Nayar, R., Mayer, L. D., Hope, M. J., & Cullis, P. R. (1984) *Biochim. Biophys. Acta 777*, 343-346.
Nishizuka, Y. (1984a) *Science (Washington, D.C.) 225*, 1365-1370.
Nishizuka, Y. (1984b) *Nature (London) 308*, 693-697.
Ohki, K., Sekiya, T., Yamauchi, T., & Nozawa, Y. (1982) *Biochim. Biophys. Acta 693*, 341-350.
Ohki, K., Shunji, N., Sagami, M., & Nozawa, Y. (1986) *Chem. Phys. Lipids 39*, 237-249.
Putney, J. W. (1986) *Cell Calcium 7*, 1-12.
Putney, J. W., Weiss, S. J., Van de Walle, C. M., & Haddas, R. A. (1980) *Nature (London) 284*, 345-347.
Ransom, J. T., & Cambier, J. C. (1986) *J. Immunol. 136*, 66-72.
Reusch, R. N. (1985) *Chem. Phys. Lipids 37*, 53-67.
Salmon, D. M., & Honeyman, T. W. (1980) *Nature (London) 284*, 344-345.
Scanlon, M., Williams, A. D., & Fay, F. S. (1987) *J. Biol. Chem. 262*, 6308-6312.
Serhan, C., Anderson, P., Goodman, E., Dunham, P., & Weissmann, G. (1981) *J. Biol. Chem. 256*, 2736-2741.
Serhan, C. N., Fridovich, J., Goetzl, E. J., Dunhan, P. B., & Weissmann, G. (1982) *J. Biol. Chem. 257*, 4746-4752.
Serhan, C. N., Broekman, M. J., Korchak, H. M., Smolen, J. E., Marcus, A. J., & Weissmann, G. (1983) *Biochim. Biophys. Acta 762*, 420-428.
Siegel, D. P. (1986) *Biophys. J. 49*, 1171-1183.
Small, E. B., Mandersloot, J. G., DeKruijff, B., & DeGier, J. (1985) *Biochim. Biophys. Acta 816*, 418-422.
Small, E. B., Mandersloot, J. G., Demel, R. A., DeKruijff, B., & DeGier, J. (1987) *Biochim. Biophys. Acta 897*, 180-190.
Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P., & Putney, J. W. (1986) *Nature (London) 319*, 514-516.
Toeplitz, B. K., Cohen, A. I., Funke, P. T., Parker, W. L., & Gougoutas, J. Z. (1979) *J. Am. Chem. Soc. 101*, 3344-3353.
Trauble, H., & Eibl, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 214-219.
Tsien, R. Y., Rink, T. J., & Poenie, M. (1985) *Cell Calcium 6*, 145-157.
Tyson, C. A., Zande, H. V., & Green, D. E. (1976) *J. Biol. Chem. 251*, 1326-1332.
Utsumi, K., Nabori, K., Terada, S., Masanobu, M., & Utsumi, T. (185) *Cell Struct. Funct. 10*, 339-348.
Von Tscharner, V., Prod'hom, B., Baggiolini, M., & Reuter, H. (1986) *Nature (London) 324*, 369-372.
Weissmann, G., Collins, T., Evers, A., & Dunham, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 510-514.
Weissmann, G., Anderson, P., Serhan, C., Samuelson, E., & Goodman, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1506-1510.
Wilschut, J., Duzgunes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.

Asymmetric Distribution of Phospholipids in Spectrin-Poor Erythrocyte Vesicles†

Jean-Yves Calvez,‡ Alain Zachowski,*,‡ Andreas Herrmann,§ Gil Morrot,‡ and Philippe F. Devaux‡

Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris, France, and Bereich Biophysik der Sektion Biologie, Humboldt-Universität zu Berlin, 1040 Berlin, GDR

Received February 12, 1988; Revised Manuscript Received March 31, 1988

ABSTRACT: We have investigated by electron spin resonance, at 37 °C, the outside–inside passage and the equilibrium distribution of spin-labeled phospholipids, respectively, in ATP-containing ghosts, in heat-treated erythrocytes, and in heat-induced vesicles. The heat-treated vesicles were spectrin depleted to ~25% of the original content and had lost almost 100% of the other cytoskeletal proteins. Yet the vesicles, as long as they contained ATP, were capable of translocating the aminophospholipids with the same efficiency as the heat-treated erythrocytes, and almost with the same efficiency as ATP-containing ghosts. In the vesicles, sphingomyelin and phosphatidylcholine analogues underwent a very slow transverse diffusion as in native cells. We conclude that spectrin and other cytoskeleton proteins are not major factors for the establishment and maintenance of phospholipid asymmetry in human erythrocytes, which may be chiefly due to the aminophospholipid translocase activity.

