

# Characteristics of Complement-Dependent Release of Phospholipid from *Escherichia coli*

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The release of <sup>32</sup>P-labeled bacterial phospholipid from a smooth *Escherichia coli* by serum components depends on complement activated by antibody. Phospholipid release in excess antibody tends to be proportional to the concentration of complement as does the release of other cellular constituents. Phospholipids are not simply stripped off during cell lysis. Whereas 94% of the total phospholipid freed from *E. coli* by mechanical lysis sediments at centrifugal forces sufficient to sediment molecules of 10<sup>6</sup> molecular weight, similar centrifugation sediments only 50% of the phospholipid released by antibody-complement. In fact, after mechanical lysis more than 50% of the phospholipid sediments at velocities sufficient to bring down cell envelopes. Although the bulk of the bacterial phospholipid is located in the cell envelopes, isolated <sup>32</sup>P-labeled cell envelopes and phenol-extracted lipopolysaccharide fails to release phospholipids in the presence of antibody-complement. Moreover, ethylenediaminetetraacetic acid, which like antibody-complement causes loss of cellular selective permeability and prepares *E. coli* cell walls for the action of lysozyme, releases only small amounts of phospholipid from *E. coli* and these are sedimentable. The most likely mechanism of phospholipid release caused by antibody-complement appears to be the activation directly or indirectly of an enzyme which is present only in the intact cells.

The release of a phosphorus-containing, hot ether-alcohol-soluble fraction from *Escherichia coli* by exposure to fresh guinea pig serum (14) and to antibody and complement "reagents" (18) has been previously reported. This fraction appears to be a phospholipid (12, 14) probably phosphatidylethanolamine (1). From previous experiments (14, 18), the release of phospholipid seems to be related to the activation of complement by either normal or hyperimmune antibody and independent of the presence of lysozyme. It has been proposed that activated complement attacks the phospholipids of membranes and lipopolysaccharides (16, 18). Moreover complement is supposed to cause 9- to 10-nm lesions in cell membranes (6) and lipopolysaccharide (LPS) (3) and loss of selective cellular permeability. Activated complement has also been shown to be responsible for a change in titratable fatty acids of antibody-complement-treated erythrocytes (13). Thus, either direct loss or modification of phospholipid in cytoplasmic membranes might result in molecular rearrangement and the formation of lesions.

In this study answers were sought to the following questions. (i) Was phospholipid release from *E. coli* a complement-dependent reaction, (ii) what was the form of the released phospholipid with regard to size, and (iii) where was this phospholipid located in undamaged bacteria?

## MATERIALS AND METHODS

**Cultures and media.** Approximately 18 hr before use, a culture of a smooth lysozyme-resistant strain of *E. coli* 0117:H27 was grown in antibiotic assay broth (Difco) from a nutrient agar maintenance slant. A complex synthetic culture medium containing amino acids, vitamins, and salts with enough inorganic phosphate to allow three to four generations was used for <sup>32</sup>P labeling and preparations of cell constituents (14, 15).

Gey's balanced salts solution (BSS) with glucose (200 mg/ml) was used as a diluent in all serum reaction mixtures (15). A tris(hydroxymethyl)aminoethane-chloride buffer (pH 8.0, 0.15 M) was used in the ethylenediaminetetraacetic acid (EDTA) experiment in place of Gey's BSS (9).

**<sup>32</sup>P labeling.** An 18-hr culture of *E. coli* (40 ml) in antibiotic assay broth was washed two times and resuspended in 5 ml of sterile water. A 40- to 60- $\mu$ Ci amount of the labeling media containing 20 to 120  $\mu$ Ci of the <sup>32</sup>P (Tracer Lab., Waltham, Mass.) was inocu-

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lated with 1.0 ml of the cell suspension and incubated for 5 to 6 hr on a reciprocal shaker water bath at 37 C. The labeled cells were then washed three times in phosphate buffer (pH 7.0,  $\mu = 0.1$ ), resuspended to volume in buffer, and stored at 10 C until used (15). When larger volumes of labeled cells were required for fractionation of cell constituents, the same volume per flask was used with only a greater number of flasks making up the required volume. This preserved uniform labeling conditions. The specific activity of the labeled *E. coli* was approximately 0.254  $\mu\text{Ci}$  per mg (dry weight).

