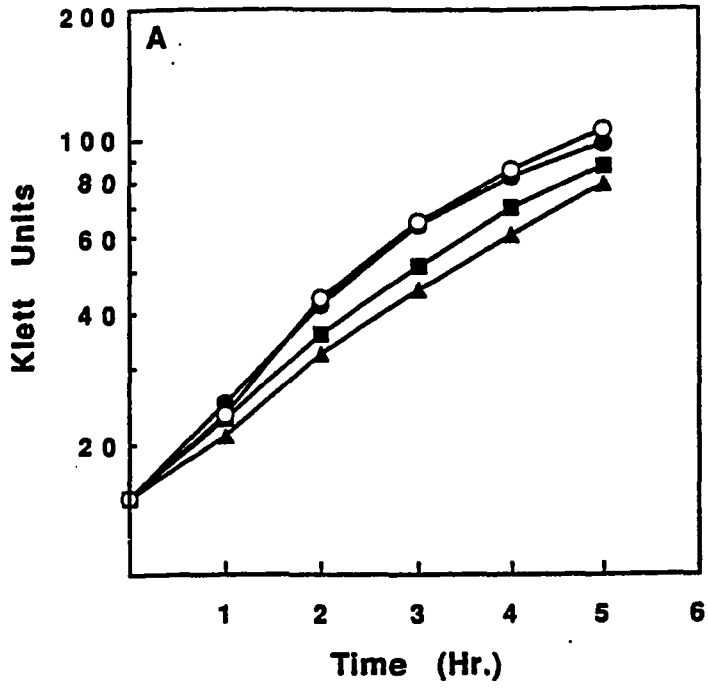

B
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
7
Time (Hr.)

Fig. 9.

**Fig. 10.** Effects of DBP and magnesium chloride on the growth of (A) LK99 (*pgsA*) and (B) LK88 (*pgsA*$^+$) cultured in CHG medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP, magnesium chloride, or both were added where indicated. The final concentrations of *rac*-DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. **Symbols:** DBP+$MgCl_2$ (○); $MgCl_2$ (●); DBP (▲); and untreated (■).


A
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)

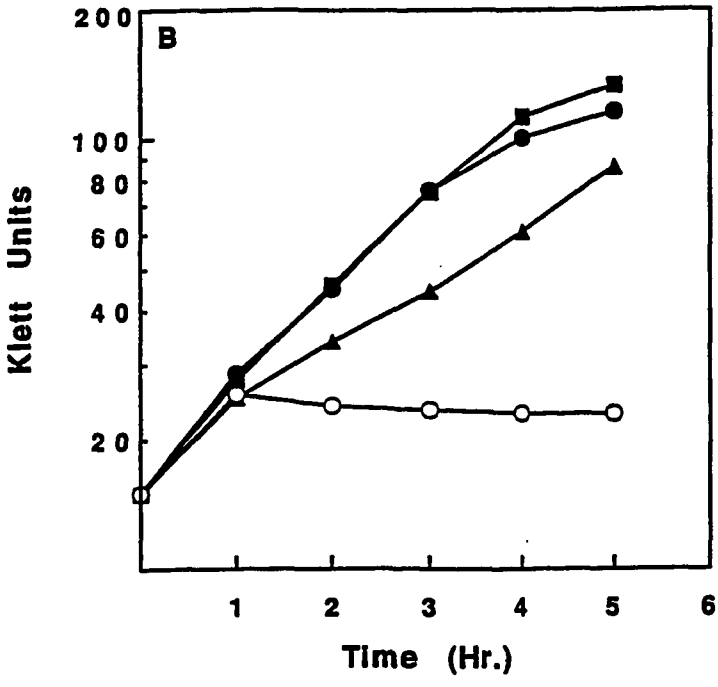

B
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)

Fig. 10.

**Fig. 11.** Effects of DBP and calcium chloride on the growth of (A) LK99 (*pgsA*) and (B) LK88 (*pgsA*$^+$) cultured in CHG medium. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP, calcium chloride, or both were added where indicated. The final concentrations of *rac*-DBP and calcium chloride were 20 $\mu$M and 20 mM, respectively. **Symbols:** DBP+$CaCl_2$ (○); $CaCl_2$ (●); DBP (▲); and untreated (■).

