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Stepwise Degradation of Membrane Sphingomyelin by Corynebacterial Phospholipases

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The mechanism of in vitro synergistic lysis of sheep erythrocytes by *Corynebacterium ovis* and *Corynebacterium equi* was investigated. Hemolysis required (i) the action of phospholipase D from *C. ovis*, (ii) the action of an extracellular protein of *C. equi*, and (iii) Mg^{2+}. Maximum lysis required imposition on the system of a fourth condition (step iv), such as chilling. Steps i, ii, and iv occur sequentially and in that order. Mg^{2+} functions in steps i and ii. The extracellular protein of *C. equi* was purified to homogeneity and found to be a phospholipase C capable of hydrolyzing ceramide phosphate, phosphatidic acid, and all of the isolated major phospholipids of mammalian erythrocyte membranes. The principal features of the synergistic hemolytic system could be reproduced in experiments involving liposomes containing either sphingomyelin or ceramide phosphate and trapped [^{14}C]glucose. We inferred that sphingomyelin of sheep erythrocytes is first converted to ceramide phosphate by *C. ovis* phospholipase D. On the basis of results with liposomes, we propose that the ceramide phosphate is then converted to ceramide by *C. equi* phospholipase C. We believe that the resulting in situ ceramide then undergoes dislocation by chilling and perhaps also by virtue of an affinity between ceramide and *C. equi* phospholipase C. The dislocation of ceramide presumably disorganizes the lipid bilayer sufficiently to result in cell lysis.

When *Corynebacterium ovis* and *Corynebacterium equi* grow in close proximity on the surface of sheep blood agar, an area of hemolysis develops, but where the growth of each organism is far from the other, hemolysis does not occur (7). It has been demonstrated that the synergistic hemolytic effect results from the action of diffusible products (8). *C. ovis* is known to produce an extracellular phospholipase D (PLD) (phosphatidylcholine phosphatidohydrolase [EC 3.1.4.4]) which, with respect to the major membrane phospholipids, is specific for sphingomyelin (16). This enzyme is also known to release choline from intact sheep erythrocytes with the formation of ceramide phosphate (*N*-acylsphingosine phosphate) (17) but without accompanying lysis. We postulated that *C. equi* produces an extracellular product (designated *equi* factor) that acts upon PLD-modified sheep erythrocytes to cause them to lyse under appropriate conditions. Since the in situ product of PLD action is ceramide phosphate, it follows that *equi* factor is an agent that interacts with this particular phospholipid. One aim of this study was to elucidate the nature and mechanism of action of *equi* factor, and one of several possibilities is that it is a phospholipase which has ceramide phosphate as its substrate. Experimental results that are consistent with this concept are presented.

MATERIALS AND METHODS

Assay of *equi* factor activity. *equi* factor activity is defined as the capacity of a solution to lyse sheep erythrocytes pretreated with *C. ovis* PLD. PLD was produced, purified, and assayed as described previously (12). Suspensions of washed sheep erythrocytes in 0.01 M Tris(hydroxymethyl)aminomethane (pH 7.2)-0.145 M NaCl (buffer I) were prepared as described previously (5). To each of a series of tubes were added 1 ml of sheep erythrocyte suspension, 0.5 ml of buffer I containing 10 mM $MgCl_2$ and 0.2% gelatin (buffer II), and 5 U of PLD (as measured by inhibition of staphylococcal β-toxin hemolytic activity) (12). The mixtures were placed in a 37°C water bath for 30 min. Starting with 0.5 ml, volumes of the test solution decreasing by about 25% were placed in the tubes, and sufficient buffer II was added to bring each tube to 2 ml. The mixtures were placed at 37°C for 30 min and then in an ice water bath for 15 min. After brief centrifugation, percent hemolysis was estimated from the hemoglobin color in the supernatants as compared with the colors of standards; 1 U of *equi* factor activity was defined as the amount of test solution causing 50% hemolysis.

Preparation of liposomes, lipid dispersions, and ceramide phosphate. Multilamellar liposomes composed of a phospholipid (sphingomyelin or ceramide phosphate), cholesterol, and dicetyl phosphate and in some experiments loaded with [^{14}C]glucose were prepared as previously described (12).

Aqueous dispersions of lipid for use as enzyme substrates were prepared by bath sonication of a dried lipid film into an appropriate buffer. When Triton X-

100 was included, the detergent was mixed in a chloroform solution of lipid before drying.

