Metabolism and Function of Bacterial Lipids

I. METABOLISM OF PHOSPHOLIPIDS IN ESCHERICHIA COLI B

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Relatively little is known about the intermediary metabolism of bacterial phospholipids. Indeed, as Aasselineau and Lederer have pointed out in their authoritative review (1), comparatively few bacterial phospholipids have been well characterized chemically by modern techniques. Interest in the chemistry and metabolism of bacterial lipids is, however, rapidly growing. In the past few years, publications from several laboratories have considerably enlarged our knowledge of the chemical structures of bacterial phospholipids, but detailed information on the metabolism of these substances is still lacking.

The occurrence of phospholipids throughout nature suggests a general role of these substances in cell physiology, but it is by no means clear what this role may be. On the one hand, phospholipids may be considered to be purely structural elements of the cell, deriving their importance as essential cell constituents from their unusual physicochemical properties. On the other hand, various dynamic functions have been ascribed to phospholipids, amongst the most attractive of which has been a postulated role as carrier substances in the active transport of ions and other substances across cell membranes. Throughout nature, metabolically active membranes, such as those of mitochondria and the endoplasmic reticulum of animal tissues, and the protoplast membrane of bacteria, are especially rich in phospholipids.

In animal tissues, phospholipids characteristically undergo a rapid rate of turnover in tissues such as liver, kidney, and intestinal mucosa, a fact which has suggested to some workers that the function of phospholipids must indeed be a dynamic one. Interpretation of turnover studies in animal tissues, however, is complicated by the presence of a diversity of cell types, and by the death and division of cells which may occur even in the tissues of adult animals. In bacteria, it has been shown that rate of turnover of cell constituents such as protein is a function of the conditions of culture (2). There is virtually no turnover of protein in Escherichia coli during the exponential phase of growth whereas the protein of resting cells undergoes an appreciable turnover. Corresponding studies on the turnover of well defined phospholipids have not to our knowledge been reported. In earlier studies such as those of Mitchell and Moyle (3) on phosphate uptake and turnover in Micrococcus pyogenes, the uptake of labeled phosphate into crude, unfractionated lipid fractions was measured, since methods available at that time did not permit the rapid and accurate characterization of the labeled lipids.

In the present paper, we wish to report the results of studies of the general features of phospholipid metabolism in E. coli B. Our experiments have been confined to that fraction of the bacterial phospholipids which can be extracted from the cells under relatively mild conditions with chloroform-methanol. (This fraction does not include the so-called "lipid A" which is part of the bacterial endotoxin complex.) Isotope tracer experiments with P32 confirmed the finding of Law (4) that phosphatidylethanolamine is the principal phosphatide in this fraction, and the report of Kurokawa et al. (5) that phosphatidylserine is also present in E. coli. In addition, we have obtained evidence for the presence of phosphatic acids, and of phosphatidyglycerol in this organism.

Experiments on the kinetics of labeling of the phospholipids of E. coli B growing exponentially after "pulse labeling" by brief exposure to orthophosphate-P32, followed by dilution with unlabeled phosphate, have revealed that phosphatidylethanolamine, once formed, undergoes a detectable turnover. Another fraction, however, accounting for about 30 to 40% of the lipid phosphorus, does rapidly lose its radioactivity after pulse labeling, even in growing cultures. This fraction has been identified as phosphatidyglycerol.

EXPERIMENTAL PROCEDURE

Materials and Methods

Conditions of Growth—Cells of a stock strain of E. coli B (the gift of Dr. Luigi Gorini) were grown in a phosphate-rich medium, containing the following components (per liter): KH2PO4, 5.35 g; K2HPO4, 10.63 g; (NH4)2SO4, 2.0 g; MgSO4·7H2O, 0.2 g; FeSO4·7H2O, 0.5 mg; and glucose (sterilized separately), 2.0 g. The phosphate-poor medium was identical with the phosphate-rich medium, except that the phosphate was replaced by 10 g of KCl plus 4.2 g of NaHCO3.

Growth of bacterial cultures was measured turbidimetrically. Extraction of Phospholipids—Aliquots of cell suspensions (usually 100 ml) were precipitated by the addition of trichloroacetic acid to a final concentration of 5% (w/v), the precipitate was collected by centrifugation, and the supernatant fluid was discarded. The precipitate was then suspended in methanol (8.0 ml) and warmed at 55° for 15 minutes in a tightly stoppered vessel. After the tubes were cooled, chloroform (16 ml) was added, and after vigorous shaking the suspension was allowed...
to stand at room temperature overnight. The suspension was filtered through a plug of glass wool, and the filtrate was equilibrated against an equal volume of aqueous 2 M KCl by vigorous shaking of the tightly stoppered vessels. The upper aqueous phase was drawn off with a capillary and discarded. The chloroform phase was washed twice more in similar fashion with 2 M KCl, and finally with water. The washed chloroform solution represented the lipid extract used for the various analytical procedures to be described.

Analytical procedures to be described.

