Occurrence of Lysophosphatides in Bacteriophage T4rII-Infected *Escherichia coli* S/6

W. E. C. BRADLEY AND L. ASTRACHAN

Department of Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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Hydrolysis of phospholipids was observed to start about 15 min after *Escherichia coli* S/6 cells were infected with T4rII bacteriophage mutants. Hydrolysis continued through the latent period and well past the time when cell lysis occurs. The hydrolytic products that accumulated were free fatty acids, 2-acyl lysophosphatidylethanolamine, and various lysocardiolipins. These products indicated the action of phospholipase A₁. From 15 to 22 min after infection, there were equivalent amounts of fatty acids and lysophosphatides in extracts of cellular lipids. Thereafter, free fatty acids were produced in excess. This suggests that lysophospholipase was active at the later time. We also observed a stoichiometric relation between loss of phosphatidylglycerol and increase of cardioliolipin plus lysocardiolipins. This continued well past the normal lysis time (25 min). The appearance of lipase activities during the latent period seems to be specific to infection with rII mutants. Neither the wild-type bacteriophage nor rI mutants produced similar activities by 22 min after infection.

From several lines of evidence, it appears that cells infected with T4rII bacteriophage mutants have faulty maintenance of the cell membrane (2, 6, 19, 33). Several laboratories therefore have studied lipid biosynthesis in infected cells. With either *Escherichia coli* B or *E. coli* K (λ), infection alters the pattern of phospholipid biosynthesis towards increased incorporation of ³²P into phosphatidylglycerol (PG) and cardioliolipin (CL; references 6, 11, 25). There is no particularly striking difference between T4r⁺ and T4rII infections except for an increased incorporation into the CL of rII-infected cells (6, 25). Even this difference has been ascribed to decreased respiration of those cells (25). Another reported consequence of phage infection is the accumulation of free fatty acids (FFA) in a suspension of infected *E. coli* B (8). The FFA appeared early after infection, but no corresponding lysophosphatides were found. On the other hand, Rampini did find lysophosphatidylethanolamine (LPE) in the suspension but only at later stages of infection (30). Josselin has suggested that the reported early appearance of FFA may be a consequence of the antiserum which had been added to prevent autosuperinfection and consequent lysis inhibition (18). In Josselin's experiments, which utilized tailless mutants to eliminate the possibility of autosuperinfection, FFA production did not start until lysis time of r⁺-infected cells but did appear before lysis when

T4rII mutants were used (*unpublished data*, cited in reference 18). A similar enhanced production of FFA with rII infection has been reported recently (3). Comparison of cultures infected with r⁺, rI, rII, or rIII mutants showed that only cells infected with an rII mutant produced significant amounts of FFA during the latent period. It was also reported that lysophospholipids were present (3).

We have further examined the production of fatty acids and the accumulation of lysophosphatides by infected cells. At later stages of the latent period, *E. coli* B or S/6 cells, infected with rII mutants, accumulate 2-acyl LPE and lesser amounts of lysocardiolipins. This effect appears specific to rII infection, because the compounds appear later and in greatly diminished amounts after T4r⁺ or rI infection.

MATERIALS AND METHODS

Bacteria and phages. *E. coli* B came originally from R. Herriott. *E. coli* strains S/6 and CR63 were provided by R. Josselin, as were bacteriophage T4D and its mutants, rdf41 (rII), amB25 (tailless), and amH26 (lysozymeless). Wild-type T4B phage and T4Br1993 (rII) were obtained from S. Benzer, and T4BeG19 (lysozymeless) came from G. Streisinger. Our T4BrI is a proflavine mutant isolated from the wild type and showed low recombination with a standard rI mutant, T4Dr48, provided by R. S. Edgar. T4Drdf41 amH26 (rIIe) was constructed as described by Steinberg and Edgar (38). T4BrIle and T4BrIe recombi-

nants were selected from the progeny of crosses in log-phase *E. coli* B. The original eG19 contained an ac marker, which was removed by appropriate crosses from all T4Be phages used in this study.

