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SPECTRIN AS A STABILIZER OF THE PHOSPHOLIPID ASYMMETRY IN THE HUMAN ERYTHROCYTE MEMBRANE

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Summary

After treatment of intact human erythrocytes with SH-oxidizing agents (e.g. tetrathionate and diamide) phospholipase A_2 cleaves approx. 30% of the phosphatidylserine and 50% of the phosphatidylethanolamine without causing hemolysis (Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353—365). These phospholipids are scarcely hydrolysed in fresh erythrocytes and are assumed to be located in the inner lipid layer of the membrane (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). The enhancement of the phospholipid cleavage is now shown to be accompanied by a 50% decrease of the membrane SH-groups and a cross-linking of spectrin, located at the inner surface of the membrane, to oligomers of $<10^6$ dalton.

Blocking approx. 10% of the membrane SH groups with *N*-ethylmaleimide suppresses both the polymerization of spectrin and the enhancement of the phospholipid cleavage. *N*-Ethylmaleimide, under these conditions, reacts with three SH groups per molecule of spectrin, 0.7 SH groups per major intrinsic 100 000 dalton protein (band 3) and 1.1 SH groups per molecule of an extrinsic protein of 72 000 daltons (band 4.2). Blocking studies with iodoacetamide demonstrate that the SH groups of the 100 000-dalton protein are not involved in the effects of the SH-oxidizing agents.

It is suggested that a release of constraints imposed by spectrin enables phosphatidylserine and phosphatidylethanolamine to move from the inner to the outer lipid layer of the erythrocyte membrane and that spectrin, in the native erythrocyte, stabilizes the orientation of these phospholipids to the inner surface of the membrane.

Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

In a previous paper [1] it has been demonstrated that incubation of human erythrocytes with agents capable of oxidizing SH groups to disulfide bonds renders all three classes of membrane glycerophospholipid sensitive to pancreatic phospholipase A_2, an enzyme unable to hydrolyse the phospholipids of fresh, intact erythrocytes. Even phosphatidylserine, supposed to be located entirely in the inner lipid layer of the membrane, was partially degraded by the enzyme. Moreover, phospholipase A_2 from *Naja naja* cleaved a higher percentage (45%) of phosphatidylethanolamine under these conditions as compared to fresh, untreated erythrocytes (20%). Alkylating or acylating SH reagents had no such sensitizing effect.

On the basis of the proposed asymmetric phospholipid arrangement in the erythrocyte membrane [2,3] (no phosphatidylserine and only 20% of the phosphatidylethanolamine in the outer lipid layer), these findings have been taken to indicate that disulfide bond formation between membrane protein SH groups perturbs protein-lipid interactions and thereby induces a reorientation of phospholipids between the inner and outer membrane lipid layer.

It was the purpose of the present study to characterize the membrane proteins which, after chemical modification by SH-oxidizing agents, perturb the asymmetric phospholipid arrangement. Moreover, it was intended to determine the minimal number of SH groups that have to be oxidized in order to enhance phospholipase susceptibility and to establish conditions under which SH groups of certain membrane proteins could be selectively modified. From such studies and experiments with alkylating SH agents that inhibit the effect of SH-oxidizing agents, strong evidence could be obtained that the major extrinsic membrane protein, spectrin, is involved in the stabilisation of the phospholipid asymmetry.

Materials and Methods

Materials

Phospholipase A_2 from porcine pancreas and from bee venom (Boehringer Mannheim GmbH. and Sigma, respectively) were used as supplied by the manufacturer. Diamide (diazinedicarboxylic acid bisdimethylamide) was obtained from Calbiochem.

Incubation procedure

Erythrocytes from freshly collected heparinized human blood were washed three times with 154 mM NaCl and 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 cells were incubated at 37°C and pH 8.0 under gentle agitation for varying times with SH-oxidizing agents (tetrathionate $(S_4O_6)^{2-}$ or diamide), washed three times and incubated for another 2 h in 10 vols of Medium A without the oxidizing agents at 37°C and pH 7.4. After a further washing they were exposed to phospholipases.

