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Metabolism of Phosphatidylglycerol, Phosphatidylethanolamine, and Cardiolipin of *Bacillus stearothermophilus*

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The total phospholipid content of *Bacillus stearothermophilus* was constant during exponential growth, increased during the transition from the exponential to stationary phase of growth, and then slowly increased during the stationary phase. The first increase was a result of an increase in phosphatidylethanolamine; the second was a result of an increase in cardiolipin. Cessation of aeration of an exponentially growing culture or suspension in a nongrowth medium resulted in an immediate reduction in the rate of total phospholipid and phosphatidylethanolamine synthesis and a quantitative conversion of phosphatidylglycerol to cardiolipin. Cardiolipin appeared to be synthesized by the direct conversion of two molecules of phosphatidylglycerol to cardiolipin. After a 20-min pulse of ^{32}P, phosphatidylglycerol showed the most rapid loss of ^{32}P followed by cardiolipin, whereas phosphatidylethanolamine did not lose ^{32}P. The loss of ^{32}P from the total lipid pool, phosphatidylglycerol, and cardiolipin was biphasic, with rapid loss during the first two bacterial doublings followed by a greatly reduced rate of loss. The major loss of ^{32}P from the total phospholipid pool appeared to be by breakdown of cardiolipin. The loss of ^{32}P from the lipid pool was energy dependent (i.e., did not occur under anaerobic conditions or in the absence of an energy source) and was dependent on some factor other than the concentration of cardiolipin in the cells. The apparent conversion of phosphatidylglycerol to cardiolipin was independent of energy metabolism. Chloramphenicol reduced the rate of turnover of both phosphatidylglycerol and cardiolipin. The rate of lipid synthesis (all phospholipid components) was constant for about 10 min after the addition of chloramphenicol but diminished markedly after 20 min. Turnover of ^{32}P incorporated into phospholipid during a 30-min period prior to the addition of chloramphenicol was more rapid after the removal of chloramphenicol than that of ^{32}P incorporated during a 30-min period in the presence of chloramphenicol.

Studies of membrane systems (especially mitochondria) of eukaryotic cells about 10 years ago suggested that membrane phospholipid might play more than a strictly structural role in membrane function (13, 30). Attempts to define specific phospholipid functions in bacterial membranes include investigations of the influence of growth conditions, metabolic inhibitors, and membrane modification on phospholipid composition and patterns of metabolism. Although for the most part details of the nature of phospholipid involvement in membrane function remain obscure, it is apparent that conditions which modify the membrane or some aspect of membrane metabolism also influence phospholipid composition or metabolism (6, 13). Likewise, conditions which influence phospholipid composition appear to influence membrane function (11, 16, 17, 45). The complexity of phospholipid metabolism is evident from several studies, especially the elegant and comprehensive investigations by White and his co-workers of *Staphylococcus aureus* (12, 17, 29, 31, 32) and *Haemophilus parainfluenzae* (25, 26, 38, 40, 43).

The major phospholipids of *Bacillus stearothermophilus* 2184 are phosphatidylglycerol (PG), phosphatidylethanolamine (PE),

and cardiolipin (CL) (4). In this paper we report the rapid conversion of PG to CL in cells under anaerobiosis or suspended in a nongrowth buffer medium. The conversion of PG to CL appears to be a nonenergy-requiring reaction involving the direct conversion of two molecules of PG to one of CL. Evidence is also provided which suggests that the loss of ^{32}P from the lipid pool occurs primarily by the breakdown of CL and is dependent on some metabolic reaction and not upon the concentration of CL in the membrane. As has been reported for *Escherichia coli* (1), *S. aureus* (31, 32), and *H. parainfluenzae* (26), there appeared to be two metabolic pools of PG and CL in *B. stearothermophilus*.

MATERIALS AND METHODS

Growth of B. stearothermophilus. Unless indicated otherwise, *B. stearothermophilus* (NCA 2184) was grown in medium consisting of 20 g of Trypticase (BBL) and 10 g of yeast extract (Difco) per liter of water. For large batches and to study lipid changes during growth, the cultures were grown in a fermentor (New Brunswick model MF 114) at 60 C. Smaller volumes were grown in 2-liter baffled flasks (200–500 ml/flask) or 250-ml baffled flasks (50 ml/flask) in a gyratory incubator shaker (New Brunswick model G-25) at 60 C. Cells were harvested by pouring the culture into a container containing an equal volume of crushed ice and separated by centrifugation.

For studying the turnover of lipid phosphorus, an exponentially growing culture at an optical density of 0.4 (at 600 nm) was routinely added to an equal volume of preheated (60 C) medium containing 0.25 mCi of $H_3{}^{32}PO_4$ per 100 ml and incubated at 60 C for one bacterial doubling (usually 20–30 min). When cells were labeled for longer periods of time, the size of the inoculum was reduced so that the optical density of the medium containing ^{32}P never exceeded 0.4 at the time the cells were harvested. After removal of the cells from the labeled medium by centrifugation, the culture was suspended to an optical density of 0.1 (or less) and incubation continued at 60 C. All samples were collected on an equal volume of crushed ice, centrifuged, and washed once with cold 0.15 M NaCl.

Anaerobic conditions were obtained by pouring the culture into Erlenmeyer flasks of just sufficient volume to hold the culture, stoppering the flask, and incubating in a 60-C water bath. At cell densities above 0.1 mg/ml (optical density of 0.2), this resulted in an almost immediate cessation of growth (see Fig. 3, 7). The oxygen concentration measured 0.5 cm below the surface of the medium with an oxygen monitor (YSI model 53) fell from 85% (water at 60 C, 100%) to 0% in less than 1 min after cessation of aeration.

Lipid extraction. The lipids were extracted by the following modification of the Bligh and Dyer (3) procedure. The cells were suspended in 10 ml of aqueous 0.3% NaCl and added to 100 ml of methanol, and the suspension was refluxed over a boiling-water bath for 5 min. The suspension was then cooled and 50 ml of chloroform and 30 ml of 0.3% NaCl were added to give final concentrations of chloroform-methanol-water of 1:2:0.8. This single-phase suspension was stirred at room temperature for at least 2 h and was then filtered and collected in a separatory funnel. Chloroform and 0.3% NaCl were added to give final concentrations of chloroform-methanol-water of 2:2:1.8, and the two phases were allowed to separate. The lipid extracts were then taken to dryness, resuspended, and stored as described previously (4). Refluxing the suspensions in methanol was necessary for quantitative extraction of the cardiolipin (Table 1).