**Isotope assay.** Isotope assay was accomplished on a Tri-Carb 3003 liquid scintillation spectrometer (Packard Instrument Corp., Downers Grove, Ill.) as previously described (15).

**Serum "reagents."** Fresh pooled, normal guinea pig serum that had been absorbed with bentonite to remove serum lysozyme (15) was used as a source of normal antibody and complement. Complement activity was removed from this serum by heat inactivation of small samples (3 ml) at 56 C for 20 min. Hyperimmune antibody, prepared by standard techniques for preparation of anti "O" antiserum in guinea pigs, was also heat inactivated and bentonite was absorbed. The titer of this preparation by tube agglutination was approximately 1:1,280. Egg white lysozyme was used to reconstitute these sera to approximately normal activity levels.

**$^{32}\text{P}$  release from labeled bacteria by serum components.** Twice washed, labeled *E. coli* cells [0.077 mg (dry weight)/ml] were added to tubes which contained optimal concentrations of the serum "reagents" listed in the tables. The reaction mixtures were incubated at 37 C for 1 hr, then centrifuged at  $6,000 \times g$  for 10 min. When not being incubated, all materials were kept in an ice bath. A 0.5-ml sample of the supernatant fluid was placed directly into 10.0 ml of Bray's scintillation medium for determination of total  $^{32}\text{P}$  released (15).

**$^{32}\text{P}$  release from labeled bacteria by EDTA.** This experiment was similar in most procedural aspects to experiments concerning  $^{32}\text{P}$  release by serum components and was based on the methods of Leive (9). The EDTA experiment differed mainly in the reagents and time of incubation (10 min). After incubation, the reaction was stopped by the addition of excess amounts of  $\text{MgCl}_2$ . A control of EDTA with  $\text{MgCl}_2$ , mixed prior to the addition of cells, was included. The final reagent concentrations are listed in Table 4.

**Ultracentrifugation.** Ultracentrifugation was carried out in a model L2-65B instrument (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) with a SW 65Ti rotor. Some of the  $6,000 \times g$  supernatant fluid from both the serum and EDTA experiments was further centrifuged at a force sufficient to clear molecules of  $10^6$  molecular weight from the supernatant fluid ( $420,000 \times g$  for 125 min). The resulting supernatant fluids were assayed in the same manner for  $^{32}\text{P}$  as the  $6,000 \times g$  supernatant fluid. Ultracentrifugation was also used to concentrate 19S antibody ( $420,000 \times g$  for 160 min) for a more efficient normal serum reagent.

**Fractionation of  $^{32}\text{P}$ -compounds.** A sample of the reaction mixture supernatant fluid ( $6,000 \times g$  or  $420,000 \times g$ ) was sequentially extracted by a modified Schmidt-Thannhauser method, which consisted of precipitation with 10% cold trichloroacetic acid followed by ether-alcohol (1:3) at 37 C for 15 min and 5% trichloroacetic acid at 90 C for 30 min (14). Two extractions with each reagent were done, and measured volumes (0.5 ml) of each fraction were placed in scintillation vials containing 10 ml of Bray's solution for counting. Whole reaction mixtures or homogenates were extracted to give a basis upon which to calculate the maximum available  $^{32}\text{P}$  of each fraction. These figures obtained experimentally were similar to those published by Roberts et al. (12). Cold trichloroacetic acid-soluble  $^{32}\text{P}$  was assumed to be due to micromolecular metabolic intermediates or breakdown products; ether-alcohol-soluble  $^{32}\text{P}$  was assumed to be due to phospholipids; and hot trichloroacetic acid-soluble materials were considered to be macromolecular nucleic acids (12, 14, 18).

