Kennedy, K. E., and Thompson, G. A., Jr. (1970), *Science 168*, 989.
Marinetti, G. V. (1962), *J. Lipid Res. 3*, 1.
McElhaney, R. N., de Gier, J., and van Deenen, L. L. M. (1970), *Biochim. Biophys. Acta 219*, 245.
Nozawa, Y., and Thompson, G. A., Jr. (1971a), *J. Cell Biol. 49*, 712.
Nozawa, Y., and Thompson, G. A., Jr. (1971b), *J. Cell Biol. 49*, 722.
Shorb, M. S., Dunlap, B. E., and Pollard, W. O. (1965), *Proc. Soc. Exp. Biol. Med. 118*, 1140.
Smith, J. D., Snyder, W. R., and Law, J. H. (1970), *Biochem. Biophys. Res. Commun. 36*, 1163.
Thompson, G. A., Jr. (1965), *J. Biol. Chem. 240*, 1912.
Thompson, G. A., Jr. (1967), *Biochemistry 6*, 2015.
Thompson, G. A., Jr. (1969), *Biochim. Biophys. Acta 176*, 330.
Thompson, G. A., Jr., and Kapoulas, V. M. (1969), *Methods Enzymol. 14*, 668.
Weinstein, D. B., Marsh, J. B., Glick, M. C., and Warren, L. (1969), *J. Biol. Chem. 244*, 4103.
Wirtz, K. W. A., and Zilversmit, D. B. (1968), *J. Biol. Chem. 243*, 3596.

A Membrane-Bound Phospholipase A1 Purified from *Escherichia coli**

Carl J. Scandella† and Arthur Kornberg‡

ABSTRACT: Phospholipase A1 bound tightly in the cell membrane hydrolyzes membrane phospholipids following heat treatment, lysis, or aging (48 hr at 0°) of *Escherichia coli* cells. This enzyme may be responsible for phospholipid breakdown and for changes in membrane integrity which have been observed following phage infection, the addition of antibody and complement, or colicin action. We purified the enzyme approximately 5000-fold to near homogeneity by solubilization with sodium dodecyl sulfate (SDS)–butanol, isoelectric precipitation, acetone fractionation, and SDS–acrylamide gel electrophoresis. The enzyme is stable in 3% SDS and tends to aggregate in the absence of detergent. Neither detergent nor the lipids which copurify with the enzyme are necessary for activity. The enzyme hydrolyzes the 1-acyl chain of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol (PG), and diphosphatidylglycerol at comparable rates. The K_m for PG is 3×10^{-7} M. Hydrolysis of L-phosphatidylcholine but not D-phosphatidylcholine or triglyceride identifies the enzyme as phospholipase A1 and distinguishes it from known lipases.

Our work on phospholipids and phospholipase activity in *Escherichia coli* was prompted by an interest in knowing how macromolecules such as proteins and DNA gain passage through the membrane. Phage gene products appear to be necessary in order to allow lysozyme to pass through the membrane of the infected cell (Mukai *et al.*, 1967; Harris *et al.*, 1967) and may be responsible for the dissolution of the membrane during the process of phage infection (Cota-Robles and Coffman, 1964).

We examined lipid extracts from phage-infected cells, hoping to find a chemical basis for this structural alteration. We found that extensive phospholipid hydrolysis accompanies phage-induced lysis. However, sonication and osmotic lysis of uninfected cells also produce this effect. We were thus led to recognize the presence of phospholipase A, an enzyme previously sought in *Escherichia coli* (Proulx and van Deenen, 1967). Recent reports that colicins, human serum, and several phages elicit phospholipase A action in *E. coli* focus additional interest on this enzyme (Cavard *et al.*, 1968; Barbu and Lux, 1969; Cronan and Wulff, 1969; Josslin, 1970; Cohen *et al.*, 1970; Reader and Siminovitch, 1971). While our work on the purification of the enzyme was in progress, Okuyama and Nojima (1969) and Fung and Proulx (1969) reported the presence of phospholipase A in *E. coli*.

Interest in the *E. coli* phospholipase extends beyond its physiologic function because the enzyme offers an opportunity to study membrane structure at the molecular level. The enzyme is bound to the membrane, yet it can be released in an active form by detergents. The enzyme is remarkably stable in the presence of sodium dodecyl sulfate[1] (SDS) and some organic solvents. These attributes together with a rapid assay for enzyme activity enabled us to work out a procedure for purifying this insoluble protein approximately 5000-fold to a nearly homogeneous state. This report describes how the enzyme is purified and some of the interesting properties and specificity of action of the enzyme in the isolated state.

Materials and Methods

E. coli B, grown by the Grain Processing Corp. of

* From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305. *Received July 9, 1971.* Supported by grants from the National Science Foundation and U. S. Public Health Service. Taken in part from a thesis submitted by C. J. S. to Stanford University.

† National Science Foundation Predoctoral Fellow, 1966–1971. Present address: Department of Chemistry, Stanford University.

‡ To whom to address correspondence.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DGP, diphosphatidylglycerol; lysoPG, monoacylglycerophosphorylglycerol; lysoPE, monoacylglycerophosphorylethanolamine; ATP, adenosine 5′-triphosphate; 1 munit = 10^{-3} unit. The reader is referred to Lennarz (1970) for a discussion of phospholipase nomenclature.

TABLE I: Purification of the Enzyme.[a]

Fraction	Total Units	Protein		Sp. Act. (U/g)	Overall Yield (%)
		mg/ml	Total (g)		
I. Cell homogenate	8.4	50	50	0.17	(100)
II. Particulate	6.7	20	10	0.67	80
III. SDS–butanol supernatant	9.4	10	4.0	2.3	110
IV. Acetate supernatant	9.0	1.0	0.40	23	110
V. SDS–butanol extract	7.4	2.2	0.080	92	88
VI. Acetone fraction	5.3		0.013	400	63
VII. Lipid extraction	5.0		0.010	500	57
VIII. Electrophoresis	4.0		0.002	2000	45

[a] These values were obtained starting with 1 lb of cell paste. Comparable yields were obtained in runs starting with 0.5–100 lb. Assays of fractions III, IV, and V were performed after precipitating the protein with 1.5 volumes of acetone. The increase in total activity in fraction III may result from removing phospholipid which competes with radioactive substrate in the assay.

Muscatine, Iowa, as described previously (Richardson *et al.*, 1964), was harvested in late-log phase.

Phage T4 was provided by Dr. A. D. Kaiser.

Methods of lipid extraction and analysis have been described in previous communications (Bertsch *et al.*, 1969; Scandella and Kornberg, 1969). Silica gel G was refluxed with methanol for 24–48 hr in a Soxhlet extractor to remove a phospholipase inhibitor present in some batches of silica. The solvent systems used in this study were (v/v): solvent A, chloroform–methanol–acetic acid–water (100:50:14:7); solvent B, chloroform–methanol–acetic acid (65:25:8); solvent C, ether–hexane (75:25); and solvent D, ligroin–ether–formic acid (60:40:1.6).

SDS was obtained from Matheson Coleman & Bell and recrystallized from ethanol. [^{35}S]SDS obtained from New England Nuclear gave a single spot (R_F 0.5) in solvent B. Acrylamide and bisacrylamide were Eastman reagents recrystallized by the method of Loening (1967). Octyl, decyl, and tetradecyl sodium sulfates and hexadecyltrimethylammonium bromide were Eastman reagents recrystallized from ethanol.

