Phospholipase A_2 as a Probe of Phospholipid Distribution in Erythrocyte Membranes. Factors Influencing the Apparent Specificity of the Reaction†

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ABSTRACT: The action of snake venom phospholipases A_2 on intact human erythrocytes was investigated in detail. The basic phospholipase from *Agkistrodon halys blomhofii* was found to induce both hydrolysis of membrane phospholipids and total cell hemolysis under certain experimental conditions. The hydrolytic action of the basic enzyme was found to consist of two sequential events: (a) hydrolysis of 70% of the total cell phosphatidylcholine without any evident hemolysis; and (b) complete hydrolysis of the remaining phosphatidylcholine, followed closely by extensive phosphatidylethanolamine hydrolysis and finally with onset of hemolysis, attack on the phosphatidylserine. At pH 7.4 and 10 m*M* Ca^{2+} only stage (a) occurred. However, a slight elevation of the pH of incubation to pH 8.0 and/or inclusion of 40 m*M* Ca^{2+} in the reaction mixture caused both events (a) and (b) to occur. The addition of glucose limited the action of the enzyme to stage (a) under any reaction conditions. An investigation into energy levels of the erythrocyte showed that enzymically induced hemolysis occurred under conditions where the intracellular ATP levels were lowered. Data are presented which suggest that stage (b) is mediated by an influx of Ca^{2+} into the cell when the levels of ATP are low. Interestingly the phospholipase from *Naja naja* venom (Pakistan) yielded results similar to those observed with the basic enzyme from *Agkistrodon* venom. However, the enzyme from *Crotalus adamanteus* and the acidic enzyme also present in the *Agkistrodon* venom produced only slight hydrolysis or hemolysis under any of the conditions studied. Other species of erythrocytes, e.g., guinea pig, monkey, pig, and rat, were tested but only those from guinea pig behaved similarly to the human cells. Pig, monkey, and rat erythrocytes underwent very limited hydrolysis and hemolysis. It is evident that the use of these phospholipases to probe the localization of phospholipids in erythrocyte membranes must be approached with caution. Certain facets of this problem are discussed.

Recent reports have appeared in which the susceptibility of mammalian erythrocyte membrane phospholipids to hydrolysis by a variety of phospholipases have been noted (Zwaal et al., 1973). The results of these studies have been interpreted to support the proposal that phospholipid classes are asymmetrically distributed in the membrane with most of the choline containing lipids residing in the extracellular portion of the lipid bilayer, while the ethanolamine and serine containing lipids are supposed to reside in the intracellular portion of the bilayer. These conclusions, if valid, have important implications with regard to the structure of the erythrocyte membrane.

We were attracted to this problem by the observations that only certain phospholipases A_2 hydrolyze phospholipids in intact erythrocytes. These include enzymes isolated from the venoms of certain snakes, *Naja naja* (cobra), *Enhydrina schistosa* (sea snake), and *Haemachatus haemachatus* (ringhals cobra), and bee venom (Ibrahim and Thompson, 1965; Condrea et al., 1970; Gul and Smith, 1972; Zwaal et al., 1973). The possible complex nature of this system is indicated by the observation of Gul and Smith (1972, 1974) that hemolysis occurs only in the presence of albumin, and the report that avian red cells are more prone to hemolysis by phospholipases when they are depleted of ATP (Gazitt et al., 1975). Furthermore, almost all studies using phospholipase A_2 as a membrane probe have employed human erythrocytes, with little attention directed toward other species, or the effects of pH and other conditions on hydrolysis. Finally, we note a report that a basic phospholipase A_2 from *Agkistrodon halys blomhofii* was considered to be directly hemolytic (Sarkar and Devi, 1968).

In view of the potential importance of the results published on phospholipid localization in membranes and the potential increased use of phospholipase A_2 to probe "sidedness" of phospholipids in membranes, we felt it was important to reexamine the action of phospholipase A_2 from several sources under different conditions on different erythrocyte species. The underlying theme to this study was to establish whether the published results are general phenomena or merely represent an isolated situation which is peculiar to a specific enzyme, to the use of human erythrocytes, and/or to the restricted conditions employed. As we show in this paper, the results obtained with phospholipase A_2 depend on a large number of factors, and suggest that caution should be exercised in the application of this enzyme to elucidate membrane structure.

Experimental Section

Materials

Dihexanoylphosphatidylcholine was prepared by the method of Cubero-Robles and Van Den Berg (1969). *Crotalus adamanteus* phospholipase A_2 was prepared by the method of Wells (1975). Blood was collected in heparinized

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Vacutainers (Beckton-Dickinson) from normal human volunteers. The cells were recovered by centrifugation at 4° for 20 min at 1200*g* in a Sorvall RC2-B. The plasma and buffy coat were removed and the cells carefully washed three times at a 50% hematocrit in isotonic NaCl. These washed cells were used either immediately or stored overnight at 4° in isotonic saline at 50% hematocrit.

Agkistrodon halys blomhoffi and *Naja naja* (Pakistan) venom were obtained from the Miami Serpentarium (Miami, Fla.). *N*-2 Hydroxyethylpiperazine-*N'*-ethanesulfonic acid (Hepes),[1] tris(hydroxymethyl)aminomethane (Tris), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) were purchased from Pierce Chemical Co. (Rockford, Ill.). DEAE- and CM-cellulose were Whatman products (Reeve Angel, Clifton, N.J.). ^{45}Ca was purchased from New England Nuclear (Gardena, Calif.).

Human erythrocytes were separated according to density by the method of Murphy (1973). The cells distributed in the gradient were divided into three fractions: the top 10%, the middle 80%, and the bottom 10%. The separated cells were washed three times with isotonic saline, and resuspended at 50% hematocrit in the same media.

Methods

Enzyme Assay. Phospholipase A_2 activity was measured by the method of Wells (1972) using 2 m*M* dihexanoylphosphatidylcholine and 1 m*M* Ca^{2+}. Plastic reaction vessels (Radiometer) were used since the *Agkistrodon* and *Naja* enzymes adhere tightly to glass vessels. Extensive washing is required between assays in order to remove enzyme which adheres to the electrodes. More efficient removal of enzyme is achieved by a brief exposure of the reaction vessel and electrodes to a solution of pepsin (10 μg/ml) in 0.05 *N* HCl (F. Kezdy, personal communication). Protein was estimated from the absorbance at 280 nm, assuming $E_{280}(1\%)$ 10.0.

Phospholipase A_2 Purification. Two sources of this enzyme, i.e., *Agkistrodon halys blomhofii* venom and *Naja naja* venom, were used in this investigation and were purified by the procedures outlined below.

A. halys blomhofii Enzymes. This venom contains two phospholipases A_2, labeled acidic and basic (Kawauchi et al., 1971), which were purified by separate procedures.

