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Characterization of the rapid phosphatidylglycerol metabolism in Bacillus stearothermophilus

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Characterization of the rapid phosphatidylglycerol metabolism in *Bacillus stearothermophilus*

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ABSTRACT

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Characterization of the rapid phosphatidylglycerol metabolism in Bacillus stearothermophilus. (71 pp.)

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The rapid metabolism of phosphatidylglycerol (PG) in Bacillus stearothermophilus was investigated by following the patterns of uptake and turnover of $H_3{}^{32}PO_4$ and [2-^{3}H]glycerol. The experiments were designed to distinguish between the following possible pathways: i. de novo synthesis and subsequent degradation of PG: ii. uptake and turnover of glycerol by an exchange reaction involving the formation of a phosphatidyl-intermediate and free glycerol: iii. turnover reactions involving a recycling of the diglyceride or phosphatidyl groups of PG. Most of the ^{3}H label (about 87%) incorporated into lipid pool was found in PG after a 1.5 min pulse of exponentially growing cells. The uptake of [2-^{3}H]glycerol in the nonacylated glycerol was faster than in the diacylated glycerol of PG during the first 0.5 min, then the label increased at the same rate in both moieties. Blocking de novo lipid synthesis by the addition of cerulenin (50 µg/ml) resulted in a cessation of uptake of both ^{32}P and [2-^{3}H]glycerol after 1 min. Label incorporated in the PG pool under this condition was found exclusively in the nonacylated glycerol. Turnover was followed in exponentially growing cells after a 10 min pulse with ^{32}P and a 2 min pulse with [2-^{3}H]glycerol. Both the total lipid and PG showed biphasic patterns of turnover in which about 50% of the label from the PG pool and 30% from total lipid pool was lost during the first 4 min of the chase period. The addition of cerulenin appeared to block the reactions leading to the loss of label from the lipid pool during the chase period but did not block the transfer of some label from the PG to the cardiolipin and phosphatidylethanolamine pools. It was concluded that the major pathway of PG turnover involved degradation of the molecule and loss of the glycerophosphoglycerol ester of PG from the lipid pool. An exchange reaction between the nonacylated glycerol and free glycerol was demonstrated but under the conditions used in this study, was a minor pathway for loss of label from the PG pool.

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Chapter I.

Introduction

Phospholipid is a major constituent of all biological membranes. There are several different type of phospholipids. Each individual phospholipid molecule is amphipathic in nature, consisting of two distinct parts, the polar head group and the hydrophobic fatty acid chains. Individual phospholipid molecules can differ from one another both in the structure of their polar head groups and in the structure of their fatty acids.

The primary function of the phospholipid component is to form a bi-layer with appropriate physical properties (61,65,72,73). The importance of the fatty acid composition (the hydrophobic portion of the phospho-lipid molecule) in determining the physical properties of the membrane is well established (see ref.21 for a review). The relative concen-trations of straight-chain saturated and unsaturated fatty acids and branched-chain fatty acids vary in response to changes in the growth temperature. The variations in fatty acid composition are correlated with changes in the phase transition temperature of the membrane. It therefore appears that the major function of the non-polar (i.e. fatty acid) component of the phospholipid molecule is to regulate membrane fluidity. The anionic phospholipids also contribute to the negative charges of cell membrane (51).

Phospholipids play more than a strictly structural role in bio-logical membranes. Enzymes which require phospholipid for activity have been obtained from many sources (9,22,34,39,41,42,50,67). Generally these membrane-bound enzymes have been solubilized, or at least de-

lipidated to the point where a lipid requirement is demonstrable. One of the systems best studied is the galactosyl transferase of S. typhimurium studied by Rothfield and coworkers (see ref. 67 for a review). These investigators have shown that the galactosyltransferase enzyme involved in lipopolysaccharide synthesis forms a tertiary complex which is needed for activity. The complex is composed of the enzyme, lipopolysaccharide and phospholipid.

Another example is Enzyme II of the phosphotransferase system studied by Roseman and coworkers (42,66). The phosphotransferase system, consisting of three protein fractions, Enzyme I and II, and a low molecular weight histidine-containing protein designated HPr, functions in the following reactions:

$$\text{Enzyme I:} \quad \text{phosphoenolpyruvate} + \text{HPr} \xrightarrow{Mg^{+2}} \text{phospho-HPr} + \text{pyruvate}$$

$$\text{Enzyme II.} \quad \text{phospho-HPr} + \text{sugar} \xrightarrow{Mg^{+2}} \text{sugar-P} + \text{HPr}$$

Enzyme I is a soluble enzyme which catalyzes the phosphorylation of an imidazole nitrogen of a histidine residue of HPr. The transfer of phospho-HPr to the carbohydrate is catalyzed by Enzyme II which is a membrane component. Phosphatidylglycerol, which is a minor component of E. coli, was found to be the lipid factor which reactivates purified Enzyme II.

Fox and coworkers (25,32,81,82) have utilized unsaturated fatty acid auxotrophs and a glycerol auxotroph to probe the function of phospholipids in the synthesis of the β-galactoside and β-glucoside transport systems. These experiments demonstrated that the unsaturated fatty acid and normal phospholipid synthesis are required for induction of the β-galactoside transport system. On the other hand, experiments of Overath, Hill and Lamnek (57) suggest that β-galactoside transport activity can be induced after the interuption of overall lipid synthesis. Since the results of

Fox and coworkers (25,32) are in conflict with those of Overath, Hill and Lamnek (57), additional studies must be done to resolve these differences. It has been pointed out by Cronan (20) that the formation of functional transport systems is not generally dependent on phospholipid synthesis. Mindich (52) has shown that net lipid synthesis is not required for the induction of lactose permease in Staphylococcus aureus. In other experiments, Willecke and Mindich (80) have shown that citrate transport can be induced in the absence of phospholipid synthesis.

At present, it is unclear why several different types of phospholipids would be formed and why these are maintained at relatively constant concentrations under steady-state growth conditions. The studies of phospholipid metabolism may shed some light on the possible functions they possess, the possible ways by which they are assembled into an assymetric membrane, and how the phospholipids compositions are regulated.

The generally accepted scheme for bacterial phospholipid synthesis was elucidated primarily by Kennedy and his co-workers in studies of E. coli (63). The biosynthetic pathways of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL) as established in E. coli are shown in Fig. 1: The precursors for phospholipid synthesis include the fatty acids, sn-glycero-3-phosphate, L-serine, and cytidine-5-triphosphate (CTP). Water soluble enzymes are involved in the synthesis of fatty acids (6,77). A double bond is formed at the C_{10} position of the fatty acid by particular β-hydroxydecanol acyl carrier protein hydrase (6,20,77). This enzyme is positioned at a metabolic point of divergence in which either palmitic acid (16:0) or palmitoleic acid and cis-vaccenic acid (18:1) are formed. sn-Glycero-3-phosphate is formed

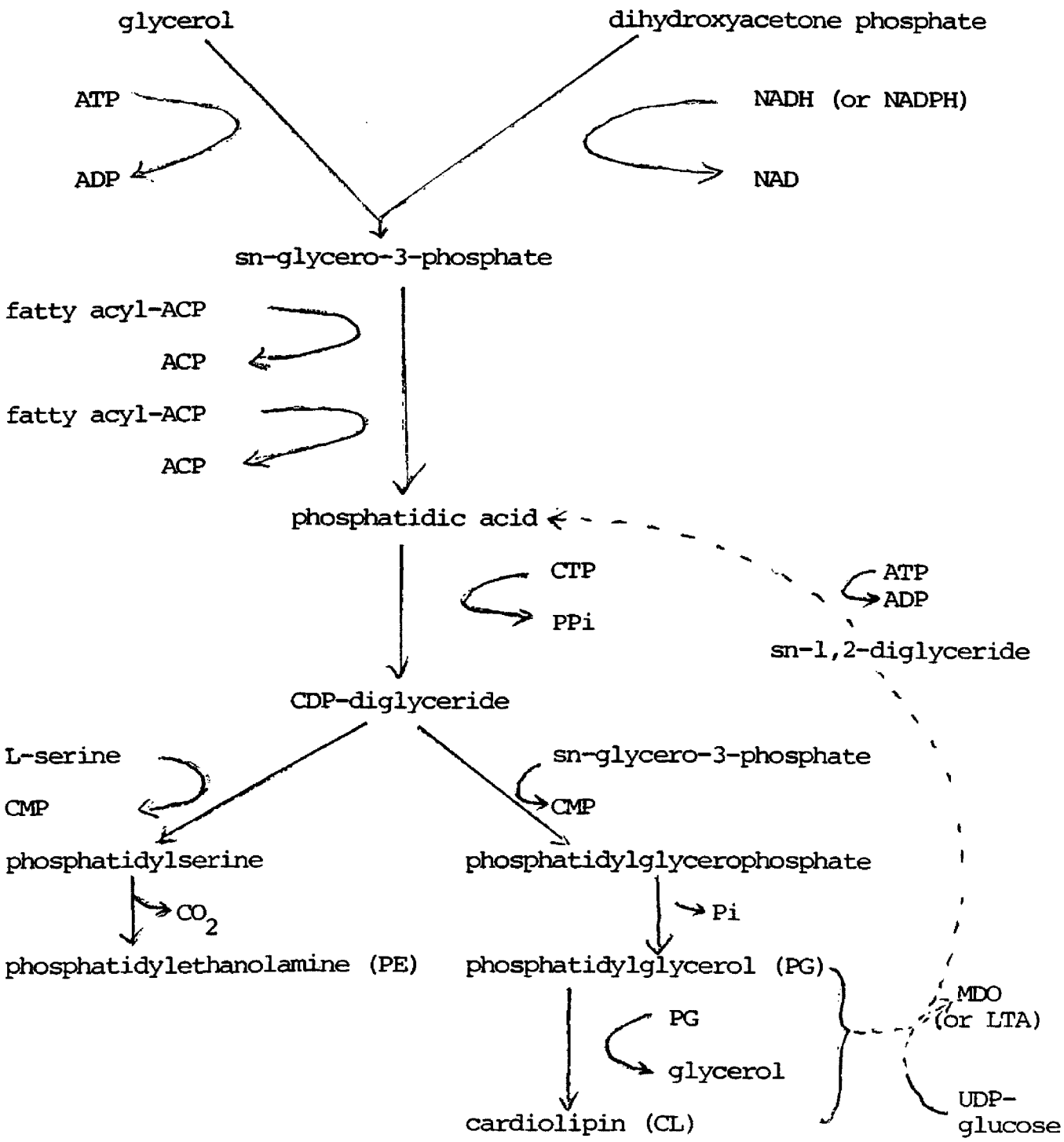


Figure 1. Enzymatic synthesis of phospholipids in Escherichia coli (63).

from dihydroxyacetone phosphate by a specific dehydrogenase which uses the reduced form of either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) (45). In the initial step, sn-glycero-3-phosphate is acylated in the first position with a saturated fatty acid by an acyl transferase which preferentially utilizes palmitoyl coenzyme A (55,56,75). Acylation of the second position occurs by a different acyl transferase which preferentially utilizes unsaturated fatty acyl coenzyme A to form phosphatidic acid (4,55,56). The product of these sequential acylations, phosphatidic acid, reacts with CTP or dCTP to form CDP-diglyceride (14). The enzyme, CDP-diglyceride synthetase, is present in all bacteria (3,14,49,59,79). At this point, CDP-diglyceride can be converted to one of two different phosphodiester products. The phosphatidyl moiety can be donated to either the hydroxyl group at position 1 of sn-glycero-3-phosphate to form phosphatidylglycerophosphate (16,38) or to L-serine to form phosphatidylserine (36). Phosphatidylserine is rapidly decarboxylated to a more stable phospholipid, phosphatidyl-ethanolamine (36,62). Phosphatidylglycerophosphate is formed by phosphatidylglycerophosphate synthetase (16,29,36,62). Phosphatidylglycerophosphate is a short lived intermediate (in fact it has never been isolated from growing cells) in the phospholipid biosynthetic pathway. It is rapidly dephosphorylated by phosphatidylglycerophosphate phosphatase to yield phosphatidylglycerol (15,33).

The subsequent metabolism of phosphatidylglycerol remains the most evasive yet most intriguing event in the phospholipid biosynthetic pathway. In contrast to eukaryotic cells, which synthesize CL from CDP-diglyceride and PG (CDP-diglyceride + PG ------> CL + CMP), prokaryotic

cells synthesize CL from two molecules of PG releasing free glycerol (2PG -------> CL + glycerol). This reaction was first suggested by Rampini et al. (64) who showed that when CDP-diglyceride formation was blocked by inhibition of energy generation, the synthesis of cardiolipin continued. Although this reaction has been demonstrated in E. coli (47, 64), Staphylococcus aureus (71), B. stearothermophilus (11,19) and several other organisms all attempts to isolate and characterize the CL-synthetase enzyme have been unsuccessful (19).

Nishijima et al. (53) constructed two mutants of which the first (pgsA444) had a partially inactivated phosphatidylglycerophosphate synthetase. It synthesized two-thirds of the normal level of phosphatidylglycerol but was not temperature sensitive. The second lesion, pgsB1, caused temperature sensitive growth and normal phosphatidylglycerol synthesis in strains harboring pgsA444. These two lesions were found to be separated yet they interacted with one another. Together these lesions caused cessation of growth, decreased phosphatidylglycerol synthesis, and accumulation of a 'lipid Y' and a 'lipid X' at non-permissive temperature. The pgsB1 mutant by itself induced an accumulation of a 'lipid X', which consisted of a diphosphorylated, glucosamine-containing disaccharide. The structure was derivatized with two amide-linked and two ester-linked 2-keto-3-deoxyoctulosonic acids (KDO) (53). It is believed that 'lipid X' is a precursor of the lipid A of lipopolysaccharide (LPS). 'Lipid Y' has been shown to be structurally similar to 'lipid X' except that 'lipid Y' has one mole extra of palmitic acid. It is suggested (53) that 'lipid Y' may be synthesized from 'lipid X' via an acylation reaction, which is stimulated by a decreased synthesis of phosphatidylglycerol. These findings suggest a previously unrecognized link between lipopolysaccharide

and phosphatidylglycerol syntheses.

In contrast to the biosynthetic pathways relatively little is known about the pathways, products, or purpose of phospholipid turnover. In most bacteria (including B. stearothermophilus) PG shows the highest turnover rate (11,35,78), whereas PE turns over slowly or is stable (1, 2,8,11,35,54,69). Several authors have also reported that different parts of the phosphatidylglycerol molecule present different rates of turnover (70,74,78). The published data indicated that the phosphate and distal glycerol groups behave similarly in this respect (70,74,78).

In 1973, van Golde et al. (76) discovered a family of related, glucose-containing oligosaccharides to which sn-glycero-1-P, succinic acid, and phosphorylethanolamine are covalently bound. This material, termed membrane-derived oligosaccharide (MDO), was localized in the periplasmic space of E. coli (69). The sn-glycero-1-P moiety is derived from the polar head groups of cardiolipin and/or phosphatidylglycerol, more likely from the latter (76). The transfer of the sn-glycero-1-P moeity of PG to MDO would generate sn-1,2-diglyceride as a byproduct which is converted back to phosphatidic acid by a membrane-bound diglyceride kinase (60).

The cell envelopes of E. coli, Salmonella typhimurium, and other gram-negative bacteria contain a major structural protein in the outer membrane, the murein lipoprotein discovered by Braun and Rehn (72). This is another possible product of PG metabolism. The NH_2-terminus of the lipoprotein is an N-acylated cysteine residue with a diglyceride moiety covalently attached to the sulfhydryl group through a thioether linkage (28). Chattopadhyay and co-workers (17) suggested that the glyceryl residue ccvalently linked to murein lipoprotein is derived from the non-acylated glycerol moiety of phosphatidylglycerol via the lipoprotein

pathway (17,18):

cysteine-lipoprotein + phosphatidylglycerol ----------→

glycerylcysteine-lipoprotein + phosphatidic acid

Lai and co-workers (43) proposed that the acylation of lipoprotein proceeds as follows:

glycerylcysteine-apolipoprotein + phospholipids ---------→

lipoprotein + lysophospholipids

The acyl moieties in phospholipids are the precursors for the fatty acids in murein lipoprotein of E. coli. Among the major glycerophosphatides in E. coli, no specificity was observed regarding the efficacy of the donor.

Gram positive organisms do not contain MDO, but do contain a water-soluble glycerophosphate polymer which has a similar structure to MDO designated as lipoteichoic acid (LTA) (44). There are two types of teichoic acids found in Gram positive bacteria: a cell-wall teichoic acid and a membrane-associated lipoteichoic acid. Membrane-associated LTA's almost always possess 1,3-phosphodiester linked chains of 25-30 glycerophosphate moieties. These are variously substituted with glycosyl or D-alanyl ester groups and terminal fatty acids. The donor of the glycerol phosphate units of wall teichoic acids appears to be CDP-glycerol (37), whereas the glycerol phosphate moieties of membrane lipoteichoic acids are proposed to be derived from phosphatidylglycerol (23,24,26,27) according to the following reaction:

PG + LTA-poly(glycerophosphate) ----------→

LTA-poly(glycerophosphate) + 1,2-diglyceride

The glycerol phosphate repeating units are transferred from phosphatidylglycerol and become linked through a phosphodiester bond to the chain farthest from or most external to the lipid end of the polymer by an

external elongation system (10).

There appear to be at least three distinct metabolic pools of PG in Bacillus stearothermophilus (12): A small pool (about 1-5% of the total membrane PG) is metabolically stable. A second pool has a turnover half-life of 10-20 minutes (0.3 to 0.5 cell doublings). The primary turnover products of this pool are CL and lipoteichoic acid (LTA). The third pool turns over very rapidly with a half-life of less than 2 minutes. The glycerol groups lost from this pool (rapid pool) do not appear to be incorporated into CL or LTA but are lost from the cell as low molecular weight products (i.e. material which is not retained by an Amicon YM-5 filter) (10).

Recently, Koga et al. (40) found that 90% of the radioactivity lost from the nonacylated glycerol moiety of PG accumulated in LTA after the chase in B. subtilis W23. The ^{32}P phosphoryl group was also transferred from phosphatidylglycerol to lipoteichoic acid almost quantitatively in B. subtilis W23. A unique metabolism of PG was found in Bacillus sp. strain A007 which lacked phosphoglycolipid and lipoteichoic acid. The turnover of phosphatidylglycerol of this organism was less extensive compared with that of B. subtilis W23, and both glycerol moieties of the lipid were metabolized at an identical rate.

