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Presence of Glycerophospholipid:Cholesterol Acyltransferase and Phospholipase in Culture Supernatant of *Aeromonas hydrophila*

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Human erythrocyte membrane glycerophospholipids are deacylated by *Aeromonas hydrophila* 13-h culture supernatants, resulting in the production of cholesterol ester, free fatty acid, and water-soluble phosphates. This activity appears to be due to the actions of an acyltransferase (phosphatide:cholesterol acyltransferase, EC 2.3.1 group) and a phospholipase (phosphatide acyl-hydrolase). The enzyme activities are produced simultaneously in late exponential/early stationary phase, are precipitated together from the culture supernatant with 85% ammonium sulfate, and are eluted together near the void volume during gel filtration on Sepharose 6B. These results suggest that *A. hydrophila* produces a multienzyme complex with an unusual mode of action on membrane lipids. The complex is distinct from the hemolytic factor aerolysin, which is also produced by *A. hydrophila*.

Although considerable information is available on the nature and properties of toxins produced by gram-positive bacteria, much less is known of the extracellular products of gram-negative bacteria (3). At least three different toxins have been isolated from culture supernatants of *Aeromonas hydrophila* (19). The hemolytic toxin, aerolysin, has been partially purified (4) and resolved into two components by isoelectric focusing (21). However, the mechanisms of its action and the actions of both the enterotoxic and cytotoxic factors are unknown. Bernheimer et al. (5) have suggested the presence of both phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) and phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1 group) in culture supernatants of *A. hydrophila*. Their evidence indicates that these enzymes are distinct from aerolysin. This report contains information concerning the phospholipase-like action of *A. hydrophila* culture supernatant on lipid dispersions and on the lipids in human erythrocyte membranes.

MATERIALS AND METHODS

Bacterial strains. *A. hydrophila* strain Ah65 was isolated from rainbow trout (*Salmo gairdneri*) in this laboratory. *A. hydrophila* ATCC 9071 was obtained from the American Type Culture Collection, Rockville, Md.

Bacterial cultivation. The bacterial cultivation conditions of Bernheimer and Avigad (4) were followed except that M9 salts (13) were added to the culture medium and incubation was at 25°C. Culture supernatants were filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) before use.

Aerolysin assay. Hemolytic activity estimations were carried out as described by Wretlind et al. (20), using 2% (vol/vol) human erythrocytes and 25-μl samples of culture supernatant for the twofold serial dilutions.

Preparation of erythrocyte ghosts and lipid suspensions. Erythrocyte membranes were isolated from outdated human blood by a procedure described earlier (7). The membranes were stored frozen in 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4. Lipid suspensions were prepared by blending them in a Vortex mixer in 0.15 M NaCl after complete removal of volatile solvents in a stream of N_2.

Enzyme assay. Conversion of membrane lipids was measured in a total volume of 0.5 ml containing 1 to 2 mg of membrane protein (0.6 to 1.2 μmol of phospholipid phosphorus), 100 mM Tris-maleate (pH 7.5), and 5×10^{-2} to 20×10^{-2} U of dialyzed enzyme fractions. Incubation was, normally, for 15 min at 37°C. One unit of activity produces 1 μmol of water-soluble phosphate per min under the conditions described. Reactions were terminated by the addition of 5 volumes of chloroform/methanol (2:1, vol/vol). After mixing and centrifugation, samples of the upper phase were used to determine water-soluble phosphate. Fatty acid and cholesterol ester were determined in samples of the lower phase.

Chromatographic procedures. Neutral lipids in the lower phase were separated on thin-layer plates of Silica Gel H, using petroleum ether/ether/acetic acid (90:10:1, vol/vol/vol). Phospholipids were separated as described by Turner and Rouser (18), using two-

dimensional thin-layer chromatography.

Protein isolation procedures. Solid ammonium sulfate was slowly added at 0°C to clear culture supernatant up to 85% saturation. After 1 h, the suspension was centrifuged for 30 min at 14,000 × *g*. The precipitate was suspended in cold water and dialyzed at 0°C against 20 mM Tris-0.02% sodium azide, pH 7.4. Insoluble material was removed by centrifugation after dialysis. The resulting supernatant was concentrated 5 to 10 times by pressure filtration through a PM 30 membrane filter (Amicon Corp., Lexington, Mass.) and applied to a Sephadex 6B column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated in 20 mM Tris-0.02% sodium azide, pH 7.4.

