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Phospholipid Metabolism in T4 Bacteriophage-infected *Escherichia coli* K-12 (λ)

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Infection of *Escherichia coli* K-12 (λ) by bacteriophage results in an altered labeling pattern of phospholipids in the host cell. Although the overall incorporation of $^{32}P_i$ into phospholipids is decreased by infection, the relative amounts of phosphatidylglycerol and cardiolipin are increased. Phospholipid changes occurring at later stages in the lytic cycle of infected bacteria are more prominent than those at earlier time intervals. The uptake of $^{32}P_i$ into phospholipids of cells infected with T4B*s* and endolysin-negative mutants was similar to that observed with the wild-type phage, suggesting that the development of resistance to lysis from without and the repair of mucopeptides are not responsible for the phospholipid changes. The metabolism of phospholipids in uninfected cells treated with cyanide was similar to that of infected cells, indicating that part of the phage-induced alterations may be a consequence of impaired respiration.

It has been demonstrated in various ways that the infection of some strains of *Escherichia coli* by T2 and T4 coliphage results in changes in the permeability of the host cell. Initially it was found that the infected cells demonstrated an increased loss of inorganic phosphorus and nicotinamide adenine dinucleotide (NAD) (15, 16). After a period of about 5 min, this accelerated leakage was ended by a postulated "sealing" reaction. Recently this was confirmed and extended by the demonstration of an increased efflux of potassium and magnesium as well as inorganic phosphorus (19). Adenosine triphosphate (ATP) also has been found to be released from phage-infected *E. coli* B (5), and infection of *E. coli* K-12 (λ) with T4*r*II mutants appears to result in altered permeability (3, 6, 7, 10).

Phospholipids are found mainly in the cell membrane, and it is reasonable to expect that changes in permeability might be reflected by changes in the metabolism of these molecules. Because of their unusual chemical and physical properties, some phospholipids have additional important biological roles. It has been demonstrated that the synthesis of cell envelope components such as lipopolysaccharides (17) and mucopeptide (4) requires phospholipids. In addition to changing the permeability of the host cell, phage infection may also require increased or altered synthesis, or both, of cell envelope components. It has been demonstrated that phage infection results in an increased resistance to lysis from without (23). Although the chemical basis for this resistance is not known, it has been suggested that new wall synthesis may be required (8) and therefore might affect phospholipid metabolism.

Recently, several reports have demonstrated alterations in the host cell metabolism of phospholipids after infection with coliphage (7, 9). It is not clear, however, whether these changes are phage gene-specific or due to a host response. It was found that the early changes in phospholipid synthesis corresponded temporally with the sealing reaction and that they were sensitive to chloramphenicol if the antibiotic was added at least 5 min before infection (9). Other experiments suggest that the sealing reaction, at least for magnesium, potassium, and phosphorus, is independent of bacteriophage genes (19). The experiments presented here were designed as attempts to determine whether the early phospholipid changes can be related to the sealing reaction, to repair of damage done to the mucopeptide during infection, or to the development of resistance to lysis from without. Data are presented that appear to relate later changes in phospholipid metabolism to an impairment of host-cell respiration.

MATERIALS AND METHODS

Bacteria and phage. *E. coli* K-12 (λ) was obtained from L. Astrachan. It is the prototroph of Yanofsky and Crawford (24), referred to as strain Ymel (25). *E. coli* B, obtained from L. Astrachan, came origi-

nally from R. Herriott, and was used for the titration of T4rII mutants. *E. coli* BB also was obtained from L. Astrachan and was used for the preparation of high-titer phage stocks. T4Bs was obtained from J. Emrich. This strain is not capable of causing the development of resistance to lysis from without (8). T4 am 8–82 (endolysin-negative) was obtained from W. Bode. *E. coli* K–12 su^- was used as the host for the production of lysozyme-free stocks T4 am 8–82. T4r^+ and T4r1993 were obtained from the Benzer collection. T4r1993 has a long deletion in the A cistron.

Bacteriophage were assayed by the methods described by Adams (1). Phage ghosts were prepared by the method of Herriott and Barlow (11).

Media. Tryptone-Na^+ contains 1% tryptone (Difco) and 0.1 M NaCl. Tryptone-Mg^{++} contains 1% tryptone and 0.08 M $MgCl_2$. Soft and hard agar for plating phage and bacteria contained tryptone-Na^+ plus 0.55 and 1.2% agar, respectively.

