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THE EFFECTS OF 3,4-DIHYDROXYBUTYL 1-PHOSPHONATE ON ESCHERICHIA COLI. A CORRELATION OF 3,4-DIHYDROXYBUTYL 1-PHOSPHONATE RESISTANCE WITH A DEFECT IN CARDIOLIPIN SYNTHESIS

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by

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ABSTRACT

THE EFFECTS OF 3,4-DIHYDROXYBUTYL 1-PHOSPHONATE ON ESCHERICHIA COLI. A CORRELATION OF 3,4-DIHYDROXYBUTYL 1-PHOSPHONATE RESISTANCE WITH A DEFECT IN CARDIOLIPIN SYNTHESIS.

by

Yu-Wen Hwang

Advisor: Professor Burton E. Tropp

Escherichia coli treated for 1 h with 100 uM rac-3,4-dihydroxybutyl 1-phosphonate (DBP), a glycerol 3-phosphate analog, die when stored at 5 °C, whereas the viability of untreated cells is relatively unaffected. This observation formed the basis of a selection procedure that was used to isolate mutants that are partially resistant to DBP. One such mutant, strain 6204, is constitutive for DBP transport, exhibits a particularly high degree of cold resistance, has the same doubling time as the parent and is similar to the parent strain in terms of incorporation of DBP into the lipid fraction. Glycerol 3-phosphate and phosphatidylglycerol phosphate synthetases obtained from 6204 and its parent were identical in terms of DBP recognition. The parent strain is killed when incubated in the presence of a combination of 70 uM rac-DBP and 0.25% deoxycholate, whereas strain 6204 continues to grow, albeit more slowly, in the presences of this combination. Strain 6204 can be distinguished from the parent strain on agar plates (low phosphate minimal medium with glucuronate as the sole carbon source) containing 15 uM rac-DBP.

The insertion of Tn10 near to the 6204 mutation has facilitated genetic manipulations. All the phenotypic effects attributed to strain 6204 appear to be due to a single mutation. Genetic analysis indicates that Tn10, inserted near to the gene responsible for DBP resistance, maps in the vicinity of min 27. Three factor crosses reveal a gene order of hemA-Dbp^r -Tn10 (zch) -trp. The only gene for phosphoglyceride metabolism, known to map in this region, is the gene associated with cardiolipin synthetase, cls. Genetic results suggest that the mutation responsible for DBP resistance maps in or very near to cls. analysis of the lipids isolated from untreated strain 6204 (and from each of the transductants prepared by P1vir mediated transfer of DBP-resistance to wild type strains) reveal that cardiolipin (CL) synthesis is defective. These results strongly suggest that the mutation responsible for DBP resistance has its primary effect on CL synthesis. To further test this hypothesis, strains with an authentic cls mutation were constructed and examined for resistance to DBP. These strains had growth properties that were identical with those of strain 6204. Wild type and mutants defective in CL synthesis were treated with DBP and 20 mM magnesium or calcium chloride. Simultaneous treatment of either cell type with DBP and divalent cation not only failed to stimulate growth, but quite the contrary, had a marked synergistic growth inhibitory effect.

To my parents
and my wife

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INTRODUCTION

Escherichia coli, like other gram-negative bacteria, has a cell envelope composed of three distinct regions (1). The inner membrane is responsible for active transport (2), membrane biogenesis (3), and energy transduction (4). It also presents an osmotic barrier. The periplasmic space is the region between the inner and outer membranes (1). The periplasmic space may contain 20-40% of the total cell mass (5). In addition to the cell wall, a rigid layer of peptidoglycan (6), the periplasmic space also contains hydrolytic enzymes (7), specific binding proteins (8,9), and membrane derived oligosaccharides (MDO) (10). The hydrolytic enzymes catalyze the breakdown of large molecules into products that can be transported into the cell (1,7). The binding proteins appear to play a part in the transport process and in the chemotactic response towards nutrients (8,9). MDO is believed to participate as an osmotic buffer between the cytoplasm and the outside environment (11). MDO synthesis increases when the cells are cultured in medium of low osmotic pressure (11). The outer membrane encapsulates the periplasmic space (1,12). The outer membrane is firmly anchored to the underlying peptidoglycan layer by a number of outer membrane proteins (13), and this is the membrane exposed to the environment. The outer membrane provides the cell with a selective barrier to substances with molecular

weights that are larger than several hundred daltons (14). The selectivity is due to the presence of various pore-forming proteins, porins (13,14). Lipopolysaccharide (LPS), the major surface antigen of gram- negative bacteria, is located exclusively on the outer leaflet of the outer membrane (12). LPS is so abundant that it has been estimated to cover about 45% of the surface of gram-negative bacteria (15). Because of the high content of hydrophilic sugar moeities in LPS, it is considered to act as the physical barrier to exclude hydrophobic compounds (16,17). This property of LPS, together with the molecular sieving character of the outer membrane, may be the reason why the gram-negative bacteria are resistant to certain dyes, chemicals, and antibiotics (1). Furthermore, LPS has been shown to play an important role in the bacterium-host and bacterium-phage interactions (17,18). The outer membrane possesses many specific receptors and channels for nutrient uptake. Some of the nutrients are too large to permeate the outer membrane (13,19). However, various biological agents, such as phages and colicins, also utilize the same pathway to enter the cell (19). Moreover, the outer membrane was found to play an essential role in cellular recognition during conjugation (20).

The physical structure of both inner and outer membranes can be pictured as a fluid lipid bilayer containing completely or partially embedded proteins as depicted by the fluid

mosaic model of biological membranes (21). Phosphoglycerides are major components of the membrane. Despite the vast differences in functions, the lipid compositions of both inner and outer membranes are rather similar (12,22). E. coli contains only three structural phosphoglycerides: phosphatidylethamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Although each of these phosphoglycerides is actually a molecular mixture containing different fatty acids, E. coli is a relatively simple system for evaluating the regulation of phosphoglyceride metabolism and for examining phosphoglyceride function (3). The metabolic pathways which lead to the synthesis of various phosphoglycerides will be discussed briefly below.

sn-Glycerol 3-phosphate (sn-G3P) is an obligatory precursor for the synthesis of all phosphoglycerides . It is an indispensable intermediate for normal cell growth. The metabolism of G3P and related compounds are under the control of glp regulon (22)- a system with many operons controlled by a single regulator. The metabolic pathways which involve G3P and related compounds are summaried in Fig 2. In the case of glp regulon, the regulator is a repressor which is coded by glpR locus (22). (For the map location of each locus on the bacterial chromosome, see Fig 1) A deletion or a mutation in the glpR locus will lead to constitutive expression of all the controlled operons (22).

In the E. coli K12 strain, the glp regulon is inducible. G3P as well as glycerol can induce the expression of at least six different genes (grouped into three operons) in the regulon: glpA (anaerobic G3P dehydrogenase), glpD (aerobic G3P dehydrogenase), glpF (glycerol facilitator), glpK (glycerol kinase), glpT (G3P permease), and the recently identified glpQ (glycerophosphodiesterase) (23). However, the induction of the glp regulon by glycerol requires the presence of an active glpK gene product-glycerol kinase (22). Glycerol kinase catalyzes the phosphorylation of glycerol to G3P at the expense of ATP. Therefore, it was assumed that the true inducer is G3P (22). Moreover, functional glycerol kinase is required for E. coli to grow on glycerol as the sole carbon source (22). This points to the fact that the metabolism of glycerol must go through G3P under normal conditions. The transport of G3P in its intact form is carried out by the G3P permease-the gene product of the glpT locus. This system can transport various G3P analogs as well as phosphate and arsenate (22,24). Among the transported G3P analogs are fosfomycin (25), L-glyceraldehyde-3-phosphate (26), and 3,4-dihydroxybutyl 1- phosphonate (DBP) (27) (see below). Except for phosphate, each of these compounds is toxic to E. coli and can therefore be employed to isolate glpT mutants (24,25,27). The glpD gene codes for aerobic G3P dehydrogenase. This enzyme catalyzes the oxidation of G3P to dihydroxyacetone phosphate and is probably the major

enzyme for generating glycolytic intermediates when the cell is using glycerol or G3P as the sole carbon source (22). Cells lacking this enzyme are not only devoid of the ability to grow on glycerol or G3P aerobically but are also inhibited by the addition of glycerol or G3P when cultured in many different media (22). The inhibition correlates with the appearance of high intracellular G3P concentrations (22). Glycerol or G3P seems to cause the depletion of the intracellular ATP concentration (28). However, the target of G3P which leads to growth stasis is still unknown. The addition of glucose to the medium can offset the inhibitory phenomenon. This effect, again, is not understood but it appears that catabolite repression is not involved (29). The glpA gene codes for the anaerobic G3P dehydrogenase (22) The gene product of glpA can only be induced under anaerobic conditions and cells lacking this enzyme can not grow anaerobically on either glycerol or G3P as the sole carbon source (22). Recent studies using a glpA-lac fusion strain (30), revealed that the expression of glpA involves another factor- a positive pleiotropic regulator coded by the fnr locus (30,31). The glpT operon can be resolved into two cistrons, glpT and glpQ (23). The promoter-distal glpQ encodes a periplasmic protein which is not required for the active transport of G3P (23). The gene product of the glpQ locus is recently demonstrated to have a glycerophosphodiesterase activity which hydroylzes glycerophosphodiesters to yield G3P and alcohol (23). The

enzyme exhibits a broad substrate specificity with respect to the alcohol moiety. The existence of the glpQ product would allow the cell to channel a wide variety of glycerophosphodiesters into the glp system. E. coli possesses a second system which can take up G3P (32). This system, ugp, is under the control of the phosphate utilitization (pho) regulon (33). Apparently, at least two genes (A and B) and a few periplasmic proteins are responsible for this activity (32,34). Despite the fact that the ugp system has a higher affinity and efficiency than the glpT system for G3P transport, G3P transported via this system can be used only as a phosphate source but not as a carbon source (33). The ugp transport system does not transport phosphate or fosfomycin (32). However, DBP is a substrate for this system (see below) (33,34).

When E. coli is cultured in medium devoid of glycerol or G3P, the G3P required for phosphoglyceride synthesis can be generated from dihydroxyacetone phosphate (DHAP). The gene product of gpsA, G3P synthetase (biosynthetic G3P dehydrogenase), catalyzes this reduction reaction (35). Mutants lacking an active gpsA gene product are auxotrophic for glycerol or G3P (36,37). This indicates that G3P synthetase is the primary pathway for producing intracellular G3P. Moreover, G3P synthetase probably plays a vital role in regulating the concentration of the potentially harmful but indispensable intermediate, G3P, by

a product feedback inhibition mechanism (38,39). This enzyme has a K_i for G3P of 0.05 mM (38). Compared to the value of 0.2-0.3 mM of normal intracellular G3P concentration (40), this suggests that even when cells grown on media without exogenous glycerol or G3P, the G3P synthetase may not function to full capacity. Furthermore, the feedback inhibition is extremely tight as evident from the fact that a strain that overproduces G3P synthetase by 60-fold still can not suppress the requirement of the plsB mutant (see below) for glycerol or G3P (41).

The pathways that lead to synthesis of various phosphoglycerides are summaried in Fig 3. G3P acyltransferase catalyzes the first committed step in phospholipid synthesis (42)- the esterification on G3P with one fatty acid. The product, lysophosphatic acid, is subsequently converted to phosphatidic acid, probably by a separate enzyme (43). The plsB gene which encodes the G3P-acyltransferase has been cloned (44,45). The purified enzyme has been shown to catalyze the conversion of G3P to lysophosphatidic acid (42). Recently, two kinetically distinguishable activities of G3P acyltransferase have been revealed by using acyl-acyl carrier protein instead of acyl-CoA as the acyl donor (46). One of the activities can be accounted for by the product of plsB gene, and the other may be the lysophosphatidic acyltransferase. However, this remains to be demonstrated. A cell carrying a plsB

mutation, which encodes for an enzyme with a 10-fold higher Km for G3P, is auxotrophic for glycerol or G3P (37). Revertants of plsB mutants that are able to grow in the absence of glycerol or G3P appear to fall into two categories (39,47): true revertants in which the acyltransferase reverts to wild type properties and pseudorevertants which map at gpsA rather than plsB (39). In the latter case, it was discovered that the mutation in gpsA relieved the enzyme from inhibition by G3P. The resistance of the G3P synthetase to feedback inhibition results in a 10-fold increase in the intracellular G3P concentration. Consequently, it phenotypically suppresses the plsB mutation without restoring the acyltransferase to the normal state (39). Again, this points to the importance of G3P synthetase in controlling the intracellular G3P concentration.

The phosphatidic acid (PA) required for synthesis of phosphoglycerides is activated by converting it to be CDP-diglyceride at the expense of CTP (48). This reaction is catalyzed by CDP-diglycerde synthetase- the gene product of the cds locus (49). The growth of one class of cds mutants is highly sensitive to alkaline pH (49). When cultures of the mutant are shifted from pH7 to pH8.5, the de novo synthesis of various metabolic downstream phospholipids (PE,PG and CL) are inhibited abruptly. Concomitantly, the PA level goes up, the liponucleotide pool

goes down, and the cell loses its viability. The conditional lethality of the cds mutation strongly suggests that the synthesis of various phospholipids must go through CDP-diglyceride. Similar to the plsB reversion experiment, the alkaline resistant cells of cds also fall in two classes: the true revertants and cells with a novel suppressor mutation (cdsS) which maps at a position unlinked to cds (50). In vitro enzyme assays indicate that the cdsS gene suppresses the pH sensitivity by stabilizing the residual CDP-diglyceride synthetase activity when the cds mutant is grown at a nonpermissive pH (50). However, the biochemistry of cds-cdsS interaction remains to be established.

CDP-diglyceride is the common precursor for the synthesis of phosphatidylserine (PS), PE, PG, and CL. PS synthetase catalyzes the synthesis of PS from CDP-diglyceride and serine (51). PS normally does not accumulate in the cell. It is decarboxylated to yield PE by a separate enzyme- PS decarboxylase (51,52). PE is the predominant phospholipid in E. coli. Approximately 75-85% the total E. coli lipids are PE under normal growth condition (3). CDP-diglyceride can also be used to synthesize various anionic phosphoglycerides. Phosphatidylglycerol phosphate (PGP) is the first product in the pathway to anionic phospholipids. The reaction is catalyzed by PGP synthetase and it requires a molecule of G3P as the cosubstrate (53). The phosphate

group on PGP is subsequently cleaved by PGP phosphatase to yield PG (54). CL synthetase catalyzes the last step in the synthetic pathway by catalyzing the condensation of two molecules of PG into one molecule of CL and glycerol at no energy cost (55). Normally, E. coli membrane contains 10-20% of PG and 5-15% of CL but no appreciable amount of PGP (3). However, the content of CL is highly dependent on the growth condition. When cell cultures near the stationary phase or growth stasis (caused by carbon source depletion), the amount of CL tends to be higher (56). CL was also reported to be accumulated during the glycerol or G3P starvation of plsB strains (57).

PS synthetase is encoded by the pss locus (58). Since PS synthetase is an essential enzyme and the isolation of PS auxotrophs is impractical, pss mutations can only be isolated as conditional lethal. The pss mutant contains more anionic phosphoglycerides (about 3 times) and less PE than pss^+ cell even at permissive temperatures (59). This is probably the cause for the hypersensitive phenotype of pss mutant to certain antibiotics (59). When cultured at non-permissive temperatures, the PE content of pss mutants is further reduced and the cell accumulates enormous amounts of anionic phosphoglycerides, especially CL (60,61). The growth inhibition of pss mutants at nonpermissive temperature can be suppressed by adding cations, particularly, divalent cations (Mg^{+2} or Ca^{+2}) to the

medium. However, neither Mg^{+2} nor Ca^{+2} restores the membrane phospholipid composition to that of the parent strain (60,61). It has been suggested that the positively-charged ions may stabilize the membrane of pss cells which contain excess amounts of anionic lipids bearing negative charges. A regulatory gene specific for controlling the pss gene has recently been identified (62). The gene, pssR, maps at a discrete location from pss locus (62). When mutated, pssR causes 5-fold induction of PS synthetase. The amount of PS and PE are hardly increased in strains that overproduce PS synthetase (63). Thus, it will be extremely interesting to find out the physiological role of the pssR gene.

The psd locus is the structural gene for PS decarboxylase (64). Strains carrying the psd mutation are temperature-sensitive for growth and accumulate large amounts of PS at non-permissive temperatures (64). PS, normally present in negligible amounts, can replace 80% of the PE in this case. However, the ratio of "PE+PS" to "PG+CL" remains constant (64). Similar to the effect of divalent cations on the pss mutant, divalent cations also have been reported to phenotypically suppress the growth inhibition of some psd cells at the restrictive temperature (65).

Colony autoradiography was used to isolate mutants defective in PGP synthetase-the pgsA mutation (66). Although cell extracts of pgsA mutants have a very low PGP synthetase activity, the mutant continues to synthesize PG, and does not have growth problems (66). The pgsB mutation, mapping at a distinct site, was isolated as a second step mutation of the pgsA strain (67,68). The pgsB lesion by itself has no effect on the synthesis of PG and does not interfere with the growth of cells (67,68). However, mutants harboring both pgsA and pgsB are unable to synthesize PG which in turn leads to growth arrest at the restrictive temperature (67,68). In addition, the double mutant accumulates two novel glycolipids, X and Y, at non-permissive temperatures (cells carrying pgsB alone only build up X) (67,68). X and Y are believed to be precursor for LPS (69). Therefore, it is possible that pgsB is involved in the synthesis of LPS (70). The chemical structure of X and Y have recently been elucidated (70). However, the nature of the pgsB gene and the interaction between PGP synthetase and LPS are obscure.

At least two enzymes are involved in catalyzing the dephosphorylation of PGP. They are encoded by the pgpA and pgpB genes (71). The pgpA gene product appears to be specific for PGP while the pgpB gene product also recognizes lysophosphatidic acid and phosphatidic acid (71). However, strains harboring both pgpA and pgpB mutations are not conditionally lethal and continue to synthesize PG.

However, PGP does accumulate in higher than normal amounts in the double mutants (71). The fact that the double mutant maintains a normal level of PG may be due to the existence of another phosphatase with the ability to cleave PGP or to the fact that the pgpA and pgpB mutations are leaky.

A mutation in CL synthetase, cls, was isolated by brute force from the collection of temperature sensitive cells which had been heavily mutagenized (72). The cls gene does not confer any observable phenotype upon the cell except for the inability to synthesize CL (72). Thus, it appears that CL synthetase is dispensable. Cells with a mutation in cls have a slightly higher content of PG which may result from the reduced turnover rate of PG (72).

PE is relatively stable whereas PG and CL are constantly turning over in growing cells (73,74). MDO (75) and lipoprotein (76) are two likely candidates which may require PG or CL for synthesis. MDO contains an unusual glycerophosphate moeity, sn-glycerol 1-phosphate (sn-G1P) (75,77). The glycerophosphate headgroups on PG have the identical stereochemical properties (3). Pulse and chase experiments using sn- [2-^{3}H] glycerol-3-[^{32}P]-phosphate suggest that PG is a precursor of the sn-G1P groups in MDO (75). Since mutants that block MDO synthesis exhibit very little turnover of PG, the transfer of the sn-G1P from PG to MDO must account for the majority

of PG turnover (74,78). The lack of MDO synthesis does not prevent cell growth (74,78). Apparently, MDO is not an essential element for cells under normal laboratory conditions. Moreover, the turnover of PG can be enhanced if the cells are cultured on medium of low osmolarity (79). Recently, the rate of synthesis of MDO has been demonstrated to be correlated with the osmolarity of the media (11). Again, this finding is consistent with the notion that the glycerophosphate moeity on MDO is derived from PG. Since the amount of MDO is not strikingly reduced in cls mutants, this result suggests that CL is probably only a minor contributor of the glycerolphosphate moeity (3).

1,2-Diacylglyceride is produced as the by-product of the transfer of sn-G1P from PG to MDO (80). E. coli can reutilize this by-product by converting it to phosphatidic acid. The enzyme that catalyzes this conversion is a membrane bound kinase, diglyceride kinase, which is the gene product of the dgk locus (80). Cells harboring the dgk mutation are not conditional lethal. However, the mutants do not grow well on medium of low osmolarity (80). In addition, cells can be induced to accumulate large amounts of the diglyceride by simultaneously stimulating MDO synthesis and blocking the activity of diglyceride kinase (81). Since diglyceride is not formed as part of the biosynthetic pathway, the diglyceride kinase cycle may only have a salvage role.

The slow but continuous turnover of PG and CL observed during the period when MDO is not being formed de novo might be attributed to the transfer of the diglyceride moeity from phosphoglycerides to lipoprotein (82). Mature lipoprotein contains various modifications which include the attachment of the digyceride moeity to the N-terminal cysteine residue via a thioether linkage (76). The diglyceride modification has been implicated as a requirement for processing the signal sequence of lipoprotein (83). However, it seems that cells are able to survive without lipoprotein (84). Another possible channel of PG turnover is the transfer of its phosphatidyl moiety to other molecules. PG has been reported to be converted to PE and this conversion is serine dependent in serine auxotrophic strains (85). However, its significance under normal physiological conditions is not yet certain.

As evident from the above discussion, much of our current knowledge in the field of phospholipid metabolism has come from genetic and biochemical studies. Despite their proven value in other biochemical areas, drugs have played a relatively minor role in the examination of phosphoglyceride metabolism and function. 3,4-Dihydroxybutyl 1-phosphonate (DBP), the phosphonic acid isostere of G3P, has been used in this laboratory to examine the pathways involved in metabolism of G3P. The analog has a methylene group in place of the oxygen molecule in the phosphoester bond (86).

This modification does not result in a significant change in either the shape or size of the analog as compared to G3P (86). However, these two compounds still possess enough differences so that they can be distinguished. For example, the methylene group is devoid of unpaired electrons whereas the oxygen in G3P has two such pairs. If electrons near the phosphate are important in enzymatic reactions, the analog might be expected to behave differently from its natural counterpart.

DBP inhibits the growth of E. coli strains which are able to actively transport it. In addition to the glpT and ugp systems (27,33), the uhp system (utilization of hexose phosphate) can also transport DBP (87,88). Since DBP is not an inducer for the glp regulon, mutations in the glpR locus are required to maintain a sufficient influx of DBP to exert its effect. Transport of DBP by the ugp and uhp transport systems have not been carefully studied. These systems geneally are considered to be less efficient carriers for the analog. DBP shares the same intracellular pool with G3P (27). Therefore, G3P can cause counterflow of intracellular DBP (27,89,90). However, the growth inhibition caused by DBP can not be offset by adding glucose to the medium (91). This indicates that the growth stasis induced by DBP and G3P probably have different mechanisms. Furthermore, DBP exerts its effect regardless of the existence of the catabolic G3P dehydrogenase (glpD). In

fact, strains that possess an active glpD gene product are more sensitive to DBP than strains without it (89). Presumably, this may be due to the fact that strains defective in the glpD locus are likely to have a higher intracellular G3P concentration.

At 30 uM, rac-DBP has only a slight growth inhibitory effect upon strain 8, a strain that is glpR and glpD, but causes an immediate 50% decrease in the rate of PG synthesis and a delayed but almost equally pronounced inhibition in the rate of PE synthesis (91,92). Analyses of the total PG, PE, and CL content of cells treated with 30 uM rac-DBP for 1 h revealed normal amounts of CL and slightly below normal amounts of PE (92). However, the PG content is drastically reduced, probably due to the continued turnover of preformed PG and to the inhibition of new synthesis (3,92). Neither the intracellular nucleotide triphosphate pool size nor the rate of macromolecule synthesis is markedly affected by the addition of 30 uM rac-DBP to a culture of strain 8 (92,93,94). In vitro examination of enzymes which directly involve G3P as the substrate, revealed that DBP is an inhibitor of G3P synthetase (K_i =0.042 mM) and a competitive substrate of PGP synthetase (K_m =0.450 mM; K_i =0.740 mM) but is not recognized by either the catabolic G3P dehydrogenase or G3P acyltransferase (95). Therefore, it is rather clear that the in vitro inhibition of PG synthesis obtained is due to the analog's inhibition

of PGP synthetase. However,it is not certain why DBP inhibits the synthesis of PE. Since DBP does not interact with PS synthetase, the likely target then becomes the G3P synthetase. It is possible that the inhibition of G3P synthetase by DBP reduces the rate of G3P formation which subsequently translates into a reduced rate of PE synthesis. In addition, a novel lipid, the phosphonic acid analog of PGP, is formed in cells treated with DBP (90). This result is not only consistent with the in vitro PGP synthetase experiment but also indicates that the various PGP phosphatase activities (71) can not recognize the PGP analog.

Recently, a previously unknown water soluble DBP metabolite was discovered (89). The observation that this substance can be labeled by [1,2-^{3}H]-DBP but not by [3-^{3}H]-DBP suggests that the substance probably originated from DBP by a dehydrogenase reaction acting on the carbon-3. The dehydrogenase can not be the gene product encoded by glpD locus because DBP is not a substrate for the catabolic G3P dehydrogenase and strain 8 synthesizes the substance after exposure to DBP (89). The substance has not yet been identified.

The multiple fates of DBP does raise a question as whether growth inibition is due to a direct effect of the analog on phosphoglyceride metabolism or some other effect caused by

its metabolites. In order to determine the primary site of DBP action, mutants that are resistant to DBP should be very helpful. In this report, I describe the isolation of a partially DBP resistant mutant and present genetic and biochemical evidence which suggests that DBP resistance is due to a single mutation in the cls gene.

MATERIALS AND METHODS

CHEMICALS: rac-3,4 -Dihydroxy [3-^{3}H] butyl 1-phosphonate ([^{3}H]-DBP) and unlabeled rac-DBP were synthesized as previously described (96). Ethyl methane sulfonate, sn-glycerol 3-phosphate (sn-G3P), adenine, L-arginine, L-histidine, L-isoleucine, L-methionine, L-threonine, L-tryptophan, casein hydrolysate, sodium deoxycholate, sodium glucuronate, lactose, maltose, galactose, glucose, δ-aminolevulinic acid, fosfomycin (disodium salt), tris(hydroxymethyl) aminomethane (tris), triethanolamine-HCl, and streptomycin were purchased from the Sigma Chemical Co., St. Louis, MO. Tryptone, yeast extract, and agar were products of Difco Lab., Detroit, Mich. Gelatin was obtained from Nutritional Biochemicals Corporation, Cleveland, OH. Microbial susceptibility test disks were products of BBL Lab., Cockeysville, Md. Sodium [1-^{14}C]-acetate and [U-^{14}C]-G3P were purchased from New England Nuclear Corp., Boston, Mass. Membrane filters (type HAWP; pore size 0.45um) were obtained from the Millipore Corp., Bedford, Mass. Whatman 3 MM chromatography paper and silica gel loaded paper (SG81) were purchased from Scientific Products, Edison, NJ. All other chemicals were reagent grade.