Phage stocks were generally grown on *E. coli* B, with *E. coli* CR63 being used for amber mutants. Purification was accomplished by differential centrifugation (15). The high-titer stocks were kept in phage dilution fluid, containing 0.1 M NaCl, 0.001 M $MgCl_2$, and 0.0001 M $CaCl_2$ over a drop of $CHCl_3$.

Media. Cells were grown in H broth (38). Most phage were plated on EHA agar (38), except for the T4Be phage which were plated on citrate agar (9).

Radioactive chemicals. Sodium acetate- $I-^{14}C$ was obtained from Amersham-Searle, and ^{32}P -orthophosphate was from New England Nuclear Corp.

Standard experimental conditions. An overnight culture of *E. coli* S/6 or B was diluted 10^2 - to 10^3 -fold into H broth. Fifteen minutes later, ^{14}C -acetate and ^{32}P i were added to final activities of 1 $\mu Ci/ml$ each. Cells were grown at 37 C with rotary shaking. At 2×10^8 cells/ml, the culture was distributed into flasks, and phage were added to give a multiplicity of 7. Under these conditions, there were 0.5 to 1.5% survivors. The lysis time, as measured by optical density changes, was 25 min for T4DamB25 (r^+ and tailless), T4DrII e^+ , and T4DrII e . Cells infected with T4DrII e phage exhibited optical density changes similar to those observed by Josslin (18) with an r^+ , tailless, lysozymeless phage. Half-maximal appearance of free T4BrII phage also occurred at 25 min.

At designated times after infection, samples were removed for lipid extraction by the Ames modification (1) of the Bligh and Dyer method (5). Either centrifuged cells or total suspensions could be extracted. No difference in results was seen when samples were extracted overnight at room temperature (8) instead of 10 min at 0 C. Extracts were evaporated under a N_2 stream and spotted on thin-layer chromatography (TLC) plates.

Phospholipase A. Lyophilized *Crotalus adamanteus* venom (Ross Allen Institute, Silver Springs, Fla.) was used, as described by van Deenen (40), to produce 1-acyl LPE from isolated phosphatidylethanolamine (PE).

Phospholipase C. *Bacillus cereus* (ATCC strain 7004) was grown in 3XD glycerol (10) supplemented with 0.2% glucose and 0.3% yeast extract. Crude phospholipase C was isolated from the medium by ammonium sulfate precipitation and dialysis (21). The sample to be hydrolyzed, either total lipid extract or isolated lipid eluted from a TLC plate, was evaporated to dryness and suspended by sonic treatment for 1 min in 0.5 ml of 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-maleate buffer, pH 7.4. To this was added 0.5 ml of crude enzyme solution (21), and 1.5 ml of ether was layered on top. The mixture was incubated at 37 C for 30 min, by which time the ether had evaporated. $CHCl_3$ and CH_3OH were added to give a final ratio of $CHCl_3-CH_3OH-H_2O$ of 1:1:0.9, and the organic phase was evaporated down and spotted on a boric acid-impregnated TLC plate. TLC chromatography with solvent 1 (see below) was used to compare the phospholipid content before and after

hydrolysis with phospholipase C. We found greater than 95% hydrolysis of PE and LPE, whereas only 45% hydrolysis was observed with PG and CL which were unresolved in the solvent system used. Approximately 60% of other lyso compounds (tentatively identified as lysocardiolipins) were hydrolyzed. More than 95% of the hydrolytic products from phospholipids and lysophospholipids were the respective diglycerides and monoglycerides.

