Biochimica et Biophysica Acta, 436 (1976) 353–365

BBA 77334

POSSIBLE RELATIONSHIP BETWEEN MEMBRANE PROTEINS AND PHOSPHOLIPID ASYMMETRY IN THE HUMAN ERYTHROCYTE MEMBRANE

C. W. M. HAEST and B. DEUTICKE

Abteilung Physiologie, Medizinische Fakultät, Technische Hochschule Aachen, D-51 Aachen (G.F.R.)

(Received December 4th, 1975)

SUMMARY

After incubation of human erythrocytes at 37 °C in the absence of glucose (A) for 24 h, (B) for 4 h with 8 mM hexanol or (C) for 3 h with SH reagents, phosphatidylethanolamine becomes partly susceptible to hydrolysis by phospholipase A_2 from *Naja naja*. The presence of glucose during the pretreatments suppresses this effect, except in the case of SH reagents that inhibit glycolysis. After incubation with tetrathionate, up to 45 % of the phosphatidylethanolamine is degraded by the enzyme, an amount considerably in excess of the 20 % attacked in fresh erythrocytes.

Pancreatic phospholipase A_2, an enzyme unable to hydrolyse the phospholipids of intact erythrocytes, partially degrades phosphatidylcholine and phosphatidylethanolamine of erythrocytes pretreated with hexanol or SH reagents. Reagents capable of oxidizing SH groups to disulfides (tetrathionate, *o*-iodosobenzoate and hydroquinone) even render susceptible to pancreatic phospholipase A_2 phosphatidylserine, a phospholipid supposed to be entirely located in the inner lipid layer of the membrane. Alkylating or acylating SH reagents have no such effect. It is postulated that disulfide bond formation between membrane protein SH groups leads to an alteration in protein-phospholipid interactions and consequently induces a reorientation of phospholipids between the inner and the outer membrane lipid layer.

INTRODUCTION

Phospholipases are valuable tools for studying the arrangement of the phospholipids in the erythrocyte membrane [1, 2]. As was shown by Verkleij et al. [2], up to 48 % of the membrane phospholipids in the human erythrocyte are hydrolysed by sequential incubation of intact cells with phospholipase A_2 from *Naja naja* and sphingomyelinase from *Staphylococcus aureus*. The phospholipids hydrolysed comprised 82 % of the sphingomyelin, 76 % of the phosphatidylcholine and 20 % of the phosphatidylethanolamine. It was concluded that these phospholipids are localized in the outer layer of the membrane lipid bilayer. This concept of a phospholipid asymmetry could be confirmed by recent investigations on the hydrolytic cleavage of the phospholipids in sealed inside-out erythrocyte membrane vesicles by phospholipases

A_2 (*N. naja*) and C (*Clostridium welchii*) [3] and in right-side-out resealed ghosts by pancreatic phospholipase A_2, trapped inside the ghosts [4]. In a previous paper [5] we have demonstrated that incubation of freshly drawn human erythrocytes in the absence of glucose at 37 °C (A) for 24 h, (B) for 1–5 h with hexanol or (C) for 1–4 h with the SH reagent, tetrathionate, greatly enhances the rate of a subsequent reaction of phosphatidylethanolamine with 2,4,6-trinitrobenzenesulfonic acid and increases the percentage of phosphatidylethanolamine labelled, as compared with cells immediately exposed to 2,4,6-trinitrobenzenesulfonic acid. These effects could be prevented when the preincubation was carried out in the presence of glucose or inosine. Inhibitors of glycolysis abolished this protection.

On the basis of these results it was suggested that in fresh as well as in glycolysing incubated erythrocytes phosphatidylethanolamine is "shielded" by protein. Changes of protein conformation "expose" the phospholipid head group towards 2,4,6-trinitrobenzenesulfonic acid. A "flip-flop" of phospholipids from the inner to the outer monolayer of the lipid bilayer was proposed to contribute to the effects observed.

In the present study the enzymatic approach towards problems of sidedness of phospholipids in the erythrocyte membrane, as affected by the above-mentioned preincubation conditions, was used. This strategy should have certain advantages over studies with group-specific reagents. Firstly, the reagents known react only with phospholipids containing free amino groups, whereas phospholipases exhibit varying substrate specificities. Secondly, it is difficult to prove the impermeability of the reagents under various incubation conditions [6]. Enzymes, however, may be safely assumed not to permeate the erythrocyte membrane. Furthermore, it was the purpose of this study to characterize in more detail the site of action of tetrathionate and the possible mechanism of the protective effect of glucose.