BACTERIAL STRAINS AND CULTURE CONDITIONS: The minimal medium (GL medium) used was that of Garen and Levinthal (97) supplemented with carbon sources at 0.4%. The media used are as follows; CH medium - 1% casein hydrolysate (pH 7.4) and 0.5% NaCl; L medium - 1% tryptone, 0.5% yeast extract and 0.5% NaCl; λ broth - 1% tryptone and 0.5% NaCl; λ ym medium - λ broth , 0.2% maltose, and 0.01% yeast extract. TMG buffer (pH 7.4) contained tris base 1.21 g, $MgSO_4$ 1.20 g, and gelatin 0.1g per liter. Saline solution contained 0.85% NaCl. λ top agar and λ plates each contained λ broth plus 10 mM $MgSO_4$. In addition, the former was solidified with 0.65% and the latter with 1% agar. All other types of agar plate were solidified with 1.5% agar. Each required amino acid and adenine were supplied at a concentration of 0.5 mM. δ-Aminolevulinic acid was added to a final concentration of 40 ug/ml. Fosfomycin was used at concentration of 15 ug/ml. Because of instability upon heating, the following compounds were sterilized by filtration; adenine, δ-aminolevulinic acid, sodium glucuronate, typtophan, and all the antibiotics. Unless otherwise stated, bacteria were cultured at 37 °C, and cell growth and viability were monitored as previously described (91,94). The susceptibility to antibiotics was determined as follows; one half ml of cells at 100 Klett Units (KU) were distributed evenly on a L plate with sterile cotton swab and allowed to dry at 37°C for 20-30 min. Antibiotic disks were then placed on the surface of the

plate. The size of the inhibitory zone of each antibiotic was measured after an overnight incubation at 37 °C. The bacterial and viral strains used are listed in Table 1.

THE MEASUREMENT OF DBP TRANSPORT AND INCORPORATIION: The method used for the DBP transport assay was a modification of that previously described (27). Cells cultured in CH medium were collected at early stationary phase by centrifugation, washed, and resuspended at a density of 100 KU in GL medium lacking a carbon source. Uptake was initiated by the addition of 50 ul of [^{3}H]-DBP (sp. act. 30 uCi/umole) to 1 ml of cell suspension at room temperature. After 1 and 5 min of aeration (200 cycles per min), 100 ul samples were withdrawn and collected on Millipore filters (0.45 um pore size). The filters were washed once with 1 ml of saline and then dried under a heating lamp. The radioactivity retained on the filters was measured in an Isocap 300 scintillation counter.

To measure the incorporation of DBP into the lipid and macromolecular fractions, 1 ml of bacterial culture at 15-20 KU was incubated with 1 uCi of [3-^{3}H]-DBP (sp. act. 30 uCi/umole) for 1 h. At the end of the incubation, an 0.1 ml sample of labeled culture was pipeted onto a Whatman 3 MM paper square. The paper was dried under a heating lamp before it was washed with trichloroacetic acid (TCA) by the procedure of van der Bosch and Vagelos (98). The washing

processes were performed including once with 10% TCA for 30 min, twice with 5% TCA (each for 15 min), and once with 1% TCA for 30 min. TCA must be kept icy-cold throughout the washing period. The paper was dried under a heating lamp and the radioactivity on the paper was measured by liquid scintillation counting in Patterson-Greene solution (99). The remaining 0.9 ml of labeled culture was treated to obtain, the phosphoglyceride fraction by a modified method of Ames (100). The method is outlined below. To 0.9 ml of culture, 3.5 ml of chloroform:methanol (1:2) mixture were added. The extract was then separated into chloroform and water layers by adding chloroform and water, each of 1 ml. The resulting chloroform extract (plus the interface) was washed once with 1 ml of 2 M $MgCl_2$, once with 1 ml of 1 mM of DBP, and twice with 1 ml of water. After the last washing, enough of methanol (about 1.5 ml) was added to form one phase. The extract was dried by blowing air before its radioactivity was measured in the toluene based scintillation fluid. The radioactivity retained on the paper and in the chloroform fractions were considered as an indication of the DBP incorporated into lipids and macromolecules together and lipids alone, respectively.

ASSAY OF G3P SYNTHETASE AND PGP SYNTHETASE: The crude extract of G3P synthetase was prepared as follows; 2 grams, wet weight, of desired strain was suspended in 20 ml of 10 mM triethanolamine-HCl buffer, pH 7.5, containing 2 mM

beta-mercaptoethanol. Cells were disrupted by five 30 sec bursts with a Branson model W140D sonifier at a setting of 8. Cell debris was removed by centrifugation at 8000 x g for 15 min. The supernatant was used without further purification for the G3P synthetase assay. The assays were performed at 30 ^{o}C exactly as previously described (95).

The preparation of PGP synthetase crude extract and the enzyme assay were performed exactly as previously described (95). However, the reaction was monitored by measuring the convertion of [^{14}C]-G3P or [^{3}H]-DBP into TCA-precipitated rather than chloroform-extractable material. At the end of the 10 min reaction period at 37 ^{o}C, an 0.1 ml reaction mixture was pipeted on Whatman 3 MM paper square and treated as described above (see DBP incorporation). The radioactivity retained on the paper was used as an indication of G3P or DBP which had been incorporated into phosphoglyceride.

LIPID ANALYSIS: Bacterial cultures (5 ml), in the late log phase, were incubated with 5 uCi of [1-^{14}C] -acetate (sp. act. 500 uCi/umole) for 1 h. The cells were collected by centrifugation, and resuspended in 1 ml of water. Cells were extracted to obtain the chloroform soluble material as described above (see DBP incorporation). Since it was found that the adding of methanol at the end of the extraction to form one phase somehow interferes with the chromatographic

separation, this step was omitted. Cell debris was removed by passing the extract through glass wool. The lipid exact was dried under a stream of nitrogen gas and redissolved in a small volume of chloroform:methanol (1:1) mixture. Before spotting, the silca gel impregnated paper (Whatman SG81) was heated for 40 min at 110 °C. A chloroform:methanol:acetic acid (65:25:8) solvent system was used to develop the chromatogram. Radioactive lipids were detected by using a Packard Chromatogram Scanner model 7220/7221. The order of lipid migration was the same as that obtained on silica gel thin layer plates. The R_f values are as follows: PE, 0.69; PG, 0.78; and CL, 0.87. The methods used to prepare and chromatograph the labeled lipid in the experiments designed to evaluate the effect of DBP on phosphoglyceride metabolism were the same as those described above except that the cultures were in the early rather than late log phase.

MUTANT ISOLATION: E. coli strain 7-1 was mutagenized with ethyl methane sulfonate by the modified procedure of Miller (102). Cells were cultured to 40-50 KU in L medium, collected by centrifugation, washed, and resuspended in half of the original volume of GL medium without a carbon source. The mutagenesis was carried out by adding 2 ml of the resuspended cells to a mixture containing 0.4 ml of ethyl methane sulfonate and 7.6 ml of GL medium then vigorously shaking for 5 min at 37 °C. Mutagen was removed by

centrifugation and resuspension before the mutagenized cells were cultured overnight for phenotypic segregation in CH medium. The next morning, the cells were diluted into fresh CH medium and cultured until the turbidity reached 15 KU. At this stage, rac-DBP was added to a final concentration of 100 uM and the culture incubated for an additional hour. The DBP treated cells were then stored at 5 °C for approximately 6 weeks during which time DBP treated cells lost viability whereas untreated cells did not (Fig 5). The surviving cells from DBP-cold treatment were cultured in fresh CH medium and the DBP-cold storage was repeated. Cells that previously had been exposed to the same treatment showed much higher viability than the untreated cells (Fig 6). To rule out transport negative mutants, cells surviving the second cycle selection were tested for the ability to use G3P as the sole carbon source and for sensitivity to fosfomycin (15 ug/ml). Cells that appeared to retain the G3P transport system were assayed for DBP uptake. Individual survivors that exhibited normal DBP transport ability were evaluated for cold sensitivity after incubation with 100 uM rac-DBP for 1 h at 37 ° C. As expected, the survival rates for most cells were considerably higher than that observed for the parent strain. Strain 6204 was chosen for further study.

GENETIC TECHNIQUES: Conjugation mapping by gradient transmission was performed according to the procedure of

Miller (102). Donor (Hfr strain) was mixed with recipient (F^- strain) in a ratio of approximately 1:20. Each cell was in the exponential phase of growth at about 30 KU. The cells were allowed to mate for 90 min at 37 °C with gentle shaking (60 cycles per min). After appropriate dilution, mating mixtures were plated to select for recipient strains that inherited an early marker from the donor strain (Table 5). Counterselection against donor strain was accomplished by using selection plates that contained 100 ug/ml of streptmycin. The recombinants were then screened for the phenotypes of various later markers and the frequency of marker cotransfer was determined (Table 5).

Plvir phage lysates were prepared either by the standard soft top agar method (102) or the quick method. In the quick method, 0.5 ml of an overnight culture of donor strain were diluted into 10 ml of L medium containing 10 mM $CaCl_2$ and then aerated until the culture reached 40 KU. At this stage, 0.2-0.3 ml of a good Plvir lysate (pfu > $1x10^9$ per ml) was added to the culture and aeration continued. The cultures were completely lysed within 2-3 h. Any surviving cells in the lysate were sterilized by adding a few drops of chloroform and vigorously shaking. Cell debris was removed by centrifugation and lysates were stored over a drop of chloroform at 5 °C. The Plvir lysates were titered by following a modified procedure of Miller (102). Cells cultured in L medium to late log phase (about 100 KU)

were collected by centrifugation, and resuspended in a solution containing 10 mM each of $MgSO_4$ and $CaCl_2$. A sample of 0.1 ml of resuspended cells was mixed with 0.1 ml of properly diluted phage (several parallel experiments were carried out, each with a different phage dilution). The phage were allowed to adsorb for 20 min at 37 °C prior to the addition of 2.5 ml L top agar (kept at 45 °C). The entire tube containing, top agar and infected bacteria, was plated on an L plate supplemented with 10 mM $CaCl_2$. The plates were incubated for overnight at 37°C. Each plaque that appeared on the plate was considered to be one plaque forming unit (pfu). Typical lysates have a titer of 10^9 pfu/ml or higher. While, the quick method saves time, it has a tendency to yield a lower titer lysate. It is, therefore, not recommended if the lysate is to be used to obtain a large number of transductants. P1vir transductions were performed in a similar fashion to that described for titering of P1vir phage described above except for the following modifications. The volume of recipient cells used in the transduction experiments were 10-20 fold higher than that used to obtain the titer, i.e. the volume of recipient cells used was 1-2 ml. The multiplicity of infection was always 1:1. In addition, the free phages in the transducing mixtures were removed by centrifugation after phage adsorption. If phenotypic seggrgation was not required, the pellets were resuspended into 1-2 ml of saline solution containing 20 mM citrate then plated directly onto

selection plates. If phenotypic segregation was required, the pellets were resuspended and incubated in the same volume of L broth containing 20 mM of citrate. At the end of a 60 min period at 37 °C, the transduced cells were either plated directly onto selection plates or switched to minimal medium before plating if removal of a nutrient was necessary for selection. Top agar was not used in the transduction throughout this study. P1vir phage stock was always propagated on a bacterial strain that was not related to any of the strains used in this study to avoid possible genetic complications. Whenever needed, the P1vir stock was then used to infect the donor strain in order to prepare the P1vir lysate.

The special phage vector, λ NK370 (Table 1), was introduced into E. coli cells to construct a random Tn10 insertion pool. The phage λ NK370 carries mutations that block phage replication (ouga), integration (b221 deletion), repression (cI::Tn10), and it is wild type for the N gene (102,103). Therefore, this phage can neither intergrate into the bacterial chromosome nor maintain itself as an autonomously replicating plasmid (102,103). In addition, the Rec-mediated general recombination (104) is inoperative because of the attachment site deletion on the λ NK370. Under these conditions, Tet^r colonies, that appeared after infection of a suppressor-free E. coli strain with λ NK370, must acquire Tn10 through an illegitimate pathway-

the transposition of Tn10 from λ phage to the bacterial chromosome. Each cell obtained in this way represents an independent transposition event in which a Tn10 was inserted at a particular site on the chromosome. If the number of independent Tet^r cells is large enough, it is possible to obtain a pool of cells in which Tn10 is inserted near each of the bacterial genes. The Tn10 can then be mobilized and used to transduce any bacterial strain by generalized transduction mediated by P1vir phage.

As stated above, λ NK370 contains an o*uga* mutation in order to render the selection of Tn10 transposition possible. Thus, λ NK370 must be propagated on a strain carrying a suppressor mutation to support phage replication. However, during the phage replication, o^+ revertants of λ NK370 are constantly appearing. The o^+ reversion will commit the phage to lytic growth (due to *c*I::Tn10) even on suppressor negative strains. This will dramatically reduce the frequency of isolating Tet^r transductants. Therefore, λ NK370 lysate stock has to be prepared from purified plaques. The procedures for preparing λ NK370 lysate and constructing Tn10 random insertion pools were modified from the described methods (105,106).

Lambda plaques were prepared as follows: The host cell, LE392, was cultured in λ ym medium to about 100 KU. A tenth ml of host culture was mixed with 0.1 ml of appropriately

diluted λ NK370 (phage was diluted in TMG buffer to about 1000 pfu/ml) and incubated for 20 min at 37 °C to allow phage adsorption. After adsorption, 2.5 ml of λ top agar (kept at 45 °C) was added, the mixture vortexed, and then poured onto a fresh λ plate. The plates were incubated at 37 °C for 8-12 h.

Lambda lysate stock was prepared as follows: Strain LE392 was cultured in λ ym medium without shaking overnight to stationary phase. Two ml of overnight culture were inoculated into 8 ml of λ broth plus a final concentration of 10 mM $MgSO_4$ and aerated at 37 °C for 1 h. An 0.6 ml sample of subcultured LE392 was placed into one test tube and 2-3 plaques of λ NK370 were added. The plaques can be cut and picked by using a sterile spatula. They should be less than 12 h old. The infected cells were incubated for 20 min at 37 °C to permit phage adsorption. A 7.5 ml sample of λ top agar (kept at 45 °C) was added to the test tube which was then vortexed and distributed onto 3 fresh plates. The plates were incubated at 37 °C for 6-8 h. All the top agar was scraped off the plates and placed into a test tube. The surface of each plate was washed with 1.5 ml TMG buffer and then combined with the top agar that had been scraped off. A few drops of chloroform were added prior to vigorously vortexing for 30 sec. The mixture was allowed to stand at room temperature for 10 min. The cell debris was removed by centrifugation (5000 x g, 10 min) and the

supernatant was saved in a sterile vial over a drop of chloroform and stored at 4 °C. The lysate was titered on strain LE392 by the same procedures as described in the section concerning preparation of λ plaques. The lysate was also titered on strain Hfr3000 to determine the frequency of o^+ revertant. The titer on strain LE392 should be at least $1x10^{10}$ pfu/ml and 5 orders of magnitude higher than on Hfr3000.

The construction of Tn10 random insertion pool was carried out by infecting strain Hfr3000 or HW22 with λ NK370. The frequency of isolating the Tet^r is about one per 10^6 to 10^7 λ NK370 phages used. The following procedures yielded more than 10^4 Tet^r cells. The host cell were cultured in λ ym medium without shaking overnight to stationary phase. The cells were harvested by centrifugation (100 ml total, 10 ml each into 10 test tubes) and resuspended in 1/20 of the original volume of λ ym medium containing 10 mM $MgSO_4$. λ NK370, at a multiplicity of infection of 0.2, were added to each tube and the infected cells were incubated at 37 °C for 50 min. Samples of 0.2 ml of the infected cells were plated directly onto tetracycline plates (L plates contained 20 ug/ml of tetracycline and 0.0025 M of pyrophosphate). The plates were incubated overnight at 37 °C.

Tetracycline resistant cells that appeared on the plates the next day were collected as follows: 1 ml saline containing 20 mM of citrate was added to each plate. The colonies were suspended with a sterile spreading rod. The cell suspensions were pooled into a single test tube by using sterile Pasteur pipets. The collected cells were washed by centrifugation and resuspended in saline containing 20 mM citrate. The washing process was repeated at least three times. A tenth ml of the washed cells were inoculated into 10 ml L broth containing 20 ug/ml of tetracycline and 20 mM of citrate. The cells were incubated at 37 ^{o}C until they reached log phase. The cells were then washed twice, and resuspended in L broth without citrate. Cells at this stage were either used to prepare P1vir lysate or stored in the freezer.

STRAIN CONSTRUCTION: The procedures used to construct strains HW10 (*glp*R zhe::Tn10), HW11 (*glp*R *glp*D zhe::Tn10), HW21 (*glp*R *glp*D zhe::Tn10 λ^{-}), and HW22 (*glp*R λ^{-}) are summarized in Chart 1. To facilitate genetic manipulations, it was necessary to insert a Tn10 into a position near the mutation that enables strain 6204 to survive cold storage. Before this could be accomplished it was necessary to construct a strain that was constitutive for the *glp* regulon and that was not lysogenic for phage. Accordingly, strain HW22 which meets these requirements was constructed from strain Hfr3000 by taking advantage of the fact that *glp*R and

glpD are adjacent (22) (Fig 1). A Tn10 random insertion pool was generated by infecting strain Hfr3000 with λ NK370. A P1vir lysate of the pooled Tet^r cells was used to transduce strain 8. Cells were selected that were resistant to tetracycline and able to use G3P as the sole carbon source. Strain HW10, which meets these requirements and is constitutive for the G3P transport system (assayed by DBP uptake), was infected with P1vir, and the lysate was used to transduce strain 8. Strain HW11 was isolated as a Tet^r transductant that is unable to use G3P as the sole carbon source. A P1vir lysate of HW11 was used to transduce strain Hfr3000 to obtain strain HW21, a Tet^r glpR glpD transductant. a P1vir lysate of strain 7 was then used to transduce strain HW21, and cells were selected for the ability to grow on G3P as the sole carbon source. Strain HW22 is a strain that lost its tetracycline resistance in concomitance with gaining the ability to use G3P as the sole carbon source.

The construction of strain HW31 (glpR Dbp^r zch::Tn10) is summarized in Chart 2. Strain HW22 was infected with λ NK370 to generate a random Tn10 insertion pool. Approximately 20,000 Tet^r cells were pooled (HW22 Tet^r Pool). A P1vir lysate, obtained from the HW22 Tet^r pool, was used to transduce strain 6204 to Tet^r . Approximately 20,000 Tet^r colonies were pooled (6204 Tet^r pool). A P1vir lysate obtained from the 6204 Tet^r pool was used to

transduce strain 7-1. Again, 20,000 Tet^r colonies were pooled (7-1 Tet^r pool). The 7-1 Tet^r pool was cultured in CH medium and treated with a combination of 70 uM rac-DBP and 0.25% deoxycholate. This combination is bactericidal to strain 7-1 but not to strain 6204 (see Results). Survivors were tested for the ability to form colonies on plates containing GL medium with glucuronate as the sole carbon source and 15 uM rac-DBP. Although this selection technique is somewhat leaky, as a general rule, I have observed that strain 6204 is able to form colonies on this plate, whereas strain 7-1 is not. Transductants that appeared to have the 6204 phenotype were tested for their ability to survive in the cold after 1 h incubation with 100 uM rac-DBP and also for their ability to grow in the presence of 70 uM rac-DBP and 0.25% deoxycholate. Presumably, cells with the 6204 phenotype obtained from the 7-1 Tet^r pool should have Tn10 inserted near the Dbp^r mutation. The closeness of Tn10 to the Dbp^r locus was verified by P1vir transduction with Dbp^r Tet^r cells as donors and strain 7-1 as the recipient. In such transductions, tetracycline resistance was always used for selection, and Tet^r cells were then tested for DBP sensitivity as described above. Strain HW31, a strain in which the closeness of Dbp^r and Tet^r was confirmed, was selected for further study.

The construction of strain HW35 (glpR glpD zch::Tn10), HW36 (glpR glpD Dbp^r zch::Tn10), HW41 (HfrH zch::Tn10), HW42 (HfrC zch::Tn10), and HW43 (HfrB7 zch::Tn10) are summarized in Chart 2. A Plvir lysate of strain HW31 was used to transduce strain 8. Tet^r transductants were then screened for DBP sensitivity on GL-succinate medium containing 30 uM rac-DBP. Strain HW35 is a DBP sensitive strain, HW36 is a DBP resistant strain. The same lysate was used to transfer Tn10 inserted near Dbp^r to different Hfr strains. Transduction of strains CSH62, KL226, and KL208 in this fashion yielded Tet^r strains HW41, HW42, and HW43, respectively.

The construction of strains HW50 (glpR glpD trp zch::Tn10) and HW60 (glpR glpD hemA zch::Tn10) are outlined in Chart 3. A Plvir lysate of HW31 was used to transfer Tn10 to strain S370, and about 300 Tet^r transductants were pooled (S370 Tet^r pool). A Plvir lysate of the S370 Tet^r pool was used to convert strain 8 to tetracycline resistance. Individual transductants were tested for a tryptophan requirement. Strain HW50 is a Tet^r, tryptophan requiring derivative of strain 8. To construct strain HW60, the S370 Tet^r pool was prescreened for strains that require δ-aminolevulinic acid. A few of the Tet^r, δ-aminolevulinic acid requiring transductants of S370 were pooled and infected with Plvir. The lysate thus obtained was used to convert strain 8 to HW60. If

tetracycline and δ-aminolevulinic acid are to be used together as in the case of selecting strain HW60, it is absolutely essential that each of the reagents is prepared fresh.

The construction of HW51 (glpR glpD Dbp^r), HW53 (glpR Dbp^r), HW55 (glpR cls), HW56 (glpR), QC104 (glpR glpD), and QC120 (glpR glpD cls) QC120 are summarized in Chart 3. Plvir-mediated transduction was used to transfer Dbp^r from strain 6204 to strain HW50 to yield HW51. Initial selection, based on the ability of transductants to grow in the absence of tryptophan, was followed by screening on GL-succinate medium plates containing 30 uM rac-DBP. Most of the Dbp^r transductants lost the tetracycline resistance. Strain HW51 is one such transductant. Strain HW50 was also used to construct strains QC104 and QC120 in a similar fashion. However, in this case, strain T1GP (cls) served as the donor, and the transductants were screened for the absence of CL by phosphoglyceride analysis. The $glpD^+$ allele of strain 7 was introduced into strains HW51, QC104, and QC120 by Plvir-mediated transduction to yield strains HW53, HW55, and HW56, respectively. These transductants were isolated on the basis of their ability to use G3P as the sole carbon source.

LYOPHILIZATION OF BACTERIA: The following procedures of lyophilization were used for long term bacteria storage.

Strips (about 6 mm x 18 mm) of Whatman 3 MM filter paper were cut to fit into Wheaton 1 ml centrally constricted ampules (10.5 x 67 mm). The strain numbers of the stock to be lyophilized and date were written on the filter paper with a pencil before the strips were placed in the ampules. The cotton-plugged ampules were autoclaved. The strains to be stored were streaked on the L plates with a toothpick and incubated overnight at 37 °C (for hemA strains, the supplement of δ-aminolevulinic acid must be added to the medium). The fully grown bacteria were transferred by means of a sterile inoculation loop and resuspended in an 0.5 ml solution containing 5% glucose and 5% Difco nutrient broth (made up by mixing equal amounts of the two 10% stock solution). A few drops of the cell suspension were added to the labeled strips of filter paper with a sterile Pasteur pipette. The paper should be saturated, but there should not be excess liquid in the ampules. After the pressure in the lyophilizer (Virtis Unitrap II) reached to 25-30 millitorr, ampules were attached to the system through drum manifolds by using No. 2 rubber stoppers as adapters. After all the ampules (2 to 3 at a time) were in place and the pressure have again been reduced to 25-30 millitorr, the ampules were sealed off with an oxygen-gas, single-jet torch. The flame must be applied evenly around the constricted region of ampule. The sealed ampules were stored at the -20 °C in the freezer.

RESULTS

Although strain 8 was used in most of the previous investigations concerning the effects of DBP on microbial physiology, strain 7-1 appeared to offer two major advantages for the present investigation. Strain 7-1 is constitutive for the hexose phosphate transport system and for G3P dehydrogenase but is otherwise isogenic with strain 8. First, as is evident from Fig 4A, 70 uM rac-DBP inhibits the growth of strain 7-1 cultured in CH medium; whereas the growth of strain 8 is not affected by these conditions (89). The constitutively produced catabolic G3P dehydrogenase of strain 7-1 probably keeps the intracellular G3P concentration much lower in this strain than it is in strain 8. Since the presence of G3P offsets the growth inhibitory effects of DBP (89,90), strain 7-1 would be expected to be more sensitive to DBP than is strain 8. The presence of the hexose phosphate transport system does not account for the difference in sensitivity (see below). The second reason for working with strain 7-1 is that it can use G3P as the sole carbon source and this provides a simple method for eliminating transport mutants. The hexose phosphate transport system provides an alternate route for DBP uptake (88) and its presence was deemed to be desirable to help eliminate transport mutants. However, in the course of this study it was discovered that while this rationale applies to

high concentrations of DBP it does not apply to concentrations beelow 100 uM. Since, revertants of strain 7-1 that have regained inducible control of glp regulon would be expected to be resistant to DBP, it was still necessary to directly measure DBP transport after preliminary screening on G3P and fosfomycin.

The direct approach to the isolation of DBP resistant mutants, mutagenesis followed by the isolation of colonies that appear on agar plates containing a high concentration of DBP yielded only transport mutants. Previous work from this laboratory demonstrated that growth inhibition caused by DBP was dependent upon a functional transport system (27) and probably involves multiple intracellular targets (95). Therefore, the probability of obtaining mutants that are completely resistant to high concentrations of DBP in a single step will be extremely low except for the mutation in the transport system. Furthermore, at low concentrations, DBP does not cause complete inhibition of cell growth in the medium used even though treated cells exhibit a marked change in phosphoglyceride metabolism (91,92). This lack of phenotype prevents the selection of resistant mutants by using a low concentration of DBP in the absence of other selective pressure. Since E. coli did not evolve to survive in the medium used in the laboratory, the failure to detect an effect of DBP on growth in synthetic medium under laboratory conditions should not have been too surprising.