Deacylation. The phospholipids were deacylated by mild alkaline methanolysis at 0 C (42).

Chromatography. The glycerol phosphate esters obtained by deacylation were separated by chromatography in two dimensions on Whatman no. 1 paper previously washed with 2 N acetic acid. The chromatograms were run for 28 cm in the first dimension by using butanol-acetic acid-water (5:3:1, vol/vol) and for 14 cm in the second dimension by using phenol-0.1 N ammonium hydroxide (100:38, wt/vol). The separated esters were located by autoradiography or with the molybdate spray as described previously (4). A typical autoradiogram of phosphate esters obtained from cells grown in ^{32}P for nine bacterial doublings is shown in Fig. 1.

The diacyl phospholipids were separated by ascending chromatography in two dimensions on silica gel-impregnated paper (Whatman SG-81) by using chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4, vol/vol) in the first dimension and chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride buffer, pH 10.4 (60:35:50:70:12, vol/vol) in the second dimension (43, 46). The separated phospholipids were located by autoradiography with X-ray film (Kodak no-screen) or with the molybdate spray described by Vaskovsky and Kostetsky (41). The separated phospholipids or glycerol phosphate esters were cut from the chromatogram (spot plus 0.5-cm border) and analyzed for radioactivity or phosphorus, or both.

Analytical procedures. Radioactivity was determined in a scintillation spectrometer (Nuclear-Chicago Unilux III) by using a fluor consisting of

TABLE 1. *Effect of heating on the extraction of phospholipids from Bacillus stearothermophilus*[a]

Procedure	Total lipid P	CL	PG	PE
A	60.7[b]	29.6	8.13	22.7
B	47.5	18.8	6.63	22.0

[a] Cells were harvested in the stationary phase of growth, washed with 0.15 M NaCl, and then divided into two equal portions of 206 mg (dry weight). Extraction procedures: A, cells were suspended in 10 ml of 0.3% NaCl added to 100 ml of methanol and then refluxed over a boiling-water bath for 5 min. The suspension was then cooled and extracted as described in the text. B, Same as in A except that the suspension was not heated.

[b] Results are given in micromoles of phosphorus per gram (dry weight).

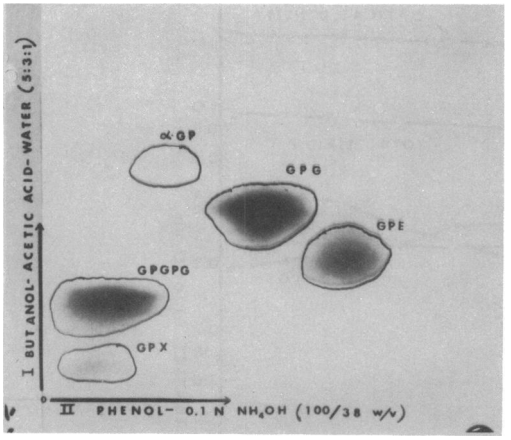

FIG. 1. *Autoradiogram of glycerol phosphate esters of phospholipids from B. stearothermophilus grown for nine bacterial doublings in medium containing* $H_3{}^{32}PO_4$. *Exponentially growing cells were harvested on ice at an optical density of 0.4 at 600 nm. The chromatogram was run for 28 cm in the first dimension and 14 cm in the second dimension with the solvent systems shown. The following abbreviations are used for the glycerol phosphate esters obtained by deacylation of the phospholipids: GPG, glycerolphosphorylglycerol from PG; GPGPG, diglycerolphosphorylglycerol from CL; GPE, glycerolphosphorylethanolamine from PE; α GP, glycerolphosphate from phosphatidic acid (PA); and GPX, a glycerolphosphate ester from an as yet unidentified phospholipid (PX).*

0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene dimethyl ester, and 0.3% 2,5-diphenyloxazole in toluene. After digestion of the samples with 70% perchloric acid, phosphorus was determined as previously described (4). To facilitate digestion of papers cut from chromatograms developed by autoradiography, 0.2 ml of the molybdate spray used for the Whatman no. 1 papers was added prior to the addition of perchloric acid (10). For phosphorus determinations, samples from silicic acid-impregnated papers were centrifuged to remove the silicic acid, after color development and before readings were made in the spectrophotometer (Coleman Jr. II). Specific activities were determined from separated phosphate esters cut from the Whatman no. 1 chromatograms. After determination of radioactivity, the papers were removed from the scintillation vials, rinsed with toluene, and then digested with 70% perchloric acid and phosphorus was determined. No detectable radioactivity remained in the scintillation vials after the paper was removed.

Materials. Tris(hydroxymethyl)aminomethane (Tris) and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo. Scintillation fluors were purchased from Packard Instrument Co., Downers Grove, Ill. $H_3{}^{32}PO_4$ was purchased from New England Nuclear Corp., Boston, Mass. Phospholipid standards and other materials were prepared or obtained as previously described (4).

RESULTS

Phospholipid composition during growth of B. stearothermophilus. During the exponential phase of growth, the concentration of total lipid phosphorus remained constant at about 50 μmol per g (dry weight) of cells (Fig. 2b). In the stationary phase (60–75 min in the experiment illustrated in Fig. 2), there was an increase in the total phospholipid to about 65 μmol of lipid phosphorus (Fig. 2b). A second increase occurred in the stationary phase to about 70 to 75% μmol of lipid phosphorus per g (dry weight). As can be seen in Fig. 2b, the first increase which occurred during the transition from exponential growth to the stationary phase was the result of an increase in the concentration of PE. It appeared that, as the growth rate decreased, the rate of PE synthesis continued at the exponential rate until growth had almost stopped (Fig. 2a). The second increase in total phospholipid which occurred during the stationary phase was the result of an increase in CL. After the culture had entered the stationary phase (about 135 min in Fig. 2a), the concentration of PG decreased, whereas PE remained constant. The slow decrease in PG and increase in CL continued for several hours (data not shown) if aeration was continued; however, if aeration was stopped there was a rapid decrease in PG and corresponding increase in CL (Fig. 3).