The finding that PE can apparently be synthesized from PG rather than from a common intermediate via branching pathways has been reported in B. megaterium. It has also been suggested that PG is capable of providing its phosphatidyl moiety for the production of PE (in response to the relief of serine limitation by addition of L-serine) in a serine auxotroph of E. coli (84). Transacylation between diacylphospholipids and 2-acyl lysophospholipids catalyzed by E. coli extract was recognized

by Homma et al. (30). The synthesis of various phospholipids from 2-acyl lysophospholipids by E. coli extracts (31) provides a minor route of phospholipid synthesis. The physiological significance of these enzymes involved in transacylation is obscure.

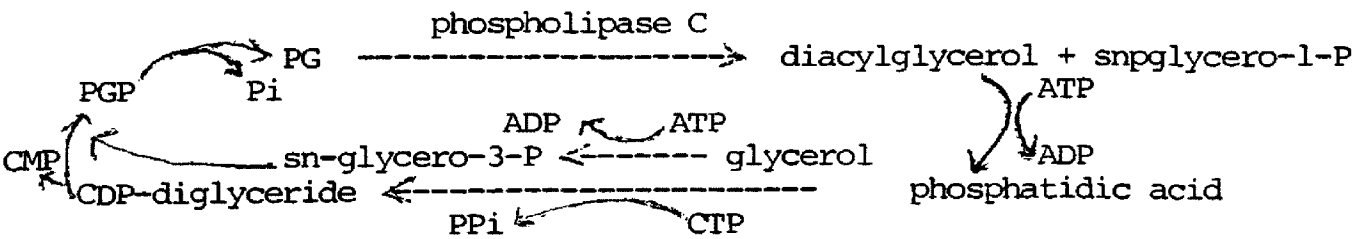
In summary, PG turnover in aerobic, Gram-positive bacteria is recognized as a complex phenomenon in which at least three kinds of reactions might be involved: (1) conversion of a whole molecule (at least the whole backbone of glycerophosphorylglycerol) of phosphatidylglycerol to another lipid (e.g., formation of aminoacylphophatidylglycerol or bis(monoacylglycerol)phosphate); (2) transfer of diacylglycerol or phosphatidyl residue to form other lipids (e.g., synthesis of phosphatidylethanolamine, cardiolipin or phosphatidyldiglycosyldiacylglycerol); (3) transfer of an unacylated sn-glycero-1-P group to non-lipid substances and reutilization of the resultant diacylglycerol for the synthesis of PG or other lipids (e.g., lipoteichoic acid synthesis).

The rapid uptake and turnover of the glycerol moiety of PG can occur either by an exchange reaction involving the formation of a phosphatidyl-intermediate and free glycerol (closed pathway), or by the rapid synthesis and degradation of a small portion of the PG pool (open pathway). The possible pathways of PG turnover are summarized below:

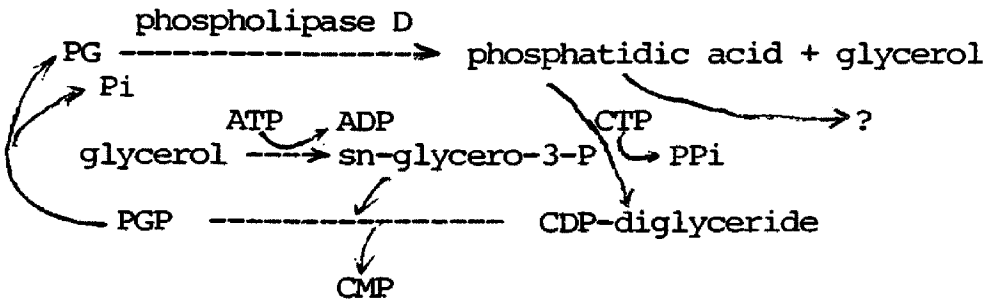
a. Exchange reaction:

PG + X <----------> phosphatidyl-X + glycerol

b. PG specific phospholipase C



C. PG specific phospholipase D



In this study we attempted to investigate the rapid turnover of PG by pulse-chase experiments. The turnover patterns of the nonacylated glycerol, diacylated glycerol, and phosphate group of PG will be different according to different pathway.

In some respects the rapid metabolism of PG appears to be similar to the phosphatidylinositol effect (PI-effect) observed with many eukaryotic cells. In response to an agonist or antigonist-receptor interaction, PI synthesis and turnover is rapidly and transiently stimulated (58). The products of PI turnover (e.g., diacylglycerol and arachidonate) appear to play central roles in the subsequent changes in cyclic nucleotide concentrations and protein kinase activities which are responsible for changes in cell metabolism or the induction of cell proliferation. Characterization of the mechanism of PG turnover may provide insight into some unrecognized functions of PG in prokaryotes (48).

Chapter II.

Materials and Methods

Materials: All biochemicals were obtained from Sigma Chemical Co., St. Louis, MO. [^{32}P]H_3PO_4 and [2-^{3}H]glycerol were purchased from ICN, Invine, Calif.. Phospholipase C (Bacillus cerus) was a product of Sigma. Cerulenin were supplied by Calbiochem.

Growth conditions: B. stearothermophilus NCA2184 was kindly supplied by Dr. George L. Card, Department of Microbiology, University of Montana, Missoula, Montana. Cells were grown either in a complex medium (TYE medium) consisting of 2% Trypticase (BBL microbiology systems), 1% yeast extract (Difco Laboratories), and 0.2% glucose, or in the low osmolarity defined minimal medium (LOTM medium) containing 5% TYE medium. The composition of the defined medium (LOTM) is shown in Table 1. This is a modification of the medium described by Rowe et al. (68). In order to observe [^{32}P]H_3PO_4 turnover within a few minutes chase period LOTM medium containing 1 mM H_3PO_4 was used in pulse-chase experiments. Cells were grown in 250- or 500-ml baffled flasks equipped with a side-arm in a gyratory incubator shaker at 60°C. In the pulse-chase experiments the flasks were incubated in a shaking water bath at 60°C. Growth was followed by measuring the optical density at 600 nm in a Coleman Junior II spectrophotometer. The pulse-chase experiments in the presence of cerulenin were performed by adding cerulenin to the cultures at the beginning of the pulse or chase. Cerulenin was initially dissolved as a 10-mg/ml solution in 95% ethanol and stored at -20°C (13). The concentration applied in this study was 50 μg/ml. In the study of the optimum condition for the

Table 1. Composition of defined medium.

stock solutions		amount of stock solution added per liter (ml)
KH_2PO	1 M	1.0
NH_4Cl	(1%)	1.0
NaCl	(1%)	1.0
L-Glutamate.HCl	(1%)	40.0
L-Glutamine	(1%)	5.0
L-Histidine	(1%)	4.2
L-Isoleucine	(1%)	10.0
L-Methionine	(1%)	5.2
L-Valine	(1%)	12.6
Biotin	(10 mg/100 ml)	0.1
Thiamine.HCl	(10 mg/100 ml)	0.1
Nicotinic acid	(6 mg/100 ml)	0.1
$CaCl_2$	(5%)	0.1
$FeCl_3$	(0.5%)	0.1
$ZnSO_4$	(5%)	0.1
$MgSC_4$	(20%)	0.1
$MnCl_2$	10 mM	0.1
Tris hydrochloride		1.0
Glucose	(20%)	20.0

rapid uptake of [2-^{3}H]glycerol, the high O.D. medium (medium of cultures grown to an O.D. of 0.4) was obtained by centrifugaion (Sorvall RC2-B, 1 x 10^4 rpm, 10 min). The low uptake cells (cells grown to an O.D. of 0.1 in TYE medium were harvested on crushed ice and collected by centrifugation (Sorball RC2-B, 1 x 10^4 rpm, 10 min).

Labeling procedure: The optimum condition for the rapid uptake of [2-^{3}H]glycerol was determined by the uptake in the following combinations of cells and medium. a. A 4 ml culture grown in TYE medium was withdrawn at O.D. 0.1, 0.2, 0.3, 0.4, and pulsed for 2 minutes with [2-^{3}H]glycerol. b. Cells withdrawn at an O.D. of 0.4 were diluted to O.D. 0.1, 0.2, 0.3, 0.4, with fresh TYE medium and with medium recovered from a culture grown to an O.D. of 0.4. c. Cells collected at O.D. 0.1, resuspended (at O.D. 0.1 ---> 0.4) in fresh medium or medium recovered from a culture grown to an O.D. of 0.4. In each case uptake was measured after a 2 minutes pulse and expressed as nmol [2-^{3}H]glycerol incorporated per mg of cells.

In pulse-chase experiments cells were grown in the LOTM medium containing 5% TYE medium to an O.D. of 0.35-0.4. The cells were pulsed with 200 μCi carrier-free $H_3{}^{32}PO_4$ and 200 μCi [2-^{3}H]glycerol for the periods indicated in the figures. The samples were taken at intervals. After the pulse, the cells were harvested on crushed ice and the radioisotope was removed by centrifugation (Sorvall RC2-B, 1 x 10^4 rpm, 10 min). The cells were washed once with same volume of ice-cold LOTM medium containing 5% TYE medium and 0.1% glycerol. The washed cells were resuspended in prewarmed (60°C) LOTM medium containing 5% TYE medium and 0.1% glycerol to an O.D. of 0.2, and distributed equally among the number of 500-ml sidearm flasks corresponding to the number of samples to be collected

during the chase period. The cultures were incubated at 60 °C in a water bath shaker during the chase period. Cerulenin was added at the time noted in each experiment.

Lipid extraction: Lipids were extracted by a modified Bligh and Dyer procedure (5). Cells were harvested on crushed ice and the non-incorporated radioisotope was removed by centrifugation. The pellet was resuspended in 0.4 ml 0.3% NaCl and transfered to a screw-capped test tube. The centrifuge tube was washed once with another 0.4 ml 0.3% NaCl and twice with 1.5 ml chloroform/methanol (1:2). The mixture gave the chloroform: methanol:water first phase Bligh and Dyer ratios of 1:2:0.8. After centrifugation in an international clinical centrifuge (model CL) at full speed for 10 minutes, the supernatants (lipid extracts) were transfered to another conical centrifuge tube. The pellet was resuspended in 0.8 ml lysozyme solution (200 µg/ml in Tris buffer with 5 mM $MgCl_2$, pH 7.8) and incubated at room temperature for 30 minutes. In the end of the incubation period 3 ml chloroform/methanol (1:2) were added and the sample was heated at 60 C for 10 minutes with vigorous shaking. The sample was then incubated at room temperature and shaken for 30 minutes. The particulate fraction and the supernatant fraction were extracted by the conventional procedure of Bligh and Dyer (5). One ml of 0.3% NaCl and one ml of chloroform were added to each tube to form the two phase system of Bligh and Dyer solvent (chloroform/methanol/0.3% NaCl ratio of 2:2:1.8). The chloroform phase of supernatant fraction and particulate fraction were removed and combined after cnetrifugation. The aqueous phase was acidified with one drop of 5 N HCl and extracted twice with 1 ml of chloroform. Chloroform extracts were pooled and dried on a Buchler Evapomix (model 2281) at 35-40°C.

Each lipid sample was then dissolved in 200 µl chloroform/methanol (1:1). Aliquots were analyzed for total radioactivity by scintilation spectrometry and for lipid composition by chromatographic techniques.

For small samples, the cells were precipitated with 10% ice-cold trichloroacetic acid (TCA) before centrifugation. Carrier cells (4 mg/ml) were added and allowed to stand for at least 10 minutes. After centrifugation the supernatant was then discarded and the pellet extracted by the procedure of Bligh and Dyer (5).

Lipid analysis: Lipids were separated by chromatography on thin layer plates (Whatman LK-6) which were developed in a one dimensional solvent system of chloroform-methanol-acetic acid (65:25:8, vol/vol). Individual lipids were located on chromatograms by autoradiography on Kodak XAR-5 film with Kodak X-Omat intensifying screens. Radioactive phospholipid spots were scraped from thin layer plates with a single edged razor blade, and recovered by successive extraction of the silicic acid with the solvents of Bligh and Dyer. Lipids were dried on a Büchler Evapomix at 35-40°C, and dissolved in 200 µl chloroform/methanol (2:1). Aliquots were analysed for radioactivity and transferred to screw-capped test tubes for phospholipase C hydrolysis.

Phospholipase C hydrolysis: The lipids recovered from thin layer plates were dried by evaporation with an air stream. The lipid was dissolved in 0.5 ml diethyl ether, and 0.4 ml of a solution containing 50 mM Tris-HCl (pH 7.5), 2.5 mM $CaCl_2$, 2.5 mM $ZnCl_2$, and 2 units of phospholipase C (Sigma). The reaction mixture was incubated for 90 minutes on a water bath shaker at 37°C. The ether was then removed by

evaporation under a stream of air; 1 ml chloroform, 1 ml of methanol, and 0.5 ml of 0.3% NaCl were added, and the contents of the tubes were mixed vigorously. The aqueous phase was reextracted twice with 1 ml of chloroform. The chloroform extracts and aqueous phase were brought to dryness on a Buchler Evapomix at 35-40°C. Water soluble fractions were resuspended in 400 µl distilled water. Chloroform soluble fractions were resuspended in 200 µl chloroform. Aliquots were analysed for radioactivity.

Analysis of radioactivity: Radioisotopes were counted in a Beckman LS-7500 programmed for dual-label ^{3}H-^{32}P counting. All samples were counted in Ready-Solv-HP (Beckman) or Aquasol II (New England Nuclear) scintillation cocktail.

Chapter III.

Results

A. Patterns of uptake and turnover in growing cells:

1. Optimum conditions for [2-^{3}H]glycerol uptake

Cells were grown in TYE medium and pulsed 2 minutes with [2-^{3}H]-glycerol (at a specific activity of 2.2 x 10^7 dpm/nmol of glycerol). The rapid uptake of [2-^{3}H]glycerol into the lipid pool at O.D. 0.1, 0.2, 0.3, 0.4 was determined and expressed as nmol [2-^{3}H]glycerol incorporated per mg of cells. As shown in column A of Table 2, the uptake did not occur at a constant rate but increased markedly at higher cell densities (O.D. 0.4). This rapid uptake may have resulted from changes in cell metabolism or changes in the medium. An approach of different combinations of cells and medium was designed to resolve the problem. If the increased uptake of [2-^{3}H]glycerol was due to the changes in cell metabolism, then cells collected at O.D. 0.4 (high uptake condition), diluted to O.D. 0.1, 0.2, 0.3, 0.4 with fresh TYE medium and with medium recovered from a culture grown to an O.D. of 0.4 (high O.D. medium) should have no difference in uptake, regardless of the medium used. As shown in column B and C of Table 2, the culture diluted with fresh TYE medium showed maximum uptake at O.D. 0.4 but the culture diluted with high O.D. medium showed maximum uptake at O.D. 0.1. So it was unlikely that the changes in cell metabolism increase the uptake of [2-^{3}H]glycerol. Cells collected at O.D. 0.1 (low uptake condition) were also resuspended in the high O.D. medium. The maximum uptake agin occured at O.D. 0.1. We can concluded from these results that the increased uptake was a result of something which accumulated or was de-

Table 2. The optimum condition for the rapid uptake of [2-^{3}H]glycerol.

O.D.	A	B	C	D	E
	(nmol of [2-^{3}H]glycerol/ mg of cells)				
0.1	1.80	0.73	8.2	5.85	0.83
0.2	2.15	0.59	5.7	4.81	0.66
0.3	2.50	0.69	4.5	5.27	0.54
0.4	5.80	5.20	4.4	3.95	0.55
0.5	4.80	-	-	-	-

Different combinations of cells and medium were pulsed for 2 minutes with [2-^{3}H]glycerol (at a specific activity of 2.2 x $10^{}$ dpm/nmol of glycerol). Lipid was precipitated by 10% of TCA and extracted by Bligh and Dyer procedure.

A. Cells were withdrawn from culture grown to O.D. 0.1, 0.2, 0.3, 0.4.

B. Cells were grown to O.D. 0.4, then diluted with fresh TYE medium

C. Cells were grown to O.D. 0.4, then diluted with high O.D. medium.

D. Cells were grown to O.D. 0.1, then diluted with high O.D. medium.

E. Cells were withdrawn from culture grown to O.D. 0.1, 0.2, 0.3, 0.4 in defined medium.

pleted from the medium. The uptake was reexamined in defined minimal medium. The data are shown in Table 2. Cells grew very slowly in this medium and the uptake of [2-^{3}H]glycerol was low after 2 minutes pulse. The highest uptake was observed at O.D. 0.1. The higher rates of uptake at low cell densities in the defined medium were probably due to more rapid growth and lipid synthesis at lower cell densities.

2. Possible pathways of PG metabolism

The possible pathways of PG metabolism are summarized in Fig. 2. The patterns of uptake and subsequent turnover of the two glycerol groups and the phosphate group of PG would be different for each possible pathway.

Pathway A is an exchange reaction in which the phosphatidyl group involves the reversible formation of a complex with a hypothetical acceptor (X). This is the only mechanism whereby glycerol could be taken directly into PG (without the conversion of glycerol to sn-glycero-3-P). Uptake or turnover would only involve the nonacylated glycerol of PG.

Pathway B represents all of the reactions in which both glycerols and the phosphate groups of PG are lost from the lipid pool. This set of undefined reactions might represent several different degradation pathways.

Pathway C and D could represent either phospholipase C (pathway C) or D (pathway D) hydrolyase activity or transferase activity. The diacylglycerol moiety of PG would be recycled.