Ceramide phosphate (*N*-acylsphingosine phosphate) was generated enzymatically as described previously (12), but separation of the product was carried out on preparative thick-layer silica gel plates rather than on paper. The product was located under ultraviolet light, the silica gel was scraped from the plate, and the lipid was recovered by the extraction procedure of Bligh and Dyer (6).

Sphingomyelinase C activity was estimated by using [^{14}C]sphingomyelin and the procedure described for *C. ovis* toxin (PLD) activity (12) taking into account the fact that the [^{14}C]phosphorylcholine product migrates more slowly (R_f = 0.12) than [^{14}C]choline.

Analytical methods. Radioactivity was estimated with a Beckman LS 7500 liquid scintillation spectrophotometer, using ACS aqueous counting scintillant (Amersham Corp., Arlington Heights, Ill.).

Lipid extraction was carried out by the method of Bligh and Dyer (6). Inorganic phosphorus was estimated by the method of Ames and Dubin (1).

Special reagents. Staphylococcal sphingomyelinase (4), phospholipase C from *Clostridium perfringens* (3), and staphylococcal delta toxin (11) were prepared as described previously.

Sphingomyelin was purchased from GIBCO Laboratories, Grand Island, N.Y., phosphatidylcholine and phosphatidic acid were from General Biochemicals, Chagrin Falls, Ohio, phosphatidylethanolamine and phosphatidylserine were from Supelco, Bellefonte, Pa., and ceramide was from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Production and purification of *equi* factor. *C. equi* strain C was maintained on sheep blood agar plates. Seed cultures were prepared in nutrient broth, whereas for production of *equi* factor a yeast diffusate casein hydrolysate medium (4) was employed. Each of three 2-liter Erlenmeyer flasks containing 530 ml of the latter medium was inoculated with 2 ml of seed culture. The cultures were grown in a 37°C shaking incubator at 150 rpm for 72 h. Under these conditions a turbidity of 9.2 to 12.3 optical density units developed, as measured by using a standard Zeiss spectrophotometer at 650 nm and a 10-mm light path. The culture supernatant usually contained 24 to 60 *equi* units per ml.

Unless otherwise noted, centrifugation was at 13,000 to 14,500 × *g*. After centrifugation for 40 min, the proteins of the supernatant were salted out with 56 g of ammonium sulfate for each 100 ml. After standing overnight in the cold, the precipitate was collected in 30 ml of 80% saturated ammonium sulfate. Subsequent steps were carried out in the cold. After centrifugation for 10 min, the precipitate was extracted with 120 ml of 30% saturated ammonium sulfate. After centrifugation for 15 min, the supernatant was discarded, and the precipitate was extracted with 45 ml of 20% saturated ammonium sulfate. The mixture was centrifuged for 30 min, the precipitate was discarded, and 11.5 g of ammonium sulfate was dissolved in the 50 ml of supernatant. The resulting precipitate was collected in 7 ml of 50% saturated ammonium sulfate.

After centrifugation at 12,000 × *g* for 10 min, the precipitate was transferred with 7 ml of 0.03 M sodium borate–0.1 M KCl (pH 8.2) to a cellophane bag and dialyzed against 1 liter of the same solution for 60 min. The dialyzed material was fractionated on a column (2.5 by 30 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals) equilibrated and eluted with 0.03 M sodium borate–0.1 M KCl (pH 8.2) at a presample flow rate of 60 ml/h. Each fraction was approximately 5 ml. The behavior of *equi* factor in a Sephacryl column is shown in Fig. 1. After the active fractions were combined, the volume was reduced by pervaporation to 24 ml, and the solution was dialyzed against 1.8 liters of distilled water for 3 h.

The solution was subjected to electrofocusing under the conditions described in the legend to Fig. 2, which provides data based on a small-scale trial of electrofocusing. The active fractions were pooled and dialyzed against 1.8 liters of distilled water for 24 h and then lyophilized. The lyophilized product was dissolved in 1.4 ml of distilled water and stored at −20°C. The recovery and specific activity at each stage of purification are shown in Table 1. In some trials the final recovery of activity was about twice that shown.

Behavior of *equi* factor in polyacrylamide gels. A sample of purified *equi* factor was allowed to stand for 2 h at 37°C with 1% sodium dodecyl sulfate–0.1% dithiothreitol in 10 mM sodium phosphate, pH 7.0. Approximately 52 *equi* units (0.083 U of absorbance at 280 nm) was subjected to electrophoresis by the method of Weber and Osborn (19). Staining revealed a single well-demarcated band (Fig. 3); the migration rate of this band compared with the migration rates of standard proteins (trypsin, ovalbumin, and bovine serum albumin) indicated a molecular weight of 74,000. The only other material visible on the gel was an area of residual ampholine just above the tracking dye.