MATERIALS AND METHODS

Phospholipases

Phospholipase A_2 (highly purified from porcine pancreas, free of proteolytic activity) and phospholipase D (cabbage), obtained from Boehringer Mannheim GmbH, were used as supplied by the manufacturer. Another phospholipase A_2 was purified from *N. naja* venom (Sigma) according to Cremona and Kearney [7]. Essentially similar preparations of the latter two enzymes have been shown to be free of proteolytic activity [8, 9].

Sphingomyelinase from *S. aureus* was purified from an 8 h culture medium of Strain 66 (a generous gift from Dr. N. Weiss, DSM München). After centrifugation of the culture media, the supernatant was adjusted to 77 % saturation with $(NH_4)_2SO_4$, allowed to stand for 16 h at 4 °C and centrifuged for 15 min at 0 °C and 27 000 $\times g$. The precipitate was dissolved in 12 ml water and dialysed for 16 h at 4 °C against water/glycerol (1 : 1, v/v). This crude enzyme preparation did not produce hemolysis when incubated with human erythrocytes for 4 h at 37 °C.

Incubation procedure

Erythrocytes from freshly collected heparinized human blood were washed three times with 154 mM NaCl. 1 vol. of the washed cells was then suspended in

10 vols. of a medium containing (mM) KCl 90, NaCl 45, Na_2HPO_4/NaH_2PO_4 10 and sucrose 44 (Medium A). The erythrocytes were incubated at 37 °C under gentle agitation in the presence or absence of 11 mM glucose in Medium A for (a) 24 h at pH 7.4 without additions, (b) 4 h at pH 7.4 with 8 mM hexanol or (c) 3 h at pH 8.0 with 5 mM sodium tetrathionate or sodium *o*-iodosobenzoate (both from Fluka AG, Buchs), ethacrynic acid (a gift from Merck, Sharpe and Dohme, Rahway), hydroquinone, *N*-ethylmaleimide and sodium iodoacetate (from Merck AG, Darmstadt) or 1 h at pH 8.0 with 0.5 mM *p*-hydroxymercuriphenylsulfonic acid (Sigma, St. Louis). Subsequently the cells were washed three times with medium A and exposed to phospholipases.

Treatment of erythrocytes with phospholipases

0.5 ml packed erythrocytes were incubated in 10 ml of a medium containing (mM) KCl 100, NaCl 50, $MgCl_2$ 0.25, sucrose 44 and glycylglycine 10. The $CaCl_2$ concentration for incubations with sphingomyelinase, phospholipase A_2 and phospholipase D were 0.25, 10 and 25 mM, respectively. The pH of the medium was 7.4, the temperature 37 °C. The amounts of enzyme added are indicated in the text.

Routinely, the incubations with phospholipase A_2 and sphingomyelinase were terminated by adding 20 ml of incubation media containing 15 and 1 mM EDTA, respectively, whereas the phospholipase D reaction was stopped by centrifugation of the cells for 2 min at $6000 \times g$.

After centrifugation, the cells were extracted according to Rose and Oklander [10] without a haemolysis step and the phospholipids separated by two-dimensional chromatography on silica plates (Merck, Darmstadt, No. 5715) according to Broekhuyse [11]. All phospholipid spots were scraped from the plates and phosphorus determined as described previously [12].

The high recovery of the extraction procedure applied [13] was not affected by the occurrence of degradation products after phospholipase treatment as indicated by a constancy of the sum of the respective phospholipids and their phosphorus-containing degradation products.

The data in the text refer to a single characteristic experiment out of a series of 2–4. The maximum variation between the extremes, in experiments of the same type, was 4 % in the amount of phospholipid degraded.

Influence of SH reagents on intracellular reduced glutathione (GSH)

1 ml of erythrocytes was incubated at 37 °C and pH 8.0 in 10 ml of Medium A in the absence or presence of glucose with various SH reagents for varying times. Subsequently, the cells were centrifuged and washed 3 times with Medium A at 4 °C. The intracellular concentration of reduced glutathione was determined according to Beutler [14].

