JOURNAL OF VIROLOGY, Apr. 1968, p. 298-307

Vol. 2, No. 4
Printed in U.S.A.

Replication of T4rII Bacteriophage in *Escherichia coli* K-12 (λ) [1]

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Received for publication 9 January 1968

The defect of T4rII replication in *Escherichia coli* K-12 (λ) can be phenotypically reversed by various supplements to the growth medium. Arginine, lysine, spermidine, and a number of diamines allowed varying levels of rII replication. The best reversion was obtained with 0.4 M sucrose in 0.002 to 0.005 M Ca^{++}. Monovalent cations severely inhibited reversion. A cell surface site of polyamine action is consistent with the fact that spermidine inhibits phage ghost-induced cell lysis and with the finding that sufficient polyamine is available within the cells to allow normal patterns of neutralization of phage deoxyribonucleic acid, as detected by the polyamine content of progeny phage. In the absence of effective supplements, rII-infected cells swelled and lost refractility. The data indicate that a leaky cell envelop is involved. No difference in mucopeptides of uninfected K-12 (λ) and K-12 was detected and, because the mucopeptide in r^+ infected cells was found to be at least partially hydrolyzed midway through the lytic cycle, it did not appear that the rII defect concerned mucopeptide synthesis. The pattern of cell phospholipid synthesis changes after phage infection, but no difference was detected between r^+ and rII with regard to biosynthesis of phosphatidylethanolamine and phosphatidylglycerol.

When a population of rapidly lysing T4 bacteriophage mutants are selected by plaque morphology on *Escherichia coli* strain B, each individual mutant maps in one of three separate gene loci. Mutants at one of these loci, the rII mutants, are readily distinguished because they cannot replicate on a host carrying lambda prophage [*E. coli* K-12 (λ)] and yet they multiply as well as wild type if the host (*E. coli* K-12) is not lysogenized with lambda. Thus, the wild-type rII gene product is required for lysis inhibition in strain B, is essential for replication in K-12 (λ), and is entirely dispensable in *E. coli* K-12 (6). The gene product and its function remain unknown, but its identity has been sought by examination of the varied biochemical consequences of infection and by study of growth medium additions which in some way can substitute for rII function and allow replication in *E. coli* K (λ).

Garen (11) demonstrated that the addition of high concentrations of Mg^{++} to the medium (0.03 to 0.08 M Mg^{++}) could overcome the rII defect in K-12 (λ). By various criteria—deoxyribonucleic acid (DNA) synthesis, protein synthesis, respiration, and ultraviolet resistance—it appeared that the reactions of phage development proceed normally in the absence of Mg^{++} for the first 10 min of infection but thereafter are blocked unless Mg^{++} is added to the medium. Other cations, such as spermine, spermidine, and a homologous series of methylene diamines from putrescine (C4) and cadaverine (C5) to octamethylene diamine, can also phenotypically correct the rII defect (2, 8) if added before the 10th min of infection (2). The different cations varied tremendously in effectiveness, both as compared to each other and as compared from one laboratory to another. In the data we present, a comparison of effective compounds indicates that the polyamines' function is not specific and is related to cationic properties.

Although the rII defect may be corrected as late as 10 min after infection, a difference between mutant and wild-type infection can be detected at 3 min. Phosphorylation of acid-soluble nucleotides is significantly lower at that time, and as a result the level of adenosine triphosphate (ATP) is decreased (27; M. Colowick, *personal communication*), which may in turn account for the decreased uptake of Mg observed at 6 min (27). The lowered ATP levels may also have some

[1] A preliminary account of this work was presented at the 1965 Annual Meeting of the American Society for Microbiology (Bacteriol. Proc., p. 102, 1965).

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bearing on the fact that monovalent cations strongly inhibit correction of the rII defect (27). Energy would be required to pump out the excess monovalent cations and to pump in Mg^{++} (27) or polyamines (30).

Even though the lowered phosphorylation rate is observed so early after infection, it is not clear that this is the primary defect. Investigation of a number of ATP-generating systems revealed no enzymatic differences between wild type and mutant (27). It is possible that faulty membrane repair or synthesis permits significant leakage and thereby leads to a cascading series of defects. Supporting this view are the reports that putrescine (2) and nicotinamide adenine dinucleotide (27) are extensively released from rII infected cells. We present data that indicate some swelling and loss of refractility in these cells. Furthermore, the fact that osmotically active compounds such as sucrose and other polyols (27) can correct the rII defect also points to a permeability problem.