Basic Phospholipase A_2. This enzyme was purified by a modification of the procedure of Kawauchi et al. (1971). All operations were conducted at 4°. Two grams of venom was dissolved in 20 ml of 0.02 *M* Tes (pH 7.0) and insoluble material removed by centrifugation at 10000*g* in a Sorvall RC2-B centrifuge. The supernatant solution was applied to a DEAE-cellulose column (2.8 × 40 cm) which had been packed and equilibrated in 0.02 *M* Tes (pH 7.0). After application of the enzyme solution, the column was eluted with 250 ml of the equilibrating buffer and then with a linear gradient formed from 1 l. of 0.02 *M* Tes (pH 7.0) and 1 l. of 0.3 *M* NaCl in the same buffer. A flow rate of 60 ml/hr was maintained with a peristaltic pump and 6.5-ml fractions were collected. Three peaks of enzyme activity were found. The first (2% of the applied activity) appeared in the initial wash and was discarded. The second peak (35% of the applied activity) was eluted at a conductivity of 2.5–4.5 mmhos (~0.05 *M* NaCl) and was the basic enzyme. The third peak (65% of the applied activity) was eluted at a conductivity of 7.5–8.5 mmhos (~0.11 *M* NaCl) and was the acidic enzyme.

The fractions containing the basic enzyme were combined and dialyzed against two changes of 0.05 *M* sodium acetate buffer (pH 5.6). This enzyme solution was then applied to a CM-cellulose column (1.4 × 23 cm) packed in the same buffer. After washing the column with 70 ml of equilibrating buffer, the column was eluted with a linear gradient formed from 400 ml of 0.05 *M* acetate buffer (pH 5.6) and 400 ml of 0.3 *M* NaCl in the same buffer. The column was run at 45 ml/hr and 4.5-ml fractions were collected. A single peak of enzyme activity (75% of applied activity) was observed, and eluted at a conductivity of 8.0–9.0 mmhos (~0.09 *M* NaCl). The overall yield was 17.3 mg and represented 26.6% of the original activity of the crude venom with a purification of 28-fold. In experiments with erythrocytes, the basic enzyme was dialyzed against distilled water, lyophilized, and dissolved in isotonic NaCl.

Acidic Phospholipase A. Initially another procedure was used for the purification of the phospholipases A_2. This procedure gave a very low yield of the basic enzyme, but represents the highest purity preparation of the acidic enzyme. Venom (1.75 g) was dissolved in 10 ml of 0.01 *M* Tes (pH 7.5), 0.1 *M* KCl, and 1 m*M* benzamidine (to inhibit proteolysis). The mixture was fractionated on a Sephadex G-75 column (2.6 × 90 cm) at room temperature. The flow rate was 12.6 ml/hr and 2.1-ml fractions were collected. The basic and acidic enzymes elute at the same point. The fractions containing enzymatic activity were pooled and dialyzed against distilled water, and lyophilized. The protein was dissolved in 25 ml of 0.05 *M* Tris (pH 7.5) and applied to a DEAE-cellulose column (1.4 × 26 cm) packed and equilibrated in the same solvent. The column was eluted (room temperature) with 100 ml of the same solvent and then with a linear gradient formed from 500 ml of 0.05 *M* Tris (pH 7.5) and 500 ml of 0.3 *M* NaCl in the same buffer. The flow rate was 45 ml/hr and 3.5-ml fractions were collected. A very small amount of the basic enzyme was eluted at a conductivity of 4 mmhos (~0.04 *M* NaCl) and the acidic enzyme at 16 mmhos (~0.18 *M* NaCl). This preparation of the acidic enzyme represented 32% of the original activity and a 6.5-fold purification.

N. naja Phospholipase A. This enzyme was partially purified by the method of Cremona and Kearney (1964) as modified by Verkleij et al. (1973). Further purification was achieved on DEAE-cellulose as follows. The active fractions obtained from the Sephadex column were combined, dialyzed against distilled water, and lyophilized. The material was dissolved in a minimum volume of 0.01 *M* Tes (pH 7.6) and applied to a DEAE-cellulose column (1.4 × 24 cm) packed and equilibrated in the same buffer. The column was eluted with 60 ml of this buffer and then with a linear gradient formed from 300 ml of 0.01 *M* Tes (pH 7.6) and 300 ml of 0.3 *M* NaCl in the same buffer. The flow rate was 60 ml/hr and 3.5-ml fractions were collected. The bulk of the activity (84% of applied activity) was eluted at a conductivity of 6–7 mmhos (~0.09 *M* NaCl). The fractions of constant specific activity were combined. This DEAE column chromatography resulted in a tenfold purification. The overall yield of activity was 77% with a purification of 62-fold.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (pH adjusted with NaOH); Tris, tris(hydroxymethyl)aminoethane (pH adjusted with HCl); Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH adjusted with NaOH); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PLA_2, phospholipase A_2; EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid.

Disc gel electrophoresis was conducted by the method of Ornstein (1964) and Davis (1964) either at pH 9.5 (Tris buffer) or pH 4.3 (β-alanine–acetic acid buffer).

Hemolysis Measurement. Unless noted all incubations were carried out at 37° using a 5% cell suspension. Hemolysis was measured in three ways: (1) 1 ml of the cell suspension was centrifuged at 1500*g* and the absorbance of the supernatant solution measured at 540 nm; (2) 25 μl of the cell suspension was added to 1 ml of the incubation media. After mixing, the absorbance at 650 nm was measured immediately; or (3) 50 μl of the cell suspension was diluted with 1 ml of the incubation media and centrifuged at 1500*g* and the absorbance of the supernatant solution measured at 430 nm. In all cases total hemolysis was measured by suspending the cells in water.