Polyacrylamide Gel Electrophoresis. Analytical SDS–acrylamide gel electrophoresis was carried out using a modified Tris–glycine discontinuous buffer system (Jovin *et al.*, 1964) incorporating 0.1% SDS in the gel and upper buffer. The gels contained 10% acrylamide and 0.267% bisacrylamide and were polymerized with ammonium persulfate (0.075%). Urea–acetic acid gel electrophoresis was performed in the system of Widnell and Unkeless (1968). In both systems preelectrophoresis (2 hr, 3 mA/gel) was required to eliminate artifacts. Gels were stained with 0.25% coomassie brilliant blue in methanol–water–acetic acid (5:5:1, v/v) for 2–5 hr at room temperature and destained with the Canalco electrophoretic destainer. Stained gels were scanned at 540 nm with the Gilford 2410 linear transport scanner. Gels containing a range of protein concentrations were scanned to ensure a linear relationship between optical density and protein concentration. Peak areas were measured by weighing Xerox copies of the recorded peaks.

The phospholipase assay depends on the relative water solubility of lysophosphatidylglycerol (lysoPG) produced in the assay (L. Bertsch, A. Kornberg, C. Scandella, unpublished results). The mixture contained 0.025 M Tris-HCl (pH 8.4), 0.05 M $CaCl_2$, Triton X-100 (0.5 mg/ml), and 5 nmoles of [^{32}P]phosphatidylglycerol (PG) in a volume of 0.2 ml. After 30 min at 37°, the reaction was terminated by the addition of chloroform (0.2 ml) and methanol (0.45 ml). The single phase that results was broken by adding chloroform (0.2 ml) and water (0.2 ml). An aliquot of the upper aqueous phase was dried on a planchet and counted in a Nuclear-Chicago gas-flow counter. At these concentrations only about 1% of the PG partitions into the upper phase, compared to 85% of lysoPG. One unit (U) of enzyme hydrolyzes 1 μmole of substrate per minute under these conditions.

Radioactive substrate for this assay was prepared by growing *E. coli* B in the S medium of Echols *et al.* (1961) containing 0.5 mM P_i and[^{32}P]P_i at 10 mCi/mmole. Phospholipids extracted from these cells were resolved in solvent B. A 500-ml culture of *E. coli* yielded 2–3 μmoles of PG. The substrate was stored at 4° as a 1 mM solution in 95% ethanol, and added to the assay as such. PG from *Bacillus megaterium* was prepared as described previously (Bertsch *et al.*, 1969).

Synthetic phospholipids were generously supplied by Dr. P. P. M. Bonsen and Dr. A. J. Slotboom.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

A. Purification of Phospholipase. I. CELL HOMOGENATE. The procedure described below (Table I) for 1 lb of cell paste has been carried out on a 100-lb scale. We are grateful to the New England Enzyme Center for the use of their facilities for steps I, II, III. Except as noted, all operations were carried out at 2–4°. The cells were suspended in 1 l. of buffer (0.1 M Tris–HCl, pH 7.5) and homogenized in the Manton-Gaulin laboratory homogenizer. Two passages achieved a 95% decrease in the optical density at 660 nm. The specific activity of crude extracts varied by less than 30% from early-log phase to stationary phase in cells grown in Tryptone broth (Difco). The specific activity of crude extracts of log-phase cells ranged from 0.13 to 0.20 U per g in cultures grown on the following media: S medium (Echols *et al.*, 1961), tryptone broth, heart broth (Difco), minimal citrate (Vogel and Bonner, 1956), and the filament-inducing medium of Weinbaum (1966).

II. PARTICULATE FRACTION. The residue collected by centrifugation (30 min at 8000*g*) was resuspended in 500 ml of

water and allowed to stand for 1 hr at 20°. Autolysis during this interval served to reduce the viscosity of the thick suspension. The particulate fraction was collected by centrifugation, resuspended in 500 ml of 0.03 M Tris-HCl (pH 8.4), and brought to pH 8.4 by the addition of Tris base.

III. SOLUBILIZATION WITH SDS AND 1-BUTANOL. EDTA and and SDS were added to final concentrations of 1 mM and 10 mg/ml, respectively. The solution was stirred for 30 min at 20° to dissolve the SDS, then chilled to 0–2°. Butanol (0.15 volume) was added to saturation. The turbid suspension was centrifuged for 1 hr at 30,000*g* using the no. 21 head of a Beckman Model ZU ultracentrifuge and the clear supernatant (500 ml) was collected.

SDS effectively solubilizes the entire cell envelope, leaving very little material in a sedimentable form. The addition of butanol precipitates approximately one-half of the protein solubilized by SDS. Much of the purification is achieved by exploiting the solubility of the enzyme in SDS solutions saturated with butanol.

IV. ACETATE PRECIPITATION. Upon addition of magnesium chloride (1 M, 1 ml) and sodium acetate (1 M, 50 ml, pH 5.2) a copious precipitate formed. After 1 hr the precipitate was removed by centrifugation (15 min, 8000*g*). The enzyme activity was recovered from the supernatant by adding 750 ml of acetone (precooled to −15°) over a 5-min interval. The solution was chilled to −15° for 30 min, then centrifuged for 15 min at 8000*g*. This precipitate can be stored as a paste for 1 year at −15° without loss of activity.

The purification at this step requires low pH, high ionic strength, and saturating amounts of butanol. The butanol concentration is the most critical parameter; the efficiency of this step drops sharply as the butanol concentration is decreased. This step appears to select proteins which have a high affinity for lipid (see Table III below). The enzyme requires a higher level of Ca^{2+} for maximal stimulation (0.05 M *vs.* 0.005 M) after this step; the enzyme also becomes more sensitive to inhibition by Tween 40. These changes recall the "allotopic" properties of another membrane-associated enzyme, the mitochondrial adenosine triphosphatase studied by Bulos and Racker (1968).

V AND VI. EXTRACTION OF ACTIVITY AND ACETONE FRACTIONATION. The activity was extracted from the pellet with a butanol-saturated buffer and treated with acetone (see Table II for details). The enzyme activity was recovered in the fraction precipitating between 0.3 and 0.5 volume of acetone added. The presence of Mg^{2+} is often desirable in acetone fractionations (M. R. Atkinson, personal communication) and we found that the addition of Mg^{2+} was essential in order to obtain reproducible results at this step.

VII. LIPID EXTRACTION WITH 1-BUTANOL. Fraction VI was resuspended in 1 ml of water and lyophilized in a 30-ml centrifuge tube. The material must be spread in a thin film in order to achieve efficient extraction of lipid. Upon addition of butanol (10 ml) a fine dispersion was obtained. After incubation for 1 hr at 20°, the insoluble material was collected by centrifugation for 5 min at 5000*g* at 20°. The material was resuspended in 1 ml of water after removing residual butanol under reduced pressure. This treatment removed 90–97% of the lipid phosphorus present and facilitated preparative gel electrophoresis by reducing the tendency of the enzyme to aggregate at the top of the gel column.

This procedure yielded an optically clear solution from the particulate product of step VI. After lipid extraction the activity became for the first time nonsedimentable in the absence of detergent; the activity did precipitate when the ionic strength exceeded 0.04 M. We were not able to purify this fraction further by ion exchange chromatography, ammonium sulfate precipitation, or acrylamide gel electrophoresis in the absence of detergent. Fraction VII could be adsorbed to DEAE-cellulose but was eluted only in the presence of detergent (*e.g.*, Triton X-100, 0.05 mg/ml, 0.2 M NaCl).

TABLE II: Acetone Fractionation of Fraction V.[a]

Fraction	Protein (mg)	Activity	
		Units	Units/g
AI	37	0.77	20
AII	13	5.3	400
AIII	6	0.18	33
Recovery, total	68	6.2	
Per cent	85	85	

[a] The precipitate from step IV (400 mg of protein) was treated with 40 ml of the extraction buffer (SDS, 10 mg/ml; EDTA, 1 mM; and 0.03 M Tris-Cl (pH 8.4); saturated with 1-butanol) for 30 min at 0° with constant stirring. The supernatant after centrifugation (10 min at 8000*g* at 0°) contained 80 mg of protein with a specific activity of 92 U/g. Magnesium chloride (0.080 ml, 1 M) and sodium acetate (4 ml, 1 M) were added and this solution was transferred to a stainless steel beaker maintained at −3°. Acetone was added with a peristaltic pump at 0.3 ml/min. The following volumes of acetone were added: AI, 12.0 ml; AII, 10.0 ml; AIII, 12.0 ml. After each addition of acetone, stirring was continued for 20 min before centrifugation. The precipitates were collected by centrifugation for 5 min at 8000*g* at −3°.