In this paper, we present our data on the effectiveness of various compounds which correct the rII defect in K-12 (λ). These and other experiments presented here indicate that a faulty cell membrane is involved in the rII defect. We found that T4 phage infection has a profound effect on phospholipid biosynthesis, but no difference was detected between r^+ and rII with regard to biosynthesis of phosphatidylethanolamine and phosphatidylglycerol. However, we do refer in the Discussion to preliminary results which reveal that r^+ and rII-infected cells can be distinguished by the extent of cardiolipin biosynthesis.

MATERIALS AND METHODS

Bacteria and phage. E. coli K-12 (λ) was obtained from I. P. Crawford. It is the K-12 prototroph of Yanofsky and Crawford (34) and has been referred to as strain Ymel (35). *E. coli* K-12, its nonlysogenic derivative, was isolated after curing with λi^{434}. *E. coli* B, used for titration of bacteriophage, came originally from R. Herriott. *E. coli* BB from D. Krieg and K12W1485 from I. P. Crawford were used to prepare high-titer stocks of bacteriophage because both allow lysis inhibition with T4rII phage mutants.

Wild-type $T4r^+$ and various rII mutants were obtained from the Benzer collection. T4r1993 has a long deletion in the A cistron, r638 has a complete deletion of the B cistron, and r1272 has both cistrons deleted. The λi^{434} and directions for curing were obtained from Dorothy Fraser. T4 phage grown on BB or W1485 were purified by differential centrifugation until they met the optical criterion of more than 2.2×10^{12} phage per optical density unit at 400 mμ (14). High-titer stocks of approximately 10^{13} phage/ml were stored in diluting fluid containing 0.1 M NaCl, 0.001 M $MgCl_2$, and 0.0001 M $CaCl_2$ over a drop of $CHCl_3$. Phage were assayed by the method described by Adams (1). Phage ghosts were prepared and assayed by the method of Herriott and Barlow (15).

Media. Tryptone-Na^+ contained 1% tryptone and 0.1 M NaCl. Tryptone-Mg contained 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.

Miscellaneous chemicals. Putrescine, cadaverine, spermine, and spermidine were purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio). Hexa-, hepta-, octa-, and deca methylene diamines came from L. Light and Co. (Colnbrook, England).

Reversion of rII defect by supplements to medium. E. coli K-12(λ) was grown to 2.5×10^8 to 3×10^8 cells/ml in tryptone-Na. Cells were infected with a multiplicity of 5 to 8 with T4 phage. After 5 or 6 min of shaking, anti-T4 serum was added for an additional 2 to 3 min. At 7 or 8 min after infection (actual times and multiplicities given with data), the cells were diluted 10^4-fold through 1% tryptone and then 10-fold into the final incubation mixture containing 1% tryptone plus the supplements at the indicated concentrations. The final mixture was aerated for 52 min longer, $CHCl_3$ was added, and the phage were titrated on *E. coli* B. The number of infected bacteria was set equal to the difference in viable cells before infection and after 7 to 8 min of infection. (Survivors ranged from 0.5 to 10% of starting cells.) "Burst size" indicates the number of phage per infected cell.

Polyamines in phage. Approximately 5×10^{13} to 1×10^{14} phage, purified as above, were hydrolyzed in 4 N HCl in sealed tubes at 105 to 106 C for 18 to 22 hr. The hydrolysates were evaporated to dryness over NaOH-$CaCl_2$ in a vacuum desiccator and were redissolved in water. After removal and washing of char at the centrifuge, a sample corresponding to 3×10^{13} to 5×10^{13} phage was chromatographed on Dowex 1-Na^+ by a method to be described (L. Astrachan and J. Miller, *in preparation*). Amines were quantitated by the ninhydrin method (22).

Phospholipids of infected and uninfected bacteria. E. coli K-12(λ) was grown with shaking at 37 C in tryptone-Mg^{++} to 3×10^8 cells/ml and then was divided into three portions of 310 ml each. At time minus 1 min, 1 mc of ^{32}P orthophosphate was added to each flask. At zero-time, the cells in two flasks were infected at a multiplicity of six with r^+ or r1993; and the third flask remained uninfected. All flasks were aerated continually at 37 C. At 5, 10, and 20 min after infection (6, 11, 21 min after ^{32}P), 100-ml samples were poured over 2.9 ml of 11 M $HClO_4$ and stirred in an ice bath. After centrifugation, resuspension in cold saline, neutralization with 1 M $NaHCO_3$, and recentrifugation, the precipitates were extracted with chloroform-methanol, 2:1 (32). The extracts were washed once with one-third volume of water, after which the chloroform phase was dried in vacuo, redissolved in chloroform, filtered through glass wool, and then chromatographed on silica gel HR (Brinkmann Instruments, Inc., New York, N.Y.) thin-layer plates with a solvent mixture of $CHCl_3$-CH_3OH-water (70:25:4). All operations were performed under N_2 in the cold wherever possible and