Measurement of Phospholipid Hydrolysis. A 5% cell suspension was incubated with phospholipase A_2 at 37° for various times and under different conditions; 2 ml of the incubation mixture was mixed with 0.3 ml of 0.5 *M* EDTA (pH 7.7) to stop enzymatic activity. The mixture was extracted by a modification of the method of Bligh and Dyer (1959) in which one-half the methanol was replaced by 2-butanol (Dittmer and Wells, 1969). Thus 2.8 ml of methanol and 2.8 ml of 2-butanol were added, followed by 5.6 ml of chloroform and 2.8 ml of water. The biphasic system was mixed thoroughly by hand, and the phases were separated by centrifugation in a clinical centrifuge. After removing the lower phase, the upper phase was reextracted with 2 ml of chloroform. The combined chloroform phases were taken to dryness under nitrogen and dissolved in chloroform–methanol 1:1 (v/v). An aliquot was taken for phosphorus analysis and the remainder fractionated by thin-layer chromatography. Ninety percent of the sample was applied to one corner of a silica gel H plate (0.25 × 20 × 20 cm) prepared using a slurry of 40 g of silica gel H in 110 ml of 1 m*M* Na_2CO_3. The remaining 10% of the sample was applied to a corner not wet by solvent, as a control for the amount of phosphorus applied to the plate and as a check on recovery. The plate was developed in the two-dimensional system of Broekhuyse (1969). After development the plates were sprayed with ninhydrin (0.33% ninhydrin in 2-propanol–acetic acid–pyridine–H_2O 90:16:4:10 (v/v)) and Zinzade reagent (Dittmer and Lester, 1964). The spots were outlined and approximately equal areas of the plate scraped into 18 × 180 mm tubes. Equal areas were used so that blanks would be comparable. After addition of two drops of 1% ammonium molybdate and 0.6 ml of perchloric acid, the samples were processed for phosphorus determination (Dittmer and Wells, 1969) in the presence of the silica gel. Before measuring the absorbance at 830 nm, the silica gel was removed by centrifugation at 1500*g*. Total recovery of applied phosphorus varied from 80 to 95%. Phospholipid composition is expressed in terms of percent of total phospholipid using sphingomyelin as an internal standard, since there was no detectable degradation of this lipid under any of the conditions employed here.

ATP Assay. A 300-μl aliquot of an erythrocyte suspension (5% hematocrit) was mixed with 0.5 ml of 11.2% perchloric acid at 4°, and the precipitate was removed by centrifugation, then 0.6 ml of the supernatant was adjusted to pH 6.0 with 3.5 *M* K_2CO_3 in 0.25 *M* triethanolamine. The resulting insoluble perchlorate salts were removed by centrifugation and the supernatant was frozen until analyzed. ATP was assayed fluorometrically by the method of Williamson and Corkey (1969).

Table I: Effect of pH on Phospholipase A_2 Induced Hemolysis of Human Erythrocytes.[a]

pH	% Hemolysis	
	With Enzyme	Without Enzyme
6.5	4.5	2.0
7.0	3.5	2.0
7.5	31.0	2.5
8.0	67.5	3.0
8.7	83.5	7.0

[a] A 5% hematocrit of freshly prepared cells was incubated at 37° in 20 m*M* Hepes, 30 m*M* $CaCl_2$, and NaCl (to make the solution isotonic) with 6.7 μg/ml of *A. halys blomhofii* basic enzyme for 2 hr. Hemolysis was measured from the absorbance at 540 nm of the supernatant solution after removal of the cells by centrifugation (subject J.M.)

Calcium Uptake Measurement. Erythrocytes (10% hematocrit) were incubated in a media containing $^{45}Ca^{2+}$. At various times 0.2-ml aliquots were layered onto 1 ml of dibutyl phthalate. The tubes were centrifuged at 8000*g* for 40 sec in a Brinkman Microfuge 3200 at room temperature. The erythrocytes pack below the phthalate layer, while the extracellular fluid remains on top of the phthalate layer. The aqueous layer was removed by suction and the top of the phthalate layer washed 10–15 times with water. Finally the phthalate layer was removed and the walls of the tube wiped carefully with tissue paper. The packed cells were suspended in 0.2 ml of water and 50 μl of the resulting hemolyzate was mixed and counted in 15 ml of Aquasol (New England Nuclear) using a Beckman scintillation counter. In order to determine the specific activity of the $^{45}Ca^{2+}$ in the incubation medium, 50 μl was counted directly without cell separation.

Cation Levels. K^+ and Mg^{2+} loss were determined after centrifugation of the cells and assaying the supernatant for K^+ at 766 nm and Mg^{2+} at 285 nm on a Beckman Model 440 atomic absorption spectrophotometer.

Results

Purity of Enzymes. The basic phospholipase A_2 from *A. halys blomhofii* showed a single band on polyacrylamide gel electrophoresis at pH 9.5 and 4.3. The acidic enzyme from this venom was judged to be approximately 90% pure on the basis of electrophoresis. The enzyme from *N. naja* venom appeared to be about 95% pure, although the existence of isoenzymes with slightly different isoelectric points (Salach et al., 1971) leads to broadening of the band. In our hands the *N. naja* enzyme prepared according to Verkleij et al. (1973) was not pure as judged by polyacrylamide gel electrophoresis and the fact that further purification was achieved on DEAE-cellulose.

Hemolysis Studies. There was some variability in the response of cells from various subjects to the treatments described below. In all cases in which a set of conditions are compared, the studies were conducted using the same preparation of cells. Preliminary experiments were conducted to characterize the pH and Ca^{2+} dependence of the basic phospholipase A_2 induced hemolysis of red cells. In this regard a number of buffers were examined with the object of finding conditions in which hemolysis of controls could be minimized. This was considered important in order to eliminate the possibility that hemolysis was induced by the action of the enzyme on hemolyzed cells produced during in-

Table II: Effect of Various Conditions on Time for 50% Hemolysis ($t_{1/2}$) of Human Erythrocytes Induced by the Basic Enzyme from *A. halys blomhofii.*[a]

I. Effect of Enzyme Concentration[b,f]		
	$t_{1/2}$ (min)	
μg of PLA_2/ml	Subject M.L.[c]	Subject M.H.[d]
6.7	91	97
16.7		77
22.0	75	
33.0		61
66.0	72	

II. Effect of Preincubation Time[b,g]	
Preincubation time (min)	$t_{1/2}$ (min)
0	90
30	72
60	54
120	51

III. Effect of $[Ca^{2+}]$ during Preincubation[e,h]	
$[Ca^{2+}]$ during Preincubation (m*M*)	$t_{1/2}$ (min)
0	95
10	97
40	95

IV. Effect of Storage[i]	
Cells	$t_{1/2}$ (min)
Fresh	110
Stored	93

[a] In each of the experiments, human erythrocytes, at 5% hematocrit, were incubated at 37° in a solution containing 20 m*M* Hepes buffer (pH 8.0), $CaCl_2$, and sufficient NaCl to make the solution isotonic. [b] Cells stored 24 hr at 4° in 0.155 *M* NaCl before use. [c] Preincubated for 1 hr at 37° in 40 m*M* Ca^{2+} before addition of enzyme. $t_{1/2}$ measured after enzyme addition. [d] Not preincubated. [e] Freshly prepared cells. [f] All incubation mixtures contained 40 m*M* Ca^{2+}. [g] Cells were preincubated at 37° for varying times before addition of 6.7 μg/ml of PLA_2. $t_{1/2}$ is measured after addition of enzyme (subject J.M.). [h] Cells were preincubated at 37° with varying concentration of Ca^{2+} for 1 hr then the cells were collected and resuspended in media containing 40 m*M* Ca^{2+} and 6.7 μg of PLA_2/ml. $t_{1/2}$ is measured after addition of enzyme (subject T.A.). [i] Cells were used immediately after collection or after storage at 4° for 24 hr in 0.155 *M* NaCl. Incubation media contained 40 m*M* Ca^{2+} and 6.7 μg of PLA_2/ml (subject T.A.).