Lipid extraction had no effect on the migration of fraction VI in gels containing SDS or the cationic detergent hexadecyltrimethylammonium bromide, suggesting that these detergents overcome lipid–protein interactions. In contrast, lipid extraction markedly influenced enzyme migration in gels containing Triton X-100 or deoxycholate (Dulaney and Touster, 1970). Fraction VII migrated as a single broad band in the Triton system of Dulaney and Touster. Triton X-100 (0.1%) had no effect on migration in the SDS–acrylamide system. The migration of fraction VII in nondetergent gels depended on the gel concentration: in high porosity gels (acrylamide <6%) all of the protein and activity migrated with the dye front; in more concentrated gels the protein and activity remained at the top of the gel. We interpret these observations to mean that SDS (or lipid) prevents the enzyme from aggregating. Porous gels allow the protein to travel with the SDS at the dye front.

Unlike the isoprenoid alcohol phosphokinase of Higashi *et al.* (1970), fraction VII was insoluble in neutral or acidic 1-butanol. The enzyme was inactivated by exposure to methanol or dimethyl sulfoxide at 0 or 20°.

VIII. PREPARATIVE GEL ELECTROPHORESIS. SDS–acrylamide gel electrophoresis was carried out in the system described in Methods. Up to 0.5 mg of protein was incubated for 60 min at 37° in 0.1 ml of 1% SDS. The sample was layered over gels (6.5 mm diameter) in upper buffer diluted fivefold, containing 10% glycerol. Electrophoresis was carried out for 3 hr at 20° at a constant current of 2 mA/gel. Gels were fractionated with a Savant Autogeldivider, using 5% butanol as the eluting buffer. The fractions were allowed to stand at

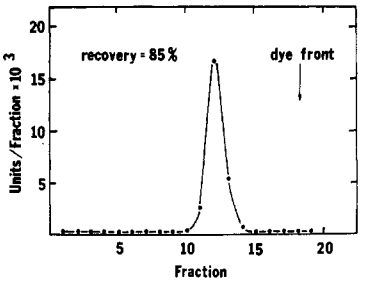

FIGURE 1: Electrophoresis with recovery of enzyme activity from SDS–acrylamide gel. Fraction VII (50 μg) was electrophoresed as described in the text. The calibration scale in Figure 2 shows that the R_F of the activity corresponds to molecular weight 29,000 ($\pm$2000). Recovery of enzyme activity was 85%.

least 10 hr at 4° before assaying. Figure 1 shows that the activity was recovered in a single band with an R_F corresponding to mol wt 29,000. Recovery of enzyme activity ranged between 55 and 100% of the applied units. The fractions were lyophilized and extracted with 1-butanol to remove the SDS.

Stability of the Enzyme. The enzyme resisted the denaturing action of SDS, alone or in combination with sulfhydryl reagents, at neutral pH. At elevated pH the enzyme lost activity. Fraction VII lost 50% of its activity in a 2 hr incubation at 37° at pH 9.5 in 1% SDS–1% 2-mercaptoethanol. The enzyme became less stable to heating after purification. The following percentages of initial activity withstood a 10-min incubation at 98° (0.02 M Tris-HCl, pH 7.4): fraction II, 120%; fraction VI, 68%; fraction VII, 52%; fraction VIII, 4%. The relative stability of the enzyme in crude extracts may be due to interactions with lipids or other proteins.

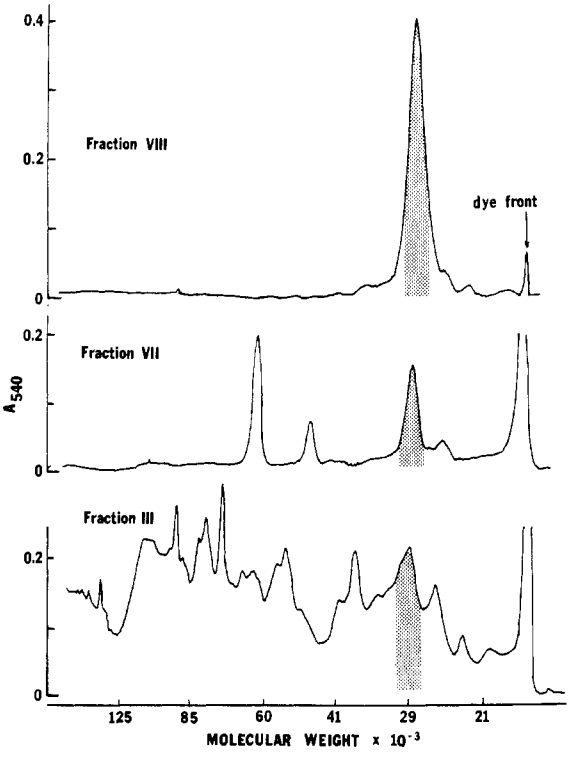

FIGURE 2: SDS–acrylamide gel electrophoresis of several enzyme fractions. Each of the enzyme fractions was electrophoresed in SDS–acrylamide gels (see Methods). Densitometry of stained gels indicated that the following percentages of the total protein in each fraction migrated with an R_F corresponding to mol wt 29,000 ($\pm$2000) (stippled region): II, 6; III, 8; IV, 13; V, 21; VI, 25; VII, 26; VIII, 90. Scans of fractions III (100 μg), VII (50 μg), and VIII (40 μg) are shown. The molecular weight scale was computed from the migration of protein standards: bovine serum albumin (mol wt 66,000), catalytic subunit of aspartate transcarbamylase (mol wt 34,000), and chymotrypsinogen (mol wt 25,000).

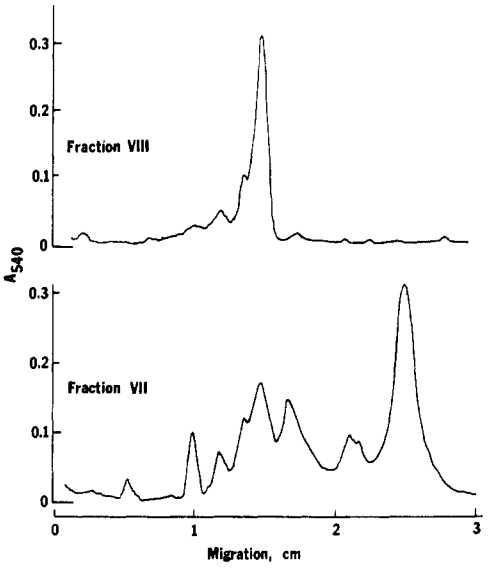

FIGURE 3: Phenol–urea–acetic acid gel electrophoresis of enzyme fractions. Fractions VII (50 μg) and VIII (10 μg) were treated with phenol–urea–acetic acid and electrophoresed in the system described by Widnell and Unkeless (1968). We thank Mr. S. M. Jazwinski for making these data available to us.

B. Electrophoretic Analysis of Enzyme Fractions. A substantial amount of the protein in each of the enzyme fractions migrated with the same R_F as the enzyme activity on SDS–acrylamide gels (Figure 2). The fraction of total protein migrating within the stippled region in Figure 2 increased from 6 to 26% in the first six steps of purification. Based on migration relative to marker proteins, the molecular weights of the proteins in this region are 29,000 ($\pm$2000). The amount of protein in this narrow molecular weight range did not increase proportionately to the increase in specific activity, suggesting that several species of protein are included within this size range. The membrane-localized lactose permease protein (Jones and Kennedy, 1969) migrates in this region of SDS gels. Pretreating enzyme fractions for 5 min at 98° in 1% SDS–1% 2-mercaptoethanol destroyed the activity but did not alter the migration of proteins in this gel system.