Analytical methods. Phosphate in aqueous phases and in separated phospholipids was determined by the method of Bartlett (2). Protein was measured by the technique of Bradford (6). Cholesterol ester, cholesterol, and fatty acids were eluted from thin-layer plates with chloroform/methanol (2:1, vol/vol). Cholesterol and cholesterol ester were quantitated by the procedure of Sperry and Webb (16). Fatty acid was measured by the colorimetric procedure of Duncombe (9). Appropriate standards were carried through chromatogaphy and extraction procedures before determination.

Materials. All chemicals were the purest commercially available. Lipid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.) and from Supelco, Inc. (Bellefonte, Pa.). Thin-layer plates and gas chromatography columns were purchased from Supelco, Inc. Silica Gel H was purchased from Brinkmann Instruments (Canada) Ltd. (Rexdale, Ontario, Canada).

RESULTS

Preliminary attempts to show the presence of phospholipase A or C in *A. hydrophila* strain Ah65 culture supernatants were unsuccessful. No breakdown of pure phospholipid suspensions occurred under any of the conditions used. When erythrocyte membranes were used as substrate, fatty acid and water-soluble phosphate were produced; however, neither lysophosphatidyl choline nor diglyceride was detected. Thin-layer chromatography of neutral lipid breakdown products indicated the presence of fatty acid and cholesterol ester (Fig. 1). Production of both of these was drastically lowered by boiling the bacterial supernatant but not reduced by heating the membranes under the same conditions (Fig. 1), suggesting that the enzyme activities resided entirely in the supernatant. Similar results were obtained with *A. hydrophila* strain ATCC 9071. Strain Ah65 was used for further studies.

When the ability to produce water-soluble phosphate, fatty acid, and cholesterol ester from erythrocyte membranes was examined in culture supernatants during growth, it was found that optimal activities for all three occurred simultaneously in late exponential/early stationary phase and declined rapidly thereafter (Fig. 2 and 3). It can be seen that the appearance of these activities preceded the appearance of aerolysin, which reaches a peak in production at 24 h (Fig. 2 and reference 4). All of the activity responsible for production of water-soluble phosphate, fatty acid, and cholesterol ester was precipitated from a 13-h supernatant in 85%-saturated ammonium sulfate. After solution of the activities in water, dialysis against 20 mM Tris-0.02% sodium azide (pH 7.4), and centrifugation, the supernatant, which typically contained 6 to 10 U of enzyme activity per mg of protein, was subjected to gel filtration on Sepharose 6B. More than 90% of the activity responsible for the formation of all three products was recovered near the void volume (Fig. 4), well separated from aerolysin, which was retarded on the column. Once again the three activities were eluted simultaneously, and no other lipid-degrading activity was observed. The column fraction was concentrated by pressure filtration and used in further experiments.

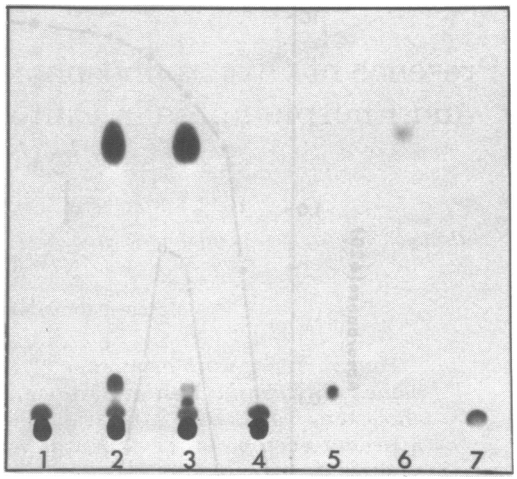

FIG. 1. *Thin-layer chromatography of membrane neutral lipids. Erythrocyte membranes (1 to 2 mg of protein) were incubated with 0.3 ml of 13-h culture supernatant for 15 min at 37°C before extraction as described in the text. (1) Zero-control; (2) 15-min incubation; (3) incubation with heated membranes (10 min, 100°C); (4) incubation with heated supernatant (10 min, 100°C); (5) palmitic acid; (6) cholesterol oleate; (7) cholesterol.*