Chemicals. All chemicals were reagent grade and all solvents were redistilled prior to use. Carrier-free ^{32}P-orthophosphate ($^{32}P_i$) was purchased from New England Nuclear Corp. (Boston, Mass.) and Tracerlab (Waltham, Mass.). The reference standards used were phosphatidylethanolamine (Nutritional Biochemicals Corp., Cleveland, Ohio), phosphatidylserine (Sigma Chemical Co., St. Louis, Mo.), phosphatidic acid, and cardiolipin (General Biochemicals Corp., Chagrin Falls, Ohio). Phosphatidylglycerol was a kind gift of John Law.

Culture conditions. An overnight culture of *E. coli* K–12 (λ) was diluted 1:100 into fresh media and was grown with aeration at 37 C to about 2×10^8 cells/ml. A 3-ml amount of this culture was then used to inoculate 1 liter of prewarmed fresh media. The culture was allowed to grow to 2×10^8 cells/ml at 37 C in a rotary shaker.

Conditions of radioactive labeling and of infection. In experiments in which the effect of infection on the distribution of $^{32}P_i$ in the phospholipids was to be studied, 2 mc of ^{32}P-orthophosphate was added to 800 ml of culture. The culture was then divided into three 250-ml portions. At 2 min after the addition of the radioactive label, two of the cultures were infected at an input multiplicity of six phages per bacterium. The third culture was used as an uninfected control. Zero-time was always designated as time of infection. In other experiments, the uptake of $^{32}P_i$ into phospholipids of infected cells was studied by infecting 250-ml samples of cells at zero-time and 2 min later adding 0.5 mc of $^{32}P_i$-orthophosphate. Infected and control cultures were then incubated at 37 C with shaking, and at various times samples were withdrawn for extraction of phospholipids. At 9 min after infection, samples were withdrawn and plated to determine numbers of bacteria surviving infection. In all experiments reported, survivors amounted to less than 5% of the initial number.

Phospholipid extraction. At various intervals after time zero, samples from both infected and control cultures were precipitated by pouring them into flasks containing sufficient trichloroacetic acid to give a final concentration of 5% (w/v). The precipitated cells were chilled in an ice bath and were then sedimented by centrifugation. The supernatant fluid was poured off and the cells were extracted by the procedure of Kanfer and Kennedy (13).

Fractionation of phospholipids. After extraction, the lipids were evaporated to dryness under reduced pressure in a stream of nitrogen gas. These lipids were then taken up in chloroform and were transferred quantitatively to a 10-ml volumetric flask. Samples were then removed for direct counting of radioactivity in unfractionated lipids and also for chromatography. The preparation of plates and the thin-layer chromatography was performed by the method of Skipski (20), mainly using the basic system III [chloroform-methanol-1 N NH_4OH (80:36:2)]. After development of the thin-layer chromatograms, the phospholipid spots were detected by the exposure of the plates to iodine vapors. The radioactivity in the individual spots was determined by quantitatively transferring the gel to vials for counting in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). To assure complete recovery of labeled phospholipids, all of the gel in a lane was routinely assayed by this procedure. The scintillation fluid used contained 5 g of 2,5-diphenyloxazole and 0.1 g of dimethyl 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 liter of toluene. Radioautography was done on X-ray film (Eastman Kodak Co., Rochester, N.Y.). The identity of the individual phospholipids was established by co-chromatographic comparison with known standards and by paper chromatography of the deacylated products obtained by mild alkaline hydrolysis (22).

RESULTS

Phospholipid composition of E. coli K–12 (λ). Several reports have appeared describing the lipid composition of various strains of *Enterobacteriaceae* (2, 12, 13). Since it is known that changes in growth conditions can alter lipid synthesis, the phospholipid composition of *E. coli* K–12 (λ) grown in tryptone media was determined. This medium, with either Na^+ or Mg^{++} salts, was used throughout this work. Figure 1 shows a representative radioautogram of $^{32}P_i$-labeled phospholipids extracted with chloroform-methanol and then fractionated by thin-layer chromatography. Phosphatidylethanolamine (PE) comprises about 71% of the total, whereas phosphatidylglycerol (PG) and cardiolipin (CL) are 15% and 2%, respectively. Other minor phospholipid bands were always present in small amounts and, although these were routinely quantitated, in this study our interests were restricted to PE, PG, and CL.