Several of the fractions were subjected to electrophoresis in the phenol–urea–acetic acid system of Widnell and Unkeless (1968). Some fractions, judged homogeneous by the criterion of SDS–acrylamide gel electrophoresis, were resolved into several components by this technique. Fraction VIII exhibited one major band (Figure 3). If this band represents the activity, then fraction VIII is >80% pure. Enzymatic activity could not be recovered after exposure to this harsh system. Prior extraction of lipid did not alter the migration of proteins in this system.

C. Sedimentation Analysis of Fraction VIII. Fraction VIII sedimented at 4.5 S in a sucrose gradient sedimentation velocity experiment (Figure 4). This value corresponds to a molecular weight of approximately 60,000 for a spherical protein. The phospholipase may exist in a multimeric form in the absence of SDS.

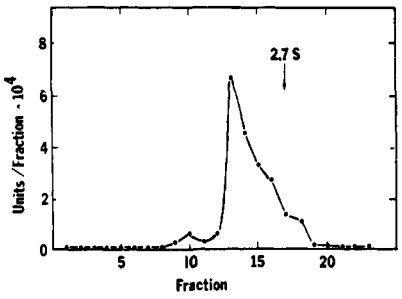

FIGURE 4: Sedimentation velocity of fraction VIII. Fraction VIII (2.6 munits) was layered over a 5–20% sucrose gradient containing 0.05 M Tris-HCl (pH 7.8) and 1 mM EDTA. The sample was centrifuged for 16 hr at 234,000*g* at 4° in the SW 50.1 head of a Beckman Model L2-65B ultracentrifuge. Tryptophan synthetase A-protein (mol wt 29,000; $s_{20,w} = 2.7$ S), kindly provided by Dr. K. Kirschner, served as a sedimentation marker. The arrow indicates the peak of tryptophan synthetase activity. Recoveries of activity were 40% (phospholipase) and 35% (tryptophan synthetase).

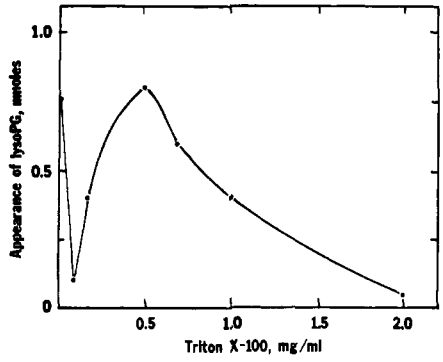

FIGURE 5: Effect of Triton X-100 on enzyme activity. Calcium chloride (0.05 M), Tris-HCl buffer (0.025 M, pH 8.4), 5 nmoles of PG, fraction VIII (0.027 munit), and Triton X-100 (0–0.4 mg) were incubated for 30 min at 37° in a volume of 0.2 ml. The amount of lysoPG formed is plotted as a function of detergent concentration.

D. Lipid and Detergent Content of Enzyme Fractions. Lipid co-purifies with the enzyme through the first six steps of purification. Fraction IV contained 2.0 (±0.4) mg of lipid per mg of protein, accounting for 40% of the lipid present in fraction II (Table III). Each of the acetone fractions (Table II) contained 2.2 (±0.5) mg of lipid per mg of protein. The composition of the lipid extractable from fraction VI expressed as per cent of total was: PE, 35; lysoPE, 20; free fatty acid, 32; other, 13. This composition reflects the lipid composition of the particulate fraction after step II and may be a consequence of phospholipid hydrolysis which takes place during the autolysis period. There was no enrichment for certain lipid components in the protein fractions obtained by this purification procedure.

E. coli phospholipids show a bias for unsaturated acyl chains at the 2 position and saturated chains at the 1 position. Free fatty acid from the enzyme fractions was enriched for palmitate while lysoPE from these fractions contained more palmitoleate than was present in PE. These observations are consistent with the idea that phospholipase A1 action generates the fatty acid and lysophospholipid.

The lipid extracted from fraction VI inhibited the delipidated enzyme by less than 10% when added to assays in amounts up to 0.5 μg. It seems unlikely that the extractable lipid contains a phospholipase inhibitor.

Fraction VI contained SDS and protein in nearly equal proportions (Table III). More than 95% of the detergent was removed by washing the acetone precipitate with cold acetone, a procedure that removed only 30% of the lipid. Apparently the protein binds lipid in preference to SDS.

E. Influence of Detergents on Enzyme Action. Triton X-100 (0.5 mg/ml) was included in the standard assay because the crude enzyme fractions were stimulated up to fivefold by this level of detergent. However, fraction VIII was fully active in the absence of detergent, as can be see in Figure 5. The crude fractions were precipitated with acetone before assaying in order to avoid inhibition by SDS. Triton enhanced the inhibitory effect of SDS (Figure 6). Table IV compares the effect of SDS and structurally related detergents. The correlation of inhibition with chain length suggests that these detergents act by competing with the substrate for hydrophobic binding sites on the enzyme. On the other hand, detergents might influence the rate of hydrolysis by altering the conformation or packing of the substrate (De Haas *et al.*, 1971).

Fraction VIII hydrolyzed PE and PG at the same rate with or without Triton X-100 but the hydrolysis of DPG was stimulated about 100-fold by the presence of Triton. Triton probably alters the structure of DPG in solution, making it more accessible to enzyme action.

Triton X-100 had a striking effect on the apparent affinity

TABLE III: Lipid and Detergent Content of Enzyme Fractions.[a]

Fraction	Protein (mg)	Lipid (mg)	SDS (mg)
II	8000	1700	5000
III	3100	1600	4200
IVa	320	1000	3000
IV	290	650	70
V	68	290	40
VI	7	12	11
VII	5.5	0.3	0.28

[a] Phospholipase was purified from 1 lb of cells using [^{35}S]-SDS (14,000 cpm/gm). Lipid was measured as chloroform-soluble phosphate. Fraction IVa is the solution before addition of acetone in step IV.

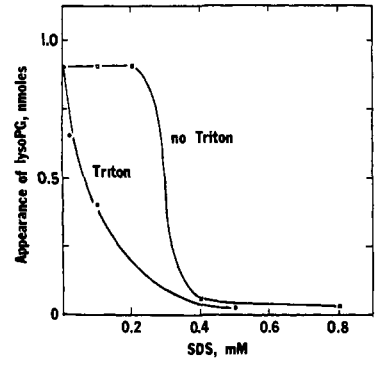

FIGURE 6: Interaction of SDS and Triton X-100 in enzyme assay. The amount of PG hydrolyzed by 0.025 munit of fraction VIII is plotted as a function of SDS concentration in standard assays, or with Triton X-100 omitted.

TABLE IV: Inhibition by SDS and Structurally Related Detergents.[a]

Chain Length	Ion	μM
8	Sulfate	700
10	Sulfate	400
12	Sulfate	100
14	Sulfate	40
16	Trimethylammonium	20

[a] These levels of detergent gave 50% inhibition of enzyme activity under standard assay conditions.

of the enzyme for PG. The K_m for PG in the absence of detergents is 3.4×10^{-7} M; adding Triton X-100 (0.5 mg/ml) increased the K_m 40-fold without changing V_{max} (Figure 7). The substrate may exist in an aggregated form at the concentrations used in these experiments (Smith and Tanford, 1971); if so, the Lineweaver–Burk plot might measure the affinity of the enzyme for phospholipid vesicles or micelles. There are several possible explanations for the effect of Triton on K_m, such as: (a) the octyl chain of Triton X-100 interacts with the enzyme or the hydrocarbon chains of phospholipids, thereby hindering the binding of substrate to enzyme; (b) the enzyme binds to phospholipid micelles more tightly than to phospholipid–detergent micelles; (c) the hydrolysis rate is limited by the diffusion of enzyme through substrate micelles, and diffusion is slower in phospholipid–detergent micelles.