In the light of the marked change in phosphoglyceride composition caused by the administration of low concentrations of DBP, it was of interest to examine the effect of DBP on E. coli under environmental stresses to which the bacteria might ordinarily be subjected. Whereas E. coli must be able to survive in the cold, it was found that E. coli strain 7-1, treated with 100 uM rac-DBP for 1 h at 37 °C, die when stored at 5 °C. As expected, untreated cells are relatively unaffected by storage in the cold (Fig 5). The differential killing after several weeks in the cold suggests the possibility of using DBP and cold in combination as a condition for selecting mutants in one of the DBP targets.

The details of the isolation of DBP resistant mutants by using the DBP-cold approach are described in the Materials and Methods section. Briefly, strain 7-1 was mutagenized, incubated in CH medium in the presence of 100 uM rac-DBP, and then stored in the cold. Survivors after 6 weeks of cold storage were subjected to the same treatment again. Cells that survived after two cycles of DBP-cold selection, demonstrated to have normal transport properties, were evaluated for cold sensitivity after incubation with 100 uM rac-DBP for 1 h at 37 °C. As expected, the survival rate for most cells was considerably higher than that for the parent strain. Strain 6204 was chosen for further study because it is constitutive for DBP transport (Table 2),

exhibits a particularly high degree of resistance to DBP-cold treatment (Fig 7), and has the same doubling time as the parent strain in the various media tested. The ability of DBP to inhibit lipid synthesis (Table 3) and to be incorporated into the lipid fraction (Fig 8) of strain 6204 and of the parent strain was compared. No major differences were detected. Furthermore, the apparent K_m and K_i vaues for DBP and G3P of PGP synthetase and the apparent K_i of DBP for G3P synthetase isolated from strain 6204 and the parent strain were examined. In agreement with the above in vivo findings, the results (Table 4) suggest that the kinetic parameters in both strains are very similar. However, the amount of DBP incorporated into the macromolecular fraction (including lipid) as measured by trichloroacetic acid precipitation was consistently lower in strain 6204 than in the parent strain (Fig 9). The susceptibility of the parent strain and strain 6204 to various antibiotics was tested (Table 5). Except for rifampin, to which strain 6204 is about twice as sensitive as the parent strain (judged by the area of the zone of inhibition), the results were largely similar in both strains. It was discovered that strain 6204 is hypersensitive to alkaline pH. When cultured in CH medium, pH 8.8, strain 6204 has a much longer lag phase and slower growth rate than the parent strain (Fig 10). Although the doubling time of strain 6204 is affected considerably less by 30-300 uM rac-DBP than that of the parent strain, the

growth of each strain is completely inhibited by 1 mM rac-DBP in CH medium (Fig 11).

The cold storage selection technique requires such a long period of time that it was not practical for genetic manipulations. Therefore, other selective pressures that can be used to differentiate strain 6204 from the parent strain were sought. As might be expected of an enteric bacteria, E. coli grows quite well in the presence of the bile salt sodium deoxycholate. However, concentrations of rac-DBP as low as 30 uM kill strain 8 cultured in GL-succinate medium containing 0.25% deoxycholate (89). The synergistic effect was applied to strain 7-1 cultured in CH medium. While rac-DBP at 70 uM is bacteriostatic to strain 7-1, simultaneous treatment of cells with 70 uM rac-DBP and 0.25% deoxycholate does cause a loss of viability (Fig 4). Interestingly, strain 6204, a mutant isolated by the DBP-cold method, was shown to have also become resistant to the combination of DBP and deoxycholate (Fig 12).

The cold sensitivity and synergistic effects, evident in liquid medium, do not translate to agar plates and it was therefore necessary to discover conditions that would permit a distinction to be made between strain 7-1 and 6204 on agar plates. Various media were tried in an attempt to achieve this goal. Strain 7-1 is considerably more sensitive to DBP than strain 6204 when cultured in GL medium containing

glucuronate as the sole carbon source. At 70 uM, rac-DBP is bactericidal to strain 7-1 cultured in the GL- glucuronate medium, whereas strain 6204 continues to grow (Fig 13). Furthermore, the parent strain does not form colonies on agar plates containing GL-glucuronate plus 15 uM rac-DBP, whereas strain 6204 does.

Because the range of DBP concentrations that permit selection on GL-glucuronate plates is narrow, the carbon source is critical, and the method is somewhat leaky, it was extremely difficult to carry out the transduction of the 6204 phenotype by P1vir phage based on the above selective conditions. It was therefore necessary to insert a transposon (107) near the Dbp^r gene. In theory, the utilization of transposon offers two advantages. First, it will greatly enhance the probability of isolating the transduced of transferring a marker from donor to recipient strain is about 10^{-6} (108), whereas, the frequency of isolating cells with a transposon near a particular gene is higher than 10^{-4} (109). Second, since the transposon and the desired gene are very close to each other, they can be treated as an entity and the transposon can be used instead of the gene itself in genetic manipulations. Therefore, it becomes possible to directly select Dbp^r transductants by combining the above selection methods (DBP plus deoxychlorate, DBP in glucuronate medium) and transposon techniques. Attempts to insert a transposon Tn10

(carrying the tetracycline resistant gene) (107) near the Dbp^r gene by starting with a random Tn10 insertion pool constructed on Hfr3000 (for genotypes of strain, see Table 1) were unsuccessful. The failure of this approach was due to a very high background interference by transport negative transductants that had received the wild type control gene for the glp regulon from strain Hfr3000. The hexose phosphate transport system was not sufficiently active at the relatively low concentration of DBP used in the selection procedures. Strain HW22 (glpR λ^-) was constructed to avoid this problem (see Materials and Methods section). A few strains that have Tn10 inserted near the Dbp^r mutation were isolated by the procedures described in the Materials and Methods section. One of these strains, HW31 was studied more carefully. A P1vir lysate prepared from strain HW31 was used to transduce strain 7-1. Approximately 60% of the transductants selected for Tet^r were found to have also been converted to the 6204 phenotype as judged by the ability to form colonies on agar plates containing GL-glucuronate medium plus 15 uM rac-DBP. Individual colonies were then tested for the ability to grow in the presence of DBP and deoxycholate and to survive storage in the cold after DBP treatment. These three properties are cotransduced with a frequency of 100% suggesting that they are all caused by the same mutational event. Moreover, strain HW31 exhibits the same patterns of susceptibility toward various antibiotics (Table 5) and hypersensitivity to alkaline pH (Fig 10) as does strain 6204.

To test whether the Dbp^r mutation is capable of being expressed in other genetic backgrounds, for example strain 8, a Plvir transduction experiment was carried out using strain HW31 as the donor and strain 8 as the recipient, Tet^r transductants were selected. Although strain 8 does not grow on plates containing GL-glucuronate or succinate media in the presence of 30-50 uM rac-DBP, approximately 60% of the Tet^r transductants selected were able to do so. Strain HW36, a Dbp^r derivative of strain 8, continues to grow in GL-succinate medium containing 0.3 mM rac-DBP. Strain HW35, lacking the mutation that confers DBP resistance but otherwise isogenic to strain HW36, is unable to grow in this medium (Fig 14). The sensitivities of strains HW35 and HW36 to DBP in GL-glucuronate were also compared. Fig 15 clearly shows strain HW36 is much more resistant to DBP than is strain HW35.

Since the Tn10 is very close to the Dbp^r locus, knowing the position of the Tn10 on the E. coli chromosome might be an important step toward understanding the nature of the Dbp^r mutation. In order to determine the map location of Tn10 inserted near to Dbp^r, the tetracycline resistance of strain HW31 was transferred to various Hfr strains by Plvir- meidated transduction. The resulting Tet^r Hfr strains, bearing a Tn10 insertion at the same site as in HW31, were mated with suitable F^- strains.

Recombination data, obtained from the conjugation of HW41 (HfrH) and HW42 (HfrC) with R477, suggest that Tn10 might be located between the origin of HfrC and his (Table 6 and Fig 1). Furthermore, when HW43 (HfrB7) was mated with strain CSH57, the transfer of Tn10 was found to be closely associated with the trp operon (Table 6). The proximity of Tn10 to the trp operon was confirmed by transduction. When strain HW31 (Tet^r trp^+) was used as the donor and strain CSH57 (trp) as the recipient, 63 of the 72 tetracycline resistant transductants were found to be trp^+ also. In contrast, a similar experiment using HW11 (zhe::Tn10 trp^+) as the donor revealed that none of the 45 Tet^r transductants tested were trp^+. The location of Dbp^r and Tn10 relative to two neighboring markers, hemA and trp were determined by three factor crosses. Because the Dbp^r phenotype can only be detected in a glpR background, it was first necessary to construct strains HW50 (glpR glpD trp zch::Tn10) and HW60 (glpR glpD hemA zch::Tn10) as described in Materials and Methods. Strains HW50 and HW60 were used as recipients and strain 6204 (Dbp^r trp^+ $hemA^+$) as the donor for P1vir-mediated transduction. HW50 and HW60 transductants were selected that no longer require tryptophan and o-aminolevulinic acid, respectively. The transductants were then scored for the ability to grow on GL-succinate plates containing 30 uM rac-DBP and on L plates containing 25 ug/ml of tetracycline. The results of these experiments are

summarized in Table 7 and Fig 16A. The order of the markers is hemA-Dbp^r-Tn10(zch)-trp.

The cls gene is the only gene known to code for an enzyme involved in phosphoglyceride metabolism that maps in the region between hemA and trp (110). We therefore examined whether strain 6204 synthesizes CL. Lipid extracted from [1-^{14}C]-acetate labeled strain 7-1 was compared with that obtained from strain 6204. The results of the lipid analysis shown in Fig 17 clearly indicate that strain 6204 either does not contain CL or has so little of this phosphogllyceride that it cannot be detected by the method used. Experiments were performed to determine whether resistance to DBP could be correlated with an absence of CL. P1vir-mediated transduction was used to transfer DBP resistance from strain 6204 to strain HW50. The phosphoglyceride composition of each of ten randomly selected transductants that were able to grow on GL-succinate plates containing 30 uM rac-DBP was examined. In each case the DBP resistant transductant was found to be unable to synthesize CL.

Because the genetic and biochemical evidence suggests that DBP resistance is related to the gene responsible for cardiolopin synthetase it was important to determine whether a cell bearing the cls mutation isolated by Pluschke et. al. (72) is resistant to DBP. In order to examine this

possibility, strain QC120 was constructed by transferring the cls mutation of strain T1GP to strain HW50, a strain with a glpR background. Since the DBP resistant phenotype was originally characterized in bacteria having an active catabolic G3P dehydrogenase, strains QC120 (glpR glpD cls) and QC104 (glpR glpD) were converted to strains HW55 (glpR cls) and HW56 (glpR), respectively (see Materials and Mehtods section). Strain HW55 is indistinguishable from strain 6204 based upon the observations that it (i) remains viable in the cold after incubation in CH medium containing 100 uM rac-DBP for 1 h (Fig 18), (ii) continues to grow in CH medium containing 70 uM rac-DBP and 0.25% deoxycholate (Fig 19) , and (iii) is able to grow in GL-glucuronate medium containing 70 uM rac-DBP (Fig 20). In contrast, strain HW56 exhibits the same sensitivity to DBP as was evident in strain 7. Moreover, strain harboring cls mutation (HW55) is normal in ability to incorporate DBP into lipid fraction but lower in ability to incorporate DBP into TCA precipitated materials (Fig 8) as compared to strain 7. This is similar to strain 6204.

The position of cls relative to Tn10 (in the zch location) and trp was mapped by a three factor cross. A P1vir lysate of strain HW55 was used to convert strain HW50 to tryptophan independence. Two unselected traits, cls and Tet^r, were then scored. In this case, detection of the cls mutation was based upon the ability of mutant cells to form colonies on

GL-succinate plates containing 30 uM rac-DBP. The results of this cross, summarized in Table 8 and Fig 16B, indicate that gene order is cls (scored as DBP-resistance)-Tn10(zch)-trp. In a parallel experiment, when a P1vir lysate of strain HW56 was used to convert HW50 to tryptophan independence, none of the transductants were able to grow on GL-succinate plates containing 30 uM rac-DBP (Table 8). The frequency of cotransduction of cls and trp is approximately 50%. These results are very similar to those obtained from the cross between strains 6204 and HW50 (see Table 7 and Fig 16) and suggest that the mutation in strain 6204 responsible for DBP resistance either lies within the structural gene for cardiolipin synthetase or is at a position very close to it. The cotransduction frequency of trp and cls that we obtained (49%) is slightly higher than the one (35%) reported by Pluschke et. al. (72).

The effect of DBP on phosphoglyceride synthesis in strains HW53, HW55 and HW56 cultured in CH medium was studied. Cells were pretreated with 30 uM rac-DBP for 20 min prior to the addition of labeled acetate. The relative concentrations of each of the phosphoglycerides, determined from the peak areas of the radioscan of the chromatogram (Fig 21), are presented in Table 9. It is evident that DBP exerts the same inhibitory effect on PG synthesis in each strain, despite the fact that DBP has much less effect on the growth of HW53 and HW55 than it does on HW56. Higher

concentrations of DBP cause even greater inhibition of PG formation.

The addition of magnesium cations to temperature-sensitive phosphatidylserine synthetase (pss) and phosphatidylserine decarboxylase (psd) mutants cultured at nonpermissive temperatures produces a partial phenotypic suppression that may be due to a stabilization of the membranes containing excess anionic lipids (60,61,65). Since DBP treatment leads to the accumulation of an abnormal anionic phosphoglyceride, the phosphonic acid analog of phosphatidylglycerol phosphate, the possibility was considered that magnesium or calcium chloride could offset the effect of DBP. However, as evident from Fig 22, the addition of 20 mM magnesium chloride to DBP treated cultures failed to offset the effect of DBP but instead had a synergistic growth inhibitory effect on both strains HW55 (cls) and HW56 (cls^+). Thus in this case the presence or absence of cardiolipin does not seem to have any effect. The addition of 20 mM calcium chloride causes the same type of growth inhibitory effect as does the addition of magnesium chloride (Fig 23).

DISCUSSION

E. coli, treated with low concentrations of DBP, exhibits major perturbations in phosphoglyceride metabolism but only slight changes in doubling time. This should not have been too surprising because E. coli did not evolve to survive in the synthetic medium used in the laboratory. Certain environmental conditions to which E. coli are ordinarily exposed are quite lethal to bacteria treated with low concentrations of DBP. E. coli normally grow very well in the presense of 0.25% deoxycholate. However, this bile salt is quite deleterious to cells that are incubated in the presence of low concentrations of DBP (Fig 4). Furthermore, strain 7-1 incubated in the presence of 100 uM rac-DBP for 1 h die when stored at 5°C, whereas untreated cells remain viable (Fig 5). These effects suggest that DBP treatment is causing a major change in membrane structure. A single mutation found in strain 6204 permits it to resist the effect of DBP under these environmental stresses (Fig 7,12). The mutation does not affect the incorporation of DBP into the phosphoglyceride fraction (Fig 8) or the ability of PGP and G3P synthetases to recognize DBP (Table 4). Furthermore, total lipid synthesis in strain 6204 is just as sensitive to the presence of DBP as is that of the parent strain (Table 3, 8). However, the incorporation of DBP into the macromolecular fraction is consistently lower in strain 6204

than in the parent strain (Fig 9). Tn10 has been inserted near the mutation responsible for DBP resistance, and transduction experiments have revealed that the DBP-deoxycholate and DBP-cold storage effects are caused by the same gene.

Experiments were performed to map the location of Tn10, inserted near the Dbp^r mutation, as well as the mutation itself. These experiments suggest that the mutation maps in or very near cls, a gene associated with CL formation (Fig 16). Consistent with this notion, strain 6204 does not form CL (Fig 17). Furthermore, Dbp^r transductants of strain 7 are also unable to synthesize CL, and a strain constructed with an authentic cls lesion has the same resistance to DBP as does strain 6204 (Fig 18,19,20).

It is not at all clear why the absence of CL should enable the cells to have enhanced resistance to DBP. Three among the many possible explanations will be briefly considered. First, it is possible that the accumulation of both CL and the PGP analog (90) might create a membrane lipid bilayer that is too anionic in character to permit normal function. In this case, the absence of CL might help to compensate for the appearance of the new anionic phosphoglyceride, the analog of PGP. In a related situation, the accumulation of anionic phospholipids in temperature -sensitive pss and psd mutants results in growth inhibition at nonpermissive

temperature (60,61,65). The growth phenotype at the restrictive temperature can be suppressed by increasing the concentration of magnesium and calcium cations (60,61,65). However, the addition of cations does not restore the cellular phosphoglyceride composition to normal (60,61,65). Therefore, the effect of magnesium and calcium cations on the growth of DBP-treated cells was examined. Contrary to expectation, the addition of either 20 mM magnesium or calcium chloride increases the sensitivity of the cells to DBP regardless of the cls genotype (Fig 22,23). This seems to argue against the buildup of anionic lipids being solely responsible for the growth inhibitory effect observed. Second, it is possible that phosphoglyceride perturbations resulting from DBP treatment raise the phase transition temperature of the bacterial membrane. Cells do not grow at temperatures below the phase transition temperature of their membranes (111). Membranes isolated from E. coli lacking CL synthetase have been shown to have a lower phase transition temperature than do membranes isolated from wild type cells (112). This has been attributed to the compensatory increment in amount of PG due to the defect in CL synthetase. PG has a much lower phase transition temperature than CL which leads to an overall lowering in membrane phase transition temperature (112). If DBP treatment raises the phase transition temperature of bacterial membranes then the absence of CL might offer some advantages. The fact that wild type cells treated with DBP

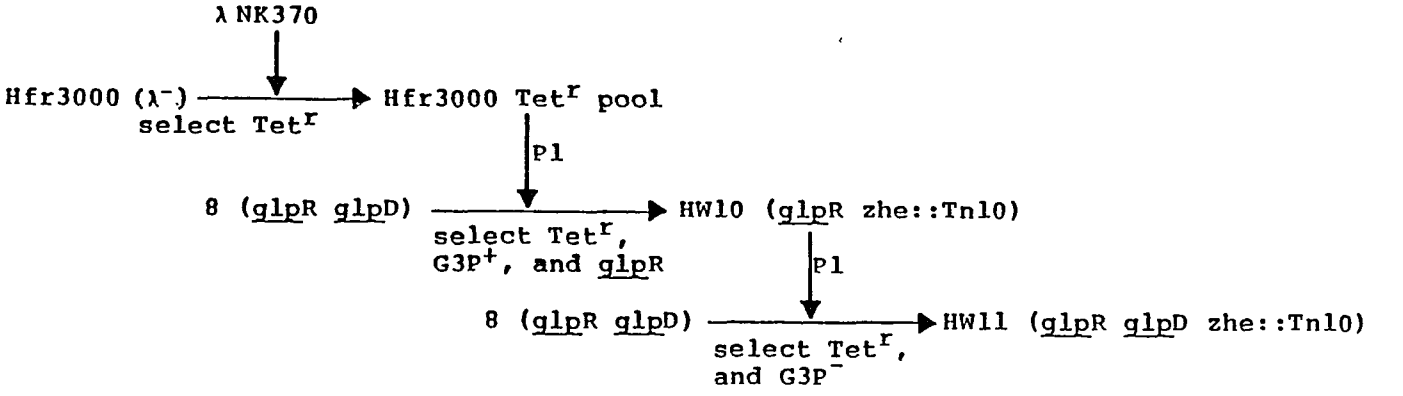
are killed during prolonged storage in the cold may be due to a DBP-induced change in membrane fluidity. In this regard, it should be noted that divalent cations increase the phase transition temperature of membranes made of synthetic PG (113). If this is also true for membranes containing the analog of PGP in place of PG then a change in membrane fluidity might also explain the synergistic effect observed when divalent ions are added to DBP treated cells. Further work is required to establish a correlation between DBP related growth phenomena and membrane fluidity. Third, it is possible that a certain amount of PG in the membrane is essential for normal cell function. In addition to its role as a membrane component, PG is also a precursor for CL (55), lipoprotein (82), and MDO (75,81). Therefore, in cells treated with DBP, a defect in CL synthetase may permit the cells to channel the scarce PG into other metabolic products. Cells with a cls mutation do indeed have a slower rate of PG turnover (72). However, it is difficult to see why channeling the PG to lipoprotein or membrane-derived oligosaccharide synthesis would cause a marked effect on cell growth. Mutants that either synthesize a lipoprotein devoid of covalently bound lipid or that are unable to synthesize lipoprotein appear to be able to grow (84,114). MDO has recently been implicated in osmotic adaptation (11), but again, it does not appear to be essential for cell growth (78,81). Numerous membrane- bound enzymes depend on the interaction of specific phospholipids for their activity

(115). In particular, the phosphoenolpyruvate- sugar phosphotransferase system was reported to require PG (116). Perhaps a defect in CL synthetase enables the cells to retain a critical membrane concentration of PG to interact with enzymes and transport systems that require it. The possibilities discussed are not mutually exclusive or the only ones that might be put forward to explain why mutants with a defect in CL synthetase exhibit enhanced resistance to DBP.

The fact that deoxycholate acts synergistically with DBP suggests that DBP treatment alters the outer membrane of the bacteria. Studies of the lipopolysaccharides of gram negative bacteria indicate that certain mutants of the genus Salmonella which have defective lipopolycaccharides are quite sensitive to deoxycholate (117,118). Lipopolysaccharide is generally thought to serve on the outer leaflet of the outer membrane of gram- negative bacteria as a physical barrier that excludes hydrophobic compounds and detergents (16,17). DBP may affect the synthesis of lipopolysaccharide, its insertion into the outer membrane or some other aspects of outer membrane structure. Further work is necessary in order to demonstrate the effect of DBP on the integrity of the outer membrane of the gram-negative bacteria. Any explanation of why deoxycholate acts synergistically with DBP must also account for the fact that cells with a lesion in cls do not exhibit this effect.

Although many questions have been raised by the observation that cells with a defect in CL synthesis exhibit enhanced resistance to DBP, a few applications of this knowledge are evident. DBP can be used to screen for the cls mutation which is otherwise silent. This could help one to study the regulation of cls gene expression or enable one to clone the cls gene. In addition, by raising the level of DBP it is possible to have cells continue to divide despite the fact that the membranes are devoid of CL and nearly devoid of PG. It should be noted that a mutation in cls does not confer complete resistance to DBP. At DBP concentrations greater than 1 mM, strains carrying the cls mutation are still completely inhibited (Fig 11). Nevertheless, strains harboring the cls mutation should be better starting strains than the wild type to isolate mutants resistant to high concentrations of DBP. The availability of such mutants should provide valuable information about the mechanisms of DBP action.