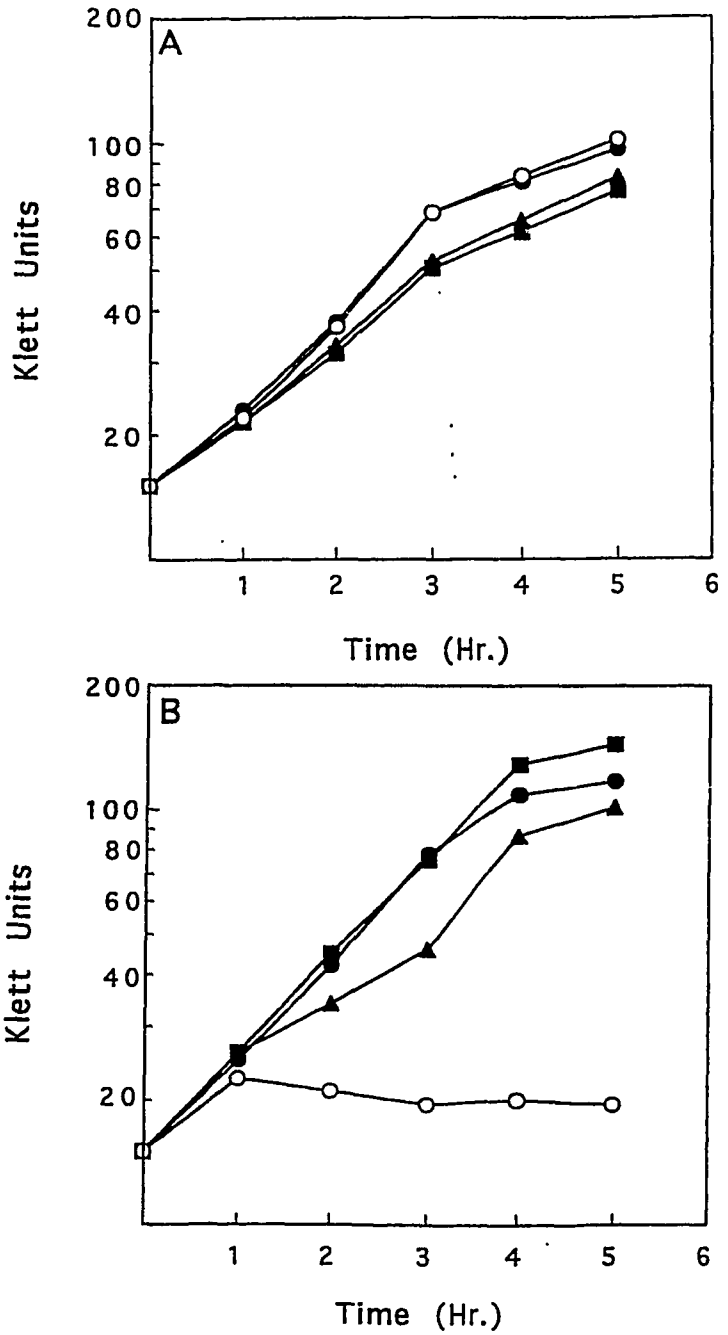

A
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)
B
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)

**Fig. 11.**

**Fig. 12.** The effect of DBP treatment on the ability of LK88 and LK99 to survive in the cold. Both strains were cultured in CHG medium. At 15 Klett units (zero time on the graph), *rac*-DBP was added to a final concentration of 100 $\mu$M. After a 1 h incubation, cells were stored at 5°C for the indicated number of days. Viability results are expressed as the ratio of $N_0$ (viability on day 0) to N (viability on a given day after exposure to DBP). LK88 treated with DBP (▲), or untreated (■); LK99 treated with DBP (△), or untreated (□).