Uptake of [^{35}S]tetrathionate by erythrocytes

Radioactive tetrathionate was prepared [15] from [^{35}S]thiosulfate (Amersham). The purity of the preparation was tested by thin-layer chromatography [16]. 1 ml of erythrocytes was suspended in 5 ml of Medium A containing tetrathionate (1 mM) and 50 μCi of [^{35}S]tetrathionate. After various periods of incubation at pH 8.0 and 37 °C the cells were centrifuged and washed 5 times with Medium A (0 °C). The

radioactivity in the incubation medium, the supernatants of the washings and in the washed cells was determined by standard liquid scintillation techniques.

RESULTS

A. Phospholipase studies

1. Influence of a 24 h preincubation on the enzymatic degradation of phospholipids. It has previously been shown [1] that in freshly drawn human erythrocytes phospholipase A_2 from *N. naja* cleaves 68 % of the phosphatidylcholine. The other glycerophospholipids are not hydrolysed. These results were confirmed in the present study.

After a 24 h preincubation in Medium A, however, in the absence of glucose, 22 % of the phosphatidylethanolamine became sensitive to hydrolysis by phospholipase A_2 from *N. naja* (Fig. 1). Preincubation in the presence of glucose reduced the subsequent hydrolysis to 5 % of the phosphatidylethanolamine. These phenomena fully agree with the enhancement, under the same condition, of the 2,4,6-trinitrobenzenesulfonic acid labelling of the phosphatidylethanolamine headgroup and the protective action of glucose on this effect [5]. Phosphatidylserine was not hydrolysed by *N. naja* phospholipase A_2 under these conditions, and the rate of degradation of phosphatidylcholine proved to be independent of the presence or absence of metabolic substrate (cf. Fig. 1). The exposition to phospholipase A_2 had to be limited to 25 min, since prolonged incubation periods produced haemolysis exceeding 2 %.

In line with the studies using phospholipase A_2, phospholipase D from cab-

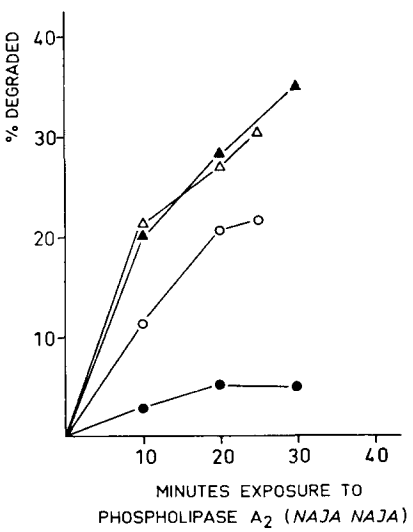

Fig. 1. Influence of a 24 h preincubation of erythrocytes on the subsequent degradation of the membrane phospholipids, phosphatidylcholine (△) and phosphatidylethanolamine (○) by phospholipase A_2 from *N. naja*. Erythrocytes were preincubated for 24 h at 37 °C and pH 7.4 in the absence (open symbols) or presence (closed symbols) of 11 mM glucose. The cells were washed and exposed to phospholipase A_2 from *N. naja* (1 I.U./ml) at 37 °C, pH 7.4 and $[Ca^{2+}]$ 10 mM (see Methods).

TABLE I

INFLUENCE OF A PRETREATMENT OF ERYTHROCYTES WITH HEXANOL ON THE SUBSEQUENT DEGRADATION OF MEMBRANE PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2

Erythrocytes were pretreated with 8 mM hexanol in the absence or presence of 11 mM glucose for 4 h at 37 °C and pH 7.4. The cells were washed and exposed to phospholipase A_2 from *N. naja* (1 I.U./ml) or pancreas (20 I.U./ml) at 37 °C, pH 7.4 and [Ca^{2+}] 10 mM (see Methods).

Hexanol pretreatment	Phospholipase A_2		% degraded			
	Source	Exposure (min)	Total phospholipids*	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine
With glucose	*N. naja*	15	12	31	10	0
	Pancreas	45	5	13	4	0
Without glucose	*N. naja*	15	14	28	19	0
	Pancreas	45	11	17	20	0

* Comprising the sum of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin.

bage, an enzyme unable to degrade the phospholipids in intact erythrocytes [17], hydrolysed 14 % of the membrane phospholipids (mainly phosphatidylcholine and some phosphatidylethanolamine) in cells preincubated in the absence of glucose, whereas in glucose-preincubated cells only 8.5 % was degraded.

In further studies, preincubated cells were exposed to pancreatic phospholipase A_2. This enzyme, which has been previously reported not to degrade the phospholipids of intact erythrocytes [1], hydrolysed only 6 % of the phospholipids of erythrocytes preincubated in the absence of glucose at 37 °C for 24 h. The presence of glucose during the preincubation period suppressed this experimentally induced susceptibility to the enzyme.