Chart 1 Pedigrees of strains HW10, HW11, HW21, and HW22



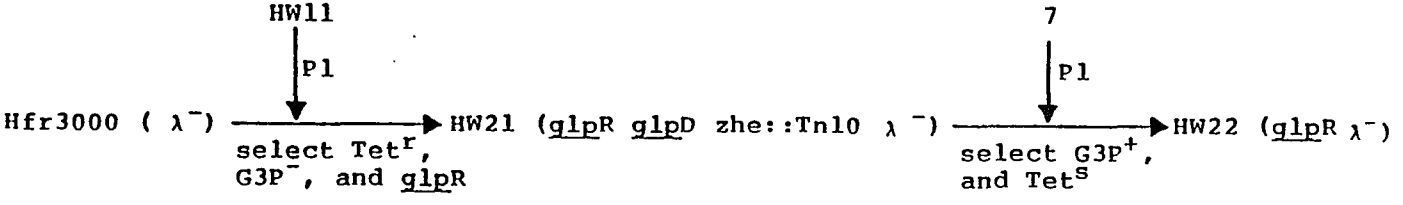
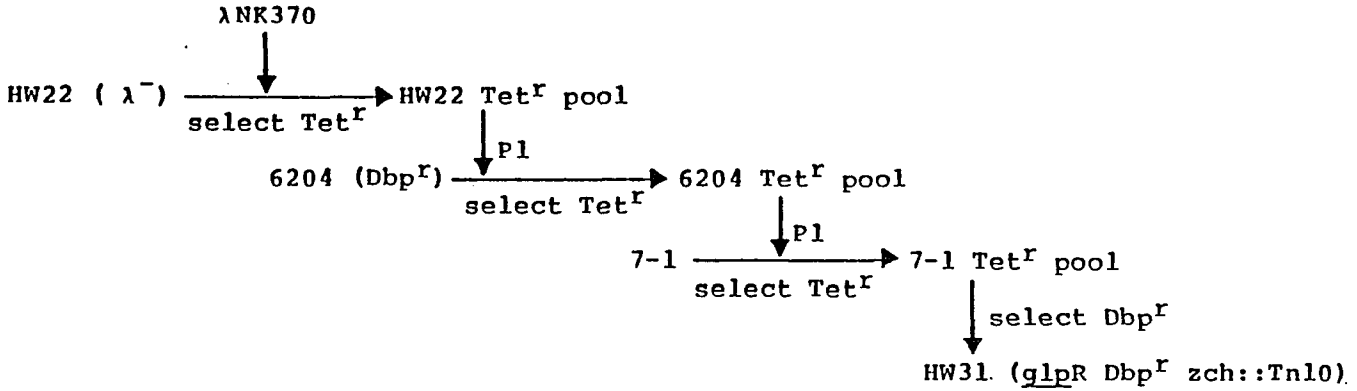
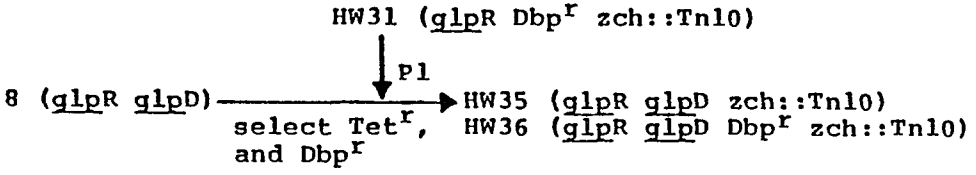


Chart 2 Pedigrees of strains HW31, HW35, HW36, HW41, HW42, and HW43





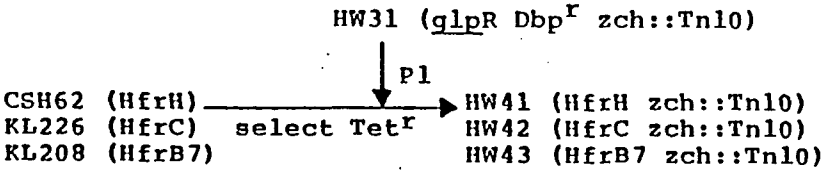
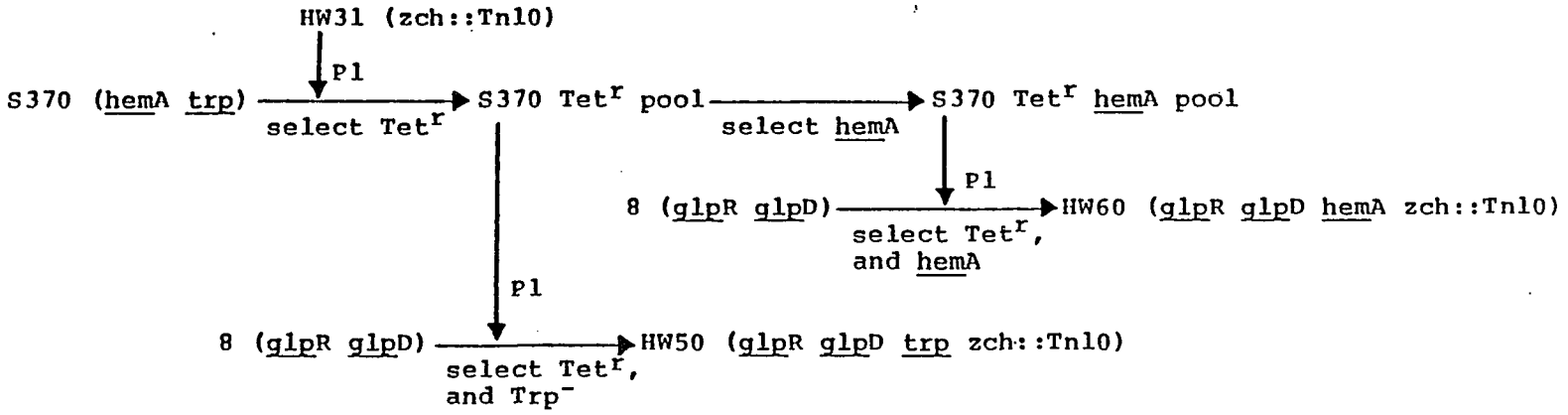
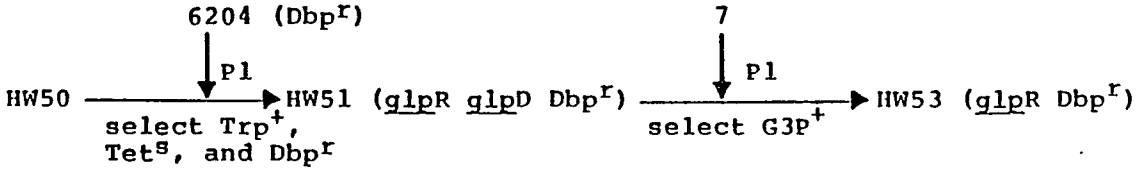


Chart 3 Pedigrees of strains HW50, HW51, HW53, HW55, HW56, HW60, QC104, and QC120





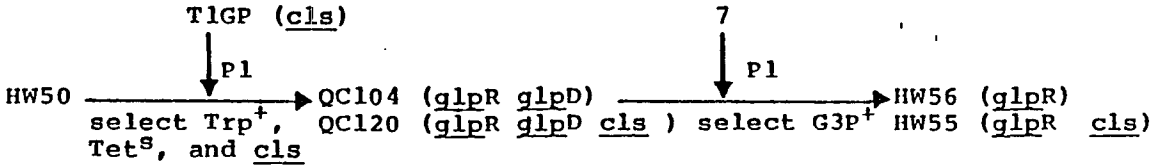


Figure 1. List of genetic markers relevant to this study.

Gene Symbol	Map (min)	Gene product or phenotypic trait
cds	4	CDP-diglyceride synthetase
cdsS	68	suppressor for cds mutation
cls	27	cardiolipin synthetase
dgk	92	diglyceride kinase
fnr	29	regulatory gene for nitrite and nitrate reductase, hydrogenase
gal	17	galactose fermentation
glpA	49	anaerobic G3P dehydrogenase
glpD	75	aerobic G3P dehydrogenase
glpF	88	facilitated diffusion of glycerol
glpK	88	glycerol kinase
glpQ	49	glycerophosphodiesterase
glpR	75	regulatory gene of glp regulon
glpT	49	G3P permease
gpsA	81	biosynthetic G3P dehydrogenase
hemA	27	δ-aminolevulinate synthetase
his	44	biosynthesis of histidine
lac	8	lactose fermentation
leu	2	biosynthesis of leucine
pgpA	10	PGP phosphatase
pgpB	28	PGP phosphatase activity
pgsA	42	PGP synthetase
pgsB	4	lipopolysaccharide synthesis
plsB	92	G3P acyltransferase
psd	95	PS decarboxylase
pss	56	PS synthetase
pssR	84	regulatory gene of pss locus
purE	12	biosynthesis of adenine
rpsL	73	ribosomal protein S12; streptomycin resistance
thr	0	biosynthesis of threonine
trp	28	biosynthesis of tryptophan
ugpA	76	G3P transport system
ugpB	76	binding protein of G3P transport system
uhp	82	hexose phosphate utilization system

The origin and direction of chromosome transfer by each of the three Hfr strains used in conjugational mapping are indicated by the arrow heads.

FIG 1

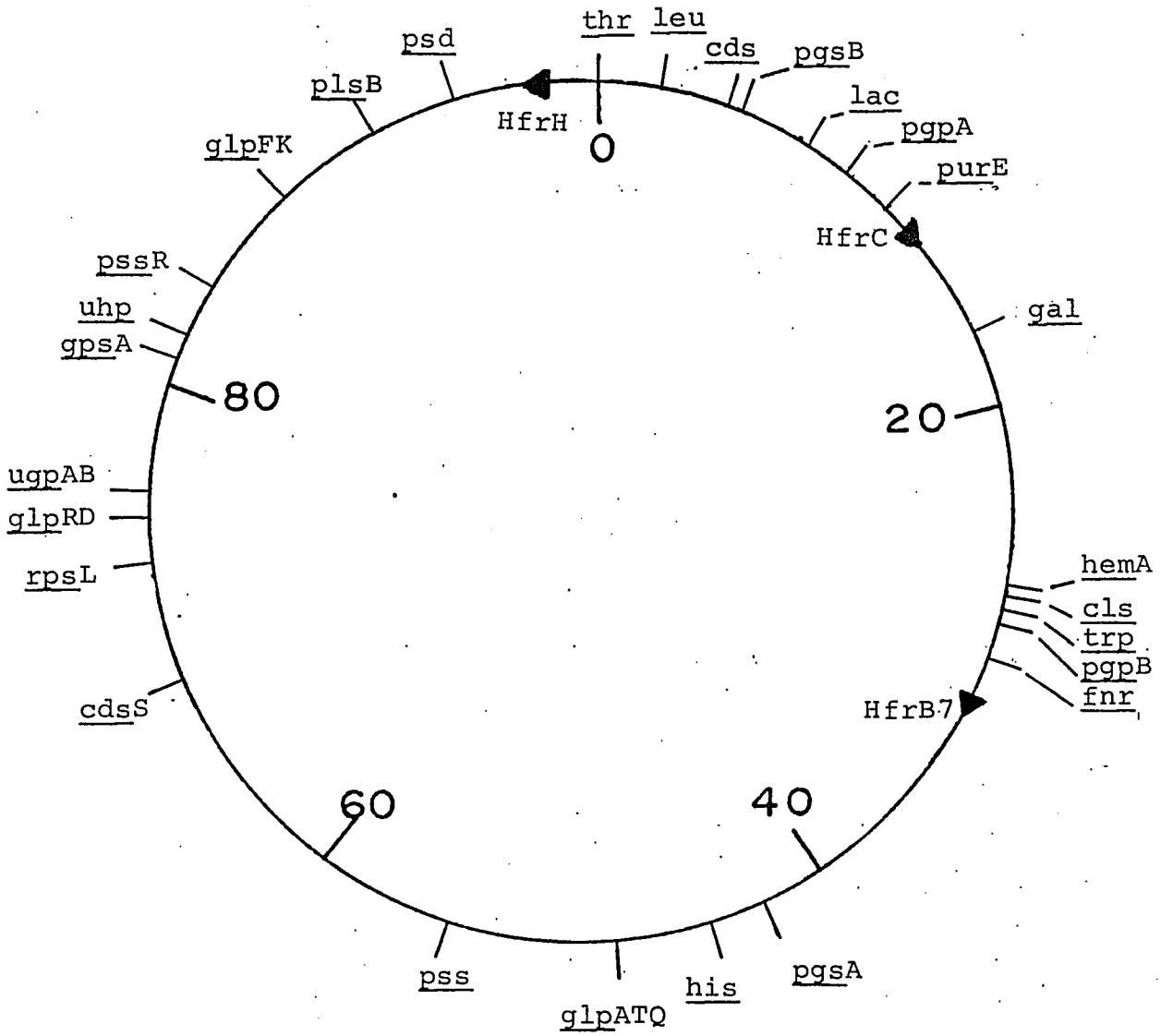
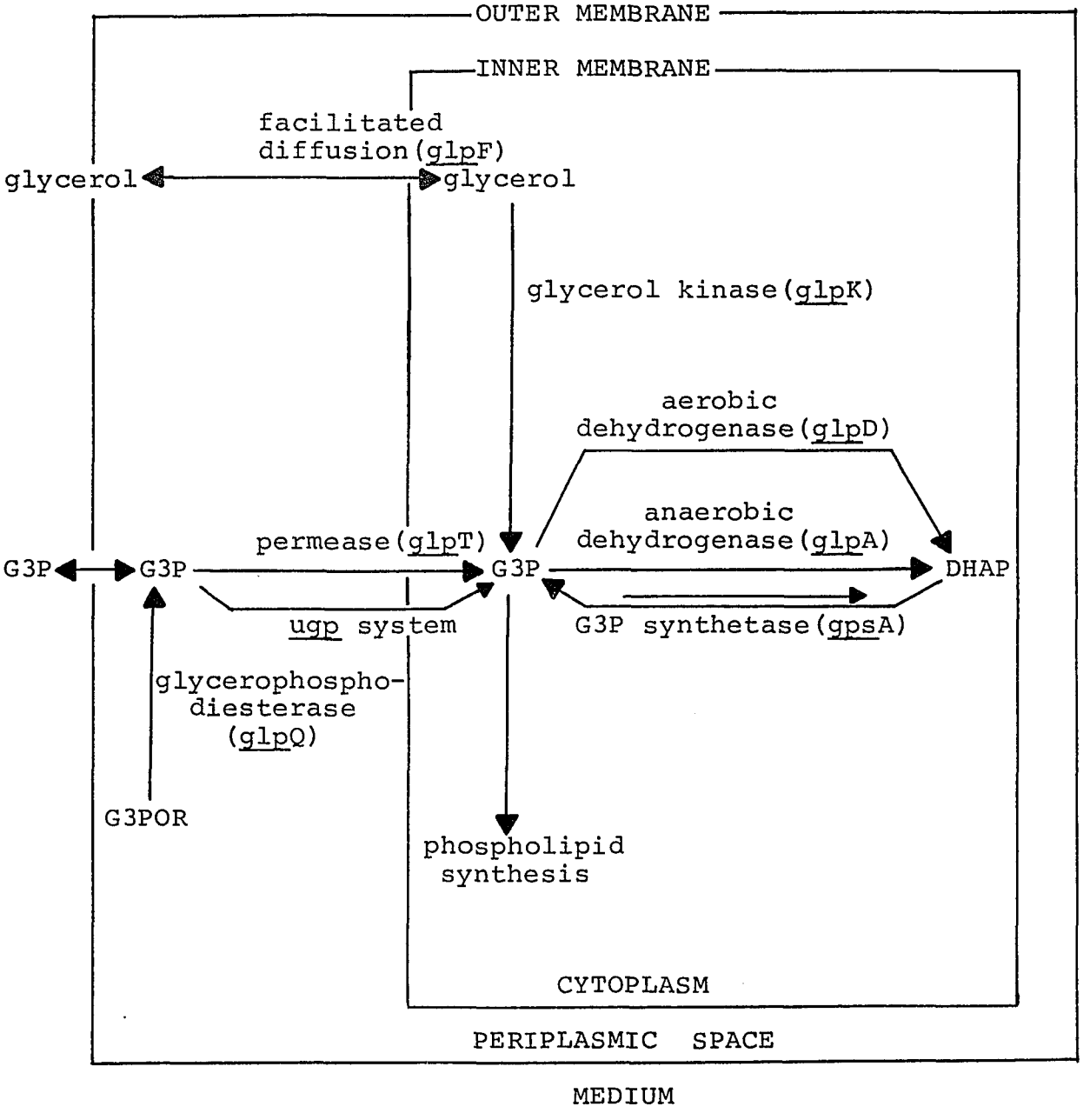

thr
leu
cds
pgsB
lac
pgpA
purE
HfrC
gal
20
hemA
cls
trp
pgpB
fnr
HfrB7
40
pgsA
his
glpATQ
pss
60
cdsS
rpsL
glpRD
ugpAB
80
gpsA
uhp
pssR
glpFK
plsB
psd
HfrH
0

Figure 2. Glycerol 3-phosphate metabolism in E. coli



DHAP:dihydroxyacetone phosphate
G3POR:glycerophosphodierter

Figure 3. Biosynthesis of membrane phospholipid in E. coli

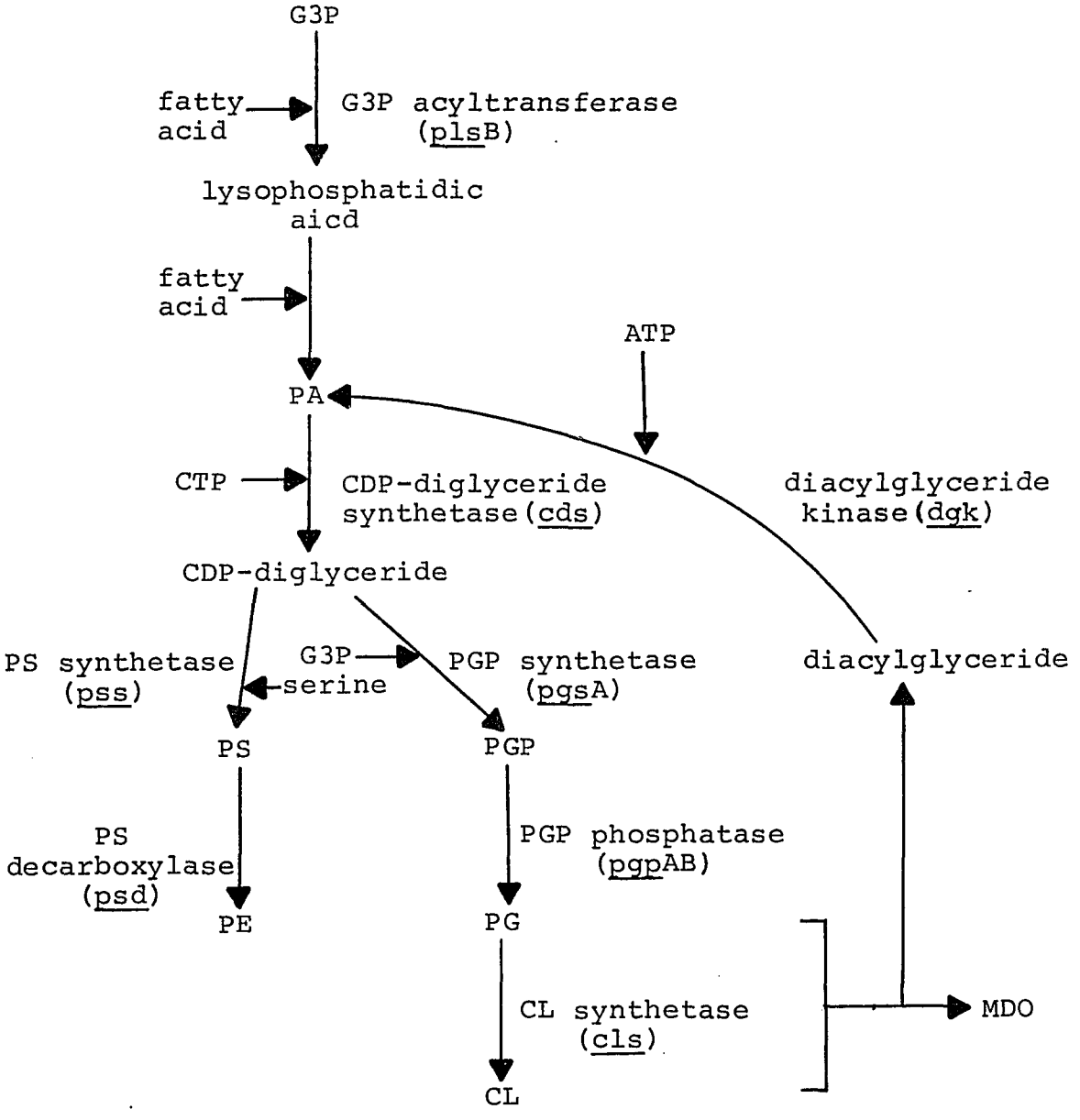
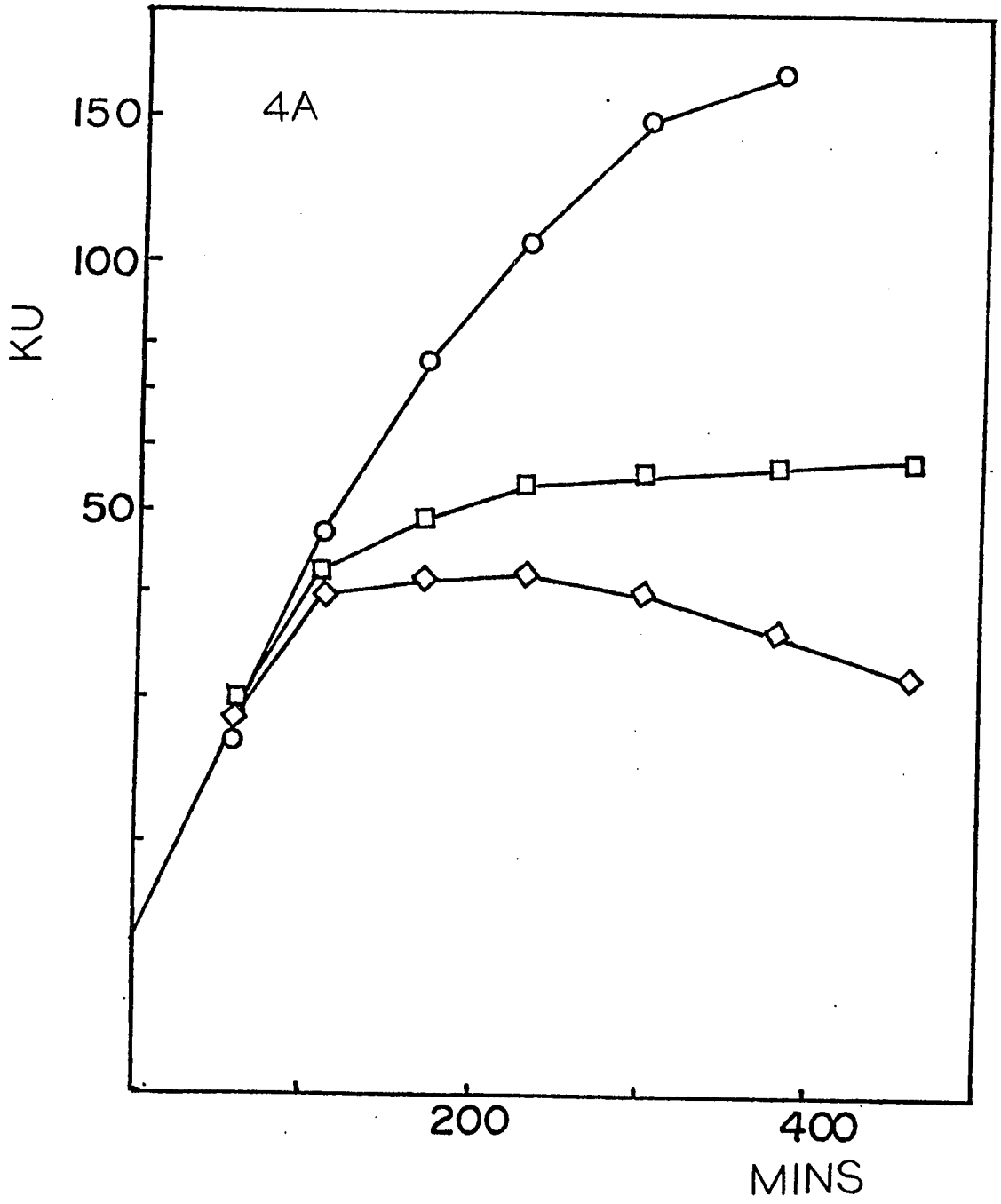


Figure 4

Effect of DBP and DBP plus deoxycholate on the growth (A) and viability (B) of E. coli strain 7-1. The bacteria were cultured in CH medium. At the time indicated as zero in the graph, 70 uM rac-DBP, 0.25% sodium deoxycholate, or both were added to growing bacterial cultures. DBP plus deoxycholate, ◇ ; DBP alone, □ ; and untreated cultures, ○ . Cultures treated with deoxycholate alone behaved identically to untreated cultures (data not shown).


4A
150
100
50
KU
200
400
MINS

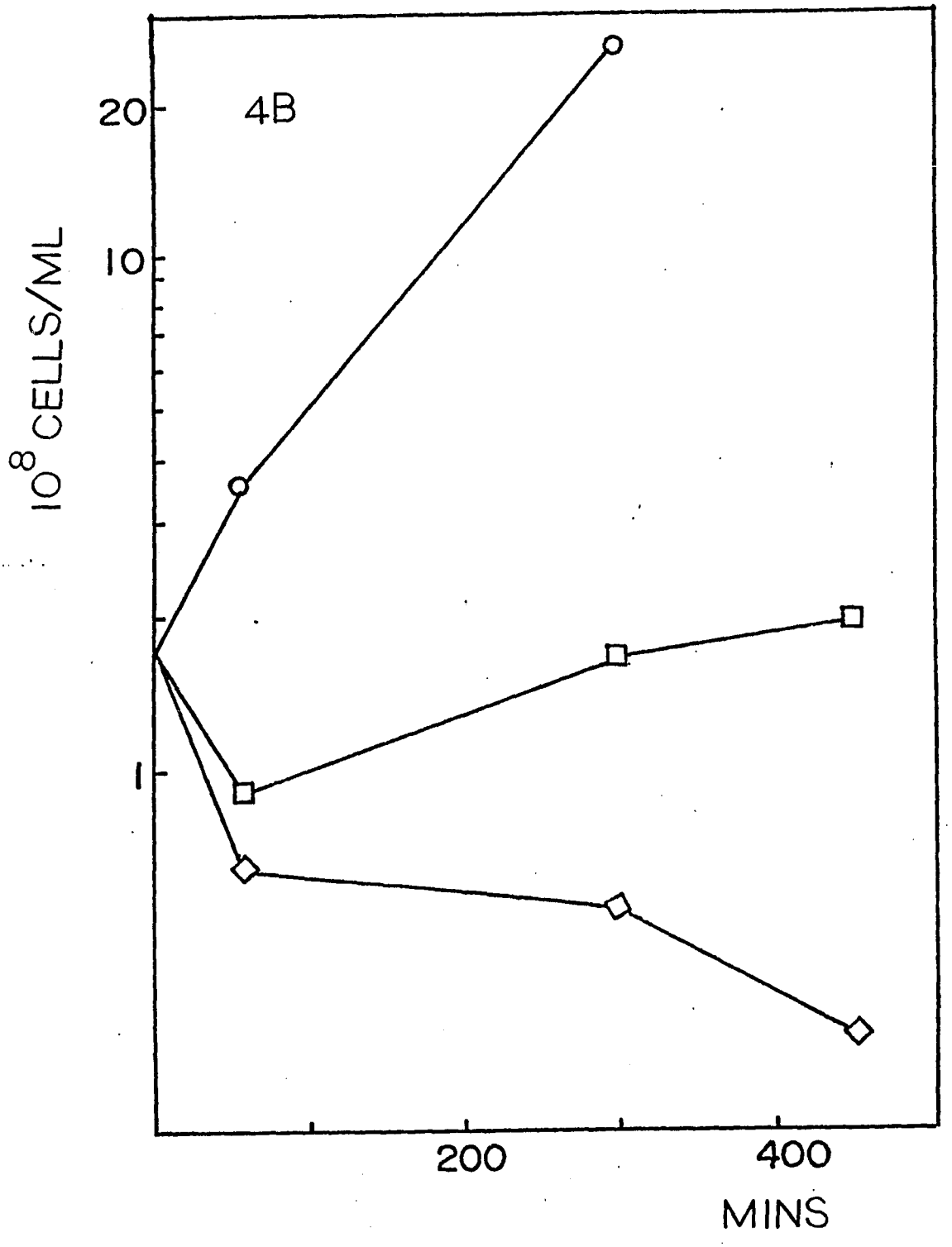

4B
20
10
1
10^8 CELLS/ML
200
400
MINS

Figure 5

The effect of cold storage on the viability of E. coli strain 7-1 that had been cultured in CH medium in the presence of 100 uM rac-DBP for 1 h at 37 ^{o}C. Viability is expressed as a ratio of N_o (viability on day 0) to N (viability on a given day after exposure to DBP). Culture treated with DBP, ◇ ; and untreated culture, o .