**Preparation of  $^{32}\text{P}$ -labeled *E. coli* LPS and cell envelopes.** LPS was prepared by the method of Westphal and Jann (17) by using highly labeled *E. coli*. Cells were labeled as described previously except that 202  $\mu\text{Ci}$  of  $^{32}\text{P}$  was added to each 60 ml of medium. Six flasks of 60 ml each were inoculated and grown as previously stated. The bacteria were then washed in water two times and resuspended in 17.5 ml of 4% glutaraldehyde fixative at room temperature for 1 hr in an effort to reduce contamination of the LPS with nucleic acids. The glutaraldehyde was removed by washing the cells two times and resuspending them to the required volume. This volume of cell suspension (17.5 ml) was added to 22.5 ml of 80% phenol at 75 C and stirred constantly at 68 to 70 C for 5 min. The mixture was cooled to 5 to 10 C with stirring and then centrifuged at  $1,500 \times g$  for 30 min at 4 C. After removal of the aqueous phase, 17.5 ml of hot water was added to the phenol phase and the extraction was repeated. The pooled aqueous phases were then dialyzed against water (4 liters) at 4 C overnight to remove residual phenol. After dialysis, the LPS was washed two times in water at  $105,000 \times g$  for 30 min and lyophilized in a tared ampoule. The LPS was adjusted prior to use to give 0.1 mg/ml. Specific activity was 0.424  $\mu\text{Ci}/\text{mg}$  (dry weight).

Cell envelopes (cell walls and plasma membranes) were prepared by the technique of Roberson and Schwab (11). The labeled cell envelopes were lyophilized in a tared ampoule and adjusted to give 0.8 mg/ml for use. Specific activity was 0.165  $\mu\text{Ci}/\text{mg}$  (dry weight).

**Release of  $^{32}\text{P}$ -phospholipid from labeled cell constituents.** The exposure of LPS and cell envelopes to serum components and the assay of  $^{32}\text{P}$  released from them was conducted in the same manner as with whole, labeled bacteria, with the exception of the sedimenting force used to wash the labeled material and separate it from the supernatant fluids. The same centrifugal forces required to isolate the LPS ( $105,000 \times g$  for 30 min) and cell envelopes ( $10,000 \times g$  for 30 min) were used in the  $^{32}\text{P}$  release experiments. The final concentrations of LPS and cell envelopes per

milliliter of reaction mixture were 0.01 mg and 0.08 mg, respectively. Both reaction mixtures contained 0.1 ml of bentonite-absorbed fresh guinea pig serum and 0.1 ml of anti-*E. coli* 0117 serum (1:100 dilution) per ml of reaction mixture. Appropriate controls of heated serum were included.

## RESULTS

**Concentration of complement: its influence on phospholipid release.** In this experiment, complement was used in increasing quantities with heat-inactivated serum added to keep the total serum content of each reaction mixture the same and to insure an equal and excess amount of normal antibody (Table 1). All cellular constituents including the ether-alcohol-soluble fraction were released in increasing quantities as the complement content increased up to a maximum level. Although lysozyme added to the system lysed the cells and released their nucleic acid, it did not change the percentage of phospholipid released.

**Physical characteristics of released bacterial phospholipid.** The resuspended sediment of guinea pig serum centrifuged to concentrate 19S antibody was extremely active even when freed of lysozyme, releasing 43% of the available  $^{32}\text{P}$  from *E. coli* (Table 2). Normal guinea pig serum with lysozyme removed released a maximum 30 to 32%  $^{32}\text{P}$  in this experiment (Table 1) and 26 to 30% in a previous experiment by using antibody and complement "reagents" freed of lysozyme (18). This active serum was used with and without lysozyme to release maximum amounts of bacterial phospholipid (Table 2). These reaction mixtures, centrifuged at  $6,000 \times g$  for 10 min to remove bacteria, provided supernatant fluids which were further centrifuged at  $420,000 \times g$  for 125 min to determine the sedimenting ability of released cellular constituents. This treatment removed very little micromolecular  $^{32}\text{P}$  from the supernatant fluids. Much of the phospholipid also was not sedimented.