Separation procedures. Deacylated lipids were prepared and then separated by ion-exchange chromatography at pH 8.6 as described by Wells and Dittmer (41). Intact phospholipids were separated by TLC procedures on commercially available silica gel plates (Mann). Before use, the plates were tapped gently to remove loose particles and then washed with acetone by ascending chromatography. For separating phospholipids, solvent 1, chloroform-methanol-water (70:25:4), and solvent 2, chloroform-methanol-glacial acetic acid (65:25:8), were used. Migration characteristics have been described for these solvents (6, 1). The minor components, phosphatidylserine (PS) and phosphatidic acid, were usually not individually isolated by systems 1 or 2, but two-dimensional TLC (1), first with solvent 3, chloroform-methanol-7 N NH_4OH (60:35:5), followed by solvent 2, revealed that they were in constant low amounts (<1%) for at least the first 22 min of infection. In solvent system 1, CL runs with PG. This was shown by two-dimensional TLC and by Dowex chromatography of the deacylated lipids (41). The fatty acid spots displayed in solvents 1 and 2 were further identified by chromatography of a sample in solvent 4, hexane-diethyl ether (9:1). A second sample was first methylated before similar chromatography. The fatty acids and their methyl esters migrated as expected for this solvent (31).

Glycerides produced by phospholipase C were separated on boric acid-impregnated TLC plates (39) which had been heat-activated at 110 C for 1 hr and then stored in a desiccator until used. The solvents used were 5, chloroform-acetone (96:4), and 6, chloroform-acetone (88:12). The migration of neutral lipids in these solvents has been described (39). In addition to the useful property of allowing separation of 1- and 2-monoglycerides, the presence of boric acid inhibits migration of the acyl group from the unstable 2-position (39).

Lipids were visualized by exposure to I_2 vapor. Preparative samples were covered with Saran Wrap while marker lanes were exposed. A subsequent 1-sec exposure outlined the samples that were then collected by scraping and elution with $CHCl_3-CH_3OH$ (1:4). All solvents were of reagent grade and not distilled before use.

Counting. Spots to be counted were scraped directly into 15 ml of scintillation fluid (8 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis-2-(5-phenyloxazolyl) benzene, 1,000 ml of toluene, and 1,000 ml of methyl cellosolve). These spots were not exposed to ninhydrin because of its quenching effect.

Chemical tests. Phospholipid solutions were evaporated to dryness and hydrolyzed with 1 ml of 11 M $HClO_4$ before assay of phosphorus (14). Amines in

PE, LPE, and PS were detected with commercial ninhydrin spray (Sigma). Vicinal diols in monoglycerides were measured in a procedure that involved periodate oxidation and subsequent chromotropic acid assay for the released formaldehyde (41). The procedure was modified in that the lipid was first adsorbed onto silica gel before oxidation with 0.02 M NaIO₄ in 0.12 M phosphate buffer at pH 7. We did not observe complete oxidation with tetraethylammonium periodate in a CHCl₃ system (29) nor with NaIO₄ in aqueous systems in which the glyceride was emulsified either by sonic treatment or with 0.01 to 0.1% sodium dodecyl sulfate. Test conditions were evaluated with 1-monopalmitin (Applied Science) and a standard serine solution.

Gas chromatography. The alkaline hydrolysis of lipids and separation of aqueous and organic extracts were performed as described by Wells and Dittmer (41). After evaporation of the organic extract, the fatty acids were dissolved in ether-methanol (9:1) (32) and methylated at 0°C by bubbling in gaseous diazomethane. The latter was produced from Diazald by a minor modification of the supplier's directions (Aldrich Chemical Co.). A persistent yellow color indicated the end point of methylation. After evaporation, the fatty acid methyl esters (FAME) were redissolved in hexane and either used directly for gas-chromatography or first purified by chromatography on silica gel with solvent 4. No significant difference was seen upon purification. The FAME were separated at 155°C on a Hewlett-Packard PC-2 column [6 ft (1.8 m) by 3 mm glass column packed with 6% diethyleneglycol succinate on 80/100 Diatoport S] with gas flow rates that had been used by the manufacturer to pretest the column. The flame ionization response of each peak was measured by the product of peak height $\times$ width at half height. The latter was measured with a Nikon 6 shadowgraph. Known mixtures of FAME (Applied Science Laboratories, State College, Pa.) were chromatographed, and plots of log retention time versus number of carbon atoms were drawn for saturated and unsaturated esters (42). We are indebted to John Law, who provided us with a sample of C₁₉ cyclopropane fatty acid and who suggested that we use diazomethane rather than acidic methanol for methylation to preserve cyclopropane fatty acid esters. Retention times for these compounds plot on the standard line for monoenoic esters. Our sample peaks were identified by retention times in reference to the standard plots. Initially, for purposes of firmer identification, the FAME were also chromatographed on a nonpolar column of silicone oil SE-30, as described by White and Cox (42). The FAME are designated by number of carbon atoms and unsaturated bonds in the fatty acid (e.g., palmitic is C16:0, oleic and *cis*-vaccenic are C18:1). Cyclopropyl compounds, which are derived from unsaturated fatty acids, are designated Δ .