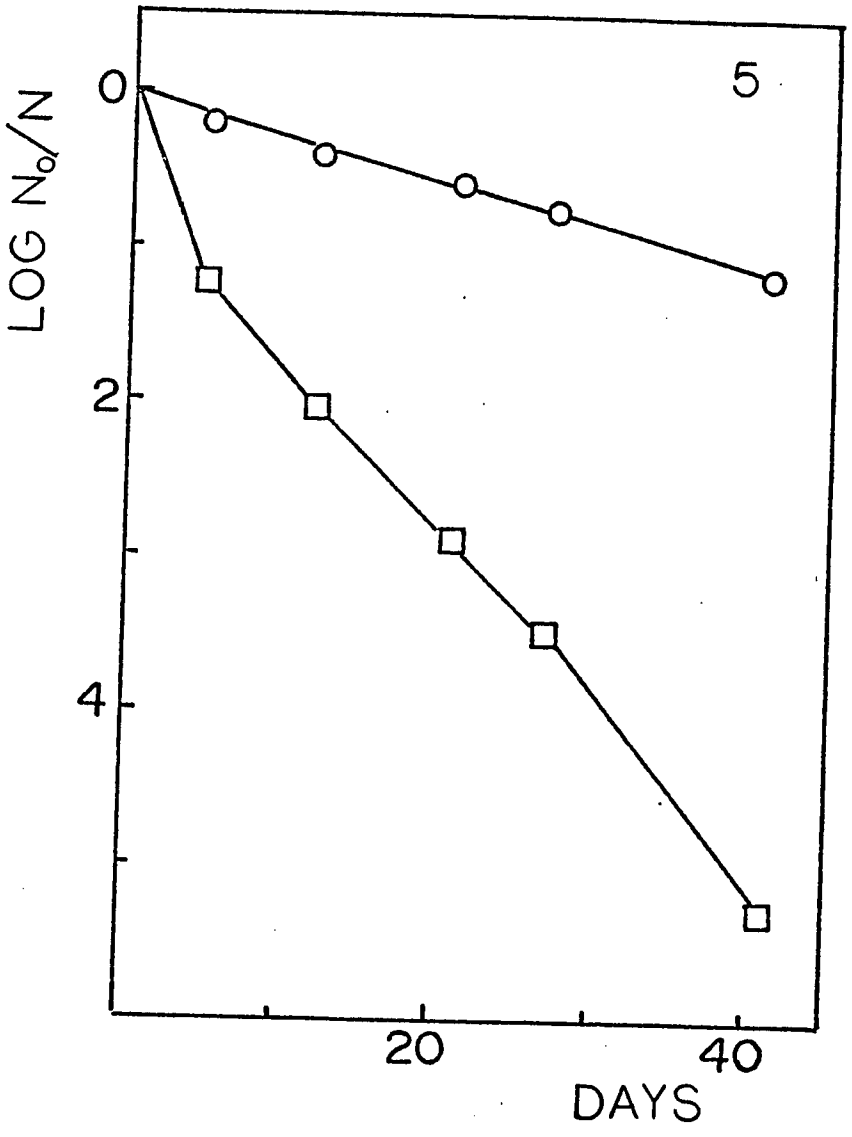

LOG N₀/N
0
2
4
5
20
40
DAYS

Figure 6

The effect of DBP-cold storage on the viability of E. coli cells that had been exposed previously to the same DBP-cold treatment. Cells were cultured in CH medium in the presence of 100 uM rac-DBP for 1 h at 37 ^{o}C before being stored at 5 ^{o}C. Viability is expressed as a ratio of N_o (viability on day 0) to N (viability on a given day after exposure to DBP). Cells which were exposed to the DBP-cold treatment for the second time, □ ; fresh culture treated with DBP, ● ; and untreated fresh culture, ○ .

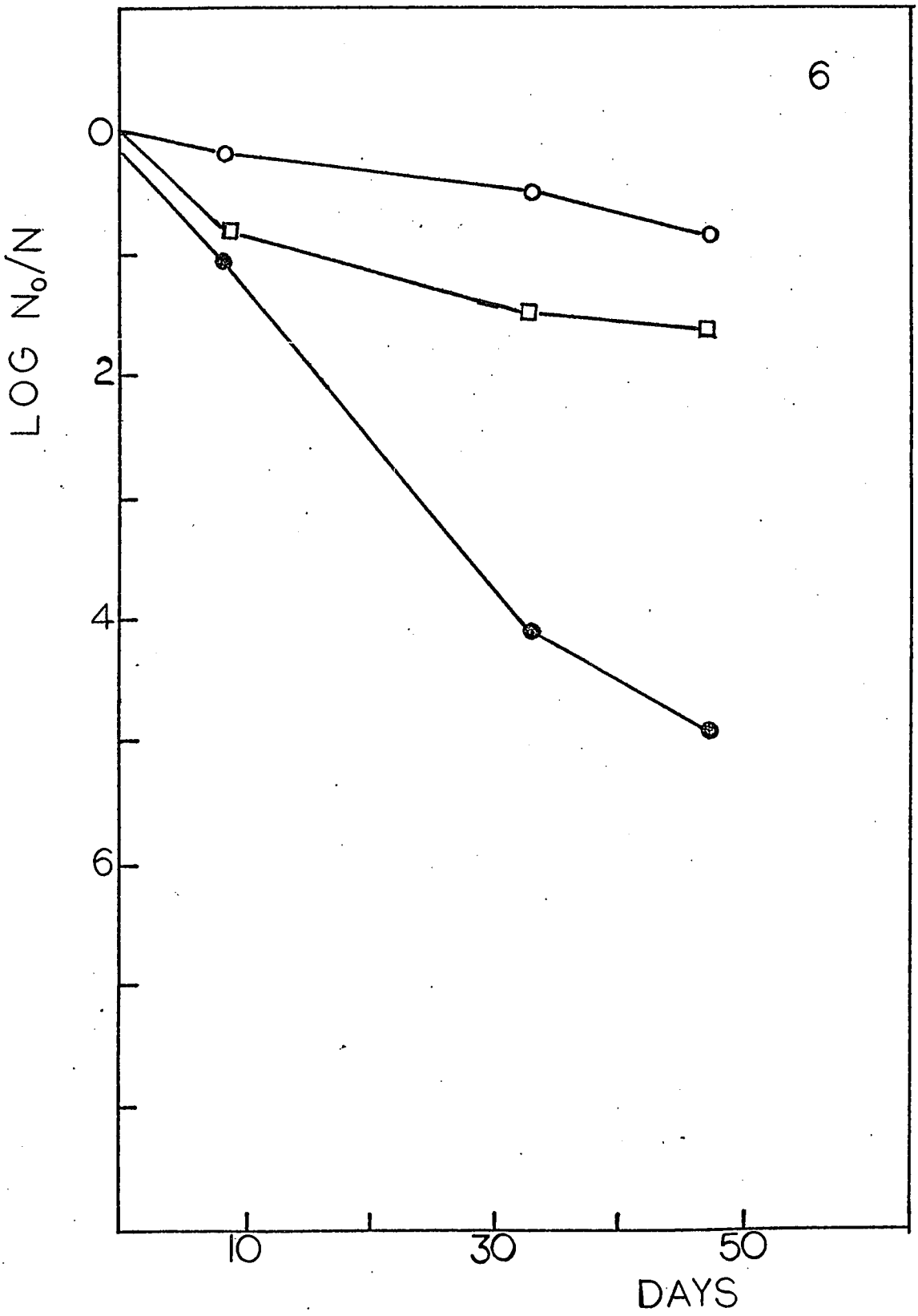

6
0
2
4
6
LOG N₀/N
10
30
50
DAYS

Figure 7

The effect of cold storage on the viability of E. coli strain 6204 (DBP resistant mutant) that had been cultured in CH medium in the presence of 100 uM rac-DBP for 1 h at 37°C. Viabilities are expressed as a ratio of N_o (viability on day 0) to N (viability on a given day after exposure to DBP). Culture treated with DBP, ■ ; and untreated culture, ● .

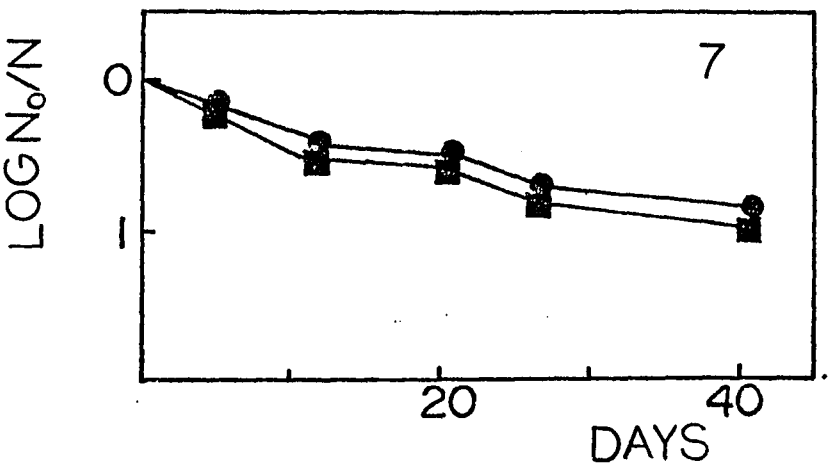

LOG N₀/N
0
1
7
20
40
DAYS

Figure 8

The incorporation of [3-^{3}H]-DBP into the chloroform-extractable fraction of E. coli strains 7-1, 6204, and HW55. At early log phase (15-20 KU), 1 uCi of [3-^{3}H]-DBP (sp. act. 30 uCi/umole) was added to 1 ml of cells and the culture incubated for 1 h at 37°C. Total lipid from 0.9 ml aliquot was extracted and radioactivity measured as described in Materials and Methods section. Strain 7-1, O ; strain 6204, Δ ; and strain HW55, □ .

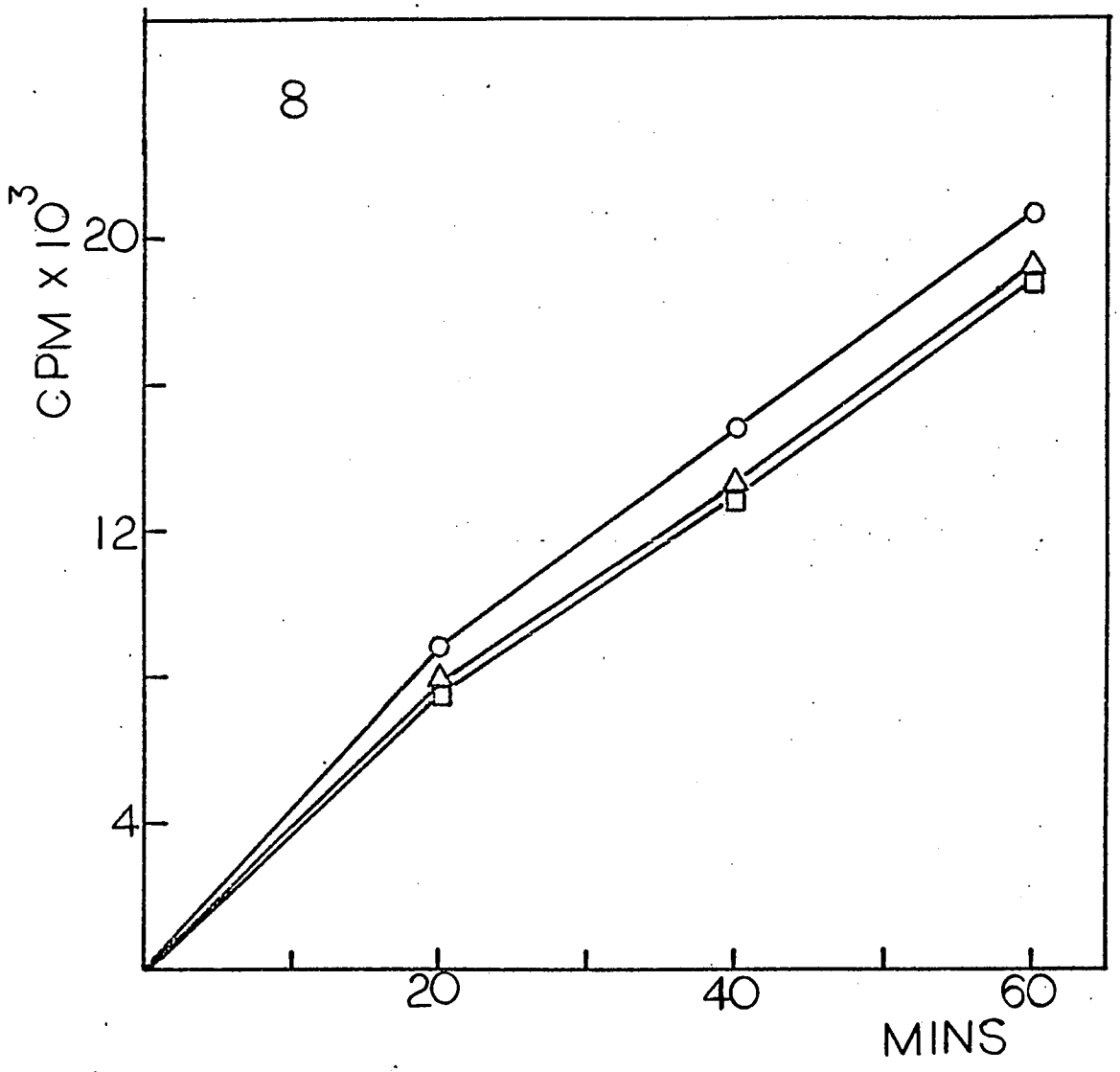

8
CPM x 10^3
20
12
4
20
40
60
MINS

Figure 9

The incorporation of [3-^{3}H]-DBP into the macromolecular fraction of E. coli strains 7-1, 6204, and HW55. At early log phase (15-20 KU), 1 uCi of [3-^{3}H]-DBP (sp. act. 30 uCi/umole) was added to 1 ml of cells and the culture incubated for 1 h at 37 ^{o}C. 0.1 ml aliquot of labeled culture was washed with trichloroacetic acid and radioactivity measured as described in Materials and Methods section. Strain 7-1, O ; strain 6204, Δ ; and strain HW55, □ .

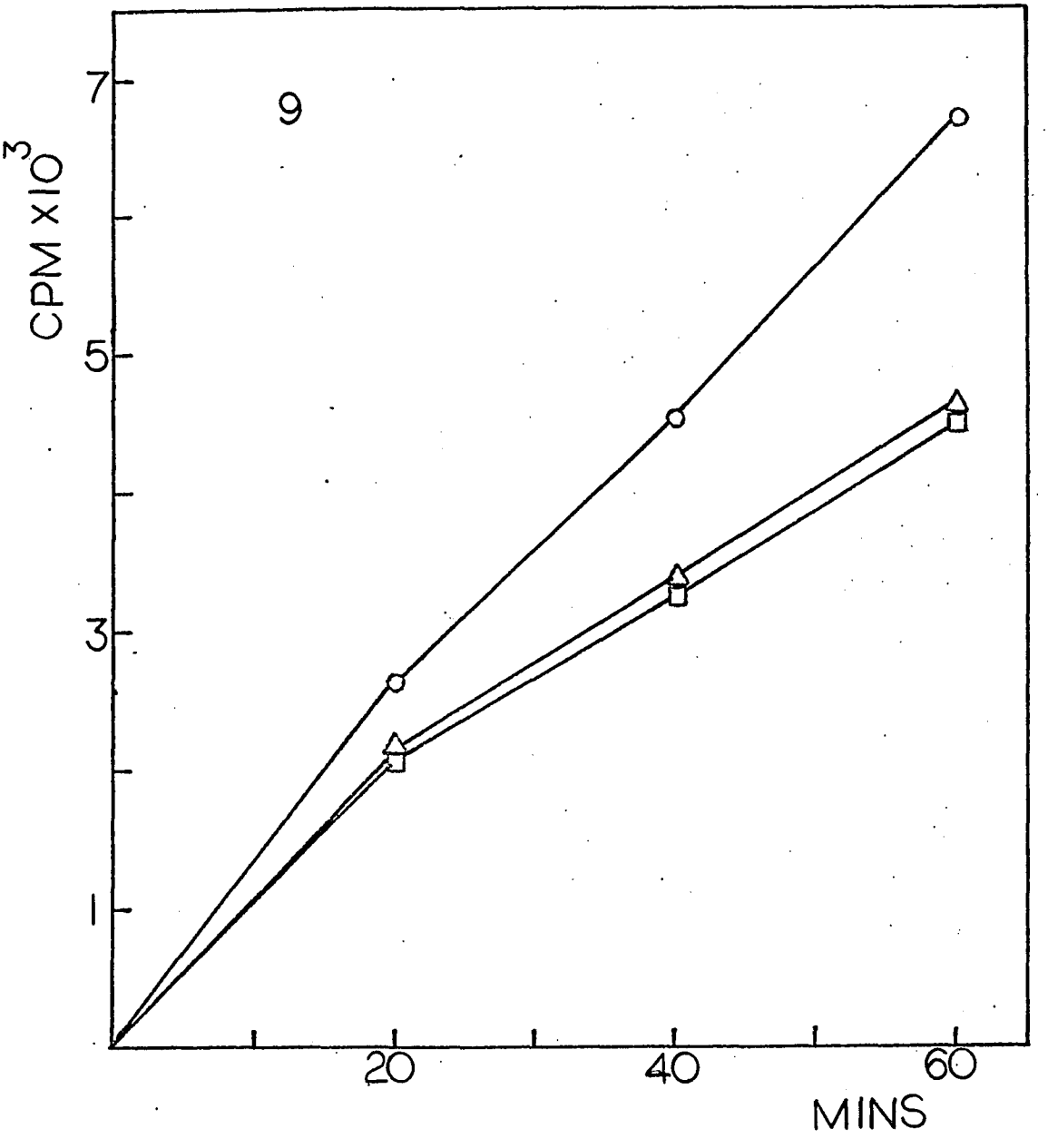

9
CPM X10 3
7
5
3
1
20
40
60
MINS

Figure 10

The effect of alkaline pH on the growth of E.coli strains 7-1, 6204, and HW31. Cells cultured in CH medium (pH 7.4) were collected by centrifugation, resuspended in one fifth of original volume of CH medium (pH 7.4). The resuspended cells were then inoculate into 10 ml of pH 7.4 and pH 8.8 CH medium, The growth of cells was monitored as previously described (91,94). Strain 7-1 at pH 8.8, ● ; Strain 6204 at pH 8.8, ■ ; and strain HW31 at pH 8.8, ▲ . Cells cultured at pH 7.4 behaved identically to each other, shown as ○ .

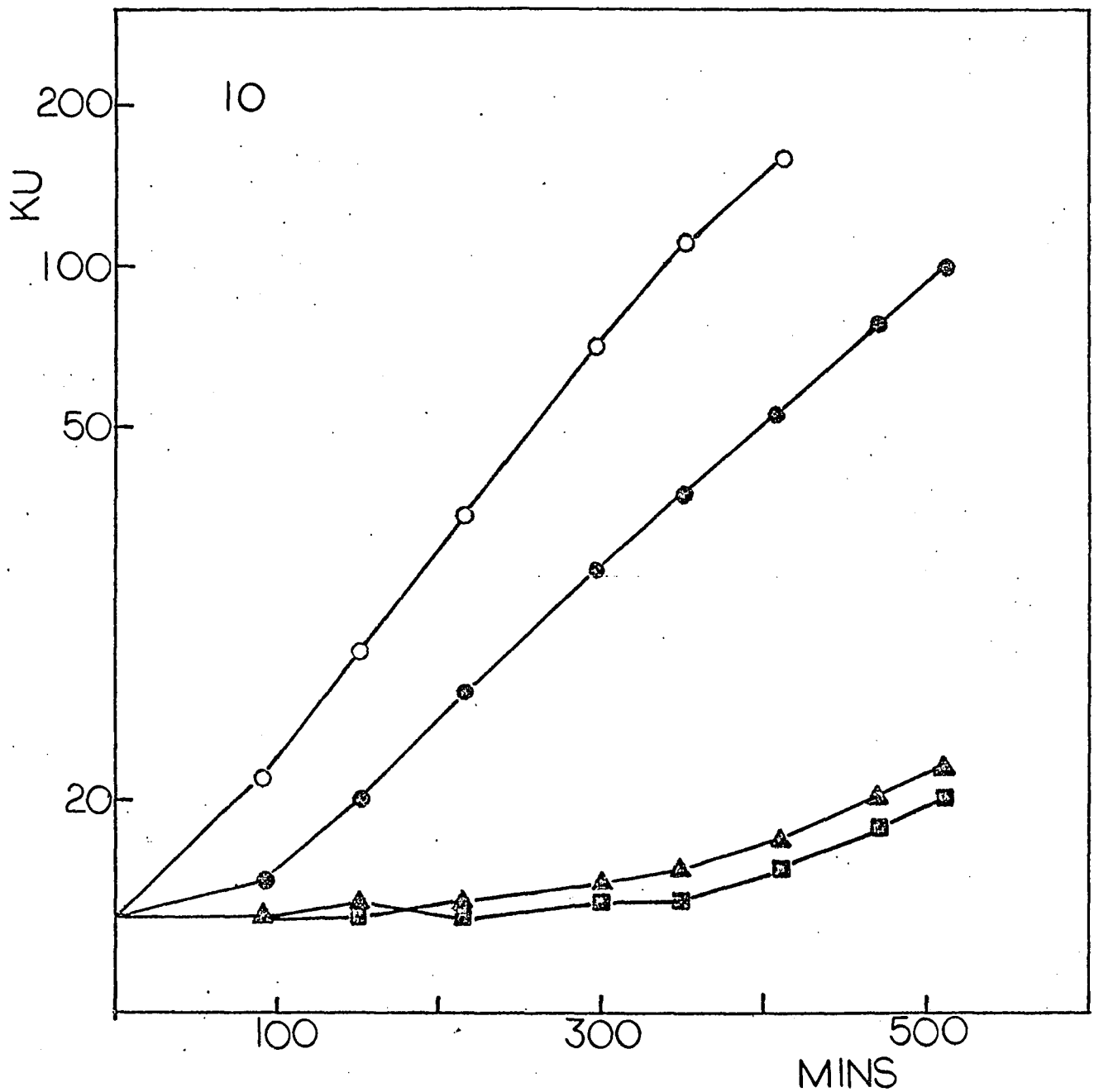

10
200
100
50
20
KU
100
300
500
MINS

Figure 11

The effect of 1 mM rac-DBP on the growth of strains 7-1, and 6204. The bacteria were cultured in CH medium at the time indicated as zero in the graph, 1 mM rac-DBP was added to growing bacterial cultures. Strain 7-1 treated with DBP, ● ; strain 7-1 untreated, ○ ; strain 6204 treated with DBP, ■ ; strain 6204 untreated, □ .