In the human erythrocyte membrane, phospholipids are asymmetrically distributed between the two leaflets: phosphatidylcholine (PC) and sphingomyelin (SM) are preferentially located on the outer layer, while the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are the main components of the cytoplasmic layer (Bretscher, 1972; Verkleij et al., 1973). Several years ago it was suggested that spectrin, the main cytoskeleton component, serves as the stabilizer of the PS and PE localization at the membrane cytoplasmic face (Haest et al., 1978, 1980; Williamson et al., 1982). More recently, an ATP-dependent outside–inside transport of spin-labeled aminophospholipids was demonstrated in our laboratory (Seigneuret & Devaux, 1984; Zachowski et al., 1986; Bitbol et al., 1987). We postulated the existence of an "aminophospholipid translocase" which would be responsible for the phospholipid asymmetry in erythrocytes. Several other investigators have now shown that when exogenous PS or PE is introduced in the erythrocyte membrane outer leaflet, they are rapidly translocated into the inner leaflet provided the red cell contains ATP (Connor & Schroit, 1987;

† This work was supported by grants from the Centre National de la Recherche Scientifique (UA 526), the Université Paris 7, and the Ministère de la Recherche et de l'Enseignement Supérieur.
* Author to whom correspondence should be addressed.
‡ Institut de Biologie Physico-Chimique.
§ Humboldt-Universität zu Berlin.

0006-2960/88/0427-5666$01.50/0

Daleke & Huestis, 1985; Tilley et al., 1986; Middelkoop et al., 1988). However one can still question whether the aminophospholipid translocase can, by itself, generate and maintain the lipid asymmetry occurring in the membrane or whether spectrin is associated in this phenomenon (Williamson et al., 1987; Middelkoop et al., 1988).

In this paper, we report on experiments performed on the translocation of spin-labeled lipids in erythrocytes and in erythrocyte-derived systems, using a new protocole which allows us to investigate the lipid translocation at 37 °C. The spin-labels used in these experiments have a short (five carbons) β-chain which bears the nitroxide radical [see the formulas in Seigneuret and Devaux (1984) and Zachowski et al. (1985a)]. The partial water solubility of these molecules has been described in other papers (Seigneuret & Devaux, 1984; King & Marsh, 1987). They can be readily incorporated in a cell surface and, if necessary, extracted back by bovine serum albumin as lysophospholipids and fatty acids can be (Haest et al., 1981). Thus, the amount of spin-label present on the outer monolayer at any given time can be estimated by BSA[1] addition to labeled erythrocytes, followed by centrifugation and ESR determination of the spin concentration in the supernatant. Reoxidation by ferricyanide allows one to compensate for possible chemical reduction due to the passage on the inner layer (Seigneuret et al., 1984). In addition to the study of ATP-containing ghosts, we have carried out experiments with heat-denaturated RBC and with heat-induced vesicles from RBC. These vesicles, prepared in an energy-rich medium, contained ATP but were devoided of most of the original spectrin molecules and cytoskeleton proteins. Thus, the involvement of spectrin in lipid asymmetry could be directly tested.