RESULTS

Occurrence and identification of LPE. *E. coli* S/6 cells, infected with T4DrIIe for 22 min, were extracted for lipids. TLC with solvent 1 revealed

the presence of three, and sometimes four, new phospholipids that differentiated T4rIIe-infected cell extracts from uninfected ones (Table 1). The major new compound (R_F 0.15) was identified as LPE. It is ninhydrin-positive and its aqueous-soluble deacylation product (mild alkaline methanolysis) elutes from Dowex-1 at the same position as glycerylphosphorylethanolamine, the deacylation product of PE (Fig. 1). The presumed LPE also co-chromatographs in solvent systems 1 and 2 with standard LPE obtained after hydrolysis of PE with snake venom phospholipase A.

Radioactivity measurements indicated a ratio of one fatty acid per phosphorus. From uninfected cells uniformly labeled with ¹⁴C and ³²P, the ¹⁴C to ³²P ratio of the new lipid averaged 0.98 when the ratio for PE was normalized to 2.00. Treatment with phospholipase C produced monoglycerides, a further indication that there was but one fatty acid per molecule. After hydrolysis of LPE, the organic-soluble material was spotted on boric acid thin-layer plates and developed in solvent system 5. The major spots, accounting for 96% of the radioactivity, migrated to the same positions as standard monoglyceride (Applied Science).

Identification of other lysophosphatides. Two other new compounds with R_F values in solvent 1 of 0.12 and 0.07 were seen in infected cell extracts (Table 1). These ninhydrin-negative compounds were partially characterized as lysocardiolipins. When the first compound (LCL-1) was deacylated, Dowex chromatography (Fig. 1) indicated that LCL-1 is a derivative of cardiolipin. Similar results were obtained with the second compound, LCL-2; 86% of the aqueous-soluble ³²P counts chromatographed in the same region. The number of fatty acids per atom of P was estimated by ¹⁴C to ³²P ratios as described above. With the ratio for PE normalized to 2.00, the ¹⁴C to ³²P ratios for LCL-1 and LCL-2 were 1.46 and 0.98, respectively. This indicates that LCL-1 and LCL-2 are cardiolipins with, respectively, 1 and 2 fatty acids removed. No further attempts at identification were essayed because the compounds are quite labile. Neither survives elution and rechromatography, giving rise instead to spots at the origin and near the solvent front.

Occasionally, a third new compound with an R_F of 0.02 in solvent system 1 was seen in the infected cell extracts. This compound was particularly labile and present in very small amounts. The only characterization attempted was Dowex chromatography after deacylation. More than 90% of the radioactivity was eluted in the cardiolipin "backbone" region. It appears that this is a third lysocardiolipin.

2-Acyl LPE. Since *E. coli* is known to have

TABLE 1. *Per cent distribution of lipid-extractable ^{32}P and ^{14}C among major cell lipid components^a*

Time after infection (min)	Label	PE	PG ^b	CL ^b	LPE	LCL-1	LCL-2	FFA
0	^{32}P	76.5	17.9	4.4	0.5	0.2	0.2	0.09
	^{14}C	76.8	17.3	4.5	0.3	0.2	0.2	
22	^{32}P	62.4	11.5	7.4	13.8	4.7	0.5	8.3
	^{14}C	63.4	10.8	7.1	6.4	3.2	0.25	
25	^{32}P	59.4	9.4	8.4	16.5	5.6	0.7	12.4
	^{14}C	57.9	8.7	7.8	8.0	4.1	0.34	
30	^{32}P	58.3	8.1	8.5	18.6	6.5	1.1	18.9
	^{14}C	53.1	6.9	7.8	8.9	4.5	0.49	
45	^{32}P	56.0	6.8	9.3	21.8	6.4	1.6	22.1
	^{14}C	48.1	5.5	6.9	10.2	4.5	0.77	

^a *E. coli* S/6 cells were labeled with $^{32}\text{P}_i$ + ^{14}C -acetate and infected with T4rIIe phage.

