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Interactions Between Aerolysin, Erythrocytes, and Erythrocyte Membranes

ALAN W. BERNHEIMER,* LOIS S. AVIGAD, AND GAD AVIGAD

Department of Microbiology, New York University School of Medicine, New York, New York 10016, and Department of Biochemistry, Rutgers Medical School, Piscataway, New Jersey 08854*

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Aerolysin, a hemolytic and lethal exotoxin of *Aeromonas hydrophila*, was analyzed for amino acids. Assuming 8 histidine residues/mol, the purified toxic protein has, by summation, a molecular weight of 49,000, a value in agreement with earlier estimates by other methods. Erythrocytes from different animal species differ greatly in sensitivity to aerolysin's lytic action. There is some correlation between sensitivity and phosphatidyl choline content. Erythrocyte membranes of different species bind the toxin, and the efficiency of binding is a function of sensitivity to lysis. Binding is temperature independent, is not dependent upon membrane sialic acid, and is decreased by prior treatment with phospholipase C and proteases. Preparations of aerolysin convert substantial amounts of membrane phosphorus to water-soluble form; the conversion is concentration and temperature dependent. Most of the conversion is attributable to contaminating phospholipase(s) that is separable from the toxin. Aerolysin purified by electrophoresis in polyacrylamide gel retains some phospholipase activity, and this activity may or may not be a contaminant.

Aerolysin, a lethal and hemolytic extracellular product of *Aeromonas hydrophila*, was early observed by Caselitz and Günther (10) and studied further by Wretlind et al. (26). Factors governing the production of this protein were subsequently investigated by Wretlind et al. (25) and by Bernheimer and Avigad (3). The latter also described a procedure for purification of aerolysin, and they partially characterized it. The present report provides information concerning its interaction with erythrocytes and their membranes.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are: Tris, 0.01 M tris(hydroxymethyl)aminomethane, pH 7.2; TS, Tris containing 0.145 M NaCl; TSG, TS containing 0.2% gelatin; and HU, hemolytic units as defined by Bernheimer and Avigad (3).

Aerolysin. Aerolysin was prepared as described earlier (3). A portion of one lot was hydrolyzed in vacuo in 6 N HCl for 24 h at 114 C, after which an amino acid analysis was carried out according to the method of Moore et al. (20). The composition of aerolysin is shown in Table 1. Assuming 8 histidine residues/mol, the molecular weight of aerolysin, by summation, is 49,000, a value that compares favorably with earlier estimates of 50,000 and 53,000 obtained by gel electrophoresis and gel filtration, respectively.

Hemolytic activity. Hemolytic activity was assayed by adding decreasing quantities of test solution to a constant quantity of washed rabbit erythrocytes contained in a final volume of 2 ml of TSG. After incubating at 37 C for 30 min and briefly centrifuging, the percentage of hemolysis was estimated from the color of the hemoglobin in the supernatant fluids. Further details are given in reference 3. Contrary to a statement by Scholz et al. (23), this lysin in our experience does not exhibit the behavior of a hot-cold hemolysin when rabbit, human, or sheep erythrocytes are used.

Preparation of erythrocyte membranes. (Method A) Eighty milliliters of a 0.7% (vol/vol) suspension of washed erythrocytes in 0.077 M NaCl and 0.067 M sodium phosphate (pH 7.0) was centrifuged at about 3,000 × *g* for 10 min. The cells were suspended in 4 ml of TS and lysed by adding 180 ml of distilled water. The lysate was centrifuged at 15,000 × *g* for 20 min, and the sedimented membranes were suspended in 3 ml of Tris.

(Method B) Five milliliters of rabbit blood was delivered into 200 ml of 0.077 M NaCl and 0.067 M sodium phosphate (pH 7.0). The cells were centrifuged at 2,000 × *g* for 10 min and washed twice, each time in 200 to 300 ml of 0.077 M NaCl and 0.067 M sodium phosphate (pH 7.0). The cells were suspended in 20 ml of TS and lysed by adding them to 800 ml of distilled water. The lysate was centrifuged at 15,000 × *g* for 30 min, and the membranes were collected and washed in 70 ml of Tris and suspended in 12 ml of Tris.