Effect of anaerobiosis on phospholipid composition. At cell densities above 0.1 mg (dry weight) per ml (optical density of 0.2), cessation of aeration resulted in an abrupt decrease in the growth rate (Fig. 3a) and a corresponding decrease in the rate of total phospholipid synthesis (data not shown, but see Fig. 7). When aeration was stopped, the relative concentration of PG decreased rapidly, with a corresponding increase in CL concentration (Fig. 3b). There was no change in the relative concentration of PE. Resumption of aeration resulted in a resumption of growth at a rate comparable to that prior to the period of anaerobiosis. There was no appreciable lag in growth if the period of anaerobiosis did not exceed about 30 min. After resumption of growth the relative concentrations of PG increased and CL decreased until each reached the steady-state levels of 26.0 ± 2 μmol of PG and 10.0 ± 1.5 μmol of CL phosphorus per g (dry weight) of cells, characteristic of exponential growth under these conditions.

A similar change in composition was observed when washed cells were suspended in a non-growth medium consisting of 0.01 M Tris-hydrochloride (pH 7.0), 0.01 M $CaCl_2$, and 0.15 M NaCl, and incubated at 60 C. There was no

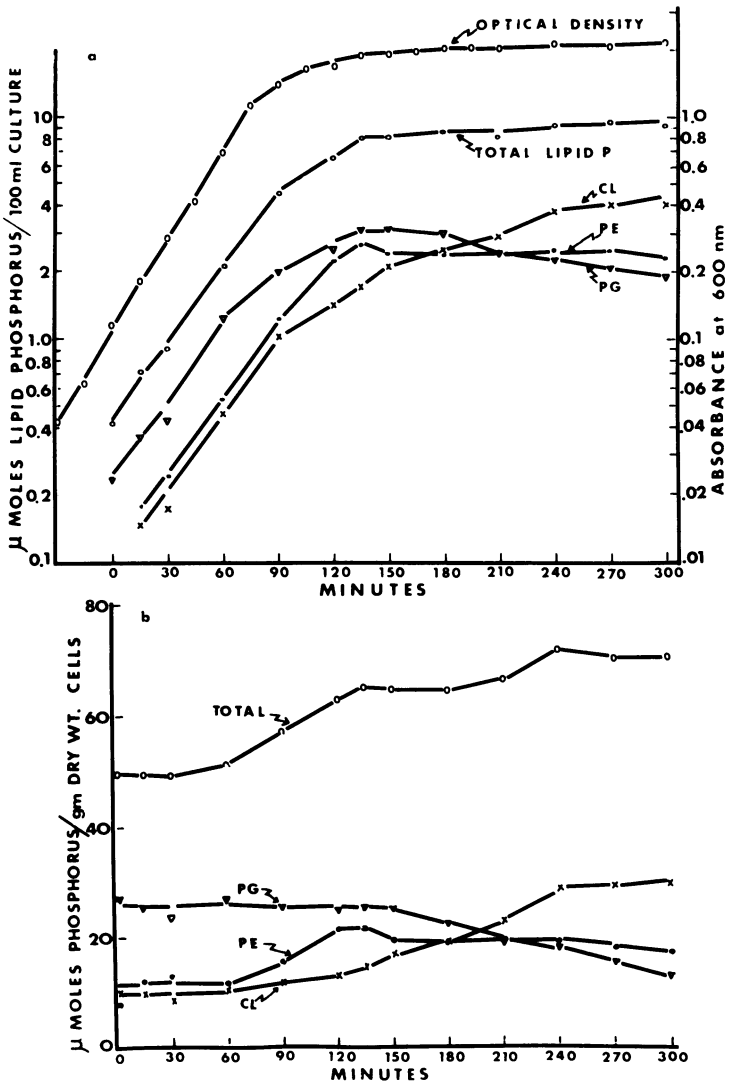

FIG. 2. *Phospholipid composition during growth of B. stearothermophilus. a, Increase in cell density, total phospholipid* (O), *CL* (X), *PE* (●), *and PG* (∇) *during growth in the fermentor. b, Concentration of total phospholipid* (O) *and relative concentrations of CL* (X), *PE* (●), *and PG* (∇) *per gram (dry weight) of whole cells. Fermentor (containing 5 liters of medium) was inoculated with 500 ml of an exponentially growing culture (an optical density of 0.25 at 600 nm) and aerated at a rate of 10 liters of air per min (drive rate 800 rpm). Glycerol phosphate esters obtained by methanolysis were separated as in Fig. 1.*

increase in total lipid phosphorus or PE under these conditions (Fig. 4). On the other hand, the initial rates of decrease in the relative concentration of PG and corresponding increase in the relative concentration of CL were comparable to the rates observed in the growing culture when aeration was stopped. Since there was no increase in total lipid phosphorus and the PG and CL conversion took place in the absence of an energy source, it appeared that CL was synthesized by the direct conversion of two molecules of PG to one CL.

Turnover of ^{32}P in phospholipids. The turnover pattern after a 20-min pulse of $H_3{}^{32}PO_4$ is shown in Fig. 5. This culture was diluted before and after the pulse of ^{32}P, to maintain exponential growth. Under these conditions PG had the highest rate of loss of ^{32}P during the first two bacterial doublings after removing the ^{32}P. CL showed an initial increase in ^{32}P and then lost