^b Amounts of PG and CL were determined by TLC separation with solvent 2.

phospholipase A₁ activity (27), we attempted to determine whether all of the LPE produced after infection is the expected 2-acyl LPE. Because of the known migration of lysophosphatide acyl groups from the 2 to the 1 position, especially during TLC on silica gel (35), a total lipid extract, rather than isolated LPE, was treated with phospholipase C. The organic soluble products, consisting mainly of a mixture of diglycerides, monoglycerides, and FFA, were chromatographed on a boric acid plate. From an extract of labeled cells sampled 25 min after infection with T4DrIIe, it was found that 94% of the monoglyceride radioactivity was in the 2-mono position.

To ensure that the relative positions of 1-mono and 2-mono-glycerides were identified correctly, the compounds were eluted and treated with periodate. 2-Acyl monoglyceride does not have vicinal hydroxyls and should resist periodate oxidation, whereas 1 mole of formaldehyde should be released per mole of 1-acyl monoglyceride. Table 2 shows reasonable agreement between observed and expected stoichiometry. Some formaldehyde was formed from 2-acyl monoglyceride but this may be, as discussed below, the result of transacylation to the 1 position.

When the 2-monoglyceride was eluted and rechromatographed in solvent 6, 30% of the material migrated as 1-acyl monoglyceride. Thus, just as with the known transesterification in lysophosphatides, the 2-acyl group of monoglycerides can also migrate to the 1-position. This raised the possibility that all (rather than 94%) of the monoesterified lipid was originally of the 2-acyl type. If this were so, then the fatty acid distributions of the 1-mono and 2-monoglycerides should be the same if it can be assumed that random trans-

FIG. 1. Chromatography of deacylated lysophosphatides. The presumed LPE and LCL-1 were eluted from the thin-layer plate, hydrolyzed, and run on Dowex-1-formate. Arrows indicate elution tubes of the deacylated products of PE (tubes 6-12), PG (22-30), and CL (53-62). Symbols: X, deacylated LPE; O, deacylated LCL-1.

esterification of fatty acids occurs. As a verification of this assumption, the fatty acids of the 1-monoglycerides that arose after rechromatography of 2-monoglycerides were compared with the fatty acids of the original 2-monoglycerides and shown to have similar contents of major fatty acids. However, the fatty acid profile of the original 1-monoglyceride was quite different from that of 2-monoglyceride, and the difference reflects the known proportion of unsaturated fatty acids in the 2-position (Table 3). This might indicate that the small observed amount of 1-acyl compound was produced *in vivo*, except that the fatty acid distribution is not representative of 1-acyl LPE. Instead of being considerably en-

riched for saturated fatty acids (16), as shown in Table 3 for authentic 1-acyl LPE produced by hydrolysis of PE with snake venom phospholipase A₂, the fatty acid profile was closer to that of the diglycerides. Therefore, we cannot be certain about the source of the 1-monoglycerides. Since diglycerides run ahead of monoglycerides, streaking of diglycerides could contribute to the monoglyceride spots. Upon rechromatography of the monoglycerides, 86% of the 1-mono and 97% of the 2-monoglyceride c/m traveled again as monoglycerides. Although streaking of diglycerides cannot be ruled out as a minor source of 1-monoglyceride material, it should also be recalled that up to 15% of the monoglyceride could come from LCL-1 and approximately 2% from LCL-2. We have not tested the possibility that the 1-position of cardiolipin might have the fatty acid distribution observed in 1-acyl monoglyceride. At any rate, it is clear that the great majority of the lysophospholipids are 2-acyl compounds and that the fatty acids have the distribution expected for that position.