(Method C) The procedure of Hoogeveen et al. (14) was used as modified and described by Low et al. (18).

Phosphorus. Unless indicated otherwise, total

TABLE 1. *Amino acid composition of aerolysin*

Amino acid	Concn (μM)	A (residues [histidine = 1])	Nearest integer to 8 × A
Aspartic acid	2.232	8.36	67
Threonine	1.200	4.49	36
Serine	1.320	4.94	40
Glutamic acid	1.776	6.65	53
Proline	1.128	4.22	34
Glycine	1.896	7.10	57
Alanine	1.560	5.84	47
Half-cystine	?	?	?
Valine	1.140	4.27	34
Methionine	0.144	~1	~8
Isoleucine	0.717	2.69	22
Leucine	1.224	4.58	37
Tyrosine	0.645	2.42	19
Phenylalanine	0.594	2.22	18
Lysine	0.867	3.25	26
Histidine	0.267	1.00	8
Arginine	0.573	2.14	17
Tryptophan	?	?	?

phosphorus was determined by the Bartlett procedure (1) as modified by Böttcher et al. (7).

Enzymes. Neuraminidase was purchased from Sigma Chemical Co. (St. Louis, Mo.), papain and trypsin from Worthington Biochemical Corp. (Freehold, N.J.), and Pronase from Calbiochem (La Jolla, Calif.). Phospholipase C from *Clostridium perfringens* was purified from solutions of dried culture filtrate by isoelectric focusing followed by gel filtration in Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.). The purified enzyme was preserved in 50% glycerol at 4 C.

Blood specimens. Rabbit and guinea pig bloods were obtained from the local animal facility, sheep, horse, and some rabbit bloods from the Public Health Research Institute (New York, N.Y.), and most of the remaining bloods from either the Animal Blood Centre (Syracuse, N.Y.) or Colorado Serum Co. Labs (Denver, Colo.).

RESULTS

Sensitivity of erythrocytes to lysis by aerolysin. Suspensions of washed erythrocytes from various kinds of animals were prepared to contain the same amount of hemoglobin as that in the standard rabbit erythrocyte suspension used for routine titrations of hemolytic activity. Purified aerolysin titrated with each gave results (Table 2) in which the lytic activity (415,000 HU/mg), obtained with the most sensitive erythrocytes, those of the rat, was set at 100%. The titers are expressed in proportion to that obtained with rat cells. Attempts to correlate these results with quantitative differences in particular membrane constituents were not notably successful. A plot of the relative amount of phosphatidyl choline against log sensitivity to lysis (Fig. 1) suggests that phospholipid might be involved as a substrate or receptor for the lysin. If this is true, the results obtained with horse, ox, and cat cells require explanation because they deviate widely from expected values.

Effect of temperature on capacity of erythrocyte membranes to inhibit aerolysin-induced hemolysis. It was earlier found (3) that osmotically prepared rabbit erythrocyte membranes inhibited aerolysin-induced hemolysis. The effect of temperature on inhibition was examined by adding, in replicate, 0.6 ml of TS containing 120 HU of aerolysin to 0.4 ml of rabbit erythrocyte membranes (method A) and placing the mixtures at several different temperatures. After 1 h they were centrifuged at

TABLE 2. *Relative sensitivity to aerolysin of erythrocytes of various species*

Species	Sensitivity of erythrocytes compared to that of rats (%)
Rat	100
Mouse	78
Dog	18
Guinea pig	18
Cat	16
Rabbit	3.1
Ox	2.4
Swine	1.1
Human	0.9
Horse	0.5
Goat	0.4
Sheep	0.3

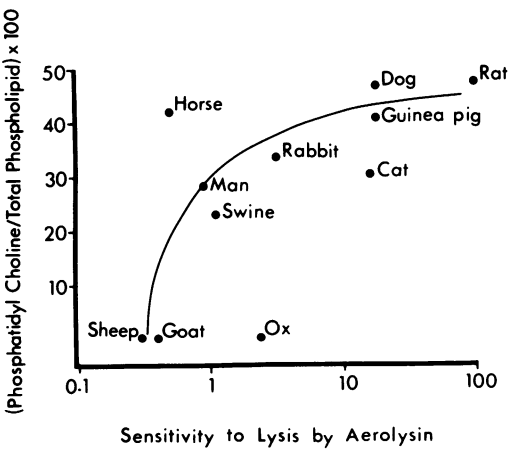

FIG. 1. *Proportion of phosphatidyl choline of total erythrocyte membrane phospholipid, plotted against log sensitivity of erythrocytes to aerolysin. Figures for phosphatidyl choline are from van Deenen (In C. Bishop and D. M. Surgenor [ed.], The Red Blood Cell, 2nd ed., in press).*