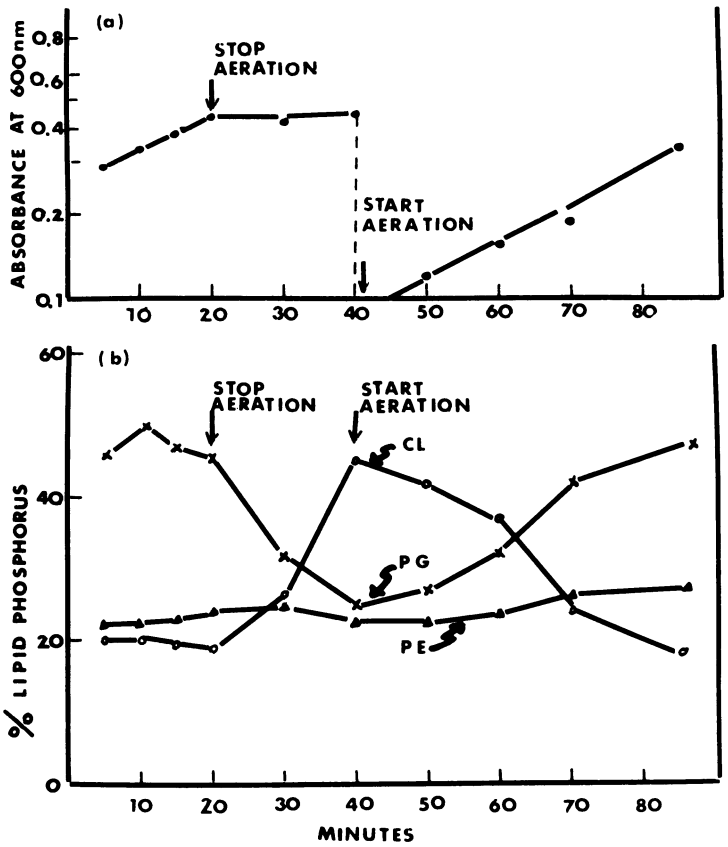

FIG. 3. *Changes in phospholipid composition during a 20-min period of anaerobiosis. a, Changes in cell density, b, Percentage of composition of PG (X), CL (O), and PE (Δ). Arrows indicate times at which aeration was stopped and resumed. Culture was diluted immediately before resumption of aeration to maintain exponential growth.*

^{32}P, whereas PE showed no turnover of ^{32}P. The biphasic nature of the turnover patterns reported for other organisms (1, 32, 40) was apparent in the loss of ^{32}P from PG and CL. The relative concentration and rates of synthesis of PE, PG, and CL remained constant throughout the time period shown in Fig. 5, which suggested that there were two metabolic pools of PG and perhaps CL.

Turnover of lipid ^{32}P in nongrowth medium. One possible explanation for the biphasic pattern of turnover would be that only a specific portion of the PG pool served as a precursor for CL synthesis, whereas the remainder was metabolically stable. If this were the situation, then we expected to see a difference in the rate of conversion of ^{32}P of PG to CL in cells which were suspended in the nongrowth medium at different times after removing the $H_3{}^{32}PO_4$ from the growth medium. This was examined in the experiment illustrated in Fig. 6. In this experiment, cells were grown in the presence of $H_3{}^{32}PO_4$ (0.25 mCi per 100 ml) for nine generations, centrifuged, suspended in preheated medium, and incubation was continued. Samples were removed at 0, 30, and 60 min after the chase, centrifuged, and suspended in the nongrowth medium. The suspensions were then incubated at 60 C and samples were collected on ice for lipid analysis. After removal of the ^{32}P, the rate of phospholipid synthesis remained constant in the growth medium (Fig. 6b) as did the relative concentrations of the individual phospholipids (Fig. 6d). The biphasic turnover pattern for PG was apparent (Fig. 6c) but less pronounced than observed with the 20-min pulse shown in Fig. 5. During the incubation period in the nongrowth suspensions for all three samples (0, 30, and 60 min after the chase), there was no loss of ^{32}P from the total lipid fraction and no increase in total lipid phosphorus. The patterns of conversion of total

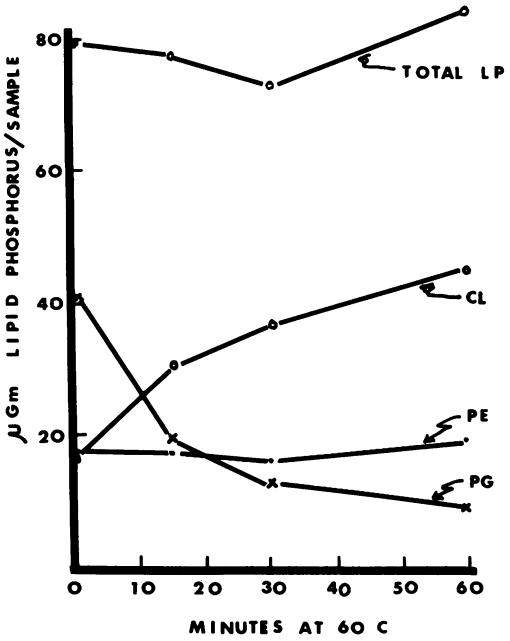

FIG. 4. *Changes in phospholipid composition during incubation in a nongrowth medium. Exponentially growing cells were harvested on ice, washed once with cold 0.15 M NaCl, and suspended in a medium consisting of 0.01 M Tris-hydrochloride buffer (pH 7.0), 0.01 M $CaCl_2$, and 0.15 M NaCl. Lipids were separated by chromatography on silicic acid-impregnated papers (Whatman SG-81) as described in Materials and Methods.*

PG to CL in the nongrowth medium were identical for the 0-, 30-, and 60-min samples and followed the pattern shown in Fig. 6e for the conversion of ^{32}P PG to ^{32}P CL for the 0-time sample. At this time the specific activity of all phospholipids was the same (9.9×10^4 counts/min of ^{32}P per μmol of P) and remained unchanged throughout the incubation period in the nongrowth medium. In contrast to what was expected, rate of loss of ^{32}P from PG for the 0-, 30-, and 60-min samples was about the same (Fig. 6e, f, g). If the ^{32}P were concentrated in a pool of PG which did not serve as a precursor to CL in the 30- and 60-min samples, there should have been a marked increase in the specific activity of the PG during incubation in the nongrowth medium. No such increase was observed. The specific activity of PG in the 30- and 60-min samples remained constant at 1.11×10^4 and 0.28×10^4 counts/min of ^{32}P per μmol of P, respectively, throughout the incubation period in the buffer suspension. As expected, the specific activity of CL approached that of the PG during the incubation period. The biphasic pattern of PG turnover was apparently not the result of a pool of PG which could not be converted to CL at least under these conditions.

The rate of loss of ^{32}P from PG under the nongrowth conditions (Fig. 6e) was about the same as the rate of loss during exponential growth (Fig. 6c). This suggested that the loss of ^{32}P from the total phospholipid fraction occurred primarily by way of the breakdown of cardiolipin. Under anaerobic conditions or in the nongrowth medium, this reaction(s) was blocked, leading to the accumulation of CL while the conversion of PG to CL proceeded at a constant rate independent of these conditions. This led us to question whether the rate of loss of ^{32}P from the total lipid pool was dependent on the concentration of CL in the membrane.

Turnover of ^{32}P after a period of anaerobiosis. In the experiment illustrated in Fig. 7, an exponentially growing culture was inoculated into preheated medium containing 0.25 mCi of $H_3{}^{32}PO_4$ per 100 ml and incubated for 20 min aerobically, followed by a 20-min period of anaerobiosis. The ^{32}P was then removed by centrifugation, the culture was suspended in unlabeled medium, and incubation was continued with aeration.