All of the pathways, except de novo synthesis and turnover by pathway B, would result in more rapid uptake and turnover of the nonacylated glycerol than of the diacylated glycerol. The nonacylated glycerol and phosphorus would be metabolized as a unit by pathway C, whereas the diacylated glycerol and phosphorus would be metabolized as a unit by pathway

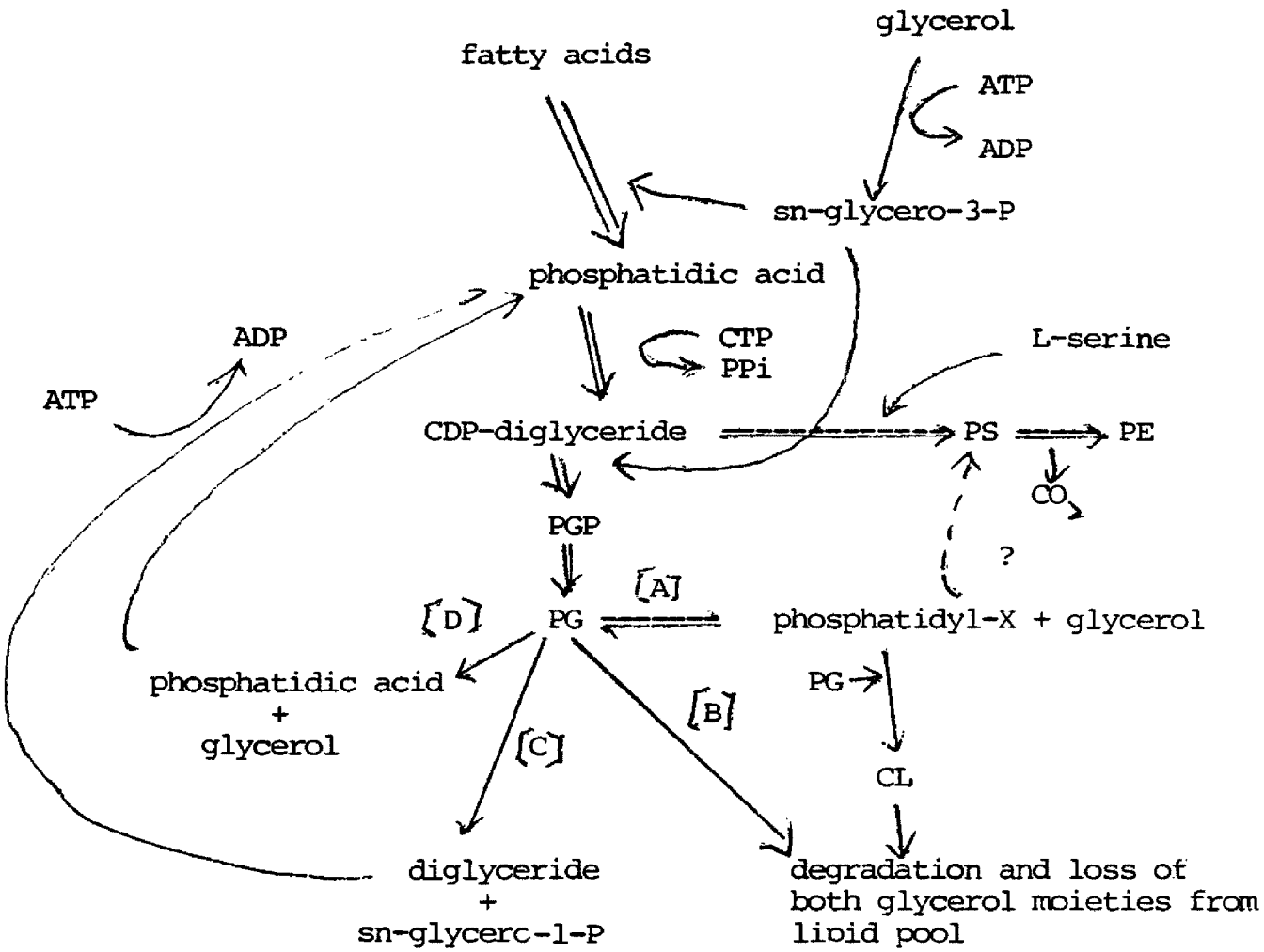


Figure 2. Possible pathways of PG metabolism.

D.

3. Uptake and distribution of [2-^{3}H]glycerol

The initial rate of uptake of [2-^{3}H]glycerol in each lipid was determined in exponentially growing cells. Cells were grown in 20 ml TYE medium to O.D. 0.4, then pulsed with 50 μCi [2-^{3}H]glycerol. The samples were taken at 0, 0.5, 1, 1.5 min. The uptake of [2-^{3}H]glycerol in the individual lipids is shown in Fig. 3. After 1.5 min pulse about 87% label incorporated into lipid pool was found in PG, 7% in phosphatidic acid and diglyceride, 4% in phosphatidylethanolamine, and 1% in cardiolipin. PG was recovered from preparative thin layer plates and the distribution of counts in the diacylated and nonacylated glycerols determined after treatment with phospholipase C as described in materials and methods. Phospholipase C cleaves the phosphodiester bond between the diglyceride and phosphate group to give sn-glycero-1-P and diacylated glycerol. After complete hydrolysis ^{32}P should be totally partitioned into water phase. Any ^{32}P found in chloroform represents nonhydrolyzed PG. The ^{3}H radioactivity measured in the chloroform phase represents both nonhydrolyzed PG and diacylated glycerol. Purified ^{32}P-labeled PG was added to determine the extent of PG hydrolysis. We usually obtained 90-100% hydrolysis of PG. The distribution of label in each glycerol was calculated using the following relationships:

$$H = \frac{W}{C + W}$$

$$L = \frac{A}{H}$$

$$n = \frac{L}{A + B}$$

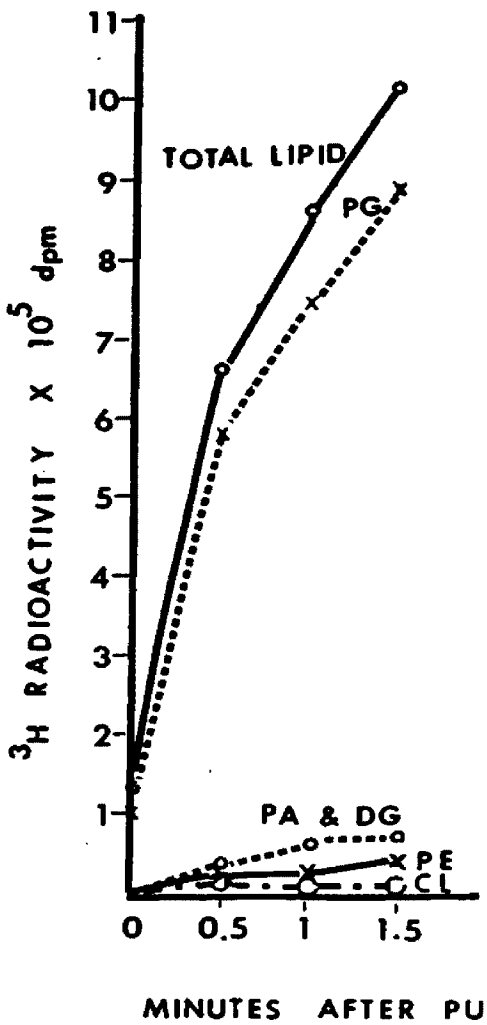


Figure 3. Pulse-labeling of phospholipid with [2-^{3}H]glycerol in B. stearothermophilus. twenty ml cells were grown to an O.D. of 0.4, then pulsed with 50 μCi [2-^{3}H]glycerol (specific acitivity = 1 μCi/10 nmol). Samples were taken at the times indicated. Lipids were extracted and separated as described in materials and methods.

$$N = T \times R \times n$$

H = fraction of hydrolysis
W = ^{32}P in water phase (sn-glycerol-1-P)
C = ^{32}P in chloroform phase (unhydrolyzed PG)
A = ^{3}H in water phase (sn-glycero-1-P)
L = total ^{3}H label in nonacylated glycerol of PG which was treated with phospholipase C
B = ^{3}H in chloroform phase (PG + diglyceride)
n = fraction of ^{3}H labeled nonacylated glycerol
T = total ^{3}H label in lipid
R = ratio of PG in total phospholipid obtained from TLC
N = ^{3}H labeled nonacylated glycerol

The amount of label in diacylated glycerol was obtained as follows:

$$D = T \times R \times (1 - n)$$

D = ^{3}H labeled diacylated glycerol

The uptake of [2-^{3}H]glycerol in nonacylated glycerol moiety of PG was faster than in the diacylated glycerol moiety in the first 0.5 min, then the label increased at the same rate in both moieties (Fig. 4).

4. Effect of cerulenin on the uptake and distribution of [2-^{3}H]glycerol and ^{32}Pi

The antibiotic cerulenin, which blocks de novo phospholipid synthesis by inhibiting β-ketoacyl-acyl carrier protein synthetase (63) was used in an effort to distinguish between uptake by a recycling of the diglyceride group (pathways C and D) or an exchange reaction (pathway A) and uptake by the primary biosynthetic pathway. The effect of cerulenin on cell growth, as measured by optical density, is shown in Fig. 5. Cerulenin was added to a final concentration of 50 μg/ml in all of the experiments described in this study.

The following labeling procedure was followed for the experiments shown in Fig. 6, 7, 8, 9, 10, 11, and 12. Cells were grown in 80 ml LOTM medium containing 5% TYE medium to O.D. 0.35 then 200 μCi $H_3{}^{32}PO_4$ were added. After eight min 200 uCi of [2-^{3}H]glycerol were added and

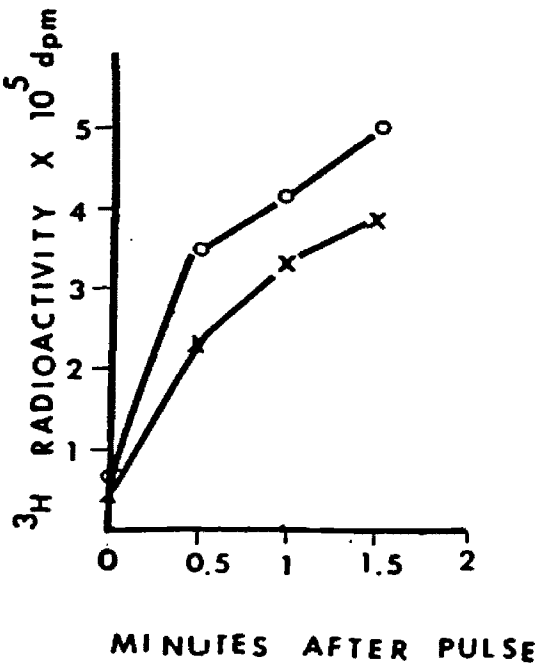


Figure 4. Distribution of ^{3}H label in nonacylated and diacylated glycerol of PG in B. stearothermophilus after pulse labeling with [2-^{3}H]glycerol. The diacylated and nonacylated glycerol moieties of PG from the experiment shown in figure 3 were separated by phospholipase C hydrolysis and their radioactivities were determined. —o— , nonacylated glycerol; —x— , diacylated glycerol.

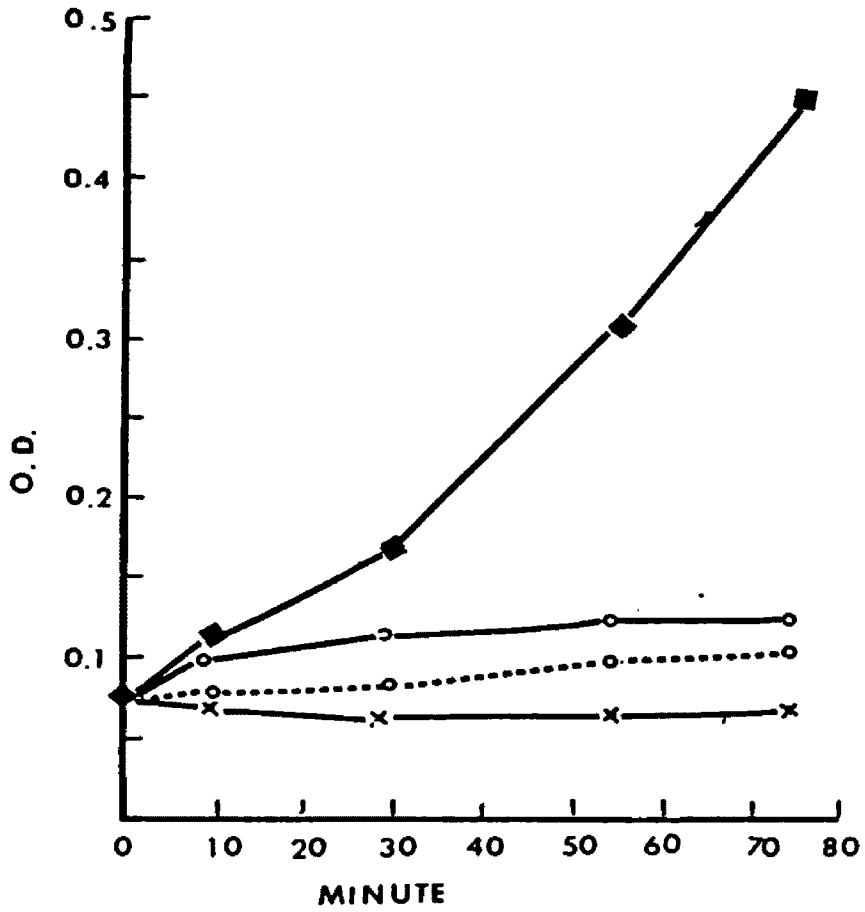


Figure 5. Effect of cerulenin on cell growth. Cells were grown in TYE medium at 60°C. Different concentrations of cerulenin were added after inoculation. The growth was monitored by optical density. The readings were taken at the times indicated. —■— , control; —o— , 10 μg/ml cerulenin; ....o.... , 50 μg/ml cerulenin; —x— ,100 μg/ml cerulenin.

after an additional 1 min cerulenin was added. In Fig. 6-12 zero min represents the time of cerulenin addition, minus 1 min the time of [2-^{3}H]-glycerol addition and minus 9 min the time of ^{32}P addition. As shown in Fig. 6 and 7 uptake of both ^{32}P and [2-^{3}H]glycerol stopped about 1 min after the addition of cerulenin. The [2-^{3}H]glycerol incorporated into the PG pool appeared to be exclusively in the nonacylated glycerol (Fig. 8). This is also evident from the ratio of [2-^{3}H]glycerol to phosphorus shown in Fig. 9. These results suggest the incorporation by acylation of sn-glycero-3-P (i.e. de novo synthesis) was blocked almost immediately after the addition of cerulenin. The loss of ^{3}H from the total lipid pool and the PG pool would be expected because the continued synthesis of cardiolipin (Fig. 6) would result in loss of glycerol from the lipid pool. The PE continued the incorporation of ^{3}H for 0.5 min. After a drop, PE was synthesized whereas PG was degraded. Ten percent of the ^{3}H was lost from total lipid and 19% ^{3}H of PG was lost after 10 min. The label which was incorporated into CL and PE accounted for 77% and 11% ^{3}H lost from PG respectively. Lipoteichoic acid (LTA) which was shown to obtain its sn-glycero-1-P from PG was not examined here. $H_2{}^{32}PO_4$ uptake was similar to [2-^{3}H]glycerol uptake (Fig. 7). The ^{3}H/^{32}P ratio of individual lipid was shown in Fig. 10. If the specific activity (dpm/mol) of the glycerol groups of each lipid were equal then the ^{3}H/^{32}P of PG divided by the PE ratio should be 2, and the ^{3}H/^{32}P ratio of CL divided by the ^{3}H/^{32}P ratio of PE should be 1.5. As shown in Fig. 10 the [2-^{3}H]glycerol was not equally distributed through the lipid pool. The specific activity of the glycerol of PG was higher than PE. The central glycerol and diacylated glycerol of CL were also separated by phospholipase C hydrolysis. The hydrolysis of CL by phospholipase C proceeds through two steps. First,

1,2-diglyceride is produced from the 3-(3-sn-phosphatidyl) group of cardiolipin, and simultaneously phosphatidylglycerol 3-phosphate is formed from the -1-(3-sn-phosphatidyl) group of cardiolipin (I) (Fig. 11) (83). Phosphatidylglycerol 3-phosphate is then hydrolyzed to the corresponding 1,2-diglyceride in the presence of a large amount of phospholipase C or for a prolonged incubation time (II). If cardiolipin was completely hydrolyzed, ^{32}P should only be partitioned into water phase. Otherwise the chloroform phase will contain cardiolipin, 1,2-diglyceride, phosphatidylglycerol 3-phosphate. The central glycerol can be obtained by the following formulas.

$$G = \frac{S}{S + T}$$

$$U = \frac{E}{G}$$

$$m = \frac{U}{E + F}$$

$$M = T \times P \times m$$

G = fraction of hydrolysis
S = ^{32}P in water phase
T = ^{32}P in chloroform phase
U = total ^{3}H in central glycerol of CL after hydrolysis
E = ^{3}H in water phase
F = ^{3}H in chloroform phase
m = fraction of ^{3}H of central glycerol
M = total ^{3}H in central glycerol of CL
T = ^{3}H in total lipid
P = ratio of CL in total lipid obtained from TLC

The diacylated glyceride was obtained by the following formula.

$$O = T \times P \times (1 - m)$$

O = total ^{3}H in 1,2-diacylated glyceride of CL

For example:

^{3}H counts in total lipid = 5963796, ratio of CL in total lipid = 0.0073

The radioactivities measured in two phases after phospholipase C hydrolysis are shown below.

	dpm	dpm
water phase:	1137.4 (^{3}H),	252,4 (^{32}P)
chloroform phase:	1979.2 (^{3}H),	179 (^{32}P)

$$G = \frac{252.4}{252.4 + 179} = 0.58$$

$$U = \frac{1137.4}{o.58} = 1943$$

$$m = \frac{1943}{1137.4+1979.2} = 0.62$$

$$M = 5963796 \times 0.0073 \times o.6237 = 27153$$

The diglyceride moieties of CL increased in the presence of cerulenin, but the central glycerol moiety of CL did not increase after 2 min (Fig. 12).

5. Turnover of [2-^{3}H]glycerol in the lipid pool

In the experiments shown in Fig. 13-24, cells were grown in 50 ml LOTM containing 5% TYE medium to O.D. 0.35, then pulsed with 200 uCi ^{32}P for 10 min and pulsed with 200 uCi ^{3}H for 2 min. After centrifugaion cells were resuspended in preheated LOTM medium containing 5% TYE medium and 0.1% glycerol. Samples were taken at 0, 1, 2, 4, 10 min. The turnover of total phospholipid and PG was biphasic. It turned over rapidly in the first four min, then remained relatively constant (Fig. 13). About 30% ^{3}H was lost from total lipid and 54% ^{3}H of PG was lost after 10 min. The label which was incorporated into CL and PE accounted for 36% and 3% ^{3}H lost from PG respectively.