The purified material was also examined by polyacrylamide gel electrophoresis under nondissociating conditions. Samples of 13.5 *equi* units each were subjected to electrophoresis for 75 min in 7% polyacrylamide gels at pH 7.2 with reversed electrodes and at a current of 2.5 mA/gel. The gel dimensions were 0.5 by 8.0 cm.

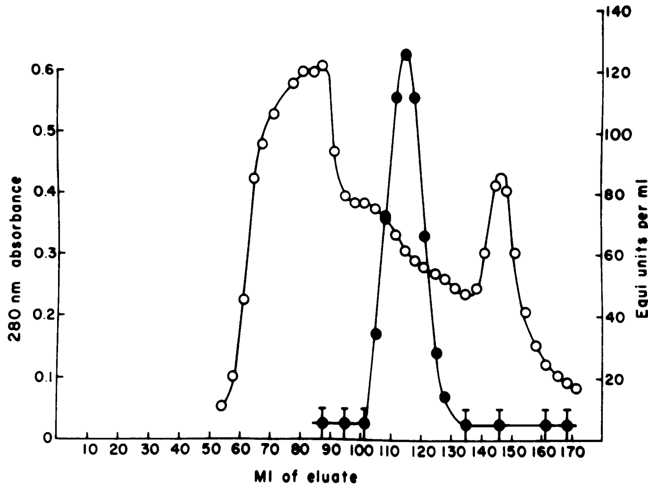


FIG. 1. *Distribution of equi activity (●) and absorbance at 280 nm (○) among fractions from Sephacryl S-200 column.*

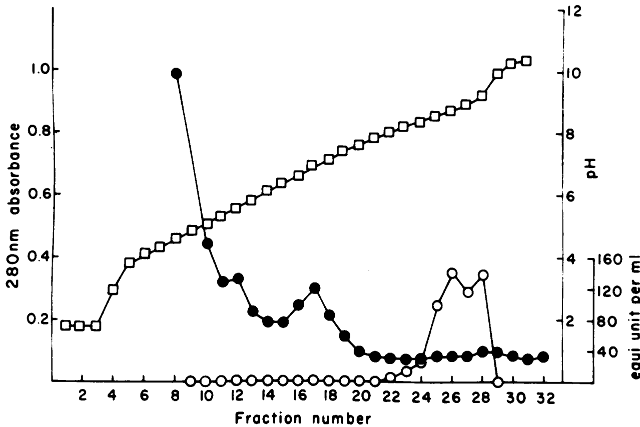


FIG. 2. *Isoelectric focusing (18) was carried out in a linear density gradient prepared from (i) a less dense solution consisting of 4 ml of 8% (wt/vol) ampholine (pH 3.5 to 10), dialyzed equi factor, and sufficient water to bring the volume to 55 ml, and (ii) a more dense solution containing 32 ml of water, 8.5 ml of 8% (wt/vol) ampholine of the same pH range, and 25 g of sucrose. Focusing was done at about 4°C for 42 h in a 110-ml electrophoresis column (LKB Instruments) with a final potential difference of 750 V. Fractions having a volume of 4 ml each were examined for absorbance at 280 nm (●), for pH (□), and for equi activity (○).*

Staining revealed a single broad band centered 4.5 mm from the origin. Tests of eluates of slices cut from an unstained gel showed a small amount of *equi* factor activity to be present at a locus coinciding with the location of the stained band.

Thermolability of *equi* factor. Heating a solution of *equi* factor in a boiling water bath for 1 to 2 min resulted in complete inactivation. Solutions diluted to contain 10 U/ml of buffer I and then heated to 60°C showed a partial loss of activity; the courses of inactivation were similar for crude and purified preparations (Table 2).