with distilled, N_2-flushed solvents. Phospholipid bands were detected by staining with I_2 vapors and by radioautography. Eight separate channels were developed for each sample. From four of the channels, the gel bands containing phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were individually transferred to Kjeldahl flasks for ashing in 11 M $HClO_4$ and subsequent phosphorus assay (12). Gel from the other four channels was transferred to vials for counting in a liquid scintillation spectrometer. Thus, the specific activities are from average values of four separate determinations each of total phosphorus and radioactivity. Preliminary experiments showed that the amounts of silica gel used did not interfere with either assay. Two additional minor phospholipids were also detected and were clearly separated on the thin-layer chromatograms. These were not further studied, other than to identify one of them as phosphatidylserine. In addition, there was always some radioactive material at the origin, but this could well have been due to the minimal washing of the chloroform-methanol extract. PE and phosphatidylserine were identified by positive ninhydrin reaction and extensive incorporation of ^{14}C-labeled serine. Since PE and PG are the major phospholipid classes of *E. coli* (17), they were readily noted by the intensity of I_2 stain and the extent of ^{32}P incorporation. They were distinguished from each other by the ninhydrin reagent and the relative amounts of phosphorus in each band (17). Furthermore, our PG co-chromatographed in the above solvent system and one other solvent system (28) with an authentic sample of phosphatidylglycerol which was kindly provided by John Law. The observed R_F values were orthophosphate, <0.05; phosphatidyl serine, 0.24; PG, 0.35; PE, 0.47; (?) (phosphatidic acid?), 0.60.

RESULTS

Growth of T4rII on K(λ) in presence o spermidine, diamines, and basic amino acids. Spermidine and putrescine are normally found in T4 coliphage (3, 4) and in bacteria (5, 10, 13, 31). In bacteriophage, these basic compounds appear to be involved in neutralization of the phosphate groups of the phage DNA. In bacteria, polyamines have been implicated in the stabilization of fragile cells (18, 19) and of protoplasts (20, 23). Spermidine and other polyamines have also been shown to stabilize ribosomal complexes (9, 36). Since spermidine in some systems behaves similarly to Mg^{++}, it occurred to a number of investigators to determine whether this polyamine could substitute for Mg^{++} in stimulating the growth of rII phages in *E. coli* K-12 (λ) (2, 8). In our experiments, spermidine was found to be effective at an optimal concentration of 0.03 to 0.05 M. The yields of rII progeny at these concentrations were usually 5 to 30 phage per infected cell, representing a 100- to 600-fold increase over the control. These results were obtained with mutants of either the A or B cistron and with r1272, an rII mutant in which both cistrons are completely deleted.

In an attempt to determine whether spermidine or Mg^{++} satisfies specific requirements, a number of other basic compounds were tested for their ability to stimulate rII replication in K-12 (λ). These supplements were all compared at a concentration of 0.03 M, which may not be optimal for each one. The results of one such experiment are presented in Table 1. In the homologous series of methylene diamines, heptamethylene diamine and cadaverine were most effective, whereas decamethylene diamine was inhibitory. Qualitatively similar results were reported by Brock (8), but she reported lower phage yields, presumably, as we shall see later, because of the presence of NaCl in the medium. Recently, the stimulatory properties of putrescine and spermidine have also been recorded (2).

Several of the basic amino acids were also tested. Of these, arginine was the most effective supplement, allowing in this experiment the production of 41 progeny phage particles per infected cell. From one experiment to another, the burst size with any of the effective compounds varied as much as recorded above for spermidine, but qualitatively the compounds were always efficacious. No stimulation of rII growth was observed with nonbasic amino acids. The action of arginine, ornithine, and lysine is of interest, because they, in contrast to the other effective compounds, offer no apparent osmotic advantage over monovalent cations for the neutralization of fixed anions. This is relevant to the site of action of the effective compounds and suggests that it is not merely neutralization of DNA.

Phage content of putrescine and spermidine. Ames and Ames (2) reported that putrescine leaks out of a cell infected with rII phage and that the

TABLE 1. *Effect of diamines, polyamines, and basic amino acids on burst size of T4r1993 in Escherichia coli K-12(λ)*[a]

Amine	Burst size	Amine	Burst size
Putrescine (C4)	3.7	Spermine	0.16
Cadaverine (C5)	54	Arginine	41
Hexamethylene diamine	28	Ornithine	9.1
Heptamethylene diamine	62	Lysine	6.1
Octamethylene diamine	22	Citrulline	0.08
Decamethylene diamine	0.006	Histidine	0.03
Spermidine	5.2	Control	0.03

[a] Multiplicity of infection = 7. Dilution and addition of 0.03 M amine at 8 min after infection.