Table III: Effect of Various Sugars on the Hemolysis of Human Erythrocytes Induced by the Basic Phospholipase A_2 from *A. halys blomhofii.*[a]

1. Incubation with 5.3 m*M* Sugars	
Sugar	$t_{1/2}$ (min)
None	97[b]
2-Deoxyglucose	95[b]
Galactose	104[c]
Mannitol	97[c]
Sucrose	97[b]
Glucose	240[b]
Mannose	240[b]

2. Effect of Glucose Concentration	
Concn	$t_{1/2}$ (min)
0	90[b]
36 μ*M*	90[b]
62 μ*M*	125[b]
124 μ*M*	190[b]
620 μ*M*	225[b]
5.3 m*M*	240[b]

[a] A 5% hematocrit of freshly prepared cells was incubated at pH 8.0 and 40 m*M* Ca^{2+} with 6.7 μg/ml of enzyme and the sugars indicated. [b] Subject S.D. [c] Subject J.Z.

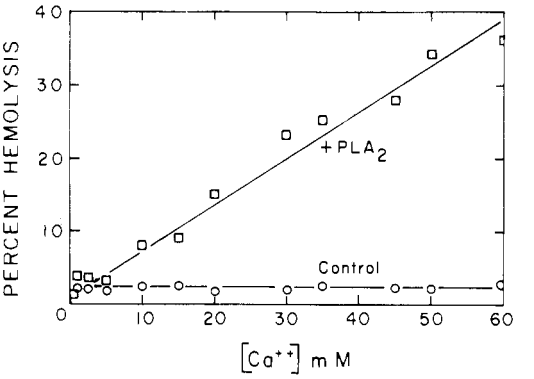

FIGURE 1: The effect of calcium concentration on the phospholipase A_2 induced hemolysis of human erythrocytes. A 5% suspension of cells (freshly prepared, subject T.A.) was incubated at 37° in 20 m*M* Hepes buffer (pH 8.0) containing the indicated amount of $CaCl_2$ and sufficient NaCl to make the solution isotonic, and 6.7 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii.* The extent of hemolysis was measured after 90 min.

cubation. It was found that 20 m*M* Hepes buffer made isotonic with various mixtures of $CaCl_2$ and NaCl gave less than 3% hemolysis in the pH range 6.5–8.0 during the time of the experiment. Therefore all subsequent experiments were conducted with this buffer. The extent of hemolysis induced by the basic enzyme depended both on pH and Ca^{2+} concentration. Some of these results are summarized in Table I, where the effect of pH at 30 m*M* Ca^{2+} is noted, and in Figure 1 where the effect of Ca^{2+} concentration at pH 8.0 is shown.

Other properties of the hemolytic process are presented in Table II. The salient points are as follows: (1) the time required for 50% hemolysis ($t_{1/2}$) depended to some extent on the concentration of the enzyme used, although this effect showed saturation characteristics; (2) cells stored overnight at 4° were more susceptible to hemolysis than freshly prepared cells; (3) incubation of the cells at 37° before adding the enzyme increased their susceptibility to hemolysis; (4) in contrast to the effect of Ca^{2+} on hemolysis, the concentration of Ca^{2+} seemed to have no effect on increased sensitivity of the cells caused by preincubation; (5) cells which had been preincubated still showed a saturation effect with regard to enzyme concentration.

In light of the effect of preincubation on susceptibility to hemolysis, it seemed possible that depletion of erythrocyte energy sources might be involved in increased sensitivity to phospholipase A_2 induced hemolysis. This possibility was investigated by adding various carbohydrates to the incubation medium, and the results are shown in Table III. It is apparent that the metabolizable sugars, glucose and mannose, afford considerable protection against hemolysis, whereas the nonmetabolizable sugars, galactose, 2-deoxyglucose, mannitol, and sucrose, have no effect. Further, as shown in Table III, considerable protection is afforded at glucose concentrations as low as 124 μ*M*. In view of these results, it did not seem likely that the glucose effect could be ascribed to a nonspecific effect of polyhydric compounds.

The relationship between the glucose effect, phospholipase A_2 induced hemolysis, and erythrocyte ATP levels was investigated and the results are presented in Table IV. The

Table IV: ATP Levels of Human Erythrocytes as Influenced by Incubation Conditions.[a]

	5.3 m*M* Glucose				No Glucose			
	With PLA_2		Without PLA_2		With PLA_2		Without PLA_2	
Time[b] (min)	ATP	% Hemolysis	ATP	% Hemolysis	ATP	% Hemolysis	ATP	% Hemolysis
				I. pH 8.0, 40 m*M* Ca^{2+} (subject W.S.)				
0	1.06	0	1.06	0	1.06	0	1.06	0
30	1.00	0			0.70	0	0.69	0
60	1.06	0	1.06	0	0.48	18		
90					0.38	78	0.52	0
120	0.93	0			0.23	87		
180	0.87	20	0.95	0	0.13	95	0.28	10
				II. pH 7.4, 10 m*M* Ca^{2+} (subject M.L.)				
0	1.20	0	1.20	0	1.20	0	1.20	0
60	1.08	0			0.78	0	0.84	
120	0.94	0	1.02	0	0.72	0	0.80	0
180			0.96	0	0.66	0	0.67	0
240	0.91	0			0.58	0		
300	0.96	0	0.90	0	0.49	0	0.54	0

[a] A 5% hematocrit of cells was incubated in various combinations with 6.7 μg of PLA_2 and/or 5.3 m*M* glucose, either at pH 8.0 or pH 7.4. ATP is expressed as μmol/ml of packed cells. [b] Cells were preincubated for 1 hr before addition of enzyme. There was no change in ATP levels during this preincubation. Time is after addition of enzyme.