Requirement for divalent cations. A 0.7% (vol/vol) suspension of washed sheep erythrocytes was treated for 30 min at 37°C with PLD at a concentration of 7.5 U/ml. The mixture was centrifuged, and the erythrocytes were suspended to their original concentration in buffer I supplemented with 0.2% gelatin. To 1-ml portions of PLD-modified erythrocytes were added 0.5 ml of buffer I containing 5 U of *equi* factor and, beginning with 0.5 ml, decreasing volumes of $MgCl_2$ solution. After the final volumes were adjusted to 2 ml with buffer I, the tubes were placed at 37°C for 30 min and then in ice water for 15 min. Estimations of the hemoglobin in the supernatants after centrifugation gave the re-

TABLE 1. *Purification of equi protein*

Stage	Vol (ml)	Total activity (U)	Total A_{280} units[a]	Sp act (U/A_{280})	% Recovery of activity
Culture supernatant	1,445	72,000	152,000	0.47	
Ammonium sulfate precipitate	30	49,800	4,140	12	69
Ammonium sulfate-fractionated material	7	25,200	280	90	35
Pervaporated and dialyzed Sephacryl eluate	24	12,800	70	183	18
Electrofocused, dialyzed, and lyophilized product	1.4	2,890	48	602	4

[a] A_{280}, Absorbance at 280 nm.

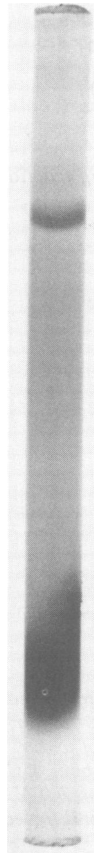


FIG. 3. *Polyacrylamide gel electrophoresis of purified equi factor. The origin was at the top, and the anode was at the bottom. See text.*

TABLE 2. *Thermolability of equi activity*

Min at 60°C	Activity of crude prepn (U/ml)	Activity of purified prepn (U/ml)
0	10	10.5
1	—	9.6
2	10	10
4	7.0	7.9
8	5.0	6.1
16	2.8	3.3

sults shown in Fig. 4, in which a requirement for Mg^{2+} is demonstrated. Substitution of $CaCl_2$ for $MgCl_2$ showed that Ca^{2+} was at least as effective as Mg^{2+}.

Demonstration of synergism in blood agar. The synergistic hemolytic effect described by Fraser (8) using cultures of *C. ovis* and *C. equi* could be duplicated by substituting for the microorganisms their respective growth products, PLD and *equi* factor (Fig. 5).

Properties of the synergistic hemolytic system and comparison of it with CAMP protein-induced hemolysis (5). As illustrated by the experiment demonstrating a requirement for divalent cations, sheep erythrocytes pretreated with PLD are modified so that they are susceptible to lysis by *equi* factor. However, when the order of treatment was reversed (that is, when sheep erythrocytes were first treated with *equi* factor, washed, and then exposed to PLD), no lysis occurred. We infer that normal sheep erythrocytes are not altered by *equi* factor.

When *equi* factor was titrated as described above, but without chilling, about three times as much *equi* factor was needed to produce lysis as was needed with chilling. Hence, a "hot-cold" effect (4) is demonstrable.

The CAMP reaction is a synergistic hemolytic system in which sheep erythrocytes modified by staphylococcal sphingomyelinase C are lysed, apparently nonenzymically, by a protein from *Streptococcus agalactiae* (5). The results of experiments designed to compare the PLD-*equi* factor system with the CAMP reaction are summarized in Table 3. The effect of staphylococcal delta toxin, which is thought to have a detergent-like action on membranes (15), was examined because it is known (11) to act synergistically with staphylococcal sphingomyelinase.

Liposome studies. Multilamellar liposomes prepared from sphingomyelin, cholesterol, and dicetyl phosphate and containing [^{14}C]glucose in the aqueous spaces were treated with PLD and *equi* factor alone and in combination to determine whether their combined effects on sheep erythrocytes could be duplicated in a simple artificial system (Table 4). All assays were done