Our enzyme assays were done under conditions which yielded <15% hydrolysis of substrate. Under these conditions the enzyme assay is linear with enzyme concentration (Figure 8) and with time for at least 60 min.

F. Optimum pH and Ionic Conditions. The pH optimum (8.4) and Ca^{2+} requirement were not affected by the presence of Triton. The activity decreased to 50% of maximal at pH 6.5 (Tris–maleate, 0.03 M) and 10% of maximal at pH 5.2 (sodium acetate, 0.03 M). The pure enzyme does not exhibit the second pH optimum (pH 5.2) reported by Proulx and Fung (1969) for phospholipase A1 action in crude extracts of *E. coli*. Raising the ionic strength by adding 0.08 M NaCl diminished the activity to 50% of maximal. Mercury(II) chloride (0.1 or 1 mM) added to the assay gave less than 15% inhibition. Chloride salts of Mg^{2+}, Zn^{2+}, Mn^{2+}, and Cu^{2+} (1 and 10 mM) did not satisfy the requirement for Ca^{2+}. Phospholipases usually require Ca^{2+}. Calcium may act by sequestering free fatty acids released by hydrolysis (Benzonana and Desnuelle, 1968). Pancreatic phospholipase A2 binds one atom of calcium per mole of enzyme (P. P. M. Bonsen, personal communication).

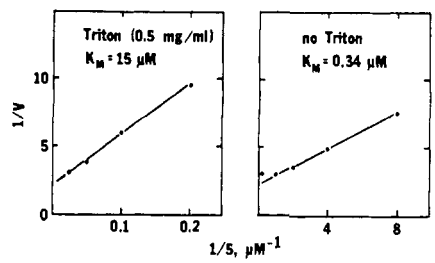

FIGURE 7: Effect of Triton X-100 on substrate affinity. Lineweaver–Burk plots measure the affinity of enzyme for PG under standard assay conditions, or with Triton X-100 omitted.

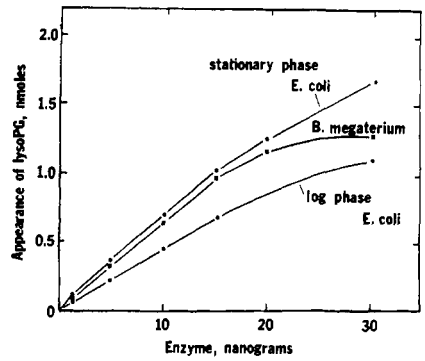

FIGURE 8: Hydrolysis of substrates bearing unsaturated, cyclopropane, and branched fatty acyl chains. PG from log-phase *E. coli*, stationary-phase *E. coli*, or log-phase *B. megaterium* was incubated with fraction VIII (1.5–30 ng) under standard assay conditions.

G. Substrate Specificity. The enzyme was identified as a phospholipase A1 by its action on synthetic substrates. Fraction VIII (0.7 munit) was incubated for 60 min at 37° in the standard assay mixture (1 ml) with (a) 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (0.1 μmole), and (b) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylethanolamine (0.1 μmole). Fatty acid analysis of the lyso derivatives formed (20–30 nmoles in each case) indicated that more than 90% of the products were 2-acyl lysophosphatides. Proulx and Fung (1969) found evidence of phospholipase A2 action as well as phospholipase A1 action in crude extracts of *E. coli*.

Fraction VIII (up to 35 munits) gave less than 2% hydrolysis of a D isomer of phosphatidylcholine, 3-oleoyl-2-stearoyl-*sn*-glycero-1-phosphorylcholine (0.1 μmole), as judged by thin-layer chromatography. Triolein (0.1 μmole) was also not hydrolyzed under our standard phospholipase assay conditions. This enzyme can be distinguished from pancreatic and fungal lipases, which also cleave the 1-acyl chain of phospholipids (Slotboom *et al.*, 1970), because it fails to degrade D-lecithin or triglyceride.

Fraction VIII (35 munits) was incubated with a phosphotriester, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-(dibenzyl)phosphoric acid (0.1 μmole), yielding 20 nmoles of a product identified as the 2-acyllyso derivative by the following criteria: cochromatography with 2-acyllyso derivative prepared by the action of pancreatic lipase (Slotboom *et al.*, 1970) in solvents C (R_F 0.2) and D (R_F 0.1); the fatty acid:phosphorus ratio was 0.7 and 80% of the fatty acid was oleate.

PG bearing mainly saturated fatty acyl chains was obtained from stationary phase *E. coli* and *B. megaterium*. More than 90% of the unsaturated fatty acids were replaced with cyclopropane derivatives in PG extracted from *E. coli* 5 hr after the end of logarithmic growth (Cronan, 1968). *B. megaterium* phospholipids contain branched-chain rather than unsaturated fatty acids (Scandella and Kornberg, 1969). These modifications in the acyl chains of the substrate had little effect on the rate of hydrolysis (Figure 8). The enzyme hydrolyzed all of the major phospholipids of *E. coli* at comparable rates (Table V).

H. Phospholipase Action in Vivo. The major phospholipid of *E. coli*, comprising 70% of the total, is PE. Figure 9 shows that lysoPE accumulates in phage-infected cells. Appearance of radioactivity in lysoPE parallelled a loss of radioactivity

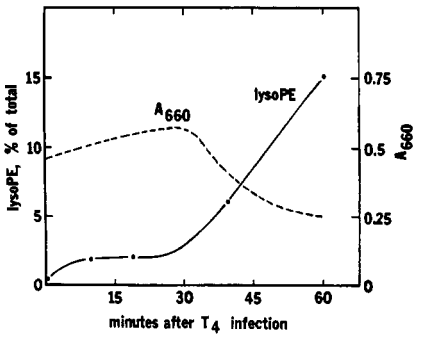

FIGURE 9: Appearance of lysoPE after phage infection. *E. coli* B, growing in tryptone broth medium (Difco) containing $^{32}P_i$ (5 mCi/mmole), was infected with bacteriophage T4 at a multiplicity of 3. Lipid extracts from centrifuged cells (10 min at 8000*g*) were applied to tlc plates and chromatographed in solvent A. Spots detected by autoradiography were scraped into planchets and counted in a Nuclear-Chicago gas-flow counter. A new spot (R_F 0.25) which appeared after phage infection was identified as lysoPE by the following criteria: (a) positive ninhydrin and negative Schiff's reaction, (b) cochromatography with authentic lysoPE in solvents A and B, (c) glycerophosphorylethanolamine produced after alkaline hydrolysis, and (d) fatty acid and phosphorus present in the ratio of 0.9 (±0.1) to 1. LysoPE is expressed as per cent of total phospholipid recovered. Up to 50% of the lysoPE partitions into the upper (aqueous) phase in the lipid extraction procedure and is not recovered in this experiment or those described in Figure 10 and Table VII. These data have not been corrected for this loss. LysoPE appears with similar kinetics after infection of *E. coli* B or W3350 with phage T4, T4rII, or phage λ, or after ultraviolet-light induction of W3350 lysogenic for phage λ.