leakage is not inhibited by Mg^{++}. If the effective compounds function by replacing putrescine in the neutralization of DNA, their presence would result in a lesser amount of putrescine packaged within the progeny phage. To test this possibility, $T4r^+$ and rII phage were grown in tryptone-Mg^{++} medium within K-12 (λ) or K-12 bacteria. After purification by differential centrifugation, the phage were hydrolyzed with acid, and the hydrolysate was chromatographed on Dowex-1 columns. The data of Table 2 show that mutant and wild-type phage have similar polyamine contents which do not vary whether the host is K-12 or K-12 (λ), or whether the mutation is in the A cistron (r1993) or B cistron (r638). Comparison of the last two lines of Table 2 further reveals that the presence of Mg^{++} in the medium does not alter phage polyamine content. It is apparent that mutant phage grown either in K-12 (λ) or in the permissive host K-12 have sufficient polyamine available to allow a normal pattern of DNA neutralization and incorporation into progeny phage. The data strongly suggest that the inability of rII to grow in K-12 (λ) under standard conditions is not due to a lethally insufficient availability of polyamines.

Admittedly, the data do not say anything about polyamine concentrations under nonpermissive conditions. This leaves the possibility that Mg^{++} may relieve a polyamine shortage by substitution elsewhere in the cell. An argument against this possibility is indicated by the stability of polyamine content in the presence or absence of Mg^{++}, and within mutant or wild-type phage.

Inhibition of ghost-induced lysis by spermidine. Since the stimulatory effect of polyamines does not appear to be related to DNA neutralization, other structures known to bind cationic compounds were considered. It has been suggested that, in this system, Mg^{++} may prevent cellular leakage of essential components (11). To decide whether a similar function can be ascribed to spermidine, we measured its effect on cell lysis by phage ghosts. In Table 3, comparison of lines 2 and 4 shows that spermidine, which has no effect on the absorbancy of untreated cells, increases the absorbancy of ghost-treated cells. This phenomenon dims but does not obscure the demonstration that spermidine inhibits ghost-induced cell lysis, behavior consistent with the idea that the effective site of polyamine action is at the cell envelope.

TABLE 2. *Putrescine and spermidine in T4 bacteriophage*

Sample			Amt per 10^{13} phage (μmoles)			
Phage	Host	Medium	Putrescine	Spermidine	Polyamine nitrogen	Molar ratio of putrescine/spermidine
r1993	K-12	Tryptone-Mg^{++}	0.97	0.36	3.0	2.7
r638	K-12	Tryptone-Mg^{++}	1.12	0.37	3.3	3.0
r1993	K-12(λ)	Tryptone-Mg^{++}	1.09	0.37	3.3	2.9
r^+	K-12	Tryptone-Mg^{++}	1.17	0.41	3.6	2.9
r^+	K-12(λ)	Tryptone-Mg^{++}	1.20	0.35	3.4	3.5
r^+	K-12(λ)	Tryptone-Na	1.26	0.38	3.6	3.3

TABLE 3. *Effect of spermidine on lysis of Escherichia coli K-12(λ) by r1993 ghosts*[a]

Incubation mixture	Optical density (660 mμ) at 15 min
Cells	0.34
Cells + ghosts	0.23
Cells + ghosts + spermidine (added at 3 min)	0.35
Cells + ghosts + spermidine (added at end)	0.28

[a] Final concentrations in incubation mixtures were 3×10^8 cells/ml, 1.6×10^9 ghosts/ml, 0.16 M NaCl, 0.05 M spermidine, and 1% tryptone. Total volume 4 ml. Mixtures were mechanically shaken at 37 C for 15 min and absorbancy was read at that time.

Replication of T4rII in presence of sucrose: effect of monovalent and divalent cations. To determine whether the cell envelope is relevant to the rII defect, we attempted to measure the effect of amine supplements on infected protoplasts. In the course of these experiments, which were unsuccessful because of excessive fragility of rII-infected cells, certain controls revealed that sucrose alone would allow growth of rII in K-12 (λ). Table 4 shows that the optimal sucrose concentration for rII growth was 0.4 M, at which concentration average burst sizes in different experiments ranged from 5 to 35 with a mode around 10. The average yield of r^+ was also increased by sucrose, but at a lower optimal concentration of 0.3 M. It appears from the inhibition of r^+ by sucrose at a concentration of 0.4 M that an optimal concentration for rII involves a balance between stimulation of rII growth and inhibition of host-cell metabolism. At higher concentrations, growth of both phages was severely restricted. Control experiments showed that the lowered yields were not caused by phage death with high sucrose concentrations.