Effect of infection on phospholipid synthesis. To examine the effect of infection on the uptake of $^{32}P_i$ by phospholipids, the cells were infected 2 min prior to the addition of the isotope. Figure 2 shows the incorporation of isotope into un-

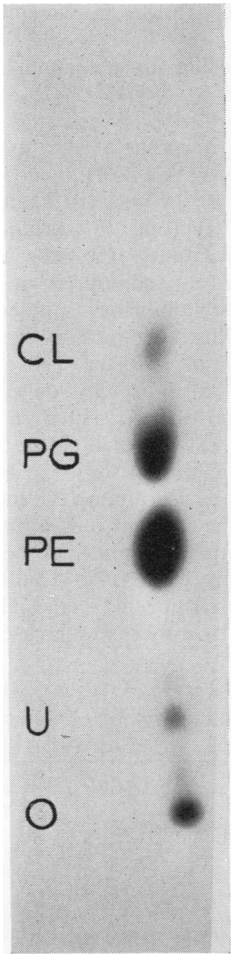

FIG. 1. *Radioautogram of the phospholipids separated by thin-layer chromatography. Abbreviations: O, origin; U, unidentified; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.*

fractionated lipids at various times after infection. It has been observed in many independent experiments of this type that infection causes a slight initial stimulation of phospholipid synthesis, as compared to uninfected cells. Figure 2 also indicates that at later times the synthesis of phospholipids by T4*r*II-infected cells is less than that by T4*r*$^+$-infected cells. This is contrary to the observation of Furrow and Pizer for *E. coli* B (9) and is more consistent with the results of Sekiguchi (18), who found that the phosphorylating ability of T4*r*II-infected cells was impaired.

Distribution of $^{32}P_i$ in the phospholipids of T4r$^+$- and T4rII-infected E. coli K-12 (λ). Various studies have suggested that *E. coli* K-12 (λ) becomes osmotically fragile after infection with *r*II mutants of T4 and is, therefore, unable to produce progeny phage (3, 6, 7, 10, 18). The possible relationship of this osmotic defect to alterations in phospholipid metabolism has been examined (7, 9). We decided to reexamine this since, in previous reports, the conditions of thin-layer chromatography of the phospholipids did not permit the separation of PG and CL. Table 1 shows the results of experiments in which the infected cells were incubated in tryptone-Mg^{++}. In these experiments, the incorporation of $^{32}P_i$ into both the unfractionated phospholipids and the individual phospholipids was determined. Phage infection again caused a decrease in the synthesis of phospholipids to an extent similar to that shown in Fig. 2. In addition, phage infection of *E. coli* also alters the relative amounts of individual phospholipids synthesized. Table 1 shows that by 10 min the percentage of the radioactivity appearing in PG is greater in infected cells than in uninfected cells. At later times, the activity is increasing also in CL of infected cells. There is, however, no difference between T4*r*$^+$- and T4*r*II-infected cells, indicating that the *r*II

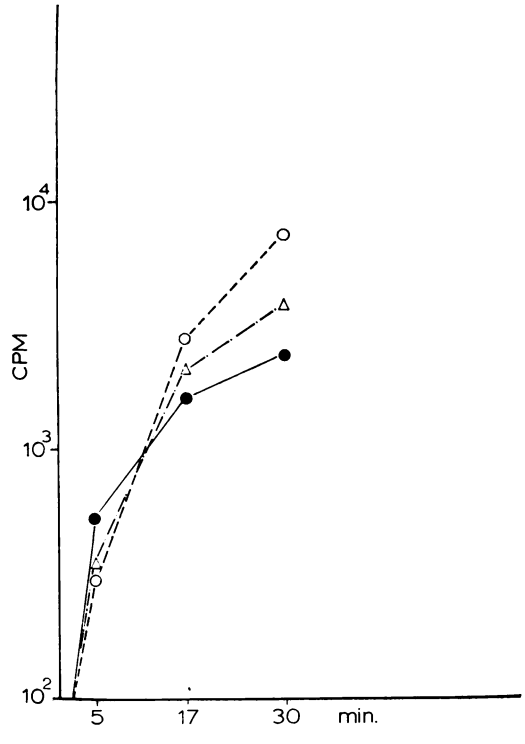

FIG. 2. *Effect of phage infection on uptake of $^{32}P_i$ into phospholipids of E. coli K-12. A portion for counting was taken directly from the volumetric flasks, as mentioned under fractionation of phospholipids. Symbols: ○, uninfected; △, infected with T4r$^+$; ●, infected with T4rII.*

