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HAEMOLYSIS OF INTACT HUMAN ERYTHROCYTES BY PURIFIED COBRA VENOM PHOSPHOLIPASE A_2 IN THE PRESENCE OF ALBUMIN AND Ca^{2+}

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SUMMARY

1. When a purified phospholipase A_2 from *Naja naja* venom is incubated with washed intact human red blood cells, phosphoglyceride hydrolysis, but no haemolysis occurs.

2. The addition of bovine albumin to the incubation mixture brings about haemolysis, which increases with increasing albumin concentration up to 40 mg/ml.

3. Ca^{2+} is essential for phospholipase action, with maximal activity at 10 mM. Complete chelation of Ca^{2+} with EDTA is required after enzyme action if maximal haemolysis is to be achieved.

4. At 0.125 % albumin, 75 % of the cleaved fatty acid was removed from the membrane without appreciable haemolysis. It is suggested that one function of the albumin in causing haemolysis is to remove lysophosphatides from the membrane.

INTRODUCTION

Purified phospholipase A_2 does not haemolyse washed intact red cells in an isotonic medium unless phospholipid or sublytic concentrations of detergents are added [1–4]. However, it is now acknowledged that whole sea snake venom [5], purified cobra venom phospholipase A_2 [6, 7] and purified bee venom phospholipase A_2 [8] will hydrolyse the red cell membrane phospholipids without causing haemolysis. In our previous report [7] we have shown that the membrane damage resulting from the non-haemolytic hydrolysis of membrane phospholipid by purified cobra venom phospholipase A_2 is such that haemolysis occurs, without any further phospholipid splitting, when albumin is added to the medium.

The present paper reports a further study of this haemolysis by phospholipase A_2 in the presence of albumin and Ca^{2+} and includes evidence that the action of albumin is not entirely due to its ability to remove cleaved fatty acid from the membrane, but may result from its action in binding and removing from the membrane the lyso derivatives produced by the enzyme. Moreover, sequestration of Ca^{2+} with equimolar EDTA is essential if an optimal degree of haemolysis is to be achieved.

MATERIALS AND METHODS

Materials

Lyophilised cobra (*Naja naja*) venom was purchased from Sigma Chemical Co., U.S.A. and phospholipase A_2 was purified as reported earlier [9]. The purified enzyme furnished a single main band and two trace bands moving towards the anode on chromatography on 7 % polyacrylamide disc gel electrophoresis [10].

Bovine plasma albumin (Fraction V Armour Pharmaceutical Co.) was defatted by the charcoal procedure [11], or alternatively fatty acid-free Fraction F albumin (Sigma Chemical Co. Ltd) was used.

Haemolysis procedure

Blood from healthy individuals was freshly collected and red cells washed as before [7].

In routine analysis 0.1-ml samples of packed erythrocytes (8.2 μg phosphorus) and 0.8 ml isosmotic glycylglycine buffer (0.1 M glycylglycine in 0.6 % NaCl) with or without 20 mg albumin were pre-incubated for 5 min at 37 °C and the reaction started by adding 20 μg enzyme in 0.1 ml 100 mM $CaCl_2$. All incubations with enzyme were for 5 min, except where otherwise stated.

In haemolysis experiments activity was terminated by the addition with rapid mixing of 5 ml ice-cold 0.9 % saline containing 2 mM EDTA and percentage haemolysis determined as previously described [7].

Lipid extraction

For the extraction of total lipids two methods were used. Method A: This method was based on the addition of 4 vol. of chloroform–methanol (2 : 1, v/v) containing 2.5 mM EDTA followed by washing the upper layer with a new aliquot of lower layer as described earlier [7]. Method B: This method consisted of adding 5 vol. methanol containing 2 mM EDTA followed by 5 vol. chloroform. The contents were mixed and centrifuged and the residue was extracted twice more in a similar fashion except that EDTA was omitted.

Both methods gave identical results for the extraction of cleaved fatty acids.

Phospholipid hydrolysis

Fatty acids cleaved by the enzyme during the 5-min incubation were extracted by Method A and expressed as a percentage of total fatty acid calculated to be esterified at the β-position, i.e. half the total fatty acid obtained from hydrolysable phosphoglycerides (phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) when 0.1 ml packed erythrocytes were either (i) digested in 4 ml methanolic KOH (4 % KOH in 95 % methanol) or (ii) extracted by extraction Method A and the extracted phospholipids transmethylated with 1 ml 0.4 % sodium methylate at 40 °C for 1 h.