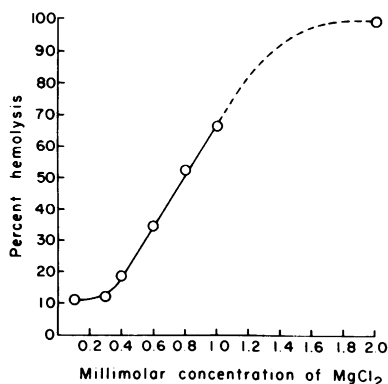


FIG. 4. *Effect of Mg^{2+} on activity of equi factor.*

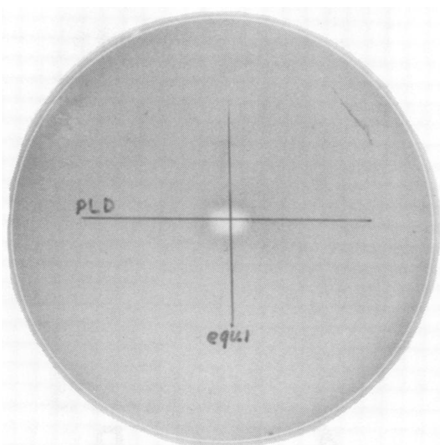


FIG. 5. *Synergistic hemolysis caused by C. ovis PLD and equi factor. A total of 65 U of C. ovis PLD contained in 5 μl was placed on the surface of a sheep blood agar plate in a single horizontal streak; 5 U of equi factor contained in 5 μl was placed in a single streak at right angles to the first streak. Incubation was at 37°C for 16 h and was followed by brief refrigeration.*

in duplicate. Spontaneous release of glucose was less than 5%. PLD at the concentration used released 26.7% of the trapped counts, whereas *equi* factor alone released 5.4%. Their combined effect (68.2%) was more than twice that of a simple additive effect. The basic feature of the synergistic hemolytic system was therefore reproduced when liposomes of appropriate composition were used instead of erythrocytes. One difference between the two systems is that PLD alone causes no leakage from erythrocytes.

[^{14}C]glucose-loaded liposomes were prepared by using ceramide phosphate as the phospholipid component. Such vesicles were less stable to the assay conditions than sphingomyelin liposomes, for they displayed approximately 35% spontaneous release of counts. Nevertheless, they were sensitive to concentration-dependent disruption when treated with small amounts of *equi* factor (Table 4). This effect was not obtained when boiled *equi* factor was used. Pretreatment of membrane sphingomyelin with PLD therefore appears to play a significant role in disruption by *equi* factor. Under the conditions used, binding of *equi* factor to liposomes with or without PLD pretreatment was not demonstrable.

Phospholipase activity of *equi* factor.

TABLE 3. *Ability of various agents to induce lysis during or after exposure of sheep erythrocytes to (i) C. ovis PLD and (ii) staphylococcal phospholipase C (sphingomyelinase C or beta-hemolysin)*

Agent tested[a]	Sheep erythrocytes treated with PLD (7 U/ml) for 30 min[b]	Sheep erythrocytes treated with phospholipase C (5 U/ml) for 30 min[b]
None	0	0
CAMP protein (100 U/ml)	0	+
equi factor (7 U/ml)	+	+
Cold (1°C for 15 min)	0	+
Staphylococcal delta toxin[c]	+	+
Ethylenediaminetetraacetate (25 mM)	0	+

[a] Tests were done at 37°C for 30 min except for the cold treatment. The diluent was buffer I containing 10 mM $MgCl_2$.

[b] 0, No hemolysis; +, hemolysis.

[c] Tested at 1 to 3 μg/ml (at these concentrations delta toxin alone is not hemolytic).

TABLE 4. *Release of trapped [^{14}C]glucose from liposomes by PLD and equi factor*

Phospholipid in liposomes	Treatment[a]	% of [^{14}C]-glucose released[b]
Sphingomyelin	PLD (29 U)	26.7
Sphingomyelin	*equi* factor (10 U)	5.4
Sphingomyelin	PLD (29 U) + *equi* factor (10 U)	68.2
Ceramide phosphate	*equi* factor (2.5 U)	29.0
Ceramide phosphate	*equi* factor (7.5 U)	42.7
Ceramide phosphate	*equi* factor (25 U)	97.0
Ceramide phosphate	*equi* factor (25 U; boiled)	3.4

[a] Liposome samples (20 μl) prepared as described in the text and containing approximately 16,000 cpm of [^{14}C]glucose were incubated at 37°C for 1 h with the agents indicated, in buffer I-10 mM Mg^{2+} in a final volume of 220 μl. Centrifugation at 17,300 × *g* for 90 min allowed recovery of the supernatant fluid and pellet for measurement of radioactivity.

[b] Values have been corrected for spontaneous release from untreated liposomes (3.53% for sphingomyelin liposomes and 34.6% for ceramide phosphate liposomes) by subtracting the spontaneous release values and normalizing to 100%.