2. Influence of a pretreatment with hexanol on the enzymatic degradation of the phospholipids. In analogy to the effects produced by a 24 h incubation, a 4 h pretreatment with hexanol in the absence of glucose brought about an increase in the amount of phosphatidylethanolamine attacked by phospholipase A_2 from *N. naja*, as compared to cells incubated with hexanol in the presence of glucose (Table I).

Moreover, pancreatic phospholipase A_2 after pretreatment of the cells in the absence of glucose hydrolysed 17 % of the phosphatidylcholine and 20 % of the phosphatidylethanolamine. Pretreatment in the presence of glucose again diminished the amount of phospholipid degraded. Phosphatidylserine was not attacked. Thus, the hexanol-induced changes in phosphatidylethanolamine "accessibility" indicated

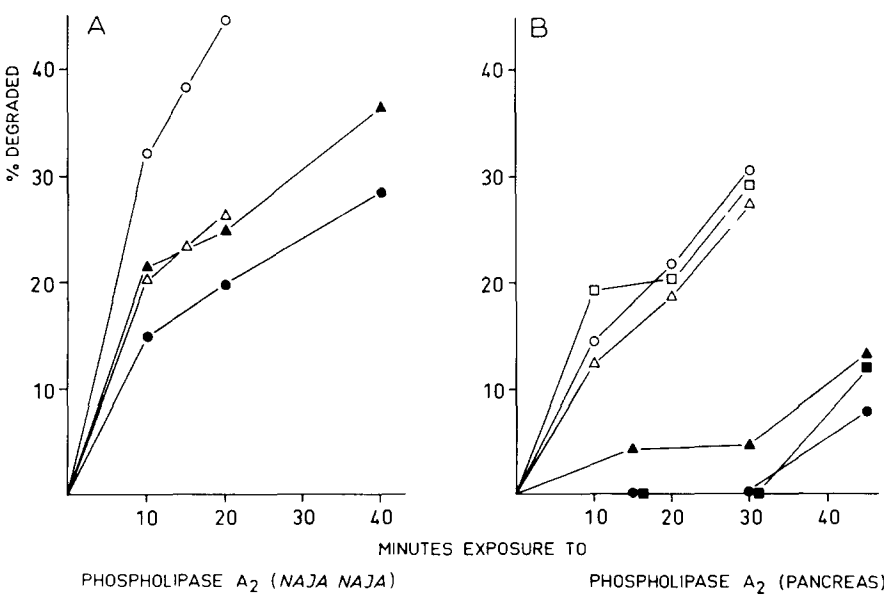

Fig. 2. Influence of pretreatment of erythrocytes with tetrathionate on subsequent degradation of membrane phospholipid, phosphatidylcholine (△), phosphatidylserine (□) and phosphatidylethanolamine (○) by phospholipase A_2 from *N. naja* and porcine pancreas. Erythrocytes were preincubated with 5 mM tetrathionate for 3 h at 37 °C and pH 8.0 in the absence (open symbols) or presence (closed symbols) of 11 mM glucose. The cells were washed and exposed to phospholipase A_2 from *N. naja* (1 I.U./ml) or pancreas (20 I.U./ml) at 37 °C, pH 7.4 and $[Ca^{2+}]$ 10 mM (see Methods).

TABLE II

INFLUENCE OF PRETREATMENT OF ERYTHROCYTES WITH TETRATHIONATE ON SUBSEQUENT DEGRADATION OF MEMBRANE PHOSPHOLIPIDS BY SEVERAL PHOSPHOLIPASES

Erythrocytes were preincubated with 5 mM tetrathionate for 3 h at 37 °C and pH 8.0 in the absence or presence of 11 mM glucose. The cells were washed and exposed to phospholipases at 37 °C and pH 7.4. Incubation media with pancreatic phospholipase A_2, *N. naja* phospholipase A_2, phospholipase D and sphingomyelinase contained, respectively, 10, 10, 25 and 0.25 mM Ca^{2+} and 20, 1, 2 and 1 I.U./ml enzyme, respectively. Haemolysis was always less than 2 %.