Liposomes prepared with egg phosphatidyl choline and cholesterol were also degraded by the column fraction, with the appearance of the three products observed with membrane substrates (Table 1). The water-soluble breakdown product was tentatively identified as glycerophosphoryl choline by thin-layer (17) and paper (8) chromatography. Liposomes prepared with phosphatidyl ethanolamine or phosphatidyl ino-

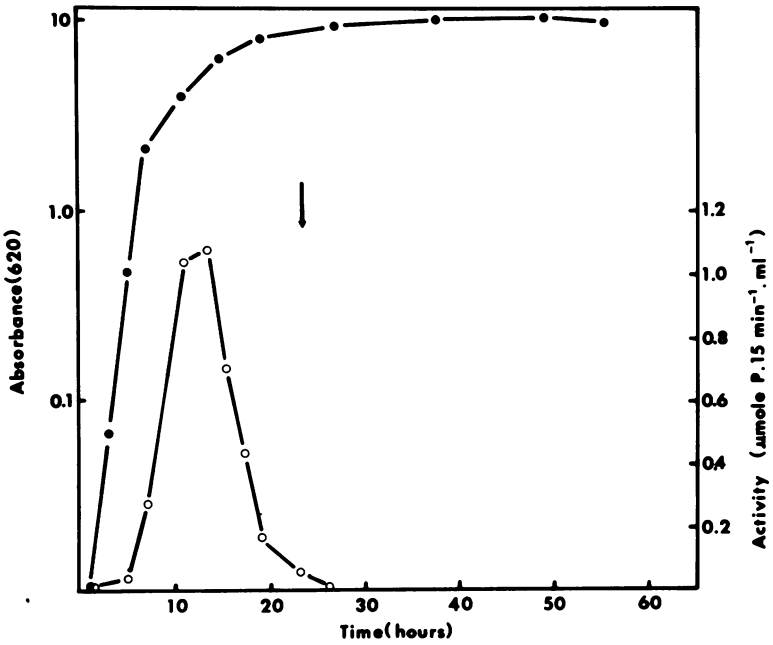

FIG. 2. *Effect of growth on culture supernatant activity—water-soluble phosphate production and aerolysin activity. Water-soluble phosphate (P) production was measured as described in the text, using 0.3-ml samples of filtered (Millipore) supernatant. The arrow indicates peak production of aerolysin (see text for details). One absorbance unit (at 620 nm) is equivalent to 0.46 mg (dry weight) per ml. Symbols: ●, growth; ○, water-soluble phosphate production.*

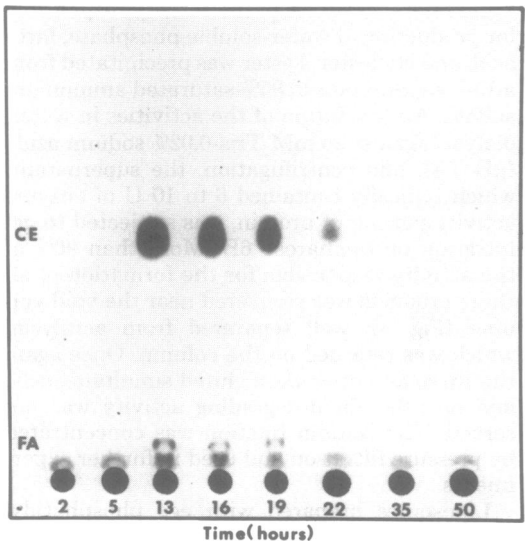

FIG. 3. *Effect of growth on culture supernatant activity—lipid conversion products. See Fig. 2 and text for details of incubation, extraction, and separation procedures. This is the same experiment as in Fig. 2. Abbreviations: CE, cholesterol ester; FA, fatty acid.*

sitol and cholesterol were equally effective as substrates. More than 30% of each was degraded in 15 min. No products were observed when either cholesterol or the glycerophospholipid was omitted or when sphingomyelin was used as the phospholipid substrate. Finally, lysophospholipids were not detected in measurable quantity (Table 1).