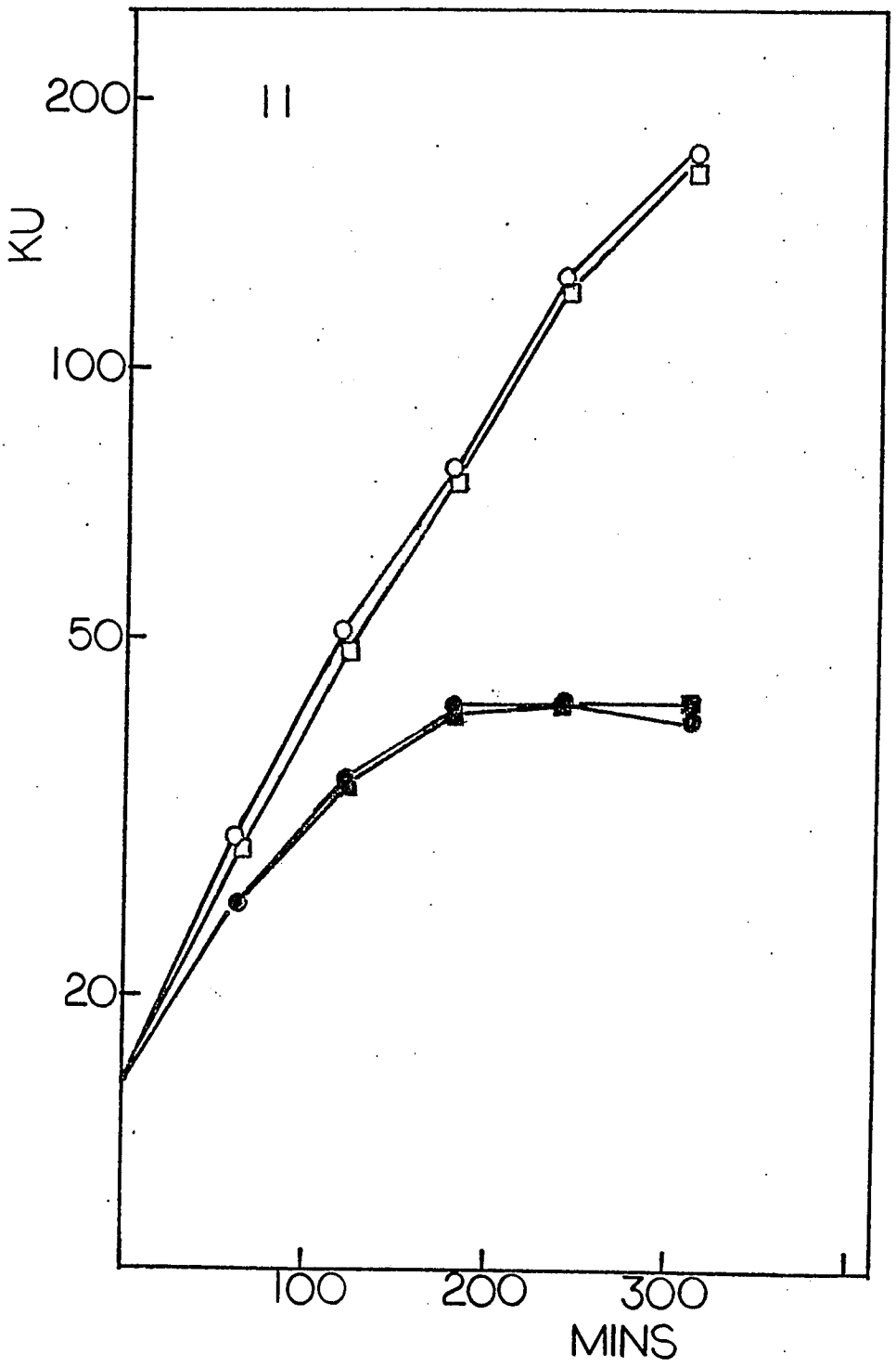
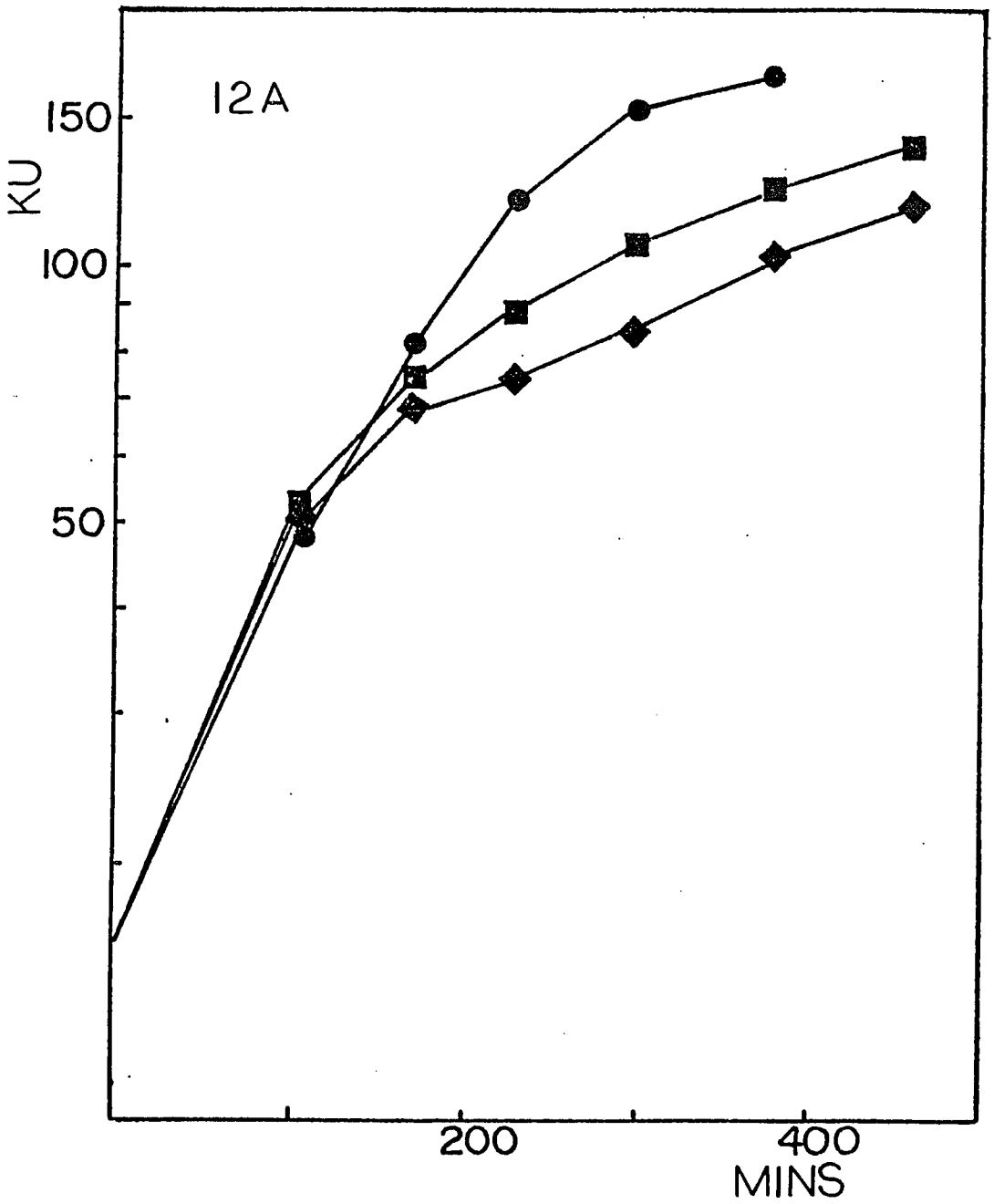

11
200
100
50
20
KU
100
200
300
MINS

Figure 12

Effect of DBP and DBP plus deoxycholate on the growth (A) and viability (B) of E. coli strain 6204 (DBP resistant mutant). The bacteria were cultured in CH medium. At the time indicated as zero in the graph, 70 uM rac-DBP, 0.25% sodium deoxycholate, or both were added to growing bacterial cultures. DBP plus deoxycholate, ◆ ; DBP alone, ■ ; and untreated cultures, ● . Cultures treated with deoxycholate alone behaved identically to untreated cultures (data not shown).


12A
150
100
50
KU
200
400
MINS

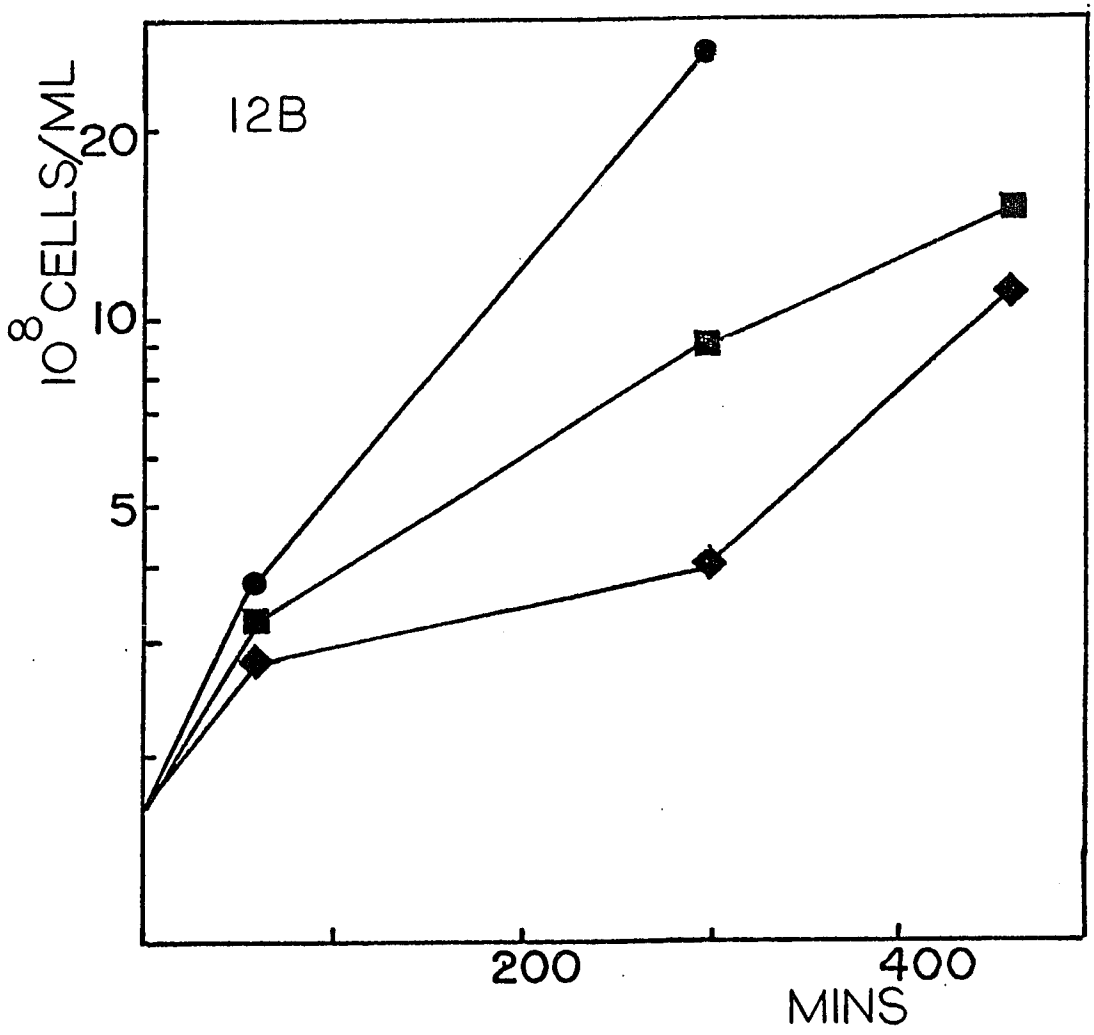
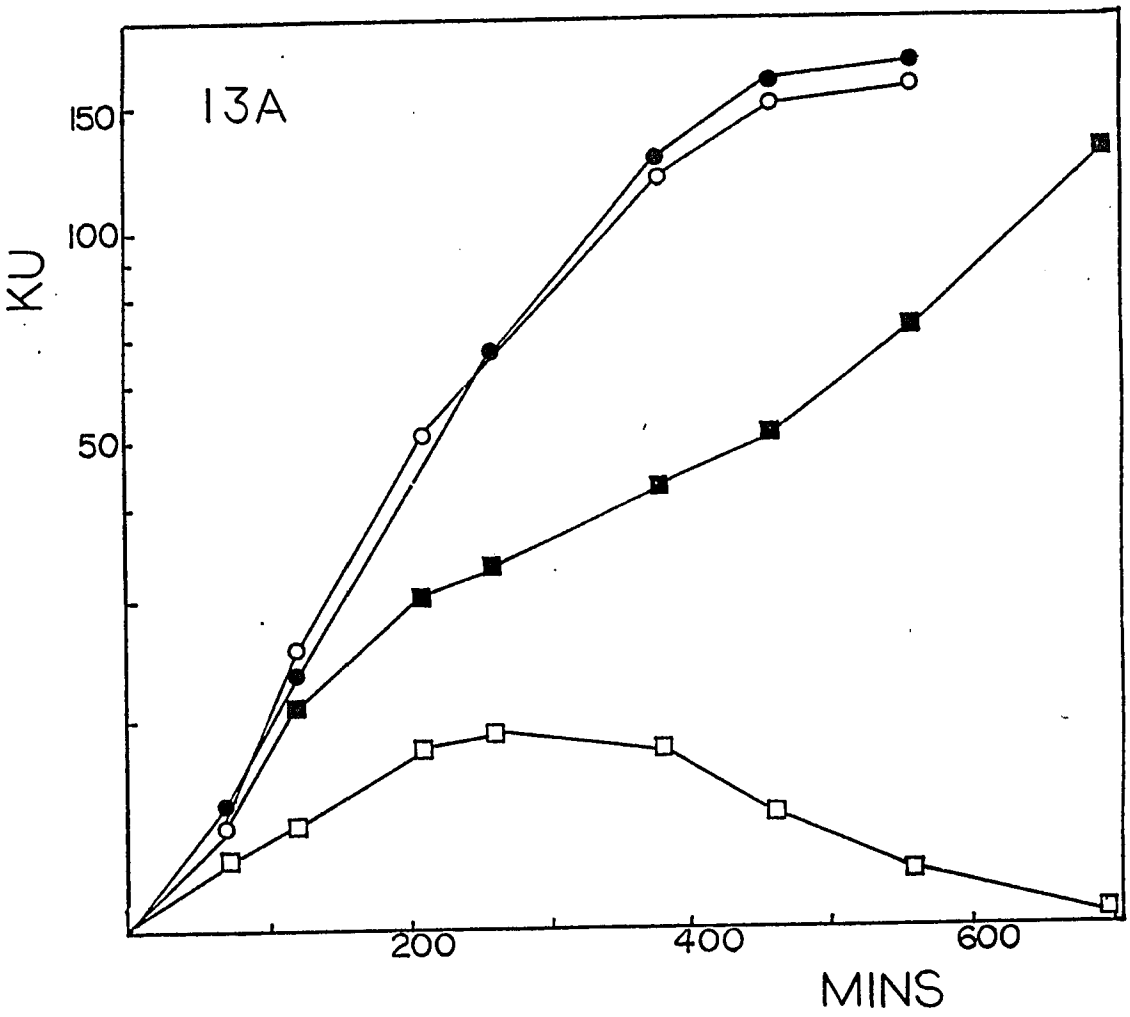

12B
10^8 CELLS/ML
20
10
5
200
400
MINS

Figure 13

Effect of 70 uM rac-DBP on the growth (A) and viability (B) of E. coli strain 7-1 and strain 6204 (DBP resistant strain) in GL medium containing glucuronate as the sole carbon source. DBP was added to growing cells at the time indicated as zero on the graph. Strain 7-1 treated with DBP, □ ; untreated strain 7-1, O ; strain 6204 treated with DBP, ■ ; and untreated strain 6204, ● .


13A
150
100
50
KU
200
400
600
MINS

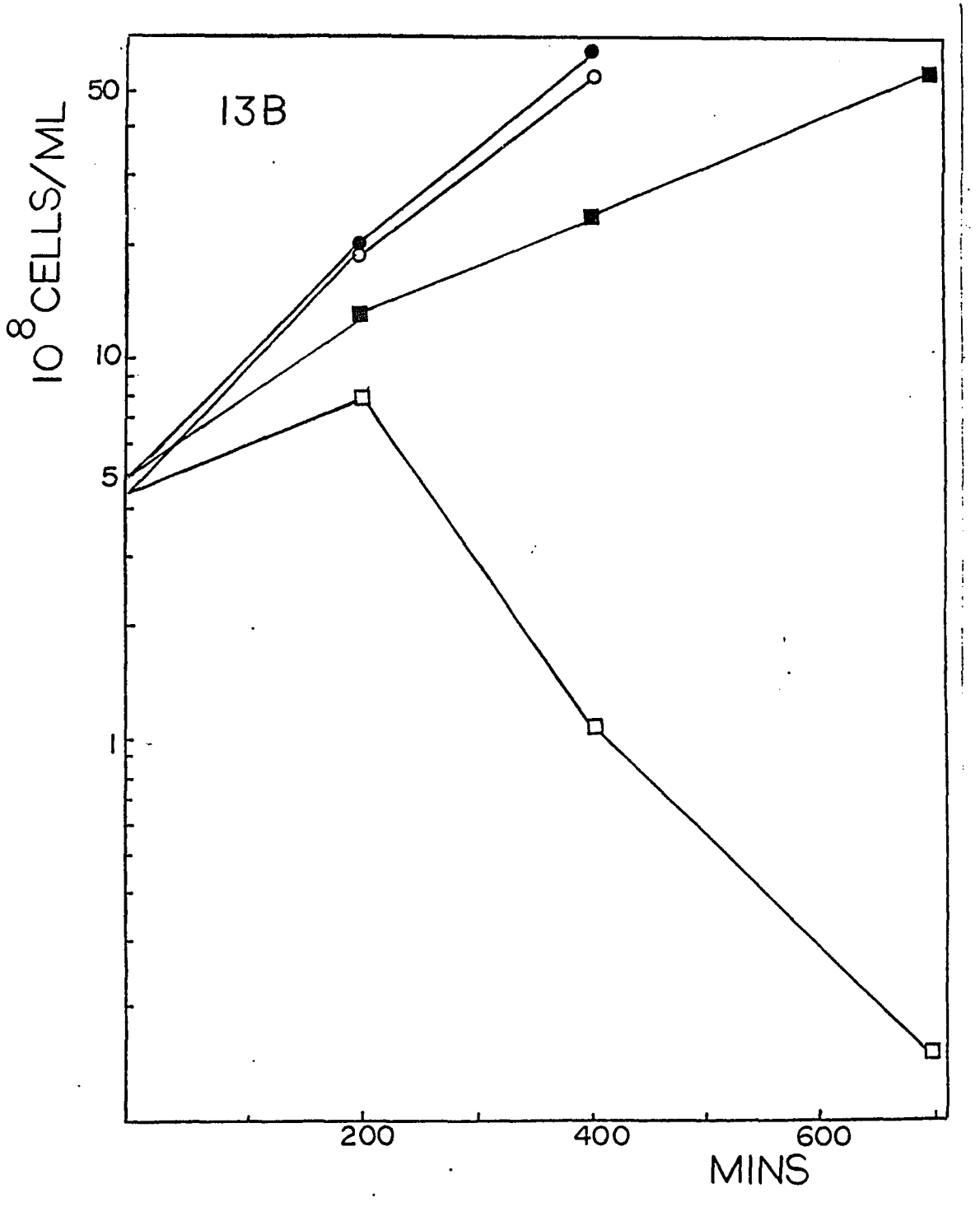

13B
10^8 CELLS/ML
50
10
5
1
200
400
600
MINS

Figure 14

The effect of 300 uM rac-DBP on the growth of E. coli strains HW35 and HW36 in GL medium containing succinate as the sole carbon source. DBP was added to growing cells at the time indicated as zero on the graph. Strain HW35 treated with DBP, ■ ; strain HW35 untreated, □ . Strain HW36 treated with DBP, ● ; strain HW36 untreated, O .

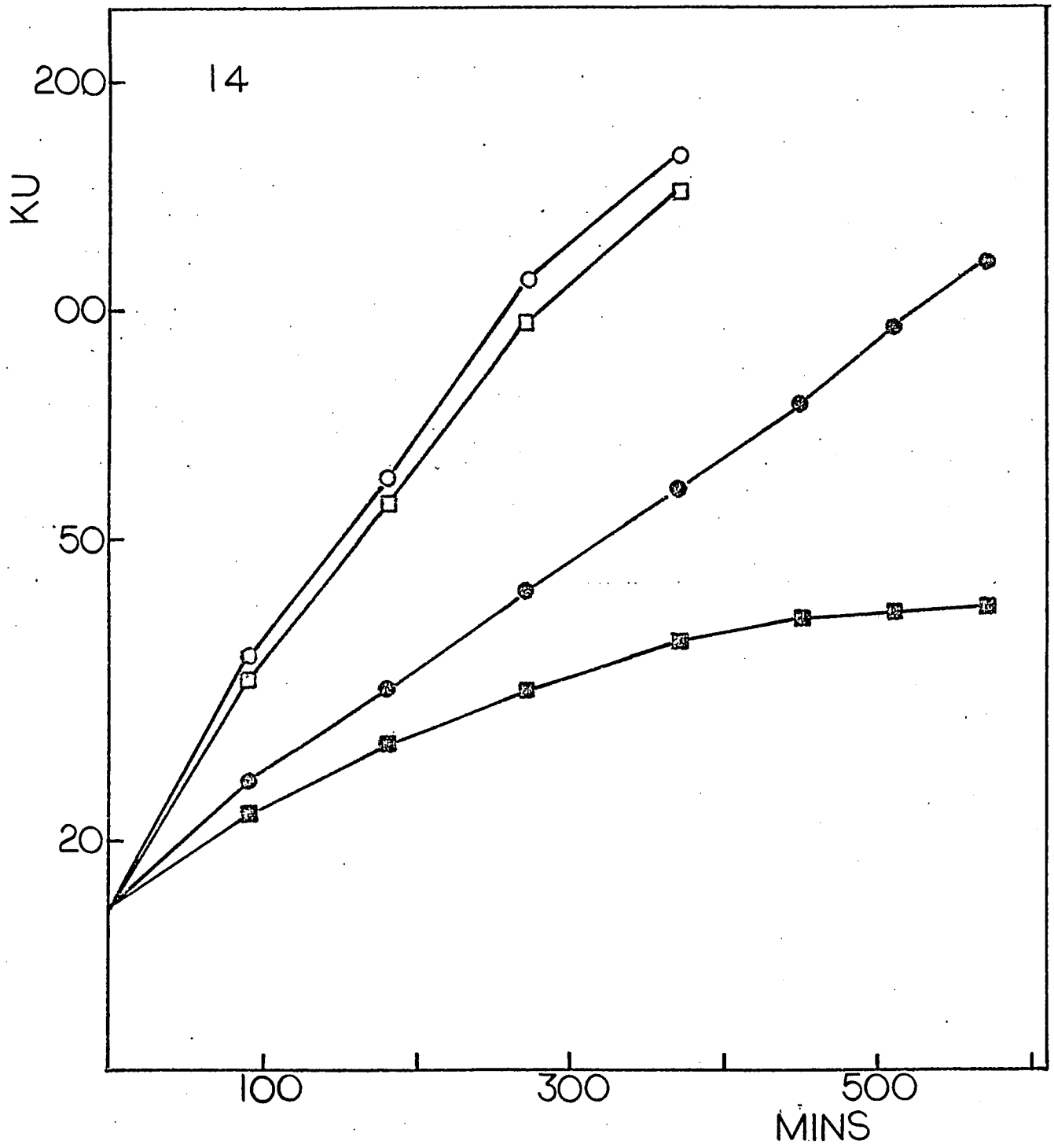

14
200
00
50
20
KU
100
300
500
MINS

Figure 15

The effect of 100 uM rac-DBP on the growth of E. coli strains HW35 and HW36 in GL medium containing glucuronate as the sole carbon source. DBP was added to growing cells at the time indicated as zero on the graph. Strain HW35 treated with DBP, ●; strain HW35 untreated, ○. Strain HW36 treated with DBP, ■; strain HW36 untreated, □.

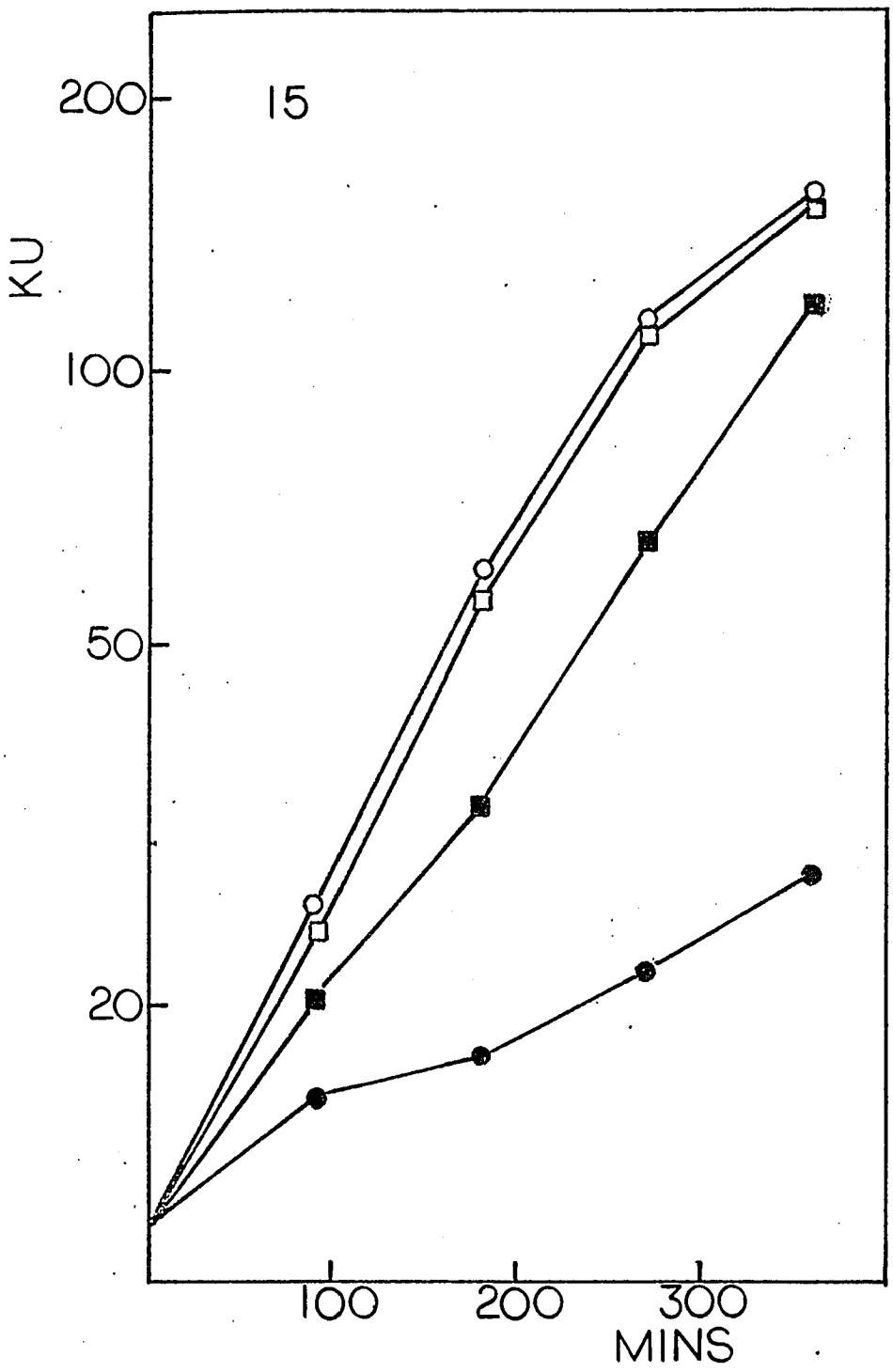
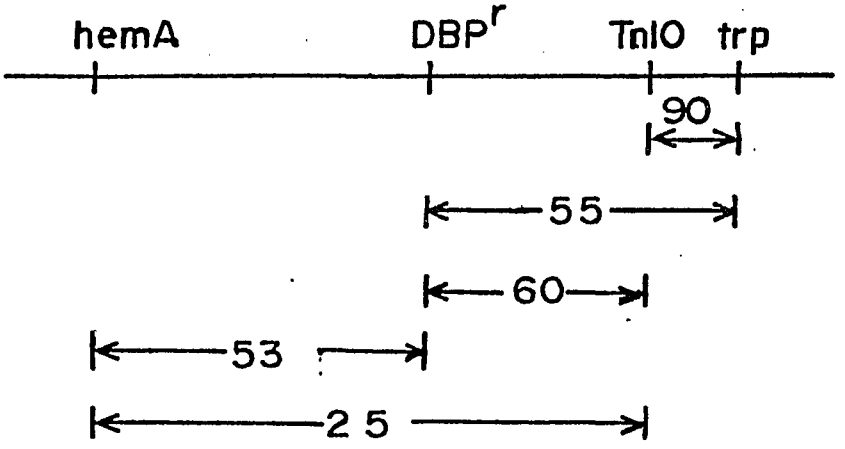

15
200
100
50
20
KU
100
200
300
MINS

Figure 16

The location and linkage of DBP-resistance (A) and cls (B) in relation to other markers in the 26-27 min map region of the *E. coli* chromosome.
The figure summarizes the data obtained from the transductional analysis presented in Tables 7 and 8. Refer to Fig 1 for the positions of *trp* and *hem*A relative to other genetic markers.