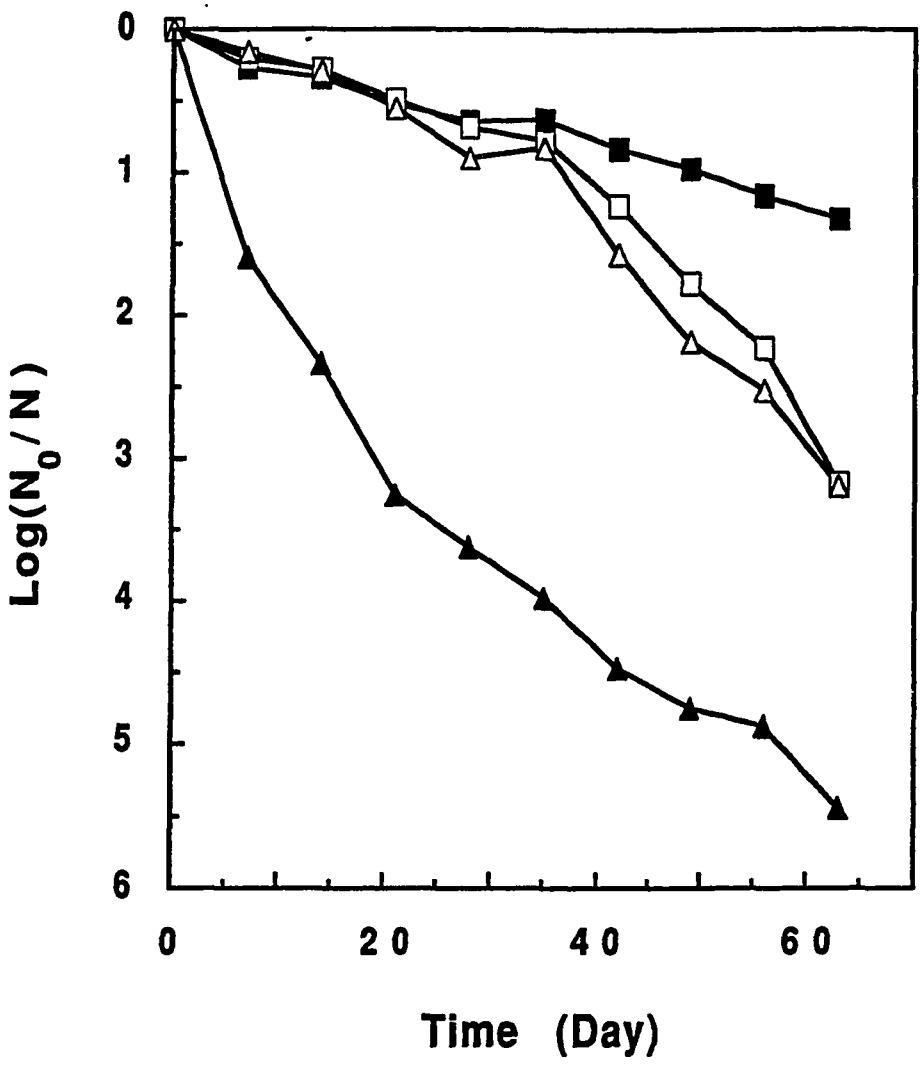



Fig. 12.

**Fig. 13.** Effects of DBP and magnesium chloride on macromolecular and phosphoglyceride synthesis in HW55 cultured in CH medium. [$^{32}$P]phosphate or L-[U-$^{14}$C]leucine (sp. act. 0.27 $\mu$Ci/mmole, 0.9 $\mu$Ci) was added when the cultures reached a turbidity of 15 Klett units (zero time on the graph). Cells were either untreated (■); or treated with DBP and magnesium chloride (○), DBP (▲), or magnesium chloride (●). The final concentrations of DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. Incorporation of [$^{32}$P]phosphate into (A) DNA, (B) RNA, (C) phosphoglycerides, and (D) [$^{14}$C]leucine into protein was followed as described in Materials and Methods.