Materials and Methods

Heat-Induced Vesicle Preparation. Freshly drawn human blood from healthy donors (Centre National de Transfusion Sanguine) was used as a source of erythrocytes. Red cells were washed 5 times in 145 mM NaCl, 5 mM KCl, 5 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.1 mM EGTA, 10 mM inosine, 10 mM glucose, and 20 mM Hepes buffer, pH 7.4 (referred to as buffer A). One volume of the final pellet was added to nine volumes of buffer A preheated at 50 °C, and the erythrocyte suspension (10% hematocrit) was incubated for 15 min at 50 °C. Exocytotic vesicles formed at this temperature (Ham et al., 1948) were removed from the cells by shear flow produced by two aspirations in a pipet with a 1.8 mm diameter orifice. Remaining cells were pelleted at 3000*g* for 4 min. The supernatant was then centrifuged for 30 min at 25000*g* to pellet the vesicles. To study the remaining translocase activity in cells after various times of heating, the same protocole was followed, except that the incubation at 50 °C lasted from 0 to 30 min and that only the remaining cells were collected.

ATP-Loaded Resealed Ghosts. Pink ghosts were prepared by the method of Schowch and Passow (1973) as modified by Seigneuret and Devaux (1984) and Zachowski et al. (1986).

Spin-Labeling. The synthesis of spin-labeled PC (PC*) was described in Seigneuret and Devaux (1984). The corresponding phosphatidylserine (PS*) and phosphatidylethanolamine (PE*) were prepared from it by the phospholipase D catalyzed base-exchange reaction as described by Comfurius and Zwaal (1977). The synthesis of spin-labeled sphingomyelin (SM*) was described in Zachowski et al. (1985a). The desired amount of spin-labeled analogue (corresponding to 1% of the total membrane phospholipids) from a chloroform solution was deposited in a tube and dried under nitrogen. The dried film was resuspended in 1 volume of buffer A by vigorous vortexing and heated at 37 °C. Translocation assay was initiated by addition of the spin-label solution to 2 volumes of preheated cell or vesicle suspension adjusted at a phospholipid concentration of ca. 1.5 mM (equivalent to a 50% hematrocrit erythrocyte suspension).

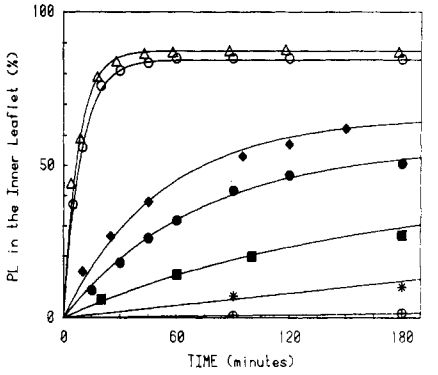

FIGURE 1: Transmembrane reorientation kinetics at 37 °C of spin-labeled phospholipids in ghosts resealed at various ATP concentrations. PS* in the presence of 0.45 (○) or 1.25 (△) mM ATP; PE* in the presence of 0.23 (■), 0.45 (●), or 2 (◆) mM ATP; PC* (*) or SM* (⊕) in presence of any ATP content. Curves are fitted to an exponential function by least-squares nonlinear regression.

Aminophospholipid Translocase Kinetics Assay. At given times varying from 0 min on, 150-μL aliquots were taken from the incubation and mixed with 70 μL of a 3.3% fatty acid free BSA solution (w/v) (Sigma). After 1 min on ice, the mixture was centrifuged at 7600*g* for 1 min in an Eppendorf tube and the supernatant kept. The amount of BSA-extractable probe at a given time was evaluated by the intensity of the ESR spectrum of the supernatant after reoxidation by potassium ferricyanide (10 mM). The difference between the signal obtained at time *t* and the signal displayed at time 0 gave the amount of spin-labeled phospholipids situated in the membrane inner layer at time *t*. Control experiments showed that ferricyanide allows the complete recovery of the nitroxides reduced by the erythrocyte cytosol. Under our experimental conditions, BSA extracted more than 95% of the labels exposed on the outer layer as inferred from the comparison of the ESR spectra of the supernatant and the pellet, when no incubation was allowed.

Measurement of Cellular ATP Content. Cell or vesicle ATP was extracted with 0.1% trichloroacetic acid and measured by the luciferin–luciferase luminescence test (Brown, 1982).

Protein Analysis. Vesicle membranes were solubilized in sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol and subjected to polyacrylamide gel electrophoresis using the discontinuous system of Laemmli (1970) with a 5% acrylamide stacking gel and a 7.5% acrylamide separating gel, at a constant current intensity of 30 mA per gel. White erythrocyte ghosts were prepared as a reference by repetitive washings in 5 mM phosphate buffer.