Exposure of E. coli B to Orthophosphate-P³²—When cultures of E. coli B are incubated with P³² of high specific activity, the readily extractable phosphatides rapidly become labeled. Application of the mild alkaline hydrolysis procedure of Dawson (7), followed by chromatographic separation of the water-soluble glycerophosphoryl derivatives, offered a convenient and very sensitive method for the analysis of the phospholipids of this organism.

Cells of E. coli B grown in the phosphate-rich medium as described in the preceding section were harvested in the early log phase of growth by centrifugation. The cells were then washed and resuspended in the phosphate-free medium, in a volume equal to that of the original growth medium (usually 100 ml) and shaken for 15 minutes at 37°C. To this suspension, carrier-free P³² was added, and the shaking was continued. At suitable time intervals, varying from 30 seconds to 30 minutes, aliquots of the suspension were removed, treated with trichloroacetic acid, and the lipids extracted as described under "Materials and Methods." Aliquots of the washed chloroform extract were plated and counted. Under these conditions the radioactivity of the lipid extract increased at a linear rate for at least 10 minutes.

Aliquots of the lipid extract were subjected to the mild alkaline hydrolysis procedure (7) and the products separated by chromatography. The radioactive phosphate esters were detected by radioautography. With use of the chromatographic System A (phenol-ammonia), the following derivatives were detected after very brief (30 seconds) exposure of the cells to P³²: glycerophosphorylethanolamine (Rf = 0.65); glycerophosphorylgllycerol (Rf = 0.37); glycerophosphorylglycerol (Rf = 0.17); and glycerophosphate (Rf = 0.09). These Rf values were closely similar to those of authentic standards run on the same chromatograms. After longer exposure to P³², the relative labeling of the glycerophosphorylglycerol and glycerophosphate was much weaker than that of the glycerophosphorylethanolamine and glycerophosphorylgllycerol. This finding is consistent with the conclusion that the parent phospholipids, phosphatidylserine and phosphatidic acid, are biosynthetic intermediates which do not accumulate in large amounts. Kanfer and Kennedy (8) have reported evidence that phosphatidylserine in this organism is derived from phosphatidic acid (via CDP-diglyceride) and is a precursor of phosphatidylethanolamine.

Further evidence for the occurrence of phosphatidylglycerol (the parent phospholipid which yields glycerophosphorylgllycerol on hydrolysis) as a metabolically active glycerophosphatide in E. coli will be given below.

Turnover of Phospholipids in E. coli B—The rate of breakdown and renewal of phospholipids in cultures of E. coli B growing in the log phase was investigated by means of the pulse labeling technique. In an experiment of this kind, the culture was labeled by exposure to P³² for 10 minutes in the phosphate-free medium as described in the preceding section. The culture was then diluted with 10 volumes of the phosphate-rich medium containing unlabeled phosphate. In this way the specific activity of the orthophosphate pool was very greatly reduced. The rate of loss of the radioactivity from the labeled lipid pool was then followed during subsequent growth for various time intervals, usually 1, 2, and 3 hours. Growth of the culture during this period was followed turbidimetrically, and it was established that growth was exponential, with a doubling time of about 40 to 50 minutes.

Of several possible hypotheses concerning the rate of loss of radioactivity from phospholipids, in an experiment of this kind, two may be particularly considered. If it is assumed that all of the labeled phospholipids are completely stable metabolically, i.e. that their function does not require constant breakdown to and renewal from orthophosphate, then the total radioactivity of the lipid extract should remain constant. On the other hand, if all of the phospholipids are rapidly broken down and renewed as a part of their function, even in log growing cultures, then radioactivity should be lost from the lipid fraction at an exponential rate.

The actual result of a typical experiment is shown in Fig. 1. The time after the dilution of the culture with unlabeled medium is shown as the abscissa. For 10 to 15 minutes after dilution, the total radioactivity of the lipid extract actually increases. This probably reflects the time necessary to reduce the specific activity of the intracellular phosphate to a low level. In the next 90 minutes about 30% of the radioactivity is lost at a comparatively rapid rate, whereas the remaining 70% appears to be quite stable. In other experiments, prolonging the growth on unlabeled medium for several hours longer did not appreciably reduce the residual 70% of radioactivity.

These results suggest that there are two principal components in the labeled phospholipid fraction, one which loses its radioactive phosphorus relatively rapidly, and another which is entirely stable when once synthesized. Accordingly, a determination of the radioactivity in the individual labeled phospholipids at various time intervals after dilution with unlabeled phosphate was carried out by the procedure of Dawson. Aliquots of the products of mild alkaline hydrolysis were chromatographed in the phenol-ammonia system, and radioautographs were made. Results of a typical experiment are shown in Fig. 2. Spot A, which is glycerophosphorylethanolamine (derived from phosphatidylethanolamine) does not fade in intensity during a period of 3 hours, whereas Spot B, which is glycerophosphorylgllycerol...
(derived from phosphatidylylycerol) representing about 30% of the initial radioactivity, rapidly fades with time, and is barely detectable after 3 hours.