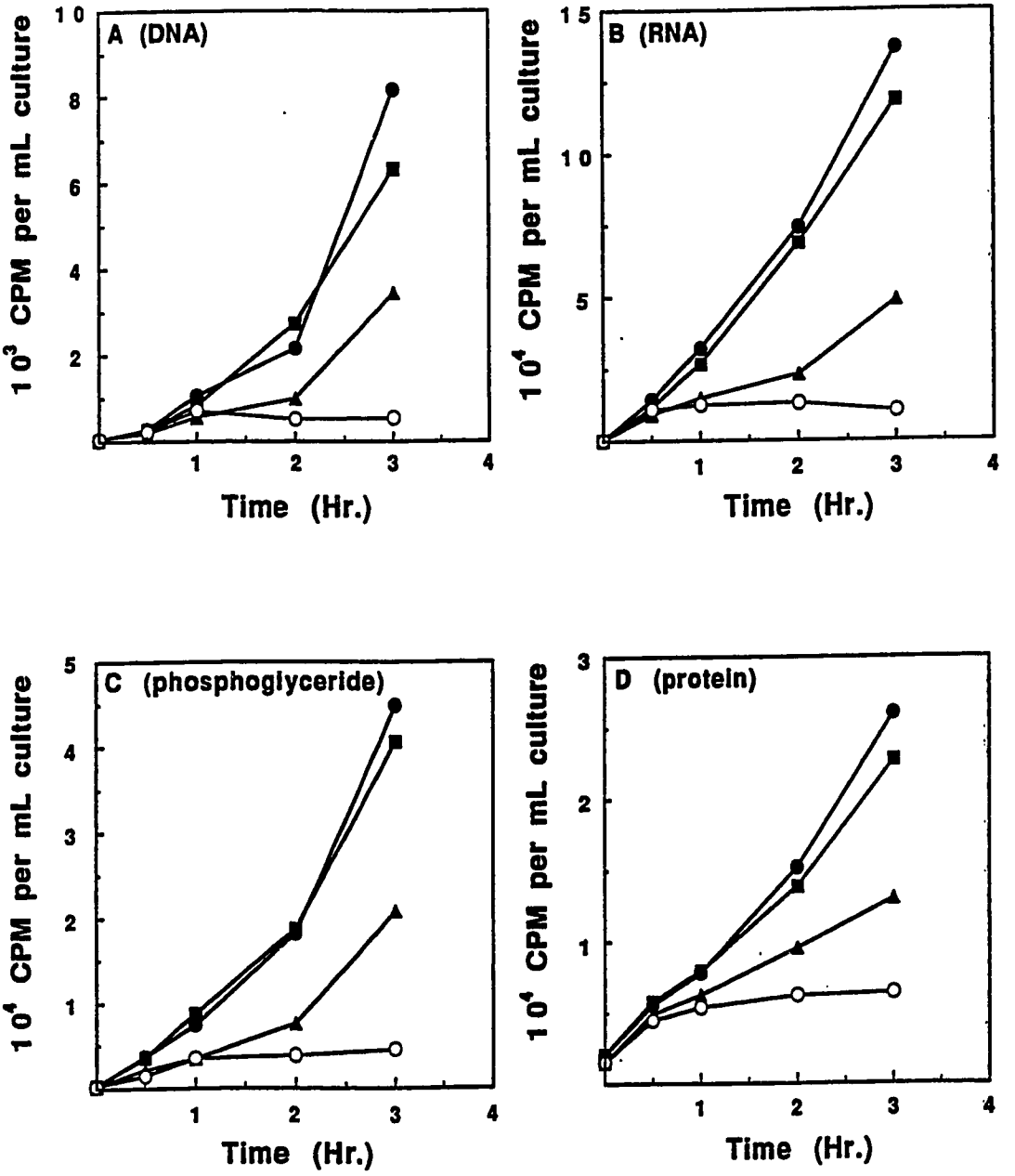

A (DNA)
$10^3$ CPM per mL culture
Time (Hr.)
B (RNA)
$10^4$ CPM per mL culture
Time (Hr.)
C (phosphoglyceride)
$10^4$ CPM per mL culture
Time (Hr.)
D (protein)
$10^4$ CPM per mL culture
Time (Hr.)