TABLE 1.  $^{32}\text{P}$ -labeled compounds released from *E. coli*<sup>a</sup> by various amounts of complement with constant amounts of antibody in the presence or absence of lysozyme

Lysozyme	Complement (ml) <sup>b</sup>	Total $^{32}\text{P}$ released	Micromolecular $^{32}\text{P}$	Lipid $^{32}\text{P}$	Macromolecular $^{32}\text{P}$
None	0.02	18 <sup>c</sup> (100) <sup>d</sup>	8 (13)	4 (14)	4 (60)
	0.1	32 (100)	12 (13)	9 (13)	8 (58)
	0.4	30 (100)	11 (15)	8 (14)	5 (59)
2 $\mu\text{g}/\text{ml}$	0.02	32 (100)	8 (14)	5 (14)	17 (59)
	0.1	65 (100)	13 (15)	11 (15)	36 (55)
	0.4	60 (100)	16 (19)	9 (12)	27 (54)

<sup>a</sup> *E. coli* labeled metabolically with  $^{32}\text{P}$ ; 0.007 mg (dry weight)/ml of reaction mixture.

<sup>b</sup> Fresh complement normal guinea pig serum absorbed with bentonite to remove lysozyme was diluted with lysozyme-free guinea pig serum heated at 56 C for 20 min to inactivate complement. Final volume of the reaction mixture was 0.4 ml.

<sup>c</sup> Per cent of  $^{32}\text{P}$  of the available  $^{32}\text{P}$  in labeled *E. coli* released in  $6,000 \times g$  supernatant fluid.

<sup>d</sup> Per cent of  $^{32}\text{P}$  available in reaction mixture after incubation.

TABLE 2. Sedimentability of  $^{32}\text{P}$ -compounds released by serum components

Centrifugation	<sup>19</sup> S antibody + complement <sup>a</sup> (ml)	Lysozyme ( $\mu\text{g}$ )	Total $^{32}\text{P}$ released	Micromolecular $^{32}\text{P}$	Lipid $^{32}\text{P}$	Macromolecular $^{32}\text{P}$
After $6,000 \times g$ for 10 min	0.0	2.0	13 <sup>b</sup>	11	0	1
	0.3	0.0	43	13	11	16
	0.3	2.0	57	23	11	18
After $420,000 \times g$ for 120 min	0.0	2.0	8 <sup>c</sup>	9	0	0
	0.3	0.0	23	14	7	1
	0.3	2.0	34	21	7	2

<sup>a</sup> Bentonite-absorbed guinea pig serum sediment from a  $420,000 \times g$  160-min centrifugation resuspended in the remaining supernatant fluid after removing the top two-thirds of the original volume. This portion was much more reactive than the bentonite-absorbed serum supernatant fluid or uncentrifuged bentonite-absorbed serum. Expressed as milliliters per milliliter of reaction mixture.

<sup>b</sup> See Table 1, footnote c.

<sup>c</sup> Per cent of  $^{32}\text{P}$  of the available  $^{32}\text{P}$  in labeled *E. coli* released in  $420,000 \times g$  supernatant fluid.

**Principal location of bacterial phospholipid.** To investigate the possibility that phospholipid existed in the bacteria in forms other than LPS and cytoplasmic membrane (e.g., as a cytoplasmic lipid pool), labeled bacteria were lysed mechanically and then centrifuged first at  $10,000 \times g$  for 30 min and then at  $420,000 \times g$  for 125 min. As shown in Table 3, cold trichloroacetic acid-soluble material did not sediment under these conditions, but most of the hot trichloroacetic acid-soluble material was sedimentable at  $420,000 \times g$ . Of the phospholipid, 60% sedimented during the  $10,000 \times g$  centrifugation and an additional 34% sedimented at  $420,000 \times g$ . Thus, over half of the phospholipid may have been associated with the cell envelope which is sedimentable at  $10,000 \times g$ , and the rest of the phospholipid, sedimentable at  $420,000 \times g$ , might have been in the form of cytoplasmic membrane or cell wall fragments. Hence, the nonsedimentable phospholipid which antibody-complement released seemed most likely to have arisen from these forms that are readily sedimented after mechanical lysis.

