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Effect of Cytochrome b_5 on the Transbilayer Distribution of Phospholipids in Model Membranes†

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ABSTRACT: The transbilayer distribution of phosphatidylethanolamine was assessed in phosphatidylcholine–phosphatidylethanolamine vesicles that contained various amounts of cytochrome b_5. The small vesicles, made by sonication, and the large vesicles, made by ethanol injection, were fractionated by centrifugation before cytochrome b_5 was asymmetrically incorporated into the bilayer. The mole ratio of phospholipid to protein ranged from 280 to 560 in the small vesicles and from 100 to 500 in the large vesicles. The phosphatidylethanolamine distribution, determined by chemical labeling with trinitrobenzenesulfonic acid, was assessed in vesicles that contained intact cytochrome b_5 molecules and in vesicles where only the hydrophobic tail remained associated with the bilayer. At every phospholipid to protein ratio examined, the transbilayer distribution of phosphatidylethanolamine in either the small or large unilamellar vesicles was not significantly different from the distribution in control vesicles that contained no protein. Ethanol was added to some cytochrome b_5–vesicle preparations (20% v/v) in an attempt to facilitate rearrangement of the phospholipids. No differences in the transbilayer distribution were observed. These results are discussed in terms of transbilayer equilibrium and the perturbation induced by the protein.

Phospholipids are usually asymmetrically distributed across biological membranes [see review by op den Kamp (1979)], but this biological phenomenon is often not observed in simple phospholipid bilayers (Nordlund et al., 1981a,b). Since proteins are another major component of biological membranes, it is possible that their interactions with the phospholipids influence the transbilayer phospholipid distribution. In a biological system, where proteins are inserted into preexisting bilayers, one plausible model for the generation of lipid asymmetry would have two components. The protein would first have to create a new transbilayer distribution through preferential interactions with specific types of lipids or by changing the packing constraints within the bilayer. Second, since transmembrane phospholipid movement (flip-flop) is slow in model systems, but fast in many biological membranes (op den Kamp, 1979; Rothman & Lenard, 1977), the protein, or other factors already present, would have to facilitate rapid flip-flop in order to allow the new system to rearrange.

In this study, we have simulated the biological condition by asymmetrically incorporating an integral membrane protein into preformed small or large unilamellar vesicles composed of phosphatidylcholine and phosphatidylethanolamine. Cytochrome b_5, an enzyme whose in vivo function is to participate in electron transfer reactions while bound to the endoplasmic reticulum (Holloway & Katz, 1972; Shimakata et al., 1972; Strittmatter et al., 1974), was chosen as a prototypic membrane protein because it possesses several characteristics that make it amenable to phospholipid asymmetry studies: it is an integral membrane protein, it forms monomeric or octameric aqueous solutions in the absence of lipid or detergent (Spatz & Strittmatter, 1971; Calabro et al., 1976), and it spontaneously inserts into phospholipid bilayers (Strittmatter et al., 1972; Sullivan & Holloway, 1973; Leto & Holloway, 1979).

The following structural and functional characteristics of cytochrome b_5 are pertinent to this study. It is an amphipathic protein comprised of a hydrophilic domain (M_r = 11 000) and a hydrophobic moiety (M_r = 5000) joined by a short flexible peptide sequence (Visser et al., 1975; Tajima et al., 1978). Denaturation studies revealed that these domains are structurally independent. The hydrophilic portion, which is stabilized by a noncovalently bound heme, unfolded first, without affecting the structure of the hydrophobic domain. Further denaturation caused the protein octamers to dissociate into monomers, suggesting that the tertiary structure of the hydrophobic domain had been altered (Tajima et al., 1976). The functional responsibilities are also segregated; proteolytic cleavage by trypsin separated the catalytic capabilities, which reside solely in the hydrophilic domain, from the membrane binding hydrophobic fragment. It is worth noting that proteolysis did not induce significant conformational changes in either the membrane-bound fragment or the soluble hydrophilic moiety (Tajima et al., 1978). The orientation of the hydrophobic domain in the bilayer has been a disputed issue, but recent experiments appear to demonstrate that the carboxyl terminus is on the same side of the membrane as the hydrophilic domain (Dailey & Strittmatter, 1981).