Fig. 13.

**Fig. 14.** The effect of DBP and magnesium chloride on phosphoglyceride synthesis in HW55 cultured in CH medium. [1-$^{14}$C]acetate (sp. act. 500 $\mu$Ci/mmole, 10 $\mu$Ci) was added when the cultures reached a turbidity of 15 Klett units (zero time on the graph). Cells either untreated (■); or treated with DBP and magnesium chloride (○), DBP (▲), or magnesium chloride (●). The final concentrations of DBP and magnesium chloride were 20 $\mu$M and 20 mM, respectively. Incorporation of [$^{14}$C]acetate into phosphoglycerides was followed as described in Materials and Methods.

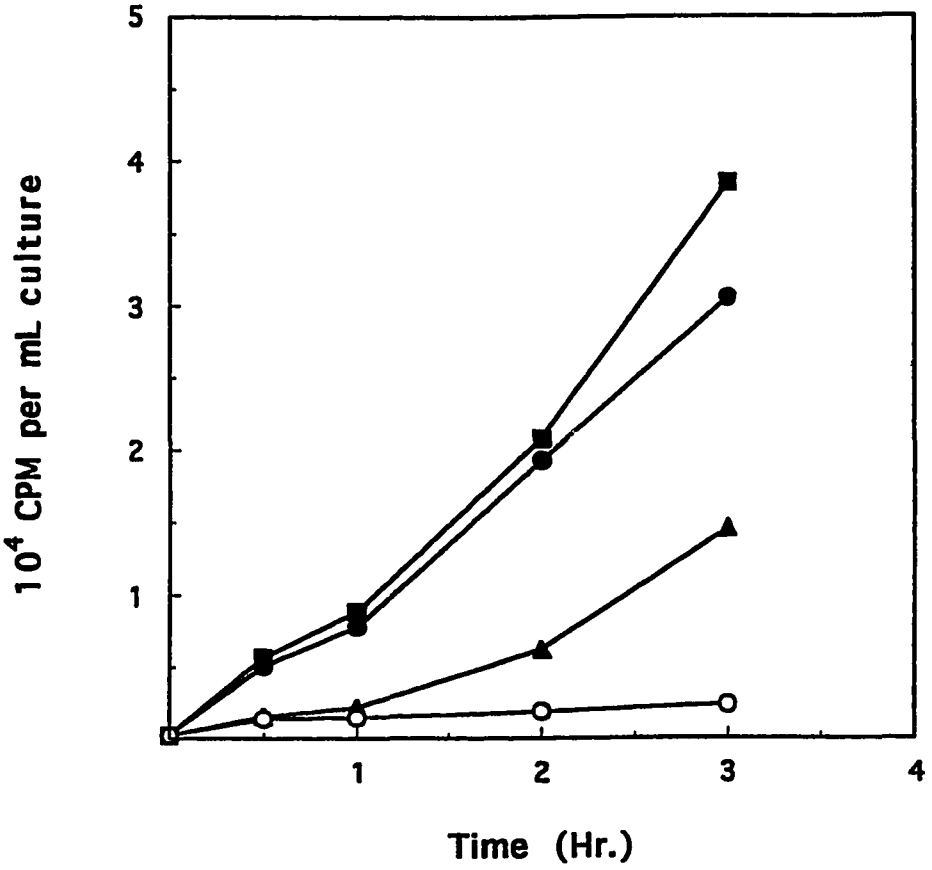



Fig. 14.

**Fig. 15.** Effects of DBP on the (A) growth and (B) viability of HW55 cultured in CH medium with spermidine or spermine. When the cultures reached a turbidity of 15 Klett units (zero time on the graph), *rac*-DBP, spermidine-HCl, spermine-HCl, or a combination of DBP and one of the polyamines were added where indicated. The final concentrations of *rac*-DBP, spermidine-HCl, and spermine-HCl were 20 $\mu$M, 0.5 mM, and 0.125 mM, respectively. **Symbols:** DBP+spermidine (△); DBP+spermine (○); spermidine (▲); spermine (●); DBP (□); and untreated (■).