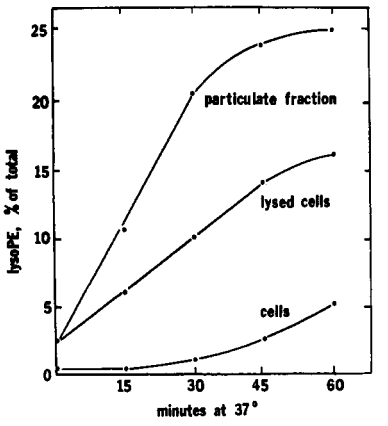

FIGURE 10: Accumulation of lysophospholipid by uninfected cells and cell extracts. Cells and cell extracts were incubated at 37° in 0.1 M Tris-HCl (pH 7.4). The accumulation of lysophospholipid was monitored as described in the legend to Figure 9. Freshly harvested cells were lysed by osmotic shock after incubation for 30 min at 20° with lysozyme (100 μg/ml) and EDTA (1 mM); particulate fraction was collected by centrifuging osmotically lysed cells for 10 min at 16,000*g*.

from PE after phage infection. The lyso derivatives of PG and DPG can be detected by tlc but they are difficult to measure quantitatively because they are recovered with low efficiency by the lipid extraction procedure. Other changes in phospholipid metabolism which follow T4 infection have been reported by Buller and Astrachan (1968) and Furrow and Pizer (1968).

Lysophospholipid production could also be elicited without adding phage. Heat treatment (Rampini *et al.*, 1969) or disruption of intact cells (Figure 10) evoked lysophospholipid production. The phospholipase activity was heat stable; incubation for 20 min at 98° diminished the activity by less than 20% (Rampini *et al.*, 1969). This activity was not removed by repeated washes with water or salt solutions, or by banding the membranes in a sucrose density gradient by the method of Miura and Mizushima (1968).

The phage-induced, latent, and extracted phospholipases have similar pH optima, requirement for divalent cation (Josslin, 1971), heat stability, response to salt (50% inhibition by 0.1 M NaCl), and preference for 1-acyl attack (Table VI) (Proulx and Fung, 1969). The phospholipase activity increased by less than twofold after T4 infection, as measured with purified PG or PE. Osmotically lysed cells accumulate lysoPE as rapidly as phage-infected cells (compare Figures 9 and 10). We tentatively conclude that the *E. coli* phospholipase which we have purified is the enzyme responsible for the bulk of the phospholipid hydrolysis after phage infection. Cohen

TABLE V: Action of the Enzyme on *E. coli* Phospholipids and LysoPE.[a]

Substrate	Hydrolysis Products
PG	LysoPG
PE	LysoPE
DPG	Triacyl-DPG and diacyl-DPG
1-AcyllysoPE	Glycerophosphorylethanolamine

[a] PG, PE, and DPG were obtained from log-phase *E. coli*. 1-AcyllysoPE and reference standards were prepared by the action of snake venom. After incubating the substrate (10 nmoles) with fraction VIII (0.035 munit) for 30 min under standard assay conditions the reaction products were analyzed by tlc in solvent A. The hydrolysis products listed were the only ones detected: lysoPG (0.81 nmole), lysoPE (1.13 nmoles), triacyl-DPG (1.2 nmoles), diacyl-DPG (0.6 nmole), and glycerophosphorylethanolamine (2.3 nmoles). Other products were present at levels of <0.1 nmole.

TABLE VI: Preference of Phage-Induced, Latent, and Purified Phospholipases for 1-Acyl Attack.[a]

Phospholipid	% Hydrolysis	Fatty Acids, Saturated: Unsaturated
1. PE	15	0.81
2. LysoPE, T4-infected cells	15	0.29
3. Lyso PE, osmotic lysis	20	0.33
4. LysoPE, *in vitro*	25	0.35
5. 1-AcyllysoPE	98+	2.7

[a] We determined the fatty acid composition of PE and several samples of lysoPE. Samples 1 and 2 were purified from *E. coli* 60 min after infection by T4 (Figure 9); sample 3, from osmotically lysed cells (Figure 10). Samples 4 and 5 were produced by treating PE with fraction VIII and snake venom, respectively. Cyclopropane fatty acids are regarded as unsaturated fatty acids in this table because they are derived from unsaturated acyl chains of phospholipids (Cronan, 1968).

TABLE VII: Factors Influencing Accumulation of LysoPE.[a]

Conditions	LysoPE, % of Total Phospholipid
Particulate fraction	
No incubation	1.8
pH 8.4	24
pH 10.2	12
pH 7.3	14
pH 7.3 + $CaCl_2$ (1 mM)	29
pH 7.3 + $MgCl_2$ (1 mM)	13
pH 7.3 + EDTA (1 mM)	2.0
pH 7.3 + Triton X-100 (0.1 mg/ml)	14
pH 7.3 + Triton X-100 (1.0 mg/ml)	3.5
pH 7.3 + ATP-generating system	2.0
Heat-treated particulate fraction	
No incubation	2.5
pH 7.3	20
pH 7.3 + ATP-generating system	21

[a] *E. coli* particulate fraction (as in Figure 10, 1 mg/ml of protein) or heat-treated particulate fraction (pretreated for 2 min at 98°) was incubated for 30 min at 37°. The buffer was Tris-HCl (0.03 M) in all cases except for sodium glycinate (0.03 M) at pH 10.2. The ATP-generating system consisted of AMP (0.16 mM), ATP (0.015 mM), phosphoenolpyruvate (4 mM), $MgCl_2$ (6 mM), pyruvate kinase (4 μg/ml), and myokinase (10 μg/ml).

et al. (1970) concluded that the host cell phospholipase catalyzed the phospholipid hydrolysis which they observed in phage-infected cells of *Salmonella typhimurium*.

Is the accumulation of lysophospholipid by lysed cells due chiefly to unmasking of a latent phospholipase or to the elimination of activities which either deacylate the lysophospholipids further (Proulx and van Deenen, 1966), or reacylate them? The particulate fraction (Figure 10) accumulated lysoPE rapidly but the addition of an ATP-generating system retarded the accumulation of lysoPE by at least 90% (Table VII). Heat pretreatment eliminated the effect of ATP on lysoPE accumulation (Table VII); thus the *E. coli* phospholipase differs from the phospholipase A2 of mitochondrial membranes (Waite *et al.*, 1969), which is inhibited by ATP. Adding ATP (10 mM) gave a tenfold stimulation of the acylation of 1-acyllysoPE by the crude lysate. Cells allowed to stand for 24–48 hr at 0° accumulated lysophospholipid when subsequently incubated at 37°. It seems likely that lysophospholipid accumulates as the membrane runs out of energy to carry out reacylation.

Discussion

A. Purification and Properties. Dissecting a firmly bound membrane enzyme and reconstructing its membrane locus should reveal some basic features of the structure of the membrane. As a first step toward this goal we have purified the *E. coli* phospholipase approximately 5000-fold[2] to near homogeneity. The answers to many questions about the structure and composition of the enzyme must await purification of a substantial amount of phospholipase in homogeneous form but some interesting properties of the enzyme deserve comment at this time.

The purified enzyme, free of lipid and detergent, tends to aggregate in aqueous solutions as judged by its behavior in acrylamide gels and by its insolubility under physiologic conditions. The solubility properties of this enzyme resemble those of the sarcoplasmic reticulum ATPase (MacLennan, 1970), cytochrome *b* of mitochondria (Goldberger *et al.*, 1961), and the "structural protein" fractions described by Green and others. Insolubility and affinity for phospholipid, two characteristics of the *E. coli* phospholipase, are among the criteria which have been used to define "structural" proteins in membranes (Criddle *et al.*, 1962; Woodward, 1968). *E. coli* phospholipase, cytochrome *b*, and the sarcoplasmic ATPase may be proteins with structural and functional roles in the membrane.

Neither detergent nor the lipids which copurify with the enzyme are necessary for activity. However, the large quantity of phospholipid present as a substrate may also act as a detergent.