The chase in the presence of cerulenin was performed by adding 50

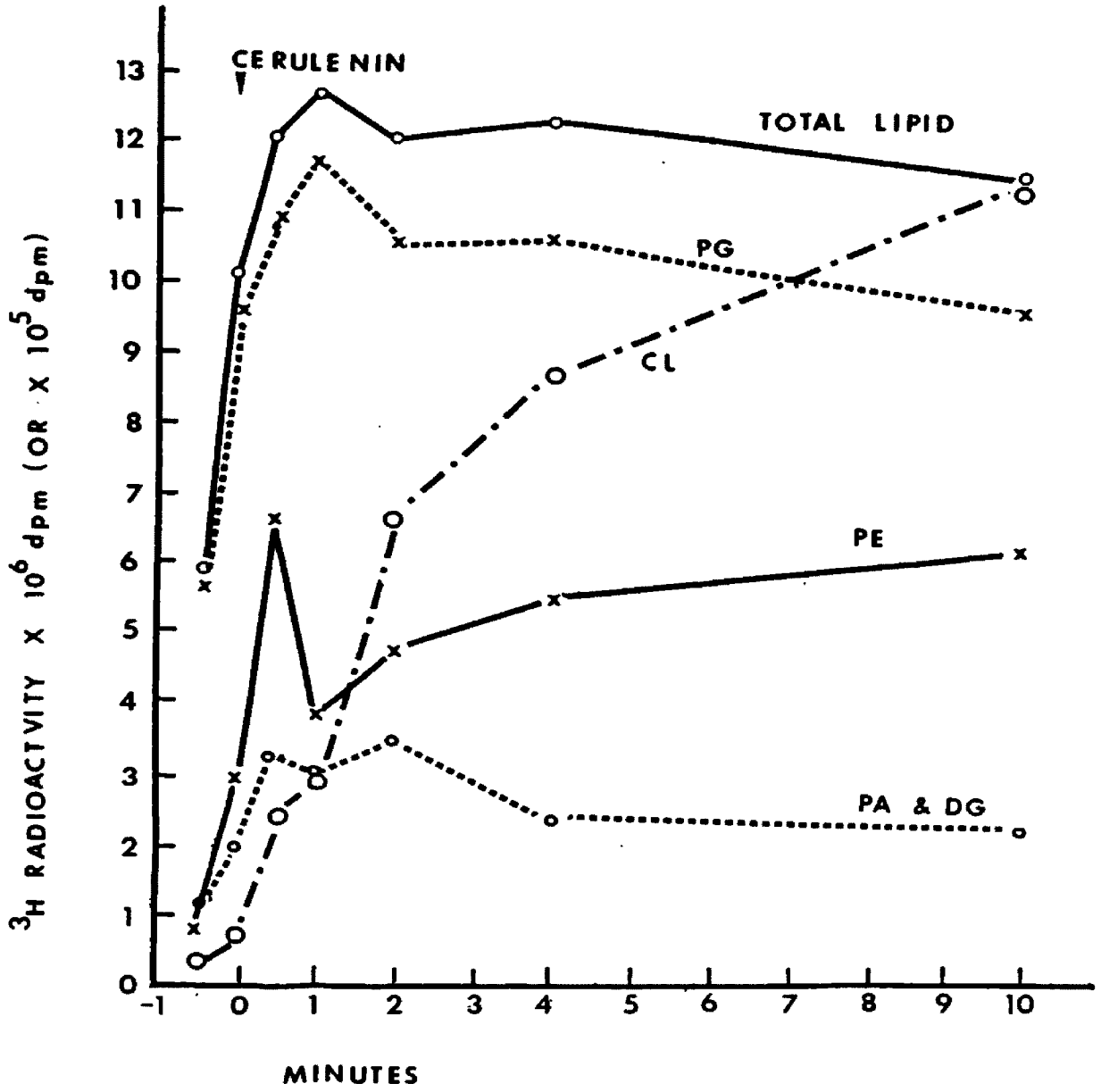


Figure 6. Pulse labeling of phospholipid with [2-^{3}H]glycerol in the precesence of cerulenin in B. stearothermophilus. eighty ml of cultures were grown at 60°C to an O.D. of 0.35, then 200 μCi $H_3{}^{32}PO_4$ was added at -9 min and 200 μCi [2-^{3}H]glycerol was added at -1 min. Cerulenin was added at a final concentration of 50 μg/ml and the addition time was indicated as 0 min. Samples were taken at the times indicated. Lipids were extracted and separated as discribed in materials and methods. Total lipid and PG were expressed as 10^6 dpm. The others were expressed as 10^5 dpm.

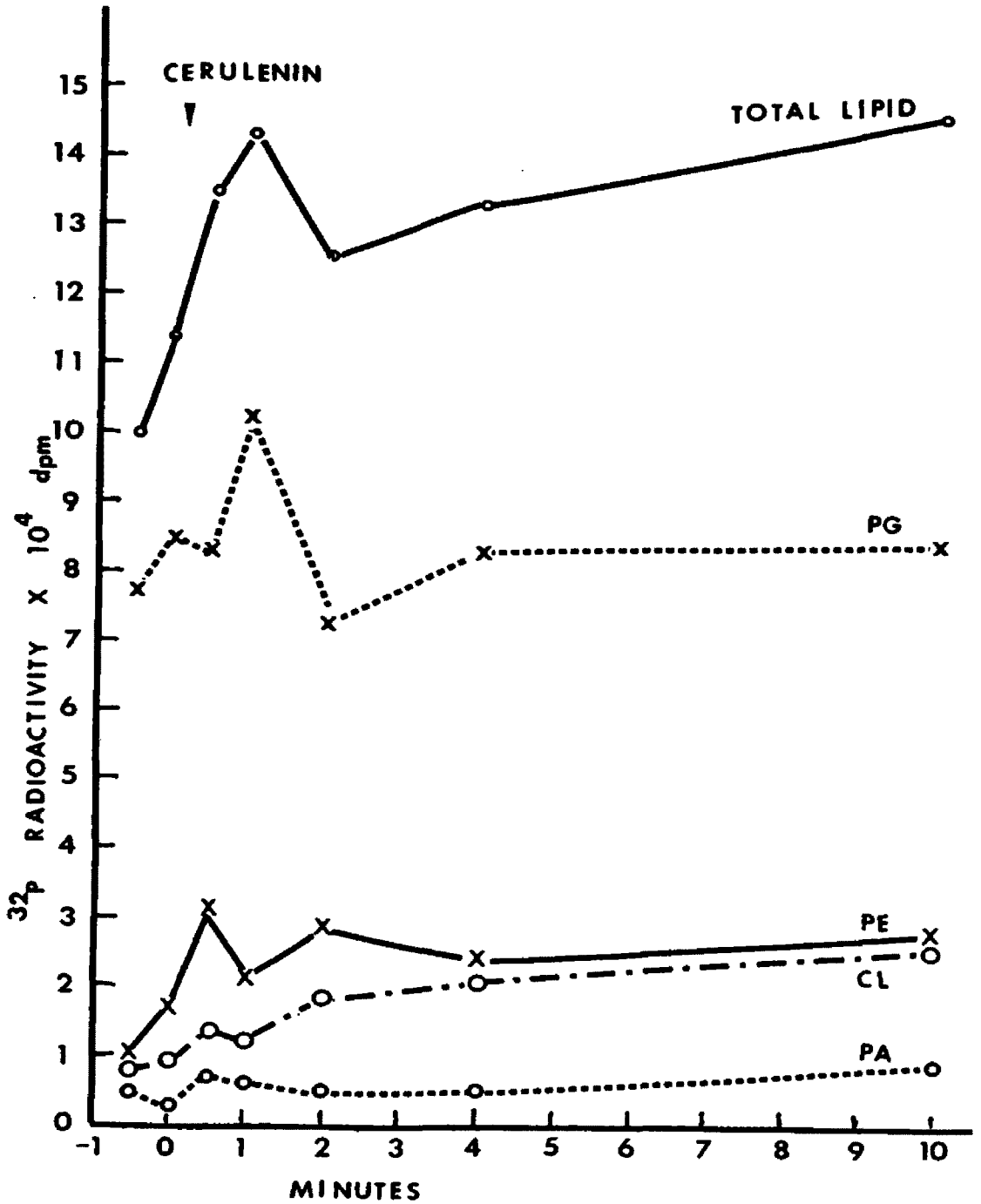


Figure 7. Pulse labeling of phospholipid with $H_3{}^{32}PO_4$ in the presence of cerulenin in B. stearothermophilus. (Samples were collected as described in figure 6.) ^{32}P radioactivities were determined.

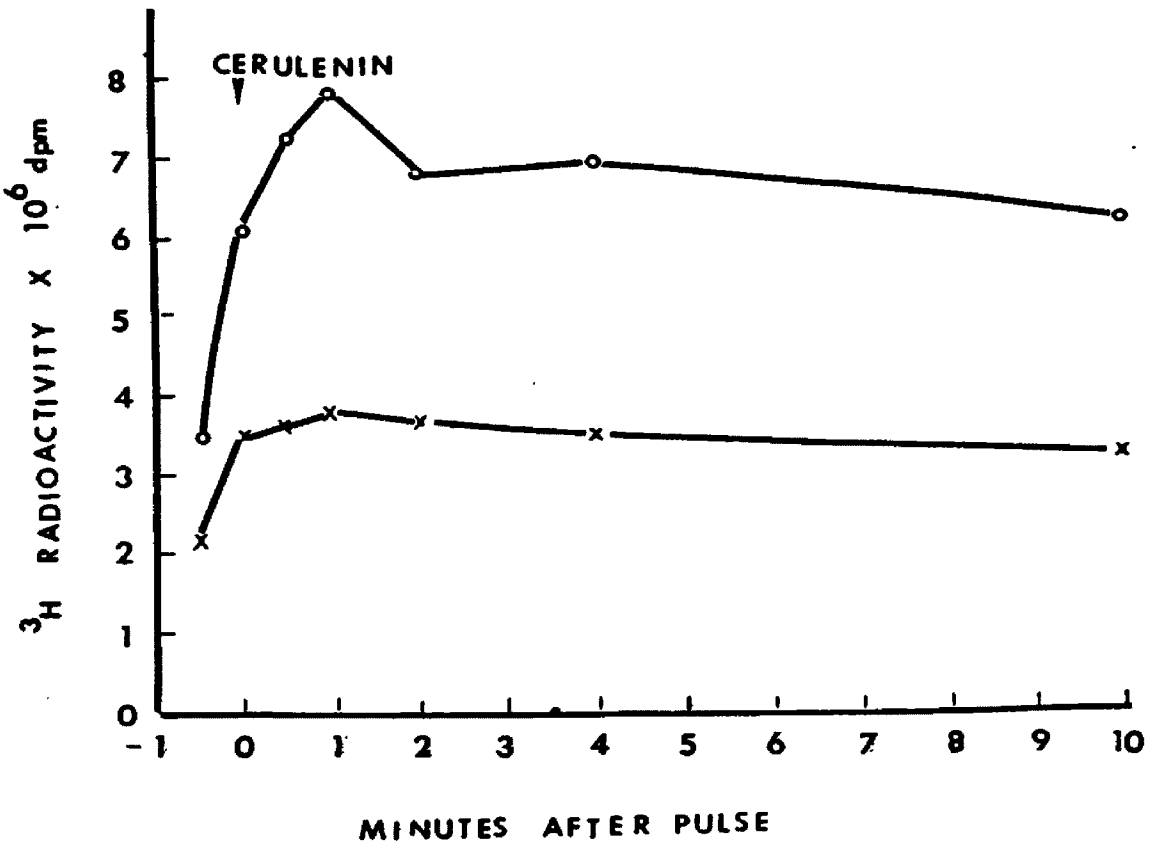


Figure 8. Distribution of ^{3}H in nonacylated and diacylated glycerol moieties of PG in B. stearothermophilus after pulse labeling in the presence of cerulenin. The diacylated and nonacylated glycerol moieties of PG from the experiment shown in figure 6 were separated by phospholipase C hydrolysis and their radioactivities were determined. —o— , nonacylated glycerol; —x— , diacylated glycerol.

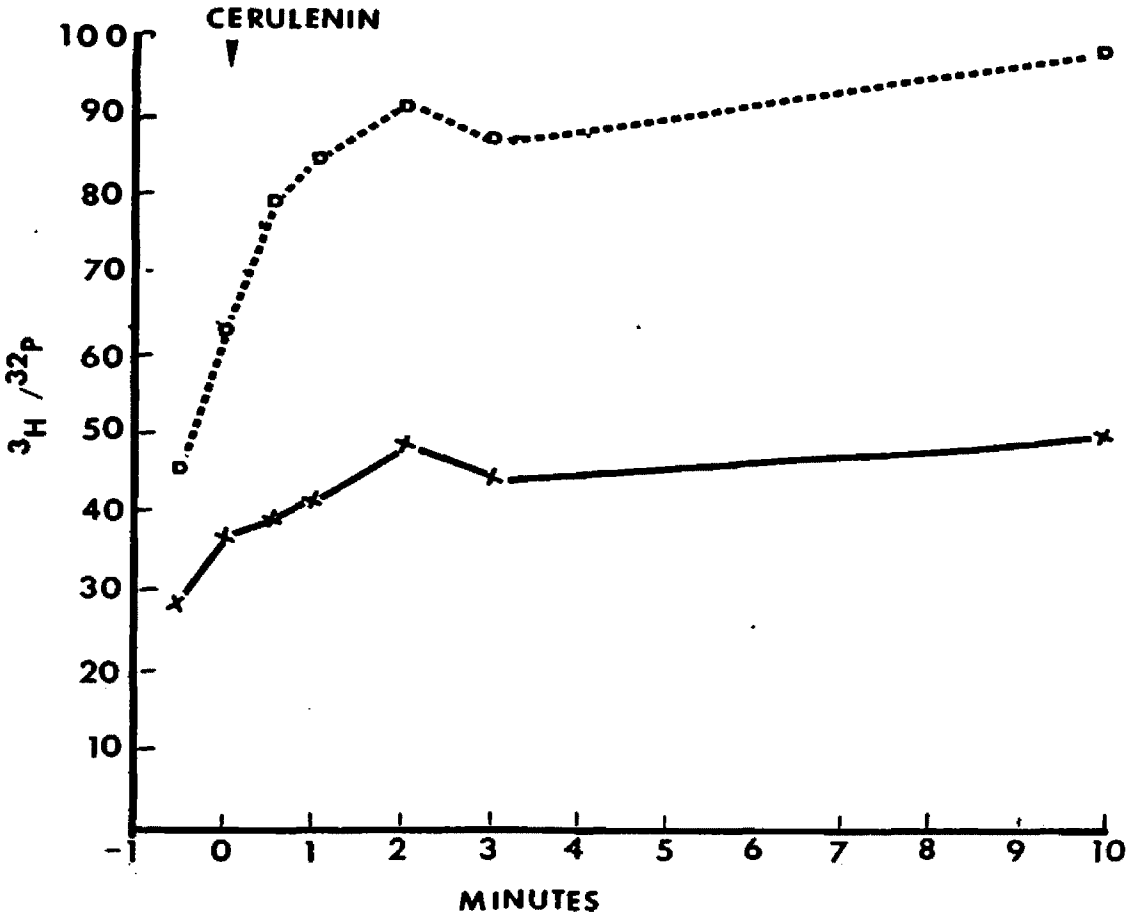


Figure 9. Relative uptake of [2-^{3}H]glycerol and ^{32}P in nonacylated and diacylated glycerol moieties of PG in the presence of cerulenin. The diacylated and nonacylated glycerol moieties of PG were separated by phospholipase C hydrolysis. The relative uptake of ^{3}H in both moieties to ^{32}P in the phosphate group are shown as the ratio of ^{3}H to ^{32}P. ...o..., nonacylated glycerol; —x—, diacylated glycerol.

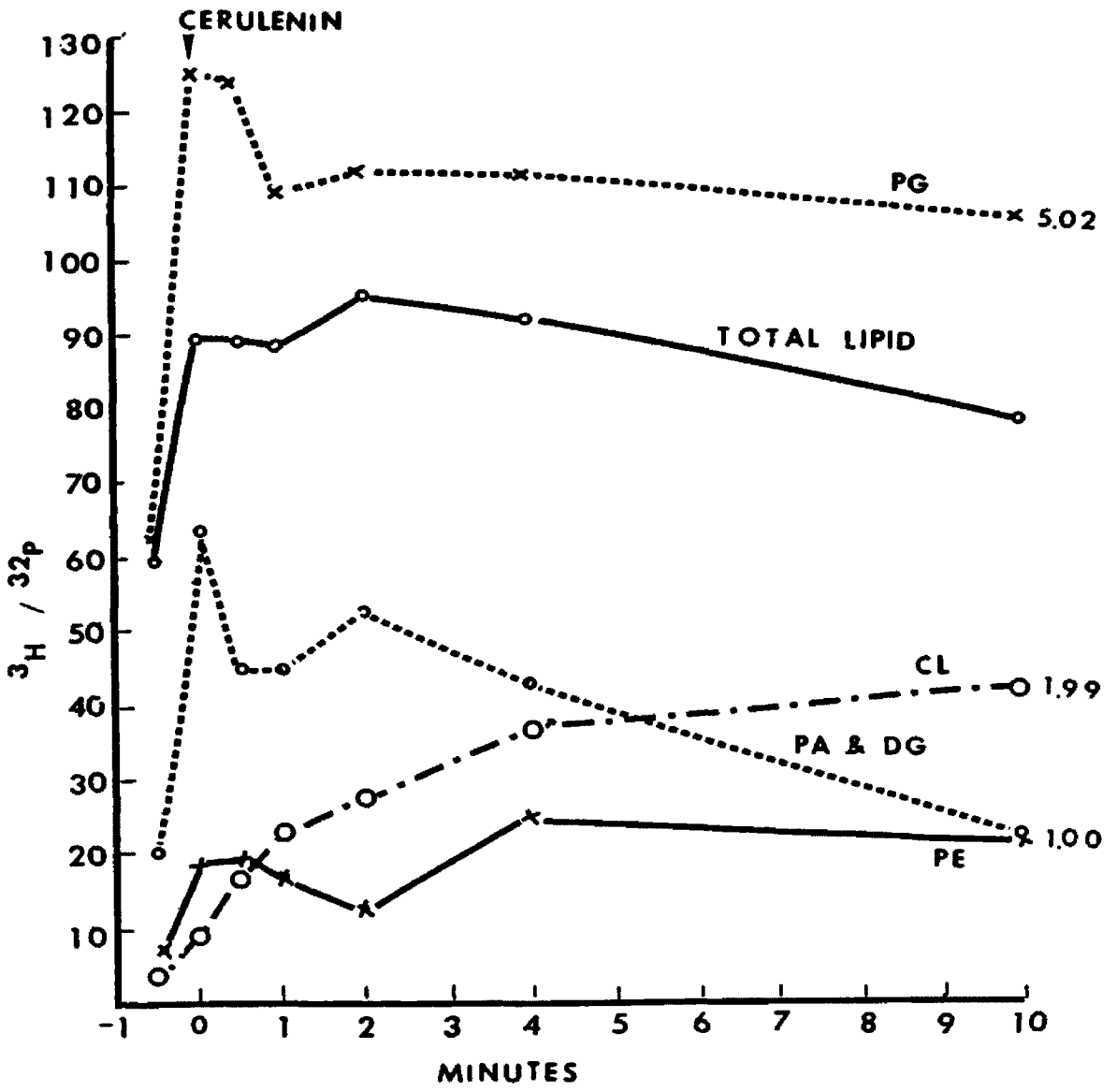
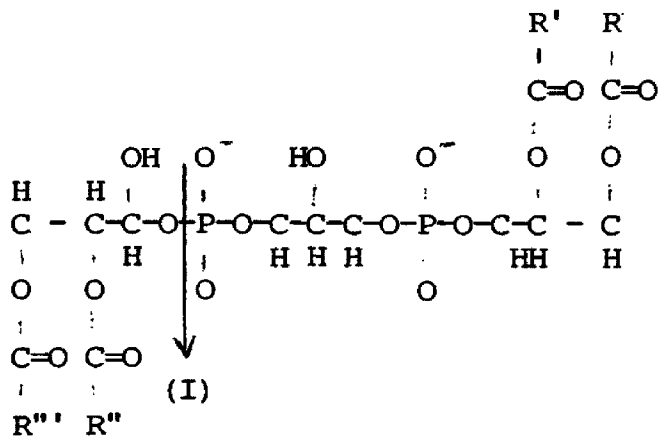


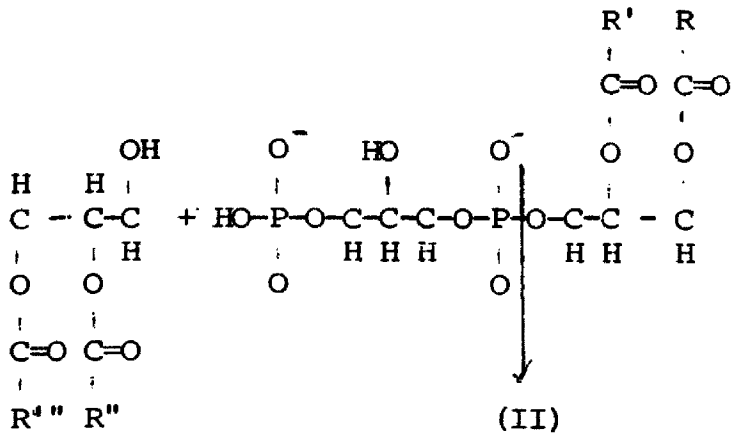
Figure 10. Relative uptake of ^3H and ^{32}P in phospholipid in B. stearothermophilus in the presence of cerulenin. The ratios of ^3H to ^{32}P in lipids from experiment shown in figure 6 were determined. The numbers indicate the relative ratios of $^3H/^{32}P$ of PG and CL to $^3H/^{32}P$ of PE.