KOH digestion and transmethylation furnished very similar values.

Thin-layer chromatography

Red cell lipid extract was chromatographed on silica gel H containing magnesium acetate [12] (50 g silica gel H and 2 g magnesium acetate were slurried in 125 ml

glass distilled water). A solvent system composed of chloroform–methanol–7 M ammonia (65 : 40 : 7, by vol.) was used in the first dimension and propanol (or butanol)–glacial acetic acid–acetone–water (50 : 20 : 5 : 10, by vol.) in the second dimension; this combination of solvent systems furnished a good separation of sphingomyelin and lysophosphatidylethanolamine. The choline-containing phospholipids move very slowly in the second solvent system. Spots were visualised with either ninhydrin, I_2, molybdenum blue reagent [13] or concentrated H_2SO_4 containing 3 % formaldehyde [12].

Phospholipid spots (including those of lysolecithin and lysophosphatidylethanolamine) were scraped off and phosphorus determined by the method of Rouser et al. [12].

RESULTS

(1) *Effect of albumin and Ca^{2+} concentrations on haemolysis*

Fig. 1 shows the effect on haemolysis of varying the albumin concentration in the presence of phospholipase A_2. Increasing the amount of albumin furnished an enhanced degree of haemolysis up to 40 mg/ml. As 20 mg/ml gave an almost identical effect and was more convenient to use than higher amounts this was the concentration chosen for most experiments. We have previously shown [7] that albumin did not activate the enzyme as judged by the degree of membrane phospholipid degradation.

Fig. 2 indicates the activating effect of Ca^{2+} concentration on the hydrolysis

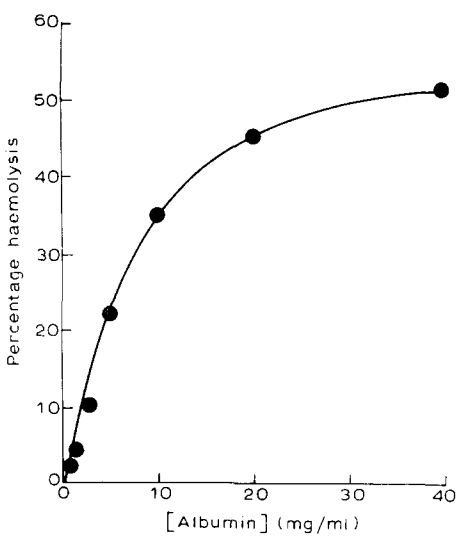

Fig. 1. Effect of albumin concentration on haemolysis.

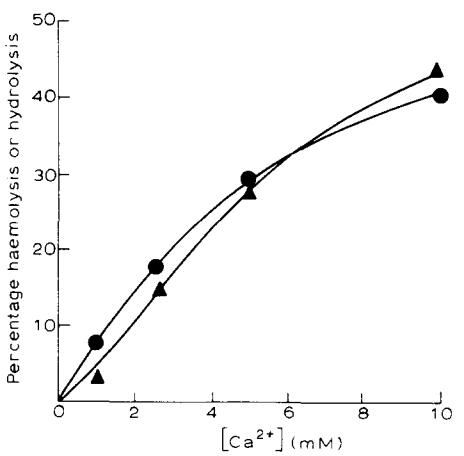

Fig. 2. Effect of Ca^{2+} concentration in the presence of 2 % albumin on hydrolysis of phospholipid and on haemolysis. ●, hydrolysis (expressed as percentage of hydrolysable phosphoglyceride as explained in text); ▲, haemolysis.

of phosphoglycerides and on the resulting haemolysis. The enhancing effect of Ca^{2+} on membrane phospholipid splitting is consistent with an earlier report [6] that Ca^{2+} can replace direct lytic factor in effecting the degradation of phospholipids in the intact erythrocyte membrane. The maximum concentration of Ca^{2+} shown in the graph (10 mM final) was earlier shown to accomplish an optimal activation when the enzyme was hydrolysing dipalmitoyllecithin. When the Ca^{2+} concentration was increased to 20 mM, haemolysis was higher (65 %) than at 10 mM, but the hydrolysis obtained was the same as at 10 mM. In view of this result, 10 mM was chosen as the concentration for use in the present experiments.

(2) *Protective effect of albumin*

Prolonged pre-incubation of red cells with albumin prior to enzyme addition resulted in rather less haemolysis, as is evident from Fig. 3. This protection of erythrocytes against haemolysis appeared to be due to stabilisation of the membrane, since the degradation of membrane phosphoglycerides was not affected by long pre-incubation times.