During the period of exponential growth, PG showed the most rapid uptake of ^{32}P, followed by PE and CL (Fig. 7c). After the shift to anaerobiosis there was an abrupt cessation of uptake of ^{32}P in the total phospholipid fraction and in the PE fraction. PG lost ^{32}P with no change in specific activity, and CL synthesis continued during the period of anaerobiosis. At the time the ^{32}P was removed and aeration resumed, the cells contained 14.2 μmol of PG and 19.7 μmol of CL phosphorus per g (dry weight), compared to 27.0 and 10.3 μmol of phosphorus per g (dry weight) of PG and CL, respectively, for a comparable experiment without the period of anaerobiosis (Fig. 5). It is apparent from a comparison of Fig. 5b and 7b that an increase in the relative concentration of CL in the cells did not lead to an increase in the rate of loss of ^{32}P from the total phospholipid fraction. In fact, in the first 30-min period after the chase, the cells with high CL concentration lost 29.5% of the total lipid ^{32}P (Fig. 7b) whereas cells with a low CL concentration (Fig. 5) lost 44.9% of the total lipid ^{32}P. The increase in PG ^{32}P observed in the first few minutes after the chase (Fig. 7c) suggested that PG might come from the CL. The products of CL turnover have not yet been characterized in this organism, but there was no increase in phosphatidic acid (PA) which remained constant at about 0.6 to 1.0% of

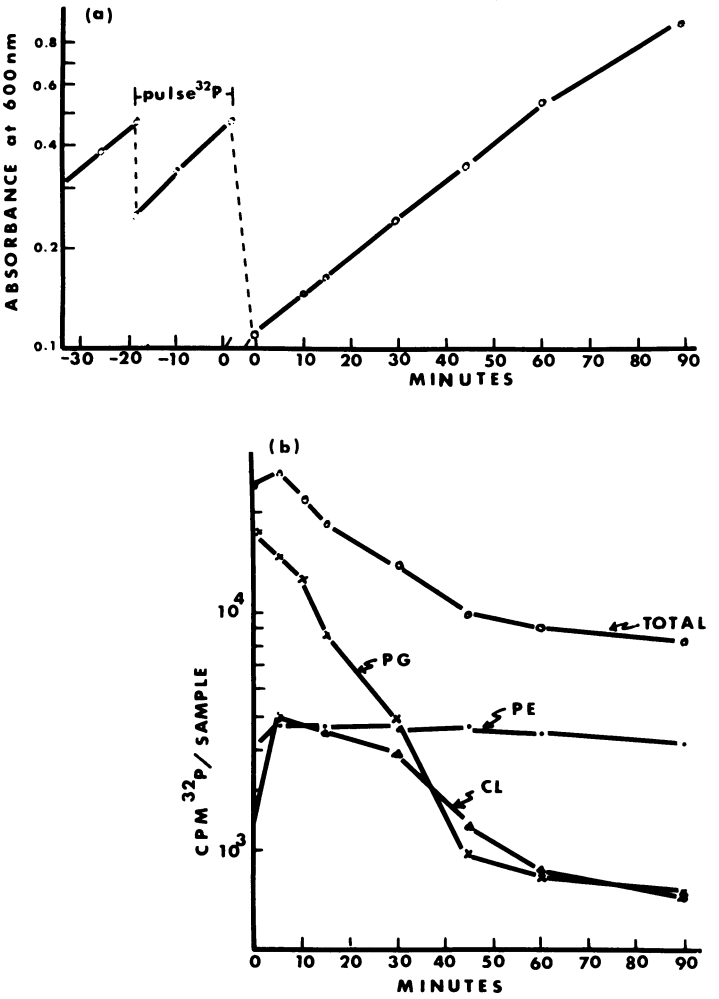

FIG. 5. *Turnover of phospholipid ^{32}P after a 20-min pulse of ^{32}P. Dotted lines indicate times at which culture was diluted to maintain exponential growth. A 200-ml amount of an exponentially growing culture was added to 200 ml of heated (60 C) medium containing 0.5 mCi of $H_3{}^{32}PO_4$. After incubation for 20 min the ^{32}P was removed by centrifugation and the cells were suspended in 1,600 ml of preheated medium (this step routinely required about 20 min). Lipids were separated as shown in Fig. 1. a, Cell density. b, Radioactivity of total lipid (O), PG (X), PE (●), and CL (Δ).*

the total lipid phosphorus) and no significant change in the specific activity of the PA fraction during the first 30 min after the chase in the experiment illustrated in Fig. 7. If the CL breaks down to PG plus PA, the PA must be very rapidly metabolized. The increase in ^{32}P in the PE fraction (Fig. 7c) might have come from PA released on CL breakdown.

Effect of chloramphenicol on ^{32}P uptake and loss. Studies by a number of investigators using different membrane systems have led to the suggestion that the bulk of the membrane lipid is present in a lipid-lipid phase (presumably a bilayer), but that a portion of the lipid may be closely associated with membrane protein (6, 34, 44). Differences in metabolism of lipid-lipid and lipid-protein-associated phospholipid might account for the complex metabolic patterns observed.

We attempted to examine this possibility by studying metabolic patterns of ^{32}P incorporated into phospholipid prior to the addition of chloramphenicol and ^{32}P incorporated in the presence of chloramphenicol. In the experiment shown in Fig. 8, an exponentially growing culture was divided into two 400-ml portions. To one portion (Fig. 8a), 0.5 mCi of $H_3{}^{32}PO_4$ was added and incubation was continued for 30 min.