Results

Phospholipid Translocation at 37 °C in ATP-Containing Ghosts. Figure 1 shows a set of curves corresponding to the progressive establishment of a stable asymmetric distribution

[1] Abbreviations: PC*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; PS*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; PE*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; SM*, *N*-(4-doxylpentanoyl)sphingomyelin; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; RBC, red blood cell.

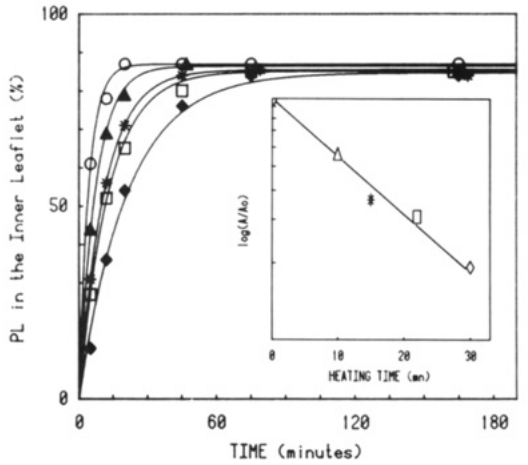

FIGURE 2: Transmembrane reorientation kinetics at 37 °C of PS* in red blood cells after various times of heating at 50 °C: (○) 0, (▲) 10, (*) 15, (□) 22, (◆) and 30 min. (Inset) Normalized initial velocity of PS* reorientation vs the time cells were heated at 50 °C (semi-logarithmic scale).

of spin-labels between outer and inner monolayers following their incorporation on the outer monolayer of erythrocyte ghosts. For PE*, three concentrations of ATP are shown; for PS*, two concentrations only are displayed. For the choline derivatives (PC* and SM*), experiments were carried out at different ATP concentrations without any indication of a significant ATP dependence (not shown). In the case of PS*, the plateaus were well determined and exhibited a very small increase between 0.45 and 1.25 mM intracellular ATP. On the other hand, the plateaus for PE* could only be obtained by extrapolation of the curves presented in this figure. The reason is that for long incubation times, i.e., for incubations exceeding 3 h, the ATP concentration was not constant, even though the ghosts were resealed in the presence of an ATP-regenerating system. This precluded a direct determination of the plateau levels as a function of ATP concentration for PE* (and SM* or PC*). Approximate plateau levels were as follows: for PS*, 85% and 87% in presence of, respectively, 0.45 and 1.25 mM ATP; for PE*, 46%, 57%, and 67%, in presence of, respectively, 0.23, 0.45, and 2 mM ATP.

Translocation Activity in RBC after Heating at 50 °C. Erythrocytes were incubated for different intervals at 50 °C, and their capacity to translocate PS* across the membrane was subsequently tested at 37 °C. Measurement of ATP content of the heated cells showed that under the conditions of the incubation (i.e., in a medium favoring ATP regeneration) no marked changes of ATP concentration occurred during the incubation at 50 °C, the cellular ATP concentration remaining at 1.24 ± 0.10 mM. On the other hand, incubation at high temperature affected the rate of PS* translocation (Figure 2): the initial velocity decreased exponentially with the duration of exposure at 50 °C (see insert of Figure 2), as can be anticipated for a protein heat–denaturation process. Note that the plateau which reflects the equilibrium distribution of PS* was only slightly decreased after heating. In order to obtain such a plateau, it is crucial to reoxidized the extracted spin-labeled phosphatidylserine with ferricyanide as explained under Materials and Methods. Without this precaution, after 3-h incubation, almost no ESR intensity can be detected in the BSA-extracted fraction.

Under the same experimental conditions, the partial inhibition by heat treatment was less pronounced for PE* translocation than for PS*; for example, after 30-min heating at 50 °C, the initial velocity of PE* translocation was only reduced by ~20% (results not shown). If cells heated for 0.5 h were subsequently incubated for 3 h at 37 °C, no reactivation could be detected (not shown).