A remarkable property of this enzyme is its stability in the presence of SDS, a reagent commonly used to denature proteins. To our knowledge, this is the first example of an active enzyme purified in the presence of SDS as a solubilizing agent. Membrane enzymes may be more resistant to the denaturing action of SDS and organic solvents owing to their hydrophobic exteriors. Weinbaum and Markman (1966) were able to identify several enzymatically active bands in SDS gels of membrane proteins. Phospholipase assays cannot be done in the presence of SDS because long-chain alkyl sulfates are potent inhibitors. SDS may convert the enzyme to a form which renatures readily, perhaps under the influence of phospholipid in the assay mixture. After crude enzyme (fraction III) was denatured by urea or guanidine hydrochloride, dialysis for at least 8 hr was necessary to recover activity. Activity was not recovered from fraction VII after urea treatment and dialysis, implying that some factor present in the crude extract, perhaps lipid, is required for proper folding of the protein.

A substantial amount of the protein in the *E. coli* membrane has a molecular weight in the vicinity of 30,000, as judged by SDS–acrylamide gel electrophoresis. Much of this protein copurifies with the phospholipase through several steps of purification, suggesting that proteins of this size share other properties. Perhaps these proteins have evolved from a common progenitor. Kiehn and Holland (1970) demonstrated heterogeneity within some molecular weight classes of mammalian membrane protein. These membranes contain more protein species than one would estimate from the number of bands in an SDS–acrylamide gel.

B. Comparison to Other Phospholipases. Phospholipase A2 enzymes have been purified to homogeneity from porcine pancreas (De Haas *et al.*, 1968), *Crotalus adamanteus* venom (Wells and Hanahan, 1969), and *Crotalus atrox* venom (Wu and Tinker, 1969). These enzymes have molecular weights of 14,000, 30,000, and 14,000, respectively. These enzymes are heat stable; their stability may be related to their high disulfide content. Phospholipase A1 enzymes (with appreciable lipase activity) have been partially purified from rat and calf brain (Gatt, 1968). The phospholipase A1 of *B. megaterium* has been purified to homogeneity and has a molecular weight of 25,000 (D. Raybin and A. Kornberg, unpublished results).

[2] The increase in specific activity is 10,000-fold but we estimate that a 2-fold increase in activity results from removing endogenous lipid which competes with radioactive substrate in assays of the crude fractions.

The *E. coli* phospholipase cleaves the 1-acyl moiety of all substrates tested. As has been found for other phospholipase A enzymes, the site of attack (1- or 2-acyl) does not depend on which position carries the unsaturated acyl chain and the enzyme accepts substrates with a variety of substituents at the head group. Hydrolysis of L-phosphatidylcholine but not D-phosphatidylcholine or triglyceride identifies the enzyme as a phospholipase A1 and distinguishes it from known lipases.

The *E. coli* phospholipase differs from other phospholipases in its high affinity for substrate ($K_m = 3 \times 10^{-7}$ M) and in its ability to hydrolyze a phosphotriester, albeit very slowly. Other phospholipases do not attack phosphotriesters, compounds lacking the negatively charged phosphate typical of a phosphodiester. This unusual lack of specificity for the head group implies that the *E. coli* enzyme interacts mainly with the fatty acyl moiety of its substrate.

C. Localization within the Membrane. We refer to this enzyme as "membrane bound" because it adheres tightly to the particulate fraction. The enzyme is deeply imbedded in the membrane, as judged (a) by its resistance to extraction by aqueous buffers, (b) its solubilization by detergents (SDS, Triton X-100) and by butanol only under conditions which completely solubilize the membrane, and (c) by its insusceptibility to several proteases. In this respect the *E. coli* phospholipase resembles the phospholipase A of the slime mold, *Dictyostelium discoideum*, which Ferber *et al.* (1970) find more tightly bound than the bulk of the membrane protein. It is possible that the *E. coli* phospholipase is one of the proteins residing within the inner hydrophobic regions of the membrane, such as those visualized by the freeze-fracture technique (Pinto da Silva and Branton, 1970). Recently developed techniques which separate the inner and outer membranes of *E. coli* (Schnaitman, 1970) should permit a better localization of the phospholipase within the envelope structure. The phospholipase activity is associated with an outer membrane fraction (R. Marco and A. Kornberg; M. J. Osborn, unpublished observations). Thus the enzyme may reside in the outer-membrane or in an inner-membrane region more firmly bound to the outer membrane.

Since the phospholipase acts on membrane phospholipids *in situ* it is of interest to calculate the effective radius of enzyme action. A rough calculation[3] indicates that there are approximately 500 molecules of phospholipase per cell and that phospholipids within a 500-Å radius of each phospholipase can diffuse to the enzyme in 30 min, implying that the membrane regions surrounding phospholipases are rather fluid. This calculation yields a minimum estimate of phospholipid diffusion in the membrane because the rate of hydrolysis *in situ* does not appear to be diffusion limited (the turnover numbers calculated for purified phospholipase *in vitro* and phospholipase acting on membrane phospholipids *in situ* are 80 and 400 molecules per min, respectively). This rate of diffusion is easily compatible with recent measurements which show that phospholipids in model membranes move a root-mean-square distance of 500 Å in 1 sec (Kornberg and McConnell, 1971).

[3] This calculation involves the following assumptions: an *E. coli* cell (a rod of 3 μ length and 1 μ diameter; see Luria, 1960) contains 2×10^{-13} g of protein, of which 0.01% is phospholipase; the phospholipases (mol wt 30,000) are distributed uniformly over the cell surface (area, 10 μ^2) in a hexagonal array; the enzyme can hydrolyze 25% of the membrane phospholipid in 30 min at 37° (Table VII).

D. Physiologic Role. This enzyme may be responsible for phospholipid breakdown and changes in membrane integrity which have been observed following phage infection, the addition of antibody and complement, or colicin action (Cavard *et al.*, 1968; Barbu and Lux, 1969; Cronan and Wulff, 1969; Reader and Siminovitch, 1971; Josslin, 1970). We find that phospholipase A activity increases dramatically preceding sporangial lysis in *B. megaterium* (D. Raybin, L. L. Bertsch, and A. Kornberg, unpublished results). In contrast, the level of phospholipase activity in cells infected with T4 phage remains nearly constant. A phage-induced phospholipase, if present, could be masked by the host cell activity. Additional work is needed to clarify the role of phospholipase(s) in the process of cell lysis.

E. coli membrane and cell wall are bound together at approximately 400 points which serve as the adsorption sites for all of the T-phages (Bayer, 1968). If the phospholipases are localized at these sites in the membrane, phospholipase action might facilitate the entry of phage DNA into the cell. One could test this hypothesis by visualizing in the electron microscope the regions of membrane breakdown in plasmolyzed cells, or by isolating a fraction containing the membrane-wall adhesions. The calcium-promoted uptake of DNA by *E. coli* (Mandel and Higa, 1970) might involve phospholipase action.

Does the enzyme function in growing cells? Although we cannot detect turnover of the acyl chains of phospholipids in logarithmically growing cells, turnover could be masked by efficient reincorporation of the fatty acyl chains released by phospholipase action (Okuyama, 1969). Acyl chain turnover has been observed in *Haemophilis parainfluenzae* (White and Tucker, 1969). *E. coli* contains membrane-bound acyltransferase (Proulx and van Deenen, 1967), acyl-CoA synthetase (Overath *et al.*, 1969), and appreciable levels of coenzyme A (Weeks *et al.*, 1969). Acyl-CoA synthetase can be eliminated by mutation (Overath *et al.*, 1969), but acyl carrier protein is localized near the membrane (van den Bosch *et al.*, 1970), and may satisfy the requirement for CoA in the acyltransferase reaction (van den Bosch and Vagelos, 1970). *E. coli* also contains a soluble lysophospholipase activity (Proulx and van Deenen, 1966) which may protect the cells from the accumulation of the toxic lyso compounds. Isolated membranes have the capacity to degrade their phospholipids extensively by phospholipase A1 action. This propensity for self-destruction may result from activation of the endogenous phospholipase, perhaps as a consequence of membrane damage, or it may reflect an inhibition of the enzymes which normally prevent the accumulation of lysophospholipid.