When the phospholipid distribution of the erythrocyte membrane was examined before and after treatment with the column fraction, decreases in absolute amounts of the three major glycerophospholipids were observed (Table 2). Sphingomyelin was not affected. Similar results were obtained by Bernheimer et al. (5), using rabbit erythrocytes and unfractionated aerolysin preparations. Neither lysophosphatidyl choline nor lysophosphatidyl serine was observed, although large quantities of their diacyl precursors disappeared during incubation (Table 2). Only one additional lipid was produced by incubation with the enzyme fraction. It stained positive with ninhydrin and comigrated with lysophosphatidyl ethanolamine, and its appearance accounted for approximately half of the loss in phosphatidyl ethanolamine which occurred during incubation (Table 2). More than half of the

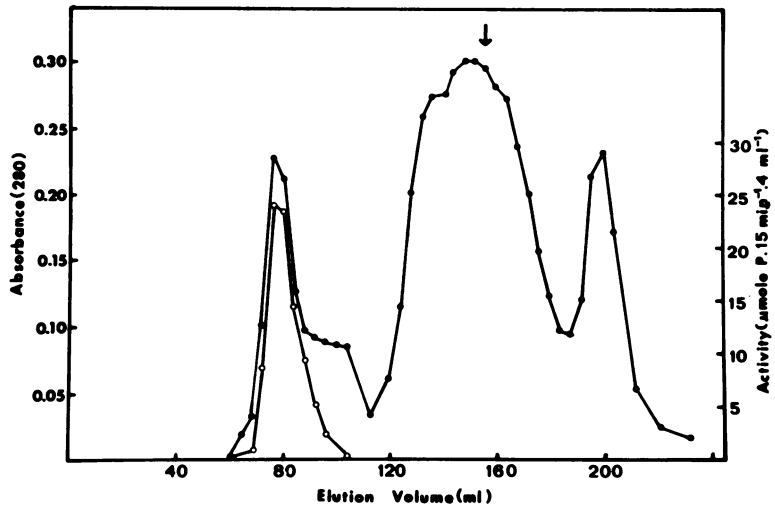

FIG. 4. *Gel chromatography of ammonium sulfate fraction. Dialyzed and filtered (Millipore) ammonium sulfate precipitate from 80 ml of culture supernatant was applied in a volume of 1.5 ml to a column (1.5 by 90 cm) of Sepharose 6B. The column was eluted in 4-ml fractions at 12 ml/h. Samples were assayed for production of water-soluble phosphate (P), cholesterol ester, and fatty acid as described in the text. Only water-soluble phosphate data are presented. Cholesterol ester and fatty acid production was confined to these fractions, which also produced water-soluble phosphate. Recovery was over 90%. Symbols: ●, absorbance (280 nm); ○, water-soluble phosphorus. The arrow corresponds to the position of aerolysin, determined in a separate experiment.*

TABLE 1. *Conversion of lipid suspensions by column enzyme preparation*[a]

Lipid suspension	Conversion products			
	FA	CE	WSP	LPL
Glycerophospholipid[b]	−	−	−	−
Cholesterol	−	−	−	−
Cholesterol + glycerophospholipid	+	+	+	−
Cholesterol + sphingomyelin	−	−	−	−
Cholesterol + lysophosphatidyl choline	−	−	−	No change[c]

[a] Incubation was for 15 min with approximately 1 U of dialyzed enzyme as described in the text except that lipid suspensions (1 mM phosphate) replaced membranes. Where indicated, the molar ratio of cholesterol to phospholipid was unity. Production of cholesterol ester (CE), lysophospholipid (LPL), and fatty acid (FA) was determined qualitatively by thin-layer chromatography. Water-soluble phosphate (WSP) was measured as described in the text against a zero-time control. (−), Lack of product formation; (+) product formation. Less than 0.01 μmol of water-soluble phosphate could be detected by these procedures.

[b] Glycerophospholipids tested included phosphatidyl choline, phosphatidyl inositol, and phosphatidyl ethanolamine. Production of WSP, where indicated, was similar with all three phospholipids (0.14 to 0.17 μmol/15 min).

[c] No change indicates same as zero-time control.