15,000 × *g* for 20 min. The supernatant fluids were diluted with 4 volumes of TSG and assayed for hemolytic activity by using rabbit erythrocytes. Compared to a no-membrane control, the amounts of activity missing from the supernatant fluid at 0, 20, and 37 C were 85, 90, and 81%, respectively. The results indicate that inhibition of hemolysis is not temperature dependent and that the inhibition evidently is due to binding of the toxin to the membranes.

Capacity of erythrocyte membranes of different species to inhibit aerolysin-induced hemolysis. Membranes were prepared from erythrocytes of various species according to method A. Volumes of membrane suspensions decreasing in steps of about 40% were added to 0.5 ml of aerolysin diluted in TSG to contain 30 HU. The mixtures were made to 1 ml with Tris, allowed to stand at 20 C for 30 min with occasional mixing, and centrifuged at 15,000 × *g* for 20 min. The supernatant fluids were diluted with 4 volumes of TSG and titrated for hemolytic activity by using rabbit erythrocytes. The inhibition end point was the quantity of membranes needed to inhibit half (15 HU) the test amount of aerolysin, and was expressed as the volume (in milliliters) of 0.7% (vol/vol) erythrocyte suspension from which that quantity of membranes was derived. The values obtained for various species are plotted in Fig. 2 against log relative sensitivity to lysis by aerolysin. The results show that the capacity of membranes to inhibit lysis is a function of the sensitivity to aerolysin-induced hemolysis of the species of erythrocytes. They are interpreted to mean that the sensitivity of cells of a given species to lysis is related to the capacity of those cells to bind the toxin.

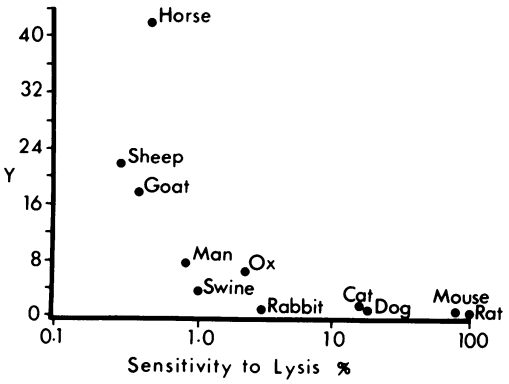

FIG. 2. *Capacity of membranes derived from erythrocytes of different species to inhibit hemolysis, as a function of log sensitivity of erythrocytes to lysis. Y, milliliters of erythrocyte suspension needed to provide membranes that will inhibit 15 HU of aerolysin.*

Effect of pretreatment of rabbit erythrocyte membranes (method A) on their capacity to bind aerolysin. (i) Divalent cations. To 0.3-ml portions of rabbit erythrocyte membrane suspension were added 0.1-ml quantities of 10 mM solutions of $CaCl_2$, $MgCl_2$, $CuSO_4$, and Zn acetate, separately, in Tris. After 10 min at 25 C, the mixtures were centrifuged at 15,000 × *g* for 20 min, and the supernatant fluids were discarded. The membranes were suspended in 0.5-ml portions of TSG, and to each was added 0.5 ml of TSG containing 30 HU of aerolysin. After 30 min at 20 C, the mixtures were centrifuged at 15,000 × *g* for 20 min. Four volumes of TSG was added to each supernatant fluid, and the resulting solutions were titrated for hemolytic activity. The results showed that $CaCl_2$ and $CuSO_4$ had no effect, whereas $MgCl_2$ and Zn acetate gave slightly increased inhibition compared to that obtained with untreated membranes. Additional experiments in which the effects of 5 mM ethylenediaminetetraacetate and 5 mM ethylene glycol-bis(β-amino ethyl ether)-*N*, *N'*-tetraacetate were studied suggested that Ca^{2+} might be necessary for binding of aerolysin to membranes. However, interpretation of the results was complicated by the fact that ethylene glycol-bis(β-amino ethyl ether)-*N*,*N'*-tetraacetate, in the absence of membranes, decreased the activity of aerolysin in one experiment, and also by the fact that this chemical is known not to be very effective in removing Ca^{2+} already bound to membranes (17).