TABLE 4. *Effect of sucrose on growth of r^+ and r1993 in Escherichia coli K-12* (λ)[a]

Sucrose concn	Burst size	
	r1993	r^+
M		
0	0.075	187
0.3	3.6	253
0.4	7.6	140
0.5	7.4	52
0.6	1.2	11
0.7	0.1	0.9

[a] Multiplicity of infection: r1993 = 12, r^+ = 6.8. Dilution and addition of sucrose at 8 min.

In our experiments, which partially overlap two other reports (8, 27), we attained rII phage yields intermediate between the results in the other two reports. The major factor contributing to the different yields was probably the amount of monovalent cation in the incubation mixture. When it is considered that 1% tryptone contains approximately 0.01 M Na^+, the shape of the curve in Fig. 1 relating rII yield in sucrose to salt concentration suggests that the yields would be considerably augmented in the absence of inherent monovalent cation. Sekiguchi (27) reported near wild-type yields of rII in sucrose, but these yields were obtained in a medium containing only 0.2% tryptone and consequently significantly less salt. This may also account for the surprising fact that Sekiguchi found 0.15 M sucrose to be so effective. With respect to spermidine, the inherent Na^+ is of no consequence, but if spermidine were added to the usual medium, which contains 0.05 to 0.1 M NaCl for phage adsorption, very few rII progeny would be formed. The effect of Mg^{++} is also greatly inhibited by monovalent cations (11).

Although phenotypic reversion of the rII defect was achieved with sucrose, the phage yields in our experiments did not approach wild-type values. Since low concentrations of Ca^{++} added to sucrose are known to stabilize osmotically fragile organelles (16), we assessed this effect in the rII system. Figure 2 shows that Ca^{++} or Mg^{++}, at concentrations too low to be of much effect by themselves, greatly augmented the burst size in the presence of sucrose. The yields of rII phage, in sucrose media containing an optimal 0.002 to 0.005 M Ca^{++}, varied from 80 to 130 phage per infected cell and were always higher than those attained with Mg^{++}. On the other hand, when Ca^{++} or Mg^{++} ions were present at concentrations (0.05 to 0.08 M) at which they produced maximal rII yields in the absence of sucrose, the yields were sharply reduced. In an experiment in which infected cells were lysed with chloroform at various times after infection, it appeared that sucrose plus 0.003 M Ca^{++} had two antagonistic effects. The mixture slowed the rate of phage synthesis but

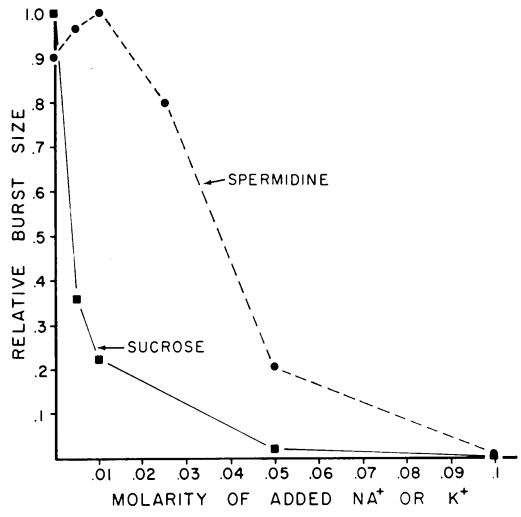

FIG. 1. *Effect of monovalent cations on sucrose- or spermidine-stimulated rII growth in Escherichia coli K-12* (λ). *Sucrose: r1993 multiplicity of infection = 5. Varying concentrations of KCl in 0.5 M sucrose. Dilution into final mixture at 8 min after infection. Ordinate, 1.0 equals a burst size of 11. Spermidine: r1272 multiplicity of infection = 7.3. Varying concentrations of NaCl in 0.03 M spermidine. Dilution at 7 min; 1.0 = burst size of 32.*

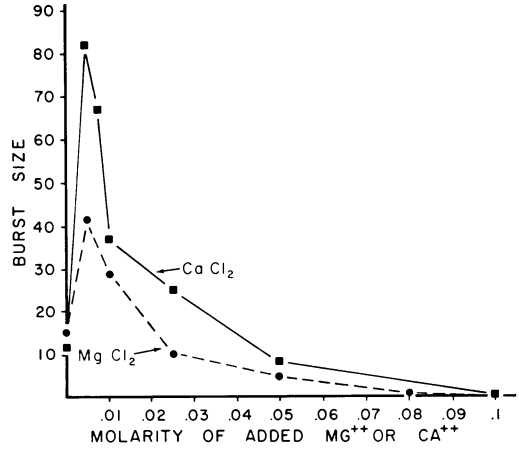

FIG. 2. *Burst size in 0.5 M sucrose of T4r1993 grown in Escherichia coli K-12* (λ). *Effect of Ca^{++} and Mg^{++}. For both experiments, multiplicity of infection = 8 and dilution into final incubation mixture was at 7 min after infection.*

delayed lysis of both r^+ and rII-infected cells so that increased yields were eventually obtained. The sucrose-Ca^{++} mixture thus appears to function by preserving the integrity of the infected cells.