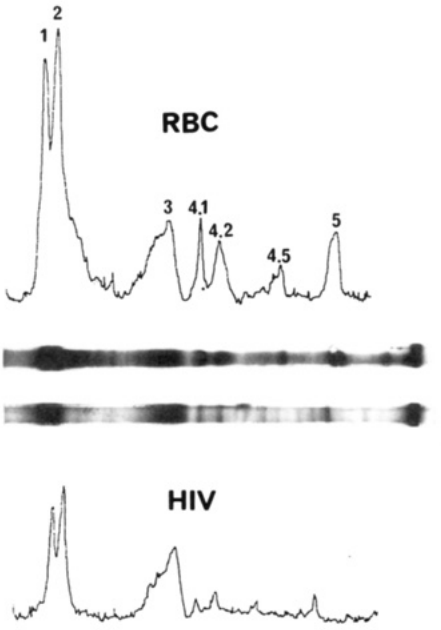

FIGURE 3: The 7.5% polyacrylamide gel electrophoretic patterns and densitometry scans of red cell membranes (top) and vesicles obtained after 15 min at 50 °C (bottom). Gels were stained with Coomassie Brilliant blue. Proteins are identified according to Steck (1974).

Heated-Induced Vesicle Characterization. The vesicle population obtained after heat treatment of erythrocytes was heterogeneous, as indicated by the vesicle pellet appearance, the top of which was white and the bottom slightly pink. The hemoglobin content of these structures can be related to the amount of erythrocyte cytoplasmic material which was entrapped in the vesicles. This is confirmed by the results of the ATP determination in the two populations of vesicles: the white vesicles were poor in ATP (less than 0.1 mM), while the pink vesicles contained up to 0.6 mM ATP. Only the pink vesicles were used thereafter. The protein composition of the pink vesicles showed a considerable decrease of the extrinsic proteins to band 3 ratio (Figure 3). When normalized to the amount of band 3 present, the vesicle membrane appeared to contain between 21 and 27% of the original amount of spectrin present in the erythrocyte membrane, while the following proteins were almost undetectable: bands 4.1, 4.2, 5, and 6. Finally, a control by negative-staining electron microscopy showed that the average vesicle diameter was 420 ± 60 nm and that the population of vesicles used for lipid translocation contained no contamination by ghosts.

Phospholipid Translocation in Heat-Induced Vesicles. Figure 4 shows the kinetics of reorientation at 37 °C of the four spin-labeled phospholipids in vesicles containing either 0.6 mM ATP (fresh vesicles) or 0.3 mM ATP (vesicles which had been incubated for 2 h at 37 °C in a metabolite-poor saline buffer). The fast transmembrane relocation of the aminophospholipids is still efficient in heat-induced vesicles. Its dependance on intravesicular ATP content is well illustrated in Figure 4: both the initial velocity and the plateau level decreased along with the ATP concentration. As to the choline-containing phospholipids, no significant differences appeared in the experiments, PC* diffusing at a slow rate (≃4% per hour)and SM* being faintly relocated. In summary, the results obtained in heat-induced vesicles are very close to those obtained in ghosts loaded with the equivalent amount of ATP. The only marked difference is a reduced initial velocity of PS* translocation, while the plateaus seem

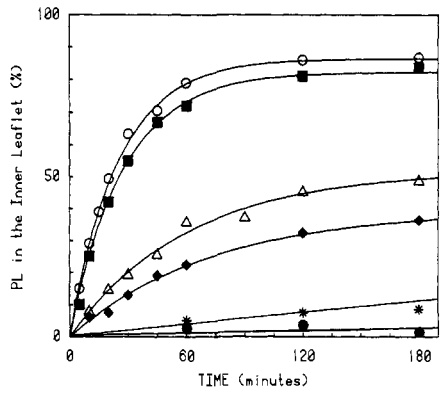

FIGURE 4: Transmembrane reorientation at 37 °C of the different phospholipid analogues in heat-induced vesicles containing two different ATP concentrations. PS* with 0.6 (○) or 0.3 (■) mM ATP. PE* with 0.6 (△) or 0.3 (◆) mM ATP. PC* (*) and SM* (●) with either ATP concentration.

very similar (compare Figures 1 and 4).