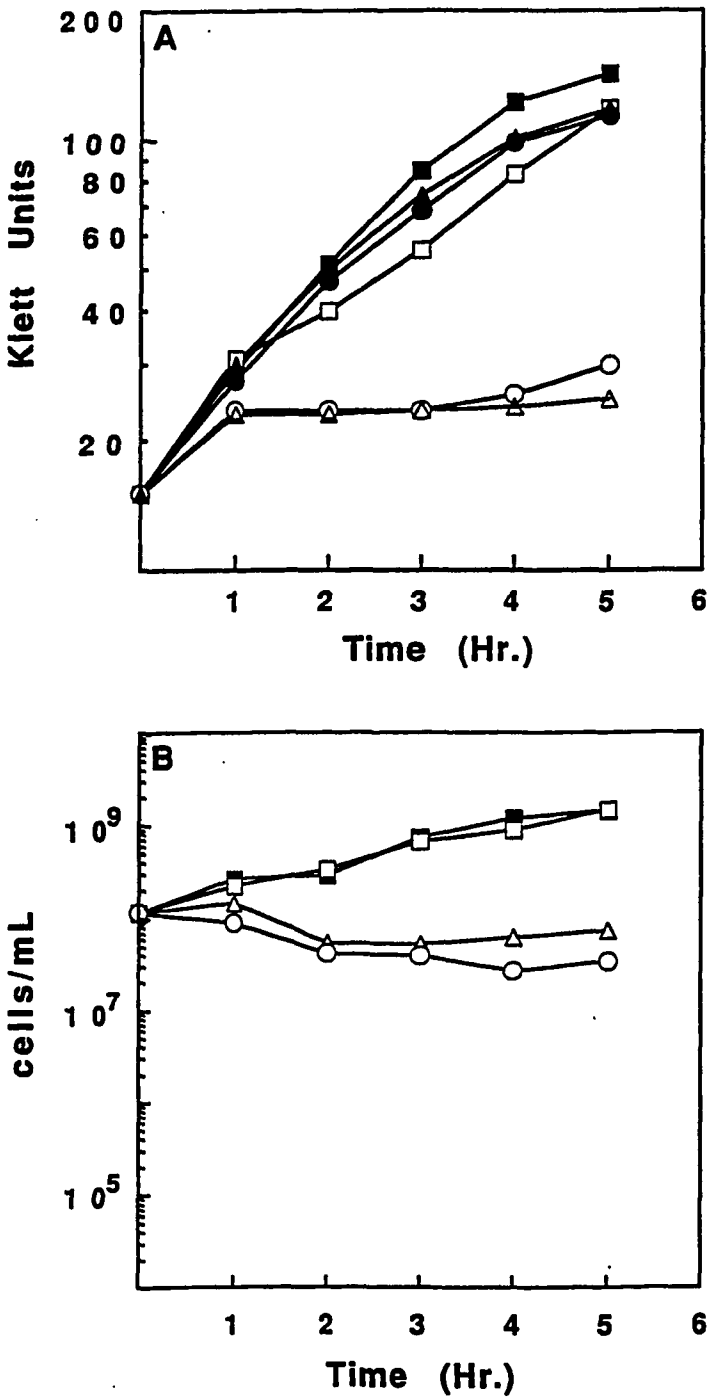

A
200
100
80
60
40
20
Klett Units
1
2
3
4
5
6
Time (Hr.)
B
10^9
10^7
10^5
cells/mL
1
2
3
4
5
6
Time (Hr.)

Fig. 15.