Treatment of erythrocytes with phospholipases

Packed erythrocytes were incubated at 37°C in 20 vols. of a medium con-

taining: (mM) KCl, 100; NaCl, 50; $MgCl_2$, 0.25; sucrose, 44; and glycylglycine, 10 (pH 7.4). The concentrations of $CaCl_2$ for the incubations with phospholipase A_2 from bee venom and from porcine pancreas were 0.25 mM and 10 mM, respectively. The amount of enzyme added was 20 I.U/ml of erythrocytes. Incubations with phospholipase were terminated by addition of an excess of EDTA or a combination of EDTA and *o*-phenanthroline and immediate centrifugation. The extent of haemolysis was always less than 2%. The cells were extracted according to Rose and Oklander [4], but without a hemolysis step, and the phospholipids were separated by two-dimensional chromatography on silica plates (Merck, Darmstadt No. 5715) using in the first dimension a mixture of (volume): chloroform/methanol/25% NH_3/H_2O (90 : 54 : 5.7 : 5.3, v/v) and in the second dimension a mixture of chloroform/methanol/acetic acid/H_2O (60 : 30 : 8 : 2.85, v/v). The amounts of the phospholipids and their degradation products were determined as described previously [1].

The data in the text refer to single characteristic experiments out of series of 2—5. The maximum variation between the extremes, in experiments of the same type, was 5% in the amounts of phospholipid degraded.

Determination of membrane SH groups

0.1 ml packed erythrocyte membranes, prepared according to [5] were solubilized with 0.1 ml SDS (10% w/v) and 0.8 ml phosphate buffer (5 mM, pH 8.0). 0.1 ml of a 1 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) was added and the increase in the absorbance at 412 nm was registered for 1 h at 37°C. The absorbance of an adequate reagent blank was subtracted from the measured absorbance. For the calculation of the SH content/mg protein, GSH was used as a reference substance. The concentration of protein in the samples was determined according to Lowry et al. [6].

Polyacrylamide gel electrophoresis

Electrophoresis was performed on gels containing 5.0% acrylamide, 0.1% *N,N'*-methylene-bisacrylamide and 1.0% SDS, following the procedure of Fairbanks et al. [7]. Samples for electrophoresis were prepared by solubilizing 0.9 vol. of packed membranes [5] with 0.1 vol. of a solution of SDS (20% w/v) followed by heating for 2 min at 100°C and addition of 100 mg sucrose/ml. No disulfide reducing agent was added to the solubilized ghost preparation, to the gel solution or to the electrophoresis buffer.

Results and Discussion

In a previous paper [8] we have demonstrated that the amount of membrane phosphatidylethanolamine that reacts with the impermeable amino reagent, 2,4,6-trinitrobenzenesulfonic acid, increases continuously with time in human erythrocytes incubated with tetrathionate. Further incubation of the cells in the absence of tetrathionate, after a 1 h exposure to the reagent, goes along with a further increase of the rate and extent of trinitrophenylation of phosphatidylethanolamine. A similar two-step mechanism could now be established for the increase in phospholipase susceptibility in tetrathionate-treated eryth-