**Phospholipid release from *E. coli* cell envelopes and LPS by activated complement.** Most if not all gram-negative bacterial phospholipid is incorporated into the cell envelope consisting of the cell wall complex and cytoplasmic membrane (2, 7). Since these two structures are inseparable, the phospholipid containing LPS, which is believed to be a substrate of complement (3, 4), was labeled with  $^{32}\text{P}$ , extracted with phenol, and then treated with antibody and complement to determine if phospholipid would be released. After exposure of the LPS to antibody-complement, no  $^{32}\text{P}$  was detected in the supernatant fluid after centrifugation at  $420,000 \times g$  for 125 min. Whether or not 9- to 10-nm lesions were present was not ascertained. However, aggregation of the LPS was observed in tubes containing antibody but not in the control tubes.

Labeled cell envelopes were exposed to serum

TABLE 3.  $^{32}\text{P}$ -compounds released by mechanical lysis

Fraction	Total $^{32}\text{P}$	Micro-molecular $^{32}\text{P}$	Lipid $^{32}\text{P}$	Macro-molecular $^{32}\text{P}$
Homogenate . . . . .	100 <sup>a</sup>	20	10	56
$10,000 \times g$ Supernatant fluid . . . .	76	18	4	42
$420,000 \times g$ Supernatant fluid . . . .	47	15	0.6	18

<sup>a</sup> Per cent of  $^{32}\text{P}$  of the available  $^{32}\text{P}$  in labeled *E. coli*.

components. Again there was no release of  $^{32}\text{P}$ -labeled phospholipid. It would appear that "free" phospholipid release occurs only from whole bacterial cells and not from isolated cell envelope constituents.

**Effects of EDTA on *E. coli* phospholipids.** EDTA releases gram-negative bacterial LPS, damages selective cell permeability, and exposes the mucopeptide of gram-negative bacteria to lysozyme (5, 9). Since antibody and complement together apparently cause similar effects (14, 18), it was decided to see if EDTA with and without lysozyme release phospholipid in a way similar to antibody-complement. The results of this experiment are shown in Table 4. EDTA readily released small-molecular-weight cell constituents both with and without lysozyme. Most of the cold trichloroacetic acid-soluble fraction was not sedimentable at the higher centrifugal force. Phospholipids and nucleic acids were released only in the presence of lysozyme. They were both sedimentable at the  $420,000 \times g$  centrifugal force.

## DISCUSSION

In our experiments, complement activation was necessary for the release of the free phospholipids from unruptured bacteria. The amount of phospholipid released was dependent on the concentration of complement and not total serum concentration. This suggested that the release was not caused by a serum enzyme. Evidence that a heat-labile serum enzyme did not release the phospholipids has been presented in a previous publication (18). Furthermore, phospholipids of cell envelopes were not released by serum components in the present experiments.

Ultracentrifugation of the  $6,000 \times g$  supernatant fluid from serum antibody-complement reaction mixtures indicated that a large amount of the released bacterial phospholipid was not sedimentable at forces sufficient to clear molecules of  $10^6$  molecular weight from the supernatant fluids. If this unsedimentable phospholipid was associated with cell wall or membrane fragments, these fragments would have to be smaller than ones produced by EDTA-lysozyme treatment or mechanical lysis of *E. coli*. Furthermore, since phospholipid release was independent of cell lysis, the cytoplasmic membrane phospholipid released in the absence of lysozyme would have to be able to diffuse through modified but unruptured cell walls which, as it turned out, would not allow the passage of the cellular nucleic acids (18). Therefore, the nonsedimentable phospholipid released by antibody-complement very likely is in the "free" form not complexed with cell mem-

TABLE 4. <sup>32</sup>P-compounds released from *E. coli*<sup>a</sup> by  $5 \times 10^{-3}$  M ethylenediaminetetraacetic acid in the presence or absence of lysozyme

Centrifugation	Lysozyme ( $\mu$ g/ml of reaction mixture)	Total <sup>32</sup> P released	Micromolecular <sup>32</sup> P	Lipid <sup>32</sup> P	Macromolecular <sup>32</sup> P
After 6,000 $\times$ g for 10 min	4 <sup>b</sup>	5 <sup>c</sup>	3	0	<1
	0	24	21	1	1
	4	32	15	6	7
After 420,000 $\times$ g for 120 min	4 <sup>b</sup>	<1 <sup>d</sup>	1	0	0
	0	14	12	<1	1
	4	14	10	1	1

<sup>a</sup> <sup>32</sup>P-labeled *E. coli*;  $10^9$ /ml of reaction mixture.