TABLE 1. *Distribution of $^{32}P_i$ in the phospholipids of T4r+- and T4rII-infected E. coli K-12 (λ)*[a]

Time after infection	Phospholipid	Per cent of total phospholipid radioactivity[b]		
		Control	T4r+-infected	T4rII-infected
min				
5	PE	72	70	69
10	PE	71	63	61
17	PE	77	69	63
5	PG	17	18	21
10	PG	14	28	25
17	PG	15	23	26
5	CL	2	2	2
10	CL	2	2	3
17	CL	2	4	5

[a] $^{32}P_i$ was added 2 min before phage infection.
[b] Mean values of two independent experiments performed in duplicate. All values are within ±5% of experimental error.

mutation has no direct effect on phospholipid metabolism. To determine whether the observed phospholipid alterations in infected cells occurred as phage gene-specific changes, similar experiments were performed using phage which had been inactivated by ultraviolet irradiation. In these experiments, the distribution of the label among the individual phospholipids was the same as for uninfected cells. Phospholipid synthesis in cells infected with ultraviolet-inactivated phage, however, demonstrated the same decrease in rate that is characteristic for cells infected with viable phage. Similar experiments were attempted with T4 ghosts, but the results were often erratic, ranging from incorporation resembling that of cells infected with irradiated phage to an almost complete inhibition of $^{32}P_i$ uptake.

To determine whether the observed changes in phospholipid metabolism are related to biosynthetic reactions which may occur in the cell envelope after infection, cells were infected with T4B*s* or T4 endolysin mutants. T4B*s* mutants are unable to cause the development of resistance to lysis from without, a phenomenon which is thought to involve cell wall synthesis (8), and endolysin mutants are unable to hydrolyze cell wall mucopeptides. As shown in Table 2, the phospholipid synthesis in cells infected with these mutants is not significantly different from that of cells infected with wild-type phage (Table 1). The development of resistance to lysis from without or the repair synthesis of mucopeptides, therefore, cannot account for detectable changes in phospholipid metabolism.

Effect of sodium on phospholipid synthesis in phage-infected E. coli K–12 (λ). The preceding experiments utilized control and infected cells suspended in tryptone-Mg^{++}, a medium that permits the growth of T4*r*II phage in *E. coli* K–12 (λ; reference 10). When T4*r*II-infected cells are incubated in tryptone-Na^+ medium, the infection is abortive unless the cells are transferred within 10 min to a sodium-free medium containing protoplast-stabilizing supplements (6, 7, 10). Table 3 illustrates the labeling pattern of the phospholipids of infected cells incubated in tryptone-Na^+ medium. In these experiments, phage infection again resulted in a diminished incorporation of $^{32}P_i$ into the phospholipid fraction, similar to that shown in Fig. 2. The distribution of the label among the individual phospholipids of the infected cells again shows a difference from that in uninfected cells, but under these conditions there is also a difference between T4*r*+- and T4*r*II-infected cells. This difference, characterized by a marked increase in the CL of

TABLE 2. *Distribution of $^{32}P_i$ into phospholipids of T4Bs- and T4 am 8-82-infected E. coli K–12 (λ)*[a]

Time after infection	Phospholipid	Per cent of total phospholipid radioactivity[b]		
		Control	T4Bs-infected	T4 am 8-82-infected
min				
4	PE	69	67	66
17	PE	78	67	66
4	PG	16	17	17
17	PG	13	21	21
4	CL	1	2	1
17	CL	2	5	5

[a] $^{32}P_i$ was added 2 min before phage infection.
[b] Mean values of two independent experiments.