Tetrathionate pretreatment	Phospholipase		% degradation of				
	Type	Exposure (min)	Total phospholipids*	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Sphingo-myelin
Without glucose	A_2 *N. naja*	20	21	26	44	0	0
	A_2 pancreas	30	22	28	31	29	0
	D cabbage	20	16	38	16	0	0
	Sphingo myelinase	15	10	0	0	0	36
With glucose	A_2 *N. naja*	20	13	25	20	0	0
	A_2 pancreas	30	1	4	0	0	0
	D cabbage	20	5	14	4	0	0
	Sphingomyelinase	15	10	0	0	0	36

* Comprising the sum of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin.

by the enhanced trinitrophenylation [5] are also confirmed by the results with phospholipases.

3. Influence of a pretreatment with tetrathionate and other SH reagents on the enzymatic degradation of the phospholipids. After a 3 h preincubation of erythrocytes with tetrathionate in the absence of glucose, 21 % of the phospholipids was hydrolysed by phospholipase A_2 from *N. naja*, comprising about 45 % of the phosphatidylethanolamine (Fig. 2A). Phosphatidylserine was not degraded and the rate of hydrolysis of phosphatidylcholine not significantly affected by the pretreatment.

Pancreatic phospholipase A_2, under the same conditions, hydrolysed about the same amount of the phospholipids, including 28 % of the phosphatidylcholine, 31 % of the phosphatidylethanolamine and 29 % of the phosphatidylserine (Fig. 2B), a phospholipid not susceptible to enzymatic cleavage in intact erythrocytes.

Phospholipase D from cabbage, which is unable to degrade the phospholipids of freshly drawn human erythrocytes [17], hydrolysed up to 16 % of the phospholipids. Moreover, tetrathionate pretreatment enhanced the amount of sphingomyelin degraded within 15 min by sphingomyelinase from 26 % to 36 %. All the consequences of tetrathionate pretreatment were suppressed, but not completely abolished, by the presence of glucose, except for the susceptibility to sphingomyeli-

TABLE III

INFLUENCE OF PRETREATMENT OF ERYTHROCYTES WITH VARIOUS SH REAGENTS ON THE SUBSEQUENT DEGRADATION OF MEMBRANE PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2

Erythrocytes were pretreated in Medium A at 37 °C for 1 h with 0.5 mM *p*-hydroxymercuriphenylsulfonic acid or for 3 h with 5 mM iodoacetate, *N*-ethylmaleimide, *o*-iodosobenzoate, ethacrynic acid or tetrathionate. The cells were washed and exposed to phospholipase A_2 from *N. naja* (1 I.U./ml) or pancreas (20 I.U./ml).

Pretreatment with SH reagent	% degradation of		
	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine
N. naja phospholipase A_2 (10 min exposure)			
None	23	0	0
Ethacrynic acid	26	16	0
Iodoacetate	24	10	0
N-Ethylmaleimide	22	18	0
o-Iodosobenzoate	15	23	0
Hydroquinone	16	25	0
Tetrathionate	20	34	0
Pancreatic phospholipase A_2 (15 min exposure)			
None	0	0	0
Ethacrynic acid	13	10	0
Iodoacetate	6	7	0
p-Hydroxymercuriphenylsulfonic acid	6	5	0
N-Ethylmaleimide	9	7	0
o-Iodosobenzoate	12	16	11
Hydroquinone	16	8	10
Tetrathionate	21	23	21

nase. Table II summarizes the maximum amount of phospholipid degraded by various phospholipases prior to haemolysis exceeding 2 %.

In order to characterize in more detail the reason for the tetrathionate-induced increase of phospholipid labelling and cleavage, the effect of pretreatments of erythrocytes with other SH reagents was tested. Besides the well known organomercurial *p*-hydroxymercuriphenylsulfonic acid and the alkylating reagents *N*-ethylmaleimide and iodoacetate, the acylating reagent ethacrynic acid, known to inhibit ATPase and glycolysis in erythrocytes [18] and *o*-iodosobenzoate and hydroquinone were investigated. The latter two reagents are able, like tetrathionate, to oxidize SH groups to disulfide bonds [19, 20]. As is evident from Table III, pretreatment with all SH reagents tested partially sensitized phosphatidylethanolamine to hydrolysis by phospholipase A_2 from *N. naja*. The amounts of phosphatidylethanolamine degraded by this phospholipase A_2 within 10 min, however, decreased in the order tetrathionate > hydroquinone > *o*-iodosobenzoate > *N*-ethylmaleimide > ethacrynic acid > iodoacetate. The rates of degradation of phosphatidylcholine were not significantly affected and phosphatidylserine was not degraded at all.