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0006-2960/82/0421-2820$01.25/0

Materials and Methods

Phospholipid Preparation. Palmitoyloleoylphosphatidylcholine (POPC)[1] and some of the palmitoyloleoylphosphatidylethanolamine (POPE) were purchased from Avanti Biochemicals (Birmingham, AL) and used without purification. The rest of the POPE was synthesized from POPC by using the transferase activity of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4). This enzyme, which was isolated from Savoy cabbage and purified through the acetone precipitation step (Yang et al., 1967), was stable for several months when stored as a lyophilized powder at −80 °C. The method used to synthesize POPE was essentially that of Comfurius & Zwaal (1977). POPC was dissolved in washed diethyl ether (20 mg/mL) and added immediately to an aqueous solution (pH 5.6) containing the enzyme, calcium, and ethanolamine hydrochloride (30% w/v). The reaction mixture was refluxed at 37 °C. After 8–12 h, 100 mM EDTA was added to stop the reaction. The ether was evaporated at room temperature under a stream of nitrogen, and the remaining aqueous layer was added to 3 volumes of methanol and refluxed for 1.5 h. The reaction mixture was filtered while hot, and the filter was washed with hot methanol. This step facilitated the separation of the product from the enzyme. The lipid was extracted according to Folch et al. (1957) and purified by column chromatography on carboxymethylcellulose using chloroform–methanol mixtures for elution. The chromatographically pure POPE fractions were washed with the ideal Folch lower phase containing EDTA to remove the residual calcium. The phospholipids were then precipitated from acetone (Kates, 1972). No detectable impurities were observed when POPC or POPE (0.1–0.2 μmol) was loaded on thin-layer silicic acid chromatography plates and developed in $CHCl_3$–CH_3OH–H_2O (65:25:4). The phospholipids, dissolved in $CHCl_3$ or $CHCl_3$–CH_3OH, were stored under argon at −20 °C. The purity of the phospholipids remained unchanged throughout the course of these experiments under these storage conditions.

Isolation of Cytochrome b_5. Cytochrome b_5, isolated from rabbit livers, was a generous gift from Thomas Markello. Coomassie Blue staining of the material separated by gel electrophoresis on 10% polyacrylamide indicated that a single band (>99%) of molecular weight 16 000 in sodium dodecyl sulfate was present. Further analysis of this preparation demonstrated that less than 0.10 mol of phospholipid and 0.01 mol of deoxycholate were present per mol of cytochrome b_5.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles were prepared by probe sonication (Huang, 1969). Mixtures of phospholipids were evaporated to dryness under argon and lyophilized for 5–6 h in the dark. The phospholipids were suspended in 10 mM borate buffer (pH 8.1) containing 50 mM KCl and 1 mM EDTA. This dispersion (2–3 mL), maintained under a nitrogen atmosphere, was sonicated intermittently at 0 °C for 2–3 min by using a Heat System W-350 sonifier. The maximum sonication time was 30 min.

A homogeneous population of small unilamellar vesicles was prepared from the crude vesicle dispersions by centrifugation for 60 min at 96500*g* (Ti-50 fixed angle rotor). Only the vesicles found in region III of the supernatant, as described by Barenholz et al. (1977), were used.

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles were obtained by modifying the ethanol injection procedure described by Batzri & Korn (1973), using a slow injection rate (Nordlund et al., 1981a). Colyophilized mixtures of phospholipids were dissolved in absolute ethanol (10–30 μmol of phospholipid/mL) and injected slowly (<3 mL/h) into rapidly stirring 10 mM borate buffer (pH 8.1) containing 50 mM KCl and 1 mM EDTA. The resulting vesicle dispersion contained less than 25% (v/v) ethanol. The ethanol was completely removed by passing the vesicles over a Sephadex G-50 column (1.6 × 15 cm). The dispersion was concentrated in an Amicon ultrafiltration cell (Lexington, MA) equipped with an XM-100 membrane. A homogeneous vesicle population was obtained by centrifugation for 38 min at 96500*g* in a Ti 50 fixed angle rotor. Only the vesicles found in region III of the supernatant, as described by Barenholz et al. (1977), were used.