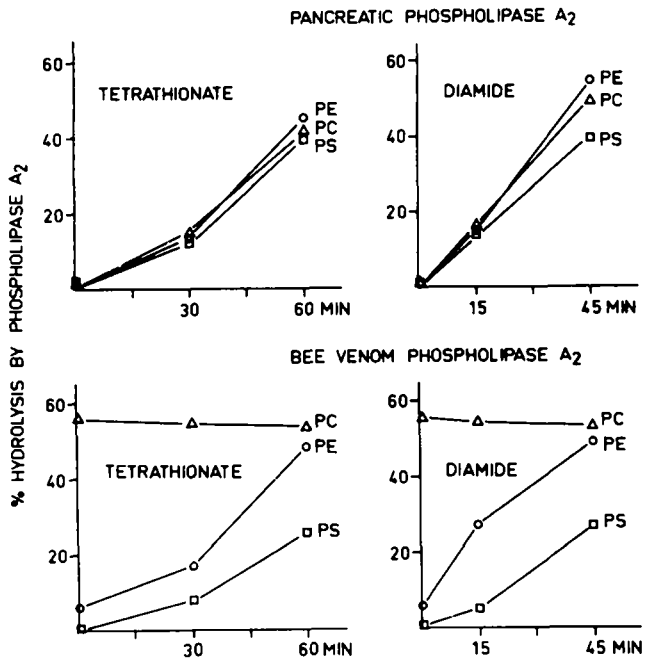

Fig. 1. Time dependency of the enhancement of the phospholipase susceptibility by SH-oxidizing agents. Erythrocytes were incubated with 20 mM tetrathionate ($S_4O_6^{2-}$) for 30 or 60 min, respectively, or with 5 mM diamide for 15 or 45 min, respectively (pH 8.0, 37°C), washed three times and incubated for another 2 h (pH 7.4, 37°C) in the absence of the SH-oxidizing agents. After a further washing the cells were exposed to phospholipase A_2 from bee venom or porcine pancreas (20 I.U./ml cells, pH 7.4, 37°C). See Materials and Methods for further details. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

rocytes. Treatment with the SH reagent (20 mM, 1 h) increases the amount of phospholipids degraded subsequently by phospholipase A_2 (bee venom or pancreas) (Table I). Further incubation in the absence of tetrathionate produces a further increase of this phospholipid cleavage. Pancreatic phospholipase A_2, unable to degrade any of the phospholipids of fresh erythrocytes, degrades a considerable amount of all three glycerophospholipids under these conditions (see also ref. 1) while phospholipase A_2 from bee venom cleaves 50% of the phosphatidylethanolamine and 30% of the phosphatidylserine in erythrocytes treated with SH-oxidizing agents, in contrast to a hydrolysis of only 5% of the phosphatidylethanolamine and none of the phosphatidylserine in fresh cells. *

Under the assumption of an asymmetric arrangement of membrane phospholipids (phosphatidylserine and 80% of the phosphatidylethanolamine in the inner lipid layer) these findings may be taken to indicate that the SH-oxidizing agents perturb membrane protein structure and thereby induce a reorientation of phosphatidylserine and phosphatidylethanolamine from the inner to the

* The involvement of exocytotic vesiculation [32,33] as an alternative explanation for our findings was suggested by one of the reviewers. According to preliminary electronmicroscopic studies such vesicles are not formed in the presence of disulfide-forming agents. Moreover, under our experimental conditions such vesicles would not have been pelleted and thus the lipid analysis of the erythrocytes would not have been falsified.

TABLE I

ENHANCEMENT OF PHOSPHOLIPID CLEAVAGE BY PHOSPHOLIPASE A_2 AFTER TREATMENT OF HUMAN ERYTHROCYTES WITH TETRATHIONATE

Erythrocytes were incubated with 20 mM tetrathionate (pH 8.0, 37°C), washed three times, and divided into two portions. One portion was incubated for another 2 h (pH 7.4, 37°C) in the absence of tetrathionate. Subsequently, both portions were exposed to phospholipase A_2 from porcine pancreas or bee venom (20 I.U./ml cells, pH 7.4, 37°C). See Materials and Methods for further details.

Pretreatment	Source of phospholipase A_2	Percentage degradation of		
		Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine
None	Porcine pancreas	0	0	0
1 h tetrathionate	Porcine pancreas	18	11	12
1 h tetrathionate, 2 h without tetrathionate	Porcine pancreas	34	36	32
None	Bee venom	58	6	0
1 h tetrathionate	Bee venom	57	33	10
1 h tetrathionate, 2 h without tetrathionate	Bee venom	56	48	32

TABLE II

DECREASE OF MEMBRANE SH GROUPS IN ERYTHROCYTES TREATED WITH SH-OXIDIZING AGENTS

Erythrocytes were incubated with tetrathionate (20 mM) or diamide (5 mM) and washed three times. Membrane SH groups were determined as described in Materials and Methods.