TABLE 2. Release of formaldehyde by periodate action on monoglycerides

Monoglyceride ^a	Amt (μmoles) of monoglyceride ^b per 10 ⁶ c/m	Amt (μmoles) of HCHO released per 10 ⁶ c/m	Amt (μmoles) of HCHO released per μmole of monoglyceride
1-Mono	2.1	2.5	1.2
2-Mono	2.1	0.33	0.16

^a As identified by position on boric acid TLC plate.

^b Original lipid extract was assayed for micromoles of lipid phosphorus and ¹⁴C radioactivity. The ratio of c/m per micromole was then used (with suitable correction for number of acyl groups) to convert radioactivity measurements to micromoles.

Production of lyso compounds and FFA with time. Labeled *E. coli* S/6 cells were grown and infected with T4DrIIe under our standard conditions. At various times, cells were collected and extracted. Figure 2 shows the change of label in four major components as a function of time after infection. Because label was present throughout growth and infection, radioactivity should be directly proportional to chemical amounts. At 15 to 17 min after infection, there is a simultaneous appearance of FFA and the lyso compounds, LPE and LCL-1. They continue to accumulate in the cells until well after normal lysis time. PE starts decreasing immediately after infection, probably as a result of lowered synthesis relative to other compounds. At 17 min, coincident with the appearance of FFA and LPE, the decrease in PE becomes steeper. From 17 until 22 min, phospholipid degradation can be attributed to the action of phospholipase A. There is good stoichiometry between the amount of FFA released and the sum of ¹⁴C counts in LPE plus one-third those in LCL-1 (when CL is hydrolyzed, three acyl residues remain in LCL-1 for each fatty acid released). Starting at 22 min, when lysis of an rIIe⁺-infected culture would begin, the production of FFA outstrips further formation of lyso compounds. It appears that the lysolipids of the cell become susceptible to a lysophospholipase at 22 min, with the result that lysolipid production plateaus, whereas FFA continues to be released.

Requirement of the rII mutation. Under our experimental conditions, the production of FFA and lysophosphatides before lysis is specific to the rII mutation. Table 4 shows the distribution at 22 min of phospholipid label in cells that were infected by various phage preparations. The rII mutants of T4B or T4D phage, acting on *E. coli* S/6 or *E. coli* B, produced large amounts of lysolipids. (The data for S/6 cells infected with T4BrIIe phage are not shown but similar results

TABLE 3. Fatty acid distribution in various cellular lipids after 25 min of infection by T4rIIe

Lipid	Per cent of fatty acids						Total saturated (%)
	C14:0	C16:0	C16:1	C17 Δ	C18:0	C18:1	
LPE.....	6	9.5	35	9		41	15.5
PE.....	4	38	20	4.5	1.5	30	43.5
1-Monoglyceride ^a	4.6	21.0	24.6	15.4	8.8	25.5	34.4
2-Monoglyceride ^a	4.6	9.6	27.6	16.3	1.6	40.6	15.8
Diglyceride ^a	1.4	31.9	14.6	6.4	5.5	40.2	38.8
FFA ^a	6.4	61.0	10.8	5.3		16.4	67.4
1-Mono LPE ^b	7.5	74.5	1.9	1.4	2.0	17.8	84

^a Total lipid extract was treated with phospholipase C.

^b LPE obtained by treatment of PE with snake venom phospholipase A₂.

FIG. 2. Time course of lipid distribution in *T4rIIe*-infected *E. coli* S/6. Cells were labeled for at least three generations with ^{32}P ; and ^{14}C -acetate, and infected at 0 min. Symbols: X, per cent ^{32}P radioactivity; O, per cent ^{14}C radioactivity.

were obtained.) Neither wild-type phage in the presence or absence of antiserum nor the rII mutant caused increased production of LPE before the cells lysed. Both T4B and T4D phage were used to ensure that effects ascribed to rII mutations were not the result of unknown mutations in our stocks.