(cardiolipin)

3-(3-sn-phosphatidyl) sn-glycerol 1-(3-sn-phosphatidyl)



1,2-diglyceride phosohatidylglycerol-3-phosphate



1,2-diglyceride

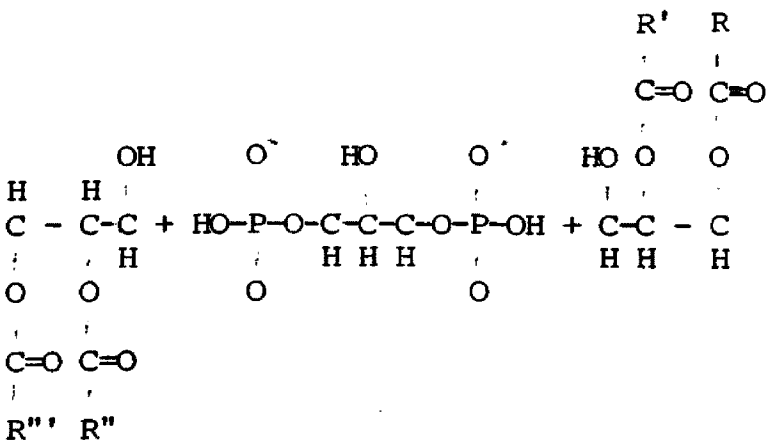


Figure 11. Hydrolysis of cardiolipin by phospholipase C. (83)

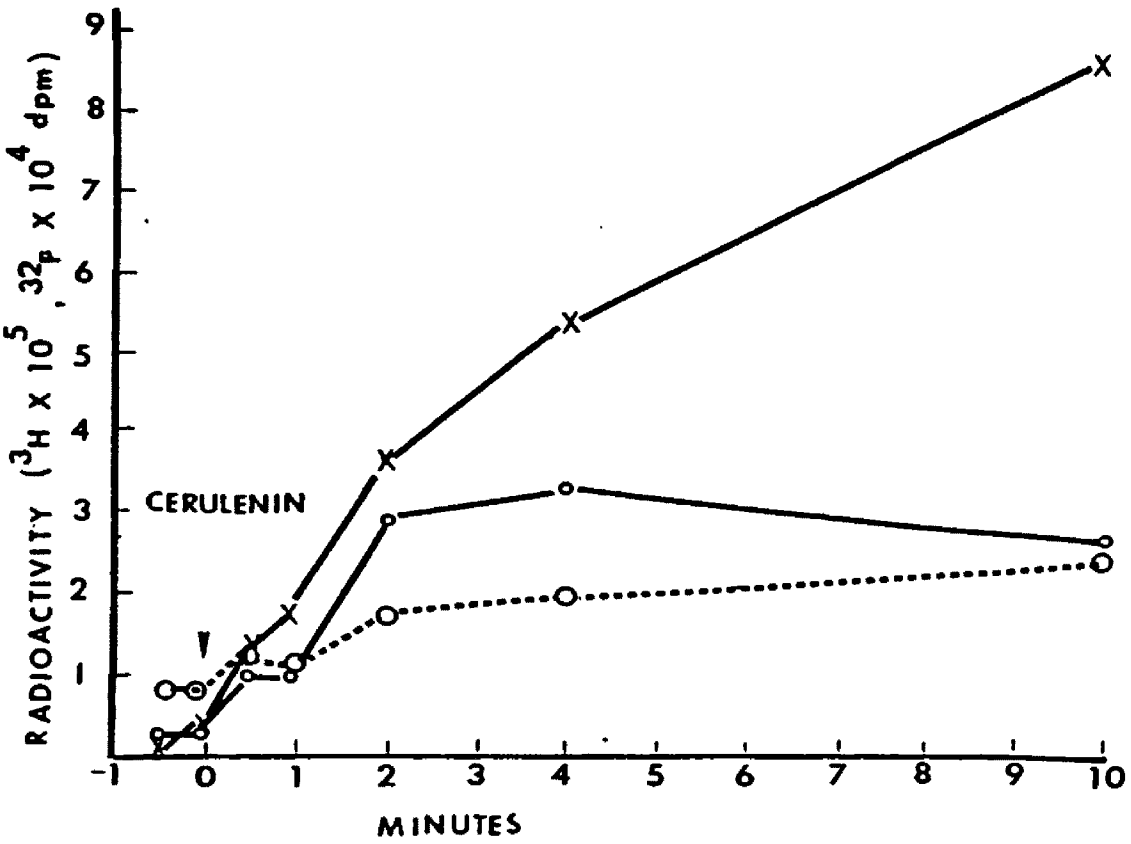


Figure 12. Distribution of ^3H in central and diacylated glycerol of CL and the uptake of ^{32}P in CL in B. stearothermophilus after pulse labeling in the presence of cerulenin. CL from the experiment shown in figure 6 was hydrolysed by phospholipase C. The radioactivities of central and diacylated glycerol were determined. —x—, diacylated glycerol; —o—, central glycerol; ····O···· , phosphate.

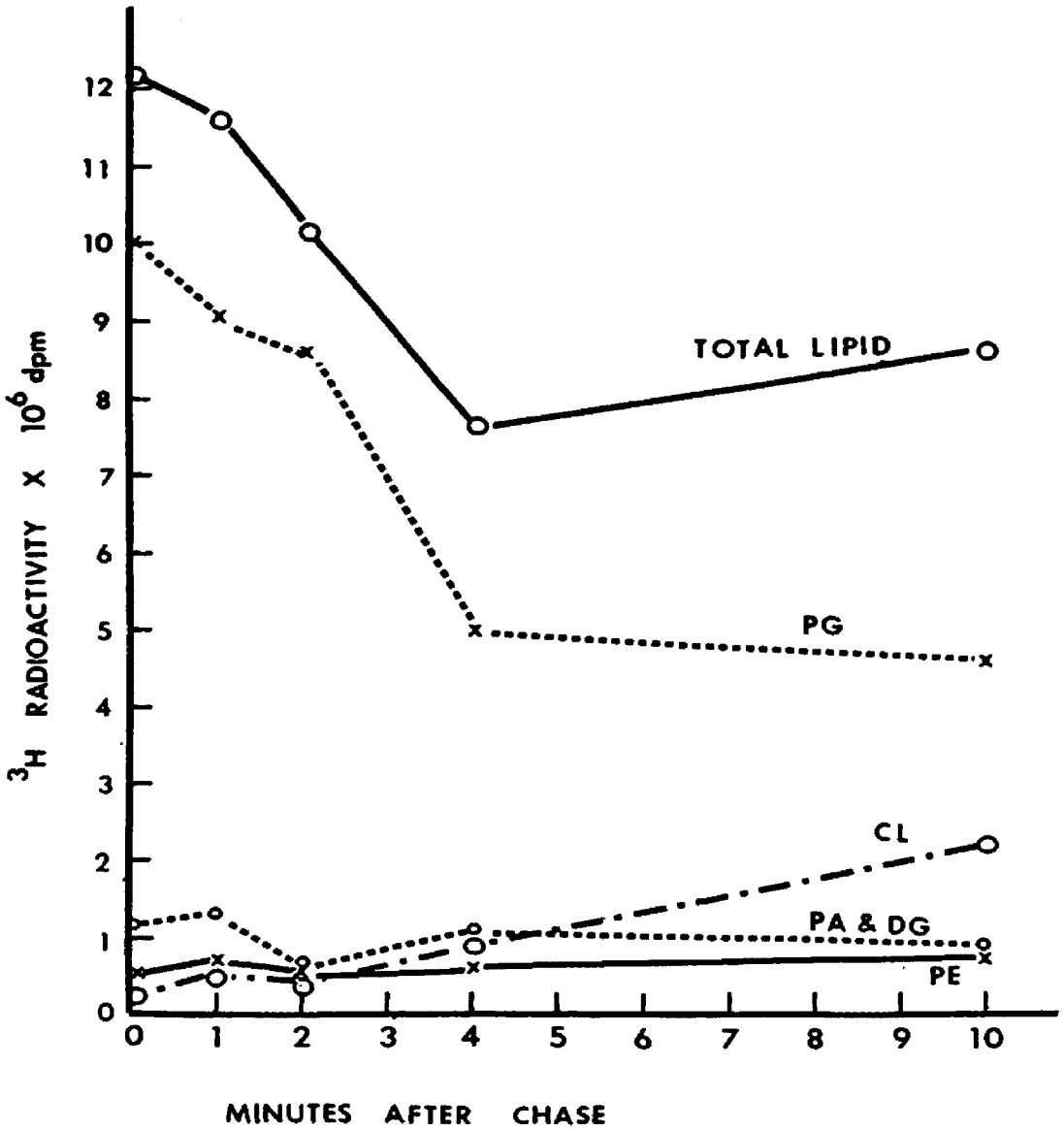


Figure 13. Fate of pulse-labeled lipids in B. stearothermophilus after chase in the absence of cerulenin. Cells were grown at 60°C to an O.D. of 0.35, then pulsed with 200 μCi $H_3{}^{32}PO_4$ for 8 min and 200 μCi [2-^{3}H] glycerol for 2 min. Cells were washed once with cold LOTM containing 0.1% glycerol. Pellet was resuspended in pre-warmed LOTM medium containing 0.1% glycerol. Samples were taken at various interval. Lipids were extracted as described in materials and methods, and their radioactivity were determined.

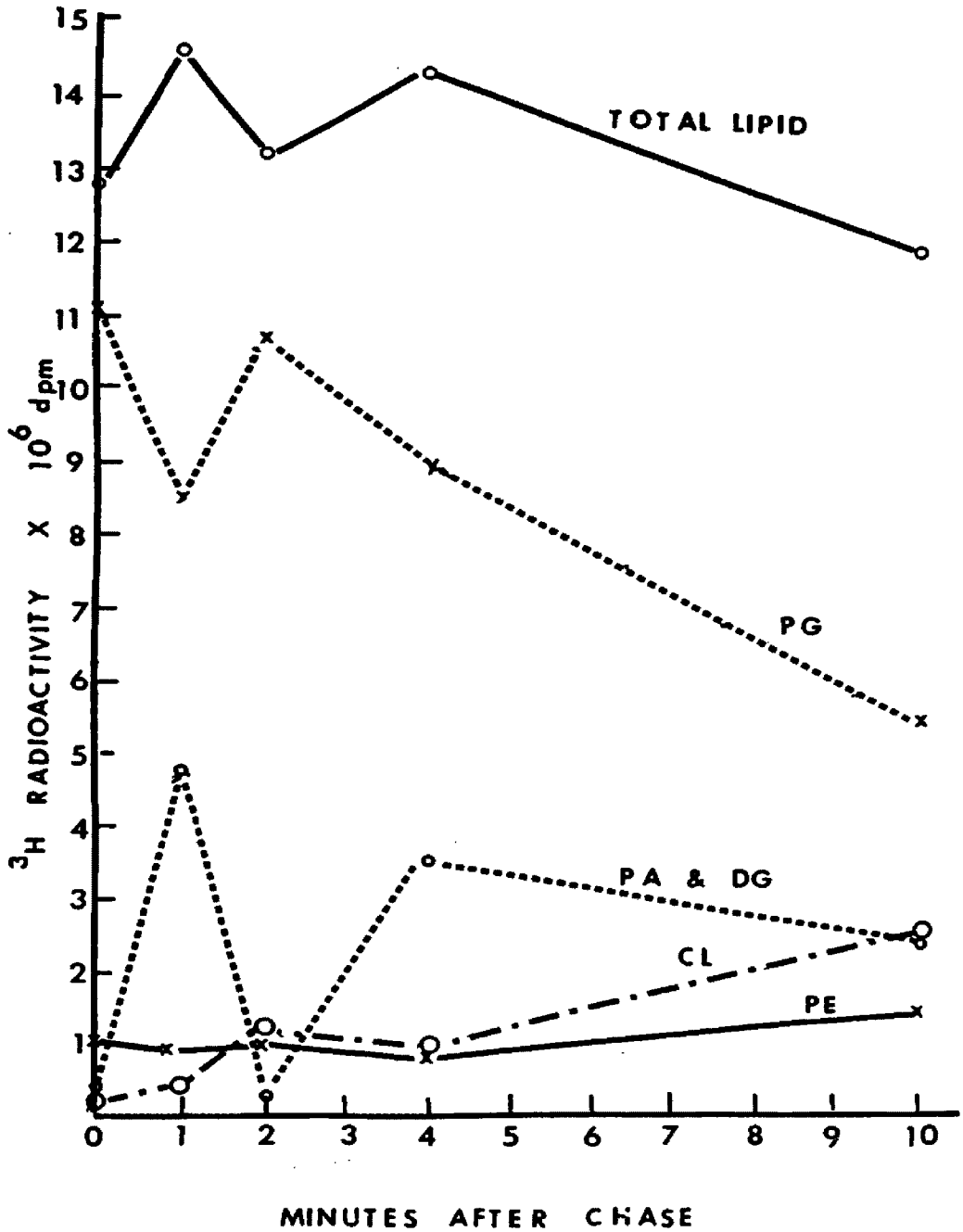


Figure 14. Fate of pulse-labeled lipids in B. stearothermophilus after chase in the presence of cerulenin. Cells (50 ml) were grown at 60°C to an O.D. of 0.35, then pulsed with 200 µCi $H_3{}^{32}PO_4$ for 10 min and 200 µCi [2-^{3}H]glycerol for 2 min. Cells were pooled on crushed ice and centrifuged to remove radioisotope. Cells were washed once with cold LOTM medium containing 0.1% glycerol. Pellet was resuspended in prewarmed LOTM medium containing 0.1% glycerol. Cerulenin was added at a concentration of 50 µg/ml. Samples were taken at the times indicated. Lipids were extracted and separated as described in materials and methods and their radioactivities were determined.

μg/ml cerulenin in the beginning of chase. The ^{3}H turnover in the lipid pool is shown in Fig. 14. It appeared that the rapid turnover in the early chase period was inhibited by cerulenin. The turnover of ^{3}H in total lipid was greatly reduced. About 8% ^{3}H was lost from total lipid after 10 min. The overall ^{3}H lost from PG and incorported into CL and PE were similar to the results of the same set of experiments in the absence of cerulenin. About 51% ^{3}H of PG was lost after 10 min, and the label which was incorporated into CL and PE accounted for 39% and 8% ^{3}H lost from PG respectively. The accumulation of PA and DG in the presence of cerulenin suggests that cerulenin inhibits the turnover of total lipid probably by blocking the pathway which leads to the formation of non-lipid products from phospholipid, most likely from PG.

6. Turnover of $^{32}PO_4^{=}$ in the absence or presence of cerulenin all showed similar patterns to the ^{3}H turnover as shown in Fig. 15 and Fig. 16. These results suggest that the turnover of phospholipid involves whole molecule degradation, or that the ^{3}H and ^{32}P turn over as a unit.

7. Distribution of label in PG during chase

The distribution of ^{3}H label in PG during chase is shown in Fig. 17. The label of nonacylated glycerol was higher than the label of diacylated glycerol moiety of PG in the beginning of chase. During the first four minutes both moieties turned over very rapidly, and with a slightly higher rate in nonacylated glycerol moiety of PG. After 4 minutes, the diacylated glycerol remained constant, whereas the nonacylated glycerol continued to turn over at a relatively low rate. The ratio of [2-^{3}H]glycerol to phosphorus remained constant during the chase in diacylated glycerol (Fig. 19). The ratios of both moieties were very close to each other. A slight decrease of the ratio during the chase was seen in nonacylated glycerol.