Cells treated with	Time of exposure (min)	Residual SH groups (%)
No additive	0	100 *
Tetrathionate	30	58 ± 6 (n = 3)
	60	39 ± 4 (n = 3)
Diamide	15	56 ± 7 (n = 3)
	45	41 ± 5 (n = 3)

* 100% = 81 ± 5 (n = 16) nmol SH/mg protein.

outer lipid monolayer of the membrane, which, once initiated, proceeds even during subsequent incubation of the cells in the absence of tetrathionate. No translocation of sphingomyelin from the outer to the inner lipid layer of the membrane seems to accompany the outward movement of phosphatidylserine (and phosphatidylethanolamine) since the amount of sphingomyelin cleaved by sphingomyelinase (a generous gift of Dr. R.F.A. Zwaal, Utrecht, The Netherlands), remained unchanged at 85% of the total.

In order to find out how many SH groups have to be modified to induce the phospholipid reorientation, erythrocytes were exposed to SH-oxidizing agents (tetrathionate, diamide) for varying times.

Incubation with tetrathionate (20 mM) for 30 min or with diamide (5 mM) for 15 min decreased membrane SH groups by about 40% (Table II). After a subsequent incubation (2 h) in the absence of the agents, however, only a minor enhancement of phospholipase susceptibility was observed (Fig. 1). Incubation with tetrathionate for 60 min or diamide for 45 min decreased membrane SH groups by about 60% (Table II) and induced a marked enhancement of phospholipase susceptibility. These findings indicate that phospholipid reorientation occurs only after oxidation of a substantial amount of membrane SH groups. The decrease of the membrane SH groups in presence of tetrathionate and diamide might be due principally to the formation of either intra- or intermolecular disulfide bonds. In ghosts, intermolecular crosslinking, by oxidizing agents, between band 3 monomers and between spectrin molecules (bands 1 and 2 according to the nomenclature of Steck [9]) has been demonstrated by several authors (see ref. 10).

According to gel electrophoretic analyses of membrane proteins, treatment of intact erythrocytes (Fig. 2 and ref. 10) with the oxidizing agents does not crosslink band 3. The spectrin bands (1 and 2) diminish, however, and a new band appears on top of the gels. Incubation of the gel slice containing this latter band with dithioerythritol and re-electrophoresis revealed that it contains only spectrin (not shown). This demonstrates the formation, by SH-oxidizing agents, of spectrin oligomers. A complete crosslinking of spectrin is only obtained after prolonged incubation of erythrocytes with tetrathionate (60 min) or diamide (45 min).