Enzymes which cleave one of the two acyl moieties of phospholipids have been found in plant and animal cells, and most recently in unicellular organisms (Okuyama and Nojima, 1969; Ono and Nojima, 1969; Ferber *et al.*, 1970). Often these activities are localized in membrane systems together with complementary acyltransferase activities. It has been proposed that a monoacyl–diacyl cycle plays a general role, yet to be elucidated, in membranes (Lands, 1965; van Deenen, 1966). Regions of the membrane undergoing active phospholipid metabolism might play a role in membrane transport; they might regulate the permeability of the membrane toward macromolecules, and they could participate in other vital membrane functions, such as growth and fusion (Lucy, 1970). *E. coli* offers a favorable system for testing these possibilities because of the wealth of biochemical knowledge available and the genetic manipulation possible with this organism.

Acknowledgments

We thank Dr. Kasper Kirschner, Dr. Alan Russell, and Dr. Pieter Bonsen for many helpful discussions.

References

Barbu, E., and Lux, M. (1969), *C. R. Acad. Sci., Ser. D 68*, 449.
Bayer, M. E. (1968), *J. Virol. 2*, 346.
Benzonana, G., and Desnuelle, P. (1968), *Biochim. Biophys. Acta 164*, 47.
Bertsch, L. L., Bonsen, P. P. M., and Kornberg, A. (1969), *J. Bacteriol. 98*, 75.
Buller, C. S., and Astrachan, L. (1968), *J. Virol. 2*, 298.
Bulos, B., and Racker, E. (1968), *J. Biol. Chem. 243*, 3891.
Cavard, D., Rampini, C. Barbu, E., and Polonovski, J. (1968), *Bull. Soc. Chim. Biol. 50*, 1455.
Cohen, L. W., Knipprath, W. G., and Allen, C. F. (1970), *Virology 41*, 430.
Cota-Robles, E., and Coffman, E. (1964), *J. Ultrastruct. Res. 10*, 304.
Criddle, R. S., Bock, R. M., Green, D. M., and Tisdale, H. (1962), *Biochemistry 1*, 827.
Cronan, J. E. (1968), *J. Bacteriol. 95*, 2054.
Cronan, J. E., and Wulff, D. E. (1969), *Virology 38*, 241.
De Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., and van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta* (in press).
De Haas, G. H., Postema, N. M., Nieuwenhuizen, W., and van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 159*, 103.
Dulaney, J., and Touster, O. (1970), *Biochim. Biophys. Acta 196*, 29.
Echols, H., Garen, A., Garen, S., and Torriani, A. (1961), *J. Mol. Biol. 3*, 425.
Ferber, P. G., Munder, P. G., Fischer, H., and Gerisch, G. (1970), *Eur. J. Biochem. 14*, 253.
Fung, C. K., and Proulx, P. (1969), *Can. J. Biochem. 47*, 371.
Furrow, M. H., and Pizer, L. I. (1968), *J. Virol. 2*, 594.
Gatt, S. (1968), *Biochim. Biophys. Acta 159*, 304.
Goldberger, R., Smith, A. L., Tisdale, H., and Bomstein, R. (1961), *J. Biol. Chem. 236*, 2788.
Harris, A. W., Mount, D. W., Fuerst, C. R., and Siminovitch, L. (1967), *Virology 32*, 553.
Higashi, Y., Siewert, G., and Strominger, J. L. (1970), *J. Biol. Chem. 245*, 3683.
Jones, T. H. D., and Kennedy, E. P. (1969), *J. Biol. Chem. 244*, 5981.
Josslin, R. (1970), *Virology 40*, 719.
Josslin, R. (1971), *Virology 44*, 94.
Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.
Kiehn, E. D., and Holland, J. J. (1970), *Biochemistry 9*, 1716.
Kornberg, R., and McConnell, H. M. (1971), *Proc. Nat. Acad. Sci. U. S.* (in press).
Lands, W. E. M. (1965), *Annu. Rev. Biochem. 34*, 314.
Lennarz, W. J. (1970), *Annu. Rev. Biochem. 39*, 359.
Loening, U. E. (1967), *Biochem. J. 102*, 251.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Lucy, J. A. (1970), *Nature (London) 227*, 811.
Luria, S. E., (1960), *in* The Bacteria, Vol. 1, Gunsalus, I. C., and Stanier, R. Y., Ed., New York, N. Y., Academic Press.
MacLennan, D. H. (1970), *J. Biol. Chem. 245*, 4508.
Mandel, M., and Higa, A. (1970), *J. Mol. Biol. 53*, 159.
Miura, T., and Mizushima, S. (1968), *Biochim. Biophys. Acta 150*, 159.
Mukai, F., Streisinger, G., and Miller, B. (1967), *Virology 33*, 398.
Okuyama, H. (1969), *Biochim. Biophys. Acta 176*, 125.
Okuyama, H., and Nojima, S. (1969), *Biochim. Biophys. Acta 176*, 120.
Ono, Y., and Nojima, S. (1969), *Biochim. Biophys. Acta 176*, 111.
Overath, P., Pauli, G., and Schairer, H. (1969), *Eur. J. Biochem. 7*, 559.
Pinto da Silva, P., and Branton, D. (1970), *J. Cell. Biol. 45*, 598.
Proulx, P., and Fung, C. K. (1969), *Can. J. Biochem. 47*, 1125.
Proulx, P., and van Deenen, L. L. M. (1966), *Biochim. Biophys. Acta 125*, 591.
Proulx, P., and van Deenen, L. L. M. (1967), *Biochim. Biophys. Acta 144*, 171.
Rampini, C., Lux, M., Cavard, D., and Barbu, E. (1969), *C. R. Acad. Sci., Ser. D 268*, 206.
Reader, R. W., and Siminovitch, L. (1971), *Virology 43*, 607.
Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., and Kornberg, A. (1964), *J. Biol. Chem. 239*, 222.
Scandella, C. J., and Kornberg, A. (1969), *J. Bacteriol. 98*, 82.
Schnaitman, C. A. (1970), *J. Bacteriol. 104*, 890.
Slotboom, A. J., de Haas, G. H., Bonsen, P. P. M., Burbach-Westerhuis, G. J., and van Deenen, L. L. M. (1970), *Chem. Phys. Lipids 4*, 15.
Smith, R. W., and Tanford, C. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1460.
van den Bosch, H., and Vagelos, P. R., (1970), *Biochim. Biophys. Acta 218*, 233.
van den Bosch, H., Williamson, J. R., and Vagelos, P. R. (1970), *Nature (London) 228*, 338.
van Deenen, L. L. M. (1966), *Ann. N. Y. Acad. Sci. 137*, 713.
Vogel, H. J., and Bonner, D. M. (1956), *J. Biol. Chem. 218*, 97.
Waite, M., Scherphof, G. L., Boshouwers, F. M. G., and van Deenen, L. L. M. (1969), *J. Lipid Res. 10*, 411.
Weeks, G., Shapiro, M., Burns, R. O., and Wakil, S. J. (1969), *J. Bacteriol. 97*, 827.
Weinbaum, G. (1966), *J. Gen. Microbiol. 42*, 83.
Weinbaum, G., and Markman, R. (1966), *Biochim. Biophys. Acta 124*, 207.
Wells, M. A., and Hanahan, D. J. (1969), *Biochemistry 8*, 414.
White, D. C., and Tucker, A. N. (1969), *J. Lipid Res. 10*, 220.
Widnell, C. C., and Unkeless, J. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1050.
Woodward, D. O. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 1167.
Wu, T., and Tinker, D. O. (1969), *Biochemistry 8*, 1558.