More lysolipid was observed with rIIe than with rII phage. This could be caused by altered retention of lysophospholipids rather than an effect of the lysozymeless mutation on the extent of lipid hydrolysis. Specifically, the lysozymeless cells might retain FFA and lysophospholipids which could be selectively lost from e^+ cells on the verge of lysis. To test this possibility, a total suspension of cells plus medium was extracted. Little difference was then found between rII- and rIIe-infected cultures (Table 5), indicating that the lysozymeless mutation does not affect the extent of lipid hydrolysis. Table 5 also shows a large excess of FFA over the corresponding lysophospholipids at 22 min after infection. This differs from our results obtained when cells alone

were extracted. Although we always observed this excess in extracts of total suspensions, the difference between FFA and lysophospholipids was usually not so large. Regardless of the varied extent of FFA production, the similarity between rII and rIIe was completely reproducible.

DISCUSSION

We could definitely identify the LPE as 2-acyl LPE despite the ease with which the acyl group migrates to the 1-position during TLC and when LPE is dissolved in chloroform. To minimize migration, borate was added where possible and assays were performed rapidly. The presence of 2-acyl LPE indicates the action of phospholipase A_1 on PE. This enzyme is known to be present in the host cell (23, 30), but products of its activity are not apparent except under special conditions such as stationary-phase cells (26) or the late stages of rII infection.

Data presented in this paper suggest the presence of two other lipase activities. In Table 1, it is seen that the PG content starts to decline late in

the latent period and continues past the normal lysis time of 25 min. The loss is almost exactly balanced by the increase in CL plus LCL-1. Enhanced CL synthesis is presumably not a continuation of the known pathway which involves cytidine diphosphate diglyceride plus PG (36), because synthesis continues after expected lysis time when the cell has no further energy supply. We suggest that a phospholipase D activity begins at about the same time as phospholipase A₁ and converts two molecules of PG directly and stoi-

chiometrically to CL. This activity has been shown for phospholipase D (37), an enzyme found in *Haemophilus* (24) and present in *E. coli* (D. C. White, *personal communication*).

Our data also suggest that another host enzyme, lysophospholipase (28), is active during infection. When the amounts of FFA and lysophosphatides were compared in rII-infected cells, there was good stoichiometry up to the time of lysis. At later times, more FFA was produced, indicative of the action of a lysophospholipase which converts lysophosphatides to FFA plus the nonlipid "backbone." Another illustration of this effect is seen in Fig. 2, in which the per cent of lipid ¹⁴C in PE falls equally with the per cent of ³²P for the first 22 min. Thereafter, the ¹⁴C values fall more rapidly. A reasonable explanation is that, starting at 22 min, LPE is hydrolyzed to GPE so that ³²P is removed from the lipid pool; the released FFA is, however, still lipid-soluble. PE would therefore have a higher percentage of lipid-extractable ³²P than it would of ¹⁴C.

There is some uncertainty about the time when lysophospholipase becomes active. The data, referred to above and obtained with cellular lipids, indicate little activity before 22 min. However, when the total suspensions of cells plus medium were assayed at or before 22 min (Table 5), more FFA was found than could be balanced by lysophosphatides. There are several possible and experimentally approachable mechanisms to account for the different results found in cells and in the total suspension. Before cell lysis, lysophospholipase could attack lysophosphatides in the medium more efficiently than those still in the cell. Another explanation would be that lysophospholipase is active in the cell before lysis, but excess fatty acids are removed, either by extraction into the medium or by controlled β oxidation.