16A



16B

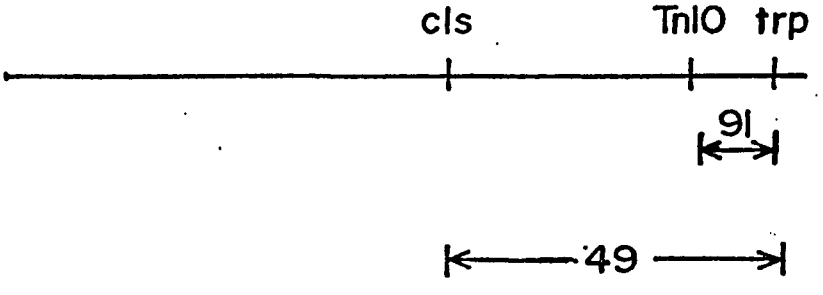


Figure 17

Phospholipid profiles of strain 7-1 (*cls*$^{+}$), 6204 (Dbp^{r}), and HW55 (*cls*). Lipids were isolated from 5 ml of late log phase cultures, labeled with 1 uCi per ml of [1-^{14}C]-acetate (sp. act. 500 uCi/umole) for 1 at 37 ^{o}C as described in the Materials and Methods section. After the lipids were spotted on silica gel loaded paper (Whatman SG81), the chromatogram was developed with a chloroform: methanol: acetic acid (65:25:8) solvent system. The radioactive lipids were detected using a Packard Chromatogram Scanner. The abbreviation SF indicates the solvent front.

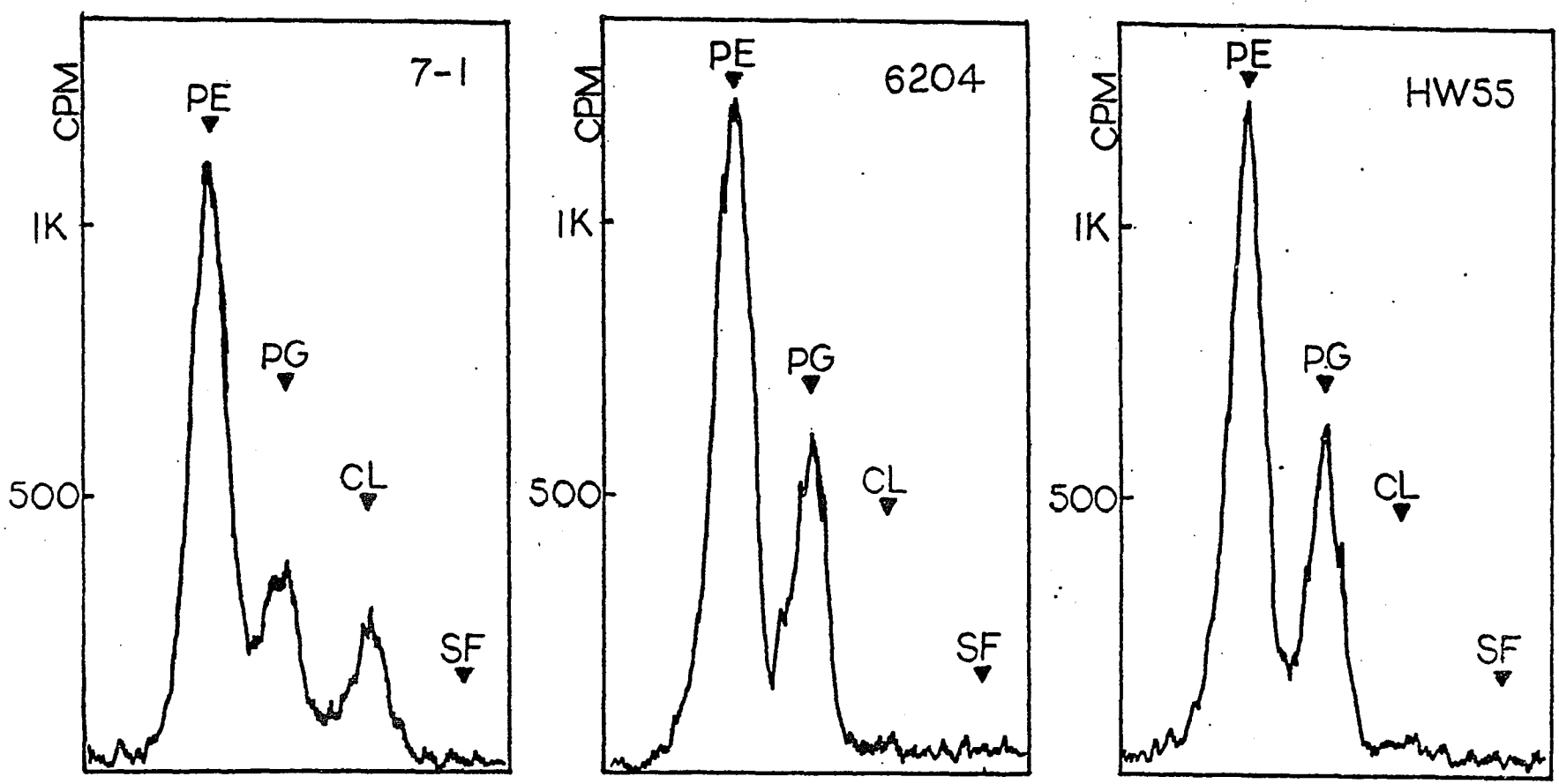

CPM
1K
500
PE
PG
CL
SF
7-1
6204
HW55

Figure 18

The effect of storage at 5°C on the viability of E. coli strain HW55 (cls) that had been cultured in CH medium containing 100 uM rac-DBP for 1 h at 37°C. The viability is expressed as the ratio of N_0 (viability on day 0) to N (viability on a given day after exposure to DBP). Culture treated with DBP, □ ; and untreated culture, ● .

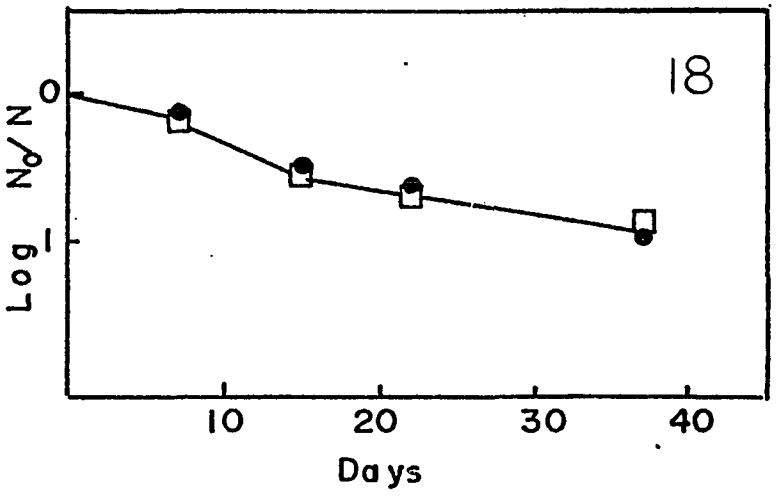
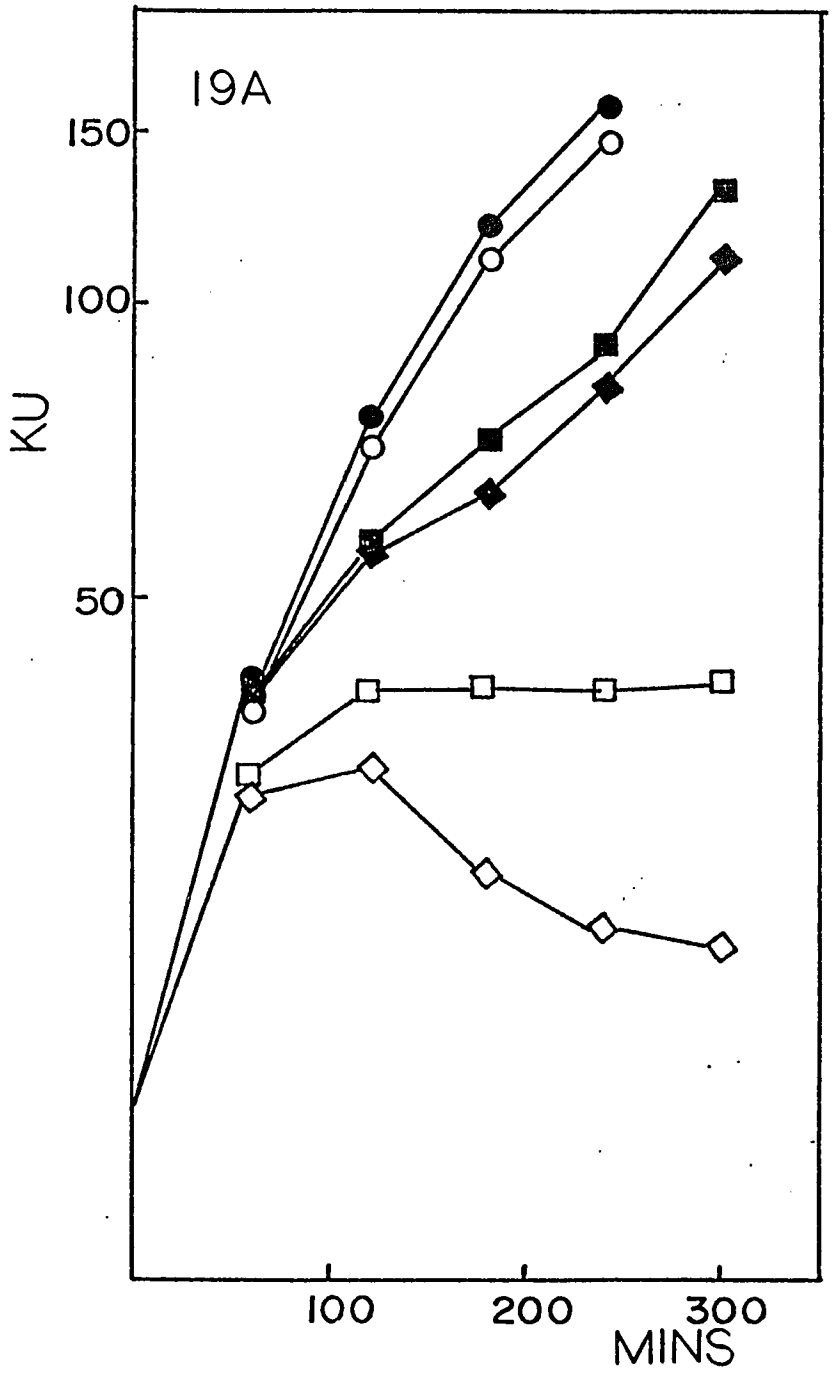

18
Log No/N
0
1
10
20
30
40
Days

Figure 19

The effect of DBP and DBP plus sodium deoxycholate on the growth (A) and viability (B) of E. coli strains HW55 (cls) and HW56 (cls^+). The bacteria were cultured in CH medium. At the time indicated as zero in the graph, 70 uM rac-DBP, 0.25% sodium deoxycholate, or both were added to growing bacterial cultures. Strain HW55 treated with DBP, ■ ; strain HW55 treated with DBP plus deoxycholate, ◆ ; and strain HW55 untreated, ● . Strain HW56 treated with DBP, □ ; strain HW56 treated with DBP plus deoxycholate, ◇ ; and strain HW56 untreated, ○ . In either strain, cultures treated with deoxycholate alone, behaved identically to untreated cultures (data not shown).


19A
150
100
50
KU
100
200
300
MINS

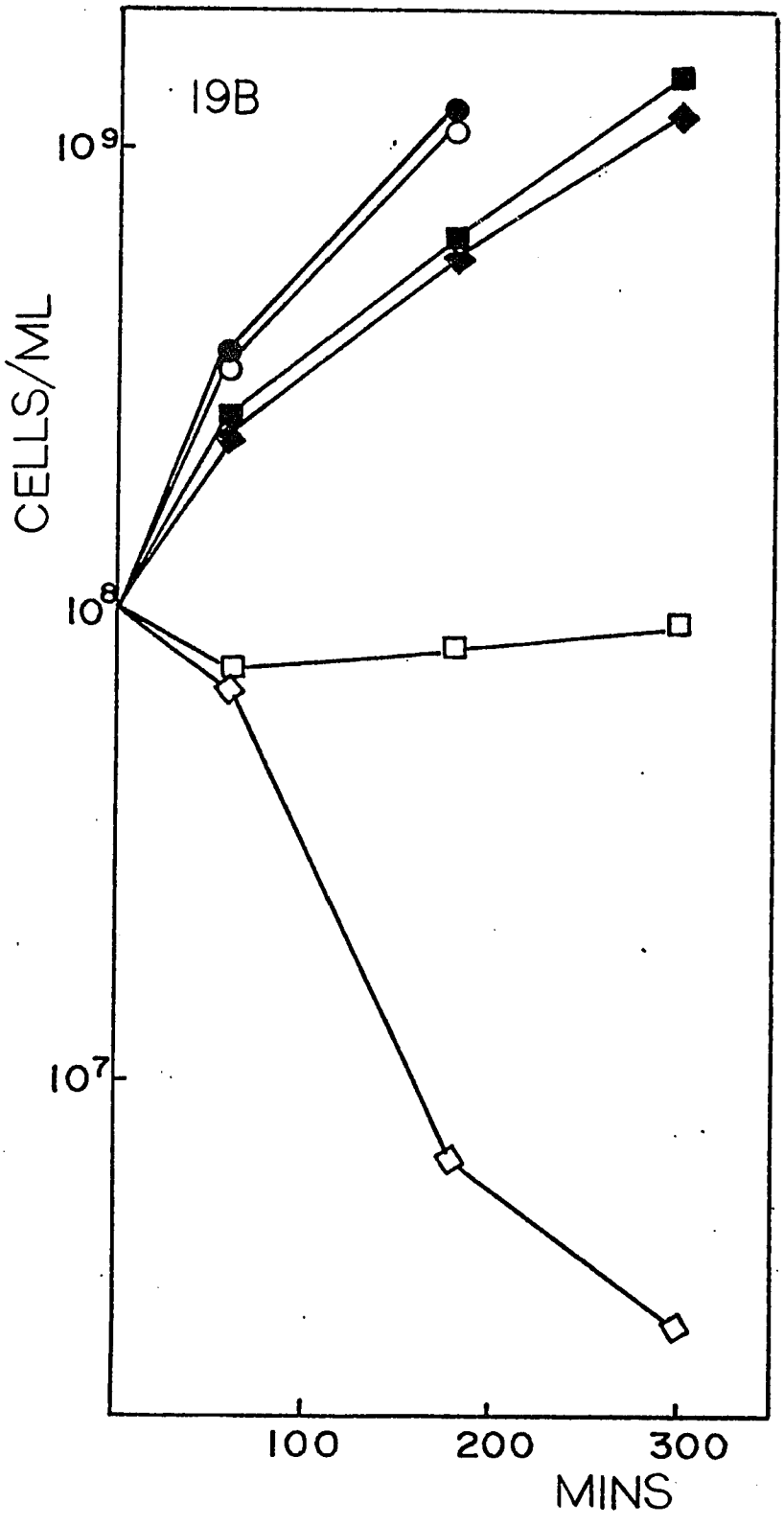
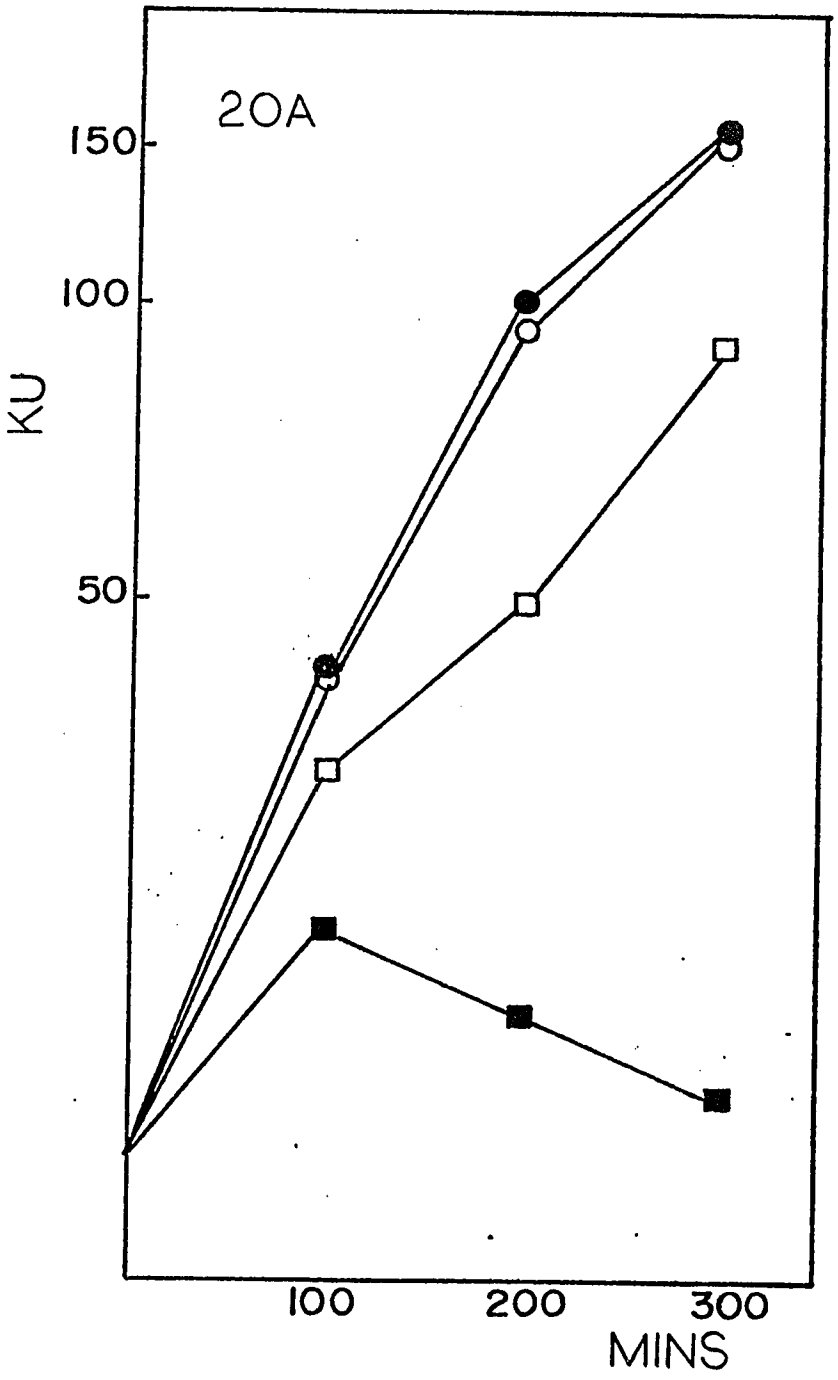

19B
10^9
10^8
10^7
CELLS/ML
100
200
300
MINS

Figure 20

The effect of 70 uM rac-DBP on the growth (A) and viability (B) of *E. coli* strains HW55 (*cls*) and HW56 (cls^+) in GL medium containing glucuronate as the sole carbon source. DBP was added to growing cells at the time indicated as zero on the graph. Strain HW55 treated with DBP, □ ; strain HW55 untreated, ○ ; strain HW56 treated with DBP, ■ ; and strain HW56 untreated, ● .


20A
150
100
50
KU
100
200
300
MINS

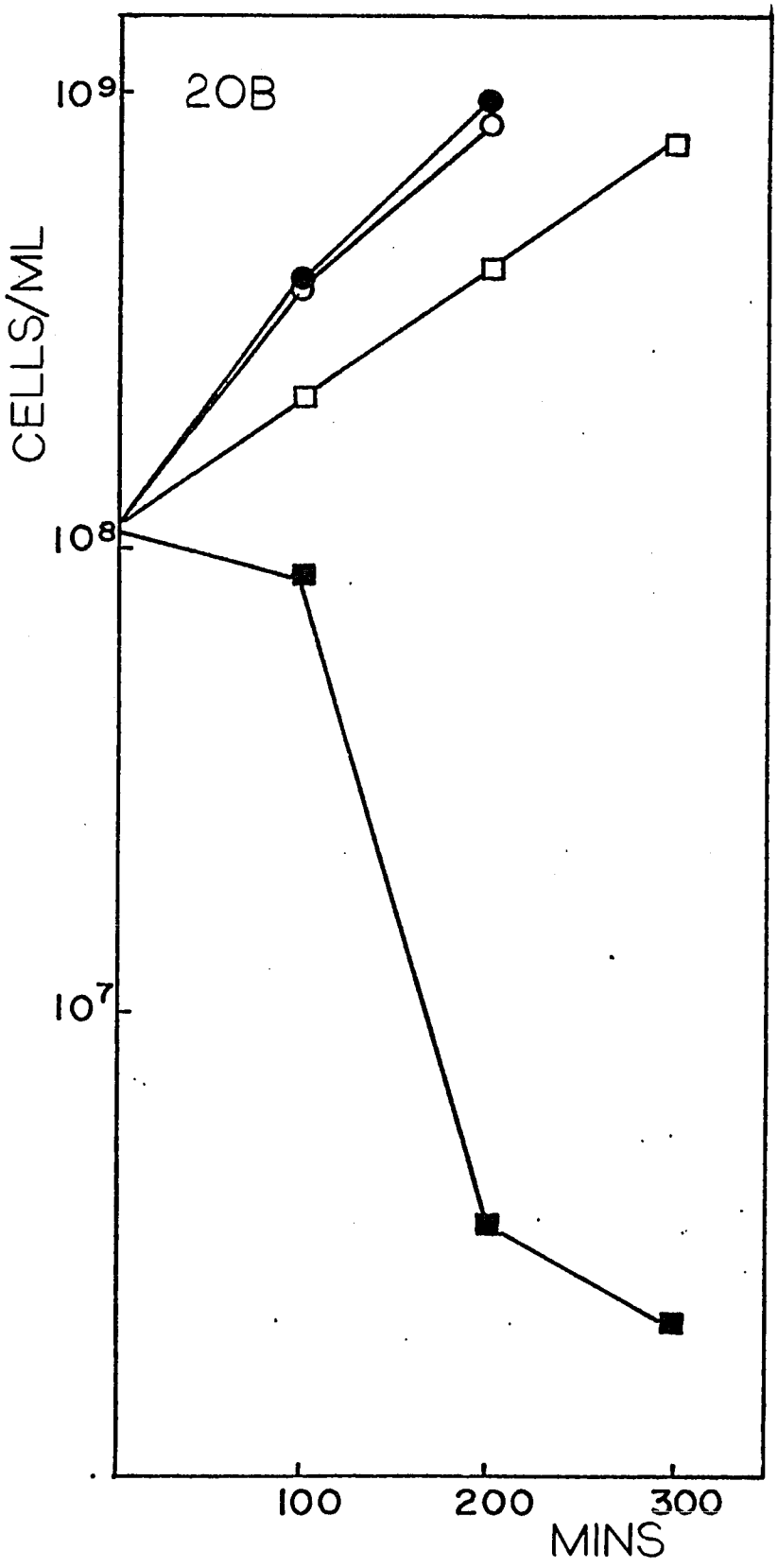

20B
10^9
10^8
10^7
CELLS/ML
100
200
300
MINS

Figure 21

Phospholipid profiles of strain 7-1 (*cls*$^{+}$), 6204 (*Dbp*^{r}), and HW55 (*cls*) upon DBP treatment. Cells (5 ml each) were treated with 30 uM rac-DBP for 20 min prior to the addition of 1 uCi per ml of [1-^{14}C]-Acetate (sp. act. 500 uCi/u mole) for 1 h at 37^{o}C. Lipids were isolated and separated on Whatman SG 81 paper as described in Fig 17. The radioactive lipids were detected using a Packard Chromatogram Scanner. The abbreviation SF indicates the solvent front.