Pancreatic phospholipase A_2, after such a pretreatment, attacked a considerable fraction of the phosphatidylcholine and the phosphatidylethanolamine. In tetrathionate-, *o*-iodosobenzoate- and hydroquinone-pretreated cells, however, this enzyme also degraded phosphatidylserine, while other SH reagents induced no such effect. It may be concluded therefore that these disulfide-bond-producing agents have a more pronounced membrane perturbing effect.

B. Studies on the mechanism of action of tetrathionate

Permeability of the erythrocyte membrane towards tetrathionate. In order to elucidate the possible localization of the SH groups involved in the "shielding" of phospholipids it is important to know whether and how fast the SH reagents used can pass the membrane and thus reach SH groups in the interior or even at the inner surface of the membrane. Ethacrynic acid, *N*-ethylmaleimide, *o*-iodosobenzoate and iodoacetate have been shown to penetrate rapidly into erythrocytes [21]. However, as yet no data on the permeability to tetrathionate are available. Previous findings that this SH reagent does not inhibit glyceraldehyde-3-phosphate dehydrogenase [22], as well as the size and charge of this anion, suggested an essential impermeability.

In order to obtain reliable evidence on this point the rate of uptake of [^{35}S] tetrathionate from the medium was studied. In addition, the concentration of GSH in erythrocytes treated with tetrathionate was used as an indicator of tetrathionate uptake. Tetrathionate entering the cell might be expected, like other SH reagents [21] to lower the concentration of this major intracellular SH compound.

In a first series of experiments erythrocytes were incubated with [^{35}S]tetrathionate at 37 °C. The uptake of tetrathionate was followed by measuring the decrease of radioactivity in the medium (see Methods). From a semilogarithmic plot of radioactivity versus time, a half time for the transfer of tetrathionate of about 15 min was estimated. This indicates that tetrathionate permeability is of the same order of magnitude as the permeability to sulfate.

Secondly, erythrocytes were incubated with tetrathionate and the concentration of intracellular GSH analysed after different periods of time. For reason of comparison the influence of other SH reagents and of a 24 h incubation of erythro-

TABLE IV

INTRACELLULAR GSH CONCENTRATIONS IN CELLS AFTER INCUBATIONS WITH SH REAGENTS

Erythrocytes were incubated for varying times at 37 °C and pH 8.0 with medium containing 5 mM ethacrynic acid, *N*-ethylmaleimide, iodoacetate or *o*-iodosobenzoate in the absence of glucose or with 5 mM tetrathionate in the absence or presence of 11 mM glucose. The cells were washed (4 °C) and the intracellular GSH content determined (see Methods). The amount of GSH in control cells was 2.60 ± 0.12 μmol/ml packed erythrocytes.

SH reagent	Exposure (min)	GSH (% of control)
Control	0	100
Ethacrynic acid	15	< 5
N-Ethylmaleimide	15	< 5
Iodoacetate	15	< 5
o-Iodosobenzoate	15	< 5
Tetrathionate (without glucose)	15	27
	30	15
	60	13
	120	12
	180	9
Tetrathionate (with glucose)	30	62
	60	52
	120	50
	180	32

cytes with or without glucose was also studied. Exposure to iodoacetate, *o*-iodosobenzoate, ethacrynic acid and *N*-ethylmaleimide decreased the GSH content to very low values within 15 min (< 5 %, cf. Table IV). This result is in agreement with other studies [21].

Incubation of erythrocytes with tetrathionate in the absence of glucose also resulted in a decrease of the intracellular GSH. This decrease, however, was less dramatic than in case of the other SH reagents (Table IV). The presence of glucose during such an incubation retarded the decline in intracellular GSH. (Protection by glucose of GSH against *N*-ethylmaleimide, ethacrynic acid and iodoacetate cannot be expected in view of the inhibitory action of these compounds on red cell glycolysis [18, 21]). Furthermore, it could be demonstrated that a 24 h incubation in the absence of glucose at 37 °C reduced the intracellular GSH concentration to about 15 % of the initial value, whereas during incubation in the presence of glucose no significant change was observed. Obviously, the availability of metabolic substrate renders possible a continuous reductive cleavage of the GSSG formed in the presence of tetrathionate. In spite of these observations a linear relationship between GSH levels and phospholipid susceptibility to phospholipase or 2,4,6-trinitrobenzenesulfonic acid seems doubtful, since our previous [5] and present studies have shown that iodoacetate, although rapidly diminishing GSH levels, has only a small stimulating effect on the subsequent labelling of phosphatidylethanolamine with 2,4,6-trinitrobenzenesulfonic acid [5] or the enzymatic degration of the phospholipids.