Swelling and absorbancy changes of infected cells. Two other lines of evidence indicate that the rII defect involves a leaky, defective cell envelope. Figure 3 shows how absorbancy changes with time after infection with r^+ or r1993. Cells, infected with rII in the presence of Mg^{++} or with r^+ in the presence or absence of Mg^{++}, followed the same pattern of increasing absorbancy until the time of lysis. The rII-infected cells in the absence of Mg^{++} followed the pattern for only 4 min, after which time a gradual decline occurred. A decrease in absorbancy has been used as an indication of mitochondrial swelling (25); in the present experiments, such a decrease is considered to indicate that the cells swell and lose refractility. A more direct indication of swelling is provided in Fig. 4, where it can be seen that rII-infected cells swell more than r^+-infected cells. For these experiments, dense cell suspensions of centrifuged log-phase cells were infected with $T4r^+$ or r1993 at multiplicities greater than 12. Throughout the course of infection, samples were centrifuged and the volume of packed cells was measured. It was necessary to shake the infected cells vigorously to prevent too early lysis. When the cells were infected with multiplicities lower than 10, no swelling was observed. This may indicate that the observed differences at higher multiplicities represent some lysis from without. It is nevertheless significant that r^+-infected cells were better able to withstand such lysis than were cells infected with rII. In some experiments, the cell volume of r^+-infected cells also increased, but always less than a parallel culture infected with rII. When Na^+ was replaced by Mg^{++}, the volumes of rII-infected cells were, as expected, the same as those of wild type.

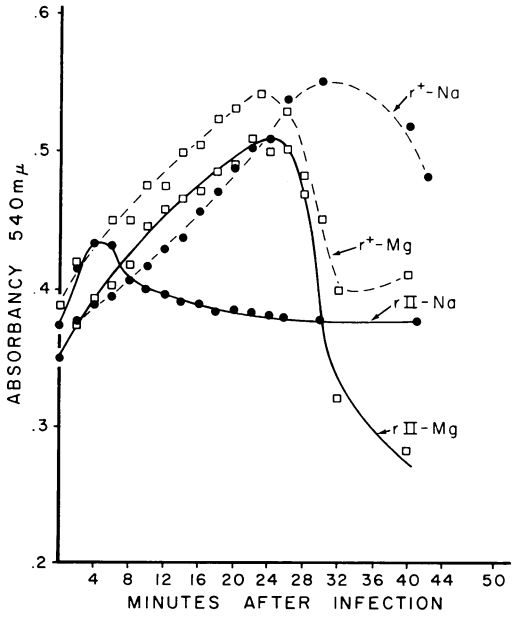

FIG. 3. *Absorbancy changes of K-12 (λ) with time after infection with $T4r^+$ or T4r1993: effect of medium. Cells at 2.5 × 10^8 in tryptone-Na^+ or tryptone-Mg^{++} were infected with $T4r^+$ or T4r1993 at a multiplicity of 6. Infected cultures were aerated at 37 C, and samples were removed at 2-min intervals. Absorbancy was read at 540 mμ in cuvettes thermostatted at 30 C.*

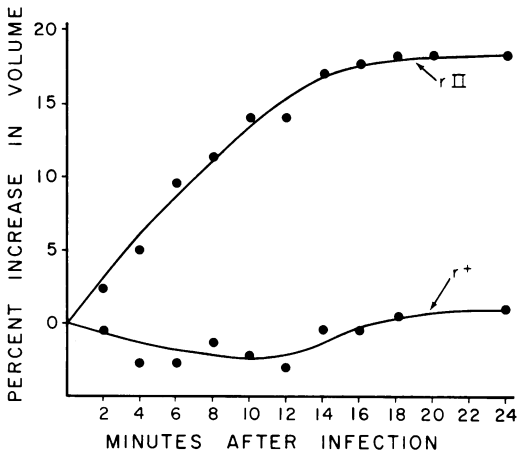

FIG. 4. *Volume changes upon infection of K-12 (λ) with r^+ or r1993 phage. Cells, grown to 3 × 10^8/ml in tryptone-Na^+, were harvested, washed, and resuspended in the same medium at 4 × 10^{10} cells/ml. A 1-ml amount of suspension in a 25-ml Erlenmeyer flask was infected with r^+ or r1993 at multiplicities of 13.5. Infected cells were shaken vigorously at 37 C. At 2-min intervals, samples were withdrawn into hematocrit capillaries, sealed with plasticene, and held in an ice bath until the end of the experiment. Capillaries were centrifuged, and the volume of packed cells was measured as a percentage of the total sample volume.*