<sup>b</sup> Control containing  $2 \times 10^{-2}$  M MgCl<sub>2</sub> in addition to lysozyme.

<sup>c</sup> See Table 1, footnote c.

<sup>d</sup> See Table 2, footnote c.

brane or wall. A smaller portion of the phospholipid released by serum may be in the form of spherules of LPS which are not sedimentable at 6,000  $\times$  g but are removed by 420,000  $\times$  g centrifugations, accounting for the drop from 11 to 7% in Table 2 (18).

Phosphatidylethanolamine, the principal phospholipid of *E. coli*, is abundant in certain *E. coli* LPS preparations and constitutes 20 to 30% of the cytoplasmic membrane (7). In cell wall or cytoplasmic membrane, phospholipid might serve as a principal target of complement activity (16). Most of the phospholipid from mechanically disrupted cells sediments with cell envelopes. The remaining small amount of phospholipid in the cytoplasmic fraction in all probability represents small fragments of cytoplasmic membrane which require high forces for sedimentation; however, they are sedimentable unlike most of the phospholipid released by antibody-complement. Thus, it would seem that there is no cytoplasmic pool of small-molecular-weight phospholipid in *E. coli*.

Both antibody-complement and EDTA make gram-negative bacteria susceptible to the action of lysozyme and cause them to lose selective permeability (9). In addition, Leive (9) found that EDTA released LPS from *E. coli*. Since we had found that antibody-complement released LPS and phospholipid from *E. coli*, it seemed possible that these two systems might share a common mechanism of action involving the chelation of divalent cations. Furthermore, this hypothesis was supported by the work of Muschel and Jackson (10) who had reported that magnesium was able to reverse the lytic action of complement, provided that it was added early in the course of the reaction. Although we confirmed the fact that EDTA tended to damage the permeability barrier of *E. coli* and expose the mucopeptide layer to the

action of lysozyme, the reaction differed in other respects from the one due to antibody-complement because phospholipid separated from the cell only when lysozyme was added to the system with EDTA and it was all precipitated at 420,000  $\times$  g.

If LPS and cytoplasmic membranes can serve as a complement substrate, and phospholipid is common to these structures, it is not clear why the treatment of isolated LPS and cell envelopes with antibody-complement did not yield the nonsedimentable (at 420,000  $\times$  g) phospholipid. Smith and Becker (13) have reported a complement-dependent modification of red blood cell (RBC) phospholipid in which fatty acids increased, whereas phospholipid decreased, provided whole cells were used in the reaction mixtures. The reaction was not evident if antibody-sensitized RBC stroma were used. These results seemed similar to the ones we obtained with isolated LPS and cell envelopes. Humphrey and Dourmashkin (6) have described 9- to 10-nm lesions in prepared LPS treated with fresh serum; however, analysis with thin-layer chromatography failed to reveal changes in the LPS phospholipids. Evidently, something more than isolated cell components is required for phospholipid release.

Since whole cells apparently are required for the complement-dependent modification or release of phospholipid, the mechanism may be dependent on the specific triggering directly or indirectly of a cellular enzyme. Support that this enzyme most likely is not membrane associated, can be found in the apparent lack of phospholipid release from preparations of bacterial cell envelopes exposed to antibody-complement. The enzyme responsible for the phospholipid release may be located between the cell wall and cytoplasmic membrane or in the cytoplasm. Release

of bacterial phospholipid from whole cells has been described in a *Bacillus cereus* autolytic system in which over 50% of the total phospholipid was released (8). Since it is not clear from our experiments whether complement-dependent phospholipid release is due to the direct enzymatic action of some activated component of complement, or some autodegradation of bacterial plasma membrane, further elucidation of the release mechanism is required.

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