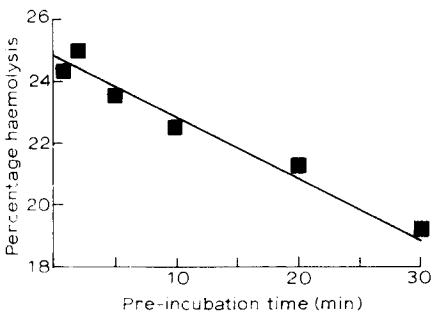

Fig. 3. Effect on haemolysis of prolonged pre-incubation of albumin with red cells. 0.1 ml packed red cells and 0.8 ml isosmotic glycylglycine buffer containing 20 mg albumin were pre-incubated at 37 °C for the times shown. The reaction was then started by adding 20 μg enzyme in 0.1 ml 100 mM $CaCl_2$, and terminated after 2 min.

(3) *Effect on haemolysis of subsequent chelation of Ca^{2+} with EDTA*

In the course of this work it became apparent that a greater degree of haemolysis was obtained when EDTA was incorporated in the saline added to stop activity. This led to the assumption that Ca^{2+}, in addition to being required for enzyme action also had a stabilising effect on the damaged red cell membrane. Experiments were therefore carried out to investigate the effect of varying the concentration of EDTA in the saline added to terminate the reaction.

The results of a typical experiment are recorded in Table I. It is obvious that complete chelation of Ca^{2+} with an equal molarity of EDTA (10 mM final) furnished an enhanced degree of haemolysis and that increasing the EDTA concentration to 20 mM did not have any further effect. The question arose as to whether, when a certain percentage haemolysis was obtained, this was due to some cells losing that percentage of haemoglobin and then re-sealing, or whether that percentage of the red cells lost all of their haemoglobin. The results in Table II, where percentage haemo-

TABLE I

EFFECT OF Ca^{2+} CHELATION ON HAEMOLYSIS MEDIATED BY PHOSPHOLIPASE A_2 IN THE PRESENCE OF ALBUMIN

Experimental details as described under Materials and Methods. Reaction was terminated with 5 ml ice-cold 0.9 % saline with or without EDTA at a final concentration as shown.

EDTA (mM)	Haemolysis (%)	Increase (%)
0	26.0	—
5	30.4	16.9
10	42.0	61.5
20	39.2	50.8

TABLE II

COMPARISON OF HAEMOLYSIS AND RED CELL DESTRUCTION

Results taken from two separate experiments. Experimental details as described under Materials and Methods. Red cell counts were carried out either by the Coulter counter or by the standard chamber counting procedure and duplicate counts agreed to within 10 %.

No. of red cells initially present	Time (min)	Haemolysis (%)	Red cells remaining after the reaction	Red cells destroyed (%)
2.1×10^5	5	38.2	1.2×10^5	43.0
2.3×10^5	1	4.6	2.2×10^5	4.4
2.1×10^5	5★	57.8★	9.5×10^4	54.8

★ 4 min without albumin and then 1 min with 1 ml buffer containing 4 % albumin.

lysis and red cell destruction reasonably equate, support the suggestion that at the effective level of EDTA (10 mM final) a certain percentage of red cells with degraded phosphoglycerides completely lost all of their haemoglobin. As discussed later, under other conditions the cells may reseal and form no ghosts [14].

(4) *Effect of albumin concentration on the removal of cleaved fatty acid*

We have earlier suggested [7] that albumin might bring about haemolysis by removing from the membrane the fatty acid hydrolysis product. If the haemolytic action of albumin arose from its ability to remove cleaved fatty acid from the membrane, the blocking by acetylation [15] of free amino groups, which may be involved in fatty acid binding, might abolish the haemolytic action of the albumin.

Table III shows the effect of such modified albumin on haemolysis. It is clear that acetylated albumin caused 55 % less haemolyis compared with control Fraction V when used at 2 % concentration. This low degree of haemolysis by acetylated albumin was not due to inhibition of the enzyme as 1 % acetylated albumin plus 1 % Fraction V furnished the same degree of haemolysis as 1 % Fraction V alone. By comparison methylated albumin used at 0.8 % concentration was less effective in causing lysis, but because of its insoluble nature, it cannot be used at higher concen-

TABLE III

EFFECT OF MODIFICATION OF ALBUMIN ON HAEMOLYSIS

Experimental details as described under Materials and Methods.