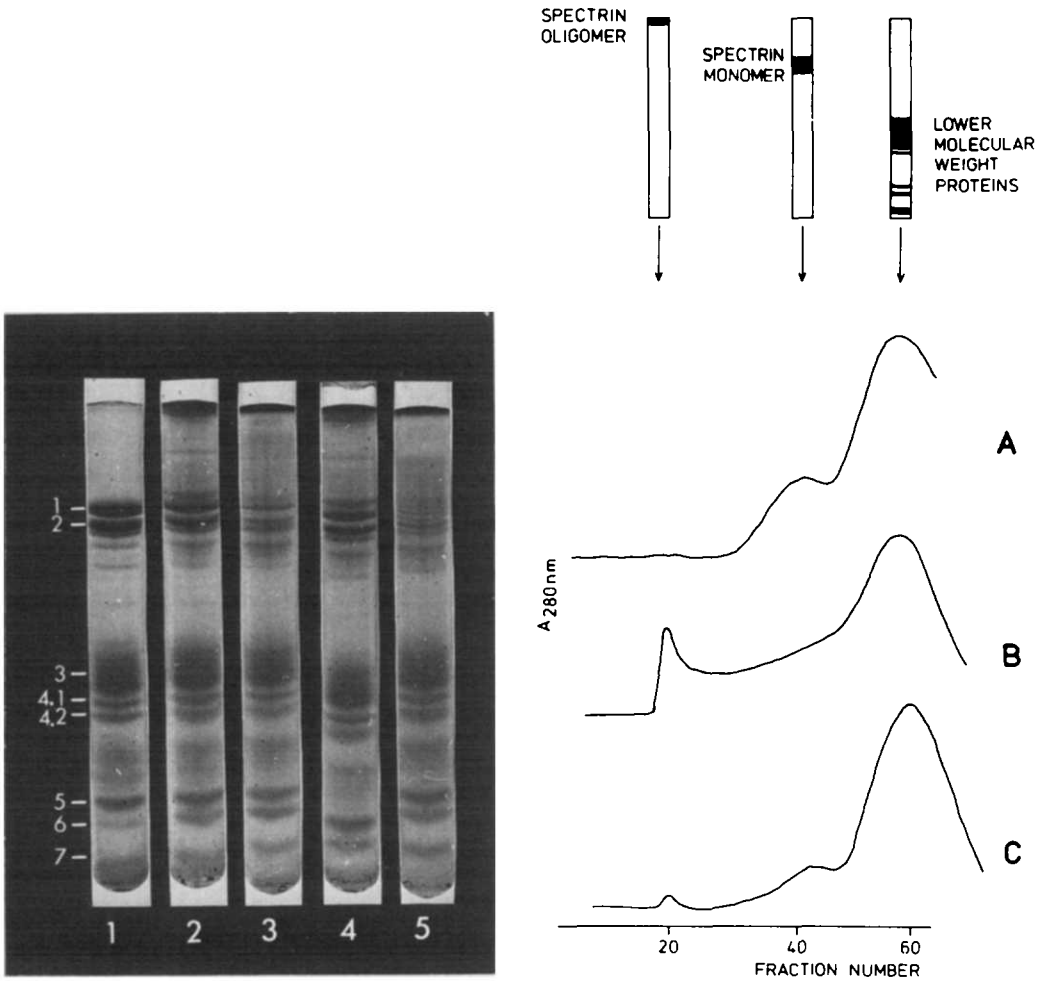

Fig. 2. Gelelectrophoretic profiles of membrane proteins from human erythrocytes exposed to SH-oxidizing agents. Erythrocytes were incubated (pH 8.0, 37°C) with 20 mM tetrathionate for 30 or 60 min, respectively, or with 5 mM diamide for 15 or 45 min, respectively and washed three times. Ghosts were prepared according to Dodge [5], solubilized with SDS and subjected to gel electrophoresis as described in Materials and Methods. 1, control; 2, tetrathionate, 30 min; 3, tetrathionate, 60 min; 4, diamide, 15 min; 5, diamide, 45 min.

Fig. 3. Chromatographic separation, on Agarose 50 m columns, of SDS-solubilized membrane proteins of erythrocytes treated with SH-oxidizing agent. Erythrocytes were incubated (pH 8.0, 37°C) with (A) no additive, (B) 20 mM tetrathionate (60 min) or (C) 0.4 mM *N*-ethylmaleimide (15 min, hematocrit 9%) followed by 20 mM tetrathionate (60 min), and washed three times. The membranes prepared from the cells [5] were solubilized in SDS (1% final concentration) and applied to an Agarose 50 m column (1 × 50 cm). The proteins were eluted with a buffer containing 20 mM Tris, 1 mM EDTA, 10 mM sodium acetate and 1% SDS (pH 8.0). The absorbance at 280 nm of the eluate was registered (lower part) using an ISCO absorbance monitor (UA-5). The SDS gel electrophoretic profiles of the peak fractions are shown in the upper part.