Preparation of Cytochrome b_5–Vesicles. Small or large unilamellar vesicles, prepared as described above, were incubated with a solution of cytochrome b_5 for as long as 4 h, but usually for 30 min at room temperature. The molar ratio of protein to phospholipid ranged from 1:565 to 1:100. In some experiments, the hydrophilic domain of the cytochrome b_5 molecule was proteolytically cleaved from the proteoliposomes. In these experiments, trypsin, isolated from bovine pancreas (Millipore Corp., Freehold, NJ), and cytochrome b_5–vesicles were incubated for 12–18 h at room temperature (molar ratio of cytochrome b_5/trypsin = 10). The components of the reaction mixture were separated on a Sephadex G-100 column (1 × 22 cm). This was verified by monitoring the eluent at 413 nm and by recording the absorption spectra of the fractions that contained vesicles. These conditions cleaved ≥94% of the heme-containing moieties, as determined from the reduced minus oxidized difference spectrum (Omura & Sato, 1964).

Determination of POPE Distribution in Cytochrome b_5–Vesicles. Cytochrome b_5–vesicles were prepared as described above and labeled with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to one of the following methods.

Method A. The outer vesicle surface phosphatidylethanolamine (PE) was determined as follows. An aliquot of a vesicle solution, containing not more than 0.25 μmol of PE, was diluted to a final volume of 1.1 mL with the vesicle buffer solution. To this was added 0.4 mL of 0.8 M $NaHCO_3$, pH 8.5. The sample was mixed. A 20-μL aliquot of 1.5% TNBS was added; then the sample was mixed and incubated in the dark at room temperature. The absorbance at 410 nm was read at 5-min intervals for up to 2 h. The total phosphatidylethanolamine content was determined in a similar manner. An aliquot, containing not more than 0.25 μmol of PE, was diluted to a final volume of 1.1 mL with the vesicle buffer solution. To this was added 0.4 mL of 1.6% Triton X-100 in 0.8 M $NaHCO_3$, pH 8.5. The sample was mixed. A 20-μL aliquot of 1.5% TNBS was added; then the sample was mixed and incubated in the dark at room temperature. The absorbance at 410 nm was read at 5-min intervals for up to 2 h. The absorbance readings were corrected for the cytochrome b_5 heme absorption at 410 nm and for the chemical labeling of the amino groups in the protein (see Results).

Method B. The cytochrome b_5–vesicles (3 μmol of phospholipid) were incubated for 12–18 h with bovine pancreatic trypsin (molar ratio of cytochrome b_5/trypsin = 10). The components of the reaction mixture were separated on a Sephadex G-100 column (1 × 22 cm), and the vesicles were labeled as described in method A.

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylethanolamine; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

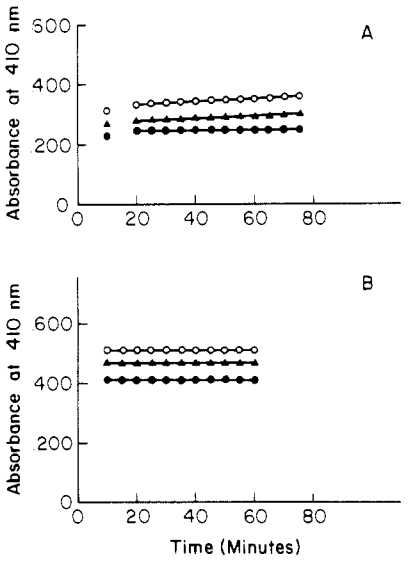

FIGURE 1: Time dependence of the TNBS reaction at various molar ratios of protein to phospholipid in small vesicles containing cytochrome b_5 (A) labeling of the outer surface and (B) labeling of the detergent-solubilized vesicles. Molar ratios of proteins to phospholipid are (●) 0, (▲) 1:560, and (○) 1:280. The experiments were performed at room temperature.