Discussion

In previous publications we have shown that spin-labeled phospholipids distribute themselves in ATP-containing ghosts between inner and outer monolayers as endogenous lipids in human erythrocytes. The experiments described in the present paper are based on a new ESR protocol which allows the study of the kinetics of spin-labeled phospholipid translocation in red cells at any temperature. Here we confirm at 37 °C our previous results obtained with ghosts at 4 °C by the ascorbate assay, experiments which showed that the rate of aminophospholipid uptake depends on the concentration of intracellular ATP (Seigneuret & Devaux, 1984). Figure 1 clearly demonstrates that, in the physiological range of intracellular ATP (i.e., ~1 mM), PE* kinetics is more sensitive to small variations of ATP concentration than PS* kinetics is.

Translocation of PS* (and PE*) in heat-treated erythrocytes was still very effective even after 0.5-h incubation at 50 °C. In particular, the plateau levels are reduced by less than 10%. Yet the heating process (15 min at 50 °C) is accompanied by a partial (50%) denaturation of spectrin (Brandts et al., 1977; Yoshino & Minari, 1987). The fact that the plateaus were barely affected while 50% of the spectrin is denaturated is a first indication that spectrin is not the major driving force for the establishment of lipid asymmetry in erythrocytes. The curves of Figure 3 show that the heating process affected primarily the initial rate of translocation (as indicated by the variation of the initial slope): this step is typically that which involves the putative aminophospholipid translocase (i.e., the "carrier protein"), while the plateau could involve stabilization by proteins of the inner layer, such as spectrin. Since the initial rate of PS* translocation was the only parameter diminished, it should be concluded that this was due to a partial denaturation of the aminophospholipid translocase and not to the denaturation of spectrin. Another argument in favor of the dominant role of the aminophospholipid translocase in maintaining lipid asymmetry is the fact that after 1-h incubation at 37 °C almost all the spin-labeled phosphatidylserine molecules present on the outer monolayer are chemically reduced and hence "invisible" without ferricyanide. This reduction reflects their passage on the inner monolayer since the endogenous reducing agent is contained in the cytosol (Seigneuret et al., 1984). Thus, the lipid transmembrane equilibrium distribution is not a fixed state with a fraction of the lipids anchored to spectrin but rather a steady state with continuous inward and outward fluxes of aminophospholipids across the membrane.

The experiments with heat-induced vesicles which are formed in the course of erythrocytes incubation bring an additional argument for our demonstration. Indeed in the vesicles, the amount of spectrin present is reduced to approximately 25% of its initial value. Furthermore, the residual spectrin is irreversibly denaturated to an extent of ~50% (Yoshino & Minari, 1987). Thus, compared to the native cells, heat-induced vesicles have only 10–15% of spectrin possibly interacting with the aminolipids. Yet, provided one takes into account the slightly reduced ATP concentration in vesicles compared to that in RBC, Figure 4 shows that the spontaneous segregation of phospholipids took place in heat-induced vesicles compared to the native cells with a time course only slightly reduced in the case of PS* and almost identical in the case of PE*, PC*, and SM*. As already pointed out, the reduced initial velocity of PS* translocation is probably due to the heat denaturation of the aminophospholipid translocase. The reason for a better protection of the mechanism responsible for PE* translocation is not known. Tentatively, it could be attributed to a better protection of the PE* binding sites on the outer monolayer by endogenous phosphatidylethanolamine. In conclusion, we suggest that spectrin and other cytoskeleton proteins are not important for the establishment and maintenance of lipid asymmetry in human erythrocytes.

How do our results compare to other data in the literature on this subject? In model systems, an interaction between spectrin and PS and PE has been demonstrated (Mombers et al., 1979, 1980). However, this interaction is strong only at acidic pH (below 6.5) and very weak at pH 7.4. At physiological pH, PE behaves as PC, a lipid which does not bind to spectrin, and PS exhibits a low level of interaction (Mombers et al., 1980). This interaction is still reduced when heat-denaturated spectrin was employed (Mombers et al., 1980). Recently, by investigating model systems, Maksymiw et al. (1987) have shown that the PS–spectrin interaction is purely electrostatic in nature and that at room temperature the binding energy is smaller than the thermal energy: the authors concluded that the spectrin–PS interaction is too weak to account for the lipid asymmetry in RBC. By studying the influence of spectrin aggregation state on the physical state of erythrocyte components, Farmer et al. (1985) also concluded from their studies on ghosts that the "interactions between the phospholipids in the inner bilayer leaflet and the skeletal network of proteins, if present in membranes in situ, were not strong".