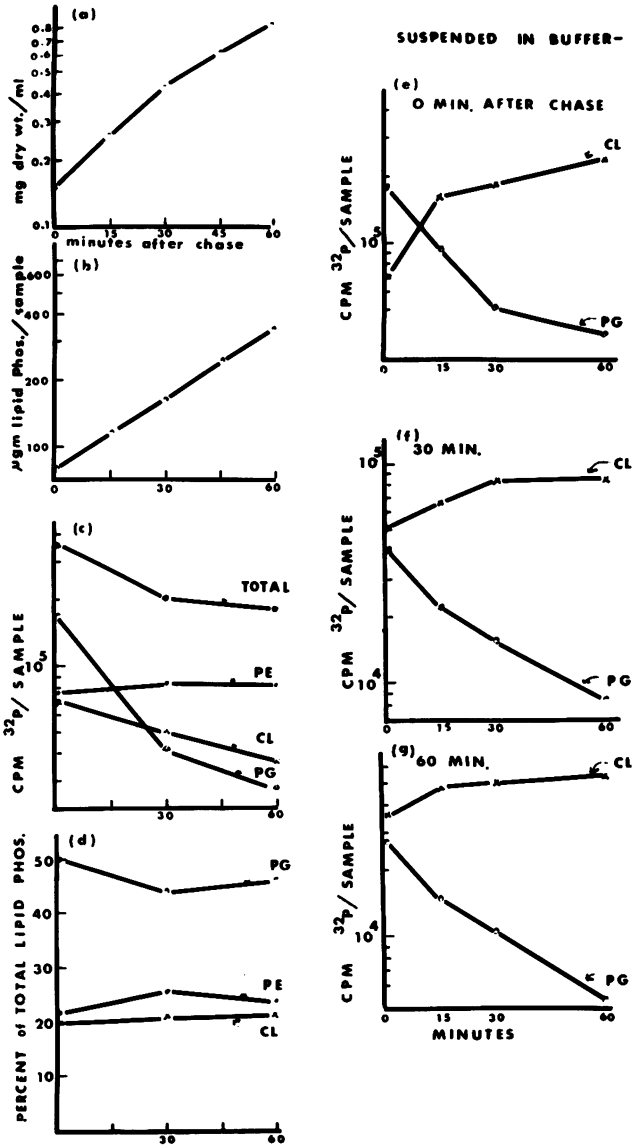

FIG. 6. *Turnover of PG and CL phosphorus in exponentially growing cells suspended in nongrowth medium 0, 30, and 60 min after removal of ^{32}P. A 500-ml culture was grown for nine generations in medium containing 0.5 mCi of $H_3{}^{32}PO_4$. The ^{32}P was removed, the cells were suspended in 1,500 ml of fresh preheated medium, and incubation was continued. At 0, 30, and 60 min after removal of the ^{32}P, 500-ml samples were collected on ice, centrifuged, and the cells were suspended in the nongrowth medium described in Fig. 4. Samples were collected and analyzed as in Fig. 1. a, Cell density; b, increase in total lipid phosphorus; c, turnover of ^{32}P of PE (●), CL (X), and PG (▽) in the growing culture; d, relative concentration of PG (○), PE (●), and CL (X) in the growing culture; e, f, g, the conversion of ^{32}P of PG to CL in the nongrowth cell suspension for cells removed from the growth medium 0, 30, and 60 min after removal of the ^{32}P. The conversion of total PG to CL followed a pattern identical to Fig. 6e for the 30- and 60-min samples.*

Both portions were then centrifuged and suspended in fresh medium containing 40 μg of chloramphenicol per ml. After 2 min, 0.5 mCi of $H_3{}^{32}PO_4$ was then added to the second portion (Fig. 8c) and incubation was continued for 30 min. Both cultures were then centrifuged and resuspended in 1,200 ml of fresh medium. After the addition of chloramphenicol, there was a rapid reduction in growth rate (Fig. 8a and 8c). Lipid synthesis continued for about 10 min after

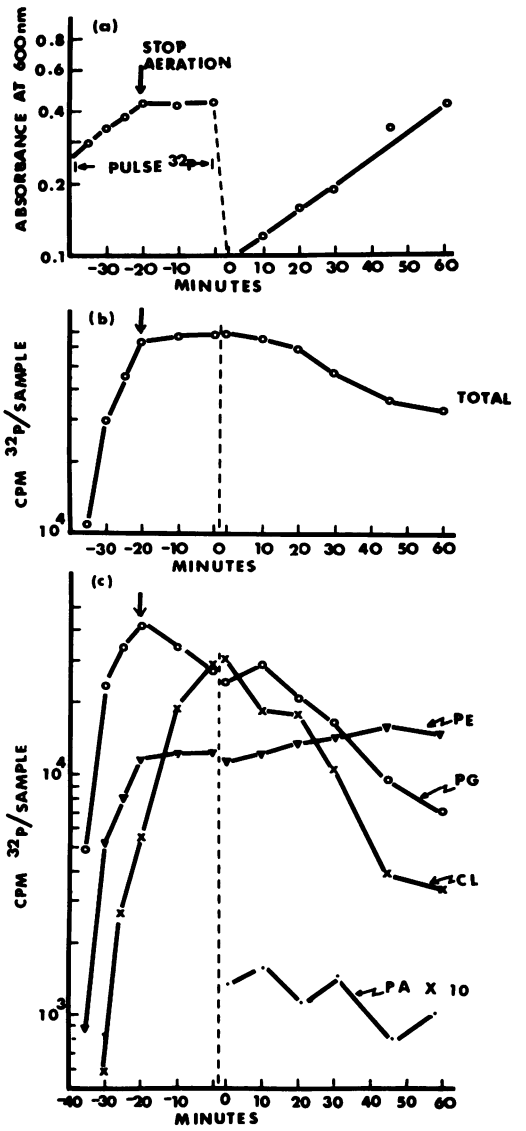

FIG. 7. *Turnover of lipid phosphorus after a short period of anaerobiosis. Experiment was the same as in Fig. 5 except that culture was held anaerobically for 20 min after 20 min of exponential growth in the medium containing ^{32}P. a, Cell density; b, uptake and subsequent turnover of total phospholipid phosphorus; c, uptake and subsequent turnover of PE (∇), PG (○), CL (X), and PA (●). PA values were multiplied by 10 for convenience in graphing.*

the addition of chloramphenicol but decreased markedly after 20 min (Fig. 8d). Loss of ^{32}P from PG and CL occurred but at a reduced rate in the presence of chloramphenicol (Fig. 8b). After removal of the chloramphenicol, the rate of turnover increased to a rate comparable to that of an untreated culture (compare to Fig. 5) if the culture had been labeled prior to the addition of chloramphenicol (Fig. 8b) but at a rate slower than that of an untreated culture if the culture had been labeled in the presence of chloramphenicol (Fig. 8d). Throughout the time periods involved, with both cultures, the relative concentrations of PG, PE, and CL remained constant. It appeared from the experiment illustrated in Fig. 8 that the ^{32}P incorporated prior to the addition of chloramphenicol turned over at a faster rate than ^{32}P incorporated in the presence of chloramphenicol. Whether this difference was the result of the state of the lipid incorporated into the membrane in the presence of chloramphenicol or some other factor such as the time at which the lipid was incorporated was not established.