In order to characterize further the molecular weight of the spectrin oligomer, SDS-solubilized membranes of untreated and of tetrathionate- or diamide-treated cells were subjected to chromatography on Agarose 50 m columns. The elution profile for control cells (Fig. 3, trace A) reveals two peaks. Gel electro-

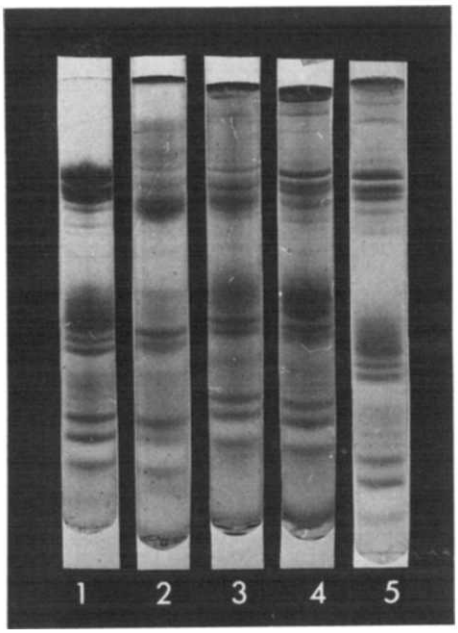

Fig. 4. Blockade by alkylating SH reagents of the SH group involved in the dimerization of band 3. Erythrocytes were incubated for 15 min (pH 8.0, 37°C) with no additive (gel 2), 0.4 mM *N*-ethylmaleimide (gel 3), 2 mM iodoacetamide (gel 4) or 10 mM iodoacetamide (gel 5) and washed three times. Ghosts prepared from these cells (according to ref. 5) were exposed to 1 mM diamide (30 min, pH 7.4, 37°C), washed three times, solubilized in SDS and subjected to gel electrophoresis in parallel to ghosts prepared from fresh cells (gel 1). See Materials and Methods for further details.

pherograms of the peak fractions demonstrate that the high molecular weight peak contains spectrin while the other one contains all other membrane proteins (Fig. 3, upper part). The profile for tetrathionate- (or diamide-) treated cells (Fig. 3, trace B) does not show the spectrin peak, but instead a peak of still higher molecular weight eluted together with Blue Dextran 2000 ($M_r = 2 \cdot 10^6$). This fraction could be shown by gel electrophoresis before and after treatment with reducing thiols, to contain only spectrin oligomers. These results suggest that under the conditions of our experiments spectrin "monomers" (M_r = 220 000 and 240 000, respectively) are cross-linked at least as a tetramer.

Further investigations served the purpose to collect more evidence that this crosslinking of spectrin is responsible for the phospholipid reorientation in the erythrocyte membrane. In this context it was of interest to find out whether alkylating or acylating SH reagents, which hardly affect the phospholipase susceptibility of erythrocytes [1], would under suitable conditions react specifically with spectrin and thereby prevent the subsequent action of SH-oxidizing agents. Although no ideal conditions could be found, blockage of about 10% of the membrane SH groups with *N*-ethylmaleimide 0.4 mM (hematocrit 9%) could be shown (Table III) to inhibit the enhancement of the phospholipase susceptibility by tetrathionate (or diamide, data not shown) as well as the polymerisation of spectrin (Fig. 3, trace C). Binding studies with labelled *N*-ethylmaleimide revealed that at these concentrations the agent reacts prefer-

TABLE III

INHIBITION BY A PRETREATMENT WITH ALKYLATING SH REAGENTS OF THE ENHANCEMENT OF PHOSPHOLIPID CLEAVAGE INDUCED BY SH-OXIDIZING AGENTS

Erythrocytes were incubated for 15 min with (a) no additive, (b) 0.4 mM *N*-ethylmaleimide, (c) 2 mM iodoacetamide or (d) 10 mM iodoacetamide (pH 8.0, 37°C). Subsequently the cells were washed three times, incubated for 60 min with 20 mM tetrathionate (pH 8.0, 37°C), washed three times and incubated without tetrathionate for a further 2 h (pH 7.4, 37°C). After one further washing these cells and control cells were exposed to phospholipase A_2 from bee venom (20 I.U./ml) cells, pH 7.4, 37°C. See Materials and Methods for further details.