TABLE 4. Effect of different T4 mutations on lipid hydrolysis at 22 min after infection^a

Host	Phage	Label	LPE	LCL-1	FFA
S/6	Uninfected	³² P	0.5	0.2	0.3
		¹⁴ C	0.2	0.2	
S/6	T4D	³² P	1.1	NM ^b	0.3
S/6	T4DrII	³² P	5.8	NM	
S/6	T4DrIIe	³² P	13.8	4.7	8.3
		¹⁴ C	6.4	3.2	
S/6	T4Dr ⁺ e	³² P	0.8	NM	0.3
S/6	T4Dr ⁺ e + anti-T4 serum ^c	³² P	1.1	0.3	
		¹⁴ C	0.6	0.2	0.2
S/6	T4Dr ⁺ e, super-infected ^d	³² P	0.9	0.2	
		¹⁴ C	0.5	0.1	0.5
S/6	T4BrIle	³² P	1.3	0.2	
		¹⁴ C	0.7	0.2	0.5
B	T4BrIIe	³² P	14.3	NM	
B	T4DrIIe	³² P	13.3	NM	

^a Cells were labeled for three generations with either ³²P_i or ³²P_i and ¹⁴C-acetate. Data are expressed as per cent of total radioactivity in phospholipid.

^b Not measured.

^c Anti-T4 serum was added at 3 min after infection (final k = 7).

^d Superinfecting phage were added at 10 min at a multiplicity of infection of 7.

TABLE 5. Comparison of lipid hydrolysis in rII⁻ and rII⁺-infected cell suspensions^a

Time after infection (min)	Label	Per cent label in									
		PE		PG + CL		LPE		LCL-1		FFA	
		rII	rII	rIIe	rII	rIIe	rII	rIIe	rII	rIIe	rII
17	³² P	71.2	68.2	21.1	20.4	1.04	2.6	0.9	1.5	—	—
	¹⁴ C	70.5	71.2	21.7	20.8	0.72	1.1	1.7	0.8	2.5	2.8
22	³² P	68.4	69.7	16.7	15.6	4.9	6.0	3.7	3.8	—	—
	¹⁴ C	61.8	58.4	16.0	12.0	2.3	2.0	2.3	1.5	15.0	22.9
45	³² P	53.8	57.6	10.3	13.2	14.9	7.8	5.3	7.3	—	—
	¹⁴ C	38.7	40.0	8.9	7.8	5.5	3.0	4.0	2.8	37.6	42.5

^a *E. coli* S/6 cells were labeled for three generations and then infected. At designated times, 1.0 ml of total suspension was removed and extracted.

We cannot now distinguish between the alternate possibilities.

It is probable that the various lipase activities are only indirectly related to the rII defect because observable differences between r⁺ and rII depend on the composition of the medium. Cronan and Wulff reported that lipid hydrolysis occurs in r⁺-infected cells that had been cultured in tryptone broth (8). We could confirm their results and also found that an important difference between H broth, which we used in our experiments, and tryptone broth is the presence of glucose in H broth. The addition of glucose to tryptone broth lowered the extent of lipid hydrolysis during r⁺ infection without affecting the results with rII-infected cells (experiments not presented).

A more compelling argument that lipase activities are not direct manifestations of the primary rII defect is that lipid hydrolysis occurs late in the infection, whereas the rII defect is observable much earlier (6, 12, 22, 34). More likely, lipid hydrolysis is a consequence of some earlier rII-induced membrane disruption which results either in a modification of substrate environment to allow lipase attack or an unmasking or release of host enzymes. How does this fit with available evidence on the rII defect? Early membrane disruption after rII infection was previously inferred from work with the restrictive host *E. coli* K (λ) (6, 12, 34), and presumably a similar but less extensive effect occurs in *E. coli* B. The release of host enzymes is inferred not only from our data but also from work with phage ligase mutants. Several authors (4, 7, 20) have reported that an rII mutation suppresses the ligase defect. Although their interpretation (4, 20) was that the rII⁺ gene product is an endonuclease, there are two reasons that favor an interpretation based on membrane disruption. First, suppression by rII mutants does not occur if the host is also ligase-defective (13). This is what we would expect if host enzyme activity is released as a consequence of membrane disruption in rII-infected cells. Secondly, the *t* mutants, whose defect appears to be a failure to lyse the cell membrane (17), are also suppressed by rII mutations (19). This can be understood readily in terms of the rII defect providing an alternate mode of membrane disruption.

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