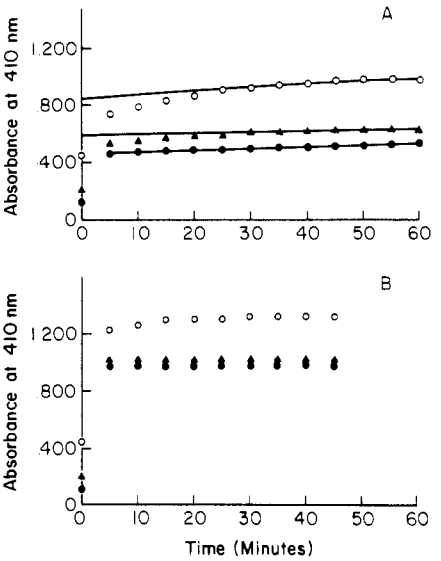

FIGURE 2: Time dependence of the TNBS reaction at various molar ratios of protein to phospholipid in large unilamellar vesicles (A) labeling of the outer surface and (B) labeling of the detergent-solubilized vesicles. Molar ratios of protein to phospholipid are (●) 1:500, (▲) 1:250, and (○) 1:100. The experiments were performed at room temperature.

Method C. The cytochrome b_5–vesicles (3 μmol of phospholipid) were incubated with ethanol (20% v/v) for 4–12 h. The ethanol was removed on a Sephadex G-50 column (1.6 × 15 cm), and the vesicles were concentrated to approximately 1.5 mL before trypsin was added. The remainder of the procedure is identical with method B.

Results

The small and the large unilamellar POPE–POPC vesicles used in this study were formed by probe sonication (Huang, 1969) and ethanol injection (Nordlung et al., 1981a), respectively. They were fractionated by centrifugation (Barenholz et al., 1977), and the PE component (30 mol % POPE) was chemically labeled by TNBS. The transbilayer distribution of this component in the small vesicles was asymmetric. The ratio of outer surface PE to total PE was 0.60 (Figure 1), similar to the PE distribution in sonicated egg PC–egg PE vesicles (Litman, 1973, 1974) and different from the outer phospholipid to total phospholipid ratio of 0.68 (C. F. Schmidt and B. J. Litman, unpublished experiments). For the POPC–POPE large vesicles, the outer PE/total PE ratio was 0.45 (data not shown). This differs from the distribution for large unilamellar egg PC–egg PE vesicles (outer PE/total = 0.49) which is symmetric (outer phospholipid/total phospholipid = 0.50; Nordlund et al., 1981a). This indicates that the POPC–POPE vesicles are not totally unilamellar but contain a small (<10%) fraction of oligolamellar vesicles (Nordlund et al., 1981a).