(ii) Sodium periodate. To destroy terminal sialyl residues, rabbit erythrocyte membranes were treated with 1 mM sodium periodate, followed by reduction with sodium borohydrate (5, 16). Capacity to bind aerolysin was not affected, indicating that intact membrane sialic acid is not required for binding of aerolysin. In line with this is also the finding that similar treatment of whole erythrocytes did not affect their sensitivity to lysis by aerolysin.

(iii) Neuraminidase, phospholipase C, and proteases. To 0.4 ml of rabbit erythrocyte membrane suspension was added 0.4 ml of Tris containing 0.5 unit of neuraminidase. After 1 h at 37 C, the membranes were centrifuged and treated as described for divalent cations. Appropriate controls were included. The inhibitory capacity of the enzyme-treated membranes was the same as that of untreated membranes.

Phospholipase C and several proteases were tested for capacity to abolish inhibition of lysis by erythrocyte membranes by using a procedure similar to that described for divalent cations. The phospholipase was allowed to react with membranes at 25 C for 60 min while the

protease-membrane mixtures were kept at 20 C for 30 min. The results (Table 3) indicate that exposure of membranes to phospholipase C, papain. Pronase, or trypsin diminished the capacity of membranes to bind aerolysin. In a separate experiment, and in agreement with earlier findings (3), proteases had no effect on the activity of aerolysin in the absence of added membranes.

Failure of certain sugars and sulfhydryl inhibitors to affect hemolytic activity. A solution of aerolysin was titrated for hemolytic activity in the presence, separately, of 5 mM galactose, lactose, and melibiose. The titers did not differ significantly from that of a control solution. This result is consistent with the assumption that terminal galactosyl moieties of membrane glycoproteins and glycolipids are not involved in the binding of aerolysin; it is consistent also with the lack of involvement of sialyl residues.

A solution of aerolysin was titrated for hemolytic activity in the presence, separately, of 0.1 mM *N*-ethylmaleimide, sodium iodoacetate, and sodium *p*-chloromercuribenzoate. No difference was found among the titers obtained in the presence and absence of these agents, indicating that sulfhydryl groups apparently are not involved in lysis.

Phospholipase C-like effect of aerolysin preparation on erythrocyte membranes. Addition of phospholipase C from *C. perfringens* (12) or *Bacillus cereus* (6) to human erythrocyte ghosts causes shrinkage of the ghosts with formation of phase-dense droplets ("black dots") consisting of diglyceride and ceramide arising from hydrolysis of phospholipids. Decreasing volumes of aerolysin solution in TSG were brought to 0.25 ml with 10 mM sodium borate buffer, pH 8.2. After addition of 0.1 ml of osmotically prepared membranes, the mixtures were allowed to stand at 25 C for 1 h or longer and then examined by phase contrast microscopy. The results of the experiments carried out in the absence and presence of 7 mM calcium acetate are in Table 4. They show that: (i) phase-dense droplets develop in rabbit and human ghosts but not in sheep cell ghosts; (ii) rabbit erythrocyte ghosts are more sensitive than human; and (iii) calcium ions intensify the effect, allowing it to manifest itself in appreciably lower concentrations of aerolysin than when calcium ions are not added. Additional data, not shown in the table, suggested that the order of sensitivity to the droplet-producing activity of the aerolysin preparation is guinea pig > rabbit ≥ horse > human > sheep.

Absence of turbidity-producing activity in diluted egg yolk. Some phospholipases C produce turbidity in dilute egg yolk. Amounts of aerolysin as great as 100 μg failed to produce turbidity in egg yolk solution with conditions described elsewhere (4) in the presence of either Ca^{2+} alone or Ca^{2+} and Mg^{2+}.