DISCUSSION

In this paper it has been demonstrated that, in agreement with 2,4,6-trinitrobenzenesulfonic acid labelling studies [5], phosphatidylethanolamine in the human erythrocyte membrane becomes partly susceptible to hydrolysis by phospholipase A_2 from *N. naja* after incubation of the cells (a) for 24 h at 37 °C, (b) for 4 h with 8 mM hexanol or (c) for 3 h with various permeable SH reagents.

As in the 2,4,6-trinitrobenzenesulfonic acid labelling studies [5], the presence of glucose diminishes the effect of the pretreatments with the exception of SH reagents that inhibit glycolysis. Furthermore, glucose retards the decline of the intracellular GSH content in the presence of tetrathionate. A linear relationship between the GSH levels and the effectiveness of the reagents could be excluded. On the other hand, GSH may still be involved in the protective action of glucose against tetrathionate if one assumes that GSH competes with membrane protein SH groups for the reaction with tetrathionate. Under these premises the higher levels of GSH in erythrocytes incubated in the presence of glucose might to some extent prevent the modification of membrane SH groups by tetrathionate and thus also counteract the subsequent enhancement of phospholipase susceptibility.

Pancreatic phospholipase A_2 has hitherto been supposed not to attack phospholipids in the intact erythrocyte. Consistent with this assumption, preincubation of erythrocytes in the presence or absence of glucose for 24 h at 37 °C did not bring about a significant hydrolysis of phospholipids. After hexanol pretreatment, however, phosphatidylethanolamine and phosphatidylcholine were partly hydrolysed by this phospholipase, too. Following a pretreatment with tetrathionate, *o*-iodosobenzoate or hydroquinone, pancreatic phospholipase A_2 even degraded a considerable fraction of all glycerophospholipids, including up to 29 % of phosphatidylserine (cf. Tables II and III). This phospholipid is supposed to be entirely located in the inner lipid monolayer of the membrane [2, 4]. Accepting this hypothesis, it is tempting to conclude that tetrathionate, *o*-iodosobenzoate and hydroquinone induce a reorientation of the phospholipids between the inner and the outer lipid monolayer. This conclusion is supported by the finding that phospholipase A_2 from *N. naja* hydrolysed up to 45 % of the phosphatidylethanolamine in tetrathionate-pretreated cells. Since according to the results of Verkleij et al. only 20 % of the phosphatidylethanolamine is present in the outer membrane lipid layer of fresh erythrocytes, the additional 25 % must have been recruited from the inner layer. Such a recruitment could occur either at the expense of other phospholipids "flipping" from the outer to the inner monolayer of the membrane, or by a net translocation of phospholipids.

The tetrathionate-induced susceptibility of membrane phospholipids to pancreatic phospholipase A_2 can be interpreted on the basis of recent studies on the hydrolysis by this enzyme of phospholipids arranged in monolayers [23]. With respect to the erythrocyte membrane, the results of these studies indicate that a high surface pressure (31–35 dynes/cm) in the phospholipid headgroup region and a particular phospholipid composition in the outer lipid layer may be responsible for the fact that certain phospholipases are able but others unable to degrade phospholipids in the intact erythrocyte [23].

In particular, pancreatic phospholipase A_2 has been shown to be unable to hydrolyse phosphatidylcholines in monolayers at high surface pressures, conditions

assumed to prevail in the outer lipid layer of the human erythrocyte membrane. In contrast, the enzyme was able to degrade, at high surface pressures, monolayers containing phosphatidylcholine and phosphatidylserine [23], a mixture that may be present in the outer membrane lipid layer of erythrocytes after pretreatment with tetrathionate. It is tempting to conclude, therefore, that an altered phospholipid composition of the outer lipid layer is responsible for the hydrolysis of the phospholipids of tetrathionate-pretreated erythrocytes. In addition, however, changes of surface pressure may also play a role.