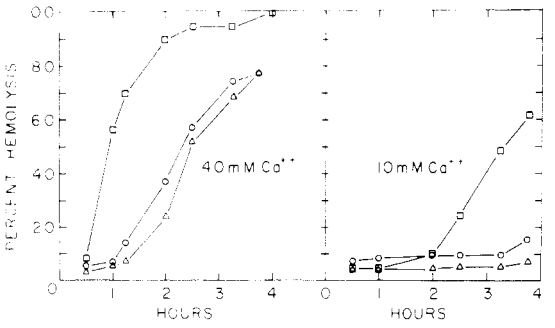

FIGURE 2: The effect of the pH of the preincubation solution on the subsequent susceptibility of human erythrocytes to hemolysis by phospholipase A_2. In all cases a 5% suspension of cells (freshly collected, subject J.Z.) was incubated for 1 hr at 37° in the absence of enzyme. The cells were collected, washed, and resuspended in the incubation media, and 6.7 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii* was added. The cells were further incubated at 37° and the extent of hemolysis was measured at the indicated times. Zero time refers to the point where enzyme was added. All solutions contained 20 m*M* Hepes buffer at the indicated pH, $CaCl_2$, and sufficient NaCl to make the solution isotonic. (Left-hand panel) All solutions contained 40 m*M* Ca^{2+}. (Right-hand panel) All solutions contained 10 m*M* Ca^{2+}. (□—□) Preincubation at pH 8.0, incubation with enzyme at pH 8.0; (○—○) preincubation at pH 7.4, incubation with enzyme at pH 7.4; (△—△) preincubation at pH 8.0, incubation with enzyme at pH 7.4.

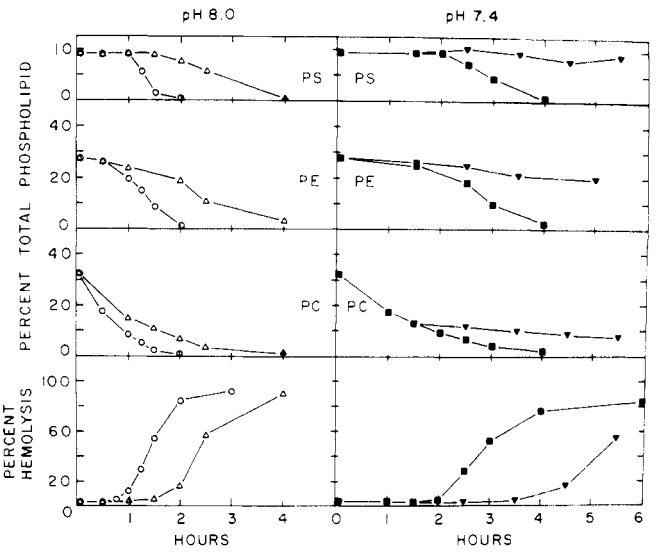

FIGURE 3: Effect of calcium concentration and pH on the rate of hydrolysis of human erythrocyte membrane phospholipids by the basic phospholipase A_2 from *A. halys blomhofii*. A 5% suspension of cells (stored 24 hr at 4°) was incubated at 37° in 20 m*M* Hepes buffer, $CaCl_2$, sufficient NaCl to make the solution isotonic, and 6.7 μg/ml of phospholipase A_2. At the indicated times, samples were taken for phospholipid analysis (see text for details). (○—○) pH 8.0, 40 m*M* $CaCl_2$ (subject T.A.); (△—△) pH 8.0, 10 m*M* $CaCl_2$ (subject T.A.); (■—■) pH 7.4, 40 m*M* $CaCl_2$ (subject M.H.); (▼—▼) pH 7.4, 10 m*M* $CaCl_2$ (subject T.A.). Results are expressed as percent total phospholipid remaining as PC (phosphatidylcholine), PE (phosphatidylethanolamine), or PS (phosphatidylserine).

depletion of ATP occurred more rapidly at pH 8.0 and 40 m*M* Ca^{2+} than at pH 7.4 and 10 m*M* Ca^{2+}, and the rate of ATP depletion was not markedly affected by the presence of the enzyme at either pH. In the presence of glucose the ATP levels were maintained at high levels regardless of the incubation conditions. Phospholipase A_2 induced hemolysis was observed only at pH 8.0 and 40 m*M* Ca^{2+} in the absence of added glucose. These data suggested that the phospholipase A_2 induced hemolysis was related to lowered ATP levels, but also indicated that lowered ATP levels in the absence of enzyme are not sufficient to induce hemolysis, at least in the time frame studied.

pH Shift Experiments. Based on the above data, one effect of increased pH during incubation was seen to be a lowering of ATP levels. In addition it was considered possible that high pH might lead to some irreversible alteration in membrane structure, which increased the exposure of the lipids to the enzyme. In order to check this possibility, cells were preincubated at pH 8.0 in either 10 or 40 m*M* Ca^{2+}, and then washed three times with pH 7.4 buffer before adding the enzyme. The results of these experiments are shown in Figure 2. It can be seen that preincubation at pH 8.0 does not cause the cells to be more readily hemolyzed at pH 7.4.

Phospholipid Hydrolysis. The extraction procedure used was developed in order to achieve consistent recoveries of total phospholipid. In particular it was necessary to add

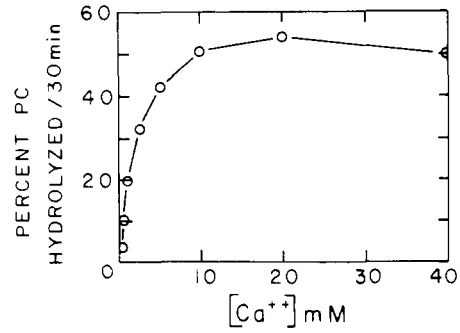

FIGURE 4: Effect of calcium ion concentration on the initial rate of human erythrocyte membrane phosphatidylcholine (PC) hydrolysis by the basic phospholipase A_2 from *A. halys blomhofii*. A 5% suspension of cells (freshly prepared, subject T.A.) was incubated for 30 min at 37°, in 20 m*M* Hepes buffer (pH 8.0), 6.7 μg/ml of enzyme, and the indicated concentration of $CaCl_2$. NaCl was added to make the solution isotonic.

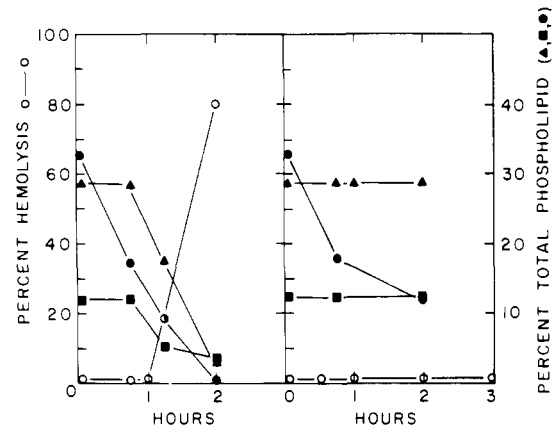

FIGURE 5: Effect of glucose on the hydrolysis of human erythrocyte membrane phospholipids by the basic phospholipase A_2 from *A. halys blomhofii*. A 5% suspension of cells (freshly prepared, subject S.D.) was incubated at 37° in 20 m*M* Hepes buffer (pH 8.0), 40 m*M* $CaCl_2$, and sufficient NaCl to make the solution isotonic. (Left-hand panel) No added glucose. (Right-hand panel) 5 m*M* glucose. Cells were preincubated for 1 hr before addition of 6.7 μg/ml of enzyme. At various times after addition of enzyme samples were analyzed for phosphatidylcholine (PC) (●—●); phosphatidylethanolamine (PE) (▲—▲); phosphatidylserine (PS) (■—■); and extent of hemolysis (o—o).