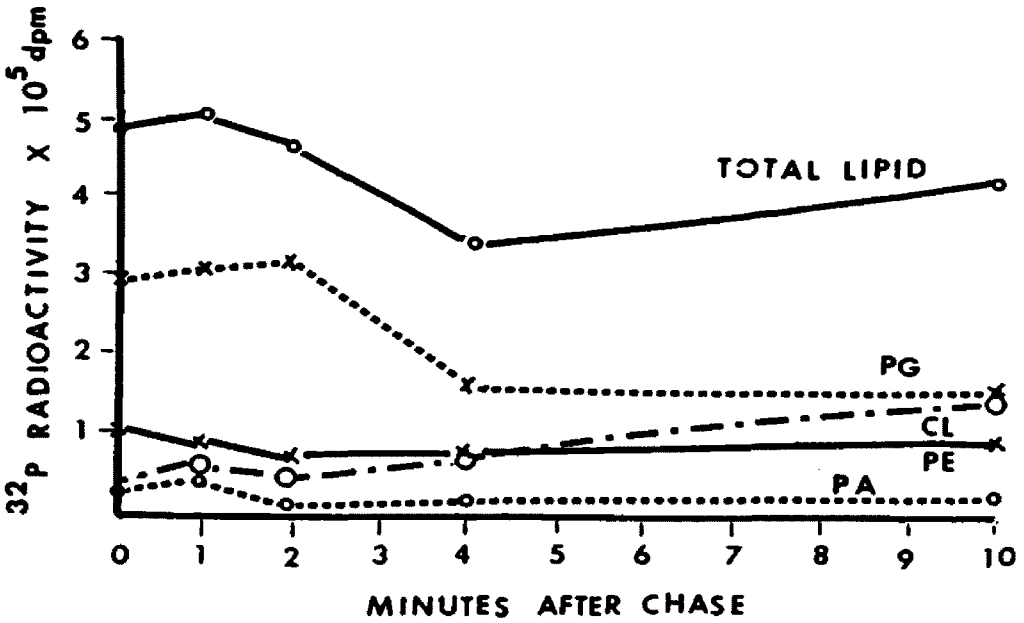


Figure 15. ^{32}P turnover of phospholipids in B. stearothermophilus in the absence of cerulenin. (as described in figure 13)

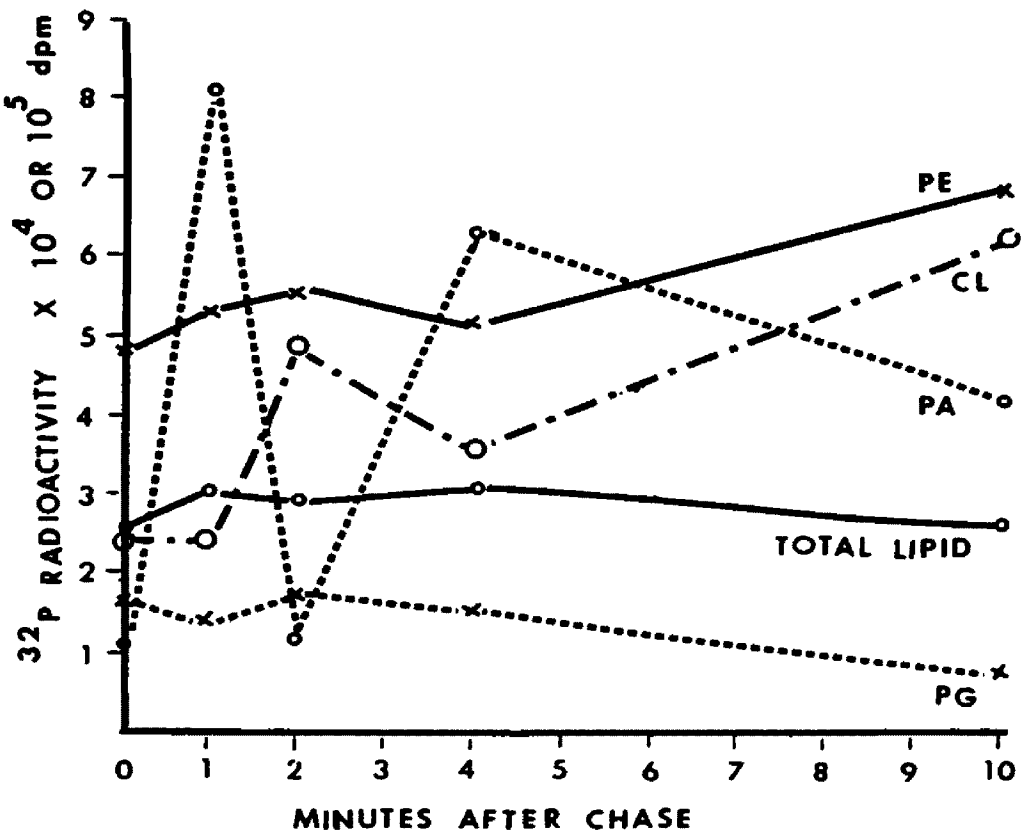


Figure 16. ^{32}P turnover of phospholipids in B. stearothermophilus in the presence of cerulenin. (as described in figure 14) Total lipid and PG are expressed as 10^5 dpm, the others are expressed as 10^4 dpm.

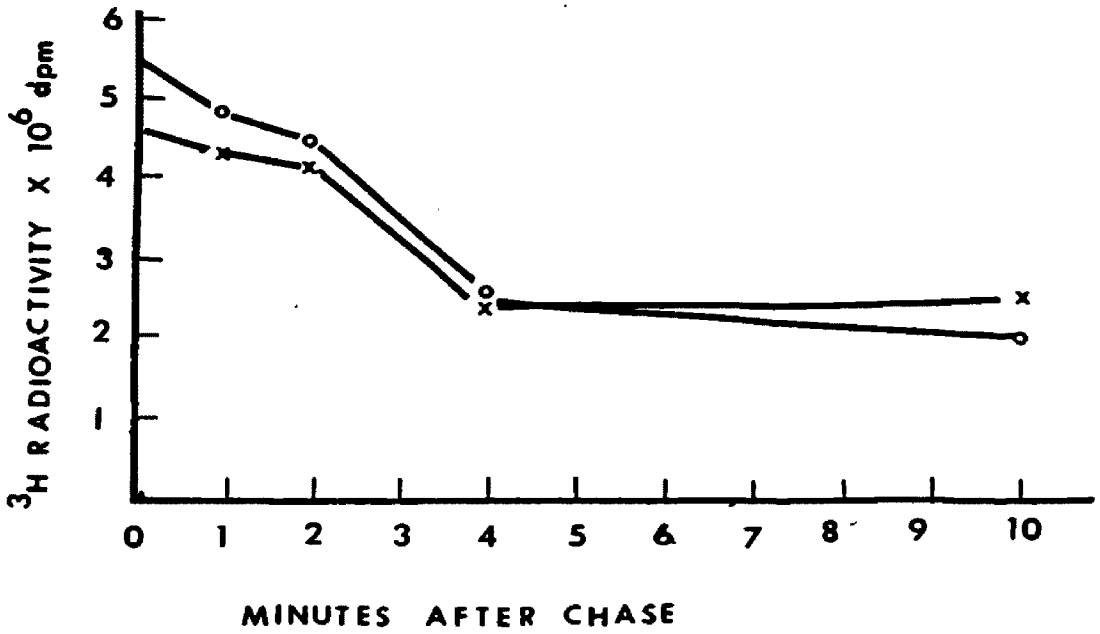


Figure 17. Turnover of diacylated and nonacylated glycerol moieties of PG in B. stearothermophilus in the absence of cerulenin. The diacylated and nonacylated glycerol moieties of PG from the experiment shown in figure 13 were separated by phospholipase C hydrolysis and their radioactivities were determined. —o— , nonacylated glycerol; —x— , diacylated glycerol.

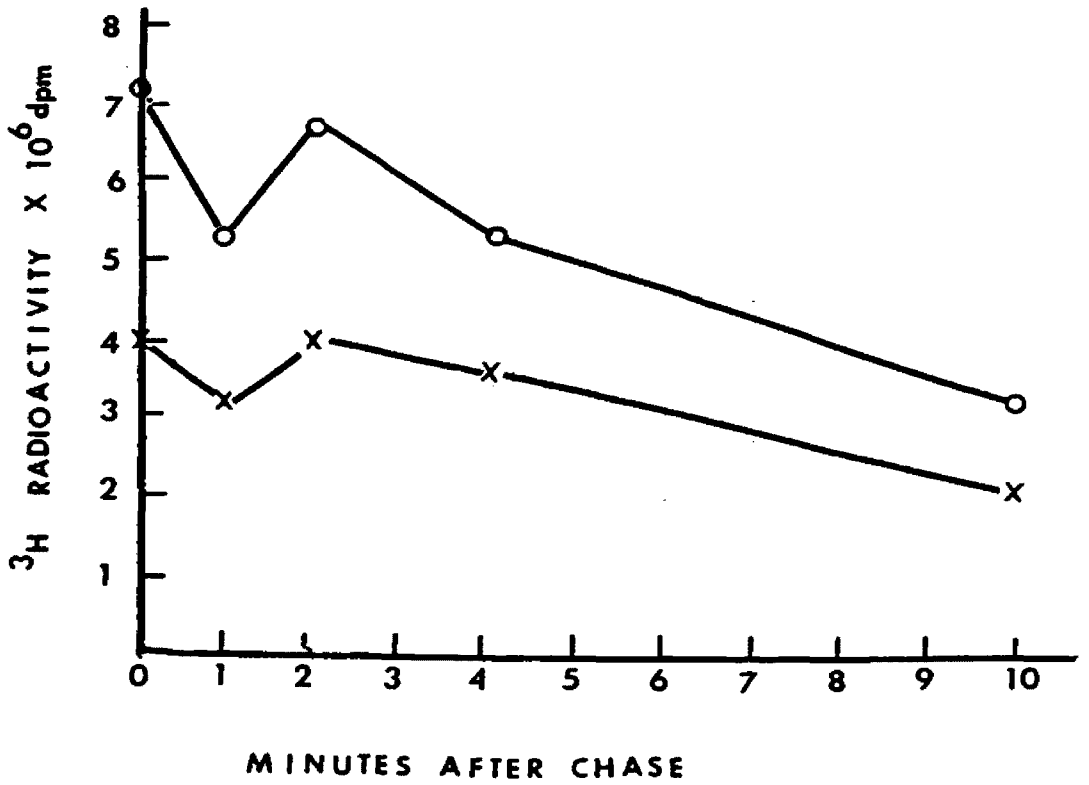


Figure 18. Turnover of diacylated and nonacylated glycerol moieties of PG in B. stearothermophilus in the presence of cerulenin. The diacylated and nonacylated glycerol moieties of PG from the experiment shown in figure 14 were separated by phospholipase C hydrolysis and their radioactivities were determined. —o— , nonacylated glycerol; —x— , diacylated glycerol.

These results suggest that at least two different pools of PG can be observed during 10 min chase, one pool undergoes very rapid turnover whereas the other pool is metabolically stable. Most of the PG turned over via whole molecule degradation. The slight difference found in non-acylated glycerol is probably due to the exchange reaction.

When cerulenin was added in the beginning of the chase, the rapid turnover appeared to be inhibited (Fig. 18). The ^{3}H was lost exclusively from nonacylated glycerol during the first 4 min. After 4 min both moieties turned over at an almost identical rate. The ratio of [2-^{3}H]-glycerol to phosphorus remained constant during the chase in diacylated glycerol (Fig. 20). The ratio decreased during the first 4 min and remained constant after 4 min in the nonacylated glycerol. Cerulenin appeared to inhibit or delay the rapid turnover of PG by some unknown mechanisms. Therefore the exchange reaction became distinguishable.

8. Distribution of label in CL during chase

The distribution of label in CL during chase are shown in Fig. 21 and Fig. 22. The ^{3}H and ^{32}P were continuously incorporated into CL, and that was not inhibited by cerulenin. If the components of CL derived from two molecules of PG, the ^{3}H label of diacylated glycerol moieties of CL should be at least two times that of central glycerol moiety of CL. It was found to be the case. It is interesting to see that the incorporation of ^{32}P was affected by cerulenin (Fig. 12, 21, 22). In the presence of cerulenin, the ^{32}P increased at a similar rate as ^{3}H increased in central glycerol of CL. In the absence of cerulenin, the ^{32}P increased at a similar rate as ^{3}H increase in diacylated glycerol of CL.

9. Relative rates of glycerol and phosphorus turnover

The $^{3}H/^{32}P$ ratio of individual lipids are shown in Fig. 23 and Fig.

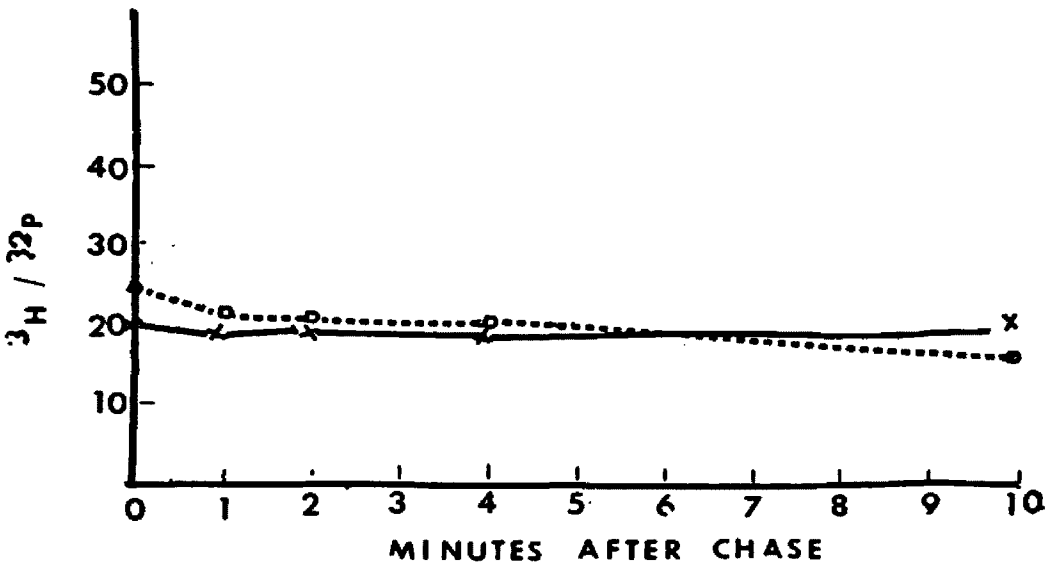


Figure 19. Turnover of diacylated and nonacylated glycerol moieties of PG relative to the turnover of phosphate group in B. stearothermophilus The diacylated and nonacylated glycerol moieties of PG were separated as described in figure 17. The relative turnover of both moieties to phosphate group are shown as the ratio of ^3H to ^{32}P. -..o..-, nonacylated glycerol; —x— , diacylated glycerol.

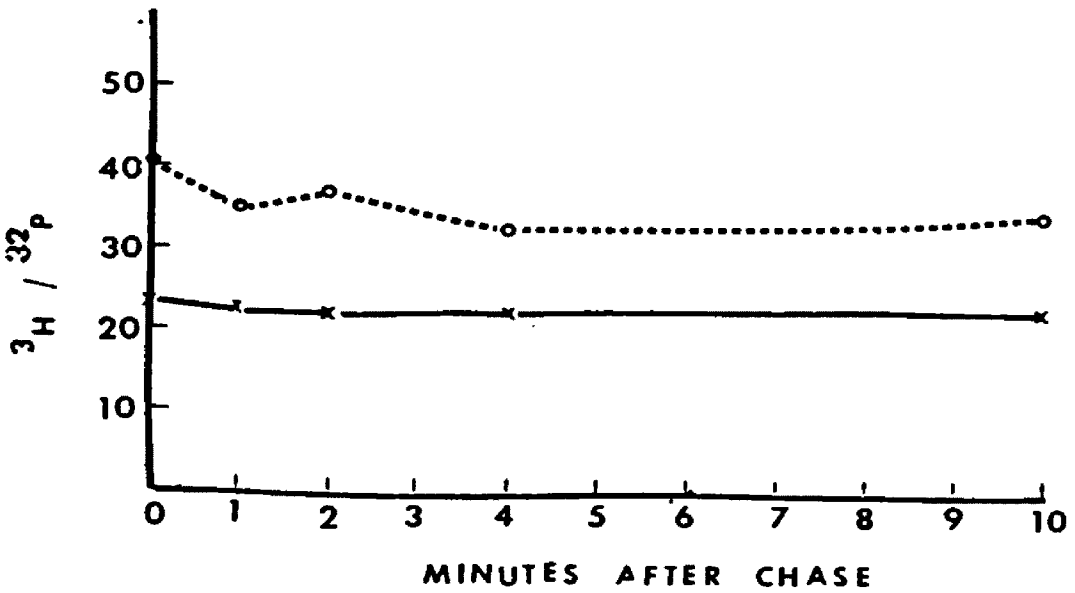


Figure 20. Turnover of diacylated and nonacylated glycerol moieties of PG relative to the turnover of phosphate group in B. stearothermophilus in the presence of cerulenin. The diacylated and nonacylated glycerol moieties of PG were separated as described in figure 18. The relative turnover of both moieties to phosphate group are shown as the ratio of ^3H to ^{32}P. ---o---, nonacylated glycerol; —x— , diacylated glycerol.

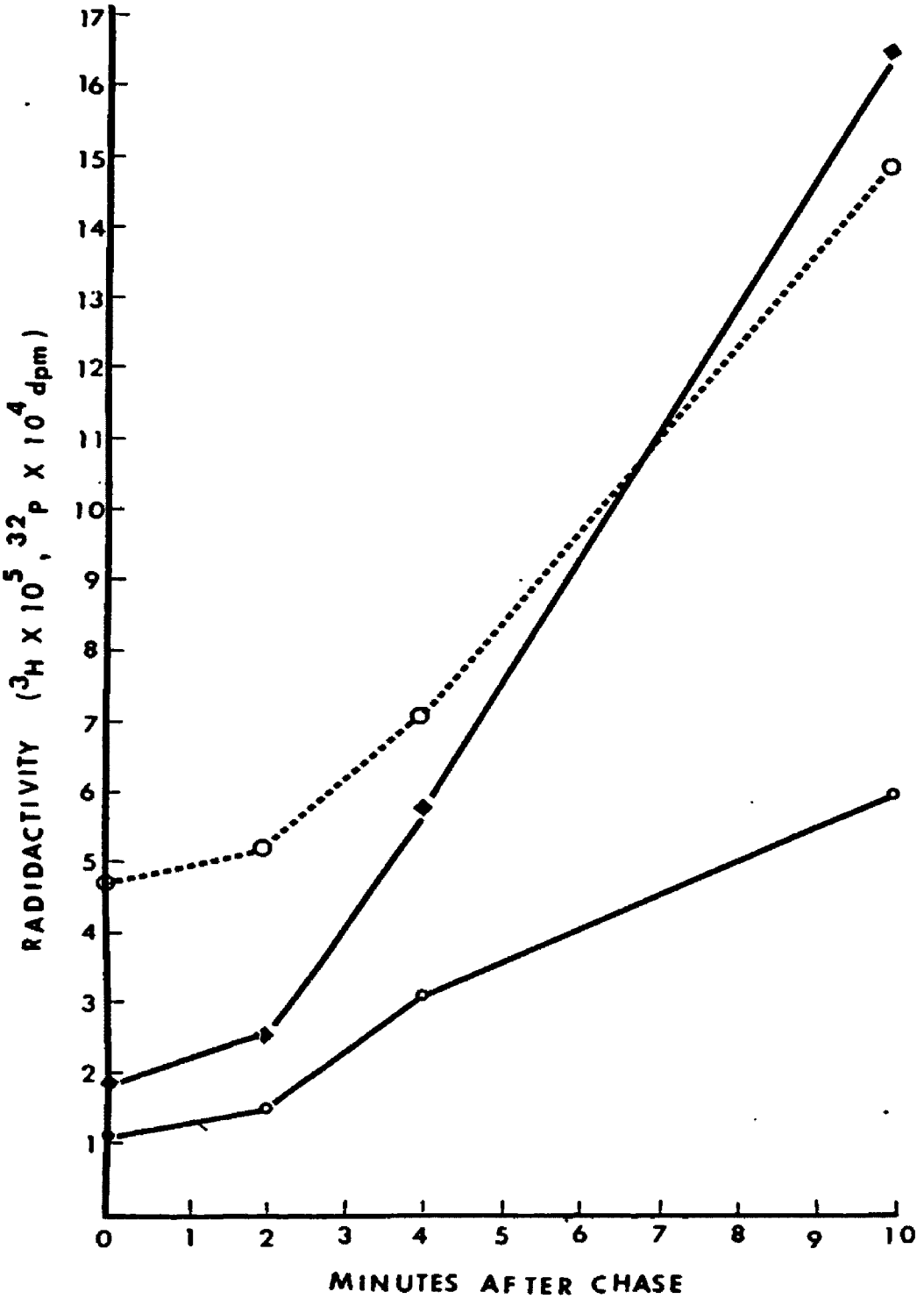


Figure 21. Turnover of diacylated glycerol moiety and central glycerol moiety of CL in B. stearothermophilus in the absence of cerulenin. CL from experiment shown in figure 13 was hydrolysed by phospholipase C. The radioactivity of central and diacylated glycerol were determined. ····o····· , phosphate;—■— , diacylated glycerol; —o— , nonacylated glycerol.