An attempt was made to alter the transbilayer distribution of PE by incorporating cytochrome b_5 asymmetrically into preformed vesicles. The time dependence of the TNBS reaction at various phospholipid to protein ratios in small vesicles containing the intact protein is plotted in Figure 1. For vesicles without protein, the maximum color development of the TNBS reaction with the outer surface PE occurred within 20 min at room temperature and remained constant for at least 55 min (Figure 1A). When protein was added to the vesicles, the TNBS reaction was essentially complete within 20 min (>90%), but the color development continued at a constant rate for the next hour, the rate being more rapid at higher protein concentrations (Figure 1A). The total PE content was determined by TNBS labeling in the presence of detergent (Figure 1B). The color development under these conditions reached its maximum value within 10 min, regardless of the protein concentration (Figure 1B). A similar trend was observed for the color development of the TNBS reaction with intact large vesicles containing cytochrome b_5 (Figure 2A) and detergent solubilized vesicles and cytochrome b_5 (Figure 2B). The data plotted in Figures 1 and 2 are the sum of the absorbances from the TNBS reaction with the amino groups in phosphatidylethanolamine and cytochrome b_5 and the absorbance of the cytochrome b_5 heme group. The latter two contributions must be corrected for before the transbilayer phosphatidylethanolamine distribution can be calculated. For small unilamellar vesicles, the absorbances from the TNBS labeling of sonicated POPC vesicles containing the appropriate amount of cytochrome b_5 were subtracted from the raw data. The corrected absorbances, which remained linear with time within the region of interest, were fit to a straight line by least-squares analysis. The line was extrapolated to the ordinate, and the transbilayer PE distribution was calculated. The POPC–cytochrome b_5 labeling experiments on small or large vesicles demonstrated that the labeling kinetics are approximately the same as those of PE, that the heme absorbance is the major correction, and that the absorbance from the TNBS labeling of cytochrome b_5 depends only on the concentration of cytochrome b_5 (data not shown). For the large vesicles, the raw data were corrected by least-squares extrapolation to zero time, subtraction of the measured zero time heme absorbance, and subtraction of a calculated contribution from cytochrome b_5 TNBS labeling.

At every phospholipid to protein ratio examined, the transbilayer distribution of phosphatidylethanolamine in either the small or large unilamellar vesicles was not significantly different from the distribution in control vesicles that contained no protein (Table I, row 1). A major difference between the large vesicle–protein and small vesicle–protein systems is that more protein can be incorporated into the large vesicles before all of the amino groups are readily accessible to TNBS. This constraint limited the range of protein concentrations inves-

Table I: Ratios of the Transbilayer Distributions (TD) of PE in Vesicles with and without Cytochrome b_5

	TD[b] (SUV + cytochrome b_5)/ TD (SUV)[c] at molar ratios of		TD (LUV + cytochrome b_5)/ TD (LUV)[c] at molar ratios of		
	560	280	500	250	100
method A – trypsin treatment	1.01	1.06	0.93	1.00	0.91
method B + trypsin treatment	1.10	1.02			1.07
method C + ethanol + trypsin treatment			1.04	1.11	

[a] Molar ratio = moles of phospholipid/moles of cytochrome b_5. [b] TD = outside PE/total PE. [c] The ratios were calculated from the results of at least two experiments. The estimated error is ±0.1.

tigated to phospholipid/protein ≥100 for the large vesicle system and phospholipid/protein ≥280 for the small vesicle system.

Corroborating results were obtained by another method (Materials and Methods, method B). In this procedure, the hydrophilic head groups of cytochrome b_5 were cleaved proteolytically from the vesicle–cytochrome b_5 complex and separated from the vesicles by a Sephadex G-50 column. The cytochrome b_5 concentration in these vesicles was assayed spectrophotometrically by measuring the oxidized minus reduced difference spectra (Omura & Sato, 1964); less than 5% of the cytochrome b_5 head groups remained associated with the vesicles. The vesicles, which now contained only the hydrophobic tail of cytochrome b_5, were labeled by TNBS. The color development of the TNBS reaction reached a maximum value within 20 min and remained constant for at least 30 min in vesicle preparations with and without added detergent (data not shown). These values, which were not corrected, were used to calculate the transbilayer phosphatidylethanolamine distribution. Once again, the PE distribution in the small and large unilamellar vesicles that contained protein was not significantly different from the PE distribution in the control vesicles (Table I, row 2).