**Fig. 16.** Effects of chloramphenicol on (A) viability of HW55 cultured in the presence of DBP and magnesium chloride, (B) [3-$^{3}$H]DBP incorporation into HW55, and (C) protein synthesis in HW55 treated with DBP and magnesium chloride. HW55 was cultured to 15 Klett units in CH medium (zero time on the graph). Then (A) *rac*-DBP and magnesium chloride were added to the cultures. Chloramphenicol was either added at zero time (▲), 0.5 hr (●), 1 hr (□), or not at all (○). (B) *rac*-[3-$^{3}$H]DBP and magnesium chloride (○), or *rac*-[3-$^{3}$H]DBP, magnesium chloride and chloramphenicol (▲) were added to the cultures. At the indicated times, samples were removed and analyzed for [3-$^{3}$H]DBP incorporation into the phosphoglyceride fraction by the filter paper method as described in Materials and Methods. (C) [$^{14}$C]Leucine was added to the cultures. Then magnesium chloride (●), magnesium chloride and DBP (○), magnesium chloride and chloramphenicol (■), or magnesium chloride, DBP and chloramphenicol (▲) were added to the cultures. At the indicated times, samples were removed and analyzed for labeled proteins as described in Materials and Methods. The

final concentrations of *rac*-DBP, magnesium chloride or chloramphenicol were 20 μM, 20 mM and 40 μg/mL, respectively.

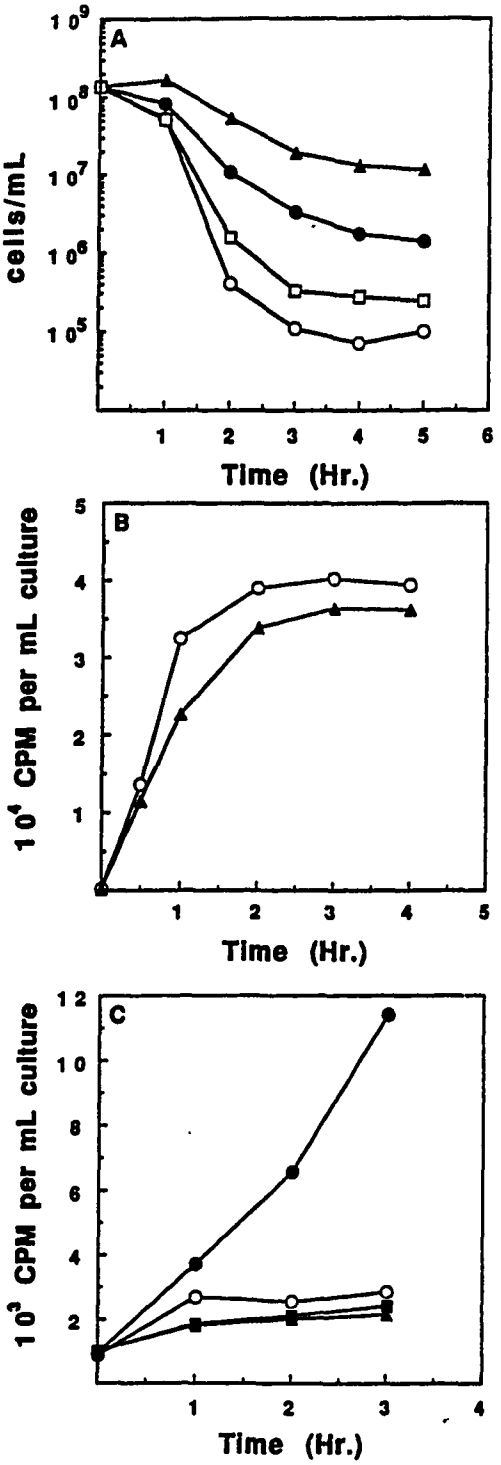

A
cells/mL
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
1
2
3
4
5
6
Time (Hr.)
B
$10^4$ CPM per mL culture
5
4
3
2
1
1
2
3
4
5
Time (Hr.)
C
$10^3$ CPM per mL culture
12
10
8
6
4
2
1
2
3
4
Time (Hr.)

Fig. 16.

## ABBREVIATIONS

cardiolipin (CL), 3,4-dihydroxybutyl-1-phosphonate (DBP), glycerol-3-phosphate (G3P), isopropyl-*β*-*D*-thiogalactoside (IPTG), kanamycin (kan), lysophosphatidic acid (LPA), *o*-nitrophenyl-*β*-*D*-galactoside (ONPG), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerol phosphate (PGP)

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