After it was established that *equi* factor has a disruptive effect on synthetic membranes containing ceramide phosphate, it was of interest to determine whether alteration of this phospholipid is enzymatic. Incubation with *equi* factor of sonicated dispersions of pure ceramide phosphate, PLD-treated sphingomyelin, or liposomes prepared from these reagents, followed by thin-layer chromatography revealed small amounts of split products. However, when the nonionic detergent Triton X-100 (final concentration, 0.1%) was included in the reacton mixtures, enzymatic breakdown was observed. Samples (0.5 mg) of sheep sphingomyelin dissolved in chloroform containing the detergent were dried in reaction vessels. Dispersion by sonication of the dried lipid-detergent film in buffer I was followed by incubation at 37°C for 1 h with either 100 U of PLD or 40 U of *equi* factor or both in the presence of 10 mM Mg^{2+}. Reaction mixtures (300 µl) were sampled for estimation of inorganic phosphorus and then extracted for total lipid. Thin-layer chromatography on silica gel G was performed on duplicate samples in solvents containing chloroform, methanol, and water (65:25:4, vol/vol) (10) and chloroform, methanol, and NH_4OH (50:5:2, vol/vol) (aqueous layer removed before use) (16). Spots were visualized by spraying with 55% (vol/vol) H_2SO_4 followed by heating. Comparisons with reference standards in both systems revealed incomplete hydrolysis of sphingomyelin to ceramide regardless of the presence of PLD, although ceramide phosphate appeared only in those tubes in which PLD was included. Analysis showed the release of 102 nmol of inorganic phosphate (corresponding to 16.9% of the total sphingomyelin phosphate) only in those tubes containing both PLD and *equi* factor. PLD alone did not give rise to inorganic phosphate. These results suggested that *equi* factor is a phospholipase C which cleaves sphingomyelin to ceramide and phosphoryl choline and is also capable of hydrolyzing the phosphate group from ceramide phosphate. The latter activity was demonstrated by using pure ceramide phosphate (Fig. 6). Figure 6, lane A represents thin-layer chromatography of a lipid extract of 0.5 mg of sheep erythrocyte sphingomyelin treated with 100 U of PLD. Much of the sphingomyelin (spot 1) was converted to ceramide phosphate (spot 2). The incubation of purifed ceramide phosphate with *equi* factor (lane B) led to virtually complete conversion to ceramide (spot 3), indicating a phospholipase C activity. The migration of reference standards in this solvent system is shown in Fig. 7.

The sphingomyelinase activity was confirmed by incubating *equi* factor (10 µl of diluted sample plus 15 µl of buffer I containing 10 mM $MgCl_2$ and Triton X-100) with 5 µl of [*N-methyl*-^{14}C]-sphingomyelin (6.7 nmol) prepared as described previously (12); 1 U of *equi* factor hydrolyzed 11.6 nmol of sphingomyelin in 30 min at 37°C. In the absence of detergent only 3.5% of this activity was observed.

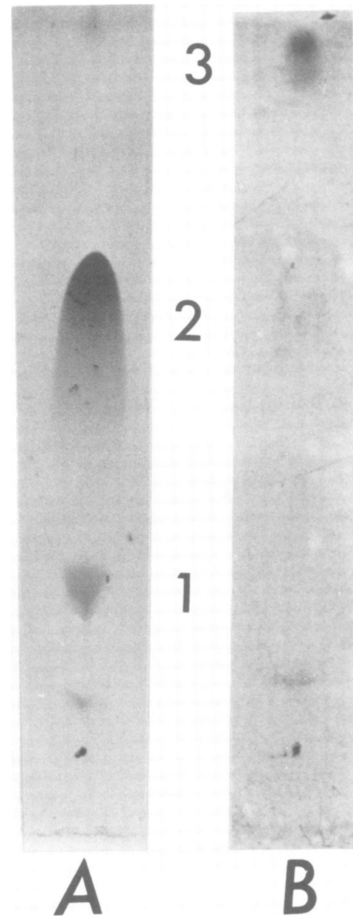


FIG. 6. Thin-layer chromatography demonstrating ceramide phosphate cleavage by equi factor. In lane A 0.5 mg of sheep erythrocyte sphingomyelin was dispersed into buffer I containing 10 mM Mg^{2+}, and 100 U of PLD (7 µl) was added (final volume, 300 µl). Incubation was at 37°C for 30 min and was followed by total lipid extraction and thin-layer chromatography in chloroform-methanol-water (65:25:4). Spots were visualized by spraying with 55% H_2SO_4 and heating. In lane B, ceramide phosphate (0.2 mg) was dispersed in buffer I containing 10 mM Mg^{2+} and 0.1% Triton X-100. Incubation was with 40 U (40 µl) of equi factor for 1 h at 37°C in a final volume of 300 µl. Extraction and thin-layer chromatography were as described above for lane A.