1st incubation	2nd incubation	Percent of degradation of		
		Phosphadityl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine
None	None	53	8	0
None	Tetrathionate	53	50	23
N-ethylmaleimide (0.4 mM)	Tetrathionate	52	18	0
Iodoacetamide (2 mM)	Tetrathionate	53	50	21
Iodoacetamide (10 mM)	Tetrathionate	53	33	12

entially with spectrin, band 3 and band 4.2. From the radioactivities measured in gel sections corresponding to complete bands (Table IV), the specific activity of the tracer, and the molecular weights of the proteins [11,12] it could be calculated that under these conditions *N*-ethylmaleimide blocks 3 SH groups per spectrin molecule, 0.7 per band 3, 1.1 per band 4.2, 0.3 per band 4.5 and less than 0.2 per band 4.1 and 7. In principle, spectrin, band 3 and band 4.2 might therefore be involved in the perturbation of the phospholipid asymmetry by SH reagents.

TABLE IV

DISTRIBUTION OF THE RADIOACTIVITY AMONG THE MEMBRANE PROTEINS OF ERYTHROCYTES TREATED WITH *N*-[*ethyl*-2-^{3}H]-ETHYLMALEIMIDE

Radioactivity of separate bands was determined by slicing of the gel according to the Coomassie Blue staining profile. Specific activity of *N*-ethylmaleimide: 1.25 mCi/mmol.

Band number	Radioactivity (cpm of band) (% of total radioactivity in parentheses)	Number of labeled SH groups per protein molecule	Number of SH groups per molecule
1 + 2	453 (48)	3	21 *
3	217 (23)	0.7	10 **
4.1	26 (3)	0.3	
4.2	91 (9)	1.1	
4.5	66 (7)	0.3	
5	25 (3)	0.2	
6	23 (3)	0.2	3 ***
7	24 (3)	0.2	

* Ref. 15.
** Ref. 16.
*** Ref. 17.

Band 3 can probably be discarded in view of the following evidence. Pretreatment of erythrocytes with iodoacetamide, in contrast to pretreatment with *N*-ethylmaleimide, does not prevent the effect of diamide on phospholipase susceptibility at a concentration of 2 mM (hematocrit 9%) and has only a minor effect at 10 mM. On the other hand, iodoacetamide at 2 mM blocks, among others, the same single SH-group on band 3 that is alkylated by 0.4 mM *N*-ethylmaleimide (hematocrit 9%, see Table III). This follows from our finding (Fig. 4) that pretreatment of erythrocytes with both reagents prevents or largely suppresses the dimerization of band 3, which can be induced in ghosts by exposure to diamide [10].

The involvement of band 4.2 seems unlikely in view of labeling studies by Allen and Cadman [13]. These authors demonstrated a preferential and intensive reaction of band 4.2 with iodoacetamide under conditions, where this agent at best partially inhibits the membrane-perturbing effect of tetrathionate.

On the basis of these findings and considerations a stabilisation of the asymmetric distribution of the phospholipids in the erythrocyte membrane by spectrin, localized at the inner surface of the membrane [12], seems to us an attractive hypothesis. Such a stabilizing effect of the erythrocyte's major "extrinsic" membrane protein on a considerable fraction of the charged phospholipids located at the cytoplasmic membrane surface would imply that the proportion of lipid in this membrane influenced by protein may be larger than the 15—20% usually assumed to act as "boundary lipids" for intrinsic membrane proteins [21—23]. Evidence that spectrin can interact with negatively charged phospholipids (phosphatidylglycerol or phosphatidylserine) has been obtained in artificial membrane systems [24—27]. In particular, recent calorimetric measurements of Mombers et al. [27] have clearly demonstrated that spectrin is able to remove phospholipids from the phase transition. Hydrophobic forces are most likely involved in this interaction according to criteria established by Papahadjopoulos et al. [28].