Several reports on erythrocytes or on vesicles derived from erythrocytes are in accordance with the results we have obtained. Dressler et al. (1984) found that on vesicles prepared from heat-treated cells no transverse reorientation of endogenous PE and only a slight one of endogenous PS occurred, results very comparable to ours. Another type of spectrin-free vesicle can be derived from cells by treatment with Ca^{2+} and A 23187: according to Raval and Allan (1984), the phospholipid asymmetry is maintained in these vesicles. A third type of vesicle devoided of spectrin is the "inside-out" vesicles, in which an asymmetric distribution of phospholipid was reported (Kahlenberg et al., 1974) although this has been contradicted by the results of another group (Dressler et al., 1984).

Finally, it is possible to alter the aminophospholipid asymmetry (principally PE) under experimental conditions where spectrin is unaffected, namely, by ATP depletion of the erythrocytes in the absence of oxygen in a Ca^{2+}-free medium (Haest & Deuticke, 1976).

Several papers concluded that spectrin is either totally or at least predominantly responsible for lipid asymmetry in RBC.

This suggestion comes largely from experiments where spectrin was altered in situ by cross-linking oxidative reagents such as diamide (Haest et al., 1978). Under these conditions, more aminophospholipids were available for attack by phospholipase A_2 externally added to the cells. However, recently Franck et al. (1986) demonstrated that the enhanced phospholipase A_2 induced hydrolysis reflects a destabilization of the bilayer and not a loss of phospholipid asymmetry. In fact, spectrin oligomers produced by tetrathionate or diamide are bound much more tightly to the membrane than is monomeric spectrin (Haest et al., 1978). Thus, if spectrin was the key component of phospholipid asymmetry, it should, in our opinion, be enhanced by these treatments. Another argument in favor of spectrin influence is, supposedly, the observed perturbation of the lipid asymmetry in reversible sickle cells (Lubin et al., 1981; Middelkoop et al., 1988). Under deoxygenating conditions, a fraction of the lipid bilayer is dissociated from the skeletal proteins. However, there is no proof that this is the only perturbation at the level of the membrane. It is possible, for instance, that hemoglobin S interacts directly with the aminophospholipid translocase and diminishes its efficiency (Zachowski et al., 1985b). Yet another argument in favor of the spectrin role is, according to Middelkoop et al. (1988), the fact that the lipid asymmetry is stable in cells depleted of ATP after asymmetry has been established. However, the stability (over a limited period), in ATP-depleted cells, can be explained on the grants of the ATP requirement for *outward* diffusion as suggested by Williamson et al. (1987) and confirmed experimently recently by us (M. Bitbol, personnal communication).

In conclusion, at the present time we think that there are more arguments in favor of the exclusive role of the aminophospholipid translocase in the establishment and stability of lipid asymmetry in red blood cells.

ACKNOWLEDGMENTS

We are indebted to P. Hervé for the kind gift of spin-labeled phospholipids and to Dr. M. Bitbol for fruitful discussions and the communication of some of his personal results.

Registry No. ATP, 56-65-5; aminophospholipid translocase, 101077-55-8.

REFERENCES

Bitbol, M., Fellmann, P., Zachowski, A., & Devaux, P. F. (1987) *Biochim. Biophys. Acta 904*, 268–282.

Brandts, J. F., Erickson, L., Lysko, K., Schwartz, A. T., & Taverna, R. D. (1977) *Biochemistry 16*, 3450–3454.

Bretscher, M. S. (1972) *Nature (London) New Biol. 236*, 11–12.

Brown, A. M. (1982) in *Red Cell Membrane. A Methodological Approach* (Ellory, J. C., & Young, J. D., Eds.) pp 223–238, Academic, London.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Connor, J., & Schroit, A. J. (1987) *Biochemistry 26*, 5099–5105.