These results indicate that cytochrome b_5, incorporated asymmetrically into preformed vesicles, does not alter the transbilayer distribution of phosphatidylethanolamine, even at rather high protein to phospholipid ratios. It is possible that the phospholipid distribution in the cytochrome b_5–vesicles is a kinetically trapped configuration. We have investigated this possibility by incubating cytochrome b_5–vesicle solutions with ethanol (20% v/v), a molecule that can increase the dielectric constant of the hydrocarbon part of the bilayer (Herskovits et al., 1961; Singer, 1962, 1971). The procedure that was used was essentially the same as method B, except that a 4–12-h incubation with ethanol (20% v/v) was included before the trypsinization step (Materials and Methods, method C). It should be noted that ethanol concentrations less than 30% (v/v) will not dissociate the heme from cytochrome b_5 (Huntley & Strittmatter, 1972). Similar TNBS color development kinetics were observed. The unadjusted values from the plateau regions in the outer surface phosphatidylethanolamine and total phosphatidylethanolamine assays were used to calculate the transbilayer distribution of phosphatidylethanolamine. No significant differences in the transbilayer phosphatidylethanolamine distribution in vesicles with or without protein were observed (Table I, row 3). Similar experiments were attempted with nonsolubilizing concentrations of sodium deoxycholate instead of ethanol, but significant quantities of detergent must have remained in the bilayer because all of the amino groups were readily accessible to TNBS, prohibiting accurate measurement of the transbilayer PE distribution.

Discussion

One model for the generation and maintenance of membrane phospholipid asymmetry would be the insertion of the protein, which establishes a new transbilayer distribution and promotes the approach to that distribution via rapid flip-flop. It should be noted, however, that continuous rapid flip-flop is not required, but only a transient rearrangement, perhaps during insertion. Rapid flip-flop, on the time scale of asymmetry assays by chemical labeling, exchange proteins, or lipases, makes the interpretation of these measurements, in terms of asymmetry, impossible.

Despite the obvious advantages of reconstituted vesicle systems for studying asymmetry, flip-flop, and their possible coupling, there have been relatively few investigations. Glycophorin, a protein that spans the bilayer, appeared to facilitate the transbilayer migration of phosphatidylcholine (deKruijff et al., 1978; Gerritsen et al., 1980) and lysophosphatidylcholine (van Zoelen et al., 1978a) in phosphatidylcholine vesicles that contained low concentrations of glycophorin (molar ratio of phospholipid/protein = 1200). Glycophorin was also reported to induce a change in the transbilayer asymmetry of lysophosphatidylcholine (15 mol %) in small, unilamellar PC vesicles (van Zoelen et al., 1978a). The transbilayer orientation of glycophorin in reconstituted vesicles is not absolutely asymmetric (van Zoelen et al., 1978b). Cytochrome *c* oxidase, incorporated asymmetrically into small vesicles by cholate dialysis, did not induce either rapid flip-flop or PC asymmetry at mole ratios as high as 130 phospholipids per protein, as assayed by an exchange protein (DiCorleto & Zilversmit, 1979). Identical results were obtained for another reconstitution, more relevant to the above model, in which the protein was inserted into preformed PC/PE/phosphatidylinositol vesicles (DiCorleto & Zilversmit, 1979). This system contained substantial but subsolubilizing levels of detergent (Eytan et al., 1976).

We have addressed the question of protein-induced phospholipid asymmetry in a detergent-free system in which the protein, cytochrome b_5, can be asymmetrically inserted into preformed vesicles. Since the exchange protein assay for asymmetry does not appear to be very precise, we have used the more accurate TNBS labeling method.[2] After the appropriate corrections were made (see Results), it was concluded that cytochrome b_5 at mole ratios as high as 100 phospholipids per protein in the large vesicles and 280 phospholipids per protein in the small vesicles did not alter the transbilayer distribution. For the small unilamellar vesicles, there was a slow, linear increase in the amount of TNBS labeling in intact vesicles (outer PE, Figure 1A). This could be interpreted as transbilayer phospholipid movement. However, we interpret