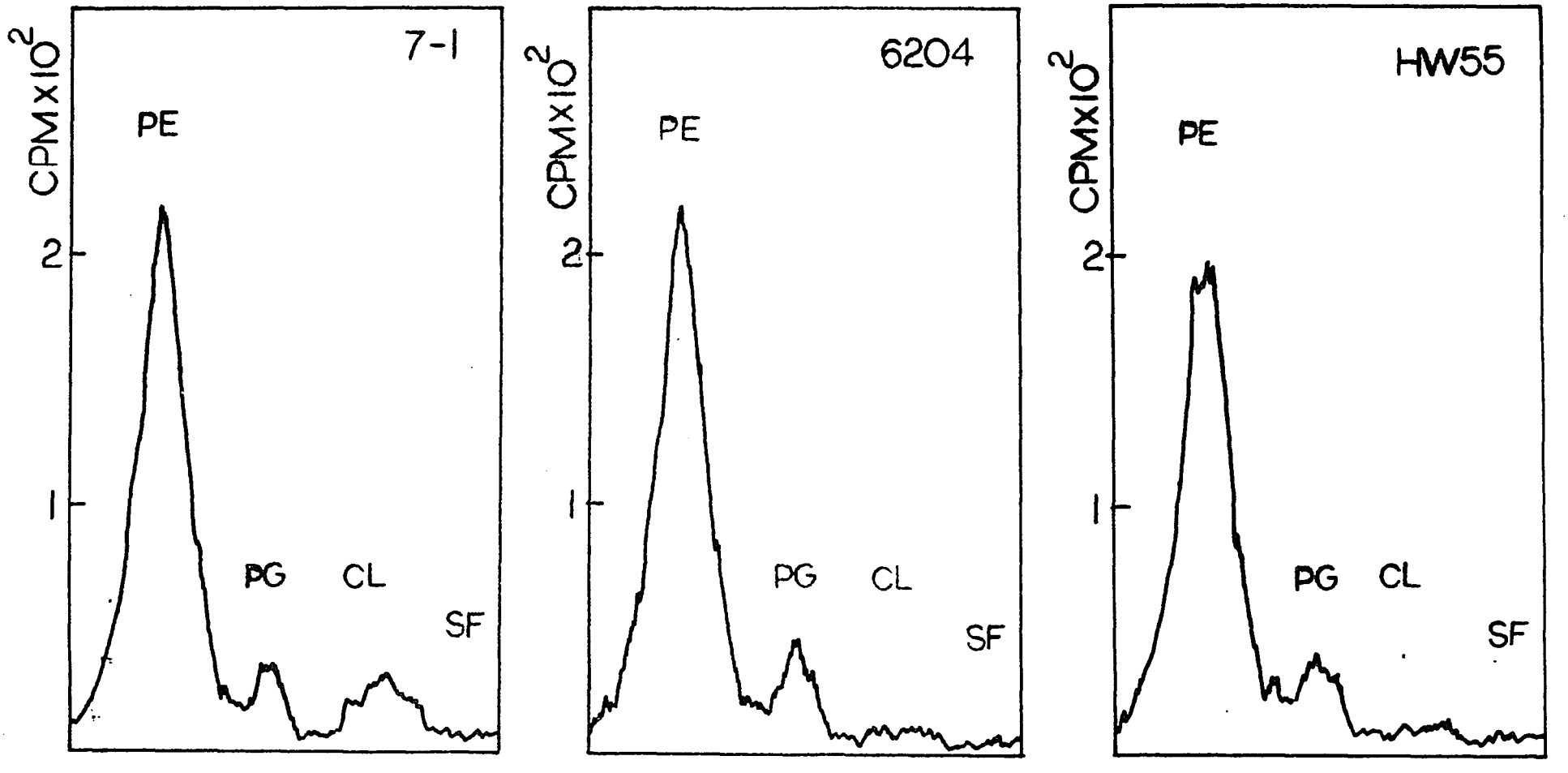
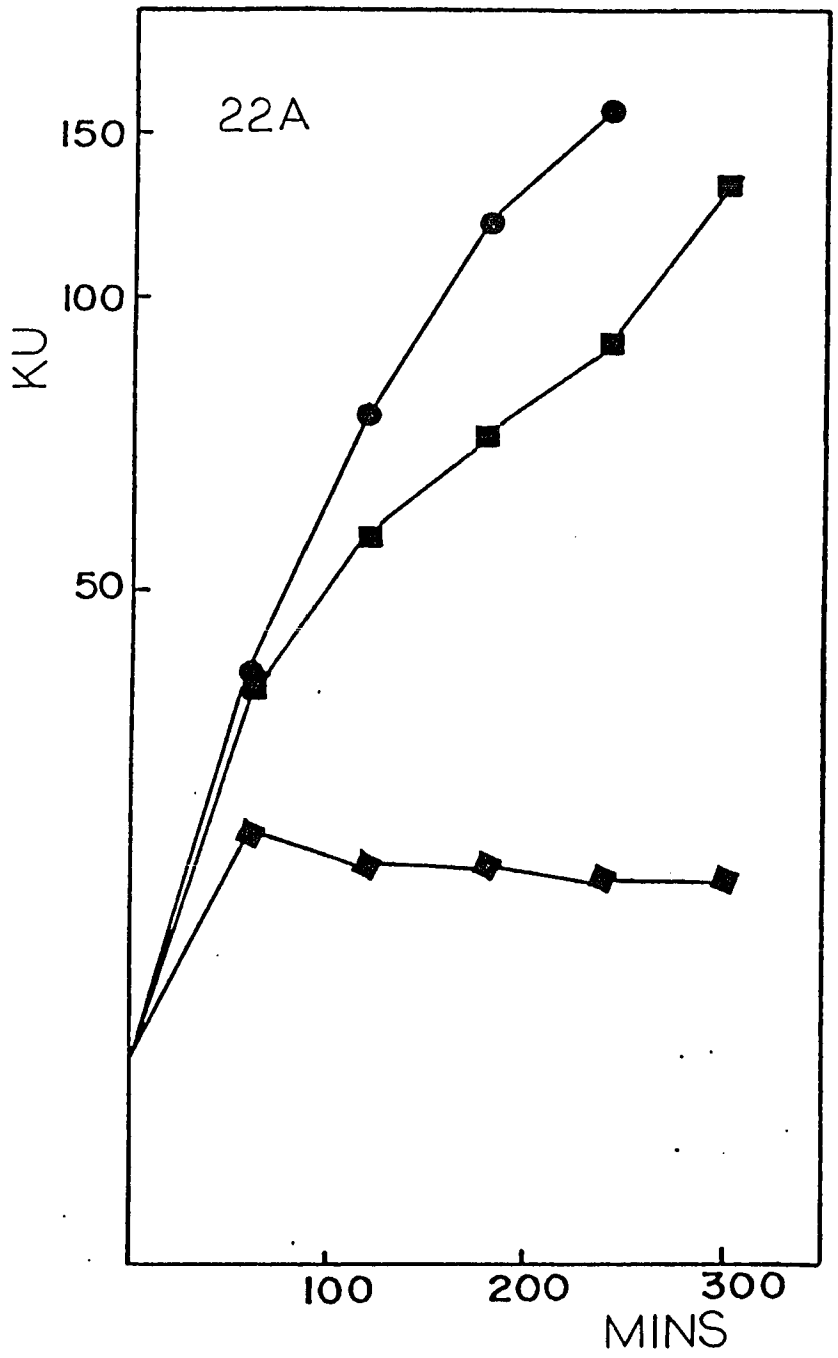

7-1
CPMX10^2
2
1
PE
PG
CL
SF
6204
CPMX10^2
2
1
PE
PG
CL
SF
HW55
CPMX10^2
2
1
PE
PG
CL
SF

Figure 22

The effect of DBP plus magnesium chloride on the growth of E. coli strains HW55 (A) and HW56 (B). The bacteria were cultured in CH medium. At the time indicated as zero on the graph, 70 uM rac-DBP, 20 mM magnesium chloride, or both were added to the growing bacterial cultures. Strain HW55 with DBP and magnesium chloride, ◆ ; strain HW55 with DBP, ■ ; strain HW55 untreated, ● ; strain HW56 with DBP and magnesium chloride, ◇ ; strain HW56 with DBP, □ ; and strain HW56 untreated, ○ .


22A
150
100
50
KU
100
200
300
MINS

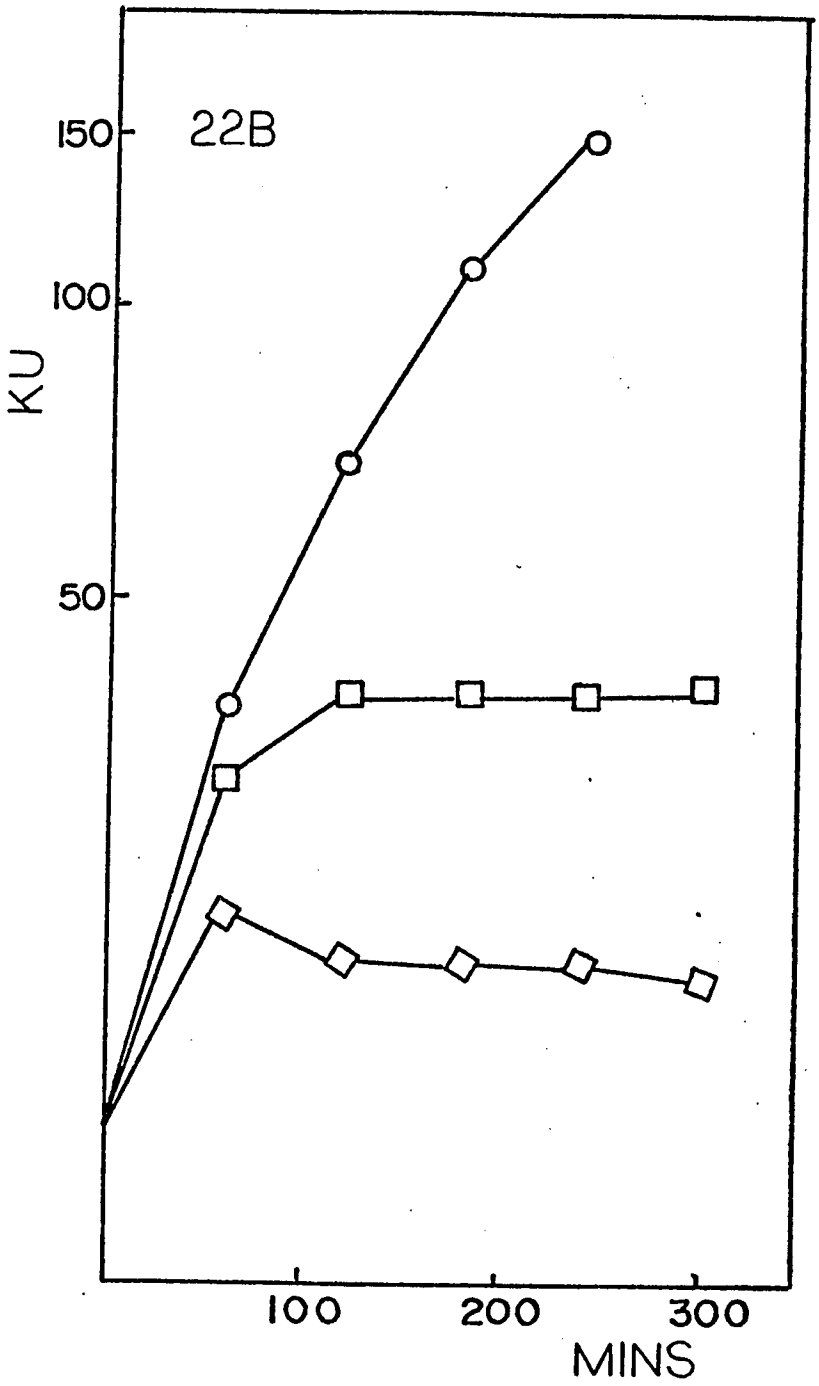
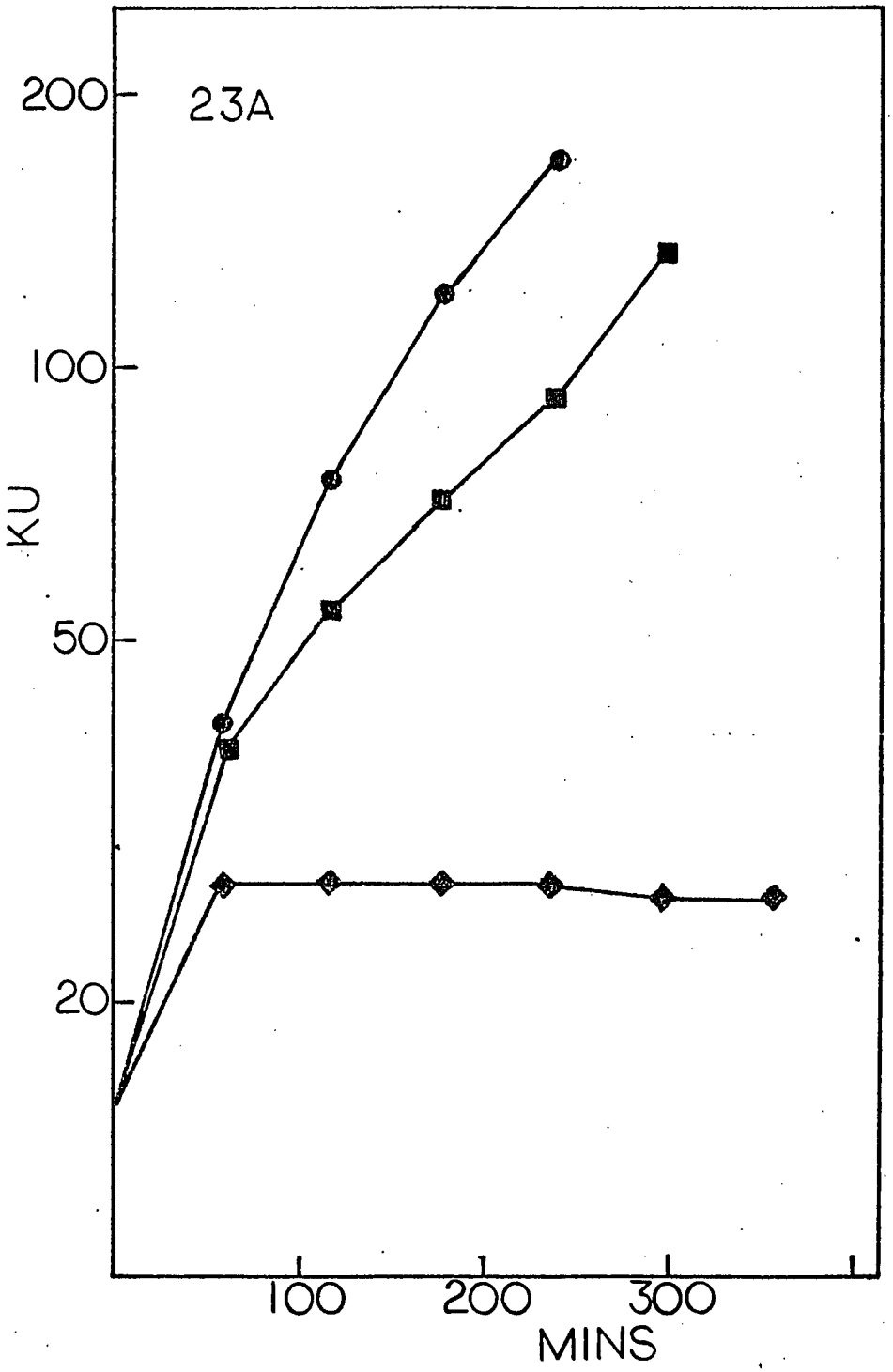

22B
150
100
50
KU
100
200
300
MINS

Figure 23

The effect of DBP plus calcium chloride on the growth of E. coli strains HW55 (A) and HW56 (B). The bacteria were cultured in CH medium. At the time indicated as zero on the graph, 70 uM rac-DBP, 20 mM calcium chloride, or both were added to the growing bacterial cultures. Strain HW55 with DBP plus calcium chloride, ◆ ; strain HW55 with DBP alone, ■ ; strain HW55 untreated, ● . Strain HW56 with DBP plus calcium chloride, ◇ ; strain HW56 with DBP alone, □ ; strain HW56 untreated, ○ .


23A
200
100
50
20
KU
100
200
300
MINS

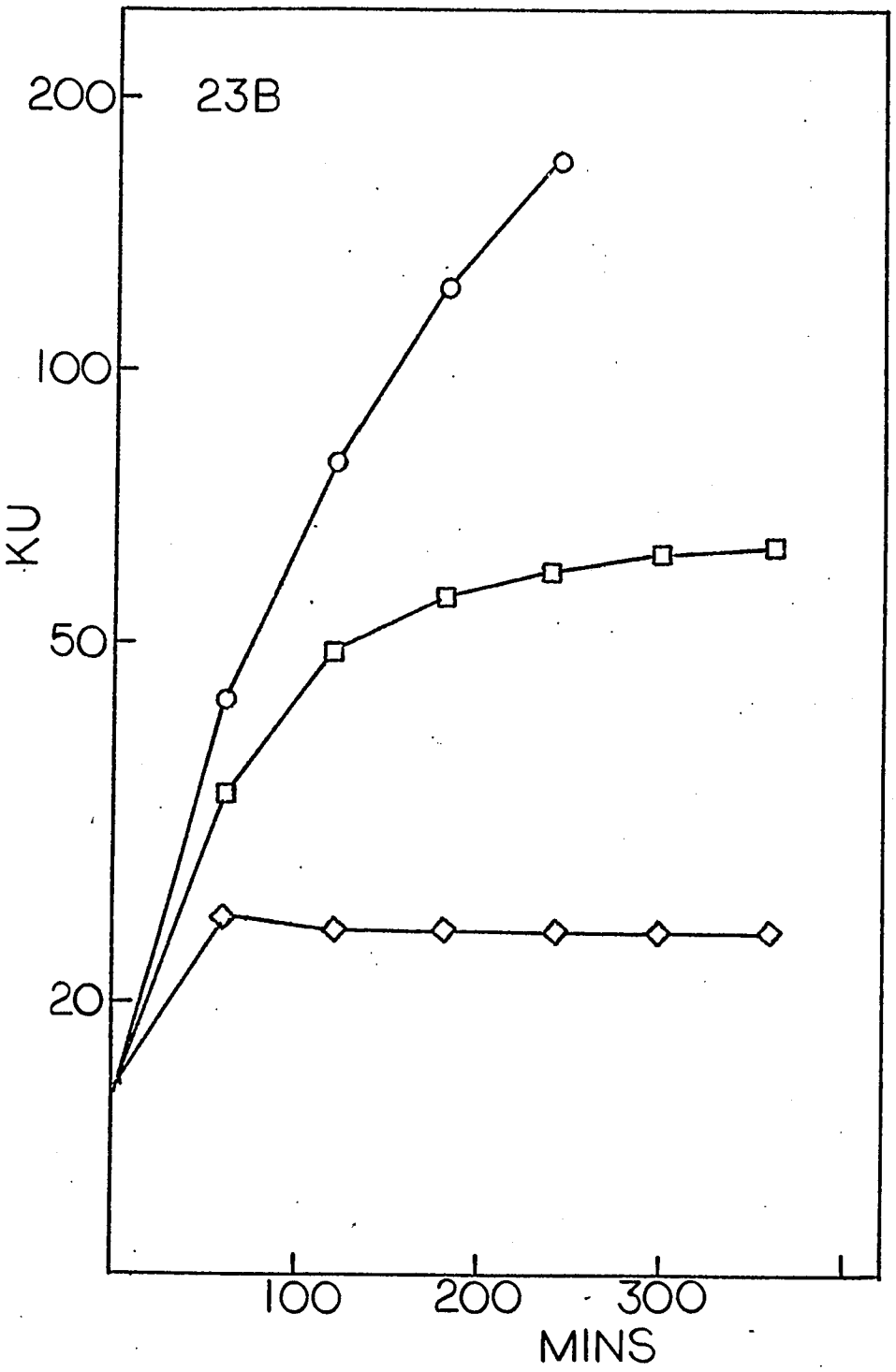

23B
200
100
50
20
KU
100
200
300
MINS

Table 1

Bacterial and viral strains

Bacterial strain	Relevant properties	Source or Reference
7	HfrC *glpR*	E. C. C. Lin (22)
7-T	strain 7 *glpT*	This study
7-1	strain 7 *uhpR*	This laboratory (88)
8	strain 7 *glpR glpD*	E. C. C. Lin (22)
6204	strain 7-1 Dbp^r	This study
CSH57	F^- *leu purE lacY trp his argG ilv metA rpsL*	(101)
CSH62	HfrH	(101)
KL226	HfrC	CGSC*
KL208	HfrB7	KL226
LE392	F^- *supF supE hsdR galK trpR metB lacY*	(106)
R477	F^- *thr1 leu6 his4 lac rpsL136*	Raetz (80)
S370	F^- *purB51 hemA30 trp45 his68 tyrA2 pyr65 lacY1 rpsL125*	CGSC*
T1GP	F^- *ilv met lacI cls*	P. Overath (72)
Hfr3000	HfrH λ^-	CGSC*
HW10	HfrC zhe::Tn10 *glpR*	This study
HW11	HfrC zhe::Tn10 *glpR glpD*	This study
HW21	HfrH zhe::Tn10 *glpR hlpD*	This study
HW22	HfrH *glpR* λ^-	This study

Table 1 (cont.)

HW31	HfrC zch::Tn10 *glpR* Dbp^r	This study
HW35	HfrC zch::Tn10 *glpR glpD*	This study
HW36	HfrC zch::Tn10 *glpR glpD* Dbp^r	This study
HW41	CSH62 zch::Tn10	This study
HW42	KL226 zch::Tn10	This study
HW43	KL208 zch::Tn10	This study
HW50	HfrC *glpR glpD trp*45 zch::Tn10	This study
HW51	HfrC *glpR glpD* Dbp^r	This study
HW53	Hfr *glpR* Dbp^r	This study
HW55	HfrC *glpR cls*	This study
HW56	HfrC *glpR*	This study
HW60	HfrC *glpR glpD hemA*30 zch::Tn10	This study
QC104	HfrC *glpR glpD*	This study
QC120	HfrC *glpR glpD cls*	This study

Viral strains

P1vir	obtained from W. Maas, NYU Medical School
Lambda NK370	*b*221 *c*I857 *c*I171::Tn10 *o*uga261 Botstein strain obtained through W. D. Nunn, University of California, Irvine, CA.

*CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Table 2

The transport assay was performed as described in Materials and Methods. The assay mixture contained 30 uM rac-DBP (sp. act. 30 uCi/umole). Strain 7-T was isolated as a spontaneous mutant resistant to 2 mM DBP.

Table 2

DBP transport in various strains

Strain	Time	
	2 min	5 min
7-1	8,165 cpm	21,289 cpm
6204	8,088 cpm	20,549 cpm
Hfr3000	965 cpm	2,410 cpm
7-T	677 cpm	904 cpm

Table 3

Cells were cultured in CH medium. At 20 Klett Units, 0.04 uCi of [1-^{14}C]-acetate (sp. act. 2.5 uCi/umole) was added to 1 ml of cells and the culture incubated for 1 h at 37 ^{o}C. rac-DBP, at a final concentration of 30 uM was added 20 min prior to the addition of labeled acetate. Total lipid was extracted and the radioactivity measured as described in the Materials and Methods section.

Table 3

Inhibition of total lipid synthesis by DBP

Strain	[^{14}C]-Acetate	[^{14}C]-Acetate + DBP
7-1	16,224 cpm	2,679 cpm
6204	17,107 cpm	3,087 cpm

Table 4

Apparent Km and Ki values of PGP and G3P synthetases. PGP synthetase and G3P synthetase were assayed as described in the Materials and Methods. DHAP, dihydroxyacetone phosphate.

Table 4

Apparent Km and Ki values of PGP and G3P synthetase

	PGP synthetase		G3P synthetase	
	7-1	6204	7-1	6204
G3P Km (mM)	0.095	0.090	---	---
DBP Km (mM)	0.500	0.500	---	---
DHAP Km (mM)	---	---	0.160	0.160
DBP Ki (mM)	0.780	0.800	0.060	0.060

Table 5

Antibiotic susceptibility of strain 7-1, 6204, and HW31. To measure antibiotic sensitivity, 0.5 ml of cells at density of 100 KU were distributed evenly over the surface of an L plate. Antibiotic disks were then placed on top of the plates which were incubated at 37 ^{o}C overnight. The diameter of the clear inhibitory zones were measured with a metric ruler and indicated in millimeter. A dash means that no inhibitory zone was observed.

Table 5

Antibiotic susceptibility

antibiotic	potency per disk	strains T7	6204	HW31
Amikacin	30 ug	20	25	25
Ampicillin	10 ug	20	20	20
Bacitracin	10 U	-	-	-
Carbenicillin	100 ug	17	18	18
Cefoxintin	30 ug	14	15	15
Cephalothin	30 ug	15	18	18
Clindamycin	2 ug	-	-	-
Erythromycin	15 ug	13	14	14
Gentamycin	10 ug	18	22	22
Kanamycin	30 ug	18	20	20
Neomycin	30 ug	12	14	14
Novobiocin	30 ug	8	11	11
Olendomycin	2 ug	-	-	-
Penicillin	10 U	-	-	-
Polymyxin B	300 U	16	16	16
Rifampin	15 ug	10	15	15
Tobramycin	10 ug	18	22	22

Table 6

Conjugational mapping was performed as described in the Materials and Methods section. Hfr and F^- strains at mid-log phase (30 KU) were mixed in a ratio 1:20 for 60 min at 37 ^oC. After proper dilution, the recombinants were selected on medium containing 100 ug per ml of streptomycin. Individual colonies with the selected phenotype were then tested for the unselected markers. A dash either means that the marker was not tested or is not applicable.

Table 6

Conjugational mapping the location of Tn10

Donor	Recipient	selected marker	% of coinheritance of unselected markers						
			thr$^+$	leu$^+$	lac$^+$	pur$^+$	gal$^+$	his$^+$	Tet^r
HW41 (HfrH)	R477	leu$^+$	-	-	32	-	-	1	5
HW42 (HfrC)	R477	leu$^+$	64	-	-	-	-	0	0
HW43 (HfrB7)	CSH57	trp$^+$	-	12	32	39	39	-	94

Table 7

A Plvir lysate of strain 6204 (Dbp^r Tet^s *trp*$^+$) was used to transduce strains HW50 (Dbp^s Tet^r *trp*) and HW60 (Dbp^s Tet^r *hem*A). Tryptophan independent colonies selected from the former transduction and δ-aminolevulinic acid independent colonies selected from the latter, were purified and scored for sensitivity to tetracycline and DBP.

Table 7

Three-factor analyses of hemA, Dbp^r, zch::Tn10, and trp by P1vir transduction

Cross	Donor	Recipient	selected marker	unselected markers	Number of co-transductants
1	6204	HW50	*trp*$^+$	Tet^r Dbp^r	3
				Tet^s Dbp^r	60
				Tet^s Dbp^s	43
				Tet^r Dbp^s	8
2	6204	HW60	*hemA*$^+$	Tet^r Dbp^r	35
				Tet^s Dbp^r	23
				Tet^s Dbp^s	4
				Tet^r Dbp^s	48

Table 8

P1 vir lysates of strains HW55 (*cls* Tet^S *trp*$^+$) and HW56 (*cls*$^+$ Tet^S *trp*$^+$) were used to transduce strain HW50. Tryptophan independent transductants were selected, purified, and scored for their sensitivity to tetracycline and DBP. It should be noted that the cls mutation was scored on the basis of DBP resistance.

Table 8

Three-factor analyses of cls, zch::Tn10, and trp by P1vir transduction

Cross	Donor	Recipient	selected marker	unselected markers	Number of co-transductants
1	HW55	HW50	trp$^+$	Tet^r Dbp^r	6
				Tet^s Dbp^r	55
				Tet^s Dbp^s	59
				Tet^r Dbp^s	5
2	HW56	HW50	trp$^+$	Tet^r Dbp^r	0
				Tet^s Dbp^r	0
				Tet^s Dbp^s	101
				Tet^r Dbp^s	9

Table 9

Five ml of early log phase cultures in CH medium of each strain was incubated with [1-^{14}C]-acetate (500 uCi/umole for 1 h at 37^{o}C. In the experiments involving DBP treatment, the cells were pretreated with 30 uM rac-DBP for 20 min prior to the addition of labeled acetate. The lipids were isolated and analyzed as indicated in the Legend to Fig 17. The concentrations of the individual phosphoglycerides were calculated from the areas in the radioscan of the chromatogram. The notation, nd indicates that the cardiolipin level was not detectable by this method. PGP analog, which appeared on the origin of the chromatogram, was not included in the calculation.

Table 9

Phosphoglyceride analysis of strains HW53,HW55,and HW56 cultured in the presence or absence of DBP

Strain	DBP	Percentage			Total lipids (cpm/ml cells)
		PE	PG	CL	
HW53 (Dbp^r)	-	75	25	nd	73,950
	+	89	11	nd	17,370
HW55 (*cls*)	-	74	26	nd	74,050
	+	89	11	nd	16,290
HW56 (*cls*$^+$)	-	73	20	7	71,170
	+	78	8	14	14,230

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