Daleke, D. L., & Huestis, W. H. (1985) *Biochemistry 24*, 5406–5416.

Dressler, V., Haest, C. W. M., Plasa, G., Deuticke, B., & Erusalimsky, J. D. (1984) *Biochim. Biophys. Acta 775*, 189–196.

Farmer, B. T., II, Harmon, T. M., & Butterfield, D. A. (1985) *Biochim. Biophys. Acta 821*, 420–430.

Franck, P. F. H., Op den Kamp, J. A. F., Roelofsen, B., & Van Deenen, L. L. M. (1986) *Biochim. Biophys. Acta 857*, 127–130.

Haest, C. W. M., & Deuticke, B. (1976) *Biochim. Biophys. Acta 436*, 353–365.

Haest, C. W. M., Plasa, G., Kamp, D., & Deuticke, B. (1978) *Biochim. Biophys. Acta 509*, 21–32.

Haest, C. W. M., Plasa, G., & Deuticke, B. (1981) *Biochim. Biophys. Acta 649*, 701–708.

Haest, C. W. M., Plasa, G., Kamp, D., & Deuticke, B. (1980) in *Membrane Transport in Erythrocytes* (Lassen, U. V., Ussing, H. H., & Wieth, J. O., Eds.) pp 108–119, Munksgaard, Copenhagen.

Ham, T. H., Shen, S. C., Fleming, E. M., & Castle, W. B. (1948) *Blood 3*, 373–403.

Kahlenberg, A., Walker, C., & Rohrlick, R. (1974) *Can. J. Biochem. 52*, 803–806.

King, M. D., & Marsh, D. (1987) *Biochemistry 26*, 1224–1231.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lubin, B., Chiu, D., Bastacky, J., Roelofsen, B., & Van Deenen, L. L. M. (1981) *J. Clin. Invest. 67*, 1643–1649.

Maksymiw, R., Sui, S.-F., Gaub, H., & Sackmann, E. (1987) *Biochemistry 26*, 2983–2990.

Middelkoop, E., Lubin, B. H., Bevers, E. M., Op den Kamp, J. A. F., Comfurius, P., Chiu, D. T.-Y., Zwaal, R. F. A., Van Deenen, L. L. M., & Roelofsen, B. (1988) *Biochim. Biophys. Acta 937*, 281–288.

Mohandas, N., Wyatt, J., Mel, S. F., Rossi, M. E., & Shohet, S. B. (1982) *J. Biol. Chem. 257*, 6537–6543.

Mombers, C., Verkleij, A. J., De Gier, J., & Van Deenen, L. L. M. (1979) *Biochim. Biophys. Acta 551*, 271–281.

Mombers, C., De Gier, J., Demel, R. A., & Van Deenen, L. L. M. (1980) *Biochim. Biophys. Acta 603*, 52–62.

Raval, P. J., & Allan, D. (1984) *Biochim. Biophys. Acta 772*, 192–196.

Schowch, G., & Passow, H. (1973) *Mol. Cell. Biochem. 2*, 197–218.

Seigneuret, M., & Devaux, P. F (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751–3755.

Steck, T. L. (1974) *J. Cell Biol. 62*, 1–19.

Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J. A. F., & Van Deenen, L. L. M. (1986) *FEBS Lett. 194*, 21–27.

Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & Van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.

Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.-R., & Schlegel, R. A. (1982) *Cell (Cambridge, Mass.) 30*, 725–733.

Williamson, P., Antia, R., & Schlegel, R. A. (1987) *FEBS Lett. 219*, 316–320.

Yoshino, H., & Minari, O. (1987) *Biochim. Biophys. Acta 905*, 100–108.

Zachowski, A., Fellmann, P., & Devaux, P. F. (1985a) *Biochim. Biophys. Acta 815*, 510–514.

Zachowski, A., Creascu, C. T., Galacteros, F., & Devaux, P. F. (1985b) *J. Clin. Invest. 75*, 1713–1717.

Zachowski, A., Favre, E., Cribier, S. Hervé, P., & Devaux, P. F. (1986) *Biochemistry 24*, 5406–5416.