Cell-envelope components. All the data presented thus far warrant investigation of the cell envelope as a possible site of the rII defect. More in the nature of eliminating a possibility, we examined the host-cell mucopeptides because they are generally considered to play an important role in the structural integrity of the cell. Since the rII defect is innocuous in *E. coli* K-12, the mucopeptides of this organism (uninfected) were extracted, purified, and compared with those of *E. coli* K-12 (λ) (21). The reason for this comparison is that K-12 does not need the rII product when infected

because it must differ in some way from K-12 (λ). Whatever the difference, it was not detected in acid hydrolysates of mucopeptides from the two organisms which contained nearly identical molar ratios of alanine, glutamic acid, lysine, diaminopimelic acid, muramic acid, and hexoseamine. Attempts were made to extend this comparison to mucopeptides of K-12 (λ) infected with r^+ and rII, but these experiments were abandoned when it was found that the mucopeptides of r^+-infected cells had already suffered some hydrolysis at 10 to 12 min after infection. (The mucopeptides were solubilized during a formic acid extraction step, which does not solubilize intact mucopeptides.) Since mucopeptide is hydrolyzed early in wild-type phage infection, it is probably not the specific structure that differentiates between mutant and wild type in the maintenance of cell integrity. This was perhaps predictable because the rII-infected cells do not swell excessively nor lyse but appear to have a permeability defect which would be more reasonably associated with the cell membrane.

Phospholipids, found in high concentrations in the cell membrane, are also known to bind polyamines. We examined the biosynthesis of phospholipids of K-12 (λ) after infection with r^+ and rII and in uninfected bacteria. Log-phase cells of *E. coli* K-12 (λ) in tryptone-Mg^{++} were exposed to ^{32}P-labeled orthophosphate. One minute later, two of three samples were infected with wild-type or mutant phage. At the times indicated in Table 5, samples were taken for phospholipid extraction, and the phospholipids were subsequently separated by thin-layer chromatography. The material in the separated bands was analyzed for radioactivity and total phosphorus. The results presented in Table 5 reveal that infection with T4 phage causes a marked inhibition of PE synthesis and a slight stimulation of PG synthesis. However, no difference was observed between r^+- and rII-infected cells. Similar results have been reported recently in experiments with *E. coli* B (M. H. Furrow and L. I. Pizer, Bacteriol. Proc., p. 26, 1967).

In later experiments, we found that 20 to 25% of the phosphorus in the PG band is in the form of cardiolipin (diphosphatidylglycerol). This could introduce a great error in the specific activities recorded for PG if ^{32}P were incorporated exclusively into either PG or cardiolipin. From preliminary measurements of the distribution of radioactivity between PG and cardiolipin, we could calculate that the recorded PG specific activities are 10% too high for the rII-infected cells and approximately 10% too low for the other two. This does not change the conclusion that T4 infection has slight effect on PG biosynthesis, whereas it markedly inhibits PE synthesis.

DISCUSSION

During infection of a host cell, a hole is made in the cell envelope to allow penetration by phage DNA. Puck and Lee (24) have shown that leakage of cellular substances occurs in T-even infections but eventually stops. With T4rII infection of *E. coli* K-12, (λ) however, loss of putrescine (2), nicotinamide adenine dinucleotide, and ATP (27)

TABLE 5. *Incorporation of ^{32}P into phospholipids of uninfected and r^+- and rII-infected Escherichia coli K-12(λ)*[a]

Min after ^{32}P	Phospholipid	Uninfected			r^+-infected			rII-infected		
		Counts/min ($\times 10^{-5}$)	Amt of P	Counts per min per μmole ($\times 10^{-5}$)	Counts/min ($\times 10^{-5}$)	Amt of P	Counts per min per μmole ($\times 10^{-5}$)	Counts/min ($\times 10^{-5}$)	Amt of P	Counts per min per μmole ($\times 10^{-5}$)
			μmoles			*μmoles*			*μmoles*	
6	PG	0.21[b]	0.11[b]	1.9	0.37[b]	0.19[b]	1.9	0.64	0.38	1.7
11	PG	1.06	0.38	2.8	1.51	0.32	4.7	1.61	0.34	4.7
21	PG	2.43	0.48	5.1	2.97	0.44	6.8	2.56	0.40	6.4
6	PE	0.30[b]	0.47[b]	0.64	0.41[b]	0.58[b]	0.7	0.53	1.03	0.5
11	PE	1.80	1.15	1.6	0.94	0.91	1.0	1.02	0.91	1.1
21	PE	4.65	1.14	4.1	1.53	1.02	1.5	1.62	1.01	1.6

[a] Phospholipids were extracted from 2.7×10^{10} bacteria (viable assay just before ^{32}P addition and infection with phage) after various durations of ^{32}P incorporation. The phospholipids were separated by thin-layer chromatography before assay of total P and radioactivity in the PG and PE. The amounts of PE and PG per bacterial cell may appear to be rather high. This is probably related to the fact that the cells were grown in tryptone-Mg^{++}, in which medium the cells are much larger than normal.