In another experiment, the radioactive spots corresponding to glycerophosphorylglycerol were located by radioautography, cut out, eluted with distilled water, and aliquots counted. In this way, the rate of loss of radioactivity from the parent phospholipid, phosphatidylylycerol, at various time intervals after dilution of the pulse labeled culture with unlabeled orthophosphate could be determined. The results are shown in Fig. 3. The radioactivity of the phosphatidylylycerol falls at a logarithmic rate, with half the radioactivity lost in 40 to 50 minutes.

Isolation and Identification of Phosphatidylylycerol—A suspension of bacteria, which had been harvested during growth in the logarithmic phase in the phosphate-free medium described above, was shaken with carrier-free $^{32}$P for 5 minutes. The labeled cells were then precipitated with trichloroacetic acid and the lipids extracted as described in “Materials and Methods.” The washed chloroform extract was taken down to dryness under...
vacuum in a rotary evaporator, taken up in dry chloroform, and chromatographed on silicic acid, using gradient elution with increasing concentrations of methanol in chloroform. The elution pattern is shown in Fig. 4. Two incompletely separated peaks were obtained. The contents of each tube were subjected to paper chromatography, and only those tubes containing a single component were pooled.

The second, and larger peak was readily shown to contain phosphatidylethanolamine, as revealed by hydrolysis according to the method of Dawson. Glycerophosphoryl ethanolamine was the sole radioactive product derived from this fraction.

The lipid in the first peak moved with an $R_f$ of 0.50, identical with that of authentic synthetic phosphatidylglycerol, upon chromatography on filter paper impregnated with silicic acid, with diisobutylketone-acetic acid-water (50:25:5) as the solvent.

Hydrolysis of the labeled lipid in the first peak with 90% acetic acid in a boiling water bath for 20 minutes yielded glycerophosphophosphate as the principal labeled product, as would be expected from phosphatidylglycerol (9). Mild hydrolysis by the method of Dawson yielded radioactive glycerophosphorylglycerol, identified by its chromatographic behavior in several solvent systems.

These results support the conclusion that the faster moving radioactive peak in Fig. 4 is phosphatidylglycerol. It may be noted that the proportion of the total radioactivity in the phosphatidylglycerol peak is roughly that to be expected from the results shown in Fig. 1.

**Relative Amounts of Phosphatidylethanolamine and of Phosphatidylglycerol in E. coli B in Logarithmic and Stationary Phases of Culture**—In a previous study of the phosphatides in E. coli, Law did not detect phosphatidylglycerol (4). Although this difference in result might be attributed to differences in the strain of bacteria used, the possibility was considered that the stage of the culture was the principal factor, since our studies were carried out with bacteria harvested during the logarithmic phase of growth, whereas Law studied cultures which had reached the stationary phase. Accordingly, the relative proportion of phosphatidylglycerol and phosphatidylethanolamine in the lipids of E. coli B was determined in cultures during logarithmic growth and in cultures which had reached the stationary phase. In the log growing cultures, phosphatidylglycerol represented 21% of the total phosphatides in the chloroform extract, but only 7% in cells harvested in stationary phase. It thus appears that the content of phosphatidylglycerol is much greater in the log growing cells than in cells in stationary phase.

**DISCUSSION**

Whatever the function of phosphatidylethanolamine may be in E. coli, it does not involve turnover of the phosphorus moiety of the molecule, since no loss of radioactive phosphorus from this fraction could be detected during several generations of rapid growth. In contrast, the rapid loss of radioactivity from phosphatidylglycerol under the same conditions of growth is most striking.

There are several explanations which may be considered to account for the relatively rapid disappearance of radioactivity from the phosphatidylglycerol fraction observed in these experiments. In the first place, it is possible that the function of this phosphatide involves its breakdown to and resynthesis from simple precursors including orthophosphate. A second possibility is that phosphatidylglycerol is an intermediate in a biosynthetic pathway which involves its conversion to some substance which is not recovered in the lipid fraction described here. The relatively amount of phosphatidylglycerol decreases as the culture approaches stationary phase, which might be consistent with the latter possibility, since the phosphatidylglycerol itself would not be an end product. A recent report by Macfarlane (10) suggests that phosphatidylglycerol may represent the lipid component of "lipo-amino acids" in *Clostridium welchi*. A structure is proposed by Macfarlane for such lipid amino acids which is more similar in chloroform might account for the findings observed here. Further experimental work is needed to test this hypothesis.

**SUMMARY**

Some general features of the metabolism of phosphatides in *Escherichia coli* B have been investigated. Phosphatidylserine and phosphatidylethanolamine have been identified amongst the phosphatides of the lipid fraction readily extractable with chloroform and methanol. The content of phosphatidylglycerol was shown to be much higher in rapidly growing cultures than in cultures in stationary phase.

Pulse labeling experiments with orthophosphate-$^{32}P$ revealed that phosphatidylethanolamine, once formed, is completely stable, undergoing no detectable turnover in rapidly growing cultures of E. coli B. In contrast, radioactivity present as phosphatidylglycerol after pulse labeling is rapidly lost under identical conditions.

Implications of these findings for theories of phospholipid function are discussed.
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