TABLE 2. *Changes in membrane phospholipid composition*

Lipid	Phosphate (μmol/mg of protein)[a]		Change (%)
	Control	Incubated	
Phosphatidyl serine	0.075	0.050	−33
Phosphatidyl choline	0.198	0.107	−45
Phosphatidyl ethanolamine	0.194	0.084	−56
Sphingomyelin	0.162	0.172	+6
Lysophosphatidyl ethanolamine	0	0.057	—[b]

[a] Phosphate analysis was performed on individual lipids after separation by thin-layer chromatography. See the text for details.

[b] —, No change.

phosphatidyl ethanolamine in the human erythrocyte is reported to be plasmalogen, with a vinyl ether linkage in position 1 in the phospholipid. Much smaller amounts of phosphatidyl serine and virtually no phosphatidyl choline are plasmalogens in the human erythrocyte (15). Thus, the appearance of lysophosphatidyl ethanolamine could reflect the inability of the enzyme activity to break the vinyl ether bond, resulting in the production of lysophosphatidyl ethanolamine plasmalogen. It should be emphasized that no diglyceride was produced under these conditions.

These results, together with the observations with liposomes, indicate a two-step conversion of erythrocyte glycerophospholipids via a cholesterol:glycerophospholipid acyltransferase and phospholipase or lysophospholipase A_1 or A_2.

DISCUSSION

The results of this work indicate that filtered culture supernatants of *A. hydrophila* have the ability to degrade erythrocyte membrane glycerophospholipids with the production of cholesterol ester, free fatty acid, and deacylated water-soluble breakdown products. The mechanism by which this occurs appears similar to that described by Owens (14) for the action of *Staphylococcus aureus* and *Serratia marcescens* on egg yolk, involving a glycerophospholipid:cholesterol acyltransferase which appears similar in action to mammalian lecithin:cholesterol acyltransferase (EC 2.3.1 group) and a phospholipase of undetermined specificity (12). Interestingly, Owens reported the absence of this activity in *Aeromonas* sp. (14), whereas we have found much higher activity in this organism than in *S. aureus* with his procedures (unpublished results). However, Owens was using much longer growth times than described here (14) and may have missed the early peak production of activity shown in Fig. 2. Some variation in findings could also have been caused by the use of different bacterial strains.

Of particular interest is the indication from our results that more than one enzyme is involved in glycerophospholipid degradation and that these enzymes are isolated together as a high-molecular-weight complex. Glycerophospholipid:cholesterol acyltransferase and phospholipase are produced simultaneously during late exponential/early stationary phase (Fig. 2 and 3), and the activities are recovered together after ammonium sulfate precipitation and gel filtration (Fig. 4). Acyltransferase and phospholipase activities were never observed separately, at any time during growth. Furthermore, both activities were quantitatively recovered together from gel filtration columns in fractions which indicate a molecular weight of over 500,000. The molecular weight of phospholipase A_1 from *Escherichia coli* is 60,000 (10), that from *Bacillus megaterium* is 29,000, and those from *Mycobacterium phlei* are 27,000 and 45,000 (1). Most bacterial extracellular proteins fall in the range of 20,000 to 60,000 daltons (11). Our results therefore indicate the likelihood of a high-molecular-weight complex of enzymes produced by *A. hydrophila* and isolated under our conditions. The mode of secretion and assembly of this complex, its occurrence in different bacterial genera, and the advantages it may provide in bacterial nutrition are attractive areas of pursuit.

Bernheimer et al. (5) have shown the presence of both phospholipase A- and phospholipase C-like activities in partially purified aerolysin preparations from *A. hydrophila*. As we have not found a distinct phospholipase A or phospholipase C in *A. hydrophila* culture superantants, it seems likely that the new complex described here could account for the activities recorded by Bernheimer et al. (5). Certainly, production of water-soluble phosphate after complete deacylation could easily be confused with phospholipase C activity.

Further work is required to establish a detailed description of the mechanism of membrane lipid conversion. Specificity in terms of bindings to membranes and substrate requirements must also be determined; it seems likely, however, that the complex will be a useful one in studies of membrane phospholipid-cholesterol interactions and a valuable tool for the modification of membrane lipid composition.

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