Liberation of water-soluble phosphorus from rabbit erythrocyte membranes. (i) As a function of aerolysin concentration. A stock solution containing 1 mg of aerolysin per ml in 0.01 M sodium borate buffer, pH 8.2, was prepared. Decreasing volumes were added to tubes (18 by 102 mm) having a wall thickness of 2 mm, and the volumes were brought to 50 μl with borate buffer. To each tube were added 1 25 ml of 3 mM $CaCl_2$ and 1 ml of rabbit erythrocyte suspension (method B). After standing at

TABLE 3. *Effect of several enzymes on the capacity of erythrocyte membranes to bind aerolysin*

Rabbit erythrocyte membrane prepn	Addition[a]	Supernatant fluid from aerolysin membrane mixture (HU/ml)
None	None	35
0.3	None	7
0.3	PLC[b] 10 λ	24
0.1	None	22
0.1	PLC 10 λ	33
0.3	Papain (100 μg) + mercaptoethanol (0.5 μg)	24
0.3	Pronase (100 μg)	28
0.3	Trypsin (100 μg)	21

[a] Aerolysin (35 HU) also added to each.
[b] PLC, Phospholipase C.

TABLE 4. *Formation of phase-dense droplets in osmotically prepared erythrocyte ghosts in absence and presence of added Ca^{2+}*

Final concn of aerolysin (HU/ml)	Ca^{2+} (mM)	Phase-dense droplets in ghosts		
		Rabbit	Human	Sheep
300	0[a]	++[b]	++	0
180	0	++	++	0
90	0	+	+	0
30	0	±	0	0
0	0	0	0	0
30	7	+++[c]	++	0
9	7	+++	0	0
3	7	+	0	0
0	7	0	0	0

[a] 0 signifies no ghosts showed one or more phase-dense droplets.
[b] ++, +, and ± signify intermediate degrees.
[c] +++ signifies every ghost showed one or more phase-dense droplets.

20 C for 30 min, the tubes were centrifuged at 15,000 × *g* for 20 min. The supernatant fluids were removed as completely as possible, and their total phosphorus as well as that of the pellets was determined. The results (Fig. 3) show that about 40% of the membrane phosphorus can be converted to water-soluble form under the conditions used.

(ii) As a function of temperature. The conditions in (i) were followed except that a constant amount (20 μg) of aerolysin was used, and the temperature of the reaction mixture was varied. The results (Fig. 4) show that the liberation of phosphorus from membranes is temperature dependent.

(iii) Effect of divalent cations. The conditions in (i) were followed except that a constant amount (10 μg) of aerolysin was used, the divalent cation was varied, and the temperature of the reaction mixture was 40 C. The phosphorus values were corrected for liberation of phosphorus in the absence of aerolysin. The results (Table 5) show that Ca^{2+} and Mg^{2+} caused a small but significant increase in the amount of phosphorus released from membranes, Zn^{2+} inhibited nearly completely, and ethylenediaminetetraacetate substantially reduced the amount of phosphorus released.

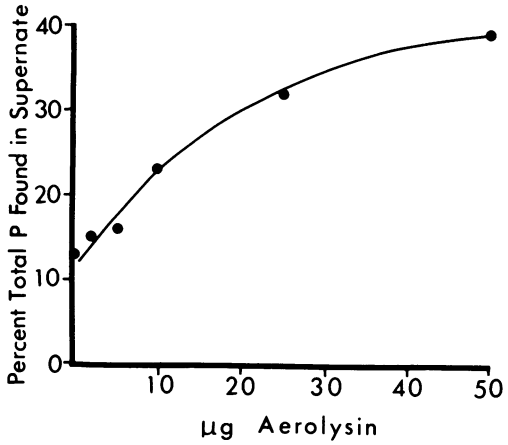

FIG. 3. *Liberation of water-soluble phosphorus (P) from rabbit erythrocyte membranes by aerolysin.*