EDTA in order to achieve reproducible yields of PS, and the use of 2-butanol was necessary in order to achieve good yields of lyso compounds. Usually the yield of total phospholipid phosphorus was 4.1–4.4 μmol/ml of packed cells (Nelson, 1972). When more than 50% hemolysis occurred, the yields were lowered by 10–15%. The composition of cells from several individuals was as follows: PC (32.4–34.7%); PE (24.9–27.5%); PS (9.5–11.5%); sphingomyelin (28.0–28.5%); traces of phosphatidic acid and lyso PC were noted on occasion.

The ability of the basic phospholipase A_2 to hydrolyze erythrocyte phospholipids was investigated with regard to the effects of pH and Ca^{2+} concentration. The results of these studies are presented in Figure 3. At pH 7.4 and 10 m*M* Ca^{2+}, conditions where limited hemolysis occurred, we obtained results similar to those of Zwaal et al. (1973), viz., about 70% hydrolysis of PC, but little or no hydrolysis of PE and PS. However, when the Ca^{2+} concentration was raised to 40 m*M* at pH 7.4, or at pH 8.0 with either 10 or 40 m*M* Ca^{2+}, different results were obtained. In these cases there appeared to be a two-step reaction. In the first step PC was hydrolyzed to the extent of about 70%, and in the second step the remaining PC and the PE and PS were hydrolyzed. The relative rates of the second phase of PC hydrolysis and the hydrolysis of PE and PS were increased by high Ca^{2+} and pH. In all cases where hemolysis was observed it seemed to begin just after the onset of PE hydrolysis. It appeared that PS hydrolysis was coincident with hemolysis.

In order to investigate the effect of Ca^{2+} concentration on the rate of phospholipid hydrolysis, it was decided to measure the rate of PC hydrolysis, since this lipid was most rapidly hydrolyzed under all conditions. The results of these experiments are shown in Figure 4. It should be noted that maximal stimulation of hydrolysis was observed at 10 m*M* Ca^{2+}. These results should be contrasted with the effects of Ca^{2+} on hemolysis (Figure 1). In particular calcium ions maximally stimulated hydrolysis at concentrations where there was minimal hemolysis, and Ca^{2+} shows saturation characteristics in the case of hydrolysis, but not in the case of hemolysis.

The results of the above experiments suggested that hemolysis induced by the basic enzyme was related to the hydrolysis of PE and PS. Since glucose was shown to protect the erythrocyte against hemolysis, it was of interest to investigate the effect of glucose on phospholipid hydrolysis. These experiments are summarized in Figure 5. The presence of 5 m*M* glucose at pH 8.0 and 40 m*M* Ca^{2+} completely protects the cells against hemolysis. In addition there is only 70% hydrolysis of PC and no hydrolysis of PE or PS. These results are comparable to those obtained at pH 7.4 and 10 m*M* Ca^{2+} in the absence of glucose. Thus it was apparent that the ability of the basic phospholipase A_2 to induce hemolysis and completely hydrolyze the phospholipids of the cell depends not only on pH and Ca^{2+} concentration, but also on the energy level of the cell.

Cation Changes. In order to further characterize the pH, Ca^{2+}, and glucose effects on the phospholipase A_2 induced hemolysis of erythrocytes, we have measured the uptake of Ca^{2+} into, and the loss of K^+ and Mg^{2+} from, the cell during incubation with the enzyme. The results are presented in Figures 6 and 7. All of these experiments were conducted at pH 8.0 and 10 m*M* Ca^{2+}, with or without 5 m*M* glucose. During the first hour of incubation with the enzyme the cells bind 0.4–0.5 μmol of Ca^{2+} above control levels, whether glucose is present or not (Figure 6, curves 1 and 2). At about 1.5 hr in the absence of glucose there is a rapid and substantial uptake of Ca^{2+} (Figure 6, curve 1) until at 2.5 hr the cells hemolyzed. In the presence of glucose this second stage of rapid Ca^{2+} uptake was not observed, and the cells did not hemolyze. In the absence of enzyme and glucose there was little uptake of Ca^{2+} until after 2.5 hr. At this time there was a slow uptake of Ca^{2+}, but only slight hemolysis (Figure 6, curve 3). When glucose was added without enzyme, the cells did not take up Ca^{2+} over the time period of the experiment (Figure 6, curve 4). When mannose was substituted for glucose, similar results were obtained but are not included here.

Schrader (1973) has questioned the measurement of Ca^{2+} uptake into erythrocytes on the basis that inorganic phosphate, which might leak out of the cell during the incubation, could precipitate calcium, and thus one might be studying this latter phenomena and not Ca^{2+} uptake by the erythrocytes. Indeed we noted that although a calcium phosphate precipitate was retained on top of the phthalate layer in the absence of cells, when cells were present, the calcium phosphate passed through the phthalate layer with the cells. In these experiments the amount of phosphate

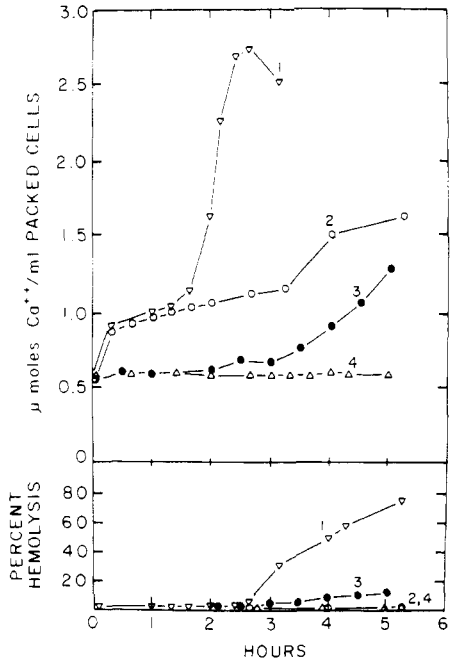

FIGURE 6: The uptake of calcium ion by human erythrocytes under various conditions. A 10% suspension of cells (freshly prepared, subject M.L.) was incubated at 37° in 20 m*M* Hepes buffer (pH 8.0), 10 m*M* $CaCl_2$, and either (1) 13.4 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii;* or (2) 13.4 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii* and 5 m*M* glucose; or (3) no additions; or (4) 5 m*M* glucose, and sufficient NaCl to make the solution isotonic. (Upper panel) μmoles of Ca^{2+} bound/ml of packed cells. (Lower panel) Extent of hemolysis. See text for details of Ca^{2+} measurements.