DISCUSSION

The phospholipid composition of *B. stearothermophilus* is similar to that of other *Bacillus* species (2, 18, 21) except for a higher concentration of cardiolipin and an apparent lack of amino acid derivatives of PG (4). The rate of synthesis of PE was independent of the growth rate (increase in total cell dry weight) and of the rate of PG and CL synthesis as the cells shifted from the exponential to the stationary phase of growth (Fig. 2). Synthesis of PE continued at the exponential rate until growth had almost stopped (Fig. 2a), resulting in about a doubling of the PE concentration in the cells. Similar changes in PE concentrations during this transition period were reported for *E. coli* (8), but were not as pronounced as reported here for *B. stearothermophilus*. After the culture had entered the stationary phase, there was no further synthesis of PE, whereas the synthesis of CL continued. It appears that PE synthesis responds to a different regulatory mechanism than PG and CL synthesis. Ballesta and Schaechter (1) concluded from studies of lipid metabolism after shift-down or the addition of inhibitors to cultures of *E. coli* that PE metabolism was related to growth and cell division, whereas PG and CL metabolism were related to membrane metablism.

The decrease in PG and increase in CL concentrations in the stationary phase of growth (Fig. 2) follows the pattern observed in several other bacteria (5, 28, 29, 32). This is not a universal phenomenom, since Morman and White (21) found that CL decreased and PG increased in the stationary phase in *B. licheniformis*. Transferring the culture of *B. stearothermophilus* to anaerobic conditions or to a nongrowth buffer medium resulted in an

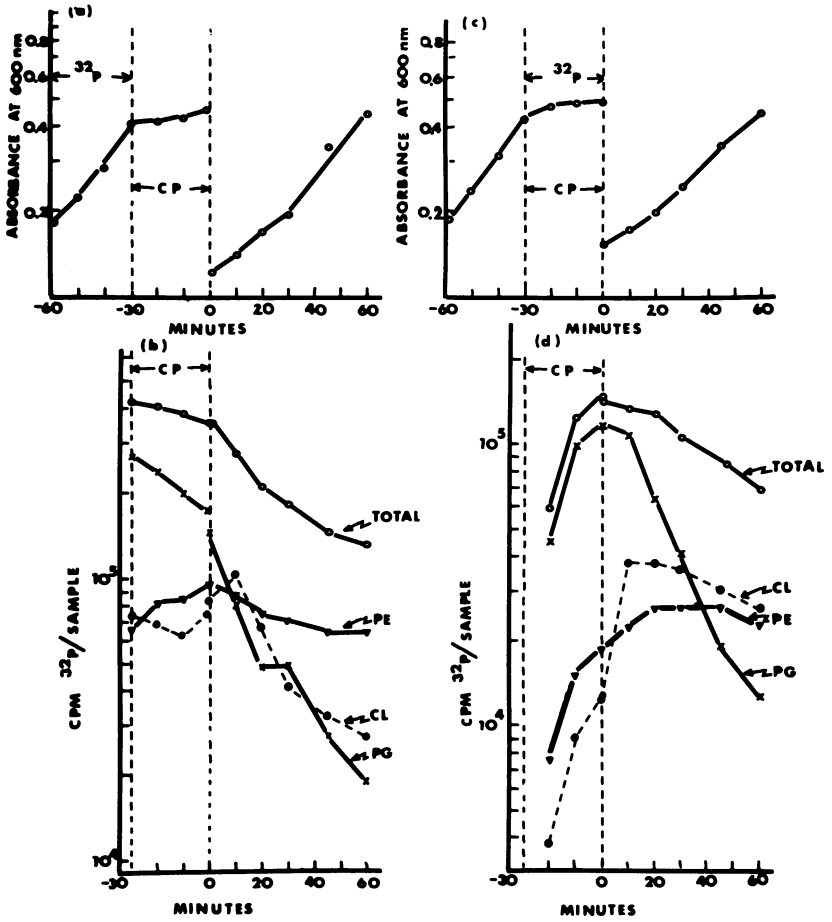

FIG. 8. *Effect of chloramphenicol on uptake and turnover of lipid phosphorus. An exponentially growing culture was divided into two portions. $H_3{}^{32}PO_4$ (0.5 mCi) was added to one portion (a and b), and incubation (of both) continued for 30 min. Both cultures were then centrifuged and suspended (20-min delay) in fresh medium containing 40 μg of chloramphenicol (CP) per ml. After 2 min of incubation, 0.5 mCi of $H_3{}^{32}PO_4$ was added to the second portion (c and d). After 30 min in medium containing CP, both cultures were centrifuged and resuspended in 1,200 ml of fresh medium. All samples were collected on ice and analyzed as in Fig. 1. a and c, cell densities of cultures labeled before and after the addition of chloramphenicol; b and d, the uptake and subsequent loss of ^{32}P from the total lipid (○), CL (●), and PE (▽), and PG (X).*

abrupt cessation of increase in total phospholipid, a rapid decrease in PG, and an increase in CL concentration (Fig. 3). These observations and the results illustrated in Fig. 6, where the rate of PG to CL conversion under nongrowth conditions was comparable to normal aerobic growth conditions, suggested that the rate of PG conversion to CL was relatively constant and that the ratio of the two lipids at any given point was dependent primarily on the rate of turnover of CL. Conditions which have been reported to effect alterations in PG and CL composition in other bacteria include infection by bacteriophage (23, 27), treatment with inhibitors (1, 22, 26) and antibiotics (37), anaerobiosis (1), temperature shifts (8, 24), exposure of sodium-sensitive mutant of *E. coli* to sodium (19) and starving fatty acid (14) or glycerol (29) auxotrophs for their respective requirements. The accumulation of CL in the membrane had a marked influence on the heat stability of whole cells (Mosley, Card, and Koostra; manuscript in preparation) and the osmotic stability of lysozyme-prepared protoplasts (Card, Mosley, and Koostra; manuscript in preparation) of *B. stearothermophilus*.