Among the SH reagents studied, only a pretreatment with tetrathionate, *o*-iodosobenzoate and hydroquinone renders phosphatidylserine susceptible to degradation by pancreatic phospholipase A_2. The specificity of these three reagents may result from the differences in the chemical reaction mechanism. SH reagents like *N*-ethylmaleimide, ethacrynic acid and iodoacetate react with protein SH groups under formation of alkyl or acyl substitutes, whereas tetrathionate, *o*-iodosobenzoate and hydroquinone produce disulfide bridges, probably via intermediates [19] (Although not all the reagents used react exclusively with SH groups, it seems safe to assume that their sites of action in the erythrocyte membrane are proteins. Any covalent attachment of one of the agents to membrane phospholipids should give rise to derivatives detectable on the thin-layer chromatograms. This was not observed. A perturbation of the lipid domain by noncovalently bound reagent, on the other hand, will not outlast the removal of these hydrophilic reagents by extensive washing [5] and is therefore not likely to account for the effects observed.). Evidence that disulfide bridges may be formed between erythrocyte membrane proteins can be adduced from results obtained by Steck [24] and Wang and Richards [25]. These investigators have shown that exposure of erythrocyte ghosts to oxidizing agents results in a dimerization of the 95 000 dalton protein and a cross-linking of the spectrin components by disulfide bridge formation. It seems promising to study whether and to what extent tetrathionate, *o*-iodosobenzoate and hydroquinone induce a cross-linking of membrane proteins, to identify these proteins and to characterize their interaction with phospholipids such as phosphatidylethanolamine and phosphatidylserine. In this way one might obtain further insight into the role that proteins play in the preservation of the asymmetrical distribution of the phospholipids in erythrocyte membranes.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160 "Eigenschaften biologischer Membranen"). The skilful and devoted technical assistance of Mrs. G. Plasa and Miss D. Kamp is gratefully acknowledged. We are indebted to Dr. N. Weiss, DSM München, for the gift of the *Staphylococcus aureus* strain.

REFERENCES

1 Zwaal, R. F. A., Roelofsen, B. and Colley, C. M. (1973) Biochim. Biophys. Acta 300, 159–182
2 Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 323, 178–193
3 Kahlenberg, A., Walker, C. and Rohrlick, R. (1974) Can. J. Biochem. 52, 803–806
4 Zwaal, R. F. A., Roelofsen, B., Comfurius, P. and van Deenen, L. L. M. (1975) Biochim. Biophys. Acta 406, 83–96

5 Haest, C. W. M. and Deuticke, B. (1975) Biochim. Biophys. Acta 401, 468–480
6 Gordesky, S. E., Marinetti, G. V. and Love, R. B. (1975) J. Membrane Biol. 20, 111–132
7 Cremona, T. and Kearney, E. B. (1964) J. Biol. Chem. 239, 2328–2334
8 Simpkins, H. and Panko, E. (1971) Biochemistry 10, 3579–3585
9 Kahlenberg, A. and Banjo, B. (1972) J. Biol. Chem. 1156–1160
10 Rose, H. G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
11 Broekhuyse, R. M. (1969) Clin. Chim. Acta 23, 457–461
12 Gerlach, E. and Deuticke, B. (1963) Biochem. Z. 337, 447–479
13 Broekhuyse, R. M. (1974) Clin. Chim. Acta 51, 341–343
14 Beutler, E., Duron, D. and Mikus Kelly, B. (1963) J. Lab. Clin. Med. 61, 882–888
15 Gmelin, L. (1906) Handbuch der Anorganischen Chemie, Band II, p. 341, Winter, Heidelberg
16 Stahl, E. (1967) Dünnschicht-Chromatographie, p. 810, Springer, Berlin
17 Gercken, G., Witt, W. and Hintze, U. (1974) Pflügers Arch. 347, R37
18 Lubowitz, H. and Whittam, R. (1969) J. Physiol. 202, 111–131
19 Means, G. E. and Feeney, R. E. (1971) Chemical modification of proteins, pp. 157–160. Holden-Day, San Francisco
20 Miller, A. and Smith, M. C. (1970) Brit. J. Haematol. 19, 417–428
21 Bide, R. W. and Myers, D. K. (1967) Can. J. Biochem. 45, 19–29
22 Duhm, J., Deuticke, B. and Gerlach, E. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1008–1016
23 Demel, R. A., Geurts van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B. and van Deenen, L. L. M. (1975) Biochim. Biophys. Acta 406, 97–107
24 Steck, T. L. (1972) J. Mol. Biol. 66, 295–305
25 Wang, K. and Richards, F. M. (1975) J. Biol. Chem. 24, 8005–8018