Albumin type	Concentration (%)	Enzyme present (μg)	Haemolysis (%)
Fraction V	2	20	36.1
Fraction V dialysed	2	20	35.6
Fraction V acetylated	2	20	16.0
Fraction V	1	20	22.3
Fraction V + acetylated	1 + 1	20	20.0
Methylated	0.8	20	6.5
Acetylated	1	0	3.1
Fraction V	2	0	0.8

tration. However, the acetylated albumin was slightly haemolytic without enzyme and at 2 % concentration was found to bind 15–20 % of the cleaved fatty acids.

These results might therefore indicate that decreasing the ability of the albumin to bind fatty acid decreased its ability to bring about haemolysis. However (Table IV) by varying the concentration of unmodified albumin it was found that up to 75 % of the cleaved fatty acids could be removed from the membrane without any significant degree of lysis (0.125 % albumin). When the albumin concentration was increased above this point (e.g. from 0.125 to 0.5 %) the degree of haemolysis increased rapidly (4–22 %) although the additional fatty acid removed was comparatively small (75–83 %). This indicates either that there is a critical point of removal of fatty acid above about 75 % or that some other factor contributes to the inducement of haemolysis by albumin.

There was a further possibility that 2 % albumin brought about some changes in red cell morphology, such as swelling, which can also be brought about by incubating the cells at pH 5.8. However, when red cells were incubated with enzyme in isomotic

TABLE IV

EFFECT OF ALBUMIN CONCENTRATION ON THE REMOVAL FROM THE RED CELL MEMBRANE OF CLEAVED FATTY ACIDS

Experimental details as under Materials and Methods. Fatty acid recovered from each supernatant after solvent extraction is expressed as percentage of fatty acid recovered from supernatant containing 2 % albumin. The data represent the mean of three experiments.

Albumin (%)	Fatty acids removed (%)
2	100
1	94.4
0.5	83.3
0.25	80.6
0.125	75.0
0.062	69.4
0.031	63.9

succinate NaCl buffer, pH 5.8 (0.034 M in 0.6 % NaCl) with and without 2 % albumin, 44.3 % haemolysis resulted in the presence and only 1.0 % in the absence of albumin. By contrast isotonic KH_2PO_4 buffer, pH 5.8, furnished 9 % haemolysis in the absence of albumin when erythrocytes were incubated with enzyme for 5 min.

(5) *Lytic and non-lytic degradation of phospholipids*

Previously we reported [7] that under experimental conditions similar to those now employed lecithin was preferentially hydrolysed. This conclusion was based on preliminary phosphorus analysis of the remaining diacylphosphoglycerides after incubation with enzyme. For the two-dimensional thin-layer chromatography the solvent system of Rouser et al. [12] was used and we were unable to achieve a good separation of sphingomyelin and lysophosphatidylethanolamine. The solvent system developed and used in the present work (see Materials and Methods) furnished a good separation of sphingomyelin and lysophosphatidylethanolamine and it was possible to carry out phosphorus estimation on lyso derivatives. Table V shows the amounts of phosphatidylcholine and phosphatidylethanolamine, together with their lyso derivatives, found after enzyme action with and without albumin, expressed as a percentage of the appropriate diacylphosphoglyceride in untreated cells. It is of interest that under lytic conditions the same amount of phosphatidylcholine as under non-lytic conditions, and only a small additional amount of phosphatidyethanolamine, is degraded, suggesting that degradation of phospholipids in ghosts is not occurring to a great extent in these short incubations.

TABLE V

DEGRADATION OF PHOSPHOLIPIDS UNDER LYTIC AND NON-LYTIC CONDITIONS

Following the method of Verkleij et al. [14], the percentages were determined by relating the amount of each compound to the amount of sphingomyelin (which is not attacked), using the values obtained by phosphorus determination after two-dimensional thin-layer chromatography. All experimental details are as described in Materials and Methods; extraction Method A was used. The figures, which are the means of four experiments, indicate for each phospholipid the amount found in enzyme-treated cells expressed as a percentage of the corresponding diacylglycerophosphatide in untreated cells.