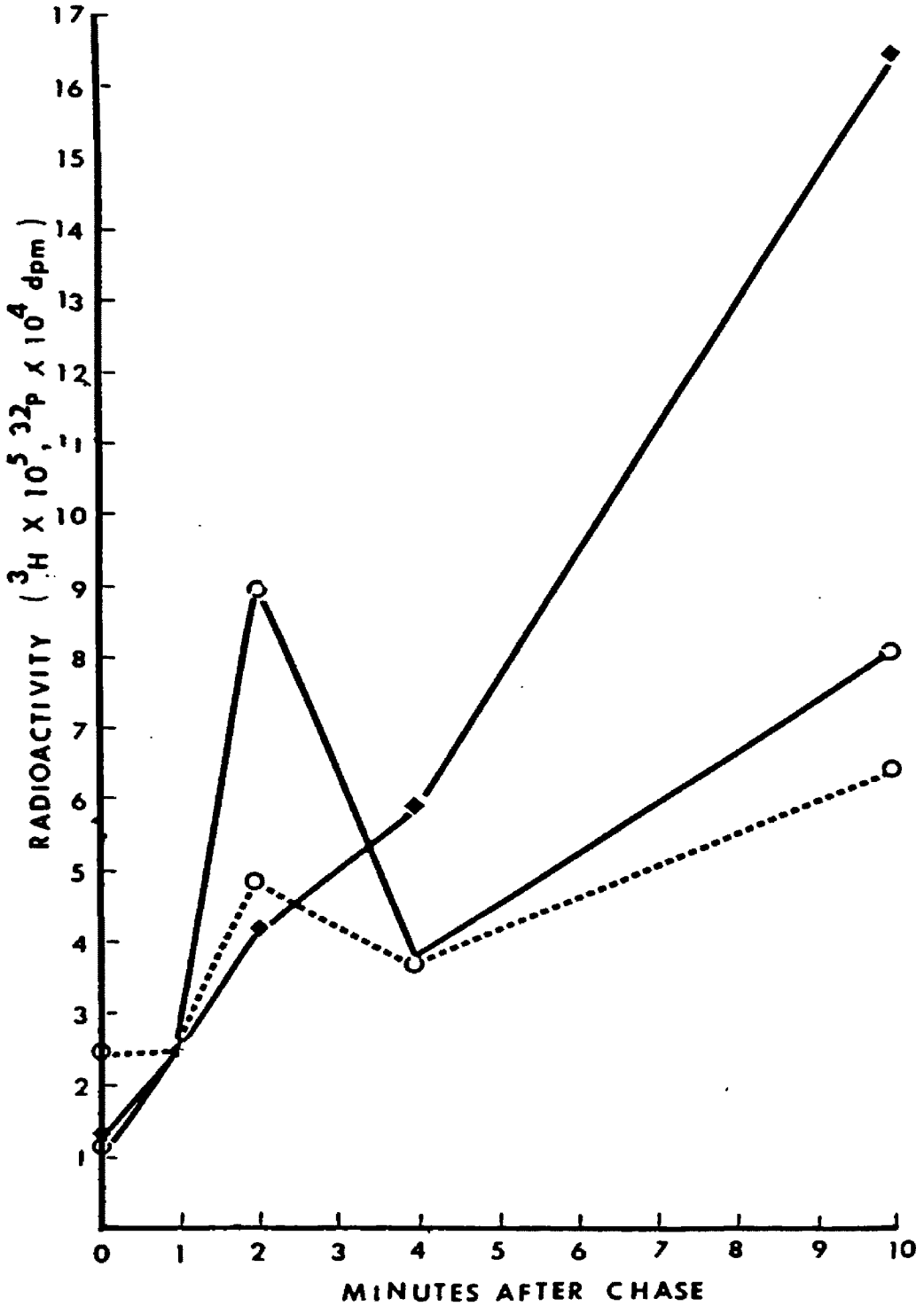


Figure 22. Turnover of diacylated glycerol moiety and central glycerol moiety of CL in B. stearothermophilus in the presence of cerulenin. CL from experiment shown in figure 14 was hydrolysed by phospholipase C. The radioactivities of central and diacylated glycerol were determined. —o—, central glycerol; —■—, diacylated glycerol; ····o····· , phosphate.

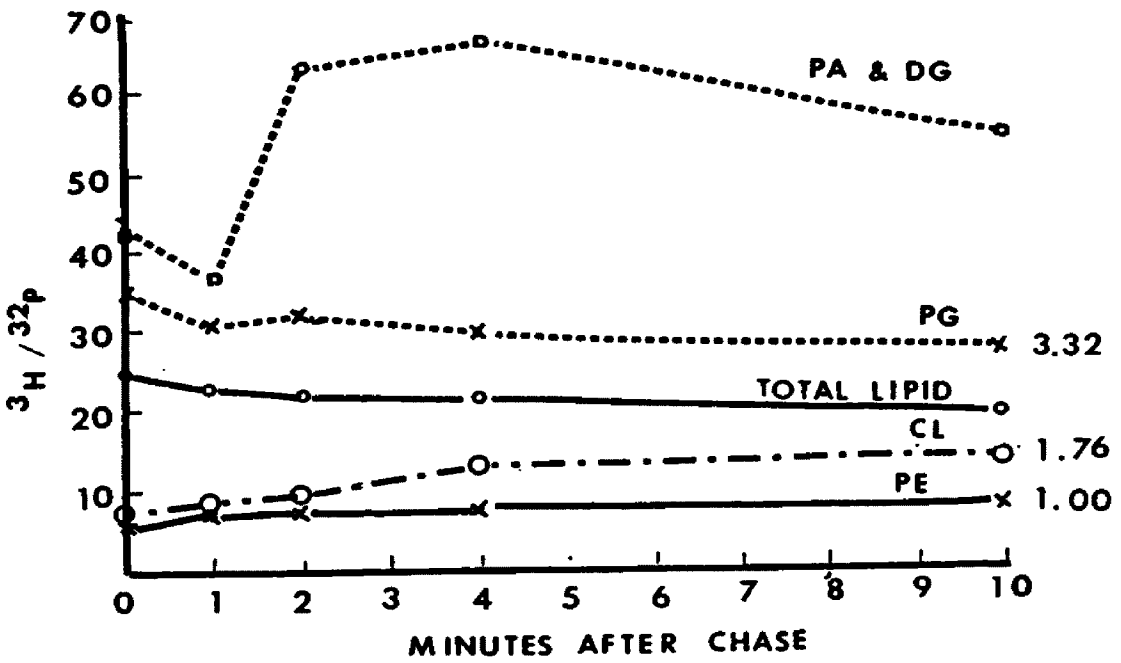


Figure 23. Relative turnover of ^3H and ^{32}P of lipids in B. stearothermophilus in the absence of cerulenin. The ratios of ^3H to ^{32}P in lipids from experiment shown in figure 13 were determined. The numbers indicate the relative ratio of $^3H/^{32}P$ of PG and CL to $^3H/^{32}P$ of PE.

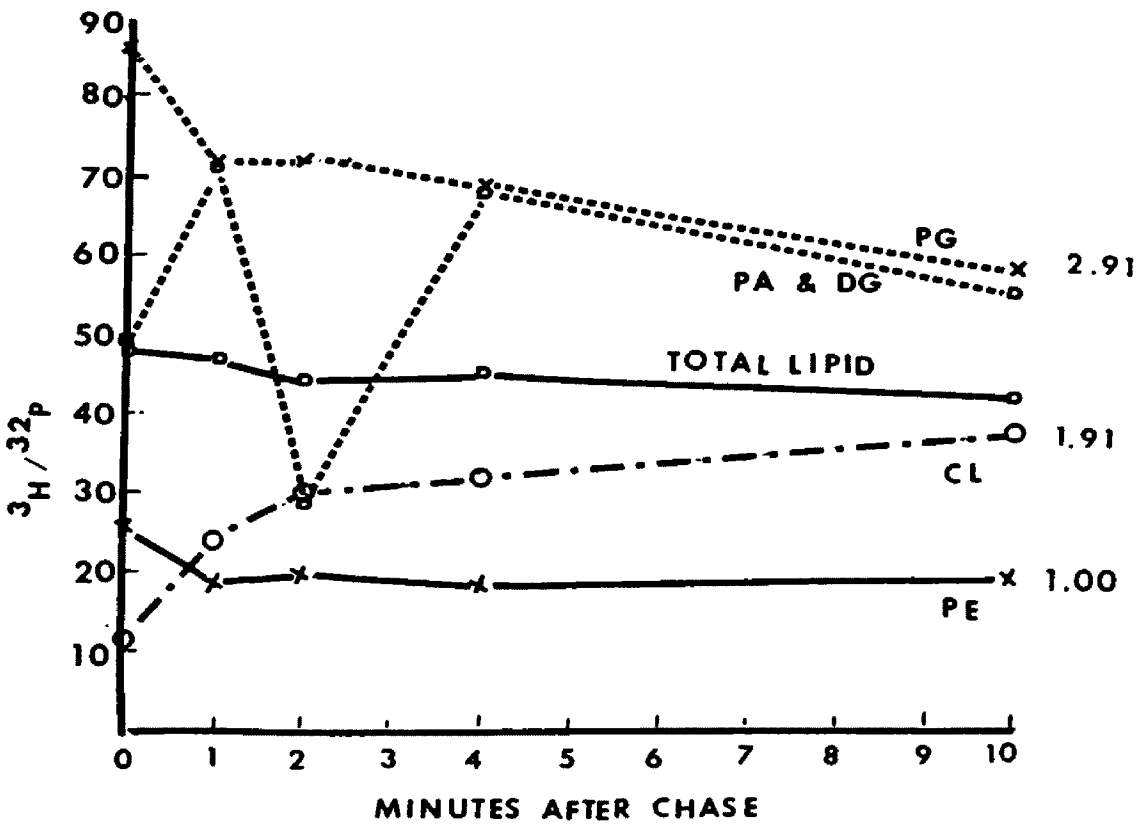


Figure 24. Relative turnover of ^3H and ^{32}P of lipids in B. stearothermophilus in the presence of cerulenin. The ratios of ^3H to ^{32}P in lipids from experiment shown in figure 14 were determined. The numbers indicate the relative ratios of $^3H/^{32}P$ of PG and CL to $^3H/^{32}P$ of PE.

24. The [2-^{3}H]glycerol was not equally distributed through the lipid pool. The specific activity of the glycerol of PG and CL were higher than PE. The exchange reaction is also evident from the relative rates of glycerol and phosphorus turnover of PG.

B. Uptake of [2-^{3}H]glycerol in isolated membrane fractions

1. optimum conditions for [2-^{3}H]glycerol uptake

The optimum conditions for [2-^{3}H]glycerol uptake were studied. The effect of divalent cations, detergent, nucleotides, cytosol, and sn-glycerol-3-phosphate is shown in Table 3. The optimum uptake of [2-^{3}H]-glycerol was obtained at pH 5.5 (Fig. 25). Divalent cations had a stimulating effect on enzyme activity in washed membrane. In contrast, enzyme activity appeared to be enhanced by the persence of EDTA in cholate extracted membrane. Triton X-100 inhibited the enzyme activity. The enzyme activity was also inhibited by cytosol, sn-glyce-o-3-P, ATP, ADP, and PO_4^{-3}. It appears that the reaction does not require energy.

2. Phosphatidylglycerol requirement for [2-^{3}H]glycerol uptake

The time course of [2-^{3}H]glycerol uptake on both membrane preparations is shown in Fig. 26. The effect of substrate concentration is shown in Fig. 27. The enzyme appeared to be saturated at concentrations of PG above 60 nmoles per 0.2 mg of membrane protein in cholate extracted membrane which was a delipidated membrane. In contrast, the [2-^{3}H]glycerol uptake decreased as the exogenous PG concentration increased in washed membrane preparations. Lipid analysis has shown that CL was formed in cholate-extracted membrane. When exogenous CL was added in the cholate-extracted membrane the [2-^{3}H]glycerol uptake was inhibited. This is probably due to the feedback inhibition by reaction product.

Tabel 3. Effect of different factors on exchange reaction

	nmol uptake in washed membrane	nmol uptake in cholate extracted membrane
control[a1,2]	14.8	-
EDTA	16.14	7.23
$MnCl_2$ (5 mM)	17.66	-
$MgCl_2$ (5 mM)	16.92	4.45
$CaCl_2$ (5 mM)	17.41	3.89
control[b]	0.4	
cytosol	0.36	
Triton X-100	0.22	
control[c]	2.95	
G-3-P	1.64	
ATP	1.52	
ADP,PO_4^{3-}	1.22	
ADP,PO_4^{3-},malate	2.22	
control[d]	1.1	
G-3-P	0.4	
ATP	1.23	
ADP,PO_4^{3-}	1.05	
ADP,PO_4^{3-},malate	1.39	

a1. Reaction mixture contained washed membrane, sodium acetate buffer (0.2 M, pH 5.5), [2-^{3}H]glycerol (1.4 x 10^5 dpm/nmol).

a2. Reaction mixture contained cholate extracted membrane, sodium acetate buffer (0.2 M, pH 5.5), unlabeled PG (60 nmol), [2-^{3}H]glycerol (2.2 x 10^5 dpm/nmol).

b. Reaction mixture contained washed membrane, sodium acetate buffer (0.2 M, pH 5.5), [2-^{3}H] glycerol (1.8 x 10^4 dpm/nmol).
c. Reaction mixture contained washed membrane, MES buffer (pH 7.0, 5 mM MgCl), [2-^{3}H] glycerol (1 x 10^4 dpm/nmol).
d. Reaction mixture contained washed membrane, cytoplasm, MES buffer (pH 7.0, 5 mM MgCl), [2-^{3}H] glycerol (1 x 10^4 dpm/nmol).

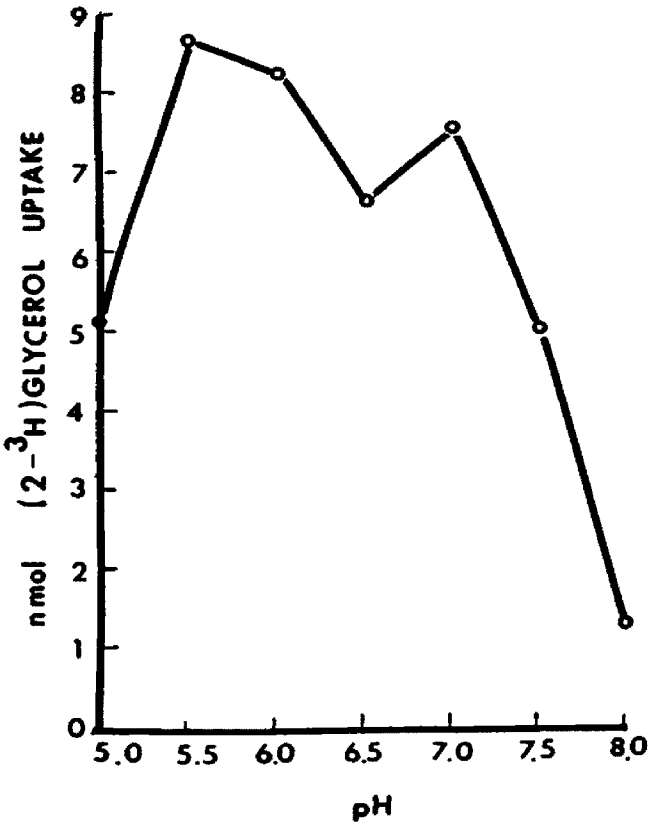


Figure 25. The effect of pH on the rapid uptake of [2-^{3}H]glycerol in washed membrane. Washed membrane was prepared by lysozyme treatment. The reaction mixture contained 10 µl $MnCl_2$ (0.1 M), 50 µl washed membrane (3.86 µg protein/µl washed membrane), 20 µl [2-^{3}H]glycerol (250 mM), and 100 µl buffer with different pH. Reaction was terminated by 10% TCA and lipids were extracted as described in materials and methods. The uptake was expressed as nmol [2-^{3}H]glycerol.