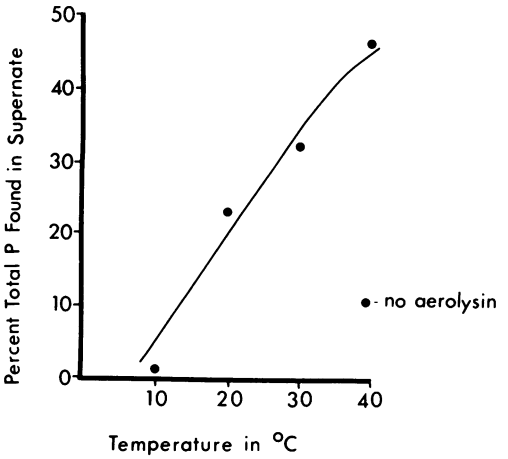

FIG. 4. *Effect of temperature on liberation by aerolysin of water-soluble phosphorus (P) from erythrocyte membranes.*

Fractionation of aerolysin preparation in polyacrylamide gels. An aerolysin preparation was subjected to electrophoresis in polyacrylamide gel, and then the gel was sliced. The slices were eluted, and the eluates were examined for hemolytic activity, black dot-forming activity, and capacity to release phosphorus from rabbit erythrocyte membranes.

Seven percent polyacrylamide gels were prepared in pH 9.5 buffer according to directions provided by the manufacturer of the Canalco model 12 electrophoresis apparatus (Canalco, Rockville, Md.) but omitting stacking gel. Aerolysin (200 μg) plus 3 μl of 0.05% bromophenol blue were electrophoresed at 4 mA per gel until the tracking dye traveled to within 3 mm of the bottom. The gel was chilled and sliced with a device (19) yielding 54 slices, each slightly less than 1 mm thick. Pairs of adjacent slices were eluted by placing each pair in 0.33 ml of 0.02 M Tris, pH 6.7, and allowing them to stand overnight in the refrigerator. The supernatant fluids were assayed for the several activities mentioned according to methods already described. An additional gel was stained for protein with Coomassie brilliant blue (Colab Laboratories, Glenwood, Ill.) according to Weber and Osborn (24) and scanned with a model 2410-S linear transport scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The results are shown in Fig. 5, in which allowance has been made for 8% swelling of the stained gel.

Degradation of erythrocyte membrane phospholipids by aerolysin preparation and

TABLE 5. *Effect of divalent cations and ethylenediaminetetraacetate on liberation of water-soluble phosphorus by aerolysin*

Additions (10 mM)	Total P[a] of reaction mixture (%) released into supernatant fluid
None	26
$MgCl_2$	31
$CaCl_2$	32
$ZnCl_2$	2
EDTA[b]	18

[a] P, Phosphorus.
[b] EDTA, Ethylenediaminetetraacetate.

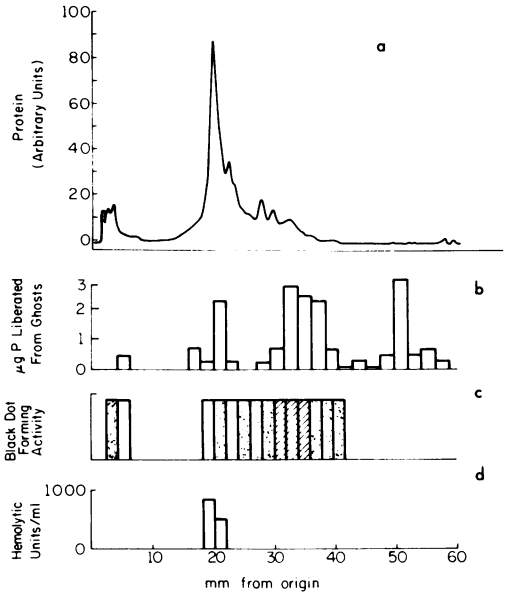

FIG. 5. *Distribution of (a) stained protein, (b) capacity to release phosphorus from erythrocyte ghosts, (c) black dot-forming activity, and (d) hemolytic activity of aerolysin preparation fractionated by electrophoresis in polyacrylamide gel.*

by two major electrophoretic fractions thereof. Aerolysin preparations (50 μg) were subjected to electrophoresis in polyacrylamide in each of 11 gels according to the conditions of the preceding experiment. One gel was stained, and ten were frozen and sliced. From 10 gels, slices between 17 and 23 mm from the origin were pooled and eluted three times each with 1 ml of 0.03 M sodium borate buffer, pH 8.2. The three eluates were pooled and designated eluate A. Slices between 31 and 41 mm, from the same 10 gels, were similarly treated and designated eluate B. Thirty percent of the input hemolytic activity was recovered in eluate A and none in eluate B. Recombination of portions of eluates of all slices along the entire length of the gel did not result in greater recovery of hemolytic activity.