[2] The outer to total PE ratio for a given batch of vesicles can normally be measured to ±0.01. For different batches, the variation is within ±0.02 (Nordlund et al., 1981a). Given this limit, if 7% of the PE in one monolayer of 30 mol % PE large vesicles (3% of the total PE, 1% of the total lipid) undergoes a net transfer to the other monolayer, then it should be detectable. For vesicles with intact cytochrome b_5, for which significant corrections were necessary (for the outer PE of the 100:1 vesicles, this was roughly half of the total absorbance), we have used a more conservative value of ±0.1 in the distribution ratio with to without cytochrome b_5. This is equivalent to ±0.04 in the outer/total ratio with cytochrome b_5, or 13% of the PE in one monolayer undergoing a net transfer.

this increase as protein-induced vesicle leakiness to TNBS, since it did not occur for large vesicles at comparable or considerably higher protein to lipid ratios or in small vesicles with trypsinized cytochrome b_5. It is therefore concluded that significant flip-flop does not occur on the time scale of the labeling experiments.

There are two possible interpretations of the absence of transbilayer rearrangement in this system. The first is that the insertion of cytochrome b_5 does not change what is thought (Nordlund et al., 1981a) to be an equilibrium phospholipid distribution. A simple calculation indicates that when cytochrome b_5 is incorporated into large vesicles at a mole ratio of phospholipid to protein equal to 100, the volume of the bilayer is expected to increase by as much as 13%.[3] As mentioned in the introduction, there is evidence which suggests that the carboxyl terminus is located on the same side of the bilayer as the hydrophilic segment (Dailey & Strittmatter, 1981). This supports a model in which the hydrophobic segment penetrates only to the middle of the bilayer (Dailey & Strittmatter, 1981). If valid, this model implies twice the calculated volume perturbation in the outer monolayer, and a large mass imbalance between the monolayers. In these terms, the apparent absence of transbilayer lipid rearrangement is quite unexpected.

The other interpretation of our results is that insertion of cytochrome b_5 creates a new transbilayer equilibrium but that the system is kinetically trapped in the initial configuration. In an attempt to explore this possibility, protein-containing vesicles were incubated with ethanol, which might lower the flip-flop barrier. But no change in the phospholipid distribution was observed. It is possible that a breakdown in the vesicle permeability barrier occurs before or simultaneously with protein-induced transbilayer rearrangement. In this case, labeling experiments such as those done here would be of limited usefulness.

It appears that the permeability barrier to TNBS in small vesicles begins to break down at phospholipid to cytochrome b_5 ratios less than 280. The calculated volume perturbation for these conditions, in contrast to the large vesicles, is less than 3%.[3] This situation presumably is a consequence of the different molecular packing densities in the inner and outer monolayers (Huang & Mason, 1978; Chrzeszczyk et al., 1977) which prevents the bilayer from expanding. This represents an additional way in which large, unilamellar vesicles differ from small vesicles. The permeability breakdown difference is presumably also related to the osmotic sensitivity of the large vesicles, in contrast to small vesicles (Lichtenberg et al., 1981), and to the decreased permeability of large vesicles to leakage of trapped compounds (Barenholz et al., 1979).

Finally, it should be mentioned that there have been many speculations that localized, transient nonbilayer phospholipid configurations may be involved in transbilayer phospholipid movement (Taupin et al., 1975; Zilversmit, 1978; Cullis & deKruijff, 1979). These configurations might be induced by local compositional changes, divalent ion interactions with charged lipids, osmotic gradients, or temperature changes, in addition to, or in combination with, protein–lipid interactions. However, finding the correct set of conditions for triggering nonbilayer configurations in an appropriate model system, such as the one used here, would be a matter of chance. This may be possible when these events are better understood and may represent the most profitable approach.

[3] The volume occupied by the cytochrome b_5 hydrophobic region was approximated by a sphere whose radius equaled 17 Å (Visser et al., 1975; Vaz et al., 1979). From an idealized representation of a cytochrome b_5–membrane complex drawn to scale, it can be surmised that the hydrophobic region of cytochrome b_5 perturbs both monolayers; however, the majority of the mass of this region is probably located in the outer monolayer. Other shapes would be expected to give similar results. The average large (small) vesicle was assumed to have a diameter equal to 700 Å (250 Å) and a bilayer thickness equal to 40 Å.