TABLE 3. *Uptake of $^{32}P_i$ into phospholipids of T4r+- and T4rII-infected E. coli K–12 (λ)*[a]

Time after infection	Phospholipid	Per cent of total phospholipid radioactivity[b]		
		Control	T4r+-infected	T4rII-infected
min				
30	PE	82	60	66
30	PG	9	14	16
30	CL	2	6	10

[a] $^{32}P_i$ was added 2 min after phage infection.
[b] Mean values of two independent experiments.

the T4*r*II-infected cells, however, probably occurs too late to be a direct consequence of the *r*II mutation.

Effect of cyanide on phospholipid synthesis by uninfected cells. One of the consequences of the T4*r*II mutation in infected cells incubated in tryptone-Na^+ is the decrease in oxygen uptake (10). To determine whether the alterations in the pattern of phospholipid synthesis in infected cells could be a consequence of impaired respiration, the effect of cyanide on the phospholipid synthesis of uninfected cells was examined. Cyanide was added to uninfected cells to give a final concentration of 3×10^{-4} M. ^{32}P-orthophosphate was added 2 min later and, at various times, the phospholipids were extracted and chromatographed. As expected, the respiratory poison caused a decrease in the rate of phospholipid synthesis. An analysis of the distribution of the label among the individual phospholipids synthesized after cyanide treatment (Table 4) indicates that the amount of label going into PG is increased to an extent similar to that occurring after phage infection.

DISCUSSION

In addition to causing a decrease in the rate of synthesis of host-cell phospholipids, phage infection also causes a change in the relative amounts of $^{32}P_i$ incorporated into individual phospholipids. The decreased rate of phospholipid synthesis occurs with ultraviolet-inactivated T4 and ghosts of T4 as well as with viable phage. Although experiments with T4 ghosts gave various results, some inhibition of phospholipid synthesis was always observed. The decreased rate of phospholipid synthesis, therefore, apparently is not a consequence of phage gene expression, and presumably occurs as a result of phage attachment and subsequent interaction with the cell envelope.

TABLE 4. *Phospholipid synthesis in uninfected E. coli K-12 (λ) after poisoning of respiration*

Time after $^{32}P_i$ addition	Phospholipid	Per cent of total phospholipid radioactivity	
		Control	Cyanide-treated[a]
min			
5	PE	78	69
17	PE	83	70
30	PE	86	71
5	PG	10	16
17	PG	10	21
30	PG	8	20
5	CL	1	1
17	CL	2	3
30	CL	2	2

[a] Cyanide was added 2 min before $^{32}P_i$.

The alterations in the relative amounts of individual phospholipids synthesized after infection did not occur after infection with ultraviolet-inactivated phage and, therefore, probably are either phage gene-specific or occur as a result of redirection of host metabolism. One of the physiological changes occurring in *E. coli* after phage infection is the decrease in oxygen uptake. This occurs in both T4*r*$^+$- and T4*r*II-infected cells at about 10 min after infection, and the inhibition is more severe in T4*r*II-infected cells when incubated in tryptone-Na^+ (10). The increase in the relative amount of PG synthesized in infected cells was found to occur at least in temporal agreement with the respiratory block. Since cyanide treatment of uninfected cells resulted in a similar increase, the alterations of PG metabolism in infected cells may be a consequence of impaired host-cell respiration.

The synthesis of CL, occurring at a faster rate in T4*r*II-infected cells than in T4*r*$^+$-infected cells when incubated in tryptone-Na^+, is surprising. In this medium, respiration is sharply inhibited in T4*r*II-infected cells (10). Furthermore, Sekiguchi (18) has found that the *r*II defect results in decreased phosphorylating ability. Since the synthesis of CL from PG requires CDP-diglyceride (21), it might be expected that CL synthesis would be inhibited. Although the relationship is not clear, we have found (*unpublished data*) that when uninfected cells are incubated under partially anaerobic conditions the pattern of phospholipid synthesis mimics that occurring in late stages of infection, demonstrating an increase in PG/PE and CL/PE ratios.

These data indicate that the development of resistance to lysis from without or the synthesis of mucopeptides does not stimulate detectable changes in phospholipid metabolism. The "sealing" reaction, occurring during the first 5 min after infection, might be expected to require phospholipid synthesis. We were unable to detect changes in relative amounts of individual phospholipids synthesized during this time. The rate of phospholipid synthesis, however, does not decrease immediately after infection (Fig. 2), and it is possible that phospholipids are participating in the "sealing" reaction during the uninhibited period of synthesis.

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