It was of interest to determine qualitatively whether *equi* factor was enzymatically active against other phospholipids, particularly those of mammalian erythrocyte membranes. Samples

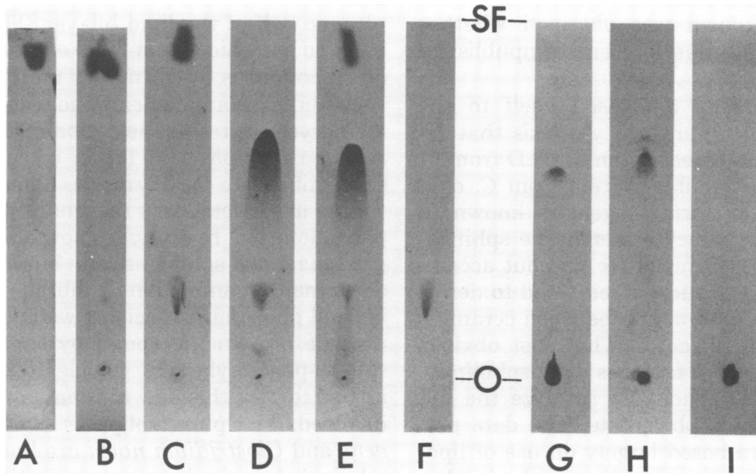


FIG. 7. *Thin-layer chromatography of the products of C. ovis PLD and equi factor hydrolysis. Sonicated dispersions of sphingomyelin (lanes B through E) and lecithin (lanes G and H) prepared as described in the text were incubated in the presence of 10 mM Mg^{2+} in buffer I for 1 h at 37°C with the agents indicated below in a volume of 300 μl. Triton X-100 (0.1%) was included in all but lanes B and G. After total lipid extraction, thin-layer chromatography on silica gel G was carried out by using chloroform-methanol-water (65:25:4) (lanes A through F) or chloroform-acetic acid (96:4) (lanes G through I). Spots were visualized by spraying with 55% H_2SO_4 and heating. Enzyme treatments were as follows: lane B, staphylococcal sphingomyelinase C; lane C, 40 U of equi factor; lane D, 100 U of PLD; lane E, 40 U of equi factor + 100 U of PLD; lane G, C. perfringens phospholipase C; lane H, 40 U of equi factor. Reference standards were included for ceramide (lane A), sphingomyelin (lane F), and lecithin (lane I). O, Origin; SF, solvent front.*

(0.5 mg) of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid were exposed to *equi* factor in the presence of detergent and Mg^{2+}; this was followed by lipid extraction and thin-layer chromatography exactly as described above for sphingomyelin, except that the chromatographic solvents used were chloroform-methanol-water (65:25:4, vol/vol) (10) and chloroform-acetic acid (96:4, vol/vol) (13). Each of the phospholipids was hydrolyzed; diglyceride was formed, and, in the case of phosphatidic acid, 510 nmol of inorganic phosphate was released. *equi* factor therefore is a phospholipase C and shows quite broad substrate specificity, provided Triton X-100 is used as an activator.

The activities of the enzyme on sphingomyelin and phosphatidylcholine are shown in the thin-layer chromatograms of Fig. 7, where they are compared with the activities of three well-characterized phospholipases. Figure 7, lanes B through E, represent the enzymatic alteration of sphingomyelin. Staphylococcal sphingomyelinase (β-hemolysin) is a phospholipase which released ceramide from sphingomyelin (lane B). *equi* factor (lanes C and E) produced the same product as the staphylococcal enzyme, whereas PLD (lanes D and E) hydrolyzed sphingomyelin to ceramide phosphate, which characteristically runs a diffuse cone-shaped spot. The hydrolysis of phosphatidylcholine by *equi* factor (lane H) resulted in the same product (diacylglycerol) as the hydrolysis by *C. perfringens* phospholipase C (lane G).

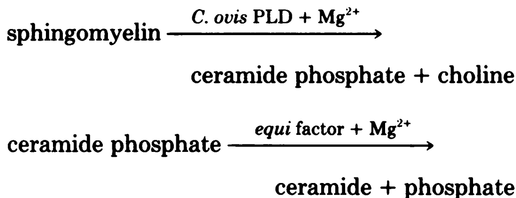
DISCUSSION

Although a considerable number of synergistic hemolytic systems are known, the biochemical mechanisms underlying them usually are not well understood. Examples of synergistic hemolysis are lysis of human erythrocytes resulting (i) from the combined action of either phospholipase C from *Bacillus cereus* or phospholipase A_2 from pancreas and a sublytic concentration of sodium deoxycholate (14) and (ii) from the combined action of cobra venom phospholipase A_2, bovine plasma albumin, and Ca^{2+} (9).