[b] We lost part of the lipid extracts from the 6-min r^+-infected and uninfected samples. This does not affect the specific activity data presented.

continue. The cell is fragile, low in ATP, unable to tolerate monovalent cations, and incapable of producing progeny phage. Some of these faults are reversed sufficiently to allow phage synthesis when any of a number of chemicals are added to the medium. The rII block can be partially relieved by arginine, spermidine, and certain diamines. These substances, like Mg^{++}, operate in a diversity of biochemical systems and, therefore, do not allow a precise localization of where or how they act to overcome the rII defect. However, they are not needed to relieve a deficiency of polyamines within the cell, since sufficient putrescine and spermidine are available for normal neutralization of DNA and packaging into progeny phage. The fact that spermidine inhibits ghost lysis suggests that the polyamine action is not localized in the cell interior. The efficacious action of sucrose, which does not cross the permeability barrier of the cell, points to an osmotic problem and thereby a faulty cell envelope associated with the rII defect. This interpretation is further supported by the finding that rII-infected cells lose refractility and swell. In fact, all of the data presented here are consistent with the idea that the rII defect is somehow associated with altered permeability. Nearly all the effective additives have, in one system or another, been shown to stabilize fragile cells or protoplasts. Furthermore, cations which affect rII multiplication have direct effects on the plasma membrane. Exposure of amoebae to Na^+ causes swelling and lowered electrical resistance of the plasma membrane, coincident with increased permeability (7). The addition of Ca^{++} or sucrose has the reverse effect. Thus, an agent which increases permeability inhibits rII multiplication, and agents with the reverse effect are stimulatory. Finally, another indication that the rII product is involved with the cell envelop is the phenomenon of lysis inhibition in *E. coli* B. This phenomenon occurs when the cell is first infected with r^+ phage and then reinfected with either mutant or wild-type phage. Since the second infection is not specific and also since it can occur so late that the superinfecting phage DNA cannot provide progeny genes, action at the surface is indicated, presumably with a product whose nature is determined by rI, rII, and rIII genes.

There seems little doubt that in some way the rII defect in *E. coli* K-12 (λ) results in a faulty cell envelope. But the unanswered questions are why is the envelope defective and how does wild-type rII product maintain it. We have presented evidence that phospholipid synthesis is altered after phage infection, as indicated by the decreased synthesis of PE and increased specific activity in the PG band. It was disappointing, if not surprising, to find no difference in phospholipid synthesis between r^+- and rII-infected bacteria. It is clear that phage infection has a profound effect on phospholipid metabolism, and this would appear to be an appropriate area of study for detecting a defect in cell-membrane synthesis. Of course, even if the rII defect were concerned with some aspect of membrane biosynthesis, the study of ^{32}P incorporation into PE and PG could only be a crude start at delineating the nature of the defect. The answer could as well come from a study of minor phospholipids, the proteins(s) of cell membrane, or the lipid components of the phospholipids. Recent results in this laboratory (M. Aghdashi and L. Astrachan, *unpublished data*) reveal that rII may be distinguished from r^+ by the fact that rII infection causes a much greater stimulation of ^{32}P incorporation into cardiolipin. This phenomenon was not detected in the presently reported experiments because the thin-layer solvent system we used does not separate PG from cardiolipin. The effect on cardiolipin synthesis is detected after separation by column chromatography of the lipids (26, 29) or of the deacylated backbones (33). Even though a difference between r^+- and rII-infected cells can be observed, this does not necessarily point directly to the rII defect. The difference could occur if the specific defect were inadequate synthesis of ATP or some other essential substrate, with the consequence that a reaction involved in membrane synthesis does not occur. For the time, the best that can be said is that a chemical phenomenon, of possible interest in the synthesis of membrane phospholipids, has been detected in association with the rII defect. It is interesting that ATP formation and cardiolipin synthesis are both associated with the rII defect, because ATP formation is so often associated with membranous structures. The identification of which phenomenon is causative (if, indeed, either one is) must await identification of a particular enzyme activity present in the wild type and absent in the mutant. Our efforts will be directed to the characterization of the phospholipid difference and, if successful, to the study of its metabolism.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant 5 R01 AI-04884 from the National Institute of Allergy and Infectious Diseases and by Public Health Service Research Career Development Award 5-K3-GM-4673 to L. Astrachan.

The technical assistance of Joan Miller is gratefully acknowledged.

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