Phospholipid	After 5 min incubation with enzyme in absence of 2% albumin	After 5 min incubation with enzyme in presence of 2% albumin
Phosphatidylcholine remaining	51	48
Lysophosphatidylcholine appearing	53	53
Phosphatidylethanolamine remaining	92	80
Lysophosphatidylethanolamine appearing	11	23

(6) *Correlation between hydrolysis and haemolysis*

Variations in the degree of haemolysis during the course of this study were encountered and could be due to the use of different batches of albumin or enzyme preparation or loss of enzyme activity or differential suspectibility of the washed erythrocytes from different individuals to haemolysis. However, when 5-min hydrolysis points from different experiments were plotted versus haemolysis (Fig. 4) a

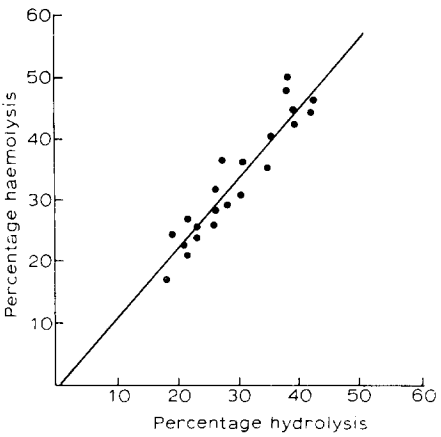

Fig. 4. Correlation between haemolysis and hydrolysis. Results of experiments with sera of different individuals and with different enzyme batches.

significant correlation ($r = 0.94$, $p < 0.001$) was found. Moreover, an equally significant correlation existed between haemolyis in the presence of albumin and hydrolysis (measured in separate tubes) in the absence of albumin; this makes it unlikely that the correlation arises from enzymic hydrolysis of phosphoglycerides in the ghosts, indicating that haemolysis was a reflection of membrane phospholipid degradation.

DISCUSSION

Our results, together with those of others [5, 6, 8] confirm that phospholipase A_2 from a number of species can degrade phospholipids in the intact washed erythrocyte membrane under isotonic conditions without causing haemolysis. We have also shown [16] that of 17 non-haemolytic whole venoms tested, only one failed to bring about phospholipid hydrolysis (as judged by the release of fatty acid) from intact red cells in the presence of 10 mM Ca^{2+}. Moreover, supplementation of the incubation medium with 2 % albumin led to a degree of haemolysis correlated with phospholipid splitting. The observation that hydrolysis of the intact red cell membrane phosphoglycerides by phospholipase A_2 occurs in the absence of haemolysis would support the hypothesis that haemolysis in the presence of albumin is a consequence of membrane phospholipid splitting.

This suggestion is also supported by the observation that an enhanced degree of haemolysis can be achieved if the enzyme is allowed to act (in the absence of albumin) on the cells for several minutes, albumin then being added and the reaction terminated after a further minute (Table II, last result, and results reported previously [7]).

It was previously thought [7] that removal of cleaved fatty acids by albumin results in a weaker membrane which can no longer retain haemoglobin. However, further investigation has shown (Table IV) that a substantial part of the cleaved fatty acids can be removed by low concentrations of albumin without any significant haemolysis occurring. This suggests that an alternative, or additional, effect of the albumin causes the haemolysis, possibly the removal of lysophosphoglycerides from the membrane. Indeed, when the supernatant was extracted with solvent after spin-

ning down the ghosts and red cells (30 min at 26 000 × *g*), lyso compounds were detected on thin-layer chromatography only when 2 % albumin was used. It has been reported [17] that 1 mole of albumin binds 1 mole of lysolecithin; 20 mg albumin used under the present conditions is just enough to bind the lyso compound produced by phospholipase A_2 on that basis.

Quantitative determination of the amount of lysolecithin removed from the enzyme-treated red cells by varying amounts of albumin would help to elucidate this point. The experiments which led to the results shown in Table III were initially designed to throw light on whether removal of fatty acid by albumin is necessary to achieve lysis. The results could be interpreted in that way, but in view of Table IV, it seems that modification of the albumin may affect not only its ability to bind fatty acid, but also some additional function such as is postulated above, possibly the binding of lysolecithin. It would therefore be of interest to determine whether acetylation interferes with the ability of albumin to bind lysolecithin.

A certain degree of resistance against haemolysis on prolonged pre-incubation of red cell suspensions with albumin (Fig. 3) could be due to the coating of the erythrocyte surface by albumin leading to less haemolysis. In this respect Williams [18] has shown that albumin can protect red cells against haemolysis by hydrodynamic shear forces and Cho and Proulx [19] have observed that pre-incubation with albumin protects red cells against lysis by lysolecithin.

It has been reported by Elsbach and Pettis [20] that some preparations of albumin are contaminated with phospholipase A. We have found no evidence that our albumin contained any phospholipase A activity as judged by the release of fatty acid from intact erythrocytes or purified dipalmitoyllecithin. Furthermore, no haemolysis results even on prolonged incubation of red cells with Ca^{2+} and albumin in the absence of added phospholipase A_2.