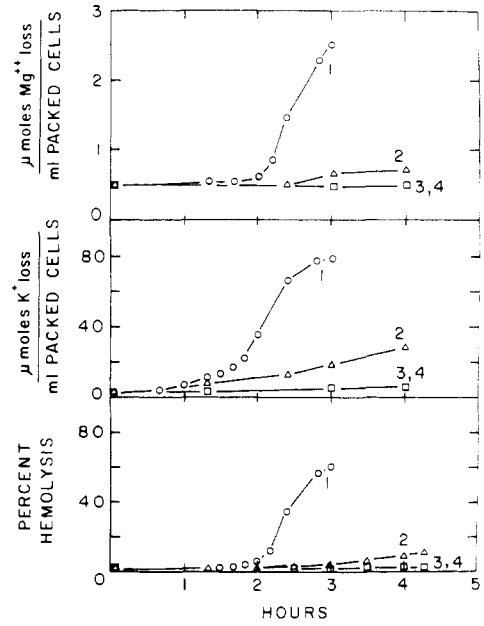

FIGURE 7: Loss of potassium and magnesium ions from human erythrocytes under various conditions. A 10% suspension of cells (freshly prepared, subject M.L.) was incubated at 37° in 20 m*M* Hepes buffer (pH 8.0), 10 m*M* $CaCl_2$, and either (1) 13.4 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii;* or (2) 13.4 μg/ml of the basic phospholipase A_2 from *A. halys blomhofii* and 5 m*M* glucose; or (3) no additions; or (4) 5 m*M* glucose, and sufficient NaCl to make the solution isotonic. (Upper panel) Mg^{2+} efflux. (Middle panel) K^+ efflux. (Lower panel) Extent of hemolysis.

added was equivalent to the total phosphorus content of the cells present in the incubations described in Figure 6. At pH 8.0, 6 m*M* EGTA dissolved 85% of the calcium phosphate precipitate which adhered to the cells. The Ca^{2+} uptake experiments were repeated, but with the addition of EGTA before separation of the cells on phthalate. Under these conditions the rapid uptake commencing at 1.5 hr was still observed. However, the initial binding was substantially reduced. In addition when the cells were 95% hemolyzed, there was no detectable Ca^{2+} in the pellet below the phthalate layer. These observations strongly suggest that the results presented in Figure 6 represent Ca^{2+} uptake by the erythrocytes and not calcium phosphate precipitation.

The loss of K^+ and Mg^{2+} from erythrocytes under these conditions is given in Figure 7. In the absence of glucose with added enzyme there is a prelytic loss of K^+, which occurs at about the same time as the uptake of Ca^{2+}, although there is not a stoichiometric relationship between K^+ loss and Ca^{2+} uptake. As in the case of Ca^{2+} uptake, glucose prevents K^+ loss from the cells. There was no significant loss of Mg^{2+}, over the zero time point, until lysis had occurred, indicating that the influx of Ca^{2+} does not replace intracellular Mg^{2+}.

Effect of Erythrocyte Age. It is known that as the cell ages, there are changes in ATP and cation levels (Brok et al., 1966; Astrup, 1971; Hanahan, 1973). It was therefore considered possible that the older cells might be more susceptible to attack by phospholipase A_2, and it might even be the case that the hemolysis of the younger cells was caused by extensive hemolysis of the older cells. As can be seen in Table V, the older (higher density) cells are more susceptible to hemolysis by the enzyme, but all ages of cells are hemolyzed by the enzyme.

Table V: Effect of Age of Human Erythrocytes on Hemolysis by Phospholipase A_2.[a]

Cells	$t_{1/2}$ (min)
Low density	122
Moderate density	100
High density	78

[a] Cells were fractionated into low, moderate, and high density cells, washed three times with isotonic saline, and incubated in 20 m*M* Hepes (pH 8.0), 40 m*M* Ca^{2+}, and sufficient NaCl added to make the solution isotonic. The hematocrit was 5% and the basic enzyme from *A. halys blomhofii* was used.

Effect of Anticoagulant Media. Erythrocytes were routinely collected in heparin, but in one case cells collected in ACD (acid citrate dextrose, U.S.P.) were checked for their response to phospholipase A_2. Using the basic enzyme, the time for 50% hemolysis was 120 min for cells collected in heparin and 200 min for those collected in ACD. Even though the cells were washed three times after collection in ACD, they had apparently taken up enough glucose to protect them for some time.

Influence of Enzyme Source. It was possible that the basic enzyme from *A. halys blomhofii,* due to its positive charge, could interact with the erythrocyte in a manner different from other phospholipases A_2, which are negatively charged. We therefore examined the ability of certain other enzymes to induce hemolysis and catalyze phospholipid hydrolysis. The results obtained using the *N. naja* enzyme are presented in Figure 8. It is apparent that this enzyme behaves in the same manner as the basic enzyme namely there is 70% hydrolysis of PC and no hemolysis at pH 7.4 and 10 m*M* Ca^{2+}, whereas at pH 8.0 and 40 m*M* Ca^{2+} there is hemolysis and hydrolysis of PC, PE, and PS. In contrast to

these results the enzyme from *C. adamanteus* and the acidic enzyme from *A. halys blomhofii* did not cause hemolysis or significant phospholipid hydrolysis under any of the conditions described above.

Behavior of Various Erythrocyte Species. Erythrocytes were collected from Rhesus monkey, rat, guinea pig, and pig. The susceptibility of these various cells to the basic phospholipase A_2 induced hemolysis and phospholipid hydrolysis were measured at pH 8.0 and 40 m*M* Ca^{2+}. Three types of results were obtained, as described below.

Guinea pig cells behaved similar to human cells. Thus in the absence of glucose there was complete hemolysis and phospholipid hydrolysis within 2 hr. In fact the guinea pig cells seemed to be even more susceptible than human cells, since they were protected from hemolysis for only 1 hr by 5 m*M* glucose, whereas human cells are protected for at least 3 hr. Monkey and rat cells gave intermediate results in that there was only about 15–20% hemolysis after 2 hr at which point the extent of hemolysis leveled off and did not increase with further incubation. At the end of 1 hr, there was approximately 30% hydrolysis of PC, 10% hydrolysis of PE, and no hydrolysis of PS. In the case of adult pig cells there was no hemolysis after 4 hr. At this point there was 40% hydrolysis of PC, 18% hydrolysis of PE, and no hydrolysis of PS.