The synthesis of CL from two molecules of PG rather than by the reaction involving cytidine 5'-diphosphate (CDP)-diglyceride (36) was suggested from the results illustrated in Fig. 3, 4, 5,

and 7. After cessation of aeration (Fig. 3, 7) or suspension in a medium lacking a metabolizable energy source (Fig. 4), PG was quantitatively converted to CL with no increase in total lipid phosphorus (Fig. 4, 7) and no uptake of ^{32}P (Fig. 7). Synthesis of CL from two molecules of PG has been demonstrated for *S. aureus* (33), *E. coli* (15), and *M. lysodeiktikus* (9). The reaction has been best characterized by Short and White (33) by using a membrane fraction from *S. aureus*. Hostetler et al. (15) reexamined CL synthesis in membranes of *E. coli* and mitochondria and found that, in mitochondria, CL was synthesized primarily by the PG plus CDP-diglyceride pathway and, in *E. coli*, by coalescence of two molecules of PG with release of glycerol. They did report, however, that at high concentrations of CDP-diglyceride, CL synthesis was stimulated in *E. coli*, suggesting that both mechanisms might be present. At the present time the synthesis of CL appears to be basically different in eukaryotic and prokaryotic cells.

After a short pulse of ^{32}P, PG showed the highest rate of loss of ^{32}P followed by CL, whereas PE did not turn over in exponentially growing cells (Fig. 5). If the concentration of CL was increased with a corresponding decrease in PG concentration by a period of anaerobiosis, CL showed the most rapid turnover (Fig. 7). The loss of ^{32}P from the total lipid pool appeared to occur primarily by the reaction(s) involving CL breakdown, because the rate of loss of ^{32}P from PG in an exponentially growing culture was identical to the rate of loss for cells suspended in a nongrowth medium where there was no loss of ^{32}P from the total lipid pool (Fig. 6).

Two metabolic pools of PG were evident from the biphasic patterns of turnover (Fig. 5, 6). As was observed by Short and White (32), this pattern of loss of ^{32}P was more pronounced at shorter labeling times. At least three factors might be responsible for these metabolic patterns: (i) a heterogenous membrane system where lipids in different portions of the membrane are metabolized at different rates; (ii) a difference in the rates of metabolism of lipid associated with protein or specific membrane protein and lipid in the bulk lipid phase; and (iii) cyclic interconversion of PG and CL and asymmetric metabolism of CL.

The membranes of several bacteria have been separated into fractions with different lipid compositions (7, 38, 39). If newly synthesized PG were incorporated into different portions of a heterogenous membrane system, then one portion might contain the enzymes responsible for turnover (presumably CL-synthetase), whereas in another portion the PG may be metabolically stable or show a low rate of turnover. Depending on the size of the membrane fractions involved and the amount of PG incorporated into each fraction during the labeling period, different patterns of turnover would be expected. This possibility does not seem likely, considering the observation that cells which were transferred to the nongrowth medium showed about the same rate of ^{32}P-PG to ^{32}P-CL conversion (70% per 30 min) regardless of whether they were transferred during the period of rapid loss of ^{32}P from PG (Fig. 6e) or during the phase of slow loss of ^{32}P from PG (Fig. 6 f, g).

The second possibility is suggested by the conclusion of Wilson and Fox (44), based on studies of β-galactoside transport in fatty acid auxotrophs of *E. coli*, that newly synthesized protein was incorporated into the membrane with newly synthesized lipid. If a portion of the PG were associated with protein and had a higher (or lower) rate of metabolism than the bulk of the PG in the lipid phase, one might see biphasic patterns. Although it was observed that the loss of ^{32}P from the total lipid pool was slower for lipid synthesized in the presence of chloramphenicol than for lipid synthesized prior to the addition of chloramphenicol (Fig. 8b, d), the significance of this observation is questionable. We have no information on either lipid-lipid or lipid-protein associations in *B. stearothermophilus*. The continued synthesis of phospholipid for some time after the addition of chloramphenicol has been reported by other workers (1, 20, 35). In contrast to growth inhibition by interference with energy metabolism (Fig. 3), inhibition with chloramphenicol did not result in any change in the relative concentrations of the different lipids, although it did reduce the rates of metabolism of each lipid (Fig. 8).

Based on the studies of White and his coworkers (26, 31, 32, 40), the third and the most likely possibility is that PG and CL are interconverted in a cyclic manner (PG $\rightarrow$ CL $\rightarrow$ PG + PA) where the two molecules of PG which form CL come from different pools and phosphorus is lost from CL breakdown in an asymmetric manner (i.e., primarily from either the PG or PA end of the CL molecule). In their comprehensive studies, White and his co-workers established that CL and PG are interconverted in a cyclic manner involving the conversion of two PG to CL (with release of glycerol; reference 33)

and the breakdown of CL to PG and PA (25). They further established that only a portion of the total pool of each of the phospholipids is involved in these interconversions (26, 32, 40). Either the conversion of PG to CL or the breakdown of CL to PG and PA could be specifically inhibited, leading to the accumulation of PG or CL in the membrane (26). The asymmetric hydrolysis of CL was demonstrated both in vivo (26, 36, 43) and in vitro (25). Furthermore, the asymmetric synthesis of CL from different pools of PG was demonstrated (32, 40). We have not yet characterized the products of CL breakdown in *B. stearothermophilus*, but, in view of the increase in ^{32}P in the PG fraction after resumption of aeration after a period of anaerobiosis (during which CL accumulated; see Fig. 7), it seems reasonable to expect a similar conversion to PG and PA. However, judging from the rate of PG turnover in the nongrowth medium compared to the exponentially growing culture (Fig. 6), PG arising from CL must be a small portion of the total PG pool. If PA is a product of CL breakdown then it must be very rapidly metabolized, without mixing with the pool of PA, because there was no increase in the total amount of PA, no turnover of PA, and no uptake of ^{32}P in the PA pool after the resumption of aeration of cells with high CL concentration (Fig. 7). The PA pool remained constant at about 0.6 to 0.8% of the total lipid phosphorus under all conditions reported here (data not shown).

Anaerobiosis had no effect on the rate of conversion of PG to CL (Fig. 3, 6, 7) but completely blocked the breakdown of CL, leading to an accumulation of CL. This suggests at least three possibilities regarding CL breakdown: (i) the reaction might be involved in some aspect of energy conversion; (ii) the breakdown might be linked to another reaction which requires energy; and (iii) less likely but just as interesting, the breakdown of CL itself might require energy. This last possibility seems especially intriguing because of lack of accumulation of PA during rapid CL metabolism (e.g., Fig. 7). Whatever the reaction which leads to loss of ^{32}P from the lipid pool, it was not dependent on the relative concentration of CL in the cells. A more comprehensive study of CL metabolism is in progress.

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