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Dynamics of Membrane–Cytoskeleton Interactions in Activated Blood Platelets†

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ABSTRACT: The dynamics of actin polymerization, cytoskeleton formation, and interaction with membrane and cytoplasmic proteins as a result of platelet activation by temperature, ADP, or thrombin were studied. The polymerization of about 30% of platelet DNase I available actin to a nonavailable state is rapid and complete within 10 s after platelet activation with ADP and thrombin. This polymerization might be related to shape change rather than to aggregation or secretion. A similar value of actin polymerization is obtained when platelets are induced to change shape by cooling. This polymerization is partially reversible upon deactivation of the platelets by apyrase, hirudin, or rewarming. Cycles of temperature-mediated activation and deactivation show a cyclic variation in the state of actin, with a tendency to refractivity to further changes after a couple of cycles. No correlation is observed between microtubule integrity and actin polymerization when studies are performed with platelets pretreated with colchicine. Analysis of the Triton residue composition shows that the cytoskeleton of resting platelets is composed mainly of actin and myosin in a 4.5:1 ratio. Activation with ADP and thrombin leads to the association and incorporation of several other proteins (actin binding protein, 95 000 daltons, three to four proteins in the 35 000-dalton region, and two proteins in the 17 000-dalton region with the cytoskeleton). The incorporation of these proteins has a dynamic nature that depends on both the state of aggregation and the reversibility of the activation. Activation leads to a significant increase in the total cytoskeletal proteins, and although low temperature also induces such an increase, the cytoskeletal pattern of cooled platelets is not different from that of resting platelets. A complete reversibility in morphology and amount of protein was observed with temperature cycling. In light of these results, the dynamic nature of the state of actin in platelets is discussed.

The cytoskeletal structure of cells was the focus of many studies in recent years. Electron microscopy and immunofluorescent studies identified actin as the main component of the cytoskeleton (Edds, 1979; Haynes & Destree, 1978; Lazarides, 1976; Heuser & Kirschner, 1980; Nachmias, 1980; Tsukita et al., 1980; Wehland et al., 1979). Cell extracts are able to develop gel–sol transformation by the specific association of actin with certain proteins (Condeelis & Taylor, 1977; Ishiura & Okada, 1979; Koenig et al., 1981; Pollard, 1976a; Pollard & Ito, 1970; Stossel & Hartwig, 1976a). This well-defined microfilamentous structure may exist permanently, as in microvilli (Mooseker & Tilney, 1975; Mukherjee & Staehlin, 1971) or skeletal muscle (Peachey, 1966), or may develop from a soluble nonfilamentous actin precursor as a result of external stimuli (Badley et al., 1980; Nachmias, 1980; Tilney et al., 1973). On the other hand, sol–gel transformation has been suggested as the basis of ameboid movement or macrophage phagocytosis (Pollard, 1976b; Stossel & Hartwig, 1976b; Taylor, 1977; Taylor et al., 1973).

Microfilament bundles with a well-defined polarity have been described in platelet pseudopods (Nachmias & Asch, 1976; Crawford, 1976). These organized structures were probably formed from soluble, nonfilamentous actin during platelet activation. Two recent publications reported that a decrease in the DNase-available actin accompanied platelet activation (Carlsson et al., 1979; Pribluda et al., 1981). This decrease in DNase-available actin (actin polymerization) was fast and reached a maximum of about 30% of total actin within 10–15 s in the case of thrombin activation. In a recent publication, Phillips et al. (1980) showed that activation and aggregation of platelets by thrombin result in the specific association of the cytoskeleton with membrane proteins. Association of actin with platelet membrane was also shown by Taylor et al. (1975). In the present paper, we show that polymerization of actin is reversible to some extent and might be associated with the platelet shape change rather than release or aggregation. In addition, we give some evidence that the

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0006-2960/82/0421-2825$01.25/0