Interactions of spectrin with membrane phospholipid are thus very likely to occur in the erythrocyte membrane. On the other hand, evidence has recently been obtained that spectrin is associated with proteins at the cytoplasmic surface of the erythrocyte membrane [29]. This association could be abolished by trypsin. The receptor protein is not yet known. Major intrinsic peptides, such as glycophorin or "Band 3", are most probably not involved.

In order to reconcile these data with our results and the evidence pointing to hydrophobic interactions with phospholipids, one might envisage an arrangement in which spectrin interacts simultaneously with both proteins and phospholipids. The complete loss of spectrin binding after trypsin treatment of the membrane is certainly difficult to reconcile with this concept. One might speculate that trypsin treatment, by an unknown indirect mechanism, also abolishes the binding to phospholipids. Alternatively, one would have to assume that, in contrast to phospholipids in artificial membranes, phospholipids in the erythrocyte membrane do not interact directly with spectrin. The effect of spectrin on phospholipid asymmetry would then have to result from an indirect effect of spectrin mediated by its interaction with intrinsic membrane proteins.

The nature of the alterations of spectrin-membrane interactions that lead to the perturbation of phospholipid asymmetry is not clear. A simple detachment

is unlikely for two reasons. (1) Spectrin oligomers produced by tetrathionate or diamide are bound much more tightly to the membrane than is "monomeric" spectrin (Haest and Kamp, unpublished results). (2) Treatment with diamide and tetrathionate greatly diminishes the shear-induced deformability of the erythrocyte which, according to present concepts of erythrocyte membrane mechanics, must be due to a tightening of a membrane-attached protein network [34]. Thus, more subtle alterations of spectrin-membrane interactions must be involved.

Our observations indicate that after perturbation of these interactions phosphatidylserine (and probably phosphatidylethanolamine) move from the inner to the outer layer of the erythrocyte membrane without a detectable concomitant inward movement of sphingomyelin or phosphatidylcholine. This statement relies on the finding of a constant extent of cleavage, by phospholipases, of these two phospholipids. In the case of phosphatidylcholine it must be kept in mind, however, that only 80% of the phosphatidylcholine supposed to be located in the outer monolayer could be cleaved. With these restrictions, the changes observed can be interpreted in terms of a transbilayer net movement of phospholipids which should lead to an accumulation of material in the outer relative to the inner leaf of the bilayer. Obviously, this transposition of about 25% of the phospholipid of the inner layer to the outer layer does not affect the stability of the membrane to any major extent, as indicated, e.g., by the absence of lysis.

The rate of this transbilayer movement of aminophospholipids (half-time approx. 2 h) is much faster than the rates hitherto observed for the "flip-flop" (assumed to be a 1 : 1 self-exchange) of phospholipids in homogeneous artificial membranes ($t_{1/2} > 10$ days, ref. 18), but of the same order of magnitude as the transbilayer movement of lecithins observed in intact human erythrocytes ($t_{1/2}$ 2—5 h, refs. 19, 20). The higher rates of transbilayer movements in the erythrocyte membrane may be due to the heterogeneity of its phospholipids and its high content in unsaturated phospholipids. Rapid transbilayer movements of unsaturated phospholipids were recently observed in artificial bilayers composed of saturated and unsaturated phospholipids and in rat erythrocyte membranes [30,31]. Alternatively, membrane proteins might be involved.

Transbilayer movements of phospholipids thus seem to be possible in the native unperturbed erythrocyte membrane. If, in spite of this principal possibility, phosphatidylserine and the bulk of phosphatidylethanolamine are confined to the inner surface of the unperturbed erythrocyte membranes, specific constraints like those provided by spectrin have to be postulated to maintain this asymmetry during the 120-day life span of the erythrocyte in the circulation.

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