Another example of synergistic hemolysis is the CAMP reaction, in which staphylococcal sphingomyelinase C and a protein from *S. agalactiae* produce hemolysis of sheep erythrocytes, although neither agent alone is lytic at 37°C. In this instance the staphylococcal enzyme converts membrane sphingomyelin to ceramide (*N*-acylsphingosine) without accompanying lysis; however, when the streptococcal protein is present, hemolysis ensues. Lysis depends upon an apparently nonenzymatic reaction between the streptococcal protein and in situ ceramide (5). In a synthetic membrane system, no evidence of

enzymatic breakdown was obtained whether neutral detergent was present (unpublished data) or not (5).

The present study addressed itself to the mechanism of sheep erythrocyte lysis that results from the combined action of PLD from *C. ovis* and an extracellular protein from *C. equi* (*equi* factor). The former agent is known to convert sheep erythrocyte membrane sphingomyelin to ceramide phosphate without accompanying lysis (17). Hence, it remained to define the nature of the interaction between ceramide phosphate and *equi* factor. The most obvious possibility is that *equi* factor is a phospholipase C which has the capacity to catalyze the hydrolysis of ceramide phosphate. The data presented, which are based largely on use of liposomes as models, are consistent with this hypothesis and suggest that the following stepwise degradation of membrane sphingomyelin may be the basis for cell lysis:



$$\text{sphingomyelin} \xrightarrow{C.\ ovis\ \text{PLD} + \text{Mg}^{2+}} \text{ceramide phosphate} + \text{choline}$$

$$\text{ceramide phosphate} \xrightarrow{equi\ \text{factor} + \text{Mg}^{2+}} \text{ceramide} + \text{phosphate}$$

In addition to the two enzymes and Mg^{2+}, a fourth condition (such as chilling or addition of CAMP protein), which is capable of causing dislocation of in situ ceramide, must be imposed on the altered cells in order for maximum lysis to be achieved.

The finding that little hydrolysis of ceramide phosphate could be detected in the absence of nonionic detergent suggests that extensive degradation of this phospholipid may not be required for lysis. This is consistent with the observation that erythrocyte ghosts treated with *equi* factor gave significant amounts of split products on thin-layer chromatography of lipid extracts only when detergent was present during hydrolysis (unpublished data).

The phospholipase C of *C. equi* (*equi* factor) was found unexpectedly to split not only ceramide phosphate but also sphingomyelin when this phospholipid was a constituent of liposomes and when a neutral detergent was present. In contrast, the *C. equi* enzyme has no demonstrable effect on intact sheep erythrocytes, presumably because the sphingomyelin of the membrane of native erythrocytes is not accessible to the enzyme. The two other major phospholipids, phosphatidylethanolamine and phosphatidylserine, are presumably even less accessible because they are located principally in the cytoplasmic half of the lipid bilayer. The inaccessibility to enzymic action of an externally located phospholipid is by no means unprecedented, a well-studied example being the resistance of human erythrocytes to the action of *Bacillus cereus* phospholipase C (14) in contrast to their susceptibility to *C. perfringens* phospholipase C.

Also unexpected was the finding that *C. equi* phospholipase hydrolyzed not only ceramide phosphate and sphingomyelin but also all of the other major mammalian membrane phospholipids and phosphatidic acid as well. Because only a single protein was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we incline toward the view that only one enzyme is involved. The phospholipases C of *C. perfringens* and *Clostridium novyi* are known to have relatively broad substrate specificities (2). Nonetheless, it may be that our preparation contained more than one enzyme.

It is possible that the effect of *equi* factor on erythrocyte membranes is more like that of CAMP protein than that of an enzyme. However, although *equi* factor can substitute for CAMP protein in the CAMP reaction, CAMP protein cannot substitute for *equi* factor in the system under consideration here (Table 3). In addition, the hot-cold effect can be interpreted as favoring the idea that ceramide is formed by virtue of enzymic action of *equi* factor, by analogy with the action of staphylococcal sphingomyelinase. It appears that further work is needed before a firm conclusion can be drawn as to the manner in which *equi* factor acts on altered erythrocyte membranes.

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