Mixtures having the composition shown in Table 6 were prepared in tubes (18 by 102 mm) with a wall thickness of 2 mm. After 1 h at 40 C, the tubes were chilled and centrifuged at 25,000 × *g* for 20 min. After removing the supernatant fluids, the lipid of each pellet was extracted by the method of Reed et al. (22). Each preparation of dried extracted lipid was dissolved in 0.4 ml of chloroform, and about 0.2 ml of each of the resulting solutions was analyzed by two-dimensional thin-layer chromatography according to Broekhuyse (8, 9). Spots were stained with iodine vapor and identified by comparison with those of authentic phospholipids and by comparison with published R_f values. The spots were scraped from the glass, 0.5 ml of concentrated HCl was added, and the mixtures were evaporated to dryness in a 110 C oven. After ashing with 70% perchloric acid, phosphorus was determined by the ascorbic acid modification (11) of the Fiske-Subbarow method.

The results (Table 7) can be interpreted as follows: (i) the unfractionated aerolysin preparation (C) degraded all the phosphatidylserine, all the phosphatidylethanolamine, and most of the phosphatidyl choline. Since no lysophosphatides were found the degradation is presumably due to a phospholipase C. (ii) Eluate A, containing all of the recovered hemolytic activity, produced slight phospholipid degradation (13.6%) compared to that (30.7%) of eluate B and that (67.1%) of the unfractionated preparation. (iii) Phosphatidylserine-degrading, phos-

TABLE 6. *Composition of reaction mixtures preliminary to thin-layer chromatographic analysis*

Addition	Packed rabbit erythrocyte membranes (method C; ml)	100 mM $MgCl_2$ (ml)	10 mM sodium borate buffer, pH 8.2 (ml)
(A) Eluate A (1 ml)	0.8	0.1	3.1
(B) Eluate B (1 ml)	0.8	0.1	3.1
(C) Aerolysin prepn (50 μg)	0.8	0.1	4.1
(D) None	0.8	0.1	4.1

TABLE 7. *Distribution of phospholipids in membrane residues*

Phospholipid	Total phospholipid (mol/100 mol)[a]			
	A[b]	B[b]	C[b]	D[b]
Phosphatidylserine	5.2	2.2	0	6.7
Sphingomyelin	17.2	17.2	17.2	17.2
Phosphatidyl choline ...	29.1	18.6	15.7	41.0
Phosphatidyl-ethanolamine	17.9	7.5	0	28.3
Lysophosphatidyl choline	4.4	11.9	0	3.0
Lysophosphatidyl-ethanolamine	8.2	11.9	0	3.7
Lysophosphatidylserine .	4.4	0	0	0
Total phospholipid found relative to D (%)	86.4	69.3	32.9	100
Total phospholipid degraded relative to D, by difference (%)	13.6	30.7	67.1	0

[a] Assuming no sphingomyelin was degraded.
[b] Reaction mixture plus addition as described in Table 6.

phatidylethanolamine-degrading, and phosphatidyl choline-degrading activities were found in both eluates, but there was substantially more in eluate B than in eluate A. (iv) Lysophosphatidyl choline was produced by eluate B but not by eluate A, suggesting that the former contains a phospholipase A. (v) Lysophosphatidyl serine was produced by eluate A, suggesting the presence of a second phospholipase A.

In general, the results can be explained by assuming (i) that eluate A contains a phospholipase A active on phosphatidylserine and phosphatidylethanolamine, and (ii) that eluate B contains a phospholipase A active on phosphatidylethanolamine and phosphatidyl choline, and also a phospholipase C, or combination of other enzymes, active on phosphatidylserine.