Regarding the degradation of phospholipid by the enzyme, our results are for the most part consistent with the distribution of phospholipid in the red cell membrane proposed by Verkleij et al. [14]. In the absence of haemolysis only 8 % of phosphatidylethanolamine is hydrolysed as against 49 % of lecithin. Zwaal et al. [8] have shown that while phospholipase A_2 from bee venom degraded 9 % of phosphatidylethanolamine, the purified enzyme from *Naja naja* did not hydrolyse this phospholipid in the intact human erythrocyte membrane. The small difference with respect to phosphatidylethanolamine between our result and that of Zwaal et al. [8] could be due to a difference in the isoenzymes resulting from the two purification procedures. We have consistently observed, however, that under our experimental conditions the amount of phosphatidylserine found upon phosphorus analysis after two-dimensional thin-layer chromatography is lower (using sphingomyelin as internal standard) after enzyme action than was found with untreated cells. As it seemed possible that this was due to incomplete extraction of phosphatidylserine from phospholipase A_2-treated cells, the extraction procedure referred to as Method B in Materials and Methods was used. When this extraction procedure was used, the amount of phosphatidylserine was still found to be lower after enzyme action on the cells, suggesting that phosphatidylserine may be degraded under the present conditions. However, the suggestion that phospholipase A_2 degrades this phospholipid in intact red cells contrasts with the results of other workers [8, 14]

and will require further investigation. It may be due to the use of glycylglycine buffer. Recently it was demonstrated that glycylglycine and Ca^{2+} form a complex with phosphatidylserine [21] and there is the possibility that the formation of such a complex exposes phosphatidylserine to phospholipase A_2 action.

Ca^{2+} might inhibit haemolysis by stabilising the membrane and since the carboxyl groups of sialic acid may be involved in binding Ca^{2+} it was envisaged that removal of these carboxyl groups by treating the erythrocytes with neuraminidase (receptor-destroying enzyme) might at least partially relieve inhibition of lysis by Ca^{2+}. We found (unpublished results) that when receptor-destroying enzyme-treated cells [22] were subsequently exposed to phospholipase A_2 action in the absence of albumin, no haemolysis occurred, and this observation is in agreement with the report of Roelofsen et al. [2]. However, when red cells treated with receptor-destroying enzyme were incubated with phospholipase A_2 in the presence of 2 % albumin, EDTA was still required to achieve maximum haemolysis though lysis in this case was 40 % more than in the case of red cells not treated with receptor-destroying enzyme.

We have observed (Table II) that in our experiments, haemolysis appears to be the result of a certain number of red cells losing all their haemoglobin. Verkleij et al. [14], recently reported that when erythrocytes were incubated with phospholipase A_2 in the presence of Ca^{2+} after an initial treatment with sphingomyelinase, 20 % haemolysis occurred without any ghost formation. It therefore appears that these two different sets of conditions give rise to different types of haemolytic behaviour.

If, as the results in Table II seem to indicate, some cells lyse totally under the present conditions, whilst others do not lyse at all, the question arises as to what extent phospholipid hydrolysis occurs in those cells that lyse compared with those that do not lyse. The possibility exists that phospholipase action occurs at a higher rate on some cells than on others, so that only those that reach a certain degree of degradation lyse. Alternatively, all cells may undergo the same degree of phospholipid degration during the incubation period, but the population of cells may show a variation in membrane stability after attack. The correlation observed between phospholipid splitting and haemolysis (Fig. 4) would thus represent the response averaged over the cell population.

The mechanism of haemolysis by lysolecithin has attracted much attention. It has been suggested that lysolecithin lyses red blood cells by displacing cholesterol from the membrane, though Reman et al. [23] have pointed out that the amount of lysolecithin required to induce haemolysis is too small to remove an appreciable quantity of cholesterol and it has also been found that the loss of up to 35 % cholesterol from pig erythrocytes apparently does not produce any haemolysis [24]. Another suggestion is that exogenous lysolecithin causes haemolysis by changing the stable bimolecular lipid leaflet structure to an unstable micellar one [25]. However, in the present work it has been shown that it is not only possible to produce an appreciable amount of endogenous lyso derivatives in the membrane without causing haemolysis but also that removal of 75 % of the cleaved fatty acid with a low concentration of albumin (with most of the lyso compounds remaining in the membrane) leaves a lipid layer capable of retaining haemoglobin. This observation poses interesting problems for future investigation.

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