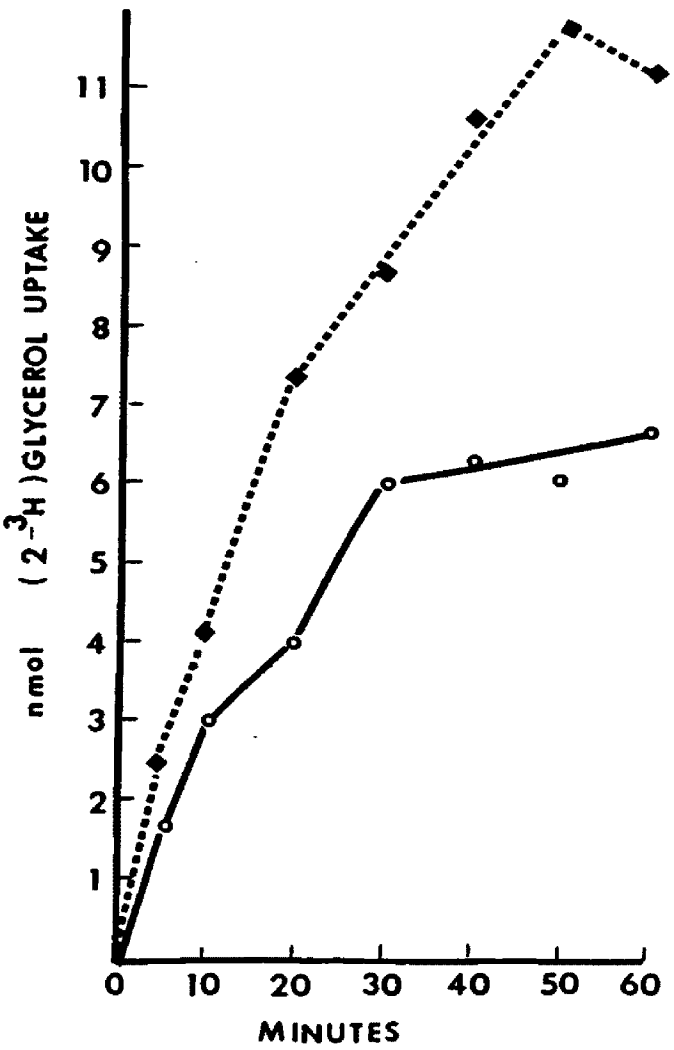


Figure 26. Time course of the uptake of [2-^{3}H]glycerol on washed membrane and cholate extracted membrane. Cholate extracted membrane was prepared by treating the washed membrane with 1.5% NaCl, 0.25 M Na SO , 0.5 M Tris-hydrochloride (PH 7.8) cholate buffer to a concentration of 1-2 mg of protein per ml. After incubation at 4 C for 60 min, solution was centrifuged, and the pellete was resuspended in 10 mM MES buffer (PH 7.0). Reaction was carried out as described in figure 25 at PH 5.5. ---●---, washed membrane; —o— , cholate extracted membrane.

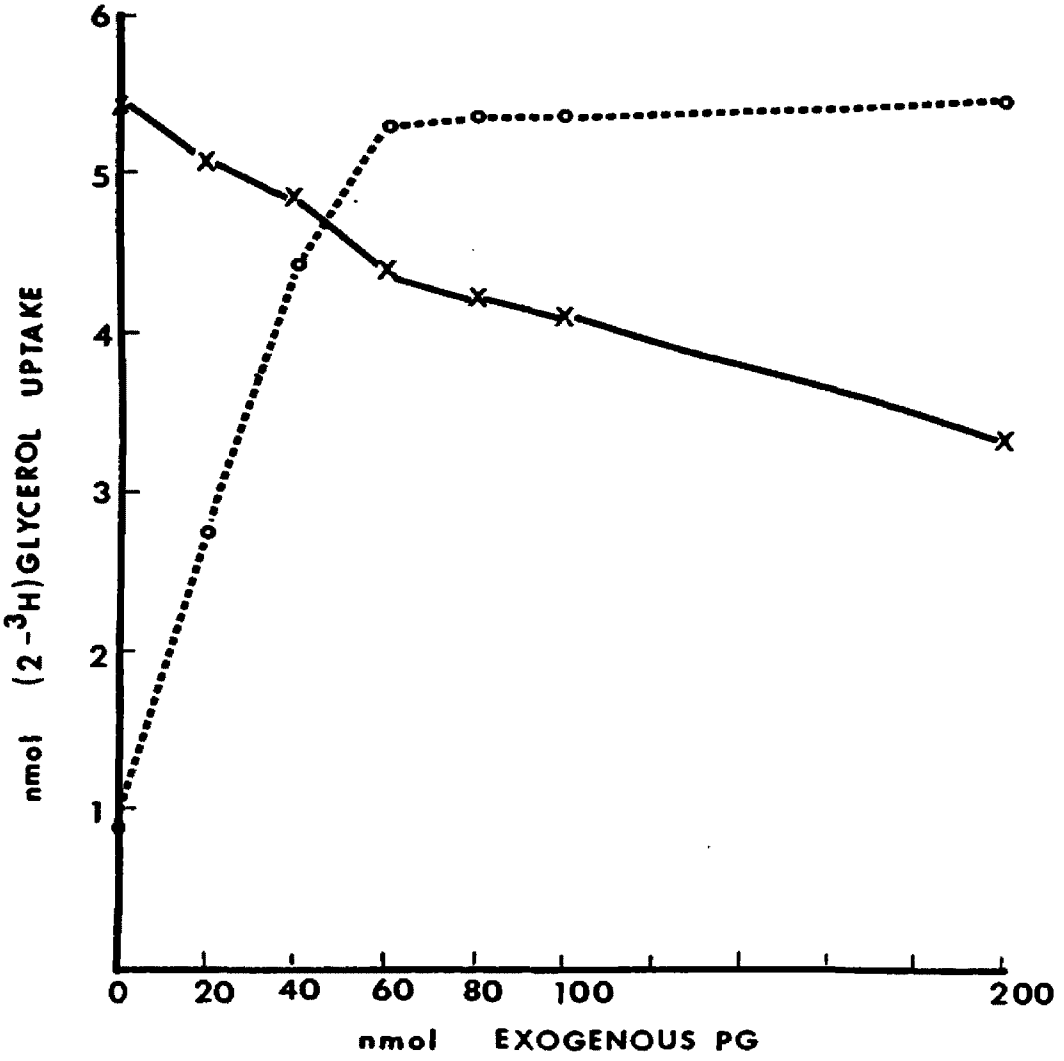


Figure 27. Effect of exogenous PG on the uptake of [2-^{3}H] glycerol in washed membrane and cholate extracted membrane. Experiment was carried out as described in figure 25 in the presence of different concentrations of exogenous PG, —x— , washed membrane; ---o--- , cholate extracted membrane.

Chapter IV.

Discussion

In this study, the maximum uptake of [2-^{3}H]glycerol by B. stearothermophilus was obtained at cell density corresponding to O.D. 0.4 with 2 minutes pulse when cells were grown in TYE medium. The rapid uptake of [2-^{3}H]glycerol was reexamined in different combinations of cells and media. Regardless of the optical density at which cells were collected, it uniformly showed the maximum uptake at low cell density (O.D. 0.1) when cells were resuspended or diluted with high O.D. medium. But the opposite results were found when cells were diluted with fresh TYE medium. These findings suggest that the increased uptake of [2-^{3}H]glycerol at high cell density was due to changes in the medium. The precise composition of TYE medium is unknown, but the simplest explanation would be that it contains glycerol or glycerol phosphate. The increased uptake at high cell density and at low cell density prepared by dilution with high O.D. medium may be simply due to the decreased amount of glycerol phosphate in medium after cells grow several generations. Cells grown in defined medium, which did not contain glycerol phosphate, showed decreased uptake with the increasing of cell density. The higher rates of uptake at low cell densities in the defined medium were probably due to more rapid growth and lipid synthesis at low cell density.

The studies with washed membrane and cholate extracted membrane have shown that the exchange reaction did occur under the condition when no net lipid synthesis could take place. The exchange activity was found exclusively in membrane fraction even after detergent treatment which indicates that this enzyme activity is highly associated with membrane.

The maximum uptake of [2-^{3}H]glycerol was obtained at pH 5.5 which was found to be the same as the optimum pH for cardiolipin synthetase (19). Divalent cations had a stimulating effect on enzyme activity in washed membrane. In contrast, enzyme activity appeared to be enhanced by the presence of EDTA in cholate-extracted membrane. The difference in divalent cation requirement between two membrane fractions is probably due to the changes in enzyme environment after delipidation. Triton X-100, which has been reported to stimulate the synthesis of lipids in cell-free systems (48), inhibited the enzyme activity. CL synthetase was also reported to be inhibited by this detergent (19). The enzyme activity was also inhibited by cytosol, sn-glycero-3-P, ATP, ADP, and $PO_4^{=}$. It appears that the reaction does not require energy. The cholate-extracted membrane was a delipidated membrane preparation. When exogenous PG was added to cholate-extracted membrane, the uptake of [2-^{3}H]glycerol increased as the amount of exogenous PG increased. Lipid analysis showed net CL synthesis. When exogenous CL was added in the cholate-extracted membrane, [2-^{3}H]glycerol uptake was inhibited. This is probably due to the feedback inhibition by reaction product. These findings provide some evidences for the proposed pathway by which CL is synthesized from PG in two stages:

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

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

where X is an intermediate carrier of the phosphatidyl moiety of PG. The ready reversibility of the first step in the presence of [2-^{3}H]glycerol might then account for the observed rapid uptake of [2-^{3}H]glycerol in PG. It is not clear that why the increased amount of exogenous PG didn't increase [2-^{3}H]glycerol uptake in washed membrane.

The mechanism of the rapid uptake was studied by dual-labeled pulse-

chase experiments. Cells were pulsed with [2-^{3}H]glycerol and $H_3{}^{32}PO_4$ for a given period of time. Because radioactive phosphate took longer time to equilibrate with the large endogenous phosphate pool than [2-^{3}H]-glycerol did. The incorporation of $H_3{}^{32}PO_4$ into phospholipid was slower than that of [2-^{3}H]glycerol. After 1.5 minutes pulse, 87% of ^{3}H was found in PG, 7% in PA plus DG, 4% in PE, and 1% in CL (Fig. 3). The label pattern of PG was biphasic even during such a short pulse time (Fig. 4). It was labeled rapidly during the first 0.5 min, then was labeled at a slower rate. The nonacylated glycerol moiety of PG was labled slightly faster than the diacylated glycerol moiety during the first 0.5 min, after which it showed the same uptake rate at both moieties (Fig. 4). The radioactivity of nonacylated glycerol moiety was 1.28 times that of diacylated glycerol moiety in the end of the 1.5 min pulse. These results suggest that [2-^{3}H]glycerol might be incorporated by both de novo synthesis from sn-glycero-3-phosphate and the exchange reaction.

In the presence of cerulenin, the incorporation of [2-^{3}H]glycerol in PG continued for one minute, and then label was lost from the PG pool (Fig. 6). PG lost 19% of ^{3}H label during the 10 min pulse period. The incorporation of ^{3}H in CL was unaffected by cerulenin. Increased ^{3}H labeling of CL accounted for 77% of ^{3}H lost from PG. The uptake of [2-^{3}H]glycerol in PE continues for 0.5 min after the addition of cerulenin and then it appeared to be synthesized at the expense of PG. This accounted for 11% of ^{3}H lost from PG. Since the synthesis of CL from PG would release free glycerol, it appeared that all of the label lost from the PG pool could be accounted for by these reactions. The ^{32}P label in individual lipid showed similar pattern (Fig. 9). The relative ^{3}H/^{32}P ratio of PG to PE and CL to PE indicate that the label is not evenly distributed

among the phospholipids. It's interesting to see that the labeling of diacylated glycerol moiety of PG ceased immediately after the addition of cerulenin (Fig. 7), but the uptake in the nonacylated glycerol moiety continued for two minutes, and then both moieties were degraded at the same rate. The $^{3}H/^{32}P$ ratio of both moieties also showed differences in the first 2 minutes (Fig. 8). These results suggest that most of the uptake took place through the regular glycerol phosphate pathway of phospholipid biosyntheses which need glycerol kinase to synthesize sn-glycero-3-phosphate from free glycerol. The glycerol kinase activity has been demostrated in E. coli and accounted for a minor source of sn-glycero-3-phosphate (63). It appears that in the presence of cerulenin the lipid biosynthesis is inhibited and that the incorporation of [2-^{3}H]-glycerol in nonacylated glycerol moiety of PG occurs through an exchange reaction by a small pool of PG. The distribution of ^{3}H in CL was analysed (Fig. 12). The ^{3}H of diacylated glycerol was greater than two times that of central glycerol. This result would be expected if the components of CL was derived from two molecules of PG.

The chase experiments were performed in the absence and presence of cerulenin. The turnover pattern of PG in the absence of cerulenin indicated that there were two different pools of PG (Fig. 13). The ^{3}H increased in CL accounted for about 36% ^{3}H lost from PG, but the ^{3}H increased in PE only accounted for 3% ^{3}H lost from PG. The phospholipid turnover in the presence of cerulenin is shown in Fig. 14. The rapid turnover of PG was inhibited by cerulenin. The label of phosphatidic acid and diglyceride was extraordinarily high. This might be significant, because the recycling of glycerol through acyl transferase after phosphatidyl transfer from PG was blocked by the inhibition of cerulenin.

The total loss of ^{3}H label from PG was not affected by cerulenin. The accumulation of ^{3}H in CL accounted for 39% ^{3}H lost from PG, and the accumulation of ^{3}H in PE accounted for 8% ^{3}H lost from PG. The decreased turnover of ^{3}H in lipid pool was due to the accumulation of PA plus DG, which accounted for 35% ^{3}H lost from PG. It appeared, therefore, that cerulenin blocked the degradation pathway which resulted in loss of label from the lipid pool. This was an entirely unexpected result and suggested either another activity for cerulenin or a link between de novo lipid synthesis and the degradation reactions. The nonacylated glycerol moiety of PG and diacylated glycerol moiety of PG turned over in the absence of cerulenin almost at the same rate (Fig. 17). The slight difference observed in the early time was probably due to the exchange reaction, which was more obvious when viewed as $^{3}H/^{32}P$ ratio of both moieties (Fig. 19). In the presence of cerulenin the $^{3}H/^{32}P$ ratio of diacylated glycerol moiety of PG remained constant, but $^{3}H/^{32}P$ ratio of nonacylated glycerol moiety decrease at the first 4 minutes and remain constant afterwards (Fig. 20). This is additional evidence for an exchange reaction.

The incorporation of ^{3}H in different portions of CL was unaffected by cerulenin (Fig. 21, 22). But the incorporation of ^{32}P was affected by cerulenin (Fig. 12, 21, 22). In the presence of cerulenin, the ^{32}P increased at a similar rate as ^{3}H increased in central glycerol of CL. In the absence of cerulenin, the ^{32}P increased at a similar rate as ^{3}H increased in diacylated glycerol of CL. The more direct way to dect the exchange reaction will be using a mutant lacking glycerol kinase activity. According to the relative rate of turnvoer of ^{3}H to ^{32}P of PG, phospholipase C appeared not to be involved in the rapid uptake of [2-^{3}H]glycerol. The phospholipase D activity should also be examined in B. stearothermophilus.

Chapter V.

Summary

Phosphatidylglycerol metabolism was studied in both growing cells and isolated membrane preparations of Bacillus stearothermophilus. Uptake of [2-^{3}H]glycerol was demonstrated by an exchange reaction in washed membrane and cholate-extracted membrane. Under these conditions net lipid synthesis could not occur. The optimum pH for the rapid [2-^{3}H]-glycerol uptake was obtained at pH 5.5 which is reported to be the optimum pH for cardiolipin synthetase. The cholate-extracted membrane was a delipidated membrane preparation. When exogenous PG was added to cholate-extracted membrane, the uptake of [2-^{3}H]glycerol increased as the amount of exogenous PG increased. Lipid analysis showed net CL synthesis. When exogenous CL was added in the cholate-extracted membrane, [2-^{3}H]glycerol uptake was inhibited. These findings are in agreement with the proposed cardiolipin synthesis pathway in which the phosphatidyl group of PG is transferred to an unknown acceptor (X) in the first stage, then the phosphatidyl-X intermediate reacts with one molecule of PG to form CL and release X in the second stage. The exchange reaction resulted from the reversible reaction in the first stage. The effects of different factors on exchange reaction was studied. The uptake of [2-^{3}H]glycerol was enhanced by EDTA in the cholate-extracted membrane. Divalent ions were not required for enzyme activity. However, divalent ions stimulated enzyme activity in washed membrane. This is probably due to changes in enzyme enviroment after delipidation. It is not clear why the increased amount of exogenous PG decreased the [2-^{3}H]glycerol uptake in washed membrane. Triton X-100 which has been reported to stimulate lipid syn-

thesis in cell free system inhibited the uptake of [2-^{3}H]glycerol. The inhibition of CL synthetase by Triton X-100 also been reported. The enzyme activity was also inhibited by cytosol, sn-glycero-3-P, ATP, ADP and PO_4^{-3}. It appears that the reaction does not requier energy.

In growing cells the optimum condition for the rapid uptake of label was determined by different combinations of cells and medium. The higher rates of uptake at low cell densities in the defined medium were probably due to more rapid growth and lipid synthesis at low cell density. The higher rates of uptake at high cell densities in the TYE medium appeared to be due to the consumption of glycerol or glycerol phosphate present in the TYE medium.

The mechanism of PG metabolism was studied in whole cells by dual labeled pulse-chase experiments in the absence or presence of cerulenin. After a 1.5 min pulse period 87% of the [2-^{3}H]glycerol incorporated was found in PG, 7% in PA plus DG, 4% in PE, 1% in CL. The nonacylated glycerol moiety was labeled more rapidly than the diacylated glycerol moiety in the first 0.5 min, then the label increased at the same rate in both moieties. After the addition of cerulenin which inhibited lipid synthesis, the ^{3}H uptake in PG continued for 1 min, then label was lost and CL and PE were synthesized. The ^{3}H label increase in CL accounted for 77% of the ^{3}H lost from PG and the ^{3}H label increase in PE accounted for 11% of the ^{3}H lost from PG. There was no significance increase of ^{3}H in diacylated glycerol of PG during the first min after the addition of cerulenin. The ^{3}H label increase in PG was in the nonacylated glycerol moiety of PG. The ^{3}H increased in the diglyceride moieties of CL in the presence of cerulenin, but it did not increase in the central glycerol

2 min.

The turnover of PG was biphasic. It appeared that two different PG pools could be observed during 10 min chase. A small pool turned over rapidly, the other pool was very stable. The nonacylated glycerol of PG turned over slightly faster than the diacylated glycerol moiety of PG, which indicated that both exchange reaction and degradation by whole cells contributed to the turnover of PG, and the exchange reaction only accounted for the minor one. In the presence of cerulenin, these two reactions could be distinguished. The $^{3}H/^{32}P$ ratio remained constant in diacylated glycerol moiety of PG, but ^{3}H turned ovef faster than ^{32}P did in nonacylated glycerol in the early chase period. The turnover rate of ^{3}H and ^{32}P revealed that exchange reaction did occur. It appeared that the [2-^{3}H]glycerol uptake also occurred via the regular biosynthetic pathway.

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