DISCUSSION

Our results show that erythrocytes from different mammals differ greatly in sensitivity to lysis by aerolysin, those from the rat being about 300 times as sensitive as those from the sheep, whereas erythrocytes from other species exhibit intermediate degrees of sensitivity or resistance. These findings contrast sharply with the results of Wretlind et al. (26), and they differ also from those of Scholz et al. (23) and of Caselitz and Günther (10), which more or less resemble Wretlind's. Wretlind et al. found only a 16-fold difference in sensitivity between cells of the most sensitive and resistant species. They found, moreover, that rat and sheep erythrocytes were the most resistant among the kinds tested, whereas we find that sheep cells are the most resistant but that rat cells are the most sensitive. Erythrocytes from two additional strains of rats exhibited sensitivity comparable to that given in Table 2. The reason for the discrepancies is not obvious but one explanation among others is that the apparent sensitivity may depend upon the state of purity of the aerolysin. This and other possible explanations are amenable to experimental test.

The possibility that sialic acid is involved in the binding of aerolysin to the erythrocyte is rendered unlikely by the lack of effect of neuraminidase and by the failure of periodate treatment, followed by reduction, to affect the capacity of membranes to bind the toxin. Other results suggest that sulfhydryl groups probably are not involved in binding. The effect of phospholipase C and of proteases, however, indicates that phospholipid and protein are directly or indirectly involved in attachment of aerolysin to the membrane. In contrast to experience with certain other lysins such as streptolysin S and staphylococcal δ-toxin, for which the receptors appear to be phospholipids (2), the action of aerolysin is not inhibited by phospholipids even in high concentration (3).

Although the major constituent of the aerolysin preparations employed is aerolysin itself, the preparations, as found earlier by electrophoretic analysis in polyacrylamide gel (3), contain numerous impurities. The phospholipase C-like effect of aerolysin on osmotically prepared erythrocyte membranes, namely, the production of phase-dense lipid droplets ("black dots") in membranes, is an effect that is due largely to protein(s) other than aerolysin itself in the preparations. The effect could be due to a phospholipase C or to the sequential action of a phospholipase D and a phosphatase. In this context both choline and diglyceride have been found to be reaction products in mixtures of phosphatidyl choline and enzyme extracts of *Aeromonas* spp. (21).

Our findings show that addition of aerolysin preparations to erythrocyte membranes releases substantial amounts of water-soluble phosphorus. The release is concentration dependent, but unlike binding of aerolysin to membranes it is also temperature dependent, suggesting the involvement of one or more enzymes. Fractionation by polyacrylamide gel electrophoresis showed that most of the phosphate-liberating activity was not associated with the hemolytic toxin, but a minor fraction of the phosphate-liberating activity was. The nonaerolysin (nontoxin) activity evidently involves phospholipases hydrolyzing phosphatidylethanolamine, phosphatidylserine, and phosphatidyl choline.

The failure of any of our preparations to produce turbidity in diluted egg yolk is surprising at first sight, in view of the aforementioned black dot-producing activity and in view of the fact that cultures of *Aeromonas* spp. and related bacteria have been shown by Esselmann and Liu (13), Owens (21), and others to produce turbidity in egg yolk-containing media. The production of turbidity in dilute egg yolk by phospholipase C from *Clostridium welchii* and from *Pseudomonas aeruginosa* has been shown by Kurioka and Liu (15) to be inhibited by a hemolysin from the latter organism, inhibition depending upon the hemolysin's detergent-like effect in solubilizing various lipids. It is conceivable that a similar mechanism may operate in our system. Another possibility is that the principal phospholipid of egg-yolk, phosphatidyl choline, may be a poor substrate for the phospholipase C-like enzyme(s) present in our *Aeromonas* preparation. It is of interest that phospholipase A activity also was detected in

the preparation. In the few instances in which phospholipase A has been found in bacteria, it usually is associated with the cell membrane.

Our results do not provide definitive information regarding the mechanism by which aerolysin brings about erythrocyte lysis. Although most of the phospholipase activity could be separated from the toxin (hemolysin), a minor fraction of the enzymatic activity fractionated along with the aerolysin. The results suggest that the substances responsible for the last two activities are closely similar in charge and molecular weight, and that their separation may therefore prove very difficult. It is tempting to suggest that the small peak visible on the right limb of the main (presumably toxin) peak of Fig. 5 is contaminating phospholipase.

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