While the above studies do not represent an exhaustive comparative study, they point out that considerable variability exists between various erythrocyte species with regard to susceptibility to phospholipase A_2.

Discussion

The results presented in this paper show that the action of phospholipase A_2 on intact erythrocytes is considerably more complex than had been previously supposed (Zwaal et al., 1973). While our results do not rule out the possibility that various classes of phospholipids are asymmetrically distributed on the extra- and intracellular sides of the membrane, they show that the susceptibility of phospholipids to hydrolysis by phospholipase A_2 is a function of a number of variables. An understanding of the manner in which these variables, viz., pH, calcium ion concentration, and intracellular ATP, cause the membrane phospholipids to become more susceptible to hydrolysis may provide important clues in elucidating the microenvironment of phospholipids in the erythrocyte membrane.

It appears that the action of phospholipase A_2 on human erythrocytes can be divided into two sequential reactions. In the first step there is hydrolysis of only phosphatidylcholine, to the extent of 70%, and the resulting membrane is stable to hemolysis under certain conditions. The membrane containing lysophosphatidylcholine is stable at pH 7.4 and low Ca^{2+} in the absence of added glucose (see also Zwaal et al., 1973), and at pH 8.0 or high Ca^{2+}, if glucose is added to the medium. Under these conditions the intracellular ATP remains high. However, of considerable importance is the observation that the cell membrane which contains lysophosphatidylcholine is only a quasi-stable situation, since additional phospholipid hydrolysis and hemolysis can occur under certain conditions, viz., high pH and [Ca^{2+}] in the absence of glucose. Under these conditions the intracellular ATP levels are low.

We propose that a phase separation occurs under these latter conditions which either (1) alters the exposure of the remaining phosphoglycerides to the enzyme, thereby leading to their hydrolysis and subsequent hemolysis of the cell,

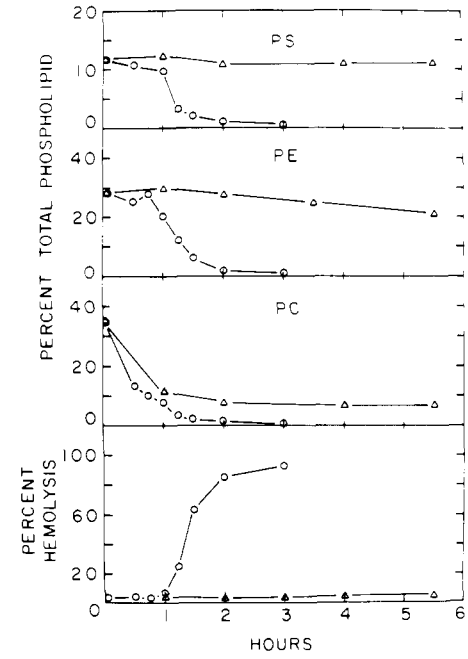

FIGURE 8: Effect of calcium concentration and pH on the rate of hydrolysis of human erythrocyte membrane phospholipids by the phospholipase A_2 from *N. naja* (Pakistan). A 5% suspension of cells (stored 24 hr at 4°, subject T.A.) was incubated at 37° either in 20 m*M* Hepes buffer (pH 8.0), 40 m*M* $CaCl_2$, and sufficient NaCl to make the solution isotonic (O—O) or 20 m*M* Hepes buffer (pH 7.4), 10 m*M* $CaCl_2$, and sufficient NaCl to make the solution isotonic (Δ—Δ). In both cases the enzyme concentration was 12 μg/ml. At the indicated times samples were taken for phospholipid analysis (see text for details). Results are expressed as percent total phospholipid remaining as PC (phosphatidylcholine), PE (phosphatidylethanolamine), or PS (phosphatidylserine).

and/or (2) leads to hemolysis of the cell and subsequent hydrolysis of the remaining phosphoglycerides. The data presented in Figure 3, which show that the hydrolysis of PE precedes hemolysis, favor proposal (1). An incisive examination of these two proposals requires stopping the reaction after the first step and studying the role of the enzyme in the second step. Thus far we have been unsuccessful in separating the enzyme from the cells.

The factors which favor the proposed phase separation, viz., high pH, high [Ca^{2+}], and low intracellular ATP, correspond to factors which have been shown to cause shape changes in intact erythrocytes (Weed and Chailley, 1972). In particular the discocyte–echinocyte transition is favored by the same conditions which lead to hemolysis of intact erythrocytes by phospholipase A_2. Based on present data we suggest that the phase separation is initiated by a high intracellular calcium concentration (see Figure 6). Accumulation of Ca^{2+} at pH 8.0 is observed when the intracellular ATP is low (Table IV). Whether increased Ca^{2+} flux at pH 8.0 is solely responsible for lowering the ATP levels is unknown at present. Further, it is possible that the increased concentration of intracellular calcium would cause magnesium to be displaced from its binding to phosphatidylserine, and that the formation of the calcium–phosphatidylserine complex could be one factor in initiating a phase separation (Ohnishi and Ito, 1974; Jacobson and Papahadjopoulos, 1975). It is important to note that if a phase separation is responsible for hemolysis, it only occurs subsequent to the initial hydrolysis of phosphatidylcholine and in the presence of low intracellular ATP. By themselves neither hydrolysis of phosphatidylcholine nor lowered ATP levels lead to he-

molysis in the presence of the basic phospholipase A_2. Other factors which might lead to the phase separation include potential effects of pH and Ca^{2+} on ionic interactions between lipids and proteins, proteins and proteins, and lipids and lipids. Although further speculation is not justified at present, it is apparent that an understanding of the mechanisms whereby the susceptibility of phospholipids to hydrolysis by phospholipase A_2 is altered by the various factors reported here represents an important piece of information in understanding the structure and stability of the erythrocyte membrane.

Additional areas for further study raised by these results are an investigation of the different reactivities of erythrocytes from various species, and the difference in the action of phospholipases A_2 from various sources on human erythrocytes. While we have not conducted an exhaustive study, it is clear that very different results are obtained with cells from different species. For example (1) both guinea pig and rat erythrocytes have a high PC content, yet the guinea pig cells are much more susceptible to hemolysis, (2) human, monkey, and pig erythrocytes have a comparable phospholipid composition, but again their reactivity with the enzyme is quite variable. Whether these differences are a function of altered lipid or protein distributions in the various erythrocytes is unknown.

It is obvious that it is necessary to exercise caution in the use of phospholipases as probes of membrane structure; nevertheless valuable information can be obtained by a thorough examination of the conditions under which experiments are conducted.

Added in Proof

The purified basic phospholipase A_2 from *Agkistrodon halys blomhofii* was tested for protease activity by the method of Murata et al. (1963). No protease activity was found using either bovine serum albumin or casein as substrate.

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